Alternative Sources of Pluripotent Stem Cells: Ethical and Scientific Issues Revisited

Maureen L. Condic¹ and Mahendra Rao^{2,3}

Stem cell researchers in the United States continue to face an uncertain future, because of the changing federal guidelines governing this research, the restrictive patent situation surrounding the generation of new human embryonic stem cell lines, and the ethical divide over the use of embryos for research. In this commentary, we describe how recent advances in the derivation of induced pluripotent stem cells and the isolation of germ-line-derived pluripotent stem cells resolve a number of these uncertainties. The availability of patient-matched, pluripotent stem cells that can be obtained by ethically acceptable means provides important advantages for stem cell researchers, by both avoiding protracted ethical debates and giving U.S. researchers full access to federal funding. Thus, ethically uncompromised stem cells, such as those derived by direct reprogramming or from germ-cell precursors, are likely to yield important advances in stem cell research and move the field rapidly toward clinical applications.

Introduction

THE FIELD OF STEM cell research has faced a number of challenges, on scientific, ethical, and practical fronts. Although the political and regulatory environment surrounding human embryonic stem cell (hESC) research continues to shift, changes in recent months have brought both greater clarity in some domains and greater uncertainty in others. As we have noted [1], the ethical and practical issues raised by research on stem cells obtained from human embryos have a negative impact on the field, limiting the availability of venture capital and investment by private industry. Contributing to this unfavorable investment climate is the 2001 policy of the Bush Administration to limit availability of federal funding to existing stem cell lines. Moreover, essentially all existing hESC lines in the United States were covered by the broad protections granted to stem cell patents held by the University of Wisconsin Alumni Research Foundation (WARF), based on the work of Dr. James Thomson, who first isolated pluripotent stem cells from human embryos. The WARF patents created a virtual monopoly in control of ESC lines. These restrictions have placed U.S. researchers at a competitive disadvantage relative to other countries [1].

In addition to the barriers imposed by limited funding for hESC research and the restrictive WARF patents, public opinion also has a negative impact on the field. The American public remains deeply divided over the ethics of research on human embryos, with views on this topic being both complex and at times contradictory [2]. The most recent Gallup

poll, conducted in early 2009, indicates that nearly 80% of respondents favor some restrictions on hESC research, with only 14% favoring no restrictions. Somewhat paradoxically, this same poll indicates that 57% of respondents view hESC research as “morally acceptable,” demonstrating that opinions are not easily characterized as either strictly in favor or strictly opposed (www.gallup.com/poll/21676/Stem-Cell-Research.aspx).

The ethical concerns regarding research on hESCs have impacted both the social climate surrounding this research and the availability of public monies. For example, the Human Embryo Research Ban (the Dickey Amendment), first enacted under the Clinton administration and reauthorized by every U.S. Congress since 1995, prohibits the use of federal funds for “the creation of a human embryo or embryos for research purposes; or ... research in which a human embryo or embryos are destroyed, discarded, or knowingly subjected to risk of injury or death” (the 2009 text of this amendment can be found under H.R.2880, section 128 at <http://thomas.loc.gov/cgi-bin/bdquery/z?d104:HR02880>). Thus, long-standing federal legislation has significantly restricted the use of public monies to support hESC research, out of concern for the destruction or harming of nascent human life.

Current Regulatory and Funding Climate

In the last year, the federal guidelines governing stem cell research have changed considerably, bringing both expanded opportunities for research and new uncertainties

¹Department of Neurobiology and Anatomy, University of Utah School of Medicine, Salt Lake City, Utah.

²Life Technologies, Frederick, Maryland.

³John Hopkins School of Medicine, Baltimore, Maryland.

regarding federal funding. On March 9, 2009, President Obama issued executive order 13505 (www.whitehouse.gov/the_press_office/Removing-Barriers-to-Responsible-Scientific-Research-Involving-Human-Stem-Cells/). This order revoked the policies of former President Bush that allowed federal funding for research on existing hESC lines and promoted funding of research using stem cells from ethically acceptable alternative sources but did not establish a new policy for stem cell research. Obama's order only stipulated that within 120 days, the Director of the National Institutes of Health (NIH), "shall review existing NIH guidance and other widely recognized guidelines on human stem cell research, including provisions establishing appropriate safeguards, and issue new NIH guidance."

After a period of public input resulting in nearly 50,000 comments, the NIH issued new guidelines for hESC research (<http://stemcells.nih.gov/policy/2009guidelines.htm>), which have drawn decidedly mixed reviews [3,4]. The new policy has reduced, but not eliminated, federal funding restrictions (notably, the Dickey amendment remains in place). Moreover, concerns regarding the eligibility of existing hESC lines for funding and the stringent requirements for informed consent have created considerable uncertainty. Thus, although the new federal policy and the NIH guidelines are likely to expand the number of hESC lines eligible for federal funding, they have not created an unrestricted funding climate for this research.

Regrettably, neither the executive order nor the new NIH guidelines adequately addresses the central ethical issues confronting the field. For example, although the guidelines "recognize the distinction, accepted by Congress, between the derivation of stem cells from an embryo that results in the embryo's destruction, for which federal funding is prohibited, and research involving hESCs that does not involve an embryo nor result in an embryo's destruction, for which federal funding is permitted" (<http://stemcells.nih.gov/policy/2009guidelines.htm>), no ethical justification for this distinction is put forward. Indeed, the NIH identifies only 2, relatively simple principles as the basis for its guidelines on human stem cell research: "1. Responsible research with hESCs has the potential to improve our understanding of human health and illness and discover new ways to prevent and/or treat illness. 2. Individuals donating embryos for research purposes should do so freely, with voluntary and informed consent." Remarkably, the guidelines do not acknowledge the significant ethical concerns raised by research on human embryos, and therefore, they do not articulate any principles for resolving these concerns. Thus, although the new policy is likely to expand funding for stem cell research, it does little to balance the ethical controversies surrounding hESC research.

In addition to the new NIH research guidelines, the legal status of the WARF patents has also been addressed within recent months. Under these patents, WARF controls the distribution of all hESC lines that have been or will be derived, as well as the methods of stem cell derivation. These broad patent protections were called into question in 2007 (www.nature.com/stemcells/2007/0711/071108/full/stemcells.2007.113.html), initiating a review of the WARF patents by the U.S. Patent and Trademark Office (USPTO). In March 2008, USPTO concluded its reexamination and ruled to uphold the validity of the WARF patents [5]. Consequently, the

derivation of new hESC lines in the United States continues to be restricted by the broad WARF protections, and relative to researchers in other countries, U.S. scientists remain at a competitive disadvantage in hESC research. Accentuating this disadvantage, the European Union has recently rejected claims related to the WARF patents, based on the fact that the European Patent Convention forbids patenting of "a method which necessarily involved the destruction of the human embryos" (text of the decision available at www.epo.org/topics/news/2008/20081127.html). To address the constraints facing U.S. scientists, it is of considerable interest to science, medicine, and industry to develop sources of pluripotent stem cells that fall outside the broad WARF patent claims and are thereby eligible to obtain the protection of new stem cell patents.

Alternative Sources of Pluripotent Stem Cells

In light of the practical and ethical issues confronting hESC research, a critical question for the future of the field is whether alternative methods of deriving pluripotent stem cells can circumvent these longstanding barriers, thereby attracting both public and private support. Sources of stem cells that simultaneously satisfy ethical concerns (thereby meeting federal funding eligibility standards) and fall outside the WARF patent claims (thereby becoming eligible to obtain the protection of new stem cell patents) would clearly be the most attractive alternatives for both research and industry. We have recently discussed the relative merits of different alternative stem cell sources in considerable detail [1,6]. In the last year, important scientific advances have been made using pluripotent stem cells derived from 2 ethically uncontroversial sources that fall outside of the WARF patent protections: induced pluripotent stem cells (iPSCs) and stem cells derived from germ-line progenitors.

Induced pluripotent stem cells

Following the seminal report from Dr. Yamanka's group [7] on the ability to reprogram adult cells to a pluripotent state using four transcription factors (Fig. 1), the field has moved rapidly. Studies replicating this result in mice [8,9], humans [10,11], other rodents [12], dogs [13], pigs [14], and monkeys [15] have extended the utility of this approach as a basic science research tool. Other investigators have shown that depending on the cell type used for reprogramming, fewer factors may be necessary [16–18], perhaps as few as one in neural stem cells [19–21], or even none [22]. Moreover, a number of groups have demonstrated that non-DNA-based factors can be used to induce pluripotency or to greatly enhance reprogramming efficiency. Altering the miRNA profile [23,24], inhibiting demethylases [25], altering telomerase [26], and manipulating the components of cell cycle biology [27–29] all appeared to enhance reprogramming. Equally important, it appears that the method of delivery for reprogramming factors is not critical; iPSC lines have been generated using retroviruses [7], lentiviruses [30–32], adenoviruses [33,34], sendai virus [35], plasmids [36–38], and protein delivery [39] of factors. Nonetheless, many papers continue to use the original four factors, so the added risk of cancer formation from iPSCs is far from solved.

A major insight from research in the last year has been the observation that the reprogramming factors are not needed

FIG. 1. Direct reprogramming. Somatic cells are obtained from a patient and expanded if necessary. Reprogramming factors are added, and the pluripotent state is induced. iPSCs are cultured in embryonic stem (ES) cell media for 1–2 weeks, after which colonies are isolated at clonal densities and expanded. iPSCs, induced pluripotent stem cells.

forever [40,41]. Indeed, once the cells are reprogrammed they express endogenous pluripotency genes and silence the exogenous ones. Thus, like ESCs, iPSCs can readily silence pluripotency-associated genes and differentiate into appropriate, mature lineages. Methods of transient delivery have allowed us to define the window of time over which reprogramming changes occur, and the sequence of application that allows for the largest numbers of cells to be reprogrammed. These observations have been utilized cleverly by several groups to develop a “zerofootprint” technology that allows one to reprogram using factors or genes that can then be permanently eliminated, leaving pluripotent cells that, theoretically at least, should be indistinguishable from ESCs derived in a conventional fashion. Such techniques include 3 general approaches: (1) using Cre-Lox [42,43], PiggyBac [37,44–46], and sleeping beauty transposons to efficiently eliminate integrating particles; (2) using plasmids, adenovirus, sendai virus, and other episomal strategies that are effectively diluted out as the cells divide; and (3) using protein and small molecules that reduce the probability of any unintended alteration of the nuclear genome to 0. Recently defined, “xeno-free” culture conditions [47] have also improved the safety of iPSCs for potential clinical applications.

Several groups have directly tested the ability of iPSCs to behave like ESCs. These experiments have included making chimeras in mice [7], demonstrating germ-line transmission [48], following F1 and F2 generations over a couple of years [48,49], using genome-wide gene expression analysis [50,51], epigenetic profiling [52,53], and miRNA expression [24] as well as functional testing in animal models of disease [54–56]. Although there are few direct side-by-side comparisons that might reveal subtle differences between iPSCs and ESCs, most of results to date largely confirm that irrespective of the path to pluripotency, once cells have entered a pluripotent state, they behave virtually identically to each other. Recent work has demonstrated that the signaling pathways controlling pluripotency in both iPSCs and ES cells are remarkably similar [57], with both cell types using Activin/Nodal signaling to control Nanog expression to maintain pluripotency and employing the same signals to differentiate

along specific cell lineages [58]. Although a recent study has indicated that iPSCs have limited differentiation capability in the hemangioblast lineage and undergo premature senescence [59], this result is contradicted by other studies that indicate iPSCs acquire high levels of telomerase activity [26] and differentiate efficiently into hematopoietic cells [60–62].

Nevertheless, some differences have been observed between ESCs and iPSCs. The frequency of karyotypic abnormalities seems to be higher in iPSCs (Cell Line Genetics, M. Rao, pers. comm.), which is not unexpected, given the genomic alterations that are known to occur with viral transduction and *in vitro* selection. Recently, Chin and colleagues characterized gene expression in iPSCs and ESCs and demonstrated that each cell type has a similar but distinct gene expression profile [51]. Similarly, miRNA expression is somewhat different in iPSCs, compared with ESCs [63]. However, whether these differences are significant and wider than normal allelic differences between ESC lines remains to be studied.

To better assess the nature of specific iPSC lines, the following areas should be carefully evaluated, in light of the different histories of the ancestor cells used for reprogramming: maternal and paternal allele-specific gene expression (imprinting), X-chromosome inactivation in lines derived from females, telomere biology and senescence (particularly for lines derived from mature or aged individuals), cell cycle regulators (p53 and rb), and the extent of DNA damage. Although initial studies have suggested that iPSCs are fully pluripotent, regardless of the ancestry of the reprogrammed cell [64,65], it is nonetheless possible that there are subtle biases in differentiation, because of the original source of the cells and incomplete reprogramming. For example, evidence suggests that teratomas from iPSCs are less complex and more cystic than those from ESCs, that the frequency and extent of chimerism in these tumors is smaller, and that there appear to be biases depending on the cell of origin [64,66]. These subtle biases are probably best tested in competitive functional assays that compare different iPS lines. Indeed, some experiments of this type are already underway [64,66]. However, such variation points out the need to characterize

each iPS line individually, until the range and source(s) of variation are better understood.

Within the last year, a number of disease-specific iPSC lines have been isolated and partially characterized. The first report was the isolation of iPSCs from an amyotrophic lateral sclerosis (ALS) patient [67]. Shortly after, Park and colleagues reported on the generation of 21 disease-specific cell lines [68], representing 10 different medical conditions (ADA-SCID, Gaucher, Duchenne MD, Becker MD, Down's, Parkinson's, Type I Diabetes, Shwachman-Bodian-Diamond, Huntington's, and Lesch-Nyhan). Since then, additional iPSC lines have been generated from patients with spinal muscular dystrophy [69], Parkinson's disease [43], Rett syndrome [70], and type I diabetes [71].

The ease of isolating disease-specific cell lines has greatly enhanced the potential for iPS-based treatment of human medical conditions. For example, a recent study of Fanconi anemia patients demonstrated that following correction of the underlying genetic defect, somatic cells can be readily reprogrammed into iPSCs lacking the defect and differentiated into hematopoietic progenitors that appear indistinguishable from those derived from unaffected individuals [62]. This study supports earlier work [54] that demonstrated "proof of concept" for iPS-based treatment of human disease. Developing iPSCs into therapeutic reagents faces a number of practical hurdles, including risks associated with cell processing, the difficulty of ensuring the purity and characteristics of the reprogrammed population and the safety and efficacy of reprogrammed cells *in vivo* [72–74]. Nonetheless, there is cause for considerable optimism that patient-specific iPS lines will both enhance the study of human disease and advance these studies toward clinical applications.

Germ-cell progenitors

Spermatogonial stem cells (SSCs) have been isolated from rodents [75–78], dogs [79], cows [80,81], primates [82], and humans [83–85]. In humans, earlier work isolated pluripotent cells from a mixture of cell types from the testes, but the precise origin of these cells remains to be determined [86–88]. These cells have also been termed germ-line-derived pluripotent stem cells [89]. *In vivo*, these cells are unipotent, but when removed from their natural environment, they spontaneously become pluripotent [90], sharing many features with ESCs, including epigenetic [91], gene expression [92], and miRNA [93] profiles. Recent work indicates that SSCs from mice can be directly transdifferentiated into multiple epithelial lineages, including prostatic, uterine, and skin epithelium, by contact with mesenchyme derived from these tissues [94]. The fact that mesenchymal cells provide instructions for the appropriate differentiation of epithelial tissues during development has been known for decades [95], but it is a novel finding that mesenchyme can direct the transdifferentiation of pluripotent stem cells. This result opens a new avenue for producing stem cell derivatives in culture using a "zerofootprint" technology that may more closely replicate the mechanisms by which cell differentiation is controlled during development. A significant scientific advantage of SSCs is that multiple lines can be obtained from a simple biopsy. A significant clinical advantage of SSCs is that they are patient specific and could be used for autologous stem cell therapies. Moreover, these cells clearly fall

outside of the WARF patent claims, and therapeutic approaches based on SSCs could potentially warrant new patent protections.

Compared with SSCs, work on female germ-line-derived stem cells (ovarian stem cells [OSCs], or sometimes, ovarian surface epithelial cells) is relatively sparse. OSCs have recently been isolated from adult mice [96–98] and humans [99–101]. Surprisingly, evidence is accumulating that female germ-cell progenitors repopulate the ovaries throughout life [96,102–104], although this finding is controversial [105]. Stem cells in the ovary appear to have a broad potency [106,107], although they do not produce teratomas when injected into nude mice [99,107], and are unlikely to be pluripotent. Moreover, these cells are difficult to propagate and differentiate *in vitro*, although recent work indicates that manipulation of the Kit ligand pathway can promote maintenance of an undifferentiated state [108]. Although OSCs can be induced to differentiate in culture [97,101], mature oocytes have not yet been obtained *in vitro*. A recently established mouse OSC line (termed "female germ-line stem cells" by the authors) are able to produce mature oocytes that give rise to viable offspring when transplanted into the ovaries of infertile female mice, even after these stem cells have been maintained in culture for relatively long periods of time [96]. Recent work has isolated a similar population of stem cells from ovarian stroma of adult mice (termed "ovary-derived colony-forming cells"), which produce oocyte-like cells in culture and teratomas in nude mice, and are therefore likely to be pluripotent [98]. An advantage of OSCs for scientific research is that such cells can be used to study the factors required for totipotency. Moreover, like SSCs, OSCs are patient specific and could be used for autologous transplant in patients. The most immediate clinical application of OSCs may be the treatment of women with premature ovarian failure or sterility due to the side-effects of cancer treatment.

Scientific, Practical, and Ethical Considerations

Stem cells from direct reprogramming and germ-cell progenitors have significant advantages relative to human embryo-derived stem cells. For potential clinical applications, patient-specific stem cells (both iPSCs and germ-line derived) have clear advantages over conventional hESCs because they avoid the serious complications of immune rejection. Although patient-specific pluripotent stem cells could theoretically be obtained from embryos produced by somatic cell nuclear transfer, iPS and germ-line-derived stem cells present scientific and practical advantages here, as well. For both iPS and germ-line-derived stem cells, a simple biopsy can produce multiple cell lines with relative ease, and derivation of such lines can utilize the full support of federal funding. Although producing both iPSCs and germ-line stem cells would require informed consent from tissue donors, they would nonetheless be subject to significant simpler regulatory requirements than those that apply to hESCs [73]. iPS and germ-line stem cells also circumvent the serious ethical issues associated with either embryo destruction or egg donation that are raised by hESC research.

Should it prove possible to fully differentiate human OSCs into mature, fertilization-competent oocytes in culture, such cells would raise significant ethical concerns that are not raised by other stem cells. An abundant supply of human

oocytes would greatly reduce the need for woman egg-donors, thereby reducing the medical risks associated with obtaining human eggs for research purposes. However, such an abundant supply of eggs would also greatly facilitate research on human embryos and human cloning (eg, see the comments of Davor Soltor in ref. [109]). Moreover, the ability to produce millions of mature human eggs in the laboratory raises the specter of producing enormous numbers of human embryos for research, or even for industrial purposes. Clearly, the isolation of stem cells from the female germ line prompts the need for renewed debate over the appropriate uses of human eggs in the laboratory and the ethics of generating human embryos solely for the purpose of scientific research.

Conclusion

In 2008, we reported that iPSCs faced “significant technical challenges that must be overcome before they can be used for treatment of human patients,” including the use of cMyc for reprogramming and the use of retrovirus [1]. In the last 2 years, research on the generation of pluripotent stem cells through direct reprogramming has advanced at a very rapid pace. Many of the initial concerns regarding safety of this procedure for clinical applications have been effectively addressed, bringing the possibility of stem cell-based therapeutic interventions much closer to a practical reality. In particular, the ability to reprogram cells without the use of integrating viruses is a major advance toward a safe (or safer) application of iPSCs in a clinical context. Although definitive evidence for clinical-grade, “zerofootprint,” personalized iPSCs that have been produced using clinically approved procedures remains to be obtained, scientists appear well on the path to success and we look forward to reporting on such results in the near future. In addition, work on germ cell-derived stem cells in both males and females is rapidly advancing. The possibility of transdifferentiating SSCs using mesenchymal tissues from different sources holds out the promise of producing tissue-specific stem cells required for the repair or regeneration of specific tissues and organs. The surprising finding that viable offspring can be produced from stem cell-derived oocytes constitutes a major advance in the study of totipotency, while raising concern that an abundant supply of oocytes could lead to the generation of large numbers of human embryos in the laboratory, solely for research purposes.

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Address correspondence to:

Maureen L. Condic

Department of Neurobiology and Anatomy

University of Utah School of Medicine

401 MREB, 20 North 1900 East

Salt Lake City, UT 84132-3401

E-mail: mlcondic@neuro.utah.edu

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