Chapter 2

# Pluripotent Adult Stem Cells: A Potential Revolution in Regenerative Medicine and Tissue Engineering

Tsz Kin Ng, Daniel Pelaez, Veronica R. Fortino,
Jordan Greenberg and Herman S. Cheung

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## 1. Introduction

Stem cells are undifferentiated cells defined by their abilities to self-renew and differentiate into mature cells. Stem cells found in fully developed tissues are defined as adult stem cells. The function of adult stem cells is the maintenance of adult tissue specificity by homeostatic cell replacement and tissue regeneration (Wagers and Weissman, 2004). Adult stem cells are presumed quiescent within adult tissues, but divide infrequently to generate a stem cell clone and a transiently-amplifying cell. The transiently-amplifying cells will undergo a limited number of cell divisions before terminal differentiation into mature functional tissue cells. The existence of adult stem cells has been reported in multiple organs; these include: brain, heart, skin, intestine, testis, muscle and blood, among others. This chapter focuses on four adult stem cell populations: hematopoietic, mesenchymal, periodontal ligament-derived, and spermatogonial (Table 1).

Hematopoietic stem cells are the most characterized adult stem cell population. They function to generate all cell lineages found in mature blood (erythroid, myeloid and lymphoid) and to sustain blood production during the entire life of an animal (Kondo et al., 2003). Adult bone marrow, umbilical cord blood and mobilized peripheral blood are sources of hematopoietic stem cells for transplantation in many blood-related diseases. Hematopoietic stem cells can be characterized by positive selection of CD34, CD45, and CD133 markers and negative selection of CD31, CD105 and CD146 markers (Tárnok et al., 2010).

Mesenchymal stem cells, also called marrow stromal cells, are another well-studied adult stem cell population. Mesenchymal stem cells were originally identified in the bone marrow, but have since been found in other systems such as adipose tissue, umbilical cord and menstrual blood (Ding et al., 2011). Mesenchymal stem cells differentiate into osteocytes, chondrocytes and adipocytes (Arita et al., 2011; Pittenger et al., 1999). Human mesenchymal stem cells can be characterized by the positive expression of CD29, CD44, CD73, CD90, CD105, CD146 and STRO-1, and the negative expression of CD31, CD34, CD45, CD49f and CD133 (Mödder et al., 2012; Tárnok et al., 2010).

| Adult stem cells | Feasible sources | Characterization |
|---|---|---|
| Hematopoietic stem cells | Bone marrow, umbilical cord blood, mobilized peripheral blood | (+): CD34, CD45, CD133 |
| | | (-): CD31, CD105, CD146 |
| Mesenchymal stem cells | Bone marrow, adipose tissue, umbilical cord, menstrual blood | (+): CD29, CD44, CD73, CD90, CD105, CD146, STRO-1 |
| | | (-): CD31, CD34, CD45, CD49f, CD133 |
| Periodontal ligament-derived stem cells | Periodontal ligament | Mesenchymal stem cell markers: CD29, CD44, CD73, CD90, CD105, CD146, STRO-1 |
| | | Neural crest cell markers: p75, nestin, Slug, SOX10 |
| Spermatogonial stem cells | Testis | (+): CD9, CD49f and GPR125 |

**Table 1.** Feasible sources and characterization of adult stem cells

Periodontal ligament, derived from the cranial neural crest, is a soft connective tissue embedded between the tooth root and the alveolar bone socket, supporting the teeth *in situ* and preserving tissue homeostasis. The periodontal ligament contains stem cell populations that can differentiate into cementum-forming cells or bone-forming cells (Seo et al., 2004). Periodontal ligament-derived stem cells are heterogeneous, composed of mesenchymal stem cells and putative neural crest cells. Therefore, human periodontal ligament-derived stem cell populations have been characterized not only by mesenchymal stem cell markers, but also by neural crest cell markers, such as p75, nestin, Slug and SOX10 (Huang et al., 2009; Mrozik et al., 2010).

Testicular spermatogonial stem cells are the germ-line cells for spermatogenesis, an ongoing process throughout the lifespan of the male animals. They are unipotent in nature and continuously generate differentiating daughter cells for subsequent production of spermatozoa (Fagoonee et al., 2011). Human spermatogonial stem cells can be purified by antibodies against cell surface markers CD9, CD49f and GPR125 (Conrad et al., 2008).

## 2. Pluripotent stem cells

Pluripotency refers to the ability of cells to self-renew and differentiate into all 3 germ layers (ectoderm, endoderm and mesoderm). Pluripotent stem cells are the origin of all somatic and germ-line cells in the developing embryo. The first pluripotent cells were derived in 1976 from a type of germ-line tumor known as a teratocarcinoma (Hogan, 1976). Embryonic stem cells, derived from the inner cell mass of a blastocyst prior to gastrulation, are still considered the gold standard for pluripotent stem cells. Even though adult cells are terminally differentiated, pluripotency has also been conferred to these cells in past studies, by the technique of somatic cell nuclear transfer (Perry, 2005), parthenogenesis of unfertilized eggs (Brevini et al., 2008), and reprogramming by cell fusion (Pralong et al., 2006). Research into adult cell pluripotency was slow to progress until a major breakthrough in 2006 brought with it the technique of "induced pluripotent stem cells". In this process adult skin fibroblasts were induced into a pluripotent state by the forced expression of key transcription factors (OCT4, SOX2, KLF4 and c-MYC; Takahashi et al., 2007) or (OCT4, SOX2, NANOG and LIN28; Yu et al., 2007). Despite the low reprogramming efficiency, this has become a convenient method for generating new pluripotent stem cell lines for research from differentiated adult cells.

Adult stem cells are thought to be tissue-specific and only able to differentiate into progeny cells of their tissues of origin. An increasing number of studies, however, report that adult stem cells are capable of giving rise to cells of an entirely distinct lineage. The concept of adult stem cell plasticity might be explained by 5 potential mechanisms: cell fusion, trans-differentiation, de-differentiation, heterogeneous stem cell populations, or pluripotency (Wagers and Weissman, 2004). Cell-cell fusion occurs at a low frequency, but is implicated in the transplantation of bone marrow cells to liver hepatocytes, cardiomyocytes and Purkinje neurons (Alvarez-Dolado et al., 2003). In cell fusion events, the stem cells acquire the mature phenotype of the tissue they are embedded within and can be easily mistaken for correct differentiation of the transplanted cells. Trans-differentiation is a direct lineage conversion by the activation of a dormant differentiation program to alter the lineage specificity of the cell. De-differentiation is another lineage conversion phenomenon in which a tissue-specific cell spontaneously de-differentiates into a more basal multipotent cell and re-differentiates to a new lineage. While the heterogeneity of the stem cell population employed can account for some of the apparent trans-differentiation and de-differentiation events observed *in vivo*, it is worth discussing as a separate factor in the resulting multi-lineage tissues, which are often seen after transplantation. The characterization of homogeneous stem cell populations that contribute to the regeneration of one cell type remains an active field of study for most cellular therapy applications. Lastly, pluripotent stem cells are present in adult tissues as minute sub-populations in certain stem cell niches. Such a population has already been identified and reported in bone marrow derived mesenchymal stem cells (Jiang et al., 2002). In addition, pluripotent stem cells in adult tissues can also arise from remnants of the migrating neural crest. The neural crest is a transient embryonic structure that affords various organs with cells which could undergo a more stochastic type of differentiation than other embryonic progenitor cells (Slack, 2008). Neural crest cells are pluripotent and may retain some of their characteristics after their migration and engraftment into their terminal sites.

## 3. Isolation of pluripotent adult stem cells

The expression of embryonic stem cell markers in some adult stem cells suggest a sub-population of pluripotent cells in these niches (Table 2). The common embryonic stem cell makers, such as OCT4, SOX2, NANOG, KLF4, LIN28, SSEA-1, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81, are all expressed in hematopoietic stem cells (Wang et al., 2010; Zhao et al., 2006; Zulli et al., 2008) and mesenchymal stem cells (Anjos-Afonso and Bonnet, 2007; Jaramillo-Ferrada et al., 2012; Riekstina et al., 2009; Sung et al., 2010). Similarly, expressions of most of these markers, except for LIN28, have been reported in periodontal ligament-derived stem cells, a tissue arising from the migrating cranial neural crest (Huang et al., 2009; Kawanabe et al., 2010). Previous studies show that spermatogonial stem cells also express most of the embryonic stem cell markers, except SSEA-3 and TRA-1-60 (Izadyar et al., 2008; Izadyar et al., 2011; Kanatsu-Shinohara et al., 2008; Panda et al., 2011; Zheng et al., 2009). These findings suggest that pluripotent stem cells exist as sub-populations in adult stem cell reservoirs.

| Embryonic stem cell marker | HSC | MSC | PDLSC | SSC |
|---|---|---|---|---|
| SOX2 | + | + | + | + |
| OCT4 | + | + | + | + |
| NANOG | + | + | + | + |
| KLF4 | + | + | + | + |
| LIN28 | + | + | | + |
| SSEA-1 | + | + | + | + |
| SSEA-3 | + | + | + | |
| SSEA-4 | + | + | + | + |
| TRA-1-60 | + | + | + | |
| TRA-1-81 | + | + | + | + |

HSC: hematopoietic stem cells; MSC: mesenchymal stem cells; PDLSC; periodontal ligament-derived stem cells; SSC: spermatogonial stem cells;

**Table 2.** Embryonic stem cell marker expression in different adult stem cell populations

The existence of cells with a defined pluripotency-associated phenotypic expression within adult tissues enables researchers to isolate and purify a homogeneous subpopulation of adult pluripotent stem cells. In fact, with the use of magnetic affinity cell sorting, adult human mesenchymal stem cells, shown to differentiate into endodermal, ectodermal and mesodermal cells, were isolated by antibody against SSEA-3 (Kuroda et al., 2010). Similarly, stem cells exhibiting the potential to generate specialized cells of the three embryonic germ layers can be isolated by positive SSEA-4 expression from human periodontal ligament (Kawanabe et al., 2010). Furthermore, human spermatogonial stem

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cells, sharing cellular and molecular similarities with human embryonic stem cells, can be purified by $\alpha_6$ integrin (CD49f) antibody (Conrad et al., 2008). Moreover, a human hematopoietic stem cell subpopulation, highly efficient in generating long-term multi-lineage grafts, can also be isolated by the same $\alpha_6$ integrin expression (Notta et al., 2011). In addition, stem cells from granulocyte colony-stimulating factor-mobilized human peripheral blood can divide indefinitely without reaching replicative senescence and differentiate into multiple lineages (Cesselli et al., 2009).

Recently, a cell surfaceome map of mouse embryonic stem cells and induced pluripotent stem cells was reported (Gundry et al., 2012). Previously unidentified cellular surface markers, such as CD31, CD49f, CD123 and CD326, indicated a purified population of pluripotent stem cells. Further analyses should be performed to determine the expression of these markers in different adult stem cell populations. Their presence in adult stem cell populations could facilitate the purification of homogeneous pluripotent stem cells within an otherwise heterogeneous pool of regenerative adult cells.

## 4. Characterization of pluripotent adult stem cells

The standard tests for pluripotency are teratoma and chimera formation assays. Teratomas can be formed when pluripotent stem cells are injected into immunodeficient animals; they consist of foci with derivatives of ectodermal, mesodermal and endodermal embryonic germs layers (Wobus et al., 1984). Chimeras can be generated when pluripotent stem cells are microinjected into mouse blastocysts and are induced to differentiate into multiple cell types during normal developmental processes (Becker et al., 1984). Teratoma formation assays can be used to test for the pluripotency of human stem cells, whereas both teratoma and chimera formation can test for the pluripotency of mouse stem cells. Spermatogonial stem cells isolated from human testis by positive expression of CD49f are able to form teratomas when injected into immunodeficient mice (Conrad et al., 2008). Mesenchymal stem cells isolated from murine bone marrow contribute to most of the somatic cell types (chimerism ranged between 0.1% and 45%) when they are singly injected into an early mouse blastocyst (Jiang et al., 2002). Moreover, human hematopoietic stem cells isolated by CD49f cell surface marker display multi-lineage chimerism when transplanted into the NOD-*scid-IL2Rgc*$^{-/-}$ mice (Notta et al., 2011). However, human bone marrow-derived mesenchymal stem cells purified by the SSEA-3 cell surface marker do not form teratomas in immunodeficient mouse testes even though cells positive for human ectodermal, endodermal and mesodermal lineage markers were detected within the injected mouse testes (Kuroda et al., 2010). Conversely, pluripotency assays of human periodontal ligament-derived stem cells isolated by SSEA-4 cell surface marker expression have not yet been reported (Kawanabe et al., 2010).

Although most of the adult stem cells are unable to form teratomas in immunodeficient mice, can they still be defined as pluripotent stem cells? Considering this apparent inability as well as the variability in teratoma formation efficiency even when using a known pluripotent stem cell line, a teratoma assay might not be a suitable assay for pluripotency of adult stem cells. Instead, *in vitro* and *in vivo* differentiation into cells of the 3 embryonic germ layers along with chimera formation in xeno-transplanted mice can be applied for testing adult stem cell potency. The conventional concept of development involves a hierarchical structure of cellular commitment extending outward from embryonic and pluripotent, to adult terminally differentiated tissues. However, recent ideas propose that all or most tissues in the postnatal body are continuously turning over and contain a pluripotent stem cell reservoir (Slack, 2008). These pluripotent stem cell populations are able to differentiate into multiple cell types depending on their microenvironmental cues. Therefore, the stem cell status should be defined by plasticity (Zipori, 2005). Pluripotency refers to the ability of cells to differentiate into any cell type of the 3 germ layers (ectoderm, endoderm and mesoderm), whereas multipotency refers to the ability of cells to differentiate only into a closely related family of cells (Ilic and Polak, 2011). All of the previously described adult stem cells (hematopoietic, mesenchymal, periodontal ligament-derived, and spermatogonial) could differentiate into specialized cells of the three germ layers: neurons (ectodermal lineage), adipocytes, cardiomyocytes, osteoblasts, and chondrocytes (mesoderm lineage), and hepatocytes and insulin-producing cells (endodermal lineage) (Conrad et al., 2008; Jiang et al., 2002; Kuroda et al., 2010; Kawanabe et al., 2010; Notta et al., 2011). Therefore, these adult stem cells could also be defined as pluripotent stem cells.

## 5. Advantages of pluripotent adult stem cells over embryonic stem cells and induced pluripotent stem cells

Human embryonic stem cells come from the inner cell mass of human blastocysts. Therefore, embryonic stem cells used for cell therapy are allogenic; the transplanted donor cells do not originate from the recipient. This raises a concern about the immunogenic response of the host, and the need for immune-suppressive therapy concurrent with embryonic stem cell transplantation (Charron et al., 2009). Moreover, embryonic stem cell-based therapy has been hampered by the moral, legal and ethical dilemma surrounding the use of human embryos for derivation of the stem cell lines (Zarzeczny and Caulfield, 2009). Furthermore, as the gold standard of pluripotent stem cells, embryonic stem cells have the potential to form teratomas in the host. Tumorigenic potential can be reduced by differentiating the embryonic stem cells into lineage-specific progenitor cells or mature tissue cells prior to transplantation (Schwartz et al., 2012). In order to better control standards of good manufacturing practices and reduce variability as much as possible, the *in vitro* manipulation of embryonic stem cells should be minimized as recommend by the Food and Drug Administration (Lysaght and Campbell, 2011). Furthermore, tumorigenic potential remains a concern if the entirety of the embryonic stem cell population does not completely differentiate into fully mature cells.

Differentiated adult cells used for the generation of the induced pluripotent stem cells can be collected from the recipient body, avoiding the contentious need for a human embryo. This also circumvents the problem of immune rejection. There are technical hurdles, howev-

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er, concerning generation of induced pluripotent stem cells (Hayden, 2011). Firstly, the delivery of reprogramming factors (OCT4, SOX2, NANOG, LIN28, KLF4 and c-MYC) relies on the use of viral vectors for delivery (Takahashi et al., 2007). Retroviral sequences could integrate into the DNA of the host cells, potentially disrupting the gene structure as well as resulting in an aberrant phenotypic expression. Ultimately this could result in pathological mutations and cancer formation. Alternative methods such as direct protein or small molecule delivery have been adopted, although the reprogramming efficiency of these techniques is lower than with viral vectors (Kim et al., 2009; Shi et al., 2008). Secondly, two of the reprogramming factors, *c-MYC* and *KLF4*, are proto-oncogenes, which raise the concern of cancer formation further. Omitting *c-MYC* would lower the reprogramming efficiency, whereas silencing *c-MYC* could lead to its reactivation. Moreover, reprogramming can induce other genomic changes, such as DNA mutations (Gore et al., 2011), copy number variations (Hussein et al., 2011) and chromosomal aberrations (Mayshar et al., 2010). Genomic instability could have unpredictable and undesirable effects on the reprogrammed cells. Furthermore, induced pluripotent stem cells carry their epigenetic signatures from the original differentiated adult cells (Lister et al., 2011). The reprogrammed cells, therefore, unlike embryonic stem cells, may not develop into some cell types. In addition, induced pluripotent stem cells can still cause immune reactions when transplanted allogeneically.

The sources of adult stem cells are multiple and feasibly obtained from various adult tissues, such as bone marrow, blood, adipose tissue, teeth and testes (Table 1). These adult stem cells can be collected from the human body at anytime throughout life. This makes them readily available and does not raise the moral and ethical issues involved with the attainment of embryonic stem cells. Moreover, pluripotent adult stem cells can easily be isolated and purified by cell surface markers, such as CD49f, SSEA-3 and SSEA4 (Conrad et al., 2008; Kuroda et al., 2010; Kawanabe et al., 2010; Notta et al., 2011). The pluripotent status of these adult stem cells is naturally acquired and does not require reprogramming by the introduction of pluripotent transcriptional factors, thus eliminating the use of viral vectors and the chance of aberrant chromosomal changes. Furthermore, transplantation of mesenchymal stem cells and periodontal ligament-derived stem cells can be autogenic or allogeneic. Immuno-suppression is not necessary since mesenchymal stem cells have strong immunomodulatory properties against alloreactivity of T lymphocytes and dendritic cells (Chen et al., 2011). Similarly, mesenchymal stem cells and periodontal ligament-derived stem cells inhibit the proliferation of peripheral blood mononuclear cells (Wada et al., 2009). Spermatogonial stem cells, however, are killed by cytotoxic T lymphocytes after transplantation (Dressel et al., 2009), whereas allogeneic hematopoietic stem cell transplantation induces graft-vs-host disease (Strober et al., 2011). Therefore, transplantation of spermatogonial stem cells and hematopoietic stem cells should only be autogenic, without the application of immunosuppressive drugs. Similar to embryonic stem cells and induced pluripotent stem cells, pluripotent adult stem cells can differentiate into specialized cells of the three germ layers. Except for spermatogonial stem cells (Conrad et al., 2008), teratoma formation was not found in pluripotent hematopoietic stem cells, mesenchymal stem cells and periodontal ligament-derived stem cells (Kuroda et al., 2010; Kawanabe et al., 2010; Notta et al., 2011). This suggests a reduction in the probabilities of tumor formation post-transplantation, and the elimination of the need to manipulate the cells into mature tissue prior to transplantation. In addition, transplanted stem cell-induced regeneration may not be due to stem cell differentiation per se (Johnson et al., 2010; Williams and Hare, 2011). Instead, a paracrine effect has been hypothesized in which the adult stem cells secrete cytokines, chemokines, or protective proteins (Bai et al., 2012; Bráz et al., 2012) that nourish the host tissue cells and facilitate the healing process. This special feature has not yet been reported with the use of embryonic stem cells or induced pluripotent stem cells in a clinical setting.

## 6. Potential applications of pluripotent adult stem cells

Stem cell clinical trials have advanced rapidly for a broad spectrum of diseases, such as diabetes, neurodegeneration, immune diseases, heart disease, and bone disease. In 2011, there were 123 clinical trials using mesenchymal stem cells (Trounson et al., 2011). It is predicted that stem cell therapy will eventually become the treatment of choice in regenerative medicine, especially the use of adult stem cells. As stem cell products become more wide-spread and maintained under various conditions, the need for global standardization and regulation of processes will become necessary for the viable application of these products in a clinical setting. The Food and Drug Administration regulates interstate commerce in human cells and tissue-based products under the Public Health Service Act and the Code of Federal Regulations for Food and Drugs (Lysaght and Campbell, 2011). Human cells and tissue-based products are defined as “articles containing or consisting of human cells or tissues that are intended for implantation, transplantation, infusion, or transfer into a human recipient” (Lysaght and Campbell, 2011). Human cells and tissue-based products must be: (1) minimally manipulated, (2) intended only for homologous use, (3) not combined with another article (except for water, or sterilization, preservation, or storage agents), and (4) either: (a) have no systemic or metabolic effect, or (b) be for autologous use, allogeneic use in first- or second-degree blood relative, or reproductive use.

Pluripotent adult stem cells fall under the criteria for human cells and tissue-based products as stated by the Food and Drug Administration. Unlike induced pluripotent stem cells, pluripotent adult stem cells can be minimally manipulated as their pluripotent state occurs naturally. Unlike embryonic stem cells, pluripotent adult stem cells are suited for autologous use. Similar to embryonic stem cells and induced pluripotent stem cells, pluripotent adult stem cells are able to differentiate into specialized cells of the three germ layers. In addition, embryonic stem cells and induced pluripotent stem cells have the potential to form teratomas (an unfavorable side-effect in clinical applications) although a recent study suggests that the teratoma-forming cells could be removed by the antibody against SSEA-5 (Tang et al., 2011). In contrast, most pluripotent adult stem cells do not form teratomas *in vivo*, eliminating the need for preemptive differentiation of pluripotent adult stem cells into mature specialized cells.

If stem cell-aided regeneration is not due to stem cell differentiation to replace damaged cells (Johnson et al., 2010; Williams and Hare, 2011), pluripotent adult stem cells are favora-

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ble over embryonic stem cells and induced pluripotent stem cells. The secretion of cytokines, chemokines, and/or protective proteins from the adult stem cells could nourish the host tissue and facilitate the healing process (Bai et al., 2012; Bráz et al., 2012).

## 7. Summary

Adult stem cells are found all over the body. They can be conveniently obtained from different accessible tissues: bone marrow, blood, adipose tissue, teeth and testes. Pluripotent adult stem cells, which reside as a subpopulation within adult stem cells, can be easily isolated by pluripotent cell surface markers, such as SSEA-3, SSEA-4 and CD49f. Moreover, pluripotent adult stem cells can be characterized by their ability to differentiate into cells of 3 germ layers (ectoderm, mesoderm and endoderm) as well as by the chimera formation in xeno-transplanted mice. Pluripotent adult stem cells are better than embryonic stem cells and induced pluripotent stem cells as they are an autologous source, require minimal manipulation and do not have the ability to form teratomas. In addition, they are more appropriate to be used as a clinical product for therapeutic treatments, as a cellular replacement or secretory protein reservoir. However, there are uncertainties that still remain unanswered. Which stem cell types are optimal for regenerative medicine? What is the optimal cell number for transplantation? Should the cells be preemptively differentiated or used as is? Further research is needed to understand the mechanisms of stem cells in regenerating damaged tissues after transplantation.

## Author details

Tsz Kin Ng[1], Daniel Pelaez[1], Veronica R. Fortino[2], Jordan Greenberg[2] and Herman S. Cheung[1,2*]

*Address all correspondence to: hcheung@med.maimi.edu

1 Geriatric Research, Education and Clinical Center, Miami Veterans Affairs Medical Center, Miami, FL, USA

2 Department of Biomedical Engineering, College of Engineering, University of Miami, Coral Gables, FL, USA

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