

Stem Cells for Regenerative Medicine

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According to the Center for Disease Control, the three major causes of death in the United States are heart disease, cancer, and stroke. As the general population relies on effective drugs to treat these diseases and improve the quality of life, one promising and emerging form of therapy is stem cell therapy. Besides heart failure, cancer and stroke, many of the degenerative or genetic diseases could benefit from stem cell therapy, e.g., spinal cord injury, Parkinson's diseases, multiple sclerosis, diabetes, leukemia, sickle cell disease, arthritis and muscular dystrophy. One well-known example of stem cell therapy is bone marrow transplantation, which replaces or restores the blood-forming stem cells in the patients with blood diseases (e.g., leukemia, sickle cell disease) or in cancer patients after radiation therapy or chemotherapy. In the past few decades, our understandings of stem cell biology and the regulation of lineage specificity have grown tremendously. Since 1998 when James Thomson and colleagues successfully derived human embryonic stem cells (ESCs),¹ public awareness of stem cells soon catapulted into mainstream media, politics, ethics, and religion. Here we will describe the major types of stem cells, including their origins and characteristics, and then illustrate several therapeutic applications of stem cells for regenerative medicine, particularly in the treatment of cardiovascular diseases, spinal cord injury and Parkinson's diseases.

Stem cell types

Stem cells are unique in their ability to both self-renew as well as give rise to daughter cells with more specialized function. Related to stem cells are progenitor/precursor cells that cannot self-renew but can give rise to daughter cells with more specialized function. Based on their plasticity of differentiation, stem cells that can differentiate into diverse range of lineages in all three germ layers are characterized as pluripotent, whereas the stem cells that are limited to several lineages are considered multipotent. Stem cells have been identified in numerous organs in the body, including bone marrow, skin, heart, muscle, liver, umbilical cord blood, adipose tissue, and kidney.

Stem cells and progenitor cells can generally be classified as of adult, fetal, or embryonic origins, including embryonic stem cells (ESCs), mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), endothelial precursor cells (EPCs), and amniotic fluid-derived stem cells. Recent advances in the successful reprogramming of fibroblasts into stem cells represents yet another breakthrough in developing stem cells for therapies and research without using ESCs.²⁻⁵ These cells can be derived autologously from the patient, allogeneically from other donors, or xenogenically from other species.

In order to identify or isolate stem cells and progenitor cells, specific phenotypic markers are usually used, particularly in

cases of when no unique marker is available for the identification of the stem cell or progenitor cell population. This phenotypic signature often results from empirical assessment, and the signature that may include cell surface proteins, transcriptional factors, and/or cytoskeletal proteins. Many of these markers are species-specific and may vary between species. In addition, some markers have diverse roles, whereas the roles of other markers are unclear, and some are expressed in multiple cell types. Nevertheless, these markers have become part of the standard process used to characterize certain stem and progenitor cells.

Here we will briefly describe the characteristics of stem cells derived from adult, fetal, and embryonic origins.

Adult Stem Cells

Adult stem cells and progenitors can be derived from many types of tissues such as bone marrow, peripheral blood, and adipose tissue. Among the various types of stem cells, the three categories of adult stem cells most widely studied for regenerative medicine are MSCs, HSCs, and EPCs. In addition to these three types, recent reports suggest that terminally differentiated organs such as the heart may harbor resident stem cells.

MSCs

MSCs are multipotent stem cells that are typically isolated from the stroma of adult bone marrow. Within the marrow, MSCs represent only 0.01% of the total nucleated cell population, but can be expanded in vitro to over million to billion-fold.⁶ In addition to the bone marrow, MSCs have also been purified from numerous tissues, including adipose tissue, amniotic fluid, and periosteum. The phenotype of MSCs appears to vary, possibly due to differences in tissue origin and species. However, human MSCs are generally characterized by positive expression of cell surface markers such as STRO-1 (a stromal cell surface antigen), CD29 (integrin β 1), CD44 (receptor for hyaluronic acid and matrix proteins), CD105 (endoglin, receptor for transforming growth factor- β (TGF- β) and integrins), and CD166 (cell adhesion molecule). In contrast, they do not express hematopoietic markers such as CD14 (monocyte surface antigen), CD34 (HSC surface antigen), and CD45 (leukocyte surface antigen).

Immunologically, MSCs have a favorable property in that they do not express major histocompatibility complex II (MHC II) antigens on their cell surface that elicit immune rejection. Additionally, evidence suggests that MSC do not acquire MHC II cell surface antigens upon differentiation along adipogenic, chondrogenic, and osteogenic lineages. Some reports have also shown that MSCs have immunosuppressive and immunomodula-

tory properties. For example, MSCs can modulate T-cell function and inhibit B cell proliferation and chemotaxis. These findings suggest that MSCs may be an immunologically favorable cell source for allogeneic cell transplantation.

The mechanism by which MSCs act appears to be multifaceted. MSCs can differentiate into the cell lineage and incorporate into the diseased organ and/or secrete growth factors that have therapeutic pro-survival or angiogenic effects. MSCs have been shown to secrete angiogenic cytokines such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF). This may account in part for the improvements in cardiac function and angiogenesis after cell transplantation into the ischemic heart, since the incidence of MSC differentiation into cardiomyocytes is very low.

HSCs

Within the bone marrow resides another class of multipotent stem cells known as HSCs. HSCs can maintain self-renewal, in addition to differentiating into myeloid and lymphoid cell lineages in the bone marrow, blood, thymus, and spleen, as was demonstrated after syngeneic transplantation of HSCs after irradiation.⁷⁻⁹ The phenotype of HSCs can be characterized by positive expression of cell surface markers such as CD34 (human), CD133 (human), CD59 (human), Thy-1 (human/mouse), C-kit (mouse), and Sca-1 (mouse). On the other hand, HSCs do not express lineage-negative (Lin-) and MSC surface markers.¹⁰

The mechanism of HSC therapeutic involvement remains unclear due to the difficulty in distinguishing between fusion (a process of two cells forming a hybrid cell) and transdifferentiation (a process of acquiring broader developmental potential).¹¹ Using enhanced green fluorescent protein (EGFP)-expressing Lin- and C-kit+ HSCs, Orlic et al. reported that the transplantation of these labeled HSCs could transdifferentiate into cardiomyocytes, ECs, and SMCs.¹² In contrast, other studies show that HSCs undergo fusion instead.^{13,14} To distinguish these two phenomena, the use of both cardiomyocyte-restricted and ubiquitously expressed reporter transgenes for cell tracking in the infarcted myocardium resulted in no detectable levels of transdifferentiation.¹⁵

EPCs

A third type of stem cell that can be found within bone marrow as well as within blood during normal circulation are EPCs. Fluorescence-activated cell sorting (FACS) analysis of surface markers CD34/Flk-1 (VEGFR2) demonstrates a prevalence of approximately 0.05% EPCs in peripheral blood. At early stages, EPCs more closely resemble HSCs in their common expression of

Flk-1 (human), CD133 (human), CD34 (human), and C-kit (mouse), whereas at later stages the EPCs adopt an endothelial-like phenotype that includes von Willebrand Factor (vWF) and vascular endothelial-cadherin (VE-cadherin) expression.¹⁶ However, conflicting studies suggest that EPCs can also originate from CD11b+ and CD14+ monocyte populations, implicating origin of EPCs from a common hemangioblast precursor.

Stem Cell Niches

In addition to MSCs, HSCs, and EPCs, stem cells have now been identified in most organs, including the kidney, liver, lung, and heart. These tissue-resident stem cells are thought to maintain homeostasis and to repair injury by replenishing the tissue in which they reside of mature cells. The activity of tissue-resident stem cells may be modulated by complex interactions with other supporting cells of the tissue through cell-cell interactions or signaling by soluble factors. For example, the heart was once believed to be a terminally differentiated organ with no intrinsic ability for repair. However, recent evidence demonstrates a rare population of cardiac stem cells or progenitor cells that reside within the heart. These cells can be purified by FACs as a side population of cells that efflux nuclear dye Hoechst33342. These cells share similarities with other stem cells in their expression of C-kit and Sca-1, although their phenotypic signature appears to differ between groups. Cardiac stem cells can proliferate unlike terminally differentiated cardiomyocytes, and can give rise to myocardial cell types such as cardiomyocytes, endothelial cells, smooth muscle cells, and fibroblasts.

ESC and Induced Pluripotent Cells

ESCs are pluripotent stem cells that are derived from the inner cell mass of a blastocyst. Unlike other stem cells, ESCs have theoretically an unlimited capacity to self-renew.¹ In addition, these cells can also give rise to cell lineages derived from the ectodermal, mesodermal, and endodermal embryonic germ layers. ESCs are characterized by enhanced telomerase activity and they express stem cell markers such as transcription factor Oct-3/4, the homeodomain protein Nanog, and the surface marker stage-specific cell embryonic antigen (SSEA)-3/4 (murine ESCs express SSEA-1 instead). To maintain self-renewing capacity, ESCs are commonly cultured in the presence of mouse embryonic fibroblast (MEF) feeder cells. Critical to maintaining ESCs in an undifferentiated state is the addition of leukemia inhibitory factor (LIF) in murine ESC media or basic fibroblast growth factor (bFGF) for human cells. To initiate differentiation, a common strategy is to culture the cells in the absence of LIF or bFGF on non-adhesive culture dishes, in which ESCs aggregate to form embryoid bodies (EBs) that can spontaneously differentiate.

Currently, federally funded human ESC cell lines have been exposed to animal cells and proteins found in the media or in contact with MEFs. These cell lines would elicit immune rejection if transplanted into patients and are therefore less favorable for clinical applications. In order to develop medical-grade cells that are free of animal components in the media, ESCs have now been successfully cultured in serum-free defined media containing human-derived components. To eliminate contamination by exposure to MEFs, ESCs are commonly grown in MEF-conditioned media on surfaces coated with Matrigel, a murine basement membrane matrix derived from Engelbreth-Holm-Swarm sarcoma.¹⁷ However, ESC culture on Matrigel would still enable their exposure to animal proteins, so recent studies have focused on the replacement of Matrigel with human-derived extracellular matrix proteins such as laminin and fibronectin as the adhesive substrate.

Traditionally, ESCs were derived from blastocysts formed by the fertilization of sperm and egg. However, the ethical issues surrounding the destruction of life has prompted the alternative strategy of somatic cell nuclear transfer (SCNT), in which the nucleus of a differentiated somatic cell is transferred to the cytoplasm of an enucleated egg, thus programming the somatic nucleus to its embryonic state. In the course of the reprogramming process, genes normally expressed in the embryonic state become reactivated and the chromatin is modified, but the mechanism by which these changes occur is still unknown. Most recently, SCNT was successfully demonstrated in primates,¹⁸ but they remain yet to be reported in humans.

Another recent development eliminates the need for embryos entirely. Yamanaka et al. and several other groups have successfully reprogrammed fibroblasts into pluripotent stem cells known as induced pluripotent stem (iPS) cells by the activation of four genes. In the case of Yamanaka's group, the factors consisted of Oct3/4, Sox2, c-Myc, and Klf4.¹⁹ Thomson's group used genes encoding Oct3/4, Sox2, Nanog and Lin28.³ Although both groups only share two factors in common, the iPS cells that were generated from their laboratories were similar to ESCs in their ability to maintain self-renewal and differentiate into cell lineages of all three germ layers. The generation of iPS cells are not limited to fibroblasts, but could also be successfully carried out using cells from bone marrow, skin, stomach and liver. Proof-of-principle of the therapeutic potential of iPS cells has been demonstrated in a murine model of sickle cell anemia in which transplantation of autologous iPS-derived hematopoietic progenitors could correct the sickle hemoglobin allele.⁴ These results suggest that iPS cells may be one of the most promising cell sources towards patient-specific stem cell therapy that minimizes ethical concerns.

However, several factors may limit the therapeutic potential of ESCs and iPS cells. First, the potential exposure of human cells to animal pathogens or virus presents safety concerns for therapeutic implantation. Second, the possibility of induced immunogenicity upon differentiation after transplantation has been demonstrated in ESCs, and this may limit their therapeutic potential. Third, the ability of ESCs and iPS cells to form benign tumors known as teratomas when implanted *in vivo* remains a concern. Finally, the intensive effort and costs in customizing patient-specific iPS stem cells may hinder their widespread use.

Amniotic Fluid Stem Cells and Umbilical Cord Blood (UCB) Stem Cells

Besides adult and embryonic tissues, stem cells and progenitor cells have been identified from fetal origins such as amniotic fluid. Amniotic fluid can be obtained during pregnancy by amniocentesis, which is routinely done if the fetus may have a congenital abnormality. The cells harvested from these sources have been shown to generate stem cells with pluripotent stem cell characteristics. In particular, fetal stem cells derived from amniotic fluid represent only two percent of the total cells, but they are thought to have high replicative capacity and doubling times. Interestingly, the phenotype of human amniotic fluid stem cells include the expression of two markers more commonly associated with ESCs, namely SSEA-4 and Oct3/4.²⁰ In addition, amniotic fluid stem cells mimic ESCs in their ability to form aggregates of EBs in suspension, with the notable exception that the EBs derived from amniotic fluid stem cells do not form teratomas *in vivo*. In addition, like ESCs, amniotic fluid stem cells can differentiate into multi-lineage cell types. The phenotype of amniotic fluid stem cells suggests that they may have greater differentiation potential than other adult stem cells, but less than ESCs.

The UCB also harbors a population of stem cells and progenitor cells. The stem cells and progenitor cells derived from cord blood have been used to treat over 6000 cases of diseases, of which the earliest successful case was demonstrated in 1989.²¹ Cord blood stem cells harbor MSCs, HSCs, EPCs and multi-lineage stem cells. UCB mononuclear cells resemble ESCs in their expression of Oct3/4 and Sox2. Besides giving rise to hematopoietic and endothelial lineages, UCB stem cells are believed to be able to transdifferentiate into other cell types such as adipocytes, osteoblasts, and neuron-like cells.

Cancer Stem Cells

The first evidence of cancer stem cells is the finding in 1997 that leukemia originates from HSCs [bonnet and Dick; weissman, 2006]. Since then cancer stem cells have

also been found in solid tumors in breast and brain. Cancer stem cells are a sub-population of cancer cells that have stem cell properties such as self-renewal and the ability to differentiate into multiple cell types. One difficulty is identifying cancer stem cells is the lack of specific molecular marker for these cells. It is hypothesized that this distinct subpopulation in cancer cells may cause relapse and metastasis by giving rise to new tumors. Therefore, it is important to understand the molecular mechanisms that regulate self-renewal and differentiation because the dysfunction of genes involved in these pathways may participate in tumor growth. Basic research of cancer stem cells will help us identify new therapeutic targets and develop new strategies to kill tumor cells.

Stem cell-based therapeutic applications

The therapeutic application of stem cells for repair or diseased or defective organs is an emerging field of research. Here we will discuss several examples of using stem cells for the treatment of cardiovascular diseases, spinal cord injury and Parkinson's diseases.

Therapies for Cardiovascular Diseases

Cardiovascular disease is one of the leading causes of death in the US. Here we will discuss stem cell therapies for myocardial infarction (MI), stroke and peripheral arterial diseases (PAD).

MI is characterized by the occlusion of coronary arteries that supply blood and oxygen to the heart, resulting in cardiomyocyte cell death. The injured myocardium then undergoes a pathological remodeling process that includes the formation of fibrous infarct scar tissue, dilatation of the chamber cavity, and thinning of the left ventricular (LV) free wall, which all contribute to reduced cardiac function and ultimately heart failure. Stem cell therapy may be an effective way to improve cardiac function and regenerate the damaged tissue. Adult stem and progenitor cells have demonstrated positive improvements in cardiac function and enhanced neovascularization after MI. For example, delivery of TGF- β -pretreated bone marrow-derived stem cells into the infarcted tissue led to enhanced fractional shortening, which is a measure of cardiac function. The TGF- β -treated cells could differentiate into a myogenic lineage, and both the pretreated and non-pretreated cells enhanced vascular density. The survival rate of transplanted MSCs can be enhanced by overexpressing the pro-survival gene, Akt1, prior to the delivery into the infarct. Besides adult stem cells, ESCs have also been delivered to the myocardium. Delivery of cardiac-enriched human ESCs or human ESC-derived cardiomyocytes could regenerate myocardium in athymic rats without the incidence of teratoma formation. Taken together, these studies demonstrate the feasibility of stem cell therapy

for treatment of MI, although it is still controversial whether transplanted stem cells differentiate into cardiac and vascular cells or enhance cardiac functions through paracrine signaling. Currently, bone marrow stem cells for cardiac repair is at the stage of clinical trial.

In stroke, occlusion of a cerebral artery leads to focal ischemia and subsequent damage in a restricted central nervous system region. In addition to the therapies to dissolve clots and prevent cell death, stem cell can be used facilitate the recovery of brain functions. Potential sources of stem/progenitor cells for stroke include fetal neural stem cells, ESCs, UCB-derived nonhematopoietic stem cells, and bone marrow-derived stem cells such as MSCs. Adult neural stem cells and HSCs have been shown to improve functional recovery after stroke. Stem cells can differentiate into neural cells and/or release biomolecules for brain tissue regeneration. Autologous bone marrow stem cells for stroke therapy are being tested for clinical trials.

PAD results from narrowing of the peripheral arteries that supply blood and oxygen to the legs and feet, leading to vascular dysfunction. Symptoms of PAD include intermittent claudication, painful ischemic ulcerations, or even limb-threatening gangrene. It is believed that the vascular endothelium, a thin layer of endothelial cells that inhabits the luminal side of all blood and lymphatic vessels, plays an important role in maintaining the health of the arterial environment. Accordingly, stem cell-based regeneration of the endothelium may be a promising approach for treating PAD. Using a hindlimb ischemia model for PAD, human ESC-derived endothelial cells can be delivered intramuscularly to the ischemic limb or into the femoral artery, which improved the rate of limb salvage, limb perfusion and the formation of capillaries and arterioles. A comparison of the therapeutic effect has been assessed between MSCs and bone marrow-derived mononuclear cells, and the results show that MSC treatment led to higher capillary density as well as incidence of differentiation into endothelial and smooth muscle cell types. Adult stem cell-based therapies for treatment of PAD are already underway in clinical trials. These clinical trials involve the delivery of bone marrow-derived stem cells such as CD34-positive EPCs.

Therapies for Spinal Cord Injury and Parkinson's Diseases

Spinal cord injury/disorders and the resulting chronic paralysis affect more than 250,000 individuals in United States, which places a heavy burden to health care system and the society. To date spinal cord regeneration remains as one of the most challenging problems in regenerative medicine, and there is no effective cure for spinal cord injury. The advance in stem cell research provides new opportunities for us to meet this challenge. The difficulty in axon regeneration in spinal cord is due to the

limited regeneration potential of adult neurons and the inhibitory factors in the spinal cord microenvironment. ESC-derived neural cells and embryo-derived glial progenitors have been used to treat nervous system disorders in animal models. There is evidence that the animals with spinal cord injury showed functional improvement after receiving transplants of ESCs, ESC-derived oligodendrocyte progenitors or glial-restricted precursors. Preclinical trials using ESC-derived oligodendrocyte progenitors have demonstrated the safety of transplanting these cells into spinal cord.

Parkinson's disease is caused by the progressive loss of the dopaminergic neurons, the cells in the brain that release dopamine—a neurotransmitter involved in regulating behavior, cognition and motor activity. As these cells die, neural communication is lost and various symptoms appear, including tremor, limb stiffness, decreased muscle activity and difficulties with gait and balance. The primary treatment for Parkinson's disease is the administration of dopamine precursors, which only relieves symptoms temporarily, but will not slow or stop the natural progression of the disease. Transplantation of dopaminergic neurons from fetal tissue has been conducted in patients and have shown some success in reducing the symptoms of the disease. Many alternative stem cell sources have been explored. ESCs have been differentiated into dopaminergic neurons. Adult neural stem cells and bone marrow stem cells also show potential to cure Parkinson's diseases. One common issue is the low survival rate of transplanted cells. It is critical to understand the signals in the brain environment that is required to allow the cells to survive, integrate and function correctly.

Another brain disease, usually occurring in people over 65 years old, is Alzheimer's disease. Alzheimer's disease is a progressive and terminal disease for which there is currently no cure. The symptoms include memory loss, confusion, anger, mood swings, and language breakdown, etc. In contrast to Parkinson's, diabetes and spinal injuries, Alzheimer's disease involves the loss of huge numbers and varieties of the brain cells and the associated synapses, which may be difficult for stem cell to recover this huge loss. However, stem cells derived from the patients with Alzheimer's diseases will help researchers to elucidate the underlying mechanism and search for the ways to prevent and cure the disease.

Conclusion

In summary, recent advances in stem cell isolation and characterization have revealed their potential to treat a wide range of diseases. The likely candidates for therapeutic repair can range from adult, fetal, and embryonic stem cells. However, whether an optimal stem cell exists for therapeutic improvement of certain diseases

remains unknown. The optimal stem cell type will likely overcome the hurdles of consistency of isolation and expansion, immune rejection, survival, and inhibition of teratoma formation. Based on advancements in stem cell research, the stem cell-based therapies have tremendous potential.

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