

Stem Cells: Paradigm and Present Concepts

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Abstract

The ultimate goal of stem cell technology is to generate and expand cells that can be used in human diseases and regenerative medicine. Diseases that might benefit from cell therapy include but are not limited to the juvenile diabetes, Parkinson's disease, myocardial necrosis, spinal cord injury, and nonunion fractures. Two major sources for human cell therapy have been adult stem (AS) cells and embryonic stem (ES) cells. Major achievement was made to generate pluripotent stem cells by inducing pluripotency in cells obtained from patients, and treat patients with patient *reprogrammed* cells. In this review we discuss sources and recent techniques for inducing pluripotency in cells for using in clinic and we will review concerns regarding potential undesired consequences of cell therapy with *reprogrammed* cells.

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Introduction

Stem cells have the unique capacity to proliferate indefinitely while they maintain their pluripotent status. Embryonic stem (ES) cells, the most common source of stem cells, are derived from the inner cell mass (ICM) of blastocyst-stage embryos (1, 2) from which ES cell lines have also been generated (3). ES cells are able to differentiate into three types of germ layer tissues, ectodermic, endodermic, and mesodermic. Although ES cells are seen as a promising source for regenerative medicine, the use of human ES cells however, is hindered by two major issues yet to overcome. First, the use of human embryos is not ethically accepted by all scientific and social institutions. Secondly, the host versus graft rejection of transplanted ES cells is still a significant concern in regenerative medicine. The other major source of stem cell is the adult stem (AS) cell extracted from many tissues including bone marrow (BM), circulating blood, skin fibroblasts, nervous tissues, and mesenchymal stem cells (MSCs). The main advantage of the AS cell is its relatively better accessibility.

Three major methods have been developed to reprogram cells to obtain engineered pluripotent cells: 1) nuclear transfer, 2) fusion with ES cells, and 3) creation of induced pluripotent stem cells (iPS cells) with retroviral-mediated induction of specific transcription factors (4, 5). Reprogramming strategies however, were developed based on previously successful methods of nuclear transfer of embryonic cells into unfertilized oocytes with generation of cloned embryos in many cases grown into an entire animal (6, 7). In this review we will summarize the present status of the major strategies to generate pluripotent and proliferating stem cells that can potentially be used in regenerative medicine and diseases that may benefit from cell therapy.

Characteristics of stem cells

Stem cells are described as pluripotent cells that proliferate indefinitely while maintaining an undifferentiated status unless placed in appropriate conditions where they differentiate into lineage specific cells for the condition. Stem cells have the unequivocal ability to form both in vitro embryoid bodies, and in vivo teratomas, with all three embryonic mesod-

ermal, endodermal, and ectodermal germ layers if transplanted into nude mice.

Stem cell pluripotency-associated genes

ES cells and stem-like cells possess genes involved with the process of self-renewal which ensure their stem cell identity. Nanog gene was first identified in pluripotent cell lines and in ES cells by Chambers and was found to be absent in differentiated ES cell (8). The Nanog protein, a transcription factor expressed in early embryos and pluripotent stem cells controls the pluripotency of stem cells by opposing the differentiation. The overexpression of Nanog in ES cells increases their hyper-proliferation and maintains the self-renewal of these cells (8, 9). Similarly in reprogrammed cells the overexpression of Nanog provokes extensive cell proliferation (8-10). On the contrary, the inactivation of Nanog results in spontaneous differentiation of ES cells indicating the loss of embryonic status (9, 11).

Two other transcription factors are involved in the maintenance of pluripotency of ES cells. These include Oct3/4 and Sox2. Oct3/4 is a major regulator of pluripotency in ES cells (12, 13). Based on the amount of Oct3/4 available for the cell, the differentiation of ES cells into one of the embryonic germ layers will selectively take place. The quantitative balance between Oct3/4 and Sox2 proteins leads to either activation or repression of the differentiation in early embryos (14). Oct3/4 might be associated with tumorigenesis since its activation results in dysplasia of gastric and intestinal epithelium (15). Oct3/4 and Sox2 genes maintain the pluripotency in ES cells and synergistically function to inhibit the differentiation of ES cells (16, 17). Sox2 and Oct3/4 proteins function by forming first a heterodimer which itself with Nanog in turn co-regulates Sox2 and Oct3/4. However Sox2 protein appears to maintain specifically the downstream Oct3/4 gene expression (18).

In addition to the above transcription factors, several oncogenes contribute to the maintenance of pluripotency and the self-renewal of ES cells. These oncogenes include c-myc, Klf4, Stat3, and E-Ras. ES cells that overexpress Klf4 (Krüppel-like factor 4) have increased capacity for self-renewal (19). C-myc and Klf4 are found also in human cancers. C-myc is able to maintain the proliferative, self-

renewing, and undifferentiated state in ES cells and possibly has similar functions in human tumors (4). Klf4 is highly expressed in cells in growth arrest but not in proliferating cells; however Klf4 is also highly expressed in differentiated epithelial cells of the skin, the GI tract, and fibroblasts cell lines (20) and has also been implicated as a negative predictor in breast cancers when it is overexpressed (21, 22). However, Klf4 is a tumor suppressor gene in gastrointestinal malignancies (23, 24). Therefore, Klf4 protein is associated with both oncogenesis and tumor suppression.

Since factors that control self-renewal and immortalization of the cancer cells appear to also control same functions in stem cells, it is suggested that cancer cells and stem cells might share the same mechanisms and the pathways of self-renewal in cancer and stem cells may overlap (25).

Epigenetic profile of pluripotent stem cells:

Epigenetic characteristics, involving aspects other than DNA sequences, are described as post-translational acetylation, phosphorylation, and methylation on histones in amino terminal in promoter regions of pluripotency-associated genes. In proliferating pluripotent cells, cell cycle genes are activated at promoter regions consisting of enriched tri-methylated H3-K4 histones, and activated chromatin. In contrast H3-K9 and H3-K27 histones are repressed with di-methylations (26). H3-K9 and H3-K27 methylations are associated with inactive chromatin and the inactivation of one X-chromosome (27). On the contrary deacetylation of H3 and H4 histones marks the gene silencing. In both ES and reprogrammed cells, when activated, promoter regions of pluripotency-associated genes change with hyperacetylation of H3, hypermethylation of H3-K4, and hypomethylation of H3-K9 and H3-H27 histones.

Strategies to obtain pluripotent stem cells

1- Adult stem cells: pluripotent stem cells can be obtained by long-term culture of cells from adult tissues. Adult progenitor cells can differentiate into various types of cells in vitro and can develop in chimera in vivo. In the BM a population of progenitor cells, mesenchymal stem cells (MSC) possess properties of stem

cells including self-renewal and pluripotency, giving rise in vivo to bone, cartilage, muscle, and fat tissues (28, 29). Pluripotent precursor cells identified in circulating blood (30- 32) were found to co-express CD34+, the hematopoietic-endothelial surface marker, and osteocalcin and bone alkaline phosphatase, the surface markers for osteoblastic cells (33, 34). Isolated circulating pluripotent cells formed mineralized nodules in vitro and when transplanted in mice, formed bone tissue (32). The promiscuity of perivascular cells and bone remodeling compartments suggests that vascular compartment might be both the source and the vehicle of pluripotent stem cells with osteogenic potential (35, 36). Perivascular cells were identified in multiple organs throughout the body that possessed pluripotency characteristics in vivo and in vitro (37, 38). The issue of multiplicity of surface markers identified in MSCs in various tissues is possibly the result of different microenvironment effects on the same type of cells and the presence of partially committed MSCs (39). It is suggested that pericytes first released by injury, become activated as MSCs, and MSCs in turn act with two functions: first prevention of autoimmune activities by cytokines secretion; second, tissue regeneration in the damaged area (40). MSCs have been the major source of adult pluripotent cells and used particularly to differentiate into bone cells in fracture repair (41, 42).

2- Nuclear transfer into oocytes: Present reprogramming strategies were developed based on successful techniques of nuclear transfer of embryonic cells into unfertilized enucleated oocytes. Cloned embryos generated with nuclear transfer had the potential of developing into a viable animal (6, 7). With advance in the field, the generation of the first cloned animal by nuclear transfer of adult somatic cell into unfertilized egg, was performed in 1996 when a cloned sheep was successfully produced using the nuclei of follicle cells (43). Thereafter, the cloning with embryonic cell nuclear transfer into enucleated frog eggs was successfully performed in frog, with generation of fertile adult frogs. Same was achieved in many mammalian species with somatic cell cloning (44). An inverse correlation between the degree of differentiation of donor cells and the success of cloning

was observed. That is, highest success rates of obtaining cloned animals was when the donor cells were not differentiated, similar to stem cells, and on the contrary lower rates were seen when fully differentiated somatic cell donors were used (45).

The success of cloning was measured by evaluating several levels of life cycles of the cloned animal, and by the rate of survival to adulthood of the derived implanted embryos, and finally by the rate of obtaining pluripotent stem cell lines from cloned blastocytes cells in cell culture (45). In planning future clinical models, investigators also created a mice model that did not require oocyte for nuclear transfer and instead the mice model was using the nucleus of somatic cells transferred into a zygote instead of oocyte (46).

3- Reprogramming by fusion with ES cells:

Miller and Ruddle in 1976 fused thymocytes and embryonic carcinoma (EC) cells and demonstrated the hybrid cells were pluripotent (47). Thymocytes fused with mouse ES cells similarly resulted in pluripotent cells with self-renewal capacity that was demonstrated by reactivation of pluripotency-associated gene Oct3/4 and inactivation of X-chromosome of somatic donor cell (48). Hybrid cells transplanted into nude mice formed teratomas with all three primitive germ layers, the unequivocal criteria for pluripotency of reprogrammed cells. In thymocyte-ES hybrid cells, the promoter region of Oct3/4 acquired the ES-like status including histone acetylation and methylation, as proof of their pluripotency (48, 49).

The Nanog protein is increased in pluripotent cells reprogrammed by fusion of neural stem cells and ES cells (10). After removal of the ES cell-derived chromosome that contains Nanog, hybrid cells remained pluripotent indicating a complete reprogramming of hybrid cells with independent expression of Nanog (50). Isolation of pure populations of neural stem (NS) cells from fetal or adult mouse brain were used to demonstrate that tissue stem cells were potentially more efficient to generate pluripotent hybrid cells than terminally differentiated cells. Despite tremendous information, in overall the molecular mechanisms behind the pluripotency of fused somatic cells have not been elucidated and importantly, the issue of potential graft

rejection remains unresolved for hybrid cells (10).

4- Nuclear reprogramming by defined factors:

Takahashi and Yamanaka delineated a set of four genes that when induced by retroviral method in embryonic or adult fibroblasts, were able to generate pluripotent proliferating cells similar to ES cells (4). Fibroblasts isolated from adult mice tail (4) and human adult dermal fibroblast (ADF) (5) were probed with β -galactosidase activity with resistance to neomycin and reprogrammed with retroviral transduction of four genes and converted to induce pluripotent stem cells (iPS). Four genes were identified by excluding sequentially one single cDNA from a pool of ten cDNAs and then induced with retroviral technique. These ten cDNA were first selected from a list of 24 candidate genes potentially involved in the survival and maintenance of pluripotent stem cells. Four selected genes sufficient to generate iPS cells included Oct3/4, Sox2, c-Myc, and Klf4 (4).

Oct3/4 and Sox2 genes, as mentioned above, were synergistically acting to maintain the pluripotency state of cells by inhibiting the differentiation of iPS cells (16, 17). The exclusion of Sox2 alone, when the three other factors were induced resulted in the generation of iPS cells with normal morphology and normal proliferation, however these cells were not pluripotent (4). A low expression or exclusion of Oct3/4 cells alone resulted in differentiation and loss of pluripotency in iPS cells (13). Oct3/4 is known to have important roles in tumorigenesis since the overexpression of Oct3/4 activates gastric and intestinal dysplastic tumors (15). C-Myc and Klf4, two known proto-oncogenes found in human cancers, were also required for the generation of iPS cells. It is speculated that the balance between c-Myc and Klf4 is essential for the activation of Nanog and suppression of p53 in ES cells, thus essential for the control of proliferation and pluripotency of ES cells (4). Klf4 protein is associated with both oncogenesis and tumor suppression (20, 21). The recovered iPS cells possessed authentic features of ES cells including self-renewal, immortal growth in culture, the population doubling time similar to ES cells, and the ability to form embryoid bodies in vitro and

teratomas in vivo (4, 5). Similar to human ES (hES) cells, human ADF-derived iPS (hiPS) had epigenetic changes including histone modifications in promoter regions of pluripotency-associated genes. The pluripotency of hiPS cells was demonstrated by differentiating in vitro into neuronal cells with dopaminergic components (5, 51) and into cardiomyocytes that expressed cardiomyocyte markers and had beating pattern, (5, 52).

Difficulties with human ADF were related to the need for surgical skin biopsies being impractical for patients and also to the prolonged and time-consuming culture with multiple passages (53). Keratinocytes were also used to generate hiPS. But keratinocytes were subjects of large variability depending on the quality of the hair follicles and the age and the medical condition of the donors (54). BM, a traditional source for MSCs necessitates BM aspirations with discomfort. Mobilized human peripheral CD34+ cells with G-CSF, transduced with doxycycline-inducible lentivirus, and CD34+ cells from healthy donors without using G-CSF, and peripheral blood mononuclear cells (PBMNC) have also been successful sources of hiPS cells that molecularly and functionally were undistinguishable from hES cells (55, 56). PBMNC-derived iPS cells were high in percentage of cells that expressed mature TCR genes indicating hiPS cells can be derived efficiently from terminally differentiated adult peripheral T cells (56, 57) and adding a new and accessible source for hiPS cells.

The issue of safety

Are human iPS cells consistent?

Considerable discrepancies were observed between various hiPS cell lines and, between hiPS and hES cell lines.

Investigators have rigorously conducted transcriptional comparison of panels of both ADF-derived hiPS and hES cell lines. In some analysis it was found that only limited genes consistently were differentially expressed between hiPS and hES cell lines and no stable change was found that distinguishes gene expression of hiPS from hES cell lines (58).

In other analysis, it was found consistent transcriptional differences between hiPS and hES cells. The “transcriptional footprint” in

hiPS cell lines generated in independent laboratories was distinct for each laboratory and was possibly related to culture conditions and standards in each laboratory (59-61). However many of the differences in expression between hiPS and hES cell lines were “stochastic in nature” and were attenuated in late passages, thus hiPS cells becoming similar to the hES cells in late passages. Authors suggested that cells with high load of mutations, because of growth disadvantage, might have been negatively selected, thus absent in late passages (61, 65).

However, meticulous comparison in sequencing of protein-coding regions of 22 hiPS cell lines and matched original fibroblast cell lines by Gore et al. identified a high load of “reprogramming-associated mutations” in hiPS cells. It was noted that hiPS cells were enriched in genes known to mutate in cancers or genes known to be functional in cancers (62). Cell lines analyzed both at early and late passages by Gore et al., were found that in overall pre-existing and new mutations, identified in early passages remained fixed in late passages (62). Estimated reprogramming-associated mutations were ten-fold higher in hiPS compared to matched fibroblasts cells (62). In addition in hiPS, DNA methylation the major epigenetic change (involving aspects other than DNA sequences), was fundamentally distinct from hES cells (63). The other types of observed anomalies were aneuploidy or abnormal chromosomal status (64), and copy number variations (65).

At this time, the functional significance of “reprogramming-associated mutations” remains unexplained and it is not clarified what step of the reprogramming protocol predisposes to mutations of cells. However these mutations might have dual functional significances: they might be a part of the reprogramming process in vitro and/or they might be the cause of malignant diseases in vivo (64, 62).

Preventive safety measures: the observations of genetic and epigenetic discrepancies in mouse and human iPS cells brought up the question of safety for patients. It is universally accepted that the ultimate goal of hSC and hiPS cells is to generate immune-compatible, and patient- and disease-specific cells that can be used in regenerative medicine for cell therapy.

Karyotyping and rigorous genetic testings were appropriately recommended to evaluate the levels of mutations in iPS cells before using these cells in patients (56, 64). Important pre-evaluations are to test the *in vitro* differentiation and the *in vivo* tumorigenicity of cloned-derived iPS cells to sort safe and unsafe clones (66, 67). These include culture and transplantations in appropriate animal models of putative hiPS and hES cells, that can unveil potential tumor-forming activities. Virus-free (68, 69) and oncogene-free (70) models were developed for future safe clinical applications. Recombinant Oct3/4, Sox2, c-Myc, and Klf4 transcription factors, were generated fused to poly-arginine transduction domain, that readily penetrated into the cells and translocated into nuclei. Thus, iPS cells expressed pluripotency-associated proteins without induction of controversial oncogenes (70). Non-integrating adenoviruses were also used to prevent potentially harmful genome-integrating viruses (69). Virus-free and oncogene-free hiPS cells described above were pluripotent, able to self-renewal, and tested positive for *in vivo* ES cell characteristics (68–70).

Examples of clinical use of stem cells and iPS cells; future models

Stem cells in bone diseases and fractures: MSCs have been the major source of pluripotent cells able to generate differentiated cells especially for fracture repair (41, 42, 71). MSCs isolated from BM of patients, were expanded *ex-vivo* on hydroxyapatite scaffolds with appropriate sizes and such autologous grafts were then implanted in patients with large bone defects. Successful results were obtained with complete fusion between the implants and the host's bone (72, 73). Autologous BM MSCs were also directly transplanted by percutaneous injection into the non-infected nonunion fractures with also successful mineralization of calluses (74). Finally, human circulating CD34⁺ cells have been transplanted in animal models where CD34⁺ cells incorporated into the fracture sites to accelerate the fracture healing (75).

Regeneration of corneal epithelium with allogeneic stem cell transplant: Corneal renewal and repair are mediated by stem cells

of the limbus area located between the cornea and the bulbar conjunctiva. If the limbus area is destroyed after severe burn, stem cells will be deficient resulting in corneal opacity and loss of vision. Autologous limbal stem cells can be obtained with biopsy from contralateral healthy eye, expanded in culture and then grafted to the blind contralateral eye. Using this method, an average of 76% success was obtained with corneal epithelium regeneration in burnt patients with unilateral eye damage (76).

In vitro model for schizophrenia using iPS cells: In schizophrenia (SCZ) there is a strong genetic component with high heritability penetrance. However the cell type affected in SCZ and the underlying molecular mechanism remains unclear. From patients afflicted by familial forms of SCZ, and normal control subjects, dermal fibroblast were obtained and primary culture and hiPS cells were generated that were positive for *in vivo* and *in vitro* criteria of pluripotency and self-renewal. Both the control hiPS and SCZ iPS cells in culture generated neural progenitor cells and neurons. However, SCZ iPS neurons had decreased neuronal connectivity compared to healthy control hiPS cells. In addition, 600 genes were differentially expressed in SCZ iPS cells. Importantly five antipsychotic drugs were assayed on SCZ iPS-derived neurons in culture and it was found that loxapine was effective in normalizing the neuronal connectivity of cultured neurons and that loxapine could also partially reverse gene expression abnormalities in SCZ iPS-derived neurons (77).

In vitro model to study cardiac arrhythmias: Congenital or acquired long QT interval can induce life-threatening ventricular arrhythmia. Timothy syndrome consists of a long QT syndrome due to a congenital missense mutation on calcium channel Ca (V) 1.2. Human iPS cells obtained from ADF of patients with Timothy syndrome were differentiated into human cardiomyocytes-iPS cells. The cardiomyocytes-iPS cells had irregular contraction, excess Ca (2⁺) influx, and prolonged action potentials. Investigators found that roscovitine, a drug that increases the voltage-dependent inactivation of Ca (V) 1.2., efficiently restored the electrical activity and Ca

(2⁺) signaling properties of cardiomyocytes iPS, derived from Timothy syndrome patients (78).

Experimental model for spinal cord injury: transplantation of neurospheres derived from iPS cells that clones were previously tested safe (66) in mice model of spinal cord injury, resulted in a significant functional recovery, without tumor formation. The grafted areas have shown a significant re-myelination compared to the control areas confirming re-myelination of the host neuronal fibers, and also shown graft-derived oligodendrocytes. The grafted areas were also positive for 5HT mostly at the distal cord, at levels significantly above control cases, confirming the presence of serotonergic nerve fibers, known to be important for the functional motor recovery. Astrocytes with bipolar morphology and long processes were also observed, associated with 5HT⁺ fibers (67). These experimental models appear encouraging for clinical applications of iPS cell transplant in spinal cord injuries.

Experimental models for pancreatic insulin-secreting cells: Kroon et al. demonstrated human ES cell-derived pancreatic cells can generate into *in vivo* insulin-secreting cells. These investigators were able to differentiate hES cells in culture through a series of developmental transitions to ultimately generate *in vitro* insulin-secreting cells. After implantation in mice, these cells differentiated further to cells functionally and morphologically similar to pancreatic islet cells that were able to protect mice against streptozotocin-induced hyperglycemia (79). Bar-Nur et al. utilized cultured human pancreatic islet mixed-cells, with multiple cell types. They labeled beta cells with Cre-recombinase fused to insulin promoter or estrogen receptor with lentivirus induction to generate human

pancreatic beta cell-derived iPS cells. Cultured beta-cell-derived cells were then transduced retrovirally with four pluripotency-inducible transcription factors to generate beta iPS cell lines. Human beta iPS cells showed typical ES cell-like phenotype and histone H3 acetylation and were maintained 30+ passages. These authors transplanted the human beta iPS cells into SCID mice which resulted in detectable serum levels of human C-peptide at 3-week post-transplantation, and insulin mRNA expression at 6-7 weeks in grafted tissues of mice (80).

In vitro premature ageing model using patient-derived iPS cells: Hutchinson-Glifford progeria syndrome (HGPS) is a rare and fatal human disease, characterized by premature ageing with arteriosclerosis and degeneration of vascular smooth muscle cells. Smooth muscle senescence is caused by accumulation of progerin, a mutant protein that also accumulates in physiological ageing leading to similar disorders of vascular smooth muscles. An *in vitro* model was created by generating HGPS-iPS cells from fibroblasts of patients with HGPS to study the pathogenesis of human premature and physiological ageing (81).

In conclusion, the generation of hiPS cells is a major achievement for transplantation of appropriate cells in regenerative medicine, for *in vitro* models from patient-provided tissues, for testing drugs and/ or investigating mechanisms of chronic and debilitating diseases. However, viral integration and induction of exogenous sequences into the host genome increases the risk of tumorigenicity. Rigorous genetic testing and appropriate preclinical steps should be conducted before transplantation in patients.

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