Stem Cell Biology

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PREFACE

The purpose of this book is to help students understand the fundamental concepts of this discipline. It is designed to motivate students to learn and prosper. I am grateful for the support of my colleagues. I would also like to acknowledge the encouragement of my family.

The cells that can divide in self-renewal to produce more of the same type of stem cells and differentiate into other types of cells are known as stem cells. There are two broad types of stem cells in mammals - embryonic stem cells and adult stem cells. The embryonic stem cells are isolated from the inner cell mass of blastocysts in early embryonic development. Adult stem cells are found in various tissues in fully developed mammals. Stem cells and progenitor cells act as a repair system for the body. Embryonic stem cells can differentiate into all the specialized cells in a developing embryo including endoderm, ectoderm and mesoderm. They also play an important role in maintaining the normal turnover of regenerative organs like intestinal tissues, blood and skin. The topics covered in this extensive book deal with the core aspects of stem cell biology. While understanding the long-term perspectives of the topics, it makes an effort in highlighting their impact as a modern tool for the growth of the discipline. Those with an interest in this field would find this book helpful.

A foreword for all the chapters is provided below:

Chapter – Introduction to Stem Cell and Stem Cell Biology

The cells of an organism that have the ability of self-renewing and developing into multiple matured and specialized cells are referred to as stem cells. The three types of stem cells are adult stem cells, embryonic stem cells and induced pluripotent cells. This is an introductory chapter which will briefly introduce all the significant aspects related to stem cell biology.

Chapter – Stem Cell: Elements and Concepts

Some of the fundamental concepts that fall under the domain of stem cell are stem cell line, stem cell genomes, stem cell marker and stem cell theory of aging. This chapter has been carefully writ¬ten to provide an easy understanding of these elements and concepts of stem cells.-

Chapter – Types of Stem Cells

Different types of stem cell include induced stem cell, dental pulp stem cell, limbal stem cell, renal stem cell, epiblast-derived stem cell, mesenchymal stem cell, amniotic fluid and amniotic membrane stem cell. The topics elaborated in this chapter will help in gaining a better perspective about these types of stem cells.

Chapter – Adult Stem Cell

The undifferentiated cells that are found throughout the body after development are known as adult stem cells. They multiply by cell division in order to replenish dying cells and regenerate the damaged tissues. Neural stem cell, endothelial stem cell, tissue-specific stem cell and spore-like cell are some of the adult stem cells. This chapter discusses these types of adult stem cells and their functions in detail.

Chapter – Stem Cell Mechanisms

There are various mechanisms in stem cell that are used for maintaining the functions of the stem cells. It includes molecular mechanisms of embryonic stem cell pluripotency, mechanisms of mesenchymal stem cell, stem cell aging, etc. This chapter closely examines these mechanisms of stem cell to provide an extensive understanding of the subject.

Chapter – Stem Cell Therapy

The utilization of stem cells for the treatment and prevention of the diseases is known as stem cell therapy. The techniques used by stem cells are peripheral stem cell transplantation, skin tissue regeneration, stem cell transplantation, hematopoietic stem cell transplantation, etc. All the diverse applications of stem cell therapy have been carefully analyzed in this chapter.

Natasha Rivera

Introduction to Stem Cell and Stem Cell Biology

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- **Properties of Stem Cell**
- **Importance of Stem Cell**
- **Pros and Cons of using Various Stem Cell**
- **Stem Cell Biology**

The cells of an organism that have the ability of self-renewing and developing into multiple matured and specialized cells are referred to as stem cells. The three types of stem cells are adult stem cells, embryonic stem cells and induced pluripotent cells. This is an introductory chapter which will briefly introduce all the significant aspects related to stem cell biology.

Stem cells are a group of cells in our bodies, with capacity to self-renew and differentiate to various types of cells, thus to construct tissues and organs. In science, it is still a challenge to understand how a fertilized egg to develop germ layers and various types of cells, which further develop to multiple tissues and organs with different biological functions. In the battle to fight against diseases, stem cells present potencies to repair tissues by cell therapy and tissue regeneration. The study of stem cells turns to be a major frontier in 21 century biology and medicine.

There are many types of stem cells, differing in their degree of differentiation and ability to self-renewing. Gametes cells (eggs or sperms) are stem cells they will develop to a whole body with various tissues after fertilizing. Embryonic cells derived from the part of a human embryo or fetus, are stem cells also with full potential to differentiation. Adult stem cells are partially differentiated cells found among specialized (differentiated) cells in a tissue or organ. Based on current researches, adult stem cells appear to have a more restricted ability of producing different cell types and self-renewing compared with embryonic stem cells.

Cancer stem cells are a sub-group of cancer cells that respond the escaping of cancer chemotherapy and the relapse of tumors. This concept has a great impact on the strategy of cancer chemotherapy and anti-cancer drug design. The new understanding of stem cell has been applied to treat leukemia (induced differentiation) and bone/blood cancer (bone marrow transplants) for many years and has achieved great success.

In the medicine applications, the induced pluripotent stem cells (iPS) reveal a special significance, as they can be induced to derive from many adult tissues or organs by treatment of protein factors.

Their features can be similar to the natural embryo stem cells. They provide the source for stem cells without an ethnic conflict.

Stem cells are certain biological cells found in all multicellular organisms. They are in small portion in body mass, but can divide through mitosis and differentiate into diverse specialized cell types and can self renew to produce more stem cells. Different types of stem cells vary in their degree of plasticity, or developmental versatility. Stem cells can be classified according to their plasticity and sources.

Table: Classification of stem cells (SCs).

Classification	Characteristics	
Sources/types	Embryonic stem cells	are pluripotent stem cells derived from the inner cell mass of the blastocyst, an early-stage embryo.
	Adult stem cells	Endodermal Origin: Pulmonary Epithelial SCs, Gastrointestinal Tract SCs, Pancreatic SCs, Hepatic Oval Cells, Mammary and Prostatic Gland SCs, Ovarian and Testicular SCs
		Mesodermal Origin: Hematopoietic SCs, Mesenchymal Stroma SCs, Mesenchymal SCs, mesenchymal precursor SCs, multipotent adult progenitor cells, bone marrow SCs, Fetal somatic SCs, Unrestricted Somatic SCs, Cardiac SCs, Satellite cells of muscle
		Ectodermal Origin : Neural SCs-Skin SCs-Ocular SCs
	Cancer stem cells	have been identified in almost all caner/tumor, such as Acute Myeloid leukemic SCs ($CD34^+/CD38^{-}$), Brain tumor SCs ($CD133^+$), Breast cancer SCs ($CD44^+/CD24^-$), Multiple Myeloma SCs ($CD138^+$), Colon cancer SCs ($CD133^+$), Liver cancer SCs ($CD133^+$), Pancreatic cancer SCs ($CD44^+/CD24^+$), Lung cancer SCs ($CD133^+$), Ovary cancer SCs ($CD44^+/CD117^+$), Prostate cancer SCs ($CD133^+/CD44^+$), Melanoma SCs ($CD4^+/CD25^+/FoxP3^+$), Gastric cancer SCs ($CD44^+$).
	Induced pluripotent stem cells	a type of pluripotent stem s artificially derived from a non-pluripotent cell, typically an adult somatic cell, by inducing a "forced" expression of specific genes.
Cell potency	Totipotent cells	Zygote, Spore, Morula; It has the potential to give rise to any and all human cells, such as brain, liver, blood or heart cells. It can even give rise to an entire functional organism.
	Pluripotent cells	Embryonic stem cell, Callus; They can give rise to all tissue types, but cannot give rise to an entire organism.
	Multipotent cells	Progenitor cell, such as hematopoietic stem cell and mesenchymal stem cell; They give rise to a limited range of cells within a tissue type.
	Unipotent cells	Precursor cell

Embryonic Stem Cells

Human embryos consist of 50–150 cells when they reach the blastocyst stage, 4-5 days post fertilization. Embryonic stem cells (ES cells) are derived from the inner cell mass of the blastocyst. They present two distinctive properties: they are able to differentiate into all derivatives of three primary germ layers (pluripotency), and they are capable of propagating themselves indefinitely, under defined conditions.

Dr. Evans first published a technique for culturing the mouse embryos in the uterus and derivation of ES cells from these embryos. Dr. Martin demonstrated that embryos could be cultured in vitro and ES cells could be derived from these embryos. In 1998, a research team led by

James Thomson reported the success of isolating and growing human embryonic stem cells in cell culture.

The studies of gene expression in these SE cells have identified many proteins associated with the "stemness" phenotype and can serve as markers for ES cells. After several decades of investigates, a list of SE-specific markers has been established (The National Institutes of Health resource for stem cell research) such as 5T4, Nanog, ABCG2, Oct-3/4, Alkaline Phosphatase/ALPL, Oct-4A, E-Cadherin, Podocalyxin, CCR4, Rex-1/ZFP42, CD9, SCF R/c-kit, CD30/TNFRSF8, sFRP-2, CDX2, Smad2, Chorionic Gonadotropin, lpha Chain (alpha HCG), Smad2/3, Cripto, SOX2, DPPA4, SPARC/Osteonectin, DPPA5/ESG1, SSEA-1, ESGP, SSEA-3, FGF-4, SSEA-4, GCNF/NR6A1, STAT3, GDF-3, SUZ12, Integrin alpha 6/CD49f, TBX2, Integrin alpha 6 beta 4, TBX3, Integrin beta 1/CD29, TBX5, KLF5, TEX19, Lefty, THAP11, Lefty-1, TRA-1-60(R), Lefty-A, TROP-2, LIN-28, UTF1, LIN-41, ZIC3, c-Myc etc.

The potential to generate virtually any differentiated cell type from embryonic stem cells (ESCs) offers the possibility to establish new models of mammalian development and to create new sources of cells for regenerative medicine and genetic disease and toxicology tests in vitro. To realize this potential, it is essential to be able to control ESC differentiation and to direct the development of these cells along specific pathways. Current embryology has led to the identification of new multipotential progenitors for the hematopoietic, neural, and cardiovascular lineages and to the development of protocols for the efficient generation of a broad spectrum of cell types including hematopoietic cells, cardiomyocytes, oligodendrocytes, dopamine neurons, and immature pancreatic β cells. Today, the most challenges are to devise and optimize effective protocols to induce differentiation of the ES cells into functional adult cells, and to demonstrate the functional utility of these cells, both in vitro and in preclinical models of human disease. For example effective protocols are expected not only to promote ES cells differentiation into hepatocytes, but also to induce hepatic functions such as albumin secretion, indocyanine green uptake and release, glycogen storage and p450 metabolism. Several recent protocols are efficient to produce high-purity (70%) hepatocytes in cultures, when these are transplanted into mice with acute liver injury, the human ES cells derived endoderm is capable to differentiate into hepatocytes and repopulated the damaged liver. However, due to the difficulty in controlling of proliferation and differential potential, and the most controversial issue on ethical concerns, the applications of human ES cells are currently limited in vitro and in animal studies.

On January 23, 2009, Phase I clinical trials for transplantation of oligodendrocytes (a cell type of the brain and spinal cord) derived from human ES cells into spinal cord-injured individuals received approval from the U.S. Food and Drug Administration (FDA), marking it the world's first human ES cell human trial. The study leading to this scientific advancement was conducted by Hans Keirstead and his colleagues at the University of California, Irvine and supported by Geron Corporation of Menlo Park, CA. In October 2010 researchers enrolled and administered ESCs to the first patient at Shepherd Center in Atlanta.

During the rapid development of medicine application of EC cells, safety is always a big concerning. The major concern is the risk of teratoma and other cancers as a side effect of ES cell applications, as their possibility to form tumors such as teratoma. The main strategy to enhance the safety of ESC for potential clinical use is to differentiate the ESC into specific cell types (e.g. neurons, muscle, liver cells) that have reduced or eliminated ability to cause tumors. Following

differentiation, the cells are subjected to sorting by flow cytometry for further purification. While ESC are predicted to be inherently safer than iPS cells because they are not genetically modified with genes such as c-Myc that are linked to cancer. Nonetheless ESC express very high levels of the iPS inducing genes and these genes including Myc are essential for ESC self-renewal and pluripotency, and potential strategies to improve safety by eliminating Myc expression are unlikely to preserve the cells' "stemness".

Embryonic Germ Stem Cells

Embryonic germ (EG) cells are derived cells from primordial germline cells (PGCs) in early development. EG cells share many of the characteristics of human ES cells, but differ in significant ways. Human EG cells are derived from the primordial germ cells, which occur in a specific part of the embryo/fetus called the gonadal ridge, and which normally develop into mature gametes (eggs and sperm).

PGCs are mainly isolated from fetal tissue in a narrowed time window. These isolated cells are subsequently allowed to grow and divide in vitro. After one to three weeks in vitro, the human PGCs had formed dense, multilayered colonies of cells that resembled mouse ES or EG cells. Cells in these colonies expressed SSEA-1, SSEA-3, SSEA-4, TRA1–60, TRA-1–81, and alkaline phosphotase. A small, variable percentage (1 to 20 %) of the PGC-derived cell colonies spontaneously formed embryoid bodies. The growth medium for embryoid body cultures lacked LIF, bFGF, and forskolin.

The range of cell types in the human PGC-derived embryoid bodies included derivatives of all three embryonic germ layers-endoderm, mesoderm, and ectoderm-based on the appearance of the cells and the surface markers they expressed. This result was interpreted to mean that the PGC-derived cells were pluripotent, however, it was not possible to demonstrate pluripotency in vivo by generating the formation of teratomas in mice.

Fetal Stem Cells

Fetal stem cells are primitive cell types found in the organs of fetuses. Fetal stem cells are capable to differentiate into two types of stem cells: pluripotent stem cells and hematopoietic stem cells. Neural crest stem cells, fetal hematopoietic stem cells and pancreatic islet progenitors have been isolated in the fetuses. Fetal blood, placenta and umbilical cord are rich sources of fetal hematopoietic stem cells.

Human fetal stem cells have been used by many people including children and adults suffering from many of mankind's most devastating diseases. Fetal neural stem cells found in the fetal brain were shown to differentiate into both neurons and glial cells. Human fetal liver progenitor cells have shown enormous proliferation and differentiation capacity to generate mature hepatocytes after transplantation in immunodeficient animals. Suzuki et al. showed that a single cell in the c-Met^{+}CD^{49}f- lowc-Kit-CD^{45}-Ter^{119}- fraction from mid-gestational fetal liver has the capacity for self-renewal in vitro and for bipotential differentiation, indicating that this defined fraction contains hepatic stem cells. Hepatic stem/progenitor cells can be enriched in mouse fetal hepatic cells based on several cell surface markers, including c-Met, Dlk, E-cadherin, and Liv2. Rat Dlk cells isolated from mid-gestational fetal liver exhibit characteristics expected for hepatic stem/progenitor cells. Thus, fetal liver cells may be suitable for overcoming the limitations in

engraftment and to allow a functional correction of the disease phenotype, as well as in use of artificial liver devices.

Hematopoietic cells are fetal stem cells in the umbilical cord after the birth of a baby. The only potential of these cells are to produce blood cells. However, in current medicine practice, they are quite effective in treating blood diseases such as leukemia and anemia. It is a mature medical service today to store the frozen umbilical cord blood of a new born baby, and to use for leukemia, anemia and other predispositions if needed in future.

The tissue rejection problems for fetal cell's application similar to those encountered in kidney and heart transplants may limit the usefulness of fetal stem cells. Further research to overcome this barrier is a hot topic in this field.

Bone Marrow (BM) Stem Cells

Adult BM mainly comprises two populations of precursor cells, hematopoietic stem cells (HSCs) and marrow stromal cells (MSCs). HSC and MSC are both multipotent stem cells. HSCs are present in circulating blood and umbilical cord blood (UCB) and are able to sustain production of all blood cells throughout life. MSCs can be isolated from several other tissues, including adipose tissue, placenta, amniotic fluid, UCB and fetal tissues are able to differentiate into osteocytes, adipocytes, chondrocytes, smooth muscle cells and haematopoietic supportive stroma.

Human HSCs have been defined with respect to staining for Lin, CD34, CD38, CD43, CD45RO, CD45RA, CD59, CD90, CD109, CD117, CD133, CD166, and HLA DR (human). In addition, metabolic markers/dyes such as rhodamine123 (which stains mitochondria), Hoechst33342 (which identifies MDR type drug efflux activity), Pyronin-Y (which stains RNA), and BAAA (indicative of Aldehyde dehydrogenase enzyme activity) have been described. The positive markers useful for MSC identification are CD106, CD105, CD73, CD29, CD44, and Sca-1.

Bone marrow transplantation (BMT) and peripheral blood stem cell transplantation (PBSCT) are the current clinical procedures to restore stem cells that have been destroyed by high doses of chemotherapy and/or radiation therapy. The isolation of a large number of potent HSC/MSC sets the basis of new methods for tissue regeneration and cell therapy. Nevertheless, the procedure of BM extraction is traumatic and the amount of material extracted is limited. Therefore, exploring new sources and isolation techniques for obtaining such cells is of great interest.

Hepatic Stem Cells

Liver transplantation is the primary treatment for various end-stage hepatic diseases, but is hindered by the source of donor organs and by complications associated with tissue rejection and immunosuppression. Thus, the regenerative capabilities of adult hepatocytes, liver progenitors and stem cells are being studied with great interest.

Adult hepatocytes remain a low mitotic rate during periods of tissue homeostasis. However, extensive documents have been established of these mature hepatic cells to re-enter the cell cycle and to restore damaged parenchyma through both cell hypertrophy and hyperplasia following acute hepatic parenchymal loss when surgical resection or hepatotoxin. Under these circumstances, liver mass is restored primarily through the activation of hepatocytes, suggesting

mature hepatocytes could serve their own physiologic precursors. As evidence, the isolated adult hepatocytes have been showed suitable for the treatment of liver diseases in both animal and human livers. After transplantation of primary adult hepatocytes into Gunn rat, an animal model for UDP-glucuronosyl transferase (UGT1A1) deficiency (Crigler-Najjar syndrome type I), the high bilirubin level is markedly reduced. This view is also supported by the current clinical practice of that the hepatocyte transplantation can cure or alleviate congenital metabolic diseases of the liver.

Liver oval cell, a blast-like cell and with the capability of self renewing and multipotent differentiation, is considered as the liver-specific stem cell. It can be identified only in the setting of chronic liver injury, when resident hepatocytes are unable to enter the cell cycle to restore liver mass. In multiple independent studies, these liver oval cells have been shown to present molecular markers of adult hepatocytes (albumin, cytokeratins 8 and 18), bile duct cells (cytokeratins 7 and 19, OV-6, A6), fetal hepatoblasts (AFP), and haematopoietic stem cells (Thy -1, Sca-1, c-kit). A recent study provides direct evidence that active Wnt/β-catenin signaling occurs preferentially during the transit amplifying of oval cell population and β-catenin clearly localizes to proliferating oval cells. Although it is not clear yet whether such a cell mass expanding in vitro is sufficient enough for clinical applications and its possible risk on carcinogenesis, oval cells isolated from the liver represent a promising source for cell-based therapy.

Human fetal liver progenitor cells have shown enormous proliferation and differentiation capacity to generate mature hepatocytes after transplantation in immunodeficient animals. Hepatic stem/progenitor cells are enriched in mouse fetal hepatic cell fraction, identified with several cell surface markers including c-Met, Dlk, E-cadherin, and Liv2. A single cell in the c-Met+CD49f- lowc-Kit-CD45-Ter119- fraction from mid-gestational fetal liver revealed the capacity of self-renewal in vitro and bipotential differentiation, indicating the containing of hepatic stem cells in this defined fraction, while the hepatic progenitor cells lack the capacity of self-renewal. As an in vitro cultivation protocol of fetal hepatic stem cells has been established, the fetal liver cells may be promised for the hepatic cell amount in engraftment and the functional correction of the disease phenotype, which should be better over the artificial liver devices.

Extra hepatic stem cells have been demonstrated to be involved in liver regeneration too in mice and rats studies. For example, cells from multiple extra hepatic tissues (including BM, umbilical cord and umbilical cord blood (UCB), and amniotic fluid) may differentiate into hepatic cells with some or many hepatic features, and some of them have shown the ability of liver repopulation in vivo. Remarkable trans-differentiation of HSCs to hepatocyte-like cells has been described, mainly in animals with BM/HSC transplantations followed by induction of liver damage. Lagasse et al demonstrated that highly purified HSCs repopulated not only the haematopoietic system, but also the livers with hereditary tyrosinaemia, rescuing these animals from liver failure. The published reports have suggested that MSCs may differentiate into hepatocyte-like cells both in vitro and in vivo. The cellular mechanism of trans-differentiation of MSCs to hepatocyte-like cells in vivo might be due to cell-fusion, while other reports suggested cell-autonomous trans-differentiation.

Pancreatic Stem Cells

Pancreatic islet transplantation has demonstrated an efficient way to achieve the long-term insulin independence for the patients suffering from diabetes mellitus type 1. However, because

of limited availability of islet tissue, new sources of insulin producing cells that are responsive to glucose are required. Development of pancreatic beta-cell lines from rodent or human origin has progressed slowly in recent years. To date, the best candidate sources for adult pancreatic stem or progenitor cells are: duct cells, exocrine tissue, nestin-positive islet-derived progenitor cells, neurogenin-3-positive cells, pancreas-derived multi-potent precursors; and mature β-cells.

The first report to describe in vitro generated insulin-producing islet-like clusters was based on the expansion of mouse pancreatic duct cells. Afterwards, Bonner-Weir et al generated the same type of insulin-producing islet-like clusters from cultivated islet buds developed from human pancreatic duct cells in vitro. Our previous study also provided evidence of that GLP-1 is able to induce pancreatic ductal cells with the expression of IDX-1 to differentiate into insulin producing cells, and is able to stimulate glucose-derived de novo fatty acid synthesis and chain elongation during cell differentiation and insulin release. These data indicated pancreatic ductal cells are potential tissue source for insulin-producing islet cells. However, at this time, the expansion capacity of these cultivated cells is still limited, and protocols for in vitro amplification need further optimization for a sufficient number of fully differentiated cells to allow a successful transplantation.

A recent genetic lineage study claimed the replication success of pre-existing β-cells and that turned to be the dominant pathway for the formation of new β-cells in adult mice. Another similar study also showed a cloned isolation of multi-potential precursor cells from mouse adult pancreas called pancreas-derived multi-potent precursors. These precursor cells arise from single islet and duct cells.

The generation of insulin-producing cells from pancreatic exocrine tissue has recently been reported. Both exocrine and endocrine pancreatic originate from a domain of the foregut endoderm, which expresses the pancreatic duodenal homeobox factor (Pdx-1) at early developmental stages. The inactivation of this gene leads to a non-pancreatic phenotype, demonstrating its major role in both exocrine and endocrine pancreatic development. In addition, signaling induced by soluble factors is a prerequisite to pancreatic lineage specification and triggers the emergence of pancreatic precursors expressing Pdx-1. Moreover, as Baeyens et al indicated, there were data suggesting the existence in vivo of acinar-islet transitional cells and the “spontaneous” trans-differentiation of acinar cells to insulin-expressing cells. Altogether, these may suggest that a population of acinar cells, in the presence of certain soluble factors, is competent to adopt an endocrine fate.

Some reports suggest that pancreatic precursor cells express nestin, an intermediate filament protein that is a marker of neural stem cells. These nestin-positive islet-derived progenitor cells also express insulin, glucagon, and Pdx-1 as well as low with levels of insulin secretion. However, other studies suggest that nestin expression is not related to pancreatic precursor identity.

Recent data indicate that Ngn-3-positive cells are endocrine progenitors both in the adult pancreas and in the embryo and that Ngn-3 expression is not seen outside the islets. Nevertheless, low levels of Ngn-3 expression within a population of duct cells are not excluded by these studies.

Pancreatic stem cells (PSCs) have the potential to differentiate into all three germ layers. Major markers present on the surface of PSCs include Oct-4, Nestin, and c-kit. DCAMKL-1 is a novel putative stem/progenitor marker, can be used to isolate normal pancreatic stem/progenitors, and potentially regenerate pancreatic tissues.

Eye Stem Cells

Human cornea is transparent and clear for vision. Unique to other human organs, there is no blood vessels to provide nutrition in corneas. It is the corneal stem cell existing in the nearby limbus ring, differentiate and move to the center of corneas to renew the transparent and clear cornea around every four months. Stem cells in human cornea play a unique and significant role to maintain the corneal function.

Human corneal stem cells locate on cornea limbus, which is between the colored and white part of the eye (where it joints the sclera). During homeostasis and following injury to the corneal epithelium, the limbal corneal stem cells divide to produce daughter transient amplifying cells that proliferate, migrate onto the central cornea and become terminally differentiated to replace the lost cells. When a stem cell divides, each new daughter cell has the potential to either remain a stem cell or become a differentiated corneal cell. The microenvironment within the corneal basement membrane is expected the primary factor responsible for the corneal terminal differentiation. However, in the case of limbal stem cell deficiency, either due to injury or diseases, it is unable for the corneal ocular repairing and regeneration. In certain corneal disorder such as Keratoconus, some stem cell markers, such as CD34, p63, were reported significantly decreased from normal to keratoconus corneas. It is speculated that many corneal disorders such as in keratoconus, anirdia and alkali burns are likely associated with the corneal stem cell deficiency.

Cornea transplantation is widely used to treat certain corneal diseases such as keratoconus. Due to the limited source of donated corneas, corneal stem cells are explored, instead of corneal buttons. In a pioneering test on cornea damage patients, stem cells were taken from the biopsied limbus tissue, grew into healthy corneal tissue in a little over two weeks, and the healthy tissue was then grafted onto the damaged eye. In the study of 112 patients between 1998 and 2006, 77% of patients had a successful first or second graft. While the opaque cornea became clear again, the vision restored. As human cornea is the most tolerant organ to accept xenograft, the corneal stem cells might be among the first large scale produced stem cells for medical application.

Another frontier of stem cell applications in human eyes is the aged-related macular degeneration (AMD). Macular degeneration is a retinal degenerative disease which causes progressive loss of central vision. The risk of developing macular degeneration increases with age. This disease most often affects people over fifties, and is the most common cause of blindness in the elderly. The impact of AMD on patients includes, but not limits, vision impairment, difficulty with daily activities, increased risk of falls, more depression and emotional distress. It affects the quality of life for millions of elderly individuals worldwide. It is not only a health challenge, but also a severe social problem across the world, no mater your ethnic group and gender.

The macula is the central portion of the retina responsible for perceiving fine visual detail. Light sensing cells in the macula, known as photoreceptors, convert light into electrical impulses and then transfer these impulses to the brain via the optic nerve. Central vision loss from macular degeneration occurs when photoreceptor cells in the macula degenerate.

During the stem cell treatment, macular patients are treated by implanting autologous (from selves) stem cells behind the eye via retrobulbar injection under local anesthesia. These reinjected stem cells have the potential to transform into multiple types of cells and are capable of

regenerating damaged tissue. Stem cell treatment is so far the most promising approach to restore the vision from AMD among many strategies.

Cancer Stem Cell

Cancer stem cells theory is a finding on stem cell biology and an application of stem cell features on cancer studies. Cancer stem cells are those stem cells in tumor mass. They specifically are with the ability to give rise to all cell types found in a cancer sample. According to the hypothesis, the original tumor is developed and formed from these cancer stem cells by self-renewal and differentiation into multiple cell types. Cancer stem cell population consists of only a small potion of tumor mass (around 0.1-1% of total mass) and can be distinguished from the other cells in tumor mass by special cell surface antigens (such as $CD34^+$). Both stem cells and cancer stem cells share the characters of stemness, the capacity of differentiation, the multi-potential differentiation. However, the unique character of cancer stem cells, different from normal stem cells, is the growth out of control. They, or their descendants, lost the behavior of "contact inhibition of growth", the most important character of a non-cancer cell.

During conventional cancer chemotherapies, the differentiated or differentiating cells are likely to be killed while the cancer stem cells, due to their stemness and inactivity, could remain untouched, therefore to escape from chemotherapies. It is believed they serve as "cancer seeds" and respond to the cancer relapse and metastasis by rising new tumors. Based on the concept of cancer stem cells, it is beneficial to include an induction of the cancer stem cell differentiation during chemotherapies. This will be expected to increase the efficacy of chemotherapies and improve the survival rate of cancer patients.

Identify Cancer Stem Cell in Various Types of Cancers

The existence of cancer stem cells has been debated for many years until the first conclusive evidence was published in 1997 in Nature Medicine. Bonnet and Dick isolated a subpopulation of acute myeloid leukemic cells that express a specific surface antigen CD34, but lacks the antigen CD38. The authors established that the subpopulation, $CD34^+/CD38^-$, is capable of initiating tumors in NOD/SCID mice that is histologically similar to the donor. Later, Blair A et al reported a similar but slightly different cancer stem cell phenotype of $CD34^+/CD71^-/HLA^-/DR^-$ in acute myeloid leukemic cells.

Evidence also comes from the rational of histology, the tissue structure of tumors. Many tumors are very heterogeneous and contain multiple types of cells. These multiple types of cells are believed to be developed from single cells (or a cluster of cells), rather than assembled by multiple cells. If the descendants of these multiple types of cells come from a same ascendant, this implies that the ancestor must have the capacity to generate multiple cell types. In other words, it possessed multi-differential potentials, the fundamental character of stem cells.

Table: Reported cancer stem cell and their surface antigens.

Tumor type	Surface antigens
Acute Myeloid leukemic	$CD34^+/CD38^-$
Brain tumor	$CD133^+$

Breast cancer	$CD44^+/CD24^-$
Multiple Myeloma	$CD138^+$
Colon cancer	$CD133^+$
Liver cancer	$CD133^+$
Pancreatic cancer	$CD44^+/CD24^+$
Lung cancer	$CD133^+$
Ovary cancer	$CD44^+/CD117^+$
Prostate cancer	$CD133^+/CD44^+$
Melanoma	$CD4^+/CD25^+/FoxP3^+$
Gastric cancer	$CD44^+$

The existence of leukemic stem cells prompted further studies in this field. Cancer stem cells have been reported in more and more other cancer types. Followed the Acute Myeloid leukemic stem cells ($CD34^+/CD38^-$), cancer stem cells have also been identified in several solid human tumors respectively.

As cancer stem cells have been identified in various organ origin cancers, it is widely accepted that cancer stem cell is a general format and fundamental concept in all cancers (or tumors).

The Origin of Cancer Stem Cells

Where the cancer stem cell comes from? The origin of cancer stem cells is still a hot topic of discussion and argument. Several camps regarding the issue have formed within the scientific community, and it is likely that the correct answer is not limited in one, depending on the tumor types and their developments. Up to date, there is not yet an experimental model has been established to demonstrate a tumor formation in lab, as cancer stem cells are usually isolated from end-stage of tumors rather than the initial stage to tumors. Therefore, describing a cancer stem cell as the cell of origin is often an inaccurate claim, and as hypothesis.

As cancer stem cells share the features of stem cells and of cancer cells, it is not wonder that some researchers believe they are the results of cell mutants from developing stem cells, including progenitor cells, adult stem cells, and the most likely from stem cell niche populations during development. The rational behind is that these developing stem populations are mutated and then expand such that the mutation is shared by many of the descendants of the mutated stem cell. These daughter stem cells are then much easier to becoming tumors, and because of the large amount of cells, there is more chance of a mutation that can cause cancer. Adult stem cells are with extremely long lifespan to accumulate mutants that drives cancer initiation. Thus, adult stem cells have also advantages on the logical backing of the theory of tumor formation.

It has also been proposed that the cancer stem cells are mutants from cancer cells after obtaining the stem cell-like features. De-differentiation is a reasonable hypothesis, which assumes these cells acquire stem cell like characteristics by reverse-differentiation from cancer cells. This is a potential alternative to any specific cell of origin, as it suggests that any cell might become a cancer stem cell.

The tumor hierarchy is another model to propose the origin of cancer stem cells. The main point of this model claims that a tumor is a heterogeneous population of mutant cells with various stages of stem cells. In this model, the tumor is made up of several types of stem cells, some stem cell lines

will be more thrive than other cell lines, as they adapt to the specific environments. Within the tumor hierarchy model, it would be extremely difficult to pinpoint the cancer stem cell's origin. It is important to bear in mind that, due to the heterogeneous nature of cancers, it is possible that any individual cancer could come from an alternative origin.

The Impact of Cancer Stem Cell Concept on Cancer Therapy

The concept of cancer stem cell has a great impact on the strategy of chemotherapy and cancer treatments. The classic view of cancer is that the tumor cell (and its progeny) arises from the progressive accumulation of mutations over time, giving it growth advantage over its neighbors. It also implies that all cells in a tumor have more or less an equivalent capacity to form another tumor - relapse or metastasis. Under the classic view of cancer, the anti-cancer drugs are designed to target rapid growth cells. However in CSC model, tumor cells have somehow been reprogrammed to be "stem-like", and thus grow slower than surrounding cells. It also implies that only CSCs have the ability to propagate new tumors. According to CSC model, the traditional therapies that target the bulk tumor are to some extent pointless, as the resulting shrinkage may look good on a CT scan, but the disease itself can still recur.

Relapse and metastasis are major challenges in current cancer treatments. During the cancer chemotherapies, the cancer (or tumor) mass is initially shrink, but barely cleared up. After a while, they usually come back (relapse) with some new drug resistance features developed. It is believed the cancer stem cells serve as "cancer seeds" with stemness and inactivity features, which help them to escape from chemotherapy and survive from drug attack. They are responding to the cancer relapse. Based on this concept of CSC, it is beneficial to include an induction of the cancer stem cell differentiation before and during chemotherapies. This will be expected to increase the efficacy of chemotherapies and improve the survival rate of cancer patients. This induced differentiation strategy has achieved significant efficacy on blood cancer treatment, such as children's acute promyelocytic leukaemia (APL). A group of pioneer scientists in China used Arsenic and retinoic acid to induce children's APL and have achieved "a complete remission in 92 - 95% of patients with this disease". However in solid tumors, the differentiation inducers and chemotherapeutic agents are difficult to penetrate into the inside of solid tumors. How to improve this penetration is still a big challenge for pharmaceutical researchers.

Properties of Stem Cell

Stem cells differ from other kinds of cells in the body. All stem cells—regardless of their source—have three general properties: they are capable of dividing and renewing themselves for long periods; they are unspecialized; and they can give rise to specialized cell types.

Stem cells are capable of dividing and renewing themselves for long periods. Unlike muscle cells, blood cells, or nerve cells—which do not normally replicate themselves—stem cells may replicate many times, or proliferate. A starting population of stem cells that proliferates for many months in the laboratory can yield millions of cells. If the resulting cells continue to be unspecialized, like the parent stem cells, the cells are said to be capable of long-term self-renewal.

Scientists are trying to understand two fundamental properties of stem cells that relate to their long-term self-renewal:

- Why can embryonic stem cells proliferate for a year or more in the laboratory without differentiating, but most adult stem cells cannot; and
- What are the factors in living organisms that normally regulate stem cell proliferation and self-renewal?

Discovering the answers to these questions may make it possible to understand how cell proliferation is regulated during normal embryonic development or during the abnormal cell division that leads to cancer. Such information would also enable scientists to grow embryonic and non-embryonic stem cells more efficiently in the laboratory.

The specific factors and conditions that allow stem cells to remain unspecialized are of great interest to scientists. It has taken scientists many years of trial and error to learn to derive and maintain stem cells in the laboratory without them spontaneously differentiating into specific cell types. For example, it took two decades to learn how to grow human embryonic stem cells in the laboratory following the development of conditions for growing mouse stem cells. Likewise, scientists must first understand the signals that enable a non-embryonic (adult) stem cell population to proliferate and remain unspecialized before they will be able to grow large numbers of unspecialized adult stem cells in the laboratory.

Stem cells are unspecialized. One of the fundamental properties of a stem cell is that it does not have any tissue-specific structures that allow it to perform specialized functions. For example, a stem cell cannot work with its neighbors to pump blood through the body (like a heart muscle cell), and it cannot carry oxygen molecules through the bloodstream (like a red blood cell). However, unspecialized stem cells can give rise to specialized cells, including heart muscle cells, blood cells, or nerve cells.

Stem cells can give rise to specialized cells. When unspecialized stem cells give rise to specialized cells, the process is called differentiation. While differentiating, the cell usually goes through several stages, becoming more specialized at each step. Scientists are just beginning to understand the signals inside and outside cells that trigger each step of the differentiation process. The internal signals are controlled by a cell's genes, which are interspersed across long strands of DNA and carry coded instructions for all cellular structures and functions. The external signals for cell differentiation include chemicals secreted by other cells, physical contact with neighboring cells, and certain molecules in the microenvironment. The interaction of signals during differentiation causes the cell's DNA to acquire epigenetic marks that restrict DNA expression in the cell and can be passed on through cell division.

Many questions about stem cell differentiation remain. For example, are the internal and external signals for cell differentiation similar for all kinds of stem cells? Can specific sets of signals be identified that promote differentiation into specific cell types? Addressing these questions may lead scientists to find new ways to control stem cell differentiation in the laboratory, thereby growing cells or tissues that can be used for specific purposes such as cell-based therapies or drug screening.

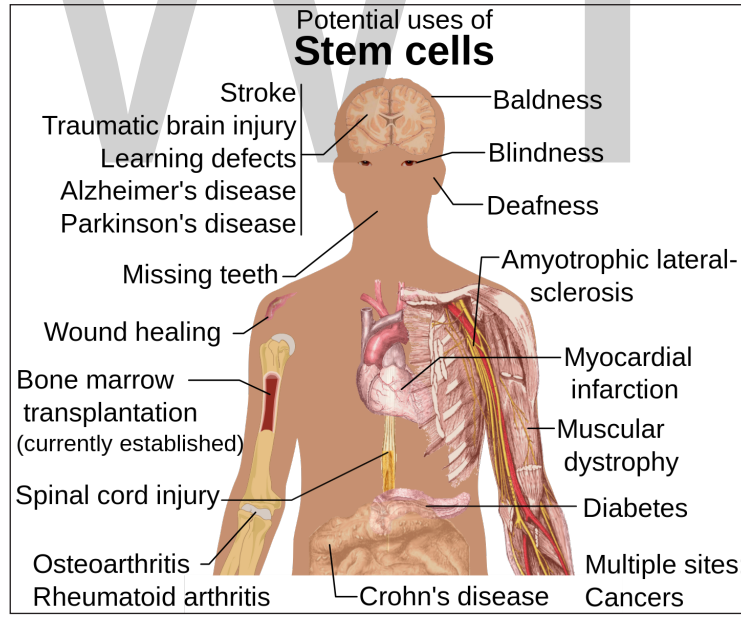
Adult stem cells typically generate the cell types of the tissue in which they reside. For example, a blood-forming adult stem cell in the bone marrow normally gives rise to the many types of blood

cells. It is generally accepted that a blood-forming cell in the bone marrow—which is called a hematopoietic stem cell—cannot give rise to the cells of a very different tissue, such as nerve cells in the brain. Experiments over the last several years have purported to show that stem cells from one tissue may give rise to cell types of a completely different tissue. This remains an area of great debate within the research community. This controversy demonstrates the challenges of studying adult stem cells and suggests that additional research using adult stem cells is necessary to understand their full potential as future therapies.

Importance of Stem Cell

Stem cells are important for living organisms for many reasons. In the 3- to 5-day-old embryo, called a blastocyst, the inner cells give rise to the entire body of the organism, including all of the many specialized cell types and organs such as the heart, lung, skin, sperm, eggs and other tissues. In some adult tissues, such as bone marrow, muscle, and brain, discrete populations of adult stem cells generate replacements for cells that are lost through normal wear and tear, injury, or disease.

Given their unique regenerative abilities, stem cells offer new potentials for treating diseases such as diabetes, and heart disease. However, much work remains to be done in the laboratory and the clinic to understand how to use these cells for cell-based therapies to treat disease, which is also referred to as regenerative or reparative medicine.



Laboratory studies of stem cells enable scientists to learn about the cells' essential properties and what makes them different from specialized cell types. Scientists are already using stem cells in the laboratory to screen new drugs and to develop model systems to study normal growth and identify the causes of birth defects.

Research on stem cells continues to advance knowledge about how an organism develops from a

single cell and how healthy cells replace damaged cells in adult organisms. Stem cell research is one of the most fascinating areas of contemporary biology, but, as with many expanding fields of scientific inquiry, research on stem cells raises scientific questions as rapidly as it generates new discoveries.

Can Doctors use Stem Cells to Treat Patients?

Some stem cells, such as the adult bone marrow or peripheral blood stem cells, have been used in clinical therapies for over 40 years. Other therapies utilizing stem cells include skin replacement from adult stem cells harvested from hair follicles that have been grown in culture to produce skin grafts. Other clinical trials for neuronal damage/disease have also been conducted using neural stem cells. There were side effects accompanying these studies and further investigation is warranted. Although there is much research to be conducted in the future, these studies give us hope for the future of therapeutics with stem cell research.

Potential Therapies using Stem Cells

Adult Stem Cell Therapies

Bone marrow and peripheral blood stem cell transplants have been utilized for over 40 years as therapy for blood disorders such as leukemia and lymphoma, amongst many others. Scientists have also shown that stem cells reside in most tissues of the body and research continues to learn how to identify, extract, and proliferate these cells for further use in therapy. Scientists hope to yield therapies for diseases such as type I diabetes and repair of heart muscle following heart attack.

Scientists have also shown that there is potential in reprogramming ASCs to cause them to transdifferentiate (turn back into a different cell type than the resident tissue it was replenishing).

Embryonic Stem Cell (ESC) Therapies

There is potential with ESCs to treat certain diseases in the future. Scientists continue to learn how ESCs differentiate and once this method is better understood, the hope is to apply the knowledge to get ESCs to differentiate into the cell of choice that is needed for patient therapy. Diseases that are being targeted with ESC therapy include diabetes, spinal cord injury, muscular dystrophy, heart disease, and vision/hearing loss.

Induced Pluripotent Stem Cell Therapies

Therapies using iPSCs are exciting because somatic cells of the recipient can be reprogrammed to en “ESC like” state. Then mechanisms to differentiate these cells may be applied to generate the cells in need. This is appealing to clinicians because this avoids the issue of histocompatibility and lifelong immunosuppression, which is needed if transplants use donor stem cells.

iPS cells mimic most ESC properties in that they are pluripotent cells, but do not currently carry the ethical baggage of ESC research and use because iPS cells have not been able to be manipulated to grow the outer layer of an embryonic cell required for the development of the cell into a human being.

Pros and Cons of using Various Stem Cell

- Abundant somatic cells of donor can be used.
- Issues of histocompatibility with donor/recipient transplants can be avoided.
- Very useful for drug development and developmental studies.

	Adult Stem Cells	Embryonic Stem Cells	Induced Pluripotent Stem Cells
Pros	• Trans differentiate and and reprogramming of these cells is possible but is not well studied. • Thought to be less likely to be rejected if used in transplants. • Success has already been demonstrated in various clinical applications.	• Can maintain and grow for 1 year or more in culture. • Established protocols for maintenance in culture. • ESCs are pluripotent cells that can generate most cell types. • By studying ESCs, more can be learned about the process of development.	• Abundant somatic cells of donor can be used. • Issues of histocompatibility with donor/recipient transplants can be avoided. • Very useful for drug development and developmental studies. • Information learned from the "reprogramming" process may be transferable for in vivo therapies to reprogram damaged or diseased cells/ tissues.
Cons	• Limitations on ASC ability to differentiate are still uncertain; currently thought to be multi or unipotent. • Cannot be grown for long periods of time in culture. • Usually a very small number in each tissue making them difficult to find and purify. • Currently there is no technology available to generate large quantities of stem cells in culture.	• Process to generate ESC lines is inefficient. • Unsure whether they would be rejected if used in transplants. • Therapies using ESC avenues are largely new and much more research and testing is needed. • If used directly from the ESC undifferentiated culture prep for tissue transplants, they can cause tumors (teratomas) or cancer development.	• Methods for ensured reproducibility and maintenance, as differentiated tissues are not certain. • Viruses are currently used to introduce embryonic genes and has been shown to cause cancers in mouse studies.
Ethical Concerns	• No major ethical concerns have been raised.	• To acquire the inner cell mass the embryo is destroyed. • Risk to female donors being consented.	• iPS cells have the potential to become embryos if exposed to the right conditions.

Stem Cell Biology

Stem cell biology is a part of 'regenerative biology'. Regenerative biology embraces regeneration in plants and animals. A striking example of regeneration in animals may be seen in starfish and

planaria. Where the idea or concept of regenerative biology is applied to humans it is called regenerative medicine. When plants produce daughter plants by vegetative propagation e.g. strawberry runners, or when cuttings are taken and grown, the term 'cloning' is used. 'Cloning', when used in relation to stem cell work in mammals is used in a specialist and defined way. There are two main driving forces behind stem cell biology as applied to humans. The first is the desire to be able to replace damaged or diseased tissue/organs without recourse to organ donation. (There are not enough suitable organs from donations to satisfy the demand, and all transplants carry some risk of rejection. Organ recipients may also be affected by taking the required immunosuppressive drugs). The second driving force is the need to be able to culture specific human stem cells to (1) study normal and disease development at the level of the cell, and (2) use stem cells for medicinal drug discovery and testing.

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Stem Cell: Elements and Concepts

2

- Stem Cell Line
- Stem Cell Genomics
- Stem Cell Theory of Aging
- Stem Cell Marker

Some of the fundamental concepts that fall under the domain of stem cell are stem cell line, stem cell genomes, stem cell marker and stem cell theory of aging. This chapter has been carefully written to provide an easy understanding of these elements and concepts of stem cells.

Stem Cell Line

Stem cell lines are the basis of a science that has been around since the early 1980s, when scientists first managed to derive stem cells from mouse embryos. Since then, the study of stem cells has evolved to a great extent, as breakthroughs were made to derive and use human stem cells from embryos and even from adults.

A stem cell line is essentially a group of stem cells that is cultured for various purposes using in-vitro methods. Stem cell lines are commonly used in genetic research and regenerative medicine, and they have already led to countless breakthroughs that would not have been possible through the use of any other type of cells. Because of stem cells' unique ability to renew themselves as they divide, they can essentially multiply to an indefinite extent, which is part of why stem cell lines are so valuable.

Stem Cell Lines and their Main Purpose

Stem cells don't multiply like immortalized cell lines that lose their ability to halt their regenerative properties due to mutation. Instead, stem cells, whether they develop in-vitro through stem cell lines, or whether they are used by the body internally to repair or build important elements of itself, propagate as a result of their inherent, natural properties. Stem cell lines retain the stem cells' original genetic qualities, while still dividing virtually indefinitely. Also, another important property, and one that could lead to major medical breakthroughs, is the fact that stem cells are multipotent, being capable of taking the role of any other specialized cells.

Types of Stem Cell Lines

There are three types of stem cell lines: Those cultured from embryos, those derived through adult stem cells and those that are generated as a result of induced stem cell research. Embryonic cells are the easiest to culture, since stem cells are present in abundance in the embryos of any species. In humans, in the case of a 5-day old embryo, these inner cells give rise to the entire cellular diversity of the body. Adult stem cells still exist, despite the fact that the adult body is fully developed. They are used by the body primarily for repairing cellular damage in certain areas, and can also be cultured in-vitro. In the case of induced stem cells, the process is more complex. These are stem cell lines derived from adult somatic, reproductive or pluripotent cell types through the use of artificial genetic reprogramming.

Methods of Culture used for Stem Cell Lines

There are many different types of culture methods used to grow stem cell lines in a lab, regardless of their origin. One of the first methods involves embryonic stem cells placed on a laboratory culture dish and allowed to multiply in a special broth with the help of mouse skin cells that were treated to prevent division. More advanced methods have done away with the mouse feeder cells, eliminating the risk of viruses infecting the cell line. Today, stem cell lines are typically cultured using special incubators under stable temperatures of 37 degrees Celsius and a steady CO_2 level of 5%.

Stem Cell Genomics

Stem cell genomics analyzes the genomes of stem cells. Currently, this field is rapidly expanding due to the dramatic decrease in the cost of sequencing genomes. The study of stem cell genomics has wide reaching implications in the study of stem cell biology and possible therapeutic usages of stem cells. Application of research in this field could lead to drug discovery and information on diseases by the molecular characterization of the pluripotent stem cell through DNA and transcriptome sequencing and looking at the epigenetic changes of stem cells and subsequent products. One step in that process is single cell phenotypic analysis, and the connection between the phenotype and genotype of specific stem cells. While current genomic screens are done with entire populations of cells, focusing in on a single stem cell will help determine specific signaling activity associated with varying degrees of stem cell differentiation and limit background due to heterogeneous populations. Single cell analysis of induced pluripotent stem cells (iPSCs), or stem cells able to differentiate into many different cell types, is a suggested method for treating such diseases like Alzheimer's disease (AD). This includes for understanding the differences between sporadic AD and familial AD. By first taking a skin sample from the patient and are transformed by transducing cells using retroviruses to encode such stem cell genes as Oct4, Sox2, KLF4 and cMYC. This allows for skin cells to be reprogrammed into patient-specific stem cell lines. Taking genomic sequences of these individual cells would allow for patient-specific treatments and furthering understanding of AD disease models. This technique would be used for similar diseases, like amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA). These stem cells developed from a singular patient would also be able to be used to produce cells affected in the diseases. As mentioned, it will also lead to patient specific phenotypes of each disease. Further chemical analyses to develop safer drugs can be done through

sequence information and cell-culture tests on iPSCs. After development on a specific drug, it can be transferred to other patient diseased cells while also being safety tested.

Included in the study of stem cell genomics, is epigenomics, genomic-scale studies on chromatin regulatory variation. These studies also hope to expand research into regenerative medicine models and stem cell differentiation. Cell-type specific gene expression patterns during development occur as the result of interactions the chromatin level. Stem cell epigenomics focuses in on the epigenetic plasticity of human embryonic stem cells (hESCs). This includes investigation into bivalent domains as promoters or chromatin regions that are modified by transcriptional initiation and related to gene silencing. They are also looking at the differences between active versus poised enhancers or enhancers that specifically control signaling-dependent gene regulation. Active enhancers are marked by acetylation of histone H3-H3K27ac and while poised are instead methylated at H3K27me3. Stem cell epigenomic studies are also looking into DNA methylation patterns, specifically characteristics of hydroxy methylation versus overall methylation and the difference between methylation of CpG-island rich and CpG poor promoters. It has been found in mouse embryonic stem cells (mESC) that implanted mESC took up similar characteristics of histone methylation of the embryos where they transplanted into, indicating that methylation may be indicative of environment. This will guide studies into the differences between induced pluripotent and embryonic stem cells. These studies hope to produce information on iPSC differentiation capacity by first needing to enhance chromatin signature reading. It also hopes to produce to look into regulatory factors that control human embryonic development. Using drug therapy techniques as mentioned earlier, epigenomics would also allow for more information on drug activity.

Stem Cell Theory of Aging

The stem cell theory of aging postulates that the aging process is the result of the inability of various types of stem cells to continue to replenish the tissues of an organism with functional differentiated cells capable of maintaining that tissue's (or organ's) original function. Damage and error accumulation in genetic material is always a problem for systems regardless of the age. The number of stem cells in young people is very much higher than older people and thus creates a better and more efficient replacement mechanism in the young contrary to the old. In other words, aging is not a matter of the increase in damage, but a matter of failure to replace it due to a decreased number of stem cells. Stem cells decrease in number and tend to lose the ability to differentiate into progenies or lymphoid lineages and myeloid lineages.

Maintaining the dynamic balance of stem cell pools requires several conditions. Balancing proliferation and quiescence along with homing and self-renewal of hematopoietic stem cells are favoring elements of stem cell pool maintenance while differentiation, mobilization and senescence are detrimental elements. These detrimental effects will eventually cause apoptosis.

There are also several challenges when it comes to therapeutic use of stem cells and their ability to replenish organs and tissues. First, different cells may have different lifespans even though they originate from the same stem cells, meaning that aging can occur differently in cells that have longer lifespans as opposed to the ones with shorter lifespans. Also, continual effort to replace the somatic cells may cause exhaustion of stem cells.

Some of the proponents of this theory have been Norman E. Sharpless, Ronald A. DePinho, Huber Warner, Alessandro Testori and others. Warner came to this conclusion after analyzing human case of Hutchinson's Gilford syndrome and mouse models of accelerated aging.

Stem cells will turn into certain cells as the body needs them. Stem cells divide more than non stem cells so the tendency of accumulating damage is greater. Although they have protective mechanisms, they still age and lose function. Matthew R. Wallenfang, Renuka Nayak and Stephen DiNardo showed this in their study. According to their findings, it is possible to track male GSCs labeled with lacZ gene in *Drosophila* model by inducing recombination with heat shock and observe the decrease in GSC number with aging. In order to mark GSCs with lacZ gene, flip recombinase (Flp)-mediated recombination is used to combine a ubiquitously active tubulin promoter followed by an FRT (flip recombinase target) site with a promotorless lacZ ORF (open reading frame) preceded by an FRT site. Heat shock is used to induce Flp recombinase marker gene expression is activated in dividing cells due to recombination. Consequently, all clone of cells derived from GSC are marked with a functional lacZ gene. By tracking the marked cells, they were able to show that GSCs do age.

Another study in a mouse model shows that stem cells do age and their aging can lead to heart failure. Findings of the study indicate that diabetes leads to premature myocyte senescence and death and together they result in the development of cardiomyopathy due to decreased muscle mass.

Behrens et al. have reviewed evidence that age-dependent accumulation of DNA damage in both stem cells and cells that comprise the stem cell microenvironment is responsible, at least in part, for stem cell dysfunction with aging.

Hematopoietic Stem Cell Aging

Hematopoietic stem cells (HSCs) regenerate the blood system throughout life and maintain homeostasis. DNA strand breaks accumulate in long term HSCs during aging. This accumulation is associated with a broad attenuation of DNA repair and response pathways that depends on HSC quiescence. DNA ligase 4 (Lig4) has a highly specific role in the repair of double-strand breaks by non-homologous end joining (NHEJ). Lig4 deficiency in the mouse causes a progressive loss of HSCs during aging. These findings suggest that NHEJ is a key determinant of the ability of HSCs to maintain themselves over time.

Hair Follicle Stem Cell Aging

A key aspect of hair loss with age is the aging of the hair follicle. Ordinarily, hair follicle renewal is maintained by the stem cells associated with each follicle. Aging of the hair follicle appears to be primed by a sustained cellular response to the DNA damage that accumulates in renewing stem cells during aging. This damage response involves the proteolysis of type XVII collagen by neutrophil elastase in response to the DNA damage in the hair follicle stem cells. Proteolysis of collagen leads to elimination of the damaged cells and then to terminal hair follicle miniaturization.

Evidence Against the Theory

Diseases such as Alzheimer's disease, end-stage renal failure and heart disease are caused by different mechanisms that are not related to stem cells. Also, some diseases related to hematopoietic

system, such as aplastic anemia and complete bone marrow failure, are not especially age-dependent. Aplastic Anemia is often an adverse effect of certain medications but as such it cannot really be considered as evidence against the stem cell theory of aging. The cellularity of the bone marrow does decrease with age and can be usually calculated by the formula 100-age, and this seems consistent with a stem cell theory of aging. A dog study published by Zaucha J.M, Yu C. and Mathioudakis G., *et al.* also shows evidence against the stem cell theory. Experimental comparison of the engraftment properties of young and old marrow in a mammal model, the dog, failed to show any decrement in stem cell function with age.

Other Theories of Aging

The aging process can be explained with different theories. These are evolutionary theories, molecular theories, system theories and cellular theories. The evolutionary theory of ageing was first proposed in the late 1940s and can be explained briefly by the accumulation of mutations (evolution of ageing), disposable soma and antagonistic pleiotropy hypothesis. The molecular theory of ageing includes phenomena such as gene regulation (gene expression), codon restriction, error catastrophe, somatic mutation (accumulation of genetic material damage) and dysdifferentiation (DNA damage theory of aging). The system theories include the immunologic approach to ageing, rate-of-living and the alterations in neuroendocrinal control mechanisms. Cellular theory of ageing can be categorized as telomere theory, free radical theory (free-radical theory of aging) and apoptosis. The stem cell theory of aging is also a sub-category of cellular theories.

Stem Cell Marker

Stem cell markers are molecules used for the identification of unspecialized, undifferentiated cells, and in the case of malignancy, presumptive cancer stem cells.

Characteristics

There is increasing evidence that subpopulations of neoplastic cells demonstrate heterogeneity with respect to proliferation, differentiation, and expression of cellular proteins characteristic of stem cells. Subpopulations of cells that express stem cell markers that can also be shown to contribute to tumor progression and resistance to chemotherapy are termed cancer stem cells. Cancer stem cells are found in very small subpopulations within tumors, in the range of 0.1–1% of the total cell number. Microscopically, cancer stem cells outwardly appear the same as any other tumor cell. Therefore, in order to identify these rare subpopulations, a number of stem cell markers have been identified and developed as a means of distinguishing stem-like cells from other cells within a cancer population.

While stem cells are best defined functionally, a number of molecular markers have been used to characterize various stem cell populations.

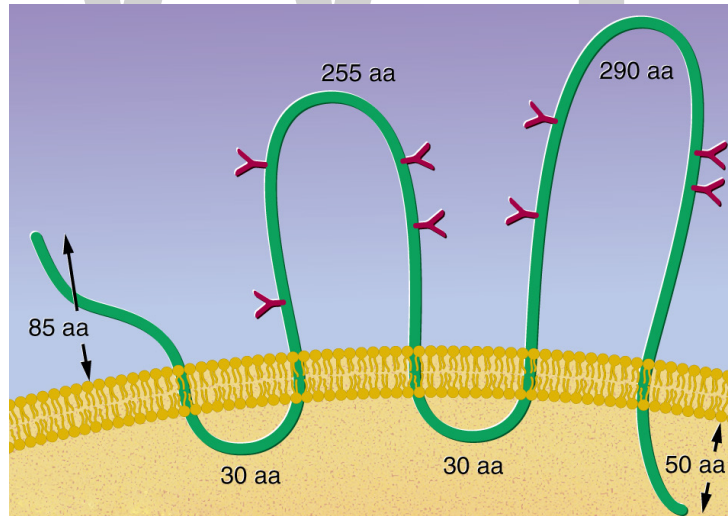
Although functions have yet to be ascertained for many of these early markers, their unique expression pattern and timing provide a useful tool for scientists to initially identify as well as isolate stem cells.

Embryonic Stem Cell Markers

Oct-4 (also termed Oct-3 or Oct-3/4), one of the POU transcription factors, was originally identified as a DNA-binding protein that activates gene transcription via a cis-element containing octamer motif.It is expressed in totipotent embryonic stem and germ cells. A critical level of Oct-4 expression is required to sustain stem cell self-renewal and pluripotency.Differentiation of embryonic stem (ES) cells results in down- regulation of Oct-4, an event essential for a proper and divergent developmental program. Oct-4 is not only a master regulator of pluripotency that controls lineage commitment, but is also the first and most recognized marker used for the identification of totipotent ES cells.

Stage Specific Embryonic Antigens (SSEAs) were originally identified by three monoclonal antibodies (Abs) recognizing defined carbohydrate epitopes associated with lacto- and globo-series glycolipids, SSEA-1, -3 and - 4. SSEA-1 is expressed on the surface of preimplantation-stage murine embryos (i.e. at the eight cell stage) and has been found on the surface of teratocarcinoma stem cells, but not on their differentiated derivatives. The oviduct epithelium, endometrium and epididymis, as well as some areas of the brain and kidney tubules in adult mice have also been shown to be reactive with SSEA-1 Abs. SSEA-3 and -4 are synthesized during oogenesis and are present in the membranes of oocytes, zygotes and early cleavage-stage embryos. Biological roles of these carbohydrate-associated molecules have been suggested in controlling cell surface interactions during development. Undifferentiated primate ES cells, human EC and ES cells express SSEA-3 and SSEA-4, but not SSEA-1. Undifferentiated mouse ES cells express SSEA-1, but not SSEA-3 or SSEA-4.

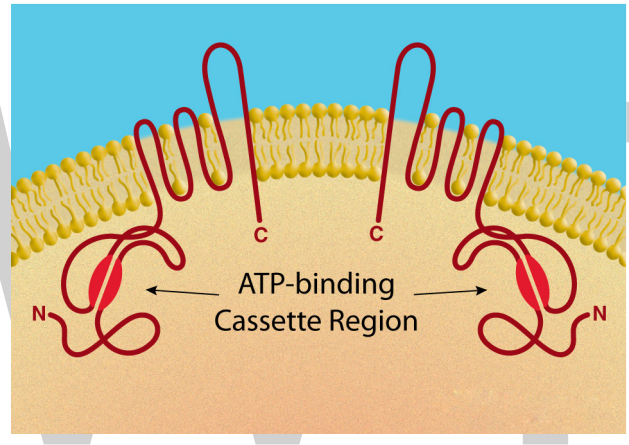
Hematopoietic Stem Cell Markers



A structure model of CD133 proposed by Miraglia S. et al. This protein has an extracellular N-terminus, 5 hydrophobic transmembrane domains, 2 small cytoplasmic loops, 2 large extracellular loops and a cytoplasmic C-termus.

CD34: The cell surface sialomucin CD34 has been a focus of interest ever since it was found expressed on a small fraction of human bone marrow cells. The $CD34^{+}$-enriched cell population from marrow or mobilized peripheral blood appears responsible for most of the hematopoietic activity.

CD34 has therefore been considered to be the most critical marker for hematopoietic stem cells (HSCs). CD34 expression on primitive cells is down-regulated as they differentiate into mature cells. It is also found on clonogenic progenitors, however, and some lineage-committed cells. Although its precise function is still unknown, the pattern of expression of CD34 suggests that it plays a significant role in early hematopoiesis. The theory of CD34 being the most primitive HSC marker, however, has recently been challenged. Osawa et al. first demonstrated that murine HSCs could be CD34 negative. In addition, a low level of engraftment and hematopoietic capacity has been demonstrated in human $CD34^-$ cells. Transplantation studies also showed repopulating activity in a $CD34^-$ cell population in fetal sheep. Additionally, studies have shown that both murine and human $CD34^+$ cells may be derived from $CD34^-$ cells. Collectively, these reports suggest the possibility that HSCs may be $CD34^+$ or $CD34^-$ and that selection of cells expressing CD34 might result in exclusion of more primitive stem cells. Nevertheless, almost all clinical and experimental protocols including ex vivo culture, gene therapy, and HSC transplantation are currently designed for cell populations enriched for $CD34^+$ cells.



The family of ABC transporters is characterized by the presence of an ATP-binding cassette region, which hydrolyzes ATP to support energy- dependent substrate exportation from the intracellular cytoplasm to the extracellular space. Full-length transporters contain two mirror image halves that are separated by a flexible linker region. Half-transporters, e.g. ABCG2, function as homo- or heterodimers and may be localized to the plasma membrane.

CD133, a 120 kDa, glycosylated protein containing five transmembrane domains, was identified initially by the AC133 monoclonal Ab, which recognizes a $CD34^+$ subset of human HSCs. A CD133 isoform, AC133-2, has been recently cloned and identified as the original surface antigen recognized by the AC133 Ab. CD133 may provide an alternative to CD34 for HSC selection and ex vivo expansion. A $CD133^+$ enriched subset can be expanded in a similar manner as a $CD34^+$ enriched subset, retaining its multilineage capacity. Recent studies have offered evidence that CD133 expression is not limited to primitive blood cells, but defines unique cell populations in non-hematopoietic tissues as well. $CD133^+$ progenitor cells from peripheral blood can be induced to differentiate into endothelial cells in vitro. In addition, human neural stem cells can be directly isolated by using an anti-CD133 Ab.

ABCG2 (ATP-binding cassette superfamily G member 2) is a determinant of the Hoechst-negative phenotype of side population (SP) cells and found in a wide variety of stem cells, including HSC.

ABCG2 is a member of the family of ABC transporters and was first identified in a breast cancer cell line. It belongs to the half-transporter group and is unique as it is localized to the plasma membrane. The expression of ABCG2 appears greatest on $CD34^-$ cells and is down-regulated with the acquisition of CD34 on the cell surface. Down-regulation in ABCG2 expression is also observed in various committed hematopoietic progenitors. ABCG2 may therefore serve as a more promising marker than CD34 for primitive HSC isolation and characterization. The expression pattern of ABCG2, however, is not limited to HSC. ABCG2 expression exclusively characterizes the Hoechst SP phenotype in cells from diverse sources, including monkey bone marrow, mouse skeletal muscle and ES cells. The potential plasticity of SP cells has been demonstrated by studies showing that cardiomyocytes and muscle can be regenerated from transplanted bone marrow-derived SP cells. Exclusive expression of ABCG2 on SP cells suggests that ABCG2 may be a potential marker for positive selection of pluripotent stem cells from various adult sources. ABCG2 has been implicated in playing a functional role in developmental stem cell biology.

Sca-1 (stem cell antigen 1, Ly-6A/E), an 18 kDa phosphatidylinositol-anchored protein, is a member of the Ly-6 antigen family. Sca-1 is the most recognized HSC marker in mice with both Ly-6 haplotypes as it is expressed on multipotent HSCs. An anti-Sca-1 Ab is frequently used in combination with negative selection for expression of a number of cell surface markers characteristic of differentiated cells of hematolymphoid lineages (Lin^-) to identify and isolate murine HSCs. $Sca\text{-}1^+$ HSCs can be found in the adult bone marrow, fetal liver and mobilized peripheral blood and spleen within the adult animal. Sca-1 has also been discovered in several non-hematopoietic tissues, however, and can be used to enrich progenitor cell populations other than HSCs. Sca-1 may be involved in regulating both B and T cell activation.

Mesenchymal/Stromal Stem Cell Markers

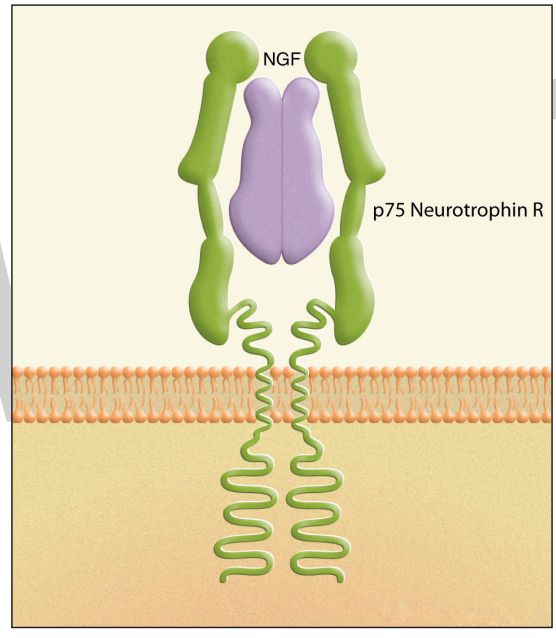
STRO-1: The murine IgM monoclonal Ab STRO-1, produced from an immunization with a population of human $CD34^+$ bone marrow cells, can identify a cell surface antigen expressed by stromal elements in human bone marrow. From bone marrow cells, the frequency of fibroblast colony-forming cells (CFU-F) is enriched approximately 100-fold in the $STRO\text{-}1^+$/Glycophorin A^- population than in the $STRO\text{-}1^+$/Glycophorin A^+ population. A $STRO\text{-}1^+$ enriched subset of marrow cells is capable of differentiating into multiple mesenchymal lineages including hematopoiesis-supportive stromal cells with a vascular smooth muscle-like phenotype, adipocytes, osteoblasts and chondrocytes. STRO-1 is a valuable Ab for the identification, isolation and functional characterization of human bone marrow stromal cell precursors, which are quite distinct from those of primitive HSCs.

Neural Stem Cell Markers

Nestin: Nestin is a class VI intermediate filament protein. Although it is expressed predominantly in stem cells of the central nervous system (CNS), its expression is absent from nearly all mature CNS cells. Nestin has been the most extensively used marker to identify CNS stem cells within various areas of the developing nervous system and in cultured cells in vitro. The role of nestin in CNS stem cell biology, however, remains undefined. Although nestin does not form intermediate filaments by itself in vitro it does co-assemble with vimentin or alpha-internexin to form and heterodimer, coiled-coil complexes that may then form intermediate filaments. Its transient expression has been suggested to be a major step in the neural differentiation pathway. Nestin

expression has also been discovered in non-neural stem cell populations, such as pancreatic islet progenitors as well as hematopoietic progenitors.

PSA-NCAM (Polysialic acid-neural cell adhesion molecule): The regulated expression of neural cell adhesion molecule (NCAM) isoforms in the brain is critical for many neural developmental processes. The embryonic form of NCAM, PSA-NCAM, is highly polysialylated and is mainly expressed in the developing nervous system. PSA-NCAM may be related to synaptic rearrangement and plasticity. In the adult, PSA-NCAM expression is restricted to regions that retain plasticity. A neuronal-restricted precursor identified by its high expression of PSA-NCAM can undergo self-renewal and differentiate into multiple neuronal phenotypes. PSA-NCAM$^+$ neonatal brain precursors are restricted to a glial fate and thyroid hormone can modulate them into an oligodendrocyte fate. Polysialic acid modification significantly decreases NCAM adhesiveness and therefore, it was originally suggested PSA-NCAM works as a purely anti-adhesive factor that modulates cell-cell interactions in promoting brain plasticity. Increasing evidence indicates that PSA-NCAM may interact with secreted signaling molecules to perform an instructive role in development.



The structure of NGF with a model of the p75 Neurotrophin Receptor. The extracellular domain of the receptor is taken from the tumor necrosis factor receptor structure and the intracellular portion contains a death domain.

p75 Neurotrophin R (NTR): p75 NTR, also named low affinity nerve growth factor (NGF) receptor, is a type I transmembrane protein that belongs to the tumor necrosis factor receptor superfamily. It binds to NGF, BDNF, NT-3 and NT-4 equally (with low affinity). p75NTR, when activated in the presence of Trk, enhances responses to neurotrophin. TrkC receptors working together with p75 NTR have been suggested to serve critical functions during the development of the nervous system. Neural crest stem cells (NCSCs) have been isolated based on their surface expression of p75NTR. Freshly isolated p75NTR$^+$ NCSCs from peripheral nerve tissues can self-renew and generate neurons and glia both *in vitro* and in vivo. In addition, neuroepithelial-derived p75NTR$^+$ cells are also able to differentiate into neurons, smooth muscle and Schwann cells in culture. Recently, p75 NTR has been used as a marker to identify mesenchymal precursors as well as hepatic stellate cells.

CD133

CD133 antigen, also known as prominin-1, is a glycoprotein that in humans is encoded by the *PROM1* gene. It is a member of pentaspan transmembrane glycoproteins, which specifically localize to cellular protrusions. When embedded in the cell membrane, the membrane topology of prominin-1 is such that the N-terminus extends into the extracellular space and the C-terminus resides in the intracellular compartment. The protein consists of five transmembrane segments, with the first and second segments and the third and fourth segments connected by intracellular loops while the second and third as well as fourth and fifth transmembrane segments are connected by extracellular loops. While the precise function of CD133 remains unknown, it has been proposed that it acts as an organizer of cell membrane topology.

CD133 is expressed in hematopoietic stem cells, endothelial progenitor cells, glioblastoma, neuronal and glial stem cells, various pediatric brain tumors, as well as adult kidney, mammary glands, trachea, salivary glands, uterus, placenta, digestive tract, testes, and some other cell types.

Today CD133 is the most commonly used marker for isolation of cancer stem cell (CSC) population from different tumors, mainly from various gliomas and carcinomas. Initial studies that showed ability of CD133-positive population to efficiently propagate tumor when injected into immune-compromised mice firstly were performed on brain tumors. However, subsequent studies have indicated the difficulty in isolating pure CSC populations. $CD133^+$ melanoma cells are considered a subpopulation of CSC and play a critical role in recurrence. Moreover, $CD133^+$ melanoma cells are immunogenic and can be used as an antimelanoma vaccination. In mice the vaccination with $CD133^+$ melanoma cells mediated strong anti-tumor activity that resulted in the eradication of parental melanoma cells. In addition, it has also been shown that $CD133^+$ melanoma cells preferentially express the RNA helicase DDX3X. As DDX3X also is an immunogenic protein, the same anti-melanoma vaccination strategy can be employed to give therapeutic antitumor immunity in mice.

Aldehyde Dehydrogenase

Aldehyde dehydrogenases (ALDH) belong to the oxidoreductase family, which catalyze the conversion of aldehydes to their corresponding acids. As a group of $NAD(P)^+$–dependent enzymes, aldehyde dehydrogenases (ALDHs) are involved in oxidation of a large number of aldehydes into their weak carboxylic acids. ALDH is found in every subcellular region such as cytosol, endoplasmic reticulum, mitochondria, and the nucleus, with some even found in more than one location.

ALDH is also found in stem cells. During early life and growth, stem cells (SCs) have a spectacular potential to develop into several cell types in the body. In many tissues, SCs behave as a kind of internal repair system, dividing essentially without limit to replenish other cells. Stem cells are distinguished from other cell types by two important characteristics: (1) Their unspecialized properties and renewal potencies; and (2) differentiation into other cell types under certain physiologic or experimental conditions. These cells are identified by their expression of a particular panel of surface molecules, with the presence of CD73, CD90, CD105, and the absence of CD14, CD34, CD45, and HLA-DR. They show no proliferative response from alloreactive lymphocytes because of the negligible levels of extracellular MHC class I and II determinants and they also have important immunomodulatory functions in all the cells involved in both the innate and adaptive immune

responses. On the other hand, cancer stem cell theory is supported by biological reason for aging. The theory postulates that cancer SCs, a small subset of tumor cells also have stem cell-like properties (epithelial-to-mesenchymal progression, differentiation and self-renewing capacity). ALDH expression has demonstrated itself to be a possibly relevant prognostic marker. For this reason, the subpopulation of cancer SCs (CSCs) can present a therapeutic target for poor-prognostic, treatment-resistant and recurrent breast cancer. Through its role in oxidizing retinol to RA, which is a modulator of cell proliferation, ALDH1 might have a role in early differentiation of SCs and stem cell proliferation.

There are several isoforms of ALDH (ALDH1A1, ALDH1A2, ALDH1A3 and ALDH8A1) that play a role in RA formation by oxidation of all-trans-retinal and 9-cis-retinal in RA cell signaling, which has been related to the "stemness" characteristics of SCs. ALDH1 is better as a marker of breast cancer SCs than $CD44^+/CD24^-$. While cellular markers including CD133 have been used to identify tumor SCs, especially for glioblastomas (GBMs) ALDH1 was desrcribed as a marker for the identification of non-neoplastic SCs and tumor stem cells (TSCs).

After $CD133^-$ GBMs are characterized to behave as brain TSCs. ALDH1 has also been described as a stem cell marker in various solid neoplasms including lung cancer, breast carcinoma colorectal cancer, and GBM. ALDH1B1 and ALDH1A1 are differentially expressed in normal human tissues. ALDH1B1 is expressed at higher levels than ALDH1A1 in human epithelial cancers. ALDH1B1 was abundantly expressed in adenocarcinomas originating from the tissue and particularly in colonic adenocarcinoma.

$ALDH^{br}$ cells can be detected with ALDEFLUOR reagent by using flow cytometry or fluorescent microscopy. Aldefluor assay is based on the conversion of fluorescent non-toxic substrate for ALDH substrate to the fluorescent reaction product. Non-toxic substrate for ALDH can freely diffuse into intact, viable cells. The BODIPY aminoacetaldehyde is converted to the fluorescent product BODIPY aminoacetate by ALDH activity. These cell populations, which are known as ALDH bright ($ALDH^{br}$) cells are isolated from adult tissues by flow sorting. $ALDH^{br}$ cells were also found in various cancer tissues including breast, liver, colon, pancreas, prostate, lung, ovarian and acute myelogenous leukemia and are related to cancer chemo resistance.

$ALDH^{br}$ population may play an important role in regenerative medicine. The regenerative potential of $ALDH^{br}$ cells obtained from different tissues was investigated in various disease models such as ischemic tissue damage, hind limb model, brain damage (spinal motor atrophy, etc.) and pancreatitis.

Aldehydes

Aldehydes are formed in various physiological processes such as catabolism of transmitters like GABA, serotonin, adrenaline, noradrenaline and dopamine, as well as catabolism of amino acids. In addition, there are more than 200 different aldehydes that are produced through lipid, and aldehydic intermediates through carbohydrate metabolism. Along with these endogenous aldehydes, there are also exogenously present aldehydes in a variety of industrial processes, including the production of polyester plastics (formaldehyde, acetaldehyde, acrolein, etc.), polyurethane, smog, cigarette smoke or motor vehicle exhaust. With their malodorous properties, some dietary and aromatic aldehydes are accepted as additives in food and cosmetics (e.g., citral, cinnamaldehyde,

benzaldehyde, and retinal), though many others are cytotoxic. Aldehydes could interact with thiol compounds of some proteins, leading to structural and functional alterations of these molecules. In order to protect the human body from the deleterious effects of aldehydes in general, and myocardium and the brain in particular, a fast aldehyde detoxification mechanism is essential. Aldehydes are significantly reactive and possess high diffusion capacities in cells, thus they can easily form complexes with DNA, proteins and lipids, of which they can alter the function and cause their inactivation. As a result of DNA damage induced by these complexes, many aldehydes are classified as mutagenic or carcinogenic, including acetaldehyde, which is derived from ethanol consumption. Over–consumption of ethanol has been related to liver disease and several gastrointestinal and upper aerodigestive cancers. Numerous other cytotoxic and reactive aldehydes have been shown to be linked with other types of diseases.

Aldehyde dehydrogenases [systematic name: aldehyde: $NAD(P)^+$ oxidoreductase] catalyze aldehyde conversion into their matching acids by $NAD(P)^+$-dependent nearly irreversible reaction. In 1949, mammalian ALDH was first discovered in ox liver. After that, many types of ALDH were distinguished according to their physico-chemical characteristics, enzymological properties, subcellular localization, and tissue distribution. They are involved in several cell functions such as proliferation, differentiation, survival as well as cellular response to oxidative stress. ALDHs are commonly delivered from bacteria and humans. Based on their physico-chemical characteristics, subcellular localization, tissue distribution and enzymological properties, a number of types of ALDH have been distinguished since the 1960s, around the time when mammalian ALDH activity was observed in ox for the first time. In 1985, 2 ALDH genes were cloned and characterized. Genes or cDNAs for more than 50 animals, fungi and bacterial ALDHs in addition to protein sequences have been discovered.

The human genome contains 19 ALDH functional genes and 3 pseudogenes. At least 5 ALDH isozymes function in the mitochondria,and all the ALDH genes are encoded in the nucleus.

All of the ALDH gene superfamily plays an important role in the enzymic detoxification of endogenous and exogenous aldehydes. They are also involved in the formation of molecules that are important in cellular processes like RA, betaine and gamma aminobutyric acid formation. Furthermore, ALDHs also have several non-enzymic functions such as binding to some hormones and other small molecules and decreasing the effects of ultraviolet irradiation in the cornea. The most important role of ALDHs is detoxification of aldehydes, which caused cytotoxicity, mutagenicity, genotoxicity, and carcinogenesis in healthy cells. Mutations in ALDH genes cause severe diseases including Sjögren-Larsson syndrome, pyridoxine-dependent seizures, and type II hyperprolinemia, and also plays a role in cancer and Alzheimer's disease.

Functions of some of these ALDHs in endobiotic and xenobiotic metabolisms have been highly reviewed before and the distinctive metabolic pathways' influences have been depicted. Because of their chemical reactivity, many distinct aldehydes are pervasive in nature and are toxic at low levels. Hence, levels of metabolic-intermediate aldehydes should be cautiously regulated. The presence of several distinct ALDH families in most studied organisms seem to have wide fundamental tissue distribution. A wide range of allelic variants within the ALDH gene family have been identified, leading to heterogeneity in pharmacogenetic characteristics between individuals, resulting distinctive phenotypes including intolerance to alcohol and increased risk of ethanol-induced cancers in most cases (ALDH2 and ALDH1A1), Sjogren-Larson

Syndrome (ALDH3A1), type II hyperprolinemia (ALDH4A1), 4-hydroxybutyric aciduria, mental retardation and seizures (ALDH5A1), developmental delay (ALDH6A1), hyperammonemia (ALDH18A1), Pyridoxine-dependent epilepsy (ALDH7A1), and late-onset Alzheimer's disease (ALDH2).

ALDH dysfunction could also be caused by drugs and environmental substances, substrate inhibition, as well as oxidative and metabolic stress. ALDH activity in drug resistance to oxazaphosphorines is one of the most vigorously studied pathways. The role of ALDH1A1 in drug resistance has been studied first in hematopoietic progenitors and more recently in lung cancer.

Cancer Stem Cells

Cancer is a class of diseases characterized by unregulated cell growth. Cancer initiation depends on genetic mutations in series that affects cellular programming. Many cancer researches have focused on the identification and characterization of these genetic and molecular properties of cancer cells. Tumors are also heterogeneous cellular entities whose growth is dependent upon dynamic interactions among the cancer cells themselves, and between cells and the constantly changing microenvironment. That kind of interaction is depent on signaling through cell adhesion molecules and different cell responses to growth factors and other external signals. All of these interactive processes act together to control cell phenotypic behaviors such as proliferation, apoptosis, and migration. There are over 100 different types of cancer, and each is classified by the type of cell that is initially affected.

According to recent statistics, cancer accounts for about 23% of the total deaths in the USA and is the second most common cause of death after heart disease.

Cancer is caused by many internal and external factors. Inherited mutations, hormones, and immune conditions are internal factors while tobacco, diet, radiation, and infectious organisms are environmental/acquired factors. In recent years, a particular sub-population of tumor cells are said to have a critical role in cancer; these cells are commonly called CSCs or tumor initiating cells (TICs). In most cancer types, CScs have been identified. CSCs are characterised by their two important properties: (1) Enhanced tumorigenicity; and (2) the capacity for self-renewal/differentiation. Thus, isolating CSCs is important in analyzing their characteristics in vitro. The isolated CSC population will not only give rise to de novo tumors with high efficiency, but will also recapitulate the tumor with both CSC and non-CSC populations.

One potential human CSC marker is the membrane antigen CD133 (Prominin) identified in subpopulations of cells in brain, colon and lung tumors. CD133+ tumor cells are also a marker identifying lung CSCs.

The expression and activity of ALDHs is determined as another potential CSC marker. ALDH1 is a marker of normal and malignant human mammary SCs and a predictor of poor clinical outcome. Aldehyde dehydrogenase enzymes participate in cellular detoxification, differentiation and drug resistance through the oxidation of cellular aldehydes.

The functional activity of ALDH has been widely used to identify and isolate CSCs found in the bone marrow, breast, lung, ovary, colon, prostate, and pancreas.

Stem Cell Markers

The derivation of SCs from adult tissues, their relative ease of isolation and enormous expansion potential in culture make them attractive therapeutic candidates. These cells are identified by their expression of a particular panel of surface molecules, with the presence of CD73, CD90, CD105, and the absence of CD14, CD34, CD45, and HLA-DR. They show no proliferative response from alloreactive lymphocytes because of the negligible levels of extracellular MHC class I and II determinants. SCs also have important immunomodulatory functions in all the cells involved in both the innate and adaptive immune responses.

ALDH as a Stem Cell Marker

In theory, ALDH isozymes including ALDH1A, ALDH1A2, ALDH1A3, and ALDH3A1, which are involved in drug resistance and RA formation, are vital in protecting SCs against toxic endogenous and exogenous aldehydes and for SCs' ability to differentiate, respectively. It is unknown what ALDH isozymes are responsible for the ALDH activity that are used to identify stem cell progenitors. In the overlap gene profile of different stem cell populations, ALDH7A1, known as antiquitin, and ALDH2 were identified, consequently, and are worthy of further investigation. There is more about ALDH to be explored as a cause of its full physiological function has remained elusive. ALDH7A1 is a green pea 26g protein, which has function in regulation of turgor pressure, and has ≥50% amino acid identity with the 3 pseudogenes in the ALDH family. It also has 69% equity with ALDH2, but nevertheless has considerably lower affinity for acetaldehyde than ALDH2. However, ALDH2, which is a mitochondrial enzyme, has been widely studied mostly for its affilitation with ethanol metabolism. Yet, there might be an extent of confusion as to how ALDH2 is associated in gene profiling studies. According to the nomenclature, this enzyme indeed is ALDH1A, related to a series of events linked to the development of dopaminergic neurons through its ability to produce RA. It was reported that ALDH2 or AHD2 expression changes during differentiation of NIH-3T3 cells into adipocytes. These studies continue to focus on ALDH1A1's role in SCs and stem cell differentiation. For hematopoietic stem cell progenitors, ALDH1A1 has been a thoroughly established marker for many years. Reseach on the role of RA in granulocyte differentiation of hematopoietic SCsdiscovered that ALDH1A1 and ALDH1B1catalyze cellular RA synthesis and are expressed in $CD34^+$ hematopoietic progenitors. They also showed that ALDH1A2 or 1A3 do not show those characteristics. For the differentiation to mature granulocytes, these 2 enzymes' expressions are necessary, however their expressions are lost once the differentiation is complete. The in vitro disulfiram treatment in which disulfiram acts as an ALDH inhibitor may inhibit granulocytic differentiation. ALDH1A1 is found in erythrocytes and has been pointed to contribute to the aldophosphamide detoxification. A study that inhibited ALDH and retinoid signaling with diethylamino-benzaldehyde (DEAB) that reported the expansion of human HSCs probably by blocking differentiation and assisting self-renewal and HSC expansion.

The cancer stem cell theory is supported by current evidence in tumor biology, which may also provide a biological reason for the age-related survival difference. The theory demonstrates that CSCs, a small subset of tumor cells with stem cell-like properties such as epithelial-to-mesenchymal progression, are capable of differentiation and self-renewal, after which leads to formation of a heterogeneous tumor cell population. Including aldehyde dehydrogenase-1 (ALDH1) activity, $CD44^+/CD24^-$, CD133, and ITGA6, a wide range of putative breast cancer stem cell markers have

been proposed. ALDH1 expression has especially demonstrated an assurance of a clinically relevant prognostic marker. In addition, the subset of CSCs is shown to be relatively insusceptible to chemo and radiotherapy by various studies. For this reason, the subpopulation of CSCs can present a statement and a therapeutic target for poor-prognostic, treatment-resistant and recurrent breast cancer. Through its role in oxidizing retinol to RA, which is a modulator of cell proliferation, ALDH1 might have a role in early differentiation of SCs and stem cell proliferation.

It is possible to isolate leukemia SCs depending on the elevated ALDH activity by using the aldefluor assay. In patient samples, the researchers encountered a population of $ALDH^+$ acute myeloid leukemia (AML) cells. In most cases, the $ALDH^+$ AML cells coexpressed $CD34^+$ (formerly determinedleukemia stem cell marker), and were introduced considerably better than the ALDH- AML cells in immunocompromised mice. In the same year, $ALDH^+$ cells from breast cancers, which had the tumorigenic and self-renewal features of CSCs, were shown to be possibly isolated. This innovative study displayed the potential applicability of quantifying ALDH activity in solid tumors. ALDH activity would be used successfully as a CSC marker for abundant cancers including liver, colon, lung, bone, prostate, pancreatic, head and neck, thyroid, bladder, brain, cervical and melanoma in the proceeding years. With one exception of a current study for melanoma, 35 demonstrate growing evidence recommending ALDH's activity to be a universal CSC marker. Nonetheless, as amounted by the aldefluor assay in various tissues and cancers, the cause of ALDH activity may differ. Essentially, determination of specific ALDH isoforms carried out commonly in certain cancers might haveprognostic suitability. Besides their valuable function in detoxification of aldehydes, ALDHs carry out other functions such as serving as binding proteins for various molecules (e.g., androgens and cholestorol), potentially act as antioxidants by NAD(P)H production, ultraviolet light absorption and/or hydroxyl radical scavenging and ester hydrolysis.

Lastly, several isoforms (ALDH1A1, ALDH1A2, ALDH1A3 and ALDH8A1), take place via RA formation by oxidation of all-trans-retinal and 9-cis-retinal in RA cell signaling, which has been related to the "stemness" characteristics of CSCs. Consequently, its supported by widening evidence that ALDH may be more than just a CSC marker and have an accomplishable role in CSC biology.

ALDH Family Members as Stem Cell Markers

ALDH proteins can be found in every subcellular region such as cytosol, endoplasmic reticulum, mitochondria, and the nucleus, with some even found in more than one location. ALDH isozymes found in organelles besides cytosol carry signal or leader sequences that make their translocation to specific subcellular regions possible. After translocation or import, while nuclear and microsomal signals remain intact, mitochondrial sequences might be removed (causing mature proteins to be shorter). Most of the ALDHs have a large tissue distribution and show distinct substrate specificity.

ALDH1A1

ALDH1A1 encodes a homotetramer that is ubiquitously distributed in the adult epithelia of several organs such as brain, testis, kidney, eye lens, retina, liver and lungs. ALDH1A1 takes its position among the three highly-conserved cytosolic isozymes, which catalyze the oxidation of the retinol metabolite, retinal (retinaldehyde), to RA. ALDH1A1 has great affinity for the oxidation of both all-trans-($Km < 0.1\ \mu M$) and 9-cis-retinal. By serving as a ligand for nuclear RA receptors (RAR)

and retinoid X receptors (RXR), RA regulates gene expression; therefore its synthesis is crucial for normal growth, differentiation, development and the maintenance of adult epithelia in vertebrate animals. In retinoid-dependent tissues (including the retina), retinal-oxidizing ALDHs have been shown to display differential expression patterns during organogenesis in rodents, reflecting that RA signaling is indeed important for embryogenesis. The in vivo function of ALDH1A1 in RA synthesis is proven by the fact that after retinol treatment, while Aldh1a1−/−mice are viable and possess normal morphology of the retina, the livers of Aldh1a1−/−mice have reduced RA synthesis and increased serum retinal levels. Surprisingly, it appeared that Aldh1a1−/− mice are protected against both diet-induced obesity and insulin resistance and this demonstrates that retinal might regulate the metabolic response to high-fat diets transcriptionally, and that the ALDH1A1 could be a candidate gene for therapeutic targeting. Supression of ALDH1A1 in cultured hepatocytes reduces both the omega-oxidation of free fatty acids and the production of reactive oxygen species (ROS). Liver ALDH1A1 levels were shown to be decreased in RXRα−/− mice, which suggests that RA binding is an activating factor in ALDH1A1 gene expression. The androgen receptor might also be included in modulation of ALDH1A1, which is recognized to be an androgen binding protein. RA is required for testicular development and ALDH1A1 is absent in genital tissues of humans with androgen receptor-negative testicular feminization. ALDH1A1 is significantly expressed in dopaminergic neurons that are known to require RA for their differentiation and development in the human brain. In these neurons, ALDH1A1 is under the control of Pitx3, a homeodomain transcription factor that, possibly through ALDH1A1 upregulation, regulates the particularization and maintenance of disassociated populations of dopaminergic neurons. Decreased levels of ALDH1A1 takes place in dopaminergic neurons of the substantia nigra of patients with Parkinson's disease (PD), as well as the ventral tegmental area in schizophrenic patients. In the central nervous system (CNS), monoamine oxidase (MAO) metabolizes dopamine to aldehyde, as it's metabolite 3,4-dihydroxyphenylacetaldehyde (DOPAL), which growing evidence suggests might be neurotoxic, and it may lead to cell death in relation to neurological pathologies when accumulated. In maintaining low intraneuronal levels of DOPAL, ALDH1A1 may undertake a critical role by catalyzing its metabolism to 3,4-dihydroxyphenylacetic acid (DOPAC). Being one of 139 genes that are differentially expressed in primary human HSCs, and through the production of RA, ALDH1A1 has been shown to promote their differentiation.

These data suggest that for the therapeutic amplification of HSCs, ALDH1A1 inhibition could potentially be used.

ALDH1A2

ALDH1A2 is a cytosolic homotetramer expressed in several embryonic and adult tissues such as brain, kidney, intestine, testis, liver, retina, lung. As ALDH1A1, ALDH1A2 also catalyzes the reaction in which both all-trans-retinal and 9-cis-retinal oxidize to RA. However, when compared with other ALDH isozymes, ALDH1A2 appears to acquire the highest specificity (Vmax/Km = 49 nmol min−1 mg−1 μM−1) for all-trans-retinal. This characteristic may be because of an uncommon discreate loop in its active site that binds all-trans-retinal in a unique manner.

Taking action in several developmental processes, ALDH1A2 might be a key regulator of RA synthesis in developing tissues. Due to defects in early heart morphogenesis, Aldh1α2−/− mice die in early embryonic stages in which they seem to lack axial rotation, incomplete neural tube closure, reduction of the trunk region and many of the properties of human DiGeorge/velocardiofacial

syndrome, a disorder characterized by cleft palate, heart abnormalities and learning disabilities. During early vascular development, aberrations in endothelial cell cycle progression have also been determined in Aldh1α2$^{-/-}$ embryos. Aldh1a2 has been determined as a key regulator in the development of many tissues including kidney, retina, lung, forebrain, pancreas, and spinal cord by miscellanous animal models.

ALDH1A3

ALDH1A3 is a cytosolic homodimer that participates in RA synthesis, oxidizes both all-trans-retinal and 9-cis-retinal (Km 0.2 μM for all-trans-retinal) to RA, and has an important role in embryonic development; including brain, retina, skeletal muscle, tooth buds, intestine, kidney, prostate, lung, liver and pancreas, it is expressed in various late-stage embryonic and adult rodent tissues. In humans, ALDH1A3 expression has been noted in stomach, salivary gland, breast, kidney and fetal nasal mucosa.Aldh1a3$^{-/-}$ mouse embryos die as a result of defects in nasal development.

It's been shown that ALDH1A3 takes part in the development of the eye, nucleus accumbens and olfactory bulbs, the forebrain, hair follicles and the cerebral cortex.

ALDH1A3 deficiency has been shown to play a critical role in cancer by a number of studies. For instance, in human breast cancer MCF-7 cells, ALDH1A3 expression is downregulated, whereas in cultured human colon cancer cells, ALDH1A3 is one of two genes that are upregulated by induction of wild type p53. In mammary tumor-susceptible BALB/cJ mice that are heterozygous for p53, Aldh1a3 is one of five candidate genes located within a region determined for its linkage to mammary tumorigenesis. In mice resistant to induced mammary tumors, (C57BL/6J), Aldh1a3 is one of the two upregulated genes. ALDH1A3 is silenced by methylation in gastric cancer cells, whereas in glioblastoma cells, it is triggered by the antitumor agent IL-13 cytotoxin.

ALDH2

ALDH2 is a tetrameric enzyme expressed profusely in lungs and liver; it is also present in organs that obligate high mitochondrial capacity for oxidative ATP generation including heart and brain. Apart from that, ALDH2 is also important in the aldehydic substrate oxidation such as 4-HNE, acrolein, and short-chain, aromatic or polycyclic carbons. To add to its dehydrogenase activity, depending on the substrates, ALDH2 can function as an esterase and reductase. More recent attention has also been focused on ALDH2 in regards to its function in the biotransformation of nitroglycerin, reducing it to 1,2-glyceryl dinitrate for the production of nitric oxide, which is a critical vasodilator.

ALDH7A1

ALDH7A1 is a homotetramer that's expressed in a large number of tissues; in rat heart, liver and kidney, increased levels of ALDH7A1 are noted, whereas in black seabream fish (sbALDH7A1), ALDH7A1 is significantly formed in the liver and the kidney, excluding the heart. In human fetal tissues, ALDH7A1 has been encountered at elevated levels in the cochlea, eye, ovary, heart and kidney. In contrast, balanced levels are detected in the liver, spleen, muscle, lung and brain.

Human ALDH7A1's primary role happens in the pipecolic acid pathway of lysine catabolism, in

which it catalyzes the oxidation reaction of alpha-aminoadipic semialdehyde (AASA) (Km180 μM) to alpha-aminoadipate. ALDH7A1 mutations form the molecular basis for pyridoxine-dependent epilepsy (PDE), an autosomal recessive disorder characterized by the aggression of tenacious seizures during infancy and early childhood and are avoidable by daily use of high-dose pyridoxine (Vitamin B6) supplementation.

Remarkably, ADH7A1 expression in the cochlea of the ear, the region dependent on the healthy upkeep of internal hydrostatic pressure, clarifies that mammalian ALDH7A1 might have an accomplishable function in osmotic regulation and in hearing disorders. However, no connection has been revealed yet, including patients with the inner-ear disorder Ménière's disease, which effects hearing and balance.

ALDH7A1 is notably and differentially expressed within the first and second meiotic stages of porcine oocyte development. Screening of the promoter region sbALDH7A1 has discovered cis-elements linked with cell cycle regulation.

ALDH in Cancer and Cancer Stem Cells

Adenocarcinoma

Adenocarcinoma is an epithelium cancer that is generated from glandular tissue. Epithelial tissue includes, but is not limited to, the surface layer of skin, glands and a variety of other tissues that line the cavities and organs of the body. Epithelium can be derived from the three germ layers ectoderm, mesoderm and endoderm during embryologic period. Adenocarcinoma classification depends on not only being a part of the gland, but also depends on having the same secretory characteristics. But, this form of carcinoma can occur in some higher mammals, including humans.

Adenocarcinomas can arise in many tissues of the body due to the ubiquitous nature of glands within the body. While each gland may not be secreting the same substance, as long as there is an exocrine function to the cell, it is considered glandular and its malignant form is therefore named adenocarcinoma. Endocrine gland tumors, such as a VIPoma, an insulinoma, a pheochromocytoma, etc. are typically not referred to as adenocarcinomas, but rather, are often called neuroendocrine tumors. If the glandular tissue is abnormal, but benign, it is called an adenoma. Benign adenomas typically do not invade other tissues and rarely metastasize, whereas malignant adenocarcinomas do both. Colon, urogenital, prostate, urachus and vagina), breast, esophagus, pancreas, stomach and throat are several examples of adenocarcinoma.

It is reported that ALDH expression marks pancreatic cancer stem cells. Also, they have mentioned that the enhanced clonogenic growth and migratory properties of ALDH-positive pancreatic cancer cells suggest a key role in the development of metastatic disease that negatively affects the overall survival of patients with pancreatic adenocarcinoma.

Breast Cancer

From high-grade, absence of hormone receptor expression to positive HER2 status and the basal-like molecular subtype, the expression of ALDH1 is in direct relation with undesired tumor characteristics in breast cancer.

Breast cancer cells with stem-cell-like properties are suggested to be responsible for metastatic spread. Aldehyde dehydrogenase 1 (ALDH1) and cluster of differentiation 44 (CD44) in addition to RhoC GTPase are among the stem cell markers that are expressed by these cells.

Breast CSCs were initially isolated, established on cell surface marker with CD24/lowCD44 expression. More currently, "functional" markers depending on stem cell properties are investigated for their plausible applications in the breast CSCs isolation. By this method, applying the aldefluor assay (Stemcell Technologies), originally designed to isolate viable HSCs and is an enzyme-based assay that recognizes ALDH activity, Ginestier et al. isolated breast CSCs. The assay is thought to precisely recognize ALDH isoform ALDH1A1 activity degree. Besides its application as a prognostic and CSC marker, ALDH activity that is primarily carried out by ALDH1A3 might be functional in breast cancer progression.

Expression of genes and tumor sphere formation in self-renewal and differentiation could be changed by adding chemical RA signaling inducers or inhibitors in breast cancer cell lines.

ALDH1 could work as a marker of breast CSCs better than $CD44^+/CD24^-$. Though we could not maintain a conclusion that ALDH1 expression was significantly related with any conventional clinicopathologic attributes, nevertheless, there is a compelling relation between ALDH1-positive breast tumors and resistance to neoadjuvant chemotherapy, because of the pCR rates being obtained, which are lower in ALDH1-positive tumors (9.5%) than ALDH1-negative tumors (32.2%). Moreover, after neoadjuvant chemotherapy, a considerable increase in the proportion of ALDH1-positive tumor cells was observed. These results are an indication of ALDH1-positive tumor cells playing an important role in resistance to chemotherapy. Because of tumor cells being more tumorigenic than $CD44^+/CD24^-$, tumor cells of breast CSCs are thought to be richer in ALDH1-positive tumor cells than in $CD44^+/CD24^-$ tumor cells. ALDH1-positive, in contrast with $CD44^+/CD24^-$, is closely associated with colony formation in the collagen gel as well. The subset of ALDH1-positive and $CD44^+/CD24^-$ tumor cells has been reported to contain the largest proportion of breast cancer stem cells (BCSCs); consequently, it is speculated to have the strongest resistantance to chemotherapy. However, pCR rates in the ALDH1-positive and $CD44^+/CD24^-$ high subset (20%, 2 of 10), are not the lowest among all the subsets consisting of the ALDH1-positive and $CD44^+/CD24^-$ low subset (0%, 0 of 11), the ALDH1-negative and $CD44^+/CD24^-$ high subset (34.1%, 15 of 44), and the ALDH1-negative and $CD44^+/CD24^-$ low subset (30.2%, 13 of 43). Adding CD44/CD24 status to ALDH1 status does not seem to positively improve the prediction of response to chemotherapy. Together, these results direct us to assume that, at least for the prediction of resistance to chemotherapy, ALDH1-positive tumor cells serve as a better marker for BCSCs than $CD44^+/CD24^-$ tumor cells. Because such tumors contain a higher proportion of CSCs, we suppose that ALDH1- positive tumors are resistant to chemotherapy. However, because ALDH1 has been shown to play an important role in the resistance to chemotherapy in hematopoietic cells, ALDH1-positive tumor cells might be involved in resistance to chemotherapy, regardless of whether they are CSCs or not. In addition to deeper illumination of ALDH1's function in chemotherapy resistance in breast cancers, obtaining a significantly specific marker for BCSCs is necessary to enlighten an authentic role of BCSCs' chemotherapy resistance.

ALDH1-positive, in contrast to $CD44^+/CD24^-$, was tremendously related to sequential paclitaxel- and epirubicin-based chemotherapy resistance, and the expression of ALDH1 increased after neoadjuvant chemotherapy, which stands for an indication of BCSCs, determined by ALDH1, indeed

having played a significant role in chemotherapy resistance. This means that ALDH1-positive appears to be a better marker than $CD44^+/CD24^-$ in identifying BCSCs, at least for the prediction of resistance to chemotherapy.

Lung Cancer

Each year, approximately 171,000 new cases of lung cancer are diagnosed, and 160,000 individuals do not survive from the disease in the United States. This high incidence and mortality makes lung cancer one of the most common cancers and the leading cause of cancer death in men. Lung cancer is still the leading cause of death from malignant diseases worldwide in spite of the advances in surgical treatment and multimodality treatments.

Cancer stem cells have attributed resistance of a smaller fraction of cells in the tumor bulk against chemotherapeutics. The isolation of CSCs is important for these reasons and have been isolated using a variety of stem cell markers and phenotypes. CD133 has recently been reported to identify tumor-initiating cells in non-small cell lung cancer (NSCLC). ABCG2 is also a stem cell marker of a variety of tissues and transporter responsible for the multidrug-resistance phenotype. However, it was demonstrated that many cells in NSCLC and SCLC cell lines show tumorigenic potential, regardless of ABCG2 and CD133 expression. Recently, ALDH activity has been used for isolation of these kinds of cells. Normal SCs were shown to contain higher levels of ALDH activity than their more differentiated progeny. ALDH-positive cells of tumors have higher proliferation rates, migration and adhesion ability, and metastatic potential than ALDH-negative cells. This may occur because that RA product of ALDHs is thought to participate in cellular differentiation and stem cell self-protection.

Ovarian Cancer

Epithelial ovarian cancer is the sixth most common cancer in women worldwide and it is still the most lethal gynecologic malignancy. Application of new technologies for detection of ovarian cancer could have an important effect on public health, but to achieve this goal, specific and sensitive molecular markers are essential. Aldehyde dehydrogenase-1A1 (ALDH1A1) has been a valid marker among several malignant and non-malignant tissues in spite of several stem cell markers to identify CSCs. ALDH plays a role in the biology of TICs as well as being a stem cell marker. Because ALDH1A1 is implicated in chemo resistance pathways, it is questioned that targeting ALDH1A1 can effect cells resistant to chemotherapy and represent a potential target for cancer stem-cell-directed therapy. In a study, ALDH1A1 was investigated in ovarian cancer cell lines and patient samples and examined whether targeting ALDH1A1 sensitizes cells to chemotherapy in both in vitro and in vivo ovarian cancer models. They showed that ALDH1A1 expression and activity have increased chemo resistant ovarian cancer cell lines. Most importantly, down-regulation of ALDH1A1 expression has sensitized normally chemo resistant tumors to both docetaxel and cisplatin in vitro and in mouse models. Besides being a stem cell marker, ALDH1A1 is also a viable target for therapy and a mediator of the aggressive phenotype.

Pancreatic Cancer

Pancreatic adenocarcinoma is a highly lethal disease, which is usually diagnosed in an advanced state, and for which there is little or no effective therapies. Therefore, finding markers to detect a

malignant cell transformation at an early stage is very important. Researches demonstrated that the pancreas possesses ALDH activity, and ALDH is also present in the pancreatic cancer cells. Different from other cancer tissues (such as ovarian and lung cancer), the activity of ALDH does not differ in pancreatic carcinoma tissue compared to normal pancreatic tissue. Additionally, serum levels of ALDH were not significantly elevated in patients with pancreatic cancer in comparison to healthy controls.

Prostate Cancer

The latest estimates of global cancer incidence show that prostate cancer has become the third most common cancer in men, with half a million new cases each year, constituting almost 10% of all cancers in men. Identifying the origin of cells in prostate cancer and its distant metastases may be important for the improvement of more effective treatment strategies and preventive therapies. Measurement of ALDH activity provides great contribution to functional identification and characterization of normal SCs and their malignant counterparts. ALDH activity is important for drug resistance, cell proliferation, differentiation, and response to oxidative stress of prostate cancer like other important cancers.

ALDH enzyme activity is used for the isolation of "stem-like" cells based on a developmentally conserved stem/progenitor cell function. In a study, high ALDH activity was used to isolate human prostate cancer cells with significantly enhanced clonogenic and migratory properties both in vitro and in vivo. Similar to other cancer tissues, the percentage of ALDHhi cells in prostate cancer cell lines are also related to tumorigenicity and metastatic behavior.

Although high expression of ALDH7A1 is shown in prostate cancer cell lines, primary cultures, and in primary prostate cancer tissue and matched bone metastases, ALDH3A2 and ALDH18A1 are not observed high ALDH activity in human prostate cancer.

Brain Cancer

Glioblastoma (GBM) is the most common primary brain tumor in adults with an approximately 15-month survival. Although there are several studies to improve the postoperative therapeutic applications within the last few years, there is not enough succes for this highly aggressive tumor. After resection, radiation, and chemotherapy regimens, relapses occur regularly. Thus, it is thought that this can be a clue to the presence of tumor stem cells (TSCs). This cellular subfraction within GBM causes continuous tumor growth and resistance to drugs and radiation. TSCs are believed to nestle in the tumor, keeping it alive and growing, providing pluripotency, self-renewal, and resistance to chemo and radiation therapy. The first malignancies from which cells could be isolated and showed the potential to self-renew and to drive tumor formation and growth were leukemias. After that, a stem cell subfraction was described in brain tumors. This was the first study that identified and showed a population with stem cell properties in pediatric solid brain tumors. Those cells were identified by their ability to proliferate under serum-free cell culture conditions and by the expression of CD133 and nestin. CD133 has long remained the most important TSC marker in malignant glioma. On the other hand, ALDH1 is a cytoplasmatic stem cell marker in a variety of malignant tumors and catalyzes the oxidation of intracellular aldehydes including the transformation of retinol to RA. As mentioned above, RA is a modulator of cell proliferation and differentiation that possibly contributes to the maintenance of an undifferentiated

stem cell phenotype. Jones et al. presented a method to isolate human cells via flow cytometry depending on the amount of cytosolic ALDH. Recently, Ginestier et al. found ALDH1 to be a stem cell marker in breast carcinoma associated with poor clinical outcomes. Since then, ALDH1 has been described as a marker of stemness in other solid malignancies including lung cancer and colorectal cancer.

Therefore, identification and isolation of these cells seem crucial for a better understanding of tumor behavior, origin, and therapy. Recently, ALDH1 has been described as a marker for the identification of non-neoplastic SCs and TSCs.

So far, cellular markers including CD133 have been used to identify TSCs in GBMs, but recently, CD133-negative GBMs are characterized to behave as brain TSCs.

Therefore, ALDH1 has also been described as a stem cell marker in various solid neoplasms including lung cancer, breast carcinoma, and colorectal cancerand GBM.

Colon Cancer

Most colon cancers are adenocarcinomas that release mucus and other cellular secretions. In the United States in 2012, estimated new cases and deaths from colon and rectal cancer are reported as: 103,170 colon cancers and 51,690 deaths. Studies showed that ALDH1B1 and ALDH1A1 are differentially expressed in normal human tissues, but ALDH1B1 is expressed at higher levels than ALDH1A1 in human epithelial cancers. ALDH1B1 was abundantly expressed in adenocarcinomas originating from the tissue and particularly in colonic adenocarcinoma. Thus it can be deduced that ALDH1B1 may be a marker for colon cancer diagnosis.

Aldefluor Activity in Stem Cells and Cancer Stem Cells

ALDHbr (ALDH-bright) cells can be detected with ALDEFLUOR reagent by using flow cytometry or fluorescent microscopy. These ALDHbr cell populations are isolated from adult tissues by flow sorting.

ALDH activity was shown in human and mouse bone marrow hematopoietic progenitor cells (HPCs) by Jones et al. for the first time. ALDH was assayed by using a new substrate with low light scatter properties with flow cytometry. Now this method was improved and known as Aldefluor assay. Aldefluor assay can be used for to measure ALDH activity of adult tissue cells, primary cancer cells and cultured cells. Aldefluor assay is based on the conversion of fluorescent non-toxic substrate for ALDH substrate to the fluorescent reaction product. Non-toxic substrate for ALDH freely diffuses into intact and viable cells. The BODIPY aminoacetaldehyde is converted to the fluorescent product BODIPY aminoacetate by ALDH activity. In this assay, a specific inhibitor of this reaction (diethylaminobenzaldehyde-DEAB) is used to control for background fluorescence. Aldehyde dehydrogenase plays a role as a cancer stem cell marker comes down to the specific isoform.

Stem and progenitor cells are identified as cells with low side scatter and high expression of ALDH. DEAB allows to distinguish between ALDH-bright cells and cells with low ALDH activity. Generally, 105-106 cells are suspended in Aldefluor assay buffer containing BODIPY aminoacetaldehyde with/without DEAB. Aldefluor was excited at 488 nm and fluorescence emission was detected at

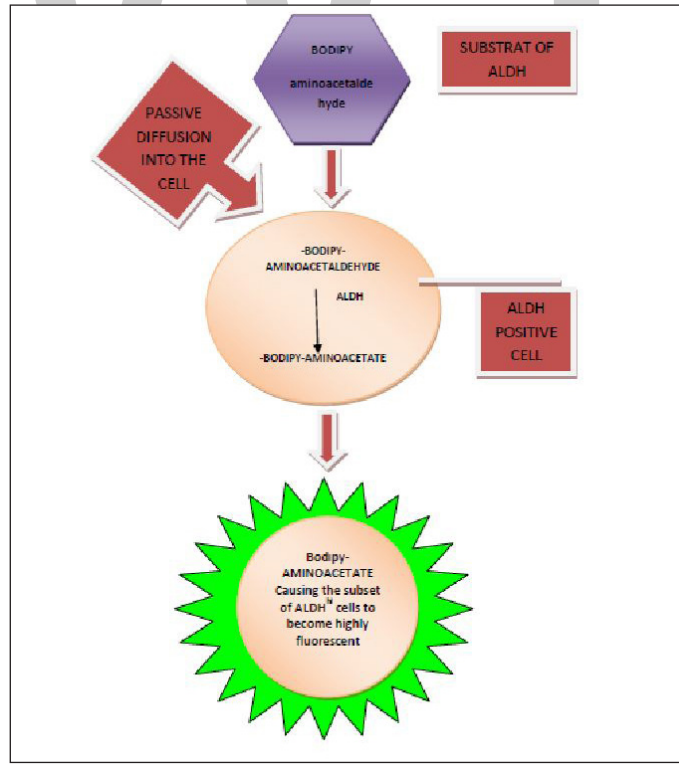
530/30. This assay provides a successful isolation of viable HSCs and more recently ALDH positive CSCs. However, aldefluor assay detects the ALDH activity of several ALDH isoforms expressed in the cells.ALDH1A1 is not the only isoform responsible from aldeflour activity. In some studies, it was demonstrated that ALDH1A1-deficient hematopoietic cells showed aldefluor activity owing to ALDH2, ALDH3A1 and ALDH9A1 isoforms.

ALDH Bright ($ALDH^{br}$) Cell

Intracellular ALDH enzymes are responsible for oxidizing aldehydes to carboxylic acids in the cell. $ALDH^{br}$ cells from different tissues express high ALDH activity and have progenitor cell activity. Firstly, HSC were defined as $SSCloALDH^{br}$ – reflecting their low orthogonal light scattering and bright fluorescence intensity by using flow cytometry. After that, high levels of the enzyme ALDH ($ALDH^{br}$) have proven to be a novel marker for the identification and isolation of SCs. In the same time angiogenic activity of $ALDH^{br}$ cells were discovered and these cells were used for regenerative medicine with preclinical models and have been used safely to treat patients in early clinical trials.

$ALDH^{br}$ cells were found in various cancer tissues including breast, liver, colon, and acute myelogenous leukemia and related with cancer chemo resistance. Human and murine HSCs and neural stem and progenitor cells have increased ALDH activity compared to non-stem-cells.

Therefore, recently the importance of ALDH activity in normal and malignant stem cell functions, and the potential diagnostic and therapeutic implications gain importance.



The Aldefluor Assay. Firstly, ALDH positive cell will uptake BODIPY-aminoacetaldehyde by passive diffusion and then convert BODIPY-aminoacetaldehyde into BODIPY-aminoacetate. Then BAAis retained inside cells, causing the subset of ALDHhi cells to become highly fluorescent.

Aldehyde Dehydrogenase in Regenerative Medicine

Studies showed that BM ALDH^{br} populations may be useful in several cell therapy applications. ALDH^{br} population may play an important role in regenerative medicine owing to RAs ALDH product. Retinoic acids could influence tissue repair by binding to transcription factors and regulating developmental programs, especially ALDH1A1 and ALDH3A1 of enzyme isoforms that produce RAs from oxidize retinaldehyde.Therefore, ALDH1a1 and ALDH1A3 may influence cell activity and proliferation by controlling intracellular retinoid concentrations and play important roles in stem cell biology.

The studies about value of ALDH^{br} cells in regenerative medicine were conducted by different researchers. The regenerative potential of ALDH^{br} cells obtained from different tissues were investigated in various disease models such as ischemic tissue damage hind limb model, brain damage and pancreatitis.

In the beginning of studies, ALDH^{br} cells were obtained from bone marrow and umbilical cord blood and normal peripheral blood. Multipotent mesenchymal progenitors and endothelial progenitor cells are concentrated in human ALDH^{br} populations. Because of potential progenitor and paracrine activities of ALDH^{br} cells, these cells especially obtained from bone marrow are important for tissue repair.

Manipulation of the graft to selectively concentrate or expand hematopoietic and/or neural stem cells prior to transplant may be a potential strategy in the future. UCBT using ALDH bright cells from the CB units have shown faster and higher engraftment in preliminary study and is being explored further. One of these studies showed that human cord blood progenitors with high ALDH activity improve vascular density in a model of acute myocardial infarction. ALDH^{br} cells were homed to the infracted anterior surface of the heart, while ALDH-low cells were in the spleen after intravenously administration.

Another study with animal model of hindlimb ischemia demonstrated that the isolated ALDH^{br} cells effectively restored blood flow to ischemic areas by mediation of local formation of new blood vessels with largeer diameter and increasing capillary density even if there was no improvement in cardiac functions.

The reason for the restoration of tissue perfusion by ALDH^{br} cells were attempted to be explained with angiogenic properties of these cell groups. Angiogenic factors secreted by transplanted ALDH^{br} cells stimulate formation of new blood vessels at sites of ischemic injury(human cord blood progenitors with high ALDH activity improve vascular density in a model of acute myocardial infarction). Paracrine mechanisms of ALDH^{br} cells can protect endothelial cells from ischemic damage and respond to ischemic tissue damage.

Another exciting finding is that ALDH^{br} cells improve formation of new vessels and increase capillary density, while ALDH^{br} cells together with ALDH-low cells did not restore tissue perfusion at all. It is suggested that ALDH-low cells can inhibit the homing and/or angiogenic activity of ALDH^{br} cells. This situation showed the importance of isolating ALDH^{br} cells from bone marrow tissue for therapeutic uses. As a result, ALDH^{br} cells may be promising for patients with ischemic heart failure and critical limb ischemia.

Table: Different tissue repair models including human $ALDH^{br}$ cells.

Tissue	Obtained from	Benefits
Neural Tissue	Rat embryonic neural tube Fetal mouse brain Subventricular and subcortical zones of adult mouse brain.	Ability to form neurospheres and retained multipotency Transplantation significantly ameliorated disease progression and extended life, but did not rescue the animals.
Skeletal Muscle	Biopsies or primary explants of human skeletal muscle.	Strong myogenic potential on IM transplantation.
Mammary Epithelium	Mammary epithelium.	Myoepithelial, luminal epithelial and mixed colonies, and ducts, when transplanted into mammary fat pads.
Pancreatic Cells	Central acinar/terminal duct cells from peripheral acinar duct units of adult mice.	Contributed to both exocrine and endocrine lineages in the developing pancreas.
Prostate Epithelium	-	Express basal epithelial and characteristic prostate progenitor cell markers.
Corneal Limbic Cells	Cadaveric human limbic tissue.	Protects the cornea from oxidative damage.

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Types of Stem Cells

3

- Amniotic Fluid and Amniotic Membrane Stem Cell
- Induced Stem Cell
- Dental Pulp Stem Cell
- Limbal Stem Cell
- Renal Stem Cell
- Epiblast-derived Stem Cell
- Mesenchymal Stem Cell

Different types of stem cell include induced stem cell, dental pulp stem cell, limbal stem cell, renal stem cell, epiblast-derived stem cell, mesenchymal stem cell, amniotic fluid and amniotic membrane stem cell. The topics elaborated in this chapter will help in gaining a better perspective about these types of stem cells.

Amniotic Fluid and Amniotic Membrane Stem Cell

Both amniotic fluid (AF) and amniotic membrane (AM) represent rich sources of stem cells that can be used in the future for clinical therapeutic applications. Ethical concerns regarding the isolation of stem cells from these sources are minimized, in contrary to the issues emerging from human embryonic stem cell (ESC) research. AF is collected during scheduled amniocenteses between 15th and 19th week of gestation for prenatal diagnosis and the excess of sample can be used for cell sourcing, whereas AM is usually collected during the caesarean sections of term pregnancies. Given the heterogeneity of the stem cell populations derived from these sources, the isolation of specific cell types is difficult and requires a detailed phenotypic and molecular characterization of the respective cells. Studies that include omics approaches are fundamental in better understanding the mechanisms of molecular expression of these cells and defining the correct methodologies for their isolation, prior to their use in therapeutic approaches.

Amniotic Fluid

AF serves as a protective liquid for the developing embryo, providing mechanical support and the required nutrients during embryogenesis. Amniocentesis has been used for many decades as a routine procedure for fetal karyotyping and prenatal diagnosis, allowing the detection of a variety of genetic diseases.

The major component of AF is water; however its overall composition varies throughout pregnancy. At the beginning of pregnancy, the amniotic osmolarity is similar to the fetal plasma. After keratinization of the fetal skin amniotic osmolarity decreases relatively to maternal or fetal plasma, mainly due to the inflow of fetal urine. More interestingly, AF also represents a rich source of a stem cell population deriving from either the fetus or the surrounding amniotic membrane. Additional investigations by several groups have been recently focused on the cellular properties of amniotic derived cells and their potential use in preclinical models and in transplantation therapies.

Amniotic Fluid Stem Cells (AFSCs)

The amniotic fluid cells (AFCs) represent a heterogeneous population derived from the three germ layers. These cells share an epithelial origin and are derived from either the developing embryo or the inner surface of the amniotic membrane, which are characterized as amniotic membrane stem cells. The AFCs are mainly composed of three groups of adherent cells, categorized based on their morphological, growth, and biochemical characteristics. Epithelioid (E-type) cell are cuboidal to columnar cells derived from the fetal skin and urine, amniotic fluid (AF-type) cells are originating from fetal membranes, and fibroblastic (F-type) cells are generated mainly from fibrous connective tissue. Both AF- and F-type cells share a fibroblastoid morphology and the dominant cell type appears to be the AF-type, coexpressing keratins and vimentins. Several studies have documented that human amniotic fluid stem cells (AFSCs) can be easily obtained from a small amount of second trimester AF, collected during routine amniocenteses, a procedure with spontaneous abortion rate ranging from 0.06 to 0.5%. Up to date, a number of different cultivation protocols have been reported, leading to enriched stem cell populations. The isolation of AFSC and the respective culture protocols were summarized in a recent review by Klemmt et al. and can be categorized as follows: (i) a single step cultivation protocol, where the primary culture was left undisturbed for 7 days or more until the first colonies appear, (ii) a two-step cultivation protocol, where amniocytes, not attached after 5 days in culture, were collected and further expanded, (iii) cell surface marker selection for CD117 (c-kit receptor), (iv) mechanical isolation of the initial mesenchymal progenitor cell colonies formed in the initial cultures, and (v) short-term cultures to isolate fibroblastoid colonies. The majority of the AFSCs, isolated following these methodologies, shared a multipotent mesenchymal phenotype and exhibited higher proliferation potential and a wider differentiation potential compared to adult MSCs.

Amniotic Membrane

The amniotic membrane, lacking any vascular tissue, forms most of the inner layer of the fetal membrane and is composed of 3 layers: (i) an epithelial monolayer consisting of epithelial cells, (ii) an acellular intermediate basement layer, and (iii) an outer mesenchymal cell layer, rich in mesenchymal stem cells and placed in close proximity to the chorion. AM was used in clinic for many decades for wound healing in burns, promoting epithelium formation and protecting against infection. Recently, the use of AM has been evaluated as a wound dressing material for surgical defects of the oral mucosa, ocular surface reconstruction, corneal perforations, and bladder augmentation.

Amniotic Membrane Stem Cells (AMSCs)

Amniotic membrane stem cells (AMSCs) include two types, the amniotic epithelial cells (AECs) and the amniotic membrane mesenchymal stem cells (AM-MSCs) derived from the amniotic epithelial and the amniotic mesenchymal layers, respectively. Both cell types are originated during the pregastrulation stages of the developing embryo, before the delineation of the three primary germ layers and are mostly of epithelial nature. A variety of protocols have been established for AECs and AM-MSCs isolation, primarily based on the mechanical separation of the AM from the chorionic membrane and the subsequent enzymatic digestion. AM-MSCs exhibited plastic adherence and fibroblastoid morphology, while AECs displayed a cobblestone epithelial phenotype. AM-MSCs shared similar phenotypic characteristics with the ones derived from adult sources. More interestingly, AM-MSCs, similarly to AF-MSCs, exhibited a higher proliferation rate compared to MSCs derived from adult sources and a multilineage differentiation potential into cells derived from the three germ layers.

Immunophenotype

Amniotic Fluid Stem Cells

The AF has recently emerged as an alternative fetal source of a variety of cells of stem cell origin. Herein, we aim to summarize the key markers that characterize AFSCs. To date, MSCs represent the best characterized subpopulation of AFSCs. The AFMSCs exhibited typical mesenchymal marker expression, such as CD90, CD73, CD105, CD29, CD166, CD49e, CD58, and CD44, determined by flow cytometry analyses. Additionally, these cells expressed the HLA-ABC antigens, whereas the expression of the hematopoietic markers CD34 and CD45, the endothelial marker CD31, and the HLA-DR antigen was undetected. More importantly, the majority of cultured AFMSCs expressed pluripotency markers such as the octamer binding protein 3/4 (Oct-3/4), the homebox transcription factor Nanog (Nanog), and the stage-specific embryonic antigen 4 (SSEA-4).

It was also reported that amniocyte cultures contain a small population of CD117 (a tyrosine kinase specific for stem cell factor present primarily in ESCs and primordial germ cells) positive cells that can be clonally expanded in culture. The differentiation properties of $CD117^{+}$ AFS were tested for the first time in vivo, proving in this way their stem cell identity. Experimental evidence suggested that AFSCs are derived from spindle-shaped fibroblastoid cells.

In an attempt to analyze the AFSCs subpopulations, our group recently identified two morphologically distinct populations of AFSCs of mesenchymal origin, with different proliferation and differentiation properties, termed as spindle shaped (SS) and round shaped (RS). Both subpopulations were expressing mesenchymal stem cell markers at similar levels. However, it was identified that SS colonies expressed higher levels of CD90 and CD44 antigens compared to RS colonies.

Amniotic Membrane Stem Cells (AMSCs): A detailed immunophenotype analysis of AMSCs revealed the expression of antigens, such as CD13, CD29, CD44, CD49e, CD54, CD73, CD90, CD105, CD117low, CD166, CD27low, stromal stem cell marker 1 (Stro-1), SSEA-3, SSEA-4, collagen I and III (Col1/Col3), alpha-smooth muscle actin (α-SMA), CD44, vimentin (Vim), fibroblast surface protein (FSP), and HLAABC antigen. However, intercellular adhesion molecule 1 (ICAM-1) was expressed in very low levels and proteins TRA-1-60, vascular cell adhesion protein 1 (VCAM1), von

Willebrand factor (vWF), platelet endothelial cell adhesion molecule (PECAM-1), CD3, and HLA-DR were not detected. One of the most abundant proteins found in AM derived cells is laminin, which plays a key role in differentiation, cell shape and migration, and tissue regeneration [54, 55]. RT-PCR analysis further showed that AMSCs expressed genes, such as Oct-3/4, zinc finger protein 42 (zfp42 or Rex-1), stem cell factor protein (SCF), neural cell adhesion molecule (NCAM), nestin (NES), bone morphogenetic protein 4 (BMP-4), GATA binding protein 4 (GATA-4), and hepatocyte nuclear factor 4α (HNF-4α) even in high passages. Brachyury, fibroblast growth factor 5 (FGF5), paired box protein (Pax-6), and bone morphogenetic protein 2 (BMP2) transcripts were not detected. Similarly, AECs were positive for CD10, CD13, CD29, CD44, CD49e, CD73, CD90, CD105, CD117, CD166, Stro1, HLA-ABC, and HLA-DQlow and negative for CD14, CD34, CD45, CD49d, and HLA-DR expressions, as determined by FACS analyses. Further investigation showed that AECs were expressing stem cell markers such as SSEA1, SSEA-3, SSEA-4, Nanog, sex determining region Y-box 2 (Sox2), Tra1-60 and Tra1-80, fibroblast growth factor 4 (FGF4), Rex-1, cryptic protein (CFC-1), and prominin 1 (PROM-1).

Transcriptomics

Amniotic Fluid Stem Cells

A functional analysis of the gene expression signature of AF-MSCs compared to bonemarrow- (BM^-), cord-blood (CB^-), and AM-MSCs was initially performed by Tsai et al. Genes expressed in MSCs from all three sources could be categorized in groups related to: (i) extracellular matrix remodeling (CD44, collagen II (COL2), insulin-like growth factor 2 (IGF2), and tissue inhibitor of metalloproteinase 1 (TIMP1)), (ii) cytoskeletal regulation (urokinase-type plasminogen activator (PLAU) and receptor (PLAUR)), (iii) chemokine regulation and adhesion (alpha actinin 1 (ACTN1), actin-related protein complex subunit 1B (ARPC1B) and thrombospondin 1 (THBS1)), (iv) plasmin activation (tissue factor pathway inhibitor 2 (TFPI2)), (v) transforming growth factor β (TGFβ) receptor signaling (caveolin 1 (Cav1), caveolin 2 (Cav2), cyclin-dependent kinase inhibitor 1A (CDKN1A)), and (vi) genes encoding E3 ubiquitin ligases (SMURF). The upregulated genes in AF-MSCs compared to BM-, CB-, and AM-MSCs included molecules involved in uterine maturation and contraction, such as oxytocin receptor (OXTR) and regulation of prostaglandin synthesis, such as phospholipase A2 (PLA2G10). Other upregulated genes in this group were involved in signal transduction related to (i) thrombin triggered response ((F2R and F2RL)), (ii) hedgehog signaling ((hedgehog acyltransferase (HHAT)), and (iii) G-proteinrelated pathways (rho-related GTP-binding protein (RHOF), regulator of G protein signaling 5 and 7 (RGS5, RGS7), and phospholipase C beta 4 (PLCB4)).

In recent studies on AFSCs, Kim et al. described for the first time the gene expression changes in total AFSC population during different passages by illumina microarray analysis. 1970 differentially expressed genes were detected and categorized according to their expression profiles into 9 distinct clusters. Genes with gradually increasing expression levels included chemokine (C-X-C motif) ligand 12 (CXCL12), cadherin 6 (CDH6), and folate receptor 3 (FOLR3). Downregulated genes were among others, cyclin D2 (CCND2), keratin 8 (K8), IGF2, natriuretic peptide precursor (BNP) B, and cellular retinoic acid binding protein 2 (CRABPII). To obtain further information, chip data analysis on aging genes was performed and revealed upregulation of gene transcripts, such as nerve growth factor beta (NGFβ), insulin receptor substrate 2 (IRS-2), insulin-like growth factor binding protein 3 (IGFBP-3), and apolipoprotein E (APOE). Expression of genes, such as

PLAU, E2F transcription factor 1 (E2F1), IGF2, breast cancer type 1 susceptibility gene (BRCA1), DNA topoisomerase 2-alpha (TOP2A), proliferating cell nuclear antigen (PCNA), forkhead box M1 (FOXM1), cyclin-A2 gene (CCNA2), budding uninhibited by benzimidazoles 1 homolog beta (BUB1B), and cyclin dependent kinase 1 (CDC2), was gradually downregulated during culture.

Wolfrum et al. performed a global gene expression analysis of AFSCs compared to iPSCs derived from AF (AFiPSC) and ESCs. Among these, genes related to self renewal and pluripotency (1299 genes e.g., POU class 5 homeobox 1 (POU5F1), Sox2, Nanog, microRNA-binding protein LIN28) and AFSCs-specificity (665 genes, e.g., OXTR, HHAT, RGS5, neurofibromatosis type 2 (NF2), protectin (CD59), tumor necrosis factor superfamily member 10 (TNFSF10), 5 -nucleotidase (NT5E)) were detected in AFSCs. Furthermore, the authors examined the expression of senescence and telomere associated genes in AFSCs of early and later passage, in order to study the effect of reprogramming on bypassing senescence observed in AFSC cultures. Sixty-four genes were identified as differentially expressed in AFSCs compared to AFiPSC lines. Of these, telomere-associated genes and genes involved in regulating cell cycle, such as the mitotic arrest deficient-like 2 (MAD2L2), the poly ADP-ribose polymerase 1 (PARP1), replication protein A3 (RPA3), the dyskeratosis congenita 1 (DKC1), the mutS homolog 6 (MSH6), the CHK1 checkpoint homolog (CHEK1), the polo-like kinase 1 (PLK1), the POU class 2 homeobox 1 (POU2F1), the CDC2, the Bloom syndrome gene RecQ helicase-like (BLM), the Werner syndrome RecQ helicase-like (WRN), the DNA methyltransferase 1 (DNMT1), the DNA methyltransferase 3 beta (DNMT3B), the lamin B1 (LMNB1), and the DNA replication factor 1 (CDT1), were downregulated in AFSCs compared to AFiPSCs and ESCs. In contrast, peptidylprolyl cis/trans isomerase (PIN1), lamin A/C (LMNA), growth arrest and DNA damage inducible alpha (GADD45A), chromobox homolog 6 (CBX6), NADPH oxidase 4 (NOX4), endoglin (ENG), histone H2B type 2-E (HIST2H2BE), CDKN1A, CDKN2A growth differentiation factor 15 (GDF15), and serine protease inhibitor 1 (SERPINE1), among others, were upregulated in AFSCs compared to AFiPSCs and ESCs.

Amniotic Membrane Stem Cells

Transcriptomic analysis using DNA microarrays has been reported for AM-MSCs These experimental data provided information on the AM-MSC gene expression pattern compared to gene expression profiles of AF, CB, and BM-MSCs. Several upregulated genes in AM-MSCs involved in immune adaptation regulation between the maternoplacental interface were identified. Among others, spondin 2 (SPON2), interferon, alpha inducible protein 27 (IFI27), bradykinin receptor B1 (BDKRB1), small inducible cytokine subfamily B member 5 and 6 (SCYB5, SCYB6), and Yamaguchi sarcoma viralrelated oncogene homolog (LYN) were found to be upregulated. In addition, other genes with increased expression in AM-MSCs compared to AF, CB, and BM-MSCs included (i) transcription factors, such as forkhead box F1 (FOXF1), heart and neural crest derivatives expressed 2 (HAND2), and transcription factor 21 (TCF21) and (ii) metabolic enzymes, such as dipeptidyl-peptidase 6 (DPP6), tryptophan 2,3-dioxygenase (TDO2), and sialyltransferases (STs).

Proteomics

Amniotic Fluid Stem Cells

Proteomic studies on the total AFSC population, including epithelioid (E-type), amniotic fluid specific (AF-type), and fibroblastic (F-type) cells, revealed 2400 spots that resulted in the identification of 432 different gene products. The majority of the proteins was localized in cytoplasm (33%), mitochondria

(16%), and nucleus (15%) and represented mainly enzymes (174 proteins) and structural proteins (75 proteins). A relatively high percentage of membrane and membrane-associated proteins were also present (7%). Among the detected proteins, 9 were corresponding to epithelial cells, such as ATP synthase D chain (ATP5H), NADH-ubiquinone oxidoreductase 30 kDa subunit (NUIM), annexin II (Anx2), annexin IV (Anx4), 40S ribosomal protein SA (Rpsa), glutathione Stransferase P (GSTP), major vault protein, and cytokeratins 19 and 7 (CK-19, CK-7), whereas 12 proteins were reported to be expressed in fibroblasts, including fibronectins, tropomyosins, transgelin (TAGLN), arp2/3 complex 34 kDa subunit (P34-arp), gelsolin (Gsn), elongation factor 1-β (EF1β), and others. Eight proteins were found to be expressed in keratinocytes, including keratins, ribonucleoproteins, Anx2, aetyl-CoA acetyl-transferase (ACAT1), and others, three to be expressed in epidermis, including tropomyosins and keratins and one in mesenchymal cell type (vimentin 1 (Vim 1)).

Recent studies provided evidence that a diversity of metabolic enzyme expression in the amnion cells is involved in metabolic and genetic syndromes, and thus, their detection might be important for prenatal diagnosis. A more detailed analysis for determining specific metabolic enzymes present in AFSCs was reported by Oh et al. Ninety-nine proteins had been identified, such as carbohydrate handing enzymes, amino acid handling enzymes, proteins of purine metabolism, and enzymes of intermediary metabolism.

A proteomic analysis was also performed on different culture passages of $CD117^{+}$ AFSCs, exhibiting variations in protein expression that mainly occurred in early passages. Twenty-three proteins were differentially expressed between early and late passages with the most sticking downregulated proteins, the Col1, the Col2, the vinculin (Vcl), the CRABP II, the stathmin (STMN1), and the cofilin1 (CFL1). In contrast, TAGLN and Col3 are increased during passages. Proteins that showed dysregulated levels along the passages were the 26S protease regulatory subunit 7 (PSMD7), the ubiquitin carboxyl terminal hydrolase isoenzyme L1 (UCH-L1), the heterogeneous nuclear ribonuclear protein H (hnRNP H), and the TAR DNA-binding protein 43 (TDP-43).

In 2007, the proteomic map of human AF-MSCs was constructed and directly compared to the one derived from BM-MSCs. 261 different proteins were identified in AF-MSCs with the majority of the proteins localized in the cytoplasm (41%), whereas others were found in the endoplasmic reticulum (8%), nucleus (13%), mitochondria (12%), ribosomes (1%), cytoskeleton (6%), cytoplasm and the nucleus (5%), and secreted (2%) proteins. AF-MSCs expressed a number of proteins related to proliferation and cell maintenance, such as ubiquilin-1 (UBQLN1), which is known to control cell cycle progression and cell growth, the proliferation associated protein 2G4 (PA2G4), a nucleolar growth-regulating protein, the secreted protein acidic and rich in cysteine (SPARC), which is regulated during embryogenesis and is involved in the control of the cell cycle and cell adhesion, and the enhancer of rudimentary homolog (ERH) that also regulates cell cycle. TAGLN and galectin 1 (Gal 1), both present in stem cells and related to differentiation, were also abundantly expressed in AF-MSCs. Other proteins expressed in high levels in AFMSCs were related to (i) development, such as Deltex-3-like (DTX3L), and (ii) cytoskeletal organization and movement, such as CFL1, the coactosin-like protein (CLP), and the enabled protein homolog (Enah). As expected, Vim was also expressed in high amounts in AF-MSCs.

We established the proteomic map of the two morphologically distinct AF mesenchymal progenitor cell types (SS and RS) by 2-DE. Twentyfive proteins were differentially expressed in the two subpopulations. Proteins upregulated in SS-AF-MSCs compared to RS-AF-MSCs included

reticulocalbin-3 precursor (RCN3), collagen α1 (I) (COL1α1), FK506-binding protein 9 precursor (FKBP9), Rho GDP-dissociation inhibitor 1 (RhoGDI), chloride intracellular channel protein 4 (CLIC4), tryptophanyl-tRNA synthetase (TrpRS), and 70 kD heat shock protein (HSP70). Peroxiredoxin 2 (Prdx2), 60 kD heat shock protein (HSP60), GSTP, and Anx4 were upregulated in RS-AFMPCs. However, proteins identified in RS-AF-MSCs only included cytokeratin-8, -18, and -19 (CK-8, -18, and CK-19), cathepsin B (CTSB), CLP, and integrin αV protein (CD51). Mesenchymal-related proteins, such as Vim, Gal, Gsn, and prohibitin (PHB), were expressed at the same levels in both populations.

Amniotic Membrane Stem Cells

A detailed approach for studying human AM proteins was described by Hopkinson et al. In this study, the authors performed a proteomic analysis of AM samples that were prepared for human transplantation, by using 2-DE gels. The wash media from the AM samples were also examined and the secreted proteins were identified. Proteins detected in both AM and the wash media suggested that partial protein release had occurred. These proteins were mostly soluble cytoplasmic proteins and were categorized according to their subcellular localization and function. One example of the most abundant and consistent proteins in AM is THBS1 which is reported to play role in wound repair, inflammatory response, and angiogenesis. Mimecan (also named osteoglycin/OGN) is another protein detected in AM that represents a small leucine-rich proteoglycan, found in the ECM of connective tissue. Mimecan is reported to maintain the tensile strength and hydration of the tissue. In addition, the larger form of mimecan was expressed in AM cells and was susceptible to proteolytic cleavage. TGF-β-induced protein ig-h3 (βIG-H3), an ECM adhesive molecule acting as a membrane-associated growth factor during cell differentiation and wound healing, and intergrin α6 (CD49f), a component of α6β4 integrin, were also present in significant amounts in AM cells. It is well known that α6β4- βIG-H3 interaction plays an important role in mediating cell adhesion and wound repair signaling pathways.

Another important study by Baharvand et al. was focused on the analysis of epithelium-denuded human AM showing both quantitative and qualitative differences compared to nontreated AM. They investigated the proteome of the human AM epithelium, which was used as a limbal stem cell niche for treating ocular surface reconstruction. 515 spots were detected in all the 2-DE gels and 43 proteins were identified using MALDI TOF/TOF MS in AM. The most abundant proteins were different isoforms of lumican (LUM) and OGN, both members of the proteoglycan (PG) family. In particular, OGN might play role in many biological processes including cell growth, angiogenesis, and inflammation. Other proteins detected included collagen VI α-1/α-2 (Col6a1/Col6a2), fibrinogen beta chain (FGB), transglutaminase 2 isoform A (TGM2A), b-actin variant (ACTB), 70 kD heat shock protein 5 (HSPA5), nidogen 2 (NID2), CD49f, βIG-H3, and tubulointerstitial nephritis (TIN). Some of the proteins identified in this study were also related to extracellular matrix (ECM). Among the detected ones, fibronectin (FN), laminins, and collagen IV (Col4) and VII were reported to promote epithelial adhesion and migration.

Secretome

Recently, significant progress has been made regarding the analysis of the secreted proteins from AFSCs. It has been documented that AFSC secretome was responsible for enhancing

vasculogenesis and was capable of evoking a strong angiogenic response in murine recipients. According to this study, a detailed analysis of the AFSC-conditioned media revealed the presence of known proangiogenic and antiangiogenic factors using Luminex's MAP Technology. Vascular endothelial growth factor (VEGF), stromal cell-derived factor 1 (SDF-1), interleukin 8 (IL-8), monocyte chemotactic protein 1 (MCP-1), and two angiogenesis inhibitors, interferon-gamma (IFNγ) and interferon gamma-induced protein 10 (IP-10), were identified as secreted proteins. It was also demonstrated that a relative small number of AFSC was enough to secrete a detectable amount of proangiogenic growth factors and cytokines. The secretion of these can be regulated in a dose-dependent manner according to the initial cell number of the cells used.

A systematic study on AFSC-secreted proteins led to the conclusion that proangiogenic soluble factors from AFSCs can mediate the recruitment of endothelial progenitors in an ischemic rat model. In particular, conditioned medium derived from AFSCs could topically deliver angiogenic growth factors and cytokines into the skin flap of the ischemic rat model and was responsible for triggering the endogenous repair by recruiting endothelial progenitor cells.

we examined the therapeutic potential of an AF-MSCs and their secreted molecules in mice with acute hepatic failure. A variety of cytokines and growth factor were detected in AF-MSC conditioned medium. Cytokines such as interleukin 10 (IL-10), interleukin 27 (IL-27), interleukin 17 family (IL-17E), interleukin 12p70 (IL-12p70), interleukin-1 beta (IL-1β), and interleukin-1 receptor antagonist (IL-1ra), responsible for inducing local and systemic downregulation of pro-inflammatory mediators, were detected. SERPINE1, MCP-1, and SDF-1, responsible for promoting tissue repair, were also secreted. Interestingly, among the highly expressed growth factors were platelet-derived endothelial cell growth factor (PD-ECGF), endostatin/collagen XVII (EN/Col17), urinary plasminogen activator (uPA), TIMP1, TIMP2, heparin-binding EGF-like growth factor (HB-EGF), fibroblast growth factor 7 (FGF7), and epidermal growth factor (EGF), responsible for liver regeneration and tissue repair.

Induced Stem Cell

Induced stem cells (iSC) are stem cells derived from somatic, reproductive, pluripotent or other cell types by deliberate epigenetic reprogramming. They are classified as either totipotent (iTC), pluripotent (iPSC) or progenitor (multipotent – iMSC, also called an induced multipotent progenitor cell – iMPC) or unipotent – (iUSC) according to their developmental potential and degree of dedifferentiation. Progenitors are obtained by so-called direct reprogramming or directed differentiation and are also called induced somatic stem cells.

Three techniques are widely recognized:

- Transplantation of nuclei taken from somatic cells into an oocyte (egg cell) lacking its own nucleus (removed in lab).
- Fusion of somatic cells with pluripotent stem cells.

- Transformation of somatic cells into stem cells, using the genetic material encoding reprogramming protein factors, recombinant proteins; microRNA, a synthetic, self-replicating polycistronic RNA and low-molecular weight biologically active substances.

Natural Processes

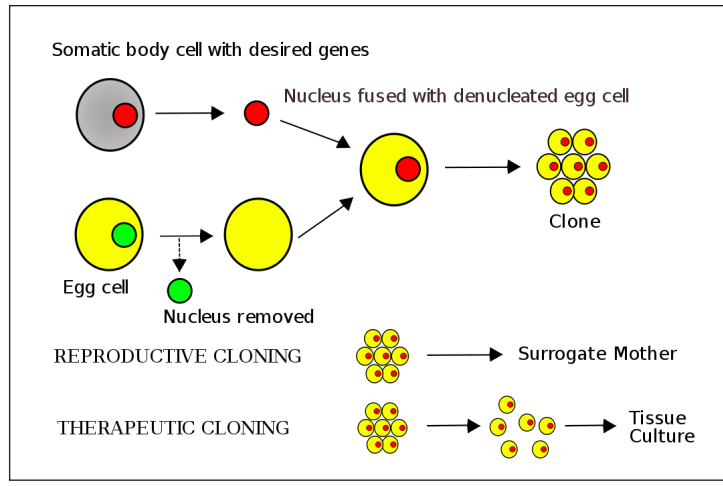
In 1895 Thomas Morgan removed one of a frog's two blastomeres and found that amphibians are able to form whole embryos from the remaining part. This meant that the cells can change their differentiation pathway. In 1924 Spemann and Mangold demonstrated the key importance of cell–cell inductions during animal development. The reversible transformation of cells of one differentiated cell type to another is called metaplasia. This transition can be a part of the normal maturation process, or caused by an inducement.

One example is the transformation of iris cells to lens cells in the process of maturation and transformation of retinal pigment epithelium cells into the neural retina during regeneration in adult newt eyes. This process allows the body to replace cells not suitable to new conditions with more suitable new cells. In Drosophila imaginal discs, cells have to choose from a limited number of standard discrete differentiation states. The fact that transdetermination (change of the path of differentiation) often occurs for a group of cells rather than single cells shows that it is induced rather than part of maturation.

The researchers were able to identify the minimal conditions and factors that would be sufficient for starting the cascade of molecular and cellular processes to instruct pluripotent cells to organize the embryo. They showed that opposing gradients of bone morphogenetic protein (BMP) and Nodal, two transforming growth factor family members that act as morphogens, are sufficient to induce molecular and cellular mechanisms required to organize, *in vivo* or *in vitro*, uncommitted cells of the zebrafish blastula animal pole into a well-developed embryo.

Some types of mature, specialized adult cells can naturally revert to stem cells. For example, "chief" cells express the stem cell marker Troy. While they normally produce digestive fluids for the stomach, they can revert into stem cells to make temporary repairs to stomach injuries, such as a cut or damage from infection. Moreover, they can make this transition even in the absence of noticeable injuries and are capable of replenishing entire gastric units, in essence serving as quiescent "reserve" stem cells. Differentiated airway epithelial cells can revert into stable and functional stem cells in vivo. After injury, mature terminally differentiated kidney cells dedifferentiate into more primordial versions of themselves and then differentiate into the cell types needing replacement in the damaged tissue Macrophages can self-renew by local proliferation of mature differentiated cells. In newts, muscle tissue is regenerated from specialized muscle cells that dedifferentiate and forget the type of cell they had been. This capacity to regenerate does not decline with age and may be linked to their ability to make new stem cells from muscle cells on demand.

A variety of nontumorigenic stem cells display the ability to generate multiple cell types. For instance, multilineage-differentiating stress-enduring (Muse) cells are stress-tolerant adult human stem cells that can self-renew. They form characteristic cell clusters in suspension culture that express a set of genes associated with pluripotency and can differentiate into endodermal, ectodermal and mesodermal cells both in vitro and in vivo.



Induced totipotent cells usually can be obtained by reprogramming somatic cells by somatic-cell nuclear transfer (SCNT).

Induced Totipotent Cells

SCNT-mediated

Induced totipotent cells can be obtained by reprogramming somatic cells with somatic-cell nuclear transfer (SCNT). The process involves sucking out the nucleus of a somatic (body) cell and injecting it into an oocyte that has had its nucleus removed.

Using an approach based on the protocol outlined by Tachibana et al., hESCs can be generated by SCNT using dermal fibroblasts nuclei from both a middle-aged 35-year-old male and an elderly, 75-year-old male, suggesting that age-associated changes are not necessarily an impediment to SCNT-based nuclear reprogramming of human cells. Such reprogramming of somatic cells to a pluripotent state holds huge potentials for regenerative medicine. Unfortunately, the cells generated by this technology, potentially are not completely protected from the immune system of the patient (donor of nuclei), because they have the same mitochondrial DNA, as a donor of oocytes, instead of the patients mitochondrial DNA. This reduces their value as a source for autologous stem cell transplantation therapy, as for the present, it is not clear whether it can induce an immune response of the patient upon treatment.

Induced androgenetic haploid embryonic stem cells can be used instead of sperm for cloning. These cells, synchronized in M phase and injected into the oocyte can produce viable offspring.

These developments, together with data on the possibility of unlimited oocytes from mitotically active reproductive stem cells, offer the possibility of industrial production of transgenic farm animals. Repeated recloning of viable mice through a SCNT method that includes a histone deacetylase inhibitor, trichostatin, added to the cell culture medium, show that it may be possible to reclone animals indefinitely with no visible accumulation of reprogramming or genomic errors. However, research into technologies to develop sperm and egg cells from stem cells raises bioethical issues.

Such technologies may also have far-reaching clinical applications for overcoming cytoplasmic defects in human oocytes. For example, the technology could prevent inherited mitochondrial

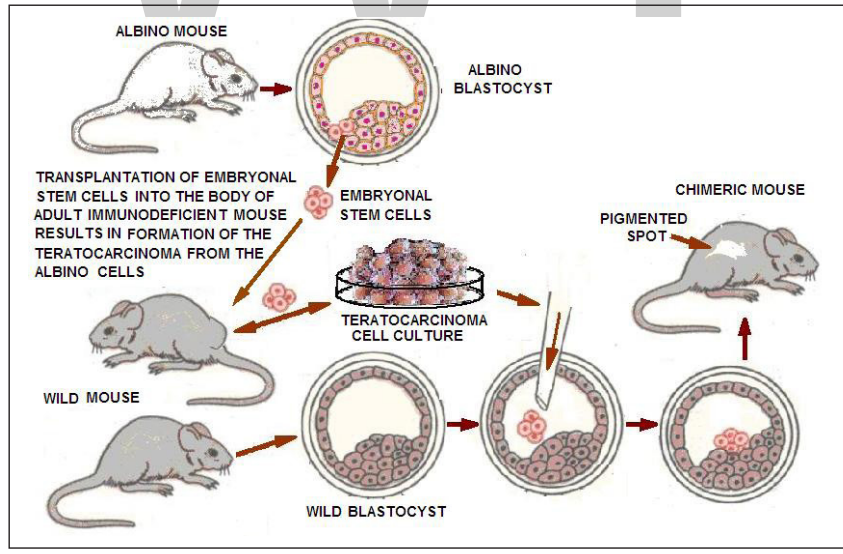
disease from passing to future generations. Mitochondrial genetic material is passed from mother to child. Mutations can cause diabetes, deafness, eye disorders, gastrointestinal disorders, heart disease, dementia and other neurological diseases. The nucleus from one human egg has been transferred to another, including its mitochondria, creating a cell that could be regarded as having two mothers. The eggs were then fertilised and the resulting embryonic stem cells carried the swapped mitochondrial DNA. As evidence that the technique is safe author of this method points to the existence of the healthy monkeys that are now more than four years old – and are the product of mitochondrial transplants across different genetic backgrounds.

In late-generation telomerase-deficient (Terc−/−) mice, SCNT-mediated reprogramming mitigates telomere dysfunction and mitochondrial defects to a greater extent than iPSC-based reprogramming.

Obtained without SCNT

Recently some researchers succeeded to get the totipotent cells without the aid of SCNT. Totipotent cells were obtained using the epigenetic factors such as oocyte germinal isoform of histone. Reprogramming in vivo, by transitory induction of the four factors Oct4, Sox2, Klf4 and c-Myc in mice, confers totipotency features. Intraperitoneal injection of such in vivo iPS cells generates embryo-like structures that express embryonic and extraembryonic (trophectodermal) markers. The developmental potential of mouse pluripotent stem cells to yield both embryonic and extra-embryonic lineages also can be expanded by microRNA miR-34a deficiency leading to strong induction of endogenous retroviruses MuERV-L (MERVL).

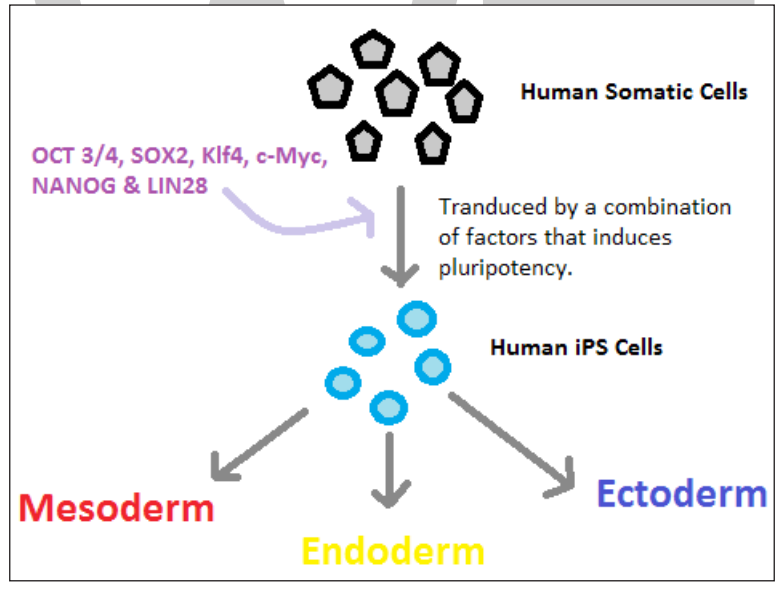
Rejuvenation to iPSCs



Transplantation of pluripotent/embryonic stem cells into the body of adult mammals, usually leads to the formation of teratomas, which can then turn into a malignant tumor teratocarcinoma. However, putting teratocarcinoma cells into the embryo at the blastocyst stage, caused them to become incorporated in the cell mass and often produced a normal healthy chimeric (i.e. composed of cells from different organisms) animal.

iPSc were first obtained in the form of transplantable teratocarcinoma induced by grafts taken from mouse embryos. Teratocarcinoma formed from somatic cells. Genetically mosaic mice were obtained from malignant teratocarcinoma cells, confirming the cells' pluripotency. It turned out that teratocarcinoma cells are able to maintain a culture of pluripotent embryonic stem cell in an undifferentiated state, by supplying the culture medium with various factors. In the 1980s, it became clear that transplanting pluripotent/embryonic stem cells into the body of adult mammals, usually leads to the formation of teratomas, which can then turn into a malignant tumor teratocarcinoma. However, putting teratocarcinoma cells into the embryo at the blastocyst stage, caused them to become incorporated in the inner cell mass and often produced a normal chimeric (i.e. composed of cells from different organisms) animal. This indicated that the cause of the teratoma is a dissonance - mutual miscommunication between young donor cells and surrounding adult cells (the recipient's so-called "niche").

In August 2006, Japanese researchers circumvented the need for an oocyte, as in SCNT. By reprograming mouse embryonic fibroblasts into pluripotent stem cells via the ectopic expression of four transcription factors, namely Oct4, Sox2, Klf4 and c-Myc, they proved that the overexpression of a small number of factors can push the cell to transition to a new stable state that is associated with changes in the activity of thousands of genes.



Human somatic cells are made pluripotent by transducing them with factors that induces pluripotency (OCT 3/4, SOX2, Klf4, c-Myc, NANOG and LIN28). This results in the production of IPS cells, which can differentiate into any cells of the three embryonic germ layers (Mesoderm, Endoderm, Ectoderm).

Reprogramming mechanisms are thus linked, rather than independent and are centered on a small number of genes. IPSC properties are very similar to ESCs. iPSCs have been shown to support the development of all-iPSC mice using a tetraploid (4n) embryo, the most stringent assay for developmental potential. However, some genetically normal iPSCs failed to produce all-iPSC mice because of aberrant epigenetic silencing of the imprinted Dlk1-Dio3 gene cluster.

An important advantage of iPSC over ESC is that they can be derived from adult cells, rather than from embryos. Therefore, it became possible to obtain iPSC from adult and even elderly patients.

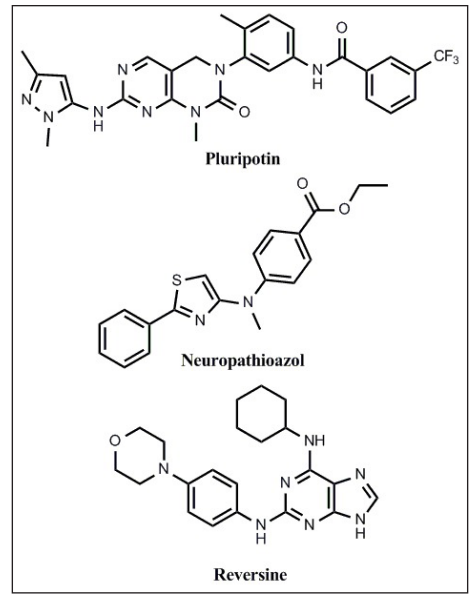
Reprogramming somatic cells to iPSC leads to rejuvenation. It was found that reprogramming leads to telomere lengthening and subsequent shortening after their differentiation back into fibroblast-like derivatives. Thus, reprogramming leads to the restoration of embryonic telomere length, and hence increases the potential number of cell divisions otherwise limited by the Hayflick limit.

However, because of the dissonance between rejuvenated cells and the surrounding niche of the recipient's older cells, the injection of his own iPSC usually leads to an immune response, which can be used for medical purposes, or the formation of tumors such as teratoma. The reason has been hypothesized to be that some cells differentiated from ESC and iPSC in vivo continue to synthesize embryonic protein isoforms. So, the immune system might detect and attack cells that are not cooperating properly.

A small molecule called MitoBloCK-6 can force the pluripotent stem cells to die by triggering apoptosis (via cytochrome c release across the mitochondrial outer membrane) in human pluripotent stem cells, but not in differentiated cells. Shortly after differentiation, daughter cells became resistant to death. When MitoBloCK-6 was introduced to differentiated cell lines, the cells remained healthy. The key to their survival, was hypothesized to be due to the changes undergone by pluripotent stem cell mitochondria in the process of cell differentiation. This ability of MitoBloCK-6 to separate the pluripotent and differentiated cell lines has the potential to reduce the risk of teratomas and other problems in regenerative medicine.

In 2012 other small molecules (selective cytotoxic inhibitors of human pluripotent stem cells – hPSCs) were identified that prevented human pluripotent stem cells from forming teratomas in mice. The most potent and selective compound of them (PluriSIn #1) inhibits stearoyl-coA desaturase (the key enzyme in oleic acid biosynthesis), which finally results in apoptosis. With the help of this molecule the undifferentiated cells can be selectively removed from culture. An efficient strategy to selectively eliminate pluripotent cells with teratoma potential is targeting pluripotent stem cell-specific antiapoptotic factor(s) (i.e., survivin or Bcl10). A single treatment with chemical survivin inhibitors (e.g., quercetin or YM155) can induce selective and complete cell death of undifferentiated hPSCs and is claimed to be sufficient to prevent teratoma formation after transplantation. However, it is unlikely that any kind of preliminary clearance, is able to secure the replanting iPSC or ESC. After the selective removal of pluripotent cells, they re-emerge quickly by reverting differentiated cells into stem cells, which leads to tumors. This may be due to the disorder of let-7 regulation of its target Nr6a1 (also known as Germ cell nuclear factor - GCNF), an embryonic transcriptional repressor of pluripotency genes that regulates gene expression in adult fibroblasts following micro-RNA miRNA loss.

Teratoma formation by pluripotent stem cells may be caused by low activity of PTEN enzyme, reported to promote the survival of a small population (0.1–5% of total population) of highly tumorigenic, aggressive, teratoma-initiating embryonic-like carcinoma cells during differentiation. The survival of these teratoma-initiating cells is associated with failed repression of Nanog as well as a propensity for increased glucose and cholesterol metabolism. These teratoma-initiating cells also expressed a lower ratio of p53/p21 when compared to non-tumorigenic cells. In connection with the above safety problems, the use iPSC for cell therapy is still limited. However, they can be used for a variety of other purposes - including the modeling of disease, screening (selective selection) of drugs, toxicity testing of various drugs.



Small molecule modulators of stem-cell fate.

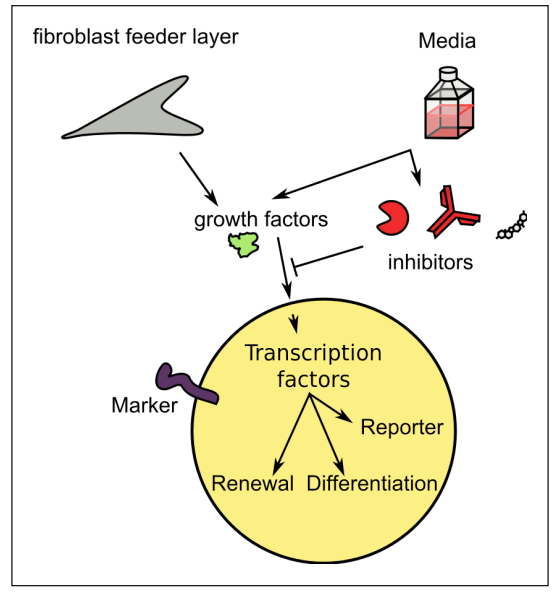
The tissue grown from iPSCs, placed in the "chimeric" embryos in the early stages of mouse development, practically do not cause an immune response (after the embryos have grown into adult mice) and are suitable for autologous transplantation At the same time, full reprogramming of adult cells in vivo within tissues by transitory induction of the four factors Oct4, Sox2, Klf4 and c-Myc in mice results in teratomas emerging from multiple organs. Furthermore, partial reprogramming of cells toward pluripotency in vivo in mice demonstrates that incomplete reprogramming entails epigenetic changes (failed repression of Polycomb targets and altered DNA methylation) in cells that drive cancer development.

Cell culture example of a small molecule as a tool instead of a protein. In cell culture to obtain a pancreatic lineage from mesodermal stem cells the retinoic acid signalling pathway must be activated while the sonic hedgehog pathway inhibited, which can be done by adding to the media anti-shh antibodies, Hedgehog interacting protein or cyclopamine, the first two are protein and the last a small molecule.

Chemical Inducement

By using solely small molecules, Deng Hongkui and colleagues demonstrated that endogenous "master genes" are enough for cell fate reprogramming. They induced a pluripotent state in adult cells from mice using seven small-molecule compounds. The effectiveness of the method is quite high: it was able to convert 0.02% of the adult tissue cells into iPSCs, which is comparable to the gene insertion conversion rate. The authors note that the mice generated from CiPSCs were "100% viable and apparently healthy for up to 6 months". So, this chemical reprogramming strategy has potential use in generating functional desirable cell types for clinical applications.

In 2015 a robust chemical reprogramming system was established with a yield up to 1,000-fold greater than that of the previously reported protocol. So, chemical reprogramming became a promising approach to manipulate cell fates.



Differentiation from Induced Teratoma

The fact that human iPSCs capable of forming teratomas not only in humans but also in some animal body, in particular in mice or pigs, allowed to develop a method for differentiation of iPSCs in vivo. For this purpose, iPSCs with an agent for inducing differentiation into target cells are injected to genetically modified pig or mouse that has suppressed immune system activation on human cells. The formed teratoma is cut out and used for the isolation of the necessary differentiated human cells by means of monoclonal antibody to tissue-specific markers on the surface of these cells. This method has been successfully used for the production of functional myeloid, erythroid and lymphoid human cells suitable for transplantation (yet only to mice). Mice engrafted with human iPSC teratoma-derived hematopoietic cells produced human B and T cells capable of functional immune responses. These results offer hope that in vivo generation of patient customized cells is feasible, providing materials that could be useful for transplantation, human antibody generation and drug screening applications. Using MitoBloCK-6 and/or PluriSIn 1 the differentiated progenitor cells can be further purified from teratoma forming pluripotent cells. The fact, that the differentiation takes place even in the teratoma niche, offers hope that the resulting cells are sufficiently stable to stimuli able to cause their transition back to the dedifferentiated (pluripotent) state and therefore safe. A similar in vivo differentiation system, yielding engraftable hematopoietic stem cells from mouse and human iPSCs in teratoma-bearing animals in combination with a maneuver to facilitate hematopoiesis, was described by Suzuki et al. They noted that neither leukemia nor tumors were observed in recipients after intravenous injection of iPSC-derived hematopoietic stem cells into irradiated recipients. Moreover, this injection resulted in multilineage and long-term reconstitution of the hematolymphopoietic system in serial transfers. Such system provides a useful tool for practical application of iPSCs in the treatment of hematologic and immunologic diseases.

For further development of this method animal in which is grown the human cell graft, for example mouse, must have so modified genome that all its cells express and have on its surface human SIRPα. To prevent rejection after transplantation to the patient of the allogenic organ or tissue, grown from the pluripotent stem cells in vivo in the animal, these cells should express two

molecules: CTLA4-Ig, which disrupts T cell costimulatory pathways and PD-L1, which activates T cell inhibitory pathway.

Differentiated Cell Types

Retinal Cells

In the near-future, clinical trials designed to demonstrate the safety of the use of iPSCs for cell therapy of the people with age-related macular degeneration, a disease causing blindness through retina damaging, will begin. There are several articles describing methods for producing retinal cells from iPSCs and how to use them for cell therapy. Reports of iPSC-derived retinal pigmented epithelium transplantation showed enhanced visual-guided behaviors of experimental animals for 6 weeks after transplantation. However, clinical trials have been successful: ten patients suffering from retinitis pigmentosa have had their eyesight restored – including a woman who had only 17 percent of her vision left.

Lung and Airway Epithelial Cells

Chronic lung diseases such as idiopathic pulmonary fibrosis and cystic fibrosis or chronic obstructive pulmonary disease and asthma are leading causes of morbidity and mortality worldwide with a considerable human, societal and financial burden. So there is an urgent need for effective cell therapy and lung tissue engineering. Several protocols have been developed for generation of the most cell types of the respiratory system, which may be useful for deriving patient-specific therapeutic cells.

Reproductive Cells

Some lines of iPSCs have the potentiality to differentiate into male germ cells and oocyte-like cells in an appropriate niche (by culturing in retinoic acid and porcine follicular fluid differentiation medium or seminiferous tubule transplantation). Moreover, iPSC transplantation make a contribution to repairing the testis of infertile mice, demonstrating the potentiality of gamete derivation from iPSCs in vivo and in vitro.

Induced Progenitor Stem Cells

Direct Transdifferentiation

The risk of cancer and tumors creates the need to develop methods for safer cell lines suitable for clinical use. An alternative approach is so-called “direct reprogramming” – transdifferentiation of cells without passing through the pluripotent state. The basis for this approach was that 5-azacytidine – a DNA demethylation reagent – can cause the formation of myogenic, chondrogenic and adipogeni clones in the immortal cell line of mouse embryonic fibroblasts and that the activation of a single gene, later named MyoD1, is sufficient for such reprogramming. Compared with iPSC whose reprogramming requires at least two weeks, the formation of induced progenitor cells sometimes occurs within a few days and the efficiency of reprogramming is usually many times higher. This reprogramming does not always require cell division. The cells resulting from such reprogramming are more suitable for cell therapy because they do not form teratomas. For example, Chandrakanthan et al., & Pimanda describe the generation of tissue-regenerative multipotent stem cells

(iMS cells) by treating mature bone and fat cells transiently with a growth factor (platelet-derived growth factor–AB (PDGF-AB)) and 5-Azacytidine. These authors claim that: "Unlike primary mesenchymal stem cells, which are used with little objective evidence in clinical practice to promote tissue repair, iMS cells contribute directly to in vivo tissue regeneration in a context-dependent manner without forming tumors" and so "has significant scope for application in tissue regeneration."

Single Transcription Factor Transdifferentiation

Originally only early embryonic cells could be coaxed into changing their identity. Mature cells are resistant to changing their identity once they've committed to a specific kind. However, brief expression of a single transcription factor, the ELT-7 GATA factor, can convert the identity of fully differentiated, specialized non-endodermal cells of the pharynx into fully differentiated intestinal cells in intact larvae and adult roundworm *Caenorhabditis elegans* with no requirement for a dedifferentiated intermediate.

Transdifferentiation with CRISPR-mediated Activator

The cell fate can be effectively manipulated by epigenome editing. In particular, by directly activating of specific endogenous gene expression with CRISPR-mediated activator. When dCas9 (that has been modified so that it no longer cuts DNA, but still can be guided to specific sequences and to bind to them) is combined with transcription activators, it can precisely manipulate endogenous gene expression. Using this method, Wei et al., enhanced the expression of endogenous Cdx2 and Gata6 genes by CRISPR-mediated activators, thus directly converted mouse embryonic stem cells into two extraembryonic lineages, i.e., typical trophoblast stem cells and extraembryonic endoderm cells. An analogous approach was used to induce activation of the endogenous Brn2, Ascl1, and Myt1l genes to convert mouse embryonic fibroblasts to induced neuronal cells. Thus, transcriptional activation and epigenetic remodeling of endogenous master transcription factors are sufficient for conversion between cell types. The rapid and sustained activation of endogenous genes in their native chromatin context by this approach may facilitate reprogramming with transient methods that avoid genomic integration and provides a new strategy for overcoming epigenetic barriers to cell fate specification.

Phased Process Modeling Regeneration

Another way of reprogramming is the simulation of the processes that occur during amphibian limb regeneration. In urodele amphibians, an early step in limb regeneration is skeletal muscle fiber dedifferentiation into a cellulate that proliferates into limb tissue. However, sequential small molecule treatment of the muscle fiber with myoseverin, reversine (the aurora B kinase inhibitor) and some other chemicals: BIO (glycogen synthase-3 kinase inhibitor), lysophosphatidic acid (pleiotropic activator of G-protein-coupled receptors), SB203580 (p38 MAP kinase inhibitor), or SQ22536 (adenylyl cyclase inhibitor) causes the formation of new muscle cell types as well as other cell types such as precursors to fat, bone and nervous system cells.

Antibody-based Transdifferentiation

The researchers discovered that GCSF-mimicking antibody can activate a growth-stimulating receptor on marrow cells in a way that induces marrow stem cells that normally develop into white

blood cells to become neural progenitor cells. The technique enables researchers to search large libraries of antibodies and quickly select the ones with a desired biological effect.

Reprograming by Bacteria

The human gastrointestinal tract is colonized by a vast community of symbionts and commensals. The researchers demonstrate the phenomenon of somatic cell reprograming by bacteria and generation of multipotential cells from adult human dermal fibroblast cells by incorporating Lactic acid bacteria. This cellular transdifferentiation is caused by ribosomes and "can occur via donor bacteria that are swallowed and digested by host cells, which may induce ribosomal stress and stimulate cellular developmental plasticity."

Conditionally Reprogrammed Cells

Schlegel and Liu demonstrated that the combination of feeder cells and a Rho kinase inhibitor (Y-27632) induces normal and tumor epithelial cells from many tissues to proliferate indefinitely in vitro. This process occurs without the need for transduction of exogenous viral or cellular genes. These cells have been termed "Conditionally Reprogrammed Cells (CRC)". The induction of CRCs is rapid and results from reprogramming of the entire cell population. CRCs do not express high levels of proteins characteristic of iPSCs or embryonic stem cells (ESCs) (e.g., Sox2, Oct4, Nanog, or Klf4). This induction of CRCs is reversible and removal of Y-27632 and feeders allows the cells to differentiate normally. CRC technology can generate 2×10^6 cells in 5 to 6 days from needle biopsies and can generate cultures from cryopreserved tissue and from fewer than four viable cells. CRCs retain a normal karyotype and remain nontumorigenic. This technique also efficiently establishes cell cultures from human and rodent tumors.

The ability to rapidly generate many tumor cells from small biopsy specimens and frozen tissue provides significant opportunities for cell-based diagnostics and therapeutics (including chemosensitivity testing) and greatly expands the value of biobanking. Using CRC technology, researchers were able to identify an effective therapy for a patient with a rare type of lung tumor. Engleman's group describes a pharmacogenomic platform that facilitates rapid discovery of drug combinations that can overcome resistance using CRC system. In addition, the CRC method allows for the genetic manipulation of epithelial cells ex vivo and their subsequent evaluation in vivo in the same host. While initial studies revealed that co-culturing epithelial cells with Swiss 3T3 cells J2 was essential for CRC induction, with transwell culture plates, physical contact between feeders and epithelial cells is not required for inducing CRCs and more importantly that irradiation of the feeder cells is required for this induction. Consistent with the transwell experiments, conditioned medium induces and maintains CRCs, which is accompanied by a concomitant increase of cellular telomerase activity. The activity of the conditioned medium correlates directly with radiation-induced feeder cell apoptosis. Thus, conditional reprogramming of epithelial cells is mediated by a combination of Y-27632 and a soluble factor(s) released by apoptotic feeder cells.

Riegel et al. demonstrate that mouse ME cells isolated from normal mammary glands or from mouse mammary tumor virus (MMTV)-Neu–induced mammary tumors, can be cultured indefinitely as conditionally reprogrammed cells (CRCs). Cell surface progenitor-associated markers are rapidly induced in normal mouse ME-CRCs relative to ME cells. However, the expression of

certain mammary progenitor subpopulations, such as $CD49f^+$ ESA^+ $CD44^+$, drops significantly in later passages. Nevertheless, mouse ME-CRCs grown in a three-dimensional extracellular matrix gave rise to mammary acinar structures. ME-CRCs isolated from MMTV-Neu transgenic mouse mammary tumors express high levels of HER2/neu, as well as tumor-initiating cell markers, such as $CD44^+$, $CD49f^+$ and ESA^+ (EpCam). These patterns of expression are sustained in later CRC passages. Early and late passage ME-CRCs from MMTV-Neu tumors that were implanted in the mammary fat pads of syngeneic or nude mice developed vascular tumors that metastasized within 6 weeks of transplantation. Importantly, the histopathology of these tumors was indistinguishable from that of the parental tumors that develop in the MMTV-Neu mice. Application of the CRC system to mouse mammary epithelial cells provides an attractive model system to study the genetics and phenotype of normal and transformed mouse epithelium in a defined culture environment and in vivo transplant studies.

A different approach to CRC is to inhibit CD47 – a membrane protein that is the thrombospondin-1 receptor. Loss of CD47 permits sustained proliferation of primary murine endothelial cells, increases asymmetric division and enables these cells to spontaneously reprogram to form multipotent embryoid body-like clusters. CD47 knockdown acutely increases mRNA levels of c-Myc and other stem cell transcription factors in cells in vitro and in vivo. Thrombospondin-1 is a key environmental signal that inhibits stem cell self-renewal via CD47. Thus, CD47 antagonists enable cell self-renewal and reprogramming by overcoming negative regulation of c-Myc and other stem cell transcription factors. In vivo blockade of CD47 using an antisense morpholino increases survival of mice exposed to lethal total body irradiation due to increased proliferative capacity of bone marrow-derived cells and radioprotection of radiosensitive gastrointestinal tissues.

Lineage-specific Enhancers

Differentiated macrophages can self-renew in tissues and expand long-term in culture. Under certain conditions macrophages can divide without losing features they have acquired while specializing into immune cells – which is usually not possible with differentiated cells. The macrophages achieve this by activating a gene network similar to one found in embryonic stem cells. Single-cell analysis revealed that, *in vivo*, proliferating macrophages can derepress a macrophage-specific enhancer repertoire associated with a gene network controlling self-renewal. This happened when concentrations of two transcription factors named MafB and c-Maf were naturally low or were inhibited for a short time. Genetic manipulations that turned off MafB and c-Maf in the macrophages caused the cells to start a self-renewal program. The similar network also controls embryonic stem cell self-renewal but is associated with distinct embryonic stem cell-specific enhancers.

Hence macrophages isolated from MafB- and c-Maf-double deficient mice divide indefinitely; the self-renewal depends on c-Myc and Klf4.

Indirect Lineage Conversion

Indirect lineage conversion is a reprogramming methodology in which somatic cells transition through a plastic intermediate state of partially reprogrammed cells (pre-iPSC), induced by brief exposure to reprogramming factors, followed by differentiation in a specially developed chemical environment (artificial niche).

This method could be both more efficient and safer, since it does not seem to produce tumors or other undesirable genetic changes and results in much greater yield than other methods. However, the safety of these cells remains questionable. Since lineage conversion from pre-iPSC relies on the use of iPSC reprogramming conditions, a fraction of the cells could acquire pluripotent properties if they do not stop the de-differentation process in vitro or due to further de-differentiation in vivo.

Outer Membrane Glycoprotein

A common feature of pluripotent stem cells is the specific nature of protein glycosylation of their outer membrane. That distinguishes them from most nonpluripotent cells, although not white blood cells. The glycans on the stem cell surface respond rapidly to alterations in cellular state and signaling and are therefore ideal for identifying even minor changes in cell populations. Many stem cell markers are based on cell surface glycan epitopes including the widely used markers SSEA-3, SSEA-4, Tra 1-60 and Tra 1-81. Suila Heli et al. speculate that in human stem cells extracellular O-GlcNAc and extracellular O-LacNAc, play a crucial role in the fine tuning of Notch signaling pathway - a highly conserved cell signaling system, that regulates cell fate specification, differentiation, left–right asymmetry, apoptosis, somitogenesis, angiogenesis and plays a key role in stem cell proliferation.

Changes in outer membrane protein glycosylation are markers of cell states connected in some way with pluripotency and differentiation. The glycosylation change is apparently not just the result of the initialization of gene expression, but perform as an important gene regulator involved in the acquisition and maintenance of the undifferentiated state.

For example, activation of glycoprotein ACA, linking glycosylphosphatidylinositol on the surface of the progenitor cells in human peripheral blood, induces increased expression of genes Wnt, Notch-1, BMI1 and HOXB4 through a signaling cascade PI3K/Akt/mTor/PTEN and promotes the formation of a self-renewing population of hematopoietic stem cells.

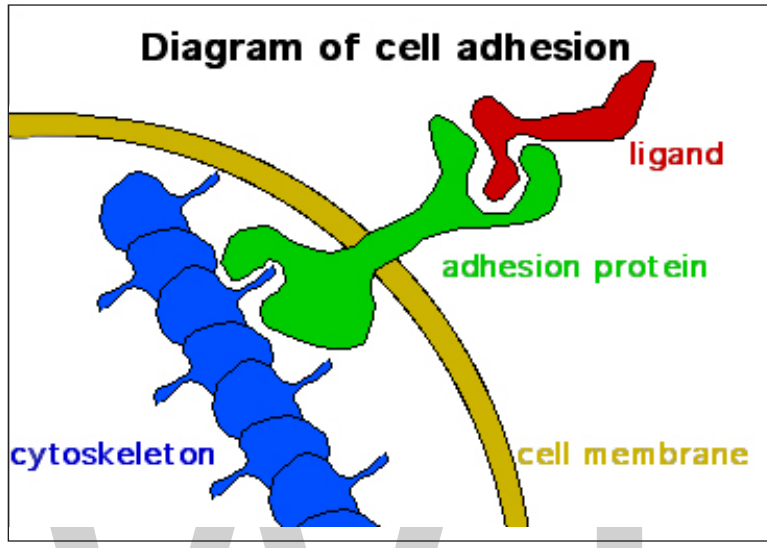
Furthermore, dedifferentiation of progenitor cells induced by ACA-dependent signaling pathway leads to ACA-induced pluripotent stem cells, capable of differentiating in vitro into cells of all three germ layers. The study of lectins' ability to maintain a culture of pluripotent human stem cells has led to the discovery of lectin Erythrina crista-galli (ECA), which can serve as a simple and highly effective matrix for the cultivation of human pluripotent stem cells.

Reprogramming through a Physical Approach

Cell adhesion protein E-cadherin is indispensable for a robust pluripotent phenotype. During reprogramming for iPS cell generation, N-cadherin can replace function of E-cadherin. These functions of cadherins are not directly related to adhesion because sphere morphology helps maintaining the "stemness" of stem cells. Moreover, sphere formation, due to forced growth of cells on a low attachment surface, sometimes induces reprogramming. For example, neural progenitor cells can be generated from fibroblasts directly through a physical approach without introducing exogenous reprogramming factors.

Physical cues, in the form of parallel microgrooves on the surface of cell-adhesive substrates, can replace the effects of small-molecule epigenetic modifiers and significantly improve reprogramming efficiency. The mechanism relies on the mechanomodulation of the cells' epigenetic state.

Specifically, “decreased histone deacetylase activity and upregulation of the expression of WD repeat domain 5 (WDR5) – a subunit of H3 methyltranferase – by microgrooved surfaces lead to increased histone H3 acetylation and methylation”. Nanofibrous scaffolds with aligned fibre orientation produce effects similar to those produced by microgrooves, suggesting that changes in cell morphology may be responsible for modulation of the epigenetic state.



Role of cell adhesions in neural development.

Substrate rigidity is an important biophysical cue influencing neural induction and subtype specification. For example, soft substrates promote neuroepithelial conversion while inhibiting neural crest differentiation of hESCs in a BMP4-dependent manner. Mechanistic studies revealed a multi-targeted mechanotransductive process involving mechanosensitive Smad phosphorylation and nucleocytoplasmic shuttling, regulated by rigidity-dependent Hippo/YAP activities and actomyosin cytoskeleton integrity and contractility.

Mouse embryonic stem cells (mESCs) undergo self-renewal in the presence of the cytokine leukemia inhibitory factor (LIF). Following LIF withdrawal, mESCs differentiate, accompanied by an increase in cell–substratum adhesion and cell spreading. Restricted cell spreading in the absence of LIF by either culturing mESCs on chemically defined, weakly adhesive biosubstrates, or by manipulating the cytoskeleton allowed the cells to remain in an undifferentiated and pluripotent state. The effect of restricted cell spreading on mESC self-renewal is not mediated by increased intercellular adhesion, as inhibition of mESC adhesion using a function blocking anti E-cadherin antibody or siRNA does not promote differentiation. Possible mechanisms of stem cell fate predetermination by physical interactions with the extracellular matrix have been described.

A new method has been developed that turns cells into stem cells faster and more efficiently by ‘squeezing’ them using 3D microenvironment stiffness and density of the surrounding gel. The technique can be applied to a large number of cells to produce stem cells for medical purposes on an industrial scale.

Cells involved in the reprogramming process change morphologically as the process proceeds. This results in physical difference in adhesive forces among cells. Substantial differences in ‘adhesive signature’ between pluripotent stem cells, partially reprogrammed cells, differentiated progeny

and somatic cells allowed to develop separation process for isolation of pluripotent stem cells in microfluidic devices, which is:

- Fast (separation takes less than 10 minutes);
- Efficient (separation results in a greater than 95 percent pure iPS cell culture);
- Innocuous (cell survival rate is greater than 80 percent and the resulting cells retain normal transcriptional profiles, differentiation potential and karyotype).

Stem cells possess mechanical memory (they remember past physical signals) – with the Hippo signaling pathway factors: Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding domain (TAZ) acting as an intracellular mechanical rheostat—that stores information from past physical environments and influences the cells' fate.

Neural Stem Cells

Stroke and many neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis need cell replacement therapy. The successful use of converted neural cells (cNs) in transplantations open a new avenue to treat such diseases. Nevertheless, induced neurons (iNs), directly converted from fibroblasts are terminally committed and exhibit very limited proliferative ability that may not provide enough autologous donor cells for transplantation. Self-renewing induced neural stem cells (iNSCs) provide additional advantages over iNs for both basic research and clinical applications.

For example, under specific growth conditions, mouse fibroblasts can be reprogrammed with a single factor, Sox2, to form iNSCs that self-renew in culture and after transplantation can survive and integrate without forming tumors in mouse brains. INSCs can be derived from adult human fibroblasts by non-viral techniques, thus offering a safe method for autologous transplantation or for the development of cell-based disease models.

Neural chemically induced progenitor cells (ciNPCs) can be generated from mouse tail-tip fibroblasts and human urinary somatic cells without introducing exogenous factors, but - by a chemical cocktail, namely VCR (V, VPA, an inhibitor of HDACs; C, CHIR99021, an inhibitor of GSK-3 kinases and R, RepSox, an inhibitor of TGF beta signaling pathways), under a physiological hypoxic condition. Alternative cocktails with inhibitors of histone deacetylation, glycogen synthase kinase and TGF-β pathways (where: sodium butyrate (NaB) or Trichostatin A (TSA) could replace VPA, Lithium chloride (LiCl) or lithium carbonate (Li_2CO_3) could substitute CHIR99021, or Repsox may be replaced with SB-431542 or Tranilast) show similar efficacies for ciNPC induction. Zhang, et al., also report highly efficient reprogramming of mouse fibroblasts into induced neural stem cell-like cells (ciNSLCs) using a cocktail of nine components.

Multiple methods of direct transformation of somatic cells into induced neural stem cells have been described.

Proof of principle experiments demonstrate that it is possible to convert transplanted human fibroblasts and human astrocytes directly in the brain that are engineered to express inducible forms of neural reprogramming genes, into neurons, when reprogramming genes (Ascl1, Brn2a and Myt1l) are activated after transplantation using a drug.

Astrocytes – the most common neuroglial brain cells, which contribute to scar formation in response to injury – can be directly reprogrammed in vivo to become functional neurons that formed networks in mice without the need of cell transplantation. The researchers followed the mice for nearly a year to look for signs of tumor formation and reported finding none. The same researchers have turned scar-forming astrocytes into progenitor cells called neuroblasts that regenerated into neurons in the injured adult spinal cord.

Oligodendrocyte Precursor Cells

Without myelin to insulate neurons, nerve signals quickly lose power. Diseases that attack myelin, such as multiple sclerosis, result in nerve signals that cannot propagate to nerve endings and as a consequence lead to cognitive, motor and sensory problems. Transplantation of oligodendrocyte precursor cells (OPCs), which can successfully create myelin sheaths around nerve cells, is a promising potential therapeutic response. Direct lineage conversion of mouse and rat fibroblasts into oligodendroglial cells provides a potential source of OPCs. Conversion by forced expression of both eight or of the three transcription factors Sox10, Olig2 and Zfp536, may provide such cells.

Cardiomyocytes

Cell-based in vivo therapies may provide a transformative approach to augment vascular and muscle growth and to prevent non-contractile scar formation by delivering transcription factors or microRNAs to the heart. Cardiac fibroblasts, which represent 50% of the cells in the mammalian heart, can be reprogrammed into cardiomyocyte-like cells in vivo by local delivery of cardiac core transcription factors (GATA4, MEF2C, TBX5 and for improved reprogramming plus ESRRG, MESP1, Myocardin and ZFPM2) after coronary ligation. These results implicated therapies that can directly remuscularize the heart without cell transplantation. However, the efficiency of such reprogramming turned out to be very low and the phenotype of received cardiomyocyte-like cells does not resemble those of a mature normal cardiomyocyte. Furthermore, transplantation of cardiac transcription factors into injured murine hearts resulted in poor cell survival and minimal expression of cardiac genes.

Meanwhile, advances in the methods of obtaining cardiac myocytes in vitro occurred. Efficient cardiac differentiation of human iPS cells gave rise to progenitors that were retained within infarcted rat hearts and reduced remodeling of the heart after ischemic damage.

The team of scientists, who were led by Sheng Ding, used a cocktail of nine chemicals (9C) for transdifferentiation of human skin cells into beating heart cells. With this method, more than 97% of the cells began beating, a characteristic of fully developed, healthy heart cells. The chemically induced cardiomyocyte-like cells (ciCMs) uniformly contracted and resembled human cardiomyocytes in their transcriptome, epigenetic, and electrophysiological properties. When transplanted into infarcted mouse hearts, 9C-treated fibroblasts were efficiently converted to ciCMs and developed into healthy-looking heart muscle cells within the organ. This chemical reprogramming approach, after further optimization, may offer an easy way to provide the cues that induce heart muscle to regenerate locally.

In another study, ischemic cardiomyopathy in the murine infarction model was targeted by iPS cell transplantation. It synchronized failing ventricles, offering a regenerative strategy to achieve

resynchronization and protection from decompensation by dint of improved left ventricular conduction and contractility, reduced scarring and reversal of structural remodelling. One protocol generated populations of up to 98% cardiomyocytes from hPSCs simply by modulating the canonical Wnt signaling pathway at defined time points in during differentiation, using readily accessible small molecule compounds.

Discovery of the mechanisms controlling the formation of cardiomyocytes led to the development of the drug ITD-1, which effectively clears the cell surface from TGF-β receptor type II and selectively inhibits intracellular TGF-β signaling. It thus selectively enhances the differentiation of uncommitted mesoderm to cardiomyocytes, but not to vascular smooth muscle and endothelial cells.

One project seeded decellularized mouse hearts with human iPSC-derived multipotential cardiovascular progenitor cells. The introduced cells migrated, proliferated and differentiated in situ into cardiomyocytes, smooth muscle cells and endothelial cells to reconstruct the hearts. In addition, the heart's extracellular matrix (the substrate of heart scaffold) signalled the human cells into becoming the specialised cells needed for proper heart function. After 20 days of perfusion with growth factors, the engineered heart tissues started to beat again and were responsive to drugs.

Reprogramming of cardiac fibroblasts into induced cardiomyocyte-like cells (iCMs) *in situ* represents a promising strategy for cardiac regeneration. Mice exposed *in vivo*, to three cardiac transcription factors GMT (Gata4, Mef2c, Tbx5) and the small-molecules: SB-431542 (the transforming growth factor (TGF)-β inhibitor), and XAV939 (the WNT inhibitor) for 2 weeks after myocardial infarction showed significantly improved reprogramming (reprogramming efficiency increased eight-fold) and cardiac function compared to those exposed to only GMT.

Rejuvenation of the Muscle Stem Cell

The elderly often suffer from progressive muscle weakness and regenerative failure owing in part to elevated activity of the p38α and p38β mitogen-activated kinase pathway in senescent skeletal muscle stem cells. Subjecting such stem cells to transient inhibition of p38α and p38β in conjunction with culture on soft hydrogel substrates rapidly expands and rejuvenates them that result in the return of their strength.

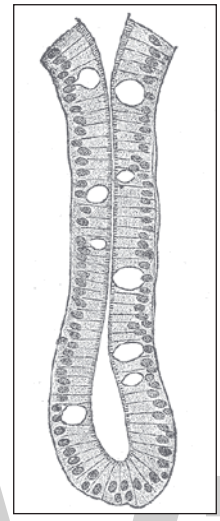
In geriatric mice, resting satellite cells lose reversible quiescence by switching to an irreversible pre-senescence state, caused by derepression of p16INK4a (also called Cdkn2a). On injury, these cells fail to activate and expand, even in a youthful environment. p16INK4a silencing in geriatric satellite cells restores quiescence and muscle regenerative functions.

Myogenic progenitors for potential use in disease modeling or cell-based therapies targeting skeletal muscle could also be generated directly from induced pluripotent stem cells using free-floating spherical culture (EZ spheres) in a culture medium supplemented with high concentrations (100 ng/ml) of fibroblast growth factor-2 (FGF-2) and epidermal growth factor.

Hepatocytes

Unlike current protocols for deriving hepatocytes from human fibroblasts, Saiyong Zhu et al., did not generate iPSCs but, using small molecules, cut short reprogramming to pluripotency to generate an induced multipotent progenitor cell (iMPC) state from which endoderm progenitor cells

and subsequently hepatocytes (iMPC-Heps) were efficiently differentiated. After transplantation into an immune-deficient mouse model of human liver failure, iMPC-Heps proliferated extensively and acquired levels of hepatocyte function similar to those of human primary adult hepatocytes. iMPC-Heps did not form tumours, most probably because they never entered a pluripotent state.



An intestinal crypt - an accessible and abundant source of intestinal epithelial cells for conversion into β-like cells.

These results establish the feasibility of significant liver repopulation of mice with human hepatocytes generated in vitro, which removes a long-standing roadblock on the path to autologous liver cell therapy.

Cocktail of small molecules, Y-27632, A-83-01 (a TGFβ kinase/activin receptor like kinase (ALK5) inhibitor), and CHIR99021 (potent inhibitor of GSK-3), can convert rat and mouse mature hepatocytes in vitro into proliferative bipotent cells – CLiPs (chemically induced liver progenitors). CLiPs can differentiate into both mature hepatocytes and biliary epithelial cells that can form functional ductal structures. In long-term culture CLiPs did not lose their proliferative capacity and their hepatic differentiation ability, and can repopulate chronically injured liver tissue.

Insulin-producing Cells

Complications of Diabetes mellitus such as cardiovascular diseases, retinopathy, neuropathy, nephropathy and peripheral circulatory diseases depend on sugar dysregulation due to lack of insulin from pancreatic beta cells and can be lethal if they are not treated. One of the promising approaches to understand and cure diabetes is to use pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced PCSs (iPSCs). Unfortunately, human PSC-derived insulin-expressing cells resemble human fetal β cells rather than adult β cells. In contrast to adult β cells, fetal β cells seem functionally immature, as indicated by increased basal glucose secretion and lack of glucose stimulation and confirmed by RNA-seq of whose transcripts.

An alternative strategy is the conversion of fibroblasts towards distinct endodermal progenitor cell populations and, using cocktails of signalling factors, successful differentiation of these endodermal progenitor cells into functional beta-like cells both in vitro and in vivo.

Overexpression of the three transcription factors, PDX1 (required for pancreatic bud outgrowth and beta-cell maturation), NGN3 (required for endocrine precursor cell formation) and MAFA (for beta-cell maturation) combination (called PNM) can lead to the transformation of some cell types into a beta cell-like state. An accessible and abundant source of functional insulin-producing cells is intestine. PMN expression in human intestinal "organoids" stimulates the conversion of intestinal epithelial cells into β-like cells possibly acceptable for transplantation.

Nephron Progenitors

Adult proximal tubule cells were directly transcriptionally reprogrammed to nephron progenitors of the embryonic kidney, using a pool of six genes of instructive transcription factors (SIX1, SIX2, OSR1, Eyes absent homolog 1(EYA1), Homeobox A11 (HOXA11) and Snail homolog 2(SNAI2)) that activated genes consistent with a cap mesenchyme/nephron progenitor phenotype in the adult proximal tubule cell line. The generation of such cells may lead to cellular therapies for adult renal disease. Embryonic kidney organoids placed into adult rat kidneys can undergo onward development and vascular development.

Blood Vessel Cells

As blood vessels age, they often become abnormal in structure and function, thereby contributing to numerous age-associated diseases including myocardial infarction, ischemic stroke and atherosclerosis of arteries supplying the heart, brain and lower extremities. So, an important goal is to stimulate vascular growth for the collateral circulation to prevent the exacerbation of these diseases. Induced Vascular Progenitor Cells (iVPCs) are useful for cell-based therapy designed to stimulate coronary collateral growth. They were generated by partially reprogramming endothelial cells. The vascular commitment of iVPCs is related to the epigenetic memory of endothelial cells, which engenders them as cellular components of growing blood vessels. That is why, when iVPCs were implanted into myocardium, they engrafted in blood vessels and increased coronary collateral flow better than iPSCs, mesenchymal stem cells, or native endothelial cells.

Ex vivo genetic modification can be an effective strategy to enhance stem cell function. For example, cellular therapy employing genetic modification with Pim-1 kinase (a downstream effector of Akt, which positively regulates neovasculogenesis) of bone marrow–derived cells or human cardiac progenitor cells, isolated from failing myocardium results in durability of repair, together with the improvement of functional parameters of myocardial hemodynamic performance.

Stem cells extracted from fat tissue after liposuction may be coaxed into becoming progenitor smooth muscle cells (iPVSMCs) found in arteries and veins.

The 2D culture system of human iPS cells in conjunction with triple marker selection (CD34 (a surface glycophosphoprotein expressed on developmentally early embryonic fibroblasts), NP1 (receptor – neuropilin 1) and KDR (kinase insert domain-containing receptor)) for the isolation of vasculogenic precursor cells from human iPSC, generated endothelial cells that, after transplantation, formed stable, functional mouse blood vessels in vivo, lasting for 280 days.

To treat infarction, it is important to prevent the formation of fibrotic scar tissue. This can be achieved in vivo by transient application of paracrine factors that redirect native heart progenitor stem cell contributions from scar tissue to cardiovascular tissue. For example, in a mouse

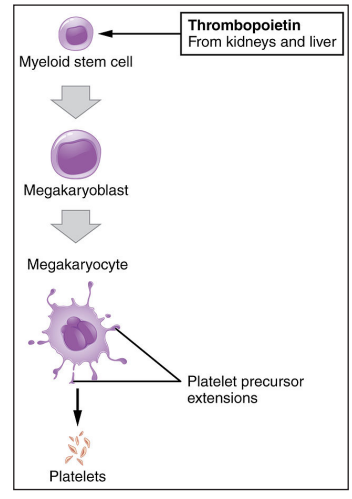
myocardial infarction model, a single intramyocardial injection of human vascular endothelial growth factor A mRNA (VEGF-A modRNA), modified to escape the body's normal defense system, results in long-term improvement of heart function due to mobilization and redirection of epicardial progenitor cells toward cardiovascular cell types.

Blood Stem Cells

Red Blood Cells

RBC transfusion is necessary for many patients. However, to date the supply of RBCs remains labile. In addition, transfusion risks infectious disease transmission. A large supply of safe RBCs generated in vitro would help to address this issue. Ex vivo erythroid cell generation may provide alternative transfusion products to meet present and future clinical requirements. Red blood cells (RBC)s generated in vitro from mobilized CD34 positive cells have normal survival when transfused into an autologous recipient. RBC produced in vitro contained exclusively fetal hemoglobin (HbF), which rescues the functionality of these RBCs. In vivo the switch of fetal to adult hemoglobin was observed after infusion of nucleated erythroid precursors derived from iPSCs. Although RBCs do not have nuclei and therefore can not form a tumor, their immediate erythroblasts precursors have nuclei. The terminal maturation of erythroblasts into functional RBCs requires a complex remodeling process that ends with extrusion of the nucleus and the formation of an enucleated RBC. Cell reprogramming often disrupts enucleation. Transfusion of in vitro-generated RBCs or erythroblasts does not sufficiently protect against tumor formation.

The aryl hydrocarbon receptor (AhR) pathway (which has been shown to be involved in the promotion of cancer cell development) plays an important role in normal blood cell development. AhR activation in human hematopoietic progenitor cells (HPs) drives an unprecedented expansion of HPs, megakaryocyte- and erythroid-lineage cells. The SH2B3 gene encodes a negative regulator of cytokine signaling and naturally occurring loss-of-function variants in this gene increase RBC counts in vivo. Targeted suppression of SH2B3 in primary human hematopoietic stem and progenitor cells enhanced the maturation and overall yield of in-vitro-derived RBCs. Moreover, inactivation of SH2B3 by CRISPR/Cas9 genome editing in human pluripotent stem cells allowed enhanced erythroid cell expansion with preserved differentiation.



Platelets extruded from megakaryocytes.

Platelets

Platelets help prevent hemorrhage in thrombocytopenic patients and patients with thrombocythemia. A significant problem for multitransfused patients is refractoriness to platelet transfusions. Thus, the ability to generate platelet products ex vivo and platelet products lacking HLA antigens in serum-free media would have clinical value. An RNA interference-based mechanism used a lentiviral vector to express short-hairpin RNAi targeting β2-microglobulin transcripts in CD34-positive cells. Generated platelets demonstrated an 85% reduction in class I HLA antigens. These platelets appeared to have normal function in vitro.

One clinically-applicable strategy for the derivation of functional platelets from human iPSC involves the establishment of stable immortalized megakaryocyte progenitor cell lines (imMKCLs) through doxycycline-dependent overexpression of BMI1 and BCL-XL. The resulting imMKCLs can be expanded in culture over extended periods (4–5 months), even after cryopreservation. Halting the overexpression (by the removal of doxycycline from the medium) of c-MYC, BMI1 and BCL-XL in growing imMKCLs led to the production of $CD42b^+$ platelets with functionality comparable to that of native platelets on the basis of a range of assays in vitro and in vivo. Thomas et al., describe a forward programming strategy relying on the concurrent exogenous expression of 3 transcription factors: GATA1, FLI1 and TAL1. The forward programmed megakaryocytes proliferate and differentiate in culture for several months with megakaryocyte purity over 90% reaching up to $2x10^5$ mature megakaryocytes per input hPSC. Functional platelets are generated throughout the culture allowing the prospective collection of several transfusion units from as few as one million starting hPSCs.

Immune Cells

A specialised type of white blood cell, known as cytotoxic T lymphocytes (CTLs), are produced by the immune system and are able to recognise specific markers on the surface of various infectious or tumour cells, causing them to launch an attack to kill the harmful cells. Thence, immunotherapy with functional antigen-specific T cells has potential as a therapeutic strategy for combating many cancers and viral infections. However, cell sources are limited, because they are produced in small numbers naturally and have a short lifespan.

A potentially efficient approach for generating antigen-specific CTLs is to revert mature immune T cells into iPSCs, which possess indefinite proliferative capacity in vitro and after their multiplication to coax them to redifferentiate back into T cells.

Another method combines iPSC and chimeric antigen receptor (CAR) technologies to generate human T cells targeted to CD19, an antigen expressed by malignant B cells, in tissue culture. This approach of generating therapeutic human T cells may be useful for cancer immunotherapy and other medical applications.

Invariant natural killer T (iNKT) cells have great clinical potential as adjuvants for cancer immunotherapy. iNKT cells act as innate T lymphocytes and serve as a bridge between the innate and acquired immune systems. They augment anti-tumor responses by producing interferon-gamma (IFN-γ). The approach of collection, reprogramming/dedifferentiation, re-differentiation and injection has been proposed for related tumor treatment.

Dendritic cells (DC) are specialized to control T-cell responses. DC with appropriate genetic modifications may survive long enough to stimulate antigen-specific CTL and after that be completely eliminated. DC-like antigen-presenting cells obtained from human induced pluripotent stem cells can serve as a source for vaccination therapy.

CCAAT/enhancer binding protein-α (C/EBPα) induces transdifferentiation of B cells into macrophages at high efficiencies and enhances reprogramming into iPS cells when co-expressed with transcription factors Oct4, Sox2, Klf4 and Myc. with a 100-fold increase in iPS cell reprogramming efficiency, involving 95% of the population. Furthermore, C/EBPa can convert selected human B cell lymphoma and leukemia cell lines into macrophage-like cells at high efficiencies, impairing the cells' tumor-forming capacity.

Thymic Epithelial Cells Rejuvenation

The thymus is the first organ to deteriorate as people age. This shrinking is one of the main reasons the immune system becomes less effective with age. Diminished expression of the thymic epithelial cell transcription factor FOXN1 has been implicated as a component of the mechanism regulating age-related involution.

Clare Blackburn and colleagues show that established age-related thymic involution can be reversed by forced upregulation of just one transcription factor – FOXN1 in the thymic epithelial cells in order to promote rejuvenation, proliferation and differentiation of these cells into fully functional thymic epithelium. This rejuvenation and increased proliferation was accompanied by upregulation of genes that promote cell cycle progression (cyclin D1, ΔNp63, FgfR2IIIb) and that are required in the thymic epithelial cells to promote specific aspects of T cell development (Dll4, Kitl, Ccl25, Cxcl12, Cd40, Cd80, Ctsl, Pax1).

Mesenchymal Stem Cells

Induction

Mesenchymal stem/stromal cells (MSCs) are under investigation for applications in cardiac, renal, neural, joint and bone repair, as well as in inflammatory conditions and hemopoietic cotransplantation. This is because of their immunosuppressive properties and ability to differentiate into a wide range of mesenchymal-lineage tissues. MSCs are typically harvested from adult bone marrow or fat, but these require painful invasive procedures and are low-frequency sources, making up only 0.001–0.01% of bone marrow cells and 0.05% in liposuction aspirates. Of concern for autologous use, in particular in the elderly most in need of tissue repair, MSCs decline in quantity and quality with age.

IPSCs could be obtained by the cells rejuvenation of even centenarians. Because iPSCs can be harvested free of ethical constraints and culture can be expanded indefinitely, they are an advantageous source of MSCs. IPSC treatment with SB-431542 leads to rapid and uniform MSC generation from human iPSCs. (SB-431542 is an inhibitor of activin/TGF- pathways by blocking phosphorylation of ALK4, ALK5 and ALK7 receptors.) These iPS-MSCs may lack teratoma-forming ability, display a normal stable karyotype in culture and exhibit growth and differentiation characteristics that closely resemble those of primary MSCs. It has potential for in vitro scale-up, enabling MSC-based therapies. MSC derived from iPSC have the capacity to aid periodontal regeneration and are a promising source of readily accessible stem cells for use in the clinical treatment of periodontitis.

Lai et al., & Lu report the chemical method to generate MSC-like cells (iMSCs), from human primary dermal fibroblasts using six chemical inhibitors (SP600125, SB202190, G06983, Y-27632, PD0325901, and CHIR99021) with or without 3 growth factors (transforming growth factor-β (TGF-β), basic fibroblast growth factor (bFGF), and leukemia inhibitory factor (LIF)). The chemical cocktail directly converts human fibroblasts to iMSCs with a monolayer culture in 6 days, and the conversion rate was approximately 38%.

Besides cell therapy in vivo, the culture of human mesenchymal stem cells can be used in vitro for mass-production of exosomes, which are ideal vehicles for drug delivery.

Dedifferentiated Adipocytes

Adipose tissue, because of its abundance and relatively less invasive harvest methods, represents a source of mesenchymal stem cells (MSCs). Unfortunately, liposuction aspirates are only 0.05% MSCs. However, a large amount of mature adipocytes, which in general have lost their proliferative abilities and therefore are typically discarded, can be easily isolated from the adipose cell suspension and dedifferentiated into lipid-free fibroblast-like cells, named dedifferentiated fat (DFAT) cells. DFAT cells re-establish active proliferation ability and express multipotent capacities. Compared with adult stem cells, DFAT cells show unique advantages in abundance, isolation and homogeneity. Under proper induction culture in vitro or proper environment in vivo, DFAT cells could demonstrate adipogenic, osteogenic, chondrogenic and myogenic potentials. They could also exhibit perivascular characteristics and elicit neovascularization.

Chondrogenic Cells

Cartilage is the connective tissue responsible for frictionless joint movement. Its degeneration ultimately results in complete loss of joint function in the late stages of osteoarthritis. As an avascular and hypocellular tissue, cartilage has a limited capacity for self-repair. Chondrocytes are the only cell type in cartilage, in which they are surrounded by the extracellular matrix that they secrete and assemble.

One method of producing cartilage is to induce it from iPS cells. Alternatively, it is possible to convert fibroblasts directly into induced chondrogenic cells (iChon) without an intermediate iPS cell stage, by inserting three reprogramming factors (c-MYC, KLF4 and SOX9). Human iChon cells expressed marker genes for chondrocytes (type II collagen) but not fibroblasts.

Implanted into defects created in the articular cartilage of rats, human iChon cells survived to form cartilaginous tissue for at least four weeks, with no tumors. The method makes use of c-MYC, which is thought to have a major role in tumorigenesis and employs a retrovirus to introduce the reprogramming factors, excluding it from unmodified use in human therapy.

Sources of Cells for Reprogramming

The most frequently used sources for reprogramming are blood cells and fibroblasts, obtained by biopsy of the skin, but taking cells from urine is less invasive. The latter method does not require a biopsy or blood sampling. As of 2013, urine-derived stem cells had been differentiated into endothelial, osteogenic, chondrogenic, adipogenic, skeletal myogenic and neurogenic lineages, without

forming teratomas. Therefore, their epigenetic memory is suited to reprogramming into iPS cells. However, few cells appear in urine, only low conversion efficiencies had been achieved and the risk of bacterial contamination is relatively high.

Another promising source of cells for reprogramming are mesenchymal stem cells derived from human hair follicles.

The origin of somatic cells used for reprogramming may influence the efficiency of reprogramming, the functional properties of the resulting induced stem cells and the ability to form tumors.

IPSCs retain an epigenetic memory of their tissue of origin, which impacts their differentiation potential. This epigenetic memory does not necessarily manifest itself at the pluripotency stage – iPSCs derived from different tissues exhibit proper morphology, express pluripotency markers and are able to differentiate into the three embryonic layers in vitro and in vivo. However, this epigenetic memory may manifest during re-differentiation into specific cell types that require the specific loci bearing residual epigenetic marks.

Dental Pulp Stem Cell

Dental pulp stem cells (DPSCs) are stem cells present in the dental pulp, which is the soft living tissue within teeth. They are pluripotent, as they can form embryoid body-like structures (EBs) *in vitro* and teratoma-like structures that contained tissues derived from all three embryonic germ layers when injected in nude mice. DPSCs can differentiate *in vitro* into tissues that have similar characteristics to mesoderm, endoderm and ectoderm layers. DPSCs were found to be able to differentiate into adipocytes and neural-like cells. These cells can be obtained from postnatal teeth, wisdom teeth, and deciduous teeth, providing researchers with a non-invasive method of extracting stem cells. As a result, DPSCs have been thought of as an extremely promising source of cells used in endogenous tissue engineering.

Studies have shown that the proliferation rate of DPSCs is 30% higher than in other stem cells, such as bone marrow stromal stem cells (BMSSCs). These characteristics of DPSCs are mainly due to the fact that they exhibit elevated amounts of cell cycling molecules, one being cyclin-dependent kinase 6 (CDK6), present in the dental pulp tissue. Additionally, DPSCs have displayed lower immunogenicity than MSCs.

Atari et al., established a protocol for isolating and identifying the subpopulations of dental pulp pluripotent-like stem cells (DPPSC). These cells are $SSEA4^+$, $OCT3/4^+$, $NANOG^+$, $SOX2^+$, $LIN28^+$, $CD13^+$, $CD105^+$, CD34-, CD45-, $CD90^+$, $CD29^+$, $CD73^+$, $STRO1^+$, and CD146-, and they show genetic stability in vitro based on genomic analysis with a newly described CGH technique.

Role in Regenerative Dentistry

The human mouth is vulnerable to craniofacial defects, microbial attacks, and traumatic damages. Although preclinical and clinical partial regeneration of dental tissues has shown success, the creation of an entire tooth from DPSCs is not yet possible.

Distraction Osteogenesis

Distraction osteogenesis (DO) is a method of bone regeneration, commonly used in the surgical repair of large craniofacial defects. The area in which the defect is present is purposely broken in surgery, allowed to heal briefly, and then the bone segments are gradually separated until the area has healed satisfactorily. A study conducted in 2018 by Song et al. found that DPSCs transfected with Sirtuin-1 (SIRT1) in rabbits were more effective in promoting bone formation during DO. SIRT1-modified DPSCs accumulated significantly higher levels of calcium after osteogenic differentiation *in vitro,* suggesting the potential role of DPSCs in enhancing the efficiency of DO.

Calcined Tooth Powder

Calcine tooth powder (CTP) is obtained by burning extracted teeth, destroying the potential infection-causing material within the tooth, resulting in tooth ash Tooth ash has been shown to promote bone repair. Although recent studies have shown that calcine tooth powder- culture media (CTP-CM) does not affect proliferation, they have shown that CTP-CM has significantly increased levels of osteo/odontogenic markers in DPSCs.

Stem Cells from Human Exfoliated Deciduous Teeth

Stem cells from human exfoliated deciduous teeth (SHED) are similar to DPSCs in the sense that they are both derived from the dental pulp, but SHED are derived from baby teeth, whereas DPSCs are derived from adult teeth. SHED are a population of multipotent stem cells that are easily collected, as deciduous teeth either shed naturally or are physically removed in order to facilitate the proper growth of permanent teeth. These cells can differentiate into osteocytes, adipocytes, odontoblast, and chondrocytes *in vitro*. Recent work has shown the enhanced proliferative capabilities of SHED when compared with that of dental pulp stem cells.

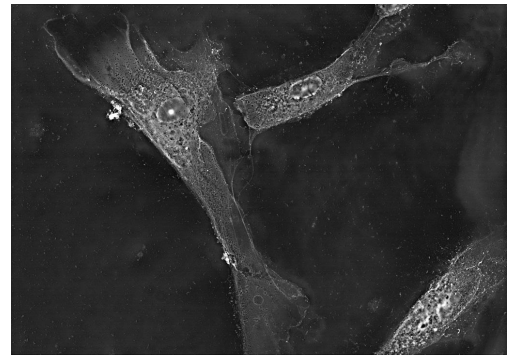
Potential Therapeutic use of SHED

Studies have shown that under the influence of oxidative stress, SHED (OST-SHED) displayed increased levels of neuronal protection. The properties of these cells exhibited in this study suggest that OST-SHED could potentially prevent of oxidative stress-induced brain damage and could aid in the development of therapeutic tools for neurodegenerative disorders. After SHED injection into Goto-Kakizaki rats, type II diabetes mellitus (T2DM) was ameliorated, suggesting the potential for SHED in T2DM therapies.

Recent studies have also shown that the administration of SHED in mice ameliorated the T cell immune imbalance in allergic rhinitis (AR), suggesting the cells' potential in future AR treatments. After introducing SHED, mice experienced reduced nasal symptoms and decreased inflammatory infiltration. SHEDs were found to inhibit the proliferation of T lymphocytes, increase levels of an anti-inflammatory cytokine, IL-10, and decrease the levels of a pro-inflammatory cytokine, IL-4.

Additionally, SHED can potentially treat liver cirrhosis. In a study conducted by Yokoyama et al. (2019), SHED were differentiated into hepatic stellate cells. They found that when hepatic cells derived from SHED were transplanted into the liver of rats, liver fibrosis was terminated, allowing for the healing of the liver structure.

Limbal Stem Cell



Limbal stem cells.

Limbal stem cells, also known as corneal epithelial stem cells, are stem cells located in the basal epithelial layer of the corneal limbus. They form the border between the cornea and the sclera. Characteristics of limbal stem cells include a slow turnover rate, high proliferative potential, clonogenicity, expression of stem cell markers, as well as the ability to regenerate the entire corneal epithelium. Limbal stem cell proliferation has the role of maintaining the cornea; for example, by replacing cells that are lost via tears. Additionally, these cells also prevent the conjunctival epithelial cells from migrating onto the surface of the cornea.

Medical Conditions and Treatments

Damage to the limbus can lead to limbal stem cell deficiency (LSCD); this may be primary - related to an insufficient stromal microenvironment to support stem cell functions, such as aniridia, and other congenital conditions, or secondary – caused by external factors that destroy the limbal stem cells, such as chemical or thermal burns, radiation, surgery, infection, use of contact lenses, or certain drugs. Signs and symptoms include : conjunctivalisation, corneal vascularisation, edema, ocular discomfort or pain, visual impairment, and blindness, which are likely associated with failure in the process of regenerating the corneal epithelium.

Immediate management aims to limit traumatic or chemical damage to the limbus, control inflammation, and help achieve a healthy corneal epithelium. Initial treatment after trauma/injury includes preservative-free artificial tears, topical steroids, 'bandage' contact lenses, and autologous eye drops (eye drops manufactured from the patient's own blood serum and plasma). Once the corneal surface has stabilized, surgery is the main approach to treatment.

Types of surgeries:

- In the case of a partial LSCD: A sequential sector conjunctival epitheliectomy (SSCE) can be performed to remove any tissue (pannus) that has grown over the cornea. This procedure is sometimes used as a temporary measure until further surgical interventions are possible.
- Transplantation of amniotic membrane from a placenta may also help. Although amniotic membrane does not have stem cells of its own, it supports regeneration of limbal stem cells. However, further surgical intervention may be needed if these approaches are unsuccessful, or when disease is more severe.

- Conjunctival limbal autograft (CLAU) involves transplantation of limbal tissue from a patient's healthy eye. As the procedure is achieved by transplanting autologous limbal stem cells from the patient's healthy eye, there is no risk of immune rejection, and hence no need for systemic immunosuppression. However, this procedure represents a risk for the donor eye, as the patient already has one eye damaged.
- In the case of bilateral LSCD, where both eyes are affected, it may be possible to transplant limbal tissue from a living donor (usually a relative). This is known as a conjunctival limbal allograft (CLAL). CLAL can be performed with both partial or total LSCD, the donor tissue is usually from a sibling or parent. As with CLAU, only a part of the donor limbus can be transplanted, as a live donor is being used. Being an allogenic transplant, immunosuppression is required, due to the risk of rejection.
- Kerato-limbal allograft (KLAL) involves transplantation from someone who has died and donated their organs. KLAL can be used for cases of bilateral LSCD when a living related donor is not available, or for patients with unilateral LSCD, who don't want to jeopardise their healthy eye. However, most of these types of transplant fail within five years. KLAL has a number of limitations: the graft is usually up to 24 hours old before retrieval and a further period of time is often required to screen the cadaver's blood before the tissue can be used; often the limbus is found to be damaged as the tissue is not immunocompatible, there is a high risk of rejection between the recipient and the donor cadaver and studies report only a temporary success in term of transplant effectiveness, with most failing after 5 years.
- A recent procedure, less invasive than CLAU, which so far has been tested only in unilateral cases, is simple limbal epithelial transplantation (SLET). In this procedure, healthy limbal tissue from the patient's good eye is cut into a number of pieces and transferred directly to human amniotic membrane covering the cornea in the damaged eye. Studies published so far have only investigated the procedure in unilaterally affected patients, and the long-term effectiveness of the technique is yet to be proven.
- Another recent innovation is cultivated limbal epithelial transplant (CLET), either autologous (where donor and recipient are the same patient) or allogenic (where donor and recipient are different patients). This approach can be used when either one or both eyes are affected, providing there is sufficient limbal tissue available (1–2 mm^2). A small sample of limbal cells is taken from a healthy part of the eye, and grown in a sterile laboratory to produce a sheet of cells sufficient for transplantation. Once transplanted, they multiply and regrow the corneal epithelium. The manufacturing process is designed to ensure implantation of the right number, size and quality of cells. CLET avoids some of the issues faced by other limbal transplantation procedures and does not pose a threat to the integrity of the donor eye. It also offers the possibility of re-grafting in case of failure of the first graft or need for a further graft.

Types

There are three types of clonogenic keratinocytes involved in the generation of the corneal epithelium: holoclones, meroclones and paraclones. 1- Holoclones: as true stem cells, have the greatest growth potential, and give rise to 2-meroclones, which have a much lower proliferative capacity,

but frequently divide. 3- Paraclones have even lower proliferative capacity. Both meroclones and paraclones are known as transient amplifying cells and their purpose is to form a stratified squamous epithelium. All three types of keratinocytes are present in the basal layer of the limbus, with holoclones in the least abundance (10%–15%). The basal layer of the cornea is populated by meroclones and paraclones at the periphery, and only paraclones in the central cornea, reflecting the above process of cell division and differentiation. Holoclones are identified by high expression of the marker p63 and are also known as p63 bright cells.

Renal Stem Cell

Renal stem cells are self-renewing, multipotent stem cells which are able to give rise to all the cell types of the kidney. It is involved in the homeostasis and repair of the kidney, and holds therapeutic potential for treatment of kidney failure.

Structure

Strong evidence suggests that renal stem cells are located in the renal papilla. Using stain-retaining assay (with bromodeoxyuridine, or BrdU), a low-cycling cell population was found in the papillary region, which was able to divide rapidly to repair the damaged caused by transcient renal ischemia. These cells are able to incorporate into other renal tissues, and was able to repeatedly form spheres in 3D cultures, and clonal analysis also exhibited its multipotency.

Other reports have suggested the renal tubule and renal capsule to be the site of stem cells. The renal capsule contain stain-retaining cells which exhibited markers for mesenchymal stem cells; after their removal, recovery was significantly slower post-ischemic injury. These evidence suggests a stem cell population exists within the renal capsule.

Development

Using *in vivo* lineage tracing techniques, *Lgr5*$^+$ cells were found to contribute to the nephron, specifically to the ascending limb of the loop of Henle and the distal convoluted tubule. Thus, *Lgr5*$^+$ cells can potentially be a marker for renal stem and/or progenitor cells.

Clinical Significance

There is much debate regarding the cells involved in repair after injury; while some suggests that stem cells are the sole driving force of repair, others suggests that cells dedifferentiate after damage to act like stem cells. Alternately, it was also reported that differentiated tubular epithelial cells are the driving mechanism for regeneration after injury, using proliferative expansion as the mechanism.

Multipotent mouse kidney progenitor cells (MKPC) were obtained from Myh9 targeted mutant mice. Injection of MKPC into mice post-ischemic injury saw the MKPC regenerating different cell lineages and was able to regenerate renal function and enhanced survival.

Renal Induced Pluripotent Stem Cells

It has been reported that endogenous kidney tubular renal epithelial cells can be dedifferentiated into induced pluripotent stem cells by the treatment of only two factors - *Oct4* and *Sox2*.

Epiblast-derived Stem Cell

After the blastocyst stage, once an embryo implanted in endometrium (in case of rodent), the inner cell mass (ICM) of a fertilized embryo segregates into two layers: hypoblast and epiblast. The epiblast cells are the functional progenitors of soma and germ cells which later differentiate into three layers: definitive endoderm, mesoderm and ectoderm. Stem cells derived from epiblast are pluripotent. These cells are called epiblast-derived stem cells (EpiSC) and have several different cellular and molecular characteristics with Embryonic Stem Cells (ESC). Pluripotency in EpiSC is essentially different from that of embryonic stem cells. The pluripotency of EpiSC is primed pluripotency: primed to differentiate into specific cell linages. Naïve pluripotent stem cells (e.g. ESC) and primed pluripotent stem cells (e.g. EpiSC) not only sustain the ability to self-renew but also maintain the capacity to differentiate. Since the cell status is primed to differentiate in EpiSC, however, one copy of the X chromosome in XX cells (female cells) in EpiSC is silenced (XaXi). EpiSC is unable to colonize and is not available to be used to produce chimeras. Conversely, XX cells in ESC are both active and can produce chimera when inserted into a blastocyst. Both ESC and EpiSC induce teratoma when injected in the test animals (scid mice) which proves pluripotency. EpiSC display several distinctive characteristics distinct from ESC. The cellular status of human ESC (hESC) is similar to primed state mouse stem cells rather than Naïve state.

Table: Comparison of Naïve and Primed pluripotent states.

Property	Naive State	Primed State
Embryonic tissue	Early epiblast	Egg cylinder or embryonic disc
Culture stem cell	Rodent ESCs	Rodent EpiSCs; primate “ESCs”
Blatocyst chimaeras	Yes	No
Teratomas	Yes	Yes
Pluripotency factors	Oct4, Nanog, Sox2, Klf2, Klf4	Oct4, Sox2, Nanog
Naive markers	Rex1, Nr0b1, Fgf4	Absent
Specification markers	Absent	Fgf5, T
Response to Lif/Stat3	Self-renewal	None
Response to Fgf/Erk	Differentiation	Self-renewal
Clonogenicity	High	Low
XX status	Both active	One X inactive

Differentiating Naïve pluripotent Stem cells into primed pluripotent stem cells (e.g. adding activin and fibroblast growth factor (FGF) in the culture medium) can be accomplished but reprogramming of Primed cells into Naïve cells is more difficult. Several approaches to reprogramming

EpiSC to achieve Naïve pluripotency have been applied. One of those methods is transfecting the primed pluripotent stem cell with a reprogramming factor, Klf4).

The reversion back to the naive-state has also been achieved by suppressing the activity of the histone methyltransferase MLL1, also known as KMT2A. The inhibition of MLL1 via the small-molecule inhibitor MM-401 in EpiSC showed marked increase in alkaline phosphatase staining as well as upregulation of "naive" markers such as Rex1 and downregulation of "primed" markers such as FGF5. Moreover, beyond the potency-state comparison, MLL1 inhibition was also shown to reactivate the silenced X-chromosome which is typically deactivated in post-implantation epiblast stem cells, suggesting an epigenetic reversion back to a more ground-level, naive state. What's more, some EpiSCs affected by the MLL1 inhibition-induced reversion were able to contribute to germline-competent chimeras, which had been considered as one of the most major differences between ESCs and EpiSCs.

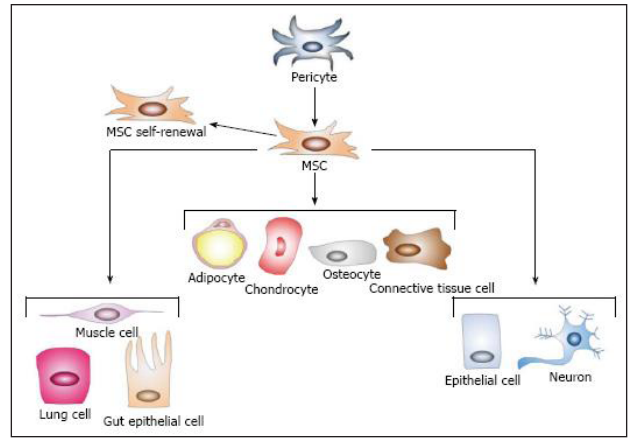
EpiLC

Scientists have been able to demonstrate the induction of EpiSC-like cells in vitro from mouse ESCs, which are referred to as Epiblast-like cells (EpiLC). Many studies have used EpiLC as suitable analogues for actual post-implantation derived epiblast stem cells, especially in attempts at reversion back to the "naive" state. Recently, overexpression of PR-domain Zinc Finger Protein 14 (PRDM14) in EpiLC was shown to cause a reversion back to an ESC-like state (with levels of Alkaline Phosphatase staining recovered to that observed in ESCs as well as more ESC-like cell morphology), with Klf2 being required for the mechanism to occur. It has been proposed that PRDM14 induces this state by activating Klf2 via active demethylation recruitment of Oct-4; such technique has yet to be demonstrated in actual epiblast-derived EpiSCs.

Mesenchymal Stem Cell

Mesenchymal stem cells (MSCs) are pleiotropic population of cells that are self-renewing and capable of differentiating into canonical cells of the mesenchyme, including adipocytes, chondrocytes, and osteocytes. They employ multi-faceted approaches to maintain bone marrow niche homeostasis and promote wound healing during injury.

MSCs were originally discovered in the 1950s as the longest surviving cells of human and mouse bone marrow monolayer cell cultures. Friedenstein et allater noted that these fibroblastic cells were very rare in the bone marrow. Over time in culture, these sparse colony-forming units divided prolifically and gave rise to expanded populations of fibroblastic clones. These spindle-shaped, fibroblastic cells were plastic adherent and were named MSCs as they could be induced in vitro and in vivo to differentiate into adipocytes, chondrocytes, connective stromal cells, and osteocytes-cells which all comprise the mesenchyme. MSC differentiation into parenchymal cells of the mesenchyme has become one of the principal criteria of establishing their identity. Additional, though controversial, reports indicate that MSCs may also be induced to transdifferentiate into cells of the endoderm (lung cells, muscle cells, and gut epithelial cells) and the ectoderm (epithelia and neurons).



In figure, basic properties of mesenchymal stem cells is shown. Mesenchymal stem cells (MSCs) are a heterogeneous population of stromal cells thought to be derived from pericytes. These cells are defined by self-renewal and the ability to differentiate into the mesodermal cells (solid lines): adipocytes, chondrocytes, osteocytes, and connective tissue cells. Though controversial (dotted lines), they may also transdifferentiate into cells of the endoderm (lung, muscle, and gut epithelial cells) and of the ectoderm (neurons and epithelial cells).

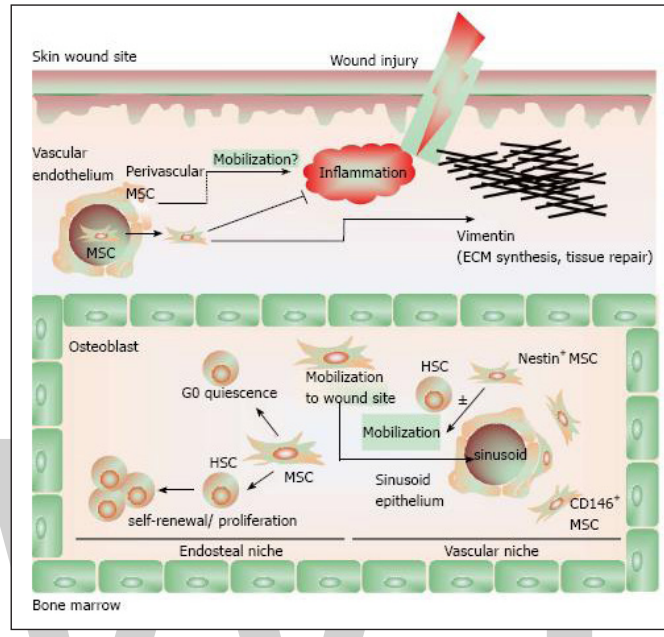
The pleiotropic nature of MSCs has presented a challenge in their identification. Their functional characteristics of self-renewal and ability to differentiate along with some widely accepted markers together form a profile to help identify them. There is consensus that MSCs, though heterogeneous, share some common features: they are uniformly negative for the expression of key hematopoietic cell markers, including CD34, CD45, CD11b, CD11c, CD14, CD19, CD79α, CD86, and MHC class II molecules. They express CD90, CD105, CD44, CD73, CD9, and very low levels of CD80. The International Society for Cellular Therapy has designated this expression pattern as the minimal criteria for human MSC discretion, but marker expression panels for MSCs continue to be updated over time.

Though MSCs were first isolated from the bone marrow, they have since been harvested from the stroma of multiple organs and tissues, including adipose, tonsils, umbilical cord, skin, and dental pulp. MSCs derived from the marrow continue to be the most frequently studied. The cellular and tissue origins of MSCs have been elusive, but in one landmark study, Crisan and colleagues suggested a pericytic origin for MSCs. Pericytes are perivascular cells that inhabit multiple organ systems. This group identified pericytes on the basis of CD146, NG2, and PDGF-Rβ expression from human skeletal muscle, pancreas, adipose tissue, and placenta. They found that these cells expressed markers typical of MSCs and could be differentiated in culture to become myocytes, osteocytes, chondrocytes, and adipocytes. Though the study did not directly track the possible in vivo transition of pericytes to MSCs, they identified pericytes as potential progenitor cells to non-bone marrow-derived MSCs.

The Physiology of MSCs

MSCs strategically form niches in perivascular spaces in almost every region of the body. It is thought that such localization allows them to detect local and distant tissue damage, as in wound infliction, and respond by migration to these sites and promoting tissue repair and

healing. While myriad studies show that exogenously administered MSCs migrate to healthy organs or to injured sites for inflammation suppression and wound healing, there has been sparse data to actually demonstrate in vivo mobilization of endogenous MSCs to sites of injury or participation in the wound healing process, due in part to lack of unique markers expressed by MSCs.



The biology of mesenchymal stem cells. In the bone marrow, mesenchymal stem cells (MSCs) aid in constructing the endosteal niche and regulate the homeostasis of HSCs. MSCs maintain HSCs in a state of quiescence defined by self-renewal and proliferation without differentiation. $CD146^+$ MSCs in the vascular niche also maintain HSC homeostasis and, along with $Nestin^+$ MSCs, regulate the mobilization of HSC into the vascular system. In response to inflammatory cues and chemokine gradients, MSCs mobilize out of the bone marrow and to peripheral sites of injury, where they suppress inflammation to facilitate wound healing. MSCs contribute to tissue reconstruction with the production and deposition of vimentin. In is incompletely understood whether perivascular MSCs may also migrate to sites of injury to contribute to wound healing.

One of the most insightful reports to address this issue utilizes a natural transplantation model of feto-maternal microchimerism, in which chimeric MSCs take up residence in maternal bone marrow in every pregnancy. Imp (It is seen that collagen-I-promoter-driven, GFP^+ MSCs derived from transgenic fetuses homed to wounds inflicted on mothers in as early as 24 h post-infliction exhibited a fibroblastic appearance, and were marked by vimentin expression, which is indicative of extracellular matrix synthesis and tissue repair. These data implicate endogenous MSCs as capable of travel from the bone marrow to wound sites for healing purposes.

Beyond their role in tissue repair and wound healing, MSCs of the perivascular niche in the bone marrow construct and maintain the hematopoietic stem cell (HSC) microenvironment. MSCs have been demonstrated to migrate and situate in the bone marrow compartment in NOD-SCID mice and differentiate into pericytes, myofibroblasts, endothelia, stromal cells,

osteocytes, and osteoblasts. In bone marrow sinusoids, $CD146^+$ MSCs are thought to create the structural framework of the hematopoietic microenvironment, as they are capable of generating this environment at heterotopic sites, along with the establishment of subendothelial cells, upon transfer to miniature bone organs. These subendothelial cells are important producers of angiopoeitin-1, which is known to contribute to HSC sustenance. MSCs in the vicinity that express Nestin are spatially associated with HSCs and may be the primary cells controlling their homeostasis. $Nestin^+$ MSCs produce high levels of HSC-maintenance factors, including CXCL-12, c-kit ligand, angiopoietin-1, IL-7, vascular cell adhesion molecule-1 (VCAM-1), and osteopontin. When HSC mobilization out of marrow is required, these MSCs down-regulate HSC maintenance genes. In response to parathyroid hormone treatment, which promotes osteoblast differentiation and HSC expansion, $Nestin^+$ MSCs proliferate and become primed towards osteoblastogenesis. When purified HSCs are transferred to lethally irradiated mice, they only efficiently home to bone marrow that is populated with $Nestin^+$ MSCs. In addition, osteoblasts derived from $Nestin^+$ MSCs form the endosteal niche that lines the surface of the trabecular bone. This niche, in concert with that formed by perivascular MSCs, regulates HSC survival, proliferation, and quiescent maintenance in the G_0 state.

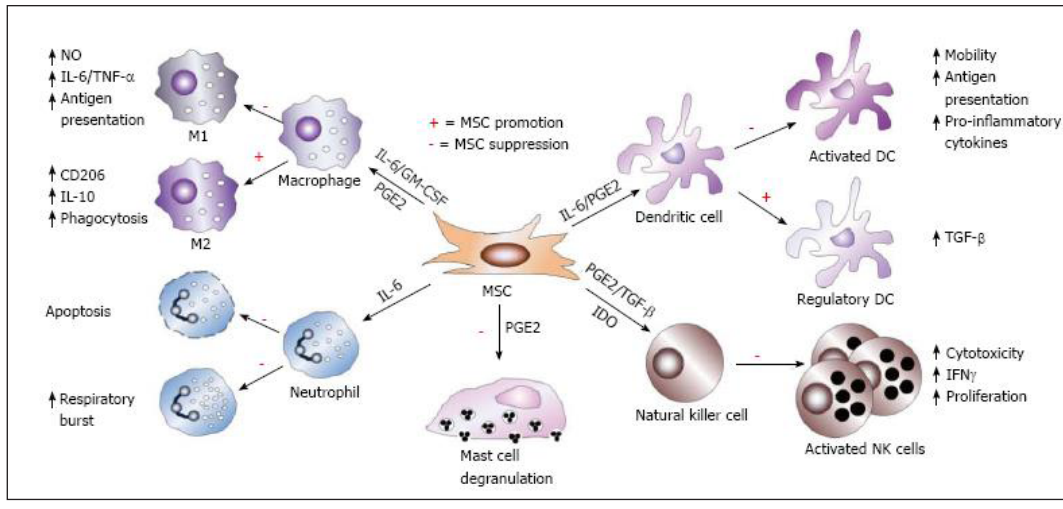
Mscs and Immunosuppression

Interest in Immuno-modulatory Properties of MSCs

A key method by which MSCs and their stromal derivatives guard the HSC microenvironment is by protecting the niche from inflammatory insults, which could cause inadvertent HSC differentiation and reserve depletion. MSC-derived fibroblasts, which also populate the HSC niche, may exert an anti-inflammatory effect by eliminating survival factors for immune cells, such as T cells, and re-calibrating chemokine gradients, as has been studied in the context of fibroblast dysfunction in the chronic autoimmune disease rheumatoid arthritis. This could promote T cell apoptosis and re-direction out of the initial site of inflammation to allow for tissue repair. In addition, MSCs and their derivatives from multiple normal sites within the body, including chondrocytes and fibroblasts from synovial joints, lungs, and skin, suppressed activated T cell proliferation and their cytokine production. MSCs may even influence T cell proliferation indirectly, as splenic stromal cells can induce nitric oxide (NO)-producing dendritic cell (DC) generation in a fibronectin-dependent fashion; these immune-regulatory DCs suppress T cell proliferation. Moreover, it is well-established that wound inflictions trigger MSC migration and suppression of inflammation to permit the proliferation of tissue-resident stromal cells, production of reconstructive molecules of the ECM, and wound healing.

Mechanisms of MSC Suppression of Innate Immune Cells

The discovery of anti-inflammatory properties of MSCs led to investigation of their use as immunosuppressive agents. Innate immune cells have important roles in tissue homeostasis and are the first line of defense against invading pathogens such as viruses and bacteria. Cells of this system respond to pathogens rapidly and do so in a relatively non-specific manner, generally responding to pathogens as a class as opposed to pathogen subtypes and strains. These cells express a multitude of pattern recognition receptors to which they can detect pathogen-associated molecular patterns and respond accordingly.



Mesenchymal stem cell immunosuppression of innate immune cells. Mesenchymal stem cells (MSCs) utilize diverse molecular mechanisms to suppress innate immune cells. MSCs suppress macrophage polarization to M1, though favors M2 polarization. MSCs inhibit mast cell degranulation of histamine-containing granules and inhibit NK cell and DC activation, differentiation, and effector functions. MSC-derived PGE2 contributes to all of these effects. MSC-produced IL-6 suppresses neutrophil apoptosis and respiratory burst and also contributes to inhibition of DC function. In the presence of IL-6 and GM-CSF, MSCs also affect macrophage function, while TGF-β and IDO suppress NK cell function. In addition, MSCs also favor the generation of regulatory DCs.

Macrophages, specifically of the M1 subset, are specialized phagocytes that engulf and digest dead cells and invading microbes such as bacteria. M1 macrophages produce pro-inflammatory cytokines and the anti-microbial molecule nitric oxide (NO), in response to interferon alone or in combination with detection of microbial stimuli such as lipopolysaccharide. However, in the presence of interleukin-4 (IL-4) and IL-13, macrophages differentiate into an alternative, immunosuppressive M2 subset, which is characterized by IL-10 production and decreased expression of IL-12 and tumor necrosis factor-α (TNF-α). Early work demonstrated that human MSCs antagonize the M1 phenotype and promote M2 polarization, as characterized by increased CD206 expression, increased IL-10 production and phagocytosis, and decreased pro-inflammatory cytokine and NO production. In transwell cultures, MSCs have also been shown to skew macrophages towards the M2 lineage, which indicates the involvement of soluble, MSC-derived factors that contribute to the polarization. In addition, MSCs reduce the expression of CD86 and MHCII on macrophages, thus diminishing their stimulatory potency. In an excisional wound repair model in mice, human gingiva-derived MSCs were shown to migrate to the wound site and polarize M2 for wound repair. One proposed mechanism is that multiple soluble factors are produced for MSCs to elicit M2 polarization. Prostaglandin E2 (PGE2) was found to be constitutively produced by human MSCs at levels able to suppress IL-6 and TNF-α expression in activated macrophages. In addition, neutralizing antibodies to IL-6 and granulocyte macrophage-colony stimulating factor (GM-CSF) showed that these cytokines synergistically promote human gingiva-derived MSC-mediated promotion of the M2 phenotype in macrophages.

In addition to macrophages, neutrophils are important phagocytes of the innate immune system. In response to detection of microbial molecules, neutrophils produce a large quantity of microbicidal

oxidative products in the so-called oxidative respiratory burst. Respiratory bursts are also closely associated with neutrophil apoptosis. MSCs inhibit neutrophil apoptosis, even under IL-8-mediated activation conditions, via MSC-derived IL-6. It is thought that MSCs may enact this effect to preserve the non-dividing neutrophil pool found in bone marrow sinusoids. MSCs also prevent respiratory bursts from neutrophils, an effect which aligns with MSC immunosuppression, but had no effect on neutrophil phagocytosis, matrix adhesion, or chemotaxis.

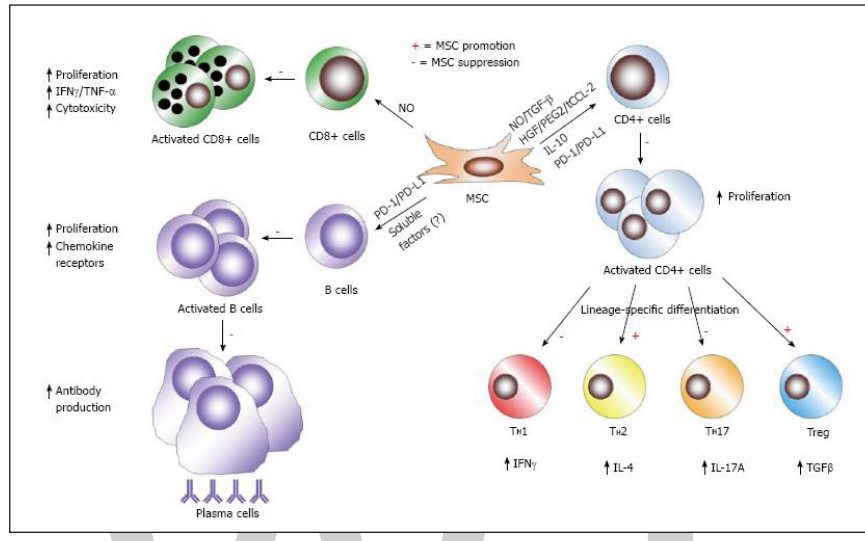
Mast cells contribute heavily to allergic responses, especially through the release of pro-inflammatory cytokines and histamine-containing granules. Co-culture studies revealed that MSCs suppressed the ability of mast cells to degranulate and produce TNF-α. In a passive cutaneous anaphylaxis in vivo model, MSCs also reduced inflammation promoted by mast cells. In these experiments, MSC-mediated immunosuppression was dependent on up-regulation of cyclo-oxygenase-2 in MSCs and their production of PGE2, which suppressed mast cells via EP4 receptor ligation.

Natural killer cells (NKs) are innate immune cells that, in addition to producing pro-inflammatory cytokines, are cytotoxic toward intracellular pathogen-infected and cancer cells. NK cytotoxicity is regulated by both inhibitory and activating receptors, in addition to target cell MHC expression levels and antibody-dependent cell cytotoxicity. Studies showed that MSCs inhibited NK proliferation activationand reduced the expression of NK activating receptors, including 2B4 and NKG2D. MSCs also reduced pro-inflammatory cytokine production by NKs. Furthermore, freshly isolated NKs were not cytotoxic towards MSCs, but acquired cytotoxicity after 4 d cultures with IL-15. Neutralization of PGE2 and transforming growth factor-β (TGF-β), both thought to contribute to MSC immunosuppression, overrode MSC-mediated suppression of NK proliferation. Indoleamine-2,3-dioxygenase expression by MSCs has also been found to inhibit NK. Taken together, these studies indicate that the inhibitory effects of MSCs on NKs may depend on NK culture duration, NK activation state, and time after which MSCs are added to NK cultures.

Dendritic cells (DCs) bridge the innate and adaptive immune systems as they function both as cytokine producers and potent antigen-presenting cells. DCs take up antigen and during maturation and activation up-regulate MHCs, increase the expression of co-stimulatory molecules (i.e., CD40, CD80, CD83 and CD86), and migrate to secondary lymphoid organs and present antigen to T cells for the generation of a primary adaptive immune response. During T cell-priming, DCs also produce a medley of cytokines that affect downstream T cell effector function. MSCs have been shown to affect most of these processes: MSCs inhibit DC endocytosis, up-regulation of MHC, CD40, CD80, CD83, and CD86 during differentiation and prevent further increase of CD40, CD83, and CD86 expression during maturation. They also interfered with DC capacity to produce IL-12 and activate allogeneic T cells. Furthermore, MSCs block the generation of dermal DCs from CD34-derived $CD14^+$ CD1a- precursors and those derived from immature monocytes. Monocytes cultured under DC-differentiating conditions in the presence of MSCs fail to proliferate and remain at the G0 state. MSC treatment inhibited in vivo DC maturation, cytokine secretion, and migration to lymph nodes, which results in insufficient T-cell priming in the lymph nodes. Diverse molecular contributions are thought to mediate MSC-modulation of DCs. For example, IL-6 has been shown to at least partially contribute to MSC-mediated inhibition of DC differentiation from bone marrow progenitors, and PGE2 from MSCs has been shown to convert mature $CD11c^+$ B220-DCs into a regulatory subset.

Mechanisms of MSC Suppression of Adaptive Immune Cells

Cells of the adaptive immune system, particularly B and T lymphocytes, are composed of billions of unique clones that, as opposed to innate immune cells, recognize highly specific molecules (usually peptides). Each clone expands upon antigen recognition and reaches an effector state in order to eliminate the pathogen present.



Mesenchymal stem cell immunosuppression of adaptive immune cells. In the context of B cells, mesenchymal stem cells (MSCs) inhibit various facets of B cells activity, including activation, proliferation, chemokine receptor expression, and differentiation to becoming antibody-secreting plasma cells. Unknown soluble factors and PD-1/PD-L1 ligation mediate these effects of MSCs on B cells. MSC have been shown to induce NO in response to inflammatory cytokine detection to suppress $CD8^+$ T cell proliferation, cytokine production, and cytotoxicity. In response to activation in specific cytokine milieus, $CD4^+$ T cells can differentiate into numerous effector populations. MSCs produce soluble factors (NO, TGF-B, HGF, PGE2, truncated CCL-2, and IL-10) and membrane-bound molecules (PD-1 ligation) to achieve suppression of $CD4^+$ T cell proliferation and the polarization of $CD4^+$ T cells towards T_H1 and T_H17 cells. MSCs favor the development of T_H2 and anti-inflammatory Treg populations.

B cells are specialized in producing antibodies, which play multiple roles in directly neutralizing pathogens, promoting opsonization for neutralization and phagocytic intake, and activation of other immune cells. Naïve B cells are activated by B-cell receptor (BCR) ligation, CD40/CD40L binding, and Toll-like receptor (TLR) binding of microbial products. In response to activation, B cells proliferate and differentiate into plasma cells, which produce antibodies. Studies have reported that MSCs inhibit B cell proliferation by arrest at the G0/G1 check point, without induction of apoptosis. In addition, MSCs reduced production of IgG, IgM, and IgA during in vitro co-culture of B cells. MSCs also suppressed chemokine receptor expression on B cells. In vivo, MSCs have also been shown to suppress B cell function. In an MRL/Lpr model of systemic lupus erythematosus, a single MSC injection along with cyclophosphamide reduced dsDNA auto-antibodies. In the context of transplantation, MSC injections led to a reduction of allo-specific antibodies and promoted long-term graft acceptance. In a proteolipid protein (PLP)-mediated form of experimental

autoimmune encephalomyelitis (EAE), a murine form of multiple sclerosis, mice given MSCs exhibited an inhibition of PLP-specific antibodies. Cell-cell contact and soluble factors synthesized by MSCs are thought to suppress B cell function. Programmed death-1 (PD-1)/PD ligand-1 (PD-L1) ligation have been shown to enact B cell suppression by MSCs, with soluble factors largely remaining unidentified.

T cells of adaptive immune systems are divided into $CD4^+$ and $CD8^+$ lineages, both of which can be sub-grouped into different effector subsets. Upon activation through unique T-cell receptors (TCRs) and co-stimulation by APCs such as DCs, T cells rapidly proliferate and differentiate into effector cells. Effector $CD4^+$ T cells develop as IFNγ-producing T_H1 cells, IL-4- and IL-13-producing T_H2 cells, IL-10-producing Treg, and IL-17-producing T_H17. $CD8^+$ T cells are mainly considered as cytotoxic T lymphocytes (CTLs) and produce cytotoxic granules that kill infected and cancerous cells; however, they can differentiate into many of the same effector subtypes as their $CD4^+$ T cell counterparts.

MSCs inhibit T cell proliferation, regardless of stimulus type, by arrest at the G0/G1 cell cycle phase. This inhibition is also MHC-independent, as both autologous and allogeneic MSCs exert this same anti-proliferative effect. T cells inhibited by MSCs also exhibit increased survival and less apoptosis, but this state can be partially reverted via IL-2. One study showed that MSCs repressed T cell proliferation via up-regulation of inducible nitric oxide synthase (iNOS), which produces the NO which produces such effect. MSCs also modulated cytokine production of T cells. It was reported that these cells suppressed IFNγ production from T_H1, promoted IL-4 secretion from T_H2, and increased the proportion of Treg present in culture. MSCs produce immune-modulatory molecules such as hepatocyte growth factor (HGF), TGF-B, and PGE2, which may enact these cellular effects. MSCs have also been reported to inhibit T_H17 development through various means, including inhibition with the effector molecules PGE2, a truncated peptide of C-C chemokine ligand-2 (CCL-2), IL-10, and PD-1/PD-L1 ligation. Importantly, MSCs must be pre-exposed to a combination of effector cytokines, including IFNγ and TNFα or IL-1β, in order to efficiently suppress T cell function. Moreover, MSCs have been shown to suppress the cytotoxicity of CTLs, presumably by a soluble factor. When administered viral peptides and tumor antigens, the cells suppress CTL killing and were not recognized as targets of infection or foreign cells, despite enhanced MHC-I expression post-IFNγ treatment.

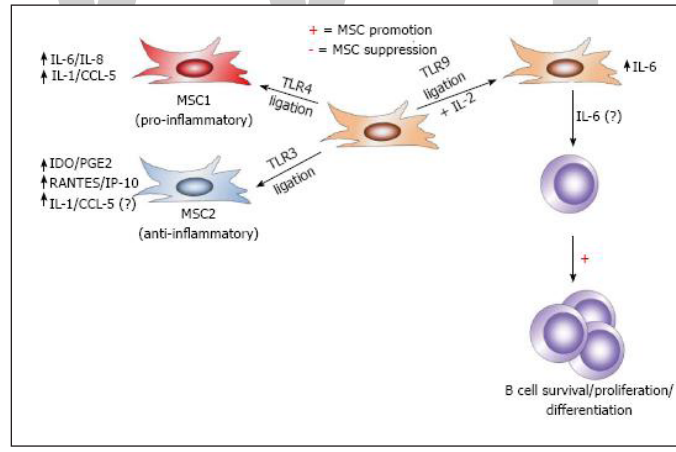
In vivo, MSCs have been extensively used in pre-clinical experimental disease settings involving pathogenic T cells. Some of the earliest reports show MSC-mediated amelioration of EAE induced by the peptide, myelin oligodendrocyte glycoprotein (MOG) 35-55, which preferentially induces a neuro-inflammatory disease mediated by T_H1 and T_H17 cells. In this setting, the polarization of these cells was inhibited in vivo, and MSC-derived HGF alone suppressed EAE while also promoting a beneficial neurotropic effect. MSCs suppressed skin-graft rejection in monkeys, which was associated with T cell suppression of proliferation. In a model of streptozotocin-induced autoimmune diabetes, MSCs inhibited T-cell mediated destruction of insulin-secreting β-cells in the pancreas. MSCs also suppressed proliferation of auto-reactive T cells in collagen-induced arthritis, in addition to decreasing TNF-α production and supporting the generation of Treg cells. These studies demonstrate immense potential for the use of MSCs in modulating the immune response in inflammatory settings for therapeutic benefit, especially of autoimmune diseases.

MSCs and Immunogenicity

Although the majority of investigations of MSC effects on immune cell function and pre-clinical immunogenic and inflammatory conditions have indicated immunosuppression, other studies have shown immunostimulatory properties.

Microbial Molecule Detection

In vivo, MSCs are present in virtually all tissues of the body and express multiple receptor types that permit detection of changes in tissue homeostasis. Differential TLR stimulation of MSCs has been shown to influence the downstream effect of MSCs on immune responses. Stimulation of TLR3 with poly (I:C), which mimics viral double-stranded RNA detection, in MSCs causes them to polarize towards an anti-inflammatory phenotype (MSC2 phenotype) characterized by increased production of the immune-regulatory factors IDO and PGE2 and of RANTES and IP-10. However, when MSCs are stimulated with LPS, a TLR4 agonist, they develop a pro-inflammatory MSC1 phenotype in which they up-regulate the pro-inflammatory cytokines IL-6 and IL-8. MSC1, but not un-primed or MSC2, support PBMC activation and proliferation. Romieu-Mourez et al found that stimulation of either TLR3 or TLR4 lead to the production of the pro-inflammatory cytokines IL-6, IL-8 IL-1, and the chemokine CCL-5; however, such differences may be due to differences in stimulation protocols, especially for MSC exposure time differences to TLR agonists. When MSCs are co-cultured with naïve and transitional B cells in the presence of IL-2 and the TLR9 agonist CpG 2006 (viral/bacterial PAMP mimic), B cell survival, differentiation, and antibody production are enhanced. Though the effect was cell-contact dependent, the MSCs produced increased IL-6 in co-culture, which is known to increase B cell proliferation. In vivo, MSCs are also postulated to not only support the viability of naïve, but also more differentiated, B cell subsets in the bone marrow.



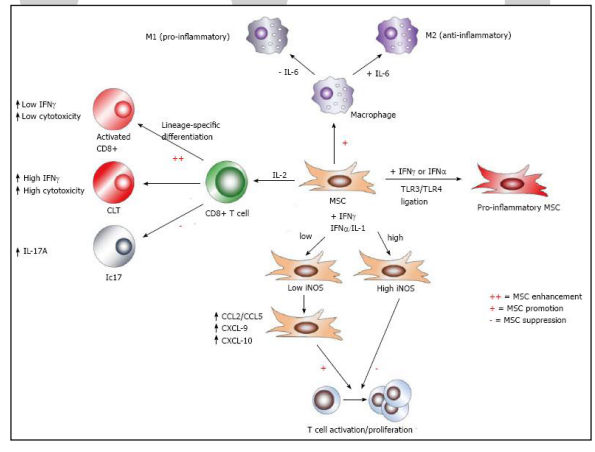
Differential toll-like receptor stimulation affects mesenchymal stem cell immune-modulation. Mesenchymal stem cells (MSCs) are situated throughout the body as sentinels in virtually all organs and the perivasculature and are equipped with pattern-recognition receptors, including Toll-like receptorS (TLRS), to detect DAMPs from dying cells and PAMPs from pathogens. In response to TLR3 signaling, MSCs maintain an anti-inflammatory MSC2 phenotype, marked by induction of IDO, PGE2, RANTES, and IP-10 (in addition to IL-1 and CCL-5). However, in response to signaling through TLR3, MSC adopt the pro-inflammatory MSC1 phenotype and up-regulate IL-6 and IL-8, in addition to IL-1 and CCL-5. In the presence of IL-2 in combination with TLR9 signaling,

MSCs have been shown to also produce IL-6, which promotes B cells survival, proliferation, and differentiation, though MSC-derived IL-6 has not been demonstrated to directly exert these effects on B cells.

The rationale for the different MSC polarization types in response to different microbial stimuli detection remains unknown. MSCs are thought to exhibit a homoeostatic default immunosuppressive phenotype for the purposes of inhibiting inappropriate HSC differentiation and potential depletion of HSC reserves in the bone marrow. However, outside of the bone marrow, they may adopt the pro-inflammatory MSC1 phenotype to aid in the formation of an immune response in tissues during early tissue damage and/or pathogen invasion. It is interesting to note that tissue necrosis and damage leads to the release of intracellular danger-associated molecular patterns (DAMPs) such as heat shock proteins, high mobility group proteins, and degraded ECM molecules, which trigger stimulation of innate immune cells through TLR4 and TLR2 ligation for resolution of tissue damage. It is possible that TLR4 stimulation of MSCs, whether derived from PAMP or DAMP, could still lead to the same pro-inflammatory outcome due to the apparent necessity of generating an inflammatory environment for the recruitment and activation of immune cells to respond to either tissue damage and/or pathogen invasion. In contrast, the MSC2 phenotype could be adopted for the down-regulation of immune responses to limit inflammatory damage to tissues and permit ECM reconstruction and healing.

Cytokine Milieu

MSCs are pleiotropic cells that are highly sensitive to different microenvironments, especially those containing cytokines. Importantly, cytokines exert immune-suppressive or immunogenic effects on cells and tissues dependent on multiple variables, including cytokine identities, combinations, and concentrations.



Effects of cytokine milieu on mesenchymal stem cell immune-modulation. Mesenchymal stem cell (MSC) modulation of immune responses is strongly affected by the makeup of cytokine milieus. Toll-like receptor (TLR) ligation in conjunction with interferon signaling drives MSCs down a pro-inflammatory route. While high concentrations of the pro-inflammatory cytokines IFNγ and either tumor necrosis factor-α (TNF-α) or IL-1 have been shown to induce iNOS and NO in MSCs to mediate suppression of T cell proliferation, low concentrations of these factors fail to fully induce iNOS, and instead enhance T cell proliferation, presumably via cytokine-induced chemokines.

Furthermore, MSCs differentially affect the polarization of effector $CD8^+$ T cell subsets: through enhanced early IL-2 expression induced by MSCs, activated $CD8^+$ T cells exhibit increased IFNγ expression and cytotoxicity, while fully differentiated cytotoxic T lymphocytes (CTLs) are largely unaffected by MSC action. In contrast, MSCs potently suppress Tc17 development. Moreover, IL-6 signaling acts as a switch for MSC immune-modulation of macrophages. In the presence of IL-6, MSCs retain promotion of M2, but favor M1 polarization in the absence of this cytokine.

In continuation of the differential TLR stimulation on MSC polarization, the downstream effects of TLR stimulation in MSCs can be affected by prior cytokine priming. Initial priming of human MSCs with either IFN-α or IFN-γ synergizes with downstream TLR3 or TLR4 stimulation to enhance the production of pro-inflammatory cytokines by MSCs. The concentration of inflammatory cytokines has also been postulated to regulate MSC polarization. IFN-γ and IL-1 or TNF-α induction of iNOS and NO production have been demonstrated as an effector mechanism MSCs used for inhibition of T cell proliferation. However, under closer scrutiny, it was discovered that their concentrations must be relatively high, for low/insufficient levels of these cytokines failed to up-regulate iNOS to adequate levels for T cell functional suppression, and led to an induction of T cell responses. In this scenario, MSCs still retained upregulation of the T-cell activity enhancing chemokines such as CCL2, CCL5, CXCL9, and CXCL10. When iNOS-/-MSCs were injected into normal C57BL/6 mice and challenged with a suboptimal dose of OVA for induction of a delayed type hypersensitivity (DTH) response, swelling occurred in injected footpads of mice. However, when these mutant MSCs were injected into CCR5 -/-CXCR3-/- mice, they could not promote the DTH response, highlighting the importance of chemokine ligation on T cells as an immune-enhancing effect of MSCs in the absence of iNOS induction. Thus high pro-inflammatory cytokine concentrations are thought to promote an MSC2 phenotype while an MSC1 phenotype may result from low level of such cytokines.

MSCs had little effect on the functions of IL-2 and IL-12-generated CTLs, increased cytokine production and cytotoxicity of non-polarized, activated $CD8^+$ T cells, and potently suppressed IL-17A-producing, Tc17 development. IFNγ-producing $CD8^+$ T cells were also cytotoxic towards MSCs, which was associated with heavily increased MHC-I expression on MSCs. These effects were associated with the early enhancement of IL-2 production, which is known to promote CTLs but antagonize the IL-17-producing program. In a the MOG_{37-50} model of EAE, which is mediated by pathogenic $CD8^+$ T cells, MSCs exacerbated the disease and increased the $CD8^+$ T cell presence in the brains of diseased mice. Here, the MSCs appeared to alter the activation program of the developing T cells, but the precise mechanisms of MSC-induced IL-2 production and downstream effector function remain undefined.

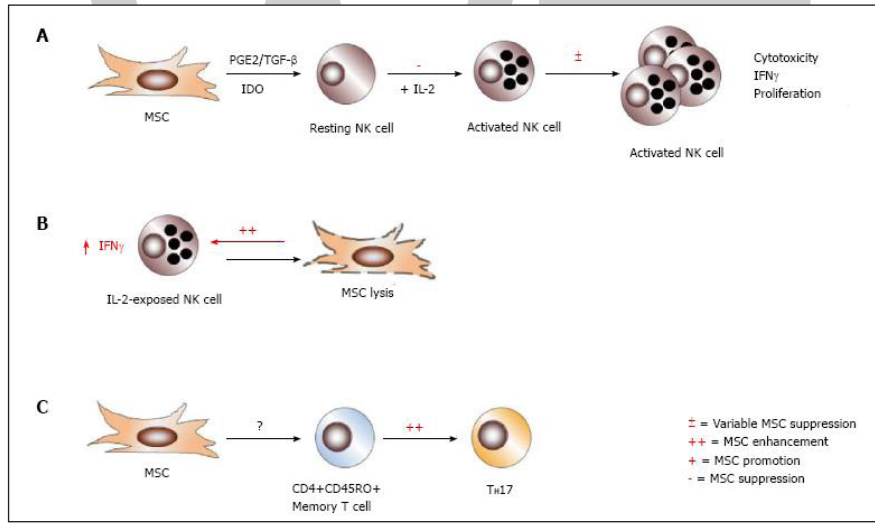
In another report of MSC modulation of neuro-inflammatory autoimmune disease, MSCs were found to ameliorate mild MOG-induced EAE, but worsen the severe form, with intracerebroventricular (ICT) injection into mice. In almost two-thirds of severe-EAE animals, these MSCs migrated into the parenchyma and formed masses characterized by focal inflammation, demyelination, axon loss, and collagen and fibronectin deposits. Importantly, these MSCs do encounter an inflammatory environment when injected ICT, and may undergo a polarization similar to the aforementioned MSC1 type, which could be dependent on the cytokine and molecular milieu.

In addition to the pro-inflammatory cytokines, production and detection of IL-6 also acts as a switch for MSCs during immune responses. This molecule, which is constitutively produced by

MSCs, polarized macrophages towards the M2 type upon cell-cell contact. This polarization was also dependent upon MSC production of IDO and PGE2. However, in the absence of IL-6, MSCs induced polarization of macrophages towards the M1 phenotype, which is characterized by IFNγ, TNF-α, and CD40L expression. In contrast, a positive correlation with IL-6 in vivo production and MSC administration in mice exhibiting collagen-induced arthritis was reported to worsen this disease. The molecular milieu that governs the production of IL-6 from MSCs in the context of macrophage polarization has not been determined, but may involve pre-exposure to certain cytokine combinations that influence MSCs in a concentration-dependent manner, as in the case of iNOS. The in vivo milieu must also be taken into account, for increased IL-6 production could theoretically enhance inflammation by promoting effector immune cell differentiation, as in the case of IL-17A-producing T cells.

Immune Cell Differentiation State

Upon activation through cell-specific receptor signaling, immune cells undergo successive stages of differentiation towards a terminal phenotype characterized by optimal effector function, usually before subsequent apoptosis or transition into memory status. The specific stage of an immune cell's differentiation may render it susceptible or refractory to any MSC action.



Effects of immune cell activation state on mesenchymal stem cell immune-modulation. The differentiation state of immune cells can render them susceptible or refractory to mesenchymal stem cell (MSC) action. Though MSCs efficiently inhibit the activation and downstream cytotoxicity of resting NK cells, they exert variable suppression on IL-2-activated NK cells, which is partially ratio dependent (A). MSCs themselves may become targets of activated NK cells for lysis, and enhance NK cell production of IFNγ in the process (B). Interestingly, MSCs promote T_H17 differentiation from $CD4^+CD45RO^+$ memory T cells, but no other $CD4^+$ or $CD8^+$ T cell population (C).

NK cells are generally in a resting state, but upon IL-2 activation, proliferate and differentiate into activated cytolytic and cytokine-producing cells capable of efficient lysis of target cells. MSCs robustly prevented resting NK cell activation and proliferation, but were only partially capable of suppressing this process on NK cells that have been pre-exposed to IL-2. Moreover, the extent of MSC suppression of NK cell proliferation in the latter case was ratio dependent, with decreasing

suppression with increasing NK:MSC ratio. IL-2-pre-exposed, but not resting, NK cells also efficiently lysed autologous and allogeneic MSCs, and exhibited increased IFNγ production with MSC co-culture. Interestingly, IFNγ-pre-exposed MSCs had a better capacity of inhibiting pre-activated NK cell activity, presumably due to increased MHC-I expression on MSCs in response to inflammatory cytokine signaling, which negatively affects NK cell function.

Under the arm of adaptive immunity, MSCs have been extensively shown to suppress T_H17 and Tc17 development, but less work has addressed MSC effects on memory T cells. Hsu and colleagues showed that MSCs specifically enhanced IL-17 expression in $CD4^+$ $CD45RO^+$ memory T cells, but not in any other populations of $CD4^+$ or $CD8^+$ T cells. These T_H17 subsequently enhanced neutrophil function. It is thought that, since these memory T cells rapidly react to a pathogen challenge in vivo, they could interact with MSCs at peripheral sites to enhance their function and increase the T cell response for efficient pathogen elimination. Thus immune cell activation state is an important factor in influencing outcome with MSC interactions.

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Adult Stem Cell

4

- Mesenchymal Stem Cell
- Hematopoietic Stem Cell
- Neural Stem Cell
- Endothelial Stem Cell
- Tissue-specific Stem Cell
- Spore-like Cell
- Induced Pluripotent Stem Cell
- Embryonic Stem Cell

The undifferentiated cells that are found throughout the body after development are known as adult stem cells. They multiply by cell division in order to replenish dying cells and regenerate the damaged tissues. Neural stem cell, endothelial stem cell, tissue-specific stem cell and spore-like cell are some of the adult stem cells. This chapter discusses these types of adult stem cells and their functions in detail.

An adult stem cell is thought to be an undifferentiated cell, found among differentiated cells in a tissue or organ. The adult stem cell can renew itself and can differentiate to yield some or all of the major specialized cell types of the tissue or organ. The primary roles of adult stem cells in a living organism are to maintain and repair the tissue in which they are found. Scientists also use the term somatic stem cell instead of adult stem cell, where somatic refers to cells of the body (not the germ cells, sperm or eggs). Unlike embryonic stem cells, which are defined by their origin (cells from the preimplantation-stage embryo), the origin of adult stem cells in some mature tissues is still under investigation.

Research on adult stem cells has generated a great deal of excitement. Scientists have found adult stem cells in many more tissues than they once thought possible. This finding has led researchers and clinicians to ask whether adult stem cells could be used for transplants. In fact, adult hematopoietic, or blood-forming, stem cells from bone marrow have been used in transplants for more than 40 years. Scientists now have evidence that stem cells exist in the brain and the heart, two locations where adult stem cells were not at first expected to reside. If the differentiation of adult

stem cells can be controlled in the laboratory, these cells may become the basis of transplantation-based therapies.

The history of research on adult stem cells began more than 60 years ago. In the 1950s, researchers discovered that the bone marrow contains at least two kinds of stem cells. One population, called hematopoietic stem cells, forms all the types of blood cells in the body. A second population, called bone marrow stromal stem cells (also called mesenchymal stem cells, or skeletal stem cells by some), were discovered a few years later. These non-hematopoietic stem cells make up a small proportion of the stromal cell population in the bone marrow and can generate bone, cartilage, and fat cells that support the formation of blood and fibrous connective tissue.

In the 1960s, scientists who were studying rats discovered two regions of the brain that contained dividing cells that ultimately become nerve cells. Despite these reports, most scientists believed that the adult brain could not generate new nerve cells. It was not until the 1990s that scientists agreed that the adult brain does contain stem cells that are able to generate the brain's three major cell types—astrocytes and oligodendrocytes, which are non-neuronal cells, and neurons, or nerve cells.

Identificaton of Adult Stem Cell

Adult stem cells have been identified in many organs and tissues, including brain, bone marrow, peripheral blood, blood vessels, skeletal muscle, skin, teeth, heart, gut, liver, ovarian epithelium, and testis. They are thought to reside in a specific area of each tissue (called a "stem cell niche"). In many tissues, current evidence suggests that some types of stem cells are pericytes, cells that compose the outermost layer of small blood vessels. Stem cells may remain quiescent (non-dividing) for long periods of time until they are activated by a normal need for more cells to maintain tissues, or by disease or tissue injury.

Typically, there is a very small number of stem cells in each tissue and, once removed from the body, their capacity to divide is limited, making generation of large quantities of stem cells difficult. Scientists in many laboratories are trying to find better ways to grow large quantities of adult stem cells in cell culture and to manipulate them to generate specific cell types so they can be used to treat injury or disease. Some examples of potential treatments include regenerating bone using cells derived from bone marrow stroma, developing insulin-producing cells for type 1 diabetes, and repairing damaged heart muscle following a heart attack with cardiac muscle cells.

Tests used to Identify Adult Stem Cells

Scientists often use one or more of the following methods to identify adult stem cells: (1) label the cells in a living tissue with molecular markers and then determine the specialized cell types they generate; (2) remove the cells from a living animal, label them in cell culture, and transplant them back into another animal to determine whether the cells replace (or "repopulate") their tissue of origin.

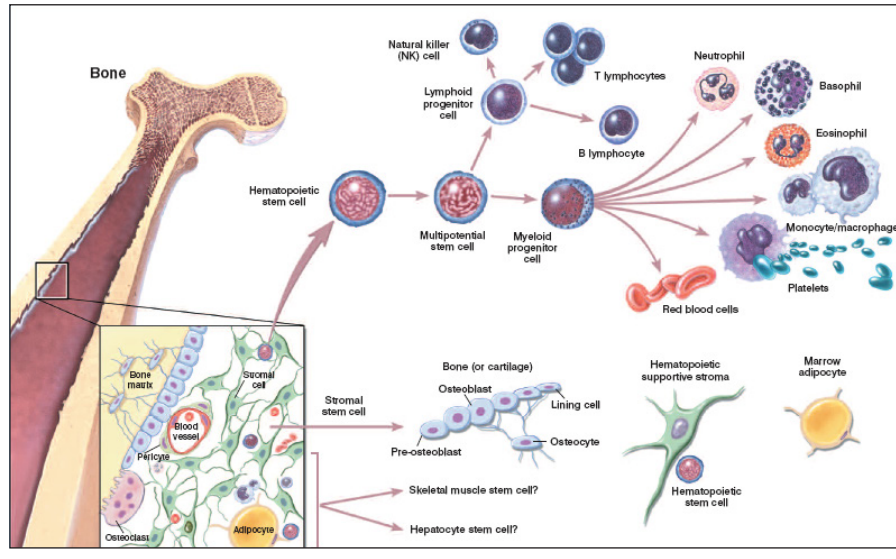
Importantly, scientists must demonstrate that a single adult stem cell can generate a line of genetically identical cells that then gives rise to all the appropriate differentiated cell types of the tissue. To confirm experimentally that a putative adult stem cell is indeed a stem cell, scientists tend to

show either that the cell can give rise to these genetically identical cells in culture, and/or that a purified population of these candidate stem cells can repopulate or reform the tissue after transplant into an animal.

Adult Stem Cell Differentiation

As indicated above, scientists have reported that adult stem cells occur in many tissues and that they enter normal differentiation pathways to form the specialized cell types of the tissue in which they reside.

Normal differentiation pathways of adult stem cells. In a living animal, adult stem cells are available to divide for a long period, when needed, and can give rise to mature cell types that have characteristic shapes and specialized structures and functions of a particular tissue. The following are examples of differentiation pathways of adult stem cells that have been demonstrated in vitro or in vivo.



Hematopoietic and stromal stem cell differentiation.

- Hematopoietic stem cells give rise to all the types of blood cells: red blood cells, B lymphocytes, T lymphocytes, natural killer cells, neutrophils, basophils, eosinophils, monocytes, and macrophages.
- Mesenchymal stem cells have been reported to be present in many tissues. Those from bone marrow (bone marrow stromal stem cells, skeletal stem cells) give rise to a variety of cell types: bone cells (osteoblasts and osteocytes), cartilage cells (chondrocytes), fat cells (adipocytes), and stromal cells that support blood formation. However, it is not yet clear how similar or dissimilar mesenchymal cells derived from non-bone marrow sources are to those from bone marrow stroma.
- Neural stem cells in the brain give rise to its three major cell types: nerve cells (neurons) and two categories of non-neuronal cells—astrocytes and oligodendrocytes.
- Epithelial stem cells in the lining of the digestive tract occur in deep crypts and give rise to several cell types: absorptive cells, goblet cells, Paneth cells, and enteroendocrine cells.

- Skin stem cells occur in the basal layer of the epidermis and at the base of hair follicles. The epidermal stem cells give rise to keratinocytes, which migrate to the surface of the skin and form a protective layer. The follicular stem cells can give rise to both the hair follicle and to the epidermis.
- Transdifferentiation: A number of experiments have reported that certain adult stem cell types can differentiate into cell types seen in organs or tissues other than those expected from the cells' predicted lineage (i.e., brain stem cells that differentiate into blood cells or blood-forming cells that differentiate into cardiac muscle cells, and so forth). This reported phenomenon is called transdifferentiation.

Although isolated instances of transdifferentiation have been observed in some vertebrate species, whether this phenomenon actually occurs in humans is under debate by the scientific community. Instead of transdifferentiation, the observed instances may involve fusion of a donor cell with a recipient cell. Another possibility is that transplanted stem cells are secreting factors that encourage the recipient's own stem cells to begin the repair process. Even when transdifferentiation has been detected, only a very small percentage of cells undergo the process.

In a variation of transdifferentiation experiments, scientists have recently demonstrated that certain adult cell types can be "reprogrammed" into other cell types in vivo using a well-controlled process of genetic modification. This strategy may offer a way to reprogram available cells into other cell types that have been lost or damaged due to disease. For example, one recent experiment shows how pancreatic beta cells, the insulin-producing cells that are lost or damaged in diabetes, could possibly be created by reprogramming other pancreatic cells. By "re-starting" expression of three critical beta cell genes in differentiated adult pancreatic exocrine cells, researchers were able to create beta cell-like cells that can secrete insulin. The reprogrammed cells were similar to beta cells in appearance, size, and shape; expressed genes characteristic of beta cells; and were able to partially restore blood sugar regulation in mice whose own beta cells had been chemically destroyed. While not transdifferentiation by definition, this method for reprogramming adult cells may be used as a model for directly reprogramming other adult cell types.

In addition to reprogramming cells to become a specific cell type, it is now possible to reprogram adult somatic cells to become like embryonic stem cells (induced pluripotent stem cells, iPSCs) through the introduction of embryonic genes. Thus, a source of cells can be generated that are specific to the donor, thereby increasing the chance of compatibility if such cells were to be used for tissue regeneration. However, like embryonic stem cells, determination of the methods by which iPSCs can be completely and reproducibly committed to appropriate cell lineages is still under investigation.

Mesenchymal Stem Cell

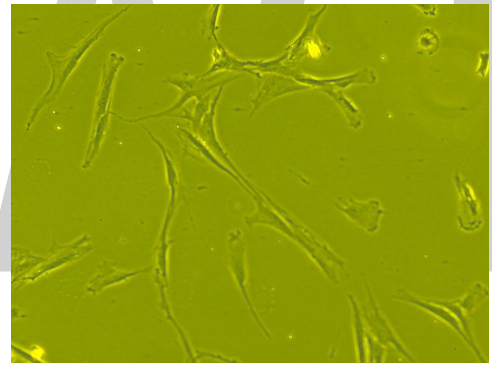
Mesenchymal stem cells are multipotent stromal cells that can differentiate into a variety of cell types, including osteoblasts (bone cells), chondrocytes (cartilage cells), myocytes (muscle cells) and adipocytes (fat cells which give rise to marrow adipose tissue).

Structure

While the terms *mesenchymal stem cell* (MSC) and *marrow stromal cell* have been used interchangeably for many years, neither term is sufficiently descriptive:

- Mesenchyme is embryonic connective tissue that is derived from the mesoderm and that differentiates into hematopoietic and connective tissue, whereas MSCs do not differentiate into hematopoietic cells.
- Stromal cells are connective tissue cells that form the supportive structure in which the functional cells of the tissue reside. While this is an accurate description for one function of MSCs, the term fails to convey the relatively recently discovered roles of MSCs in the repair of tissue.
- The term encompasses multipotent cells derived from other non-marrow tissues, such as placenta, umbilical cord blood, adipose tissue, adult muscle, corneal stroma or the dental pulp of deciduous baby teeth. The cells do not have the capacity to reconstitute an entire organ.

Morphology



Human bone marrow derived Mesenchymal stem cell showing fibroblast-like morphology seen under phase contrast microscope (carl zeiss axiovert 40 CFL) at 63 x magnification.

Mesenchymal stem cells are characterized morphologically by a small cell body with a few cell processes that are long and thin. The cell body contains a large, round nucleus with a prominent nucleolus, which is surrounded by finely dispersed chromatin particles, giving the nucleus a clear appearance. The remainder of the cell body contains a small amount of Golgi apparatus, rough endoplasmic reticulum, mitochondria and polyribosomes. The cells, which are long and thin, are widely dispersed and the adjacent extracellular matrix is populated by a few reticular fibrils but is devoid of the other types of collagen fibrils.

Location

Bone Marrow

Bone marrow was the original source of MSCs, and still is the most frequently utilized. These bone marrow stem cells do not contribute to the formation of blood cells and so do not express the hematopoietic stem cell marker CD34. They are sometimes referred to as *bone marrow stromal stem cells*.

Cord cells

The youngest and most primitive MSCs may be obtained from umbilical cord tissue, namely Wharton's jelly and the umbilical cord blood. However MSCs are found in much higher concentration in the Wharton's jelly compared to cord blood, which is a rich source of hematopoietic stem cells. The umbilical cord is available after a birth. It is normally discarded and poses no risk for collection. These MSCs may prove to be a useful source of MSCs for clinical applications due to their primitive properties.

Adipose Tissue

Adipose tissue is a rich source of MSCs (or adipose-derived mesenchymal stem cells, AdMSCs).

Molar Cells

The developing tooth bud of the mandibular third molar is a rich source of MSCs. While they are described as multipotent, it is possible that they are pluripotent. They eventually form enamel, dentin, blood vessels, dental pulp and nervous tissues. These stem cells are capable of producing hepatocytes.

Amniotic Fluid

Stem cells are present in amniotic fluid. As many as 1 in 100 cells collected during amniocentesis are pluripotent mesenchymal stem cells.

Function

Differentiation Capacity

MSCs have a great capacity for self-renewal while maintaining their multipotency. Beyond that, there is little that can be definitively said. The standard test to confirm multipotency is differentiation of the cells into osteoblasts, adipocytes and chondrocytes as well as myocytes and neurons. MSCs have been seen to even differentiate into neuron-like cells, but there is lingering doubt whether the MSC-derived neurons are functional. The degree to which the culture will differentiate varies among individuals and how differentiation is induced, e.g., chemical vs. mechanical; and it is not clear whether this variation is due to a different amount of "true" progenitor cells in the culture or variable differentiation capacities of individuals' progenitors. The capacity of cells to proliferate and differentiate is known to decrease with the age of the donor, as well as the time in culture. Likewise, whether this is due to a decrease in the number of MSCs or a change to the existing MSCs is not known.

Immunomodulatory Effects

MSCs have an effect on innate and specific immune cells. MSCs produce many molecules having immunomodulatory effects. These include prostaglandin E2 (PGE2), nitric oxide, indolamin 2,3-dioxigenase (IDO), IL-6, and other surface markers - FasL, PD-L1 / 2.

MSCs have an effect on macrophages, neutrophils, NK cells, mast cells and dendritic cells in innate immunity. MSCs are able to migrate to the site of injury, where they polarize through PGE2

macrophages in M2 phenotype which is characterized by an anti-inflammatory effect. Further, PGE2 inhibits the ability of mast cells to degranulate and produce TNF-α. Proliferation and cytotoxic activity of NK cells is inhibited by PGE2 and IDO. MSCs also reduce the expression of NK cell receptors - NKG2D, NKp44 and NKp30. MSCs inhibit respiratory flare and apoptosis of neutrophils by production of cytokines IL-6 and IL-8. Differentiation and expression of dendritic cell surface markers is inhibited by IL-6 and PGE2 of MSCs. The immunosuppressive effects of MSC also depend on IL-10, but it is not certain whether they produce it alone, or only stimulate other cells to produce it.

MSC expresses the adhesion molecules VCAM-1 and ICAM-1, which allow T-lymphocytes to adhere to their surface. Then MSC can affect them by molecules which have a short half-life and their effect is in the immediate vicinity of the cell. These include nitric oxide, PGE2, HGF, and activation of receptor PD-1. MSCs reduce T cell proliferation between G0 and G1 cell cycle phases and decrease the expression of IFNγ of Th1 cells while increasing the expression of IL-4 of Th2 cells. MSCs also inhibit the proliferation of B-lymphocytes between G0 and G1 cell cycle phases.

Antimicrobial Properties

MSCs can produce antimicrobial peptides (AMPs). These include human cathelicidin LL-37, β-defensines, lipocalin 2 and hepcidin. MSCs effectively decrease number of colonies of both gram negative and gram positive bacteria by production of these AMPs. In addition, the same antimicrobial effect of the enzyme IDO produced by MSCs was found.

Clinical Significance

Mesenchymal stem cells in the body can be activated and mobilized if needed. However, the efficiency is low. For instance, damage to muscles heals very slowly but further study into mechanisms of MSC action may provide avenues for increasing their capacity for tissue repair.

Autoimmune Disease

Clinical studies investigating the efficacy of mesenchymal stem cells in treating diseases are in preliminary development, particularly for understanding autoimmune diseases, graft versus host disease, Crohn's disease, multiple sclerosis, systemic lupus erythematosus and systemic sclerosis. As of 2014, no high-quality clinical research provides evidence of efficacy, and numerous inconsistencies and problems exist in the research methods.

Other Diseases

Many of the early clinical successes using intravenous transplantation came in systemic diseases such as graft versus host disease and sepsis. Direct injection or placement of cells into a site in need of repair may be the preferred method of treatment, as vascular delivery suffers from a "pulmonary first pass effect" where intravenous injected cells are sequestered in the lungs.

Detection

The International Society for Cellular Therapy (ISCT) has proposed a set of standards to define

MSCs. A cell can be classified as an MSC if it shows plastic adherent properties under normal culture conditions and has a fibroblast-like morphology. In fact, some argue that MSCs and fibroblasts are functionally identical. Furthermore, MSCs can undergo osteogenic, adipogenic and chondrogenic differentiation *ex vivo*. The cultured MSCs also express on their surface CD73, CD90 and CD105, while lacking the expression of CD11b, CD14, CD19, CD34, CD45, CD79a and HLA-DR surface markers.

Hematopoietic Stem Cell

Hematopoietic stem cells (HSCs) are the stem cells that give rise to other blood cells. This process is called haematopoiesis. This process occurs in the red bone marrow, in the core of most bones. In embryonic development, the red bone marrow is derived from the layer of the embryo called the mesoderm.

Haematopoiesis is the process by which all mature blood cells are produced. It must balance enormous production needs (the average person produces more than 500 billion blood cells every day) with the need to regulate the number of each blood cell type in the circulation. In vertebrates, the vast majority of hematopoiesis occurs in the bone marrow and is derived from a limited number of hematopoietic stem cells that are multipotent and capable of extensive self-renewal.

Hematopoietic stem cells give rise to different types of blood cells, in lines called myeloid and lymphoid. Myeloid and lymphoid lineages both are involved in dendritic cell formation. Myeloid cells include monocytes, macrophages, neutrophils, basophils, eosinophils, erythrocytes, and megakaryocytes to platelets. Lymphoid cells include T cells, B cells, and natural killer cells. The definition of hematopoietic stem cell has evolved since they were first discovered in 1961. The hematopoietic tissue contains cells with long-term and short-term regeneration capacities and committed multipotent, oligopotent, and unipotent progenitors. Hematopoietic stem cells constitute 1:10.000 of cells in myeloid tissue.

HSC transplants are used in the treatment of cancers and other immune system disorders.

Structure

They are round, non-adherent, with a rounded nucleus and low cytoplasm-to-nucleus ratio. In shape, hematopoietic stem cells resemble lymphocytes.

Location

Hematopoietic stem cells are found in the bone marrow of adults, especially in the pelvis, femur, and sternum. They are also found in umbilical cord blood and, in small numbers, in peripheral blood.

Stem and progenitor cells can be taken from the pelvis, at the iliac crest, using a needle and syringe. The cells can be removed as liquid (to perform a smear to look at the cell morphology) or they can be removed via a core biopsy (to maintain the architecture or relationship of the cells to each other and to the bone).

Subtypes

A colony-forming unit is a subtype of HSC. (This sense of the term is different from colony-forming units of microbes, which is a cell counting unit.) There are various kinds of HSC colony-forming units:

- Colony-forming unit–granulocyte-erythrocyte-monocyte-megakaryocyte (CFU-GEMM).
- Colony-forming unit–lymphocyte (CFU-L).
- Colony-forming unit–erythrocyte (CFU-E).
- Colony-forming unit–granulocyte-macrophage (CFU-GM).
- Colony-forming unit–megakaryocyte (CFU-Meg).
- Colony-forming unit–basophil (CFU-B).
- Colony-forming unit–eosinophil (CFU-Eos).

The above CFUs are based on the lineage. Another CFU, the colony-forming unit–spleen (CFU-S), was the basis of an *in vivo* clonal colony formation, which depends on the ability of infused bone marrow cells to give rise to clones of maturing hematopoietic cells in the spleens of irradiated mice after 8 to 12 days. It was used extensively in early studies, but is now considered to measure more mature progenitor or transit-amplifying cells rather than stem cells.

Isolating Stem Cells

Since Hematopoietic stem cells cannot be isolated as a pure population, it is not possible to identify them in a microscope. Hematopoietic stem cells can be identified or isolated by the use of flow cytometry where the combination of several different cell surface markers (particularly CD34) are used to separate the rare Hematopoietic stem cells from the surrounding blood cells. Hematopoietic stem cells lack expression of mature blood cell markers and are thus, called Lin. Lack of expression of lineage markers is used in combination with detection of several positive cell-surface markers to isolate Hematopoietic stem cells. In addition, Hematopoietic stem cells are characterised by their small size and low staining with vital dyes such as rhodamine 123 (rhodamine ^{lo}) or Hoechst 33342 (side population).

Function

Haematopoiesis

Hematopoietic stem cells are essential to haematopoiesis, the formation of the cells within blood. Hematopoietic stem cells can replenish all blood cell types (i.e., are multipotent) and self-renew. A small number of Hematopoietic stem cells can expand to generate a very large number of daughter Hematopoietic stem cells. This phenomenon is used in bone marrow transplantation, when a small number of Hematopoietic stem cells reconstitute the hematopoietic system. This process indicates that, subsequent to bone marrow transplantation, symmetrical cell divisions into two daughter Hematopoietic stem cells must occur.

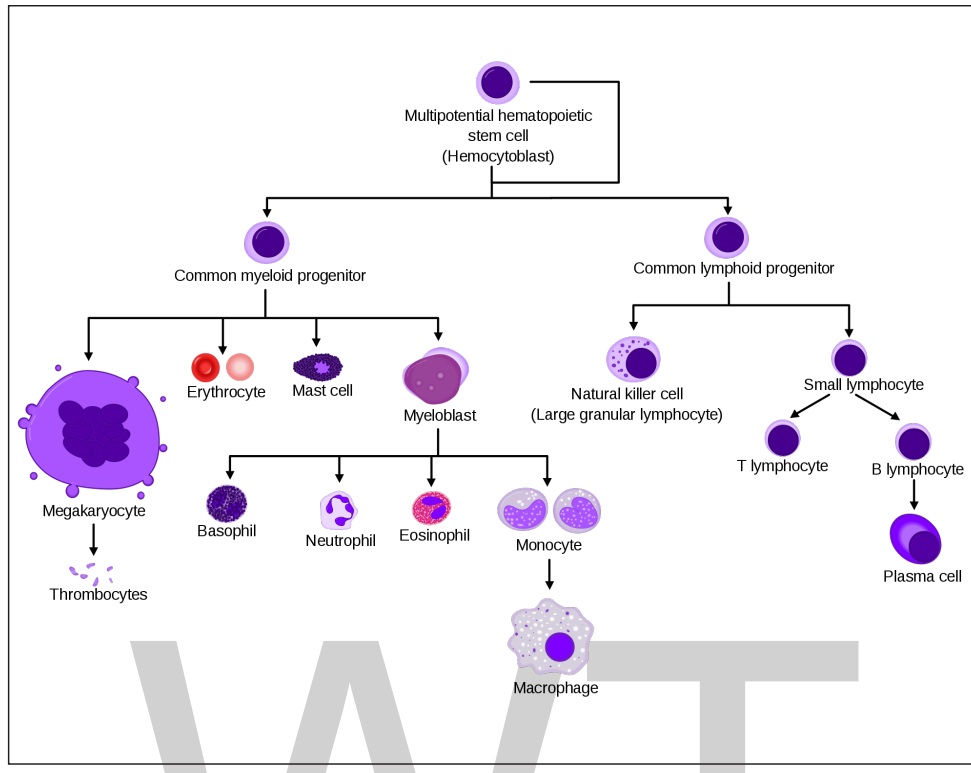


Diagram of cells that arise from Hematopoetic stem cells during the process of hematopoiesis.

Stem cell self-renewal is thought to occur in the stem cell niche in the bone marrow, and it is reasonable to assume that key signals present in this niche will be important in self-renewal. There is much interest in the environmental and molecular requirements for HSC self-renewal, as understanding the ability of HSC to replenish themselves will eventually allow the generation of expanded populations of HSC *in vitro* that can be used therapeutically.

Quiescence

Hematopoietic stem cells, like all adult stem cells, mostly exist in a state of quiescence, or reversible growth arrest. The altered metabolism of quiescent HCSs helps the cells survive for extended periods of time in the hypoxic bone marrow environment. When provoked by cell death or damage, Hematopoietic stem cells exit quiescence and begin actively dividing again. The transition from dormancy to propagation and back is regulated by the MEK/ERK pathway and PI3K/AKT/mTOR pathway. Dysregulation of these transitions can lead to stem cell exhaustion, or the gradual loss of active Hematopoietic stem cells in the blood system.

Mobility

Hematopoietic stem cells have a higher potential than other immature blood cells to pass the bone marrow barrier, and, thus, may travel in the blood from the bone marrow in one bone to another bone. If they settle in the thymus, they may develop into T cells. In the case of fetuses and other extramedullary hematopoiesis, Hematopoietic stem cells may also settle in the liver or spleen and develop.

This enables Hematopoietic stem cells to be harvested directly from the blood.

DNA Damage with Aging

DNA strand breaks accumulate in long term Hematopoietic stem cells during aging. This accumulation is associated with a broad attenuation of DNA repair and response pathways that depends on HSC quiescence. Non-homologous end joining (NHEJ) is a pathway that repairs double-strand breaks in DNA. NHEJ is referred to as "non-homologous" because the break ends are directly ligated without the need for a homologous template. The NHEJ pathway depends on several proteins including ligase 4, DNA polymerase mu and NHEJ factor 1 (NHEJ1, also known as Cernunnos or XLF).

DNA ligase 4 (Lig4) has a highly specific role in the repair of double-strand breaks by NHEJ. Lig4 deficiency in the mouse causes a progressive loss of Hematopoietic stem cells during aging. Deficiency of lig4 in pluripotent stem cells results in accumulation of DNA double-strand breaks and enhanced apoptosis.

In polymerase mu mutant mice, hematopoietic cell development is defective in several peripheral and bone marrow cell populations with about a 40% decrease in bone marrow cell number that includes several hematopoietic lineages. Expansion potential of hematopoietic progenitor cells is also reduced. These characteristics correlate with reduced ability to repair double-strand breaks in hematopoietic tissue.

Deficiency of NHEJ factor 1 in mice leads to premature aging of hematopoietic stem cells as indicated by several lines of evidence including evidence that long-term repopulation is defective and worsens over time. Using a human induced pluripotent stem cell model of NHEJ1 deficiency, it was shown that NHEJ1 has an important role in promoting survival of the primitive hematopoietic progenitors. These NHEJ1 deficient cells possess a weak NHEJ1-mediated repair capacity that is apparently incapable of coping with DNA damages induced by physiological stress, normal metabolism, and ionizing radiation.

The sensitivity of haematopoietic stem cells to Lig4, DNA polymerase mu and NHEJ1 deficiency suggests that NHEJ is a key determinant of the ability of stem cells to maintain themselves against physiological stress over time. Rossi et al. found that endogenous DNA damage accumulates with age even in wild type Hematopoietic stem cells, and suggested that DNA damage accrual may be an important physiological mechanism of stem cell aging.

Clinical Significance

Transplant

Hematopoietic stem cell transplantation (HSCT) is the transplantation of multipotent hematopoietic stem cells, usually derived from bone marrow, peripheral blood, or umbilical cord blood. It may be autologous (the patient's own stem cells are used), allogeneic (the stem cells come from a donor) or syngeneic (from an identical twin).

It is most often performed for patients with certain cancers of the blood or bone marrow, such as multiple myeloma or leukemia. In these cases, the recipient's immune system is usually destroyed with radiation or chemotherapy before the transplantation. Infection and graft-versus-host disease are major complications of allogeneic HSCT.

In order to harvest stem cells from the circulating peripheral blood, blood donors are injected with a cytokine, such as granulocyte-colony stimulating factor (G-CSF), that induces cells to leave the bone marrow and circulate in the blood vessels. In mammalian embryology, the first definitive Hematopoietic stem cells are detected in the AGM (aorta-gonad-mesonephros), and then massively expanded in the fetal liver prior to colonising the bone marrow before birth.

Hematopoietic stem cell transplantation remains a dangerous procedure with many possible complications; it is reserved for patients with life-threatening diseases. As survival following the procedure has increased, its use has expanded beyond cancer to autoimmune diseases and hereditary skeletal dysplasias; notably malignant infantile osteopetrosis and mucopolysaccharidosis.

Behavior in Culture

A *cobblestone area-forming cell (CAFC)* assay is a cell culture-based empirical assay. When plated onto a confluent culture of stromal feeder layer, a fraction of Hematopoietic stem cells creep between the gaps (even though the stromal cells are touching each other) and eventually settle between the stromal cells and the substratum (here the dish surface) or trapped in the cellular processes between the stromal cells. Emperipolesis is the *in vivo* phenomenon in which one cell is completely engulfed into another (e.g. thymocytes into thymic nurse cells); on the other hand, when *in vitro*, lymphoid lineage cells creep beneath nurse-like cells, the process is called pseudoemperipolesis. This similar phenomenon is more commonly known in the HSC field by the cell culture terminology *cobble stone area-forming cells (CAFC)*, which means areas or clusters of cells look dull cobblestone-like under phase contrast microscopy, compared to the other Hematopoietic stem cells, which are refractile. This happens because the cells that are floating loosely on top of the stromal cells are spherical and thus refractile. However, the cells that creep beneath the stromal cells are flattened and, thus, not refractile. The mechanism of pseudoemperipolesis is only recently coming to light. It may be mediated by interaction through CXCR4 (CD184) the receptor for CXC Chemokines (e.g., SDF1) and $\alpha 4\beta 1$ integrins.

Repopulation Kinetics

Hematopoietic stem cells (HSC) cannot be easily observed directly, and, therefore, their behaviors need to be inferred indirectly. Clonal studies are likely the closest technique for single cell in vivo studies of HSC. Here, sophisticated experimental and statistical methods are used to ascertain that, with a high probability, a single HSC is contained in a transplant administered to a lethally irradiated host. The clonal expansion of this stem cell can then be observed over time by monitoring the percent donor-type cells in blood as the host is reconstituted. The resulting time series is defined as the repopulation kinetic of the HSC.

The reconstitution kinetics are very heterogeneous. However, using symbolic dynamics, one can show that they fall into a limited number of classes. To prove this, several hundred experimental repopulation kinetics from clonal Thy-1^{lo} SCA-1^{+} lin^{-} c-kit^{+} HSC were translated into symbolic sequences by assigning the symbols "+", "-", "~" whenever two successive measurements of the percent donor-type cells have a positive, negative, or unchanged slope, respectively. By using the Hamming distance, the repopulation patterns were subjected to cluster analysis yielding 16 distinct groups of kinetics. To finish the empirical proof, the Laplace add-one approach was used to determine that the probability of finding kinetics not contained in these 16 groups is very small. By

corollary, this result shows that the hematopoietic stem cell compartment is also heterogeneous by dynamical criteria.

It was originally believed that all Hematopoietic stem cells were alike in their self-renewal and differentiation abilities. This view was first challenged by the 2002 discovery by the Muller-Sieburg group in San Diego, who illustrated that different stem cells can show distinct repopulation patterns that are epigenetically predetermined intrinsic properties of clonal Thy-1^{lo} Sca-1^{+} lin^{-} c-kit^{+} HSC. The results of these clonal studies led to the notion of lineage bias. Using the ratio $\rho = L / M$ of lymphoid (L) to myeloid (M) cells in blood as a quantitative marker, the stem cell compartment can be split into three categories of HSC. Balanced (Bala) Hematopoietic stem cells repopulate peripheral white blood cells in the same ratio of myeloid to lymphoid cells as seen in unmanipulated mice (on average about 15% myeloid and 85% lymphoid cells, or $3 \leq \rho \leq 10$). Myeloid-biased (My-bi) Hematopoietic stem cells give rise to very few lymphocytes resulting in ratios $0 < \rho < 3$, while lymphoid-biased (Ly-bi) Hematopoietic stem cells generate very few myeloid cells, which results in lymphoid-to-myeloid ratios of $\rho > 10$. All three types are normal types of HSC, and they do not represent stages of differentiation. Rather, these are three classes of HSC, each with an epigenetically fixed differentiation program. These studies also showed that lineage bias is not stochastically regulated or dependent on differences in environmental influence. My-bi HSC self-renew longer than balanced or Ly-bi HSC. The myeloid bias results from reduced responsiveness to the lymphopoetin interleukin 7 (IL-7).

Subsequently, other groups confirmed and highlighted the original findings. For example, the Eaves group confirmed in 2007 that repopulation kinetics, long-term self-renewal capacity, and My-bi and Ly-bi are stably inherited intrinsic HSC properties. In 2010, the Goodell group provided additional insights about the molecular basis of lineage bias in side population (SP) SCA-1^{+} lin^{-} c-kit^{+} HSC. As previously shown for IL-7 signaling, it was found that a member of the transforming growth factor family (TGF-beta) induces and inhibits the proliferation of My-bi and Ly-bi HSC, respectively.

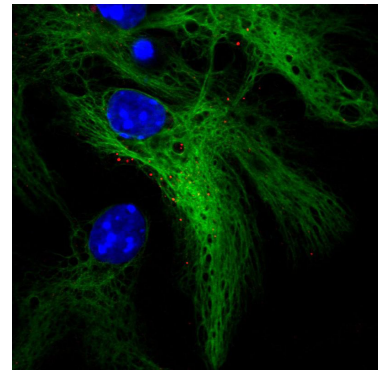
Neural Stem Cell

Neural stem cells (NSCs) are self-renewing, multipotent cells that firstly generate the radial glial progenitor cells that generate the neurons and glia of the nervous system of all animals during embryonic development. Some neural progenitor stem cells persist in highly restricted regions in the adult vertebrate brain and continue to produce neurons throughout life.

Stem cells are characterized by their capacity to differentiate into multiple cell types. They undergo symmetric or asymmetric cell division into two daughter cells. In symmetric cell division, both daughter cells are also stem cells. In asymmetric division, a stem cell produces one stem cell and one specialized cell. NSCs primarily differentiate into neurons, astrocytes, and oligodendrocytes.

Development

There are two basic types of stem cell: adult stem cells, which are limited in their ability to differentiate, and embryonic stem cells (ESCs), which are pluripotent and have the capability of differentiating into any cell type.



Neural stem cells differentiating to astrocytes (green) and sites of growth hormone receptor shown in red.

Neural stem cells are more specialized than ESCs because they only generate radial glial cells that give rise to the neurons and to glia of the central nervous system (CNS). During the embryonic development of vertebrates, NSCs transition into radial glial cells (RGCs) also known as radial glial progenitor cells, (RGPs) and reside in a transient zone called the ventricular zone (VZ). Neurons are generated in large numbers by (RGPs) during a specific period of embryonic development through the process of neurogenesis, and continue to be generated in adult life in restricted regions of the adult brain. Adult NSCs differentiate into new neurons within the adult subventricular zone (SVZ), a remnant of the embryonic germinal neuroepithelium, as well as the dentate gyrus of the hippocampus.

Adult NSCs were first isolated from mouse striatum in the early 1990s. They are capable of forming multipotent neurospheres when cultured *in vitro*. Neurospheres can produce self-renewing and proliferating specialized cells. These neurospheres can differentiate to form the specified neurons, glial cells, and oligodendrocytes. In previous studies, cultured neurospheres have been transplanted into the brains of immunodeficient neonatal mice and have shown engraftment, proliferation, and neural differentiation.

Communication and Migration

NSCs are stimulated to begin differentiation via exogenous cues from the microenvironment, or stem cell niche. Some neural cells are migrated from the SVZ along the rostral migratory stream which contains a marrow-like structure with ependymal cells and astrocytes when stimulated. The ependymal cells and astrocytes form glial tubes used by migrating neuroblasts. The astrocytes in the tubes provide support for the migrating cells as well as insulation from electrical and chemical signals released from surrounding cells. The astrocytes are the primary precursors for rapid cell amplification. The neuroblasts form tight chains and migrate towards the specified site of cell damage to repair or replace neural cells. One example is a neuroblast migrating towards the olfactory bulb to differentiate into periglomercular or granule neurons which have a radial migration pattern rather than a tangential one.

Aging

Neural stem cell proliferation declines as a consequence of aging. Various approaches have been taken to counteract this age-related decline. Because FOX proteins regulate neural stem cell homeostasis, FOX proteins have been used to protect neural stem cells by inhibiting Wnt signaling.

Function

Epidermal growth factor (EGF) and fibroblast growth factor (FGF) are mitogens that promote neural progenitor and stem cell growth *in vitro*, though other factors synthesized by the neural progenitor and stem cell populations are also required for optimal growth. It is hypothesized that neurogenesis in the adult brain originates from NSCs. The origin and identity of NSCs in the adult brain remain to be defined.

During Differentiation

The most widely accepted model of an adult NSC is a radial, astrocytes-like, GFAP-positive cell. Quiescent stem cells are Type B that are able to remain in the quiescent state due to the renewable tissue provided by the specific niches composed of blood vessels, astrocytes, microglia, ependymal cells, and extracellular matrix present within the brain. These niches provide nourishment, structural support, and protection for the stem cells until they are activated by external stimuli. Once activated, the Type B cells develop into Type C cells, active proliferating intermediate cells, which then divide into neuroblasts consisting of Type A cells. The undifferentiated neuroblasts form chains that migrate and develop into mature neurons. In the olfactory bulb, they mature into GABAergic granule neurons, while in the hippocampus they mature into dentate granule cells.

Epigenetic Modification

Epigenetic modifications are important regulators of gene expression in differentiating neural stem cells. Key epigenetic modifications include DNA cytosine methylation to form 5-methylcytosine and 5-methylcytosine demethylation. These types of modification are critical for cell fate determination in the developing and adult mammalian brain.

DNA cytosine methylation is catalyzed by DNA methyltransferases (DNMTs). Methylcytosine demethylation is catalyzed in several distinct steps by TET enzymes that carry out oxidative reactions (e.g. 5-methylcytosine to 5-hydroxymethylcytosine) and enzymes of the DNA base excision repair (BER) pathway.

During Disease

NSCs have an important role during development producing the enormous diversity of neurons, astrocytes and oligodendrocytes in the developing CNS. They also have important role in adult animals, for instance in learning and hippocampal plasticity in the adult mice in addition to supplying neurons to the olfactory bulb in mice.

Notably the role of NSCs during diseases is now being elucidated by several research groups around the world. The responses during stroke, multiple sclerosis, and Parkinson's disease in animal models and humans is part of the current investigation. The results of this ongoing investigation may have future applications to treat human neurological diseases.

Neural stem cells have been shown to engage in migration and replacement of dying neurons in classical experiments performed by Sanjay Magavi and Jeffrey Macklis. Using a laser-induced damage of cortical layers, Magavi showed that SVZ neural progenitors expressing Doublecortin, a critical molecule for migration of neuroblasts, migrated long distances to the area of damage and

differentiated into mature neurons expressing NeuN marker. In addition Masato Nakafuku's group from Japan showed for the first time the role of hippocampal stem cells during stroke in mice. These results demonstrated that NSCs can engage in the adult brain as a result of injury. Furthermore, in 2004 Evan Y. Snyder's group showed that NSCs migrate to brain tumors in a directed fashion. Jaime Imitola, M.D and colleagues from Harvard demonstrated for the first time, a molecular mechanism for the responses of NSCs to injury. They showed that chemokines released during injury such as SDF-1a were responsible for the directed migration of human and mouse NSCs to areas of injury in mice. Since then other molecules have been found to participate in the responses of NSCs to injury. All these results have been widely reproduced and expanded by other investigators joining the classical work of Richard L. Sidman in autoradiography to visualize neurogenesis during development, and neurogenesis in the adult by Joseph Altman in the 1960s, as evidence of the responses of adult NSCs activities and neurogenesis during homeostasis and injury.

The search for additional mechanisms that operate in the injury environment and how they influence the responses of NSCs during acute and chronic disease is matter of intense research.

Regenerative Therapy of the CNS

Cell death is a characteristic of acute CNS disorders as well as neurodegenerative disease. The loss of cells is amplified by the lack of regenerative abilities for cell replacement and repair in the CNS. One way to circumvent this is to use cell replacement therapy via regenerative NSCs. NSCs can be cultured *in vitro* as neurospheres. These neurospheres are composed of neural stem cells and progenitors (NSPCs) with growth factors such as EGF and FGF. The withdrawal of these growth factors activate differentiation into neurons, astrocytes, or oligodendrocytes which can be transplanted within the brain at the site of injury. The benefits of this therapeutic approach have been examined in Parkinson's disease, Huntington's disease, and multiple sclerosis. NSPCs induce neural repair via intrinsic properties of neuroprotection and immunomodulation. Some possible routes of transplantation include intracerebral transplantation and xenotransplantation.

An alternative therapeutic approach to the transplantation of NSPCs is the pharmacological activation of endogenous NSPCs (eNSPCs). Activated eNSPCs produce neurotrophic factors, several treatments that activate a pathway that involves the phosphorylation of STAT3 on the serine residue and subsequent elevation of Hes3 expression (STAT3-Ser/Hes3 Signaling Axis) oppose neuronal death and disease progression in models of neurological disorder.

Generation of 3D in Vitro Models of the Human CNS

Human midbrain-derived neural progenitor cells (hmNPCs) have the ability to differentiate down multiple neural cell lineages that lead to neurospheres as well as multiple neural phenotypes. The hmNPC can be used to develop a 3D *in vitro* model of the human CNS. There are two ways to culture the hmNPCs, the adherent monolayer and the neurosphere culture systems. The neurosphere culture system has previously been used to isolate and expand CNS stem cells by its ability to aggregate and proliferate hmNPCs under serum-free media conditions as well as with the presence of epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF2). Initially, the hmNPCs were isolated and expanded before performing a 2D differentiation which was used to produce a single-cell suspension. This single-cell suspension helped achieve a homogenous 3D structure of

uniform aggregate size. The 3D aggregation formed neurospheres which was used to form an *in vitro* 3D CNS model.

Bioactive Scaffolds as Traumatic Brain Injury Treatment

Traumatic brain injury (TBI) can deform the brain tissue, leading to necrosis primary damage which can then cascade and activate secondary damage such as excitotoxicity, inflammation, ischemia, and the breakdown of the blood-brain-barrier. Damage can escalate and eventually lead to apoptosis or cell death. Current treatments focus on preventing further damage by stabilizing bleeding, decreasing intracranial pressure and inflammation, and inhibiting pro-apoptoic cascades. In order to repair TBI damage, an upcoming therapeutic option involves the use of NSCs derived from the embryonic peri-ventricular region. Stem cells can be cultured in a favorable 3-dimensional, low cytotoxic environment, a hydrogel, that will increase NSC survival when injected into TBI patients. The intracerebrally injected, primed NSCs were seen to migrate to damaged tissue and differentiate into oligodendrocytes or neuronal cells that secreted neuroprotective factors.

Galectin-1 in Neural Stem Cells

Galectin-1 is expressed in adult NSCs and has been shown to have a physiological role in the treatment of neurological disorders in animal models. There are two approaches to using NSCs as a therapeutic treatment: (1) stimulate intrinsic NSCs to promote proliferation in order to replace injured tissue, and (2) transplant NSCs into the damaged brain area in order to allow the NSCs to restore the tissue. Lentivirus vectors were used to infect human NSCs (hNSCs) with Galectin-1 which were later transplanted into the damaged tissue. The hGal-1-hNSCs induced better and faster brain recovery of the injured tissue as well as a reduction in motor and sensory deficits as compared to only hNSC transplantation.

Assays

Neural stem cells are routinely studied *in vitro* using a method referred to as the Neurosphere Assay (or Neurosphere culture system), first developed by Reynolds and Weiss. Neurospheres are intrinsically heterogeneous cellular entities almost entirely formed by a small fraction (1 to 5%) of slowly dividing neural stem cells and by their progeny, a population of fast-dividing nestin-positive progenitor cells. The total number of these progenitors determines the size of a neurosphere and, as a result, disparities in sphere size within different neurosphere populations may reflect alterations in the proliferation, survival and/or differentiation status of their neural progenitors. Indeed, it has been reported that loss of β1-integrin in a neurosphere culture does not significantly affect the capacity of β1-integrin deficient stem cells to form new neurospheres, but it influences the size of the neurosphere: β1-integrin deficient neurospheres were overall smaller due to increased cell death and reduced proliferation.

While the Neurosphere Assay has been the method of choice for isolation, expansion and even the enumeration of neural stem and progenitor cells, several recent publications have highlighted some of the limitations of the neurosphere culture system as a method for determining neural stem cell frequencies. In collaboration with Reynolds, STEMCELL Technologies has developed a collagen-based assay, called the Neural Colony-Forming Cell (NCFC) Assay, for the quantification of neural stem cells. Importantly, this assay allows discrimination between neural stem and progenitor cells.

Endothelial Stem Cell

Endothelial stem cells (ESCs) are one of three types of stem cells found in bone marrow. They are multipotent, which describes the ability to give rise to many cell types, whereas a pluripotent stem cell can give rise to all types. ESCs have the characteristic properties of a stem cell: self-renewal and differentiation. These parent stem cells, ESCs, give rise to progenitor cells, which are intermediate stem cells that lose potency. Progenitor stem cells are committed to differentiating along a particular cell developmental pathway. ESCs will eventually produce endothelial cells (ECs), which create the thin-walled endothelium that lines the inner surface of blood vessels and lymphatic vessels.

Development

ECs were first thought to arise from extraembryonic tissues because blood vessels were observed in the avian and mammalian embryos. However, after histological analysis, it was seen that ECs were in the embryo. This meant that blood vessels come from an intraembryonic source, the mesoderm.

Role of Insulin-like Growth Factors in Endothelium Differentiation

ECs derived from stem cells are the beginning of vasculogenesis. Vasculogenesis is the new production of a vascular network from mesodermal progenitor cells. This can be distinguished from angiogenesis, which is the creation of new capillaries from vessels that already exist through the process of splitting or sprouting. This can occur "in vitro" in embryoid bodies (EB) derived from embryonic stem cells; this process in EB is similar to "in vivo" vasculogenesis. Important signaling factors for vasculogenesis are TGF-β, BMP4, and VEGF, all of which promote pluripotent stem cells to differentiate into mesoderm, endothelial progenitor cells, and then into mature endothelium.

It is well established that insulin-like growth factor (IGF) signaling is important for cell responses such as mitogenesis, cell growth, proliferation, angiogenesis, and differentiation. IGF1 and IGF2 increase the production of ECs in EB. A method that IGF employs to increase vasculogenesis is upregulation of VEGF. Not only is VEGF critical for mesoderm cells to become an EC, but also for EPCs to differentiate into mature endothelium. Understanding this process can lead to further research in vascular regeneration.

Function

Self-renewal and Differentiation

Stem cells have the unique ability make identical copies of themselves. This property maintains unspecialized and undifferentiated cells within the body. Differentiation is the process by which a cell becomes more specialized. For stem cells, this usually occurs through several stages, where a cell proliferates giving rise to daughter cells that are further specialized. For example, an endothelial progenitor cell (EPC) is more specialized than an ESC, and an EC is more specialized than an EPC. The further specialized a cell is, the more differentiated it is and as a result it is considered to be more committed to a certain cellular lineage.

Blood Vessel Formation

Blood vessels are made of a thin layer of ECs. As part of the circulatory system, blood vessels play a critical role in transporting blood throughout the body. Consequently, ECs have unique functions such as fluid filtration, homeostasis and hormone trafficking. ECs are the most differentiated form of an ESC. Formation of new blood vessels occurs by two different processes: vasculogenesis and angiogenesis. The former requires differentiation of endothelial cells from hemangioblasts and then the further organization into a primary capillary network. The latter occurs when new vessels are built from preexisting blood vessels.

Markers

The vascular system is made up of two parts: 1) Blood vasculature; 2) Lymphatic vessels.

Both parts consist of ECs that show differential expression of various genes. A study showed that ectopic expression of Prox-1 in blood vascular ECs (BECs) induced one-third of LEC specific gene expression. Prox-1is a homeobox transcription factor found in lymphatic ECs (LECs). For example, specific mRNAs such as VEGFR-3 and p57Kip2 were expressed by the BEC that was induced to express Prox-1.

Lymphatic-specific vascular endothelial growth factors VEGF-C and VEGF-D function as ligands for the vascular endothelial growth factor receptor 3 (VEGFR-3). The ligand-receptor interaction is essential for normal development of lymphatic tissues.

Tal1 gene is specifically found in the vascular endothelium and developing brain. This gene encodes the basic helix-loop-helix structure and functions as a transcription factor. Embryos lacking *Tal1* fail to develop past embryonic day 9.5. However, the study found that *Tal1* is actually required for vascular remodeling of the capillary network, rather than early endothelial development itself.

Fetal liver kinase-1 (Flk-1) is a cell surface receptor protein that is commonly used as a marker for ESCs and EPCs.

CD34 is another marker that can be found on the surface of ESCs and EPCs. It is characteristic of hematopoietic stem cells, as well as muscle stem cells.

Role in Formation of Vascular System

The two lineages arising from the EPC and the hematopoietic progenitor cell (HPC) form the blood circulatory system. Hematopoietic stem cells can of course undergo self-renewal, and are multipotent cells that give rise to erythrocytes (red blood cells), megakaryocytes/platelets, mast cells, T-lymphocytes, B-lymphocytes, dendritic cells, natural killer cells, monocyte/macrophage, and granulocytes. A study found that in the beginning stages of mouse embryogenesis, commencing at embryonic day 7.5, HPCs are produced close to the emerging vascular system. In the yolk sac's blood islands, HPCs and EC lineages emerge from the extraembryonic mesoderm in near unison. This creates a formation in which early erythrocytes are enveloped by angioblasts, and together they give rise to mature ECs. This observation gave rise to the hypothesis that the two lineages come from the same precursor, termed hemangioblast. Even though there is evidence that corroborates a hemangioblast, the isolation and exact location in the embryo has been difficult to

pinpoint. Some researchers have found that cells with hemangioblast properties have been located in the posterior end of the primitive streak during gastrulation.

In 1917, Florence Sabin first observer of blood vessels and red blood cells in the yolk sac of chick embryos occur in close proximity and time. Then, in 1932, Murray detected the same event and created the term "hemangioblast" for what Sabin had seen.

Further evidence to corroborate hemangioblasts come from the expression of various genes such as CD34 and Tie2 by both lineages. The fact that this expression was seen in both EC and HPC lineages led researchers to propose a common origin. However, endothelial markers like Flk1/VEGFR-2 are exclusive to ECs but stop HPCs from progressing into an EC. It is accepted that VEGFR-2+ cells are a common precursor for HPCs and ECs. If the *Vegfr3* gene is deleted then both HPC and EC differentiation comes to a halt in embryos. VEGF promotes angioblast differentiation; whereas, VEGFR-1 stops the hemangioblast from becoming an EC. In addition, basic fibroblast growth factor FGF-2 is also involved in promoting angioblasts from the mesoderm. After angioblasts commit to becoming an EC, the angioblasts gather and rearrange to assemble in a tube similar to a capillary. Angioblasts can travel during the formation of the circulatory system to configure the branches to allow for directional blood flow. Pericytes and smooth muscle cells encircle ECs when they are differentiating into arterial or venous arrangements. Surrounding the ECs creates a brace to help stabilize the vessels known as the pericellular basal lamina. It is suggested pericytes and smooth muscle cells come from neural crest cells and the surrounding mesenchyme.

Role in Recovery

ESCs and EPCs eventually differentiate into ECs. The endothelium secretes soluble factors to regulate vasodilatation and to preserve homeostasis. When there is any dysfunction in the endothelium, the body aims to repair the damage. Resident ESCs can generate mature ECs that replace the damaged ones. However, the intermediate progenitor cell cannot always generate functional ECs. This is because some of the differentiated cells may just have angiogenic properties.

Studies have shown that when vascular trauma occurs, EPCs and circulating endothelial progenitors (CEPs) are attracted to the site due to the release of specific chemokines. CEPs are derived from EPCs within the bone marrow, and the bone marrow is a reservoir of stem and progenitor cells. These cell types accelerate the healing process and prevent further complications such as hypoxia by gathering the cellular materials to reconstruct the endothelium.

Endothelium dysfunction is a prototypical characteristic of vascular disease, common in patients with autoimmune diseases such as systemic lupus erythematosus. Further, there is an inverse relationship between age and levels of EPCs. With a decline in EPCs the body loses its ability to repair the endothelium.

The use of stem cells for treatment has become a growing interest in the scientific community. Distinguishing between an ESC and its intermediate progenitor is nearly impossible, so research is now being done broadly on EPCs. One study showed that brief exposure to sevoflurane promoted growth and proliferation of EPCs. Sevoflurane is used in general anesthesia, but this finding shows the potential to induce endothelial progenitors. Using stem cells for cell replacement therapies is known as "regenerative medicine", which is a booming field that is now working on transplanting cells as opposed to bigger tissues or organs.

Clinical Significance

Role in Cancer

Understanding more about ESCs is important in cancer research. Tumours induce angiogenesis, which is the formation of new blood vessels. These cancerous cells do this by secreting factors such as VEGF and by reducing the amount of PGK, an anti-VEGF enzyme. The result is an uncontrolled production of beta-catenin, which regulates cell growth and cell mobility. With uncontrolled beta-catenin, the cell loses its adhesive properties. As ECs get packed together to create the lining for a new blood vessel, a single cancer cell is able to travel through the vessel to a distant site. If that cancer cell implants itself and begins forming a new tumour, the cancer has metastasized.

Tissue-specific Stem Cell

Tissue-specific stem cells (also referred to as somatic or adult stem cells) are more specialized than embryonic stem cells. Typically, these stem cells can generate different cell types for the specific tissue or organ in which they live.

For example, blood-forming (or hematopoietic) stem cells in the bone marrow can give rise to red blood cells, white blood cells and platelets. However, blood-forming stem cells don't generate liver or lung or brain cells, and stem cells in other tissues and organs don't generate red or white blood cells or platelets.

Some tissues and organs within your body contain small caches of tissue-specific stem cells whose job it is to replace cells from that tissue that are lost in normal day-to-day living or in injury, such as those in your skin, blood, and the lining of your gut.

Tissue-specific stem cells can be difficult to find in the human body, and they don't seem to self-renew in culture as easily as embryonic stem cells do.

Spore-like Cell

Spore-like cells were proposed to be pluripotent cells that lie dormant in animal tissue and become active under stress or injury as adult stem cells, exhibiting behavior characteristic of spores. They were proposed in 2001 by brothers Charles and Martin Vacanti and colleagues. Further work in collaboration with Japanese researchers led to the apparent discovery of STAP cells, in which the pluripotent cells were newly created by stress or injury. This work was published in 2014, but soon found to be due to fraudulent work by Haruko Obokata.

Characteristics

Spore-like cells were said to be a specific class of stem cells in adult organisms, including humans, which are small, versatile, and most frequently remain in a dormant "spore-like" state as the rest of

the cells of the organism divide, grow, and die. Despite their dormancy, they apparently retain the ability to grow, divide, and differentiate into other cell types expressing characteristics appropriate to the tissue environment from which they were initially isolated, if some external stimulus should prompt them to do so. This capacity to continue to regenerate new cells has been shown in in vitro conditions for some animals in which all other cells have died, especially if the animal died from exposure to cold elements.

Spore-like cells were said to remain viable in unprepared tissue (using no special preservation techniques), frozen at -86 °C and then thawed, or heated to 85 °C for more than 30 minutes. This has led researchers to try to revitalize spore-like cells from tissue samples of frozen carcasses deposited in permafrost for decades (frozen walrus meat more than 100 years old, and mammoth and bison in Alaska estimated to be 50,000 years old). Vacanti et al. believed that these unique cells lie dormant until activated by injury or disease, and that they have the potential to regenerate tissues lost to disease or damage. Because the cell-size of less than 5 micrometers seems rather small as to contain the entire human genome the authors speculate on the "concept of a minimal genome" for these cells.

Later Work

Charles Vacanti continued to work on these cells when he moved to Harvard, including with thoracic surgeon Koji Kojima who identified them in lung tissue. Working with a graduate student Haruko Obokata in his lab at Harvard from 2008, Vacanti later refined this theory to suggest that stress or injury could actually trigger the development of pluripotency in somatic cells. He first proposed this to Obokata and Masayuki Yamato at a conference in Florida in 2010; Yamato had independently come to the same conclusion. Obokata returned to Japan and continued this work at RIKEN. Vacanti presented these results in July 2012 at the Society of Cardiovascular Anesthesiologists conference, and then in January 2014 two reports suggesting that a simple acid treatment could cause mouse blood cells to become pluripotent. The *Boston Globe* reported that "His discovery is a reminder that as specialized as science is, sometimes, a little ignorance may be a virtue. A stem-cell expert would probably never have even bothered to try the experiment Vacanti has been pursuing, on and off, since the late 1990s." Both STAP reports were retracted in July 2014 after an investigation by RIKEN concluded that the data were fabricated.

Researcher Mariusz Ratajczak has linked spore-like cells to his idea of Very small embryonic-like stem cells, also proposed to be very small adult stem cells.

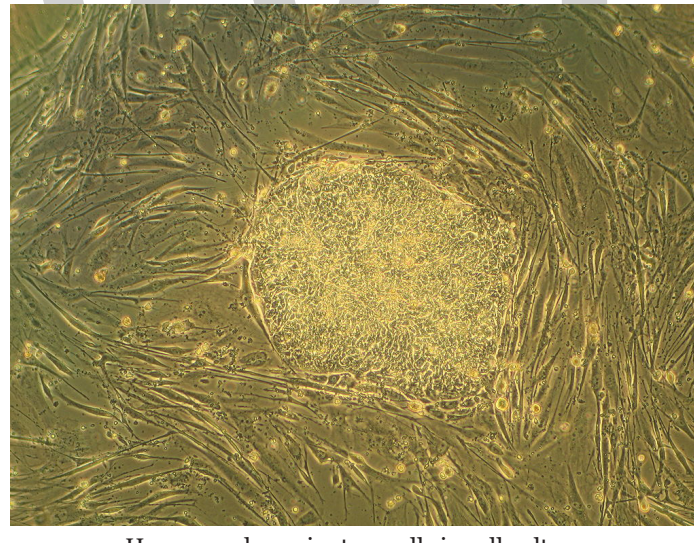
Induced Pluripotent Stem Cell

Induced pluripotent stem cells (iPSCs) are adult cells that have been genetically reprogrammed to an embryonic stem cell–like state by being forced to express genes and factors important for maintaining the defining properties of embryonic stem cells. Although these cells meet the defining criteria for pluripotent stem cells, it is not known if iPSCs and embryonic stem cells differ in clinically significant ways. Mouse iPSCs were first reported in 2006, and human iPSCs were first

reported in late 2007. Mouse iPSCs demonstrate important characteristics of pluripotent stem cells, including expressing stem cell markers, forming tumors containing cells from all three germ layers, and being able to contribute to many different tissues when injected into mouse embryos at a very early stage in development. Human iPSCs also express stem cell markers and are capable of generating cells characteristic of all three germ layers.

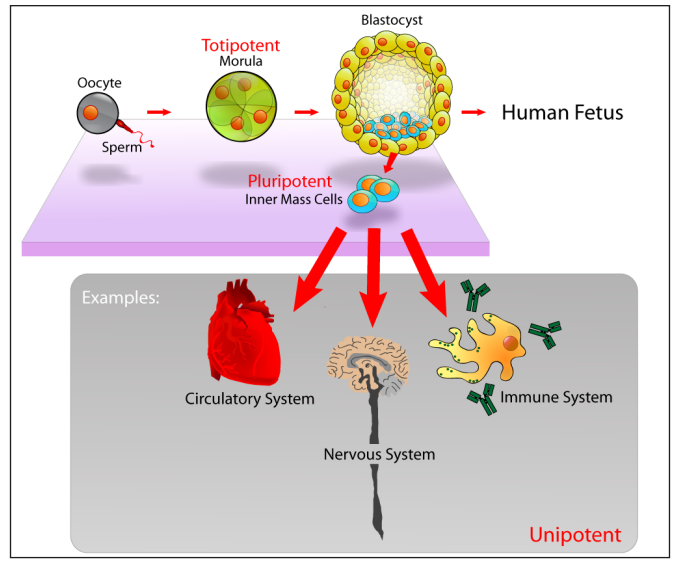
Although additional research is needed, iPSCs are already useful tools for drug development and modeling of diseases, and scientists hope to use them in transplantation medicine. Viruses are currently used to introduce the reprogramming factors into adult cells, and this process must be carefully controlled and tested before the technique can lead to useful treatment for humans. In animal studies, the virus used to introduce the stem cell factors sometimes causes cancers. Researchers are currently investigating non-viral delivery strategies. In any case, this breakthrough discovery has created a powerful new way to "de-differentiate" cells whose developmental fates had been previously assumed to be determined. In addition, tissues derived from iPSCs will be a nearly identical match to the cell donor and thus probably avoid rejection by the immune system. The iPSC strategy creates pluripotent stem cells that, together with studies of other types of pluripotent stem cells, will help researchers learn how to reprogram cells to repair damaged tissues in the human body.

Embryonic Stem Cell



Human embryonic stem cells in cell culture.

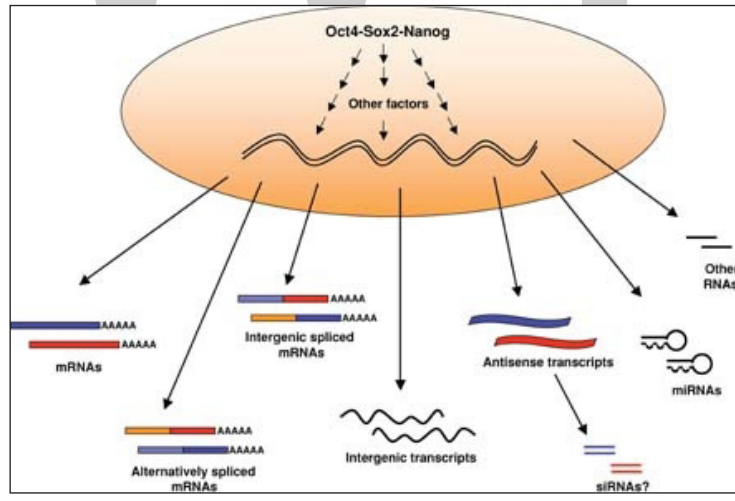
Embryonic stem cells (ES cells or ESCs) are pluripotent stem cells derived from the inner cell mass of a blastocyst, an early-stage pre-implantation embryo. Human embryos reach the blastocyst stage 4–5 days post fertilization, at which time they consist of 50–150 cells. Isolating the embryoblast, or inner cell mass (ICM) results in destruction of the blastocyst, a process which raises ethical issues, including whether or not embryos at the pre-implantation stage should have the same moral considerations as embryos in the post-implantation stage of development.



Pluripotent: Embryonic stem cells are able to develop into any type of cell, excepting those of the placenta. Only embryonic stem cells of the morula are totipotent: able to develop into any type of cell, including those of the placenta.

Researchers are currently focusing heavily on the therapeutic potential of embryonic stem cells, with clinical use being the goal for many laboratories. Potential uses include the treatment of diabetes and heart disease. The cells are being studied to be used as clinical therapies, models of genetic disorders, and cellular/DNA repair. However, adverse effects in the research and clinical processes such as tumours and unwanted immune responses have also been reported.

Properties



The transcriptome of embryonic stem cells.

Embryonic stem cells (ESCs), derived from the blastocyst stage of early mammalian embryos, are distinguished by their ability to differentiate into any embryonic cell type and by their ability to self-renew. It is these traits that makes them valuable in the scientific and medical fields. ESCs have a normal karyotype, maintain high telomerase activity, and exhibit remarkable long-term proliferative potential.

Pluripotent

Embryonic stem cells of the inner cell mass are pluripotent, meaning they are able to differentiate to generate primitive ectoderm, which ultimately differentiates during gastrulation into all derivatives of the three primary germ layers: ectoderm, endoderm, and mesoderm. These germ layers generate each of the more than 220 cell types in the adult human body. When provided with the appropriate signals, ESCs initially form precursor cells that in subsequently differentiate into the desired cell types. Pluripotency distinguishes embryonic stem cells from adult stem cells, which are multipotent and can only produce a limited number of cell types.

Self-Renewal

Under defined conditions, embryonic stem cells are capable of self-renewing indefinitely in an undifferentiated state. Self-renewal conditions must prevent the cells from clumping and maintain an environment that supports an unspecialized state. Typically this is done in the lab with media containing serum and leukemia inhibitory factor or serum-free media supplements with two inhibitory drugs ("2i"), the MEK inhibitor PD03259010 and GSK-3 inhibitor CHIR99021.

Growth

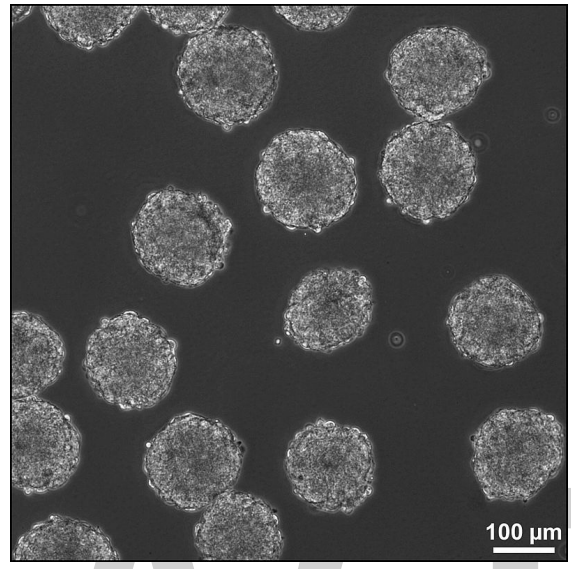
ESCs divide very frequently due to a shortened G1 phase in their cell cycle. Rapid cell division allows the cells to quickly grow in number, but not size, which is important for early embryo development. In ESCs, cyclin A and cyclin E proteins involved in the G1/S transition are always expressed at high levels. Cyclin-dependent kinases such as CDK2 that promote cell cycle progression are overactive, in part due to downregulation of their inhibitors. Retinoblastoma proteins that inhibit the transcription factor E2F until the cell is ready to enter S phase are hyperphosphorylated and inactivated in ESCs, leading to continual expression of proliferation genes. These changes result in accelerated cycles of cell division. Although the shortened G1 phase has been linked to maintenance of pluripotency, ESCs grown in serum-free 2i conditions do express hypo-phosphorylated active Retinoblastoma proteins and have an elongated G1 phase. Despite this difference in the cell cycle when compared to ESCs grown in media containing serum these cells have similar pluripotent characteristics. Pluripotency factors Oct4 and Nanog play a role in transcriptionally regulating the ESC cell cycle.

Uses

Due to their plasticity and potentially unlimited capacity for self-renewal, embryonic stem cell therapies have been proposed for regenerative medicine and tissue replacement after injury or disease. Pluripotent stem cells have shown promise in treating a number of varying conditions, including but not limited to: spinal cord injuries, age related macular degeneration, diabetes, neurodegenerative disorders (such as Parkinson's disease), AIDS, etc. In addition to their potential in regenerative medicine, embryonic stem cells provide a possible alternative source of tissue/organs which serves as a possible solution to the donor shortage dilemma. There are some ethical controversies surrounding this though. Aside from these uses, ESCs can also be used for research on early human development, certain genetic disease, and *in vitro* toxicology testing.

Utilizations

"Human embryonic stem cells have the potential to differentiate into various cell types, and, thus, may be useful as a source of cells for transplantation or tissue engineering."



Embryoid bodies 24 hours after formation.

However, embryonic stem cells are not limited to cell/tissue engineering.

Cell Replacement Therapies

Current research focuses on differentiating ESCs into a variety of cell types for eventual use as cell replacement therapies (CRTs). Some of the cell types that have or are currently being developed include cardiomyocytes (CM), neurons, hepatocytes, bone marrow cells, islet cells and endothelial cells. However, the derivation of such cell types from ESCs is not without obstacles, therefore current research is focused on overcoming these barriers. For example, studies are underway to differentiate ESCs into tissue specific CMs and to eradicate their immature properties that distinguish them from adult CMs.

Clinical Potential

- Researchers have differentiated ESCs into dopamine-producing cells with the hope that these neurons could be used in the treatment of Parkinson's disease.
- ESCs have been differentiated to natural killer (NK) cells and bone tissue.
- Studies involving ESCs are underway to provide an alternative treatment for diabetes. For example, D'Amour *et al.* were able to differentiate ESCs into insulin producing cells and researchers at Harvard University were able to produce large quantities of pancreatic beta cells from ES.
- Translational process of generating human embryonic stem cell-derived cardiac progenitor cells to be used in clinical trials of patients with severe heart failure.

Drug Discovery

Besides becoming an important alternative to organ transplants, ESCs are also being used in field of toxicology and as cellular screens to uncover new chemical entities (NCEs) that can be developed as small molecule drugs. Studies have shown that cardiomyocytes derived from ESCs are validated in vitro models to test drug responses and predict toxicity profiles. ES derived cardiomyocytes have been shown to respond to pharmacological stimuli and hence can be used to assess cardiotoxicity like *Torsades de Pointes*.

ESC-derived hepatocytes are also useful models that could be used in the preclinical stages of drug discovery. However, the development of hepatocytes from ESCs has proven to be challenging and this hinders the ability to test drug metabolism. Therefore, current research is focusing on establishing fully functional ESC-derived hepatocytes with stable phase I and II enzyme activity.

Models of Genetic Disorder

Several new studies have started to address the concept of modeling genetic disorders with embryonic stem cells. Either by genetically manipulating the cells, or more recently, by deriving diseased cell lines identified by prenatal genetic diagnosis (PGD), modeling genetic disorders is something that has been accomplished with stem cells. This approach may very well prove valuable at studying disorders such as Fragile-X syndrome, Cystic fibrosis, and other genetic maladies that have no reliable model system.

Yury Verlinsky, a Russian-American medical researcher who specialized in embryo and cellular genetics (genetic cytology), developed prenatal diagnosis testing methods to determine genetic and chromosomal disorders a month and a half earlier than standard amniocentesis. The techniques are now used by many pregnant women and prospective parents, especially couples who have a history of genetic abnormalities or where the woman is over the age of 35 (when the risk of genetically related disorders is higher). In addition, by allowing parents to select an embryo without genetic disorders, they have the potential of saving the lives of siblings that already had similar disorders and diseases using cells from the disease free offspring.

Repair of DNA Damage

Differentiated somatic cells and ES cells use different strategies for dealing with DNA damage. For instance, human foreskin fibroblasts, one type of somatic cell, use non-homologous end joining (NHEJ), an error prone DNA repair process, as the primary pathway for repairing double-strand breaks (DSBs) during all cell cycle stages. Because of its error-prone nature, NHEJ tends to produce mutations in a cell's clonal descendants.

ES cells use a different strategy to deal with DSBs. Because ES cells give rise to all of the cell types of an organism including the cells of the germ line, mutations arising in ES cells due to faulty DNA repair are a more serious problem than in differentiated somatic cells. Consequently, robust mechanisms are needed in ES cells to repair DNA damages accurately, and if repair fails, to remove those cells with un-repaired DNA damages. Thus, mouse ES cells predominantly use high fidelity homologous recombinational repair (HRR) to repair DSBs. This type of repair depends on the interaction of the two sister chromosomes formed during S phase and present together during

the G2 phase of the cell cycle. HRR can accurately repair DSBs in one sister chromosome by using intact information from the other sister chromosome. Cells in the G1 phase of the cell cycle (i.e. after metaphase/cell division but prior the next round of replication) have only one copy of each chromosome (i.e. sister chromosomes aren't present). Mouse ES cells lack a G1 checkpoint and do not undergo cell cycle arrest upon acquiring DNA damage. Rather they undergo programmed cell death (apoptosis) in response to DNA damage. Apoptosis can be used as a fail-safe strategy to remove cells with un-repaired DNA damages in order to avoid mutation and progression to cancer. Consistent with this strategy, mouse ES stem cells have a mutation frequency about 100-fold lower than that of isogenic mouse somatic cells.

Clinical Trial

On January 23, 2009, Phase I clinical trials for transplantation of oligodendrocytes (a cell type of the brain and spinal cord) derived from human ES cells into spinal cord-injured individuals received approval from the U.S. Food and Drug Administration (FDA), marking it the world's first human ES cell human trial. The study leading to this scientific advancement was conducted by Hans Keirstead and colleagues at the University of California, Irvine and supported by Geron Corporation of Menlo Park, CA, founded by Michael D. West, PhD. A previous experiment had shown an improvement in locomotor recovery in spinal cord-injured rats after a 7-day delayed transplantation of human ES cells that had been pushed into an oligodendrocytic lineage. The phase I clinical study was designed to enroll about eight to ten paraplegics who have had their injuries no longer than two weeks before the trial begins, since the cells must be injected before scar tissue is able to form. The researchers emphasized that the injections were not expected to fully cure the patients and restore all mobility. Based on the results of the rodent trials, researchers speculated that restoration of myelin sheathes and an increase in mobility might occur. This first trial was primarily designed to test the safety of these procedures and if everything went well, it was hoped that it would lead to future studies that involve people with more severe disabilities. The trial was put on hold in August 2009 due to FDA concerns regarding a small number of microscopic cysts found in several treated rat models but the hold was lifted on July 30, 2010.

In October 2010 researchers enrolled and administered ESTs to the first patient at Shepherd Center in Atlanta. The makers of the stem cell therapy, Geron Corporation, estimated that it would take several months for the stem cells to replicate and for the GRNOPC1 therapy to be evaluated for success or failure.

In November 2011 Geron announced it was halting the trial and dropping out of stem cell research for financial reasons, but would continue to monitor existing patients, and was attempting to find a partner that could continue their research. In 2013 BioTime, led by CEO Dr. Michael D. West, acquired all of Geron's stem cell assets, with the stated intention of restarting Geron's embryonic stem cell-based clinical trial for spinal cord injury research.

BioTime company Asterias Biotherapeutics (NYSE MKT: AST) was granted a $14.3 million Strategic Partnership Award by the California Institute for Regenerative Medicine (CIRM) to re-initiate the world's first embryonic stem cell-based human clinical trial, for spinal cord injury. Supported by California public funds, CIRM is the largest funder of stem cell-related research and development in the world.

The award provides funding for Asterias to reinitiate clinical development of AST-OPC1 in subjects with spinal cord injury and to expand clinical testing of escalating doses in the target population intended for future pivotal trials.

AST-OPC1 is a population of cells derived from human embryonic stem cells (hESCs) that contains oligodendrocyte progenitor cells (OPCs). OPCs and their mature derivatives called oligodendrocytes provide critical functional support for nerve cells in the spinal cord and brain. Asterias recently presented the results from phase 1 clinical trial testing of a low dose of AST-OPC1 in patients with neurologically-complete thoracic spinal cord injury. The results showed that AST-OPC1 was successfully delivered to the injured spinal cord site. Patients followed 2–3 years after AST-OPC1 administration showed no evidence of serious adverse events associated with the cells in detailed follow-up assessments including frequent neurological exams and MRIs. Immune monitoring of subjects through one year post-transplantation showed no evidence of antibody-based or cellular immune responses to AST-OPC1. In four of the five subjects, serial MRI scans performed throughout the 2–3 year follow-up period indicate that reduced spinal cord cavitation may have occurred and that AST-OPC1 may have had some positive effects in reducing spinal cord tissue deterioration. There was no unexpected neurological degeneration or improvement in the five subjects in the trial as evaluated by the International Standards for Neurological Classification of Spinal Cord Injury (ISNCSCI) exam.

The Strategic Partnership III grant from CIRM will provide funding to Asterias to support the next clinical trial of AST-OPC1 in subjects with spinal cord injury, and for Asterias' product development efforts to refine and scale manufacturing methods to support later-stage trials and eventually commercialization. CIRM funding will be conditional on FDA approval for the trial, completion of a definitive agreement between Asterias and CIRM, and Asterias' continued progress toward the achievement of certain pre-defined project milestones.

Adverse Effects

The major concern with the possible transplantation of ESC into patients as therapies is their ability to form tumors including teratoma. Safety issues prompted the FDA to place a hold on the first ESC clinical trial, however no tumors were observed.

The main strategy to enhance the safety of ESC for potential clinical use is to differentiate the ESC into specific cell types (e.g. neurons, muscle, liver cells) that have reduced or eliminated ability to cause tumors. Following differentiation, the cells are subjected to sorting by flow cytometry for further purification. ESC are predicted to be inherently safer than IPS cells created with genetically-integrating viral vectors because they are not genetically modified with genes such as c-Myc that are linked to cancer. Nonetheless, ESC express very high levels of the iPS inducing genes and these genes including Myc are essential for ESC self-renewal and pluripotency, and potential strategies to improve safety by eliminating c-Myc expression are unlikely to preserve the cells' "stemness". However, N-myc and L-myc have been identified to induce iPS cells instead of c-myc with similar efficiency. More recent protocols to induce pluripotency bypass these problems completely by using non-integrating RNA viral vectors such as sendai virus or mRNA transfection.

Ethical Debate

Due to the nature of embryonic stem cell research, there are a lot of controversial opinions on the topic. Since harvesting embryonic stem cells necessitates destroying the embryo from which those cells are obtained, the moral status of the embryo comes into question. Some people argue that the 5-day old mass of cells is too young to achieve personhood or that the embryo, if donated from an IVF clinic (which is where labs typically acquire embryos from), would otherwise go to medical waste anyway. Opponents of ESC research counter that an embryo is a human life, therefore destroying it is murder and the embryo must be protected under the same ethical view as a more developed human being.

- 1964: Lewis Kleinsmith and G. Barry Pierce Jr. isolated a single type of cell from a teratocarcinoma, a tumor now known to be derived from a germ cell. These cells isolated from the teratocarcinoma replicated and grew in cell culture as a stem cell and are now known as embryonal carcinoma (EC) cells. Although similarities in morphology and differentiating potential (pluripotency) led to the use of EC cells as the *in vitro* model for early mouse development, EC cells harbor genetic mutations and often abnormal karyotypes that accumulated during the development of the teratocarcinoma. These genetic aberrations further emphasized the need to be able to culture pluripotent cells directly from the inner cell mass.

Martin Evans revealed a new technique for culturing the mouse embryos in the uterus to allow for the derivation of ES cells from these embryos.

- 1981: Embryonic stem cells (ES cells) were independently first derived from mouse embryos by two groups. Martin Evans and Matthew Kaufman from the Department of Genetics, University of Cambridge published first in July, revealing a new technique for culturing the mouse embryos in the uterus to allow for an increase in cell number, allowing for the derivation of ES cells from these embryos. Gail R. Martin, from the Department of Anatomy, University of California, San Francisco, published her paper in December and coined the term "Embryonic Stem Cell". She showed that embryos could be cultured *in vitro* and that ES cells could be derived from these embryos.
- 1989: Mario R. Cappechi, Martin J. Evans, and Oliver Smithies publish their research which details their isolation and genetic modifications of embryonic stem cells, creating the first "knockout mice". In creating knockout mice, this publication provided scientists with an entirely new way to study disease.

- 1998: A team from the University of Wisconsin, Madison (James A. Thomson, Joseph Itskovitz-Eldor, Sander S. Shapiro, Michelle A. Waknitz, Jennifer J. Swiergiel, Vivienne S. Marshall, and Jeffrey M. Jones) publish a paper titled "Embryonic Stem Cell Lines Derived From Human Blastocysts". The researchers behind this study not only create the first embryonic stem cells, but recognize their pluripotency, as well as their capacity for self-renewal. The abstract of the paper notes the significance of the discovery with regards to the fields of developmental biology and drug discovery.
- 2001: President George W. Bush allows federal funding to support research on roughly 60—at this time, already existing—lines of embryonic stem cells. Seeing as the limited lines that Bush allowed research on had already been established, this law supported embryonic stem cell research without raising any ethical questions that could arise with the creation of new lines under federal budget.
- 2006: Japanese scientists Shinya Yamanaka and Kazutoshi Takashi publish a paper describing the induction of pluripotent stem cells from cultures of adult mouse fibroblasts. Induced pluripotent stem cells (iPSCs) are a huge discovery, as they are seemingly identical to embryonic stem cells and could be used without sparking the same moral controversy.
- January, 2009: The US Food and Drug Administration (FDA) provides approval for Geron Corporation's phase I trial of their human embryonic stem cell-derived treatment for spinal cord injuries. The announcement was met with excitement from the scientific community, but also with wariness from stem cell opposers. The treatment cells were, however, derived from the cell lines approved under George W. Bush's ESC policy.
- March, 2009: Executive Order 13505 is signed by President Barack Obama, removing the restrictions put in place on federal funding for human stem cells by the previous presidential administration. This would allow the National Institutes of Health (NIH) to provide funding for hESC research. The document also states that the NIH must provide revised federal funding guidelines within 120 days of the order's signing.

Techniques and Conditions for Derivation and Culture

Derivation from Humans

In vitro fertilization generates multiple embryos. The surplus of embryos is not clinically used or is unsuitable for implantation into the patient, and therefore may be donated by the donor with consent. Human embryonic stem cells can be derived from these donated embryos or additionally they can also be extracted from cloned embryos using a cell from a patient and a donated egg. The inner cell mass (cells of interest), from the blastocyst stage of the embryo, is separated from the trophectoderm, the cells that would differentiate into extra-embryonic tissue. Immunosurgery, the process in which antibodies are bound to the trophectoderm and removed by another solution, and mechanical dissection are performed to achieve separation. The resulting inner cell mass cells are plated onto cells that will supply support. The inner cell mass cells attach and expand further to form a human embryonic cell line, which are undifferentiated. These cells are fed daily and are enzymatically or mechanically separated every four to seven days. For differentiation to occur, the human embryonic stem cell line is removed from the supporting cells to form embryoid bodies, is co-cultured with a serum containing necessary signals, or is grafted in a three-dimensional scaffold to result.

Derivation from other Animals

Embryonic stem cells are derived from the inner cell mass of the early embryo, which are harvested from the donor mother animal. Martin Evans and Matthew Kaufman reported a technique that delays embryo implantation, allowing the inner cell mass to increase. This process includes removing the donor mother's ovaries and dosing her with progesterone, changing the hormone environment, which causes the embryos to remain free in the uterus. After 4–6 days of this intrauterine culture, the embryos are harvested and grown in *in vitro* culture until the inner cell mass forms "egg cylinder-like structures," which are dissociated into single cells, and plated on fibroblasts treated with mitomycin-c (to prevent fibroblast mitosis). Clonal cell lines are created by growing up a single cell. Evans and Kaufman showed that the cells grown out from these cultures could form teratomas and embryoid bodies, and differentiate *in vitro,* all of which indicating that the cells are pluripotent.

Gail Martin derived and cultured her ES cells differently. She removed the embryos from the donor mother at approximately 76 hours after copulation and cultured them overnight in a medium containing serum. The following day, she removed the inner cell mass from the late blastocyst using microsurgery. The extracted inner cell mass was cultured on fibroblasts treated with mitomycin-c in a medium containing serum and conditioned by ES cells. After approximately one week, colonies of cells grew out. These cells grew in culture and demonstrated pluripotent characteristics, as demonstrated by the ability to form teratomas, differentiate *in vitro,* and form embryoid bodies. Martin referred to these cells as ES cells.

It is now known that the feeder cells provide leukemia inhibitory factor (LIF) and serum provides bone morphogenetic proteins (BMPs) that are necessary to prevent ES cells from differentiating. These factors are extremely important for the efficiency of deriving ES cells. Furthermore, it has been demonstrated that different mouse strains have different efficiencies for isolating ES cells. Current uses for mouse ES cells include the generation of transgenic mice, including knockout mice. For human treatment, there is a need for patient specific pluripotent cells. Generation of human ES cells is more difficult and faces ethical issues. So, in addition to human ES cell research, many groups are focused on the generation of induced pluripotent stem cells (iPS cells).

Potential Method for New Cell Line Derivation

Research found a way to extract embryonic stem cells without destroying the actual embryo. This technical achievement would potentially enable scientists to work with new lines of embryonic stem cells derived using public funding in the USA, where federal funding was at the time limited to research using embryonic stem cell lines derived prior to August 2001. In March, 2009, the limitation was lifted.

Induced Pluripotent Stem Cells

The iPSC technology was pioneered by Shinya Yamanaka's lab in Kyoto, Japan, who showed in 2006 that the introduction of four specific genes encoding transcription factors could convert adult cells into pluripotent stem cells. He was awarded the 2012 Nobel Prize along with Sir John Gurdon "for the discovery that mature cells can be reprogrammed to become pluripotent."

In 2007 it was shown that pluripotent stem cells highly similar to embryonic stem cells can be generated by the delivery of three genes (*Oct4*, *Sox2*, and *Klf4*) to differentiated cells. The delivery of these genes "reprograms" differentiated cells into pluripotent stem cells, allowing for the generation of pluripotent stem cells without the embryo. Because ethical concerns regarding embryonic stem cells typically are about their derivation from terminated embryos, it is believed that reprogramming to these "induced pluripotent stem cells" (iPS cells) may be less controversial. Both human and mouse cells can be reprogrammed by this methodology, generating both human pluripotent stem cells and mouse pluripotent stem cells without an embryo.

This may enable the generation of patient specific ES cell lines that could potentially be used for cell replacement therapies. In addition, this will allow the generation of ES cell lines from patients with a variety of genetic diseases and will provide invaluable models to study those diseases.

However, as a first indication that the induced pluripotent stem cell (iPS) cell technology can in rapid succession lead to new cures, it was used by a research team headed by Rudolf Jaenisch of the Whitehead Institute for Biomedical Research in Cambridge, Massachusetts, to cure mice of sickle cell anemia.

On January 16, 2008, a California-based company, Stemagen, announced that they had created the first mature cloned human embryos from single skin cells taken from adults. These embryos can be harvested for patient matching embryonic stem cells.

Contamination by Reagents used in Cell Culture

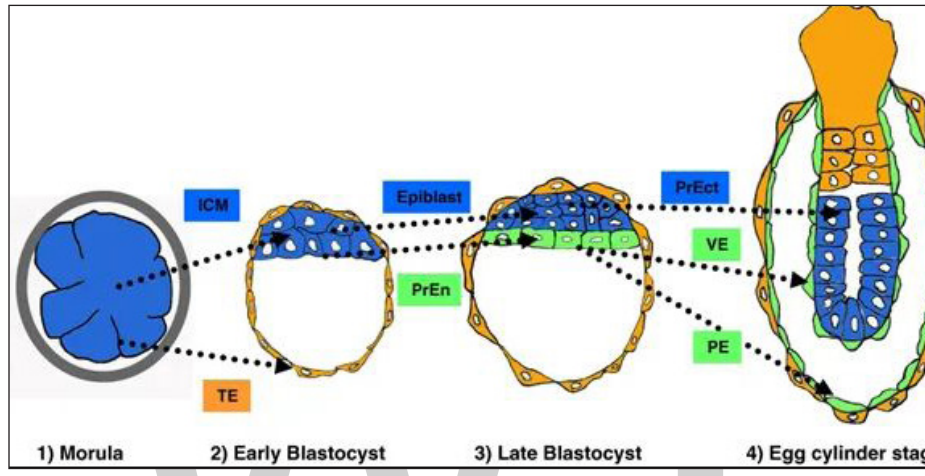
The online edition of *Nature Medicine* published a study on January 24, 2005, which stated that the human embryonic stem cells available for federally funded research are contaminated with non-human molecules from the culture medium used to grow the cells. It is a common technique to use mouse cells and other animal cells to maintain the pluripotency of actively dividing stem cells. The problem was discovered when non-human sialic acid in the growth medium was found to compromise the potential uses of the embryonic stem cells in humans, according to scientists at the University of California, San Diego.

About a new stem cell line that was derived from human embryos under completely cell- and serum-free conditions. After more than 6 months of undifferentiated proliferation, these cells demonstrated the potential to form derivatives of all three embryonic germ layers both *in vitro* and in teratomas. These properties were also successfully maintained (for more than 30 passages) with the established stem cell lines.

Molecular and Biological Properties of Pluripotent Embryonic Stem Cell

At the early blastocyst stage of development, the mammalian embryo is comprised of three predominant cell populations. First, the trophectoderm (TE), which gives rise to extraembryonic tissues, such as placenta, which are critical for supporting embryonic development by facilitating exchange of nutrients and oxygen with the mother. The second is a ball of approximately 20 cells known as the inner cell mass (ICM). The ICM is comprised of pluripotent cells, which amplify rapidly during the epiblast stage of development and subsequently differentiate into the three embryonic germ layers (ectoderm, mesoderm and definitive endoderm)—the founders of all adult

tissues. The ICM is surrounded by a third cell type known as primitive endoderm (PrEn). Formation of PrEn around the ICM denotes the epiblast stage of development, which extends until gastrulation. As the epiblast develops past the ICM stage to the egg cylinder stage, a cavity forms at the core of the pluripotent cells resulting in the formation of a single layer of pseudostratified epithelia known as primitive ectoderm (PrEct). Primitive ectoderm is pluripotent but represents a different stage of the pluripotent continuum that exists during pre-gastrulation development. As pluripotent cells commit toward one of the three germ layers, they lose pluripotency and just prior to or coinciding with this, the germ cells are segregated away into the allantois where they will preserve the germ line by migrating to the genital ridge.



An overview of peri-implantation mouse development. (1) Morula: around embryonic day 2.5 (E2.5), embryonic compaction occurs—cell boundaries at this stage are obscure. (2) Early blastocyst has two distinct cell types; ICMs (blue) and TE (orange). ICMs are composed of pluripotent cells and give rise to embryonic stem cells via adaptation of ICM cells to in vitro culture. Two types of TE exist at this stage of development: polar TE, which gives rise to trophoblast stem cells (located adjacent to the ICM) and mural trophoectoderm. (3) Late blastocyst: ICMs form two types of cells: epiblast (pluripotent) and PrEn. PrEn gives rise to the yolk sac to support embryonic development. PrEn differentiates toward visceral and parietal endoderm. (4) Egg cylinder stage: the embryonic epiblast is composed of a single layer of pseudostratified epithelia called PrEct. ICM, inner cell mass; PrEct, primitive ectoderm; PrEn, primitive endoderm; TE, trophectoderm.

Embryonic Stem Cells and Pluripotency

Murine embryonic stem cells (ESCs) were first isolated in 1981 from blastocyst stage embryos and exhibit two remarkable features in culture. First, under the appropriate conditions, they can be propagated indefinitely as a stable self-renewing population where at every cell division, both mother and daughter cell retain stem-cell identity following a symmetric cell division. This immortalized phenotype allows ESCs to be cultured over extended periods of time. Upon differentiation, this feature is lost and progeny succumb to cellular aging mechanisms (Hayflick limit) as has been well documented for all other non-transformed primary cells. This self-renewing phenomenon seems to be developmentally regulated but it is not clear if it is inherently tied into the pluripotent state. A second feature of more developmental relevance is that during extended culture, ESCs retain their pluripotency and can differentiate into the same range of cell types as those formed in

the embryo from the ICM. For the purposes of this discussion, we describe pluripotency as being the ability to generate all adult cell types and totipotency as the ability to form all adult, germ line and extra-embryonic tissues. The latter definition is usually reserved for fertilized eggs since ESCs can not contribute to TE or PrEn when injected into blastocysts, but do differentiate into other lineages. A stringent test for the developmental potential for mESCs is their ability to contribute to the germ line and all tissues of an adult animal following injection into recipient blastocysts. A common alternate assay for ESC potency, particularly for hESCs where embryo transfer is not practical, is to inject ESCs into immunocompromised mice where they form mixed cell tumors known as teratomas. The ability of injected ESCs to generate a tumor comprising mesoderm, ectoderm and endoderm lineages is indicative of their multipotency.

While functional analysis of ESC developmental potential is the gold-standard for ESC analysis, molecular markers are often used as readouts for the stem-cell state because of practical issues. Many of these markers are transcription factors expressed in the ICM and ESCs and which have demonstrated roles in maintenance of ESCs and/or the ICM. The best-characterized examples include the POU domain transcription factor Oct3/4, the homeodomain transcription factor Nanog and the high-mobility group protein protein Sox2. Transcription factor networks involved in ESC pluripotency have been reviewed elsewhere. Other signatures include high TERT expression and presentation of characteristic cell-surface antigens such as the glycomarker SSEA1, the tetraspanin CD916 and the carbohydrate epitope N-acetylgalactosamine.

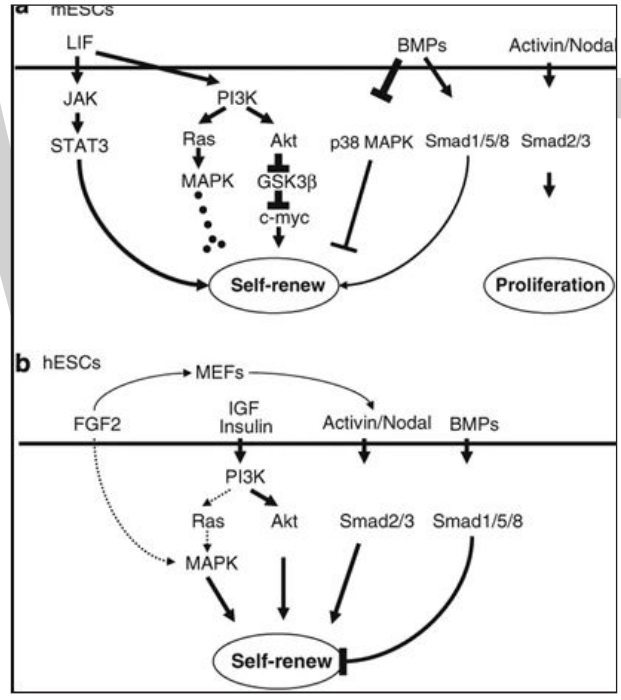
Most of the transcription-factor regulatory networks responsible for maintenance of pluripotency appear to have been conserved between human and mouse ESCs. Striking differences do emerge however, when cell-surface markers of ESCs are compared. hESCs do not exhibit high SSEA1 reactivity, but instead are identified based on elevated SSEA3, -4 and TRA-1–60, TRA-1–81 antigens. In contrast to mouse ESCs, N-acetylgalactosamine epitopes recognized by the lectin DBA are not a feature of hESCs (SD unpublished). The central question that again arises is: Are human and mouse ESCs representative of different stages of development or, are mouse and human pluripotent cells different in many respects? While human and murine ESCs pack tightly together and individual cells exhibit a high nuclear-to-cytoplasmic volume ratio, gross morphological differences exist between the structures of ESC colonies. For example, mESCs grow as a three-dimensional dome-shaped colonies whereas hESCs grow in colonies as thin layers, often monolayers. Self-renewal signals required for hESCs and mESCs maintenance are quite differ.

Isolation of pluripotent cells from mammalian embryos has clearly focused on the pre-implantation (ICM) stage of development where clear success has been obtained, resulting in the successful isolation of ESC-like cells from several species. Successful isolation of self-renewing populations from post-implantation stages had not been successful until recently when two groups reported the isolation of stem cells from the murine late-stage epiblast. Establishment of epiblast stem cells (EpiSCs) does not require the same cocktail of media components as for mESCs. Although EpiSCs can be maintained on mouse embryonic fibroblasts (MEFs) (as can mESCs and hESCs), a major difference between mESCs and EpiSCs is that under feeder-free conditions, mESCs have a requirement for IL6 family member cytokines such as leukemia inhibitory factor (LIF) to maintain self-renewal and pluripotency. This is not the case with EpiScs where LIF is not required. Moreover, the colony morphology of EpiSCs is more reminiscent of hESCs than mESCs since they grow as flat, epithelial colonies. In common with hESCs, EpiSCs have a requirement for Activin/Nodal

signaling to promote self-renewal and they respond to bone morphogenetic protein-4 (BMP4) by differentiating into TE. Although EpiSCs can differentiate into the three embryonic germ layers, their ability to contribute to the germ line has not been tested and so the differentiation capacity of these cells is yet to be fully characterized. These are very exciting findings and indicate that ICM is not the only embryonic stage from which self-renewing stem cells can be isolated. It should be pointed out that successful attempts have been made to isolate mESCs and hESCs from different stages of pre-implantation stages of development, but not from post-implantation stages. The parallel between EpiSCs and hESC did not escape the authors' attention and the possibility that they represent a developmentally equivalent cell type was raised.

Signaling Pathways Required for Murine ESC Self-renewal

As elucidated briefly already, the signaling requirements for maintenance of human and murine ESCs differ considerably. Why should this be? We will discuss the literature and try to reconcile these differences by considering known signaling pathways implicated in ESC self-renewal.



Key signaling pathways required for maintaining pluripotency of (a) mESCs and (b) hESCs. (a) LIF signaling activates JAK–STAT3 to induce target genes essential for pluripotency, such as c-myc. c-myc is also regulated negatively by glycogen synthase kinase-3 (GSK3)β via inhibitory phosphorylation. LIF also induces MAP kinase activation, which antagonizes self-renewal. BMP signals potentially function in two ways: (i) activation of Smad1/5/8-Id gene and (ii) suppression of p38 MAP kinase. Activin A has been shown to contribute mESCs proliferation but not pluripotency. (b) FGf2 is an essential factor for hESCs self-renewal and functions in part at least by inducing Activin secretion from MEFs. Activin/Nodal signaling is essential to support hESCs self-renewal via activation of Smad2/3, resulting in upregulation of Nanog and Oct3/4 transcription. In contrast to mESCs, BMP promotes hESCs differentiation toward trophectoderm.PI3K–Akt pathway is also essential for hESC self-renewal although downstream effectors and targets have not been defined.

BMP, bone morphogenetic protein; ESCs, embryonic stem cells; Fgf, fibroblast growth factor; LIF, leukemia inhibitory factor; MEFs, mouse embryonic fibroblasts.

The best-characterized effector of mESC self-renewal is LIF. LIF is a member of the IL6 family of cytokines that plays a key role in maintaining mESC self-renewal and functions by engaging the LIF/gp130 heterodimeric receptor, thereby recruiting and activating STAT3, a transcription factor that translocates to the nucleus and regulates genes required for 'stemness'. While LIF can activate JAK–STAT3 and Ras–MAPK pathways in mESCs, studies in mice indicate that genetic inactivation of LIF signaling has no major effect on development. This may be due to compensation by other IL6 family members, such as ciliary neurotrophic factor, which can also signal through LIF/gp130 receptors. LIF/STAT3 seems to be important for maintenance of the blastocyst during delayed implantation, although this is not relevant to human development.

Several efforts have been made to understand the mechanism of STAT3-dependent self-renewal in mESCs. One of the most promising targets identified is the proto-oncogene c-myc, a helix–loop–helix transcription factor that is a direct transcriptional target of STAT3. Following LIF withdrawal, c-myc transcript levels decrease due to inactivation of STAT3. Maintenance of myc levels using inducible transgenes can maintain self-renewal in the absence of LIF indicating that myc is a major target of the LIF–STAT3 self-renewal pathway in mESCs. A second pathway that controls myc levels involves glycogen synthase kinase-3 (GSK3)-dependent phosphorylation. When LIF signaling ceases, GSK3 is rapidly activated and phosphorylates c-myc on threonine, triggering its ubiquitination and proteosome-dependent degradation. How GSK3 activity is suppressed in mESCs is unclear, but is likely to involve phosphatidylinositol 3 kinase (PI3K) activity either directly or indirectly as a consequence of LIF signaling. Another intriguing connection between GSK3 and self-renewal was made when the efficiency of mESC derivation was shown to be markedly enhanced in the presence of BIO, a chemical inhibitor of GSK3. Hence, low GSK3 activity could be an absolute requirement for pluripotency and ESC self-renewal.

A second pathway implicated in mESC self-renewal involves BMP signaling. Although BMP is generally not added as a recombinant factor, as in the case of LIF, BMP in fetal calf serum (FCS) appears to have a pro-maintenance effect at least under some culture conditions. Under these conditions, BMP acts by promoting Id gene expression, which serves to block neural differentiation. The report by Ying et al. was the first to seriously raise the issue that self-renewal must be a coordinated series of events that involves maintenance of the pluripotent state and the blockade of differentiation pathways. In the case of BMP signaling, ectoderm specification is inhibited. By this model, other factors would work in collaboration with BMP to restrict differentiation pathways for mesoderm and endoderm. Qi et al. have alternative explanations for how BMP impacts on mESCs self-renewal. In their experiments, they show that BMP blocks differentiation by suppressing p38 MAP kinase. Since different laboratories use different culture conditions including FCS which is a huge variable, it seems likely that BMP contributes to suppression of differentiation by context-dependent mechanisms. The main outcome however, is to suppress pro-differentiation signaling pathways.

Phosphatidylinositol 3 kinase is involved in many aspects of cell behavior such as proliferation, apoptosis and differentiation. A major effector of PI3K signaling is protein kinase B (PKB)/AKT1. There is a large body of evidence demonstrating that PI3K signaling is crucial for mESC self-renewal. Inhibition of PI3K signaling by small molecule inhibitors such as LY294002 promotes differentiation even in the presence of LIF. As mESCs differentiate, PI3K and AKT activities

decline, consistent with this signaling pathways being important for self-renewal. Sustained AKT activity, achieved by ectopic expression of a constitutively active mutant, significantly delays differentiation of murine and monkey ESCs. Although PI3K/AKT seems to be crucial for mESC self-renewal, the factors promoting their activity have not been clearly defined. Candidates include serum components such as insulin-like growth factor (IGF) or even LIF, a known activator of PI3K signaling through LIF-gp130 receptor complexes. Mechanistically, PI3K/AKT may function by suppressing GSK3, a known antagonist of pro-self renewal regulators such as c-myc.

Increased ERK activity is though to be correlated with early differentiation of mESCs following LIF withdrawal and suppression of its activity by addition of PD98059 reduces the level of LIF required to maintain mESC self-renewal. LIF itself promotes ERK activity but this is balanced by self-renewal signals generated at the LIF-gp130 receptor. Recent work indicates that fibroblast growth factor (Fgf)4 secreted by mESC primes cells for differentiation by acting through ERK and that suppression of this signal compromises differentiation.

In summary, LIF–STAT3 is critical for mESCs self-renewal. In conjunction with additional signals in serum, self-renewal is promoted. Besides LIF, PI3K/AKT appears to be most critical, and may be activated as part of the LIF signaling pathway or from other factors in media (for example insulin, IGF). The absence of defined media formulations has compounded the definition of self-renewing signaling pathways in mESCs. Part of the problem relates back to the different culture conditions used by laboratories in the field and the nemesis of many tissue culture systems, variability in batches of FCS.

Signaling Pathways Required for hESC Self-renewal

It did not take long for the field to realize that culture conditions required for mESC self-renewal are quite different to that required for hESC maintenance. Although human and murine ESCs can be maintained on MEF feeder layers in FCS (which has to be carefully batch tested), differences clearly emerge under feeder-free conditions. LIF is clearly not required for hESC self-renewal, but instead several other factors have been identified such as Fgf2, Activin A and activators of PI3K signaling such as IGF/insulin.

From the onset of discussions relating to hESC culture, it should be clearly stated that first generation feeder-free conditions utilized MEF-CM, a complex mixture of secreted factors and FCS/synthetic serum-replacement formulations. The complexity of MEF-CM again raises question in terms of defining the critical factors. For example, Xu et al. identified BMP activity in serum-replacement media associated with serum albumin. Clearly, there is a great need to progress toward defined media that can sustain hESC self-renewal independently of MEFs, serum and other undefined media components.

From the early experiments using MEF-CM, it was clear that supplementation with Fgf2 had profound effects on hESC stability. Since then, Fgf2 (basic Fgf; bFGF) has been consistently used in both MEF-CM-based and -defined media formulations for hESCs. Fgf2 may promote hESC self-renewal in two ways. First, by directly activating signaling pathways required for self-renewal—perhaps through transcriptional networks. Second, it could work indirectly by stimulating autocrine effects. Since Fgf2 is added at the time of MEF-media conditioning, it may serve to promote secretion of factors from MEFs. Since Fgf2 is a key component of defined media, where MEFs and MEF-CM are absent, the first possibility certainly seems likely.

Members of the transforming growth factor-β family such as Activin A also seem to play a role in maintaining hESC self-renewal perhaps in collaboration with Fgf2. This may involve a mechanism where Activin A signals directly through Smads to promote transcription of genes encoding transcription factors required for self-renewal such as Nanog and Oct3/4. In contrast, Activin A does not appear to be involved in mESC self-renewal and would probably interfere with BMP-dependent self-renewal pathways in this system because of reciprocal antagonism. In contrast to the situation in mESCs, BMP promotes differentiation into hESCs and antagonism of BMP signaling by growth differentiation factor-3 can promote hESC self-renewal.

Evidence is emerging that PI3K signaling is crucial for hESCs self-renewal. We previously showed that for specification signals such as Activin A to promote hESCs differentiation, PI3K signaling must first be inactivated. Although the mechanism for this has not been resolved, it appears that PI3K antagonizes signaling pathways required for cell-fate commitment as well as by promoting self-renewal regulatory circuits. In the case of Activin A-dependent definitive endoderm specification, PI3K can be suppressed by chemical inhibitors such as LY 29402 or by removing/reducing FCS or serum supplements. Removal of insulin/IGF type molecules seems to be important for reducing PI3K signaling in this context. Addition of PI3K agonists such as IGF and insulin to defined media formulations would therefore play two roles: (i) promote self-renewal by suppressing differentiation and (ii) promote cell survival. Another anticipated outcome of PI3K signaling would be to suppress GSK3 activity. This is consistent with reports from Sato et al., who showed that suppression of GSK3 is central to hESCs self-renewal in short-term assays.

In contrast to what has been described for mESCs, ERK activity is inhibited during hESCs differentiation when cells are cultured in MEF-CM. This raises questions about the generality of ERK in self-renewal/differentiation and it is unclear if these differences represent differences between species, culture conditions or whether this can be attributed to hESCs and mESCs representing different phases of development.

Now that defined media formulations are being widely used to propagate hESCs, the key growth factors and signaling pathways are now being revealed. Activin A, Fgf2 and insulin/IGF seem to be the consensus players revealing key roles for Smad, PI3K signaling and possibly ERK signaling in hESC self-renewal. This clearly portrays a different picture to that which has emerged from studies on mESCs.

Cell-cycle Control in ESCs

Perhaps one of the most-striking features of mESCs is their mode of cell-cycle control. To place this in context, it is important to understand cell division in the peri-implantation stages of mouse development. Just preceding gastrulation, pluripotent cells of the epiblast have a cell-cycle length of ~6 h. Once differentiation into the embryonic germ layers occurs, this rate of cell division slows down dramatically (>20 h). Similar changes in cell-cycle dynamics occur as ESCs differentiate, indicating that cell-cycle changes are intimately associated with loss of pluripotency. Pluripotent cells divide rapidly since they do not spend appreciable amounts of time in G1 phase. As cells exit mitosis, they commit to DNA replication without a significant G1 delay period that is normally devoted to cell growth and integration of mitogenic signals. Consequently, ESCs spend ~65% of their time in S phase because of the very short periods of time devoted to the gap phases. Differentiation is associated with increased generation times and the establishment of a fully formed G1 phase. At the

molecular level, this has an intriguing basis. mESCs exhibit elevated, constitutively active Cdk activities. Most notably, Cdk2–cyclin E is not under cell-cycle control until cells differentiate—in part due to changes in the expression of Cdk inhibitors such as p16INK4a p27Kip1 and p21cip1. The only Cdk complex under cell-cycle control in mESCs is Cdk1–cyclin B. The constitutive activity of Cdk complexes results in hyperphosphorylation of Rb family members and constitutive transcription of E2F target genes. By definition, pluripotent cells do not have an intact restriction (R-) point and only acquire one as part of a developmental program associated with gastrulation/cell-fate commitment. Detailed molecular characterization of the cell-cycle machinery has not been performed in hESCs but they do exhibit a similar cell-cycle structure to mESCs, with an S-phase fraction of ~50–60%. hESCs however, cycle much more slowly than mESCs with generation times of 30–38 h being common. The reasons for this are not clear but are likely to reflect differences in the absolute levels of Cdk activities. It should be emphasized that no evidence to date has directly linked this unusual mode of cell-cycle regulation in the embryo or ESCs to the pluripotent state.

Transcriptional Networks that Control Pluripotency

A network of transcription factors including Oct3/4, Nanog and Sox2 have been shown to be crucial for development of pluripotent cells in the peri-implantation embryo and for maintenance of pluripotency of human and murine ESCs. This has been the subject of numerous reviews. However, a few important features of this network will be briefly discussed. First, chromatin immunoprecipitation, on chip array analysis indicates that Oct3/4, Nanog and Sox2 appear to work together in protein complexes by binding promoters of genes that are expressed and repressed. The interpretation of these findings is that these transcription factors activate genes required for self-renewal and repress other genes required for differentiation. It should be mentioned however, that functional validation of this hypothesis has not been definitively provided. Another major unknown is how the activity of these factors is controlled in the self-renewing state. Presumably, signaling pathways required for self-renewal sustain Oct3/4, Nanog and Sox2 expression but how this occurs is a mystery.

Takahashi et al. recently showed that coexpression of four transcription factors by retroviral transduction could dedifferentiate MEFs back to an ESC-like state. The factors required were Sox2, Oct3/4, the Kruppel-like factor 4 (Klf4) and c-myc. Nanog was not required for dedifferentiation but its expression was re-established by the four named factors. Further investigation showed that Sox2 is dispensable so long as Oct3/4 levels are elevated further. How could these factors participate in re-estab-lishment of an ESC-like state? c-myc for example is known to be a global gene regulator and plays an active role in gene activation and repression. In particular, it is known to promote global acetylation of chromatin. Establishing an open chromatin state by enhanced histone acetylation could then facilitate complexes of Oct3/4, Sox2 and Klf4 to bind target genes required for establishment and maintenance of pluripotency. Similar experiments could not reproduce this effect in human cells however.

In summary, genetic and biochemical analysis has established a role for Oct3/4, Nanog, Sox2, c-myc and Klf4 in ESC cell identity. However, how the transcription factors co-operate on reprogramming event needs to be addressed.

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Stem Cell Mechanisms

5

- **Molecular Mechanisms of Embryonic Stem Cell Pluripotency**
- **Mechanisms of Mesenchymal Stem Cell**
- **Regulatory Mechanisms in Stem Cell Biology**
- **Stem Cell Aging**

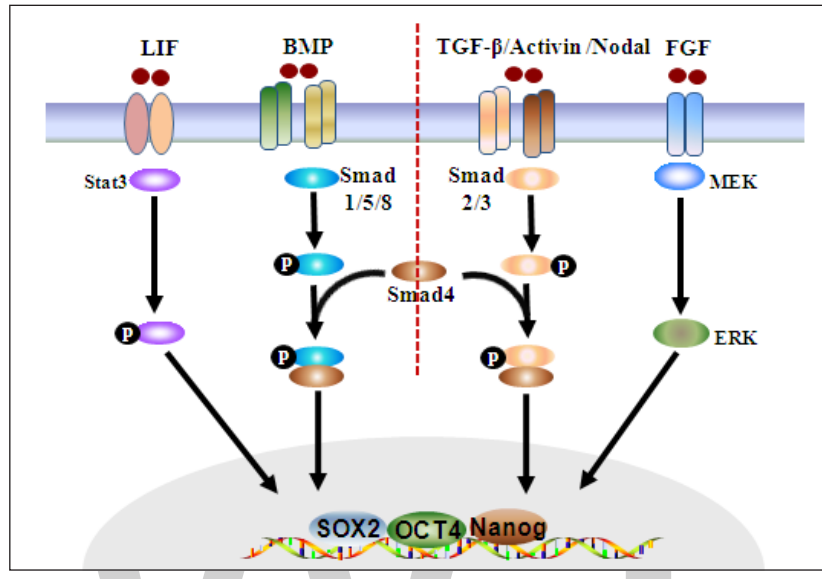
There are various mechanisms in stem cell that are used for maintaining the functions of the stem cells. It includes molecular mechanisms of embryonic stem cell pluripotency, mechanisms of mesenchymal stem cell, stem cell aging, etc. This chapter closely examines these mechanisms of stem cell to provide an extensive understanding of the subject.

Molecular Mechanisms of Embryonic Stem Cell Pluripotency

Embryonic stem (ES) cells isolated from the inner cell mass (ICM) of blastocysts possess the defining pluroptency: unlimited self-renewal and giving rise to all cells of the organism. Thus, ES cells hold great promise for regenerative medicine to treat many diseases including heart failure, diabetes, Alzheimer's and Parkinson's disease by replacing the damaged cells with ES cell-derived healthy ones. The recent advent of induced pluripotent stem (iPS) cells reprogrammed from somatic cells has the potential to revolutionize the field of regenerative medicine since patient-derived iPS cells, in principle, circumvent the ethical problems and immune rejection associated with human ES cells. Nevertheless, the future clinical translation of ES cells and iPS cells is facing numerous hurdles. Understanding the molecular mechanisms that impart ES cells with pluripotency may help address some of these challenges. The past few years have seen tremendous progress in understanding of mechanisms which govern ES cell pluripotency.

Signaling Pathways of ES Cells

ES cells require extrinsic growth factors to maintain their pluripotency in culture. These extrinsic growth factors act on different signaling pathways to regulate intrinsic transcription factor networks to sustain ES cells in the undifferentiated state. The signaling pathways required to support pluripotency in mouse ES cell are distinct from those in human ES cells.



Exogenous growth factors signal through distinct signaling pathways to regulate transcription factors for ES cell pluripotency.

LIF/JAK/STAT3 Pathway

Mouse ES cells were originally cultured on feeder layers derived from mouse embryonic fibroblasts (MEF). Later it was found that Leukaemia Inhibitory Factor (LIF), a member of the Interleukin-6 cytokines produced by MEFs, was the key factor to maintain pluripotency of mouse ES cells by inhibiting their differentiation. Upon LIF binding, the LIF receptor recruits gp130 to form a heterodimer which subsequently activates Janus kinase (JAK) through transphosphorylation. Activated JAK then phosphorylate gp130, creating a docking site to bind the SH2 domain of Signal Transducers and Activators of Transcription 3 (STAT3). Once STAT3 binds to the gp130 docking site, JAK then phosphorylates the recruited STAT3. Phosphorylated STAT3 forms a homodimer, which subsequently translocate into the nucleus, where it binds to gene enhancers to regulate target gene expression.

Although the LIF/JAK/STAT3 pathway has been well documented to maintain pluripotency of mouse ES cells in the presence of serum, the mechanisms by which activated STAT3 functions in this regard is poorly understood. Recently, studies in identification of STAT3 target genes have improved our understanding of activated STAT3 in maintaining pluripotency. Chen et al identified 718 STAT3-bound genomic sites that were co-occupied by pluripotency transcription markers (Oct4, Sox2 and Nanog) by using chromatin immunoprecipitation sequencing (ChIP-seq). In addition, Kidder and colleagues found that STAT3 target genes enriched in ES cells were downregulated in differentiated cells by mapping STAT3 binding targets in mouse ES cells and differentiated embryoid bodies (EBs). Along with these results, it has been demonstrated that knocking down

STAT3-target genes induces activation of endodermal and mesodermal genes, supporting the conclusion that STAT3 prevents mESC differentiation by suppressing lineage-specific genes.

Interestingly, the LIF receptor and gp130 are also expressed in human ES cells and human LIF can induce STAT3 phosphorylation and nuclear translocation in human ES cells. However, human LIF is unable to maintain the pluripotent state of human ESs, suggesting that mouse and human ES cells require distinct signaling mechanisms to govern their pluripotency.

TGF-β Signaling

TGF-β superfamily consists of more than 40 members, including TGF-β, Activin, Nodal, and bone morphogenetic proteins (BMPs). The TGF-β members transduce signals by binding to heteromeric complexes of serine/threonine kinase receptors, type I and type II receptors, which subsequently activate intracellular Smad proteins. Smads 2 and 3 are specifically activated by activin, nodal and TGF-β ligands, whereas Smads 1, 5 and 8 are activated by BMP ligands. The TGF-β-related signaling pathways play complex roles in regulating the pluripotency and cell fate of ES cells.

BMP Signaling Pathway

Bone Morphogenetic Protein (BMP) is a subset of the TGF-β superfamily. When BMP ligands bind to type II BMP receptors (BMPRII), BMPRII then recruits and phosphorylates type I BMP receptors (BMPRI). Activated type I receptors subsequently phosphorylate BMP-responsive SMAD1/5/8 which then forms a complex with SMAD4 and translocates into nucleus to regulate target gene expression. In mouse ES cells, LIF can substitute MEF feeder layers in maintaining pluripotency in the presence of animal serum by activating the transcription factor STAT3. However, in serum-free cultures, LIF is insufficient to block neural differentiation and maintain pluripotency. Recently, Ying et al reported that BMP was able to replace serum to maintain pluripotency of mouse ES cells in the presence of LIF. BMP has been shown to phosphorylate SMAD1/5 and activate inhibitors of differentiation (Id) genes, which block neural differentiation by antagonizing neurogenic transcription factors. In the absence of MEF and serum, exogenous LIF, in combination with BMP4 proteins, can sufficiently maintain the pluripotency of mouse ES cells derived from "permissive" mouse strains.

In contrast to a maintenance role in mouse ES cell pluripotency, BMP has been shown to promote human ES cells differentiation to trophoblasts, and inhibiting BMP signaling with the BMP antagonist, Noggin, sustains the undifferentiated state of human ES cells. In consistence, dorsomorphin and DMH1, small molecule BMP inhibitors previously identified in our lab, were shown to promote long-term self-renewal an pluripotency of human ES cells, presumably by inhibiting BMP induced extraembryonic lineage differentiation.

TGF-β/Activin/Nodal Signaling Pathway

Although MEFs feeder layers were initially used to co-culture both mouse and human ES cells, signal factors secreted from MEFs to maintain pluripotency of the two types of ES cells are fundamentally different. Sato et al first discoveried that TGF-β and Nodal genes were highly expressed in undifferentiated human ES cells. Beattie et al later reported that Activin A, a member of the TGF-β superfamily, was secreted by MEFs, and medium enriched with activin A can replace MEF

feeder-layers or MEF-conditioned media to maintain human ES cells in an undifferentiated state. In consistence, James et al demonstrated that the TGF-β/Activin/Nodal pathway was activated through the transcription factors Smad2/3 in undifferentiated human ES cells. The notion that TGF-β/Activin/Nodal signaling supports human ES self-renewal and pluripotency is further supported by the fact that recombinant Activin or Nodal stimulation induces higher levels of pluripotent protein expression (Oct4 and Nanog), while inhibition of TGF-β/Activin/Nodal signaling with Lefty or Follistatin decreases expression of these pluripotent proteins in human ES cells.

Recent studies have focused on understanding the molecular mechanisms of TGF-β/Activin/Nodal signaling in retaining human ES cells pluripotency. Xu and colleagues showed that TGF-β/Activin/Nodal signaling activated Smad2/3 which subsequently binds to the Nanog promoter in undifferentiated human ES cells to induce expression of Nanog, a pluripotent transcription factor. Additionally, mutating the putative Smad-binding sites reduced the response of Nanog to modulation of TGF-β signaling. Nanog was also shown to coordinate with Smad2 in a negative-feedback loop to inhibit human ES cell differentiation. In contrast to its important role in maintaining human ES cell pluripotency, the TGF-β/Activin/Nodal signaling is not essential for pluripotency of mouse ES cells. Although this pathway was shown to be active in undifferentiated mouse ES cells as assessed by phosphorylation of smad 2/3, inhibition of smad 2/3 phosphorylation by SB431542 had no effect on the undifferentiated state of mouse ES cells. However, the TGF-β/Activin/Nodal signaling may play a role in mouse ES proliferation. A recent study showed that Inhibition of TGF-β/Activin/Nodal signaling by Smad7 or SB-431542 dramatically decreased mouse ES cell proliferation without effect on their pluripotency.

Growth and Differentiation Factor 3 (GDF-3)

GDF-3 is another TGF-beta superfamily member that plays opposite roles in mouse and human ES cells. GDF-3, which acts as a BMP antagonist by direct binding to BMP-4, is specifically expressed in the pluripotent state of both mouse and human ES cells. Ectopic expression of GDF-3 leads to the maintenance of pluripotency in human ES cells, whereas a similar effect is observed in mouse ES cells when GDF-3 levels are decreased. In the absence of LIF, GDF-3-deficient mouse ES cells can still sustain pluripotent markers. These results are consistent with previously discussed BMP signals which can promote pluripotency of mouse ES cells, but cause differentiation of human ES cells. Thus lower concentrations of BMP antagonists, such as GDF-3, may enhance pluripotency in mouse ES cells, whereas higher levels of GDF-3 may favor pluripotency of human ES cells by abrogating BMP signaling.

FGF/MEK Signaling

The importance of Fibroblast growth factor (FGF) signaling for human ES cells pluripotency is highlighted by the facts that human ES cells are traditionally cultured in the presence of Fibroblast growth factors (FGFs) either on fibroblast feeder layers or in fibroblast-conditioned medium. Studies have demonstrated that all four FGF receptors (FGFR1, FGFR3 and FGFR4) and several components (SOS1, PTPN11 and RAF1) of their downstream activation cascade are significantly upregulated in undifferentiated human ES cells, in comparison to differentiated human ES cells. In consistence, withdrawal of FGFs or inhibition of FGF signaling by a FGFR inhibitor, SU5402, rapidly induces human ES cell differentiation.

Although the pluirpotency maintenance role of exogenous FGFs in human ES cell has been known for a long time, the molecular mechanisms by which they function remain unclear. FGFs signal by binding to FGF receptors (FGFRs), and activate multiple signaling cascades, including Mitogen-Activated Protein Kinases (MAPKs), the Janus kinase/signal transducer and activator of transcription (Jak/Stat), phosphatidylinositol 3-kinase (PI3K) and phosphoinositide phospholipase C (PLCg) pathway. Several studies have highlighted the FGF contribution to the maintenance of human ES cells mainly through the FGF/MEK pathway. Studies have showed that FGF2 induces feeder layer cells to secret TGFβ1 and insulin-like growth factor 2 (IGF2), which can subsequently promote the undifferentiated state of human ES cells. Bendall et al further reported that the function of exogenous FGFs in promoting ES self-renewal could be replaced by addition of IGF2 alone, suggesting an indirect role of FGFs for human ES cell growth. However, this model was challenged in subsequent publications from Wang et al who reported that exogenous IGF2 alone was insufficient to maintain undifferentiated growth of human ES cells, and they proposed that FGFs may play a direct role in blocking caspase-activated apoptosis through anoikis in human ES cells. Recently, Eiselleova and colleagues postulated a new model whereby endogenous FGF-2 signaling maintained the undifferentiated state and survival of human ESCs, while exogenous FGF-2 mainly suppress cell death and apoptosis genes, thus indirectly contributing to the maintenance of human ES cell pluripotency.

FGF signaling in mouse ES cells has also been extensively investigated. Mouse ES cells genetically deficient in Fgf4 and extracellular-signal regulated kinase-2 (Erk2) differentiate inefficiently. These results can be reproduced using inhibitors of FGF receptor and ERK, suggesting blockage of the FGF/MEK signaling pathway promotes mouse ES cell pluripotency. Indeed, serum-free mouse ES cell medium supplemented with FGF/MEK inhibitors and LIF permits the derivation of mouse ES cells in the absence of feeders from strains normally considered non-permissive. In addition, a recently identified compound, Pluripotin/SC1, has been shown to maintain mouse ES pluripotency by inhibiting ERK1 and activating the phophoinositide-3 kinase (PI3K) pathway through blocking RasGAP. Although inhibition of FGF/MEK pathway can attenuate ES cell differentiation, it is insufficient to support mouse ES cell self-renewal. Combination of the MEK inhibitor PD0325901 with the Glycogen synthase kinase-3 (GSK-3) inhibitor CHIR99021 (known as 2i) can efficiently sustain the pluripotency of mouse ES cells in the absence of exogenous cytokines. Several groups demonstrated that improvement of mouse ES cell pluripotency by inhibition of GSK-3 occurred via Wnt/β-catenin signaling, whereas many others argued that GSK3 was likely to exert β-catenin independent effects in ES cells.

As demonstrated above, human and mouse ES cells are both derived from blastocyst-stage embryos, but they require different biological signals for maintaining pluripotency. In general, mouse ES cells maintain their pluripotency by activating LIF/STAT3 and BMP signaling, while human ES cells require TGF-β/Nodal and FGF/MEK pathways. Interestingly, several pathways, such as BMP and FGF/MEK, have completely oppositing effects on maintaining the pluriotency of mouse and human ES cells. Activation of BMP signaling and inhibition of the FGF/MEK pathway promote mouse ES self-renewal, whereas inhibition of BMP signaling and activation of FGF/MEK pathway sustain human ES cell pluripotency. These distinct signaling effects on pluripotency may reflect intrinsic differences between mouse and human ES cells. Recent studies have demonstrated that conventional human ES cells do not represent the “ground or naïve state” of stemness, but rather a more developmentally mature “primed state” resembling mouse epiblast stem cells (mEpiSCs)

found in the post-implantation, pre-gastrulation stage of embryos. Conventional human ES cells exhibit numerous similarities to the mouse EpiSCs over mouse ES cells. For instance, conventional human ES cells and mouse EpiSCs display flattened cell colonies and epigenetic X-chromosome inactivation (XiXa), and require Activin and FGF for pluripotency maintanince. In contrast, mouse ES cells exhibit dome-shaped colony morphology and epigenetic activation of both X-chromosome (XaXa), and require LIF/STAT3 signaling to promote self-renewal. Subsequent studies have demonstrated that the medium containing "2i" (MEK inhibitor and GSK-3 inhibitor), when supplemented with other factors (such as forskolin), can efficiently convert conventional human ES cells into a ground or "naïve" state with display of hallmark features of mouse ES cells. This medium can also maintain human ES cell pluriptoency at the naïve state.

The Regulatory Network of Pluripotency Factors

ES cell pluripotency is conferred by a unique transcriptional network. Early global transcriptional profiles and genetic studies have identified several critical transcription factors that are required for the pluripotency of ES cells, such as Oct4, Sox2, Nanog, Foxd3 and Id, etc.

OCT4 and SOX2

OCT4 (also known as Oct3), a POU domain-containing transcription factor, was one of the first transcription factors identified as essential for both early embryo development and pluripotency maintenance in ES cells. The expression of Oct4 is activated at the 8-cell stage and is later restricted to the inner cell mass (ICM) and germ cells in early mouse embryogenesis in vivo. Oct4 is highly expressed in both human and mouse ES cells, and its expression diminishes when these cells differentiate and lose pluripotency. Oct4 regulates a broad range of target genes including Fgf4, Utf1, Opn, Rex1/ Zfp42, Fbx15, Sox2 and Cdx2. Repression of Oct4 activity in ES cells upregulates Cdx2 expression, leading to ES cell differentiation into trophectoderm. Oct4 is also known to activate downstream genes by binding to enhancers carrying the octamer–sox motif (Oct–Sox enhancer), for synergistic activation with Sox2. In contrast with its target genes, little is known about Oct4 upstream regulators. The Oct4 promoter contains conserved distal and proximal enhancers that can either repress or activate its expression depending on the binding factors occupying these sites. The precise level of Oct4 is important for ES cell fate determination. Loss of Oct4 causes inappropriate differentiation of ES cells into trophectoderm, whereas overexpression of Oct4 results in differentiation into primitive endoderm and mesoderm.

Sox2 is an HMG-box transcription factor that is detected in pluripotent cell lineages and the nervous system. Inactivate Sox2 in vivo results in early embryonic lethality due to the failure of ICM maintenance. Sox2 can form a complex with the Oct4 protein to occupy Oct–Sox enhancers to regulate target gene expression. Oct–Sox enhancers are found in the regulatory region of most of the genes that are specifically expressed in pluripotent stem cells, such as Oct4, Sox2, Nanog, Utf1, Lefty, Fgf4 and Fbx15.

Nanog

Nanog is another homeobox-containing transcription factor that is specifically expressed in pluripotent ES cells. The essential role of Nanog in maintaining the pluripotency of ES cells is highlighted by the facts that Nanog-deficient ES cells are prone to differentiation, whereas forced expression of

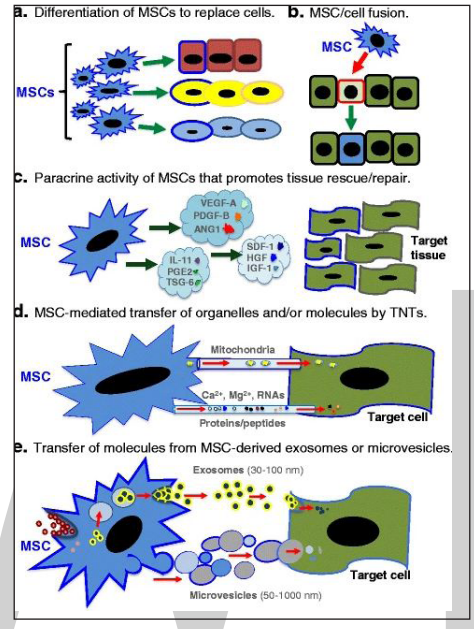
Nanog partially renders ES cells self-renewal potential in the absence of LIF. How Nanog regulates stem cell pluripotency remains entirely unknown. Studies have indicated that Nanog may maintain ES cell pluripotency by 1) downregulating downstream genes essential for cell differentiation such as Gata4 and Gata6 and 2) activating the expression of genes necessary for self-renewal such as Rex1 and Id. Although it is widely accepted that Nanog, like Oct4 and Sox2, play a central role in pluripotency maintenance, this dogma has been challenged by a subsequent report that Nanog protein levels are undetectable in a fraction of ES cells that express Oct4, and the pure populations of Nanog−/− ES cells can be propagated without losing expression of other pluripotency markers.

Little is known about the mechanism by which Nanog is regulated in ES cells. Recently, Suzuki et al showed that Nanog expression was upregulated by BrachyuryT and STAT3 in mouse ES cells. In human ES cells and in mouse EpiSCs, Vallier et al reported that Activin/Nodal signaling stimulated expression of Nanog, which in turn prevents FGF-induced neuroectoderm differentiation. In addition, several studies indicated that the Oct4/Sox2 complex was directly bound to the Nanog promoter to regulate target gene expression. Genomic studies have revealed that Oct4, Sox2, and Nanog frequently bind the same regulatory regions in undifferentiated mouse and human ESCs, and that these binding sites are often in close proximity to one another. These results indicate that Oct4, Sox2, and Nanog may physically interact with each other and coordinately regulate target genes in some cases. Additionally, Goke and colleagues reported that combinatorial binding sites of the Oct4/Sox2/Nanog were more conserved between mouse and human ES cells than individual binding sites were.

Mechanisms of Mesenchymal Stem Cell

Mesenchymal stem cells, also referred to as multipotent stromal cells or mesenchymal stromal cells (MSCs), have been the subject of intense scientific investigation since their initial discovery by Alexander Friedenstein in the late 1960s. In their early studies, Friedenstein and colleagues demonstrated that MSCs, likely originating from the mesoderm, had the capacity to differentiate into a variety of mesenchymal tissue lineages such as osteoblasts, chondrocytes, and adipocytes. These observations sparked a substantial degree of interest in the potential application of MSCs for the repair of serious connective tissue trauma and disease. It was originally hypothesized that, upon administration, MSCs would migrate to sites of injury, engraft, and differentiate into functional cells, resulting in regeneration of damaged or diseased connective tissues. Surprisingly, results from hundreds of animal studies and many human trials conducted over the past few decades have challenged this classic paradigm. In short, while MSCs were found to exhibit a remarkable degree of efficacy in a variety of disease models, it became increasingly apparent that the cells did not engraft in significant numbers or for durations sufficient to explain the results in terms of tissue replacement. More surprisingly, MSCs were reported to engraft and differentiate into functional cells of tissues that did not originate from mesoderm, questioning the long-established dogma that differentiation of adult stem cells is typically restricted to tissues derived from their germ layer of origin. Later studies confirmed that the majority of results describing cross-germ line differentiation of MSCs could be ascribed to limitations in methodology or cell fusion events. Still largely unsolved, the mystery of efficacy without long-term engraftment, especially in non-mesodermal tissues, remains a source of considerable debate. In retrospect, a partial explanation for

the benefits of MSC administration traces back to some of the very first observations made with bone marrow stromal cells. In the 1970s, Dexter and colleagues were the first to demonstrate that adherent stromal cells from bone marrow (later identified as MSCs) could sustain the growth, viability, and multipotent status of hematopoietic stem cells in long-term co-cultures that lacked growth factor supplementation. Of particular interest was that the cultures achieved homeostasis with the self-renewal of progenitor cells balanced against the development of committed hematopoietic cells. These initial studies suggested that MSCs had the capacity to sustain the growth and viability of certain cell types through secretion of so-called trophic factors and even presented the notion that they could regulate certain facets of the immune system.



MSCs rescue and/or repair injured cells and tissues by diverse mechanisms. a Differentiation into replacement cell types. b Rescue of damaged or dying cells through cell fusion. c Secretion of paracrine factors such as growth factors, cytokines, and hormones. VEGF vascular endothelial growth factor, PDGF platelet-derived growth factor, ANG1 angiopoietin-1, IL-11 interleukin-11, PGE2 prostaglandin E2, TSG-6 TNF-stimulated gene-6, SDF-1 stromal-derived factor-1, HGF hepatocyte growth factor, IGF-1 insulin-like growth factor-1. d Transfer of organelles (e.g., mitochondria) and/or molecules through tunneling nanotubes (TNTs). Ca^{2+} calcium, Mg^{2+} magnesium. e MSC-mediated transfer of proteins/peptides, RNA, hormones, and/or chemicals by extracellular vesicles such as exosomes or microvesicles. Exosomes are generated through the endocytic pathway and released through exocytosis. By contrast, microvesicles are produced by cell surface budding and released directly from the plasma membrane. Note that the figure is not drawn to scale. Also, use of mechanisms a–e is not equivalent. For example, for MSCs administered intravenously, use of mechanism c is likely more relevant than are mechanisms (a) or (b).

In an effort to reconcile discrepancies between the modest frequency and duration of engraftment with their remarkable healing properties, a contemporary view of MSC functionality is taking form. Rather than assuming long-term engraftment and differentiation, new hypotheses indicate that MSCs heal injured and diseased tissues/organs using alternative modes of rescue and repair that enhance cell viability and/or proliferation, reduce cell apoptosis, and, in some cases,

modulate immune responses. The alternative modes of repair by MSCs include paracrine activity of secreted growth factors, cytokines, and hormones, cell–cell interactions mediated by tunneling nanotubes, and release of extracellular vesicles (EVs) that contain reparative peptides/proteins, mRNA, and microRNAs.

Paracrine Effects of Administered MSCs

Immune Modulation by MSCs

Some of the first evidence that MSCs could actively blunt immune responses originated from the results of mixed lymphocyte reaction (MLR) assays performed ex vivo. These assays are based on the observation that T cells from preparations of immunologically mismatched peripheral blood mononuclear cells proliferate rapidly when mixed together under appropriate conditions. Results from MLR assays showed that T-cell expansion could be inhibited by the addition of MSCs to MLRs. While the majority of cell culture studies to date agree that such observations are mediated by MSC-derived soluble factors that do not cause T-cell apoptosis, several alternative mechanisms have also been proposed. Di Nicola et al. employed a series of antibody blocking assays to implicate the role of transforming growth factor beta (TGFβ) and hepatocyte growth factor (HGF) whereas Aggarwal et al. proposed a role for prostaglandin E2 (PGE2) based on their ability to ablate inhibitory responses with cyclooxygenase 2 (COX2) inhibitors. the secretion of PGE2 and related factors induced dendritic cells to up-regulate the anti-inflammatory cytokine interleukin (IL)10 while reducing the secretion of pro-inflammatory tumor necrosis factor alpha (TNFα) and IL12. This, in turn, initiates a shift in the ratio of T helper (Th) cells from a pro-inflammatory Th1 subtype to an anti-inflammatory Th2 subtype. This was accompanied by the differentiation of naive T cells to an immunoregulatory regulatory T cell (Treg) phenotype, thereby reducing the overall number of Th cells. Similarly, MSCs could induce apoptosis of inflammatory T cells through activation of the Fas–Fas ligand axis. During this process, MSCs recruited additional T cells by secretion of monocyte chemotactic protein-1 (MCP-1) as part of a positive feedback loop.

Apoptotic T-cell debris then activated phagocytes to secrete TGFβ, resulting in the differentiation of naive T cells into Treg cells that can promote systemic immune tolerance. In an alternative model, an intriguing mechanism whereby MSC-derived indoleamine-2,3-dioxygenase (IDO) catalyzes the conversion of tryptophan to kynurenine in an interferon gamma-dependent manner. In turn, the kynurenine inhibits T-cell proliferation. This mechanism was later confirmed by utilizing the IDO antagonist 1-methyl-L-tryptophan. In a series of experiments, it was reported that MSCs could be induced to express enhanced levels of IDO and PGE2 by transient stimulation of toll-like receptor (TLR)3 with polyinosinic-polycytidylic acid (poly I:C). MSC-mediated IDO activity has also been shown to enhance kidney allograft tolerance in mouse models through a mechanism involving Treg up-regulation, demonstrating that IDO-mediated mechanisms of immune modulation can indeed occur in vivo. Nitric oxide, galectin-1 and semaphorin-3A have also been implicated as MSC-derived modulators of T-cell proliferation, but it is noteworthy to add that nitric oxide has only been shown to function as an MSC modulator in the murine system.

MSCs also have the capacity to modulate the activity of macrophages. This effect was initially described ex vivo using macrophage cultures stimulated with TLR ligands such as lipopolysaccharide (LPS), zymozan, or polyinosine-polycytidylic acid (poly I:C); these simulate the effects

of bacterial or viral infection. When macrophages are challenged with such agents, they secrete inflammatory factors such as TNFα, IL1β, IL6, and reactive oxygen species. In the presence of MSCs, however, the ability of activated macrophages to secrete inflammatory factors was attenuated. Of interest, these observations were explained, in part, by MSC-mediated secretion of the extracellular protein TNFα-stimulated gene protein (TSG)6. In this model, exposure to zymozan caused cultured macrophages to secrete high levels of TNFα and other inflammatory mediators via the TLR2–nuclear factor kappa-B (NFkB) axis. TNFα activates TSG6 expression by MSCs and engages a negative feedback loop by inhibiting NFkB via activation of the CD44 receptor. Several in vivo studies have confirmed that MSC-derived TSG6 acts via the CD44 receptor to inhibit NFkB activity in macrophages, dendritic cells, and Th cells in models of peritonitis, diabetes, and corneal transplant rejection. In addition to the action(s) of TSG6, MSC-derived PGE2 has also been demonstrated to have potent effects on macrophages in vivo. In a murine model of sepsis, Nemeth et al. demonstrated that, upon activation by LPS or TNFα, MSCs secreted PGE2. This caused the release of anti-inflammatory IL10 by macrophages and improved cell survival. Indeed, the role of PGE2 in MSC-mediated macrophage modulation is a common theme in many culture models. In an alternative mechanism proposed by Chen et al., placental human MSCs inhibited the interaction of TLR4 with a key effector molecule, MyD88, resulting in inhibition of secretory factors by macrophages. This process was inhibited by addition of a COX2 inhibitor, suggesting that the process was PGE2-dependent.

MSCs were reported to modulate the proliferation, differentiation, and immunoglobulin secretion of B cells without induction of apoptosis. Transwell assays separating the two cell types but allowing for exchange of secreted factors showed that such MSC-mediated effects derived, in part, from the paracrine activity of soluble factors secreted by MSCs. These experimental results have since been replicated using purified B cells and unpurified preparations of peripheral blood mononuclear cells; however, the paracrine mechanism was recently challenged by a co-culture study that suggested physical interaction between T cells and MSCs was necessary for MSCs to inhibit the activities of B cells. Using a mouse model of allergy, Nemeth et al. reported that MSC-derived TGFβ was critical in suppressing B-cell mediated allergic responses in vivo. They speculated that MSCs may recruit Treg cells that down-regulate allergy-specific cytokine and immunoglobulin production as well as lung eosinophil infiltration. Consistent with their immune-modulatory properties, efficacy with MSC treatment has been demonstrated in a variety of inflammatory models of disease, including arthritis, Crohn's disease, multiple sclerosis, myocardial infarction, diabetes, graft versus host disease, and corneal rejection.

Promotion of Cell Survival by MSCs

In addition to the paracrine effects of MSCs on immune cells, they also secrete a diverse repertoire of factors that support cell survival, including growth factors, cytokines, and extracellular matrix (ECM). Together, the components of the MSC secretome have the theoretical capacity to rescue injured cells, reduce tissue damage, and accelerate repair. This is exemplified by their natural roles as reticular cells that support the hematopoietic stem cell niche and as vascular pericytes that support endothelial cells. The observation that MSCs can be isolated from a wide variety of tissues, such as bone marrow, adipose, ligament, skin, placenta, dental pulp, synovium, placenta, umbilical cord, and other fetal tissues, lends support to the concept that they function endogenously as stromal support cells.

The pro-survival effect(s) of the MSC secretome on other cell types was first recognized through studies of long-term bone marrow cultures and embryonic cells. Collectively, these cell culture studies provide for an attractive, paracrine-based explanation for the ability of MSCs to promote healing across a broad range of developmentally unrelated tissues and for myriad diseases and injury types. Detailed analysis of the MSC transcriptome and proteome has confirmed that they secrete a vast repertoire of paracrine pro-survival factors commonly referred to as trophic factors or mediators. Of interest, the MSC-secreted factors comprise a diverse group of soluble peptides and proteins with complementary set(s) of biological activities that can accelerate progenitor cell self-renewal, stimulate angiogenesis, and minimize apoptosis and/or inflammation. Despite several decades of research and progress, the specific paracrine mechanisms by which administered MSCs improve cell survival and self-renewal under particular contexts of tissue rescue/repair remain largely undefined.

In line with the traditional model of paracrine biology whereby cells secrete factors that regulate adjacent cells, it was initially thought that engrafted MSCs readily migrated into injured tissue and then remained to orchestrate repair. For many models of tissue injury, however, what was originally perceived as "MSC migration" turned out to be far less directed (e.g., non-specific, transient trapping of MSCs within the microvasculature and capillary network). Of particular interest, depending on their relative size (i.e., diameter), the majority of intravenously administered MSCs will typically lodge in the lung microvasculature upon the first pass through the circulation, regardless of the presence or absence of lung-specific injury. Notably, after intravenous MSC infusion, paracrine factors released into the blood by circulating MSCs or from trapped MSCs may indirectly influence survival signaling and the fate of distal cells previously compromised by injury or disease. Thus, for effect, paracrine factors produced by MSCs appear not to depend on long-term MSC engraftment, nor do they require the unlikely differentiation of mesodermal progenitors into tissues of ectodermal or endodermal lineages.

Some of the best evidence supporting an indirect role for MSCs in the repair of tissues/organs originates from studies of heart with infarction. In a rat model of myocardial infarction, MSCs modified with the gene encoding protein kinase B (a.k.a. Akt) engrafted into the myocardium, reduced pathological remodeling, and improved cardiac function. The observed efficacy was later attributed to a paracrine effect mediated by secreted frizzled related protein (sFRP), a Wnt signaling inhibitor that reduces cardiomyocyte apoptosis. Since these studies, a number of additional mechanisms for the paracrine action of MSC-derived factors on cardiac repair have been proposed, including secretion of angiogenic factors, stromal cell derived factor-1 (SDF-1), and Jagged/Notch signaling. Of interest, MSC-mediated improvements in cardiac function could be achieved without long-term engraftment of MSCs. Using a different approach, MSC-conditioned medium was employed to prime cardiac stem/progenitor cells prior to cardiac grafting in a rat model of myocardial infarction. The conditioned medium (CM) improved cardiac stem cell engraftment through mechanisms involving connective tissue growth factor and insulin signaling.

The role of MSCs in the protection of other damaged tissues has also been demonstrated. For example, intraperitoneally and intravenously administered MSCs from murine bone marrow and adipose tissue had a protective effect in a cisplatin-induced acute kidney injury (AKI) model, as evidenced by a reduction in the apoptosis of tubule cells and improved renal function. This effect appeared to be mediated by secreted factors since the results could be repeated by intraperitoneal

administration of CM generated from the MSCs (MSC-CM). In contrast, Xing et al. reported that murine MSC-CM containing HGF, vascular endothelial growth factor (VEGF)-A and insulin-like growth factor (IGF)-1 failed to protect the kidneys of mice against ischemia-reperfusion injury, whereas live MSCs had a significant protective effect. This is one of several examples in the field where apparently minor differences in the cell source, the culture conditions, duration of medium conditioning, and dosage can profoundly affect outcome. Such complexities have made elucidation of the mechanism(s) responsible for the protective effect of MSCs on kidney tissue challenging, but some progress has been made. For example, Zarjou et al. demonstrated that the stress-responsive enzyme heme-oxygenase-1 (HO-1) played a role by utilizing MSC from bone marrow of HO-1-/- mice. HO-1+/+ MSC-CM rescued pathology associated with cisplatin-induced AKI, while HO-1-/- MSC-CM was ineffective. immunological and transcriptional blocking experiments both confirm a protective role for VEGF-A and IGF-1 in mice with AKI and for VEGF-A in rats with cerebral ischemia (stroke).

The utility of MSCs and their secreted products to protect cells and to foster tissue repair has been demonstrated in numerous efficacy-based studies across a broad range of tissue injury and disease models. some key examples of MSC-derived benefits include facilitation of wound healing, improved treatment of diabetes, enhancement of bone repair, and effect(s) on cancer.

Effects of MSCs on Fibrosis

Fibrosis is generally defined as a an accelerated accumulation of ECM factors (predominantly collagen type I) that prevents the regeneration of tissue. It can occur in virtually any tissue as a result of trauma, inflammation, immunological rejection, chemical toxicity, or oxidative stress. Current clinical strategies generally have poor outcomes in terms of efficacy and adverse effects. Given the immunomodulatory and trophic properties of MSCs, they have become attractive candidates for the treatment of fibrosis and preclinical studies suggest they have a promising level of efficacy in a variety of models. While the anti-fibrotic effects of MSCs are likely to overlap with their anti-inflammatory and angiogenic properties, the specific mechanisms remain poorly understood. Nevertheless, a comprehensive review suggests that their modes of action seem to fall under four categories: i) immune modulation, ii) inhibition of TGFβ-mediated differentiation of various cells types into ECM-secreting myofibroblasts by epithelial to mesenchymal transition, iii) inhibition of oxidative stress, and iv) matrix remodeling. For example, Ortiz et al. demonstrated that systemic murine MSC administration attenuated fibrosis in a bleomycin-induced lung injury model. This was achieved through MSC-mediated secretion of IL1 receptor antagonist, which reduced infiltration of lymphocytes and neutrophils and their production of inflammatory and fibrotic mediators such as IL1 and TNFα. Using the same model, it was recently reported that MSCs had the capacity to inhibit fibrosis through the action of the secreted protein stanniocalcin-1 (STC-1). STC-1 acted in multiple ways by reducing the secretion of collagen by fibroblasts, by reducing TGFβ output by endothelial cells and also through alleviating oxidative stress by uncoupling mitochondrial respiration via the induction of uncoupling protein 2. Using a model of chronic kidney injury, Huuskes et al. demonstrated that MSCs improved kidney morphology and functionality when co-administered with the putatively anti-fibrotic hormone recombinant human relaxin (serelaxin). In this system, MSCs and serelaxin acted synergistically to reduce TGFβ-induced myofibroblast differentiation and collagen deposition while increasing the level of matrix metalloproteinase 2 (MMP2), a collagen-degrading enzyme.

Transfer of Mitochondria by TNTs and Microvesicles

Discovery of TNTs

Rustom et al. first reported TNTs as a communicating intercellular transport network formed in cultures of transformed cells (human 293 cells and rat PC12 cells) as well as primary cells from rat kidney. Endocytic organelles (lysosomes) and vesicles were shown to move through thin, 50–200 nm diameter filaments that stretched between cells. Incubation of cells in the inhibitor latrunculin B demonstrated a requirement for polymerized F-actin in TNT formation. Onfelt et al. reported TNTs in human immune cells (e.g., natural killer cells, macrophages, and B cells) and later demonstrated that TNTs between macrophages had different properties and potentially differing functions; they observed thin filaments containing F-actin and also a thicker subset (0.7 microns) that contained both F-actin and microtubules. The thicker TNT subset was shown to transport mitochondria and lysosomal vesicles. Other studies demonstrated that some TNTs were actinomyosin-dependent. For example, the Gerdes group showed that kidney cells treated with S-(-)-blebbistatin, a myosin II-specific inhibitor, increased the number of TNTs formed and also organelle transfer, whereas a general myosin inhibitor increased TNT number but significantly reduced organelle transfer.

Discovery of Mitochondrial Transfer by Cultured MSCs

The first evidence that transfer of mitochondria might benefit injured target cells came from studies of human MSCs co-cultured with a unique lung epithelial cell line that lacked functional mitochondria (A549rho cells). Utilizing a complementation screen to detect mitochondrial transfer and resulting cell growth, the Prockop group reported that human MSCs could restore aerobic respiration to A549rho cells by transfer of mitochondria or mitochondrial DNA (mtDNA). Mitochondrial transfer from MSCs to rescued A549rho cells was demonstrated by tracking genetic tags (i.e., mtDNA and nuclear DNA) and by time-lapse photomicroscopy of MSCs transduced with lentiviral vectors to target DsRed2 to mitochondria. MSCs are now understood to transfer mitochondria to several different cell types, including epithelial cells, endothelial cells, and cardiac myocytes. Such transfers are particularly evident when the potential target cells are injured or under stress. For example, MSCs were recently shown to prevent apoptosis in endothelial cells by transferring mitochondria during hypoxic/ischemic stress.

TNT Formation and Mitochondrial Transfer in Vivo

The first evidence that TNTs could form in vivo came from studies of the eye. Using wild-type, eGFP chimeric mice, and Cx3cr1(GFP) transgenic mice and confocal microscopy tracking, Chinnery et al. documented membrane nanotubes that formed between bone marrow-derived MHC class II(+) cells in whole-mounted corneal tissue. Notably, they observed an increase in TNT frequency during corneal injury or inflammation. In a follow-up study with live imaging of myeloid cells in inflamed corneal explants from Cx3cr1(GFP) and CD11c(eYFP) transgenic mice, Seyed-Razavi et al. showed de novo formation of nanotubes at a rate of 15.5 μm/min. These results demonstrated that TNTs could form in the absence of actual cell–cell contact and, furthermore, that they could then be directed from one cell toward another. Additional evidence for in vivo mitochondria or mtDNA transfer between cells came from studies of a remarkable canine transmissible venereal tumor that had persisted in feral dog populations for about 10,000 years. Rebbeck et al. showed

that the transmitted tumor cell line had obtained mitochondria (mtDNA) from multiple canine hosts over time. They suggested that fitness/persistence of canine transmissible venereal tumor benefited from the acquisition of host-derived mtDNA and through shedding of mutant and/or damaged mtDNA that could negatively impact mitochondrial biogenesis. Importantly, multiple research groups have shown that intercellular transfer of organelles and mtDNA is not limited only to the animal kingdom. Intercellular organelle trafficking and horizontal gene transfer in plants has been reported for both plastids and mitochondria.

Proteins Shown to Control Transfer of Mitochondria by MSCs after Tissue Injury

Several recent studies have provided compelling evidence that administered MSCs can transfer mitochondria in vivo and, furthermore, that mitochondria transfer from MSCs can rescue injured pulmonary cells and ameliorate lung injury. Islam et al. demonstrated that airway instillation of human MSCs could reduce LPS-mediated lung injury, in part, through transfer of mitochondria. Using live optical imaging, they documented transfer of vesicles containing labeled mitochondria from MSCs to alveolar epithelial cells that increased alveolar ATP levels and cell survival. Unlike wild-type MSCs, MSCs genetically modified for connexin 43 that were incapable of forming gap junctions and MSCs with dysfunctional mitochondria did not reduce acute lung injury.

Recent data from a cigarette smoke-induced model of lung injury suggest that donor source and age may affect repair by mitochondria transfer by MSC. Li et al. found that transplantation of MSCs derived from induced pluripotent stem cells may provide enhanced repair after transplantation by virtue of increased TNT formation and mitochondria transfer relative to adult-derived MSCs.

Using loss- and gain-of-function approaches, Ahmad et al. elegantly demonstrated that Miro-1, an outer mitochondrial membrane Rho-like GTPase, regulated the amount of mitochondrial transfer from MSCs to cultured lung epithelial cells. Enhanced expression of Miro-1 was shown to increase transfer of mitochondria from MSCs and treatment of mice with MSCs overexpressing Miro-1 reduced Rotenone lung injury and airway hyperresponsiveness and negative remodeling in several models of asthma.

Regulators of mitochondria transport identified in other cell types that may orchestrate mitochondrial transfer by MSCs:

In addition to Miro-1, other proteins known to regulate intracellular mitochondrial dynamics (e.g., fusion, fission, tethering, and trafficking) may also promote or inhibit intercellular mitochondria transfer. Miro-1 and Miro-2 belong to a group of dynamin-related proteins that regulate mitochondrial division and fusion. They interact with TRAK1 and TRAK2 (identified as Milton in Drosophila), adaptor proteins that recruit kinesin motor proteins to mitochondria. The resulting adaptor–motor protein complex shuttles mitochondria along microtubules and was demonstrated to be critical for neuronal transport of mitochondria to axons, dendrites, and synapses. Mitofusin 1 and 2 may also regulate mitochondria transfer as they are known to interact with Miro-1 and Miro-2 as well as TREK1/TREK2 in the adaptor–motor protein complex. Perhaps not surprising, motor proteins are likely to be required for generation of some forms of TNTs. Myo-X (Myo10) is a myosin motor protein that localizes to the ends of cellular filapodia. It is unique in that it does not require substrate attachment to induce filapodia extension. Co-culture studies in neuronal cells

demonstrated that Myo10 was required for TNT formation from filapodia and overexpression of Myo10 resulted in increased TNT formation and vesicle transfer between cells.

Although the damage/injury signals that initiate mitochondrial transfer have yet to be identified, it is plausible that differences in intracellular Ca^{+2} or energy stores (e.g., glucose, ATP) may play a role in directing one cell to transfer mitochondria to another. For example, intracellular movement of mitochondria is highly sensitive to cytosolic Ca^{+2} levels. Wang and Schwartz elegantly demonstrated that Ca^{+2} promotes Miro to interact with the motor domain of kinesin, thus blocking kinesin from the microtubule. Accordingly, mitochondria transfer from cell to cell may be affected by differences in intracellular Ca^{+2} concentration and/or localization. Consistent with this concept, TNTs have been shown to transfer Ca^{2+} and even electrical signals to neighboring cells through TNT-associated gap junctions. In addition, the level of available nutrients can alter movement of mitochondria. In neurons, Pekkurnaz et al. reported that extracellular glucose and the enzyme O-GlcNAc transferase (OGT) affect mitochondrial motility by altering GlcNAcylation of Milton, an OGT substrate. As OGT activity is dependent on glucose, increased glucose was shown to decrease mitochondrial motility.

Of special interest, several reports indicate regulatory overlap or some form of integration between TNT formation and endosomal trafficking, as both interact with components of the exocyst complex that regulates vesicular transport from the Golgi apparatus to the plasma membrane. For example, Hase et al. reported that M-sec, part of the exocyst complex, interacted with the small GTPase RalA and was required for TNT formation in a macrophage cell line. Furthermore, they showed that M-sec expression could induce cell protrusions de novo, some of which formed TNTs with adjacent cells. Subsequently, Schiller et al. found that the transmembrane MHC class III protein leukocyte specific transcript 1 (LST1) was also required for TNT formation. At the cell membrane, LST1 was shown to interact with M-Sec, myosin, and myoferlin and also to recruit RalA, promoting its interaction with the exocyst complex. Notably, some mechanisms (e.g., proteins) controlling TNT formation and/or mitochondrial transfer may be specific to specialized cell types such as neurons. However, in light of the conserved nature of intracellular adaptor/kinesin motor protein complexes, mitochondrial dynamics, and endosomal trafficking, it is probable that many mechanisms that control TNT formation and/or mitochondrial transfer are similar between many cell types, including MSCs.

Modifying Mitochondrial Transfer and Mitochondria for Clinical Application

For future clinical application, harnessing mitochondrial transfer in a controlled and predictable manner will likely require further mechanistic insight. Importantly, recent advances in targeting of DNA to mitochondria may provide new tools to track or even perhaps to genetically alter mitochondria by modifying mtDNA as opposed to nuclear genes for proteins targeted to mitochondria (e.g., genes for mitochondrial membrane proteins). For example, Yu et al. restored ATP synthesis in cells carrying mutant mtDNA for human NADH ubiquinone oxidoreductase subunit 4 (ND4) by infecting cells with an adeno-associated virus capsid (VP2) fused to a mitochondrial targeting sequence and the wild-type ND4 mitochondrial gene sequence. Following recent successful testing in non-human primates and human eyes ex vivo, the innovative method may soon be applied in clinical trials for treatment of Leber hereditary optic neuropathy, a disease caused by a mutation in the ND4 mitochondrial gene.

Despite the potential benefits of mitochondrial transfer or other TNT-mediated effects, it is worth noting that cell–cell communication by way of TNTs may also have some negative consequences. In contrast to their potential therapeutic benefits, TNTs also have potential to act as disease vectors for transmission of HIV/AIDS, bacteria, Prions, and oncogenic miRNAs.

Transfer of RNAs and other Molecules by EVs

The general term "extracellular vesicle" (EV) refers to membrane-bound vesicles released from most, if not all, somatic cell types. Together, the EVs include exosomes, 30–100-nm plasma membrane-coated vesicles of endocytic origin; microvesicles, 50–1000-nm vesicles of non-endocytic origin; and apoptotic bodies, 1–5-μm vesicles released during membrane blebbing of apoptotic cells.

Cellullar exosomes are released when multivesicular bodies traffic to and fuse with the plama membrane in a regulated manner. Exosomes were first identified and isolated from cultures of normal and transformed cells during the 1980s. Valadi et al. made a key contribution when they demonstrated that both mRNA and miRNA could be exchanged between cells by virtue of exosomal transfer. Studying xenogenic co-cultures, they observed expression of various mouse proteins in human mast cells after exosomal transfer from murine cells, indicating successful translation of exosomally delivered mRNA into protein. As with exosomes isolated from diverse cell types, MSC-derived exosomes are reported to contain lipid raft domains and tetraspanins known to alter the fusion state of cell membranes (e.g., CD9, CD81), Alix, a calcium-binding protein with roles in both endosomal trafficking and cell death, and TSG101, a tumor suppressor protein. Compared with exosomes, which are relatively homogenous upon release, microvesicles are heterogenous in both size and composition. Furthermore, regulatory mechanisms for microvesicular shedding from the membrane surface remain poorly understood.

Exosomes purified from MSCs have garnered tremendous interest in the field of regenerative medicine based on their ability to reduce apoptosis/necrosis in rodents after ischemic injury to the heart, brain, lung, liver, or kidney. In addition, exosomal transfer from MSCs is reported to reduce inflammation and to increase cell proliferation during tissue repair. Tomasoni et al. showed that MSCs transferred exosomes with mRNA for IGF1R and IGF1 to cisplatin-damaged proximal tubular cells; this resulted in their expression of IGF1R, thereby increasing sensitization to IGF-1. The exosomal transfer improved renal cell survival and increased proliferation during repair after injury. In multiple drug-induced models of liver injury, treatment with MSC exosomes at the time of injury increased the number of proliferating cell nuclear antigen-positive proliferation cells while reducing the number of hepatocytes undergoing apoptotic cell death. Treatment of a murine carbon tetrachloride-based injury model with exosomes from human umbilical cord-derived MSCs was shown to reduce liver fibrosis. Following stroke in rats, treatment with MSC-derived exosomes was shown to promote angiogenesis, neurogenesis, neurite outgrowth, and recovery by virtue of transfer of miR-133b. In addition to RNAs, exosomes and microvesicles can deliver peptide/protein-based paracrine effectors such as growth factors, cytokines, and hormones. For example, transfer of Wnt4 by exosomes from human umbilical cord-derived MSCs improved repair of skin wounds in rats by altering cell proliferation.

Currently, many investigators and clinicians are interested in the potential of MSC-derived EV therapeutics for repair of injured and diseased tissue and to treat cancer. Most studies with

exosome-based treatment of injured tissues/organs report positive outcomes, However, whether or not MSC-mediated transfer of exosomes, microvesicles, and/or their constituents promote or inhibit the activities of transformed cells in a way that would positively or negatively impact cancer remains context-dependent and controversial. For example, bone marrow MSCs were shown to reduce the growth of cultured breast cancer cells by transferring miR-127, -197, -222, and -223 through gap junctions and exosomes; these miRNAs are known to target CXCL12 (a.k.a. SDF-1). Lee et al. suggested that exosomes from MSCs might suppress angiogenesis based on their containing miR-16, a miRNA that targets VEGF and was shown to reduce its expression in a breast cancer cell line. By contrast, Zhu et al. reported that exosomes from human MSCs actually promoted tumor growth in vivo by inducing VEGF expression in tumor cells. Boelens et al. reported cross-talk between stromal cells and breast cancer cells whereby stromal exosomes induced paracrine antiviral signals and stimulated juxtacrine Notch3 signaling that increased the number of therapy-resistant tumor-initiating cells. As with other paracrine effects of cell-based therapy or treatments based on administration of signaling agonists (e.g., growth factors), it is clear that care must be taken to avoid potential off-target treatment effects of administered EVs to avoid cancer cell propagation and/or metastasis.

Towards standardization of exosome-based therapy using MSCs or any cell type, identification of the most reliable and consistent vesicle isolation methods will be critical so that different laboratories can effectively compare their results. At present, several different methods of isolation are widely used, including centrifugation, filtration, immunoaffinity isolation with beads, and microfluidics. Notably, exosomes isolated from the same source by different methods may differ in amount and/or content.

Research aimed at improved understanding of mechanisms controlling cargo loading of exosomes will also be important. For protein-based cargo, Shen et al. have reported some progress using expressed plasma membrane anchors. For miRNA-based cargo, Villarroya-Beltri et al. recently identified specific miRNA sequence motifs that direct their loading into exosomes. Furthermore, they determined that sumoylated heterogenous nuclear ribonucleoprotein (hnRNPA2B1) was required for sorting of miRNAs into exosomes based on the specific motifs. Detailed characterization of MSC exosome content under various conditions and from all tissues will likely aid in a more predictable product in terms of therapy. For example, MSCs isolated from various tissues differ in terms of exosome content and MSCs from bone marrow with multiple myeloma were reported to differ in miRNA content relative to MSCs from control bone marrow.

Regulatory Mechanisms in Stem Cell Biology

Stem cells are a subject of intense and increasing interest because of their biological properties and potential medical importance. Unfortunately, the field has been difficult for the nonspecialist to penetrate, in part because of ambiguity about what exactly constitutes a stem cell. A working definition is useful in order to pose the important questions in stem cell biology. A minimalist definition is that stem cells have the capacity both to self-renew and to generate differentiated progeny. Although this is in many respects inadequate, it immediately highlights some important problems: How at each cell division is a stem cell able to pass on its “stem” properties to at least

one of its two daughters? And what determines whether stem cell divisions will be self-renewing, or differentiating?

The focus on hematopoiesis and neurogenesis reflects the fact that these systems are the ones in which stem cells have been most rigorously and directly identified. Hematopoietic stem cells (HSCs) have been isolated using antibodies to cell surface antigens, and their functional properties have been established by transplantation into lethally irradiated host animals under conditions where the progeny of a single stem cell can be identified. The self-renewal properties of these cells have been demonstrated by serial transfer into secondary recipients.

The brain has not traditionally been considered a stem cell system because of the dogma that this tissue is incapable of regeneration. Recently, however, there has been a rediscovery of Altman's original observations that some regions of the adult brain exhibit ongoing neurogenesis, and this has been accompanied by a surge of activity in identifying the progenitor cells responsible for both embryonic and postnatal neural development. Stem cells in the neural crest and embryonic central nervous system (CNS) have been identified using in vitro assays in which the differentiation and self-renewal capacity of single progenitor cells have been demonstrated by subcloning experiments. It is not yet clear, however, whether any of these neural stem cells can generate all the different classes of neurons found in the adult CNS or PNS, nor is it clear whether the stem cells isolated from adult brain tissue manifest their multilineage differentiation capacity under physiological conditions in vivo.

The existence of stem cells in the gut, gonads, skin, and olfactory epithelium has been demonstrated indirectly by mosaic in vivo lineage–marking experiments, anatomical studies, or in vitro experiments. Although the standard of proof defined for HSCs or neural stem cells has not yet been achieved, one can proceed on the assumption that stem cells exist in these tissues. It has also been proposed that stem cells exist in the liver a tissue which can regenerate in response to injury, although this is controversial because under most conditions differentiated cell types reenter the cell cycle and contribute the preponderance of regeneration.

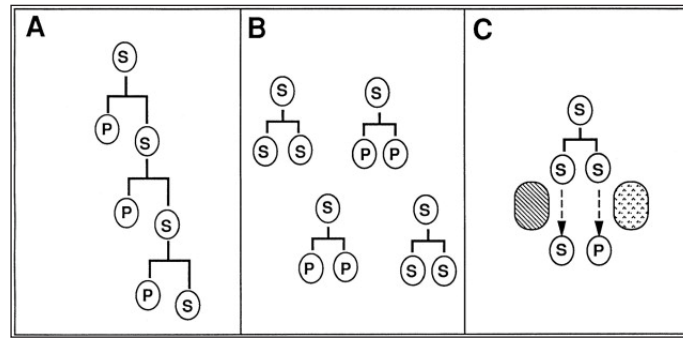
Properties of Stem Cells

A number of properties besides self-renewal and differentiation potential are frequently ascribed to stem cells, including the ability to undergo asymmetric cell divisions, exhibit extensive self-renewal capacity, exist in a mitotically quiescent form, and clonally regenerate all of the different cell types that constitute the tissue in which they exist. many of these properties are exhibited by stem cells in some tissues or organisms, but not in others. This helps to distinguish the most fundamental questions in stem cell biology from questions that are highly relevant but specific to certain systems. It also illustrates the difficulty in arriving at a universally applicable definition of a stem cell. a certain tolerance of ambiguity in the definition of stem cells is necessary in order to remain focused on the mechanistic questions and avoid semantic arguments.

Symmetric versus Asymmetric Divisions

Stem cells are often thought to undergo repeated, intrinsically determined asymmetric cell divisions that produce one differentiated (progenitor) daughter and another daughter that is still a stem cell. While there are clear examples of such lineages in Hirudo medicinalis, Drosophila

melanogaster, and Caenorhabditis elegans, in mammalian systems there is stronger evidence that stem cells divide symmetrically. Symmetric divisions allow the size of the stem cell pool to be regulated by factors that control the probability of self-renewing versus differentiative divisions.



Possible Patterns of Cell Division in Stem Cell Lineages.

Self-Renewal Capacity

Murine HSCs do not have unlimited self-renewal potential, although a subset is able to self-renew for the lifetime of a mouse. However, in larger, longer-lived animals, such as humans, it is not at all clear that HSCs self-renew for an entire lifespan; rather, successive subsets of stem cell clones may become activated with increasing age. Even in small, shorter-lived organisms, there is clear evidence that stem cells have lifetimes less than that of the entire animal. For example, one of the two somatic stem cells in the Drosphila ovary dies or differentiates after about 26 days. Thus, not all stem cells have unlimited self-renewal potential.

In tissues where serial transplantation of isolated cells is not technically possible, it is often difficult to assess the self-renewal capacity of putative stem cells in vivo. The mere existence of progenitor cells in an adult tissue is not de facto evidence that these cells have undergone extensive self-renewal, as is sometimes assumed, because they may simply have persisted in quiescent form. There are, moreover, clear cases of stem cells that exist only transiently during development, such as fetal and embryonic HSCs. Oocyte production ceases by birth, while that of sperm continues into adulthood, yet both cells derive from primordial germ cells (PGCs) whose stem cell properties are indistinguishable in males and females early in gestation. Thus, not all stem cells self-renew into adulthood, and not all adult stem cells reflect self-renewal of fetal cells. Finally, in some cases, adult stem cells may derive neither by self-renewal nor by persistence of fetal cells, but rather may represent a distinct stem cell class that develops from a transient fetal stem cell population. This makes the entire concept of self-renewal capacity "for the lifetime of the organism" precarious as a criterion for stem cells.

Mitotic Quiescenc

Another property shared by some, but not all, stem cells is that they divide slowly or rarely. This is thought to be true for stem cells in the skin and bone marrow. Other kinds of stem cells, however, divide more rapidly. Somatic stem cells in the Drosophila ovary and mammalian intestinal crypt stem cells have been estimated to divide every 12 hr. It may be generally true that stem cells in adult tissues are more likely to cycle slowly, but this quiescence is not an obligatory property of stem cells.

Mother of all Cells

Another characteristic attributed to stem cells is the ability to regenerate clonally the entire adult tissue from which they derive, meaning all cell types that constitute that tissue. In practice, this is an extremely difficult criterion to satisfy. Even in the hematopoietic system, for example, certain classes of blood cells—such as some kinds of T cells—are only produced during fetal life and are maintained in the adult by proliferation of committed cells. Therefore, adult HSCs can replace most, but not all, blood cells found in the adult tissue. The mature olfactory epithelium consists of neurons and sustentacular (glial) cells, but retroviral lineage analysis has shown that only the neurons are regenerated from stem cells in the basal layer. These examples illustrate cases where stem cells regenerate only a subset of the differentiated cell types in a given tissue. We suggest that stem cells include all self-renewing progenitor cells that have the broadest developmental potential available within a particular tissue at a particular time.

Some authors do not consider all self-renewing pluripotent progenitors to be stem cells, reserving this category only for the subset with the "most primitive" characteristics. This results in a trend to restrict incrementally the stem cell definition to smaller and smaller subsets of cells. The concept of a most primitive progenitor is inherently ambiguous because it often is based on largely untested expectations about the properties that correlate with primitiveness. If we are to understand the biology of self-renewal and pleuripotency, then all self-renewing pluripotent progenitors in a given tissue should be studied.

Regenerative Capacity

It has been argued that only regenerative tissues can have stem cells. The most significant problem with this definition is that certain tissues or at least certain cell types exhibit regenerative capacity only during limited windows of ontogeny. It seems arbitrary to exclude certain classes of progenitor cells from consideration simply because they display their regenerative capacity at one stage of development but not at others. The failure of regeneration in the adult may be due not to the absence of pluripotent, self-renewing cells, but to the inability of the injured tissue to accomodate or promote their differentiation, as may well be the case in most areas of the brain.

These considerations reinforce the idea that there are basic common properties of stem cells that extend across diverse species, tissues, and developmental stages: the capacity to self-renew and to generate progeny that are fated to differentiate into mature cells. This raises the question of whether there are common molecular mechanisms, shared by all stem cells, that underly these properties. Other properties, such as the ability to divide asymmetrically, to undergo extensive self- renewing divisions, to exist in a quiescent rather than mitotically active state, and to generate a multiplicity of differentiated derivatives, are exhibited by some classes of stem cells, but not by others.

Control of Self-renewal

Self-renewal potential is the most fundamental property of stem cells. However, to understand self-renewal it is not sufficient simply to understand how stem cell proliferation is controlled, because not all cell divisions involve self-renewal. Are there specific signals that couple mitogenesis to maintenance of the stem cell state? Or are proliferation and maintenance of the stem cell state

regulated independently by distinct signals? These issues are important because although the size of the stem cell pool remains nearly constant in many tissues under steady-state conditions, it can expand rapidly in response to tissue damage.

Extrinsic Regulation of Self-renewal

What limits the number of stem cells under steady-state conditions? One possibility is that stem cells can only exist in a restricted microenvironment in each tissue, which provides factors that maintain them and excludes factors that induce differentiation. For example, intestinal epithelium stem cells appear to be localized to a narrow ring of tissue near the base of the crypts. If the amount of space in such microenvironments (or "niches") is limited, the number of stem cells would be limited by the number that can fit in that space. Stem cells generated in excess of the available space would differentiate. Evidence for such a mechanism is scant in mammals, but in C. elegans the self-renewal of germ line stem cells requires proximity to the distal tip cell, which produces a ligand that promotes stem cell divisions. Not all stem cell systems, however, utilize such local control mechanisms. For example, PGCs self-renew while migrating to the genital ridges.

The proliferation of stem cells also increases in response to tissue damage. For example, in the sensory epithelia of the nose and the inner ear, damage to the primary sensory neurons induces the proliferation of cells that regenerate the lost neurons. In principle, the induction of division in such systems could be promoted either by the release of mitogens from dying cells, or by relief from inhibitors normally produced by healthy neurons (or both); no evidence yet exists to distinguish among these possibilities. It is also assumed that such feedback control of stem cell proliferation is local, either by direct signaling to the stem cells or by indirect signaling via intermediate progenitor compartments.

Identity of Factors that Control Stem Cell Self-renewal and their Mechanisms of Action

In C. elegans, the germ line stem cells require activa- tion of the Notch-related receptor GLP-1 to retain self- renewal potential. The ligand for GLP-1, LAG-2, is membrane bound and expressed only by the neighboring distal tip cell. In glp-1 mutants, germline stem cells not only cease self-renewing mitoses, but also undergo meiosis and differentiate into gametes. Thus, LAG-2 appears to be necessary both to maintain proliferation and prevent differentiation of stem cells. By contrast, genetic studies of Notch (a glp-1-related gene) in Drosophila have been interpreted to suggest that its primary role is to maintain cells in an undifferentiated state, whether or not those cells are actively dividing. Consistent with this, activated forms of mNotch, a murine homolog of GLP-1, inhibit differentiation of myogenic and neurogenic cell lines without a detectable effect on cell proliferation However, lineage-specific expression of an activated form of human Notch, tan-1, is found in tumors of primitive lymphoid cells in humans. Taken together, these data suggest that Notch and its homologs can regulate proliferation or maintenance of the undifferentiated state, or both, depending on context.

Although a number of growth factors can drive quiescent HSCs into cycle, despite a vigorous search no factors have yet been identified that (singly or in combination) are capable of maintaining self-renewing divisions of these stem cells in vitro. In the nervous system, EGF promotes proliferation of stem cells from the adult CNS, and basic fibroblast growth factor (bFGF) promotes the

self-renewal of embryonic as well as adult CNS stem cells. bFGF also promotes proliferation of primordial germ cells in culture, although it also appears to broaden their developmental potential. While these studies have been performed in vitro, they demonstrate that factors do exist that can cause stem cells to self-renew repeatedly when they would otherwise remain quiescent or differentiate.

Stem cell self-renewal can also be negatively regulated by locally acting or long-range factors. In tissues where stem cells have a restricted location, locally acting factors have been sought. For example, proliferation of primordial germ cells and intestinal crypt stem cells is thought to be inhibited by local sources of transforming growth factor β (TGFβ). Both short- and long-range feedback mechanisms are hypothesized to regulate negatively HSC self-renewal. Macrophage inhibitory protein 1α, constitutively produced by macrophages, has been shown to inhibit the proliferation of multipotent progenitors; whether this inhibition occurs locally or at long range is not yet clear. Since HSCs are segregated among different bones and organs throughout the body, at least some factors that regulate self-renewal must act at long range for the stem cell pool to be regulated in a coordinated fashion.

Factors that regulate stem cell self- renewal can induce or inhibit proliferation, and can act locally or at long range. Few of the factors involved have been identified. In cases where factors have been identified, it is usually not known what cells produce them, or how their production is regulated. It will be interesting to determine whether there are systematic differences in stem cell regulation between tissues with relatively invariant architecture, like intestinal crypts, and those with more flexible architecture, like the hematopoietic system.

Do Stem Cells have Intrinsic Limitations on their Self-renewal Capacity?

The self-renewal capacity of certain stem cells may exceed the extent of self-renewal that they actually undergo in vivo. Does that mean that self-renewal capacity is unlimited, or are there limitations on self-renewal capacity even when that capacity exceeds actual self-renewal fate? The hematopoietic system clearly exemplifies that not all pluripotent stem cells have equivalent self-renewal capacities. Individual HSCs can exhibit either transient (< 8 weeks) or long-term (> 16 weeks) self-renewal capacity. This difference was proposed to depend on the environment encountered by intrinsically similar cells. However, fractionation of HSCs by surface marker expression has revealed distinct subpopulations that exhibit different self-renewal capacities even when the cells are exposed to equivalent environments in vivo, implying that these differences are cell intrinsic.

The molecular basis of self-renewal capacity remains to be elucidated. Even in cases where this has been shown to be an intrinsic property of stem cells, the molecules need not act in a purely cell-autonomous way. For example, differential expression of adhesion molecules could cause different HSC subpopulations to home to different bone marrow microenvironments that specify different self-renewal fates. Entirely cell-autonomous mechanisms may, however, be at work as well. Telomerase expression widely correlates with self- renewal potential in many cell types. Recently, about 70% of fetal liver or bone marrow HSCs, but only rare non-self-renewing multipotent progenitors, were shown to exhibit telomerase activity. Unlike tumor cells, HSCs are not immortal, and human HSCs show decreasing telomere length with increasing age. Thus, telomerase may regulate self-renewal capacity by reducing the rate at which telomeres shorten. Stem cells with

long telomeres could, nevertheless, be caused to differentiate and exit the stem cell pool by other factors.

Maintenance of the Uncommitted State by Intrinsic Factors

There is strong evidence for cell-intrinsic factors that can maintain the uncommitted nature of the stem cell state without influencing proliferation. Germline progenitors in the C. elegans embryo undergo asymmetric divisions that maintain the germline lineage and produce a series of progenitor cells that become committed to various somatic fates. This asymmetric segregation of daughter cell fates appears to be determined by the nuclear protein PIE-1, which is maternally inherited and asymmetrically distributed to the germline daughter cells. PIE-1 represses the transcription of embryonic genes that cause commitment to particular somatic fates. Thus, one mechanism for maintaining the stem cell state is to actively repress genes required for commitment. Transmission of this state to daughter stem cells would require a mechanism for maintaining expression of such active repressors.

Evidence for Asymmetric Cell Divisions

It is often assumed (incorrectly) that all stem cell lineages necessarily involve intrinsically asymmetric divisions. There are several well-documented examples of such lineages in invertebrates, including C. elegans germline blastomeres and Drosophila neural precursors. However, in mammals, there are very few examples of asymmetric stem cell divisions. In the ferret cerebral cortex, time-lapse films have revealed that some progenitor cells divide to generate one daughter that remains in the ventricular zone, and another that migrates away, presumably to differentiate to a neuron. Such asymmetric divisions are correlated with an orientation of the mitotic spindle perpendicular to the surface of the ventricle. The further observation that a mammalian homolog of Notch1 is asymmetrically distributed on some ventricular zone cells prior to cytokinesis suggests that at least some molecules are unequally distributed to the daughter cells (although it does not mean that the orientation of this distribution is independent of environment). Asymmetric divisions of multipotent hematopoietic progenitors have also been observed in clone-splitting experiments.

Molecular Determinants of Asymmetry

In Drosophila neuroblasts, asymmetric cell divisions are dependent upon correct mitotic spindle orientation, as well as on the asymmetric distribution of several proteins, such as numb and prospero. The asymmetric distribution of numb and prospero is in turn controlled by additional regulators, such as inscuteable. Mammalian homologs of numb have been isolated, and one is asymmetrically distributed in some cortical progenitor cells (as well as in cells in other, non-neural tissues), suggesting that some asymmetric divisions in mammals may also be intrinsically determined. Distinct molecular determinants of asymmetric cleavages have also been identified in C. elegans and yeast, but whether these have been conserved in mammals as well is not yet known. Apparently asymmetric divisions can also reflect intrinsically symmetric divisions that place the daughter cells in different environments that confer different fates. While such a mechanism has been shown to control the fate of somatic blastomeres in C. elegans embryos at the four-cell stage, direct evidence for such a process in vertebrates is lacking.

Are asymmetric cell divisions the rule or the exception? Despite the recent attention to asymmetric stem cell divisions, the available evidence favors a predominance of symmetric divisions in

mammalian stem cell systems. In strictly asymmetric stem cell lineages, no regulation of stem cell number is possible. But there is ample evidence for such changes in the size of stem cell populations in mammals, implying that symmetric divisions must occur. The absolute number of fetal liver HSCs doubles daily during mid-gestation, and during adult life in mice there is a more than five-fold increase in the absolute number of long-term self-renewing HSCs. Primordial germ cells undergo at least five rounds of symmetric self-renewing divisions while they migrate into the genital ridges during fetal development.

Some mammalian stem cell populations may undergo both symmetric and asymmetric divisions, depending on their circumstances. Indeed, neural progenitors in the ferret cortex undergo both symmetric and asymmetric divisions. The relative proportion of symmetric divisions appears to change over time, with symmetric divisions predominating at early time points when the stem cell pool would be expected to be expanding. Whether this indicates that a single cell can switch from a symmetric to an asymmetric mode of cell division is not yet clear.

Control of Stem Cell Survival

As mentioned earlier, the persistence of stem cell populations throughout adulthood likely depends on the survival of quiescent cells, as well as on the ability of cycling cells to self-renew. Evidence for quiescent stem cells has been presented in the liver, the brain, and in bone marrow. However, it is still not clear whether such apparently quiescent cells are really in G0 or whether they are just moving very slowly through G1. Are there factors that promote stem cell survival, but not necessarily self- renewal? By itself, steel factor (also known as stem cell factor) promotes the survival, but not the proliferation, of HSCs and primordial germ cells; however, the regulation of these effects is likely to be complex, since steel factor is not required for the survival of HSCs and can synergize with other factors to promote stem cell proliferation. Intestinal crypt and liver stem cells are also regulated by steel factor. These data raise further questions about the regulation of steel factor expression and its combinatorial action with other factors. As more factors are identified, the control of stem cell survival is likely to become an increasing focus of investigation.

Control of Stem Cell Differentiation

What sets the repertoire of potential fates available to a stem cell in a given tissue? How do stem cells choose to exit the stem cell state and begin to differentiate? In cases of multipotent stem cells, how is the choice of a particular differentiated fate made?

Determination of the Repertoire of Potential Stem Cell Fates

The overall developmental potential of a stem cell is defined by all the types of differentiated progeny it can ultimately give rise to. How is this property encoded in the stem cell in molecular terms? One possibility is that multipotent stem cells might express a set of transcription factors which individually specify different lineages or combinations of lineages. For example, mutations in the ikaros gene, which encodes a zinc finger protein present in HSCs, prevent the development of multiple lymphoid derivatives. However, it is not yet clear whether ikaros acts in HSCs themselves, or is independently required in multiple lymphoid sublineages at later stages of development. The entire developmental repertoire of a given multipotent stem cell could also, in theory, be specified by a single determining factor that sits at the top of a regulatory hierarchy. A targeted mutation

in the bHLH transcription factor SCL prevents the development of all hematopoietic derivatives, but it is not yet known whether SCL is expressed in HSCs, and, if so, required for their formation, self-renewal, or differentiation. From an evolutionary standpoint, mutations that increased the developmental repertoire of stem cells could lead to increased cellular diversity in a tissue by "duplication and modification" of cell types.

In tissues where different cell types are generated from a multipotent progenitor on a relatively precise schedule, such as the retina, multipotent cells may be competent to generate only one or two specific fates in a given period of development. For example, all retinal cell types derive from multipotent progenitors, but the competence of these progenitors to respond to environmental signals changes over time. There are clear cases where competence is determined by the expression of receptors necessary to respond to fate-determining signals, but this need not always be so; in principle, competence may also be determined by expression of signal transduction molecules or transcription factors. However, there are few specific examples of this type.

How do Stem Cells Initiate the Differentiation Process?

The differentiation of stem cells involves both exit from the uncommitted state and entry into a particular developmental pathway. Evidence from C. elegans indicates that these two aspects are independently controlled. Exit from the stem cell state requires loss of PIE-1, a zinc finger protein that represses the expression of genes involved in commitment to differentiation. This loss occurs by asymmetric distribution of PIE-1 to stem cell daughters at each blastomere division. However, the absence of PIE-1 in somatic blastomere daughters is insufficient to initiate a program of differentiation: positive-acting transcriptional regulators, such as SKN-1, are also required to promote entry into a particular somatic lineage.

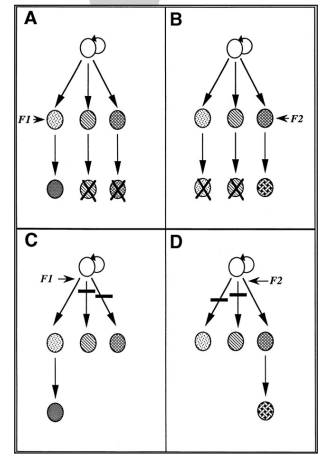
It is not yet clear whether exit from the stem cell state and initiation of differentiation are also independently controlled in mammals. At one extreme, differentiation might be a "default" pathway executed by the stem cell when it is removed from a microenvironment that promotes maintenance of the uncommitted state. At the other extreme, specific signals might promote differentiation and consequently exit from the stem cell state. There is evidence that both mechanisms operate in the nervous system. In vitro, CNS stem cells undergo self-renewing divisions in bFGF, but upon withdrawal of this growth factor they rapidly differentiate to neurons. On the other hand, the differentiation of cultured neural crest stem cells to autonomic neurons is promoted by BMP2. These examples leave open the question of whether the effect of such environmental signals is to regulate transcription factors that maintain the stem cell state (analagous to PIE-1), or factors that promote entry into particular lineages, or both. In either case, such factors are likely to be subject to both negative and positive regulation by environmental signals, which may explain the different effects of such signals on cell fate decisions by CNS and PNS neural stem cells.

How do Multipotent Stem Cells Select a Particular Differentiation Pathway?

The choice of fate by a multipotent stem cell could, in principle, be controlled from inside or outside the cell. There is ample evidence from invertebrate systems that such choices can be determined nonautonomously by local cell-cell interactions. For example, in C. elegans, an EGF-like signal produced by the gonadal anchor cell specifies the fate of vulval precursor cells. Similarly,

in Drosophila, the choice between cone (glial) and photoreceptor cell fates is determined by a transmembrane ligand, BOSS, presented by the R8 photoreceptor. While these examples concern cells that do not exhibit the self-renewal capability necessary to fit our definition of stem cells, they nevertheless provide important examples of how extrinsic signals can regulate fate determination in multipotent progenitors.

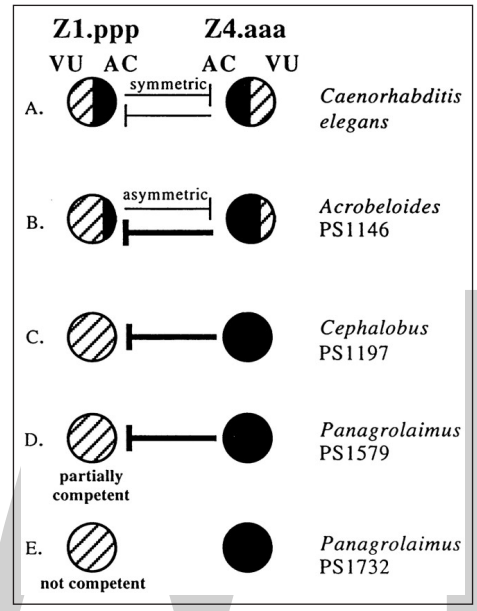
Selective versus instructive actions of growth factors on mammalian stem cells: In mammalian systems, there is considerable evidence that growth factors and cell–cell interactions can influence the outcome of fate decisions by multipotent progenitors at the population level. This raises a problem not encountered in invertebrate systems where the fates of individual cells are easily monitored. Specifically, growth factors could influence individual stem cells in a selective or instructive manner. In a selective mechanism, the stem cells commit to a particular lineage independently of the growth factors, and the factors act subsequently to control the survival or proliferation of such committed progenitors. In an instructive mechanism, the growth factor causes the progenitor to choose one lineage at the expense of others. In hematopoiesis, the relative contributions of these two mechanisms remain controversial. Forced expresson of bcl-2 in an immortalized hematopoietic progenitor cell line yielded multilineage differentiation in the absence of cytokines, implying that these growth factors act selectively. In the neural crest, by contrast, serial observation of individual clones in vitro has indicated that differentiation to each of three cell types—autonomic neurons, Schwann (glial) cells, and smooth muscle—can be instructively promoted by three signals: BMP2, GGF (a neuregulin), and TGFβ, respectively. Similarly, the differentiation of CNS stem cells to astrocytes is instructively promoted by CNTF. It remains to be determined whether growth factors influence stem cells in the nervous system and hematopoietic system in fundamentally different ways, or whether instructive differentiation signals for HSCs have simply not yet been identified owing to lack of appropriate assays.



The Difference Between Selective and Instructive Mechanisms of Growth Factor Influences on Stem Cell Fate Decisions.

Instructive factors can influence differentiation choices whose outcomes are stochastic. Instructive environmental signals may increase or decrease the probabilities of choosing a particular fate, rather than promote or repress them in an all-or-none manner. In nematodes, the binary decision between ventral uterine (VU) and anchor cell (AC) fates by neighboring precursor cells is controlled by lateral signaling, mediated by the NOTCH-like protein LIN-12 and its ligand LAG-2. In some species, such as Cephalobus, this cell–cell interaction produces a deterministic

(invariant) outcome: the same precusor always adopts the VU fate in every animal of the species. In others (Acrobeloides), a similar cell–cell interaction produces a stochastic (probabilistic) outcome exhibiting bias: one precursor becomes the anchor cell roughly 80% of the time Finally, in C. elegans, the outcome is stochastic and unbiased: each precursor has a 50:50 probability of adopting either fate. In all three cases, the cell–cell signaling is instructive, since in the absence of one precursor the other always adopts the AC fate. Thus, in different species, instructive signaling can exert a range of bias strengths on stochastic cell fate decisions. Similarly, it has been proposed that the engagement of MHC molecules with either the CD4 or CD8 coreceptors may exert a bias on a stochastic decision by T-cell progenitors between helper and killer cell fates.



Phylogenetic Variation in the Control of a Binary Cell Fate Decision in Nematodes.

It is sometimes assumed that if differentiation is stochastic and unbiased, a cell-autonomous mechanism must be at work. However, in C. elegans, the unpredictability of the outcome of the AC/VU decision derives from the equivalent strength of the reciprocal inhibitory interactions between AC/VU precursors. Similarly, where cell-autonomous mechanisms have been inferred from the apparently stochastic behavior of hematopoietic progenitors in vitro, the cells are usually cultured in complex media containing serum and other sources of undefined factors, and the collective influence of such environmental factors could cause the cells to behave in an apparently unpredictable (stochastic) manner.

Autonomous Control of Cell Fate

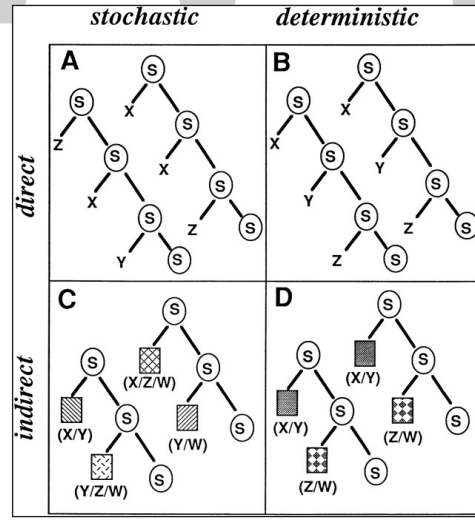
A selective action of environmental factors implies that the initial choice of differentiated fate by a stem cell is controlled by a cell-autonomous mechanism. Such an intrinsic mechanism may yield a stochastic outcome, as has been suggested for HSCs, or a deterministic outcome. In yeast, the mating-type switch is a cell-autonomous fate decision that appears stochastic at the population level, but is deterministic for individual cells according to their previous history. In early C. elegans embryos, the assignment of somatic blastomere fate is determined in an autonomous and deterministic manner by the asymmetric partitioning of transcription factors at successive cleavages.

Currently there are no clear examples of such cell-autonomous mechanisms operating in a mammalian stem cell.

There are, of course, many examples of transcription factors required for the development of particular mammalian lineages. Although once expressed these factors may impose a cell-heritable and autonomous state of determination on a progenitor cell, the initial decision to express such factors may be nonautonomously controlled. For example, the bHLH transcriptional regulator myoD is able to confer a cell-heritable state of myogenic determination, owing to its autoregulatory properties, when transfected into cultured fibroblasts. However, in vivo, the expression of this protein in somitic mesoderm is induced by a combination of signals from neighboring tissues, such as the notochord and neural plate. Moreover, the execution of the muscle differentiation program in determined myoblasts is still regulated by growth factors. Thus, the involvement of lineage-specific transcription factors does not imply that either selection or execution of specific fates are autonomously controlled.

Order and Pattern in the Segregation of Different Lineages from Stem Cells

In principle, multipotent stem cells could generate different derivatives in a random manner, or according to a predictable sequence or hierarchy. There is evidence for both mechanisms in different systems. In grasshopper, the midline neuroblast sequentially produces neurons, glia, and neurons again. In the vertebrate retina, different cell types emerge on a predictable schedule, although whether individual progenitors generate their differentiated progeny in a fixed order is not yet clear. In contrast, clone-splitting experiments in vitro have suggested that there is no perceptible order or pattern to the emergence of different lineages from multipotent hematopoietic progenitors, although since no lymphoid differentiation was detected it is not clear whether these conclusions apply to HSCs.



Alternative Modes of Differentiation by Multipotent Stem Cells.

A related question is whether the immediate progeny of stem cells are committed to a single fate, or restricted to a subset of fates. CNS stem cells generate some progeny fated to produce only neurons, but whether these unifatent cells are truly committed was not determined. Committed neuronal progenitors have been identified in the PNS, but whether these are directly generated from

stem cells is not yet clear. In the hematopoietic system, progenitors committed to single lineages (e.g., B cell or T cell) have been shown to be derived from partially restricted lymphoid progenitors. Analagous partially restricted progenitors have been suggested to exist in the neural crest based on in vitro clonal analyses, but whether these cells are truly committed to a subset of lineages has not been rigorously tested by exposure to appropriate instructive signals. The existence of partially restricted intermediates raises the additional question of whether their developmental potentials are assorted randomly, or in an ordered, hierarchical manner. The hematopoietic system may employ both strategies, depending upon the stage of lineage diversification. An ordered or hierarchical segregation of lineages at the cellular level may reflect the action of transcription factors that coordinately specify multiple sublineages; for example, there are lymphoid progenitors restricted to B and T sublineages and several transcription factors, such as ikaros and E2A, required for both sublineages.

Formation of Stem Cells

Stem cells in the hematopoietic system, nervous system, gonads, liver, and intestine form de novo during fetal life. The progenitors of stem cells are sometimes referred to as pre-stem cells. Pre-stem cells can be defined as cells whose progeny contribute to tissues other than that derived from the particular stem cell they generate, and that produce stem cells only during a defined interval of development. While the sites of stem cell formation during mammalian fetal development are generally known, the identities of the pre-stem cells are usually not known; furthermore, little is known about the events that regulate the acquisition of stem cell competence.

Are there any genes identified that are required for the formation of stem cells? In Drosophila, asymmetrically dividing CNS progenitors, which are in many ways like stem cells, delaminate from a group of neuroectodermal precursor cells. Within this group, the bHLH transcription factors ACHAETE-SCUTE confer competence to generate the progenitor. A single progenitor is selected from the group of competent cells by lateral inhibition, mediated by Notch proteins and their ligands. Recent data indicate that a similar process underlies the selection of neuronal precursors during primary neurogenesis in Xenopus. Although such amphibian neuronal precursors have not been defined as stem cells, a similar mechanism may be employed in the mammalian CNS, where stem cells have been clearly identified. Genes encoding both transcription factors and extracellular signals that are involved in the formation of the hematopoietic system have been identified, but whether these act at the level of stem cell formation is not yet known. Genetic screens in zebrafish may identify more such molecules.

There is evidence that different classes of stem cells can exist simultaneously in the same tissue. Stem cells from different positions along the cephalocaudal axis of the gut exhibit position-specific differences in terms of the differentiated cells they give rise to. When explants from different portions of the intestine were transplanted subcutaneously, the regional differences appeared to persist, providing some evidence that the differences may be intrinsic to the stem cells. There is also evidence for regional differences among central nervous system progenitor cells. Mouse basal ganglion progenitors, but not ventral mesencephalic progenitors, were able to differentiate into striatal cells upon transplantation into rat striatum, suggesting that the progenitors differed in their ability to adopt the fates of their new tissues. Such differences are correlated with the region-specific expression of transcriptional regulators in the neuroepithelium from the earliest stages of brain development, suggesting an intrinsic component to such progenitor cell diversity.

On the other hand, there are several cases where neural precursors adopt a correct identity when transplanted from one region into another, suggesting that intrinsic differences may not always irreversibly commit such cells to a given fate.

The developmental potential of stem cells for a given tissue can differ in time as well as in space. Fetal liver HSCs are thought to give rise to adult bone marrow stem cells. Yet fetal liver stem cells are able to give rise to several classes of blood cells that adult bone marrow stem cells do not themselves produce. These differences are intrinsic to the stem cells since they persist even when fetal liver stem cells are transplanted into adult bone marrow, or when both stem cell types are transplanted into culture. The mechanisms underlying such stage-specific differences in developmental potential are not known.

Stem Cell Aging

Stem cells persist throughout life, replacing cells lost to homeostatic turnover, injury, and disease. However, their functions decline with age, which contributes to degeneration and dysfunction. The molecular mechanisms involved in the aging of stem cells are the same as the ones involved in the aging of somatic cells, including telomere shortening, oxidative stress, epigenetic dysregulation, miRNAs changes, alterations of DNA, RNA, proteome, and various cellular organelles. Aging impacts various pathways, such as insulin/insulin-like growth factor 1 (IGF-1), mTOR, FoxO, AMP-activated protein kinase (AMPK), sirtuin, and many others, resulting in senescent stem cells that exhibit functional and numerical impairment. Stem cells have developed special mechanisms to prevent age related damage accumulation and to sustain their stemness properties, however, these mechanisms lose their effectiveness over time. The most fatal consequence of this is found in the immune system, where both innate and adaptive immunity are affected, exhibiting a plethora of defects, including increased autoimmune disease occurrence, elevated tolerance to cancer and chronic inflammatory status. Stem cell therapies call for the best quality of stem cells grafts. Stem cell products should be devoid of cells containing a senescent phenotype, thus a comprehensive knowledge of the biology behind the senescence of stem cells should be taken into account in every cell based therapy.

An 70 kg adult human body consists of approximately 3.72 × 1013 cells. These trillions of cells are not permanent and a majority of them are constantly renewed throughout our lifetime, although some of them – such as cells in the lenses of our eyes and some of the neurons of our central nervous system – are thought to be an exception. The frequency of renewal depends on the function of the cells and may vary from several hours to several years.

The renewal of adult tissues is enabled by specialized cells that function over the lifetime of an organism, i.e., the stem cells (SCs). They persist throughout life in numerous mammalian tissues, replacing cells lost to homeostatic turnover, injury, and disease. Stem cells reside in specific anatomic reservoirs, such as bone marrow, and circulate in the organism when needed. SCs represent a very small proportion in adult tissues. It is estimated that the bone marrow of a 70 kg adult human contains around $1.5–1.7 \times 10^{12}$ cells, among them only $45–120 \times 10^{6}$ are true hematopoietic stem cells (HSCs) that give rise to more frequent progenitors. The frequencies of stem cells in other tissues are even lower and still a matter of debate.

Adult SCs can typically self-renew and differentiate into multiple cell types within a developing and adult body. Due to their self-renewal capacity they were regarded as immortal reservoirs of youth, however, they are nonetheless susceptible to the age related damages. To prevent or reverse the accumulation of age related damage and epigenetic changes, SCs developed special mechanisms to maintain long telomeres, enhance proteostasis, avoid ROS production and defend against toxic substances. In spite of that, their functions decline with age in a number of tissues, including blood, forebrain, skeletal muscle, skin and all the other tissues.

Declines in stem cell functions not only contribute to degeneration and dysfunction of aging tissues, but also negatively affect the life span of the organism. Some strong evidence for SCs as regulators of longevity comes from animal studies. For instance, if in C. elegans germline stem cells (GSCs) are eliminated, this almost doubles its lifespan and such a phenomenon is highly conserved. Similarly, if the fruit flies are modified with overexpression of a PGC-1α homolog or a heat-shock response transcription factor and moderate repression of insulin/IGF or JNK signaling, this directly extends their life span, implying that improved stem cell function leads to better tissue function, and that stem cell aging underlies the aging of tissues and organs.

In humans, there is considerable evidence supporting the fact that young stem cells perform better than old ones. Proof of this concept is best documented is the recent multicenter study on the success of hematopoietic stem cell transplantation, which is currently the most popular and efficient cell therapy for malignant diseases. In more than 6000 cases of allogeneic bone marrow transplantation between 2007 and 2011, it was clearly shown that patient survival was significantly better after grafts from young donors (aged 18–32 years) were used. For every 10-year increment in donor age, there was a 5.5% increase in the hazard ratio for overall mortality. This is probably one of the most important findings in this field, suggesting that for regenerative purposes, and other stem cell therapies, grafted stem cells should be young and devoid of senescent defects.

The Biology of Stem Cell Aging

Adult stem cells express several characteristic features that are specific to stem cells, as well as certain features that are found in any other somatic cell in the body. They express telomerase – an enzyme required for telomere extension that is essential for repeated self-renewal, they cycle between phases of quiescence and activation needed for the production of progeny, their chromatin exists in a bivalent state primed for self-renewal or differentiation, they have unique metabolic requirements, they distribute their macromolecules asymmetrically during asymmetric cell divisions, and they reside in niches that regulate their behavior.

The molecular mechanisms that are involved in the aging of adult stem cells are the same as the ones involved in the aging of the somatic cells. Traits and mechanisms that are affected by aging are present in various populations of stem cells. The age-related decline of stem cells is mainly functional, but in some cases, a decline in stem cell numbers can also be observed. Since many of these mechanisms appear simultaneously, it is practically impossible to trace or determine a single initial damaging agent that causes the cascade of other detrimental sequences. Therefore authors agree that aging is probably the result of multifactorial derangements caused by several causative factors that act in parallel, including the formation of damaging reactive oxygen species (ROS), telomere attrition, DNA damage and mutations, epigenetic changes (alterations of histones, DNA and the consequent dysregulation of gene expression), mitochondrial DNA mutations with

mitochondrial decline, changes of microRNAs, ribosomal changes and defects of RNA splicing, changes of proteostasis, changes in cellular polarity, changes in nutrient sensing and metabolism, niche deterioration, improper accumulation of various circulating factors, stem cell pool exhaustion, cellular senescence with cell cycle arrest, and altered intercellular communication.

Table: Multifactorial causes of stem cell aging.

1.	Formation of damaging reactive oxygen species (ROS).
2.	Mitochondrial DNA mutations, decline of mitochondrial integrity and biogenesis.
3.	Nuclear damage and nuclear DNA mutations.
4.	Telomere shortening/attrition.
5.	Epigenetic changes/alterations of histones and DNA and consequent dysregulation of gene expression.
6.	Changes of microRNAs.
7.	Changes of RNA splicing and ribosomal machinery.
8.	Changes of proteostasis.
9.	Changes of cell polarity.
10.	Metabolism and nutrient sensing.
11.	Niche deterioration.
12.	Accumulation of various circulating factors.
13.	Stem cell pool exhaustion.
14.	Cellular senescence – arrest of the cell cycle.
15.	Altered intercellular communication.

Formation of Damaging Reactive Oxygen Species (ROS) and Oxidative Stress

The free radical theory of aging has been long accepted as the most plausible explanation for the aging process. It was first formulated in the 1950s by Harman who hypothesized that an accumulation of endogenous oxygen radicals (reactive oxygen species, or ROS) occurs, which in turn causes further mitochondrial deterioration and the global cellular damage responsible for the aging and death of all living beings. This theory was then revised in 1972 when mitochondria were identified as being responsible for the initiation of most of the free radical reactions. It was also postulated that life span was determined by the rate of free radical damage to the mitochondria. Mitochondrial respiration, the basis of energy production in all eukaryotes, generates ROS by leaking intermediates from the electron transport chain. In all aerobic organisms, age-related oxidative stress is generated either by exposure to endogenous metabolites or exogenous sources such as radiation (UV, X-ray), and ROS accumulation is the result of an imbalance between free radical production and antioxidant defenses, such as superoxide dismutase that is responsible for scavenging superoxide anions. In fact, oxidative modifications have been shown to occur in DNA, protein, and lipid molecules.

Whereas young stem cells contain a spectrum of antioxidant mechanisms, aged stem cells display an inadequate anti-oxidant defense that is associated with functional impairment, including decreased responsiveness to physical environmental cues and decreased resistance to oxidative stress. In several studies, aging stem cells from bone marrow and adipose tissue showed a significantly reduced capacity for coping with oxidative stress with increasing donor age. Therefore,

oxidative stress is still recognized as the fundamental underlying component of the aging process, leading to dysregulation of various cellular pathways and the subsequent accumulation of toxic aggregates and cellular debris, and ultimately to the activation of cell death/survival pathways leading to apoptosis, necrosis, or autophagy.

However, recent developments have forced an intense re-evaluation of the mitochondrial free radical theory of aging after the unexpected observation that increased ROS may paradoxically prolong the lifespan of yeast and Caenorhabditis elegans. In mice, genetic manipulations, which increased mitochondrial ROS and oxidative damage, did not accelerate aging as one would expect. Furthermore, manipulations that increased antioxidant defenses did not extend longevity, and lastly, genetic manipulations that impaired mitochondrial function but did not increase ROS, accelerated aging. There has also been other solid evidence that in response to physiological signals and stress conditions, ROS triggered proliferative and survival signals.

The mitochondrial theory of aging has also been challenged as it has become clear that there exists a rather complicated interplay between various other cellular compartments. Dysfunctional mitochondria can contribute to aging independently of ROS, as demonstrated by studies with mice deficient in DNA polymerase γ. This could happen through a number of mechanisms, for example, mitochondrial deficiencies may affect apoptotic signaling by increasing the propensity of cell's death through mitochondrial membrane permeabilization in response to stress, and trigger inflammatory reactions by favoring ROS-mediated and/or permeabilization- facilitated activation of inflammasomes. Also, mitochondrial dysfunction may directly impact cellular signaling and interorganellar crosstalk, by affecting mitochondrion-associated membranes that constitute an interface between the outer mitochondrial membrane and the endoplasmic reticulum.

The mitochondrial ROS that were considered the main cause of age related defects actually contribute positively to various signaling pathways and normal cellular responses, such as adaptation to hypoxia, cellular differentiation, autophagy, inflammation, and immune responses, meaning that ROS are also beneficial for cellular biology.

Mitochondrial DNA Mutations, the Decline of Mitochondrial Integrity and Biogenesis

Mitochondrial function has a profound impact on the aging process. Mitochondrial dysfunction can accelerate aging in mammals. It was generally believed that age-related pathology was caused by defects of mitochondria related to oxidative stress, leading to the accumulation of irreparable changes of nucleic acids, proteins, and lipid molecules. But there are also other defects of mitochondria that develop during normal aging. Similar to the nuclear DNA, mitochondrial DNA (mtDNA) is exposed to mutations and deletions in aged cells, which are not found in nuclear DNA, and which also contribute to aging. This is aggravated by the oxidative microenvironment of the mitochondria and the limited efficiency of the mtDNA repair mechanisms.

The mutations that can lead to mitochondrial dysfunction and death are now detectable in generated induced pluripotent stem cell (iPSC) lines, i.e., expanded clones from each individual skin or blood cell. As a result, every cell in the iPSC line contains the same mitochondrial DNA (mtDNA) mutations as the original adult cell, and can for this reason be easily sequenced. We now know that to ensure healthy mitochondrial genes, we must screen stem cells for mutations or collect them at

a younger age. This may help illuminate the role of mutated mitochondria in degenerative diseases and to assess the patient-derived regenerative products destined for clinical applications.

Interestingly, most mtDNA mutations in adult or aged cells appear to be caused by replication errors early in life, rather than by oxidative damage. These mutations may undergo polyclonal expansion and cause respiratory chain dysfunction in different tissues. Studies of accelerated aging in HIV-infected patients treated with anti-retroviral drugs, which interfere with mtDNA replication, have supported the concept of clonal expansion of mtDNA mutations that originated early in life.

Aging also affects the biogenesis of mitochondria. Mitochondrial biogenesis is the process by which cells increase their individual mitochondrial mass and copy their number to increase the production of ATP, as a response to greater energy needs. With aging, the reduced efficiency of mitochondrial bioenergetics may be a result of multiple converging mechanisms, including reduced biogenesis of mitochondria. For instance, in telomerase-deficient mice, it can be a consequence of telomere attrition with subsequent p53-mediated repression of PGC-1α and PGC-1β (peroxisome proliferator-activated receptor gamma coactivator 1 –alpha and –beta, which are the master regulators of mitochondrial biogenesis). This mitochondrial decline also occurs during physiological aging in wild-type mice and can be partially reversed by telomerase activation. Sirtuin 1 (SIRT1) modulates mitochondrial biogenesis through a process involving the transcriptional co-activator PGC-1α and the removal of damaged mitochondria by autophagy. SIRT3, which is the main mitochondrial deacetylase, targets many enzymes involved in energy metabolism, including components of the respiratory chain, tricarboxylic acid cycle, ketogenesis and fatty acid β-oxidation pathways. SIRT3 may also directly control the rate of ROS production by deacetylating manganese superoxide dismutase, a major mitochondrial antioxidant enzyme. Collectively, these results support the idea that sirtuins may act as metabolic sensors to control mitochondrial function and play a protective role against age-associated diseases.

Interestingly, endurance training and alternate-day-fasting may improve healthspan through the capacity to avoid mitochondrial degeneration. It is tempting to speculate that these beneficial effects are mediated, at least in part, through the induction of autophagy, for which both endurance training and fasting constitute potent triggers. However, autophagy induction is probably not the sole mechanism through which a healthy lifestyle can retard aging, since, depending on the precise diet reduction regime, additional longevity pathways can be activated.

The combination of increased damage and reduced turnover in mitochondria, due to lower biogenesis and reduced clearance, may contribute to the aging process. Some other mechanisms can also affect the mitochondrial bioenergetics and contribute to the aging mitochondrial phenotype, among them the mutations and deletions in mtDNA, oxidation of mitochondrial proteins, destabilization of the macromolecular organization of respiratory chain, defects of the lipid membranes, and defective autophagy that targets deficient mitochondria.

In conclusion we could say that the importance of mitochondria in the basic biology of aging and the pathogenesis of age-associated diseases is stronger than ever, although the emphasis has moved from ROS to other causative aspects. Obviously, besides the mitochondrial dysfunction due to ROS, there exists a complex interplay of several other factors of aging, such as mDNA mutations, changes of lysosome processing, endoplasmic reticulum stress, genomic instability,

telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, altered intercellular communication, mitochondrial biogenesis and turnover, energy sensing, apoptosis, senescence, and calcium dynamics. Mitochondria do play one of the key roles in the pathophysiology of aging and events that lead to the aged phenotype, therefore they will increasingly be targeted to prevent and treat chronic diseases and to promote healthy aging. We expect that future studies will determine whether genetic manipulations that decrease the load of mtDNA mutations and other damaging factors, are able to extend lifespan.

Nuclear Damage and Nuclear DNA Mutations

It is clear that in aged humans and model organisms, somatic mutations accumulate over time within all cells. Other forms of DNA damage, such as chromosomal aneuploidies, copy-number variations and increased clonal mosaicism for large chromosomal anomalies have also been found to be associated with aging. Each time a stem cell replicates its DNA and divides, the likelihood of DNA defects and oncogenic transformations increases. Therefore the lifetime risk of cancer development in a tissue correlates with the number of divisions the stem cells of this particular tissue have undergone. A variety of these DNA alterations can finally affect the essential genes that control the key transcriptional pathways. Such defect cells should be normally eliminated by apoptosis or senescence, however, if this does not happen it may jeopardize tissue and organismal homeostasis. This is especially important in stem cells because the DNA damage has a detrimental impact on their functional competence, i.e., on their role in tissue renewal.

An accumulation in DNA damage and mutations leading to stem cell aging has been one of the earliest theories of aging. DNA damage can be caused by external factors (ionizing radiation, ultraviolet radiation or environmental toxins), or by internal factors (ROS and errors in DNA replication). These factors can lead to various DNA lesions such as modifications of bases or sugar residues, the formation of DNA adducts, cross-linking of the DNA strands or the appearance of single and double-strand breaks. Among these lesions, DNA double-strand breaks (DSBs) are particularly lethal because they result in physical cleavage of the DNA backbone. DSBs can occur through replication fork collapse, during the processing of interstrand crosslinks, or following exposure to ionizing radiation. In spite of the fact that cells have evolved at least six different DNA repair pathways to deal with these distinct types of DNA damage, there is convincing evidence that with aging, stem cell DNA is also subject to damage. In HSCs, histone H2AX phosphorylation and comet tails, both of which are measures of DNA damage, increase with age. Phosphorylation of H2AX, one of several genes coding for histone H2A (one of the five main histone proteins involved in the structure of chromatin in eukaryotic cells), accumulates with age in satellite cells, i.e., stem cells of the muscles. Moreover, aged HSCs display a history of replication stress and decreased expression of DNA helicases, further sensitizing them to future replication challenges.

Since mutations are a common daily occurrence, our cells could not survive without DNA repair mechanisms. There are two groups of repairing mechanisms, the first acting to repair DNA single-strand breaks (mismatch repair mechanism, base excision repair mechanism, nucleotide excision mechanism), and the second acting to synchronously repair DNA double-strand breaks, i.e. homologous recombination and non-homologous end joining (NHEJ). In spite of the repair mechanisms our DNA accumulates mutations, since the genes of repair mechanisms are themselves subject to mutations.

Evidence that DNA damage plays a causal role in the aging process includes the observation that mice with defects in DNA damage repair display some aspects of premature aging, whereas enhancing DNA repair through increased expression of SIRT6 increases lifespan. In some situations, DNA damage may also reduce stem cell numbers by causing them to undergo apoptosis, senescence or differentiation, although it is not yet confirmed whether these effects are due to an increase in stem cell longevity.

It is also known that deficiencies in DNA repair mechanisms cause accelerated aging in mice and underlie several human progeroid syndromes such as Werner syndrome, Bloom syndrome, xeroderma pigmentosum, trichothiodystrophy, Cockayne syndrome, or Seckel syndrome. Moreover, transgenic mice overexpressing multidomain protein kinase BUBR1 (budding uninhibited by benzimidazole-related 1), a mitotic checkpoint component that ensures the accurate segregation of chromosomes, exhibit an increased protection against aneuploidy and cancer, and display an extended healthy lifespan. These experimental data prove that artificial reinforcement of nuclear DNA repair mechanisms could delay aging.

In addition to genomic damage affecting nuclear or mitochondrial DNA, there is evidence that certain defects in the nuclear lamina can also change nuclear architecture and thereby cause genomic instability. Nuclear lamins participate in genome maintenance by providing a scaffold for tethering chromatin and protein complexes that regulate genomic stability. Mutations in genes encoding protein components of this structure, or factors affecting their maturation and dynamics, cause accelerated aging syndromes such as the Hutchinson-Gilford and the Néstor-Guillermo progeria syndromes. Alterations of the nuclear lamina and production of an aberrant prelamin A isoform called progerin have also been detected during normal human aging. Since telomere dysfunction also promotes progerin production in normal human fibroblasts upon prolonged in vitro culture, this suggests that there exist intimate links between telomere maintenance and progerin expression during normal aging. In addition to these age-associated changes in A-type lamins, lamin B1 levels decline during cell senescence, pointing to its utility as a biomarker of this process.

Telomere Shortening

Although accumulation of DNA damage affects the genome near-to-randomly, there are some chromosomal regions that are particularly susceptible to age-related deterioration. Telomeres are repetitive TTAGGG sequences and associated nucleoproteins at the ends of a chromosome that play a critical role in protecting chromosomes from degradation, undesirable recombination, and chromosome fusion. With each somatic cell division, telomeres shorten and this exposes cells to the aging phenotype. Due to inability of the normal DNA replication machinery to completely replicate the telomeric sequences, telomeres in somatic cells shorten with each cell division, and are thus markers for cellular aging and replicative capacity.

Mice with shortened or lengthened telomeres exhibit decreased or increased lifespans, respectively. Telomere shortening is observed during normal aging both in humans and mice. In humans, recent meta-analyses have indicated a strong relation between short telomeres and mortality risk, particularly at younger ages.

In contrast to somatic cells, embryonic and adult stem cells express telomerase, a reverse transcriptase enzyme, which catalyzes the extension of telomeric sequences, thereby avoiding

telomere attrition and prolonging cellular proliferative life span. While the telomerases are normally absent from most somatic cells, they are active in the stem cells and most cancer cells. Mammalian telomerase consists of a telomerase RNA component (TERC) and a telomerase reverse transcriptase (TERT) component. The latter catalyzes the synthesis of new telomeric repeats. Nevertheless, consistent decline in telomere length with age does occur in adult stem cells, suggesting that telomerase activity is insufficient to maintain the replication of these cells indefinitely. So the telomeres of various stem cells, i.e., hematopoietic, neural, germinal and other, do shorten with age.

Telomere exhaustion also explains the limited proliferative capacity of some types of in vitro cultured cells, the so-called replicative senescence or Hayflick limit. Indeed, as shown already in the 1990s, ectopic expression of telomerase confers immortality to otherwise mortal cells, without causing oncogenic transformation. Similarly, telomerase deficiency in humans is associated with the premature development of diseases, such as pulmonary fibrosis, dyskeratosis congenita and aplastic anemia, which involve the loss of the regenerative capacity of different tissues.

Recent evidence also indicates that aging can be reverted by telomerase activation. In particular, the premature aging of telomerase-deficient mice can be reverted when telomerase is genetically reactivated in these aged mice. Moreover, normal physiological aging can be delayed without increasing the incidence of cancer in adult wild-type mice by pharmacological activation or systemic viral transduction of telomerase.

This correlation between telomere length, telomerase activity and age is not completely clear. For example, while telomere length is negatively correlated with age in humans up to 75 years, it is positively correlated with age in the elderly, suggesting that long telomeres contribute to survival in old age. Furthermore, telomere length predicted survival in elderly twins, suggesting that telomeres contribute to longevity in humans even when controlling for the influence of genetic background.

There is a good correlation between the expression of human TERT mRNA and the presence of telomerase activity in extracts from tissue culture cells, and normal and cancer tissues, suggesting that human TERT expression is the primary and rate-limiting determinant of telomerase activity. This is important in stem cell therapies, so we have already investigated the importance of longer telomere length of the CD34+ cell grafts used for cell therapy and found that longer telomere length and higher telomerase expression agree with CD34+ cell's increased functional capacity, however the patients with longer CD34+ telomere length did not favorably respond to autologous CD34+ cell transplantation therapy.

Epigenetic Changes and Consequent Dysregulation of Gene Expression

The regulation of the chromatin state is important for stem cell function. In Waddington's epigenetic landscape theory, stem cells stand at an undifferentiated epigenetic summit above multiple cell fates. During the differentiation and aging of cells, numerous chromatin and gene expression changes appear progressively in response to cell stress, most notably in response to DNA damage signals. The changes in epigenetic modification of chromatin and histones lead to dysregulation of gene expression. The epigenetic modifications that are observed to change during aging are histone acetylation, histone methylation, and DNA methylation.

Of all other different types of DNA damage, the one that has the greatest lasting effect on chromatin is the double-strand breaks, which cause a dramatic redistribution of chromatin factors. This is a part of the response to damage that is not fully restored after the repair. Thus, changes in chromatin caused by DNA damage might underlie the skewed lineage phenotypes exhibited by aged stem cells.

The epigenetic changes have now been cataloged. In mice, it has been observed that the level of histone deacetylase SIRT1 decreases with age and that decrease of SIRT1 expression correlates with premature aging in mice with increased p53 activity.

The expression levels of chromatin modifiers, including components of the SWI-SNF (switch/sucrose non-fermentable) and PRC (polycomb repressive complex) complexes, histone deacetylases (HDACs) including sirtuins, and DNA methyltransferases, also change with age in stem cells. These changes may underpin declining stem cell function. Indeed, the overexpression of enhancer of zeste homolog 2 (EZH2), a component of PRC2, improves long-term repopulating potential in HSCs. Additionally, in aged HSCs, clusters of genes increase in expression levels based on chromosomal location, suggesting that epigenetic dysregulation engenders regional loss of transcriptional silencing. Taken together, these findings suggest that changes in epigenetic modifications are a general trait of stem cell aging, which impacts their function.

It is interesting that with aging appear changes that reinforce self-renewal. Sun et al. conducted a comprehensive integrated genomic analysis of young (4 mo) and old (24 mo) murine HSCs by profiling the transcriptome, DNA methylome, and histone modifications. Transcriptome analysis indicated reduced transforming growth factor beta (TGF-β) signaling and perturbation of genes involved in HSC proliferation and differentiation. Aged HSCs showed increased DNA methylation at transcription factor binding sites associated with differentiation-promoting genes, combined with a reduction at genes associated with HSC maintenance. When they profiled the principal regulatory chromatin marks with the use of chromatin immunoprecipitation sequencing (ChIP-seq) they found that the H3K4me3 mark, an activating histone modification, increases with age at loci that regulate HSC self-renewal, potentially underlying the increase in HSC number observed with aging.

In satellite cells of muscles, H3K4me3 levels modestly decrease with age, whereas levels of the repressive modification H3K27me3 significantly increase with age. It has also been shown that the expression levels of histones themselves decrease with age. The levels of H4K16Ac, another activating modification, decrease with age in HSCs; inhibition of cell division control protein 42 homolog (CDC42) restores H4K16Ac levels to that of young HSCs and reverses phenotypes of HSC aging in transplantation assays.

It is not known whether the epigenetic changes in stem cell products affect their clinical efficiency. The epigenetic landscape of different cell products can tell us little about the functional capacity and regenerative properties of CD34+ cells.

Changes of MicroRNA

Impairments in stem cell function that occur during aging are globally mirrored in the epigenome and transcriptome of HSCs, including the microRNAs. MicroRNAs (miRNAs) are small noncoding evolutionarily conserved RNAs that regulate gene expression primarily at the posttranscriptional

level. They act by binding to specific sequences in the 3′ untranslated region of their target genes and causing the transcripts to be degraded by the RNA-induced silencing complex (RISC). The human genome encodes over 1000 miRNAs that appear to target about 60% of other genes. MiRNAs are important posttranscriptional regulators of gene expression and play important and diverse roles in almost all biological and metabolic processes, including early development, cell proliferation, cell cycle regulation, apoptosis, fat metabolism, signal transduction, aging and diseases.

In stem cells, miRNAs influence properties such as potency, differentiation, self-renewal, and senescence. Different kinds of stem cells possess distinct miRNA expression profiles. Among other things, miRNAs regulate a number of cell functions such as defense mechanisms against ROS, DNA repair, and apoptosis. These properties, and the assumption that miRNAs act as some kind of general switch, make them highly relevant in research on aging, especially since specific miRNA expression profiles could be used to terminally differentiate cells from stem cells in order to treat various diseases, including myocardial infarction, neurodegenerative diseases, blood diseases, and muscle diseases.

miRNAs regulate the state of stem cells by directly targeting three prime untranslated region (3′-UTR) of pluripotency factors in the section of messenger RNA. For instance, miR-145 miRNA represses the pluripotency of human embryonic stem cells (ESCs) through targeting octamer-binding transcription factor 4 (Oct4; also known as Pou5f1), sex determining region Y-box 2 (Sox2), and kruppel-like factor 4 (Klf4). In addition, miRNAs target the coding regions of transcription factors to modulate stem cell differentiation. miR-296, miR-470, and miR-134 regulate mouse ESC differentiation by targeting the coding regions of Nanog, Oct4, and Sox2. Other classified miRNAs also regulate pluripotency, self-renewal, reprogramming, and differentiation of stem cells.

miRNAs act as key regulators of hematopoiesis during the proliferation and differentiation of HSCs in mammals. Ectopic expression of AAAGUGC seed-containing miRNAs enhance the primary hematopoietic progenitors. miR-181, miR-223, and miR-142 are preferentially expressed in hematopoietic tissues, with miR-181 significantly promoting B-lymphocyte differentiation. miR-125a is conservatively expressed in long-term HSCs and can increase the number of HSCs by targeting the apoptosis factor Bax1. Furthermore, overexpression of miR- 125b leads to lethal myeloid leukemia in mice.

Besides regulating the ESCs, miRNAs exert several other actions that indirectly impact stem cells and regeneration. For instance, let-7 family and miR-15a/16-1 cluster function as regulators of the cell cycle and tumor suppressors. While miR-29a and miR-29b regulate progression through the cell cycle, miR-9 and miR-124a play a critical role in specification of the neural progenitors from ESCs.

On the other hand, miRNAs also modulate development of other tissues, such as cardiovascular differentiation of cardiomyocyte progenitor cells and stem cells, including the differentiation of cardiomyocytes, vascular smooth muscle cells, and endothelial cells. They are involved in the regulation of cardiovascular differentiation of human-derived cardiomyocyte progenitor cells, the cardiovascular differentiation of ESCs and iPSCs, in cardiac differentiation of ESCs after myocardial infarction, vascular endothelial growth factor (VEGF) signaling and angiogenesis, which has great therapeutic value for the future regenerative medicine, by Li et al. Some other observations comment on the important role miRNAs play in brain development, as well as in later stages of

mammalian neuronal maturation and synapse development. Conversely, dysregulation of miRNAs expression has been implicated in developmental defects, cancers and nervous system diseases, as recently reviewed by Murashov.

Lee et al. have measured the expression levels of 521 small regulatory miRNAs in young and old animals of six mouse strains and found that expression levels of three miRNAs (miR-203-3p, miR-664-3p, and miR-708-5p) were associated with lifespan. Pathway analysis of binding sites for these three miRNAs revealed enrichment of key target genes involved in aging and longevity pathways including mechanistic target of rapamycin (mTOR), forkhead box protein O (FOXO) and mitogen-activated protein kinase (MAPK), most of which also demonstrated associations with longevity.

In conclusion, one could infer that miRNAs have critical roles in stem cell reprogramming, pluripotency maintenance and differentiation, as well as some other important cellular functions. In the future, miRNAs may greatly contribute to stem cell clinical therapy and have potential applications in regenerative medicine.

Changes in RNA Splicing and Ribosomal Machinery

RNA splicing is the editing of the nascent precursor messenger RNA (pre-mRNA) transcript into a mature messenger RNA (mRNA). After splicing, introns are removed and exones are joined together. Splicing usually takes place immediately after transcription, and is carried out in a series of reactions catalyzed by the spliceosome, a complex of small nuclear ribonucleoproteins (snRNPs). This results in an mRNA molecule, which can be translated into protein. Splicing enables one gene to generate multiple proteins allowing organisms to generate complexity from a relatively limited number of genes.

In healthy aging, splicing homeostasis takes place, while deregulation of the splicing machinery is linked to several age-related chronic illnesses. Certain studies point out that defective splicing machinery and de-regulation of RNA splicing acts as a driver of the aging process itself. Studies on the roundworm C. elegans show that with age they lose muscle mass, their cutickle thickens, they wrinkle, and they experience declines in fertility and immune functions. The pre-mRNA splicing homeostasis is a biomarker and predictor of life expectancy in this worm. Recently, Heintz and her colleagues found that splicing could also play a major role in the aging process of humans. Using transcriptomics and in-depth splicing analysis in young and old animals they found defects in global pre-mRNA splicing with age that are reduced by caloric restriction via one particular component of the splicing apparatus, called splicing factor 1 (SFA-1)—a factor also present in humans. They also showed that SFA-1 is specifically required for lifespan extension by caloric restriction and by modulation of the target of rapamycin complex 1 (TORC1) pathway components 5′ AMP-activated protein kinase (AMPK), RAGA-1, and ribosomal protein S6 kinase (RSKS-1/S6 kinase), and demonstrated that overexpression of splicing factor 1 (SFA-1) extends lifespan. Together, these data demonstrate a role for RNA splicing homeostasis in caloric restriction longevity and suggest that modulation of specific spliceosome components may prolong healthy aging.

The ribosomal machinery that is responsible for protein synthesis (translation), i.e., linking amino acids in the order specified by mRNA molecules, consists of two major components: the small ribosomal subunit, which reads the RNA, and the large subunit, which joins amino acids to form a polypeptide chain. Ribosomes contain ribosomal RNA (rRNA) molecules and a variety of highly

conserved ribosomal proteins, and similar to other cellular compartments, these are particular targets of aging. After a comprehensive integrated genomic analysis of young and aged cells, consisting of the profiling of transcriptome, DNA methylome, and histone modifications of young and old murine HSCs, Sun et al. found an increased transcription of ribosomal protein and RNA genes, and hypomethylation of rRNA genes. Indeed, inhibition of ribosomal proteins or their regulators has been shown to extend life span in yeast and worms. Although the research has not been focused on the plicing in stem cells we can expect that the splicing homeostasis in stem cells is similarly affected by aging.

Proteostasis

The proteostasis or homeostasis of the proteome is a complex system that takes care of the proper folding, functioning, and degradation of cellular proteins. Mechanisms, by which proteostasis is ensured, include regulated protein translation, chaperone assisted protein folding, and protein degradation pathways. Adjusting each of these mechanisms to the requirements of proteins, which need to be correctly folded, is essential for maintaining all cellular functions.

In previous paragraphs it has been already explained that in aged subjects, stem cells display a thoroughly altered proteome. Many studies have demonstrated that proteostasis is altered with aging and that accumulation of misfolded or damaged proteins is an important determinant of the aging process. Indeed, many different proteins involved in cytoskeletal organization, anti-oxidant defense, and other functions are age-dependent and associated with functional impairment of the cell functions, including decreased responsiveness to physical environmental cues and decreased resistance to oxidative stress. Chronic expression of unfolded, misfolded or aggregated proteins contributes to the development of some age-related pathologies, such as Alzheimer's disease, Parkinson's disease and cataracts. Since the passage of altered proteins to progenitor cells during asymmetric division could compromise development and cause aging, proteostasis maintenance in stem cells has an important role in organismal aging.

During the evolution the cells developed a variety of mechanisms that maintain and promote proteostasis and slow down the aging. This is performed by an array of quality control mechanisms that preserve the stability and functionality of the proteome. Various mechanisms for the correction of folded proteins have developed, such as the heat-shock family of proteins, as well as the corrective mechanisms for the degradation of misfolded proteins in proteasome or the lysosome. Moreover, there are regulators of age-related proteotoxicity, such as modifier of protein aggregation (MOAG-4), that act through an alternative pathway distinct from molecular chaperones and proteases. The stress-induced synthesis of cytosolic and organelle-specific chaperones is significantly impaired in aging. All these systems function in a coordinated fashion to restore the structure of misfolded polypeptides or to remove and degrade them completely, thus preventing the accumulation of damaged components and assuring the continuous renewal of intracellular proteins.

There are several approaches for maintaining or enhancing proteostasis aimed at activating protein folding and stability mediated by chaperones. A number of animal models support a causative impact of chaperone decline on longevity. In particular, transgenic worms and flies overexpressing chaperones are long-lived. Also, mutant mice deficient in a co-chaperone of the heat-shock family exhibit accelerated aging phenotypes, whereas long-lived mouse strains show a marked up-regulation of some heat-shock proteins.

Moreover, activation of the master regulator of the heat-shock response, the transcription factor heat shock factor 1 (HSF-1), increases longevity and thermotolerance in nematodes, while amyloid-binding components can maintain proteostasis during aging and extend lifespan. Pharmacological induction of the heat-shock protein Hsp72 preserves muscle function and delays progression of dystrophic pathology in mouse models of muscular dystrophy.

Small molecules may be also employed as pharmacological chaperones to assure the refolding of damaged proteins and to improve age-related phenotypes in model organisms.

For the degradation of unneeded and misfolded proteins there are special protein complexes, named proteasomes, which degrade them with proteolysis, a chemical reaction that breaks peptide bonds. The degradation process yields peptides of about seven to eight amino acids long, which can then be further degraded into shorter amino acid sequences and used in synthesis of new proteins.

Stem cells can also maintain high levels of autophagy and proteasome activity to clear damaged proteins. For example, autophagy is greater in HSCs and skin stem cells than in surrounding differentiated cells. Although proteasome activity has yet to be characterized in adult stem cells, it has been shown that human ESCs exhibit high proteasome activity. Fly oocytes, which require similar long-term proteome-protection mechanisms as stem cells, maintain high activity of large multi-protein complex 26S proteasome with age, despite the decline of its activity in the somatic cells.

The activities of the two principal proteolytic systems implicated in protein quality control, namely, the autophagy-lysosomal system and the ubiquitin-proteasome system, decline with aging, supporting the idea that collapsing proteostasis constitutes a common feature of old age. In relation to the proteasome, activation of epidermal growth factor (EGF) signaling extends longevity in nematodes by increasing the expression of various components of the ubiquitin-proteasome system activators accelerates the clearance of toxic proteins in human cultured cells. Moreover, increased expression of the 26S proteasome subunit RPN-6 by the FOXO transcription factor DAF-16 confers proteotoxic stress resistance and extends lifespan in C. elegans.

Regarding autophagy, transgenic mice with an extra copy of the chaperone-mediated autophagy receptor lysosome-associated membrane protein 2a (LAMP2a) do not experience aging-associated decline in autophagic activity and preserve improved hepatic function with aging. This is a promising example of genetic manipulations that improve proteostasis and delay aging in mammals. Functional decline in the cellular proteolytic machinery leads to the formation of an autofluorescent protein called lipofuscin, which can be used as a biomarker of aging. Based on the given data it is obvious that SCs are a subject of age related changes of proteostasis and further studies will probably focus on proteostasis maintanance in SCs.

Changes of Cell Polarity

In order to prevent the accumulation of damaged components, stem cells developed diverse mechanisms such as the asymmetric segregation of damaged proteins and enhanced proteostasis. After a symmetric division, stem cells produce two daughter cells with the same fate, whereas after asymmetric division they produce one daughter stem cell and one differentiating daughter cell. During the asymmetric division, damaged components such as damaged DNA, replicating circular DNA, carbonylated proteins and damaged organelles are distributed into the differentiating cell, whereas the daughter stem cell remains youthful. In a similar way, stem cells have been

shown to asymmetrically segregate damaged proteins and mitochondria into the progeny, which retains the stemness of the mother cell. A similar evolutionary principle enables that the parental strand of DNA is always sequestered in the daughter stem cell, whereas the strand synthesized during S phase, which might contain errors from replication, is directed to the differentiating daughter cell. In this way the non-random strand segregation serves to avoid mutations and to control the inheritance of epigenetic state. It was shown that the distribution of epigenetic modifications on mitotic chromosomes differs, which means that the bias is generated non-randomly during chromatid segregation. In Drosophila male GSCs, the histone modifications present in the stem cells are distinct from those in the differentiating daughter cells, which helps to retain pre-existing histones in the mother stem cell while imparting newly synthesized histones to the daughter cell. This retention of pre-existing histones in the stem cells is a prerequisite for maintaining their ability to self-renew. Different epigenetic modifications potentially lead to variations in the otherwise equivalent chromatids that segregate during asymmetric cell divisions.

There is accumulating evidence that other organelles are also non-randomly distributed between daughter cells. Numerous organelles have been widely studied for their asymmetric segregation in non-mammals and mammals, such as mitochondria, centrioles of the centrosome, and midbody, as well as different protein complexes.

The asymmetric division of stem cells first requires that a cell be polarized and several studies demonstrate that aged germinal stem cells (GSCs) and HSCs are less able to perform such polarized divisions, suggesting that loss of polarity contributes to stem cell aging. Other data on HSCs suggest that changes in age-related Wnt signaling are a cause of this loss of polarity. This process also appears to occur in satellite cells. There is certain disagreement as to whether polarized division occurs in other stem cell populations, such as intestinal, hair follicle, neural or germline stem cells.

Changes in Metabolism and Nutrient Sensing

Metabolic status plays an important role in stem cell aging. Similar to other cells, stem cells generate energy via glycolysis or oxidative phosphorylation. Quiescent stem cells generally rely upon glycolysis, perhaps because this reduces the abundance of ROS. Many adult stem cells also reside in hypoxic niches, perhaps as a part of a mechanism to limit ROS production.

For the provision of necessary energy, proliferating stem cells rely on the oxidative phosphorylation, which predisposes them to oxidative damage and cellular dysfunction. Therefore the molecules that scavenge ROS or enable the overexpression of the transcription factor NRF2, which regulates the response to oxidative stress, reduce the aged phenotype of old cell.

Caloric Restriction

The most robust longevity-extending intervention across species is caloric restriction (CR). For example, CR increases the abundance of satellite cells in muscles and improves the function of many stem cell populations, including HSCs in mice and GSCs in flies.

CR also promotes ISC self-renewal in mice by induction of the enzyme BST1 in Paneth cells, which form the niche. BST1 then converts NAD+ to the paracrine signal cyclic ADP ribose (cADPR), which is sensed by the ISCs. Pathways and factors implicated in mediating the response of stem cells to

CR that extend lifespan, include insulin and IGF-1 signaling (IIS) pathway, target of rapamycin (TOR) signaling, AMPK, sirtuins and FOXO transcription factors.

Glucose Metabolism

Recent studies also show that HSCs and satellite cells increase glucose and glutamine metabolism during activation— an alteration that mimics the Warburg effect in cancer cells. Similarly, in skeletal muscle, aging is associated with pseudohypoxia and Warburg-like metabolism, which compromise cellular function and promote oncogenic transformation.

Glucose is the main nutrient in the cell, whereas insulin informs cells about the presence of glucose. The intracellular signaling pathway that governs insulin is the same as that elicited by IGF-1, which is, together with the growth hormone (GH), produced by the anterior pituitary, and is the secondary mediator of the somatotrophic axis in mammals. For this reason, IGF-1 and insulin signaling are known as the "insulin and IGF-1 signaling" (IIS) pathway. GH and IGF-1 levels decline during normal aging, as well as in mouse models of premature aging. Remarkably, the IIS pathway is the most conserved aging-controlling pathway in evolution and among its multiple targets are the FOXO family of transcription factors and the mTOR complexes, which are also involved in aging and conserved through evolution. Similarly, genetic polymorphisms or mutations that reduce the functions of GH, IGF-1 receptor, insulin receptor or downstream intracellular effectors such as protein kinase B (PKB), also known as AKT, mTOR and FOXO, influence longevity both in humans and in model organisms, further illustrating the major impact these pathways have on longevity.

Multiple genetic manipulations of the IIS pathway, which attenuate signaling intensity at different levels, consistently extend the lifespan of worms, flies and mice. Genetic analyses indicate that this pathway mediates part of the beneficial effects of CR on longevity.

Mice with an increased dosage of the tumor suppressor protein phosphatase and tensin homolog (PTEN) exhibit a general down-modulation of the IIS pathway and an increased energy expenditure that is associated with improved mitochondrial oxidative metabolism, as well as with an enhanced activity of the brown adipose tissue. In line with other mouse models with decreased IIS activity, PTEN-overexpressing mice, as well as hypomorphic phosphatidylinositol-3-kinase (PI3K) mice show an increased longevity.

Organisms with a constitutively decreased IIS pathway can live longer because they have lower rates of cell growth and metabolism, and a lower rates of cellular damage. Similarly, the aged organisms decrease their IIS pathway in an attempt to extend their lifespan. However, defensive responses against aging eventually exhaust and later on they even aggravate aging.

Other nutrient-sensing systems: mammalian target of rapamycin (mTOR), AMP-activated protein kinase (AMPK) and sirtuins: Besides the IIS pathway, three additional related and interconnected nutrient-sensing systems that participate in glucose –sensing: mammalian TOR (mTOR), for the sensing of high amino acid concentrations; AMPK that senses low energy states by detecting high AMP levels; and sirtuins, which sense the low energy states by detecting high NAD^+ levels.

The mTOR kinase is part of two multiprotein complexes, mTORC1 and mTORC2, that regulate essentially all aspects of anabolic metabolism. Genetic down-regulation of mTORC1 activity in yeast,

worms and flies extends longevity and attenuates further longevity benefits from CR, suggesting that mTOR inhibition phenocopies CR. In mice, treatment with rapamycin also extends longevity in what is considered the most robust chemical intervention to increase lifespan in mammals.

Genetically-modified mice with low levels of mTORC1 activity, but normal levels of mTORC2 activity, have an increased lifespan, and mice deficient in ribosomal protein S6 kinase beta-1 (S6 K1), which is a main mTORC1 substrate, are also long-lived. This means that the down-regulation of mTORC1/S6 K1 acts as the critical mediator of longevity in relation to mTOR.

It seems that the intense trophic and anabolic activity, signaled through the IIS or the mTORC1 pathways, is a major accelerator of aging. Although inhibition of TOR activity clearly has beneficial effects during aging, it also has some undesirable side-effects, such as impaired wound healing, insulin resistance, cataract formation and testicular degeneration in mice. In order to determine the extent to which beneficial and damaging effects of TOR inhibition can be separated from each other, it will be crucial to understand the mechanisms involved.

There are two another nutrient sensors, AMPK and sirtuins, which act in the completely opposite direction of the IIS and mTOR. Instead of signaling nutrient abundance and anabolism, they signal nutrient scarcity and catabolism. Accordingly, their up-regulation promotes a healthy aging. AMPK activation has multiple effects on metabolism and, remarkably, shuts off mTORC1. There is evidence indicating that AMPK activation may mediate lifespan-extension following metformin administration to worms and mice.

In addition, SIRT1 can deacetylate and activate the PPARγ co-activator 1α (PGC-1α). PGC-1α orchestrates a complex metabolic response that includes mitochondriogenesis, enhanced anti-oxidant defenses, and improved fatty acid oxidation. Moreover, SIRT1 and AMPK can engage in a positive feedback loop, thus connecting both sensors of low-energy states into a unified response.

Collectively, currently available evidence strongly supports the idea that anabolic signaling accelerates aging, and decreased nutrient signaling extends longevity. Consistent with the relevance of deregulated nutrient-sensing as a hallmark of aging, CR increases lifespan or healthspan in all investigated eukaryote species, which are unicellular and multicellular organisms of several distinct phyla, including non-human primates. What is more, a pharmacological manipulation that mimics a state of limited nutrient availability, such as rapamycin, can extend longevity in mice. All of these reflects in stem cells, however, the exact mechanisms in the metabolism of stem cells awaits further clarification.

Niche Deterioration

In the context of a tissue, adult stem cells reside in a special microenvironment referred to as the "niche". The niche allows interaction between the stem cells and different extrinsic signals. In some instances, these signals are mediated via direct cell to cell communication or cell to matrix interaction. Another category of signals comprises of diffusible signaling ligands which regulate various transcription programs in the stem cells. These interactions are crucial, as they are able to regulate whether stem cells are quiescent, self-renew, or commit to differentiation.

Similarly to the stem cells themselves, the BM niche changes substantially with age. The niche consists of mesenchymal stem cells (MSCs), stromal cells, osteoblasts, adipocytes, and other cells, as

well as extracellular matrix. The proliferative capacity of human MSCs has been shown to decline with age. Certain other authors noticed a prominent increase in adipocytes in the aged BM, which is associated with lower HSC potential.

Mechanisms of niche aging are probably the same as in other cells. Khatri et al. recently showed that accumulation of excessive ROS in BM stromal cells suppress BM cellularity by affecting microenvironment in aged mice. Treatment of these mice with a polyphenolic antioxidant curcumin has quenched ROS, rescued stromal cells from oxidative stress-dependent cellular injury, and improved hematopoietic reconstitution in old (18 months) mice. This implicates the role of ROS in perturbation of stromal cells function upon aging, which in turn affects BM's reconstitution ability in aged mice. Rejuvenation therapy using curcumin, prior to transplantation of HSCs and progenitor cells could be an efficient strategy for successful marrow reconstitution in older mice.

The question remains as to whether aged BM niche cells induce age-related changes in HSCs. Evidence suggests that aging in the microenvironment influences HSC engraftment, as aged HSCs demonstrate a lower engraftment after transplantation. Hematopoietic cells engrafted in subcutaneous implantation of BM stroma from both aged and young mice exhibit lower spleen colony-forming units (CFU-S) capacity. Furthermore, young HSCs transplanted to aged niches exhibit impairment in homing and decreased potential for differentiation, failing to efficiently repopulate an old niche.

Another characteristic of aged HSCs is an altered differentiation potential tending toward higher myeloid/platelet output and lower lymphoid output. Skewing toward myeloid differentiation is attributed to the niche microenvironment, since the transplantation of young HSCs to aged recipients resulted in a tendency toward higher myeloid output. Transplantation experiments on old recipients show that granulocyte-macrophage progenitor (GMP) expansion is comparable regardless of donor age. Also, the differentiation of B-cells depends on the BM microenvironment and it was shown that aged HSCs occupy different niches to young HSCs.

One of the mechanisms of aging in the hematopoietic system are the changes in adhesion between HSCs and niche cells. Expression of various adhesion molecules in HSCs alters with age so the aged HSCs express low levels of integrin α4, integrin α5 and VCAM-1, and high levels of P-selectin and integrin α6 compared to young HSCs. In Drosophila, the age-dependent E-cadherin decline in the stem cell-niche junction that regulates the adhesion of GSCs to the niche was shown to contribute to the aging of stem cells. Another authors similarly showed that the aged HSCs exhibit less adhesion to the stromal cells compared to the young ones. Another group has shown that an overexpression of CDC42, a small Rho GTPase that is involved in adhesion signaling, causes premature aging phenotypes in these cells.

Age-related changes in niche cells may also be attributed to changes in their metabolic state. MSCs obtained from old human BM have an elevated level of ROS along with p21 and p53 expression, indicating cellular senescence. As already mentioned, high oxygen tension causes senescence in cultured human BM MSCs, whereas the continuous hypoxia make the human MSCs to exhibit higher self-renewal divisions without cellular senescence. Compared to MSCs cultured in low oxygen, MSCs cultured in higher oxygen levels utilize oxidative phosphorylation, suggesting that the generation of ROS might influence MSC senescence.

Age-related changes in the stem cell niches can influence HSC mobilization from the BM, which is extremely important in the clinical settings. Several authors, including ourselves, have noted that the collection of stem cells from aged patients results in low yields of mobilized HSCs intended for therapy. It is interesting that in various animal models an opposite effect was demonstrated since the granulocyte colony-stimulating factor (G-CSF)-induced mobilization resulted in increased numbers of HSCs in aged mice. The authors deduce that differences in mobilization potential according to age are influenced mainly by the niche in which the HSCs reside and that the clonality of HSCs may largely be influenced by specific niche cells at different anatomical sites.

Various studies utilizing heterochronic transplantation and parabiosis experiments showed that aging can be also be caused by extrinsic mechanisms, i.e., it is caused by factors external to the cell itself. This was shown in satellite cells, NSCs, and GSCs. In flies, the cells that form the niche of the GSCs themselves decline in abundance with age, possibly because of decreased self-renewal.

Aged niche cells can also fail to send proper signals to stem cells, namely through morphogen and growth factor signaling, thereby affecting cell fate decisions. For example, increased fibroblast growth factor 2 (FGF2) in the aged satellite cell niche of mouse muscle impairs self-renewal. Markers of inflammation also increase in the aging niche, for example in hair follicle stem cells, and impair stem cell function.

Taken together, stem cells require support cells that constitute the niche to maintain proper function. Thus, aging of the stem cell niche can also critically modulate stem cell function.

Influence of Various Circulating Factors

The concentrations of various circulating factors exerts important influences on stem cell aging. Many of these factors have been identified by rejuvenating effects of blood or plasma derived from either young or calorically restricted animals. Among such factors are insulin and IGF-1, which have been already discussed. Reduced signaling from these molecules is believed to mediate much of the longevity-extending effects of CR in mice. An opposite example is the TGF- β molecule, the levels of which increase during aging in mouse and human sera, which impairs the function of satellite muscle cells and NSCs. By contrast, growth differentiation factor 11 (GDF11) has been suggested to improve the function of satellite cells and NSCs, and its levels appear to decrease during aging. The validity of the effects of GDF11 on satellite cells, however, has been questioned by other studies, although it is worth noting that the dose of GDF11 and the skeletal muscle injury models used in the various studies differed. Whether GDF11 actually declines with age has also been questioned, based in part on the argument that GDF11 detection methods cross-react with myostatin (ibid.), although a recent study using additional methods and controls also reports that GDF11 declines with age in mice. Finally, the latest reports infer that high levels of GDF11 cause reductions in body and heart weight in both young and old animals, suggestive of a cachexia effect with the conclusion that elevating blood levels of GDF11 in the aged might cause more harm than good.

An important debate regarding the decline in stem-cell function is the relative role of cell-intrinsic pathways compared to cell-extrinsic ones. Recent work has provided strong support for the latter. In particular, CR increases intestinal and muscle stem functions through cell-extrinsic mechanisms. Similarly, when muscle-derived stem cells from young mice are transplanted to progeroid mice, this extends their lifespan and improves degenerative changes even in tissues where donor cells

are not detected, suggesting that their therapeutic benefit may derive from systemic effects caused by secreted factors. Furthermore, parabiosis experiments have demonstrated that the decline in neural and muscle stem cell function in old mice can be reversed by systemic factors from young mice.

There is also an ancient system in each cell that relates to the homeostasis of intracellular calcium (Ca^{2+}), which in normal cell sustains a 20,000 fold concentration gradient to the exterior of the cell, resulting in the extracellular Ca^{2+} acting as cellular regulator when it enters the cell via the Ca^{2+} channels. This gradient is sustained by specific pumping and transporting mechanisms consisting of protein molecules. Anomalies of these proteins results in an increase of intracellular calcium which can cause various diseases. With age, the hampered calcium homeostasis can lead to different muscle, immune and neural related defects.

Stem Cell Exhaustion

Although stem cells are regarded as immortal, as they are not subject to replicative senescence, they are susceptible to damage accumulation over time. Besides many other changes, a decline in their relative numbers and changes in subpopulations were observed. The group of dormant and active stem cells, existing in the niches of an organism that can be considered a pool of regenerative reserve, plays an important role in prevention of disease, in regeneration and aging. For instance, a decline in CD34+ circulating progenitor cells was reported with advancing age. When 100 octogenarians were observed for 7 and 10 years it was demonstrated that the number of their circulating CD34+ cells better predicted their lifespan and cardiovascular (CV) issues related mortality then the classic cardiovascular risk factors (hypertension, smoking, hypercholesterolemia), levels of inflammatory markers, or levels of cholesterol, or some other traditional cardiovascular indexes such as FRS and CVFRs. The chances of reaching an older age depended on higher numbers of CD34+ cells at baseline, thus the number of CD34+ cells could be considered as a biomarker of longevity in the elderly over 80 years.

On the other hand, there are reports that in certain tissues the numbers of adult stem cells even increase with age, however the number of their parent clones decreases, meaning that fewer pluripotent stem cells give rise to more frequent progeny, in order to compensate for the decrease of numbers. Ruzankina and Brown suggest that mammals in fact do have a finite number of stem cell replications per life and that aging of the hematopoietic system, which is due to a finite doubling capacity of stem cells, degrades its regenerative potential as well as the potential for preventing cancer.

Verovskaya used cellular barcoding combined with multiplex high-throughput sequencing to demonstrate clonal behavior of young HSCs transplanted to older organisms. In their study, the majority of transplanted clones steadily contributed to hematopoiesis in the long-term, although the clonal output in granulocytes, T cells, and B cells was substantially different. The final pool of old HSCs was composed of multiple small clones, whereas the young HSC pool was dominated by fewer, but larger, clones.

Holstege et al. have showed that the contents of a stem cell compartment actually deplete with old age. In the nonrepetitive genome of a 115-year-old centenarian woman they found approximately 450 somatic mutations that accumulated in the last years of her life, and the distribution of these

mutations suggested that the majority of her peripheral white blood cells were offspring of only two HSC clones that were still active in her old age. The telomeres of her white blood cells were significantly shorter than the telomeres from other tissues, suggesting that the HSCs have a finite lifespan, which is the cause of hematopoietic clonal evolution at extreme ages.

Several recent studies have confirmed that clonal hematopoiesis is almost a "normal" part of aging, with recent reports showing 0.8%, 11% and 19.5% of normal individuals aged <60, >80 and >90 years, respectively, having demonstrable clonal hematopoiesis – so called age-related clonal hematopoiesis. Clonal hematopoiesis (CH) arises when a substantial proportion of mature blood cells is derived from a single dominant hematopoietic stem cell lineage. It was recently shown, in the study on 11,262 elderly Icelanders which used whole-genome sequencing, that somatic mutations in candidate driver genes are thought to be responsible for at least some cases of CH.

At the same time there is ample evidence that there exist many dormant HSCs, and even some other and more "primitive" types of stem cells, such as for instance the VSEL stem cells with "primitive" embryonic characteristics, which co-inhabit the BM. These VSEL cells exhibit some characteristics of long-term repopulating HSCs (LT-HSCs), they may differentiate into organ-specific cells (e.g., cardiomyocytes), and probably have a role in aging since the number of these cells positively correlates with longevity in several murine models. Along with others, we have found similar cells in the reproductive organs.

It is now becoming obvious that maintaining robust stem cell pools seems to extend not only lifespan but also healthspan.

Cellular Senescence – A Stable Arrest of the Cell Cycle

Cellular senescence can be defined as a stable arrest of the cell cycle coupled to typical phenotypic changes. This phenomenon was originally described by Hayflick in human fibroblasts serially passaged in culture. The senescence that was observed by Hayflick was caused by telomere shortening and some other aging-associated stimuli that trigger senescence independently of the telomeric process. It is for instance well known that the non-telomeric DNA damage and de-repression of the INK4/ARF locus, both of which progressively occur with chronological aging, are also capable of inducing senescence.

The accumulation of senescent cells with age is a simple mathematical result of the increase in the rate of generation of senescent cells and/or a decrease in their rate of clearance. In normal physiology this has detrimental consequences, but in some circumstances it also has useful effects. For instance, there is good evidence that the senescent tumor cells are subjected to strict immune surveillance and are efficiently removed by phagocytosis.

Among other functions, the senescent cells manifest dramatic alterations in their secretome, which is particularly enriched in pro-inflammatory cytokines and matrix metalloproteinases, which is referred to as the "senescence-associated secretory phenotype". This pro-inflammatory secretome may contribute to aging.

Studies on aged mice have revealed an overall decrease in HSC cell cycle activity, with old HSCs undergoing fewer cell divisions than young HSCs. This correlates with the accumulation of DNA

damage and with the overexpression of cell cycle-inhibitory proteins such as p16INK4a. In fact, old p16INK4a−/− HSCs exhibit better engraftment capacity and increased cell cycle activity compared with old wild-type HSCs (ibid.). Telomere shortening is also an important cause of stem cell decline with aging in multiple tissues.

The accumulation of senescent cells in aged tissues has been often inferred using surrogate markers such as DNA damage. Some studies have directly used senescence-associated β-galactosidase (SABG) to identify senescence in tissues. Of note, a detailed and parallel quantification of SABG and DNA damage in liver produced comparable quantitative data, yielding a total of ~8% senescent cells in young mice and ~17% in very old mice. Similar results were obtained in the skin, lung and spleen, but no changes were observed in heart, skeletal muscle and kidney. Based on these data, it is clear that cellular senescence is not a generalized property of all tissues in aged organisms.

Some authors think that the amount of senescent cells increases with age and that senescence contributes to aging, but this probably undervalues the primary purpose of senescence, which is to prevent the propagation of damaged cells and to trigger their removal by the immune system. They explain that senescence is a beneficial compensatory response that contributes to clearing tissues of damaged and potentially oncogenic cells. This however requires an efficient cell replacement system that involves clearance of senescent cells and mobilization of stem cells and their progenitors to re-establish cell numbers. In aged organisms, this turnover system may become exhausted, resulting in the accumulation of senescent cells that aggravate the damage and contribute to aging.

Deficient proliferation of stem and progenitor cells is obviously detrimental for the long-term maintenance of the organism, but excessive proliferation of stem and progenitor cells can also be deleterious by accelerating the exhaustion of stem cell niches, which can be compensated by stem cell quiescence over the long-term. This has been demonstrated in Drosophila ISCs, where excessive proliferation leads to exhaustion and premature aging and in p21-null mice, which present premature exhaustion of HSCs and NSCs.

Recent studies have shown that an increase in FGF2 signaling in the aged muscle stem cell niche results in the loss of quiescence, stem cell depletion and diminished regenerative capacity, whereas the suppression of this signaling pathway reverses these defects. This opens up the possibility of designing strategies aimed at inhibiting FGF2 signaling to reduce stem cell exhaustion during aging.

As a mechanism to protect themselves from acquiring damage, many stem cells are resting for a long time in a quiescent state. During this time they are protected from replicative damage, but they are more susceptible to mutations. However, although proliferating stem cells are more likely to encounter DNA damages, they repair that damages more accurately than do quiescent stem cells.

In addition to DNA damage, excessive mitogenic signaling is the other stress most robustly associated with senescence. A recent account listed more than 50 oncogenic or mitogenic alterations that are able to induce senescence. The number of mechanisms that implement senescence in response to this variety of oncogenic insults has also grown, but still, the originally reported p16INK4a/Rb and p19ARF/p53 pathways remain, in general, the most important ones. The relevance of these pathways for aging becomes even more striking when considering that the levels of p16INK4a (and to a lesser extent also p19ARFARF) correlate with the chronological age of essentially all tissues

analyzed, both in mice and humans. INK4a/ARF locus was actually determined as being genetically linked to the highest number of age-associated pathologies, including several types of cardiovascular diseases, diabetes, glaucoma, and Alzheimer's disease. Although the activation of p53 and INK4a/ARF is a beneficial compensatory response that prevents the propagation of damaged cells, under the stress conditions the p53 and INK4a/ARF responses can become deleterious and even accelerate aging.

Taken together, cellular senescence is a beneficial compensatory response to damage, but it becomes deleterious and accelerates aging when tissues exhaust their regenerative capacity. A moderate enhancement of the senescence-inducing tumor suppressor pathways may extend longevity, whereas at the same time, elimination of senescent cells in an experimental progeria model delays age-related pathologies. Therefore, two interventions that are conceptually opposite are able to extend healthspan.

Altered Intercellular Communication

Beyond intrinsic cellular alterations, aging also involves changes at the level of intercellular endocrine, neuroendocrine or neuronal communication. As during the aging inflammatory reactions increase, immunosurveillance against pathogens and premalignant cells declines, and the composition of the peri- and extracellular environment changes, neurohormonal signaling (i.e., renin-angiotensin, adrenergic, insulin/IGF-1 signaling) is consequently deregulated, which affects various mechanical and functional properties of all tissues.

An important age-associated pathological finding in the intercellular communication in mammals is so called "inflammaging," i.e., an appearance of pro-inflammatory phenotype that accompanies aging. Several authors proposed that aging is accompanied by a chronic up-regulation of several pro-inflammatory responses. Inflammaging may result from multiple causes such as the accumulation of pro-inflammatory substances, tissue damage, the failure of the aged immune system to effectively clear pathogens and remove dysfunctional host cells, the secretion of pro-inflammatory cytokines by aged immune cells, the enhanced activation of the NF-κB transcription factor, or from a defective autophagy response. These defects and alterations result in an enhanced activation of the NLRP3 inflammasome and other pro-inflammatory pathways, finally leading to increased production of interleukin 1ß (IL-1ß), tumor necrosis factor and interferons. Inflammation is also involved in the pathogenesis of obesity and type 2 diabetes, two conditions that contribute to, and correlate with aging in the human population. Likewise, defective inflammatory responses play a critical role in atherosclerosis.

Another link between inflammation and aging derives from the finding that inflammatory and stress responses activate NF-κB in the hypothalamus and induce a signaling pathway that results in reduced production of gonadotropin-releasing hormone (GnRH) by neurons. This GnRH decline can contribute to numerous aging-related changes such as bone fragility, muscle weakness, skin atrophy, and reduced neurogenesis. These findings suggest that the hypothalamus may modulate systemic aging by integrating NF-kB-driven inflammatory responses with GnRH-mediated neuroendocrine effects.

Besides chronic inflammation, aged immune cells are prone to a multitude of deteriorating factors. Age related defects of innate immunity are observed not only in the macrophage/monocyte

compartment, which is probably the main "culprit" of inflammaging, but also in other cells, i.e., NK cells, dendritic cells, and granulocytes, whereas the defects of adaptive immunity are observed in both the B-cell and the T-cell compartments. Aging of the immune system or "immunosenescence" is characterized by a time-dependent functional alteration of immunity leading to immunodeficiency that manifests in chronic inflammation, reduced resistance to infections, poor responses to vaccination, and increased incidence of autoimmunity and cancers. Similarly, the involvement of immune processes in clinical conditions, such as atherosclerosis, diabetes, and dementia, have been described. The impairment of the immune system exerts an influence on the increased morbidity and mortality observed in human subjects as they age.

There is also accumulating evidence indicating that aging-related changes in one tissue can lead to aging-specific deterioration of other tissues. Typical case are the inflammatory cytokines that can cause so called "contagious aging". In certain bystander effects senescent cells induce senescence in neighboring cells via gap junction-mediated cell-to-cell contacts and processes involving ROS. The microenvironment contributes to the age-related functional defects of CD4 T cells, as assessed by using an adoptive transfer model in mice. Likewise, impaired kidney function can increase the risk of heart disease in humans. Conversely, lifespan-extending manipulations targeting one single tissue can delay the aging process in other tissues.

Defective intercellular communication underlying aging processes, including genetic, can be restored by nutritional or pharmacological interventions that may improve the cell–cell communication properties lost with aging. Of special interest in this regard are the CR approaches to extend healthy lifespan and the rejuvenation strategies based on the use of blood-borne systemic factors identified in parabiosis experiments. Moreover, the long-term administration of anti-inflammatory agents, such as aspirin, may increase longevity in mice and healthy aging in humans. Finally, it also appears possible to extend lifespan by manipulating the composition and functionality of the intestinal bacterial ecosystem of the human body. The near future research will undoubtedly bring spectacular results in this field of human physiology that will also be translated to the clinical medicine.

Stem Cell Therapy

6

- Stem Cell Therapy in Brain Trauma
- Cancer Stem Cell
- Tissue Engineering for Skin Replacement Methods
- Peripheral Stem Cell Transplantation
- Skin Tissue Regeneration for Burn Injury
- Stem Cell Transplantation for Articular Cartilage Repair
- Hematopoietic Stem Cell Transplantation
- Clinical uses of Mesenchymal Stem Cell

The utilization of stem cells for the treatment and prevention of the diseases is known as stem cell therapy. The techniques used by stem cells are peripheral stem cell transplantation, skin tissue regeneration, stem cell transplantation, hematopoietic stem cell transplantation, etc. All the diverse applications of stem cell therapy have been carefully analyzed in this chapter.

Pluripotent Stem Cells

Pluripotent stem cells have not yet been used therapeutically in humans because many of the early animal studies resulted in the undesirable formation of unusual solid tumors, called teratomas. Teratomas are made of a mix of cell types from all the early germ layers. Later successful animal studies used pluripotent cells modified to a more mature phenotype which limits this proliferative capacity. Cells derived from pluripotent cells have been used to successfully treat animals. For

example, animals with diabetes have been treated by the creation of insulin-producing cells responsive to glucose levels. Also, animals with acute spinal cord injury or visual impairment have been treated by creation of new myelinated neurons or retinal epithelial cells, respectively. Commercial companies are currently in negotiations with the FDA regarding the possibility of advancing to human trials. Other animal studies have been conducted to treat several maladies such as Parkinson's disease, muscular dystrophy and heart failure.

Scientists hope that stem cell therapy can improve cardiac function by integration of newly formed beating cardiac myocytes into the myocardium to produce greater force. Patches of cardiac myocytes derived from human embryonic stem cells can form viable human myocardium after transplantation into animals, with some showing evidence of electrical integration.Damaged rodent hearts showed slightly improved cardiac function after injection of cardiac myocytes derived from human embryonic stem cells. The mechanisms for the gain in function are not fully understood but it may be only partially due to direct integration of new beating heart cells. It is more likely due to paracrine effects that benefit other existing heart cells.

Multipotent Stem Cells

Multipotent stem cells harvested from bone marrow have been used since the 1960's to treat leukemia, myeloma and lymphoma. Since cells there give rise to lymphocytes, megakaryocytes and erythrocytes, the value of these cells is easily understood in treating blood cancers. Recently, some progress has been reported in the use of cells derived from bone marrow to treat other diseases. For example, the ability to form whole joints in mouse models has been achieved starting with mesenchymal stem cells that give rise to bone and cartilage. In the near future multipotent stem cells are likely to benefit many other diseases and clinical conditions. Bone marrow-derived stem cells are in clinical trials to remedy heart ailments.

Pluripotent vs. Multipotent

Pluripotent and multipotent stem cells have their respective advantages and disadvantages. The capacity of pluripotent cells to become any cell type is an obvious therapeutic advantage over their multipotent kin. Theoretically, they could be used to treat diseased or aging tissues in which multipotent stem cells are insufficient. Also, pluripotent stem cells proliferate more rapidly so can yield higher numbers of useful cells. However, use of donor pluripotent stem cells would require immune suppressive drugs for the duration of the graft while use of autologous multipotent stem cells (stem cells from ones' self) would not. This ability to use one's own cells is a great advantage of multipotent stem cells. The immune system recognizes specific surface proteins on cells/objects that tell them whether the cell is from the host and is healthy. Autologous, multipotent stem cells have the patient's specific surface proteins that allow it to be accepted by the host's immune system and avoid an immunological reaction. Pluripotent stem cells, on the other hand, are not from the host and therefore, lack the proper signals required to stave off rejection from the immune system. Research is ongoing trying to limit the immune response caused by pluripotent cells and is one possible advantage that iPS cells may have.

The promises of cures for human ailments by stem cells have been much touted but many obstacles must still be overcome. First, more human pluripotent and multipotent cell research is needed since stem cell biology differs in mice and men. Second, the common feature of unlimited

cell division shared by cancer cells and pluripotent stem cells must be better understood in order to avoid cancer formation. Third, the ability to acquire large numbers of the right cells at the right stage of differentiation must be mastered. Fourth, specific protocols must be developed to enhance production, survival and integration of transplanted cells. Finally, clinical trials must be completed to assure safety and efficacy of the stem cell therapy. When it comes to stem cells, knowing they exist is a long way from using them therapeutically.

Stem Cell Therapy in Brain Trauma

Traumatic brain injury (TBI) is a major health problem worldwide. Currently, there is no effective treatment to improve neural structural repair and functional recovery of patients in clinic. Recent studies suggest that adult neural stem/progenitor cells residing in the neurogenic regions in the adult mammalian brain may play regenerative and reparative roles in response to CNS injuries or diseases. Alternatively, cell transplantation is a potential strategy to repair and regenerate the injured brain.

Approximately 350,000 individuals in the United States are affected annually by severe and moderate TBI that may result in long-term disability. This rate of injury has produced more than 3 million disabled citizens in the United States alone. Despite generally improving rates of survival after TBI, approximately 80,000 individuals in the United States annually sustain TBIs that result in significant long-term disability. These impairments involve both memory and behavior and can result in a total vegetative state. Most of these 3 million survivors depend upon others for daily care. Many clinical and animal model studies have now shown that severe and even moderate TBI is characterized by both neuronal and white matter loss with resultant brain atrophy and functional neurological impairment. Injury may be in the form of focal damage because it typically occurs after acute subdural hematoma, or it may be diffuse with widespread delayed neuronal loss as it typically occurs after diffuse axonal injury. To date, there is no effective treatment for TBI. Current therapies are primarily focused on reducing the extent of secondary insult rather than repairing the damage from the primary injury. After TBI, the hippocampus is particularly vulnerable to the secondary insults. Hippocampal injury associated to learning and memory deficits are the hallmarks of brain trauma and are the most enduring and debilitating of TBI deficits because they prevent reintegration of patients into a normal lifestyle by impairing employment and social interactions. Spontaneous cognitive improvement is not uncommon but is greatly limited and not normally seen past the second year postinjury. This natural recovery, however, does suggest that innate mechanisms for repair and regeneration are present within the brain.

Recent findings reveal that multipotent neural stem cells/progenitor cells (NSCs/NPCs) persist in selected regions of the brain throughout the life span of an animal, rendering the brain capable of generating new neurons and glia. Furthermore, increasing evidence indicates that these endogenous NSCs/NPCs may play regenerative and reparative roles in response to central nervous system (CNS) injuries or diseases. In support of this notion, heightened levels of cell proliferation and neurogenesis have been observed in response to brain trauma or insults suggesting that the brain has the inherent potential to restore populations of damaged or destroyed neurons. This raises the

possibility of developing therapeutic strategies aiming at harnessing this neurogenic capacity to repopulate and repair the damaged brain. Recent experimental successes in cell replacement in models of Parkinson disease and other neurodegenerative diseases have inspired TBI researchers to investigate this approach for treating the injured brain. The therapeutic prospects of cell transplantation are based on the potential for transplanted cells to differentiate into region-specific cells and integrate into the host tissue to replace lost cells in the injured brain; alternatively, transplanted cells could provide neurotransmitters or trophic support to the host tissue to facilitate survival or regeneration.

These two approaches, through modulating endogenous NSCs or using exogenous stem cells, are gaining increasing attention in the field of neural regeneration.

Potential of Endogenous Nscs for Brain Repair

Extent of Endogenous Neurogenesis in the Normal Brain

The mature mammalian brain is traditionally considered an organ without regenerative capacity. Recently, this statement was revised after the discovery of multipotent NSCs that are capable of generating neurons and glial cells residing in the mature mammalian brain. The region of neurogenesis in the mature brain is primarily confined to the subventricular zone (SVZ) surrounding the lateral ventricle and the dentate gyrus (DG) of the hippocampus. The majority of the SVZ progeny are neuroblasts that undergo chain migration along the rostral migratory stream to the olfactory bulb, where they differentiate into olfactory interneurons. Another subpopulation of these cells migrate into cortical regions for reasons yet to be identified, but evidence suggests they may be involved in repair or cell renewal mechanisms. Likewise, the newly generated cells of the DG migrate laterally into the granule cell layer and exhibit properties of fully integrated mature dentate granule neurons. Most importantly, the newly generated DG granule neurons form synapses and extend axons into their correct target area, the CA3 region.

Multiple studies have quantified the degree of cytogenesis occurring in these regions and have clearly shown that large numbers of new cells are regularly produced. Specifically, the rat dentate gyrus produces ~9,000 new cells per day, which equates to ~270,000 cells per month. Considering that the total granule cell population in the rat is 1–2 million cells, this degree of new cell addition is certainly large enough to affect network function. A more recent study has found that in the olfactory bulb almost the entire granule cell population in the deep layer and half of the super layer was replaced by new neurons over a 12-month period. The same study also reported that in the hippocampus, the adult-generated neurons comprised about 10% of the total number of dentate granule cells and they were equally present along the anteroposterior axis of the DG. However, studies have also found that in normal adult rodent brains, many newly generated neurons in the DG and nonolfactory-bound SVZ cells have a transient existence of 2 weeks or less. Although this interval is long enough for supportive glial roles, neuron formation and integration into an existing network takes approximately 10–14 days. It must be noted, however, that a small population of these cells are sustained for months to years, strongly supporting the theory of network integration. Furthermore, this dramatic loss of newly generated cells might be a recapitulation of network pruning seen in early mammalian development. Whether the limited life span represents network pruning or merely distinct cell-specific roles is yet to be understood.

Functions of Adult-generated Neurons

In the normal hippocampus, the newly generated granular cells in the adult DG can become functional neurons by displaying passive membrane properties, generating action potentials and functional synaptic inputs as seen in mature DG neurons. Increasing evidence has also shown that adult hippocampal neurogenesis is involved in learning and memory function. For example, mouse strains with genetically low levels of neurogenesis perform poorly on learning tasks when compared with those with higher level of baseline neurogenesis. Conversely, physical activity stimulates a robust increase in the generation of new neurons and subsequently enhances spatial learning and long-term potentiation. Additionally, diminished hippocampal neurogenesis, as observed after the administration of antimitotic drugs such as methylazoxymethanol acetate, cytosine-β-D-arabinofuranoside, by irradiation or by genetic manipulation, was associated with worse performance on hippocampus-dependent trace eye blink conditioning, contextual fear conditioning, and long-term spatial memory function tests. Collectively, these studies provide compelling evidence that adult born neurons in the hippocampus play a critical role in many important hippocampal-dependent functions in normal adult brain. Compared with the evident role of hippocampal neurogenesis in hippocampal-dependent functions, the function of SVZ-olfactory neurogenesis is less certain. Thus far, limited studies have found that adult-generated neurons in the olfactory bulb have a critical role in olfactory tissue maintenance and are involved in olfactory discrimination and olfactory perceptual learning functions.

The proliferation and maturational fate of cells within the SVZ and DG is modulated by a number of physical and chemical cues. For example, biochemical factors such as serotonin, glucocorticoids, ovarian steroids, and growth factors tightly regulate the proliferative response, suggesting that cell proliferation within these regions have physiologic importance. In addition, certain physical stimuli produce alterations in cell production suggesting a role in network adaptation. For example, environments that are cognitively and physically enriched increase cell proliferation and neurogenesis in both the SVZ and DG, whereas stress reduces this type of cellular response. Nevertheless, a functional role for these new cells is dependent upon a significant number of cells being generated, and their survival, differentiation, and ultimate integration into existing neuronal circuitry.

Neurogenesis in the Human Brain

Compared with rodent brains, the degree of adult neurogenesis in human brain is less clear. The most well-characterized neurogenic region in the adult human brain is the SVZ lining the lateral ventricle, where a ribbon of SVZ astrocytes have been identified that proliferate in vivo and behave as multipotent progenitor cells in vitro. In rodents and primates, neurons born in the SVZ migrate in chains through the rostral migratory stream to replace interneurons of the olfactory bulb currently. In contrast, there is no evidence for chains of migrating neuroblasts in the human SVZ. It has been estimated that in normal humans less than 1% of astrocytes within the SVZ ribbon are undergoing cell division and although these endogenous NSCs can be expanded in culture, their response to injury in patients has not been studied. In another neurogenic region in humans, the hippocampal DG neurogenesis in vivo was demonstrated on histological sections obtained in patients who had died of cancer but for which BrdU staining was used for diagnostic purposes. A recent study has found that the generation and migration of new neurons is very much limited to

the early childhood. Less well-characterized in the human brain are proliferating NPCs in the hippocampus, white matter, and other regions, where cells isolated from the adult human brain are capable of generating both neurons and glia under culture conditions.

Response of Endogenous NSCs to Brain Injury and the Role of these Cells for Brain Repair

The regenerative capacity of the SVZ and DG is of particular interest with regard to TBI. Because adult-generated neurons from both regions have functional roles, harnessing this endogenous population of stem cells to repopulate the damaged brain is an attractive strategy to repair and regenerate the injured brain. In the injured brain, including diffuse and focal injury models. the juvenile brain has more robust neurogenic response after injury than the adult and aged brain. Such increased levels of cell proliferation with increased generation of new neurons likely contribute to the better functional recovery in juvenile animals after TBI. Furthermore, injury-induced newly generated granular cells integrate into the existing hippocampal circuitry, and this endogenous neurogenesis is associated to the innate cognitive recovery after injury. In human brain specimens, a recent study has found an increased number of cells expressing NSCs/NPCs markers in the perilesion cortex in the injured brain. These studies strongly indicated the inherent attempts of the brain to repair and regenerate after injury through endogenous NSCs.

The degree of endogenous neurogenesis can be enhanced via exogenous means and augmentation of endogenous neural stem cells could be a potential therapy for treating the injured brain. So far, many factors have been shown to enhance neurogenesis particularly in the hippocampus. Studies have found that various types of growth factors and drugs can enhance neurogenesis and improve functional recovery of the injured brain after trauma. For example, intraventricular administration of growth factors basic fibroblast growth factor or epidermal growth factor can significantly enhance TBI-induced cell proliferation in the hippocampus and the SVZ, and drastically improve cognitive functional recovery of the injured adult animals. Other studies have found that infusion of S100β or vascular endothelial growth factor can also enhance neurogenesis in the hippocampus and improve the functional recovery of animals after TBI. Several drugs that are currently used in clinical trials for treating TBI or other conditions have shown effects in enhancing neurogenesis and cognitive function in TBI animals including statins, erythropoietin, progesterone, and the antidepressant imipramine. Other strategies that have beneficial effects for TBI such as hypothermia and environment enrichment are also shown enhanced hippocampal neurogenesis in injured animals. Collectively, these studies suggest the therapeutic potential of augmenting the endogenous repair response for treating TBI.

Stem Cells as Cell Source for Neural Transplantation for Brain Repair and Regeneration

Because of the limited capacity of the injured brain to repair and replace the damaged neurons, neural transplantation is a prospective therapy for TBI as transplanted cells may differentiate into region-specific cells and integrate into the host tissue to replace the lost cells in the injured brain. Additionally, transplanted cells could provide trophic support to the host tissue to facilitate regeneration. Over the past few decades, researchers have explored a wide array of cell sources for neural transplantation. These cells include embryonic stem cells isolated to fetal or embryonic

tissue, mesenchymal stromal cells such as bone marrow stromal cells and umbilical cord cells, adult NSCs, and more recently, induced pluripotent stem cells (iPSCs).

Embryonic Stem Cells

Embryonic stem (ES) cells are pluripotent stem cells that have unlimited capacity of self-renewal and can give rise to cells of all three primary germ layers. Because of their high plasticity, ES cells are the ideal cell source for neural transplantation. When transplanted into normal or damaged CNS, human ES cells can differentiate, migrate, and make innervations. Thus, ES cells derived from human or mice fetal brains have been tested as a transplantation cell source for TBI treatment in animal studies in different TBI models with different results reported.

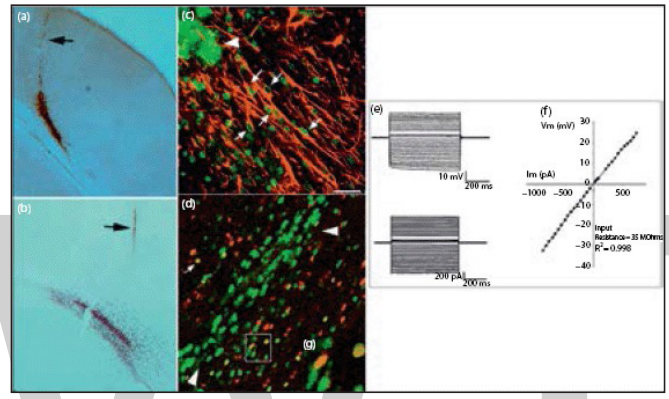
NSCs from human ES cells isolated from fetal brain were capable of surviving for an extended period of 6 weeks, migrating to the contralateral cortex, and differentiating into neurons and astrocytes when transplanted into the injured brain after a cortical contusion injury. Gao et al. have reported that NSCs from human ES cells survived and differentiated to neurons after transplantation into the injured brain when examined at 2 weeks after cell injection, and the injured animals with cell transplantation had improved cognitive functional recovery. In a more recent study, Skardelly et al. transplanted predifferentiated human fetal ES cells into injured rat brain after a severe controlled cortical injury. They observed a transient increase in angiogenesis and reduced astrogliosis together with improved long-term motor functional improvement, brain injury lesion volume reduction, and increased neuronal survival in the border zone of the lesion. Shear et al. assessed the long-term survival, migration, differentiation, and functional significance of NSCs derived from mice fetal brain after transplantation into the injured brain up to 1 year posttransplantation. They found that the injured animals receiving transplants showed significant improvement in motor and spatial learning functions, and the transplanted cells migrated widely in the injured brain, with the majority of transplanted cells expressing NG2, an oligodendrocyte progenitor cell marker, but not neuronal markers. Post-TBI neural transplantation of immortalized fetal ES-derived NSCs (C17.2 cells) has also shown improved motor function with the transplanted cells surviving for up to 13 weeks and differentiating into mature neurons and glial cells. In vitro–modified ES cells either predifferentiated into mature neurons expressing neurotransmitters or with overexpression of growth factors such as glial cell line–derived and brain-derived neurotrophic factor showed beneficial effects when transplanted into the injured animals by promoting motor and cognitive improvement of the injured animals concomitant with better graft survival and neuronal differentiation.

Taken together, these data suggest that post-TBI transplantation using ES-derived cells can restore motor and cognitive functions of the injured animals. However, the beneficial effect of the transplanted cells may be associated with the neural trophic effect of the transplanted cells rather than direct neural replacement as long-term survival and neuronal differentiation is rather limited. Further studies are needed to improve survival and functional neural replacement by modulating the injured host environment. Caution must be exercised when working with multipotent ES cells as undifferentiated ES cells have a potential risk of tumor formation.

Adult NSCs

Recent findings show that the mature mammalian CNS harbors multipotent stem cells capable of differentiation into a variety of specialized cells throughout life. In the adult mammalian CNS, the

NSCs/NPCs are primarily confined to the SVZ surrounding the lateral ventricle and the DG of the hippocampus. Aside from these major neurogenic regions, adult neurogenesis in rodents has also been reported in other regions in the CNS including the striatum, the substantia nigra, the cortex, and the spinal cord. These adult-derived NSCs express low levels of the major histocompatibility complex antigens, display high survival rates, and become region-specific cells when transplanted into normal adult rat brains. When transplanted into the injured brain in a rat experimental TBI model, we found that the adult derived NSCs can survive for an extended period in the injured brain. Many cells migrated out of the injection site into the surrounding areas expressing markers for mature astrocytes or oligodendrocytes. Electrophysiological studies showed that the transplanted cells possessed typical mature glial cell properties demonstrating that adult-derived NSCs became region-specific functional cells.



Survival and functional differentiation of transplanted adult neural stem and progenitor cells after TBI. Cultured adult rat NSCs/NPCs were labeled with BrdU in vitro 3 days before being used for transplantation and subsequently identified with BrdU immunostaining. (a) Coronal section from an animal sacrificed 2 weeks after transplantation. Grafted cells were mostly located at the cortex–white matter interface. (b) Coronal sections taken from an animal sacrificed at 4 weeks after transplantation. Compared to 2 weeks, at 4 weeks many BrdU-labeled cells migrated out of the injection site to the surrounding areas. Arrow indicates the injection needle tract. (c) Confocal micrograph showing that many BrdU-labeled transplanted cells (green) migrated away from the injection center (arrowhead) along white matter tracts and differentiated into spindle-shaped glial fibrillary acidic protein–labeled astrocytes (red, arrows). Bar scale = 50 μm. (d) Arrows indicate many BrdU+ transplanted cells (green) away from the injection center (arrowhead) were colabeled with mature oligodendrocyte marker Olig2 (red). Merged image shows colocalization of BrdU and Olig2 (f). (g) Enlarged image of the boxed area in (f) showing colabeling of BrdU and Olig2. (e) Electrophysiological properties of transplanted cells showing membrane potential response (top) to current injection (bottom). (f) The amplitude of the membrane potential responses is plotted against the current injected. The input resistance was low and the current-voltage relationship was linear, consistent with mature glial cell but not neuronal electrophysiological properties.

In humans, multipotent stem/progenitors cells have been identified and successfully isolated from various regions of adult human brain including the hippocampus, SVZ, neocortex, and subcortical white matters from neurosurgical resection tissues. These raise the possibility of using such cells as an autologous cell source for transplantation therapy. Indeed, Brunet and colleagues have demonstrated that adult monkey NSCs/NPCs derived from cortical biopsy survived for at least

3 months and displayed a neuronal phenotype after reimplantation into the normal or ibotenic acid excitotoxic-lesioned motor cortex of the donor brains. These cells may also restore the anatomy and function of the injured CNS as shown in a study after grafting adult human NSCs/NPCs into the demyelinated rat spinal cord.

To date, very few studies have attempted to examine the behavior of adult-derived human NSCs/NPCs in the injured mature CNS. Olstorn and colleagues recently reported that a small portion (4 ± 1%) of adult human NSCs/NPCs can survive for 16 weeks after transplantation into the posterior periventricular region in normal adult rats or rats with hippocampal CA1 ischemic injury. Although the results of this study are promising, questions remain whether these cells become anatomically and functionally integrated into the injured brain and whether the proportions of surviving cells can be increased by transplanting NSCs/NPC's at a different developmental stage.

Bone Marrow Stromal Cells

Because of ethical and immunological concerns as well as the risk of tumorigenesis, the translational value of using ES cells for clinic application is limited. Autologous transplantation of NSCs isolated from neurosurgical removal of brain tissue from TBI patients is an attractive strategy; however, so far, the success of long-term cell survival and functional outcomes of these cells in the treatment of experimental TBI is rather limited. Because of these limitations, adult-derived mesenchymal cells, particularly bone marrow stromal cells, (BMSCs) have received much attention.

BMSCs are undifferentiated cells with mixed cell population including stem and progenitor cells. These cells can be easily isolated from the mononuclear fraction of patients' bone marrow and be expanded in culture without ethical and technical concerns. Another advantage of considering BMSCs for cell transplantation is the low antigenicity because of their low expression of the major histocompatibility complex antigens (class II). In addition, these cells produce high levels of growth factors, cytokines, and extracellular matrix molecules that could have potential neurotrophic or neuroprotective effect in the injured brain. As a matter of fact, all studies using BMSCs for neural transplantation have demonstrated that the beneficial effects of BMSCs are attributed to their neurotrophic or neuroprotective effect rather than direct cell replacement.

The potential of BMSCs for treating TBI have been extensively assessed in experimental TBI models. Cells were delivered either focally to the injured brain, or systemically through intravenous or intraarterial injections at the acute or subacute phase after TBI and significant reduction of neurological deficits including motor and cognitive deficits were reported. For example, intracranial injection of rat BMSCs into the brain region adjacent to the brain lesion site or intravenous injection of cells at 24 hours after a controlled cortical contusion injury in rats and it was found that the injured animals had improved sensory motor functional. When human BMSCs were combined with collagen scaffolds and transplanted into the injury cavity at 4 or 7 days after TBI, animals had significantly improved sensorimotor and spatial learning functions together with reduced brain lesion volume and enhanced focal brain angiogenesis. The effect of BMSCs in improving sensorimotor function of injured animals was reported even when delivered at 2 months after TBI. Further studies have demonstrated that the beneficial effort of BMSCs in the injured brain is due largely by their production of bioactive factors, which facilitates the endogenous plasticity and remodeling of the host brain thus promoting functional recovery. Although a low number of BMSCs can be found in the injured brain expressing neuronal or glial markers, no study has demonstrated that BMSCs

can become fully differentiated functional neurons in vivo. Taken together, extensive experimental studies have demonstrated the beneficial effects of BMSCs in the injured brain and highlighted the potential use of BMSCs

Other Potential Types of Stem Cells for Cell Replacement Therapy

Apart from the previously mentioned stem cells, researchers have recently explored several other types of stem or stem-like cells for TBI application. Published data have reported that the use of human amnion–derived multipotent progenitor cells can significantly attenuate axonal degeneration and improve neurological function and brain tissue morphology of the injured rats. Intravenous administration of human adipose–derived stem cells or the derived culture medium into a controlled cortical impact rat model significantly improved motor and cognitive functions and reduced focal tissue damage and hippocampal cell loss.

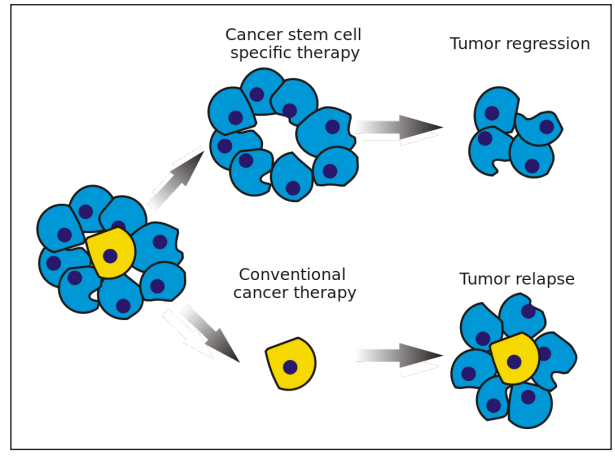
Human umbilical cord blood is an abundant source of multiple stem cells, including hematopoietic stem cells, mesenchymal stem cells, unrestricted somatic stem cells, and embryonic-like stem cells. These cells can be easily harvested without ethical controversy and could be an attractive source of stem cells for brain repair. These studies have shown that these cells can survive in injury sites and promote survival of local host neurons in ischemic and spinal cord injury animal models. In a recent study, Wang et al. have conducted a small-scale clinical trial using these cells for treating TBI patients. The authors reported that the patients treated with umbilical cord stem cells had improved neurological function and self-care compared with control group.

Recent development of somatic cell reprogramming that generates iPSCs provides prospects for novel neural replacement strategies. Human iPSCs possess the dual properties of unlimited self-renewal and the pluripotent potential to differentiate into multilineage cells without ethical concerns. More importantly, patient-specific iPSCs can serve as an autologous cell source for transplantation without encountering graft rejection. These unique properties of iPSCs have raised the widespread hope that many neurological diseases including TBI might be cured or treated. Thus far rapid progress has been made in the field of reprogramming; however, the optimal source of somatic cells used for applications in neurological disorders has not been identified yet.

Cancer Stem Cell

Cancer stem cells (CSCs) are cancer cells (found within tumors or hematological cancers) that possess characteristics associated with normal stem cells, specifically the ability to give rise to all cell types found in a particular cancer sample. CSCs are therefore tumorigenic (tumor-forming), perhaps in contrast to other non-tumorigenic cancer cells. CSCs may generate tumors through the stem cell processes of self-renewal and differentiation into multiple cell types. Such cells are hypothesized to persist in tumors as a distinct population and cause relapse and metastasis by giving rise to new tumors. Therefore, development of specific therapies targeted at CSCs holds hope for improvement of survival and quality of life of cancer patients, especially for patients with metastatic disease.

Existing cancer treatments have mostly been developed based on animal models, where therapies able to promote tumor shrinkage were deemed effective. However, animals do not provide a complete model of human disease. In particular, in mice, whose life spans do not exceed two years, tumor relapse is difficult to study.

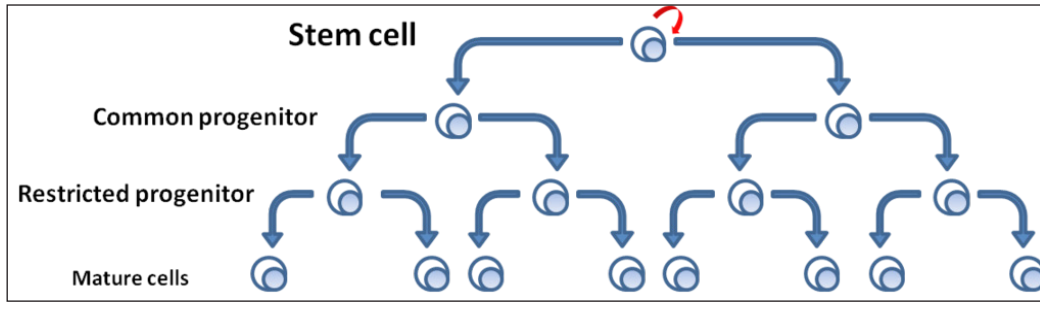


Stem cell specific and conventional cancer therapies.

The efficacy of cancer treatments is, in the initial stages of testing, often measured by the ablation fraction of tumor mass (fractional kill). As CSCs form a small proportion of the tumor, this may not necessarily select for drugs that act specifically on the stem cells. The theory suggests that conventional chemotherapies kill differentiated or differentiating cells, which form the bulk of the tumor but do not generate new cells. A population of CSCs, which gave rise to it, could remain untouched and cause relapse.

Cancer stem cells were first identified by John Dick in acute myeloid leukemia in the late 1990s. Since the early 2000s they have been an intense cancer research focus. The term itself was coined in a highly cited paper in 2001 by biologists Tannishtha Reya, Sean J. Morrison, Michael F. Clarke and Irving Weissman.

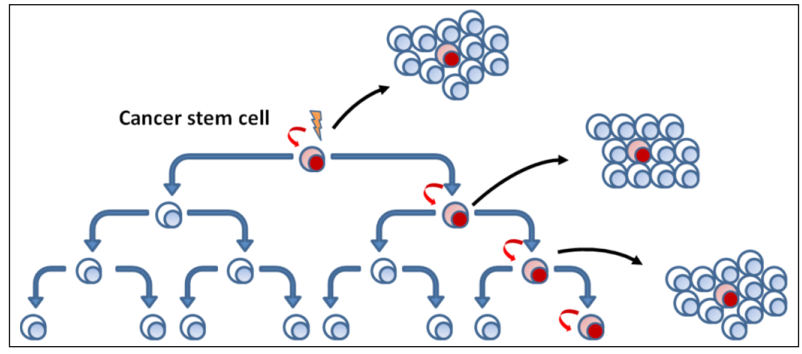
Tumor Propagation Models



A normal cellular hierarchy comprising stem cells at the apex, which generate common and more restricted progenitor cells and ultimately the mature cell types that constitute particular tissues.

In different tumor subtypes, cells within the tumor population exhibit functional heterogeneity and tumors are formed from cells with various proliferative and differentiation capacities. This functional heterogeneity among cancer cells has led to the creation of multiple propagation models

to account for heterogeneity and differences in tumor-regenerative capacity: the cancer stem cell (CSC) and stochastic model. However, certain perspectives maintain that this demarcation is artificial, since both processes act in complementary manners as far as actual tumor populations are concerned.



In the cancer stem cell (CSC) model, only the CSCs have the ability to generate a tumor, based on their self-renewal properties and proliferative potential.

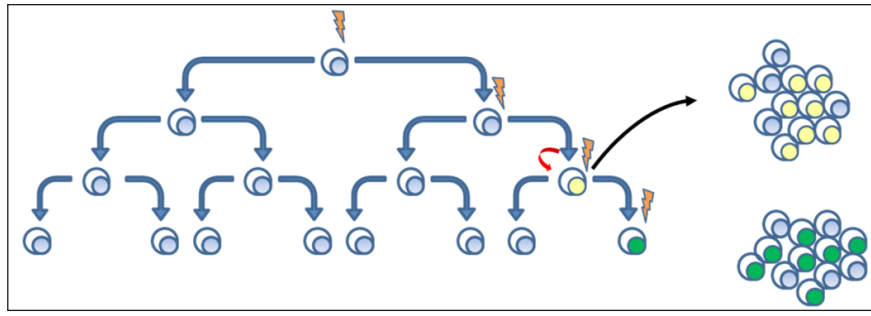
The Cancer Stem Cell Model

The cancer stem cell model, also known as the Hierarchical Model proposes that tumors are hierarchically organized (CSCs lying at the apex. Within the cancer population of the tumors there are cancer stem cells (CSC) that are tumorigenic cells and are biologically distinct from other subpopulations. They have two defining features: their long-term ability to self-renew and their capacity to differentiate into progeny that is non-tumorigenic but still contributes to the growth of the tumor. This model suggests that only certain subpopulations of cancer stem cells have the ability to drive the progression of cancer, meaning that there are specific (intrinsic) characteristics that can be identified and then targeted to destroy a tumor long-term without the need to battle the whole tumor.

Stochastic Model

In order for a cell to become cancerous it must undergo a significant number of alterations to its DNA sequence. This cell model suggests these mutations could occur to any cell in the body resulting in a cancer. Essentially this theory proposes that all cells have the ability to be tumorigenic making all tumor cells equipotent with the ability to self-renew or differentiate, leading to tumor heterogeneity while others can differentiate into non-CSCs. The cell's potential can be influenced by unpredicted genetic or epigenetic factors, resulting in phenotypically diverse cells in both the tumorigenic and non-tumorigenic cells that compose the tumor. According to the "stochastic model" (or "clonal evolution model") every cancer cell in a tumor could gain the ability to self-renew and differentiate to the numerous and heterogeneous lineages of cancer cells that compromise a tumor.

These mutations could progressively accumulate and enhance the resistance and fitness of cells that allow them to outcompete other tumor cells, better known as the somatic evolution model. The clonal evolution model, which occurs in both the CSC model and stochastic model, postulates that mutant tumor cells with a growth advantage outproliferate others. Cells in the dominant population have a similar potential for initiating tumor growth.

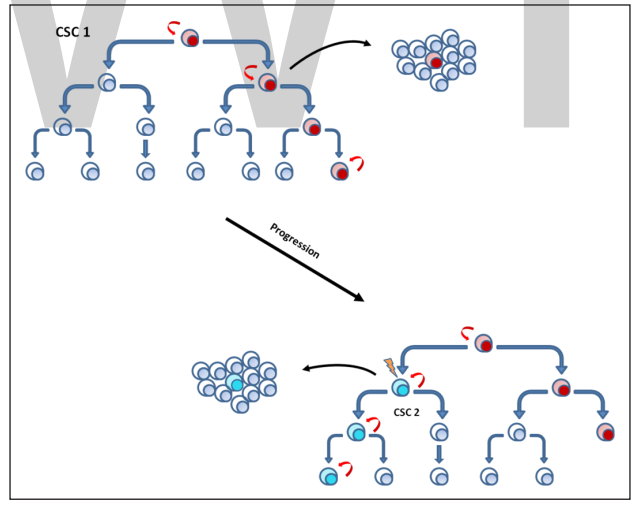


In the clonal evolution model, all undifferentiated cells have similar possibility to change into a tumorigenic cell.

These two models are not mutually exclusive, as CSCs themselves undergo clonal evolution. Thus, the secondary more dominant CSCs may emerge, if a mutation confers more aggressive properties.

Tying CSC and Stochastic Models Together

A study in 2014 argues the gap between these two controversial models can be bridged by providing an alternative explanation of tumor heterogeneity. They demonstrate a model that includes aspects of both the Stochastic and CSC models. They examined cancer stem cell plasticity in which cancer stem cells can transition between non-cancer stem cells (Non-CSC) and CSC via in situ supporting a more Stochastic model. But the existence of both biologically distinct non-CSC and CSC populations supports a more CSC model, proposing that both models may play a vital role in tumor heterogeneity.



Both tumor models may play a role in the maintenance of a tumor. Initially, tumor growth is assured with a specific CSC (CSC1). With tumor progression, another CSC (CSC 2) may arise due the clonal selection. The development of a new more aggressive CSC may result from the acquisition of an additional mutation or epigenetic modification.

The Cancer Stem Cell Immunology Model

This model suggests that immunological properties may be important for understanding tumorigenesis and heterogeneity. Such, CSCs can be very rare in some tumors, but some researchers found that a large proportion of tumor cells can initiate tumors if transplanted into severely immunocompromised mice, and thus questioned the relevance of rare CSCs. However, both stem cells

and CSCs possess unique immunological properties which render them highly resistant towards immunosurveillance. Thus, only CSCs may be able to seed tumors in patients with functional immunosurveillance, and immune privilege may be a key criterion for identifying CSCs. Furthermore, the model suggests that CSCs may initially be dependent on stem cell niches, and CSCs may function there as a reservoir in which mutations can accumulate over decades unrestricted by the immune system. Clinically overt tumors may grow if: A) CSCs lose their dependence on niche factors (less differentiated tumors), B) their offspring of highly proliferative, yet initially immunogenic normal tumor cells evolve means to escape immunosurveillance or C) the immune system may lose its tumorsuppressive capacity, e.g. due to ageing.

Debate

The existence of CSCs is under debate, because many studies found no cells with their specific characteristics. Cancer cells must be capable of continuous proliferation and self-renewal to retain the many mutations required for carcinogenesis and to sustain the growth of a tumor, since differentiated cells (constrained by the Hayflick Limit) cannot divide indefinitely. For therapeutic consideration, if most tumor cells are endowed with stem cell properties, targeting tumor size directly is a valid strategy. If CSCs are a small minority, targeting them may be more effective. Another debate is over the origin of CSCs - whether from disregulation of normal stem cells or from a more specialized population that acquired the ability to self-renew (which is related to the issue of stem cell plasticity). Confounding this debate is the discovery that many cancer cells demonstrate a Phenotypic plasticity under therapeutic challenge, altering their transcriptomes to a more stem-like state to escape destruction.

Evidence

The first conclusive evidence for CSCs came in 1997. Bonnet and Dick isolated a subpopulation of leukemia cells that expressed surface marker CD34, but not CD38. The authors established that the $CD34^+/CD38^-$ subpopulation is capable of initiating tumors in NOD/SCID mice that were histologically similar to the donor. The first evidence of a solid tumor cancer stem-like cell followed in 2002 with the discovery of a clonogenic, sphere-forming cell isolated and characterized from adult human brain gliomas. Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers *in vitro*. Cancer stem cells isolated from adult human gliomas were shown to induce tumours that resembled the parent tumour when grafted into intracranial nude mouse models.

In cancer research experiments, tumor cells are sometimes injected into an experimental animal to establish a tumor. Disease progression is then followed in time and novel drugs can be tested for their efficacy. Tumor formation requires thousands or tens of thousands of cells to be introduced. Classically, this was explained by poor methodology (i.e., the tumor cells lose their viability during transfer) or the critical importance of the microenvironment, the particular biochemical surroundings of the injected cells. Supporters of the CSC paradigm argue that only a small fraction of the injected cells, the CSCs, have the potential to generate a tumor. In human acute myeloid leukemia the frequency of these cells is less than 1 in 10,000.

Further evidence comes from histology. Many tumors are heterogeneous and contain multiple cell types native to the host organ. Tumour heterogeneity is commonly retained by tumor metastases.

This suggests that the cell that produced them had the capacity to generate multiple cell types, a classical hallmark of stem cells.

The existence of leukemia stem cells prompted research into other cancers. CSCs have recently been identified in several solid tumors, including:

- Brain,
- Breast,
- Colon,
- Ovary,
- Pancreas,
- Prostate,
- Melanoma,
- Multiple Myeloma,
- Non-melanoma skin cancer.

Mechanistic and Mathematical Models

Once the pathways to cancer are hypothesized, it is possible to develop predictive mathematical models, e.g., based on the cell compartment method. For instance, the growths of abnormal cells can be denoted with specific mutation probabilities. Such a model predicted that repeated insult to mature cells increases the formation of abnormal progeny and the risk of cancer. The clinical efficacy of such models remains unestablished.

Identification

CSCs, now reported in most human tumors, are commonly identified and enriched using strategies for identifying normal stem cells that are similar across studies. These procedures include fluorescence-activated cell sorting (FACS), with antibodies directed at cell-surface markers and functional approaches including side population assay or Aldefluor assay. The CSC-enriched result is then implanted, at various doses, in immune-deficient mice to assess its tumor development capacity. This *in vivo* assay is called a limiting dilution assay. The tumor cell subsets that can initiate tumor development at low cell numbers are further tested for self-renewal capacity in serial tumor studies.

CSCs can also be identified by efflux of incorporated Hoechst dyes via multidrug resistance (MDR) and ATP-binding cassette (ABC) Transporters.

Another approach is sphere-forming assays. Many normal stem cells such as hematopoietic or stem cells from tissues, under special culture conditions, form three-dimensional spheres that can differentiate. As with normal stem cells, the CSCs isolated from brain or prostate tumors also have the ability to form anchor-independent spheres.

Heterogeneity (Markers)

CSCs have been identified in various solid tumors. Commonly, markers specific for normal stem cells are used for isolating CSCs from solid and hematological tumors. Markers most frequently used for CSC isolation include: CD133 (also known as PROM1), CD44, ALDH1A1, CD34, CD24 and EpCAM (epithelial cell adhesion molecule, also known as epithelial specific antigen, ESA).

CD133 (prominin 1) is a five-transmembrane domain glycoprotein expressed on $CD34^+$ stem and progenitor cells, in endothelial precursors and fetal neural stem cells. It has been detected using its glycosylated epitope known as AC133.

EpCAM (epithelial cell adhesion molecule, ESA, TROP1) is hemophilic Ca^{2+}–independent cell adhesion molecule expressed on the basolateral surface of most epithelial cells.

CD90 (THY1) is a glycosylphosphatidylinositol glycoprotein anchored in the plasma membrane and involved in signal transduction. It may also mediate adhesion between thymocytes and thymic stroma.

CD44 (PGP1) is an adhesion molecule that has pleiotropic roles in cell signaling, migration and homing. It has multiple isoforms, including CD44H, which exhibits high affinity for hyaluronate and CD44V which has metastatic properties.

CD24 (HSA) is a glycosylated glycosylphosphatidylinositol-anchored adhesion molecule, which has co-stimulatory role in B and T cells.

CD200 (OX-2) is a type 1 membrane glycoprotein, which delivers an inhibitory signal to immune cells including T cells, natural killer cells and macrophages.

ALDH is a ubiquitous aldehyde dehydrogenase family of enzymes, which catalyzes the oxidation of aromatic aldehydes to carboxyl acids. For instance, it has a role in conversion of retinol to retinoic acid, which is essential for survival.

The first solid malignancy from which CSCs were isolated and identified was breast cancer and they are the most intensely studied. Breast CSCs have been enriched in $CD44^+CD24^{-/low}$, SP and $ALDH^+$ subpopulations. Breast CSCs are apparently phenotypically diverse. CSC marker expression in breast cancer cells is apparently heterogeneous and breast CSC populations vary across tumors. Both $CD44^+CD24^-$ and $CD44^+CD24^+$ cell populations are tumor initiating cells; however, CSC are most highly enriched using the marker profile $CD44^+CD49f^{hi}CD133/2^{hi}$.

CSCs have been reported in many brain tumors. Stem-like tumor cells have been identified using cell surface markers including CD133, SSEA-1 (stage-specific embryonic antigen-1), EGFR and CD44. The use of CD133 for identification of brain tumor stem-like cells may be problematic because tumorigenic cells are found in both $CD133^+$ and $CD133^-$ cells in some gliomas and some $CD133^+$ brain tumor cells may not possess tumor-initiating capacity.

CSCs were reported in human colon cancer. For their identification, cell surface markers such as CD133, CD44 and ABCB5, functional analysis including clonal analysis and Aldefluor assay were used. Using CD133 as a positive marker for colon CSCs generated conflicting results. The AC133 epitope, but not the CD133 protein, is specifically expressed in colon CSCs and its expression is

lost upon differentiation. In addition, $CD44^+$ colon cancer cells and additional sub-fractionation of $CD44^+EpCAM^+$ cell population with CD166 enhance the success of tumor engraftments.

Multiple CSCs have been reported in prostate, lung and many other organs, including liver, pancreas, kidney or ovary. In prostate cancer, the tumor-initiating cells have been identified in $CD44^+$ cell subset as $CD44^+\alpha2\beta1^+$, TRA-1-60$^+$CD151$^+$CD166$^+$ or $ALDH^+$ cell populations. Putative markers for lung CSCs have been reported, including $CD133^+$, $ALDH^+$, $CD44^+$ and oncofetal protein $5T4^+$.

Metastasis

Metastasis is the major cause of tumor lethality. However, not every tumor cell can metastasize. This potential depends on factors that determine growth, angiogenesis, invasion and other basic processes.

Epithelial-mesenchymal Transition

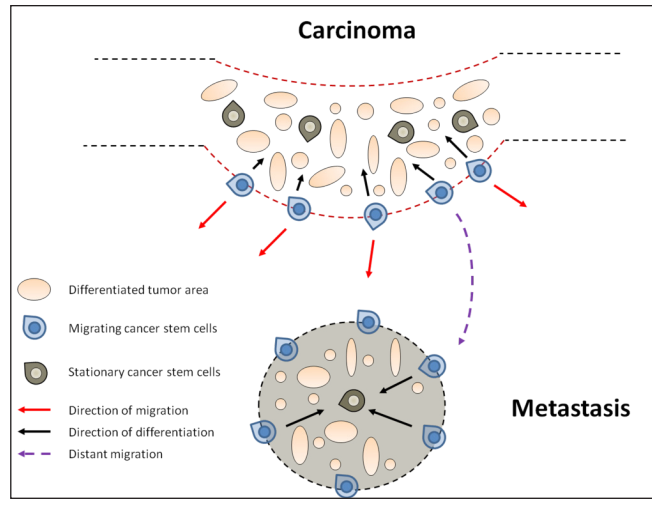
In epithelial tumors, the epithelial-mesenchymal transition (EMT) is considered to be a crucial event. EMT and the reverse transition from mesenchymal to an epithelial phenotype (MET) are involved in embryonic development, which involves disruption of epithelial cell homeostasis and the acquisition of a migratory mesenchymal phenotype. EMT appears to be controlled by canonical pathways such as WNT and transforming growth factor β.

EMT's important feature is the loss of membrane E-cadherin in adherens junctions, where β-catenin may play a significant role. Translocation of β-catenin from adherens junctions to the nucleus may lead to a loss of E-cadherin and subsequently to EMT. Nuclear β-catenin apparently can directly, transcriptionally activate EMT-associated target genes, such as the E-cadherin gene repressor SLUG (also known as SNAI2). Mechanical properties of the tumor microenvironment, such as hypoxia, can contribute to CSC survival and metastatic potential through stabilization of hypoxia inducible factors through interactions with ROS (reactive oxygen species).

Tumor cells undergoing an EMT may be precursors for metastatic cancer cells, or even metastatic CSCs. In the invasive edge of pancreatic carcinoma, a subset of $CD133^+CXCR4^+$ (receptor for CXCL12 chemokine also known as a SDF1 ligand) cells was defined. These cells exhibited significantly stronger migratory activity than their counterpart $CD133^+CXCR4^-$ cells, but both showed similar tumor development capacity. Moreover, inhibition of the CXCR4 receptor reduced metastatic potential without altering tumorigenic capacity.

Two-phase Expression Pattern

In breast cancer $CD44^+CD24^{-/low}$ cells are detectable in metastatic pleural effusions. By contrast, an increased number of $CD24^+$ cells have been identified in distant metastases in breast cancer patients. It is possible that $CD44^+CD24^{-/low}$ cells initially metastasize and in the new site change their phenotype and undergo limited differentiation. The two-phase expression pattern hypothesis proposes two forms of cancer stem cells - stationary (SCS) and mobile (MCS). SCS are embedded in tissue and persist in differentiated areas throughout tumor progression. MCS are located at the tumor-host interface. These cells are apparently derived from SCS through the acquisition of transient EMT.



The concept of migrating cancer stem cells (MSC). Stationary cancer stem cells are embedded in early carcinomas and these cells are detectable in the differentiated central area of a tumor. The important step toward malignancy is the induction of epithelial mesenchymal transition (EMT) in the stationary cancer stem cells (SCS), which become mobile or migrating cancer stem cells. Stem cells divide asymmetrically. One daughter cell will begin proliferation and differentiation. The remaining MCS migrates a short distance before undergoing a new asymmetric division, or disseminates through blood vessels or lymphatic vessels and produces a metastasis.

Implications

CSCs have implications for cancer therapy, including for disease identification, selective drug targets, prevention of metastasis and intervention strategies.

Treatment

CSCs are inherently more resistant to chemotherapeutic agents. There are 5 main factors that contribute to this:

- Their niche protects them from coming into contact with large concentrations of anti-cancer drugs.
- They express various transmembrane proteins, such as MDR1 and BCRP, that pump drugs out of the cytoplasm.
- They divide slowly, like adult stem cells tend to do, and are thus not killed by chemotherapeutic agents that target rapidly replicating cells via damaging DNA or inhibiting mitosis.
- They upregulate DNA damage repair proteins.
- They are characterized by an overactivation of anti-apoptotic signaling pathways.

After chemotherapy treatment, surviving CSCs are able to repopulate the tumor and cause a relapse. Additional treatment targeted at removing CSCs in addition to cancerous somatic cells must be used to prevent this.

Targeting

Selectively targeting CSCs may allow treatment of aggressive, non-resectable tumors, as well as prevent metastasis and relapse. The hypothesis suggests that upon CSC elimination, cancer could regress due to differentiation and/or cell death. The fraction of tumor cells that are CSCs and therefore need to be eliminated is unclear.

Studies looked for specific markers and for proteomic and genomic tumor signatures that distinguish CSCs from others. In 2009, scientists identified the compound salinomycin, which selectively reduces the proportion of breast CSCs in mice by more than 100-fold relative to Paclitaxel, a commonly used chemotherapeutic agent. Some types of cancer cells can survive treatment with salinomycin through autophagy, whereby cells use acidic organelles such as lysosomes to degrade and recycle certain types of proteins. The use of autophagy inhibitors can kill cancer stem cells that survive by autophagy.

The cell surface receptor interleukin-3 receptor-alpha (CD123) is overexpressed on CD34+CD38- leukemic stem cells (LSCs) in acute myelogenous leukemia (AML) but not on normal CD34+CD38- bone marrow cells. Treating AML-engrafted NOD/SCID mice with a CD123-specific monoclonal antibody impaired LSCs homing to the bone marrow and reduced overall AML cell repopulation including the proportion of LSCs in secondary mouse recipients.

A 2015 study packaged nanoparticles with miR-34a and ammonium bicarbonate and delivered them to prostate CSCs in a mouse model. Then they irradiated the area with near-infrared laser light. This caused the nanoparticles to swell three times or more in size bursting the endosomes and dispersing the RNA in the cell. miR-34a can lower the levels of CD44.

A 2018 study identified inhibitors of the ALDH1A family of enzymes and showed that they could selectively deplete putative cancer stem cells in several ovarian cancer cell lines.

Pathways

The design of new drugs for targeting CSCs requires understanding the cellular mechanisms that regulate cell proliferation. The first advances in this area were made with hematopoietic stem cells (HSCs) and their transformed counterparts in leukemia, the disease for which the origin of CSCs is best understood. Stem cells of many organs share the same cellular pathways as leukemia-derived HSCs.

A normal stem cell may be transformed into a CSC through disregulation of the proliferation and differentiation pathways controlling it or by inducing oncoprotein activity.

BMI-1

The Polycomb group transcriptional repressor Bmi-1 was discovered as a common oncogene activated in lymphoma and later shown to regulate HSCs. The role of Bmi-1 has been illustrated in neural stem cells. The pathway appears to be active in CSCs of pediatric brain tumors.

Notch

The Notch pathway plays a role in controlling stem cell proliferation for several cell types including hematopoietic, neural and mammary SCs. Components of this pathway have been proposed to act as oncogenes in mammary and other tumors.

A branch of the Notch signaling pathway that involves the transcription factor Hes3 regulates a number of cultured cells with CSC characteristics obtained from glioblastoma patients.

Sonic Hedgehog and Wnt

These developmental pathways are SC regulators. Both Sonic hedgehog (SHH) and Wnt pathways are commonly hyperactivated in tumors and are necessary to sustain tumor growth. However, the Gli transcription factors that are regulated by SHH take their name from gliomas, where they are highly expressed. A degree of crosstalk exists between the two pathways and they are commonly activated together. By contrast, in colon cancer hedgehog signalling appears to antagonise Wnt.

Sonic hedgehog blockers are available, such as cyclopamine. A water-soluble cyclopamine may be more effective in cancer treatment. DMAPT, a water-soluble derivative of parthenolide, induces oxidative stress and inhibits NF-κB signaling for AML (leukemia) and possibly myeloma and prostate cancer. Telomerase is a study subject in CSC physiology. GRN163L (Imetelstat) was recently started in trials to target myeloma stem cells.

Wnt signaling can become independent of regular stimuli, through mutations in downstream oncogenes and tumor suppressor genes that become permanently activated even though the normal receptor has not received a signal. β-catenin binds to transcription factors such as the protein TCF4 and in combination the molecules activate the necessary genes. LF3 strongly inhibits this binding *in vitro,* in cell lines and reduced tumor growth in mouse models. It prevented replication and reduced their ability to migrate, all without affecting healthy cells. No cancer stem cells remained after treatment. The discovery was the product of "rational drug design", involving AlphaScreens and ELISA technologies.

Tissue Engineering for Skin Replacement Methods

The skin is the biggest structure of the body, and it plays a significant role in maintaining the unity of the body environment. The skin is important for the endurance of the organism as an outer coat for the thermal regulation and hydration preservation. With the intention of helping these significant utilities, the skin continually experiences regeneration and holds the capability to overhaul wound by repair and regeneration of several kinds of skin stem cells. Noteworthy, development has been accomplished throughout the recent times in the generation of engineered skin alternates which imitate human skin cells in vitro for replacement or modeling. Conversely, existing new skin alternatives do not reinstate completely the healthy skin anatomy and suffer from deficiency of natural supplements in skin covering, sebaceous glands, hair follicles, and sweat glands. Improvements in stem cell biology and skin morphogenesis show significant potentials to evidently advance the engineering of skin replacements which would preferably be vague from normal skin.

Tissue engineering is growing as a novel area in biomedical engineering which purposes to redevelop newfangled material for substituting problematic or injured tissues. In order to accomplish this, not only is a basis of cells necessary but also a simulated extracellular matrix (ECM) that the cells which may be reinforced should exist. Human skin signifies about one-tenth of the body form, and injuries like physical distress, infection, burn, disease, or operation to a portion of this main

organ carry intense penalties. Tissue engineering of skin substitutes signifies a potential foundation of improved treatment in fighting acute and chronic skin wounds. Currently, there are no significant prototypes of engineered skin which entirely duplicate the composition, structure, organic constancy, or visual environment of healthy skin. Skin alternates should carry some important physiognomies that comprise being simple to use and implement to the wound location; deliver vital blockade utility with suitable aquatic fluidity; be willingly adherent; have fitting corporeal and mechanical possessions; experience regulated deprivation; be disinfected, nontoxic, and nonantigenic; and induce negligible inflammatory effect. Moreover, they should join to the congregation with nominal damaging and agony and ease angiogenesis, whereas yet being cost operative. The eventual aim of tissue engineering is to gratify most if not all of these standards when creating original, clever skin replacement.

Structural and Progressive Provisions

The skin covers epidermal and dermal sheets pervaded via a multifaceted vascular and nervous system. The hypodermis is located underneath, made by moveable linking tissue and fat. Epidermal basal cells and stem cells existing in the basal layer and hair follicles are in control of an unremitting progression of epidermal regeneration. Additional cell varieties that exist in the epidermis comprise melanocytes, Langerhans cells, and Merkel cells.

The dermis carries two stratums: a superior papillary layer carrying a reedy organization of collagen fibers and a dense inferior reticular layer with profuse collagen fibers similar to the superficial of the skin. The dermal extracellular matrix is made mostly of collagen, elastin, and reticular fibers. The key constituent of the dermis is the fibroblasts that deliver continuous excretion of the collagen and proteoglycan matrix.

Fetal wound repair shows a lack of scarring and fibrosis. This progression is categorized via negligible irritation and renewal of healthy collagen deposition and skin adnexa. The development dynamic outline in fetal renewing skin is significantly diverse from the adult one, being described via advanced intensities of transforming growth factor (TGF)-β3 and minor stages of platelet-derived growth factor (PDGF), TGF-β1, and TGF-β2.

Aims of Skin Tissue Engineering

Tissue-contrived skin is a noteworthy improvement in the arena of wound healing. It has primarily been advanced related to the limits linked with the utilization of autografts and allografts where the contributor site agonizes from aching, contamination, and blemishing. Lately, engineered skin substitutes have been covering extensive submission, particularly in the circumstance of injuries, where the main preventive issue is the obtainability of autologous skin. The expansion of an imitated skin enables the action of patients with burns and several skin-associated disorders. The existing review contributes an inclusive outline of the improvements and upcoming forecasts of skin alternates for tissue overhaul and renewal.

Current Skin Substitutes

Autologous keratinocytes may be obtained and cultivated into interconnected layers of the epithelium which may be displaced onto big skin deficiencies on the suffering individual. Clonogenic

keratinocytes, defined as holoclones, may be obtained from the skin and consecutively proliferated in culture for more than 140 replications and have revealed to be bona fide multipotent stem cells founded on their aptitude to renovate manifold lines in the skin.

These embedded stem cells inside of these epithelial expanses provide repair and regeneration of the epidermis. Developing the epidermal stem cells over fibrin environments or allogeneic dermis has established to be beneficial. The funding materials have significantly enhanced the receiving amounts of the implants, advanced the affluence of managing and operation of the implants, and reduced the wound refutation and scarring. Cultivation of autologous epidermal stem cells simplifies to obtain large epithelial areas for transfer from a minor skin biopsy; therefore, this method needs more than a few weeks.

Mounting the stem cells on a substance drops the period necessary to brand outsized epithelial layers from a minor skin biopsy for the epithelia on the material which does not necessitate to attain full confluence of the previous replacement. Furthermore, epidermal stem cells on fibrin environments or allogeneic dermis converse the capability to renew the usual rolled dermal/epidermal connection and the artificial ration of the dermis, named the papillary dermis. Nevertheless, these epidermal stem cell implants lack renovation of a complete practical skin. Epidermal adjuncts, containing hair follicles, sebaceous glands, or sweat glands, are not redeveloped after transferring these implants of epidermal stem cells, signifying that multifaceted epithelial and mesenchymal connections are essential to generate additions. Additionally, the implantations do not reinstate the automated possessions or visual form of the novel skin. Improvements in stem cell biology and skin morphogenesis have the prospective to expand the manufacturing of the skin that may interchange the typical utility and esthetics of healthy skin.

Skin Stem Cells

Up to now, scientists have recognized numerous diverse sorts of skin stem cell covering epidermal stem cells, hair follicle stem cells, melanocyte stem cells, mesenchymal stem cells, and recently identified human newborn foreskin stem cells. Epidermal stem cells are in charge for routine regeneration of the dissimilar stratums of the epidermis. These stem cells exist in the basal layer of the epidermis. Hair follicle stem cells safeguard continuous renewal of the hair follicles. They can also restore the epidermis and sebaceous glands in case of injury. Hair follicle stem cells originated through the hair follicles.

Melanocyte stem cells are in control of melanocyte revival which is a kind of pigment cell. Melanocytes generate the pigment melanin and so carry a significant part in skin and hair follicle pigmentation. It is not yet clear where these stem cells are located. Studies also indicate another type of stem cell, known as mesenchymal stem cells, which can be established in the dermis and hypodermis. Mesenchymal stem cells conquer lymphocyte production in vitro and extend skin graft endurance in vivo.

Another stem cell that resides in the skin is recently established and named as human newborn foreskin stem cells. They carry pluripotency, and they are capable to turn into different cell types. They show fibroblastic shape; however, they express both mesenchymal stem cell markers and some of the hematopoietic stem cell markers.

In Vivo Applications

Autologous skin transfer is now the scientific main protocol for full-breadth skin injuries covering burn damages. Before grafting, primary editing is a significant portion of the handling of burn wounds, as skin temperature-denatured proteins have to be detached to avoid numerous difficulties like contamination, manifold organ impairment condition, hypertrophic mutilation development, unrestrained inflammatory reaction, or infection with pathogenic microorganisms. Microbes could utilize the eschar as a basis of nutriments and are particularly damaging to seriously burnt individual, as this damage also triggers a provisional destruction of cell-related and humoral immunity.

Autologous split skin grafts (SSGs) are reaped with a dermatome which separates the epidermis and an artificial portion of the dermis. Residual epidermal cells in the enduring dermis of the SSG giver site will recreate an epidermis. Subsequent to the submission of an SSG to a full-width wound, its vessels unite with the capillary system in the removed wound. This "graft take" is vital for a correct source of nourishment and brings implant endurance. The divided skin contributor place patches up in 1 week and may be utilized for SSG collecting up to four times; though, continual reaping is linked with blemishing at the contributor sites in addition to long hospital visits. Furthermore, in the situation of a wider damage, contributor sites are tremendously restricted and might leave the individual with very small unharmed skin to produce sufficient autologous SSGs. An initial and enduring wound healing is wanted, as it outcomes in negligible or no scarring difficulties, poorer impermanence, and improved practical extended duration outcomes. Oppositely, wound healing postponement is straightly relative to vigorous hypertrophic scarring. In order to indicate the difficulty of restricted SSG reaping sites, an interconnecting method is applied that expanses the implant and so may cope a superior wounded region at the expenditure of cosmetic and practical result.

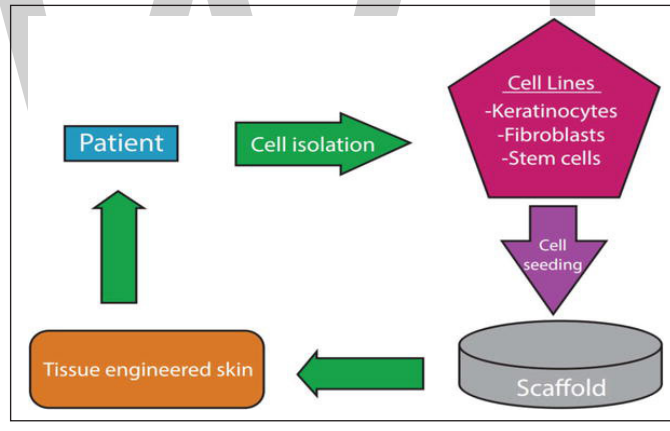
Additional option is the usage of allografts, for a provisional deterrence of liquid loss or infection of the wound. Allografts integrate into serious injuries and deliver ache relief. Therefore, moral as well as protection matters endure, as the severe broadcast for virus-related illnesses and consistent disinfection methods cannot entirely eradicate the probability of infectious mediator conduction. When compared to autologous SSGs, a leading trouble of allografts is that they consent the patients for a long time with wounds likely to problems. Ultimately, allografts experience immunogenic refusal, and the location of wound requires to be enclosed with an autologous SSG. Deferred refusal may happen in people with wide injuries because of their clinically repressed immune reaction and, nonetheless, finally may be activated via the extremely immunogenic epithelial cells of the allograft throughout its vascularization. Consequently, there is a countless requirement for a substitute that may deliver a more enduring clarification.

Tissue-engineered Skin Alternates

Manufactured cell free along with allogeneic cell comprising skin alternates delivers a conceivable resolution to the difficulty of donor implant scarcity. The engineered skin alternatives propose defense from liquid loss and infection while transporting dermal matrix constituents, cytokines, and evolution elements to the wound bed, increasing usual host wound therapeutic answers. Bioengineered skin alternatives may be utilized as impermanent covers once wound damaged tissue up to there is an autograft accessible. Subsequent to assimilation, these assemblies persevere in the

wound throughout healing or even afterward. Cell-free biomaterial-related skin alternates may be utilized in integration with autografts as a defensive cover over interconnected autografts to fund their income in addition to arouse the wound bed in the spaces or to expand implant engraftment in parts of pressure. Nevertheless, in contradiction of autografts, tissue-engineered allogeneic skin implants may tolerate the danger of conveying like hepatitis B virus (HBV) or human immunodeficiency virus (HIV). One benefit over autologous in vitro engineered skin alternates is that they have decreased industrial prices.

In order to manufacture epidermal replacements, a skin biopsy of 2–5 cm^2 must be picked up from the individual. This may be joined with the first debridement of the injured person. Consequently, the epidermis is detached from the dermis, and solitary keratinocytes are chemically discharged and cultivated on mitotically incapacitated mouse fibroblasts. The utilized development media cover fetal calf serum and other essential additions; conversely, it is also likely to enlarge these cells in xenogeneic-free situations. There are numerous revisions analyzing epithelial allografts such as Celaderm; conversely, the efficiency and protection of these harvests have to be established in organized scientific trainings. Along with these custom-built concepts, there have been several laboratories manufacturing cultivated epithelial allografts. Allogeneic crops carry the benefit of abridged industrial charges equated to autologous crops. Yet, an inadequacy of both harvests is that they demonstrate deprived attachment levels that may bring the creation of wounds.



Representation of perspectives of skin tissue engineering. Primary keratinocytes, fibroblasts, and stem cells are obtained from human contributor tissues that are afterward in vitro expanded previously to seeding onto appropriate scaffold materials.

On behalf of the management of full-width burns, mutually the epidermal and the dermal layers of the skin require to be substituted, as the action with expanded epidermal (keratinocyte) layers would end in a mediocre conclusion. In contradiction of cultured epidermal layers, engineered dermal concepts can inhibit wound shrinkage, and they deliver a better constancy. The dermal and epidermal counterparts should be submitted successively, as decent dermal vascularization via the debrided wound bed requires to be attained previously to submission of the epidermal stratum. There is an extensive diversity of advertised dermal concepts, both natural and artificial. Some of these alternates are chemical-treated allografts such as Alloderm, deficient for cellular rudiments that are important for the immunogenic refusal. Besides, Dermagraft contains human foreskin fibroblasts, expanded in a fissionable polyglactin network. In these alternates, cells secrete extracellular matrix (ECM) proteins, a variation of growth factors and cytokines into the wound till they experience usual programmed cell death 1 or 2 weeks post-embedding.

The most progressive and refined concepts that are accessible for scientific utilization are alternates that imitate epidermal along with dermal sheets of the skin. Even though imitating the histo-architecture of healthy skin, the epidermal/dermal skin alternates would be thought as provisional structurally effective wound layers. Skin replacements deliver growth factors, cytokines, and ECM for host cells; control wound remedial; and may consequence in active pain relief. Main drawbacks are the elevated industrial charges and their insufficiency to heal the wound enduringly regarding to tissue refusal. The immunogenic acceptance of a host in the direction of allogeneic fibroblasts is controversially deliberated. There are different revisions sustaining the theories that allogeneic fibroblasts are individual autologous keratinocytes and are passable for the creation of a perpetual epidermal-dermal skin alternate.

In Vitro Applications

Tissue-engineered human skin has been technologically advanced to replicate the main fundamental and practical features of normal skin. In this background, they allow not only the examination of essential procedures in the skin but also the risk valuation of several chemicals which are locally presented to the skin deprived of the necessity to utilize animal models. Outcomes obtained from experimentations showed in animal models are mostly restricted regarding the alterations in the metabolism and the functional architecture. In vitro tests in two-flat monolayer cultivation of human cells are also of nominal significance because of the absence of multifaceted cell-cell and cell-ECM connections. Conversely, manufactured skin alternates may eliminate these difficulties via utilizing human cells which are organized in a 3D physical background, letting the interface of the dissimilar cell sorts with one additional and nearby matrix.

Table: In vitro skin tissue engineering models.

In vitro skin models	Cell foundation
Melanoma model	Melanoma mesenchymal cells, fibroblasts, keratinocytes
UV radiation and phototype	Keratinocytes, melanocytes, and fibroblasts
Wound healing model	Fibroblasts and keratinocytes
Psoriasis model	Keratinocytes and fibroblasts
Full-thickness model	Epithelial sheath, fibroblasts, and keratinocytes
Ex vivo model	No requirement of cells

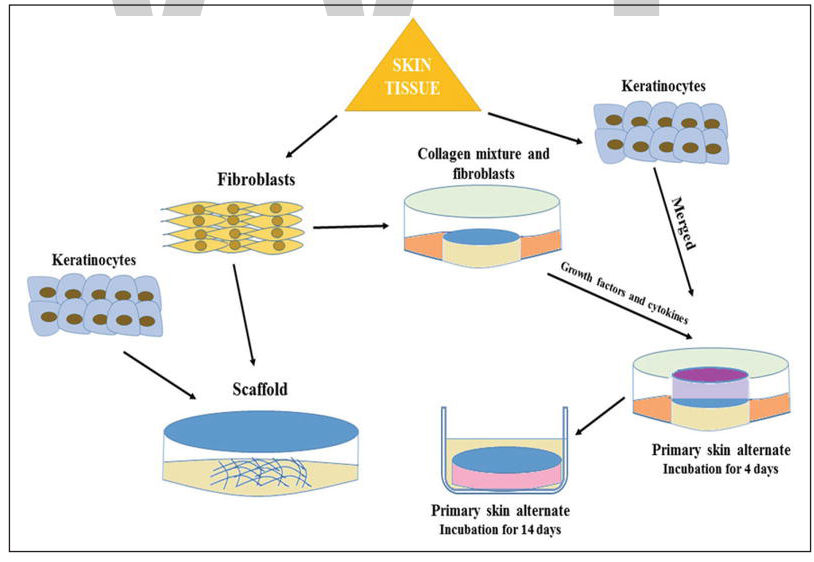
Up to the present time, several sorts of skin alternatives have been established via dissimilar scientific groups. These skin alternatives may be categorized in two kinds. The primary one contains keratinocytes applied on an artificial or collagen transporter faking only the human epidermis. The subsequent one contains a dermal sheet of human fibroblasts entrenched in numerous types of scaffolds.

Full-thickness in Vitro Models

Although the excessive mainstream of the skin alternates utilized in pharmaceutical investigation is made of an epidermal sheet, these skin replacements could then be developed via the totaling of a dermal stratum covering fibroblasts. In this perspective, fibroblasts have only lately started to

have more consideration. It was revealed that skin fibroblasts are distant from being uniform, and it was guessed that a few of the chronic wounds are related to an alteration in the arrangement of the fibroblasts. It was displayed that fibroblasts clearly effect keratinocyte development in vitro, most probably because of the circumstance that these cells discharge solvable growth factors. In normal skin, the communication among fibroblasts and keratinocytes covers a key role in progressions like wound healing and the creation of the base membrane.

Utilizing skin alternatives, it was established that fibroblasts carry a vital part in the normal epidermal histology. In the absence of fibroblasts, the keratinocyte differentiation harshly changes and outcomes only in some sheets of extremely distinguished epithelial cells. Fascinatingly, keratinocytes carry a significant influence on the expansion of fibroblasts. This interface of epidermal and dermal cells is theorized because of a double paracrine tool that controls the development of keratinocytes and fibroblasts. Due to the theory, keratinocytes discharge IL-1 which rouses the skin fibroblasts to secrete keratinocyte growth factor (KGF) and granulocyte-monocyte colony-stimulating factor (GM-CSF) that sequentially effect the production of the keratinocytes. Moreover, dermal fibroblasts carry an important part in the renovation of the skin and in the tightening of acute wounds, and they can upturn the struggles of keratinocytes to toxic chemicals. Regarding the outcomes, one could determine that for obtaining significant data from toxicological in vitro experiments. Oppositely, epidermal replacements might be more appropriate for the determination of the diffusion constant through the skin. In monotonous in vitro diffusion studies, a specific part of skin alternates divorces a contributor from an acceptor compartment. Collagen-related full-breadth skin alternates are not ultimate for that kind of tests since they do not cover the entire external part regarding to a low mechanic pliability, consequently finishing in open superiorities, over that the constituent under study may flexibly diffuse.



Utilization of skin fibroblast and keratinocyte for skin tissue engineering.

Skin Gene Therapy

The aptitude to hereditarily alter cells utilized to organize skin alternates allows ex vivo gene treatment methods to treat cutaneous illnesses and damages. Nonetheless, gene therapy stays as an unsatisfied potential of cell treatments with skin alternatives. Still, designated analysis of gene

therapy in skin wounds has been accomplished recently. Initial models utilizing overexpression of angiogenic growth factors with duplication-incompetent retroviral vectors established viability for constructive distribution of physically dynamic composites such as VEGF and PDGF, with a capacity for prompt wound healing in diminished wounds. Therefore, usage of retroviral gene transmission for the action of hematopoietic syndromes was linked with expansion of leukemia regarding the addition of mutagenesis, an outcome which has fundamentally banned the method from potential deliberation.

Parallel threats have been recognized in lentiviruses which have also decreased their forthcoming custom in therapeutics. Replacements to viral tools have been industrialized that comprise plasmid transfection for expression of endogenous antimicrobial peptides like cathelicidin. Additional lately advanced methods such as gathered frequently interspaced short palindromic repeat (CRISPR) arrangement enable site-specific genome editing, greatly reducing the risk of insertional mutagenesis. In addition if gene therapies are managed in allogenic cells that are eradicated immunologically after a restricted period of time, then threats can be minimalized. Nonviral skin gene transfer techniques are listed in Table individually.

Table: Nonviral skin gene therapy methods.

Transfer techniques	Therapeutic mediator	Submission reasons
Direct injection	Cytosine-phosphate-guanine class C/immunestimulatory sequence oligodeoxynucleotides	Tumor treatment
Electroporation	Antisense oligodeoxynucleotides	Wound healing
Electroporation	Chimeric RNA/DNA oligodeoxynucleotides	Hair follicle manipulation
Topical	Liposome-coated DNA	Expressions of growth factors, cytokines, and hormones
Biolistic	Naked DNA	Immunization

Induced pluripotent stem (iPS) cells may progress the competence of genome excision. The application of iPS cells includes deterioration of donor cells to a pluripotent state in vitro, growth of cell quantities, and adjustment of cell populace in the direction of a distinguished phenotype of concern. The arrangement of genetic adjustment methods, permitting accumulation of healthy genes or alteration of mutated genes, with iPS knowledge delivers the aptitude to fix hereditary illnesses.

Skin alternates consequent to skin stem cells also carry a potential for practicable gene therapy for inactivating inherited illnesses of the skin, like epidermolysis bullosa. The epidermis is systematized into epidermal multiplying components which are self-renovated via at least one epidermal stem cell an propose that transport of epidermal stem cells from the basal stratum of the epidermis for gene treatment might consequence in enduring expression of the transgene. Ex vivo transduced keratinocytes of holoclones have been revealed to have transgene expression which continues for more than 150 cell productions in culture and, more significantly, have been publicized to express the transgene protein once implanted in epidermal stratums in vivo. Autologous epidermal stem cells obtained in culture via development of holoclones were retrovirally interacted with laminin 5 and were effectively relocated in people with junctional epidermolysis bullosa. The implants renewed a healthy epidermis at day 8, and the usual epidermis was preserved during 1 year of continuation.

Scaffold Biomaterials in Tissue Engineering of the Skin

In order to manufacture a body-compatible scaffold, it is important that the scaffold does not cause any acute or chronic response in the body. The scaffold must have a surface that is suitable for cell attachment so that it can replace the damaged tissue and help creating new tissue. If the biomaterial used in the making of the scaffold is biodegradable, newly regenerated tissue can replace the scaffold. Therefore, it is crucial that the scaffold is compatible with the skin tissue. For a scaffold to hold, it must have certain physical and mechanical properties and have a certain chemical structure in the surface. Researchers may use different biomaterials such as collagen, chitosan, hyaluronic acid, and poly(lactic acid) (PLA) in tissue engineering to build scaffolds.

Chitosan

Chitosan is one of the materials that is used in tissue engineering, which is used in wound healing. It is biodegradable, biocompatible, and nontoxic. In addition, it has hemostatic activity. It is also advantageous that chitosan is antibacterial. Chitosan can be used in stimulating collagen synthesis, and its electrostatic function can speed up the healing process. Sponges and gels that are made from chitosan are utilized in the healing process of full-width burn wounds. Chitosan loses its effect in acidic environments, and since wound healing is an acidic incident, cross-link agents may be used to stabilize chitosan structures.

Hyaluronic Acid

Hyaluronic acid is a lineal polysaccharide made of repeating disaccharide elements of N-acetyl-glucosamine and n-glucuronic acid. Hyaluronic acid can be found in human skin and is known to be speeding up the healing process. Apart from these, it is observed that hyaluronic acid amount is increased in fetal skin and wounds in case of scar-free healing. Hyaluronic acid is a material with so much perks in scaffolding such as expanding the possibilities of cross-linking, delaying the biodegrading of materials, and more control over mechanical aspects of the process. Also, hyaluronic acid offers more incorporation of cell adhesion ligands and growth factors in the making of scaffolds. Aquatic uptake ability, flexibility, and biocompatibility of the scaffold are some of the properties that are made possible and enhanced by HA.

Collagen

Collagen is a naturally found protein that can enhance the structural integrity. Collagen can be found in human skin tissue and mostly created by fibroblasts and myofibroblasts. In the body parts that are under stress and used often, for example, the skin, tendons and bones, collagen can be found in fibrils. One of the most common types of collagen which is also seen widely in scar tissues as well as the dermis, fasciae, and tendons is type I collagen. There are 20 variations of collagens, and only types II, III, V, and XI can make up fibrils. Collagen is one of the most used materials that have been utilized in skin tissue engineering, and only recently it has been possible to create a model that can promote human capillary-like network.

It is an excellent material for scaffolding because of its ability to boost cell attachment, migration, proliferation, and differentiation. It is preferred in medical applications as a primary material since it is excellent in biocompatibility, biodegradability, and weak in antigenicity.

Scientific researches provided recombinant human collagen, and it proves to be a more dependable foundation for collagen which is not animal based. Human-based collagens are used in scaffolding, and they show promising results in efficiency for manufacturing skin, cartilage, and periodontal ligaments. Permeable collagen matrices with specific structural, biochemical, and biotic characteristics are interesting materials for tissue engineering, and introducing glycosaminoglycans may add to these characteristics because they are constituents of ECM proteins.

Silk

Silk is a biopolymer that is found in nature and has been used in medical applications for centuries. It contains filament core protein, named fibroin, and a glue-similar coating with sericin proteins. Silk can be composed into many forms such as films, fibers, meshes, and sponges, and these forms have been used in many incidents, show great promise in supporting stem cell union, multiplying, and distinction in vitro, and are known to be boosting tissue repair in vivo. Skeletal structures such as bone, ligament, cartilage, and connective tissues such as the skin have been engineered using 3D silk fibroin scaffolds in stem cell-related tissue manufacturing.

Fibrin Glue

For some time now, fibrin glue has been used for medical applications such as plastic surgery and reconstructions as an adhesive compound. It is antibacterial, as well as it boosts hemostasis. Apart from these, fibrin helps grow keratinocyte and fibroblast in vitro and in vivo and therefore promotes cellular movement in the wound. It has been observed that endogenous fibrin clots to create a temporary matrix in a purpose of promoting angiogenesis in the primary stage of wound healing. It is recognized that some growth factors are increased during the wound healing process to promote angiogenesis. Vascular endothelial growth factor (VEGF) is one of these. Furthermore, if fibrin is preferred as a dermal substrate for an alternative of cultivated skin, it upsurges the discharge of VEGF, thus indorsing angiogenesis.

Artificial Fragmental Polymers

A few of the artificial fragmentable polymers utilized as permeable scaffolding constituents cover polyethylene glycol (PEG), poly(lactic acid) (PLA), polyglycolide (PGA), poly(lactic-co-glycolic acid) (PLGA), polycaprolactone (PCL), poly(d,l-lactic acid or d,l-lactide) (PDLLA), polyester elastomer (PEE) founded on polyethylene oxide (PEO), and polybutylene terephthalate (PBT). There are some synthetic polymers with biodegradable properties that are highly preferred as permeable scaffolding constituents such as polyethylene glycol (PEG), poly(lactic acid) (PLA), polyglycolide (PGA), poly(lactic-co-glycolic acid) (PLGA), polycaprolactone (PCL), poly(D,L-lactic acid or D,L-lactide) (PDLLA), polyester elastomer (PEE) based on polyethylene oxide (PEO), and polybutylene terephthalate (PBT).

A superlative absorbent scaffold in skin tissue engineering should be the one which imitates the normal surroundings for skin development over suitable cell penetration, propagation, and differentiation. It should be biodegradable and penetrable to oxygen, aqua, and nutrition interchange and must be defensive contrary to contamination and injury. Up to the present time, there have been numerous kinds of absorbent scaffolds defined for skin tissue renewal, and most of them may

be branded as fibrous permeable scaffolds. Nevertheless, there are some spongy or foamy scaffold sorts with advanced absorbency that may be utilized as operative concepts for skin renewal. Supreme of these scaffolds has collagen as a foundation, and then keratinocytes or fibroblasts are planted into the scaffolds.

When choosing a porous scaffold to be used in skin tissue engineering, one must look for some properties and characteristics to create optimum conditions that resemble the usual background for skin development of the most over suitable cell permeation, creation, and distinction. The ideal scaffold also should safeguard against contamination and injury. There have been plenty of porous scaffolds with various forms to this day that are described for the regeneration of skin tissue, and most of these may be seen as fibrous absorbent scaffolds. Other than these scaffolds, there are also various types of spongy or foamy scaffolds which have higher porosity and can be used in skin regeneration. Collagen is the main ingredient in most of these porous scaffolds with keratinocytes or fibroblasts that are seeded into the scaffolds.

Scaffolds are designated in severe burns and skin deficiencies persuaded because of the elimination of tumors or skin implanting in patients experiencing necrotizing fasciitis owing to bacterial contaminations. Meanwhile, some original sponge scaffolds in arrangement with biomaterials like human keratin and polyvinyl alcohol/chitosan have also been described for their utilization as operative skin alternatives. Scaffolds have been used in medical applications such as acute burns and skin defects. Apart from these, some novel sponge scaffolds when used with biomaterials like human keratin and polyvinyl alcohol/chitosan have also been used since they are so effective as skin substitutes.

Nanofibrous scaffolds are extensively utilized for firming along with lenient tissue engineering submissions, and they also perform as tools for the regulated distribution of drugs and numerous biological particles in the arrangement of proteins and DNA. Numerous usual and synthetic polymers have been applied for nanofiber constructions to generate fibrous scaffolds for biomedical applications. These nanofibers are occasionally precisely functionalized via a basic interference or coating method or with superficial implanting polymerization by adding ligands and adhesive proteins on the nanofiber shallow. Combination of drugs, development factors, and genes straightly into the polymer elucidation throughout electrospinning is also a training for precise discharge possessions. Current methods for integrating therapeutic mediators or bioactive particles comprise coaxial electrospinning, suspension electrospinning, and alterations by external absorption or chemical conjugation. In lieu of soft and hard tissue engineering and its submissions, nanofibrous scaffolds are widely used as well as deliver drugs in a controlled manner. Synthetic and natural polymers have been used for nanofiber manufactures to harvest rubbery scaffolds for biomedical presentations.

Peripheral Stem Cell Transplantation

Peripheral blood stem cell transplantation (PBSCT), also called “Peripheral stem cell support”, is a method of replacing blood-forming stem cells destroyed, for example, by cancer treatment. PBSCT is now a much more common procedure than its bone marrow harvest equivalent, this is in-part

due to the ease and less invasive nature of the procedure. Studies suggest that PBSCT has a better outcome in terms of the number of hematopoietic stem cell (CD34+ cells) yield.

Immature hematopoietic stem cells in the circulating blood that are similar to those in the bone marrow are collected by apheresis from a potential donor (PBSC collection). The product is then administered intravenously to the patient after treatment. The administered hematopoietic stem cells then migrate to the recipient's bone marrow, a process known as stem cell homing, where the transplanted cells override the previous bone marrow. This allows the bone marrow to recover, proliferate and continue producing healthy blood cells.

The transplantation may be autologous (an individual's own blood cells saved earlier), allogeneic (blood cells donated by someone else with matching HLA), or syngeneic (blood cells donated by an identical twin). The apheresis procedure typically lasts for 4–6 hours, depending on the donor's total blood volume.

Preparation Prior to PBSC Collection

GCSF

Granulocyte colony stimulating factor (GCSF) are naturally occurring glycoproteins that stimulate white blood cell proliferation. Filgrastim is a synthetic form of GCSF produced in *E.coli*. PBSC donors are given a course of GCSF prior to PBSC collection, this ensures a better outcome, as stem cell proliferation increases, thus increasing the number of peripheral stem cells in circulation. The course is usually given over a 4-day period prior to PBSC collection. Mild bone pain usually results due to the excessive stem cell crowding within the bone marrow.

Complications

Since allogeneic PBSCT involves transformation of blood between different individuals, this naturally carries more complications than autologous PBSCT. For example, calculations must be made to ensure consistency in the amount of total blood volume between the donor and recipient. If the total blood volume of the donor is less than that of the recipient (such as when a child is donating to an adult), multiple PBSCT sessions may be required for adequate collection. Performing such a collection in a single setting could result in risks such as hypovolemia, which could lead to cardiac arrest, thus health care providers must exercise careful precaution when considering donor-recipient matching in allogeneic PBSCT.

Skin Tissue Regeneration for Burn Injury

The skin is the largest organ of the body, which meets the environment most directly. Thus, the skin is vulnerable to various damages, particularly burn injury. Skin wound healing is a serious interaction between cell types, cytokines, mediators, the neurovascular system, and matrix remodeling. Tissue regeneration technology remarkably enhances skin repair via re-epidermalization, epidermal-stromal cell interactions, angiogenesis, and inhabitation of hypertrophic scars and keloids. The success rates of skin healing for burn injuries have significantly increased with the use of various skin substitutes.

Burns remain as one of the most common injuries worldwide, with more than one million patients annually in the USA alone. A burn ensues after the skin is damaged by heat, radiation, electricity, or chemicals. Serious complications of deep or widespread burns can happen, e.g., sepsis due to bacterial infection, shock caused by hypovolemia, or scaring tissue contraction after improper wound healing. The skin damage causes the death of skin cells, leading to an enormous loss of body fluids that is followed by dehydration, electrolyte imbalance, and renal and circulatory failure. Another serious threat to lives of burn patients is an infection. The burned skin is extremely susceptible to bacteria and other pathogens, due to the loss of protection by intact layers of the skin. Each of these complications can be fatal or make a patient suffer. Therefore, it is critical to promptly cover a burn injury using an appropriate approach to prevent them and save patients' lives, besides providing intravenously fluids and nutrients to offset dehydration and replace lost proteins.

The survival rates of patients with burns have significantly improved due to the application of various skin grafts over the last decades. Despite wide use, autologous skin grafts are deficient in the treatment of severe burns for patients with limited donor site area. Skin substitutes, especially cell-based ones, play critical role in overcoming this scarcity. The cumulative effect of cell-sheets, scaffolds, cell-scaffolds, and hydrogels with healing promoting factors triggers, accelerates, and enhances wound healing and re-epithelialization that leads to a reduction in scar formation and prevention of burn injury complication. Skin substitutes have shown high efficacy and cost-effectiveness compared to autologous skin replacement.

The skin plays an important role which cannot be overestimated; its functioning ensures homeostasis and protects us from aggressive and causative agents in the environment. It is constantly involved in numerous processes: water balance and temperature regulation, signal perception, hormone, neuropeptide and cytokine production and activation, etc. The skin is formed by three main layers (the epidermis, the dermis, and the hypodermis) with its appendages (hair, sweat and sebaceous glands, sensory neurons, blood and lymph vessels, etc.). The entire skin tissue contains various cells (epidermal, stromal, endothelial, and neuronal cells) and the extracellular matrix (ECM). Cells, growth factors, and matrix are the basic elements for use in the skin regeneration and replacement after an injury.

Skin Anatomy

The skin is a complex tissue, and its structure is presented by the epidermis, the dermis, the hypodermis, and skin appendages.

The external first layer—the epidermis—is the main barrier between the environment and internal organs and tissues. It is structured in layers (strata): horny layer (stratum corneum), clear layer (stratum lucidum), granular layer (stratum granulosum), spinous layer (stratum spinosum), and basal layer (stratum basale). The epidermis is thin and stratified and consists of cell populations such as keratinocytes, Merkel cells, melanocytes, and Langerhans cells. Keratinocytes are the major cell component of the epidermis and responsible for its stratified structure; they form numerous and tight intercellular junctions. Melanocytes, which synthesize melanin (pigment absorbing UV radiation and protecting from its negative effects), are located in the basal layer (stratum basale) and form dendrites that can reach the spinous layer (stratum spinosum). Merkel cells, which are responsible for the mechanic perception, are also found in the stratum basale (above the basement

membrane). Langerhans cells are distributed in the stratum spinosum and involved in immune protection: they act as an antigen-presenting cell and engulf pathogens or other foreign matter.

Being the main cell component in all epidermal layers, keratinocytes ensure keratinization due to their differentiation starting in the basal layer. While differentiating and migrating towards a skin surface, keratinocytes become anucleated and have clustered keratin in the stratum granulosum. Then they flatten and die in the stratum corneum. Corneocytes (differentiated keratinocytes) have tight intercellular junctions that prevent water evaporation and skin dehydration, but they are expulsed because of the desmosome loss. This process is involved in desquamation (i.e., skin peeling). However, the epidermis has no direct blood supply, and delivery of nutrients and elimination of residuals occur due to the diffusion from the underlying dermis through the epidermal basement membrane. The basement membrane is a semipermeable layer which is formed by ECM components such as collagen type IV, nidogen, laminin, and perlecan.

Beneath the epidermis, there is the dermis which forms a thick layer mainly consisting of the connective tissue and ECM. It is more heterogeneous than the epidermis, and different structures like blood and lymph vessels, sweat and sebaceous glands, and hair follicles are located there. It can be divided into two layers: papillary and reticular. The first one is thin and superficial and presented by the flowing connective tissue, which includes reticular, elastic, and non-organized collagen (mostly type III) fibers and capillaries. The latter one is thick and deep and presented by the compact connective tissue, which has crosslinked elastic and well-organized collagen (type I and III) fibers and large blood vessels. The connective tissue mainly consists of collagen, which enables the skin's strength, but there are also elastin (elasticity and flexibility) and proteoglycans (hydration and viscosity). It is constantly remodeled because of the action of proteolytic enzymes (matrix metalloproteinases) synthesized by fibroblasts, neutrophils, keratinocytes, etc., and involved in numerous processes in the skin. The main cell type of the dermis is fibroblasts, which produce components of the ECM (collagen, elastin, and proteoglycans) and secrete various growth factors (TGF-β), cytokines (TNF-α), and matrix metalloproteinases. This "cocktail" ensures the formation of the ECM and keratinocyte proliferation and differentiation. Therefore, fibroblasts are essential for the skin remodeling and wound healing. Moreover, various immune cells (e.g., dendritic cells, leukocytes) are found and can migrate through the dermis.

Between the dermis and muscles, the hypodermis (subcutaneous tissue) is located. It protects the internal tissues and organs from cold and trauma, provides energy, and participates in the hormone synthesis (e.g., estrone, leptin). The hypodermis is formed by adipocytes structured in lobules. These lobules are separated with the septa from the connective tissue and contain nerves and lymphatic and microvascular network, which ensures nutrient and oxygen delivery.

Moreover, the skin structure also includes the skin appendages, e.g., nails, hair follicles, sweat glands, and sebaceous glands. Hair follicles, which are distributed all around the body (except palms and soles), are formed by basal cells in the basement membrane and responsible for the body temperature control and mechanic perception. Keratinized and dead cells compose nails. At the base of the hair follicles, there are sebaceous glands which produce sebum (oily substance), which ensures the skin and hair lubrication and waterproofness. Sweat glands secrete sweat onto a skin surface, and ceruminous and mammary glands are the changed sweat glands that are responsible for the cerumen and milk (respectively) production.

The recent findings have shown that the skin has its own stem cells which are rather heterogeneous and can be divided into various subtypes: epidermal, follicular, hematopoietic, melanocyte and sebaceous gland stem, mesenchymal stem-like, and neuronal progenitor cells.

Skin Tissue Regeneration Processes

Skin would healing is a systematic process, traditionally including four overlapping classic phases: hemostasis (coagulation), inflammation (mononuclear cell infiltration), proliferation (epithelialization, fibroplasia, angiogenesis, and formation of granulation tissue), and maturation (collagen deposit or scaring tissue formation). Several factors influence skin healing after burn injuries, e.g., the causes, the degree and size of burn, and the patient's general condition and types of the graft or materials for covering burn wounds.

Depending on burn severity, the healing process may result in different consequences. Superficial burns recover within two weeks and cause minimal scarring. The re-epithelization of partial thickness burns is ensured by keratinocyte migration from skin dermal appendages within a few hours of the injury. In deeper burns, the healing starts around the edges, but not at the center because of the necessity of rapid wound closure. The acceleration of early cell proliferation ensuring the rapid burn healing occurs due to dendritic cells releasing various factors. So, agents enhancing dendritic cells are considered as therapeutics improving burn wound care. Angiogenesis during burn healing is induced by hypoxia-inducible factor 1 and angiogenic cytokines such as VEGF and CXCL12 and ensured by the increase in endothelial progenitor cell blood level correlating with the skin area burnt. The increased contraction is ensured by the activation of the TGF-β pathway that causes remodeling and scar formation.

Compared to other wound types, burns may have systemic effects, influencing almost all body systems and causing changes in lung, kidney, heart, liver, gastrointestinal tract, bone marrow, and lymphoid organ functioning and multiple organ dysfunction syndrome. At the burn site, inflammatory mediators such as tumor necrosis factor alpha (TNF-α) and interleukins 6, 8, and 1-beta, responsible for systemic effects, are released. Their concentration in serum correlates with the burn surface area. The rise in their concentrations is considered to increase risk of infections, multiple organ dysfunction syndrome, and death.

Moreover, burn healing is followed by significant immune imbalance. At an early stage, the suppression of bone marrow leads to lymphoid and myeloid immune cell dysfunction, which makes infections resistant to common therapy, and may even cause sepsis. These infectious complications result in wound healing delay. Neutrophils are shown to overexpress heat shock proteins, leading to an increase in oxidative activity and a decrease in apoptosis. Thus, the inflammation phase is prolonged, and the wound site overexposed with growth factors and inflammatory mediators.

Cell Types used in Skin Regeneration

Cells are the main component of the tissue-engineered skin used for burn therapies. They include both stem and somatic cells and can be divided into three main groups: autologous, allogeneic, and xenogeneic. One of the main trends in choosing a cell type for patient treatment is the use of autologous cells as they do not cause immune rejection and their tumorigenicity is low due to the absence of epigenetic manipulations. Nowadays, animal cells are not widely used for skin tissue

regeneration, only ECM or its components that they synthesize. Plant stem cells, which are commonly applied in cosmetics, can be interesting as they have no use limitations when compared to animal and human cells. Of course, they cannot be used in skin substitute development as a cell component; but they can provide bioactive substances, which can improve the wound healing process.

Table: Somatic and stem cells used in skin tissue regeneration.

Cell types (Refs.)	Origin	Source	CT	Examples of commercial products and their indications
Fibroblasts	Allogeneic	Skin	Yes	Apligraft - Venous leg ulcers - Diabetic foot ulcers OrCel - Partial-thickness burns
		Neonatal foreskin	Yes	TransCyte - Full-thickness and deep partial-thickness burns - Partial-thickness burns Dermagraft - Full-thickness diabetic foot ulcers
		Fetus	Yes	ND
	Autologous	Skin	Yes	TissueTech Autograft System - -Diabetic foot ulcers Hyalograft 3D - Diabetic ulcer - Cartilage engineering
Keratinocytes	Allogeneic	Skin	Yes	Apligraft - Venous leg ulcers - Diabetic foot ulcers OrCel - Partial-thickness burns
		Neonatal foreskin	Yes	Lyphoderm - Chronic venous ulcer - Partial-thickness burns
		Fetus	Yes	ND
	Autologous	Skin	Yes	Epicel - Deep dermal or full thickness burns. TissueTech Autograft System - Diabetic foot ulcers Bioseed-S - Chronic venous leg ulcers Cell-spray - Partial and deep partial-thickness burns Karocells - Partial and deep partial-thickness burns
		Outer root sheath of scalp hair follicles	Yes	EpiDex - Recalcitrant vascular leg ulcers - Partial-thickness burns
ESC	Autologous	Epidermis (basal layer)	Yes	ND
MMSC	Allogeneic	Adipose tissue	Yes	ND
		Umbilical cord	Yes	ND
		Bone marrow	Yes	ND
	Autologous	Bone marrow	Yes	ND
		Adipose tissue	Yes	ND
Stromal vascular fraction	Autologous	Adipose tissue	Yes	ND
BMSC	Autologous	Bone marrow	Yes	ND
USC and secretome	Autologous or Allogeneic	Urine (kidney)	Yes	ND
iPSC	Autologous	Skin	No	ND
Vascular progenitor cells	Allogeneic	Vessels	Yes	ND

Cell types (Refs.)	Origin	Source	CT	Examples of commercial products and their indications
EPC	Allogeneic	Vessels	Yes	ND
Mononuclear cells	Autologous	Bone marrow	Yes	ND

Fibroblasts and keratinocytes are common cells used in products for wound and burn healing. Keratinocytes are the major cell component of the epidermis and responsible for its stratified structure and form numerous tight intercellular junctions. Fibroblasts are the main cell type of the dermis and produce ECM components and secrete various growth factors (TGF-β), cytokines (TNF-α), and matrix metalloproteinases, which ensure the ECM formation and keratinocyte proliferation and differentiation. Commercial products such as Epicel, Cryoskin, and BioSeed-S contain keratinocytes; Dermagraft, TransCyte and Hyalograft 3D—fibroblasts; and Apligraf, Theraskin, and OrCell—a combination. The use of these cells enables the large-scale production of standardized product batches. However, these materials are mostly non-permanent bioactive dressings, which provide cytokines, ECM, and growth factors for the successful skin reparation. Immune rejection is commonly reported with allogeneic fibroblasts and keratinocytes, but this is mostly shown for allogeneic keratinocytes that can be explained by the difference in HLA expression and cytokine production. Fetal fibroblasts are of particular interest because they can significantly improve skin repair due to the high expansion ability, low immunogenicity, and intense secretion of bioactive substances such as basic fibroblast growth factor, vascular endothelial growth factor, and keratinocyte growth factor. However, ethical issues limit their application.

Epidermal stem cells (ESC) are of particular interest for skin tissue regeneration as they have favorable features such as high proliferation rate and easy access and keep their potency and differentiation potential for long periods. They are one of the skin stem cell types, either heterogeneous or autogenous origins. ESC are mostly connected to the process of skin regeneration. They are rare, infrequently divide and generate short-lived and rapidly dividing cells, which are involved in the regeneration process. Their main population, responsible for skin repair, is located in the basal layer of the epidermis; however, they can also be revealed in the base of sebaceous glands and the bulge region of hair follicles. However, while working with ESC culture, we may face progressive aneuploidy or polyploidy and mutation accumulation after several passages. Moreover, as they can be easily derived from the patient's skin and transplanted to the same patient, ESC are not restricted by ethical issues. Grafts containing autologous holoclones ESC have proven to be effective in treating vast skin defects: epidermolysis, skin and ocular burns, etc.

Table: Subtypes of skin stem cells.

Cell type (Refs)	Localization	Specific markers
Epidermal stem cells	Basal layer of the epidermis	$b1^{high}$/melanoma chondroitin sulfate proteoglycan positive, $\alpha6^{high}/CD71^{dim}$, p63
Melanocyte stem cells	Follicle bulge region and hair germ	Dct, Pax3, Sox
Follicular stem cells	Follicle bulge region	CD34, CD200, K15, K19, Lgr5, Lhx2, NFATC1, NFIB, PHLDA1, Sox9
Hematopoietic stem cells	Follicle dermal papillae	CD34 for lymphoid and hematopoietic progenitor cells

Sebaceous gland stem cells	Sebaceous glands and infundibulum	Blimp1
Mesenchymal stem-like cells	Dermis	$CD70^+$, $CD90^+$, $CD105^+$, CD34-
Neural progenitor cells	Follicle dermal papillae	S100 for schwannomas, peripheral neural tissue astrocytes; HMB45, a neuraminidase-sensitive oligosaccharide side chain of a glycoconjugate

Mesenchymal stromal cells (MSC) have similar (not identical) features as ESC and can be derived from various tissues, even the skin as mentioned previously. They have a high differentiation potential and a certain degree of plasticity and may generate cells of mesodermal, ectodermal, and endodermal lineages. Moreover, paracrine, trophic, and immunomodulatory MSC properties enable their clinical use. MSC can migrate to the injured tissues, differentiate, and regulate the tissue regeneration by the production of growth factors, cytokines, and chemokines. Their immunomodulatory activity is based on the release of anti-inflammatory cytokines and the inhibition of proliferation of $CD4^+$ and $CD8^+$ natural killer cells, T cells, and B cells. MSC are considered to be hypoimmunogenic because they do not express class I and II molecules of the major histocompatibility complex (MHC) and co-stimulatory proteins (e.g., CD40, CD80, CD86). Therefore, the transplantation of allogenic MSC has a low risk of the immune rejection. In burn therapy, adipose-derived stromal cells refined from the stromal vascular fraction are widely applied because of their easy access and isolation procedure and inspiring improvement of the healing processes. They are showed to preserve their therapeutic effects after freezing that ensures their multiple use. It is worth mentioning that even the freshly isolated stromal vascular fraction is showed to be effective in burn therapy, but compared to adipose-derived stromal cells, it can release high concentrations of inflammatory mediators. However, the number of randomized controlled preclinical and clinical trials remains insufficient.

Among the MSC derived from other tissues (adipose tissue, umbilical cord, etc.) the MSC derived from bone marrow (BMSC) requires special attention. They also possess plasticity and can differentiate into tissues of mesodermal, ectodermal, and endodermal origin. BMSC are considered to participate in the skin development. It has been reported that bone marrow can generate not only hematopoietic and mesenchymal cells but also fibroblast-like cells that are located in the dermis and actively proliferate in the skin during the regeneration processes. The possible disadvantages of BMSC are that the tumor microenvironment may induce changes in the angiogenesis ability and anti-tumor response. Moreover, they may generate tumor-associated fibroblasts and shift a normal immune cell phenotype to an immunosuppressive and tumor promoting one.

However, nowadays, the greatest interest in tissue regeneration belongs to induced pluripotent stem cells (iPSC); using somatic cell reprogramming like a magic wand, we can develop patient-specific cells with a tailored phenotype and apply them in clinics. The most commonly used cells for cell reprogramming are dermal fibroblasts, melanocytes, and keratinocytes since they can be easily accessed and isolated from punch biopsies. Research has shown that both murine and human iPSC can be differentiated into dermal fibroblasts, keratinocytes, and melanocytes, opening a door for iPSC technology into dermatology applications. The interesting fact is that fibroblasts achieved via this technique may show increased properties compared to those of the parental fibroblasts, e.g., the exceeded ECM production. This might be related to the changed epigenetic signature that

occurs during iPSC differentiation and is critical for their use in skin tissue regeneration. However, when cells are reprogrammed with tumorigenic c-Myc and this transgene remains in iPSC, the risk of tumor formation increases, because c-Myc might be reactivated. Since modern methods for cell purification cannot ensure the full separation of differentiated cells from iPSC, undifferentiated and partly differentiated cells may be implanted into a patient and increase the possibility of tumor formation.

Growth Factor Therapy

Growth factor therapy is to administrate pro-epidermal growth factors to promote wound healing. These growth factors are bioactive molecules secreted by the body whose function is to stimulate the growth and propagation of cells involved in skin wound healing and inflammation. The use of extra-growth factor increases the number of wound-healing cells, causing faster wound healing. Despite their variety, there are five types commonly used as invigorating molecules in wound healing and regaining via benign tissue repair processes. They include compounds influencing epidermal tissue regrowth (epidermal growth factor (EGF); hepatocyte growth factor (HGF)), anti-scarring (transforming growth factor (TGF-ß3)), pro-angiogenesis (vascular endothelial growth factor (VEFG); platelet-derived growth factor (PDGF)), and stromal cell growth (fibroblast growth factor (FGF)). A combination of multiple growth factors may efficiently improve cellular functions: proliferation, migration, differentiation, collagen remodeling, inhibition of fibroblast overgrowth, ECM deposition, etc. Therefore, strategies to control growth factors release may prompt skin tissue regeneration. To optimize substance delivery and loading, bioactivity, therapeutic functionality, dosage form stability, etc., it is vital to develop platforms such as hydrogels, microbeads, or tissue-engineered constructs.

Table: Growth factor therapy for skin tissue repair.

GFs (Refs)	Delivery approach	Dose	In vivo experiment	Outcomes
EGF	Topically HA-EGF conjugate immobilized within HA films	1 µg per patch once	SD rat (full-thickness dorsal skin excision)	Being secreted by the platelets and macrophages; - Stimulating proliferation of fibroblasts, the cells that produce collagen; - Reducing the healing time of wounds when applied topically.
	Topically rhEGF-loaded lipid nanoparticles	20 µg per scar tissue twice a week	White pig (full-thickness dorsal skin excision)	
KGF	Topically KGF covalently attached to a fluorescent matrix-binding peptide encapsulated within fibrin	500 ng/ml	Athymic mouse (full-thickness dorsal skin excision)	Promotes keratinocytes growth.
TGF-β1	Topically Incorporated into polyoxamer gel	1 µg per wound	SD rat (full-thickness skin excision)	Stimulating growth and migration of keratinocytes and fibroblasts to the affected area - Promoting the growth of new blood vessels (angiogenesis), ensuring adequate blood supply to the healing wound.
TGF-β2	Subcutaneous implantation Gelatin microspheres	0.5 µg per implant	Athymic rat (subcutaneous implantation)	
HGF	Subcutaneous injection	2 mg per scar tissue once	Rabbit (full-thickness skin excision)	Reducing scarring.

GFs (Refs)	Delivery approach	Dose	In vivo experiment	Outcomes
VEGF	Implantation VEGF-loaded alginate microspheres	2 and 4 μg	Wistar rat (small incision in the groin)	Enabling the most extensive blood vessel formation with microspheres containing 4 μg of VEGF.
PDGF Regranex	Topically Carboxymethyl-cellulose hydrogel	100 μg/g	Patients with type 1 or type 2 diabetes suffering from chronic ulcers	Being secreted by the platelets, - Attracting fibroblasts and macrophages to the area of injured tissue.
TGF-β3	Topically BMSC overexpressing TGF-β3	0.5 ml (1.3×10^5 cells/ml)	Rabbit (full-thickness skin excision)	Reducing scar depth and density.
bFGF	Topically Poly(ethylene glycol)-poly(dl-lactide) microfibrous mats containing bFGF	ND	Diabetic SD rat (full-thickness dorsal skin excision)	Enabling higher complete wound closure rate - Stimulating collagen deposition and ECM remodeling bFGF-loaded mats.
HGF+bFGF	Topically Collagen/gelatin sponge	10 μg/cm^2 + 7 μg/cm^2	C57BL/6JJcl mouse (full-thickness dorsal skin excision)	Dual release of HGFC and bFGF ensured re-epithelization and angiogenesis.
Platelet-rich fibrin extract	Topically Gelatin gel	3.3 ml of blood per defect	Wistar rat (full-thickness dorsal skin excision)	Promoting neovascularization and formation of granulation tissue. - Epidermalization started in 1 week.
VEGF+PDGF+ bFGF+EGF	Topically Collagen-HA membrane	0.1 μg/mg (each)	Diabetic SD rat (full-thickness dorsal skin excision)	Increasing wound healing rate - Enhancing the collagen deposition and maturation of vessels.

To improve re-epithelialization after a burn injury, growth factors such as EGF and HGF are applied. EGF and HGF are shown to enhance epithelial cell proliferation, growth, and migration. Their potential in skin tissue regeneration is intensively studied, and various approaches to deliver them are under investigation. For example, Lee at al. achieved the improved wound healing of laser-induced burn after treatment with recombinant EGF conjugated with low molecular weight protamine. Regarding HGF, there are few in vivo studies. The EGF efficacy was proven in clinical trials.

Angiogenesis in a defect site can be promoted by PDGF and VEGF. PDGF-BB is approved by FDA for diabetic ulcer treatment, but it has low success in clinics probably due to its damage by proteolytic enzymes or low expression of PDGF-receptors. VEGF showed high efficacy in experiments in vivo (e.g.,) and passed a phase I trial proving its safety and efficacy in treatment of chronic wounds. To promote vessel formation, both PDGF and VEGF require constant application during a treatment period that has induced research to develop delivery systems with sustained release. For instance, Tan et al. revealed VEGF-loaded collagen scaffolds significantly improved the wound healing processes in diabetic rats followed by the increase in VEGF level in tissue and induced angiogenesis. Moreover, Gorkun et al. showed that VEGF-induced spheroids from adipose-derived stromal cells encapsulated within modified fibrin gel can form tubule-like network that might be interesting as a new approach to enhance angiogenesis in a wound and improve skin tissue regeneration.

The increased stromal cell growth can be achieved by the application of FGF. FGF-2 (bFGF) was shown to control ECM formation, and its use enabled the decreased scar formation and inhibition of TGF-β1/SMAD-dependent pathway. Treating deep partial-thickness burns in humans, Ma et al.

revealed that recombinant aFGF accelerated the healing rate and the healing process required less time.

The main anti-scarring agent is TGF-ß3. In TGF family, TGF-β1 and TGF-β2 stimulate fibroblast differentiation, contraction, ECM synthesis and deposition, and scarring and TGF-β3 enables the reduction in scar formation. The concentration of TGF-β isoforms varies in the fetal and adult wound healing process; in the first, the TGF- β 3 concentration is high, but TGF-β1 and TGF-β2 isoforms are absent or in a small amount, while in the second, the situation is opposite and the high TGF-β1 and TGF-β2 concentrations are caused by the platelet degranulation and synthesis in monocytes during inflammation. When TGF-β1 and TGF-β2 isoforms were blocked and TGF-β3 isoform was externally added, the wound healing occurred with the less remarkable scar formation than that in control. However, blocking all three isoforms did not ensure the scarring decrease that the complexity of molecular pathways shows. Clinical trials showed that avotermin (TGF-β3) ensured scar reduction and was well tolerated.

Growth factors for skin wounds are often applied locally (topically). One advantage of growth factor therapy is that it uses the body's own cells to promote healing. Its use may also speed up the time it takes for wounds to heal, resulting in a greater reduction of disability or discomfort for the patient. Various delivery systems are offered to ensure growth factor stability and controlled release in wounds: particulate systems, scaffolds, hydrogels, and their combinations. Moreover, devices such as microneedles and jet injectors are of potential interest although to date no studies where they have been applied to treat burns were found.

Since it is often applied topically, the incidence of systemic side effects is minimal. However, for example, high VEGF serum level causes anasacra, edema, and edema-associated burn complications although, in general, VEGF is considered to promote burn healing. EGF and PDGF can lead to the hypertrophic scarring. Also, theoretically, growth factor therapy of wounds may induce oncogenesis (for instance, TGF-β can be both a pro-oncogenic and tumor suppressing factor, and VEGF is involved in tumor formation, but in pre-clinical and clinical trials, tumor development was not revealed. Further long-term trials are required to confirm and strengthen growth factors safety.

In some cases, the use of a single growth factor may be insufficient because of the complexity of molecular pathways and wound chronicity that reveals a need to develop multiple growth factor systems with sustained release. For example, Lai et al. designed a collagen-HA membrane with immobilized VEGF, PDGF, bFGF, and EGF and showed that it efficiently induced the increase in wound healing rate by enhancing collagen deposition and neovasculogenesis compared to the control group.

Scaffold for Skin Wound Healing

Biomaterials are a crucial part of the different dressings and tissue-engineered constructs used in burn therapy. The main idea in using them is to imitate the skin ECM formed by collagen, elastin, proteoglycans, nidogen, laminin, and perlecan and its properties: the skin's strength is enabled by collagen, elastin ensures its elasticity and flexibility, and proteoglycans provide hydration and viscosity. In skin grafts and substitutes, biomaterials of various origins (natural, synthetic, or semi-synthetic) are used and their choice in the scaffold fabrication is essential because this can influence the in situ regeneration, with their features regulating cell behavior and enabling new

tissue formation. The main requirements are biodegradability, temporary mechanical support, and permeability. Depending on the approach, scaffolds may be with or without cells, and the latter can be divided into dermal, epidermal, and epidermal-dermal composites.

Table: Scaffolds applied in the skin tissue regeneration and wound healing.

Scaffolds (Refs)	Origin	BD	Cell component	CA	Example of commercial products
Decellularized material-based					
Small intestine, acellular lyophilized	Porcine	Yes	Not included	Yes	OASIS Wound Matrix
Dermis, acellular lyophilized	Allogeneic	Yes	Not included	Yes	AlloDerm, Karoderm, SureDerm
Dermis, acellular pre-meshed	Allogeneic	Yes	Not included	Yes	GraftJacket
Dermis, acellular lyophilized, coated with elastin hydrolysate	Bovine	Yes	Not included	Yes	Matriderm
Dermis, acellular diisocyanate cross-linked	Porcine	Yes	Not included	Yes	Permacol Surgical Implant
Collagen-based scaffolds					
Collagen	Bovine	Yes	Allogeneic keratinocytes and fibroblasts	Yes	Apligraft
			Autologous keratinocytes and fibroblasts	Yes	PermaDerm
Collagen, aldehyde cross-linked reconstituted	Porcine	Yes	Not included	Yes	EZ Derm
Collagen, sponge	Bovine	Yes	Allogeneic keratinocytes and fibroblasts	Yes	OrCel
Collagen, cross-linked Glycosaminoglycan Polysiloxane	Bovine/synthetic	Yes/no	Not included	Yes	Integra Dermal Regeneration
Collagen, cross-linked Glycosaminoglycan Polysiloxane	Bovine/synthetic	Yes/no	Autologous adipose-derived regenerative cells	No	ND
Collagen, lyophilized cross-linked sponge, heat-denatured Silicone	Bovine/synthetic	Yes/no	Not included	Yes	Terudermis
Atelocollagen Silicone/silicone fortified with silicone gaze TREX	Porcine/synthetic	Yes/no	Not included	Yes	Pelnac Standard/Pelnac Fortified
Collagen Silicone, film Nylon, mesh	Porcine/synthetic	Yes/no	Allogeneic fibroblasts	Yes	Biobrane/Biobrane-L, TransCyte
Hyaluronic acid-based					
Hyaluronic acid membrane (microperforated)	Recombinant	Yes	Autologous keratinocytes and fibroblasts	Yes	TissueTech Autograft System, LaserSkin (Vivoderm)
	Allogeneic	Yes	Autologous fibroblasts	Yes	Hyalograft 3D
HYAFF, derivative of hyaluronan Silicone, membrane	Allogeneic/synthetic	Yes/no	Not included	Yes	Hyalomatrix PA
Other biopolymer-based					
Silk fibroin/alginate, sponge	Xenogeneic/synthetic	Yes/no	Not included	No	ND

Cellulose, nanofibrils	Recombinant	No	Not included	No	ND
Synthetic material-based					
Polyethylene oxide terephthalate/ Polybutylene terephthalate	Synthetic	No	Autologous keratinocytes and fibroblasts	Yes	PolyActive
Polyglycolic acid/polylactic acid Extracellular matrix, derived from fibroblasts	Synthetic	Yes	Allogeneic fibroblasts	Yes	Dermagraft

To date, most products available on the world market contain collagen or decellularized tissues. This is not surprising because one of the main skin component is collagen types I and III, and therefore, the product design will be more similar to the native tissue than others. However, collagen possesses poor mechanical properties, and most scientists and manufacturers try to improve them via cross-linking or reinforcing with synthetic materials such as polylactide, polycaprolactone, and their copolymers. For instance, in TranCyte, the collagen gel is fortified with nylon mesh and covered with a silicone film; the latter enables the maintenance of moist environment. The products based on decellularized materials have more clinical limitations than collagen-based scaffolds mainly because they require specific raw materials (especially, allogeneic) and can evoke the strong immune response and calcification (especially, xenogeneic). What is remarkable is that the first FDA-approved skin substitute, Apligraf, contains bovine collagen.

It is worth mentioning that hydrogel has proven to provide the most favorable conditions for the burn healing process and is widely applied in tissue engineering. The abovementioned collagen is also a gel, and apart from collagen, gels such as fibrin, hyaluronic acid, chitosan, and alginate are used in the skin substitute and bio-ink production. The structure and properties of hydrogels (3D network, hydrophilicity, etc.) can be easily modified and are similar to those of the native ECM, enabling not only cell proliferation and differentiation but also in situ cell recruitment. Gels provide adequate moist environment that is favorable for the burn healing process. Moreover, they can deposit and deliver bioactive compounds, which then enhance the healing process.

Table: Hydrogels for cell and growth factor delivery in the skin tissue regeneration.

Polymer type	Hydrogels (Refs)	Origin	BD	CA	FDA approved	Commercial product
Protein	Collagen	Xenogeneic	Yes	Yes	Yes	Apligraf
	Gelatin	Xenogeneic	Yes	Yes	No	ND
	Fibrin	Allogeneic	Yes	Yes	No	AcuDress
Polysaccharide	Chitosan	Xenogeneic	No	Yes	No	ND
	Hyaluronic acid	Recombinant, allogeneic	Yes	Yes	Yes	LaserSkin
	Dextran	Xenogeneic (microbial)	Yes	Yes	No	ND
	Alginate	Xenogeneic	No	Yes	Yes	Kaltostat
	Glycosaminoglycan	Xenogeneic, allogeneic	Yes	Yes	No	ND
Polyether	Polyethylene glycol diacrylate	Synthetic	No	Yes	No	ND

Delivery Approaches

Currently, dressings are the most common form of cell-based products used in burn therapy. Their shape, however, does not provide a possibility to treat large and complex wounds with a heterogeneous surface profile. Therefore, such technologies as cell spraying and three-dimensional (3D) bioprinting were developed for these applications.

3D bioprinting is a multitasking platform that enables in situ cell deposition according to the wound pattern. In 3D bioprinting, cells are distributed within gels, and these mixtures are used as bio-inks. Commonly, the procedure involves printing hydrogel layers, which are further cross-linked via UV, enzymes, ions, etc., to give better support for cells.

In situ 3D bioprinting was first proposed by Campbell and Weiss for an inkjet bioprinter and is particularly interesting as a delivery approach since it can ensure the full-thickness tissue restoration followed by vasculogenesis due to progenitor cell migration and angiogenesis. Nevertheless, despite promising results, the number of studies, where this technology is used, is limited. This may be caused by the complexity of the equipment and commercial non-availability. For skin tissue applications, there are only two studies performed with human fibroblasts and keratinocytes and amniotic fluid-derived stem cells encapsulated within fibrin-collagen hydrogel and transplanted into a full-thickness wound in nude mice. Thus, after solving technical issues the idea of in situ skin bioprinting could be considered attractive for clinical translation.

Another promising delivery technology is cell spraying that allows clinicians to treat large deep burns. In most studies, scientists used autologous epidermis-derived cells. Cells are not cultured but suspended in saline. The required amount can be derived only from a small donor site. A cell suspension is sprayed homogenously onto a wound so that cells proliferate and improve re-epithelialization. Cell spraying cannot replace common autografting but can be applied easily and early to deep partial thickness burns. Many complications (poor esthetic outcome, hypertrophic scarring, contracture, etc.) may be avoided or decreased due to the early re-epithelialization after a cell spray. Nevertheless, this technology is expensive and needs special equipment, aseptic rooms, and highly qualified personnel as 3D bioprinting.

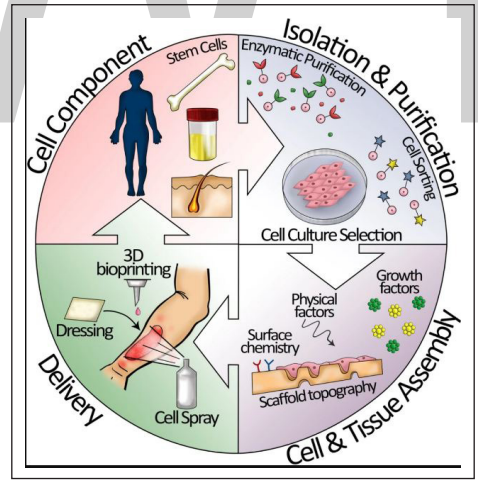
Challenges and Future Directions

To date, despite imperfections, the existing dressings and tissue-engineered skin substitutes have significantly improved clinical insight into burn treatment, allowing clinicians to treat severe cases that increase patients' survival rates and quality of life. Most of them only aim to temporarily protect the denuded tissue from the aggressive environment and provide cytokines and growth factors to enhance the wound healing process. There is no doubt that commercial products based on autologous cells (fibroblasts and keratinocytes) are close to the native skin and enable the successful skin repair but they cannot fully replace the injured tissue.

Many issues limit the introduction and rapid expansion of new products for cell-based therapies. First of all, their production is time- and labor-consuming and requires complex and specific equipment. To cover the extensive burn areas, a huge number of cells is needed, and if they are not autologous or hypoimmunogenic, a substitute can be rejected. These products should be transported and stored under certain conditions, which are hard to maintain, and their shelf-life is short. When autologous cells are applied, the work of cell culture facilities and surgeons should

be well coordinated. Moreover, the cost of treatment with skin substitutes is high but the only one function, protective, can be replaced with them: all these tissue-engineered constructs cannot restore thermoregulation, sensation, UV-protection, excretion, perspiration, etc.

Nowadays, in the design of skin substitute, there are three main approaches: cell-based, biomaterial-based, and delivery-based. In the first, scientists try to fabricate skin equivalents using not only fibroblasts and keratinocytes, but also melanocytes and endothelial cells in order to imitate native tissue morphology. In many studies, stem cells derived from various sources are used for their properties such as hypo-immunogenicity and high differentiation potential. The use of autologous and allogeneic cells still remains questionable. Although there are studies showing that only autologous cells can promote rapid wound healing, a bank of allogeneic cells can provide a possibility to treat quickly patients suffering extensive and deep second-degree burns, and in this case, the most preferable cells are stem cells (e.g., adipose-derived or bone-marrow derived stem cells) possessing hypo-immunogenicity. Moreover, attempts to reproduce skin appendages (for instance, hair follicles and sebaceous glands) in vitro and integrated into skin substitutes are made. The second approach tries to functionalize scaffolds with different methods. For example, the immobilization of signaling molecules on their surface can promote the cell proliferation and differentiation and control cell-matrix adhesion. According to the third approach, researchers try to develop a new delivery system or to improve the existing ones. To ensure burn healing, cells can be injected intravenously, or, more often, they are immobilized on various materials and applied topically as dressings. For sure, dressings are the most common system, but they cannot be precisely adjusted to the wound surface profile. Therefore, technologies such as cell spray and bio-printing are of particular interest and are able to solve this issue.



Procedure of autologous stem cell-based therapy on burn injury.

Moreover, the stem cells described above can enable true skin regeneration and decrease scar formation and have the clear manipulation step procedure for autologous use. Preclinical and clinical studies have shown that bone marrow, urine, adipose-derived, and other stem cells can significantly improve the wound healing process in chronic wounds. However, despite these successful results, still FDA has not approved any stem cell-based skin substitute for wound treatment, and for them to make the approval, certain points such as optimal cell type and population and time and way of administration should be clarified. There is an essential need to find out the mechanisms of cell action, survival, and incorporation after transplantation and their stability and differentiation

features in the wound microenvironment. Moreover, the delayed postoperative outcomes should be studied in large-scale clinical trials to prove the safety of stem cell-based products. Thus, stem cells are a promising tool for skin substitute design and fabrication for advanced burn therapies.

As the number of findings proving its safety and efficacy is growing, cell-based therapy is becoming a great alternative in burn care. However, some essential points required to standardize all related procedures and prepare guidelines for clinicians are still unclear. In most studies, cells and cell-based products are applied once topically, but these measures can be insufficient in case of extensive burns causing systemic inflammation and hypohydration and only intravenous injections of cells can improve the patient's condition. Autologous cells are considered to be preferable although their use is impossible in large burns because of the lack of donor sites and time. Moreover, especially in case of acute burns, the successful outcomes of cell-based therapy depend on intervention timing defined by coordination between clinicians and cell facilities staff. Thus, despite outstanding results of cell applications in burn care, the mentioned above issues should be solved to exploit the whole potential of cell-based therapy.

Stem Cell Transplantation for Articular Cartilage Repair

Mesenchymal stem cells (MSCs) are multipotent cells found in multiple human adult tissues including bone marrow, synovial tissues, and adipose tissues. Since they are derived from the mesoderm, they have been shown to differentiate into bone, cartilage, muscle, and adipose tissue. MSCs from embryonic sources have shown promise scientifically while creating significant controversy. As a result, many researchers have focused on adult stem cells, or stem cells isolated from adult humans that can be transplanted into damaged tissue.

Because of their multi-potent capabilities, mesenchymal stem cell (MSC) lineages have been used successfully in animal models to regenerate articular cartilage and in human models to regenerate bone. Recent research demonstrates that articular cartilage may be able to be repaired via percutaneous introduction of mesenchymal stem cells (MSC's).

Research into MSC's has exploded in recent years. As an example, a PubMed search for the year 1999 reveals about 90 papers published under the MESH heading of "Mesenchymal Stem Cells", the same search ran for the year 2007 reveals more than 4,000 entries. The most commonly used source of MSC's is bone marrow aspirate. Most of the adult bone marrow consists of blood cells in various stages of differentiation. These marrow components can be divided into plasma, red blood cells, platelets, and nucleated cells. The adult stem cell fraction is present in the nucleated cells of the marrow. Most of these cells are CD34+ heme progenitors (destined to differentiate into blood components), while very few are actually MSC's capable of differentiating into bone, cartilage, or muscle. As a result, that leaves the very small number of MSC's in the marrow as cells capable of differentiating into tissues of interest to joint preservation. Of note, this may be one of the reasons that commercially available centrifuge systems that concentrate marrow nucleated cells have not shown as much promise in animal research for cartilage repair as have approaches where MSC's are expanded in culture to greater numbers.

Mesenchymal Stem Cell Applications

Marrow nucleated cells are used every day in regenerative orthopedics. The knee microfracture surgery technique relies on the release of these cells into a cartilage lesion to initiate fibrocartilage repair in osteochondral defects. In addition, this cell population has also been shown to assist in the repair of non-union fractures. For this application, bed side centrifugation is commonly used. Again, these techniques produce a very dilute MSC population, usually a yield of 1 in 10,000–1,000,000 of the nucleated cells. Despite this low number of MSC's, isolated bone marrow nucleated cells implanted into degenerated human peripheral joints have shown some promise for joint repair. As the number of MSC's that can be isolated from bone marrow is fairly limited, most research in cartilage regeneration has focused on the use of culture expanded cells. This method can expand cell numbers by 100-10,000 fold over several weeks. Once these MSCs are ready for re-implanation, they are usually transferred with growth factors to allow for continued cell growth and engraftment to the damaged tissue. At some point, a signal is introduced (either in culture or after transplant to the damaged tissue) for the cells to differentiate into the end tissue (in this discussion, cartilage).

Recent Developments

Until recently, the use of cultured mesenchymal stem cells to regenerate cartilage has been primarily in research with animal models. There are now, however, two published case reports of the above technique being used to successfully regenerate articular and meniscus cartilage in human knees. This technique has yet to be shown effective in a study involving a larger group of patients, however the same team of researchers have published a large safety study (n=227) showing fewer complications than would normally be associated with surgical procedures.

Another team used a similar technique for cell extraction and ex vivo expansion but cells were embedded within a collagen gel before being surgically re-implanted. They reported a case study in which a full-thickness defect in the articular cartilage of a human knee was successfully repaired.

While the use of cultured mesenchymal stem cells has shown promising results, a more recent study using uncultured MSC's has resulted in full thickness, histologically confirmed hyaline cartilage regrowth. Researchers evaluated the quality of the repair knee cartilage after arthroscopic microdrilling (also microfracture) surgery followed by post-operative injections of autologous peripheral blood progenitor cells (PBPC) in combination with hyaluronic acid (HA).

Hematopoietic Stem Cell Transplantation

Hematopoietic stem cell transplantation (HSCT) is the transplantation of multipotent hematopoietic stem cells, usually derived from bone marrow, peripheral blood, or umbilical cord blood. It may be autologous (the patient's own stem cells are used), allogeneic (the stem cells come from a donor) or syngeneic (from an identical twin).

It is most often performed for patients with certain cancers of the blood or bone marrow, such as multiple myeloma or leukemia. In these cases, the recipient's immune system is usually destroyed

with radiation or chemotherapy before the transplantation. Infection and graft-versus-host disease are major complications of allogeneic HSCT.

Hematopoietic stem cell transplantation remains a dangerous procedure with many possible complications; it is reserved for patients with life-threatening diseases. As survival following the procedure has increased, its use has expanded beyond cancer to autoimmune diseases and hereditary skeletal dysplasias; notably malignant infantile osteopetrosis and mucopolysaccharidosis.

Medical Uses

Indications

Indications for stem cell transplantation are as follows:

Malignant (Cancerous)

- Acute myeloid leukemia (AML),
- Chronic myeloid leukemia (CML),
- Acute lymphoblastic leukemia (ALL),
- Hodgkin lymphoma (HL) (relapsed, refractory),
- Non-Hodgkin lymphoma (NHL) (relapsed, refractory),
- Neuroblastoma,
- Ewing sarcoma,
- Multiple myeloma,
- Myelodysplastic syndromes,
- Gliomas, other solid tumors.

Non-malignant (Non-cancerous)

- Thalassemia,
- Sickle cell anemia,
- Aplastic anemia,
- Fanconi anemia,
- Malignant infantile osteopetrosis,
- Mucopolysaccharidosis,
- Immune deficiency syndromes,
- Autoimmune diseases.

Many recipients of HSCTs are multiple myeloma or leukemia patients who would not benefit from

prolonged treatment with, or are already resistant to, chemotherapy. Candidates for HSCTs include pediatric cases where the patient has an inborn defect such as severe combined immunodeficiency or congenital neutropenia with defective stem cells, and also children or adults with aplastic anemia who have lost their stem cells after birth. Other conditions treated with stem cell transplants include sickle-cell disease, myelodysplastic syndrome, neuroblastoma, lymphoma, Ewing's sarcoma, desmoplastic small round cell tumor, chronic granulomatous disease, Hodgkin's disease and Wiskott-Aldrich syndrome. More recently non-myeloablative, mini transplant (microtransplantation)," procedures have been developed that require smaller doses of preparative chemotherapy and radiation. This has allowed HSCT to be conducted in the elderly and other patients who would otherwise be considered too weak to withstand a conventional treatment regimen.

Number of Procedures

In 2006, a total of 50,417 first hematopoietic stem cell transplants were recorded worldwide, according to a global survey of 1,327 centers in 71 countries conducted by the Worldwide Network for Blood and Marrow Transplantation. Of these, 28,901 (57 percent) were autologous and 21,516 (43 percent) were allogeneic (11,928 from family donors and 9,588 from unrelated donors). The main indications for transplant were lymphoproliferative disorders (55 percent) and leukemias (34 percent), and the majority took place in either Europe (48 percent) or the Americas (36 percent).

The Worldwide Network for Blood and Marrow Transplantation reported the millionth transplant to have been undertaken in December 2012.

In 2014, according to the World Marrow Donor Association (WMDA), stem cell products provided for unrelated transplantation worldwide had increased to 20,604 (4,149 bone marrow donations, 12,506 peripheral blood stem cell donations, and 3,949 cord blood units).

Graft Types

Autologous

Autologous HSCT requires the extraction (apheresis) of hematopoietic stem cells (HSC) from the patient and storage of the harvested cells in a freezer. The patient is then treated with high-dose chemotherapy with or without radiotherapy with the intention of eradicating the patient's malignant cell population at the cost of partial or complete bone marrow ablation (destruction of patient's bone marrow's ability to grow new blood cells). The patient's own stored stem cells are then transfused into his/her bloodstream, where they replace destroyed tissue and resume the patient's normal blood cell production. Autologous transplants have the advantage of lower risk of infection during the immune-compromised portion of the treatment since the recovery of immune function is rapid. Also, the incidence of patients experiencing rejection is very rare (and graft-versus-host disease impossible) due to the donor and recipient being the same individual. These advantages have established autologous HSCT as one of the standard second-line treatments for such diseases as lymphoma.

However, for other cancers such as acute myeloid leukemia, the reduced mortality of the autogenous relative to allogeneic HSCT may be outweighed by an increased likelihood of cancer relapse and related mortality, and therefore the allogeneic treatment may be preferred for those conditions.

Researchers have conducted small studies using non-myeloablative HSCT as a possible treatment for type I (insulin dependent) diabetes in children and adults. Results have been promising; however, as of 2019 it was premature to speculate whether these experiments will lead to effective treatments for diabetes.

Allogeneic

Allogeneic HSCT involves two people: the (healthy) donor and the (patient) recipient. Allogeneic HSC donors must have a tissue (human leukocyte antigen, HLA) type that matches the recipient. Matching is performed on the basis of variability at three or more loci of the HLA gene, and a perfect match at these loci is preferred. Even if there is a good match at these critical alleles, the recipient will require immunosuppressive medications to mitigate graft-versus-host disease. Allogeneic transplant donors may be *related* (usually a closely HLA matched sibling), *syngeneic* (a monozygotic or 'identical' twin of the patient – necessarily extremely rare since few patients have an identical twin, but offering a source of perfectly HLA matched stem cells) or *unrelated* (donor who is not related and found to have very close degree of HLA matching). Unrelated donors may be found through a registry of bone marrow donors, such as the National Marrow Donor Program in the U.S. People who would like to be tested for a specific family member or friend without joining any of the bone marrow registry data banks may contact a private HLA testing laboratory and be tested with a blood test or mouth swab to see if they are a potential match. A "savior sibling" may be intentionally selected by preimplantation genetic diagnosis in order to match a child both regarding HLA type and being free of any obvious inheritable disorder. Allogeneic transplants are also performed using umbilical cord blood as the source of stem cells. In general, by transfusing healthy stem cells to the recipient's bloodstream to reform a healthy immune system, allogeneic HSCTs appear to improve chances for cure or long-term remission once the immediate transplant-related complications are resolved.

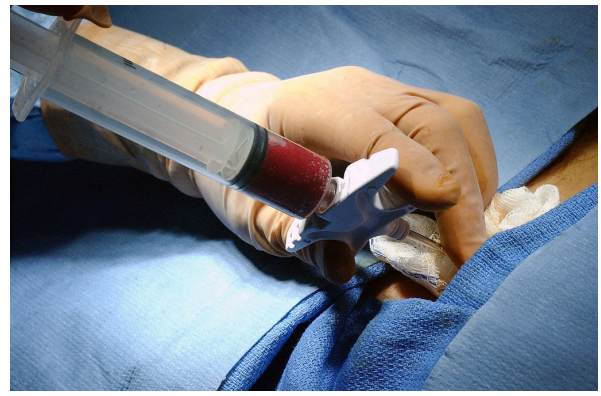
A compatible donor is found by doing additional HLA-testing from the blood of potential donors. The HLA genes fall in two categories (Type I and Type II). In general, mismatches of the Type-I genes (i.e. HLA-A, HLA-B, or HLA-C) increase the risk of graft rejection. A mismatch of an HLA Type II gene (i.e. HLA-DR, or HLA-DQB1) increases the risk of graft-versus-host disease. In addition, a genetic mismatch as small as a single DNA base pair is significant so perfect matches require knowledge of the exact DNA sequence of these genes for both donor and recipient. Leading transplant centers currently perform testing for all five of these HLA genes before declaring that a donor and recipient are HLA-identical.

Race and ethnicity are known to play a major role in donor recruitment drives, as members of the same ethnic group are more likely to have matching genes, including the genes for HLA. As of 2013, there were at least two commercialized allogeneic cell therapies, Prochymal and Cartistem.

Sources and Storage of Cells

To limit the risks of transplanted stem cell rejection or of severe graft-versus-host disease in allogeneic HSCT, the donor should preferably have the same HLA-typing as the recipient. About 25 to 30 percent of allogeneic HSCT recipients have an HLA-identical sibling. Even so-called "perfect matches" may have mismatched minor alleles that contribute to graft-versus-host disease.

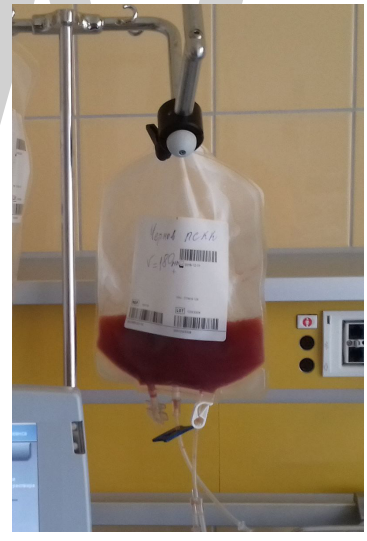
Bone Marrow



Bone marrow harvest.

In the case of a bone marrow transplant, the HSC are removed from a large bone of the donor, typically the pelvis, through a large needle that reaches the center of the bone. The technique is referred to as a bone marrow harvest and is performed under local or general anesthesia.

Peripheral Blood Stem Cells



Peripheral blood stem cells.

Peripheral blood stem cells are now the most common source of stem cells for HSCT. They are collected from the blood through a process known as apheresis. The donor's blood is withdrawn through a sterile needle in one arm and passed through a machine that removes white blood cells. The red blood cells are returned to the donor. The peripheral stem cell yield is boosted with daily subcutaneous injections of granulocyte-colony stimulating factor, serving to mobilize stem cells from the donor's bone marrow into the peripheral circulation.

Amniotic Fluid

It is also possible to extract stem cells from amniotic fluid for both autologous or heterologous use at the time of childbirth.

Umbilical Cord Blood

Umbilical cord blood is obtained when a mother donates her infant's umbilical cord and placenta after birth. Cord blood has a higher concentration of HSC than is normally found in adult blood. However, the small quantity of blood obtained from an umbilical cord (typically about 50 mL) makes it more suitable for transplantation into small children than into adults. Newer techniques using *ex vivo* expansion of cord blood units or the use of two cord blood units from different donors allow cord blood transplants to be used in adults.

Cord blood can be harvested from the umbilical cord of a child being born after preimplantation genetic diagnosis (PGD) for human leukocyte antigen (HLA) matching in order to donate to an ill sibling requiring HSCT.

Storage of HSC

Unlike other organs, bone marrow cells can be frozen (cryopreserved) for prolonged periods without damaging too many cells. This is a necessity with autologous HSC because the cells must be harvested from the recipient months in advance of the transplant treatment. In the case of allogeneic transplants, fresh HSC are preferred in order to avoid cell loss that might occur during the freezing and thawing process. Allogeneic cord blood is stored frozen at a cord blood bank because it is only obtainable at the time of childbirth. To cryopreserve HSC, a preservative, dimethyl sulfoxide, must be added, and the cells must be cooled very slowly in a controlled-rate freezer to prevent osmotic cellular injury during ice crystal formation. HSC may be stored for years in a *cryofreezer,* which typically uses liquid nitrogen.

Conditioning Regimens

Myeloablative

The chemotherapy or irradiation given immediately prior to a transplant is called the *conditioning regimen*, the purpose of which is to help eradicate the patient's disease prior to the infusion of HSC and to suppress immune reactions. The bone marrow can be *ablated* (destroyed) with dose-levels that cause minimal injury to other tissues. In allogeneic transplants a combination of cyclophosphamide with total body irradiation is conventionally employed. This treatment also has an immunosuppressive effect that prevents rejection of the HSC by the recipient's immune system. The post-transplant prognosis often includes acute and chronic graft-versus-host disease that may be life-threatening. However, in certain leukemias this can coincide with protection against cancer relapse owing to the graft-versus-tumor effect. *Autologous* transplants may also use similar conditioning regimens, but many other chemotherapy combinations can be used depending on the type of disease.

Non-myeloablative

A newer treatment approach, non-myeloablative allogeneic transplantation, also termed reduced-intensity conditioning (RIC), uses doses of chemotherapy and radiation too low to eradicate all the bone marrow cells of the recipient. Instead, non-myeloablative transplants run lower risks of serious infections and transplant-related mortality while relying upon the *graft versus tumor*

effect to resist the inherent increased risk of cancer relapse. Also significantly, while requiring high doses of immunosuppressive agents in the early stages of treatment, these doses are less than for conventional transplants. This leads to a state of mixed chimerism early after transplant where both recipient and donor HSC coexist in the bone marrow space.

Decreasing doses of immunosuppressive therapy then allow donor T-cells to eradicate the remaining recipient HSC and to induce the graft-versus-tumor effect. This effect is often accompanied by mild graft-versus-host disease, the appearance of which is often a surrogate marker for the emergence of the desirable graft versus tumor effect, and also serves as a signal to establish an appropriate dosage level for sustained treatment with low levels of immunosuppressive agents.

Because of their gentler conditioning regimens, these transplants are associated with a lower risk of transplant-related mortality and therefore allow patients who are considered too high-risk for conventional allogeneic HSCT to undergo potentially curative therapy for their disease. The optimal conditioning strategy for each disease and recipient has not been fully established, but RIC can be used in elderly patients unfit for myeloablative regimens, for whom a higher risk of cancer relapse may be acceptable.

Engraftment

After several weeks of growth in the bone marrow, expansion of HSC and their progeny is sufficient to normalize the blood cell counts and re-initiate the immune system. The offspring of donor-derived hematopoietic stem cells have been documented to populate many different organs of the recipient, including the heart, liver, and muscle, and these cells had been suggested to have the abilities of regenerating injured tissue in these organs. However, recent research has shown that such lineage infidelity does not occur as a normal phenomenon.

Chimerism monitoring is a method to monitor the balance between the patient's own stem cells and the new stem cells from a donor. In case the patient's own stem cells are increasing in number post-treatment, this might be a sign the treatment did not work as intended.

Complications

HSCT is associated with a high treatment-related mortality in the recipient, which limits its use to conditions that are themselves life-threatening. (The one-year survival rate has been estimated to be roughly 60%, although this figure includes deaths from the underlying disease as well as from the transplant procedure.) Major complications include veno-occlusive disease, mucositis, infections (sepsis), graft-versus-host disease, and the development of new malignancies.

Infection

Bone marrow transplantation usually requires that the recipient's own bone marrow be destroyed (myeloablation). Prior to the administration of new cells (engraftment), patients may go for several weeks without appreciable numbers of white blood cells to help fight infection. This puts a patient at high risk of infections, sepsis and septic shock, despite prophylactic antibiotics. However, antiviral medications, such as acyclovir and valacyclovir, are quite effective in prevention of HSCT-related outbreak of herpetic infection in seropositive patients. The

immunosuppressive agents employed in allogeneic transplants for the prevention or treatment of graft-versus-host disease further increase the risk of opportunistic infection. Immunosuppressive drugs are given for a minimum of 6-months after a transplantation, or much longer if required for the treatment of graft-versus-host disease. Transplant patients lose their acquired immunity, for example immunity to childhood diseases such as measles or polio. For this reason transplant patients must be re-vaccinated with childhood vaccines once they are off immunosuppressive medications.

Veno-occlusive Disease

Severe liver injury can result from hepatic veno-occlusive disease (VOD), newly termed sinusoidal obstruction syndrome (SOS). Elevated levels of bilirubin, hepatomegaly, and fluid retention are clinical hallmarks of this condition. There is now a greater appreciation of the generalized cellular injury and obstruction in hepatic vein sinuses. Severe cases of SOS are associated with a high mortality rate. Anticoagulants or defibrotide may be effective in reducing the severity of VOD but may also increase bleeding complications. Ursodiol has been shown to help prevent VOD, presumably by facilitating the flow of bile.

Mucositis

The injury of the mucosal lining of the mouth and throat is a common regimen-related toxicity following ablative HSCT regimens. It is usually not life-threatening but is very painful, and prevents eating and drinking. Mucositis is treated with pain medications plus intravenous infusions to prevent dehydration and malnutrition.

Hemorrhagic Cystitis

The mucosal lining of the bladder could also be involved in approximately 5 percent of the children undergoing HSCT. This causes hematuria (blood in urine), frequent urination, abdominal pain, and thrombocytopenia.

Graft-versus-host Disease

Graft-versus-host disease (GVHD) is an inflammatory disease that is unique to allogeneic transplantation. It is an attack by the "new" bone marrow's immune cells against the recipient's tissues. This can occur even if the donor and recipient are HLA-identical because the immune system can still recognize other differences between their tissues. It is aptly named graft-versus-host disease because bone marrow transplantation is the only transplant procedure in which the transplanted cells must accept the body rather than the body accepting the new cells.

Acute graft-versus-host disease typically occurs in the first 3 months after transplantation and may involve the skin, intestine, or the liver. High-dose corticosteroids, such as prednisone, are a standard treatment; however this immunosuppressive treatment often leads to deadly infections. *Chronic graft-versus-host disease* may also develop after allogeneic transplant. It is the major source of late treatment-related complications, although it less often results in death. In addition to inflammation, chronic graft-versus-host disease may lead to the development of fibrosis, or scar tissue, similar to scleroderma; it may cause functional disability and require prolonged

immunosuppressive therapy. Graft-versus-host disease is usually mediated by T cells, which react to foreign peptides presented on the MHC of the host.

Graft-versus-tumor effect

Graft-versus-tumor effect (GVT) or "graft versus leukemia" effect is the beneficial aspect of the graft-versus-host phenomenon. For example, HSCT patients with either acute, or in particular chronic, graft-versus-host disease after an allogeneic transplant tend to have a lower risk of cancer relapse. This is due to a therapeutic immune reaction of the grafted donor T lymphocytes against the diseased bone marrow of the recipient. This lower rate of relapse accounts for the increased success rate of allogeneic transplants, compared to transplants from identical twins, and indicates that allogeneic HSCT is a form of immunotherapy. GVT is the major benefit of transplants that do not employ the highest immunosuppressive regimens.

Graft versus tumor is mainly beneficial in diseases with slow progress, e.g. chronic leukemia, low-grade lymphoma, and in some cases multiple myeloma. However, it is less effective in rapidly growing acute leukemias.

If cancer relapses after HSCT, another transplant can be performed, infusing the patient with a greater quantity of donor white blood cells (donor lymphocyte infusion).

Oral Carcinoma

Patients after HSCT are at a higher risk for oral carcinoma. Post-HSCT oral cancer may have more aggressive behavior with poorer prognosis, when compared to oral cancer in non-HSCT patients.

Prognosis

Prognosis in HSCT varies widely dependent upon disease type, stage, stem cell source, HLA-matched status (for allogeneic HSCT), and conditioning regimen. A transplant offers a chance for cure or long-term remission if the inherent complications of graft versus host disease, immunosuppressive treatments and the spectrum of opportunistic infections can be survived. In recent years, survival rates have been gradually improving across almost all populations and subpopulations receiving transplants.

Mortality for allogeneic stem cell transplantation can be estimated using the prediction model created by Sorror *et al.*, using the Hematopoietic Cell Transplantation-Specific Comorbidity Index (HCT-CI). The HCT-CI was derived and validated by investigators at the Fred Hutchinson Cancer Research Center in the U.S. The HCT-CI modifies and adds to a well-validated comorbidity index, the Charlson Comorbidity Index (CCI). The CCI was previously applied to patients undergoing allogeneic HCT, but appears to provide less survival prediction and discrimination than the HCT-CI scoring system.

Risks to Donor

The risks of a complication depend on patient characteristics, health care providers and the apheresis procedure, and the colony-stimulating factor used (G-CSF). G-CSF drugs include filgrastim (Neupogen, Neulasta), and lenograstim (Graslopin).

Drug Risks

Filgrastim is typically dosed in the 10 microgram/kg level for 4–5 days during the harvesting of stem cells. The documented adverse effects of filgrastim include splenic rupture, acute respiratory distress syndrome (ARDS), alveolar hemorrhage, and allergic reactions (usually experienced in first 30 minutes). In addition, platelet and hemoglobin levels dip post-procedure, not returning to normal until after one month.

The question of whether geriatrics (patients over 65) react the same as patients under 65 has not been sufficiently examined. Coagulation issues and inflammation of atherosclerotic plaques are known to occur as a result of G-CSF injection. G-CSF has also been described to induce genetic changes in agranulocytes of normal donors. There is no statistically significant evidence either for or against the hypothesis that myelodysplasia (MDS) or acute myeloid leukaemia (AML) can be induced by G-CSF in susceptible individuals.

Access Risks

Blood is drawn from a peripheral vein in a majority of patients, but a central line to the jugular, subclavian, and femoral veins may be used. Adverse reactions during apheresis were experienced in 20 percent of women and 8 percent of men, these adverse events primarily consisted of numbness/tingling, multiple line attempts, and nausea.

Clinical Observations

A study involving 2,408 donors (aged 18–60 years) indicated that bone pain (primarily back and hips) as a result of filgrastim treatment is observed in 80 percent of donors. Donation is not recommended for those with a history of back pain. Other symptoms observed in more than 40 percent of donors include muscle pain, headache, fatigue, and difficulty sleeping. These symptoms all returned to baseline one month post-donation in the majority of patients.

In one meta-study that incorporated data from 377 donors, 44 percent of patients reported having adverse side effects after peripheral blood HSCT. Side effects included pain prior to the collection procedure as a result of G-CSF injections, and post-procedural generalized skeletal pain, fatigue and reduced energy.

Severe Reactions

A study that surveyed 2,408 donors found that serious adverse events (requiring prolonged hospitalization) occurred in 15 donors (at a rate of 0.6 percent), although none of these events were fatal. Donors were not observed to have higher than normal rates of cancer with up to 4–8 years of follow up. One study based on a survey of medical teams covered approximately 24,000 peripheral blood HSCT cases between 1993 and 2005, and found a serious cardiovascular adverse reaction rate of about 1 in 1,500. This study reported a cardiovascular-related fatality risk within the first 30 days of HSCT of about 2 in 10,000.

Donor Registration and Recruitment

At the end of 2012, 20.2 million people had registered their willingness to be a bone marrow donor

with one of the 67 registries from 49 countries participating in Bone Marrow Donors Worldwide. 17.9 million of these registered donors had been ABDR typed, allowing easy matching. A further 561,000 cord blood units had been received by one of 46 cord blood banks from 30 countries participating. The highest total number of bone marrow donors registered were those from the U.S. (8.0 million), and the highest number per capita were those from Cyprus (15.4 percent of the population).

Within the U.S., racial minority groups are the least likely to be registered and therefore the least likely to find a potentially life-saving match. In 1990, only six African-Americans were able to find a bone marrow match, and all six had common European genetic signatures.

Africans are more genetically diverse than people of European descent, which means that more registrations are needed to find a match. Bone marrow and cord blood banks exist in South Africa, and a new program is beginning in Nigeria. Many people belonging to different races are requested to donate as there is a shortage of donors in African, mixed race, Latino, aboriginal, and many other communities.

Two registries in the U.S. recruit unrelated allogeneic donors: NMDP or Be the Match, and the Gift of Life Marrow Registry.

Other Autoimmune Neurological Diseases

HSCT can also be used for treating selected, severe cases of other autoimmune neurological diseases such as neuromyelitis optica, chronic inflammatory demyelinating polyneuropathy, and myasthenia gravis.

Clinical uses of Mesenchymal Stem Cell

Adult mesenchymal stem cells (MSCs) are being used by researchers in the fields of regenerative medicine and tissue engineering, to artificially reconstruct human tissue which has been previously damaged. Mesenchymal stem cells have the capacity to become any type of fully developed cell, which can contribute to replacing muscle tissues or internal organs. To help discover the therapeutic uses of these stem cells they are grown in laboratories or by using medication to stimulate new cell growth within the human body. In MSC therapy the cells are extracted from the adult patient's bone marrow via a procedure called bone marrow aspiration. This usually involves inserting a needle into the back of the patients hip bone and removing the sample from there. These cells are then grown under controlled *in vitro* conditions in a lab, so that they can multiply and same time mature(also referred to as differentiated. This process may take two to three weeks. The kind of mature, fully differentiated cell phenotype and the number of those cells created though this can be influenced in three ways. Firstly by varying the initial seed density in the culture medium, secondly through changing the conditions of the medium during expansion, and lastly through the addition of additives such as proteins or growth hormones to the culture medium. They are then harvested and put back into the patient through local delivery or systemic infusion.

Therapeutic Properties

MSCs possess many properties that are ideal for the treatment of inflammatory and degenerative diseases. They can differentiate into many cell types including bone, fat, and muscle which allow them to treat a large range of disorders. They possess natural abilities to detect changes in their environment, such as inflammation. They can then induce the release of bioactive agents and the formation of progenitor cells in response to these changes. MSCs have also been shown to travel to sites of inflammation far from the injection site.

MSCs can be easily extracted through well-established procedures such as bone marrow aspiration. Also, transplanted MSCs pose little risk for rejection as they are derived from the patients own tissue, so are genetically identical, however graft versus host disease is a possibility, where the cells change enough while outside the patient's body that the immune system recognizes them as foreign and can attempt to reject them. This can lead to symptoms such as itchiness, sensitive/raw skin and shedding or dry skin.

Advantages Over Embryonic Stem Cells

Several different forms of stem cells have been identified and studied in the field of regenerative medicine. One of the most extensively studied stem cell types are embryonic stem cells (ESCs). ESCs possess many of the same therapeutic properties as MSCs, including the ability to self-regenerate and differentiate into a number of cell lineages. Their therapeutic abilities have been demonstrated in a number of studies of autoimmunity and neurodegeneration in animal models.

However, their therapeutic potential has been largely limited by several key factors. Injected ESCs have been shown to increase the risk for tumor formation in the host patient. Also, the host's immune system may reject injected ESCs and thus eliminate their therapeutic effects. Finally, research has been largely limited due to the ethical issues that surround their controversial procurement from fertilized embryos.

Treated Disorders

MSCs have been used to treat a variety of disorders including cardiovascular diseases, spinal cord injury, bone and cartilage repair, and autoimmune diseases.

Treatment for Multiple Sclerosis

A vast amount research has been conducted in recent years for the use of MSCs to treat multiple sclerosis (MS). This form of treatment for the disease has been tested in many studies of experimental allergic encephalomyelitis, the animal model of MS, and several published and on-going phase I and phase II human trials.

Treatment Requirements

Current treatments are unable to prevent the accumulation of irreversible damage to the central nervous system (CNS). MS patients experience two major forms of damage, damage resulting from on-going autoimmune induced processes and damage to natural pair mechanisms. Therefore, an

ideal treatment must possess both immunomodulating properties to control irregular autoimmune responses to prevent further damage and regenerative properties to stimulate natural repair mechanisms and replace damaged cells.

Therapeutic Mechanisms

The exact therapeutic mechanisms of MSCs in the treatment of MS are still very much up to debate among stem cell researchers. Some of the suggested mechanisms are immunomodulation, neuroprotection, and neuroregeneration.

Immunomodulation

MSCs exhibit immunmodulatory properties through the release of bioactive agents such as cytokines that can inhibit autoimmune responses. In patients with MS, autoreactive lymphocytes such as T and B cells cause damage to the CNS by attacking myelin proteins. Myelin proteins make up the myelin sheath that functions in protecting nerve axons, maintaining structural integrity, and enabling the efficient transmission of nerve impulses. By suppressing the unregulated proliferation of T and B cells, MSCs can potentially minimize and control on-going damage to the CNS.

MSCs can also produce an immunomodulating effect by stimulating the maturation of antigen presenting cells. Antigen presenting cells trigger the immune system to produce antibodies that can destroy potentially harmful material. This property allows MSCs to actively contribute to neutralizing harmful autoreactive by-products of MS.

Neuroprotection

MSCs can promote neuroprotection in the CNS of patients with MS which may prevent the progression of the disease to chronic disability. MSCs contribute to neuroprotection through several different mechanisms. These mechanisms include inhibiting apoptosis which will prevent the death of healthy cells and prevent gliosis which will prevent the formation of a glial scar. They can also stimulate local progenitor cells to produce replacement cells that can assist in rebuilding the myelin sheath.

Neuroregeneration

The CNS's regenerative abilities are greatly decreased in adults, impairing its ability to regenerate axons following injury. In addition to this natural limitation, MS patients exhibit even greater decreases in neuroregeneration coinciding with increases in neurodegeneration. In particular, MS patients experience a significant decrease in the number of neural stem cells which are responsible for producing large numbers of progenitor cells that are necessary for normal maintenance and function. Decreases in the neural stem cells results in severe damage to the CNS's ability to repair itself. This process results in the amplified neurodegeneration exhibited in MS patients.

MSCs have the ability to stimulate neuroregeneration by contributing to cell replacement through differentiating into neural stem cells in response to inflammation. The neural stem cells can then promote the repair of damaged axons and create replacement cells for the damaged tissue.

Regeneration and repair of damaged axons has been shown to occur naturally and spontaneously in the CNS. This shows that it is an environment capable of unassisted, natural healing. naturally possesses an environment that is susceptible to regeneration. MSCs contribute to this regenerative environment by releasing bioactive agents that inhibit apoptosis and thus create an ideal regenerative environment.

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PERMISSIONS

We would like to thank the editorial team for lending their expertise to make the book truly unique. They have played a crucial role in the development of this book. Without their invaluable contributions this book wouldn't have been possible. They have made vital efforts to compile up to date information on the varied aspects of this subject to make this book a valuable addition to the collection of many professionals and students.

This book was conceptualized with the vision of imparting up-to-date and integrated information in this field. To ensure the same, a matchless editorial board was set up. Every individual on the board went through rigorous rounds of assessment to prove their worth. After which they invested a large part of their time researching and compiling the most relevant data for our readers.

The editorial board has been involved in producing this book since its inception. They have spent rigorous hours researching and exploring the diverse topics which have resulted in the successful publishing of this book. They have passed on their knowledge of decades through this book. To expedite this challenging task, the publisher supported the team at every step. A small team of assistant editors was also appointed to further simplify the editing procedure and attain best results for the readers.

Apart from the editorial board, the designing team has also invested a significant amount of their time in understanding the subject and creating the most relevant covers. They scrutinized every image to scout for the most suitable representation of the subject and create an appropriate cover for the book.

The publishing team has been an ardent support to the editorial, designing and production team. Their endless efforts to recruit the best for this project, has resulted in the accomplishment of this book. They are a veteran in the field of academics and their pool of knowledge is as vast as their experience in printing. Their expertise and guidance has proved useful at every step. Their uncompromising quality standards have made this book an exceptional effort. Their encouragement from time to time has been an inspiration for everyone.

The publisher and the editorial board hope that this book will prove to be a valuable piece of knowledge for students, practitioners and scholars across the globe.

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