

## Editorial: Our Top 10 Developments in Stem Cell Biology over the Last 30 Years

LYLE ARMSTRONG,<sup>a</sup> MAJLINDA LAKO,<sup>a</sup> NOEL BUCKLEY,<sup>b</sup> TERRY R.J. LAPPIN,<sup>c</sup> MARTIN J. MURPHY,<sup>d</sup> JAN A. NOLTA,<sup>e</sup> MARK PITTENGER,<sup>f</sup> MIODRAG STOJKOVIC<sup>g</sup>

<sup>a</sup>The Institute of Genetic Medicine, Newcastle University, The International Centre for Life, Central Parkway, Newcastle upon Tyne, United Kingdom; <sup>b</sup>Department of Neuroscience, Institute of Psychiatry, King's College London, London, United Kingdom; <sup>c</sup>Centre for Cancer Research and Cell Biology, Queen's University, Belfast, Northern Ireland, United Kingdom; <sup>d</sup>AlphaMed Consulting, Inc., North Carolina Division, Durham, North Carolina, USA; <sup>e</sup>University of California, Davis School of Medicine, Davis, California, USA; <sup>f</sup>Johns Hopkins University School of Medicine, Severna Park, Maryland, USA; <sup>g</sup>SPEBO Medical, Leskovac, Serbia

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### ABSTRACT

To celebrate 30 years of peer-reviewed publication of cutting edge stem cell research in *Stem Cells*, the first journal devoted to this promising field, we pause to review how far we have come in the three-decade lifetime of the Journal. To do this, we will present our views of the 10 most significant developments that have advanced stem cell biology where it is today. With the increasing rate of new data, it is natural that the bulk of these developments would have occurred in recent years, but we must not think that stem cell biology is a young science. The idea of a stem cell has actually been around for quite a long time having appeared in the scientific literature as early as 1868 with Haeckels' concept of a *stamzelle* as an uncommitted or undifferentiated cell responsible for producing many types of new cells to repair the body [Naturliche Schopfungsgeschichte, 1868; Berlin: Georg Reimer] but it took many years to obtain hard evidence in support of

this theory. Not until the work of James Till and Ernest McCulloch in the 1960s did we have proof of the existence of stem cells and until the derivation of embryonal carcinoma cells in the 1960s–1970s and the first embryonic stem cell in 1981, such adult or tissue-specific stem cells were the only known class. The first issue of *Stem Cells* was published in 1981; no small wonder that most of its papers were devoted to hematopoietic progenitors. More recently, induced pluripotent stem cells (iPSCs) have been developed, and this is proving to be a fertile area of investigation as shown by the volume of publications appearing not only in *Stem Cells* but also in other journals over the last 5 years. The reader will note that many of the articles in this special issue are concerned with iPSC; however, this reflects the current surge of interest in the topic rather than any deliberate attempt to ignore other areas of stem cell investigation. *STEM CELLS* 2012;30:2–9

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### GOING WITH THE FLOW—FINDING AND ISOLATING ADULT STEM CELLS

July 16, 1945 was not an auspicious day. At the White Sand Proving Grounds, New Mexico at 5:29 a.m. local time, the world entered the nuclear age with the detonation of a 20-kiloton plutonium implosion device that although puny by the standards of later weapons changed our atmosphere forever by adding the first of many increments of radioactive nuclides. After 3 weeks, broadly similar devices were detonated over Japan,

bringing an end to World War II and starting an enormous program of research into the effects of radiation on the human body. One of these effects turned out to be destruction of the cells in the bone marrow (BM) leading to catastrophic anemia in individuals exposed to high doses of ionizing radiation; a logical inference from this was that transplantation of unaffected BM might be a treatment for radiation sickness. The next logical step was treatment of hematological malignancies by radiation-induced ablation followed by transplantation of BM taken from donating individuals. It should come as no surprise, with the benefit of hindsight that these early attempts were

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Correspondence: Lyle Armstrong, PhD., The Institute of Genetic Medicine, Newcastle University, The International Centre for Life, Central Parkway, Newcastle upon Tyne NE1 3BZ, United Kingdom. Telephone: 00+44 191 241 8695; Fax: 00+44 191 241 8666; e-mail: Lyle.Armstrong@ncl.ac.uk or Majlinda Lako, PhD, The Institute of Genetic Medicine, Newcastle University, The International Centre for Life, Central Parkway, Newcastle upon Tyne NE1 3BZ, United Kingdom. Telephone: 00+44 191 241 8688; Fax: 00+44 191 241 8666; e-mail: Majlinda.Lako@ncl.ac.uk Received November 28, 2011; accepted for publication November 28, 2011; first published online in *STEM CELLS EXPRESS* December 7, 2011. available online without subscription through the open access option. © AlphaMed Press 1066-5099/2011/\$30.00/0 doi: 10.1002/stem.1007

unsuccessful due to immune rejection both from the recipient and the incoming donated BM (graft versus host disease); however, a detailed discussion of the development of the entire field of BM transplantation is beyond the scope of this article. For our present discussion, it is sufficient to note that the hematopoietic tissues of the BM produce a continuous supply of differentiated blood cells whose functions are essential for life. The resulting cells have short lifetimes and do not self-renew; therefore, replacing these cells relies upon the existence of cells that can not only produce new copies of themselves but also give rise to new differentiated cells to maintain blood function. The basis of this system is the hematopoietic stem cell (HSC) whose existence was first demonstrated by James Till and the late Ernest McCulloch in 1963 [1] in culmination of their earlier work on the radiation sensitivity of mouse BM cells by showing that limiting numbers of BM cells could give rise to clonal colonies of erythroid and myeloid cells in the spleens of the irradiated hosts. At this stage all we knew was that each spleen colony was genetically unique and had thus arisen from a single cell but we did not know much about the phenotype of the cells in question. Moreover, we had no protocols to isolate them; however, the development of flow cytometry by the 1980s permitted enrichment of the HSC from BM and so qualifies as the first major breakthrough in the history of our journal.

Flow cytometry was a well-established technique by the late 1980s by which time the original principles used to build early coulter cell counters had been advanced considerably. Fluorometry of a single cell as it traversed a beam of exciting light could be used to give quantitative information about the amounts of fluorescent dyes bound to the cell surface, and since a number of excitation wavelengths could be used, the number of possible combinations of dyes was large. Conjugation of such dyes to monoclonal antibodies directed against known terminally differentiated hematopoietic cell markers (such as those on B or T cells) meant that fluorescence-activated cell sorting could be used to separate these cells (lineage depletion) from less-committed progenitor cells. Development of antibodies against antigens present on the progenitor populations allowed us to fractionate this population still further. The investigations of Irving Weissman [2] showed that a population of mouse BM cells expressing the surface antigens Thy1 (low) and Sca1 from which lineage differentiation markers were absent (Lin negative) was able to rescue irradiated BM far more effectively than unsorted BM cells. Injection of as little as 30 cells into a lethally irradiated mouse rescued half of the recipients from BM failure whereas 13,000–33,000 unsorted BM cells were needed to achieve the same effect, indicating that the proposed stem cells were greatly enriched by this protocol. Many studies followed, defining flow-based isolation methods for human stem cells, assessed by xenotransplantation into mice that lacked the capacity for immune rejection. Flow cytometry is now used to enrich many types of adult stem cells and the differentiated progeny of pluripotent cells; without this technique, stem cell biology would be enormously difficult, and so even though its uses are not restricted to the stem cell arena, we have no hesitation in placing it high on our list of major stem cell breakthroughs.

### THE FIRST EMBRYONIC STEM CELLS FROM MICE

Winning a Nobel Prize does not guarantee inclusion in our list but it does help! Quite apart from that, the work of Martin Evans, Matthew Kaufmann, and Gail Martin [3] was a tremendous boost to the study of pluripotent cells because it provided a seemingly stable and immortal cell line that was not

transformed like the immortal lines derived from cancers. By the time mouse embryonic stem cells (mESCs) were derived, the concept of pluripotency, the ability of a single cell type to differentiate into multiple other types of cells, was far from new. The concept arose hand-in-hand with the theoretical proposition that stem cells were responsible for tissue repair in the adult from detailed histological studies of teratocarcinomas which are neoplasms composed of seemingly undifferentiated cells randomly interspersed with somatic tissues. The nature of these somatic tissues was fascinating because they appeared to represent various stages of differentiation. However, the fact that a single cell suspension produced from teratocarcinomas could give rise to completely new neoplasms in experimental animals suggested the presence of stem cells in their tissue mass. Lewis Kleinsmith and Barry Pierce [4] were the first to develop conditions to isolate and culture these cells and they coined the term “embryonal carcinoma cells” (EC cells) in 1964. What did Evans and Kaufmann do that was so special? They showed for the first time that it was possible to isolate the inner cell mass (ICM) of a mouse embryo at the blastocyst stage and coax those cells to survive and develop into colonies of cells that could be cultured as cell lines. This was a lot more difficult than it seems since several groups had attempted unsuccessfully to grow cells from the ICM, although much of this work was done to gain more understanding of embryonic development [5].

By the mid-1970s, we had learned that mouse embryos could complete at least some of their development outside the uterus and that the cells of the trophectoderm could proliferate in the absence of the ICM, but all attempts at independent culture of the ICM had failed. Michael Sherman observed that an enriched culture medium containing 10% heat-inactivated fetal calf serum promoted 90% of mouse blastocysts to hatch from their zona pellucidae and attach to the culture dish but he was unable to prevent differentiation of the ICM. Although the ICM cells expanded in culture to overgrow the trophoblast cells, they seemed to differentiate primarily to epithelial cells. Evans and Kaufmann solved this problem by removing the ICMs as they began to develop into egg cylinder-like structures and disaggregating them into single cell suspensions. These cells were passaged onto irradiated fibroblasts, whereupon colonies of cells that were morphologically similar to EC cells appeared [6]. Unlike EC cells, the mESCs (as they were called by Evans) had normal karyotypes and were able to form embryoid bodies when cultured away from their feeder fibroblasts. Prior to this development, EC cells were widely used to model developmental processes and early embryonic cell differentiation but within a few years, these were supplanted by mESCs principally because the latter are thought to be more representative of those cells present in the ICM (a snapshot of early development, if you will). A major contributing factor to the success of mESCs was the introduction of leukemia inhibitory factor (LIF) into mESC culture media to prevent differentiation and allow them to grow under feeder cell-free conditions [7]. This works because of the diapause phenomenon which permits several rodent species to generate a new batch of fertilized embryos while still pregnant. The new embryos arrest at the blastocyst stage of development until the uterus is vacated, whereupon they implant and resume their development.

Most of the last 10 years has seen the notion of pluripotency evolve as one of a “ground state,” a view protagonized by many notably Austin Smith and Rudi Jaenisch. This perspective sees the job of pluripotency factors as maintaining this ground state (at least in part) by inhibiting differentiation. In an elegant recent review [8] in which Loh and Lim challenge this view and present an alternative view of

pluripotency factors maintaining pluripotency (at least in part) by acting as mutually antagonistic lineage specifiers. As long as all factors are present and correct then this results in a metastable state that is pluripotency. Which view prevails, and whether stem cell biology is broad enough to accommodate both views, only time will tell.

### COPYING GENOMES—THE DEVELOPMENT OF MAMMALIAN CLONING

One might not immediately think of mammalian cloning as a stem cell-related topic but enforcing epigenetic plasticity on a somatic genome, the essential basis of the cloning technique, told us a great deal about the molecular basis of pluripotency. Also, the technique was hailed as a possible means of producing ESCs for individual patients and since a wealth of data was derived from cloning experiments that led, albeit indirectly, to improved techniques for reprogramming somatic cells, cloning has its place in our list. Briggs and King [9–12] demonstrated that somatic cell nuclear transfer (SCNT) could be used to clone frogs. Using oocytes and donor nuclei from *Rana pipiens*, they found that the “reconstructed” embryos were capable of developing to at least the early cleavage stages and in some experiments as far along as the tadpole stage. The use of nuclei from blastomere cells was instrumental in this process because such cells are relatively unspecialized [13, 14]. In retrospect, it was not surprising that early attempts to use SCNT to clone frogs from adult somatic cells met with failure. However, later work by Gurdon [15] using intestinal cells from tadpoles demonstrated that differentiated somatic cells were capable of producing viable embryos. In contrast, SCNT in mammals was more difficult and for many years it was believed that the cells of adult vertebrates were simply too specialized to revert to a totipotent state. This opinion was decisively contradicted with the cloning of “Dolly” in 1996 [16] by fusion of a mammary gland epithelial cell from a Finn Dorset ewe with the enucleated oocyte from a separate donor. Many studies have now shown the possibility of SCNT in various mammalian species [17–19] but for a while the interest lay with mouse SCNT because it proved possible to derive ESC from blastocysts obtained from SCNT of murine fibroblasts into early-stage oocytes [20]. For a while, such nuclear transfer (nt) ESCs were hailed as the solution to the problem of immune rejection of differentiated cells generated from human ESC but so far derivation of nt-ESC from human SCNT blastocysts has been elusive. Even if such derivation is achieved, supply problems with human oocytes may render the technique only marginally useful.

### HUMAN EMBRYONIC STEM CELLS

Derivation of human ESC (hESC) was not rapid following mESC since it took 17 years before James Thomson at the University of Wisconsin reported his first five hESC lines. Fresh or frozen early cleavage stage human embryos, produced by in vitro fertilization (IVF) for clinical purposes, were donated by individuals after informed consent. Embryos were cultured to the blastocyst stage, 14 ICMs were isolated, and five hESC lines originating from five separate embryos were derived, essentially as described for nonhuman primate ESC (which was published 3 years earlier [21]). These cells were more difficult to grow in culture than mESCs but behaved in a broadly similar fashion in terms of their apparent immortality, expression of key surface antigens, and their

ability to generate teratomas in immune compromised mice. Naturally, it was impossible to examine germline transmission following injection into blastocysts since manipulation of human embryos in this manner is illegal.

The method used by Thomson et al. was actually very similar to that of Evans in his mESC derivation work; however, the delay in obtaining hESC may be attributed to problems with the ethical issues and the availability of the necessary human embryos. The original article describing the first five hESC lines was published in *Science* in November 1998 [22] and although it is a fairly brief report in its own right, the significance of this development cannot be underestimated since it is the forerunner of hundreds of new hESC lines derived in the 13 following years. Subsequent research has shown that hESC while similar to mESC have many unique characteristics for modeling human development.

### WHEN GOOD CELLS GO BAD—THE CONCEPT OF CANCER STEM CELLS

We have come a long way in developing methods to kill cancer cells that form a variety of malignancies but relapse is an ongoing problem, along with the development of metastatic tumors at sites remote from that of the original tumor. One suggestion to account for these phenomena is the existence of a tumorigenic stem cell that is capable of regenerating all the differentiated cell types present in the original tumor. Most chemotherapeutic treatment strategies kill the replicating differentiated cells that form the bulk of the tumor mass and these may not be able to destroy all of the rare quiescent putative cancer stem cells (CSCs). If these CSC have self-renewal and expansion characteristics similar to nontumorigenic stem cells, it would only require a few survivors to generate a whole new tumor. The key paper supporting the CSC hypothesis from the laboratory of John Dick appeared in 1997, in which he and Dominique Bonnet demonstrated that an isolated cell type was capable of initiating acute myeloid leukemia [23]. These cells were exclusively CD34<sup>+</sup>CD38<sup>−</sup> similar to normal hematopoietic progenitors suggesting that normal primitive cells rather than the more committed hematopoietic cell types are responsible for leukemic transformation.

### INDUCED PLURIPOTENT STEM CELLS

The pluripotency of ESC makes them a potentially attractive resource for generating clinically useful somatic cells but for the problem of immune rejection. Transplanting differentiated cells obtained from ESC lines is the same as transplanting those cells from the individual from whom they were derived. There have been several attempts to avoid this problem, not the least of which was the therapeutic cloning approach described above, but the field was excited in 2006 by the generation of induced pluripotent stem cells (iPSCs) from the laboratory of Shinya Yamanaka which demonstrated the reprogramming of mouse somatic cells to pluripotency. Retroviral transduction of just four genes (*Oct4*, *Sox2*, *Klf4*, and *c-Myc*) reprogrammed mouse embryonic fibroblasts and adult tail fibroblasts to show characteristics reminiscent of mESC. The resulting cells were named iPSCs and they were capable of contributing to chimeric animals with germline transmission and contribution to all tissues of the resulting offspring indicative of their pluripotency [24, 25].

Yamanaka's group extended their earlier work and showed that human adult dermal fibroblasts could be

reprogrammed by retroviral transduction of *Oct4*, *Sox2*, *Klf4*, and *c-Myc* *OCT4*, *SOX2*, *KLF4*, and *C-MYC* with good efficiency [26]. James Thomson's laboratory found that a slightly different set of factors, *OCT4*, *SOX2*, *NANOG*, and *LIN28*, were sufficient to allow iPSC generation from fetal and adult fibroblasts [27] via lentiviral, rather than retroviral transduction, allowing the transduction of nondividing cells, which was not previously possible using retroviruses. iPSCs are a truly remarkable development which could open the way to patient-specific regenerative medicine and so they qualify as one of the most significant events in our list of the top 10 developments in stem cells but they are not without problems. Initial iPSC studies used retroviruses for gene transfer into target cells, since it was believed these genes would be silenced [28, 29], but alongside their inability to infect nondividing cells [30], it was noted that gene silencing was not maintained in iPSC raising the risk of tumorigenesis [31, 32]. Constitutive lentiviral use, in which transgene silencing is poor [29, 33, 34] was superseded by inducible lentiviral methods which hoped to attain full silencing of transgene expression upon attainment of the pluripotent state. However, the common problem with these vector types is the possibility of mutations upon integration or reactivation of the transgenes, which has been shown to lead to tumorigenesis [25]. New vectors have been developed that can be removed from the reprogrammed genome and further developments of RNA-based reprogramming systems such as those using micro-RNAs, isolated proteins, and small molecules show some promise for deriving integration free iPSC lines but there are still concerns that the somatic genome may not have been fully reprogrammed to pluripotency [35–38]. Some of the concerns are manifested as an epigenetic trace left over from the cell of origin. Even though this trace may be erased over extended passage, the observation raises the concern over the impact and importance of cell-of-origin and its subsequent translational value. Another comfortably forgotten fact in the iPSC field is the representative nature of a single iPSC line—this again points to “not all iPSCs are equal” and again should act as a caution in considering patient-specific iPSCs (see below). These problems could restrict the clinical utility of iPSCs and are under intense investigation by many groups. However, generation of in vitro models of human disease using patient-specific iPSCs is allowing investigation and the generation of a wealth of data that promises to make a major impact on science and medicine.

As an illustration of the use of iPSCs for modeling disease in a dish we can turn to the work of Rusty Gage. At a time when Pharma is retreating from neurodegeneration/neuropsychiatric research because of cost and paucity and inadequacy of animal models, the use of iPSCs as “disease in a dish” assume massive potential. Gage derived iPSC from fibroblasts of schizophrenic patients and subsequently differentiated them into neurons and showed reduced connectivity and synapse formation. Most dramatic was the rescue of this “disease phenotype” by application of the antipsychotic, loxapine [39]. There is a long road between disease in a dish and cures for psychiatric disorders, but at least iPSCs offer a tractable system to interrogate cellular and molecular mechanism.

## MESENCHYMAL STEM CELLS

Number 7 in our top 10 is devoted to the study of mesenchymal stem cells (MSCs). The reasons behind the inclusion of MSCs are simply that they are currently the most prolific source of potential therapeutic strategies for human disease

and numerous clinical trials are underway using this versatile source of stem cells.

MSCs may be isolated from human BM and the first experimental evidence for the existence of a stem cell population in this tissue compartment other than the HSCs arose in the 1960s. This predates the focus of our review but it is worth mentioning to set the later studies in context. Transplantation of decellularized bone to ectopic sites demonstrated that cells from other tissues could generate bone. Pursuing the source of this potential, Friedenstein identified adherent fibroblast-like cells from BM capable of osteogenesis in vivo [40]. Such cells were believed to be a component of the BM stroma needed to support and nurture the hematopoietic functions of the BM but their ability to differentiate into other cells types, such as chondrocytes and adipocytes demonstrated additional lineage potentials. At this point, the multipotency of such adherent fibroblastic cells was recognized and they became known as MSCs rather than simply marrow stromal cells [41].

The name change proved to be quite fortuitous because it soon became apparent that MSCs could be derived from sources other than BM. By the late 1990s, it seemed that umbilical cord blood, in addition to being a valuable source of HSCs, also contained multipotential cells similar to those found in the BM [42]. While it should have been no surprise that the umbilicus would require the presence of stromal cells to generate a niche capable of supporting its HSCs, some sources of MSC were less obvious. For example, cells very similar to MSCs have been isolated from adipose tissues, amnion, placenta, and even the deciduous teeth of younger individuals [43–45]. An entire field of medicine now centers on the use of adipose-derived MSCs for tissue repair. The degree of similarity between MSCs from such seemingly diverse sources is still a matter of some debate despite early suggestions that they are present in the connective tissues of many organs and surround blood vessels as pericytes and may contribute to maintenance of organ integrity.

We may find that other adult or tissue-specific stem cells are capable of similar feats but to date the evidence for this is not so strong and the ubiquitous nature of MSCs suggests they may have a degree of plasticity not enjoyed by other stem cell types. The ease of obtaining MSCs means they have been the subject of more intensive investigation which has brought them closer to the point of medical or commercial application than many other types of stem cell. A recent example in which MSCs were applied to tissue engineering is given in our next top 10 topics.

## TISSUE ENGINEERING WITH STEM CELLS

Growing stem cells in the laboratory is fine for investigating their molecular characteristics and differentiation ability; however, long-term goals of making whole human organs for transplant into patients requires some different approaches. One of the major problems of growing stem cells enriched from the body is that they are no longer in the three-dimensional (3D) microenvironment that supports and nurtures the cells and encourages their efficient function. For these reasons, it is difficult to get stem cells to re-create the complex 3D structures of organs, especially since the patterns of most organs were laid down during embryonic development and the stem cells were only required to replace cells lost from the existing structure. One way around this problem is to build a scaffold onto which the stem cells (and other types of cell) can be engrafted. Artificial scaffolds have been created



from collagen, hydroxyapatite, and various biodegradable polymers most of which have been used for building artificial bone.

The technique of tissue or organ decellularization strips away cells and antigens, to leave a scaffold composed mostly of the extracellular matrix deposited by the cells in the original structure. This is an interesting concept since it suggests that all the positional information required to build an organ is present in its extracellular matrix “skeleton.” This implies the presence of signals to tell specific cell types where they need to attach and this was the basis of attempts to construct animal hearts from cadaveric examples from which the cellular material had been removed by perfusion with detergent solutions. A specific example of this type of experiment was performed by the group of Doris Taylor at the University of Minnesota in which beating rat hearts were generated by recellularization of the extracellular matrix scaffolds using suspensions of neonatal cardiac cells [46]. Taylor’s group thus introduced the concept of tissue decellularization as “nature’s platform” for rebuilding organs. Decellularized tissues were prominent in the 1980s when bioengineers were comparing their polymer materials to natural products. Small intestinal submucosa has been used since 1960s, and decellularized pig valves were used clinically for many years. An exciting extension of this technique is Anthony Atala’s use of collagen–polyglycolate polymers as scaffolds on which smooth muscle cells obtained from a biopsy taken from a diseased bladder could be seeded followed 48 hours later by urothelial cells from the same biopsy. After 3–4 days, these cells had colonized the scaffold sufficiently well to allow the whole structure to be transplanted into the patient [47]. The patients’ cells remodeled and replaced the foreign materials and the resulting bladders functioned well in the recipient patients.

The most successful and well noted clinically used decellularized tissue engineering product was the artificial trachea transplanted into a patient in Barcelona in 2008 (to replace the patient’s left bronchus) [48]. This procedure was exciting because the research group used a cadaveric trachea that had been decellularized. This scaffold was readily colonized by the patient’s own epithelial cells and chondrocytes derived from MSCs and provided a functional section of airway that was an immediate replacement for the patients damaged bronchus. The patient is healthy 3 years after surgery and several other attempts to transplant sections of trachea made outside the body have been recorded worldwide and are now extending to pediatric patients.

Some organ structures are more complex than others so it remains to be seen how effective these techniques will be in future studies especially since many cell types are needed to build the various parts of the organs. These problems are being addressed in various investigations worldwide, such as in lung and liver bioengineering, where reconstructed organs based on the innate decellularized tissue can survive for at least 2 months in vivo [49, 50] and if they can be overcome, this could be a most interesting method of generating new organs for transplant, and hence this development has been included in our top 10 list.

ably obtain a cell line with a stable integrated reporter. The trick was not to be too liberal with the trypsin because pluripotent cells did not like being on their own very much. Single hESC will adhere to feeder cells or extracellular matrix monolayers but their survival rates are low. Another significant problem was that one could never be sure quite where the transfected construct was going to integrate into the genome meaning that expression of the transgene could be unpredictable over time due to atypical epigenetic changes occurring near its site of integration. There was also the complication of multiple integrations of the transgene per cell and disruption or activation of other genes, creating a nonisogenic experimental setting.

Targeting gene constructs to specific genomic loci offers the possibility of specific permanent editing of the genome providing a truer representation of genetic behavior in its native environment. The best way to achieve this uses homologous recombination by delivering a DNA template with long regions of homology to the target locus. This technique has been routinely used for successful and efficient gene targeting in mouse ESC with a homologous recombination rate of 1 in  $10^3$  cells, to knock genes in and out, and generate transgenic lines, which have been important for elucidating gene function. Applying the same procedure to hESC only achieves a recombination rate of 1 in  $10^6$  cells because successful transfection with the gene targeting construct relies on a single cell suspension. This is not a problem for mESC because we can prevent their differentiation with LIF but since this does not apply to hESC, the procedure does not work well and following the first report of human gene targeting by Thomas Zwaka [51] only a few publications reported the use of this technique.

The recent development of zinc finger nucleases (ZFNs) promises to change this state of affairs and so are worthy of inclusion into our top 10 list. ZFNs [52, 53] are designed to recognize specific DNA sequences by combining C2H2 zinc finger proteins [54] into a customized array [55]. This zinc finger domain is linked to the nonspecific FokI nuclease which cleaves the DNA into a double-strand break (DSB) [56–59]. For its activity, the FokI domain needs to dimerize [60]; thus ZFN pairs are required and hence designed to bind to the region of interest in the opposite orientation. DSBs are bad for genome stability so the cell proceeds to repair them as quickly as possible using either one or both of two available methods. Homologous recombination is more accurate but relies on the presence of the homologous sequence from the undamaged sister chromatid as a template. It has been shown that the HDR apparatus can use a supplied donor DNA plasmid which contains homology arms as a surrogate template. This approach allows for gene correction of single nucleotide changes from an exogenous episomal donor to the endogenous locus. Larger sections of DNA can also be inserted into the genome at a desired location using this technique making this one of the most powerful methodologies available for manipulation of pluripotent stem cell genomes (for a comprehensive review of ZFN-based gene targeting see [61]).

## IMPROVING GENETIC MANIPULATION

Until a few years ago, what you saw was largely what you got with ESC, at least as far as their genomes were concerned. It was possible to insert constructs with reporter genes and as long as you did not break up the colonies of cells too much, you could put them under antibiotic selection and prob-

## GETTING CLOSER TO CURES

The ultimate objective of all stem cell research is to understand human biology and use this knowledge to cure human diseases but how close are we to this goal? In truth, stem cell transplants have been used for many years in the treatment of leukemias (BM transplant) but given the amount of media

attention devoted to the field since the first derivation of hESC, it is important to demonstrate that clinical application of stem cell biology is possible. For the final entry in our top 10 list, we have included a number of examples of ongoing clinical trials to demonstrate that stem cell-based cures are not merely some hypothetical concept that are always, “just a few years down the line.”

Our first example illustrates an application of adult tissue-specific stem cells to restore sight and results from the efforts of several groups worldwide. The human cornea is maintained by a population of stem cells residing in the limbus which is the border between the transparent region of the cornea and the opaque conjunctiva. In cases of chemical, mechanical, or thermal injury to the eye, the stem cells can be destroyed leading to reduced corneal maintenance which in turn permits the conjunctiva to grow over the spaces they leave behind. The excessive growth of opaque tissue naturally occludes vision but is also very painful. The global term for this condition is limbal stem cell deficiency (LSCD) and it often requires long-term, costly treatment with frequent clinic visits and intensive hospital admissions. The vision loss due to LSCD makes this disease not only costly but also often requires social support due to the enormous impact on the patient's quality of life. This is further magnified by the fact that LSCD mostly affects young patients. If the damage is unilateral, it is possible to excise a small amount of tissue from the limbus of the healthy eye and after appropriate culture, colonies enriched in limbal stem cells can be derived. Attachment of these cells to small pieces of human amniotic membrane or culture of single cell suspension on mitotically inactivated feeder cells allows them to be engrafted into the surgically exposed limbus of the damaged eye whereupon the stem cells can recolonize their intended niche [62, 63] eventually restoring sight. The technique also avoids the need for drugs to suppress immunity and means there is no chance of the implanted cells being rejected.

Our next example of a stem cell-related therapy takes us back to the MSC arena. Cellular therapies for myocardial infarction (MI) are currently emerging that include i.v. delivery of culture-expanded BM-derived MSC. It was initially hoped that MSCs would differentiate to the tissue of interest, but their potent secretion of factors to help heal and revascularize tissues is emerging as the more important mechanism. A great advantage of MSCs is their seemingly low immunogenicity which permits the use of allogeneic cells. This is a significant benefit since expansion and application of limited numbers of batches, or “lots” of MSC would be more cost-effective and well-controlled in a Good Manufacturing Practice setting than isolation and expansion of MSC from every patient requiring treatment of this type. Clinical trials have taken place which established the safety and efficacy of allogeneic MSC transplantation, first for graft versus host disease [64] and then for MI treatment [65]. In terms of pulmonary function and cardiac performance, MSC-treated MI patients showed significant improvement relative to those treated with placebo, and there was no evidence that i.v. administration of MSC resulted in formation of tumors or ectopic tissues. In addition, there was no evidence of organ damage due to MSC lodging in the microvasculature. Thousands of patients have been safely treated with expanded MSCs worldwide, and phase II and III clinical trials for many indications are ongoing. However safe, there remains significant room for improvement in the engraftment of MSCs, as only 1%–2% are detectable in the recipients after a short period (1–2 weeks).

The progress toward stem cell cures is not always straightforward as shown by the recent controversy surrounding the Geron Corporation's phase I clinical trial to examine the safety and efficacy of hESC-derived oligodendrocyte progenitor cells in treating spinal cord injuries (SCIs). Approximately 12,000

people in the U.S. sustain SCIs every year caused by trauma to the spinal cord that results in a loss of such functions as locomotion, sensation, or bowel/bladder control. A traumatic blow to the spine can fracture or dislocate vertebrae that may injure the nerve fibers and the glial cells that insulate the nerve fibers in the spinal cord. SCIs do not repair spontaneously but oligodendrocyte progenitor cells have demonstrated remyelinating and nerve growth stimulating properties leading to restoration of function in animal models of SCI. This works because oligodendrocytes naturally synthesize the myelin that wraps around the axons of neurons to enable them to conduct impulses in a manner analogous to the insulation surrounding electrical wires. Oligodendrocytes also produce several neurotrophic factors that promote the survival of neurons and preclinical studies have shown that injecting oligodendrocyte precursor cells (made from ESC) into rats with SCI allows new oligodendrocytes to colonize the injury site where they proceed to generate new myelin and promote neuronal growth [66]. These data encouraged the idea that SCI might be treatable using ESC-derived oligodendrocytes and Geron has pressed hard in recent years to push the concept to a clinical trial so it was surprising that the company called a halt to the study in mid-November 2011 citing economic reasons. The company claimed that further development would cost \$25 million per year and this was too large a drain on its resources to justify supporting a research program from which no products have yet arisen. We understand that Geron is seeking alternative business partners to continue the project so we can only hope that this would be resolved quickly and that this important pioneering translational work resumes.

Whereas Geron's approach in repairing the damaged nervous system is based on transplantation, as is much of the work on “simple” neurodegenerative diseases such as Parkinson's Disease, it is much less clear what transplantation has to offer more complex neurodegenerative disorders such as Huntington's or Alzheimer's disease. An alternative strategy is to recruit the endogenous neural stem cell machinery that lies within the neurogenic niches of the forebrain; no transplantation, no rejection of heterologous transplants, no trauma. An even more radical idea is to recognize the latent neurogenic capacity of parenchymal “non-niche” reactive astrocytes—reactive astrocytes are by definition at the site of injury/degeneration—right where you need to initiate repair. Magdalena Gotz has led the way on this over the last decade [67] and has clearly shown that reactive astrocytes possess a latent neurogenic capacity that is clear in a dish—the challenge is overcoming the inhibitory signals from the brain that block this capacity in vivo.

## CONCLUSION

There is not enough space in this review to include all the exciting developments that are currently taking place in the use of stem cells to improve the lives of patients suffering from a broad spectrum of diseases throughout the world. We hope that this brief listing of our top 10 developments over the last 30 years of *Stem Cells*' history has convinced the reader that far from being an obscure academic discipline, the study of stem cell biology is making significant contributions to the quality of human life. While we are pardonably proud of the prominent role that *Stem Cells* has played in helping to prosecute the peer-reviewed progress of the past three decades, we are all the more mindful of the abiding responsibilities we shoulder as the first and oldest journal devoted to discovering the universe of secrets still enwrapped within stem cells. We accept it as a sacred trust and, as we did at the

outset 30 years ago, we ask you, our readers, and our authors, for your collegial collaboration.

We are grateful to our four Founding Editors, Donald Metcalf, Fumimaro Takaku, and the late Laszlo Lajtha who, with Martin Murphy serving as Editor-in-Chief, founded the Journal in 1981, “by scientists in the service of science” at the dawn of what may be truly called the Stem Cell Era. *Stem Cells*, first published by Karger (1981–1983), was published by AlphaMed Press as *The International Journal of Cell Cloning* from 1983 to 1994 when AlphaMed Press reclaimed its founding name, *STEM CELLS*®, which was then trademarked on the Primary Register. We are grateful to Curt Civin, who led *Stem Cells* from 2000 to 2007, to Donald Phinney and Miodrag Stojkovic, who were its coeditors from 2007 to 2009, and to Miodrag Stojkovic, who served the Journal as editor from 2009 to 2011. Now, in January of 2012 at the dawn of its third decade, we warmly welcome Jan Nolte as she takes up her role as the Journal’s Editor ... with the same mission and renewed commitment to publishing excellence by scientists in the service of science.

Finally, we pause to acknowledge our gratitude and our respect for our publisher and managing editor, Ann Murphy, who has been the guiding hand behind every issue of the Journal for three decades. She is the glial element that has bound us together and, by her example, reminds us to devote our very best to this very special journal.

We would be delighted to hear from you, our readers. What is the one scientific advance that you believe—evidence-based—should have been included in your top 10? The best will be published as Letters to the Editor, as space allows.

Working together, the best is yet to come!

### DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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## Concise Review: Embryonic Stem Cells Versus Induced Pluripotent Stem Cells: The Game Is On

MIRA C. PURI,<sup>a,b</sup> ANDRAS NAGY<sup>a,c,d</sup>

<sup>a</sup>Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada; <sup>b</sup>Department of Medical Biophysics, <sup>c</sup>Department of Molecular Genetics, and <sup>d</sup>Institute of Medical Science, University of Toronto, Toronto, Ontario, Canada

**Key Words.** Embryonic stem cells • Induced pluripotent stem cells • Pluripotency • Disease modeling • Genome integrity • Tumorigenicity

### ABSTRACT

Extraordinary advances in pluripotent stem cell research have initiated an era of hope for regenerative strategies to treat human disease. Besides embryonic stem cells, the discovery of induced pluripotent stem cells widened the possibility of patient-specific cell therapy, drug discovery, and disease modeling. Although similar, it has become clear that these two pluripotent cell types display significant differences. In this review, we explore current knowledge of

the molecular and functional similarities and differences between these two cell types to emphasize the necessity for thorough characterization of their properties as well as their differentiation capabilities in the pluripotent state. Such comparative studies will be crucial for determining the more suitable cell type for future stem cell-based therapies for human degenerative diseases. *STEM CELLS* 2012;30:10–14

Disclosure of potential conflicts of interest is found at the end of this article.

### INTRODUCTION

Since the advent of human embryonic stem cells (hESCs) in 1998 [1], stem cell research has been developing at a breathtaking pace. The pluripotent nature of these cells renders them the ability to differentiate into any cell type—including into those with therapeutic potential—after practically unlimited self-renewal in the stem cell state. ESCs hold enormous promise as tools for understanding normal development and disease, and just as importantly, for cell therapy applications to treat devastating and currently incurable disorders, such as spinal cord injury, neurological disease, blindness, and type 1 diabetes. On the other hand, the use of human embryos to derive these cells has ignited a diverse ethical debate rooted in the complex background of human historical, cultural, and religious differences. In this review, we are not going to pursue a discussion of ethical issues but rather focus on the potential of pluripotent cells in general to cure disease and eliminate human suffering.

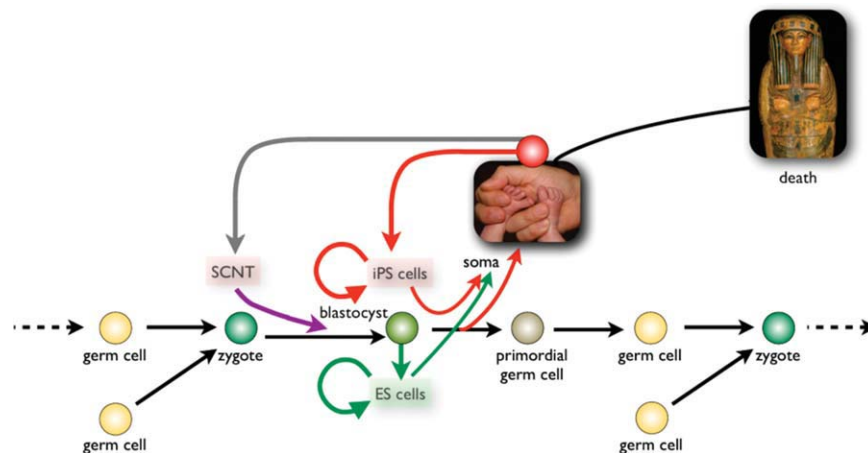
Following the characterization of the first hESC lines in the late 1990s, standard protocols have steadily been developed to accommodate future clinical applications, including maintenance of these cells in the absence of animal-derived culture components. Furthermore, guided by insights gained from decades of research on the molecular genetic basis of mammalian development, detailed protocols have emerged for the reproducible generation of enriched populations of various differentiated cell lineages in mouse and human, including neurons, cardiomyocytes, and hematopoietic cells [2]. Numer-

ous preclinical animal studies have demonstrated that the differentiated derivatives of ESCs can provide functional replacements for diseased tissues, such as for Parkinson's disease [3], and clinical trials are currently underway for hESC-based cellular therapy for spinal cord injury and macular degeneration in the U.S. and U.K. [4].

Six years ago, Takahashi and Yamanaka astonished the world by showing that enforced expression of four key transcription factors, Oct4, Sox2, Klf4, and c-Myc, can reprogram mouse somatic cells such as fibroblasts to pluripotency, and achieve similar developmental potential as ESCs, without the requirement for an embryo [5]. They named these new cells “induced pluripotent stem cells” or iPSCs. A year later, several groups, including Yamanaka's, reported the successful generation of iPSCs from human somatic cells [6, 7]. With this step forward, a race was initiated. The expectation that iPSCs will offer the same therapeutic potential as hESCs and the robust, reproducible method of deriving iPSCs have spawned hundreds of studies addressing in vitro disease modeling and cell therapy strategies in preclinical animal models. Indeed, iPSC cell lines have now been generated from patients of several monoallelic and complex genetic disorders (reviewed in [8]). These developments have brought the field a hopeful step closer to the promises of in vitro disease modeling, disease-specific pharmacological treatment testing, and in some cases individualized cell replacement therapy. Several examples of the differentiation of disease-specific iPSCs into the cell types that are implicated in the disorder's pathogenesis have been reported, and therefore this technology is particularly attractive for the diseases for which animal models are

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Correspondence: Andras Nagy, Ph.D., Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 60 Murray Street, Box 40, Toronto, Ontario M5T-3L9, Canada. Telephone: 416-586-4800, ext. 3246; Fax: 416-586-5130 <http://www.mshri.on.ca/nagy/>; e-mail: [nagy@lunenfeld.ca](mailto:nagy@lunenfeld.ca) Received October 13, 2011; accepted for publication November 2, 2011; first published online in *STEM CELLS EXPRESS* November 18, 2011. © AlphaMed Press 1066-5099/2011/\$30.00/0 doi: 10.1002/stem.788



**Figure 1.** Schematic representation of the germ-soma conflict theory of August Weismann and the journey of the ES, iPS, and SCNT cell genome. The black arrows show the journey of the genome in the germ line. Red and green arrows show where iPSCs and ESCs could acquire genetic alterations, respectively. The semicircle arrows show the self-renewal/expansion of iPSCs and ESCs. Purple arrow represents the reprogramming after SCNT. Abbreviations: ESCs, embryonic stem cells; iPSCs, induced pluripotent stem cells; SCNT, somatic cell nuclear transfer.

either not available or do not accurately represent the human disease etiology.

The following question thus arises can iPSCs replace ESCs in clinical application and disease modeling? Although all of us would likely welcome a “yes,” it is clear that we are not yet in the position to answer this question, despite the unprecedented speed of development in the iPSC research area. Our understanding of the full characteristics of iPSCs and the mechanistic details of reprogramming to pluripotency is far from complete [9]. Although several analyses indicate that iPSCs share many key properties with ESCs including morphology, pluripotency, self-renewal, and similar gene expression profiles, there are just as many published examples that point out their differences. There is an urgent need to shed more light on the complex landscapes of safety, efficacy, economy, and disease coverage associated with clinical use of these new and exciting pluripotent cell types. At the current state of knowledge, we promote the view that parallel, direct studies on iPSCs and ESCs, including detailed characterization of mechanisms of pluripotency and differentiation are required to make the promise of stem cell-based therapeutics for human disease a reality.

### GENOME INTEGRITY

During expansion and prolonged passage, hESC lines frequently acquire abnormal karyotypes such as trisomy 12 and 17 [10, 11] as well as genetic amplification at 20q11.21, which has been associated with oncogenic transformation [12, 13]. iPSC lines are also subjected to similar selective forces, resulting in cell culture adaptation frequently manifested in karyotypic abnormalities [14]. So in this respect, ESCs and iPSCs are likely equivalent.

The distinct cellular origin, however, could lead to significant differences between these two pluripotent stem cell types. ESCs are derived from the inner cell mass of a blastocyst-stage embryo before the soma and the germ cell lineages separate. iPSCs, however, are derived from somatic cells.

It was August Weismann who in 1889 first recognized that in most organisms, the somatic and germ cell lineages separate very early in development and pointed out the evolutionary consequences of this separation [15]. Weismann postulated that hereditary information moves only from germ

cells to somatic cells. The reverse direction, soma to germ line, would be impossible. Moreover, the genome of the germ cell lineage is passed to the new generation and in this respect it is immortal. On the other hand, the genome of the somatic cells is mortal, as it is discontinued with the death of the organism. Therefore, mutations generated in the soma are not subjected to evolutionary selective forces, such as natural selection or genetic drift. Instead, the immortal germ cell genome remains mostly locked into the germ cell lineage with a brief passing through the very early developmental stages (preimplantation and early postimplantation in mouse and human), prior to the germ-soma separation (Fig. 1).

As evolutionary selective forces act only on mutations in the germ line genome, the expectation is that the strength of genome integrity protection might therefore be different between germ line and soma. A putative differential genome protection could have significant consequence regarding the genome integrity of ESCs versus iPSCs. ESCs derived from the inner cell mass of the blastocyst have never had a journey through a stage in the soma.

John Gurdon’s somatic cell nuclear transfer (SCNT) in frogs showed that with experimental manipulation it is possible to return the genome of a somatic cell to the germ line [16]. This discovery was later followed by success in sheep [17], mice [18], and numerous agricultural species [19]. SCNT reprograms the somatic cell genome into a totipotent cell state (Fig. 1). As SCNT has become a routine procedure in many mammalian species, it has become evident that cloned animals suffer increased risk of abnormalities ranging from prenatal death to altered development [20]. It is still not completely clear what proportion of these abnormalities is due to incomplete epigenetic reprogramming or due to permanent genetic changes occurring during somatic cell development or during the reprogramming process (see below).

The generation of iPSCs by reprogramming using enforced expression of a finite number of transcription factors is similar in this respect. The genome of a fully differentiated somatic cell is returned to pluripotency, which theoretically includes germ line competence (Fig. 1). Therefore, iPSCs can acquire genetic alterations at two additional phases: during somatic differentiation and during reprogramming. It is likely that none of these phases have developed genome-protecting mechanisms responding to evolutionary pressure.

Several recent studies have demonstrated that the reprogramming process leads to genomic instability and genomic abnormalities, with a notable proportion of lesions mapping to known cancer causative loci [14, 21, 22]. Reprogramming causes genomic copy number variations (CNVs) to occur early in iPSC passage leading to mutations and a mosaic iPSC population [21, 22]. During passage, iPSCs undergo strong selection pressure against most of the mutations and reach a CNV load similar to that of ESCs. Nevertheless, hiPSCs contain *de novo* mutations that are not detected in hESCs, suggesting that certain mutations are selected for and are advantageous to reprogramming [14, 21, 22]. Taken together, the available data suggest that reprogrammed cells indeed likely pose a greater risk for accumulation of deleterious genomic mutations. Furthermore, when the reprogramming factors are not silenced, iPSCs are predisposed to additional genomic instability [23]. These findings underscore the critical requirement for detailed characterization of the genome integrity of iPSCs in comparison to that of ESCs and the human genome for correct interpretation of experimental results using these cell lines, and also for safe future therapeutic applications.

### GENETIC AND EPIGENETIC REGULATION OF THE PLURIPOTENT STATE

Comparisons of iPSCs and ESCs have indicated that major features of the ESC epigenome are reproduced in iPSCs, including genome-wide methylation patterns and the establishment of bivalent histone marks at specific loci [24–26]. However, some analyses of reprogramming in mouse cells have shown that differences in gene expression and differentiation potential are observed specifically in early passage iPSCs and have led to the concept that an “epigenetic memory” of previous fate persists in these cells [27–31]. Epigenetic memory has been attributed to the incomplete removal of somatic cell-specific DNA methylation at regions in proximity to CpG islands known as “shores” [28, 32]. The residual DNA methylation pattern and resulting gene expression of the somatic cell of origin are lost upon continued serial passage of derived iPSCs and after treatment with molecular inhibitors of DNA methyltransferase activity [28, 29] suggesting that epigenetic memory also identifies cells that are incompletely reprogrammed. On the other hand, these findings suggest that cell type of origin could affect results in disease modeling as iPSCs show distinct cellular and molecular characteristics based on the cell type of origin. However, it has been noted that this property may improve the prospects of generating some cell types for cell replacement therapy, in particular for those that are difficult to generate by differentiation from ESCs, including insulin producing pancreatic beta cells [27].

### GENE EXPRESSION

In agreement with the epigenetic similarity of the two pluripotent cell types, comparative transcriptome analyses using microarray also indicate that hESCs and hiPSCs are highly alike on a global scale, with gene expression patterns clustering together, and separate from the originating somatic cells [9]. iPSCs may retain, however, a unique gene expression signature, including that of microRNAs and long noncoding RNAs [33–37]. In addition, a few studies have noted that some transcriptional differences can also be attributed to

latent expression of the four reprogramming factors, to genetic background, and to differences in *in vitro* microenvironment and handling conditions in different laboratories [24, 38]. These findings collectively suggest that detailed analyses and standardization of reprogramming and cell culture protocols will be required to validate whether small variations in gene expression seen between iPSCs and ESCs have biological significance.

### DEVELOPMENTAL POTENTIAL VERSUS DISEASE RISK

As mouse ESCs have the capacity to generate an entire normal adult mouse, they are considered as the gold standard against which all other cell types are compared with respect to pluripotency. The ability to significantly contribute to chimeras is considered the most stringent test of pluripotency for mouse iPSCs. Interestingly, available data suggest that compared with ESCs, only a small percentage of mouse iPS cell lines can contribute to strong chimeras or quite infrequently form completely iPSC-derived animals in tetraploid embryo complementation [39]. Furthermore, the earliest studies on iPSC-derived chimeric mice demonstrated that they were prone to cancer and attributed this property to the re-expression of the *c-myc* reprogramming factor [40]. *C-myc* is a well-studied oncogene, and the expression of the other three reprogramming factors has been associated with several forms of human cancer [41]. For this reason, substantial efforts have been made to find reprogramming methods that do not require permanent transgene integrations. During the last 3 years, several such factor delivery methods have been developed using adenovirus, the piggyBac transposon, as well as direct protein transduction among others [42].

Pluripotency of ESCs and iPSCs, as defined by the ability to differentiate into tissues of all three germ layers, is also assessed using the *in vivo* teratoma assay, the only pluripotency test available for the study of human pluripotent cells. Detailed pathological characterization of teratomas in immunocompromised mice has recently revealed surprising differences between hESCs and iPSCs. iPSC-induced teratomas were more aggressive, with a shorter latency than ESCs and frequently contained areas with more aggressive teratocarcinoma characteristics [43]. It remains to be determined whether such pathological features can be directly attributed to alterations at the genome level during reprogramming and prolonged passage *in vitro*. Recent analyses suggest that the pluripotent and tumorigenic capacity of ESCs may be governed by different cell signaling pathways [44], a property that most likely also applies to iPSCs. This necessitates a thorough molecular understanding of the differences between ESCs and iPSCs with respect to their developmental potential and risk of ill behaving if their derivatives were grafted into an individual.

Ironically, the vast proliferation and tissue differentiation potential of iPSCs and ESCs *in vivo* is considered to be one of their main obstacles for clinical use. For example, formation of teratoma-like tumors was observed in one of the tests for the efficacy of hESCs in a mouse model of Parkinson's disease and interfered with the ability of grafted cells to restore dopaminergic neural function [3]. Furthermore, a survey of teratoma formation by grafted neural tissue obtained from iPSCs that were derived from different cellular sources and with different methods has identified another important aspect of the safety of cellular therapy. Tumor formation was positively correlated only with the residual presence of

undifferentiated cells but, interestingly, not with the presence of c-myc or with other variables in the iPSC derivation process [45]. These reports demonstrate that the elimination of residual pluripotent cells is a major challenge and an issue that is equally potent for ESCs as is for iPSCs. With current protocols, it is very difficult to produce completely pure populations of differentiated derivatives from ESC or iPSC cultures for transplantation. In the future, stringent cell surface marker-based cell separations, or depletion of undifferentiated cells, or modifications of the starting iPSC or ESC populations that permit deletion of undifferentiated cells *in vivo* will have to be considered.

## DIFFERENTIATION AND DISEASE MODELING

For clinical applications, reprogramming is the first step with the ultimate goal being reproducible differentiation and maximum enrichment to specific cell lineages. While this property is established for ESCs, albeit still with technical barriers, very recent studies have begun to address the differentiation capacity of human iPSCs and the functionality of their differentiated derivatives. Although multiple protocols have been developed to derive specific cell types *in vitro*, there is considerable variability in the efficiency of generating differentiated lineages among independent hESC and iPSC lines [46]. The production of hemangioblast cells and other derivatives occurred at a much lower efficiency from hiPSCs than from hESCs [47]. Similarly, hiPSCs differentiate to neural lineages at a much lower frequency than ESCs regardless of the means of derivation [48]. The molecular signature of iPSCs can be influenced by the cell type of origin, and in one case, can explain this biased differentiation potential [27]. Premature senescence of differentiated endothelial cells and retinal pigment epithelium from iPSCs have also been observed [49, 50] suggesting that the differentiated progeny of iPSCs may also display significant functional differences that could undermine their therapeutic utility. Thus, it is important to consider that genetic or epigenetic features that affect iPSCs during differ-

entiation could also do so after transplantation, generating cells with gene expression patterns or phenotypic characteristics that are different from ESC-derived transplants.

## CONCLUSION

Permanent cell lines of pluripotent ESCs and iPSCs and our increasing ability to direct them into any cell type for therapeutic potential holds enormous promise for future regenerative medicine. ESCs are considered to be the gold standard of pluripotency, while iPSCs offer the development of cells from any adult individual, which advances the possibility of curing devastating degenerative diseases using cell or tissue grafts with perfect histocompatibility match. This potential calls for efforts to characterize and compare the nature of these pluripotent cell types in great detail. Only such deep studies can give us sufficient insight into the potential, efficacy, and safety to reach a decision; which one will be more favorable for future clinical applications. At the current state of knowledge, we are not in a position to make such a decision. The game between ESCs and iPSCs is still on with no obvious indication of the winner.

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## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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## Concise Review: Oct4 and More: The Reprogramming Expressway

JARED STERNECKERT,<sup>a</sup> SUSANNE HÖING,<sup>a</sup> HANS R. SCHÖLER<sup>a,b</sup><sup>a</sup>Department of Cell and Developmental Biology, Max Planck Institute for Molecular Biomedicine, Münster, NRW, Germany; <sup>b</sup>Faculty of Medicine, University of Münster, Münster, NRW, Germany**Key Words.** Reprogramming • Oct4 • Induced pluripotent stem cells • Germline stem cells • Transdifferentiation

## ABSTRACT

Through cellular differentiation, a single cell eventually gives rise to all the various lineages of an organism. This process has traditionally been viewed as irreversible. However, nuclear transfer experiments have demonstrated that differentiated cells can be reprogrammed to form even an entire organism. Yamanaka electrified the world with the discovery that expression of only four transcription factors was sufficient to induce pluripotency in differentiated somatic cells of mammals. Expansion of this work has shown that expression of the master pluripotency gene *Oct4* is

sufficient to induce pluripotency in neural stem cells. In contrast to somatic cells, germline cells express *Oct4* and can acquire pluripotency without the addition of exogenous transcription factors. More recently, it has been possible to also induce an alternative cell fate directly by the transdifferentiation of cells mediated by the introduction of specific transcription factors, including *Oct4*. Therefore, we suggest that *Oct4* is the gatekeeper into a reprogramming expressway that can be directed by altering the experimental conditions. STEM CELLS 2012;30:15–21

Disclosure of potential conflicts of interest is found at the end of this article.

## INTRODUCTION

All mammalian organisms begin as a single cell—the zygote. This single cell will go on to differentiate into every cell lineage and pattern the various cells into a fully functional organism. This ability is referred to totipotency and is retained through the first few cleavage divisions. Within a few days of fertilization, the zygote has divided and differentiated to form a blastocyst, which is composed of trophectoderm and inner cell mass (ICM) cells. Even at this early stage of development, trophectoderm cells have already committed to a developmental fate and will generally not regain the potential to differentiate into other cell types. Although the ICM cells retain a broad developmental potential to form every lineage of the embryo proper, they have lost the ability to organize all the cell types independently into an organism. As such, these cells are no longer totipotent—that is, they are pluripotent. After gastrulation, all cells have committed to either a germ cell or a particular germ layer fate. Only germ cells retain the ability to form a totipotent cell through fertilization.

Although fate commitment cannot normally be reversed in vivo during development, technologies have emerged that are capable of reprogramming mammalian somatic cells to totipotency and pluripotency in vitro. Gurdon [23] firmly established that fate commitment is reversible by showing that nuclei from differentiating endodermal cells from different developmental stages, ranging from blastulae to swimming tadpoles, consistently gave rise to swimming tadpoles when introduced into enucleated oocytes (Table 1). In 1996, the birth of Dolly proved that reversing differentiation in mam-

malian species was also possible [24]. More recently, direct induction of pluripotency in somatic cells, such as fibroblasts, has become possible [3, 19]. Finally, transdifferentiation of one cell type directly into an alternative cell lineage, such as reprogramming a fibroblast directly into a neuron, suggested that totipotent or pluripotent cells may not even be necessary intermediates. However, reprogrammed cells have been shown to retain the epigenetic memory of their tissue of origin [25, 26]. This indicates that reprogramming technologies must be improved and that careful consideration be given to the technology to be used, such as cell therapy or drug discovery, so as to obtain the appropriate result.

## INDUCTION OF PLURIPOTENCY WITH EXOGENOUS OCT4 AND OTHER TRANSCRIPTION FACTORS

Pluripotent stem cells are also capable of reprogramming somatic cells. Embryonic stem cells (ESCs) are the best-known pluripotent stem cells and were first derived in 1981 from mouse (Fig. 1) [28, 29] and in 1998 from human blastocysts [30]. ESCs can be grown as pluripotent cell lines without losing their differentiation potential. ESCs form teratomas when introduced into immunocompromised mice; these teratomas are composed of cells that have differentiated into derivatives of all three germ layers. In contrast to a totipotent cell, an ESC is not capable of autonomously developing into an embryo. However, ESCs readily incorporate into the ICM and

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Correspondence: Hans R. Schöler, Ph.D., Department of Cell and Developmental Biology, Max Planck Institute for Molecular Biomedicine, Röntgenstrasse 20, 48149 Münster, NRW, Germany. Telephone: +49-251-70365-300; Fax: +49-251-70365-399; e-mail: office@mpi-muenster.mpg.de Received April 20, 2011; accepted for publication September 29, 2011; first published online in STEM CELLS EXPRESS October 18, 2011. © AlphaMed Press 1066-5099/2011/\$30.00/0 doi: 10.1002/stem.765

**Table 1.** Summary of reprogramming methods, species, factors used, and results

Species	Tissue origin	Resulting cell type	Factors <sup>a</sup>	Efficiency (%)	Required time	References
Cell fusion						
<i>Mus Musculus</i>	Thymocytes	Tetraploid pluripotent stem cells	EC cells	ND	ND	[1]
<i>Mus Musculus</i>	Thymocytes	Tetraploid pluripotent stem cells	ESCs	ND	2 days	[2]
Induced pluripotency						
<i>Mus Musculus</i>	MEF-Fbx15	iPSCs	OSKM	0.01–0.05	16 days (2 wk)	[3]
<i>Mus Musculus</i>	MEF-Fbx15	iPSCs	OSK	ND	16 days (2 wk)	[3]
<i>Mus Musculus</i>	MEF-Fbx15	iPSCs	OKM	ND	16 days (2 wk)	[3]
<i>Mus Musculus</i>	MEF-Nanog	iPSCs	OSKM	0.001–0.03	12 days (2 wk)	[4]
<i>Mus Musculus</i>	MEF-Oct4	iPSCs	OSKM	0.08	16 days (2 wk)	[5]
<i>Mus Musculus</i>	MEF-Nanog	iPSCs	OSKM	0.05	16 days (2 wk)	[5]
<i>Mus Musculus</i>	NSC	iPSCs	OSKM	3.6 ± 0.5	2 wk	[6]
<i>Mus Musculus</i>	NSC	iPSCs	OK	0.11 ± 0.02	2–3 wk	[6]
<i>Mus Musculus</i>	NSC	iPSCs	O	0.014	4–5 wk	[7]
<i>Mus Musculus</i>	MEF	iPSCs	OSKM + Brg1 + Baf155	4.5%	12 days	[8]
<i>Homo sapiens</i>	Fibroblast	iPSCs	OSKM	0.02	3–4 wk	[9]
<i>Homo sapiens</i>	Fibroblast	iPSCs	OSLN	0.02	3 wk	[10]
<i>Homo sapiens</i>	NSC	iPSCs	OK	0.006	7–8 wk	[11]
<i>Homo sapiens</i>	NSC	iPSCs	O	0.004	10–11 wk	[11]
<i>Homo sapiens</i>	Keratinocytes	iPSCs	OSKM	1	10–13 days	[12]
<i>Homo sapiens</i>	Keratinocytes	iPSCs	OSK	0.06	20 days	[12]
Cell culture						
<i>Mus Musculus</i>	PGCs	EG cells	–	5%	3–5 days	[13]
<i>Mus Musculus</i>	PGCs	EG cells	–	ND	3–5 days	[14]
<i>Mus Musculus</i>	Testis cells	ES-like cells	–	1 in 1.5 × 10 <sup>7</sup> cells	4–7 wk	[15]
<i>Mus Musculus</i>	gSCs	gPS cells	–	0.01	3–4 wk	[16]
<i>Homo sapiens</i>	PGCs	EG cells	–	ND	7–21 days	[17]
Transdifferentiation						
<i>Mus Musculus</i>	10T1/2 cell line	Myoblast	<i>MyoD</i>	ND	ND	[18]
<i>Mus Musculus</i>	MEF	Neurons	<i>Ascl1, Brn2, Myt1l</i>	15–20%	5 days	[19]
<i>Mus Musculus</i>	Fibroblast	Cardiomyocytes	<i>Gata4, Mef2c, Tbx5</i>	~ 5%	7 days	[20]
<i>Mus Musculus</i>	MEF	Cardiomyocytes	OSK + defined culture conditions	15–20%	11 days	[21]
<i>Homo sapiens</i>	Fibroblast	Multilineage blood progenitors	O + cytokines	~ 1%	21 days	[22]

<sup>a</sup>Factor abbreviations: K = *Klf4*; L = *Lin28*; M = *c-Myc*; N = *Nanog*; O = *Oct4*; S = *Sox2*.

Abbreviations: EC: embryonal carcinoma; EG: embryonic germ; ESCs: embryonic stem cells; gPS: germline pluripotent stem; gSC: germline stem cell; iPSCs: induced pluripotent stem cells; MEF: mouse embryo fibroblast; ND: not determined, NSC: neural stem cell; NT: nuclear transfer; PGC: primordial germ cell; TF: transcription factor mediated; wk: weeks.

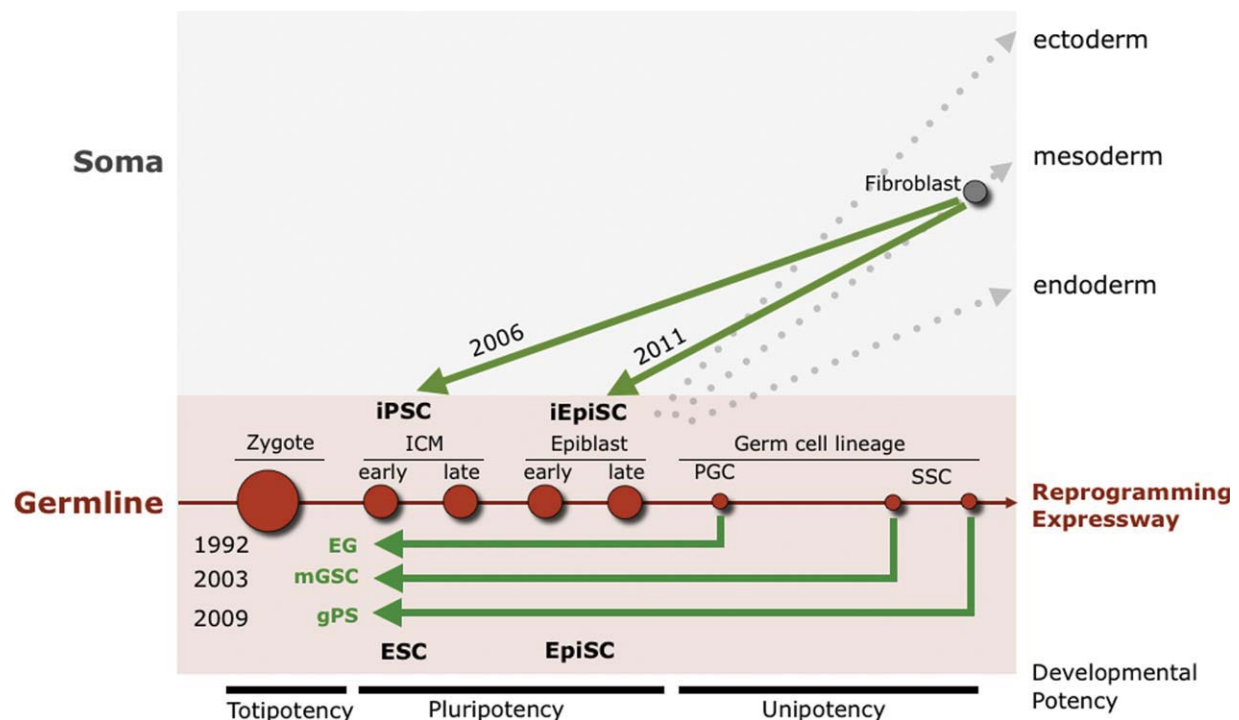
form chimeras when aggregated with morula-stage embryos or injected into blastocysts. The most stringent test for pluripotency, termed tetraploid complementation, is when mice are produced entirely from cells that had been aggregated with tetraploid embryos. Whereas the tetraploid components form extraembryonic lineages, the diploid cells—if pluripotent and without major mutations—give rise to the embryo proper [31].

Cell fusion experiments first demonstrated that pluripotent cells were capable of reprogramming somatic cells (Table 1). Miller and Ruddle [1] demonstrated that when embryonic carcinoma (EC) cells, which are related to ESCs but are derived from tumors, were fused with thymocytes, the resulting hybrid cells morphologically resembled the EC cells and had a silenced thymocyte marker *Thy1*. When ESCs were fused with thymocytes, the somatic nucleus adopted characteristics of the pluripotent cells, including X-chromosome reactivation (in female cells), early replication timing, unstable Xist transcription, and *Oct4* promoter utilization. The ES–thymocyte hybrids formed teratomas, confirming their pluripotency [2].

As ESCs can reprogram somatic cells by cell fusion, Takahashi and Yamanaka [3] sought to identify genes expressed in ESCs that would be sufficient to induce the formation of induced pluripotent stem cells (iPSCs) (Table 1). An initial list of 24 candidate genes was compiled from existing data and

these genes were cloned into retroviral expression vectors. When embryonic fibroblasts were infected with these expression vectors, iPSCs were generated. These cells expressed stage-specific embryonic antigen-1 (SSEA-1) and *Nanog*, formed teratomas when injected into immune compromised mice, and contributed to different tissues of developing embryos on blastocyst injection. Of significance, the retroviral transgenes of these iPSCs were methylated and their expression was silenced. However, these iPSCs also showed aberrant expression of key pluripotency genes, as well as incomplete demethylation of pluripotent gene promoters, and failed to either generate full-term chimeras or give rise to germ cells. Just a couple of months later, iPSCs selected using either the *Nanog* or *Oct4* promoter were shown to more closely resemble ESCs than the iPSCs originally generated by Takahashi and Yamanaka both in gene expression and chimera formation [4, 5]. After 2 years, viable mice were generated entirely from iPSCs following tetraploid complementation [32–34].

In 2007, iPSCs were successfully derived from human fibroblasts through expression of the four Yamanaka factors *Oct4*, *Sox2*, *Klf4*, and *c-Myc*, as well as by the combination *Oct4*, *Sox2*, *Nanog*, and *Lin28*—the latter is the only protein of the combination that is not a transcription factor [9, 10]. These human iPSCs closely resemble human ESCs in gene



**Figure 1.** The “Reprogramming Expressway.” The germline passes genetic information from one generation to the next and ensures its continuation by re-establishing both totipotency and pluripotency from the unipotent germ cells, oocyte, and sperm. Cells along the diploid phase of the germline can be converted by only using specific culture conditions [13–17]. In contrast, somatic cell lineages can be reprogrammed to pluripotency through the expression of specific transcription factors [3, 27]. The year indicates the year in which the respective pluripotent stem cells were established. Abbreviations: EG cells: embryonic germ cells; EpiSCs: epiblast stem cells; ESC, embryonic stem cell; gPS cells: germline-derived pluripotent stem cells; ICM: inner cell mass; iEpiSC: induced epiblast stem cells; iPSCs: induced pluripotent stem cells; mGSC, multipotent germline stem cells; PGCs: primordial germ cells; SSCs: spermatogonial stem cells.

expression, promoter methylation, and differentiation potential. To date, iPSCs have been derived from numerous somatic cell populations [12].

An important topic in reprogramming is identifying the minimum number of transgenes required for iPSC formation. Takahashi [3] derived iPSC-like colonies from mouse fibroblasts using *Oct4*, *Sox2*, and *Klf4*, (no *c-Myc*), or *Oct4*, *Klf4*, and *c-Myc* (no *Sox2*). Kim et al. [6] demonstrated that by starting with neural stem cells, which exhibited endogenous expression of two of the four Yamanaka factors at levels comparable to ESCs, the expression of only two genes, *Oct4* and *Klf4*, was sufficient to induce iPSC formation. Through further optimization, *Oct4* alone was found to be sufficient to induce iPSC formation in both mouse and human neural stem cells [7, 35].

Even different strategies used to induce reprogramming have consistently found that only a small fraction of cells will become iPSCs. Therefore, a major goal in reprogramming research is to increase the efficiency of iPSC derivation. Reduction of p53 signaling, which acts as a barrier to reprogramming by limiting cell cycling and inducing apoptosis, has been reported to significantly increase the efficiency of reprogramming [35, 36]. By screening nuclear fractions from extracts of pluripotent mouse cells, Singhal et al. [8] identified the ATP-dependent Brg1/Brm-associated factor (BAF) chromatin-remodeling complex as a factor that substantially increases reprogramming efficiency when used together with the four factors.

Problems with epigenetic memory appear to be a general feature of reprogramming. Nuclear transfer of B6C3F1 female mice resulted in abnormal obesity not found in the donor mice [37]. After subsequent mating, the obesity phenotype was not transmitted to the progeny, which suggests that it was

an epigenetic error that occurred during reprogramming. A hypomorphic DNA methyltransferase 1 (*DNMT1*) allele, which reduced epigenetic memory by decreasing global DNA methylation, significantly improved the efficiency of blastocyst formation after nuclear transfer [38]. Similarly, residual DNA methylation signatures have been found in iPSCs, which lead to restricted differentiation into cells with a different fate from the tissue of origin [25]. Interestingly, these problems are most prominent in iPSCs of an early passage and are largely attenuated upon further passaging [26].

Recently, Bock et al. [39] systematically compared the genome-wide gene expression and DNA methylation of 20 human ESC and 12 iPSC lines. They found that the vast majority of genes exhibiting significant variability between iPSC lines were similarly variable between ESC lines. No specific locus that discriminated ESCs and iPSC could be detected. Using a statistical model, those authors concluded that somatic memory does not contribute to more than 0.01%–0.001% of the variation seen in human iPSC lines [39]. Therefore, many of the findings regarding epigenetic memory in iPSCs appear to result from the epigenetic diversity inherent to pluripotent stem cells.

#### INDUCTION OF PLURIPOTENCY WITHOUT EXOGENOUS TRANSCRIPTION FACTORS IN CELLS WITH ENDOGENOUS OCT4

In contrast to somatic cells, germ cells retain the ability to form pluripotent cells through embryogenesis. Transcription



factors required for pluripotency, such as Oct4, are already expressed within cells of the germ lineage and do not need to be added exogenously to induce pluripotency. Therefore, germline cells are potentially a rich source of patient-specific pluripotent stem cells that, by their very nature, retain no epigenetic memory of the somatic cells, are likely to have fewer mutations than somatic cells, and their derivation requires no genetic manipulation. The relative ease of inducing pluripotency in germline cells has enabled the derivation of such cells almost 15 years before pluripotency could also be induced in somatic cells (Fig. 1). Because of the amazing reprogramming capacity and capability of germline cells in vivo (establishment of totipotency after fertilization and induction of pluripotency in the preimplantation embryo) and in vitro in unipotent germ cells (see below), we consider the germline to represent a reprogramming expressway. The reprogramming power of germline cells is also highlighted by the transfer of somatic cell nuclei into oocytes and by the dominant nature of pluripotent cells in fusion experiments as described above.

In 1992, two groups reported that pluripotent stem cells could be generated from primordial germ cells (PGCs) derived from 8.5-day-old mouse embryos [13, 14]. PGCs are unipotent cells in vivo, as they only differentiate to form germ cells. However, in contrast to embryonic fibroblasts, which require exogenous transcription factors to induce pluripotency, embryonic PGCs can be converted into pluripotent stem cells in culture through the addition of specific growth factors, such as Fgf2, leukemia inhibitory factor (LIF), and Steel, with an efficiency of about 5%. The resulting cells, termed embryonic germ (EG) cells, are morphologically indistinguishable from ESCs. Moreover, both EG cells and ESCs express markers, such as SSEA-1 and alkaline phosphatase, and both form teratomas composed of cells from all three germ layers after injection into immunocompromised mice. When introduced into blastocysts, EG cells readily form chimeras comparable to ESCs. In 1998, Shamblo et al. [17] demonstrated that EG cells could be derived from human PGCs using conditions similar to those for EG derivation in the mouse.

In 2004, Kanatsu-Shinohara et al. [15] generated pluripotent stem cells from neonatal mouse testis. Although pluripotent EG cells can be derived from PGCs, these cells are only available from embryos. Mouse spermatogonial stem cells can be derived from mouse testis and directed to self-renew in vitro as germline stem cells (GSCs). Under these conditions, GSCs are unipotent and are only able to differentiate into sperm. On transplantation into the seminiferous tubules of infertile mice, GSCs are capable of engrafting, reconstituting the testicular tissue with new gonocytes, and forming fully functional germ cells that are in turn capable of fertilizing oocytes. Teratomas are not observed, which demonstrates that GSCs are not pluripotent. However, when testis cells were cultured under ESC conditions, pluripotent stem cells were obtained in 4 of 21 experiments. The overall frequency of formation ES-like cells was rare, at  $1$  in  $1.5 \times 10^7$ , which is the equivalent of about 35 newborn testes. Removal of the gene *p53* increased the efficiency of derivation of ES-like cells from neonatal testis and enabled the derivation of ES-like cells from adult testis. These cells expressed all of the markers of pluripotency comparable to ESCs and formed teratomas after transplantation, instead of sperm. Like ESCs, germline-derived pluripotent stem (gPSCs) were capable of forming chimeras. In contrast to ESCs, tetraploid complementation was not successful. This could have been due to either a male imprinting pattern or an aberrant DNA methylation at some imprinted loci such as *Peg10* in the gPSCs.

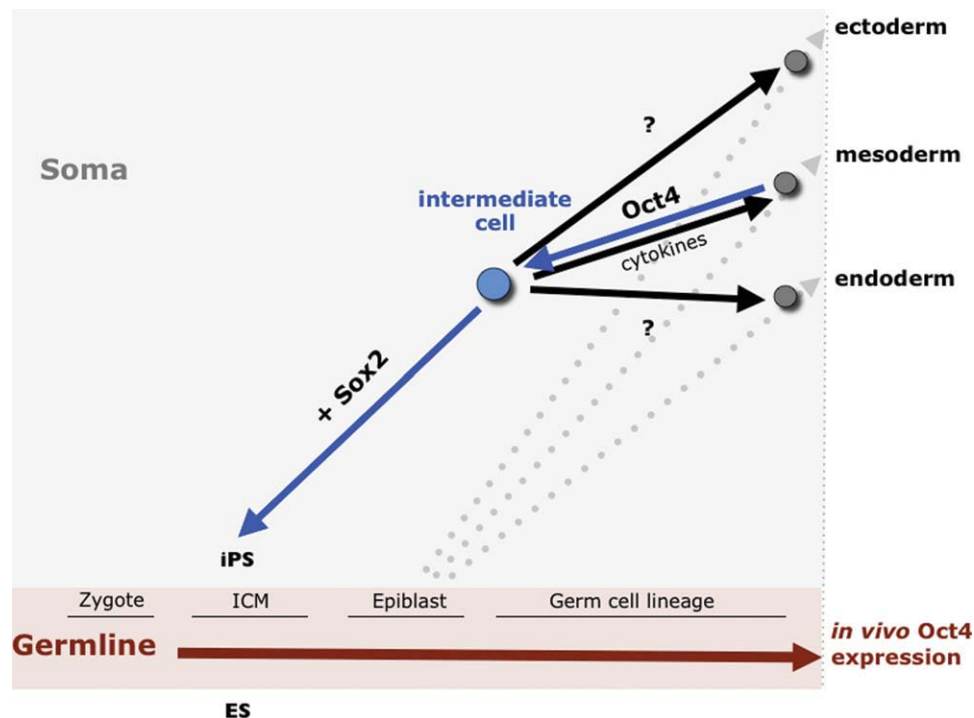
It is given that a very limited amount of source material will usually be available for the generation of patient-specific pluripotent stem cells in cell culture. Ko et al. [16] provided proof of principle for the conversion of adult GSCs into pluripotent stem cells. In a subsequent study, Ko et al. [40] demonstrated that self-renewing GSCs could be obtained even from small biopsies, at least from the mouse. These GSCs could then be reprogrammed into pluripotent stem cells under specific culture conditions, including a microenvironment dependent on the number of plated GSCs and the length of culture. The pluripotency of these gPS cells was confirmed by chimera formation and in vitro differentiation into functional neurons and cardiomyocytes. Using such an approach, pluripotent stem cells could be clonally derived from very limited source material. Therefore, in principle, such an approach could be applied to human biopsied material for the generation of patient-specific pluripotent stem cells. Although several reports have described the derivation of pluripotent cells from human testis, the results of these studies are controversial [41–45]. For example, Conrad et al. [41] claimed to have derived pluripotent cells from human testis, but further examination demonstrated that the cells in question were more likely to be fibroblasts or fibroblast-like cells [45–47].

## INDUCED TRANSDIFFERENTIATION

It is also possible to induce cells directly into an alternative fate through transdifferentiation by introducing specific transcription factors. This was first demonstrated in 1987 when David et al. converted fibroblasts into myoblasts by expressing the transcription factor *MyoD* [18]. This suggests that other lineages could also be formed through a similar approach (Table 1). Thomas Vierbuchen et al. [19] used exactly the same approach as Yamanaka and demonstrated that mouse fibroblasts could be directly transdifferentiated into neurons. After testing an initial collection of 19 neuronal lineage-specific transcription factors, a combination of only three factors, *Ascl1*, *Brn2*, and *Myt1l*, was found to be sufficient in inducing neuronal differentiation in fibroblasts, with more than 19% efficiency in about 12 days. Normal electrophysiological function was observed in the generated neurons. Interestingly, fibroblast transdifferentiation into neurons could be induced with only *Ascl1*, but the efficiency was significantly lower and the neurons failed to electrically mature.

Similarly, Masaki Ieda et al. [20] demonstrated that a combination of three cardiac-specific transcription factors, *Gata4*, *Mef2c*, and *Tbx5*, could directly induce mouse fibroblast transdifferentiation into cardiomyocytes. As with previous approaches, a cocktail of 14 transcription factors was initially tested for the ability to induce transdifferentiation. *Gata4*, *Mef2c*, and *Tbx5* induced  $\alpha$ major histocompatibility complex (MHC) - green fluorescence protein (GFP) reporter expression in more than 20% of mouse fibroblasts. A significant fraction of these  $\alpha$ MHC-GFP-positive cells formed functional cardiomyocytes and exhibited spontaneous contraction.

Efe et al. [21] used an alternative strategy to directly convert mouse embryonic fibroblasts into cardiomyocytes, by overexpressing *Oct4*, *Sox2*, *Klf4*, and *c-Myc*. However, instead of the normal pluripotency cell culture conditions, the authors cultured the cells under alternative conditions that favored cardiomyocyte formation. By day 18, spontaneously beating colonies were observed. Between 15% and 20% of the cells expressed cardiac markers, such as *Fli1*, *Nkx2.5*, and *cTnT*. Subsequent analysis revealed that the cells had directly



**Figure 2.** In 2010, specific transcription factor cocktails were defined that directly differentiate somatic cells into other somatic cells without the cells first having to pass through a pluripotent state [48]. Interestingly, one of those three studies used *Oct4* as the only exogenous transcription factor, and only after 3 weeks hematopoietic cytokines were added to CD45-positive colonies [22]. The important question concerns whether an intermediate cell exists that is induced by *Oct4* and that can then be pushed along any of the germ layer lineages or instead back to the pluripotent state by the presence of defined factors. Abbreviations: ESCs, embryonic stem cells; ICM, inner cell mass; iPSCs, induced pluripotent stem cells.

transdifferentiated into the cardiac lineage instead of forming a transient pluripotent intermediate.

Using a similar approach, Szabo et al. directly converted human dermal fibroblasts into multilineage blood progenitors [22]. Overexpression of *Oct4* resulted in a population of round hematopoietic-resembling cells expressing the hematopoietic marker CD45 but not pluripotent markers. After changing the culture conditions to those supporting early hematopoiesis, hematopoietic precursors were isolated that were capable of forming granulocytic, monocytic, megakaryocytic, and erythroid lineages, as well as supporting *in vivo* engraftment. A pluripotent cellular intermediate appeared not to be required to generate these hematopoietic cells (Fig. 2). It would be an amazing scientific accomplishment and potentially of enormous practical medical relevance if such intermediate cells could be not only defined but also stabilized in culture. This cell in principle could represent an artificial state not found *in vivo*. This is certainly true also for other cells kept in culture, the most famous example being ESCs. Indeed, it is an amazingly flexible feature of ESCs that they can be taken out and be brought back to the germline.

The power of defined culture conditions in specifying cell fate has also been demonstrated with respect to the induction of pluripotency. Depending on the culture conditions, fibroblasts can be reprogrammed by the Yamanaka cocktail to either iPSCs or induced epiblast stem cells (Fig. 1) [27].

Both nuclear transfer and induction of pluripotency has resulted in cells that retain the epigenetic memory of the donor cell origin. Although it is not known whether this also holds true for induced transdifferentiation, we extrapolate from these results and therefore argue that this is likely to be the case, if not even more so than with iPSCs.

### OCT4 AND THE REPROGRAMMING EXPRESSWAY

Because of these new reprogramming experiments, we propose that *Oct4* is more than a master regulator of pluripotency—it is the master regulator all along and into the reprogramming expressway. It is well known that *Oct4* is specifically expressed in pluripotent cells, and expression of *Oct4* is sufficient to induce pluripotency in somatic cells [3]. However, *Oct4* is also expressed in cells committed to each of the three germ layers of gastrulation-stage embryos [49]. This suggests that *Oct4* plays an important role in the commitment of pluripotent cells to somatic lineages. Indeed, ESCs overexpressing *Oct4* undergo rapid differentiation and lose pluripotency [50]. Recently, Thomson et al. [51] have shown that *Oct4* and *Sox2* are critical for germ layer fate choice. This appears to be accomplished by differentiation signals that continuously and asymmetrically modulate *Oct4* and *Sox2* protein levels, thus altering their binding pattern to the genome. Therefore, *Oct4* expression in somatic cells may lead to the induction of progenitor cells that are committed to a particular germ layer, as well as give rise to iPSCs when cultured under specific conditions. As such, *Oct4* would not simply be a “reprogramming factor,” but rather the gatekeeper into and out of the reprogramming expressway that can be directed by altering the experimental conditions.

The results of recent transdifferentiation experiments suggest that simply modifying the experimental conditions can influence the trajectory of reprogramming. For example, when using epiblast culture conditions, which require Fgf and Activin signals, epiblast stem cells are directly formed [27]. When

Fgf is used in combination with Egf instead of Activin, neural progenitors, which are the ectodermal lineage, are readily induced in mouse fibroblasts [52]. Efe et al. [21] demonstrated that the use of serum in the absence of LIF results in cardiomyocytes commitment, which are the mesodermal lineage. Therefore, by modifying the culture conditions, either iPSCs, induced epiblast stem cells, induced neural cells, or induced cardiac cells are formed using the same factor combination, which includes *Oct4*. We suggest that these results are likely to be extended to other lineages in the future, and that *Oct4* is likely to be the key factor in inducing transdifferentiation, as it was for inducing iPSC formation.

## SUMMARY

Through reprogramming technologies—nuclear transfer, cell fusion, induced pluripotency, and transdifferentiation—various cell types can be created from donor tissues, including those

from patients with known pathologies. *Oct4* is capable of inducing transdifferentiation of somatic cells into multiple cell types, including committed progenitors, when used under different experimental conditions. Therefore, we argue that *Oct4* is the gatekeeper into a reprogramming expressway.

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## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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## Concise Review: Genomic Stability of Human Induced Pluripotent Stem Cells

KRISTEN MARTINS-TAYLOR,<sup>a,b</sup> REN-HE XU<sup>a,b</sup>

<sup>a</sup>Stem Cell Institute, University of Connecticut, Farmington, Connecticut, USA; <sup>b</sup>Department of Genetics and Developmental Biology, University of Connecticut Health Center, Farmington, Connecticut, USA

**Key Words.** Induced pluripotent stem cells • Embryonic stem cells • Chromosomal aberrations • Karyotype • Genomic stability • Copy number variations • cytogenetics

### ABSTRACT

The usefulness of human induced pluripotent stem cells (hiPSCs) in research and therapeutic applications highly relies on their genomic integrity and stability. Many laboratories including ours have addressed this concern by comparing genomic (at both karyotypic and subkaryotypic levels) and epigenomic abnormalities of hiPSC lines (derived via either DNA- or non-DNA-based methods), as well as human embryonic stem cell lines during long-term culture. A variety of methods have been used for this purpose, such as karyotyping and fluorescent in situ hybrid-

ization to detect karyotypic abnormalities, array-based comparative genomic hybridization to detect copy number variations (CNVs), single-nucleotide polymorphism-based microarrays to detect both CNVs and loss of heterozygosity, analysis of integration sites in the genome, and whole genome sequencing for protein-coding exome and DNA methylome profiling. Here, we summarize the progresses in this dynamically evolving field and also discuss how the findings apply to the study and application of hiPSCs. *STEM CELLS* 2012;30:22–27

Disclosure of potential conflicts of interest is found at the end of this article.

### INTRODUCTION

Although induced pluripotent stem cells (iPSCs) share great similarities with embryonic stem cells (ESCs), the somatic cell reprogramming methods used for iPSC derivation have caused both scientific interest and concerns about the new cell type. For example, do iPSCs have complete competence and similar efficiency as ESCs to generate lineage-specific cell types? How does incomplete reprogramming affect the differentiation potential of iPSCs? Can parental cell memory predispose iPSCs to a differentiation bias? On the other hand, there has been an equally important biosafety concern about the genomic integrity and stability of iPSCs, as well as ESCs, during their derivation and prolonged culture. Certain genomic or epigenomic abnormalities may not only compromise the differentiation potential but also cause tumorigenesis in the recipients of iPSC-based therapies. Genomic abnormalities have been observed as karyotypic aberrations such as changes in chromosomal number and structures, copy number variations (CNVs) such as subkaryotypic or subchromosomal amplifications and deletions, loss of heterozygosity (LOH) due to acquired uniparental disomy, and random or site-specific integration of alien DNA into the host genome. This review attempts to summarize the findings reported thus far for the studies of the genomic, as well as epigenomic, integrity, and stability of iPSCs.

### GENOMIC STABILITY OF hESCs

It is impossible to analyze genomic stability of human iPSCs (hiPSCs) without referring to similar studies conducted previously and concurrently on human ESCs (hESCs). Although most hESCs that have been cultured for extended periods retain a normal karyotype [1–3], some develop chromosomal instability often marked by aneuploidy or translocations (Table 1). G-banded karyotyping has detected recurrent aneuploidies that are acquired by hESCs during prolonged culture and include trisomy 12, trisomy X (in female hESC lines), and amplification of 17q [4–6]. Increased dosage of genes within 17q and chromosome 12 may provide a selective advantage for hESCs. Genes implicated in apoptosis and differentiation, such as *SURVIVIN* [7], as well as the homologs of *STAT3* and *GRB2* that are implicated in self-renewal and differentiation in mouse ESCs [8] are encoded on 17q. Furthermore, *NANOG*, one of the key pluripotency genes in ESCs [9], is encoded with 12p.

The development of high-resolution array-based comparative genomic hybridization (aCGH) has increased the resolution of conventional karyotyping (when compared with chromosome-based CGH), enabling the detection of small regions of amplification or deletion, or CNVs. CNVs that affect a single gene may result from the repair of DNA double-strand breaks by nonallelic homologous recombination and/or nonhomologous end joining (NHEJ) [10]. Moreover, replication stress,

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Correspondence: Ren-He Xu, M.D., Ph.D., Department of Genetics and Developmental Biology, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, Connecticut 06030, USA. Telephone: 1-860-679-3363; Fax: 1-860-679-8345; e-mail: renhexu@uchc.edu Received May 2, 2011; accepted for publication July 15, 2011; first published online in *STEM CELLS EXPRESS* August 5, 2011. © AlphaMed Press 1066-5099/2011/\$30.00/0 doi: 10.1002/stem.705

**Table 1.** Summary of recurrent changes in human embryonic stem cells and human induced pluripotent stem cells following prolonged culture

Recurrent changes	hESCs	hiPSCs	References
Trisomy 12 or Amplification of 12p	+	+	[4–6, 33, 35–37]
Trisomy X	+	+	[4, 33]
Trisomy 17	+	–	[4, 5, 33]
Amplification of 17q	+	+	[6, 14]
Amplification of 20q11.21	+	+	[11, 12, 35, 37–39]
Isodicentric X	+	–	[13]
Deletion of 18q12.1	+	–	[11]
Amplification of 1p36.13	+	–	[15]
Amplification of 1p36.33	+	–	[15]
Amplification of 2p11.2	+	–	[15]
Amplification of 7q35	+	–	[15]
Amplification of 14q32.32	+	–	[15]
Deletion of 15q11.2	+	–	[15]
Amplification of 21q11.2	+	–	[15]
Amplification of 22q11.22	+	–	[15]
Deletion of 22q11.21	+	–	[15]
Trisomy 8	+	+	[33]
Trisomy 20q (isochromosome 20q)	+	+	[33]
Amplification of 1q31.3	–	+	[35]
Deletion of 17q21.1	–	+	[35]
Deletion of 8q24.3	–	+	[35]

Abbreviations: hESC, human embryonic stem cells; hiPSC, human induced pluripotent stem cells.

such as DNA replication fork stalling and collapse, may also contribute to CNV formation [10]. CNVs also tend to be clustered in regions of the genome with complex genomic architecture, such as common fragile sites, centromeres, and subtelomeric regions [10], suggesting that chromosome architecture plays an important role in CNV formation.

Recurrent CNVs detected in hESCs following prolonged culture include the amplification of 20q11.21 [11, 12]. This region often includes *ID1* and *BCL2L1*, which encodes the anti-apoptotic protein BCL-X. The region of amplification of 20q11.21 can also extend to include *DNMT3B*, a known important pluripotency-associated factor. Other recurrent CNVs include isodicentric X chromosome [13], amplification of 17q [6, 14], and deletion of 18q12.1 [11].

High-resolution single-nucleotide polymorphism (SNP) arrays have further expanded the repertoire of CNVs commonly acquired by hESCs during extended periods in culture. Seven amplified and two deleted regions on chromosomes 1, 2, 7, 14, 25, 21, and 22 have been identified as recurrent CNVs in hESCs following prolonged culture [15]. Furthermore, SNP arrays also permitted for the first time the detection of LOH of 16q in hESCs during prolonged culture [15].

## GENOMIC STABILITY OF hiPSCs

hiPSCs are generated through the reprogramming of somatic cells into a pluripotent state with defined reprogramming factors. Two groups independently derived the first hiPSC lines by using two partially different combinations of reprogramming factors via retro- or lentiviral vector transduction [16, 17]. New methods using other viral or nonviral vectors [18], protein [19, 20], mRNA [21, 22], and miRNA [23–25] have been used to successfully derive iPSC lines, avoiding DNA

integration into the host genome. However, genomic aberrations still occur in iPSCs during their derivation and long-term culture, regardless how they were derived (Table 1).

## Analysis of Integration Sites in the Genome

During iPSC derivation using lentiviral or retroviral transduction, the viral vectors that express the reprogramming factors integrate into the genome of the somatic cells. MiRNA-based reprogramming also relies on viral vectors to deliver the miRNA [23–25]. Several studies have mapped the integration sites of the viral vectors. In one study, the number of integration sites varied between eight individual iPSC clones and there were no integration sites in common between different iPSC clones [26]. However, another study revealed that 30% of lentiviral integrations occurred in the vicinity of the telomeres in one hiPSC line [27]. Although hiPSC derivation using protein [19, 20] or mRNA [21, 22] causes no genetic disruption, these methods are not as efficient as the viral transduction methods. On the other hand, the derivation efficiency for episomal transfection methods has become as high as that for the viral transduction methods, and no alien DNA integration has been found in the genome of the episomally derived iPSC lines [28–32]. It remains unclear if the integration of viral vectors into the genome contributes to CNV formation and genomic instability. However, it is clear that recurrent abnormalities also occur in iPSC lines derived using episomal vectors [33] and RNA [34].

## Karyotyping

We [35] and others have found that hiPSC lines derived from fibroblasts with normal karyotypes generally maintain normal karyotypes as detected by G-banded karyotyping, suggesting that the reprogramming process does not induce massive genomic instability. However, some abnormal lines were detected [35]. It remains to be determined if these abnormal hiPSC lines were generated during reprogramming or during early adaptation to culture. Following prolonged culture, few aneuploidies were detected in the original karyotypically normal hiPSCs, and one of fifteen hiPSC lines developed trisomy 12 [35], as observed in other reports [33, 36, 37]. These results suggest that most hiPSC lines are karyotypically normal during reprogramming and maintenance in culture. A recent report has added trisomy eight and trisomy 20q (isochromosome 20q) to the repertoire of recurrent chromosomal abnormalities observed in both hiPSCs and hESCs [33]. Only one of the 219 hiPSC lines karyotyped became trisomy X, suggesting that this abnormality may be rare in hiPSCs [33]. To date, there have been no reports of trisomy 17 in hiPSCs, indicating that this abnormality may be unique to hESCs.

## Subkaryotypic Analysis

Closer examination of the genomic stability of hiPSCs using higher resolution techniques has revealed that hiPSCs can acquire subkaryotypic changes during reprogramming and subsequent expansion in culture. G-banded karyotyping can only detect large chromosomal aberrations over 5 mb in size, and fails to detect small regions of amplification or deletion. The first CNV analysis was performed on three hiPSCs lines using high-resolution aCGH [38]. There were no shared CNVs detected among the hiPSC lines, which may be due to the small sample number. Notably, one of the CNVs detected in the hiPSC lines was the amplification of 20q11.21, a commonly acquired CNV in hESCs during extended culture [11, 12]. Amplification of 17q12 and 20q11.21 were detected in hiPSC lines assayed using high-resolution aCGH with custom microarrays that had extended coverage over stem cell associated genes and cancer-related genes [39]. These regions

of amplification are often acquired by hESCs following extended periods in culture. The custom arrays also detected other regions of amplification, including amplification of the reprogramming factors *SOX2*, *KLF4*, and *C-MYC* [38].

Global gene expression meta-analysis was also able to reveal that hiPSCs can acquire chromosomal aberrations [36]. These aberrations were classified into three categories: (a) aberrations shared by parental somatic cells, (b) aberrations present at early passage but absent in corresponding parental somatic cells, and (c) aberrations that are acquired during prolonged culture. The subchromosomal amplifications or deletions that were detected in early passage hiPSCs were absent in the somatic cells and in other clones from the same somatic source, and may have originated during the derivation or early culture of the hiPSCs. Following extended periods in culture, hiPSCs have a high incidence for amplification of chromosome 12. Trisomy 12 is also a recurrent aneuploidy acquired by hESCs during prolonged culture [4–6].

The types of CNVs acquired by hiPSCs as a function of time in culture have been further examined via high-resolution SNP arrays [37]. In general, this study revealed that deletions tended to occur in early passage hiPSCs and involve tumor-suppressor genes, while amplifications tended to occur in later passage hiPSCs and included oncogenic genes. This suggests that deletions may occur either during reprogramming or early adaptation to culture. As observed in other reports [38, 39], amplifications observed in hiPSCs after extended periods in culture included duplication of 20q11.21 or chromosome 12. Moreover, hiPSCs were found to contain more deletions while hESCs had more gains, than somatic cells [37].

Moreover, early passage hiPSCs have been shown to acquire significantly more CNVs than later passage hiPSCs, their parental somatic fibroblasts, or hESCs [40]. The *de novo* CNVs detected only in the hiPSCs were further classified into three groups: type A CNVs included homozygous deletions that were present only in early passage hiPSCs, type B CNVs were homozygous deletions that were detected only in later passage hiPSCs, and type C CNVs included those deletions that remained during passage. The CNVs in the early passage hiPSCs created mosaicism and were selected against during propagation. These CNVs may have rendered a selective disadvantage to the affected cells. Moreover, the CNVs in hiPSCs may have been generated as a consequence of replication stress. The CNVs in early passage hiPSCs occurred more frequently in regions of genomic fragility, specifically in common fragile sites and subtelomeric regions, than in other parts of the genome, and occurred more frequently in these regions in hiPSCs than in the parental fibroblasts or hESCs. The strong selection that early passage hiPSCs endured resulted in the loss of the majority of the CNVs generated during the reprogramming process or early culture, leading to a CNV load in later passage hiPSCs that was similar to that in hESCs.

We used high-resolution aCGH analysis to examine the types of subchromosomal abnormalities recurrently acquired by hiPSCs [35]. Unique CNV signatures for hiPSC lines derived from specific sources of parental fibroblasts were identified and categorized into two classes [35]: class I CNVs were comprised of CNVs that were shared between hiPSCs and their respective parental fibroblasts, and class II CNVs were unique CNVs that were only detected in hiPSCs derived from a specific source of parental fibroblasts and thus possibly acquired during reprogramming or early culture. Recurrent CNVs at 1q31.3 and 17q21.1 were shared by >25% of hiPSCs. Furthermore, the loss of 8q24.3 was unique to hiPSCs, and was observed in 12% of the hiPSCs. Similar to other reports [37–39], an amplification of 20q11.21 was also detected in hiPSCs [35].

## Protein-Coding Mutations

Other regions of genomic variation have also been elucidated in hiPSCs, including mutations in protein-coding exons (exomes) [34]. The majority of the mutations included missense, nonsense, or splice variants. Most of the missense mutations were predicted to alter protein functions [41] and were enriched in genes that are mutated or have causative effects in some cancers [42, 43]. However, most hiPSC lines derived from the same parental fibroblast source did not share common mutations. About half of the reprogramming-associated mutations have already existed at low frequencies in the parental fibroblasts, while the rest occurred during reprogramming or culture of the hiPSCs. Moreover, the reprogramming-associated mutations in early passage hiPSCs were maintained during prolonged culture. Additional mutations were detected following extended culture. However, to date, there is not a similar report on hESCs.

## Genomic Integrity of Mitochondria

Three reports have addressed whether somatic mitochondria within hiPSCs acquire hESC-like features during reprogramming or retain the phenotype of the parental cells [44–46]. All the three studies concluded that the mitochondria in hiPSCs revert to an immature hESC-like state in morphology, distribution, and function. Both hESCs and hiPSCs contain very few mitochondria, unlike somatic cells. This number increases during differentiation. Moreover, hESCs and hiPSCs are thought to defend their genomic integrity by maintaining low levels of reactive oxygen species. Thus, hiPSCs and hESCs share similar mitochondrial properties and reprogramming represses the senescence-related mitochondrial oxidative stress pathway. During *in vitro* differentiation, the mitochondrial properties of the hiPSCs returned to their preprogrammed state, similar to that of their parental cells [44, 45], suggesting that specific cell types are more suitable than others for derivation of iPSCs that will be differentiated to the same cell types for transplantation studies. However, mitochondria in cells differentiated *in vivo* from hiPSCs (in a teratoma assay) had dramatic functional improvements when compared with those in the parental fibroblasts [46]. Thus, the proper function of mitochondria in hiPSC-derived cells will be likely a consideration in future therapeutic applications.

## DNA Damage Response

HiPSCs share numerous similarities with hESCs in their DNA damage response including cell cycle arrest in G<sub>2</sub>/M, efficient DNA repair, and high expression of genes that mediate the DNA damage signaling and repair [47]. Both hESCs and hiPSCs repair double-strand breaks through homologous recombination repair (HRR) and NHEJ. However, the relative contributions of HRR and NHEJ to CNV formation in hESCs and hiPSCs remain to be clarified.

It has been suggested that the absence of silencing of ectopic reprogramming factors in established iPSCs may enhance the genomic instability of iPSCs [47]. However, comparison of iPSCs derived with viral vectors and those with synthetic mRNA suggests that the changes observed in both genome and epigenome were a function of reprogramming but not of the reprogramming methods or factors or the parental cell types [34]. Moreover, hiPSC lines derived using viral or episomal methods can both become trisomy eight or trisomy 12, regardless of the reprogramming methods [33]. Thus, genomic instability is associated with the reprogramming process and/or early adaptation to culture, but has not yet been associated with any particular derivation method.

**Table 2.** Comparisons of methods used to study genomic stability

Method	Advantages	Disadvantages
G-banded karyotyping	Detects gross abnormalities quickly. Can detect mosaicism and structural aberrations or inversions	Limited to changes >5 Mb. Cannot detect functions of affected genes
FISH and SKY	Identifies and classifies structural aberrations	Limited to changes >5 Mb. Cannot detect functions of affected genes
CGH arrays, SNP arrays	Highly sensitive with resolution to change >50 Kb. Can detect mosaicism. SNP arrays can also detect loss of heterozygosity	Expensive and time consuming. Cannot detect functions of affected genes. Samples can only be compared when run on the same platform
Whole genome sequencing	Highly sensitive with resolution to single bases. Can detect mosaicism	Expensive and time consuming. Cannot detect functions of affected genes. Samples can only be compared when run on the same platform
Global gene expression meta-analysis	Tests genomic abnormalities functionally	Limited to changes > 10 Mb and to genes expressed in samples. Cannot detect mosaicism

Abbreviations: CGH, comparative genomic hybridization; FISH, fluorescent in situ hybridization; SKY, spectral karyotyping; SNP, single-nucleotide polymorphism.

### EPIGENOMIC ANALYSIS OF hiPSCs

Additional studies comparing the DNA methylomes of hESCs, hiPSCs, and parental somatic cells have revealed important similarities and differences in the methylomes of hESCs and hiPSCs. Targeted bisulfite sequencing analysis of CpG islands in hiPSCs and parental fibroblasts has revealed changes in DNA methylation following reprogramming [49]. However, when compared with hESCs, hiPSCs appeared to have enhanced methylation, suggesting that reprogramming may be associated with aberrant methylation.

Closer examination of DNA methylation in hiPSCs, their parental somatic fibroblasts, and hESCs by comprehensive high-throughput array-based relative methylation analysis using custom arrays that included both CpG islands and CpG island “shores,” regions of comparatively low CpG density that are located near traditional CpG islands, identified differentially methylated regions (DMRs) that distinguish hiPSCs from fibroblasts and hESCs [50]. Many regions were found to be differentially methylated between the hiPSCs and the fibroblasts, and were termed reprogramming-specific DMRs (R-DMRs). The majority of the R-DMRs were associated with CpG-island shores, rather than with CpG islands. Furthermore, the R-DMRs in hiPSCs overlapped with known tissue-specific DMRs that are involved in normal development. Comparisons of the methylomes of hESCs and hiPSCs revealed that several DMRs were specific to hiPSCs. These hiPSC specific DMRs generally were hypermethylated and included genes involved in key developmental processes. Certain loci in the genome of hiPSCs remained incompletely reprogrammed, while others were aberrantly reprogrammed. Thus, the methylation pattern of hiPSCs differs both from the parental somatic cells and hESCs.

Whole-genome profiles of DNA methylation at a single-base resolution of hiPSC lines revealed that the methylomes of hESCs and hiPSCs are very similar to one another, and highly distinct from the parental somatic cells from which the hiPSC lines were derived [51]. Interestingly, partially methylated domains in the somatic fibroblasts were fully methylated following induction of pluripotency. Moreover, highly methylated CpG islands found in the somatic cells were predominately demethylated in hiPSCs as in hESCs. DMRs have also been identified between hESCs and hiPSCs in CpG islands (CG-DMRs) that are proximal to gene promoters and transcriptional start sites. The CG-DMRs were either the result of a failure to fully reprogram the parental somatic cell

methylation patterns that resulted in somatic “memory”, or specific events observed only in the hiPSCs—termed iPSC-specific DMRs (iDMRs). Many of the CG-DMRs were shared between independent hiPSC lines, suggesting that these loci are perhaps hotspots for aberrant methylation during the reprogramming process. Notably, both the memory CG-DMRs and the iDMRs were maintained following the differentiation of hiPSCs at high frequency. Furthermore, megabase-scale DMRs in non-CpG methylated regions were repeatedly resistant to reprogramming, and were also associated with altered histone modifications and transcriptional activity. These non-CpG mega-DMRs were in close proximity to centromeres and telomeres. Together, these iDMRs may be used as important diagnostic markers to evaluate iPSC reprogramming.

### COMPARISON OF METHODS USED FOR GENOMIC STABILITY STUDY

A comparison between the methods used to assay genomic stability is summarized in Table 2. G-banded karyotype analysis of hiPSCs has several important advantages. The analysis provides a snapshot of the entire genome. Gross abnormalities can be detected quickly. Mosaicism can also be detected in a subpopulation of the sample. Moreover, G-banded karyotyping is not as labor intensive or as expensive as other technologies, such as aCGH or SNP array analyses. Furthermore, structural abnormalities such as balanced translocations and inversions can only be detected by karyotyping. As amplifications or deletions may not occur in these scenarios, the balanced structural abnormalities cannot be detected by other methods. Fluorescent in situ hybridization (FISH) or spectral karyotyping, a technique that uses chromosome-specific fluorescently labeled probes, can be used to identify structural aberrations including translocations or duplications. However, one important disadvantage of karyotyping is that only large abnormalities greater than five megabases in size can be detected. Other methods are required for the detection of small regions of variation.

CGH arrays, SNP arrays, and whole genome sequencing methods are highly sensitive. Regions, as small as 50 kilobases, can be detected by the CGH and SNP arrays. Moreover, LOH can be detected by the SNP arrays. Single base changes can be detected in whole genome sequencing methods. These methods can also detect regions of variation found within only in a subpopulation in the culture, permitting the



detection of mosaicism. However, there are several limitations. Although these methodologies can detect small regions of change, it is not immediately apparent if the CNVs are directly correlated with changes in the expressional levels of genes within the affected region. The degree of mosaicism found in the samples must be determined by other methods, such as FISH. Moreover, the expression of genes outside of the affected regions may also be altered, if the regions contained important distal regulatory elements. Thus, results from CGH or SNP arrays should be validated through gene expression analysis. In addition, samples can only be directly compared with each other in CGH and SNP arrays if they are run on the same platform.

Expression-based analysis has several important advantages. The functionality of genomic abnormalities becomes immediately apparent in the aberrant expression of genes within the affected region. Thus, gene expression profiling is correlated with genomic integrity. Although this method is more sensitive than karyotyping, it is not as sensitive as other methods, such as CGH and SNP arrays. Only relatively large abnormalities (>10 megabases) can be detected based on the design. A confidence call is made for an amplification or deletion of a genomic region only when the expression of most, if not all, genes from this region is upregulated for amplification or downregulated for deletion, compared with the median across the entire dataset [36]. Moreover, mosaicism cannot be detected and the number of genes expressed in the samples limits the resolution of expression-based analysis.

## CONCLUSION

There are important similarities and differences in genomic and epigenomic stabilities between hiPSCs and hESCs. When compared with parental somatic fibroblasts and hESCs, hiPSCs more closely resemble hESCs. On a genome scale, the DNA methylomes and gene expression profiles of hiPSCs and hESCs closely resemble each other. HiPSCs can recurrently acquire many of the chromosomal aberrations observed in hESCs during prolonged culture, including trisomy 12, trisomy 8, and amplification of 20q11.21. However, important differences are observed in hiPSCs, such as the unique CNVs that are recurrently acquired by only hiPSCs during prolonged culture. These differences occur during both the reprogramming process and prolonged culture. There may be important implications in basic research and clinical applications, given

that these differences are likely to influence the interpretation of biological and therapeutic studies that use hiPSCs.

Several factors may contribute to the differences observed between hESCs and hiPSCs, including (a) derivation source (embryonic vs. somatic cells), (b) derivation methods (direct isolation vs. reprogramming), and (c) culture conditions (no reported studies have compared genomic stability of all analyzed hESC and hiPSC lines under identical culture conditions). Moreover, the results of a genomic stability study can be influenced by the references and platforms used for data analysis, as well as the sample size. All these impact factors should be considered when comparing genomic stability of various pluripotent cell lines.

It is also important to note that genomic abnormalities occur in any cell culture. Nonpathogenic variations may also occur *in vivo* such as in blood or bone marrow cells that have been used for transfusion or transplantation to treat patients for decades. Such variations may be unpreventable and harmless to patients who receive hiPSC-based therapy. Taken together, to have a complete snapshot of the genomic integrity and stability of hiPSCs, multiple methods are needed to assess the nature of the genomic abnormalities in hiPSCs and hiPSC-derivatives. Epigenomic analysis is also important to identify abnormalities in DNA methylation, histone modification, etc., in hiPSCs. Correlations between genomic/epigenomic abnormalities and gene expression should be analyzed in not only hiPSCs but also their progeny to assess the functionality of the abnormalities in both pluripotent and differentiated states. These analyses will serve as important measures to distinguish and track hiPSCs best suited for research and future therapeutic applications.

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## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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## Concise Review: The Magic Act of Generating Induced Pluripotent Stem Cells: Many Rabbits in the Hat

GUSTAVO MOSTOSLAVSKY

Section of Gastroenterology, Department of Medicine and Center for Regenerative Medicine (CReM), Boston University School of Medicine, Boston, Massachusetts, USA

**Key Words.** Pluripotent stem cells • Reprogramming • Induced pluripotency • Induced pluripotent stem cells

### ABSTRACT

Since the seminal discovery by Yamanaka et al. demonstrating that four transcription factors were capable of inducing nuclear reprogramming to a pluripotent state, a plethora of publications have followed aimed at improving the efficiency, simplicity, and safety of the original methodology that was based on the use of integrating retroviruses. A better understanding of the basic mechanisms behind reprogramming as well as an improvement in tissue culture conditions have allowed for the development of new tools based on different molecular approaches, such as excisable

and nonintegrating vectors, RNA, proteins, and small compounds, among others. In most instances, a dynamic interplay exists between each method's efficiency of reprogramming versus overall safety, and these points need to be considered when choosing a particular approach. Regardless, the fast pace at which this field has advanced in recent years attracted many investigators to enter into the induced pluripotent stem cell (iPSC) world and has made the process of nuclear reprogramming and iPSC generation a routine lab technique. *STEM CELLS* 2012;30:28–32

Disclosure of potential conflicts of interest is found at the end of this article.

Similar to the beauty we experience the first time we see a good magic act, the day the seminal description of reprogramming by Takahashi and Yamanaka was published in *Cell* [1], most people in the field thought: this must be magic. But as with any magic trick, this “act” of nuclear reprogramming was the result of a series of elegant and rigorous experiments performed to convince an incredulous audience that this phenomenon was real and not magic.

In this review, I will attempt to give a brief overview of the different methodologies for the generation of induced pluripotent stem cells (iPSCs), that have emerged since that seminal publication, reviewing each methods' properties, advantages, and disadvantages. The overall goal is to provide an understanding of the rapid evolution of the reprogramming field that has taken place in the last 5 years and to give a glimpse of where we are heading in the future.

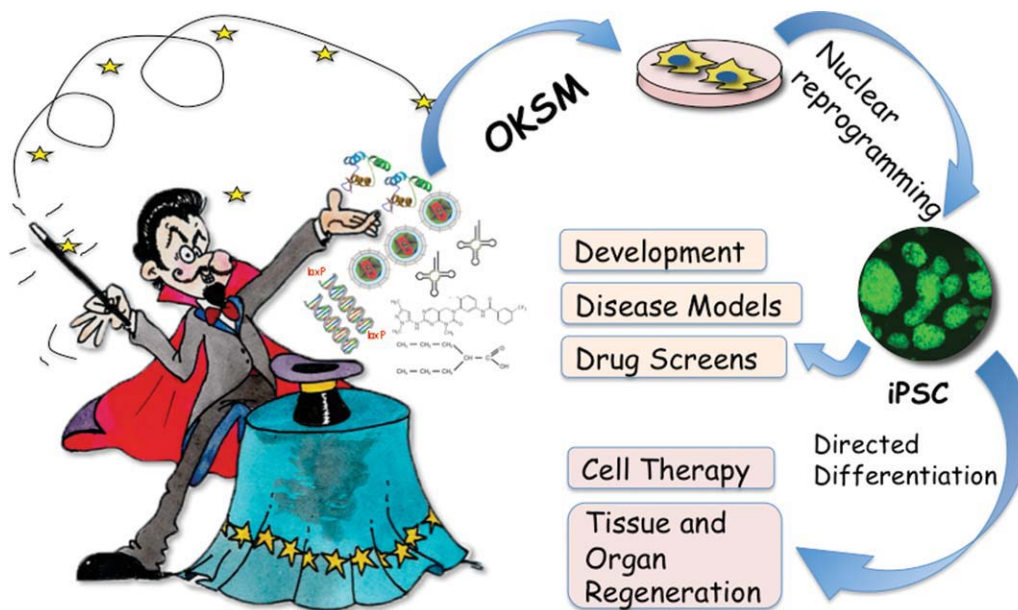
In their original manuscript, Yamanaka and colleagues chose to use gammaretroviruses (also known generally as simple retroviruses) derived from the Moloney murine leukemia virus to introduce 24 individual transcription factors and ultimately the famous four “Yamanaka factors,” namely Oct3/4, Klf4, Sox2, and cMyc (OKSM). It is important to note that in this study [1], Yamanaka and colleagues were able to obtain iPSC by selecting for Fbx15-driven antibiotic resistance in ESC culture conditions. Fbx15, however, is not essential for the maintenance of the pluripotent state, which, together with the timing of drug selection may explain why the first iPSC lines failed to generate adult chimeric mice and exhibited a global gene-expression profile that was not identical to that of

ESC. Indeed, by changing the selection method and culture conditions, the follow-up studies that also relied on the use of gammaretroviruses were able to derive germline-competent iPSC from mouse fibroblasts [2–4].

The successful use of gammaretroviruses was no coincidence, and I will even dare to say that the experiment would have failed if it were not for the use of retroviruses. Retroviral vectors are by far the most well studied and used vectors for gene transfer into mammalian cells, due in part to their ability to integrate their genomes into the host chromosomes, which enables efficient and long-term gene expression. Integrating viruses have evolved over million of years to optimally use the host transcription machinery for expression of the viral transduced transgenes. However, retroviral vectors are prone to epigenetic silencing [5, 6] and herein lies the basis for my second assertion. Had Yamanaka chosen a different methodology such as lentiviral vectors whose constitutive promoters are less sensitive to silencing he would have likely failed to obtain iPSC able to properly differentiate, form mouse chimeras, and contribute to mouse germline. This would have made it difficult to convince himself and the rest of the stem cell world that his reprogrammed cells were truly pluripotent. One can speculate as to why he did not use a more transient or nonintegrating methodology and, as I will explain in the next few pages that would have also fallen short of his expectations. In summary, by using retroviruses, Yamanaka found a good balance of sufficient amounts of gene expression, that last just long enough (due to silencing) to allow for the emergence of the “magic” iPSC.

Author contributions: G.M.: conception and design and manuscript writing.

Correspondence: Gustavo Mostoslavsky, M.D., Ph.D., Section of Gastroenterology, Department of Medicine and Center for Regenerative Medicine (CReM), Boston University School of Medicine, 650 Albany Street, Suite 513, Boston, Massachusetts 02118, USA. Telephone: 617-638-6532; Fax: 617-638-7785; e-mail: gmostosl@bu.edu Received June 9, 2011; accepted for publication July 22, 2011; first published online in *STEM CELLS EXPRESS* September 21, 2011. © AlphaMed Press 1066-5099/2011/\$30.00/0 doi: 10.1002/stem.742



**Figure 1.** The “magic act” of nuclear reprogramming. A toolbox full of tricks is now available for scientists to achieve reprogramming of somatic cells to generate normal and disease-specific iPSC, which will open new avenues of research in human disease modeling, drug discovery and therapy. Abbreviations: iPSC, induced pluripotent stem cell; OKSM, Oct3/4, Klf4, Sox2, and cMyc.

But a good balance is not a perfect one. The low efficiency associated with premature silencing and the use of multiple individual retroviruses, together with the desire for developing more “user-friendly” approaches, prompted investigators including myself to look for alternative reprogramming methodologies [7] (Fig. 1). Moreover, the fact that these vectors do integrate, while convenient in terms of appropriate levels of gene expression, posed a critical issue in terms of safety, specifically when aberrant expression of cMyc and probably also of the other reprogramming transgenes is known to induce oncogenic transformation [3].

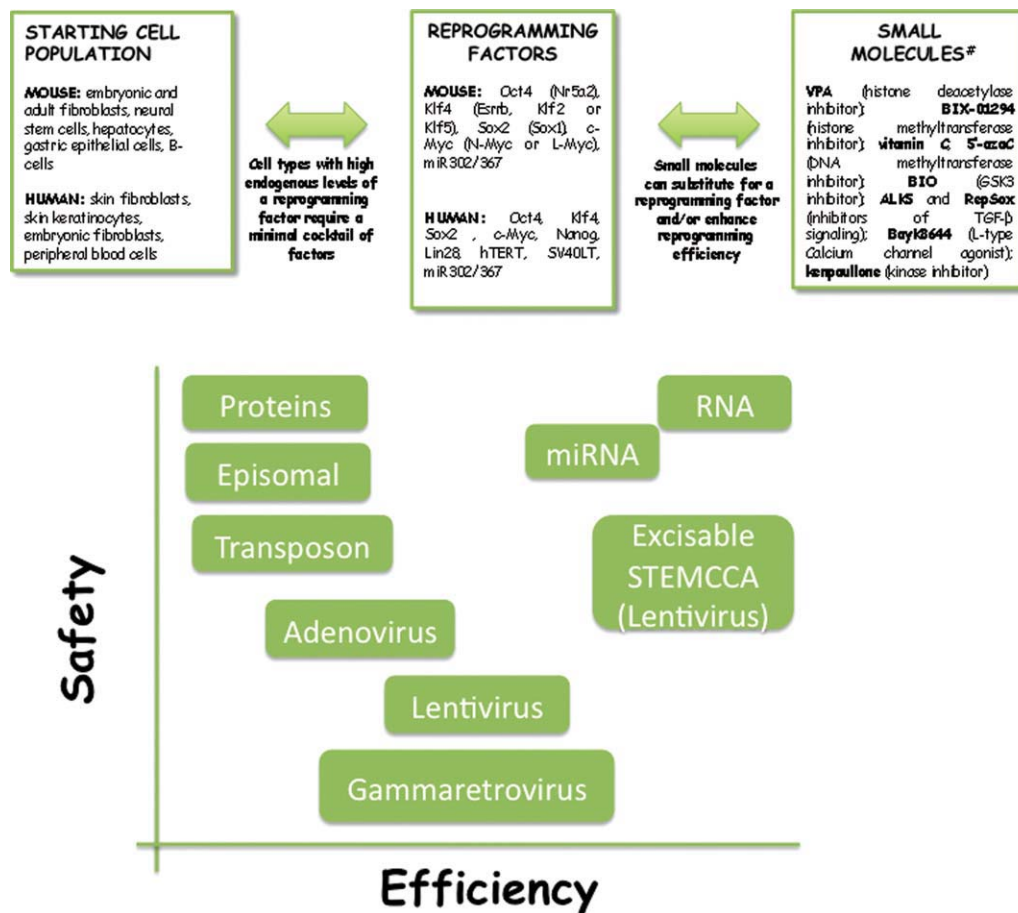
The first to use lentiviruses for iPSC generation, a close cousin of gammaretroviruses, was the laboratory of Ramalho Santos [8]. In contrast to gammaretroviruses, lentiviruses can transduce nondividing cells and they are capable of transducing human cells more efficiently than gammaretroviruses. However, in that original study the efficiency of reprogramming was not much improved and it was not clear as to whether using a lentivirus with a constitutive promoter allowed for the generation of fully pluripotent cells, capable of differentiating appropriately in the presence of constitutive overexpression of the reprogramming factors. Generation of individual lentiviral vectors carrying inducible (tetracycline responsive) promoters was a clear improvement to this technology [9, 10]. The ability to shut down expression of exogenous transgenes by simply removing doxycycline from the culture media allowed confirmation that nuclear reprogramming was achieved through activation of the endogenous stem cell transcription machinery and also proved to be a valuable tool for the development of secondary iPSC systems and the study of the dynamics and molecular mechanisms underlying nuclear reprogramming.

Remarkably, the fact that shortly after the original Yamanaka’s report, again Yamanaka’s laboratory and teams led by James Thomson in Wisconsin and George Daley in Boston were able to produce iPSCs from human fibroblasts using a similar experimental design [11–13] served to confirm the robustness of Yamanaka’s findings and to give a major push forward to the use of iPSC in regenerative medicine. The

resulting human iPSCs were strikingly similar to human ESCs, judged by morphology, surface marker expression, methylation status in the promoter regions of pluripotency-associated genes, in vitro differentiation, and teratoma formation. Following these first studies, retroviruses were used to reprogram somatic cells from patients with a variety of diseases [14, 15] including amyotrophic lateral sclerosis, Parkinson’s disease, type 1 diabetes mellitus, Huntington’s disease, and Down syndrome, providing an unprecedented opportunity for disease modeling and drug screening.

An important step to bring this technology closer to the clinics was achieved with the design of polycistronic vectors expressing all factors from a single construct. Different versions were published approximately at the same time, either as a transfectable plasmid by Yamanaka himself [16], a platform that suffered from very low efficiency, or as lentiviruses by our laboratory at Boston University and Rudolf Jaenisch at Massachusetts Institute of Technology [17, 18]. While the concept was the same, significant differences in terms of efficiency of reprogramming were found among these different vectors (ranging from 0.0001% to 0.5%), mostly due to the specific engineering design dependent on either the combination of 2A peptides with an internal ribosomal entry site (IRES) element versus the use of tandem 2A peptides alone. In our hands, the latter suffered from inefficient “cleavage” in the downstream 2A peptides affecting the overall production of the multiple protein products (unpublished), which could impart a disadvantage compared to the other design that relies on 2A peptides and IRES. Furthermore, the specific order of the genes within the polycistronic cassette, which allows for a characteristic stoichiometry of protein expression, likely played a role in the efficiency of reprogramming [16]. Indeed, the specific design of our STEMCCA vector has allowed us and others to consistently achieve reprogramming of both mouse and human cells to obtain iPSC clones containing a single vector integration [18–20]. An obvious immediate consequence and application was the addition of loxP sites to make the polycistronic cassette excisable on Cre exposure, a design demonstrated again by a few different laboratories [20,





**Figure 2.** Overview of reprogramming methodologies. Depending on the starting cell population, reprogramming requires different factors, whose biological activity can be enhanced by using small molecules and chemicals. Different reprogramming methods show a dynamic range between efficiency of reprogramming and overall safety in terms of genetic modifications and potential tumorigenesis. Ultimately, selection of a specific method should be made according to the application for which induced pluripotent stem cells are generated (see text for details); \*reprogramming using only small molecules has not been reported yet. Abbreviation: miRNA, micro RNA.

21]. In contrast to multiple vectors, the use of a polycistronic vector appears to achieve a more reliable reprogramming to generate iPSC that are transcriptionally closer to ESC [22]. It must be noticed, however, that when using excisable lentiviruses, even after removal of most exogenous sequences, a residual inactive long terminal repeat (LTR) (~200 bp) remains integrated within the host chromosome. Its potential safety threat by insertional mutagenesis, while still present, could be minimized by further sequencing of the proviral integration site. Importantly for its future application in the clinical arena, there is to date no published data supporting an oncogenic risk based on the presence of an integrated inactive LTR. Quite the opposite, the use of inactive LTRs has been shown to significantly diminish those risks [23]. Furthermore, in a seminal but often forgotten study by the laboratory of Verma and colleagues, the oncogenic potential of virally mediated integration was 100% correlated with the transduced transgene (the common receptor  $\gamma$  chain in that specific study) and absent when the same integrating control lentiviral backbone was used [24]. There will be a need in the future to carefully weigh the benefits of using integrating methodologies against their potential risks when attempting to move iPSC technology forward to the bedside. In this regard, having a small residual genetic tag (such as the inactive LTR) could serve a beneficial purpose and could be welcomed by regulating agencies, as it will allow investigators to more rigorously define

the contribution, distribution, and in vivo function of the pluripotent derived cells (see letter by Ellis et al. [25]).

A major breakthrough based on a modification of this approach was the use of excisable transposon elements, such as *piggyBac* transposons, expressing all reprogramming factors also from a polycistronic message [26, 27]. Transient exposure of the cells to a specific transposase achieves seamless excision of the reprogramming cassette and the generation of genetically unmodified iPSC. This approach was received with much excitement by the stem cell community, however, the overall low efficiency together with the need for a laborious screening before and after transposon removal and the potential genomic toxicity mediated by transposase activity have so far limited its generalized applicability.

Significant efforts have been devoted to develop approaches to induce nuclear reprogramming using nonintegrating methodologies. These include nonintegrating viral vectors such as Adenovirus and Sendai virus, as well as direct transfection of plasmids, RNA, proteins, and finally, the use of chemicals and small molecules aimed at recapitulating the reprogramming role of the OKSM factors. The first methodology to demonstrate that genomic integration was not necessary for reprogramming to occur was the study by Stadtfeld, Hochedlinger and coworkers using individual Adenoviruses [28], followed almost at the same time by Yamanaka's group using transfection of DNA plasmids [16]. Both methods, however,

were limited by orders of magnitude of lower efficiencies due to the transient nature of gene expression. Adenoviral vectors have been shown to be able to reprogram human cells as well [29]. The use of nonintegrating RNA Sendai viruses appeared to improve these methodologies, by achieving both the generation of genetically unmodified iPSC and a relatively high efficiency of reprogramming [30, 31]. The latter might well be the result of the very high number of viral copies obtained in each infected cell and the availability of viral "in sense" RNA ready-to-be translated into the reprogramming proteins. Time will serve to confirm the benefits of this approach as more publications reporting the use of this methodology appear in the near future. Other integration-free vectors have been described, including self-replicating selectable episomes [32] and minicircle vectors [33], the former requiring the use of additional factors such as SV40LT. Recently, a long sought-after method was reported by the laboratory of Rossi and coworkers [34], in which a sophisticated use of modified RNAs encoding OKSM factors achieved high efficiency of reprogramming while minimizing the adverse effects of interferon mediated anti-RNA responses. While some of the highly stringent technical aspects of this methodology may prevent its widespread use by laboratories in the field, it holds great promise for its use in clinical applications.

The use of purified proteins to achieve reprogramming has been hailed by many as the ultimate method to give the iPSC field its chance to have an impact at industrial scale. Indeed, for biotechnology and pharmaceutical companies the use of purified proteins represents a more appealing way to scale up this technology for commercialization. So far, only a few laboratories have succeeded in demonstrating the feasibility of obtaining reprogramming of mouse and human cells using either Tat-mediated transfection of recombinant purified OKSM proteins [35], or using whole cell extracts from cells expressing the reprogramming factors [36, 37], albeit at such a low efficiency that its practical implications and general applicability for now appear far from reality.

The low efficiency obtained with most nonintegrating methods has prompted investigators to screen for chemical compounds and small molecules that promote reprogramming. Indeed some molecules were found to increase the efficiency of reprogramming in the context of OKSM overexpression and even to replace individual factors, giving rise to the tempting idea of generating iPSC solely with chemicals (reviewed in [7, 38, 39]). But once again, low efficiency of reprogramming limited the use of chemicals, and so far, reprogramming solely with chemicals has been unsuccessful. Importantly, it must be cautioned that most of these molecules are potent modifiers of DNA and chromatin, and therefore, they may introduce undesired epigenetic abnormalities in the resultant iPSC. Finally, a most recent study by Morrissey and coworkers demonstrated a novel and different mechanism for nuclear reprogramming based on expression of a single miRNA cluster [40]. In this study, lentiviral-mediated expression of miR302/367 was shown

to induce reprogramming of both mouse and human cells at higher efficiency and more rapidly than compared to other methodologies. The exact mechanisms underlying miRNA-mediated reprogramming is still unknown but appears to be mediated in part by direct activation of OCT4 expression and suppression of Hdac2 [40].

## CONCLUSION

Few fields have enjoyed such a prolific record of publications in a relatively short amount of time as the emerging field of reprogramming. The diversity of methodologies devised by the creative minds of stem cell scientists can at times be overwhelming (Fig. 2). However, I believe we are now reaching an equilibrium where most researchers are settling on specific techniques to start asking more significant questions regarding the mechanisms behind nuclear reprogramming and how to induce robust cell-lineage-specific differentiation. In this regard, learning from the signals and cues that work during normal embryonic development, as has been done for the generation of several tissue specific cell-lineages derived from ESC, may be key to obtain reproducible differentiation protocols from iPSC.

The ultimate decision on which reprogramming methodology to use will likely depend on the specific application for which iPSC are generated. In most cases, using integrating polycistronic vectors that are highly efficient and relatively simple to produce will be sufficient and practical. Later on, as we move toward potential clinical applications, it may be worth considering methodologies that while more complex offer improved safety characteristics.

Perhaps, the most fascinating aspect of iPSCs is their utility to study any human disease and even to provide new treatments derived from just a few readily accessible somatic cells from the patients themselves [41–45]. Moving iPSC from bedside to bench, for example, obtaining fibroblasts from human patients and generating iPSC, has now become a routine technique. This progress coupled with some recent impressive developments in the design of new tools for gene correction [46] as well as in the construction of bioengineered tissues [47–50] make me believe that it is only a matter of time until the iPSC world moves back from bench to bedside.

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## Concise Review: Induced Pluripotent Stem Cells Versus Embryonic Stem Cells: Close Enough or Yet Too Far Apart?

JOSIPA BILIC,<sup>a</sup> JUAN CARLOS IZPISUA BELMONTE<sup>a,b</sup>

<sup>a</sup>Center of Regenerative Medicine in Barcelona, Barcelona, Spain; <sup>b</sup>Gene Expression Laboratory, Salk Institute for Biological Studies, La Jolla, California, USA

**Key Words.** Self-renewal • Reprogramming • Pluripotent stem cells • Embryonic stem cells

### ABSTRACT

The state of a cell is defined by the genes it transcribes and the epigenetic landscape that regulates their expression. Pluripotent cells have markedly different epigenetic signatures when compared with differentiated cells. Permissive chromatin, high occurrence of bivalent domains, and low levels of heterochromatin allow pluripotent cells to react to distinctive stimuli and undergo changes of cell state by differentiating into various tissues. Differentiated cells can be reprogrammed by a set of transcription factors to induced pluripotent stem cells (iPSC) that convert their transcriptional and epigenetic state to pluripotency

and thus closely resemble embryonic stem cells (ESC). However, questions remain on whether the epigenetic reprogramming is complete or if there are some recurring iPSC specific aberrations that impede their full pluripotency potential. For this reason, iPSC need to be closely compared with ESC, which is used as a golden standard for in vitro pluripotency. Transcribed genes, epigenetic landscape, differentiation potential, and mutational load show small but distinctive dissimilarities between these two cell types. *STEM CELLS* 2012;30:33–41

Disclosure of potential conflicts of interest is found at the end of this article.

### INTRODUCTION

In 2006, the researchers Takahashi and Yamanaka opened a completely new venue in stem cell research by showing that the forced expression of only four transcription factors (Oct4, Sox2, Klf4, and c-Myc) was sufficient to convert fibroblast cells into embryonic stem cell (ESC)-like cells, which were named induced pluripotent stem cells (iPSC) [1]. Many subsequent articles have since confirmed that the timed expression of master regulator factors can change differentiated cells into iPSC, a process called reprogramming. By now a variety of starting cell types, different combinations of main transcription factors and different delivery techniques of these factors into the cells have been used successfully for this.

Reprogramming starts by the binding of few master pluripotency transcription factors to regulatory elements of many genes, thereby affecting their expression. The epigenetic landscape of somatic cells is refractory to the total control of these transcription factors, but their prolonged expression and positive-feedback regulatory loops [2] slowly modify the epigenetic landscape and new pluripotent circuits are being established, changing the cell state. When looking at the total population of cells expressing reprogramming factors in a limited time frame, the reprogramming process is highly inefficient. It depends on poorly understood stochastic events in the cells and requires cell division [3, 4]. The final result is an iPSC colony with newly restructured epigenetic marks driving expression of the endogenous transcription factors and chro-

matin regulators that further sustain and balance the achieved pluripotent state. However, questions remain whether this drastic cell change leaves epigenetic “scars.” A closer inspection of the epigenetic state, stability, mutational load, and full developmental potential of iPSC is still in progress.

Pluripotent stem cells allow the study of embryonic development and cell differentiation and offer much hope for regenerative medicine. Besides once again proving cell plasticity, iPSC have drawn special attention to both the public and scientific community because they avoid the handling of embryonic material and can be patient tailored. But how close are they really to the gold standard ESC, and related to it—how “safe” are they for future clinical use? Avoiding the integration of reprogramming factors into the genome during iPSC generation is already achieved using different methodologies [5–9]. This is a first important step toward safer cells. The goal of this review is to provide an up-to-date overview of the epigenomic, transcriptional, and genomic states of iPSC, together with their differentiation potential, by comparing iPSC with ESC and to the somatic cells that they are derived from.

### SETTING THE STANDARDS FOR IPSC PLURIPOTENCY

Thoroughly erasing the differentiation specific epigenetic marks in iPSC and returning to the ESC pluripotency “ground state”

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Correspondence: Juan Carlos Izpisua Belmonte, Ph.D., Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037. Telephone: 858-453-4100; Fax: 858-453-2573; e-mail: belmonte@salk.edu or izpisua@cmrb.eu Received May 9, 2011; accepted for publication July 7, 2011; first published online in *STEM CELLS EXPRESS* December 23, 2011; available online without subscription through the open access option. © AlphaMed Press 1066-5099/2011/\$30.00/0 doi: 10.1002/stem.700



is believed to give the highest chance for successful subsequent differentiation. This is corroborated by the fact that iPSC are generally less successful in generating high percentage chimeras and even less efficient in generating live mice in tetraploid complementation when compared with ESC [10].

During reprogramming, often a number of colonies appear, including ones that are highly proliferative but not pluripotent. The first selection for a “good quality” iPSC colony is usually done by morphology criteria [11]. The morphological appearance, proliferation rate, the reactivation of endogenous pluripotency genes followed by silencing of transgenes used for reprogramming, and the ability to form teratomas are some of the basic criteria a cell line has to meet to be considered true iPSC. Furthermore, when injected into blastocysts they should contribute to the embryo tissues, including the germ line. Ultimately, the ability of iPSC to form a whole animal via tetraploid complementation is a clear indication of iPSC pluripotency and a nearly identical state to ESC [12, 13] (Fig. 1). The problem is that such rigorous pluripotency tests are difficult to perform routinely on many lines.

In human, the most rigorous tests for pluripotency can not be performed for obvious ethical reasons. This lowers the standard for pluripotency and increases the heterogeneity of obtained iPSC lines. Even differences in the culturing conditions between different labs can contribute to the heterogeneity of the lines [14, 15]. One example is the X chromosome inactivation in human iPSC lines of female origin. There are reports of “ground state” lines, where both X chromosomes are again active [16, 17], while others show persistence of X chromosome inactivation [18]. But there is a general ambiguity associated with the human pluripotent lines isolated so far. Namely, human ESC/iPSC share several important features with mouse stem cells isolated from postimplantation embryo epiblasts, called epiblast embryonic stem cells (EpiESC) [19]. Epiblast stem cells present the next stage in development and therefore have a more limited developmental potential. They show poor success in generating chimeras and can manifest expression of early lineage commitment markers [20, 21]. Thus, the similarity of mouse EpiESC to human ESC/iPSC, together with the limited pluripotency tests available in human lines, raise questions on whether those cells are capable of producing whole embryos and about their general level of pluripotency. The possibility of directly converting human pluripotent cells into mouse-like ESC is tempting. So far it has been achieved by constitutive expression of transcription factors, producing either metastable cells without proper epigenetic activation of major pluripotency regulators [22], or cells stable for only several passages [23]. Possibly, the optimal isolation and culture conditions required for human ESC culture have not yet been met. Alternatively, the observed differences in human and mouse ESC simply reflect the intrinsic species differences.

### TRANSCRIPTIONAL COMPARISON OF ESC AND iPSC

The transcription profiles of good quality iPSC and ESC are nearly identical. Chin et al. [24] showed that a small group of genes is continuously differentially expressed between several iPSC and ESC lines. Even though those genes couldn't be categorized by gene ontology analysis to the same functional group, they could point to iPSC as being a distinct subtype of pluripotent cells. In contrast to this finding, two other groups compared iPSC lines with slightly different statistical algo-

ritms and found that some difference between iPSC and ESC expression profiles does exist, but is not consistent through all the lines and points rather to different laboratory culture conditions [14, 15]. Also, focused profiling on only miRNA expression does not segregate iPSC from ESC [25]. Therefore, it seems that iPSC do not form a different new class of pluripotent stem cells distinct from ESC in their gene expression signature. Or if they do, the difference can not be pinpointed by transcriptome analysis because of the high noise in existing gene expression data and the possible heterogeneity in the quality of the tested iPSC lines [26].

However, when one looks at the individual reprogramming experiments instead of focusing on all differentially expressed genes between multiple iPSC lines and ESC, a statistically significant difference and logic can be seen. Namely, the common features of the deviant transcription come from (a) iPSC not efficiently silencing the expression pattern of the somatic cell from which they are derived and (b) failing to induce some ESC specific genes to the level of expression in ESC, akin to epigenetic memory [24, 27, 28] (Fig. 2).

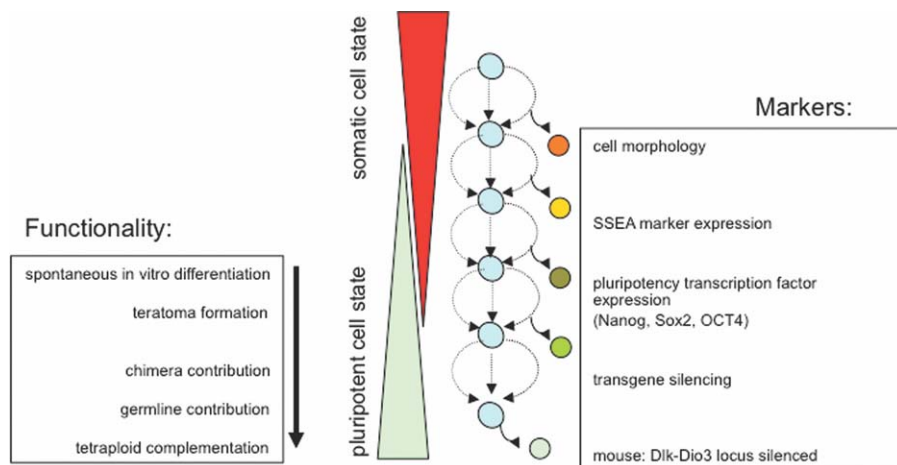
By using ESC and iPSC with identical genetic background and reprogramming factors integrated into the same genetic locus, it is possible to minimize the genetic and reprogramming methodology “noise” and to concentrate exclusively on the intrinsic differences between the two pluripotent cell lines [10]. Surprisingly, mouse iPSC and ESC obtained in this way have only two differentially expressed transcripts—non-coding RNA Gtl2 and small nucleolar RNA Rian. They localize to the imprinted *Dlk1-Dio3* gene cluster on mouse chromosome 12 and are maternally expressed. Its aberrant regulation is implicated in murine impaired development [29]. Epigenetically, the locus is fully methylated in many iPSC lines, while some lines have only one allele silenced, as is the case in ESC. Functionally, it seems that iPSC with the *Dlk1-Dio3* locus fully silenced can not form tetraploid complementation animals, and chimerism is also significantly lower when doing blastocyst injections, when compared with ESC [10, 30].

In human iPSC, the *Dlk1-Dio3* locus is not silenced, suggesting a different iPSC state/reprogramming. It would be interesting and useful to find similar marker in human cells. The search for such marker is convoluted by the possibility that epigenetic memory or aberrations during reprogramming may affect some genes which are not expressed in the pluripotent state, but whose expression would be relevant during differentiation.

Hence, in reprogramming experiments a wide palette of different quality iPSC lines have emerged. Clearly, the correlation between *Dlk1-Dio3* imprinting and a high degree of pluripotency needs more research. If confirmed, the strong advantage of the *Dlk1-Dio3* test lies in the fact that instead of having a diverse panel of pluripotency tests, this one is rather simple and technically manageable in most laboratories. Thus, even though reprogramming seems to be stochastic, there are some defined milestone steps that need to be taken sequentially, and directly analyzing for the final step(s) allows for a more simple and focused analysis in order to select true iPSC (Fig. 1).

### DIFFERENTIATION POTENTIAL OF iPSC

Another way to test the pluripotency of iPSC is by controlled in vitro differentiation. This is particularly true in case of human iPSC where their contribution to embryo formation can not be tested. Despite the recent report of a potential immunological response to iPSC in mice [31], directed differentiation with relatively high efficiency and production of



**Figure 1.** Reprogramming as a directed stepwise transition from the somatic to the pluripotent stem cell state. Although reprogramming is regarded to have stochastic elements, along the path to pluripotency, there seem to be certain milestones that need to be sequentially reached. Finding the link between cell markers and a function of a “pluripotency grade” would ease the analysis necessary to standardize and/or select for high-quality induced pluripotent stem cells.

functionally adequate cells are the crucial preliminary steps necessary for their future clinical use. There is a plethora of articles describing the potential of iPSC to differentiate into a particular cell type, including cardiomyocytes, neurons, hematopoietic progenitors, endothelia, osteoclasts, hepatocyte-like cells, islet-like cells, and retina. iPSC have passed these tests of differentiation and again defended their pluripotency status. But are they equivalent to ESC?

The direct comparison of the differentiation potential of various cell lines can be difficult. As different laboratories use different culture conditions and/or differentiation protocols, the lines can be compared only in the same work. If there is a significant variation from experiment to experiment the best comparisons are done with all the lines differentiated in parallel. This poses a problem when working with a large number of lines. Finally, evaluating the outcome of differentiation can be approached in different ways. One way is to score for the efficiency of the differentiation, that is, the quantity of cells obtained with a particular differentiation marker. Another important parameter is the quality or the identity of the final differentiated cell. This requires detailed tests for as many cell specific functions as possible. One example is the differentiation into neuronal cells where the full characterization of obtained neurons is still poorly addressed. In the end, it needs to be stressed that overall optimized differentiation protocols are still lacking. Although already more than a decade has passed since hESC were first established, there are few reproducible protocols that give functionally transplantable cells and that could be used as standards to compare the differentiation potential of pluripotent lines.

However, using available protocols, a side-by-side comparison of iPSC with ESC counterparts shows certain variations (Table 1). iPSC show either equal performance to ESC or in some cases inferior performance, especially when comparing the efficiency of their turnover into differentiated cells. Surprisingly, taking into account their degree of characterization—that is, the measures taken to work with “good quality” iPSC, or (although here the data is much more scarce) transgene free cells—there seems to be no correlation with the differentiation efficiency or the quality of the final cells [52]. This aspect of occasional iPSC low performance can perhaps be explained by the fact that the differentiation protocols are mainly established with ESC. Additionally, epigenetic memory and aberrations might make some iPSC more refractory

to external differentiation signals. It also has to be taken into account that adopting the cells for in vitro culture can already elicit certain aberrations in the cell state. The in vitro derived ESC used as a pluripotency standard are thus somewhat artificial and also showed significant variation in the differentiation potential between themselves [53, 54].

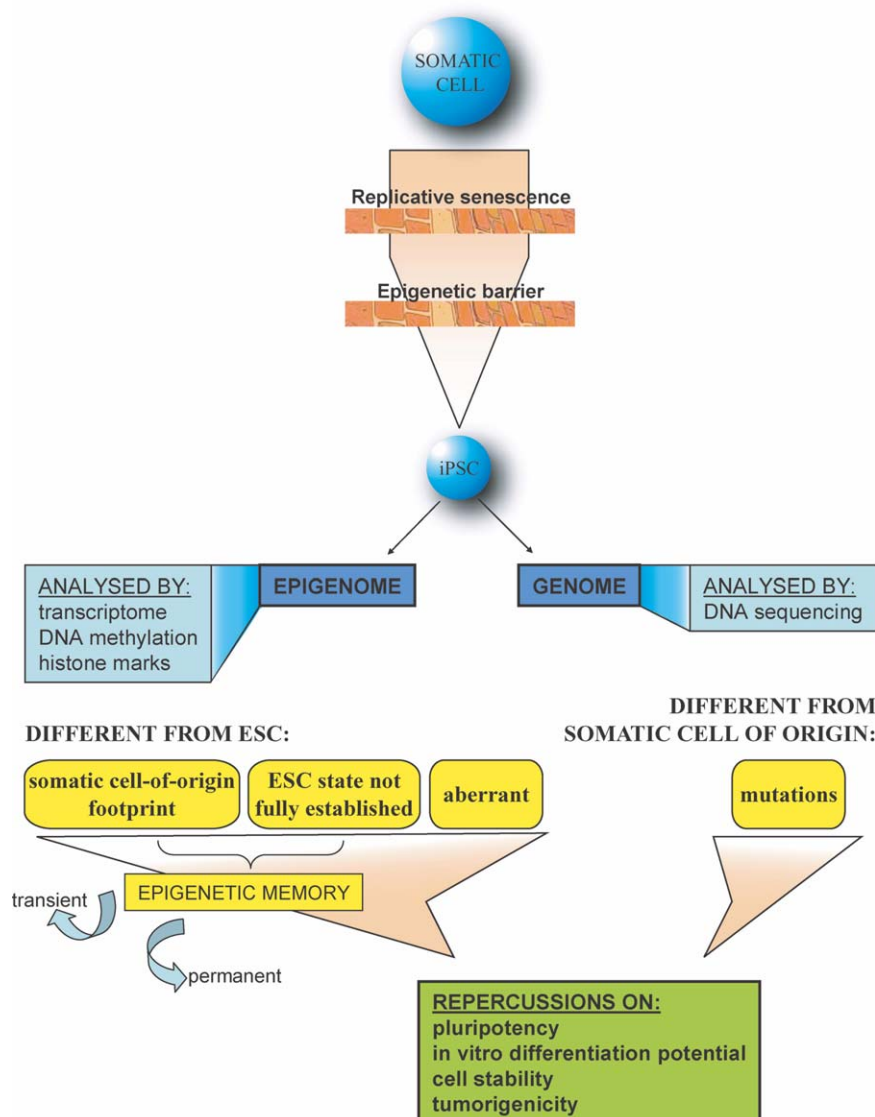
The degree of differentiation deviance of some iPSC stresses the need of having robust and relatively simple tests to screen the iPSC. Recently, such an attempt has been made by comparing the DNA methylation, transcriptome and spontaneous in vitro differentiation potential of a pellet of human ESC and iPSC. By doing so, the authors developed so called “scorecards” against which any pluripotent cell line can be checked to measure its potential to differentiate toward a particular lineage [55]. Tests like that can, in a reasonable experimental setting, select among various pluripotent lines the most receptive one for a particular use.

### EPIGENETIC COMPARISON BETWEEN iPSC AND ESC

Detailed insight into epigenetic differences between iPSC and ESC was made possible by the development of high-throughput sequencing technologies and by the generation of single-nucleotide genome-wide maps of DNA methylation.

The DNA methylation pattern is very similar between iPSC and ESC when compared with nonpluripotent lines, such as fibroblasts. However, hierarchical clustering performed on the methylation level of cca 66,000 CpG sites, besides clearly clustering fibroblasts from ESC/iPSC, also distinguishes iPSC from ESC [56]. One analysis on the whole genome scale found 71 differentially methylated regions (DMR) between three iPSC lines and three ESC lines (and 2,179 between fibroblasts and iPSC) [57]. Almost half of the DMRs show incomplete epigenetic reprogramming of the differentiated cell-of-origin genome, which is in agreement with the gene expression data [24] and epigenetic memory [58]. However, not all the DMR belong to the cell-of-origin memory, indicating that iPSC also accumulate novel aberrant epigenetic states [57, 59].

Compared with the ESC standard, both hypermethylated and hypomethylated CpG sites are found in iPSC, but the



**Figure 2.** Roadblocks in reprogramming bring forward epigenetic and genetic aberrations of the induced pluripotent stem cells (iPSC). Epigenetic dissimilarities between embryonic stem cell and iPSC can be analyzed by directly comparing DNA methylation and histone marks, or indirectly by gene expression analysis. Epigenetic memory of iPSC arises by cells being refractory to complete reprogramming from its original state. Together, epigenetic and genetic aberrations have repercussions on cell differentiation potential and tumorigenicity. Abbreviations: ESC, embryonic stem cell; iPSC, induced pluripotent stem cell.

balance is tipped toward hypomethylated CpGs [59]. This indicates that rather than the absence of an appropriate DNA demethylase (for example oocyte enriched) there is an inefficient methylation (or instruction of methylation) during the reprogramming [60]. These CpG methylation aberrations are not transient because they are observed in high passage number iPSC and are transmitted with high frequency through the differentiation to trophoblasts [59].

During reprogramming, iPSC regain non-CpG methylation, which is specific for pluripotent ESC. Also, several regions with aberrant methylation can be found (again mainly in the form of absence of the methylation mark when compared with ESC). Curiously, non-CpG aberrations are rather big, around 1 Mb, and are proximal to centromeres and telomeres [59].

Regarding histone methylation, there are few reports unable to find significant genome-wide differences between iPSC and ESC lines [14, 24, 61]. Using CHIP-on-CHIP, Chin

et al. analyzed H3K27me3 and H3K4me3 around the promoters (-5.5 to +2.5 kb from transcription start site) of 17,000 genes. Guenther et al. [14] did a more comprehensive CHIP-Seq analysis covering the whole genome of six iPSC and 6 ESC lines and did not observe significant variation to discriminate ESC from iPSC. Both types of cell lines showed characteristic pluripotent epigenetic landscapes with decreased H3K27me3, H3K4me3 enriched at promoters of actively transcribed genes and both of the marks on many bivalent domains. The variation in gene occupancy of those histone markers were the same between different iPSC and ESC lines as the variations within the same cell group.

Another comparative analysis was done on histone three lysine methylation by CHIP-Seq, with an improved computational method to detect not only peaks, but also long stretches of these marks [62]. This analysis highlighted the difference between human fibroblasts and pluripotent cells by showing that fibroblasts have more repressive chromatin due to the

**Table 1.** In vitro differentiation potential of various human and mouse induced pluripotent stem cell lines compared with the embryonic stem cell as a standard

Species	Cell type reprogrammed	Reprogramming factors	Transgene integration	Passage number of iPSC	iPSC characterized	Number of lines: ESC/iPSC	Differentiated to lineage	Efficiency	Functionality	Reference
Mouse	Fibroblasts	Oct4, Sox2, KLF4, Myc	Yes (retro)	ND	No; selection of Oct4 endogenous expression	1/1	Cardio-myocytes	iPSC delayed	Equal	[32]
Mouse	Fibroblasts	Oct4, Sox2, KLF4, Myc	Yes (retro)	ND	Yes (chimera)	1/2	Cardio-myocytes	iPSC lower	Similar, iPSC delayed in differentiation	[33]
Mouse	Fibroblasts	Oct4, Sox2, KLF4, $\pm$ Myc	Yes (retro)	8–15	No (nanog selection, SSEA1 expression)	1/3	Cardio-myocytes	iPSC lower (one iPSC line differentiated much worse)	iPSC lower (1 iPSC line differentiated much worse)	[34]
Mouse	Embryonic fibroblasts	Oct4, Sox2, KLF4, $\pm$ Myc	Yes (lenti)	ND	Yes, (chimera embryos)	0/6 (three in number; Myc+3 with myc)	Cardio-myocytes	iPSC with Myc differentiate worst	iPSC with Myc behave worst	[35]
Mouse	Fibroblasts	Oct4, Sox2, KLF4, Myc	Yes (retro)	ND	Yes (germline transmitting chimeras)	1/1	Cardio-myocytes, hemato-poietic	Equal (one protocol to CM; iPSC better)	Equal	[36]
Mouse	Fibroblasts	Oct4, Sox2, Klf4, Myc	Yes (retro)	ND	Yes (chimera embryos)	1/1	Neural stem cells	ND	Similar (different markers in iPSC vs. ESC)	[37]
Mouse	Embryonic fibroblasts	Oct4, Sox2, KLF4, Myc	Yes (retro)	ND	Yes (chimera, germ-line competent)	1/1	Renal lineage	iPSC lower	ND	[38]
Human	Fibroblasts	Oct4, Sox2, Nanog, Lin28	Yes (lenti)	ND	Yes (teratoma)	2/4	Cardio-myocytes	Variability <sup>a</sup>	Variability <sup>a</sup>	[39]
Human	Fibroblasts	Oct4, Sox2, Nanog, Lin28	Yes (lenti)	ND	Yes (teratoma)	1/1	Cardio-myocytes	Equal	Equal	[40]
Human	Fibroblasts	Oct4, Sox2, Nanog, $\pm$ Lin28	Yes (lenti and retro)	9–45	Yes (teratoma)	5/7	Hemato-poietic precursors, endothelia	Variability <sup>a</sup>	Variability <sup>a</sup>	[41]
Human	Fibroblasts	Oct4, Sox2, KLF4, Myc or Oct4, Sox2, Nanog, Lin28	Yes (retro) (lenti)	ND	Yes (teratoma)	14/8	Hemangi-oblast, endothelial, hemato-poietic precursors	iPSC lower (more than 50x)	iPSC lower (apoptosis and senescence in differentiated cells)	[42]
Human	Mesenchymal stem cells	Oct4, Sox2, Klf4, Myc, (hTERT, SW40 largeT)	Yes (retro)	ND	Yes (in vitro differentiation)	2/1	Hemato-poietic precursors, osteoclasts	Variation <sup>a</sup>	iPSC equal to best ESC	[43]
Human	Fibroblasts (IMR90 and adult)	Oct4, Sox2, Nanog, Lin28	Yes (lenti)	13–32	Yes/no (pluripotency markers, spontaneous in vitro differentiation)	1/2	Red blood cells	iPSC lower (5–10x less number of erythroid cells produced)	Equal	[44]
Human	IMR90 adult fibroblasts neonatal lung	Oct4, Sox2, Nanog, Lin28, lenti and episomal	Yes (lenti, retro) + no (episomal)	ND	Yes (teratomas)	5/12	Neurons	iPSC lower (~20%) than ESC (~90%)	Equal	[45]
Human	Adult fibroblasts	Oct4, Sox2, KLF4, Myc, $\pm$ Nanog	Yes (retro)	ND	No (pluripotency markers)	1/2	Motor neurons	iPSC lower (~4x lower)	Equal	[46]
Human	Fibroblasts	Oct4, Sox2, Nanog, Lin28	Yes (lenti)	ND	Yes (teratoma)	1/4	Retinal pigmented epithelium	Equal	Equal	[47]
Human	Adult fibroblasts	Oct4, Sox2, KLF4, $\pm$ Myc	Yes (retro)	ND	Yes (teratoma)	2/4	Adipocytes	Equal (minor variations between all the lines)	Equal	[48]



TABLE 1. (Continued).

Species	Cell type reprogrammed	Reprogramming factors	Transgene integration	Passage number of iPSC	iPSC characterized	Number of lines: ESC/iPSC	Differentiated to lineage	Efficiency	Functionality	Reference
Human	Fibroblasts	Oct4, Sox2, KLF4, Myc	Yes (retro)	ND	Yes (teratomas)	1/4	islet-like cells	Ipsc lower; two lines failed to differentiate	Equal (iPSC that did differentiate)	[49]
Human	Adult fibroblasts	Oct4, Sox2, KLF4, UTF1	Yes (lenti)	4–19	No (pluripotency markers)	1/2	Hepatocytes	Equal	Equal	[50]
Human	Hepatocytes	Oct4, Sox2, Klf4, Myc	Yes (retro)	ND	Yes (teratomas)	2/3	Hepatocytes	Equal	Equal	[51]

The level of induced pluripotent stem cell (iPSCs) pluripotency characterization and passage number used in experiments where iPSCs are available are given. The efficiency of differentiation was scored either by taking the percentage of differentiated cells expressing reference differentiation marker (by fluorescence-activated cell sorting) or by analyzing the total expression levels of a particular differentiation marker (by quantitative polymerase chain reaction). Functionality represents the characteristics or performance of the differentiated cell in a specific test for a differentiated cell type. The table features only articles that compare side by side iPSCs and embryonic stem cells to evaluate their relative differentiation capabilities; a number of articles showing the differentiation potential of iPSCs alone were not included to avoid possible confusion.

<sup>a</sup>Variability indicates both embryonic stem cell and induced pluripotent stem cell (iPSC) lines show same variability (iPSC do not group to better/worst performing category). Abbreviations: CM, cardio-myocytes; ESC, embryonic stem cell; iPSC, induced pluripotent stem cell; ND, not determined.

large expansion of H3K27me3 and H3K9me3 repressive marks. The study also demonstrated further differences between hiPSC and ESC. Namely, even though iPSC are more similar to ESC, they have longer repressive domains in H3K27me3 and H3K9me3, similar to fibroblasts. In addition, differentially marked genomic regions are mainly in nongene areas (88% for H3K9me3 and 79% for H3K27me3), raising the question of the functional significance of the main body of those epigenetic marks. H3K9me3 modification is less represented throughout the genome, but it shows more differences between iPSC and ESC, mostly being present in “unique” regions in iPSC, that is, regions not characteristic for ESC or fibroblasts [62]. The same group also associated differences in the gene expression analysis by Chin et al. to the H3K9me3 mark, rather than to the H3K27me3 mark.

### EPIGENETIC MEMORY OF iPSC

By now, iPSC have been derived from many different somatic cell types including fibroblasts, keratinocytes, B-lymphocytes, stomach cells, and hepatocytes [5, 63–65]. Are there differences between lines based on their cell of origin? The analysis of gene expression profiles of various human iPSC lines supports this hypothesis by showing significant and persistent donor-cell gene expression in iPSC [24, 27, 28]. So far, all experiments point to three major characteristics when focusing on the distinction between iPSC and ESC. One is the aberrant silencing of somatic genes in cells undergoing reprogramming, another is the weak activation of ESC specific pluripotency genes and the third are unspecific aberrations distinct from either the cell of origin or ESC (Fig. 2). The first two groups led to the belief that there is an epigenetic memory present in the iPSC.

Furthermore, mouse iPSC derived from distinct tissues had marked differences in the frequency of teratoma formation, when differentiated into neurospheres and transplanted into the brain [66]. An explanation for this observation is not yet clear, but could lie in aberrant epigenetic memories of iPSC that reflect different epigenetic states of the donor cells.

Recently, two articles analyzed the epigenetic memory of mouse iPSC in more detail [58, 67]. iPSC derived from the same cell of origin can be clustered together on the basis of their gene expression and DNA methylation. More interestingly, there is a functional significance of the donor cell gene expression, where iPSC differentiation back into the cell of origin brings an advantage over iPSC differentiation into an unrelated lineage [58, 67]. The epigenetic basis of this memory is linked either to DNA methylation [58] or to histone modifications [67].

Importantly, all the analyses in the two articles were performed with iPSC with low passage numbers (p4–p6). It seems that reprogramming takes longer than previously thought, and goes on for several passages even after the appearance of ESC morphological features and the expression of pluripotency markers. Possibly, it occurs through a more passive and cell division dependent resetting of the epigenetic cell state. This is corroborated by several facts—higher passage number iPSC (p16 in mice) lose the differences in gene expression and can no longer be clustered together by their cells of origin [24, 67]. The preferential differentiation capabilities of iPSC to its tissue of origin are dispersed either by longer passaging [67], chemical treatment influencing epigenetic machinery (5-azacytidine, Trichostatin A), or sequential differentiation-reprogramming cycles into the desired direction [58].

One important point arising from those two articles is the possibility to use the epigenetic memory in order to obtain cells whose differentiation protocols are not yet optimized, as is the case for blood differentiation in human. However, care must be taken because ESC still differentiated more efficiently to blood precursors than early passage blood iPSC in mice [58]. Thus, at the moment the reported epigenetic memory brings rather a disadvantage in the differentiation to any other lineage different from its origins. Early-passage iPSC may not have acquired ESC-like responsiveness to react to differentiation clues. Nevertheless, it remains to be shown whether partially reprogrammed iPSC could be stabilized into a state that has an epigenetic memory of origin and additionally, whether they harbor a certain plasticity that in combination will give an advantage in differentiation capabilities, compared with ESC. Supporting this idea, a recent article reported epigenetic memory in human iPSC lines derived from retinal pigmented epithelial cells [68]. Several iPSC lines differentiated back into their cell of origin with 5- to 10-fold higher efficiencies compared with ESC. iPSC with early passage numbers were not required for this memory.

### MUTATIONAL LOAD OF iPSC

Besides epigenetic aberrations it is reported that iPSC also bear genomic mutations [69–72]. These could arise from the reprogramming itself and from the in vitro expansion of cells afterward. So far iPSC reprogramming is a very inefficient process, where in the end just few single cells get reprogrammed. Mutations could bring certain advantages for the change of cell fate, thus representing a strong mutagenic factor. Subsequent proliferation and adaptation to the in vitro culture conditions is another important cause of mutations, although common for other cell lines too, including the ESC where gross mutations have already been noted [73–75].

Observed iPSC mutations range from chromosomal aneuploidy, subchromosomal deletions or duplications to single base mutations. From the selected number of iPSC analyzed, as many as 20% had gross chromosomal aberrations, including complete trisomies (9% of total) [69]. Another study focused on copy number variation (CNV) (approximately 0.6–12,000 kb stretches of genomic DNA) of a large number of pluripotent and somatic samples. iPSC had on average 17 CNV per sample [70]. As a comparison ESC also had 17 and nonpluripotent samples had 12 CNV. Focusing only on the exome, an iPSC line has on average about six mutations, most of which are predicted to alter protein function [72]. Surprisingly, in all the studies focusing on the genetic aberrations so far there was no correlation between the extent of genetic aberrations and the reprogramming method (combination of transcription factors used, Myc oncogene, the genomic integration vs. nonintegrative methods) or iPSC propagation method (mechanical or by trypsin).

Importantly, some of the aforementioned mutations were shown to be already present in the (small) fraction of the somatic cells that the iPSC were derived from [49, 70, 72]. Another interesting study showed that early passage iPSC bear a significant number of CNV that actually attenuates during subsequent intermediate length passaging, finally descending to the average number of CNV per ESC lines or fibroblasts [71]. The elimination of the CNV in iPSC population is possible because many are present in mosaic fashion (i.e., only a certain part of the cell population has the mutation). Three conclusions can be made from that observation; the

low efficiency and long duration of reprogramming increases the mutational load that could help in breaching some roadblocks along the way to pluripotency. Next, some of these changes seem to be deleterious for the final homeostatic state of the cell, and are therefore eliminated from the population. Finally, it stresses the importance of cell-cell communication and signaling during reprogramming. As the mutations are mosaic in the iPSC, they bring advantages to the whole population of cells, including the mutation-free sister cells that reach the pluripotent state together with the mutation bearing ones.

Categorizing the genes in iPSC affected by mutations brings forward, if any, genes implicated in the cell cycle regulation and tumors [46, 70]. Although the spread of the mutations in the genome of iPSC is pretty wide, there are some more frequent mutations and chromosomal aberrations that are common to late passage human ESC, too. Particularly, chromosomes 12, 20, and parts of chromosome 1 are being affected in both cell types, as well as isolated genomic regions close to *DNMT3B*, *NANOG*, and *GDF3*. Overall, even though iPSC obviously need to pass through one additional selection process, reprogramming, iPSC and ESC are not drastically different considering the mutational load. In addition, there is some correlation between mutations found in late passage human ESC, iPSC, and cancers cells [75–77]. Thus, pluripotent cells cultured in vitro are by definition proliferating and can acquire specific aberrations that support growth advantage, similar to tumor progression, that eventually take over the population. Currently, a lot of research effort is put into the better understanding of reprogramming processes and into increasing its efficiency, which could further diminish genomic instability in iPSC. For example, as many as 10% of fibroblasts get reprogrammed by the *miRNA302/367* cluster [78]. It will be interesting to see whether these iPSC have fewer mutations accumulated when compared with the conventional iPSC (that get around 0.1% of the starting cell population reprogrammed). In addition, profiling of the mutational and epigenetic aberrations has to be performed to distinguish those that pose clinical risk from those that are actually harmless.

### CONCLUSION

iPSC must have convinced even the most skeptical minds of their developmental potential and pluripotency when tetraploid complementation resulted in viable adult mice [12, 13]. It is a definite proof of principle that four transcription factors are able to modify a differentiated cell all the way to the pluripotent state of ESC. The current problem with iPSC lies in their low efficiency of derivation and the heterogeneity of the obtained colonies.

Not all mouse iPSC lines are able to successfully complement 4n blastocysts [10]. In the reprogramming process only a fraction of colonies appear that are considered “good quality” iPSC. The first important step is therefore to select only for the iPSC that have reached this fully reprogrammed state. As they are morphologically and transcriptionally very similar to the lesser quality iPSC, a detailed analysis is currently required for the selection of the good ones. Alternatively, a good marker is needed. One such marker might be *Dkl1-Dio3* locus [10, 30].

The majority of epigenetic aberrations in iPSC are only present in early passage numbers, and therefore can be considered transient epigenetic memory [58, 67]. However, it has also been noted that some transcripts and chromatin marks

are persistent in the later passage number iPSC and even in their differentiated progeny [59]. In addition to this failure to erase all the features of the differentiated cell state, iPSC also seem to be able to accumulate ESC-dissimilar transcripts and chromatin marks that are not related to the cell of origin. They can not be grouped under the same nominator, corroborating their stochastic nature. Additionally, genomic mutations can arise during reprogramming. All of these aberrations likely appear because of the imperfection of the reprogramming procedure, as illustrated by the low efficiency of reprogramming. Efforts have to be made to improve culture conditions and factors [79] available to the cells during reprogramming, which could lower the number of stochastic steps the cell needs to breach in order to achieve pluripotency. This would likely increase the ratio of true versus bad quality iPSC colonies and lower the aberrations present in the cells.

In the end, iPSC continue to offer much promise for both clinical applications with personalized medicine, and for basic research in developmental and cell biology. The iPSC research field is still unfolding, and with the current attention

it holds in the scientific community, the iPSC safety issues discussed here should be addressed in the near future.

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## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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## Concise Review: Induced Pluripotent Stem Cell-Derived Mesenchymal Stem Cells: Progress Toward Safe Clinical Products

YUNJOON JUNG,<sup>a,b</sup> GERHARD BAUER,<sup>b,c</sup> JAN A. NOLTA<sup>a,b,c</sup>

<sup>a</sup>Department of Biomedical Engineering, <sup>b</sup>Stem Cell Program and Institute for Regenerative Cures,

<sup>c</sup>Department of Internal Medicine, Division of Hematology/Oncology, University of California, Davis, Sacramento, California, USA

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### ABSTRACT

Adult stem cell therapies have provided success for more than 50 years, through reconstitution of the hematopoietic system using bone marrow, umbilical cord blood, and mobilized peripheral blood transplantation. Mesenchymal stem cell (MSC)-mediated therapy is a fast-growing field that has proven safe and effective in the treatment of various degenerative diseases and tissue injuries. Since the first derivation of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), there has been impressive progress toward developing safe clinical applications from PSCs. Recent successes in transgene-free iPSC reprogramming

have brought attention to the potential of clinical applications of these pluripotent cells, but key hurdles must be overcome, which are discussed in this review. Looking to the future, it could be advantageous to derive MSC from iPSC or human ESC in cases where genetic engineering is needed, since in the PSCs, clones with “safe harbor” vector integration could be selected, expanded, and differentiated. Here, we describe the status of the progress of the use of MSC and PSCs in clinical trials and analyze the challenges that should be overcome before iPSC-derived MSC therapy can be used widely in the clinic. *STEM CELLS* 2012;30:42–47

Disclosure of potential conflicts of interest is found at the end of this article.

### INTRODUCTION

An emerging approach to treat disorders requiring the replacement of injured or dying cells is to replace those cells with healthy ones generated from stem cells, which have the potential to differentiate into multiple mature cell types. In particular, adult stem cell-based therapies have been successful for several decades, with the first hematopoietic stem cell (HSC) transplantation occurring over 50 years ago [1]. Recent discoveries based on embryonic stem cells (ESCs) [2] and induced pluripotent stem cells (iPSCs) [3] escalate the hope for future regenerative medicine applications, with one human ESC-based therapy already being tested in a first-in-man Phase I clinical trial. In spite of the great potential, there are technical challenges to be overcome before PSCs can be applied to clinical applications in a broader fashion.

In this review, we highlight the potential of iPSC-derived MSCs and several other iPSC derivatives currently developed as stem cell therapy candidates and provide an evaluation of challenges to overcome potential barriers toward clinical usage of PSC-based products.

### MESENCHYMAL STEM CELLS

For decades, cell therapies using adult stem cells have rescued thousands of patients from induced or genetic disorders [1].

Bone marrow (BM)-derived HSC therapy was first delivered to patients in 1956, following extensive testing in a canine model [4], afterward becoming a standard clinical procedure, particularly as a treatment for leukemia and lymphoma (reviewed in ref. [5]). MSCs were first described by Friedenstein and colleagues as an adherent fibroblast-like subset of the BM microenvironment called the “marrow stromal cells,” which was capable of supporting hematopoiesis. Later, these fibroblast-like cells were found to have adult stem cell properties as they could differentiate into cartilage, bone, fat, and tendon [1]. MSCs have been evaluated for regenerative medicine applications either through direct differentiation into these tissues or indirectly through protein or cytokine secretion and immune suppression [1, 6–9]. MSCs are a promising tool for cell therapies because they are easily accessible from various tissue sources such as BM, fat, umbilical cord, and others, easily isolated, show robust in vitro expansion to clinical scale and allow for cryostorage with minimal loss of stem cell characteristics. MSCs have demonstrated systemic migration capabilities after i.v. transplantation, in particular to areas of hypoxia or tissue damage [10]. Even systemic administrations of allogeneic MSCs do not cause any adverse effects, in part due to immune-modulatory effects [11, 12]. MSCs have been considered safe as they do not show tumor formation after transplantation [13] and have been widely tested and

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Correspondence: Jan A. Nolta, Ph.D., Stem Cell Program and Institute for Regenerative Cures, University of California, Davis, 2921 Stockton Boulevard, Room 1300, Sacramento, California 95817, USA. Telephone: 916-703-9308; Fax: 916-703-9310; e-mail: jan.nolta@ucdmc.ucdavis.edu Received June 8, 2011; accepted for publication August 16, 2011; first published online in *STEM CELLS EXPRESS* September 2, 2011. © AlphaMed Press 1066-5099/2011/\$30.00/0 doi: 10.1002/stem.727

proven efficacious in preclinical and clinical studies for cardiovascular [14] and neurodegenerative [15] diseases, graft-versus-host disease (GvHD) [11], and autoimmune disease. Because of the ability of MSCs to differentiate to osteoblasts, Caplan and colleagues initiated clinical trials for osteogenesis imperfecta using allogeneic BM transplantation with MSC [16]. Le Blanc et al. pioneered a study to investigate immunomodulatory effects of MSC transplantation therapies for steroid-resistant GvHD [11], and similar methods were applied to other diseases [1]. These early studies established a good clinical record of safety for systemic MSC transplantation.

Several papers have demonstrated that MSCs can be efficiently transduced with retroviral and lentiviral vectors and maintain transgene expression throughout many passages and lineage-specific differentiations, with fewer complications caused by viral integrations [13, 17, 18]. MSCs genetically modified to secrete cytokines and other growth factors have been successfully used in animal models of tissue repair and various other diseases and are therefore poised to be tested in human clinical trials [9]. Clonal analysis of transduced MSCs have shown that MSCs often contain several thousand copies of transgene RNA per cell and can maintain transgene expression for up to 6 months [19]. However, the risk of tumor formation due to insertional mutagenesis by viral vector integrations still raises caution for human clinical applications [20] (discussed in a later section). Identification and utilization of genetically modified MSCs, which have “safe harbor” integrations of the desired transgenes, is restricted due to the limited lifespan of primary MSCs during *in vitro* expansion. Aging, moreover, significantly reduces the survival and differentiation potential of BM-MSCs [21]. In contrast, using human PSC (hESC or iPSC) to generate MSCs, a vector integration site could be mapped and cells with safe harbor integrations could potentially be expanded nearly indefinitely.

## HUMAN PLURIPOTENT STEM CELLS

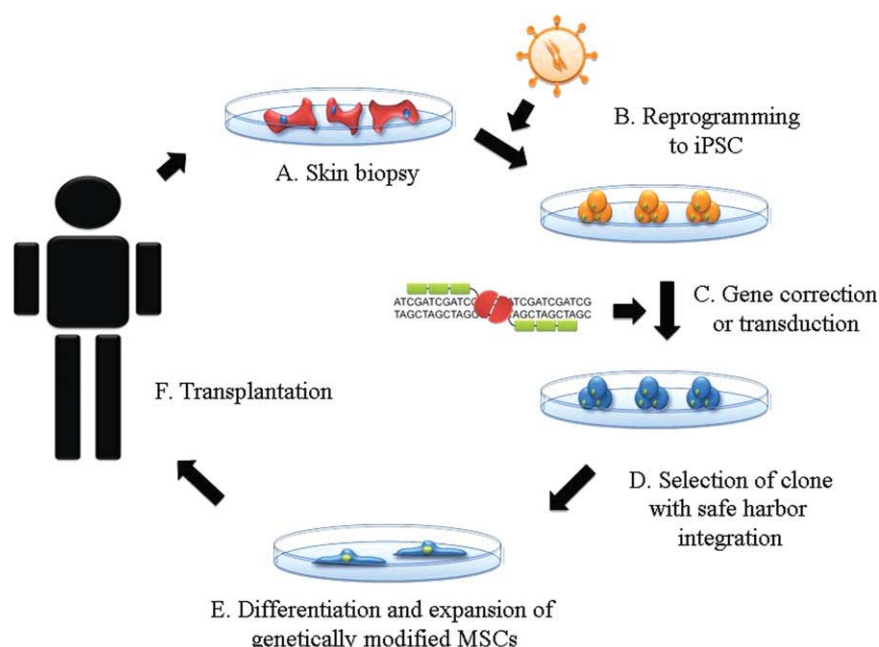
hESCs have the potential to differentiate into all types of adult human tissues and to grow indefinitely [2]. Since their initial derivation, hESCs have become promising tools for developmental biology and regenerative medicine. However, concerns related to ethical objections regarding the use of human embryos for hESC derivation have dramatically restricted funding of research using these cells and therefore have set back the development of hESCs for clinical trials. Because of their allogeneic nature, immune rejection of cells and tissues derived from hESCs is another potential drawback to their use in transplantation. Immunosuppressive drug regimens, similar to those used for current human tissue and organ transplant procedures, might lessen the severity of the anticipated immune rejection, but at the same time, can also put the tissue recipient at an increased risk of infections. This risk can be lessened by application of human leukocyte antigen-matched tissue, as is currently being practiced in organ transplantation, or could be completely eliminated by the use of the patient's own tissue. The latter possibility can now be achieved by application of autologous iPSCs, the patient's own somatic cells, “reprogrammed” to become pluripotent cells [3].

Following groundbreaking work by Yamanaka and colleagues demonstrating that mouse fibroblasts could be converted into iPSCs by retroviral delivery of four transcription factors (Oct4, Sox2, Klf4, and Myc), other groups reported that terminally differentiated human somatic cells could be reprogrammed into a pluripotent state using retroviral or lentiviral vectors transferring the same four transcription factors. In many ways, iPSCs are similar to hESCs, in their morphol-

ogy, gene expression, *in vitro* differentiation potential, and teratoma formation. However, inherent “epigenetic memory” of the starting cells may influence specific differentiation and *in vivo* functionality of tissues derived from such reprogrammed cells. More research in this area is needed to determine the best starting somatic cell for iPSC generation that allows for reproducible differentiation into different types of functional tissues for human clinical applications. iPSCs hold great potential for regenerative medicine, as can already be demonstrated in mouse models of Parkinson's disease [22] and sickle cell anemia [23]. Disease-specific iPSC lines for modeling “diseases in a dish,” screening new drug compounds, and developing new therapies have been used successfully [24–26] (detailed review in Shinya Yamanaka and Masato Nakagawa paper in this issue). However, clinical applications of iPSCs have been criticized because of the possibility to form tumors by integrated oncogenes, c-Myc in particular [27], by insertional mutagenesis that has the potential to cause cancers [28] or disrupt tumor suppressor genes [20], and recently, for epigenetic memories and genomic aberrations in the reprogrammed cells [29]. Therefore, to manufacture iPSCs for clinical applications, several precautions need to be taken, as discussed in detail in the following section.

As BM-MSCs can easily be harvested from adult sources and cultured *in vitro*, many preclinical and clinical studies have used BM-MSCs [1]. Although easy access to BM-MSCs is recognized as a great advantage, extended *in vitro* culture reduces the differentiation potential of MSCs, which limits their therapeutic efficacy [30]. To overcome this shortfall, MSCs derived from iPSCs may therefore be considered for human cell and gene therapy applications as iPSCs have the potential to be expanded indefinitely without senescence (Fig. 1). Several laboratories, including ours, have already shown that MSCs derived from hESC have the same *in vitro* and *in vivo* characteristics as MSCs derived from adult sources [31, 32]. Our group reported that hESC-derived MSCs were karyotypically stable, had the same cell surface phenotype as MSCs isolated from adult BM, and could home similarly to areas of hypoxic injury in a hind-limb ischemia model [32]. Lian et al. [33] showed that MSCs derived from human iPSCs can be generated in clonal expansion cultures and can be differentiated into osteoblasts, adipocytes, and chondrocytes and promote vascular and muscle regeneration. This team also described a greater regenerative potential of MSCs derived from iPSCs, which may be attributed to superior survival and engraftment after transplantation, because of higher telomerase activity and less senescence as compared to BM-MSCs. In these studies, iPSC- or hESC-derived MSCs were comparable to BM-MSCs in surface marker expression, differentiation potential, and in *in vivo* regenerative potential in the hind limb ischemia mouse model. Future studies should examine the efficiency of MSC derivation based on different clinically relevant protocols or cell sources, with term follow-up of *in vivo* safety and efficacy studies.

Genetically manipulated MSCs may also serve as cellular therapeutics since MSCs can be used as targeted drug delivery vehicles (detailed review in ref. [9]). For instance, MSCs could be transduced with a transgene expressing vascular endothelial cell growth factor (VEGF) to stimulate revascularization in ischemic heart and peripheral limb tissue [34]. Previous direct injection of VEGF protein and gene therapy vectors carrying VEGF showed promise in Phase I–II clinical trials but did not achieve significance in Phase III trials [34]. MSCs, however, migrate to ischemic areas, remain there for an extended period of time, as has been demonstrated in preclinical animal models [9, 35], and could continuously deliver VEGF. This could become a cellular therapy using highly tested allogeneic, transduced MSCs. These MSCs could be



**Figure 1.** Schematic diagram of iPSC therapy. (A): Fibroblasts from skin biopsy are cultured from patients. (B): Patient-specific cells can be reprogrammed by viral delivery of induction factors or nonintegrating methods. (C): Gene correction can be accomplished by vector-mediated gene transfer or gene exchange by homologous recombination. (D): Gene-corrected iPSCs can be screened by sequencing to find a clone with proper gene correction or integration into a safe harbor site. (E): MSCs with integration into the controlled site can be tested, expanded, and purified in a good manufacturing practice facility and could then be transplanted to the patient, following appropriate clearance by all regulatory agencies. Abbreviations: iPSC, induced pluripotent stem cell; MSCs, mesenchymal stem cells.

generated from iPSCs that were created in an integration-free system and transduced with a VEGF vector; these could be selected for safe harbor integrations of the transgene to exclude the possibility of tumor formation due to insertional mutagenesis. Other diseases such as Huntington's disease or other neurodegenerative disorders, could also be targeted with this type of approach, using highly characterized iPSC-derived MSC batches engineered to produce neurotrophic factors [15].

### REGULATORY ISSUES FOR FUTURE SAFE THERAPIES FROM HUMAN PSCs

The US Food and Drug Administration (FDA) regulates the clinical application of cell and gene therapy. The final cellular product administered into a patient must meet important safety and efficacy criteria, such as identity, purity, potency and clinical safety, and efficacy [36]. Besides criteria that all cellular products must meet, such as sterility, viability and freedom from endotoxin, particular concerns for stem cells are (1) characterization of the product, including in vitro and in vivo potency, (2) freedom from cell differentiation to undesired cell types, (3) in vivo cell migration/trafficking to non-target site(s), (4) potential uncontrolled cell proliferation or tumorigenicity, (5) immunogenicity, (6) graft-versus-host effects, (7) interactions with devices, other tissues or drugs in vivo, and (8) for gene-modified cells, potential uncontrolled biological activity of the transgene, alteration of expression of the non-transgenes, and insertional mutagenesis. In this section, we discuss individual areas of consideration for iPSC-derived cellular products in the path to the clinic.

### Development of Clinically Relevant iPSCs and Their Derivatives

At this point, clinically applicable iPSC do not yet exist, but are under development. For iPSC-based therapies, several

categories must be considered to meet regulatory requirements. One of the most important goals for the manufacturing of a safe stem cell product is the prevention of tumor formation after transplantation. Tumors could be generated in iPSC-mediated clinical applications by insertional mutagenesis caused by transgenes used for reprogramming [37], by enhancer effects caused by particular viral sequences found in retroviral or lentiviral vectors [28], and by disruptions of essential genes caused by integrated vector cassettes [20].

Teratomas could be caused by undifferentiated cells contaminating the differentiated final product. Integrated c-Myc delivered by a retroviral vector has been shown to cause tumor formation in 40% of mice due to the reactivation of silenced genes [37]. In adult stem cell therapies, genetically modified cells can carry the risk of tumor generation. An HSC gene therapy clinical trial to treat X-linked severe combined immunodeficiency disease (X-SCID) using a retrovirus caused 4 out of 11 children to develop leukemia [28, 38] and 1 out of 10 Wiskott–Aldrich syndrome gene therapy clinical trial patients developed an acute lymphocytic leukemia [39] due to transgene integrations in the proximity of the *LMO2* proto-oncogene promoter. Numerous other stem cell gene therapy clinical trials using retro- or lentiviral vectors that were not carrying a growth factor receptor gene, however, have avoided this outcome [40]. Another concern may be cell transformation caused by gene disruption. An HSC therapy paper claimed that integrated lentiviral vector had disrupted a tumor-suppressor gene leading to premature termination of endogenous genes that could cause tumor formation [20]. This effect could be monitored in in vitro cell immortalization assays and by serial transplantation experiments in vivo [13, 41].

MSCs derived from iPSCs with safe harbor therapeutic gene integrations, or gene corrections by homologous recombination, could significantly reduce the chance of tumor formation as these cells can be screened to avoid gene disruptions or oncogene activation. iPSC colonies can be

specifically selected for proper gene insertion, can be highly tested, and can then be expanded at large scale for master cell bank generation prior to directed differentiation to MSCs or other lineages. Gene-modified iPSC-derived MSCs could be used for safe administration of a therapeutic gene product to specific sites of injury or inflammation, as MSCs are known to migrate to such areas in vivo [9, 15, 42].

Improving reprogramming technology for safe iPSC derivation is important for human therapeutic applications, and permanent transgene integrations for reprogramming should be avoided. Recent papers have described many approaches to accomplish this, such as adenoviral vector transductions, DNA plasmid vector transfections, Cre-LoxP excision of reprogramming vector cassettes transferred by a lentiviral vector, transposons, episomal Epstein-Barr virus, mRNA transfections, and protein transfections [43]. All of these methods avoided transgene integration or persistence, and tumor formation in chimeric mice could not be observed (detailed review in Gustavo Mostoslavsky paper in this issue). Additionally, small molecule-mediated reprogramming has become interesting for clinically relevant iPSC generation [44]. A small molecule approach could be simpler and may not be associated with the same side effects as an RNA approach. However, such approaches are currently rather inefficient in the generation of iPSCs and are under further development.

### Epigenetic Memory and Genetic Aberrations

Another important concern for cellular therapies is whether the transplanted cells may become unstable or could be transformed into tumors. A number of studies have demonstrated that iPSCs contain abnormalities at the genetic and epigenetic level and that these defects are often related to oncogenic pathways [29, 45–47]. The epigenetic memory of iPSCs with its incomplete epigenetic reorganization and skewed differentiation potential also raises the question of whether such cells may actually be suitable for therapeutic applications (detailed review in Ren-He Xu paper, Juan Carlos Izpisua Belmonte paper, Hans Schoeler, and Jared Sternecker paper in this issue). These issues will be addressed in iPSC derived cellular therapies currently under development.

### Cell Culture Conditions

Even though iPSCs can be reprogrammed by integration-free methods, there are still a number of concerns to be addressed before any of these methods can be applied to generate a clinical grade cellular product. Current FDA regulations mandate the derivation and manufacture of cell and gene therapy products to be compliant with current Good Tissue Practice (cGTP) and Good Manufacturing Practice (cGMP) regulations, which include collecting, storing, and recovery of patient samples, derivation, culturing and differentiation of tissues, screening, testing, validating of products and procedures, packaging, labeling, and distribution of final products [36]. However, a Phase I clinical trial applying hESC-derived neuronal tissues for the treatment of spinal cord injury was recently approved by the FDA. The hESCs were not derived under GMP conditions and had been cultured on mouse feeder cells; however, they were highly tested for communicable xenogeneic diseases [48]. Nonetheless, it will be in the best interest of the laboratory manufacturing an iPSC- or hESC-derived cellular product to be in compliance with cGTP and cGMP regulations, otherwise the product will not be able to progress to Phase II or III clinical trials, and will have to be rederived. Additionally, to generate a safe and clinically acceptable iPSC-derived product, xeno-free cell culture conditions should be used to minimize the risk of transmitting disease or causing human immune reactions [36]. In the past, hPSCs have been derived and cultured

using media containing animal-based serum replacements and a mouse embryonic fibroblast feeder layer. Martin et al. [49] found that both xenogeneic serum replacement and feeder cells are sources of nonhuman sialic acid Neu5Gc, which causes immunological reactions involving human antibodies. Therefore, human clinical applications of iPSCs should use cultures with either human feeder cells or a feeder-free system applying a chemically defined matrix.

### Consistency of iPSC Derivation and Differentiation

Standard Operating Procedures (SOPs) are mandated in a GMP environment. These SOPs guarantee that a safe and efficacious cellular product will be manufactured in a reproducible manner. Although there are several clinically applicable reprogramming technologies, the consistency of iPSC-derived products is still a concern. SOPs cannot eliminate variations in cell reprogramming, expansion and differentiation efficiencies, but rather, will have to adapt to these properties inherent in iPSCs and iPSC-derived products. In all likelihood, well growing colonies and differentiated tissues will have to be selected under GMP conditions, and appropriate tests will have to be performed to assure their safety and efficacy in the planned human clinical application [29, 47, 50]. Single cell clonal expansion of human iPSCs have shown low survival rates compared to mouse counterparts [51]; therefore, developing reliable and reproducible standard protocols to differentiate and select iPSC-derived cellular products is a pressing issue.

There are indications that due to epigenetic memory or incomplete reprogramming, some iPSC lines favor specific differentiation pathways [52]. Differentiated cells can be thought of as a heterogeneous population of desired, differentiated cells mixed with undesired, undifferentiated cells, in spite of the application of efficient direct-differentiation methods [53]. To eliminate undifferentiated PSCs within the population of differentiated cells, several techniques have to be assessed. Cell sorting using a clinical grade flow cytometric cell sorter under GMP conditions can be one of the solutions. Introduction of a suicide gene only expressed in undifferentiated cells and antibodies directed against stem cell-specific surface markers could be used to selectively kill or capture and remove undifferentiated PSCs.

### Safety and Efficacy

Safety of a cellular product remains the most important criterion for human applications. Therefore, toxicology studies must be performed on the proposed final product. Such studies must be evaluated in acute and chronic in vivo models and must encompass the examination of major organs, neighboring tissues, blood chemistry, and blood cell counts after the transplantation into the in vivo models [36].

Efficacy and functional consistency of an iPSC-derived cellular product is also important. Specific efficacy and potency tests need to be developed for each product. Such tests could be in vitro or in vivo tests, testing a specific function of the final product. The in vivo functionality and efficacy of the cellular product could be evaluated in a transplantation model and could be correlated with the in vitro assay. If the assays show consistent correlation, then the in vitro assay could later be used as a surrogate marker for the in vivo assay. The same model could possibly be applied to the measurement of cell surface markers associated with established efficacy in an in vivo model. In later phase clinical trials, the efficacy assay is mandated by the FDA [36].

Currently, it is not clear whether fully differentiated cells or progenitor cells would be more suitable for engraftment and functionality. Recently, a fetal neural stem cell transplantation paper raised the issue of tumorigenicity. Fetal neural



stem cell injections into an ataxia telangiectasia patient caused tumor formation 4 years after transplantation. Tissue analysis confirmed that those tumors originated from donor tissues [54]. Fully differentiated cellular products have low potential to form tumors. However, they also have low engraftment efficiency and poor durability in most cases. Therefore, systematic *in vivo* studies applied to the state of differentiation of specific iPSC-derived cellular products should be conducted before human applications are considered.

There is no standard protocol prescribing the number of cells to be transplanted for maximum effectiveness. Any such number will have to be derived for the particular cellular product and the tissue to be treated. However, given the low engraftment and survival rates of transplanted cells, in the past, large numbers of cells have been transferred into patients. Delivery of cells to certain anatomic locations may require novel procedures or novel delivery devices, and care needs to be taken not to disrupt the local environment (detailed review in ref. [8]). If injected intravenously in very high numbers, cells run the risk of clogging blood vessels and potentially causing pulmonary emboli or infarctions. Therefore, an initial safe dose regimen and dose escalations, based on animal data, must be used with careful consideration of the route of administration and the dose schedule.

In early phase MSC preclinical and clinical trials, safety of transplanted MSC was well documented in animal models and in human trials, but *in vivo* efficacy was controversial in humans [1]. While some MSC trials have shown clear efficacy, others have not achieved significant outcomes [55–59]. Mixed results from early MSC clinical trials may be due the lack of fundamental MSC biology and low engraftment efficiency, which can lead to low therapeutic efficacy [7, 8]. Recently, several preclinical studies have shown enhanced efficacy of MSC therapies by overexpression of trophic factors, preconditioning with hypoxic environment, and surface antigen modifications [60, 61]. Among others, our laboratory demonstrated that hypoxic preconditioned MSCs prior to transplantation improves their tissue regenerative potential in mice with hindlimb ischemia [60]. The recent work by Sackstein and colleagues improved homing efficiency of BM-MSCs into the bone; they chemically modified the MSC surface antigen CD44 and intravenously injected such manipulated MSCs into nonobese diabetic/SCID mice. Transplanted MSCs were found to have homed into the bone and showed sustained survival as compared to unmanipulated MSCs [62]. Further long-term *in vivo* studies must be conducted to assure that such surface antigen modifications are safe and can increase the homing and therapeutic

efficacy of transplanted MSCs, derived from both adult tissues and from pluripotent sources.

## CONCLUSION

hPSCs have enormous potential for regenerative medicine. Adult stem cell (HSC)-based therapies have demonstrated safety and efficacy for several decades, and adult MSC therapies are showing efficacy in some trials, with mixed results such as only transient effects in others due to poor cellular retention or other factors that need to be further optimized. Creation of large batches of iPSC-derived MSCs would allow full *in vivo* testing in preclinical studies. Although iPSCs might offer great hope for stem cell therapies, there are important safety issues to be considered before these cells are suitable for clinical trials. However, the potential future ability to generate stem cell lines matched to a particular patient, and to perform homologous gene correction or targeted transgene insertion into a safe harbor site in the genome prior to further expansion and differentiation offers amazing potential for future regenerative medicine therapies and is the goal of major research efforts worldwide.

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## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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## Concise Review: Cord Blood Banking, Transplantation and Induced Pluripotent Stem Cell: Success and Opportunities

MAHENDRA RAO,<sup>a</sup> LARS AHRlund-RICHTER,<sup>b</sup> DAN S. KAUFMAN<sup>c</sup>

<sup>a</sup>National Center for Regenerative Medicine, NIH, Bethesda, Maryland, USA; <sup>b</sup>Department of Women's and Children's Health, Karolinska Institutet, Stockholm, Sweden; <sup>c</sup>Department of Medicine and Stem Cell Institute, University of Minnesota, Minneapolis, Minnesota, USA

**Key Words.** Hematopoietic stem cell transplantation • Hematologic malignancies • Bone marrow • Cord blood

### ABSTRACT

Hematopoietic cell transplantation (HCT) has become a standard practice to treat a number of malignant and non-malignant hematologic diseases. Bone marrow, mobilized peripheral blood, and umbilical cord blood can all serve as primary sources of cells for HCT. The number of cord blood units currently stored is large, although it represents only a fraction of potential collections. With much of the collection being sequestered in private banks for possible autologous use, there is a reason to expect that public banks may not be able to provide for the demand in coming years as use of cord blood for treatment of patients with diseases such as leukemia and lymphoma continues to increase. We suggest that a possible solution to encourage private banks to share their valuable units is to apply

recent methodologies to generate induced pluripotent stem cells from cord cells and to optimize techniques to generate hematopoietic lineages from them. This strategy would allow us to take advantage of the units already collected under appropriate regulatory guidelines, to access a pristine cell that can be converted to a pluripotent cell at a much higher efficiency and in a shorter time period than other cells. The ability to potentially replenish a used cord unit with new cells, as well as extend the potential utility of cord blood for additional therapeutic applications, should allow banks to develop an appropriate business model for both private and public cord blood banks to flourish. *STEM CELLS* 2012;30:55–60

Disclosure of potential conflicts of interest is found at the end of this article.

### INTRODUCTION

Hematopoietic cell transplantation (HCT), also commonly referred to as bone marrow (BM) transplantation, was first performed successfully 40 years ago [1, 2]. Currently, 50,000 patients per year receive HCT typically to treat malignant diseases such as leukemia, lymphoma, or multiple myeloma [3], and there are now approximately 11 million human leukocyte antigen (HLA)-typed donors in international donor registries [4]. Despite the development of marrow registries, approximately one-third of patients who need an allogeneic HCT are currently unable to find an appropriate “adult” donor match.

Following the successful transplant of cord blood to treat Fanconi anemia in 1989 [5], umbilical cord blood (UCB) has emerged as an alternative rich source of hematopoietic stem cells [6, 7]. This has translated to a now rapidly developing medical field, described in several recent reviews [4, 8–10]. There have been more than 15,000 cord blood transplants worldwide by 2009, and in the United States, more than half of all stem cell transplants from unrelated donors in children now use cord blood (<http://www.nationalcordbloodprogram.org>).

The use of UCB for HCT provides some potential advantages compared with the use of BM or mobilized peripheral blood (PB). Advantages include prompt availability, decreased

risk of transmissible viral infections, reduced incidence of graft-versus-host disease (GVHD), and ease of collection with little to no risk to the mother or newborn [4, 11]. In contrast to BM or PB that generally require a high degree of HLA match between donor and patient [11, 12], UCB only needs to be matched at four of six HLA class I and II molecules. This reduced incidence of GVHD with partially HLA-mismatched UCB is likely due to the lower numbers of T cells and the relatively immunologically naïve status of the lymphocytes in units of UCB [11, 13].

Initial trials using UCB for HCT focused on pediatric patients for two main reasons. One logistic reason was that for the first clinical use of UCB, the donated unit was obtained from an HLA-matched sibling. Sibling donors are preferentially used for BM and PB HCT as complications are fewer and survival is improved compared with the use of unrelated allogeneic donors. Second, pediatric recipients are small, and there are enough hematopoietic stem cells (HSCs) (as measured by CD34<sup>+</sup> cells) in a single unit of UCB to engraft in a pediatric patient in acceptable amount of time to prevent complications. In contrast, in adults it took a month or more to engraft and resulted in significant morbidity and mortality [14–16].

To expand the utility of this rich source of HSC, considerable effort has been invested in developing methods to make

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Correspondence: Mahendra Rao, National Center for Regenerative Medicine, NIH, Bethesda, Maryland, USA.; e-mail: mahendra.rao@nih.gov Received June 30, 2011; accepted for publication October 13, 2011; first published online in *STEM CELLS EXPRESS* November 8, 2011. available online without subscription through the open access option. © AlphaMed Press 1066-5099/2011/\$30.00/0 doi: 10.1002/stem.770

UCB more suitable for adults. One pursuit has been to define conditions for *ex vivo* expansion of the HSCs in a unit of UCB so that more cells that provide long-term multilineage engraftment can be obtained. Most efforts to support expansion of UCB (or PB or BM) lead to production of hematopoietic progenitor cells that may provide some improved short-term engraftment of myeloid cell lineages [17, 18]. While this is potentially beneficial, studies to more effectively expand true HSCs capable of life-long engraftment remain a priority in hematopoiesis research and constitute an intriguing challenge for UCB stem cell biology [10].

A second approach to improve clinical use of UCB for adults, pioneered at the University of Minnesota, has been the infusion of two units of UCB to one patient. In current clinical studies, patients are given two units that are both at least four of six HLA-matched to the patient and each other. The combined cell dose allows substantially improved time-to-engraftment for adults compared with the use of a single UCB unit and this “double UCB transplants” (DUCBT) has been a remarkable clinical success [4, 15, 19, 20]. A recent clinical study of 536 adult patients with hematologic malignancies at the University of Minnesota and Fred Hutchinson Cancer Center (Seattle, WA) compared results of HCT using DUCBT, HLA-matched related donors, matched unrelated donors, or one-antigen mismatched unrelated donors as cell sources [20]. This analysis demonstrated that leukemia-free survival was similar for patients who received allogeneic cells from cord blood or adult donors. Indeed, risk of relapse was lower in the DUCBT patients.

In parallel to the increasing clinical use of UCB worldwide, an entire industry to collect and store UCB has developed. Two competing models have developed a public cord blood bank model supported by public funds akin to the blood bank and BM programs and a competing private cord banking business where commercial “private” entities offer to store UCB for use by a particular child or family. One recent report tallies 36 public, nonprofit UCB banks in 36 countries and at least 150 private, commercial UCB banks worldwide [21]. For a list of accredited public cord blood facilities worldwide, see <http://www.factwebsite.org/> and for the presently most comprehensive list of private cord blood bank sites worldwide, see <http://parentsguidecordblood.org/>. Despite the growth of the collection industry, total UCB collections represent less than 5% of potentially available cords and increasing donation rates by even a small percentage could significantly increase the number of available units.

### ISSUES OF PRIVATE AND PUBLIC UCB BANKING

Registries of potential allogeneic adult donors, as well as UCB units in public banks, have become instrumental to facilitate allogeneic HCT for patients who do not have a suitable HLA-matched related (typically sibling) donor. Considerable societal benefit is garnered from this UCB donation and public banking. Efforts in the United States to increase collection of UCB units have been partially supported by recent legislation such as the Stem Cell Therapeutic & Research Act of 2005. To date, access to UCB has generally not been a significant problem for patients who need this therapy. However, with the success of clinical trials using DUCBT for adults, including the use of reduced intensity conditioning to benefit older patients or those with comorbidities that make fully myeloablative conditioning too risky, the existing system of

UCB collection, storage, and distribution could become strained in the future.

Collection of donated UCB by public banks is in direct competition with private banks that have prospered by catering to parents and guardians who may wish to do everything for the future benefit of their children. Private UCB banks aggressively market the collection and storage of UCB to expectant parents, and these individuals have a high motivation to consider the opportunity to store UCB as offered by private cord blood banks rather than donate for the common good. These private banks charge a fee for processing the sample and its subsequent storage. In the process, private cord blood banks spend significantly to educate the public about HCT and establishing relationships with clinics to ensure a supply chain. Private cord blood banks have grown rapidly, although as would be expected collections are restricted to more affluent segments of society.

Indeed, the total number of cord units in private banks far exceeds the number preserved in public banks while the majority of units actually used for therapy come largely from the public banks. This is understandable as on an individual level, the probability of using the stored cord blood unit is relatively low (fortunately for the individual). By various estimates, the chance of an individual receiving his/her own UCB as treatment for one of many hematological disorders where HCT plays a role ranges from probabilities of 1:2,500–1:200,000 [21, 22], a more precise estimate being difficult to discern. This use may be slightly more frequent if donor cells were also available to family members, although it must be noted that a single unit of UCB is not typically sufficient or optimal for adult HCT. Moreover, it is possible that if a child develops a childhood malignancy that could be treated by UCB, there may be malignant cells in the UCB itself rendering the treatment ineffective [21–24]. Based on these calculations, the vast majority of cord blood units stored for autologous use in private UCB banks will not be used and may potentially be wasted. Furthermore, if a potential recipient of a stored autologous unit of UCB has grown to greater than approximately 50 kg, then two units of UCB will likely be needed to ensure prompt engraftment. Therefore, having one stored unit of UCB may be helpful, but this may not be sufficient for HCT.

Differences in the process of UCB collection and the failure to type samples by private cord blood banks make it difficult to search for unrelated HLA-matched donors in private banks or transfer units from private banks to public banks even if one wished to do so. Regulatory guidelines defining processes of transferring a family-bank stored product to a public bank do not exist. Given that samples frozen for autologous use (i.e., private/family banking) are not always comprehensively characterized (e.g., typed for HLA), transfers may prove difficult particularly for the majority of UCB units that are stored in single compartment bags where no sample can be removed for HLA typing or other characterization. Transfers of existing samples to public banks may require further testing to fulfill the proper requirements for nonautologous use. This will add costs for analysis and regulatory compliance and in many instances may not be feasible.

In addition, the recent changes in regulations suggest more regulatory burden than in the past, and there are significant concerns regarding the private banking model [21, 22]. The idea of private blood banking has sparked numerous ethical debates and a number of professional societies worldwide have issued statements/policies that address the conflicting interests between public and private UCB banking (reviewed in ref. [21]). Uniformly, these groups discourage the use of private UCB banking and encourage families to donate UCB to public UCB banks. Many of us in the field contend that



health choices should be egalitarian as has been espoused in the laws governing organ and marrow transplant, where selling organs is illegal. Private cord blood banking is likely to reduce the amount of sample availability in the public banks and appears to be encouraging clinicians involved in the collection process to endorse an unproven therapy. As samples themselves are unlikely to be used, banks have indulged in exaggerated or even false claims to promote the idea of banking. The situation is thus suboptimal from both ethical and commercial standpoints.

Nevertheless, private cord blood banks spur initiative and have to large extent pioneered storage and cryopreservation procedures and the establishment of collection facilities. Proponents point out that they have collected a large repository at considerable expense that may be of value to society and more importantly may provide insurance for people with the means to afford it. As with any private enterprise, free market forces are working to open up this use to an ever larger fraction of the population and make private banking an option for ever more individuals. It also appears that prohibiting parents from donating to private banks is not a reasonable approach.

### INDUCED PLURIPOTENT STEM CELL DERIVATION FROM UCB

The ability to reprogram any adult cell using defined factors was pioneered by Yamanaka and colleagues in 2006 [25] and the field has been seen extraordinary rapid progress and numerous novel breakthroughs, as recently reviewed [26–28]. Overall, the large number of independent publications and meta-analysis of the published data suggest that induced pluripotent stem cells (iPSCs) closely resemble ESC derived from blastocysts and that like ESC, iPSCs can contribute to the germline in chimeras in mice and that gene expression profile shows no greater variation than that seen among different ESC lines [29, 30]. Human iPSC as such may be functionally interchangeable with hESCs and like hESCs can generate diverse hematopoietic lineages [31–33]. These findings make human iPSCs potentially useful for novel hematopoietic and immune-based therapies as well as studies of genetic diseases that effect hematopoietic development [34].

The initial derivation of iPSC relied on using retrovirus and lentivirus transformation of cultured fibroblasts derived from patients. Since that initial description there have been many advances that have both reduced the possibility of deleterious effects of integration, persistent expression or reactivation of the inducing genes as well as increasing the efficiency of the induction process. These techniques range from using excisable all-in-one constructs (e.g., CRE-LOX flanked or piggyback or *Sleeping Beauty* transposon-based vectors), episomal vectors (plasmids, minicircles, and episomal viruses such as baculovirus or sendai), using RNA, protein, or small molecules that activate the specific pathways [27, 29, 30, 35]. Researchers have also examined the cell types that may be the most amenable to induction of pluripotency, and in general, it appears that most cell types can be induced to become pluripotent but the frequency and efficiency depend on the age of the sample (younger is usually better). Cell types that already express some of the pluripotency genes appear to be reprogrammed more efficiently or with fewer exogenous factors being required [27, 29, 30, 35].

Overall, the large number of independent publications and meta-analysis of the published data suggest that iPSCs superficially resemble ESC derived from blastocysts and that like ESC iPSCs can contribute to the germline in chimeras in

**Table 1.** Benefits to use UCB for iPSCs

1. Tissue sourcing is well organized and processes are validated.
2. HLA typing data are already being collected by public banks.
3. Existing samples can be used without compromising their ultimate use.
4. A remuneration model already exists.
5. Integration-free methods work well with cord blood cells.
6. A xenofree media and protocol has been developed so clinical-grade iPSC lines can be made.
7. Cord blood is the youngest source of stem cells one may obtain reliably and easily.
8. Stem cells in general have specific mechanisms to maintain genomic integrity, delay senescence, and protect against transformation.
9. The process can be easily extended to blood banks and marrow-derived CD34+ cells using the same infrastructure.
10. Somatic memory and differentiation bias may work in our favor for early therapeutic efforts.
11. May stimulate the cord blood banking business.

Abbreviations: HLA, human leukocyte antigen; iPSC, induced pluripotent stem cell.

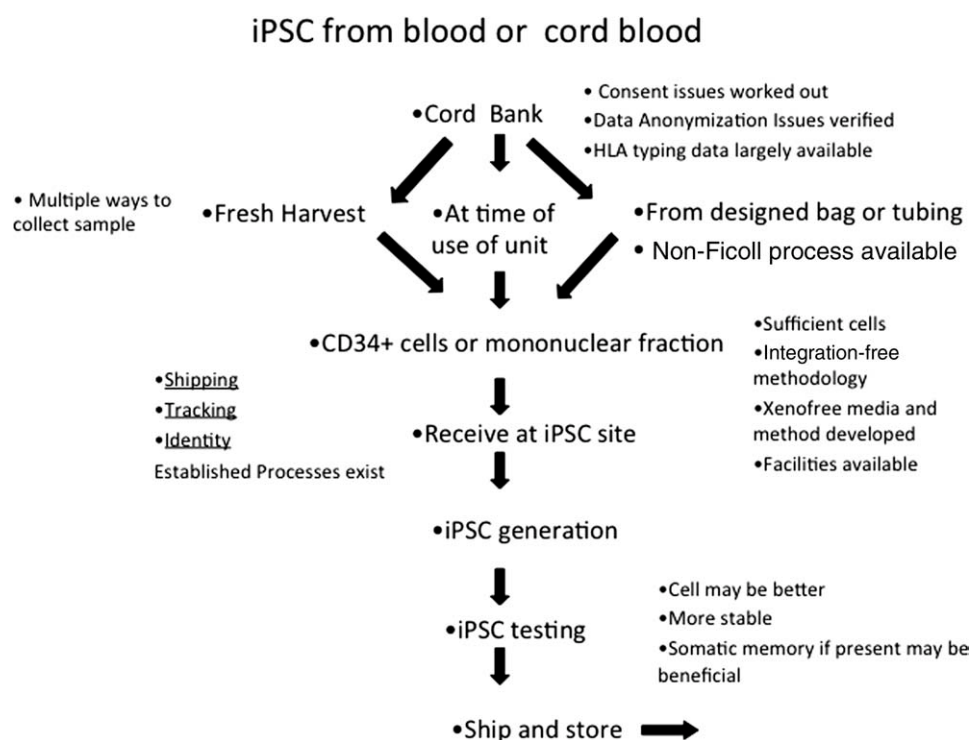
mice and that gene expression profile shows no greater variation than that seen among different ESC lines. iPSC as such may be functionally interchangeable with ESC even though more subtle differences may be present.

Investigators have suggested that such subtle difference and their consequence may not be detected in standard assays but may have profound consequences on long-term survival or behaviors of iPSC particularly after transplantation. These include issues such as long-term karyotypic stability, rate of accumulation of mutations, ability to maintain telomeric ends and protect against senescence, the activity of imprinted genes, and overall epigenomic profile, mitochondrial integrity and number, and differential immune response in transplantation assays. Despite possible subtle differences between ESC and iPSC, the relative ease with which iPSC can be obtained, the ability to prospectively identify a ideal donor, and the ability to make multiple lines from the same donor all make iPSCs occasionally more useful and often more practical to generate than ESC for many applications.

The availability of well-characterized, HLA-typed cells collected with appropriate consent coupled with recent breakthroughs in iPSC generation suggest that cord blood cells may represent a good source of cells for such an effort (Table 1). Recently, protocols for transformation of cord blood cells to iPSC have been reported by several groups [36–39].

Two important points are worth emphasizing. The efficiency of generating iPSC from cord blood-derived stem cells is not only higher but also faster, as the absolute number of cells that can be obtained from a small fraction of the cord blood aliquot far exceeds what is required for a cell line generation. In addition, if good patient history is available and if consent forms are well written as is common with all blood bank registries it is also possible to collect additional cells or additional data for further follow-up.

On a practical level, it is likely that the residual blood that is present in the tubing at the time of collection contains sufficient amount of cells to be frozen separately so the entire sample need not be thawed for this purpose. Of course, a small aliquot of cord blood could also easily be frozen separately at the time of collection, or iPSC generation could be planned at the time of use of a cord blood unit. These options provide a system to generate iPSCs that can be included



**Figure 1.** A hypothetical process of iPSC generation from cord or marrow cells is diagrammed. Note that cord blood samples for iPSC generation could be collected at different time points and thus would not affect cord blood processing for CD34<sup>+</sup> cells. Abbreviations: HLA, human leukocyte antigen; iPSC, induced pluripotent stem cell.

readily in the workflow without any major changes to current processes (see Fig. 1).

iPSC generation also may allow private blood banks to be able to share their samples without compromising on the contracts they have entered into with the donors who pay to have their samples stored for their own use. It may also allow private blood banks to consider additional sources of revenue and increase the use of their stored sample which in turn is likely to increase the number of potential donors willing to store samples. Current penetration rate of cord blood storage is under 5% of total possible collections.

One can imagine a workflow process where cord blood is shipped to a facility, a small aliquot is removed, and iPSC lines are generated at the same site using a zero-footprint process such as plasmids, or minicircles or Sendai virus (each of which can be manufactured using a GMP qualified process) and stored in a regulated environment, thawed, and then used when required. Alternatively, a small sample is separated from an existing stored unit when there is need for such a specific HLA-typed sample and this is then processed to generate an iPSC line for potential therapeutic use or when a cord blood unit is shipped for use a small sample is retained and iPSC lines are made to provide a replenishment for the used unit. Other methods of integration-free iPSC derivation also exist. These include protein-based methods, use of synthetic RNA, conditioned media, and excisable vectors (reviewed in ref. [41]). The efficiency of these methods using cord blood as a starting material remains to be determined.

The incentive for private cord blood banks is obvious. The procedure allows them to address the ethical issues of private storage by making available cells for the public good. It provides them with additional incentive to expand their storage efforts and extend the potential utility of their stored samples to treat a variety of additional conditions as pluripo-

tent cells can theoretically be used to treat many more conditions than cord blood cells alone. Furthermore, pluripotent cells represent an insurance value if you will as technical advances suggest that one may be able to replace the UCB unit used for therapy with HSC derived from the pluripotent cells generated from that unit [34, 40, 41].

Likewise, the incentive for public banks is clear. The ability to make iPSC from UCB will allow them to recover some additional costs and enhance the utility of the samples collected and allow them access to the samples stored by private banks as well. Given that samples have been stored for many years, prospective history (from time of collection) available from donors may allow for an added layer or selection criteria that is unavailable with other samples used for iPSC generation.

## SUMMARY

Clinical use of UCB has markedly increased over the past several years and this has led to the development of an entire banking industry. Scientific evidence has suggested that these cells are not only an alternative to BM but also perhaps even superior in some applications. The utility of UCB has been expanded by the findings that multiple units can be used, thus circumventing problems of small volume and HSC (CD34<sup>+</sup> cell) quantity. Studies to show that additional stem cell populations, in addition to hematopoietic stem/progenitor cells, may be present in UCB suggest that the future use of cord blood may be even greater in the future. For these reasons, it is important to advocate banking of UCB. As the field has evolved, there has been increasing controversy between public and private banking but we would argue that this controversy

overshadow the potential utility of this source of HSCs. Rather one should consider ways to make these stored units more widely accessible.

Developing additional uses of stored and freshly collected cord blood cells would represent an incremental cost for a potentially huge benefit. We propose that generating iPSC from cord blood has many advantages over conventional methods and that one can leverage the infrastructure of the cord blood banking industry to accelerate the transition to therapy. Wider collection of cord blood and targeted collection of marrow stem cells from individuals with appropriate genetic profiles will allow one to generate banks of iPSC customized for large-scale screening and therapeutic purposes. Equally important, providing private cord blood banks with an incentive to offer an opportunity to make a limited resource collected for the use of one individual more widely available will reduce the ethical controversy that has surrounded private cord blood banking. We hope this article stimulates conversation and perhaps leads to enterprising groups demonstrating the viability of this concept. Preemptive planning by existing private banks, for HLA typing, coordination of storage requirements between public and private banks, and working with the regulatory authorities to devise

appropriate storage and banking processes to ensure regulatory compatibility would go a long way to resolve some of these practical issues related to implementing such a program.

## ACKNOWLEDGMENTS

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## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest. Dr. Rao was a full-time employee of Life technology which supplies tools and reagents to stem cells scientists. Dr. Lars Ahrlund-Richter is a part-time employee of a biotechnology company focused on preclinical development of anti-cancer drugs. Dr. Kaufman participates in hematopoietic and cord blood transplant studies.

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## Concise Review: A Chemical Approach to Control Cell Fate and Function

WENLIN LI,<sup>a</sup> KAI JIANG,<sup>b,c</sup> SHENG DING<sup>b,c</sup>

<sup>a</sup>Department of Chemistry, The Scripps Research Institute, San Diego, California, USA; <sup>b</sup>Gladstone Institute of Cardiovascular Disease and <sup>c</sup>Department of Pharmaceutical Chemistry, University of California, San Francisco, California, USA

**Key Words.** Stem cells • Small molecules • Differentiation • Self-renewal • Reprogramming

### ABSTRACT

Stem cells are essential for maintaining tissue homeostasis and mediating physiological and pathological regeneration. Recent breakthroughs in stem cell biology have generated tremendous enthusiasm and hope for the therapeutic potential of stem cells in regenerative medicine. However, this research is still in an early development stage. An improved understanding of stem cell biology is required to precisely manipulate stem cell fate and to harness these cells for regenerative medicine. Small molecules, targeting specific signaling pathways and mechanisms, are powerful

tools for manipulating stem cells for desired outcomes. Those small molecules are increasingly important in probing the fundamental mechanisms of stem cell biology and facilitating the development of therapeutic approaches for regenerative medicine. These could involve cell replacement therapies with homogenous functional cells produced under chemically defined conditions in vitro and the development of small-molecule drugs that modulate patient's endogenous cells for therapeutic benefit. *STEM CELLS* 2012;30:61–68

Disclosure of potential conflicts of interest is found at the end of this article.

### INTRODUCTION

Stem cells possess two fundamental characteristics: they can self-renew themselves and can differentiate into an array of specific cell types. They have essential roles in generating the hierarchical cellular lineages during development, maintaining tissue homeostasis, and mediating physiological/pathological regeneration in adults. These properties and functions make stem cells excellent model systems to study the basic biology of human development and tissue homeostasis and also offer significant promise for developing treatments for devastating human diseases and injuries.

However, before we can realize the promise, several obstacles must be overcome. For example, renewable sources of stem cells must be developed for any therapeutic applications. Although significant progress has been made in maintaining embryonic stem cells (ESCs) when compared to the past decades, many substantial challenges remain in isolating and expanding most tissue-specific adult stem cells. To fully harness their clinical potential, functional expansion of these therapeutically valuable adult stem cells is needed. In addition, although ESCs can self-renew infinitely and generate any cell types under appropriate conditions, they are prone to cause teratomas and cannot be directly used to repopulate host tissues in vivo before they differentiate into tissue-spe-

cific cells. Great efforts are still required to improve our ability to coax stem cells, especially the ESCs, into the desired developmental stages (e.g., lineage-specific stem/progenitor cells) or functional cells for disease therapy.

Small molecules, modulating specific target(s) in the signaling pathways or epigenetic mechanisms, are emerging as valuable tools with distinct advantages for manipulating stem cell fates [1, 2]. For example, regulating protein functions is much easier with small molecules than by genetic manipulation. Importantly, the effects of small molecules are typically rapid and reversible and can be fine tuned by varying concentrations and combinations of small molecules. These characteristics provide temporal and flexible regulation of complex signaling networks. In addition, virtually unlimited structure and functionality diversity endowed by synthetic chemistry provide small molecules with theoretically unlimited potential for precisely controlling cell phenotypes, which could be extensively explored by phenotype-based high-throughput screening.

As a nascent field, stem cell research will continue to benefit from its crossover with chemistry. In this review, we discuss the new developments of chemical approaches to stem cell biology and regenerative medicine. The examples are not intended to be comprehensive. Rather, we want to emphasize the conceptual points, current challenges, and potential opportunities for this emerging research field.

Author contributions: W.L. and K.J.: manuscript writing, S.D.: manuscript writing and conception and design.

Correspondence: Sheng Ding, Ph.D., Gladstone Institute of Cardiovascular Disease and Department of Pharmaceutical Chemistry, University of California, San Francisco, 1650 Owens Street, San Francisco, California 94158, USA. Telephone: 415-734-2717, Fax: 415-355-0141; e-mail: sheng.ding@gladstone.ucsf.edu Received June 23, 2011; accepted for publication September 29, 2011; first published online in *STEM CELLS EXPRESS* October 25, 2011. available online without subscription through the open access option. © AlphaMed Press 1066-5099/2011/\$30.00/0 doi: 10.1002/stem.768

## STEM CELL SELF-RENEWAL

The derivation of ESCs from mice and subsequently from human and other species represents one of the major milestones in genetics, developmental biology, and human biomedical research [3–5]. Extensive efforts have been made to develop better ways to maintain self-renewal of these versatile cells.

Supporting ESC self-renewal with small molecules under chemically defined conditions has particular advantages. By using green fluorescent protein (GFP) expression under control of the *Oct4* promoter as a primary indicator of pluripotency, we screened synthetic small-molecule libraries under chemically defined conditions in the absence of feeder cells, serum, and leukemia inhibitory factor (LIF) [6]. A novel compound, pluripotin/SC1 (Table 1; S1), was identified that maintains long-term self-renewal and germline competence of mouse ESCs (mESCs) in vitro by dual inhibition of two endogenously expressed differentiation-inducing proteins, RasGTPase activating protein and extracellular signal-regulated kinase-1 (ERK1) [6]. This proof-of-concept study demonstrated that modulators of stem cell fate can be identified by carefully designed phenotypic screens. More importantly, the fact that pluripotin maintains ESC self-renewal, independent of the exogenous activation of conventional self-renewal pathways, by simply inhibiting the activity of endogenous differentiation-inducing proteins has provided a fundamental new view on the mechanism of ESC self-renewal. Thus, ESCs have an intrinsic ability to maintain pluripotency and do not require exogenous stimulation. A more recent study supports this conceptual advance. A combination of specific chemical inhibitors (CHIR99021 and PD0325901, Table 1; S2 and S3) of glycogen synthase kinase-3 (GSK3) and mitogen-activated protein kinase/ERK kinase (MEK) similarly supported the derivation and long-term self-renewal/germline competence of mESCs in the absence of exogenous cytokines [7]. Those small molecules provide a platform for generating pluripotent cell lines from refractory mouse strains or other species, for example, pluripotent cell lines from nonobese diabetes/severe combined immunodeficiency (NOD-SCID) and SCID beige mice [8, 9], and rats [10–13]. Notably, these small molecules are also used to capture the naïve, mESC-like human pluripotent stem cells (hPSCs). Conventional human ESCs (hESCs) correspond very closely to epiblast stem cells, which are derived from the postimplantation egg cylinder-stage epiblasts of mouse [14, 15], and display very different gene expression and signaling dependency for self-renewal/differentiation from mESCs, which are derived from inner cell mass of preimplantation blastocysts. For example, both LIF and bone morphogenetic protein 4 (BMP4) are typically used for maintaining the pluripotency of mESCs [16, 17]. Also, inhibition of the MEK-ERK pathway promotes mESC self-renewal [18]. In contrast, hESCs typically depend on basic fibroblast growth factor (bFGF) and activin A for long-term self-renewal, and LIF does not promote hESC self-renewal [19], and BMP4 induces differentiation of hESCs [20]. By combining genetic reprogramming and cell signaling modulation by small molecules that favor the naïve pluripotent state, mESC-like human induced PSCs (m-hiPSCs) were generated from human fibroblasts by expressing reprogramming factors in culture medium that contains human LIF [10]. m-hiPSCs form small domed colonies and display stable long-term self-renewal when cultured in the presence of three chemicals, PD0325901, A-83-01, and CHIR99021. A-83-01 is a small-molecule inhibitor of the transforming growth factor  $\beta$  (TGF $\beta$ )/activin receptors (Table 1; S4). Recently, another study reported that hESCs

could be stably maintained under the combination of bFGF, CHIR99021 and PD0325901 [21]. However, the cells cultured under this condition seemed to resemble the conventional hESCs. This study also showed that undifferentiated hESCs were maintained only under low concentration of CHIR99021, and hESCs would differentiate at higher concentrations of CHIR99021. This could be due to specificity of CHIR99021 and/or dosage effect of signaling pathway modulation. Such differential dose-dependent effects are not uncommon for both small molecules and growth factors/cytokines. Nevertheless, considerations on small molecule's specificity must be taken when interpreting their affected biological phenotype and mechanism. For a more thorough overview of small molecules that maintain ESC self-renewal, readers are encouraged to examine comprehensive reviews on the topic [1, 22–24].

In contrast to robust ESC self-renewal conditions, long-term self-renewal of tissue-specific stem cells remains challenging. Here, we discuss new developments and possible strategies for expanding tissue-specific stem cells, which are directly applicable to regenerative medicine. Although tissue-specific stem cells exist in many adult tissues, and many of them have considerable self-renewing capacity under physiologically or pathologically regenerative conditions, it is technically challenging to expand most types of tissue-specific stem cells *ex vivo*. These challenges might reflect currently limited understanding of the extremely complex *in vivo* stem cell microenvironment. Before thorough dissection of the mechanisms for stem cell microenvironment, which is essential to rationally devise appropriate conditions for stem cell self-renewal, phenotypic screening (e.g., using the expression of stem cell markers as readout) of small-molecule libraries represents a fertile approach to identify the conditions that expand tissue-specific stem cells.

Using expression of CD34 as a readout to screen small-molecule libraries, Boitano et al. [25] identified a purine derivative, SR1 (Table 1; S5), that promotes *ex vivo* expansion of primary CD34-positive hematopoietic stem cells (HSCs) from human cord blood. Treatment with SR1 led to a 50-fold expansion of CD34-positive cells and a 17-fold increase in cells that functionally repopulated the hematopoietic system of NOD/SCID mice. Follow-up studies showed that SR1 promotes HSC expansion by directly antagonizing the aryl hydrocarbon receptor, representing a new mechanism to modulate HSC expansion. This study again reinforces the notion that unbiased phenotypic screen is useful to probe novel mechanisms for controlling stem cell fate.

Because of limited donor cell sources and often invasive nature of cell isolation from adults, an alternative approach to obtain tissue-specific stem cells is to differentiate PSCs (e.g., ESCs) that have unlimited supplies. Similarly, capturing and stably expanding hESC-derived tissue-specific stem/progenitor cell types remain a significant challenge for translating hESCs toward various *in vitro* and therapeutic applications. Recently, we identified novel combinations of small molecules for either inducing or expanding primitive neural stem cells (pNSCs) from hESCs in culture [26]. We found that synergistic inhibition of GSK3, TGF $\beta$ , and Notch signaling pathways by small molecules efficiently converted monolayer-cultured hESCs into homogenous primitive neuroepithelium within 1 week under chemically defined conditions. Importantly, these pNSCs represent the prerosette stage neuroepithelia and stably self-renew in the presence of LIF, GSK3 inhibitor (CHIR99021) and TGF $\beta$  receptor inhibitor (SB431542, Table 1; S6), which are distinct from previously identified neural precursor cells that typically depend on bFGF and epidermal growth factor (EGF) as mitogens. Most remarkably, after long-term passages under the small molecule condition, these pNSCs maintain highly

**Table 1.** Structures of small-molecular modulators of stem cell fate

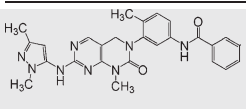
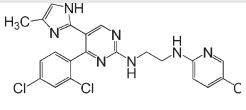
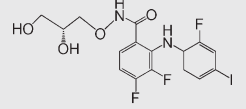
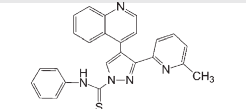
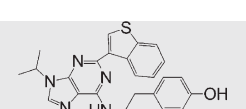
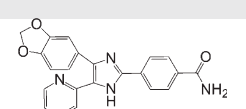
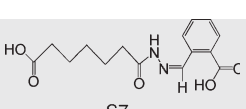
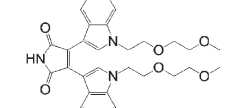
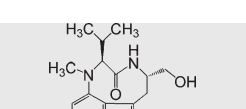
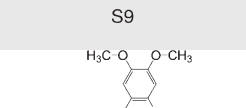
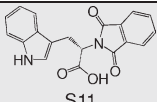
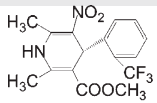
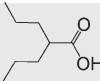
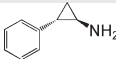
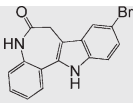
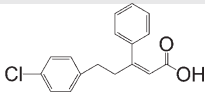
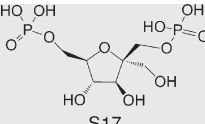
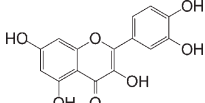
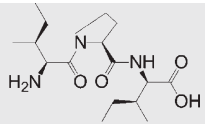
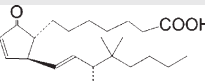
Molecule	Name	Target(s)/function	Effect(s)
 <p>S1</p>	Pluripotin/SC1	RasGAP and ERK1 inhibition	Supports mESC self-renewal
 <p>S2</p>	CHIR99021	GSK3 inhibition	Supports mESC self-renewal; promotes reprogramming
 <p>S3</p>	PD0325901	MEK inhibition	Blocks differentiation pathway of mESCs
 <p>S4</p>	A-83-01	ALK5, ALK4, and ALK7 inhibition	Supports rat iPSC long-term self-renewal when combined with CHIR99021 and PD0325901
 <p>S5</p>	SR1	Aryl hydrocarbon receptor inhibition	Promotes ex vivo expansion of CD34-positive human hematopoietic stem and progenitor cells
 <p>S6</p>	SB431542	ALK5, ALK4, and ALK7 inhibition	Promotes reprogramming; induces neural differentiation of hESCs together with Noggin
 <p>S7</p>	IDE1	Unknown	Induces endoderm differentiation of ESCs
 <p>S8</p>	1m	GSK3 inhibition	Induces endoderm differentiation of ESCs
 <p>S9</p>	(-)-Indolactam V	PKC activation	Enhances the pancreatic differentiation of ESC-derived definitive endoderm
 <p>S10</p>	BIX-01294	G9a histone methyltransferase inhibition	Promotes reprogramming of NPCs and MEFs transduced by Oct4/Klf4

TABLE 1. (Continued).

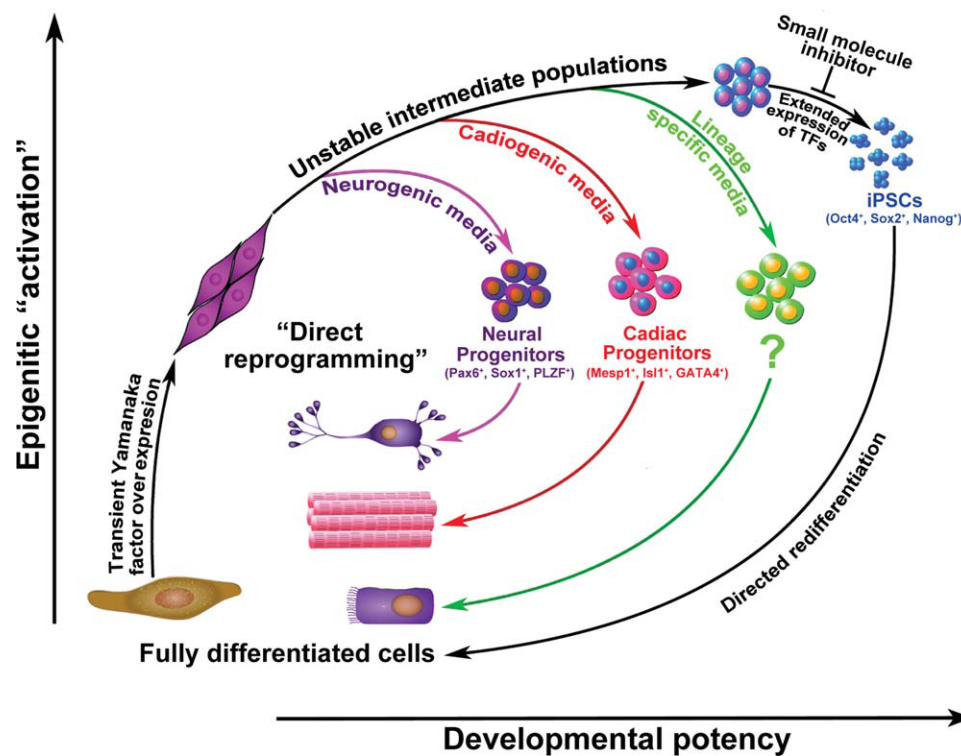
Molecule	Name	Target(s)/function	Effect(s)
 S11	RG-108	DNA methyltransferase inhibition	Promotes MEF reprogramming
 S12	(+)Bayk 8644	L-type Ca <sup>2+</sup> channel agonism	Promotes MEF reprogramming
 S13	Valproic acid	Histone deacetylase inhibition	Promotes reprogramming, replaces Klf4 to induce human fibroblast reprogramming
 S14	Parnate	Lysine-specific demethylase 1 inhibition	Enables reprogramming of human keratinocytes transduced with Oct4/Klf4
 S15	Kenpauillone	CDKs and GSK3 inhibition	Replaces Klf4 to induce MEF reprogramming
 S16	PS48	3'-Phosphoinositide-dependent kinase-1 activation	Enables reprogramming of human cells transduced with Oct4 together with sodium butyrate, A-83-01 and PD0325901
 S17	Fructose 2,6-bisphosphate	Phosphofructokinase 1 activation	Promotes human primary cell reprogramming
 S18	Quercetin	Hypoxia-inducible factor-1 activation	Promotes human primary cell reprogramming
 S19	Diprotin A	Dipeptidylpeptidase inhibition	Enhances recruitment of CXCR4 positive circulating endothelial progenitors to myocardium
 S20	dmPGE2	PGEs pathway activation	Enhances HSCs homing <i>in vivo</i>

Abbreviations: ALK, activin receptor-like kinase; CDK, cyclin-dependent kinase; CXCR4, C-X-C chemokine receptor type 4; dmPGE2, 16,16-dimethyl-PGE2; ERK1, extracellular signal-regulated kinase-1; GAP, GTPase activating protein; GSK3, glycogen synthase kinase-3; hESCs, human embryonic stem cells; HSC, hematopoietic stem cells; IDE, iPSC, induced pluripotent stem cells; MEF, mouse embryonic fibroblast; MEK, mitogen-activated protein kinase/ERK kinase; mESC, mouse embryonic stem cells; NPC, neural progenitor cells; PGE2, prostaglandin E2; PKC, protein kinase C.

neurogenic differentiation propensity, remain plastic to instructive regional patterning cues toward midbrain and hindbrain neuronal subtypes, and exhibit *in vivo* functions. This study

provided a “check-point” strategy to get around the issues that the typical hESC differentiation is a nonstop process and impurities of differentiated cells from each step of





**Figure 1.** The model of direct reprogramming. Transient overexpression of reprogramming factors in fibroblasts leads to the rapid generation of epigenetically activated cells (unstable intermediate populations), which can then be coaxed to relax back into various differentiated state(s), ultimately giving rise to fully differentiated cells entirely distinct from the starting population. Aside from restricting iPSC formation by drastically limiting Yamanaka factor expression, the reprogramming process can be made to overwhelmingly favor transdifferentiation by using small-molecule modulators of signaling, for example, Janus Kinase inhibitor that prevents the establishment and maintenance of pluripotency. Using empirically determined media and culture conditions, neural, cardiac, and possibly other lineage-specific cells can be obtained. Importantly, progenitor populations belonging to these lineages are generated in the process and can perhaps be isolated. Abbreviations: iPSCs, induced pluripotent stem cells; TFs, transcription factors.

differentiation are carried over leading to low efficiency and significant heterogeneity in terminally differentiated cells.

## STEM CELL DIFFERENTIATION

Typically, applications based on hPSCs (e.g., ESCs or iPSCs) require their *in vitro* differentiation into a desirable cell population. Although significant progress has been made over the years on ESC differentiation into a wide variety of cell types [27], we focus here on some of the existing challenges and newly developed strategies by applying small molecules. In addition to the strategy of capturing and maintaining the intermediate stem/progenitor cells during hPSC differentiation discussed above, substantial efforts are highly desirable to more efficiently induce hPSC differentiation in a homogenous manner under chemically defined conditions.

Chemical approaches have been particularly useful for accelerating differentiation process, increasing differentiation efficiency, and normalizing different differentiation propensity of diverse hPSC lines [28]. Based on known mechanisms of neural development and hESC differentiation, Chambers et al. [29] developed an efficient neural induction method for hPSCs that bypasses the conventional embryoid body formation. They found that combination of Noggin (a natural BMP antagonist) and SB431542 (TGF $\beta$  receptor inhibitor) promotes rapid neural induction of more than 80% of hESCs in a monolayer fashion. Those two signaling pathway inhibitors appear to function synergistically to destabilize self-renewal

of hESCs (e.g., TGF $\beta$  signaling is essential for self-renewal of hESCs), promote neural induction, and prevent cells from differentiating into trophectoderm, mesoderm, and endoderm lineages (for which BMP and TGF $\beta$  signaling have an inductive effect). This study suggested that directed PSC differentiation toward a specific lineage can be achieved by deliberately combining the inductive signals for the desired cell lineage and the inhibitory signals blocking PSC self-renewal and their differentiation toward undesired lineages.

Recent efforts have also focused on discovery approaches to identify small molecules for certain steps during ESC differentiation toward specific lineage. Using mESCs stably transfected with the dTomato reporter gene under the control of the *Sox17* promoter, Borowiak et al. screened a collection of 4,000 compounds for small molecules that could induce definitive endoderm (DE) induction in the absence of activin A (a typically used DE inducer). Two structurally similar small molecules, IDE1 (Table 1; S7) and IDE2, were found to induce DE differentiation in up to 80% of mESCs (or 50% of hESCs) in the absence of activin A [30]. Similar to activin A, both IDE1 and IDE2 induce Smad2 phosphorylation in mESCs, while their targets remain unknown. However, IDE1 and IDE2 seem to share some properties with Im (Table 1; S8), a GSK3 inhibitor that can transiently upregulate NODAL expression and induce DE from hESCs under chemically defined condition [31]. The endoderm-like cells induced by IDE1 and IDE2 were shown to have the ability to differentiate into pancreatic lineage when they were subsequently treated with another small molecule, Indolactam V (Table 1; S9), which was identified in a separate screen for small molecules that can induce Pdx1

expression from hESC-derived DE cells [32]. Indolactam V is an activator of protein kinase C (PKC), revealing a potential role of PKC during pancreatic development.

## REPROGRAMMING

iPSCs generated from somatic cells by overexpression of defined transcription factors have attracted enormous interest [33, 34]. The simplicity of such genetic reprogramming approach has opened up unprecedented opportunities to generate patient-specific cells for disease modeling and potential therapeutic applications without the controversies associated with hESCs. However, there are critical concerns that the genetic technique initially used to generate iPSCs might result in genome modifications by oncogenes and potentially harmful genetic and epigenetic alterations in target cells. Some key advances toward overcoming these safety concerns have been achieved with nonintegrating gene delivery methods [35–37], using cell penetrating recombinant proteins or repeated transfection of synthetic reprogramming mRNAs [38–40]. Nevertheless, new methods for generating iPSCs with better qualities (e.g., as identical to ESC as possible) through improved efficiency and specificity in the process are highly desirable.

An alternative method to using transcription factors is to activate endogenous reprogramming mechanisms through small molecules that not only can provide a better nongenetic reprogramming approach but also ultimately will fundamentally change the reprogramming (toward a directed and specific process). We and others have identified small molecules with various mechanisms of action that can exert powerful effects on enhancing reprogramming and replacing transcriptional factors [22]. Using formation of compact colonies that express GFP under the control of Oct4 promoter as a readout, we first screened chemical collections in neural progenitor cells for reprogramming small molecules and identified a small-molecule inhibitor of G9a histone methyltransferase, BIX-01294 (Table 1; S10), that can substitute Oct4 and significantly improve reprogramming efficiency [41]. It was further demonstrated that BIX-01294 can also enable the reprogramming of mouse embryonic fibroblasts (MEFs) into iPSCs in the absence of Sox2 expression by only two exogenous factors Oct4 and Klf4 [42]. A subsequent chemical screen in fibroblasts with BIX-01294 identified a DNA methyltransferase inhibitor, RG108 (Table 1; S11), and a L-type calcium channel agonist, BayK8644 (Table 1; S12), that can work synergistically with BIX-01294 to increase reprogramming efficiency [42]. Consistent with epigenetic mechanisms in reprogramming, several studies also showed other commonly used, small-molecule inhibitors of epigenetic enzymes, including histone deacetylase inhibitors (e.g., valproic acid, Table 1; S13) could improve mouse and human somatic cell reprogramming [43–45]. In particular, valproic acid enabled reprogramming of human fibroblasts into iPSCs with only two factors (Oct4 and Sox2) [44] and MEF reprogramming with recombinant cell-penetrating reprogramming proteins [39]. In addition to these direct epigenetic modulators, we found in another study that GSK3 inhibitor CHIR99021 can facilitate reprogramming of MEFs by only Oct4 and Klf4. When combined with Parnate (Table 1; S14), a lysine-specific demethylase 1 inhibitor, CHIR99021 could enable reprogramming of human primary keratinocytes by only Oct4 and Klf4 [46]. Lyssiotis et al. [47] identified another GSK3 inhibitor, kenpaullone (Table 1; S15), that can replace Klf4 in reprogramming MEFs transduced with Oct4, Sox2, and c-Myc. However, because kenpaullone was shown to inhibit various other kinases, its mechanism in facilitating reprogramming remains elusive. Through a hypothesis-driven study, we

found that the dual inhibition of MEK and TGF $\beta$  by PD0325901 and SB431542 dramatically improved (>100-fold) the generation of iPSCs from human fibroblasts within 7 days of treatment (14 days of transfection) with an efficiency of >1% by enhancing mesenchymal–epithelial transition [48]. Other concurrent studies showed that TGF $\beta$  receptor inhibitors can replace Sox2 in MEF reprogramming [49, 50].

Notably, we recently identified a small-molecule activator of 3'-phosphoinositide-dependent kinase-1 PS48 (Table 1; S16) that enabled the reprogramming of human primary cells transduced with only Oct4 when combined with sodium butyrate (a histone deacetylase inhibitor), A-83-01, and PD0325901 [51]. In-depth mechanistic studies revealed that PS48 acts at the early phase of reprogramming at least in part by inducing a metabolic switch from mitochondrial oxidation (differentially used by adult somatic cells) to glycolysis (almost exclusively used by PSCs) during the reprogramming process. It was further demonstrated that additional small molecules that promote glycolytic metabolism also enhance reprogramming, including fructose 2,6-bisphosphate (Table 1; S17) (an activator of phosphofructokinase 1, a key rate-limiting enzyme of glycolysis), and *N*-oxaloylglycine and Quercetin (Table 1; S18) (both stimulate glycolytic genes by activating hypoxia-inducible factor-1). In contrast, a specific glycolysis inhibitor (2-deoxy-D-glucose) inhibits reprogramming without altering cell proliferation, which can be potentially used for eliminating undifferentiated PSCs from their differentiation culture. This study suggested that metabolism modulation represents another fundamental mechanism in somatic cell reprogramming, in addition to other direct epigenetic and signaling mechanisms.

Induced pluripotency is established in a stepwise and stochastic fashion [52, 53]. Only a rare subset among various intermediate cells finally becomes pluripotent under extended expression of reprogramming factors and favorable culture conditions. We reasoned that it might be possible to guide those initially epigenetically unstable cells (induced by the iPSC-reprogramming factors) into lineage-specific cell types under favorable condition without traversing pluripotency (Fig. 1). We found that through temporally restricting ectopic overexpression of iPSC factors in fibroblasts, epigenetically “activated” cells could be generated rapidly, which can then be coaxed to “relax” back into certain differentiated state by each specific culture conditions (that favor lineage-specific cell types and simultaneously inhibit the establishment of pluripotency), ultimately giving rise to somatic cells entirely distinct from the starting population. For example, we found that with as little as 4 days of the iPSC-factor expression (far shorter than what is required for induction of pluripotency), MEFs can be directly reprogrammed to spontaneously contracting cardiomyocytes over a period of 11–12 days under the treatments with a small molecule Janus Kinase (JAK) inhibitor for the first 9 days (that blocks establishment of pluripotency by inhibiting the LIF signaling) [54], and BMP4 from day 9 onward (that mediates cardiac mesoderm induction). Interestingly, extending JAK inhibitor treatment beyond 9 days to overlap with BMP4 treatment was detrimental for the induction of cardiomyocytes. This observation is consistent with previously reported requirement for JAK/signal transducer and activator of transcription signaling in cardiomyogenesis [55, 56]. Applying the same concept and approach, neural and definitive endodermal cells were directly reprogrammed from fibroblasts rapidly and efficiently using transient expression of iPSC factors and treatments with FGFs/EGF (toward neural cells) [57] or activin A (toward definitive endodermal cells) (Fig. 1). In comparison to transdifferentiation using overexpression of tissue-specific transcription factors [58, 59], our iPSC-factor-based transdifferentiation paradigm has a number of advantages: it is a single combination of transcription factors that is applicable to induce reprogramming

toward various lineage-specific cell types; its transient expression could be more easily replaced by nonintegrating or nongenetic methods; and most significantly, progenitor populations belonging to these lineages are generated in the process, which can be isolated and expanded for various applications [57]. Such direct reprogramming to proliferating progenitors will dramatically increase the utility of this transdifferentiation paradigm.

It is worthy to note that the functions/effects of many small molecules discussed could be highly dependent on the specific culture conditions. Various elements, including the presence of undefined supplements (such as serum) or even the protein concentration in culture media, could have impact on the effectiveness of small molecules in a specific context.

### SMALL MOLECULE THERAPEUTICS MODULATING STEM CELLS

Chemical approaches could facilitate translation of stem cell research into clinical applications in at least two ways. First, as mentioned above, chemical approaches could provide robust tools to precisely manipulate stem cell fate and function in vitro to generate sufficient number of safe, homogenous, and functional cells for cell therapy. However, development and manufacture of cell-based therapy typically are more complex and such therapy also costs more for patients than conventional small-molecule and protein therapeutics. Many issues in cell-based therapy even with transplantable cells, including immune-related ones, cell homing, engraftment, and long-term maintenance of transplanted cells' functions in the target tissue remain challenging. Alternatively, chemical approaches also offer a complementary strategy by directly modulating endogenous tissue-specific stem/progenitor (or even more differentiated) cells in vivo for therapeutic benefits.

Recently, Zaruba et al. [60] described a small-molecule-based regenerative strategy for myocardial infarction by enhancing the recruitment of endogenous bone marrow stem/progenitor cells to the heart through inhibition of CD26/dipeptidylpeptidase IV in vivo via a chemical compound, ultimately increasing the formation of new blood vessels and improving heart functions. In ischemic heart tissue, stromal cell-derived factor 1 $\alpha$  (SDF-1 $\alpha$ ) is the major chemokine attracting endogenous endothelial progenitors expressing SDF-1 $\alpha$  receptor (C-X-C chemokine receptor type 4, CXCR4) homing to the heart. However, SDF-1 $\alpha$  is sensitive to a number of protease (including CD26) cleavages. The authors demonstrated that combined administration of granulocyte-colony stimulating factor (functions to mobilize stem/progenitor cells, including endothelial progenitors, from bone marrow) and a CD26 inhibitor Diprotin A (Table 1; S19) intraperitoneally enhanced recruitment of CXCR4-positive stem/progenitor cells to myocardium and improved myocardial function by increasing neovascularization, leading to increased animal survival. This study represents an excellent example of using small molecule in vivo to modulate endogenous stem/progenitor cells behavior (i.e., homing to injury site) for tissue repair. Similar strategies might entail modulation of endogenous stem/progenitor cell fate (e.g., survival, expansion, differentiation, and reprogramming), behavior (e.g., migration and niche interac-

tions), and state/function (e.g., quiescence and polarization) by small-molecule and/or protein therapeutics to achieve tissue repair and regeneration.

To avoid in vivo systemic exposure of small-molecule drugs that may have side effects on other tissues/organs given their ability to modulate key developmental signaling pathways, another strategy is to modulate stem/progenitor cells ex vivo to enhance their functions for transplantation. North et al. screened a collection of biologically active compounds using zebrafishes to identify the modulators of HSC induction in the zebrafish aorta-gonad-mesonephros region, where the first definitive HSCs primarily arise. They found a number of small molecules that enhance prostaglandin E2 (PGE2) synthesis, and PGE2 itself, increased HSC numbers in zebrafish [61, 62]. Ex vivo temporal treatment of murine and human HSCs with 16,16-dimethyl-PGE2 (dmPGE2) (Table 1; S20), a more stable analog of PGE2, enhanced their engraftment in vivo possibly through induction of genes involved in HSC homing, including CXCR4. These findings led to rapid clinical studies of short-term ex vivo-treated human cord blood cells by dmPGE2 for improved transplantation in adult patients with hematologic malignancies [63]. Similar clinical studies with human cord blood cells temporally treated with a CD26 inhibitor are also ongoing based on the HSC homing mechanism on the SDF-1 $\alpha$ -CXCR4 axis.

### CONCLUSION

Although stem cell research and regenerative medicine are still in an early development stage, they have had substantial growth in recent years. In particular, the iPSC technology has generated tremendous enthusiasm and efforts to explore their various applications. Now, chemical approaches are becoming increasingly accessible and valuable in discovery biology and have already played an essential role in stem cell research and regenerative medicine. It is clear that chemical approaches in precisely controlling cell fate, behavior, and state/function will continue to open up new opportunities for the field of stem cell biology and regenerative medicine.

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### DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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## Concise Review: Inner Ear Stem Cells—An Oxymoron, But Why?

MOHAMMAD RONAGHI,<sup>a,b</sup> MARJAN NASR,<sup>a,b</sup> STEFAN HELLER<sup>a,b</sup>

<sup>a</sup>Department of Otolaryngology-Head and Neck Surgery and <sup>b</sup>Department of Molecular and Cellular Physiology, Stanford University School of Medicine, Stanford, California, USA

**Key Words.** Adult stem cells • Aging • Nervous system • Notch • Tissue regeneration • Tissue-specific stem cells

### ABSTRACT

Hearing loss, caused by irreversible loss of cochlear sensory hair cells, affects millions of patients worldwide. In this concise review, we examine the conundrum of inner ear stem cells, which obviously are present in the inner ear sensory epithelia of nonmammalian vertebrates, giving these ears the ability to functionally recover even from repetitive ototoxic insults. Despite the inability of the mammalian inner

ear to regenerate lost hair cells, there is evidence for cells with regenerative capacity because stem cells can be isolated from vestibular sensory epithelia and from the neonatal cochlea. Challenges and recent progress toward identification of the intrinsic and extrinsic signaling pathways that could be used to re-establish stemness in the mammalian organ of Corti are discussed. *STEM CELLS* 2012;30:69–74

Disclosure of potential conflicts of interest is found at the end of this article.

### INTRODUCTION

Mechanosensitive sensory hair cells represent an evolutionary successful concept used in many different mechanoreceptive organs ranging from the lateral line of aquatic animals to the complex inner ears of mammals with specialized vestibular and auditory organs. Despite the great morphological and functional variations of hair cell-bearing organs, the requirement of certain key genes for mechanosensory cell development is evolutionary conserved. The basic helix-loop-helix genes *atonal* and *atonal homolog 1* (*Atoh1*), for example, are essential for invertebrate chordotonal mechanoreceptor and vertebrate hair cell development, respectively [1]. Based not only on such genetic evidence but also on comparative anatomical studies, it is generally accepted that the inner ears, particularly those of amniotes, including reptiles and birds, as well as mammals, are homologous organs [2].

Despite their common ancestry, there is a crucial difference in the ability of adult vertebrate inner ears to regenerate lost hair cells. The most robust generation of hair cells happens in the vestibular organs of amphibians and fish that display permanent addition of new sensory cells leading to continuous growth of the sensory epithelial patches [3]. Mature avian vestibular sensory epithelia do not grow, but there is a robust turnover of hair cells as well as a robust regenerative response after induced hair cell loss [4, 5]. In contrast, mammalian vestibular sensory epithelia do not turn over hair cells and show only very limited mitogenic replacement of hair cells after drug-induced loss of hair cells [6].

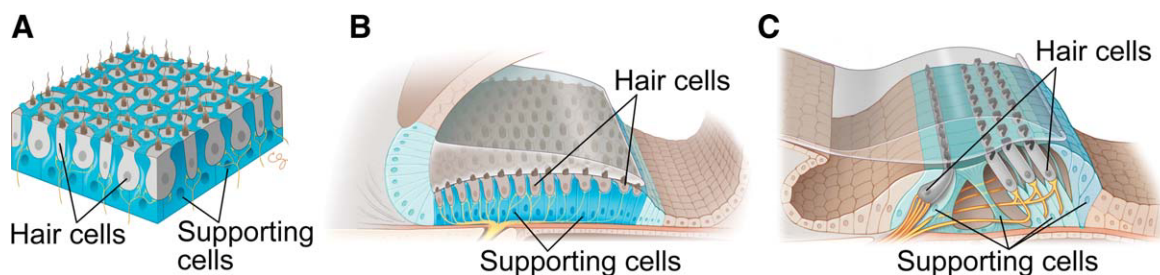
The difference in regenerative capability becomes even more obvious in case of the auditory organs. The avian

cochlea, also known as the basilar papilla, does not turn over hair cells, but it robustly responds to ototoxic insults with hair cell regeneration and functional recovery [7–9]. This regenerative capacity does not exhaust even after repeated deafening or at old age [10, 11]. The mammalian organ of Corti, conversely, does not replace lost hair cells. Continuous wear and tear, combined with the effects of aging as well as environmental threats such as loud noise and ototoxic drugs, result in an incessant diminishment of hearing at older ages. Approximately one-third of seniors over the age of 60 suffer from hearing loss (<http://www.nidcd.nih.gov>). Besides acquired hearing loss, approximately 2–3 out of 1,000 born babies are diagnosed with hereditary hearing loss and a similar high number of children lose their hearing before their teenage years (<http://www.nidcd.nih.gov>). This situation leads to an increasing health problem affecting hundreds of millions of patients worldwide. Undoubtedly, this number will continue to rise due to growing noise pollution, environmental factors, lifestyle choices such as listening to loud music, and worldwide increase of aminoglycoside use particularly in the third world, where these drugs are often the only affordable first-line treatments for life-threatening diseases such as tuberculosis [12].

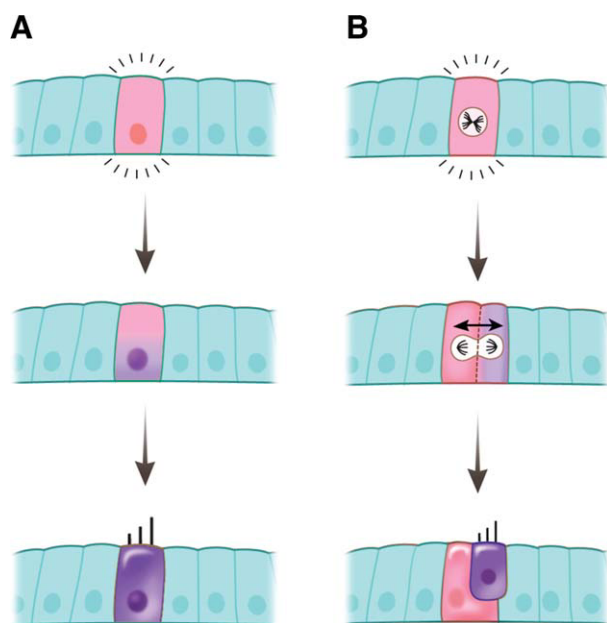
In this review, we will compare the avian vestibular and auditory organs with their mammalian counterparts. We will start with describing anatomical and cellular differences as well as similarities. We will summarize the known regenerative mechanisms and the pathways involved in regeneration, and finally, we attempt to explain why supporting cells should be regarded as inner ear stem cells, how stemness is successively lost in the mammalian cochlea, and what options exist for re-establishing regenerative capacity in the adult mammalian cochlea.

Author contributions: M.R., M.N., and S.H.: conception and design, manuscript writing, and final approval of manuscript.

Correspondence: Stefan Heller, Ph.D., Department of Otolaryngology-Head and Neck Surgery/Molecular and Cellular Physiology, Stanford University, 801 Welch Road, Stanford, California 94305-5739, USA. Telephone: 650-724-8086; Fax: 650-725-8502; e-mail: [hellers@stanford.edu](mailto:hellers@stanford.edu) Received August 5, 2011; accepted for publication October 27, 2011; first published online in *STEM CELLS EXPRESS* November 18, 2011. © AlphaMed Press 1066-5099/2011/\$30.00/0 doi: 10.1002/stem.785



**Figure 1.** Inner ear sensory epithelia. (A): A generic illustration of avian and mammalian vestibular sensory epithelia. (B): A drawing of the avian basilar papilla. (C): The mammalian organ of Corti.



**Figure 2.** Hair cell regeneration. (A): A supporting cell differentiates into a hair cell. This process is also referred to as transdifferentiation. The lost supporting cell replaced via a mitotic division of another supporting cell. (B): An illustration of asymmetric supporting cell division giving rise to a new hair cell and a supporting cell.

### ANATOMICAL AND CELLULAR COMMONALITIES AND DIFFERENCES BETWEEN AVIAN AND MAMMALIAN INNER EAR SENSORY EPITHELIA

In general, the sensory epithelia of the vestibular organs (utricle, saccule, and cristae) consist of a mosaic of sensory hair cells and surrounding supporting cells (Fig. 1A). Supporting cells reach from the apical surface to the basilar lamina. The hair cells, however, do not contact the basilar membrane and are basolaterally ensheathed by supporting cells. Anatomically, there are no major differences between avian and mammalian vestibular epithelia, but after hair cell loss, the differences become quite obvious. Avian vestibular hair cells readily regenerate, while proliferative hair cell regeneration in mammalian vestibular epithelia only happens at a very low rate [6]. This difference is also apparent in cultured chicken hatchling utricle sensory epithelia, which show high proliferative capacity [13], whereas cultured neonatal mammalian utricle sensory epithelia display only limited proliferative capacity [14].

Although avian and mammalian vestibular organs have similar anatomy, the anatomical differences between the basilar papilla and the mammalian cochlea are considerable. The avian hearing organ harbors a drawn-out patch of hair cells that is several millimeters long and has a width of more than 60 hair cells at its widest point (Fig. 1B). The hair cells are afferently innervated from the cochleo-vestibular ganglion underlying the auditory epithelium. Afferent nerve fibers connect to the sensory epithelium laterally, from the so-called neural side, and innervate the cylindrical “tall” hair cells located toward the neural side. The shape of hair cells changes gradually across the avian basilar papilla and the abneural “short” hair cells are mostly innervated by efferent fibers [15]. It is presumed that the tall hair cells are equivalent to the inner hair cells of the mammalian organ of Corti that will be described in the next paragraph, whereas the short hair cells are presumably involved in feedback and gain control [15, 16]. Basilar papilla supporting cells are anatomically not substantially different from vestibular supporting cells and appear homogenous and without cytomorphological specializations.

In contrast, the organ of Corti, which is the sensory epithelium of the mammalian cochlea, has two highly specialized hair cell types and comprises a variety of supporting cell types with distinct cytomorphologies (Fig. 1C). A single row of afferently innervated inner hair cells extends from the base of the coiled cochlea to its apex. The inner hair cell row is accompanied by three rows of mainly efferently innervated outer hair cells, which fulfill amplification and frequency tuning functions. Highly specialized supporting cells that are organized in an orderly structured pattern are interspersed between the hair cells [17]. Organ of Corti supporting cells appear to have evolved at least in part to provide mechanical support and filtering to the highly dynamic and actively moving tissue. Many supporting cells contain cytoskeletal specializations that are probably necessary for maintenance of cell shape.

### REGENERATIVE RESPONSES IN AVIAN INNER EAR SENSORY EPITHELIA

Upon hair cell loss, the supporting cells of the basilar papilla regenerate hair cells [7, 8]. Within a day after induced hair cell loss, supporting cells start to re-express developmental genes that are normally found in prosensory progenitors [18, 19]. Many supporting cells do not re-enter the cell cycle but rather begin to differentiate directly into new hair cells (Fig. 2A). It has been hypothesized that this process, which is also referred to as direct transdifferentiation, triggers a second response phase in which remaining supporting cells re-enter the cell cycle and replenish the supporting cells lost due to transdifferentiation [20]. In parallel, supporting cells are able to respond to hair cell loss by asymmetric division, giving

rise to pairs of replacement hair and supporting cells [21, 22] (Fig. 2B). This latter behavior resembles a bona fide somatic stem cell response. It is unclear which of the two regenerative processes is the dominating one in vivo, but it has been suggested that direct transdifferentiation might be a strong early phase regenerative response to massive hair cell loss [20, 23]. The degree of each regenerative mechanism in vitro appears to depend on the culture conditions and the nature of the ototoxic insult [13, 24]. In vivo, there is evidence for both processes happening in the basilar papilla after gentamicin-induced hair cell loss [20], whereas the major regenerative response in vestibular sensory epithelia appears to be happening via asymmetric supporting cell division [25]. Overall, it is clear that the regenerative processes that happen in the damaged basilar papilla are the direct result of activation of a resident population of stem cells. Plenty of questions, however, remain open. For example, it is not clear whether all supporting cells are stem cells or whether a subpopulation of stem cells exist. Likewise, the mechanisms that trigger direct transdifferentiation and asymmetric supporting cell division are unknown, although research on this topic is making progress as explained in the following paragraphs.

Coculture experiments of chicken utricle sensory epithelia with damaged chicken vestibular epithelia suggested that the regenerating epithelia secrete an activity that is able to trigger increased proliferation in the target tissue [26]. Likewise, the same study reported evidence for a soluble mitotic inhibitor that is secreted by the undamaged chicken utricle. A number of growth factors have been discussed as candidates for promoting proliferation in regenerating avian sensory epithelia [27, 28], and recent gene array analyses suggested the possible involvement of Notch, transforming growth factor beta (TGF $\beta$ ), Wnt, activator protein 1 (AP-1), Pax, nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B), and insulin-like growth factor 1 (IGF)/insulin pathways [18]. Using an RNA interference (RNAi)-based method for screening different transcription factors, Alvarado et al. [29] showed that inhibition of components of the AP-1 (Cebpg, Lrp5, JunD), Pax (Pax2 and Pax5), and Wnt (Wnt4) pathways, as well as c-Jun N-terminal kinase (JNK) and mitogen-activated protein kinase (MAPK) inhibitors were able to interfere with regenerative supporting cell proliferation. Downstream targets of TGF $\beta$  signaling such as Cutl1 were also upregulated during sensory epithelia regeneration [18] and small interfering RNA (siRNA) to Cutl1 resulted in inhibition of supporting cell proliferation. Cutl1 is a suppressor of p27<sup>Kip1</sup>, a cell cycle inhibitor that suppresses proliferation of supporting cells in the mammalian organ of Corti [30, 31]. Cutl1 has been put forward as a potential mediator of a regenerative response leading to downregulation of cell cycle inhibitors such as p27<sup>Kip1</sup> at the onset of mitogenic hair cell regeneration in birds [18, 29].

Another pathway that has been implicated in triggering cell proliferation in the avian basilar papilla is mediated by protein kinase A, a direct target of cAMP. It has been demonstrated that increase of cAMP levels triggers a robust proliferative response in supporting cells of undamaged avian basilar papilla sensory epithelia [32]. Likewise, supporting cell proliferation in response to aminoglycoside treatment is strongly but not completely attenuated in cultured chicken basilar papillae when protein kinase A inhibitors were present. These observations suggest that one of the signals capable of triggering supporting cell proliferation in avian sensory epithelia could be acting either via receptor tyrosine kinases or via G-protein-coupled receptors leading to an increase in intracellular cAMP.

Besides secreted factors, there has been considerable interest in the role of cell-to-cell signaling mediated by the Notch pathway or more classic cell adhesion proteins. Notch signal-

ing plays multiple roles in the avian inner ear such as during hair cell regeneration as well as in development, where the Notch pathway is important for early steps of prosensory specification, but also later on, in its classic role as mediator of lateral inhibition [33]. In the adult chicken basilar papilla, the Notch pathway is active during regeneration and manipulation of Notch signaling, for example, by inhibition of gamma secretase results in an overproduction of hair cells [34]. In contrast, in the mature mouse cochlea, Notch pathway genes become downregulated and remain silent, even after aminoglycoside-induced hair cell loss [35]. During development, Notch and its ligands are expressed in the emerging prosensory domains of the inner ear [36, 37], and activation of Notch in neighboring nonsensory regions appears to be sufficient for prosensory induction [38, 39]. Nevertheless, conditional disruption of the canonical Notch signaling mediator recombining binding protein suppressor of hairless J kappa in the developing mouse inner ear revealed that although Notch activation is sufficient, the RBPj $\kappa$ -mediated canonical pathway does not appear to be essential for prosensory induction in the mouse cochlea [40]. In the avian inner ear, blockade of Notch activation leads to loss or reduction of prosensory domains, but induction of early prosensory markers such as *Serrate1* does not appear to be dependent on Notch signaling [41].

Overall, it is obvious that the avian basilar papilla maintains a resident population of stem cells that are capable of fully regenerating the damaged auditory sensory epithelia. We are just beginning to understand the mechanisms how the regenerative potential of these normally quiescent cells is regulated, and how the cells become active after ototoxic damage leading to hair cell loss. Restored sensory epithelia are subsequently innervated and properly connected to the central nervous system, which functionally restores the auditory system [9, 10]. Nevertheless, an open question remains, which is whether the apparent stemness of supporting cells is a universal feature of avian vestibular and auditory supporting cells, or whether the sensory epithelia maintain a specialized niche for a distinct somatic stem cell subpopulation [42]. The unequivocal identification of these stem cells and the unraveling of the ensuing mechanisms for regeneration are somewhat limited in the avian system, particularly because of the lack of routine genetic manipulations. A possible alternative model system for such studies is zebrafish. Hair cell regeneration in the zebrafish lateral line system, however, appears to follow yet another variation of regeneration program where a supporting cell divides symmetrically into two hair cells [43]; the lost supporting cell subsequently is very likely replaced by symmetric division of another supporting cell. Another open question, equivalent to the one raised in the regenerating chicken sensory epithelia, is whether lateral line supporting cells are randomly chosen to replace lost hair cells or whether there is a local niche maintained for a population of distinct stem cells.

### LACK OF ROBUST REGENERATIVE RESPONSES IN MAMMALIAN INNER EAR SENSORY EPITHELIA

Evolutionary, it is inconceivable why the mammalian inner ear has lost its regenerative capacity. One argument that has been put forward is to achieve the structural specializations of the organ of Corti, which presumably extend the range of hearing into the higher frequencies, that the stemness and its ensuing regenerative potential of supporting cells was traded off for structural complexity [44]. Another argument is that, evolutionary,



the preservation of regenerative capability was not under strong selective pressure because acquired hearing loss and ototoxic insults are mainly the product of the industrial revolution [45]. All these speculations, however, cannot explain why the mammalian vestibular system has such a restricted regenerative capacity when compared with birds, reptiles, and fish. Anatomically and functionally, the differences between the vestibular organs of mammalian and nonmammalian amniotes appear small. At the cellular level, however, either the signals that trigger regeneration or the factors that provide competence to the responding cells, or both, are no longer featured.

Although mitotic hair cell regeneration in adult mammalian vestibular sensory epithelia does only happen on rare occasions [6], there are some indications that adult vestibular supporting cells have regenerative capacity, which can be activated when the cells are dissociated and cultured in conditions that were originally developed to stimulate neurosphere formation from neural stem cells [46, 47]. Dissociated utricle sensory epithelium cells are able to grow clonally into spheres, albeit with low yields. Stringent tests showed that sphere-forming cells from the adult utricle sensory epithelium were self-renewing and able to give rise to cell types from all three germ layers [47], which indicates that the adult vestibular sensory epithelia harbors stem cells. Open questions remain. First, as with avian supporting cells, it is not clear whether stemness is a possible feature of all mammalian vestibular supporting cells or whether there is a subpopulation of sensory epithelial cells that maintain stemness and are the source of the limited regenerative capacity. Second, the number of stem cells with this ability is low: a few dozen per sensory epithelium, which, however, is more than the few mitotic cells that can be detected in vivo after an ototoxic insult [6]. This finding suggests that a group of supporting cells might be competent to respond to a regenerative trigger, but that the lack of appropriate signals or the presence of an inhibitor might contribute to the low regenerative capacity of adult mammalian vestibular epithelia. It is interesting in this regard that neonatal mouse balance sensory epithelia display a higher propensity for sphere formation than the adult tissue [46]. This suggests that young vestibular sensory epithelia harbor more cells that are able to re-enter the cell cycle provided an adequate trigger is supplied. Nevertheless, it is important to point out that presence of stem cells does not necessarily mean that the organ displays substantial regenerative capacity. In the mammalian central nervous system, for example, regenerative responses to injury or disease are limited, despite the existence of neurogenic niches. Conversely, neural stem cells are not always and necessarily quiescent and have been shown to become active in certain situations [48]. It is consequently important to distinguish between stemness and regenerative potential, which not always go hand-in-hand.

Although the molecular nature of activators or inhibitors of mammalian hair cell regeneration are not known, some candidates are emerging. For example, brief exposure of explants of neonatal rat vestibular sensory epithelia to forskolin led to a significant increase of cell cycle re-entry of supporting cells, which indicates that tissue dissociation is not absolutely necessary to evoke S-phase re-entry [14]. Moreover, the S-phase re-entry in these cultures was only occurring in the presence of serum or mitogenic growth factors and was not observed when receptor trafficking to the plasma membrane was blocked. These observations suggest that transient elevation of cAMP levels in neonatal vestibular supporting cells very likely results in an increase of growth factor receptor density in the plasma membrane, which in turn leads to a

higher number of supporting cells that are competent to respond to mitogenic stimulators in form of growth factors or serum components. Possible growth factor or cell contact-based signaling cascades involved in triggering S-phase re-entry include the phosphatidylinositol 3-kinase cascade culminating in activation of mammalian target of rapamycin (mTOR) because inhibition of elements of this signaling cascade interferes with mitotic cell proliferation in neonatal rat vestibular sensory epithelia [49]. It appears that intact neonatal vestibular sensory epithelia in vivo do not contain sufficient amounts of mitogenic stimulators, hence the cell cycle quiescence of supporting cells that otherwise would be readily responsive to mitogens. Furthermore, maturing and aging supporting cells might lose growth factor receptors and consequently the competence to respond to mitogenic growth factors.

The adult mammalian organ of Corti completely lacks regenerative potential. In contrast, neonatal mouse organ of Corti-derived cells have a rather solid mitogenic capacity, which is reflected in their ability to give rise to clonal spheres or colonies [46, 50]. Mitogenic capacity, however, is not necessarily an indication whether neonatal organ of Corti-derived cells have the ability to generate progenitor cells that give rise to hair and supporting cells. Cell sorting experiments have shown that the cells with the highest capacity to give rise to hair cell- and supporting cell-marker positive cells are the pillar cells as well as the supporting cells that are most closely associated with hair cells [50, 51]. Nevertheless, other cell types that reside in the neonatal cochlea also have potential to proliferate and to differentiate into hair cells and supporting cells, albeit with less efficacy [51]. These observations are in support of the hypothesis that organ of Corti maturation and the distinct cytomorphological differentiation of cochlear supporting cells are accompanied by downregulation of signaling molecules and presumably also their receptors and intracellular signaling components. It is conceivable that the organ of Corti never establishes a proper stem cell niche and that the atavistic stemness found in neonatal cochlear supporting cells disappears when the cells become fully differentiated. Consequently, there is no regenerative capacity detectable in the mature organ of Corti. The question remains whether competence to respond to regenerative triggers can be restored in adult organ of Corti supporting cells. Research on other organ systems, such as the heart, is promising in this respect. The mammalian heart, like the inner ear, lacks robust regenerative capacity whereas the hearts of non-mammalian vertebrates such as fish can regenerate cardiomyocytes and can restore function [52]. Nevertheless, recent findings established a lineage relationship between stem cells that reside in the epicardial layer of the adult mouse heart and functional cardiomyocytes that differentiate de novo from the resident epicardial stem cells after myocardial infarction [53]. This mobilization and differentiation required pretreatment with thymosin  $\beta_4$ , a peptide that has been previously shown to stimulate re-expression of developmental genes in presumptive stem cells in the epicardium [54]. Another example is the restoration of cell loss in a mouse model of stress-mediated muscle atrophy by treatment with the food and drug administration (FDA)-approved drug losartan [55]. Cells with stem cell characteristics in these organs can evidently be tweaked to display a certain degree of regenerative potential, which is providing some reason for careful optimism. Nevertheless, particularly for the infarcted heart, many roadblocks need to be solved before functional restoration by activation of the regenerative potential of resident stem cells could become a feasible therapy option. Translated to the inner ear, it is plausible that the discovery of small molecule activators that evoke re-expression of developmental genes would be a promising route toward developing novel therapies for hearing loss [45, 56]. It would be interesting



to investigate whether such a strategy would instigate localized developmental processes that lead to hair cell regeneration or even restoration of the anatomical intricacies of the organ of Corti. Although research in this regard is just beginning and translation into the clinic is probably decades away, it is obvious that a longer life paired with a lifetime of ototoxic insults causes a steady increase of the number of patients worldwide who await novel treatments for hearing loss. The apparent loss of stemness in the mammalian inner ear when compared to nonmammalian vertebrates remains puzzling, thereby making the term “inner ear stem cells” truly an oxymoron for patients who are dearly affected by the inability of the cochlea for self-repair.

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## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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## Concise Review: Human Cell Engineering: Cellular Reprogramming and Genome Editing

PRASHANT MALL,<sup>a,b</sup> LINZHAO CHENG<sup>a,c</sup>

<sup>a</sup>Stem Cell Program, Institute for Cell Engineering and <sup>c</sup>Division of Hematology, Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA; <sup>b</sup>Graduate Program in Biomedical Engineering, The Johns Hopkins University, Baltimore, Maryland, USA

**Key Words.** Human cell reprogramming • Pluripotent stem cells • Gene targeting • Genome editing • Genome engineering • Gene therapy • Disease modeling

### ABSTRACT

Cell engineering is defined here as the collective ability to both reset and edit the genome of a mammalian cell. Until recently, this had been extremely challenging to achieve as nontransformed human cells are significantly refractory to both these processes. The recent success in reprogramming somatic cells into induced pluripotent stem cells that are self-renewable in culture, coupled with our increasing ability to effect precise and predesigned genomic editing, now readily permits cellular changes at

both the genetic and epigenetic levels. These dual capabilities also make possible the generation of genetically matched, disease-free stem cells from patients for regenerative medicine. The objective of this review is to summarize the key enabling developments on these two rapidly evolving research fronts in human cell engineering, highlight unresolved issues, and outline potential future research directions. *STEM CELLS* 2012;30:75–81

Disclosure of potential conflicts of interest is found at the end of this article.

### INTRODUCTION

The overarching goal of regenerative medicine is to develop processes for creating functional tissues to enable the repair or replacement of damaged and diseased tissues. The realization of this goal is typically envisioned through a two-step process: the first being to derive disease-free starting cells, ideally stem cells that are genetically matched to the recipient; and the second being to modulate these through appropriate differentiation and assembly to achieve a transplantable tissue form. However, in practice, efficacious fulfillment of each of these steps presents serious obstacles and is thus the subject of active research by biomedical scientists. Specifically, in the quest for a suitable source of cells, pluripotent stem cells, such as embryonic stem (ES) cells derived from early embryos (Fig. 1A), offer a particularly attractive avenue to explore. This is because they possess two key features: one, an indefinite self-renewal capability in culture and, two, a very broad differentiation potential to generate all cell types [1]. Hence, in theory, if one can efficaciously derive such cells and then efficiently do gene therapy in them to correct all underlying disease causing mutations, then the resulting cells can serve as the desired inexhaustible source of healthy stem cells. These can subsequently be directed to differentiate into any desired cell type of choice, which can ultimately serve to repair the damaged or diseased tissue of interest. This review aims to provide an overview of the first step in the above cascade, specifically approaches toward engineering

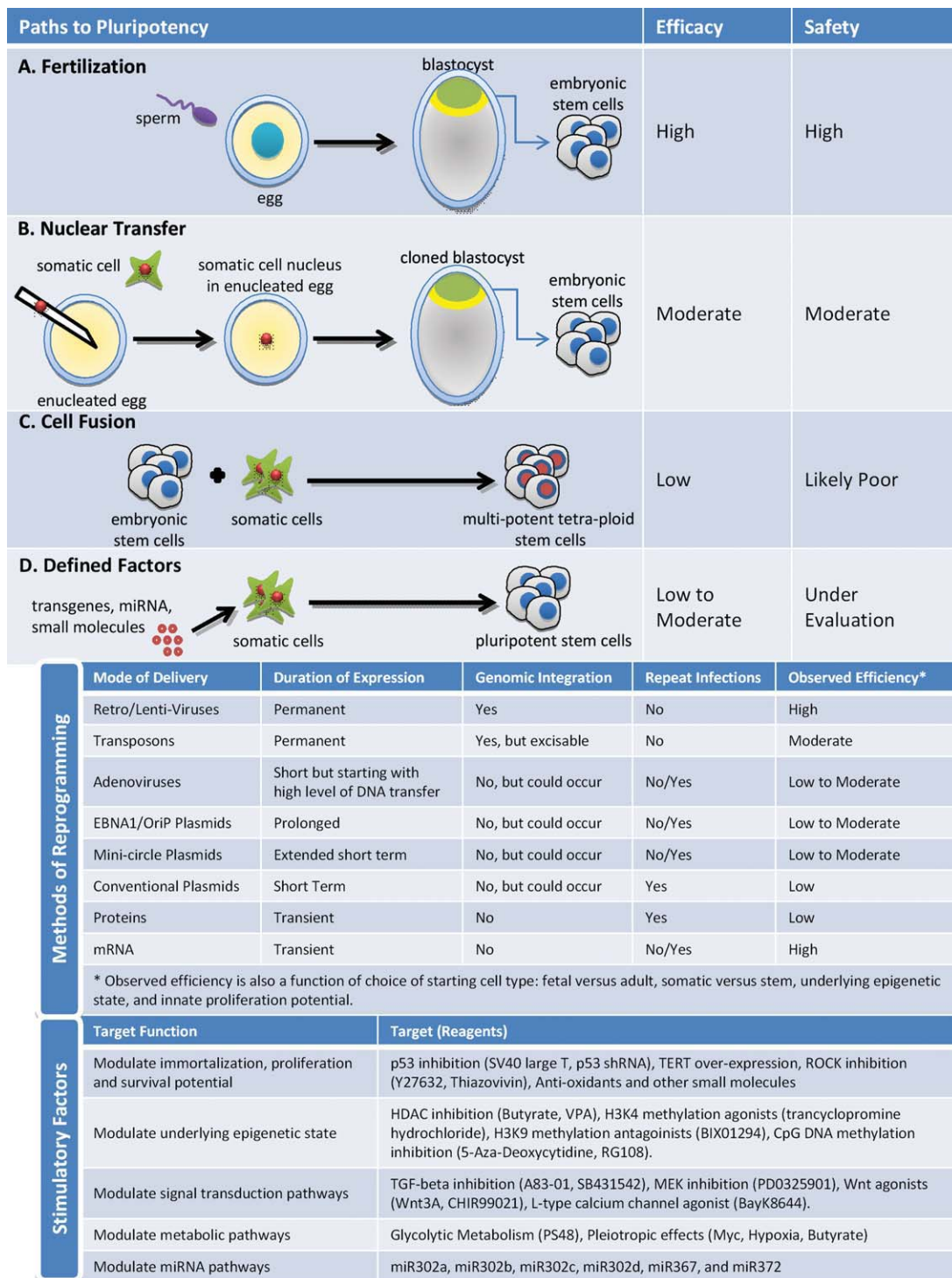
disease-free human stem cells that can serve as a viable source of cells for cell-based therapies.

### REJUVENATING CELLS FOR REGENERATIVE MEDICINE

During the course of embryonic and subsequent development, cells starting from a pluripotent state differentiate into various cell types with progressively narrower developmental potential. Their cellular and epigenetic programs gradually become less flexible and more defined, resulting in the acquisition of a stable phenotype [2, 3]. Drawing an analogy using Waddington's epigenetic landscape of mammalian development [3], akin to marbles that lose potential energy on going downhill, cells too during the course of development (starting from the unicellular zygote stage) progressively lose their degree of multipotentiality. The marbles eventually settle into valleys that correspond to local minima's and thus represent cell types with stable phenotypes that will be normally found during homeostasis. Occasionally cells in response to external stimuli may crossover to local minima's in their immediate vicinity, if an intervening barrier is not too high. However, to affect a movement uphill toward the top or into a distant valley, two distinct processes that are termed, respectively, as dedifferentiation or transdifferentiation (and referred collectively as cellular reprogramming), a sustained stimulus or driving force is needed.

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Correspondence: Linzhao Cheng, Ph.D., Broadway Research Building 747, 733 North Broadway, Baltimore, Maryland 21205, USA. Telephone: 410-614-6958; e-mail: lcheng@welch.jhu.edu Received May 31, 2011; accepted for publication July 24, 2011; first published online in *STEM CELLS EXPRESS* September 8, 2011. © AlphaMed Press 1066-5099/2011/\$30.00/0 doi: 10.1002/stem.735



**Figure 1.** Paths to pluripotency. (A): Fertilization of an egg by a sperm results in a totipotent cell that gives rise to the entire embryo proper and to the extraembryonic tissues. This is the process that nature takes and is associated with near perfect efficiency of reprogramming. (B): Alternatively one can introduce nuclei of somatic cells into oocytes, however, only a few percent of embryos develop to term. Furthermore, both these processes are also saddled by serious ethical and technical concerns. (C): One can also achieve reprogramming by simple cell fusion of somatic cells to embryonic stem cells, but the resultant cells, although multipotent, have tetraploid nuclei and hence are of little clinical relevance. (D): Most recently, in vitro reprogramming of somatic cells into pluripotent stem cells was achieved in pioneering experiments by Yamanaka and coworkers, which relied on just the forced expression of four transcription factors: *Oct4*, *Sox2*, *c-Myc*, and *Klf4*. This defined recipe remarkably suffices to restart the cells endogenous pluripotency network. This approach has since been refined and several techniques to achieve reprogramming have now been developed. The appended table summarizes these. In vitro reprogramming back to pluripotency is thus now feasible by various means, but it is also important to note that each of these reprogramming approaches can still have limitations (such as process associated introduction of genetic mutations, incomplete epigenetic reprogramming, etc.) and these need to be fully deciphered and resolved before any attempt at clinical translation. Abbreviations: EBNA, Epstein-Barr nuclear antigen; HDAC, histone deacetylase; miRNA, microRNA; shRNA, small hairpin RNA; TERT, telomerase reverse transcriptase; VPA, valproic acid.



For somatic cells, the ascent (dedifferentiation) has classically been achieved by one of two means: either nuclear transfer into oocytes [4] (Fig. 1B), or fusion with ES cells [5] (Fig. 1C). Transplantation of a somatic cell nucleus into an enucleated oocyte can initiate a striking conversion to an embryonic phenotype. Akin to the use of blastocysts for human ES cell derivation, this process is, however, also saddled by serious ethical and technical concerns [6]. However, it must be noted that not only is cloning inefficient because most cloned embryos die shortly after implantation but also the few that survive to birth frequently have developmental abnormalities and usually a short lifespan. This implies that compared with a fertilized egg from natural mating, the reprogramming of the transplanted nucleus is relatively incomplete. A similar result is also achieved by fusion of somatic cells to ES cells (Fig. 1C); however, the resulting multipotent cells have tetraploid nuclei and thus possess only limited developmental and clinical potential. It has also been found that exposure of somatic cells or nuclei to cell extracts from ES cells or embryonal carcinoma cell lines (roughly a tumor-version of ES cells) can lead to reprogramming to a ES cell-like or more undifferentiated state [7, 8]. Together, these experiments demonstrate that nuclear reprogramming is indeed possible through several means. However, the precise identity and nature of the underlying players for somatic cell reprogramming using these techniques is not easily elucidated.

Merely 5 years ago, a fourth method was developed by Takahashi and Yamanaka[9], first with the murine system in 2006 (Fig. 1D). This approach relied simply on the forced gene expression of four transcription factors: *Oct4*, *Sox2*, *Klf4*, and *Myc* (OSKM) to restart the pluripotency network (Fig. 1D). The resulting cells called induced pluripotent stem (iPS) cells are phenotypically and functionally very similar to ES cells in that they can self renew indefinitely and are pluripotent. This pioneering work and subsequent early publications [10–12] presented the first successful approach to reprogram a mammalian genome to a pluripotent state using defined factors.

### OVERCOMING HURDLES TOWARD HUMAN CELL ENGINEERING

The publication of this seminal work by the Yamanaka laboratory for mouse cells sparked a frenzy of activity to extend it to the human system. However, it quickly became evident that a direct translation of this approach to human cells was plagued by multiple roadblocks. First, the reprogramming efficiencies for human cells were found to be significantly lower (typically one colony per  $10^4$  input cells or even lower) [13–16]. Second, the derivation of iPS cells from these also took a significantly longer duration of time, typically 4 weeks or even more (as opposed to just 2 weeks in mouse cells). Both may reflect the fact that normal human cells proliferate much slower than murine cells in culture. Together, these aspects made derivation from human cells a technically challenging process in early days. Furthermore, adult cells were observed to be typically significantly more refractory than embryonic or fetal cells to reprogramming [15]. However, it was imperative for biomedical research to be able to do this reprogramming efficiently in cells from adult or postnatal tissue sources.

Towards addressing these issues, it is instructive to first consider the following analogy for the pluripotency network: the four transcription factors OSKM can be considered as key nodes (genes) of the ES cell regulatory network graph from which it is possible to efficiently reach (activate) all other nodes, that is, kick-start the pluripotency network and effect

reprogramming of a somatic cell to a pluripotent state. Now, although these four factors form a sufficient set, however, they need not represent a necessary or optimal starting set. Specifically, judicious inclusion of additional factors (nodes) could hasten this graph traversal, that is, speed up reprogramming, as also would conditions that improve overall graph connectivity, for instance, modulation of the epigenetic state of the somatic cell type. Finally, appropriate choice of a starting cell type that already has a partially activated pluripotency network or favorable epigenetic status would also make it highly amenable to this reprogramming process.

Consequently, toward the goal of improving reprogramming efficiency and efficacy approaches exploring each of these possibilities have been considered by researchers, and entail introduction of additional stimulatory factors to the basic four-factor cocktail. Per the above, these fall into four broad categories (Fig. 1D, table): first, factors that promote cell immortalization, proliferation, and improvement of survival potential of cells, such as SV40 large T antigen, telomerase reverse transcriptase, and reagents that reduce *p53* levels [15, 16]; second, modulation of the underlying epigenetic state of the cells to promote active chromatin marks, specifically, histone deacetylase inhibition (butyrate or valproic acid) [17, 18], H3K4 methylation agonists (tranylcypromine hydrochloride), H3K9 methylation antagonists (BIX01294) [19], and CpG methylation inhibitors (5-aza-deoxycytidine or RG108); third, modulation of key signal transduction and metabolic pathways known to be active in ES cells: specifically, MAPK/ERK kinase inhibition (PD0325901), Wnt agonists (*Wnt3A* or CHIR99021), L-type calcium channel agonist (BayK8644), transforming growth factor  $\beta$  inhibition (A83-01 or SB431542), promotion of glycolytic metabolism (PS48) [20], and factors with pleiotropic effects on metabolism as well as global gene expression (such as *Myc*, hypoxia, and butyrate) [21]; and finally fourth, modulation of microRNA (miRNA) pathways based on those selectively expressed in the pluripotent state [22]. In fact, in a recent publication, enforced expression of few exogenous miRNAs alone was shown to be sufficient to reprogram mouse and human somatic cells to a pluripotent state [23].

Successful reprogramming of somatic cells requires prolonged overexpression of reprogramming factors. Consequently, retroviruses/lentiviruses were the initial preferred choice of delivery vectors, because upon infecting a cell, they can efficiently integrate into the genome and thus provide the required stable and high levels of transgene expression. However, this feature results in permanent modification of the genome, and hence also significantly raises the risk of insertional mutagenesis [24–28]. Moreover, it was soon realized the expression of reprogramming genes are required only transiently; indeed, they have to be adequately silenced in successfully reprogrammed iPS cells to avoid interference of differentiation programs [11, 12, 29]. Thus, for iPS cells to be relevant in a clinical setting, it was imperative that one derives them using techniques that result in minimal genomic alterations. Consequently, there have also been significant efforts in exploring alternative reagents and approaches to enable derivation of integration-free iPS cells. Specifically, several virus-free and integration-free methods were reported, which generated mouse and human iPS cells by using purified proteins, modified mRNAs, and novel plasmid systems [29–36]. However, as expected, the reprogramming efficiencies using some of these approaches were vanishingly small, and in other cases (such as using proteins or mRNAs), needed cumbersome serial delivery (daily and up to 3 weeks) of adding multiple reprogramming molecules to reprogram fibroblasts. However, recent literature has highlighted that certain

cell types such as fetal neural stem cells [31] and postnatal blood cells (after stimulation in culture) [37, 38] are easy cell types to reprogram to the ES cell-like state and thus also amenable to facile reprogramming even using transient stimulatory techniques as above. Taken together, the above advances have enabled robust and reproducible derivation of human iPS cells from most accessible sources.

If the thus derived iPS cells are to be eventually relevant in a regenerative medicine paradigm, it is also critically important to rid them of any underlying mutations that cause diseases. As an alternative approach of correcting the mutation in iPS cells, one could do gene therapy in the starting somatic cells and subsequently reprogram them to a pluripotent state. However, the lack of self-renewal ability of most somatic cell types makes selection and expansion of rare corrected clones difficult and thus this approach is often not feasible. Thus ES/iPS cells are typically the cell type of choice for effecting genetic mutations. Precise gene targeting by homologous recombination (HR) has played a critical role in genetic studies of various systems, including the generation of knockout/knockin transgenic mouse models using mouse ES cells. However, the efficiency of HR-mediated gene targeting in human ES cells, as in nontransformed human cells, remains low even after nearly a decade since its first report [39]. Only a few studies have been published to date using methods that are commonly performed in mouse ES cells. Using standard plasmid-based systems, the current HR rates are approximately  $10^{-6}$  and usually even lower in normal human ES cells and other nontransformed mammalian cells (Fig. 2). This is further compounded by the fact that human ES cells and iPS cells grow very poorly when plated as single cells (a practice required for selection of rare targeted clones), compared with mouse ES cells. A promising approach toward the same has been the use of zinc-finger nuclease (ZFN) generated site-specific double stranded breaks to stimulate HR efficiencies. Simply speaking, ZFNs are engineered sequence-specific nucleases comprising of two domains: a customized array of zinc-fingers (engineered to bind to a specific DNA sequence) fused to the DNA endonuclease domain from the FokI restriction enzyme [40, 41]. Each zinc-finger domain recognizes 3–4 bp of DNA and a three-finger ZFN can thus recognize approximately 9–10 bp of DNA sequences. When two ZFNs bind cognate target sequences in the proper orientation, the FokI domains can dimerize and create a nuclease that makes a DNA double-stranded break (DSB) between the two cognate sequences. The use of a ZFN pair in this manner also increases the overall sequence specificity, enabling them to precisely target a single unique locus in the genome ( $\geq 18$  bp by a pair of ZFNs, each with three zinc finger domains). Stimulated by ZFN-induced DSBs, endogenous loci can be targeted with high efficiency by either HR (in the presence of an exogenous donor DNA fragment serving as a repair template) or error-prone nonhomologous end-joining (especially in the absence of a DNA template). Thus, ZFNs have been used to make site-specific genomic modifications with high efficiencies in a variety of (mammalian and plant) cell lines and small organisms. Indeed, now several studies have also reported successful gene targeting by HR-mediated gene insertion at a few selective loci in normal or disease-specific human iPS cells [42–46].

### LOOKING FORWARD TO THE FUTURE

Human iPS cells that are derived from adult somatic cells hold great promise as a renewable cell source for developing

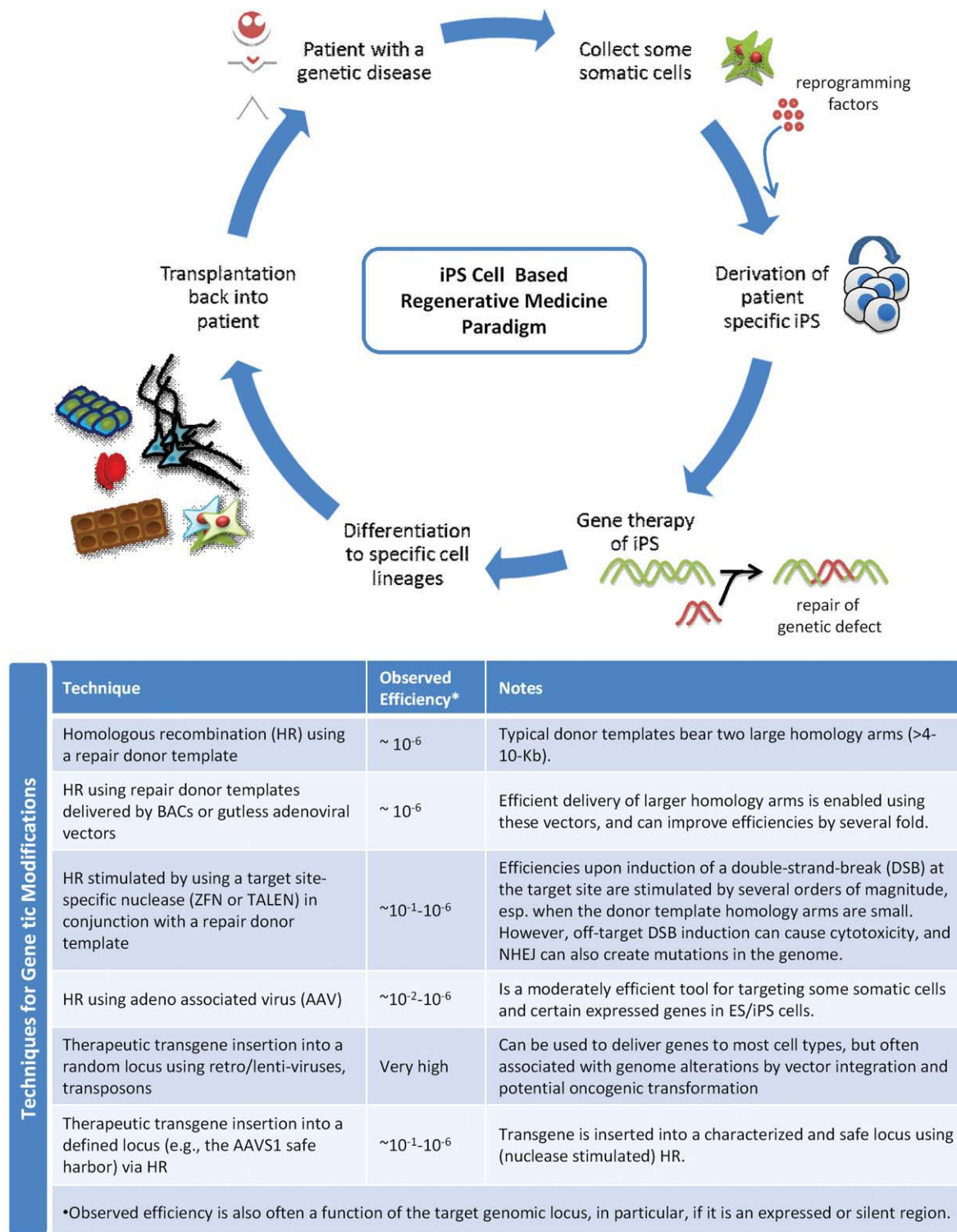
patient-specific cell therapies. As we look to understand the issues that still need to be overcome before clinical translation becomes feasible, we recapitulate below the three key steps toward enabling such a regenerative medicine paradigm. These are, first, efficient and efficacious derivation of patient-specific pluripotent stem cells from accessible somatic cell types; second, subsequent facile correction of all underlying genetic mutations to obtain disease-free stem cells; and finally third, scalable differentiation to a functional tissue form suitable for transplantation. Below we analyze the first two steps (the focus of this review) in detail and identify in particular the critical aspects that still need to be addressed and also potential directions that may be explored toward these.

With regards to the first step, that is, derivation of human iPS cells, efficient reprogramming is currently no longer an impeding research issue. Recent improvements have resulted in development of protocols that enable highly robust derivation of integration-free human iPS cells from multiple postnatal human cell types in a research laboratory (Fig. 1B). The facile method by episomal vectors after one round transfection of plasmid DNA (that can be cheaply produced and are stable) for generating high-quality, integration-free iPS cell lines from blood [37, 38] is a particularly attractive approach towards development of a robust technology compliant to future clinical uses.

Recent articles have suggested that early passage iPS cells may also retain a degree of epigenetic memory of their starting somatic cell types which may influence their differentiation ability [47–49]. Preliminary studies into the potential tumorigenicity and other aberrant properties of early versions of iPS cell lines have also been initiated [50–52]. Thus, development of assays for qualifying the efficacy (especially, the safety quotient) of derived iPS *vis-à-vis* ES cells, which are the gold standards for pluripotency, will be a key next step for the reprogramming field. It is important to point out that for most applications in somatic cell regenerative medicine, iPS cells do not need to be identical to ES cells with an epigenetic signature of an embryonic cell. For instance, the residual epigenetic memory left in derived iPS cells could also provide advantages to differentiate back to the original cell type where the iPS cell line is derived from [47–49].

With regards to the second step, that is, facile correction of genomic mutations in iPS cells, the field still has a long way to go. While the use of ZFNs can stimulate HR rates significantly, it is still not high enough that the step of gene targeting or correcting can be assumed to be either facile (especially for transcriptionally silent loci) or of short enough duration to be adaptable to a clinical setting. Thus, it is important to look beyond just ZFNs and explore other technologies. A few of note are (Fig. 2): the recently emerging TAL effector nucleases (TALENs) (which are significantly more modular than ZFNs) [53, 54], adeno-associated viruses (which are efficient at targeting several human somatic cell types) [55, 56], gutless adenoviral vectors that allow high-level gene transfer and large cargos of longer homology arms for increasing HR [57], and bacterial artificial chromosome-based plasmid vectors with extremely large homology arms [58] are all active avenues that merit further exploration. We believe that development of enabling technologies on this front will be a very intense field of research in the near future, and progress here will have far reaching impact not just in regenerative medicine but also in the general field of gene therapy and disease modeling.

Ultimately, targeted differentiation and assembly into a transplantable tissue form of the disease-free iPS cells will be critical to achieve the goal of cell-based patient-specific therapies. Although there are still several hurdles to surpass and not



**Figure 2.** A human induced pluripotent stem (iPS) cell-based regenerative medicine paradigm. This approach proceeds in three steps and entails, first, collecting a patients somatic cells (such as their skin or blood cells); next, directly converting them into pluripotent stem cells (that is into cells which now have the ability to differentiate into all three germ layers); and then correcting their endogenous disease causing mutations to obtain immune-matched disease-free stem cells suitable for potential cell-based therapies. The appended table lists the various techniques for performing genetic modifications. Finally, the thus derived healthy stem cells are modulated through appropriate differentiation and assembly into a transplantable tissue form. Note that, the derived iPS cells can also serve as a valuable tool for basic science research, enabling disease modeling, and potential drug screening and toxicological studies of human cells that are otherwise not directly feasible in human subjects. Abbreviations: AAV, adeno-associated virus; BAC, bacterial artificial chromosome; DSB, double-stranded break; ES cell, embryonic stem cell; HR, homologous recombination; iPS cell, induced pluripotent stem cell; TALEN, TAL effector nuclease; ZFN, zinc-finger nuclease.



all differentiation paradigms are equally mature, nonetheless, examples of attainment of functional differentiated tissues are regularly emerging (such as in instances of neural, gut, and retinal differentiation, to name but a few) [59, 60]. Clearly, advances in the years to come will lead to further refinement of these technologies making them more efficacious and also eventually scalable to enable ready clinical use.

Of particular relevance has also been the recent growing interest in the field of transdifferentiation or lineage conversion, that is, the process of converting one somatic cell type to another. Demonstrations of successful reprogramming on this front have been rapidly increasing in recent literature. While the earliest among these were the conversion of fibroblasts into muscle cells decades ago [61], lately conversion of B lymphocytes into macrophages [62] and more recently of fibroblasts into neurons [63] and blood progenitors [64] has also been successfully effected. This was achieved following the forced expression of a few transcription factors that provide the necessary transformative force to the target tissue type of interest. While maturity of the derived tissues as well as overall scalability of these processes still needs to be demonstrated, these studies offer us an unprecedented insight into the key players that govern tissue specification. More importantly, we believe these studies will eventually guide us to development of techniques for both harvesting and subsequent faithful and sustained in vitro culturing of adult human tissues or tissue progenitors. This could potentially obviate the very need to ever reprogram cells! As in such a scenario, barring the step of correcting underlying mutations, one would simply need to transiently culture such tissues to the desired scale of

expansion before their eventual transplantation back into the patient to enable repair or replacement directed cell-based therapies.

Overall, we thus look forward to an exciting future in this highly interdisciplinary field of research. Several basic science discoveries are at the cusp of being unraveled as our understanding of human development and aging rapidly expands. Most importantly, this improved understanding is directly impacting the development of clinically translatable technologies for regenerative medicine.

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## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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## Concise Review: Multidimensional Regulation of the Hematopoietic Stem Cell State

IL-HOAN OH,<sup>a</sup> R. KEITH HUMPHRIES<sup>b</sup>

<sup>a</sup>Catholic High Performance Cell Therapy Center and Department of Medical Lifescience, The Catholic University of Korea, Seoul, Korea; <sup>b</sup>Terry Fox Laboratory, British Columbia Cancer Agency, Vancouver, British Columbia, Canada

**Key Words.** Stemness • Hematopoietic stem cell • Microenvironment • Epigenetic • Intrinsic regulators

### ABSTRACT

Hematopoietic stem cells (HSCs) are characterized by their unique function to produce all lineages of blood cells throughout life. Such tissue-specific function of HSC is attributed to their ability to execute self-renewal and multilineage differentiation. Accumulating evidence indicates that the undifferentiated state of HSC is characterized by dynamic maintenance of chromatin structures and epigenetic plasticity. Conversely, quiescence, self-renewal, and differentiation of HSCs are dictated by complex regulatory mechanisms involving specific transcription factors and

microenvironmental crosstalk between stem cells and multiple compartments of niches in bone marrows. Thus, multidimensional regulatory inputs are integrated into two opposing characters of HSCs—maintenance of undifferentiated state analogous to pluripotent stem cells but execution of tissue-specific hematopoietic functions. Further studies on the interplay of such regulatory forces as “cell fate determinant” will likely shed the light on diverse spectrums of tissue-specific stem cells. *STEM CELLS* 2012;30:82–88

Disclosure of potential conflicts of interest is found at the end of this article.

### INTRODUCTION

#### The Concept of Stem Cell “State” As Applied to Hematopoietic Stem Cells

The adult body is now known to retain multiple types of stem cells that are dedicated to the life-long maintenance and potential regeneration of a wide range of specific tissues. These cells not only have tissue-specific features such as location, growth factor responsiveness, cell surface characteristics, and differentiation potential but also share key functional properties of self-renewal, and in general, multipotential differentiation capacity. Hematopoietic stem cells (HSCs) have become a stem cell paradigm with their ability both to produce a multiplicity of functionally distinct blood cells throughout life and to reconstitute the hematopoietic system in myeloablated hosts. This picture of hematopoiesis and the central role of stem cells has also now been extended to the concept of leukemic stem cells as critical components in a leukemic cell hierarchy [1]. Recent studies point both to heterogeneity, or subsets, within the normal stem cell compartment [2] and to lineage-restricted progenitors as targets of leukemic stem cell transformation [3]. Such observations have led to the concept of considering stem cells as occupying a functional “state or sub-state” rather than connoting a particular stage of differentiation [4]. This point of view focuses

attention on the essential mechanisms that underlie the stem cell state and that potentially overlap between multiple types of stem cells. In the following sections, we briefly review emerging evidence notably from studies of hematopoietic stem cells indicating that the state of “stemness” is a complex outcome of multidimensional interactions between the stem cell and its environment that ultimately and critically impact on the epigenetic status of the HSC.

### INTRINSIC REGULATORS OF HSC

A large and still not fully characterized repertoire of molecules ranging from cell surface receptors through signal transduction molecules and a myriad of transcription factors are now recognized for their regulatory roles in HSCs. Among these so-called “intrinsic regulators,” transcription factors have attracted much attention given their essential roles in the initial development, expansion, and maintenance of HSCs [5]. Such attention has been reinforced by the understanding that many of these key transcription factors such as mixed-lineage leukemia (MLL), Runt-related transcription factor (AML1), and stem cell leukemia are also major players in leukemogenesis. Moreover, the engineered overexpression in normal HSC of HOXB4 or the variant fusion of HOXA10 and NUP98 among other transcription factors has provided a potent new

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Correspondence: Il-Hoan Oh, M.D., Ph.D., Catholic High Performance Cell Therapy Center, Department of Medical Lifescience, The Catholic University of Korea, 505 Banpodong, Seocho-Ku, Seoul 137-701, Korea. Telephone: 82-2-2258-8268; Fax: 82-2-591-3994; e-mail: iho@catholic.ac.kr; or R. Keith Humphries, M.D., Ph.D., Terry Fox Laboratory, British Columbia Cancer Agency, 675 West 10th Avenue, Vancouver, British Columbia, Canada V5Z 1L3. Telephone: 604 675 8140; e-mail: khumphri@bccrc.ca Received June 2, 2011; accepted for publication October 13, 2011; first published online in *STEM CELLS EXPRESS* November 14, 2011. © AlphaMed Press 1066-5099/2011/\$30.00/0 doi: 10.1002/stem.776

avenue to enhance the self-renewal of HSC for basic investigations and potentially clinical application [6–9]. Very recently the application of next generation sequencing to genome-wide analysis of hematopoietic transcription factors has provided remarkable new evidence that they operate in a complex combinatorial manner [10, 11]. These hitherto unrecognized multidimensional interactions between transcription factors and their targets provide new insights into the regulatory processes at play in HSC and place new demands on integrated analysis approaches for their study [12]. Such findings have also focused increased attention on the importance of epigenetic regulation as a way of coordinating the expression and activity of such transcription factors in both normal and leukemic stem cells.

## EPIGENETICS AND THE HSC STATE

### Epigenetic Regulation As a Critical Coordinator of Gene Expression Patterns

As described above, numerous studies have identified key transcription factors involved in the self-renewal of HSCs [13], and gene-expression patterns specific to primitive hematopoietic cells were identified [14, 15]. However, given that HSCs can undergo such an extensive spectrum of cell fate decisions from self-renewal to differentiation down myriad specialized pathways, a major question emerges—how on the one hand can a specific gene-expression pattern be maintained consistent with self-renewal and retention of multipotentiality versus gene-expression changes associated with loss of self-renewal and restriction of potential? Studies have shown that epigenetic modifications can change the expression of large sets of genes with changes in the chromatin structures [16], which can influence the accessibility of transcription factors to DNA and alter the transcription profile of cells [17]. The modification of chromatin structures is largely regulated by specific post-translational modifications of histones acting as switches between permissive or repressive chromatin [18]. The modification of histones includes acetylation, methylation, phosphorylation, sumoylation, and ubiquitylation [19]. In general, hyperacetylation of histone is associated with “open” chromatin, whereas histone deacetylation is associated with “condensation” and heterochromatin formation. Acetylation of histone is catalyzed by histone acetyl transferases including Gcn5-related N-acetyltransferases, MYSTs, and p300/c-AMP response element-binding protein, whereas deacetylation is catalyzed by four distinct families of histone deacetylases (HDACs). Histone is also modified by methylation on arginine or lysine residues. Although arginine methylation is usually associated with gene activation, lysine methylation is related to activation as well as repression depending on the specific residues modified (reviewed by Rice et al. [19]). For example, methylation in the H3K4, H3K36, and H3K79 is related to transcriptional activation, whereas methylation in H3K9, H3K27, and H4K20 is related to repression [20]. The methylation of H3K4 residues is catalyzed by MLL proteins to activate transcription, whereas methylation of H3K27 is catalyzed by polycomb (PcG) repressive complex (PRC)-2,3, which recruits PRC1 to establish repressive chromatin structures [19].

Methylation of CpG in DNA comprises another major category of epigenetic regulation. DNA methylation in promoter regions is associated with transcriptional silencing of genes by promoting the binding of MeCP2 [21], a transcriptional repressor that recruits HDACs to the methylated promoters [22]. DNA methylation is catalyzed by DNA methyltransfer-

ases (Dnmt)1 or Dnmt 3a/3b for maintenance or de novo methylation of DNA, respectively [17]. Thus epigenetic modification and alterations of chromatin structures are important mechanisms that can permit the establishment, maintenance, and changes of “en block” gene expression patterns likely critical to the determination of functional state and cell fates.

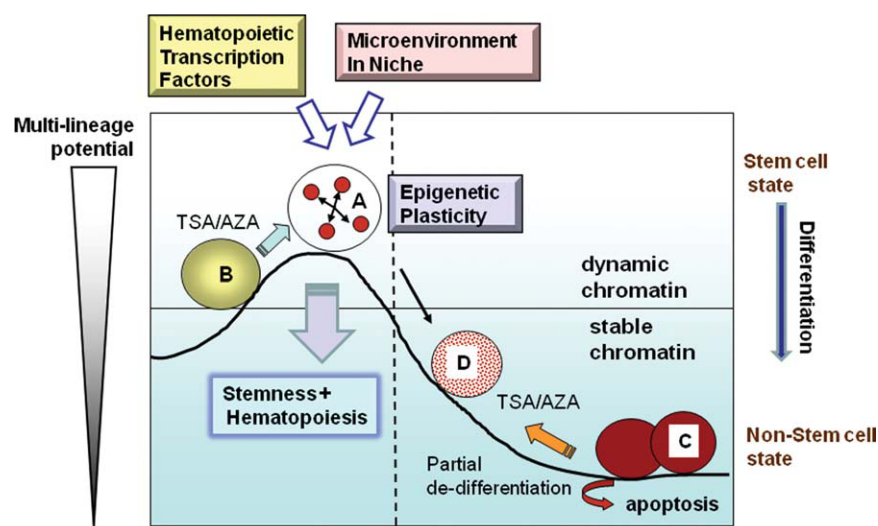
### Epigenetic Signature for Undifferentiated State of Stem Cells

Major insight into the possible roles of epigenetics in stem cell state has emerged from studies focused on the epigenetic status of undifferentiated pluripotent embryonic stem cells (ESCs). ESCs can be characterized by less-condensed chromatin structures [23, 24] leaving the chromatin more accessible to multiple transcription factors. Moreover, the pluripotent state of ESCs is characterized by “poised,” that is, “primed but held-in-check,” expression of lineage-associated regulatory genes. Such poised gene expression was primarily mediated by a bivalent mode of histone modification; that is, a positive regulatory chromatin mark (H3K4-methylation) is juxtaposed to a repressive chromatin mark (H3K27-trimethylation), where the methylation of H3K27 is catalyzed by PcG group proteins [23–25].

Of note, studies on histone modification of undifferentiated cells showed that chromatin exists in a dynamic equilibrium between open and “closed” states, maintaining “fluidity” of chromatin [26], and that these dynamic changes in the chromatin states may be mediated by nucleosome remodeling and histone acetylation [27]. Moreover, efficient acquisition of pluripotent state from somatic cells was dependent on the open chromatin state maintained by chromatin remodeling factor such as chd1 [28], and the cell reprogramming process was facilitated by chemical treatment that can cause decondensation of the chromatin structure [29, 30]. Thus poised expression and dynamic remodeling of chromatin comprise characteristics of pluripotent stem cells.

### Epigenetic Signature for HSCs

Growing evidence also point to a key role for epigenetic “signatures” in relation to HSCs and hematopoietic differentiation [19, 31]. For example, hematopoietic progenitor cells exhibit a promiscuous, low-level expression of lineage-specific genes before commitment [32, 33]. In addition, hematopoietic differentiation correlates to a stepwise decrease in the transcriptional accessibility of multilineage-affiliated genes [32, 34], and changes in the expression of lineage-specifying genes in hematopoietic progenitors were correlated with changes in chromatin structures in the promoter regions during differentiation [35, 36]. Analysis of lineage-associated genes in various stages of murine hematopoietic progenitors also revealed concerted epigenetic modifications of the selected hematopoietic genes by DNA methylation and histone modification [37]. Moreover, recent genome-wide analyses of hematopoietic progenitors and lineage-specific progenitors using comprehensive high-throughput array-based relative methylation revealed that lineage-specific differentiation is associated with modulation of DNA methylation [38]. Interestingly, differential DNA methylation with hematopoietic differentiation was more strongly correlated with DNA methylation in the CpG shore (regions within 2 kb of island) than in CpG islands. In addition, myeloid commitment involved less global DNA methylation than lymphoid commitment, which was supported by the finding for a myeloid shift of progenitors following methyltransferase inhibition [38]. Similar hypomethylation of myeloid cells was observed in a study using human hematopoietic progenitor cells, wherein distinct methylation patterns were also observed between young- and old-age progenitor cells



**Figure 1.** Schematic illustration of the model for the epigenetic integration of hematopoietic stem cell (HSC) state. Undifferentiated hematopoietic cells (A) have dynamic chromatin structures with high turn-over rate of epigenetic modification, being permissive to alterations in chromatin structures (as represented by multiple arrows in A). Treatment of undifferentiated cells with epigenetic chemicals (AZA/TSA) further enhance self-renewal potential of hematopoietic progenitors (B), whereas similar treatment of mature cells (C) leads to limited dedifferentiation (D) and apoptosis. Therefore, the undifferentiated state of HSCs can be represented by epigenetic plasticity and multilineage potential. In addition, hematopoietic transcription factors and microenvironmental regulation in the stem cell niche regulate tissue-specific hematopoietic functions of HSCs. Thus, the stem cell states of HSCs are determined by multidimensional integration of tissue-specific hematopoietic functions and the epigenetic plasticity of undifferentiated cells. Abbreviations: AZA, 5-azacytidine; TSA, trichostatin A.

[39]. These results show that DNA methylation is involved in the regulation of lineage-specific differentiation as well as aging-associated changes of hematopoietic progenitors.

Recently, one of us (I.-H. Oh) analyzed the genome-wide DNA methylation of undifferentiated human hematopoietic cells (CD34+) in comparison to differentiated cells (CD34-) and showed that undifferentiated cells were characterized by undermethylation at the transcription start site of the promoter region (a so call dip) and overmethylation of flanking regions [40]. Interestingly, the regions of undermethylation dip in CD34+ cells were significantly enriched with genes encoding nuclear proteins for chromatin remodeling, suggesting that the genes involved in the dynamic changes of chromatin structures are primed in the undifferentiated status. Moreover, we found that undifferentiated human and murine hematopoietic cells displayed less-condensed chromatin structures and exhibited a higher rate of histone acetylation in pulse-chase experiments, indicating that undifferentiated cells are in a state of higher turn-over of chromatin structures than differentiated cells [40]. This is highly reminiscent of the observation in ESCs that exhibit hyperdynamic chromatin proteins in a pluripotent state, but these proteins were immobilized on chromatin in the differentiated state [41]. Thus, it is possible that the undifferentiated state of hematopoietic cells can be characterized by a higher turn-over rate of epigenetic modifications to maintain dynamic state of chromatin, compared with more differentiated cells (schematically shown in Fig. 1).

### Functional Impact of Epigenetic Signature

The apparent unique epigenetic status of undifferentiated hematopoietic cells suggests important roles of epigenetic modifications in conferring HSC properties. In support of this view, lack of functional Dnmts was shown to cause defective self-renewal of HSCs. Specifically, conditional disruption of Dnmt3a and 3b, two enzymes responsible for de novo DNA methylation, did not overtly affect later hematopoietic progenitors or more primitive cells capable of tran-

sient lymphomyeloid engraftment. However, major defects were apparent in the long-term reconstitution of HSCs, thus indicating that de novo DNA methylation is required for self-renewal of HSCs [42]. Similarly, conditional disruption of Dnmt1, the enzyme for maintenance methylation of DNA, led to loss of HSC self-renewal and defective production of mature bone marrow (BM) cells over multiple lineages [43]. Interestingly, another study using a hypomorphic Dnmt1 allele revealed somewhat different outcomes with defective hematopoiesis both in myeloablative and nonmyeloablative conditions [44], whereas complete deletion of Dnmt1 led to a defective repopulation only in the "stressed" (transplantation into conditioned recipient) but not in the "steady" condition [43]. Moreover, donor cells from hypomorphic Dnmt1 exhibited a total lack of B-lymphopoiesis with a moderate decrease of myeloid reconstitution, whereas HSCs from Dnmt1-deleted mice exhibited profound decrease in myeloid potential but retained T- and B-lymphoid repopulating potential. In addition, the hypomorphic Dnmt1 allele did not show any defect in competitive homing into BMs, whereas deletion of Dnmt1 led to a defect in homing of HSCs exhibiting lower retention of HSC in the niche. The reasons for these differences in functional outcomes remain unclear, but it remains to be determined whether distinct biological impacts can be caused with respect to their differences in DNA methylation levels. Interestingly, DNA hypomethylation was also associated with defects in the self-renewal of leukemic stem cells with the Dnmt1 hypomorphic allele manifesting defective development of B-lymphoid leukemia and decreased leukemic stem cell self-renewal [44]. Taken together, these studies point to a critical role of DNA methylation in normal and malignant HSCs for regulation of self-renewal and hematopoiesis.

Similar to the influence of DNA methylation changes, histone modifications also exert diverse effects on HSC function. Some important examples include the observed loss of HSC self-renewal and HSC exhaustion on disruption of BMI1, a PcG protein in PRC1 [45]. Similarly, loss of long-term



repopulating HSCs was also observed after disruption of Mph1/Rae28, another PRC1 complex protein [46]. In contrast, loss of Ring1b or mice with hypomorphic mutations of Eed or Suz12, the components of the PRC2 complex, exhibited upregulated hematopoietic activities, indicating a crucial interplay between histone modifications and HSC regulation (reviewed by Konuma et al. [47]).

Further pointing the key roles for DNA methylation and histone modification on HSCs, series of studies have shown that treatment of hematopoietic progenitors with chemicals inhibiting DNA methylation and histone deacetylation during *in vitro* culture was associated with higher maintenance of the undifferentiated state and increased expansion of hematopoietic progenitors [48–50]. Moreover, *in vivo* administration of the epigenetic inhibitors trichostatin A (TSA, inhibitor of HDAC) and 5-azacytidine (AZA, inhibitor of Dnmt) resulted in enhanced self-renewal of transplanted HSCs [40]. Together, these studies suggest that tethering of chromatin into a “less dense” state can indeed promote higher maintenance of undifferentiated status and self-renewal of HSCs. Interestingly, the response of hematopoietic cells to such epigenetic modifiers was dependent on the degrees of maturation of the target hematopoietic cells. For example, when more mature ( $L^+S^-K^-$  or  $Lin^+$ ) hematopoietic cell populations were similarly treated with TSA or AZA, limited dedifferentiation was observed but was accompanied by extensive levels of apoptosis, the extent of which was inversely correlated to the degrees of undifferentiation [40]. This raises an interesting speculation that the more undifferentiated the cells are, the greater is their potential flexibility to epigenetic alterations of chromatin structures, and that such epigenetic flexibility is correlated to the hierarchy of undifferentiated state (model in Fig. 1).

### MICROENVIRONMENTAL REGULATION OF HSCs

#### Stem Cell Niche in BM

Although the precise transcriptional program operative and epigenetic modifications exert crucial regulatory influences on the HSC-state, growing evidence also point to the important regulatory role of the HSC microenvironment, or niche, in the BM. The microenvironmental regulation of HSCs mostly occurs in a specialized architecture of BMs, referred to as the stem cell niche, where the majority of HSCs reside and are regulated for self-renewal, quiescence, survival, and differentiation (reviewed by Oh [51]).

Current evidence has pointed to the existence of two types of niches in the BM, an endosteal osteoblastic and a vascular/perisinusoidal niche. The vascular niche is composed of reticular cells around the sinusoid or a subendothelial (adventitial) layer of sinusoidal walls, projecting a reticular process in close contact with HSCs in human BM [52]. Recent studies have indicated that sinusoidal endothelial cells (SECs) also constitute an endothelial niche, that is, infusion of endothelial progenitor cells was associated with higher recoveries of HSCs [53] and inhibition of vascular endothelial growth factor receptor 2 (VEGFR2) signaling during the recovery of BM prevented not only the regeneration of SECs but also the reconstitution of transplanted HSCs [54]. Although the osteoblastic and vascular niches share a common cellular origin as well as common growth factors, evidence also shows that HSCs are distinctively localized in these two types of niches depending on the physiological conditions of BMs [55, 56] suggesting that these two niches might have distinct functions for HSCs (concisely reviewed in [51]).

#### Microenvironmental Crosstalk in the Stem Cell Niche

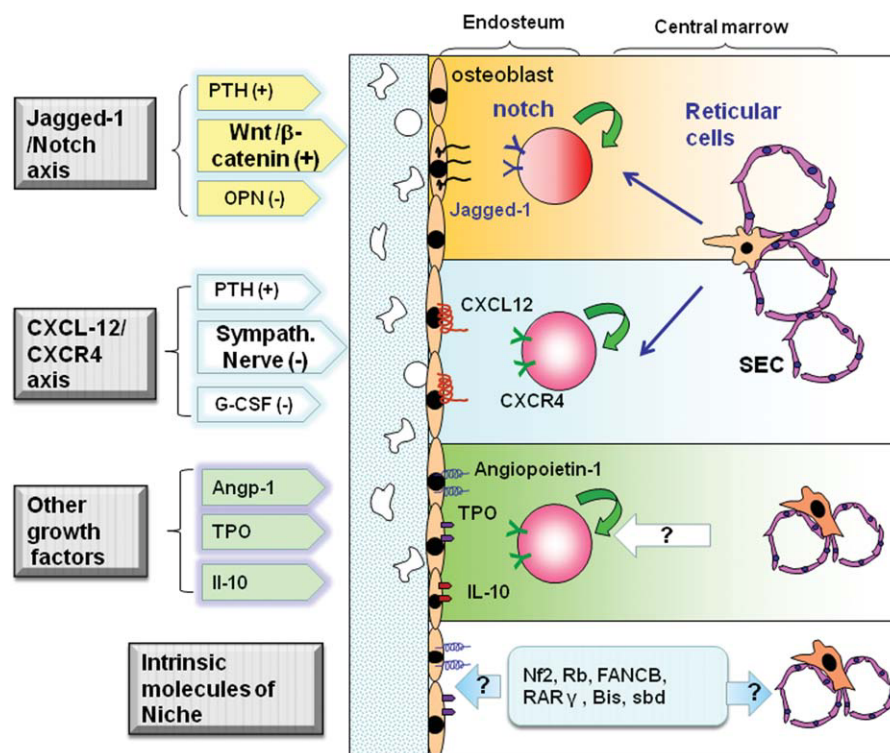
A large number of potential regulators have been identified and found to share some common modes of action in triggering of crosstalk between the niche and HSCs. Notable among these are crosstalk between jagged-1/notch signaling and convergence of signals to the chemokine (C-X-C motif) ligand 12/C-X-C chemokine receptor 4 (CXCL12/CXCR4) signaling axis (schematically shown in Fig. 2).

**Jagged-1/notch Axis in the Niche.** Parathyroid hormone (PTH)/PTH-related protein [57] has been shown to induce jagged-1 expression in osteoblasts being associated with increased HSC numbers and hematopoietic activity [57]. Wnt/ $\beta$ -catenin is also linked to jagged-1/notch axis in stroma [58]. Of note, our recent work has shown that distinct biological outcomes can be caused by wnt/ $\beta$ -catenin signals depending on the target site of their activation. Thus direct stabilization of  $\beta$ -catenin in HSCs resulted in the loss of their repopulating activity, whereas stabilization of  $\beta$ -catenin in the stroma led to enhanced self-renewal of HSCs in a contact-dependent manner [58]. Stromal activation of wnt/ $\beta$ -catenin signaling leads to induction of notch ligand and exerts a stimulatory effect on HSC in a notch signal-dependent manner, revealing a functional crosstalk in the stem cell niche [58]. Moreover, direct intrafemoral injection of  $\beta$ -catenin-activated MSCs stimulated self-renewal of transplanted HSCs several fold higher than the HSCs injected along with naïve MSCs [59] (further reviewed by Oh [60]). Osteopontin (OPN) is another signal implicated in notch regulatory axis. In an OPN-null microenvironment, the number of HSCs is increased in association with elevated stromal Jagged-1 and Angiopoietin-1 expression [61]. Of note, a study showed that endothelial cells, in addition to osteoblast, use the notch axis, that is, adenoviral gene E4–open reading frame–immortalized endothelial cells supported the expansion of the long-term repopulating HSCs in a manner dependent on notch signal activation in HSCs [62].

**CXCL-12/CXCR4 Signaling.** As described, reticular cells expressing high levels of CXCL-12 (CXCL-12 abundant reticular cells) are in contact with 90% of HSCs and its production is increased in the presence of DNA damaging agents (irradiation, 5-fluorouracil, cyclophosphamide) or by PTH activation [63]. In addition, recent studies in mice with defective nerve conduction showed that HSC mobilization by granulocyte colony-stimulating factor is dependent on the intact adrenergic nerve system and that norepinephrine downregulates osteoblast expression of CXCL-12 [64]. Thus, the CXCL-12/CXCR4 axis may serve as an important modulator of niche activity and, hence, HSCs, in response to environmental conditions or stress.

#### Other Growth Factors

In addition to crosstalks described above, a growing number of hematopoietic growth factors are being identified as microenvironmental factors. For example, thrombopoietin is produced in osteoblasts that are in close contact with long-term HSCs [65]. Similarly, angiopoietin-1 was shown to be expressed in the osteoblastic niche as well as in the reticular cells in the vascular niche of BM [52]. Of note, our study showed that interleukin 10, a pleiotropic cytokine regulating immune reaction, also functions as a growth factor promoting self-renewal of murine HSCs, and its production is induced in the endosteal osteoblast in response to the radiation stress on BM [66].



**Figure 2.** Microenvironmental regulation of hematopoietic stem cells (HSCs) in multiple niches of bone marrow. The microenvironmental regulation in niche can be categorized into several axis of crosstalk. First, extrinsic factors such as PTH signaling or canonical wnt/ $\beta$ -catenin induce jagged-1 in the stromal niche to activate notch in HSCs, whereas OPN suppresses the induction of jagged-1. Thus jagged-1/notch axis represents one of conserved elements of crosstalk in the stem cell niche (yellow area). Second, several signals including PTH or 5-FU induce the expression of CXCL-12, whereas G-CSF or sympathetic nerve system downregulates CXCL-12 to release HSCs, indicating that CXCL-12/CXCR4 axis represents another axis of crosstalk (blue area). Third, growth factors such as angiopoietin-1, TPO, or IL-10 exert their effects in conjunction with stroma in the bone marrow niche (green area). Finally, intrinsic molecules such as Nf2, Rb, FANCB, RAR- $\gamma$ , Bis, or Sbd influences niche activities in a yet poorly defined manner but modulate hematopoietic activity in a stroma-dependent manner (white area). Notably, Jagged-1 or CXCL-12 axis is also similarly shared by cells in the vascular niche (reticular cells or SEC; marked by blue line arrows). (+) represent upregulation, (–), downregulation, curved arrows represent self-renewal of HSCs. Abbreviations: CXCL, chemokine (C-X-C motif) ligand; CXCR, C-X-C chemokine receptor 4; FU, 5-fluorouracil; G-CSF, granulocyte colony-stimulating factor; IL, interleukin; OPN, osteopontin; PTH, parathyroid hormone; SEC, sinusoidal endothelial cell; RAR $\gamma$ , retinoic acid  $\gamma$ ; TPO, thrombopoietin.

**Intrinsic Molecules That Control the HSC Niche.** In addition to extrinsic growth factors playing a role in the HSC niche, recent studies are beginning to identify intrinsic molecules that can regulate the activity or integrity of the hematopoietic niche. For example, in mice with a disruption in Nf2/merlin, HSC frequencies are increased and shifted into the circulation with an associated increase in trabecular bone mass and stromal cell numbers, as well as vascularity and VEGF levels [67]. Conversely, our recent study showed that targeted disruption of bis, the gene encoding antiapoptotic protein interacting with Bcl-2, led to loss of HSCs with selective deterioration of the vascular niche accompanied by loss of CXCL12 expressing stromal cells in BM but without affecting the osteoblastic niche [68]. Similarly, loss of the murine homolog of FANCB led to microenvironmental defects mimicking the hematological signs of Fanconi anemia, that could be rescued by the adoptive transfer of wild-type MSCs [69].

Of note, alteration of the niche can also lead to a pathological microenvironment leading to abnormal hematopoiesis. For example, mice deficient in retinoic acid  $\gamma$  develop a myeloproliferative syndrome in a microenvironment-dependent manner [70]. Similarly, the disruption of Rb causes a defective interaction in hematopoietic cells with the microenvironment leading to the myeloproliferative disease of BMs and mobilization of primitive cells into extramedullary organs

[71]. More recently, deletion of Dicer1 specifically from the osteoprogenitor cells reduced expression of sbd gene, which led to BM dysfunction and myelodysplasia due to stromal dysfunction [72]. Taken together, these findings now implicate the microenvironment as a new entity that has the ability to mediate the regulation of the hematopoietic activity of HSCs during physiological as well as abnormal disease conditions.

## CONCLUSION

Hematopoietic stem cells can be considered to occupy a unique functional and molecular state represented by maintenance of multilineage differentiation potential and self-renewal capacity. As reviewed, the undifferentiated state of HSCs is maintained by unique epigenetic signatures including epigenetic plasticity and bivalent modifications, akin to signatures of pluripotent stem cells. However, the unique tissue-specific functions of HSCs are also regulated by hematopoietic transcription factors and microenvironmental factors being integrated into HSC identity. Emerging evidence also suggest functional connection between extrinsic growth factors and epigenetic modifications and of extrinsic factors and transcription factors. Thus HSCs exist and function by virtue

of multidimensional regulatory mechanisms to simultaneously carry out the two opposing properties of HSCs, that is, maintenance of undifferentiated state analogous to pluripotent stem cells but execution of tissue-specific hematopoietic functions. Thus the identify of stemness in HSCs should be considered as a net interplay of those genetic, epigenetic, and microenvironmental elements integrated together, rather than a master regulatory force by limited regulatory forces (schematically drawn in Fig. 1). It is also likely that such interplay of multiple regulatory forces as a “determinant” of cell fate could be also extrapolated toward diverse spectra of tissue-specific stem cells. Further studies on the interplay of such regulatory mechanism will shed the light into stemness and regenerative function of tissue-specific stem cells.

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## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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## Concise Review: Cancer Stem Cells and Minimal Residual Disease

GABRIEL GHIAUR,<sup>a</sup> JONATHAN GERBER,<sup>a</sup> RICHARD J. JONES<sup>a,b</sup>

<sup>a</sup>Johns Hopkins School of Medicine, Department of Medicine, Division of Hematology, Baltimore, Maryland, USA; <sup>b</sup>The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, Maryland, USA

**Key Words.** Cancer • Leukemia • Lymphoma • Adult stem cells • Clinical translation

### ABSTRACT

Evidence gathered over the past two decades confirms earlier reports that suggested that hematologic malignancies exhibit a hierarchical differentiation structure similar to normal hematopoiesis. There is growing evidence that some solid tumors may also exhibit a differentiation program similar to the normal tissue of origin. Many excellent reviews on the topic of cancer stem cells (CSCs) document the recent explosion of information in the field, particularly highlighting the phenotypic and functional characteristics of these putative cells in vitro. Accordingly, here we only briefly discuss these concepts, and instead

primarily examine the potential clinical relevance of CSCs, arguably the major unresolved issue in the field. Although it is generally accepted that CSCs are resistant to chemotherapy in vitro, only recently have data surfaced that suggest a role for these cells in disease relapse. Importantly, cancer cells with a stem cell phenotype have been found to be enriched in minimal residual disease of several malignancies. If the role of CSCs in relapse is confirmed, targeting these cells would hold substantial potential for improving the outcome of cancer patients. *STEM CELLS* 2012;30:89–93

Disclosure of potential conflicts of interest is found at the end of this article.

### HISTORICAL PERSPECTIVE

First formulated by Nordling in 1953 [1], the theory that cancer results from an accumulation of DNA mutations was further refined by Ashley [2], Knudson [3] and Nowell [4]. In this model of carcinogenesis, inherited mutations and/or environmental carcinogens lead to the development of premalignant clones. These cells further accumulate genetic hits until one cell reaches a critical genetic or epigenetic state that confers a growth and/or survival advantage over its normal counterparts. Over time, if it can evade the immune system, this abnormal cell would give rise to a malignant tumor. In the purest sense, the cell that suffered the “critical insult” is the primordial cancer-initiating cell and the tumor is its clonal expansion.

As postulated by Ashley, a cancer-initiating cell must survive long enough to accumulate three to seven genetic mutations necessary to generate cancer [2]. Moreover, it must already manifest proliferative capacity or, alternatively, develop it anew as a consequence of genetic mutation(s). Nowell [4] hypothesized that the inherent longevity and extensive proliferative capacity of a tissue stem cell make it an ideal candidate cancer-initiating cell. In contrast, most terminally differentiated cells are neither long-lived nor possess the ability to produce tumors with the limited number of divisions remaining in their differentiation program. Such cells could only acquire the multiple genetic mutations required for malignant tumor growth if such mutations occurred simultaneously or in rapid succession (e.g., as in the generation of induced pluripotent

stem cells). However, longevity and extensive proliferative capacity are not traits restricted to classic normal tissue stem cells. To some degree, myeloid progenitors beyond the level of hematopoietic stem cells (HSCs) also retain these properties [5]. Moreover, within the lymphoid system, self-renewal capacity is preserved during differentiation through the memory lymphocyte stage to maintain life-long immunity [6].

The cancer stem cell (CSC) concept would explain why only a minority of cells from most hematologic malignancies and solid tumors are clonogenic in vitro and in vivo. In this CSC model, the cancer-initiating event, while conferring some advantages to the original cancer cell, does not completely alter its differentiation program; the malignant tumor would thus consist of a heterogeneous population of cells including the differentiated progeny of the original cell, mimicking to an extent the hierarchical structure of the normal tissue of origin. Since the primordial cancer-initiating cell or one of its progeny in this model possesses self-renewal capability and at least some differentiation potential—two of the defining features of normal stem cells—this cell naturally came to be called a CSC. Alternatively, it is also conceptually possible that the low clonogenicity of cancer is the result of all cells within a cancer retaining the capacity to proliferate but only at a low rate. Which of these two scenarios account for the low clonogenicity of most cancers has been debated for years. The first evidence supporting the CSC concept was published more than 40 years ago, when Fialkow et al. [7] demonstrated clonal hematopoiesis involving both the erythroid and myeloid lineages in patients with chronic myeloid leukemia (CML).

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Correspondence: Richard J. Jones, M.D., The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Room 244, Bunting-Blaustein Cancer Research Building, 1650 Orleans Street, Baltimore, Maryland 21231, USA. Telephone: 443-287-7104; Fax: 410-502-7213; e-mail: rjjones@jhmi.edu Received September 30, 2011; accepted for publication October 6, 2011; first published online in *STEM CELLS EXPRESS* November 1, 2011. © AlphaMed Press 1066-5099/2011/\$30.00/0 doi: 10.1002/stem.769

**Table 1.** Phenotype of AML CSC

Phenotype	Xenograft model	Comments	References
CD34+CD38–	NOD/SCID		Lapidot et al. [13]
CD34+CD38+	NOD/SCID, NOD/SCID/ $\beta 2m^{-/-}$ , NOD/SCID/IL2R $\gamma^{-/-}$		Taussig et al. [14]
CD34+CD123+	NOD/SCID		Jordan et al. [15]
CD44+	NOD/SCID	Neutralizing antibodies reduces AML CSC	Jin et al. [16]
CD96+	NOD/SCID	Neutralizing antibodies reduces AML CSC	Jin et al. [17]
CD34+CD38–CLL1+	Newborn Rag2–/– $\gamma c^{-/-}$		Hosen et al. [18]
CD34+CD38–CLL1+	NOD/SCID		van Rhenen et al. [19]
CD34–	NOD/SCID/ $\beta 2m^{-/-}$ , NOD/SCID/IL2R $\gamma^{-/-}$	Only NPM1+AML	Taussig et al. [20]
Lin–CD38–	NOD/SCID/IL2R $\gamma^{-/-}$	Lin–CD38– fraction had the highest AML CSC frequency but all populations showed some AML CSC activity	Sanjiv et al. [21]

Abbreviations: AML, acute myeloid leukemia; CD, cluster of differentiation; CLL1, C lectin like molecule 1; CSC, cancer stem cell; IL2R $\gamma^{-/-}$ , interleukin 2 receptor gamma knock out; NOD/SCID: nonobese diabetes/severe combined immunodeficiency; NPM1, nucleophosmin; Rag2–/–, recombination activating gene 2 knock out;  $\beta 2m^{-/-}$ , beta-2 microglobulin knock out.

## IDENTIFYING AND CHARACTERIZING CSCs

### Myeloid Malignancies

Probably not surprisingly, given that hematopoiesis is the best characterized somatic stem cell system, CSCs have been best characterized in hematologic malignancies. The stem cell origin of CML was confirmed nearly 20 years ago when several groups, using characteristics known to define normal HSCs, identified and isolated CML cells capable of expansion *ex vivo* [8–10]. Dick and colleagues extended these observations, showing that primitive HSCs purified from patients with CML would generate leukemia *in vivo* when injected into nonobese diabetes/severe combined immunodeficiency (NOD/SCID) mice [11]. Moreover, the expression patterns of CML stem cells closely resemble those of normal HSCs [12]. Thus, the accumulated evidence over the last 15 years suggests that CML stem cells share many properties with, and likely arise from, normal HSCs. Thus, there is now universal agreement that the cancer-initiating event in CML, the Philadelphia (Ph) chromosome, occurs in an early hematopoietic cell if not the HSC itself.

Acute myeloid leukemia (AML) was the first cancer in which malignant cells with the ability to recapitulate the disease in a NOD/SCID mouse were identified [13]. These AML stem cells not only reproduced the disease in NOD/SCID mice but also possessed self-renewal capacity and exhibited an HSC phenotype. However, the exact surface phenotype of AML stem cells continues to be a subject of debate, possibly because of the heterogeneity of AML. Nevertheless, most studies suggest that, like CML, most cases of AML arise from phenotypic HSCs. Thus, markers of HSCs, including CD34, absence of CD38 and lineage-specific markers, CD133, and expression of aldehyde dehydrogenase (ALDH) have been widely used to identify and isolate putative AML stem cells (Table 1).

### Other Hematologic Malignancies

The first modern use of the term cancer or tumor stem cells was probably by Bergsagel and Valeriote [22], who found that only a minority mouse multiple myeloma cells were capable of clonogenic growth. Subsequent studies by Hamburger and Salmon [23] confirmed these findings with clinical myeloma specimens, revealing a cloning efficiency ranging from approximately 1:1,000 to 1:100,000 cells. Insufficient

tools existed at the time to distinguish whether this low clonogenic potential was the result of proliferative capacity exclusively restricted to a small subset of cancer cells or by all cancer cells retaining the capacity to proliferate but only at a low rate. Work from our laboratory suggests that the cancer-initiating cells in myeloma are found within the memory B-lymphocyte population, with the CD138<sup>+</sup> plasma cells terminally differentiated progeny of these malignant myeloma B cells [24]. These malignant CD138<sup>neg</sup> myeloma B cells expressed CD19, CD20, and CD27, along with high levels of ALDH. Moreover, myeloma CSCs and the plasma cells that comprise the bulk of the tumor exhibited disparate drug sensitivities. The CSCs seem to be resistant to most clinically active agents (e.g., dexamethasone, lenalidomide, bortezomib), perhaps in part by co-opting normal stem cells' intrinsic defense mechanisms such as quiescence, efflux pumps, and detoxifying enzymes [24, 25].

Hodgkin and Reed-Sternberg (HRS) cells, the hallmark of classic Hodgkin lymphoma (HL), also belong to the B lymphoid lineage. However, they are unlike any normal cells of that lineage, and their limited proliferative potential belies the clinical aggressiveness of the disease. More than 20 years ago, Newcom et al. [26] identified a population of cells that phenotypically resembled B cells and appeared to be responsible for the propagation of an HL cell line *in vitro*. Our group recently confirmed these findings in several other HL cell lines [27]. Moreover, clonotypic memory B cells with a similar phenotype to myeloma CSCs could be isolated from the peripheral blood of most newly diagnosed HL patients, regardless of stage, and these B cells and the patients' HRS cells exhibited identical clonal immunoglobulin gene rearrangements. Clonotypic CD19<sup>+</sup>CD5<sup>+</sup>ALDH<sup>high</sup> B cells were also identified in human mantle cell lymphoma (MCL) cell lines, as well as in patients with newly diagnosed MCL [25]. These cells were found to be relatively quiescent and resistant to many classic chemotherapeutic agents used to treat this condition.

### Solid Tumors

Identification and characterization of CSCs from hematologic malignancies was founded on decades of biologic experience in human hematopoiesis, including well-understood purification methodology and both *in vivo* and *in vitro* functional assays. Limited understanding of the biology of their normal counterparts has hampered the study of solid tumor CSCs, if they indeed exist. Thus, initial research into CSCs in solid

**Table 2.** Phenotype of cancer stem cell in various human solid malignancies

Cancer type	Phenotype	Xenograft model used	References
Breast	CD44+CD24–Lin– ALDH1+	NOD/SCID NOD/SCID	Al-Hajj et al. [28] Ginestier et al. [29]
Brain	CD133+	NOD/SCID	Singh et al. [30]
Glioblastoma	CD133+	nu/nu	Bao et al. [31]
Lung	CD133+Ep-CAM+	NOD/SCID	Eramo et al. [32]
Prostate	Side population	NOD/SCID	Patrawala et al. [33]
	CD44+	NOD/SCID	Patrawala et al. [34]
	CD44+/ $\alpha 2\beta 1$ + /CD133+	Methylcellulose progenitor assay	Collins et al. [35]
Colon	CD133+	NOD/SCID	O'Brien et al. [36]
	CD44+ /Ep-CAM+	NOD/SCID	Ricci-Vitiani et al. [37]
Melanoma	ABC5+	NOD/SCID	Schatton et al. [38]
	1:4 unselected cells	NOD/SCID/IL2R $\gamma$ -	Quitana et al. [39]
Liver	CD90+CD44+	SCID/Beige, BALB/c	Yang et al. [40]
Pancreas	ALDH1+	NOD/SCID	Rasheed et al. [41]
	CD133+	NMRI-nu/nu	Hermann et al. [42]
	CD44+CD24+ESA+	NOD/SCID	Li et al. [43]
Head and neck	CD44+Cytokeratin 5/14+	NOD/SCID	Prince et al. [44]

Abbreviations: ABCB5, ATP-binding cassette subfamily B member 5; ALDH1, aldehyde dehydrogenase 1; CD, cluster of differentiation, Ep-CAM, epithelial cell adhesion molecule; ESA, epithelial specific antigen; IL2R $\gamma$ -/-, interleukin 2 receptor gamma knock out; Lin, lineage; NOD/SCID, nonobese diabetes/severe combined immunodeficiency; NMRI, Naval Medical Research Institute; nu/nu mice, homozygous nude mice.

tumors was based on findings in liquid malignancies (Table 2). Accordingly, breast CSCs, initially described as CD44<sup>+</sup>CD24<sup>low</sup>, were identified by their ability to generate tumors in immunodeficient mice [28]. This description was followed quickly by the discovery of CSCs expressing CD133 in brain cancers [45]. Since then, although the importance of any specific marker for CSC identification remains unclear, multiple malignancies have been shown to contain a stem-cell like population capable of initiating tumors in a xenograft model (Table 2). Similar to hematologic CSCs, solid tumor CSCs have been found to be relatively more resistant to cytotoxic therapy than the differentiated cells that make up the bulk of the tumor mass [31].

### Controversy

Although cells meeting the definition for CSCs have now been described in many malignancies, there remains healthy skepticism about their true biologic significance. In fact, many investigators have proposed that CSCs may be nothing more than laboratory curiosities, simply reflecting the limitations of NOD/SCID mice for assessing tumorigenic potential [39, 46, 47]. This controversy is highlighted by a study which compared the growth of primary melanoma cells in NOD/SCID mice with the more immunocompromised NOD/SCID interleukin-2 receptor gamma chain null (NOG) mice. Although only about 1:100,000 unselected melanoma cells produced tumors in NOD/SCID mice, as few as 1:4 melanoma cells were tumorigenic when transplanted into NOG mice [39]. However, despite being considered the gold standard assay for CSCs by many in the field, there is no reason to assume that growth in immunocompromised mice is in fact a relevant assay for CSC activity.

Analogous to HSCs' dependence on their interactions with the stem cell niche [48], the microenvironment is likely critical for CSCs. For instance, a malignant niche rich in proinflammatory cytokines (e.g., interleukin [IL]-1, IL-6, tumor necrosis factor  $\alpha$ ) might promote and maintain cells from a variety of cancers [49]. CSCs are likely to cultivate continuous interactions with their microenvironment via a variety of surface molecules, including CD44, epithelial cell adhesion molecule, CD24, and CXCR4. It has also been shown that

high levels of IL-4 and IL-10 in the malignant niche can protect CSCs from Fas–Fas ligand-mediated apoptosis [50]. It is within this nurturing microenvironment that CSCs grow, endure chemotherapy, and possibly evade immune surveillance to initially form a tumor and later cause disease relapse. However, essential interactions between CSCs and their malignant niche are likely disrupted in xenograft models. Accordingly, it is possible that injecting human tumor cells into a mouse primarily tests metastasis-initiating cells rather than cancer-initiating cells. Recent findings have also implicated the microenvironment in determining the pattern of metastatic spread [51]. Interestingly, while circulating cancer cells can be found early in the clinical course of malignancies [27], most cases of relapse occur at the site of the original tumor. The lack of an adequate “premetastatic niche” may explain why metastases are not more of a regular occurrence in the presence of circulating tumor cells.

### Minimal Residual Disease and CSCs

Presumably, the most clinically important cancer cells are those that survive therapy and lead to relapse, whether they are tumorigenic in immunocompromised mice or not. Even if every cell in a cancer possessed tumorigenic potential, the presence of a discrete subset responsible for treatment resistance—perhaps as a result of stem cell properties—would have undeniable clinical significance. The CSC concept potentially explains not only the low clonogenic capacity of most malignancies but also why complete treatment responses rarely translate into cures for cancer patients: initial responses in cancer represent therapeutic effectiveness against the bulk cancer cells, while rarer but more resistant CSCs theoretically are responsible for relapse. However, even in the case of leukemia where the most evidence for the CSC concept exists, there is little proof that CSCs have any relevance to clinical practice.

If CSCs are indeed more resistant to therapy than the bulk tumor cells and thus responsible for relapse, then minimal residual disease (MRD) after treatment should be enriched for these cells. Furthermore, the presence of CSCs after therapy should predict recurrence. Indeed, it has recently been found that residual breast tumor cell populations persisting after

conventional treatment are enriched for phenotypic breast CSCs [52]. Similarly, patients with deletion 5q myelodysplastic syndrome (MDS) continue to have a population of phenotypically distinct MDS stem cells (CD34<sup>+</sup>CD38<sup>low</sup>CD90<sup>+</sup>), even in complete clinical and cytogenetic remissions [53]; these cells appear resistant to lenalidomide treatment and may account for disease relapse. Our group also showed that there was a strong and significant association between myeloma CSC numbers and progression-free survival in patients after treatment with rituximab [54]. Interestingly, rituximab was detected on the surface of circulating myeloma CSCs in patients who progressed; thus, rituximab was able to target but not kill the myeloma CSCs in those patients. Our recent data also demonstrate that MRD in AML has a stem cell phenotype, and the presence or absence of AML CSCs after therapy correlates with progression-free survival [55].

These data, perhaps for the first time, provide evidence of clinical relevance for CSC's. They also suggest that studying MRD may prove to be an excellent tool for better defining CSCs. Screening for CSCs after treatment might provide an early window into prognosis and help personalize treatment.

## CONCLUSIONS

There remains a healthy skepticism regarding the CSC concept. The uncertainty is based on discrepant phenotypic findings, conflicting results from the current gold standard xenograft transplant assay, and limited evidence for clinical significance. However, CSCs need not phenotypically mirror normal stem cells or be homogeneous within a tumor type. Moreover, xenograft transplantation may not be the optimal model for testing cancer initiation and may more aptly measure metastasis-initiating cells.

Importantly, new data suggest that cancer cells with stem cell characteristics are enriched in the MRD responsible for disease relapse. If CSCs are indeed proven to be clinically relevant, targeting these cells holds substantial translational

potential. First, there may be a role for intensification of treatment in patients with persistent CSCs after initial therapy. Second, emerging data suggest that CSCs across a wide spectrum of malignancies exhibit similar stem cell biology and rely on similar mechanisms to outcompete the normal tissue (e.g., efflux pumps, Hedgehog signaling [56], and telomerase expression [57]). While the bulk cells of various tumors have distinct biology and thus require distinct treatments, the therapies targeting CSCs of different malignancies may prove to be more universally applicable. Using such treatments either in addition to debulking therapy in the upfront setting, or as subsequent maintenance therapy, may improve cure rates. Third, similar to normal tissue stem cells, the microenvironment may play a crucial role in the behavior of CSCs. Accordingly, microenvironment-directed therapies may impact disease biology and improve clinical outcomes. Finally, tools developed through CSC research may allow a better understanding of key cancer-initiating events, such as the influence of chronic inflammation, environmental exposures, and nutrition. Such studies have proved difficult when looking only at the bulk tumor. Ultimately, the clinical translation of ongoing investigations into CSC biology will provide the final verdict as to whether CSCs are really just laboratory curiosities or truly represent a relevant part of cancer biology.

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## DISCLOSURE OF POTENTIAL CONFLICT OF INTEREST

The authors indicate no potential conflicts of interest.

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