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Regulatory issues for personalized pluripotent cells

Maureen L. Condic^{1,*} and Mahendra Rao^{2,3}

¹ University of Utah School of Medicine, UT 84132

² Invitrogen Corporation, 1600 Faraday Ave, Carlsbad CA 92008

³ John Hopkins School of Medicine, MD 21231

Abstract

The development of personalized pluripotent stem cells for research and for possible therapies holds out great hope for patients. Yet such cells will face significant technical and regulatory challenges before they can be used as therapeutic reagents. Here we consider two possible sources of personalized pluripotent stem cells; embryonic stem cells derived from nuclear transfer (NT-ESCs) and induced pluripotent stem cells (iPSCs) derived from direct reprogramming of adult somatic cells. Both sources of personalized pluripotent stem cells face unique regulatory hurdles that are in some ways significantly higher than those facing stem cells derived from embryos produced by fertilization (ESCs). However, the outstanding long-term potential of iPSCs and their relative freedom from the ethical concerns raised by both ESCs and NT-ESCs makes direct reprogramming an exceptionally promising approach to advancing research and providing therapies in the field of regenerative medicine.

Keywords

iPSC; ESC; SCNT; pluripotency; reprogramming

Immune rejection of transplanted tissues presents a serious challenge for regenerative medicine. Just as for organ transplant, if stem cell-derived tissues are not a close MHC-match to the patient, life-long immune suppression is required (reviewed in¹). Immune rejection is an even more serious concern for potential stem-cell based therapies than for conventional tissue transplant, because the dispersed integration of transplanted stem-cell derivatives precludes their surgical removal should immune issues arise.

Estimates of the number of embryonic stem cell lines (Figure 1A; hereafter, embryonic stem cell lines derived from embryos produced by fertilization are referred to as “ESCs”) required to provide a good match for specific patient populations vary enormously, from several hundred^{2, 3} to several million^{1, 4} lines, depending on the genetic diversity of the population under consideration and on the definition of what constitutes an “acceptable” degree of immune mismatch. In all likelihood, the number of ESC lines required to treat the genetically diverse US patient population would be large⁵, and would far exceed the number of lines that could be generated from excess embryos currently available from assisted reproductive clinics (IVF

*Corresponding Author: Maureen L. Condic, University of Utah School of Medicine, Department of Neurobiology and Anatomy, 401 MREB, 20 North 1900 East, Salt Lake City, UT 84132-3401, email: E-mail: mlcondic@neuro.utah.edu.

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Maureen L. Condic: Conception and design, financial support, administrative support, manuscript writing, final approval of manuscript, other (production of artwork).

Mahendra Rao: Conception and design, financial support, manuscript writing, final approval of manuscript.

'spares'; <http://www.rand.org/news/press.03/05.08.html>). Producing ESCs from donated oocytes and sperm could expand the genetic diversity of available stem cell lines, but would not substantially reduce the number of lines required for therapeutic purposes.

In contrast to ESCs derived from embryos produced by fertilization, patient-specific stem cell lines would offer compatibility at both major and minor antigenic sites. Thus, developing an efficient method of generating patient-matched pluripotent stem cells for possible therapeutic applications is a key component of developing effective stem-cell therapies. Here we compare the two major methods of generating of patient-matched pluripotent stem cells; somatic cell nuclear transfer (SCNT) to produce stem cells from cloned embryos (NT-ESCs) and direct reprogramming to yield induced pluripotent stem cells (iPSCs).

There have been various attempts over the last ten years to obtain patient-matched pluripotent stem cells by reprogramming adult cells to an embryonic state (reviewed in⁶). The feasibility of reprogramming was first (and most dramatically) demonstrated by SCNT or cloning (see Figure 1B;⁷). Although SCNT has proven to be more challenging in primates than in other mammalian species, two embryonic stem cell lines have been isolated from cloned macaque monkey blastocysts⁸, and SCNT-derived human blastocysts have also recently been reported⁹, suggesting it will soon be possible to derive patient-specific lines from nuclear-transfer human embryos.

As an alternative to reprogramming by nuclear transfer, Yamanaka's laboratory¹⁰ recently demonstrated that adult mouse skin cells can be reprogrammed into a state that is similar to an embryonic stem cell by expression of only four factors (Oct 4, Klf4, Sox2 and c-Myc). Such reprogrammed cells (see Figure 1C) have been termed "induced pluripotent stem cells" (iPSCs). Rapid advances in the reprogramming technique have generated pluripotent cells that are not only similar to mouse ESCs, but functionally identical; capable of self renewal and germ-line transmission^{11–13}. Recent work demonstrates that similar to ESCs, mouse iPSCs are able to differentiate into multiple cell types *in vitro*^{14, 15}. Four independent groups have recently produced human iPSCs^{16–19}, making patient-specific pluripotent stem cell lines a possibility for the first time.

Producing patient-specific stem cell lines by direct reprogramming is relatively straightforward, albeit inefficient; initial studies reported that less than 1% of adult cells are transformed, although recent work reports increased efficiency when additional factors are used^{20, 21}. A strong advantage of this approach compared to SCNT is that direct reprogramming circumvents not only the ethical concerns over the use of human embryos for research, but also the ethical and practical concerns raised by human egg donation (see^{6, 22}). However, iPSCs present significant technical challenges and regulatory issues that must be addressed before they can be used for treatment of human patients, and in several respects, these challenges are distinct from those presented by SCNT.

The scientific hurdles that must be overcome in developing human ESC-based therapies have been extensively reviewed²³. Recently, Halme and Kessler²⁴ (see also²⁵), have considered the regulatory requirements likely to be applied to ESC-based therapies in light of four important questions: Does the donor pose a risk of transmitting infectious or genetic diseases? Does cell-processing pose a risk of contamination or damage? What is the purity of the cells in the final product? Will the product be safe and effective *in vivo*? These same questions must be asked of iPSCs and NT-ESCs if they are to be developed as therapeutic agents. Here we consider the hurdles confronting the development of patient-specific stem cells for human therapies, and contrast the challenges facing iPSCs to those facing NT-ESCs (Table 1).

Donor-associated risks

Because SCNT requires the use of donated oocytes, NT-ESCs would be subject to regulatory requirements for assessment of donor associated risk, and oocyte donors would need to be screened for both genetic and transmissible disease. Moreover, although NT-ESCs would be patient-specific, in most cases they would not be autologous (i.e. they would have nuclear DNA identical to the patient, but the mitochondrial genes would be from the oocyte donor). Because mitochondrial gene defects cause a large number of diseases^{26–28}, egg donors would need to be screened to ensure safety of donated oocyte mitochondria. It is also likely that mitochondria from the patient's somatic cell will be retained following NT, resulting in mitochondrial heteroplasmy. Such multiparental mitochondria have already been demonstrated in ooplasm transfer cases^{29–31} and in sheep derived from NT³², making the health risks associated with heteroplasmy (mitochondrial dysfunction due to aberrant interactions between mismatched nuclear and mitochondrial genes)³³ a reasonably legitimate concern.

One of the strong advantages of direct reprogramming is the ability to generate patient-specific cell lines with relative ease^{16, 17}. Because such patient-matched lines are autologous, they would be subject to significantly simpler donor screening requirements; donors would not have to be rigorously screened for genetic or infectious disease if the reprogrammed cells were intended only for transplant back to the patient from whom they originated, though the quality of the cells will still need to be evaluated, as discussed below.

Cell processing-associated risks

Generating both iPS and NT-ES cells requires significant manipulation in the laboratory. Thus, both types of pluripotent cells would be subject to the provisions of the Code of Federal Regulations (C.F.R.) governing transplant of laboratory-processed human cells and tissues (21 C.F.R. 1271), and would have to be screened for contamination that may have been introduced during all stages of preparation and processing.

An added concern regarding iPSCs is that they are currently generated by retroviral insertion of at least three gene products, with an estimated 20 integration events for each cell-line founder¹⁷, although recent results have suggested this number can be lowered considerably (T.M. Townes, unpublished data). The use of retrovirally transformed cells is permitted in human clinical trials under established NIH guidelines (<http://www4.od.nih.gov/oba/rac/guidelines/guidelines.html>), yet such genetically modified cells carry an increased risk for patients. As transformed cells, iPSCs would be regulated by both the FDA and also by the NIH Office of Biotechnology Activities Recombinant DNA Advisory Committee (the "RAC"). Consequently, developing potential therapies using iPSCs would carry increased technical and regulatory challenges associated with establishing the safety of the methods used for virus production, determining the safety of the site of viral insertion and the safety of the inserted genes themselves.

The additional regulatory concerns facing iPSCs may ultimately be obviated by modifications of the reprogramming technique that avoid the use of retroviral vectors (*i.e.* non-integrating vectors or non-DNA based reprogramming). Indeed, recent work has shown that transgene expression is only required for the first 10–12 days of the reprogramming process^{34, 35}, suggesting that expression of reprogramming factors from episomal viruses may be sufficient to transform cells. Moreover, treating cells with molecular inhibitors of either the G9a histone methyltransferase, mitogen-activated protein kinase kinase (MAPK/ERK kinase or MEK)³⁶ or histone deacetylase³⁷ can both increase reprogramming efficiency and reduce the number of transforming factors required. However, at the current time, the use of retroviral vectors presents additional technical and regulatory challenges for iPSCs, compared to NT-ESCs.

A possible application of both of iPSCs and NT-ESCs would be to derive patient specific cells and subsequently use genetic engineering methods to correct an inherited genetic defect. Genetically corrected cells could then be differentiated into the appropriate cell type and transplanted back to the patient to treat (or correct) the underlying genetic disease. The feasibility of this approach has been tested in animal models for both NT-ESCs³⁸ and iPSCs³⁹, with varying degrees of success. Such a gene-correction approach would involve a second stage of genetic manipulation, and therefore raise additional safety concerns, requiring additional regulation and testing.

Cell type and cell-purity risks

Both SCNT and direct reprogramming use nuclei that have aged and therefore undergone significant developmental changes. These changes include alteration in telomerase activity, shortening of telomeres, tissue and lineage specific methylation, imprinting and other epigenetic modulation. DNA damage is also likely to have occurred in aged nuclei, and it has been shown that such damage varies with the exposure to environmental agents and with tissue type⁴⁰. Given that each of these changes can have potential deleterious effects on the behavior of cells generated from such nuclei, it is likely that both NT-ESCs and iPSCs will require much more stringent scrutiny than ESCs derived from embryos produced by fertilization. Moreover, it is well established that ESCs accumulate genetic and epigenetic abnormalities over time in culture^{41–43}, and this problem is likely to face iPSCs and NT-ESCs as well. Therefore, pluripotent lines derived from SCNT or reprogramming would need to be tested for possible effects both of aging and of instability in the laboratory; *i.e.* DNA damage, changes in imprinting and epigenetic changes.

Patient-specific lines from either reprogramming or SCNT would not be universal therapeutic agents. Consequently, regulatory authorities are likely to treat each line as a separate product and testing will have to be rigorous for each line generated. Specific concerns about the safety of NT-ESCs and iPSCs that stem from the methods used to produce these cells are discussed below.

The efficiency of generating NT-ESCs is typically low (between 1 and 10%), with live birth rate of cloned animals being even lower^{44, 45}. Recent work (S. Wakayama, personal communication) suggests the live birth rate can be increased to nearly 40% by serial transfer of nuclei (first producing a NT-ESC line, then using the NT-ESC nuclei for a second round of SCNT), although other studies have not seen an improvement in developmental capability using this approach^{46, 47}. While human NT-ESC lines have not yet been derived, efficiency of producing genetically normal lines is likely to be relatively poor compared to efficiency of generating lines from embryos produced by fertilization. In the single report of successfully deriving NT-ESCs from non-human primates, two lines were derived from 304 monkey oocytes (0.7% overall derivation efficiency), with one line being karyotypically abnormal⁸. Work in other species also raises concern regarding the normalcy of NT-ESC lines. While some studies have demonstrated that NT-ESC lines are indistinguishable from lines derived from fertilized oocytes^{48, 49}, others have documented significant abnormalities in cloned animals and in NT-ESC lines^{42, 50–53}. For patient-matched NT-ESCs, it would therefore be necessary to establish a comprehensive screening procedure to select lines with normal karyotype and normal epigenetic characteristics.

An added concern for both NT-ESCs and iPSCs is large offspring syndrome (LOF). LOF is one of several abnormalities that lead to a reduction in the number of viable births following successful implantation. LOF is associated with a range of embryo manipulations in culture^{54–56}, including SCNT^{57–59}. The basis of LOF appears to be culture-induced epigenetic changes that are not readily detected at early stages *in vitro*⁶⁰. It is therefore a

reasonable concern that such epigenetic abnormalities will affect NT-ESC derived cells. Although detailed testing of epigenetic state has not been done for multiple iPSC lines, the preliminary results are encouraging^{11, 61, 62}. Nonetheless, epigenetic changes may also affect iPSCs. Importantly, assessing which iPSC or NT-ESC line is epigenetically normal may prove difficult, but would be a necessary regulatory requirement.

The nature of somatic cell reprogramming raises unique concerns regarding iPS cell type and cell purity. Recent work has shown that integration of retrovirus into specific sites in the genome is not required for reprogramming⁶³. Therefore, as long as retroviral vectors are used for reprogramming, each iPSC line will have a unique genetic constitution, and each iPSC line will need to be independently characterized to determine its precise properties. While NT-ESCs from mouse have been extensively compared to conventional mouse ESC lines (as noted above), this data has not yet been obtained for mouse (or human) iPSCs. It will be important to determine whether every specific iPSC line is indeed ESC-like, in order to make use of the large amount of data obtained from blastocyst-derived ESCs. Alternatively, safety and efficacy data, as well as stability and heritability of the induced pluripotent state and absence of bias in lineage differentiation, will have to be generated independently for every iPSC line.

In light of the low efficiency of reprogramming, a further concern regarding iPSCs is the method used for selecting reprogrammed cells. Currently, mouse iPSCs have been selected based on activation of a neomycin resistance gene inserted into the Nanog or Oct4 locus^{11–13} or by morphological criterion that can be applied to cells without introducing selectable markers that allow for identification of the altered cells^{64, 65}. All four laboratories isolating human iPSCs based their selection on morphology^{16–19}, but this approach will need to be tested and validated prior to approval in any GMP (good manufacturing practice) protocol.

Safety and efficacy in vivo

A challenge common to all pluripotent cells is the difficulty of differentiating them into stable, mature cell types. ESCs have been studied since the early 1980's, and yet it has proven challenging to induce them to form functional replacement tissues that are therapeutically useful for the treatment of disease and injury. While ESCs are clearly capable of generating multiple tissue types in chimeric embryos and in tumors, producing clinically useful cells in the laboratory has been not been routinely achieved. Similar challenges are likely to face iPSCs and NT-ESCs as well.

In most cases, survival of tissue derived from pluripotent stem cells is poor following transplantation. While there has been some degree of success for long-term survival of hematopoietic progenitors generated either from NT-ESCs^{38, 66}, or from iPSCs³⁹, success with blood appears to be the exception rather than the rule. For example, a recent study using monkey ESCs in a Parkinson's disease model showed only 1% survival of transplanted neuronal cells at 14 weeks⁶⁷. Similar results have been seen for multiple tissues differentiated from ESCs, including (among others) hepatocytes⁶⁸, cardiomyocytes⁶⁹, and pancreatic islet cells⁷⁰. Although data from NT-ESCs and iPSCs is limited at this time, it is likely that patient-specific stem cells will face similar challenges in terms of producing tissues that survive long-term following transplantation. The efficacy *in vivo* of tissue derived from pluripotent stem cells therefore remains questionable for most cell types, other than blood.

Similarly, safety *in vivo* presents a significant challenge for development of pluripotent-stem cell therapies. It is well established that undifferentiated pluripotent stem cells form tumors when transplanted to mature tissues, while stably differentiated cells do not. However, numerous studies have shown tumor formation even in cases where ESCs have been subjected to rigorous differentiation protocols, suggesting that the purity and stability of differentiated cell populations can be difficult to predict or to control⁷¹. For example, human ESCs

differentiated into dopaminergic neurons and transplanted into a rat model of Parkinson's disease showed substantial phenotypic instability and reverted to undifferentiated, tumorigenic neuroepithelial cells⁷². Similarly, animals transplanted with mature dopaminergic neurons generated from ESCs showed frequent tumor-related deaths unless the transplanted population was first presorted to remove sox1-positive, neuronal progenitors that persisted despite an extended differentiation protocol⁷³. Indeed, pre-differentiation of human ESCs for up to 23 days *in vitro* reduced, but did not entirely eliminate tumor formation⁷⁴. Teratoma formation has been observed for ESCs differentiated along multiple lineages, including (among others) hematopoietic⁷⁵ pancreatic^{70, 76}, hepatic^{68, 77} and retinal⁷⁸.

Together, these results suggest that efficacy and safety *in vivo* has not yet been rigorously established for most tissues derived from ESCs. Although similar data is not currently available for human iPSC or NT-ESC lines, it is fair to assume that until contrary data is accumulated, the transplant issues identified with ESCs will apply to NT-ESCs and iPSCs as well. Indeed it is likely that the frequency of tumor formation will be higher with patient-specific lines, as there is no immune mismatch which would co-opt the immune system in rejecting cells with immature or inappropriate phenotypes. Increased numbers of ESC-derived tumors were noted in SCID mice when human fetal tissue containing hESCs was transplanted, compared to mice receiving hESCs alone, suggesting that the lack of immune rejection can enhance tumor formation^{79, 80}. In addition it has been shown for mouse iPSCs that some of the genes used to induce transformation are oncogenic; chimeric mice containing iPSC-derived tissues formed head and neck tumors which were attributed to the myc gene used to reprogram fibroblasts into iPSCs. Subsequent experiments have suggested that this may not be true when other cell types are used for reprogramming⁶³ and that reprogramming can be accomplished without the use of the myc gene^{18, 81} yet increased propensity towards tumor formation remains a concern for iPSCs, as they are currently generated.

Conclusion

Although patient-specific stem cells (both derived from SCNT and from direct reprogramming) have clear advantages over ESCs derived from embryos produced by fertilization, the very nature of the processes used to create such lines will create additional regulatory hurdles.

Embryonic stem cells produced by SCNT would be patient-specific, and thereby avoid the serious complications of immune rejection. However, because NT-ESCs require the use of human eggs, they would be subject to regulatory requirements governing egg-donor selection and screening. In addition, NT-ESCs raise serious ethical and practical issues that are not easily resolved. While many Americans accept the generation of stem cell lines from excess IVF embryos (embryos that will "die anyway"), the production of embryos intended solely for research (through either fertilization or NT) has far less public support (for example, a poll conducted in early 2008 showed 47% in favor of using excess IVF embryos for research, while only 18–30% support creation of embryos solely for research purposes; *cf.* Questions 9 and 13–15 in⁸²). The medical risks to women associated with egg donation also raise significant ethical concerns for NT-ESCs (as well as for ESCs produced by fertilization of donated oocytes). Human cloning itself presents ethical issues not raised by other forms of stem cell research. Finally, because generation of human ESCs and NT-ESCs requires destruction of human embryos, many oppose this research on either ethical or religious grounds⁶. On a purely practical level, the Human Embryo Research Ban (the Dickey Amendment) that has been continuously authorized by Congress since 1995 prevents the use of federal funds for generation of human NT-ESC lines (text for the current fiscal year is available under section 509 of H.R.2764; <http://thomas.loc.gov/cgi-bin/query/C?c110:/temp/~c110zhKoHu>), and this situation seems unlikely to change in the foreseeable future.

In contrast, iPSCs offer several advantages over both conventional ESCs and NT-ESCs, including the relative ease with which multiple cell lines can be obtained from a single biopsy, the eligibility of iPSC research for federal funding and the freedom from the ethical and practical burdens associated with either embryo destruction or egg donation^{6, 22}. Since iPSCs are entirely patient derived, the burdens of demonstrating immune compatibility and donor safety are much lower than for ESCs or NT-ESCs. However, it is unlikely that other regulatory requirements can be lowered. As a new, and still unproven technology that incorporates both genetic manipulation and pluripotent cell propagation, the regulatory hurdles for iPSCs will be high, particularly in the light of previous experience with gene therapy.

Despite the remaining technical and regulatory challenges, none of the hurdles facing iPSCs are insurmountable. Direct reprogramming technology represents a fundamental breakthrough that provides a novel path for generating multiple differentiated cell types with an isogenic gene profile that would be virtually impossible to obtain in therapeutically relevant numbers otherwise. Direct reprogramming thus offers an exceptionally promising method for advancing research and providing therapies in the field of regenerative medicine.

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A: ESCs from IVF embryos

B: ESCs from nuclear transfer

C: iPSCs from direct reprogramming

Figure 1.

Table 1

Relative advantages and disadvantages of current stem cell approaches.

	ESC	ESC (NT)	iPSC
Donor eggs required	Yes (No if using IVF 'spares')	Larger number	No
Embryo destroyed for stem cells	Yes	Yes	No
Embryo created for research	Yes (No if using IVF 'spares')	Yes	No
Potential mitochondrial incompatibility	No	Yes	No
Aging issues with donor nuclei	No	Yes	Yes
Time to obtain pluripotent cells *	1 week	1-2 weeks	2-3 weeks
Use of viral vectors	No	No	Yes
Stability and heritability of pluripotent state	High	May be lower	May be lower
Immune issues	Yes	No	No
Ethical issues	High	Higher	Lowest
Potential to form teratomas/tumors after transplantation	Yes	May be higher	May be higher
Survival and persistence after transplantation	Poor	Likely same as ESC	Likely same as ESC
Potential Regulatory burden	Medium	High	High

ESCs can be obtained from either excess IVF embryos (IVF 'spares') or from embryos produced by fertilization of donated oocytes.

* For all three methods, expansion, subcloning and characterization of the cells to establish a stem cell line takes at least an additional three weeks.