Lazarus's Gate: Challenges and Potential of Epigenetic Reprogramming of Somatic Cells

JT Henderson[1]

The past year has seen tremendous advances in epigenetic reprogramming of somatic cells. Direct genesis of pluripotent stem cells, in contrast to earlier somatic cell nuclear transfer (SCNT) techniques, removes significant ethical and regulatory concerns regarding the utilization of human oocytes and zygotes, and represents a significant step toward the development of nonxenogeneic production methods. While significant technical hurdles remain, this and related technologies are enabling new approaches toward clinical treatments, basic research and diagnostics, and drug evaluation.

THERAPEUTIC POTENTIAL

The potential for utilizing endogenous mammalian stem cells to enhance repair and renewal of damaged organs and tissues has received much attention over the past decade.[1,2] However, only recently have technical advances in the field proceeded to the point where the therapeutic potential of stem cells can be efficiently realized. These advances will enable researchers to directly examine processes in embryonic germ layer differentiation, cell senescence, and tissue repair in humans in ways which would not have been possible even a few years ago. Immediate benefits of induced pluripotent stem (iPS) cell lineages include: (i) direct derivation of iPS lineages containing a variety of somatic pathogenic mutations enabling a wide array of experimental analyses into disease mechanisms in humans; (ii) development of patient-specific (immune compatible) cell-based therapies for the treatment of acute and chronic tissue injury; (iii) high-throughput drug testing, utilizing populations of primary human cells for a variety of disease states; (iv) direct analysis of early developmental processes in humans using *in vitro* cell culture and animal–human embryo hybrid methods; and (v) development of nonviral gene therapy and individual gene repair methods in humans. In addition, the development of iPS cells will significantly advance research on the molecular mechanisms that regulate the differentiation of stem and multipotential progenitors, as well as a variety of somatic cell lineages. Understanding such signaling interactions will be critically important to the development of advanced artificial tissues and organs. Each of the above endeavors poses unique benefits and challenges. In order to appreciate these, it is important to consider the technical elements underlying recent advances in iPS.

GENERATION OF STEM CELL LINEAGES

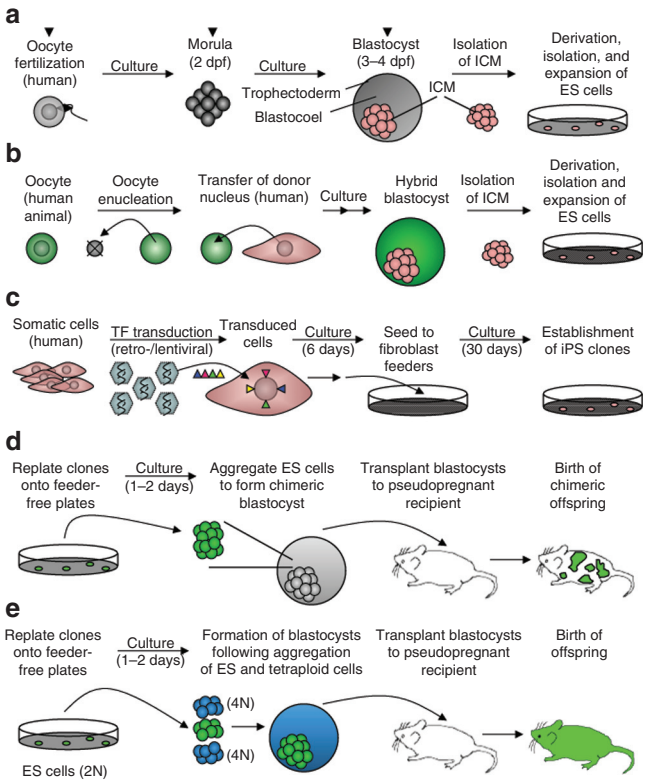
Epigenetic reprogramming of somatic cells has, until recently, employed the process of somatic cell nuclear transfer (SCNT), in which a somatic cell nucleus is placed within properly prepared enucleated oocytes (typically stage MII) or zygotes.[3] Numerous studies have demonstrated that germ cell cytoplasm induces reprogramming of somatic chromosomes, resulting in the establishment of a hybrid cell line exhibiting enhanced potentiality.[4,5] After the first successful demonstration of this methodology in mammals through production of the cloned sheep "Dolly" in late 1995,[6] SCNT methods have proliferated, becoming customized for specific cloning applications.[3] Although the frequency of live births using SCNT remains low, this method has successfully generated viable clones for a variety of mammalian species.[7–10] The process of SCNT provided an important first step in our understanding of epigenetic reprogramming. Subsequently, in 2006, the work of Takahashi and Yamanaka demonstrated that enforced expression of transcription factors, such as Oct3/4, Sox2, c-Myc, and Klf4, induced murine fibroblasts to exhibit properties of pluripotent stem cells.[11] These cells, termed iPS cells by the authors, exhibited numerous similarities, but not identity with, bona fide embryonic stem (ES) cells. The differences included alterations in the pattern of gene expression between iPS and ES cells, and the aberrant development of chimeric iPS embryos in blastocyst aggregates.[12] These differences raised questions regarding the relationship between iPS and ES cells. In July 2007, a laboratory team headed by Rudolph Jaenisch similarly demonstrated, using *Nanog* and *Oct4* expression as markers to identify pluripotent clones, that enforced expression of the Oct3/4, Sox2, c-Myc, and Klf4 transcription factors

[1]Department of Pharmaceutical Sciences, Leslie Dan Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada. Correspondence: JT Henderson (jeff.henderson@utoronto.ca)

Received 8 January 2008; accepted 11 February 2008; advance online publication 26 March 2008. doi:10.1038/clpt.2008.51

in murine fibroblasts resulted in the formation of ES cell-like colonies.[13] The induced pluripotent clones exhibited a normal chromosomal karyotype and produced live-born chimeras when introduced into host blastocysts (see **Figure 1**). These chimeras were subsequently shown to exhibit germline transmission of genes specific to iPS clones. Because chimeric aggregates can mask subtle genetic abnormalities within the adoptive (iPS) clone, the developmental potential of iPS cells was further examined using tetraploid aggregations. In this technique, the embryo proper develops solely from the introduced iPS cells[14] (**Figure 1**). The observation of viable late-term embryos in tetraploid aggregates of iPS cells indicates that these clones possess sufficient developmental potential to generate the entire embryo and are free of significant functional abnormalities. Consistent with this, the expression and methylation patterns of a substantial majority of the genes examined were observed to be similar in induced and ES cell clones, suggesting that the discrepancies originally observed may reflect differences in the conditions under which the induction and culture were carried out.[13,15]

Recently (November 2007), the Yamanaka and Thompson groups demonstrated that iPS cells could be derived from adult human somatic cells.[16,17] Again, the pattern of gene expression in iPS cell clones exhibits extensive similarity to (but also significant differences from) that observed in established human ES cell lines. Differentiation of these clones as embryoid bodies resulted in the generation of differentiated cells arising from all three germinal layers, and iPS cells exhibited cell doubling times (43–49 h) similar to those observed in human ES cells.[3,18]



MECHANISM AND SAFETY OF INDUCED PLURIPOTENT CELLS

While the precise mechanism by which enforced expression of *Oct3/4*, *Sox2*, *c-Myc*, and *Klf4* induces somatic reprogramming remains unclear, evidence suggests that expression of *c-Myc* and *Klf4* alters chromatin structure.[13,16] This, in turn, may enhance the access of Oct3/4 and Sox2 (factors known to be critically important for the maintenance of pluripotency) to their transcriptional targets. With respect to direct therapeutic use of iPS cells in humans, concerns have been raised regarding the presence of retroviral DNA sequences (used for expressing Oct3/4, Sox2, Klf4, and c-Myc) in iPS cells and derivatives, and ectopic expression of the oncogene c-Myc.[13,17] With respect to the first concern, the temporal requirements of Oct3/4, Sox2, Klf4, and c-Myc expression have not yet been firmly established. There is considerable evidence (including the observed hypermethylation/inactivation of retroviral target sequences in derived iPS cells) to suggest that ectopic expression of these factors is required only acutely for establishing the initial iPS colony. Therefore, nonviral DNA transduction methods including direct transgenesis (see later text), or short-acting introduction of the required transcription proteins may prove feasible for the initial establishment of patient-specific iPS clones. With respect to c-Myc, it has recently

Figure 1 Overview of technical procedures described. (**a**) Derivation of human embryonic stem (ES) cells. Prepared oocytes (typically MII phase) can be fertilized and cultured *in vitro*, initially resulting in morula (4–16 cells that are totipotent), followed by blastocyst formation (16–40-cell stage). During this period, the inner cell mass (ICM) becomes apparent. The isolation of pluripotent cells of the ICM from the trophectoderm (often through immunosurgery) allows their culture to permanent ES cell lines. The majority of current ES cell lines have been established in this manner. Vertical arrowheads indicate points where human material can be donated to generate ES cells. dpf, days post fertilization. (**b**) Derivation of ES cells using somatic cell nuclear transfer. Using a prepared enucleated oocyte (frequently murine), nuclear material from human somatic cells is transferred to the recipient cytoplast, forming a cytoplasmic hybrid (cybrid). The isolation of the ICM from the resulting chimeric blastocyst allows the establishment of human ES cell lines as in the first example. The effects of exposing the ES cell lines so created (and all ES cell lines during their culture and differentiation) to xenobiotic materials such as fetal bovine serum albumin is currently a subject of investigation. (**c**) Generation of induced pluripotent stem (iPS) cell lineages. Somatic cells are transduced with a minimal set of transcription factors required for cellular reprogramming (current protocols use Oct4, Sox2, and Klf4). Current methods use retroviruses to maximize cellular transfection and reprogramming, but other transduction methods appear feasible. As with ES cells, after nuclear reprogramming, the colonies are typically transferred onto fibroblast feeder layers (however, feeder-free methods also exist). The cultures are subsequently incubated in standard ES media plus basic fibroblast growth factor for 30 days to establish iPS cell colonies. (**d**) Construction of chimeric embryos. After the ES cells have been thawed and cultured onto fibroblast feeders, they are replated onto feeder-free plates (1–2 days), and ES cell clusters (8–15 cells in size) are injected into or aggregated with host embryos to form a chimeric blastocyst. These are transferred to a pseudopregnant recipient so as to generate chimeric embryos. Germline transmission from chimeric founder animals allows ES cell characteristics to be passed to every cell of the subsequent generation. (**e**) Construction of tetraploids. ES cell aggregates of normal diploid (2n) karyotype are sandwiched between clusters of tetraploid (4n) cells, typically generated through electrofusion at the two-cell stage, and subsequently cultured (eight-cell stage) before aggregation with ES cells. Because tetraploid cells cannot contribute to the embryo proper (instead, they form the extraembryonic tissues), embryos will be derived only from the ES cells provided.

been reported that iPS cell clones can be established (albeit at significantly lower efficiency) without the use of c-Myc.[19] Therefore, methods of addressing these concerns may yet prove straightforward. However, it is important to note that when current induction methods are used, iPS cells do not exhibit identical patterns of gene expression to ES cells derived from the blastocyst inner cell mass.[12,13] The significance of this finding will have to await functional analysis of iPS cells and derivatives using murine model systems. This will be a critical test of the ability of iPS cells to truly replace endogenous cell functions.

One further aspect of the creation of iPS cells bears consideration. While prior SCNT investigations have demonstrated the capability of germ cell cytoplasm to reprogram somatic cell nuclei, the relatively small percentage (≤0.1%) of pluripotent clones retrieved from the initial (somatic) population may suggest that recovered iPS cell clones arise from rare multipotential progenitors that exist within skin cell populations.[20] Alternatively, derived clones could reflect the occurrence of additional (rare) events required for pluripotency. These could include retroviral integration at a specific chromosomal locus, or specific genetic changes induced in culture, resulting in the activation or inhibition of additional genetic components.[12,16] These issues should be clarified in the near future, because each can be addressed experimentally in a straightforward manner. Finally, the infrastructure necessary for good manufacturing practices and the protocols governing iPS cell derivation, culture, storage, and differentiation will need to be clearly defined and standardized within the scientific community before this new technology can be put to clinical use. A key issue is likely to pertain to guidelines regarding exposure of iPS cells to xenobiotic constituents during culture and derivation, in view of the fact that these could potentially provide a mechanism toward the development of new cell pathogens.

NEW APPROACHES TO GENE/CELL THERAPY

As indicated earlier, iPS cell lines exhibit sufficient developmental potential to generate viable full-term embryos. This suggests that human iPS cells and their cellular derivatives may prove useful in an array of therapeutic cell transplantation strategies, utilizing both endogenous and genetically modified cell lines. In support of this, autologous iPS cells derived from skin fibroblasts have recently been utilized to correct a murine model of sickle cell anemia.[21] For such "gene therapy" applications, the issue of safety of the patient should always be paramount. Traditional gene therapy approaches have relied upon viral transduction to introduce the transgene into host lineages, principally because of the limitations relating to availability of host material and renewal capability. The drawbacks of such methods are potential immunologic consequences[22] and the variability of chromosomal target integration in different cells,[23] potentially producing undesirable consequences such as oncogenic transformation. In view of their renewal capabilities, the development of iPS cell lines offers a potential solution to these problems. Using well-defined homologous/site-specific recombination strategies (**Figure 2**), precise genetic modifications can be performed in iPS cell lines.[24] Following the identification, selection, and expansion of the desired modified clone, key genetic elements of concern could be sequenced prior to the introduction of cells into patients, thereby enhancing safety. In the event that recombination/selection sequences require removal after

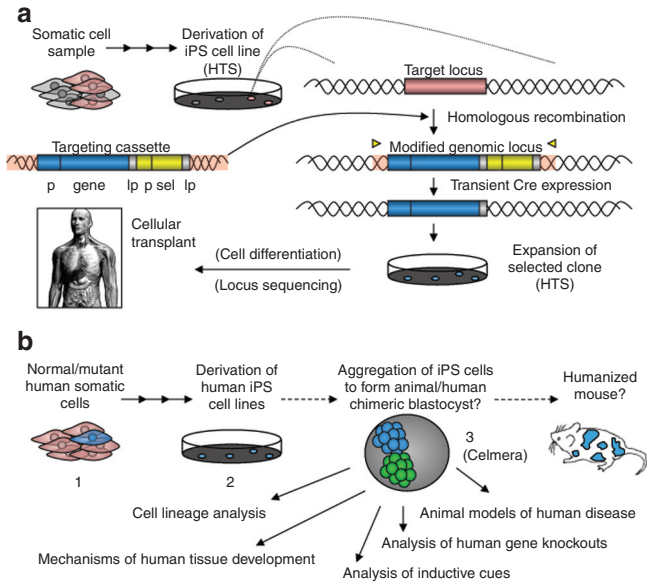


Figure 2 Potential therapeutic role of induced pluripotent stem (iPS) cells. Shown in the figure are potential clinical and research advances directly enabled through the development of iPS cell lines. (**a**) Patient-specific gene therapy. Derivation of iPS cells in conjunction with homologous recombination and high-throughput gene sequencing will significantly enhance development of patient-specific gene therapy. In the example shown, iPS cell clones are derived from the somatic cells of a given patient. High-throughput sequencing (HTS) of DNA from these clones at gene loci of interest/concern identifies those of optimal character (red) from suboptimal (gray) candidates. The proliferative potential of iPS cell clones allows homologous targeting at specific gene loci, thereby preventing complications associated with the use of viral methods (see text). Human genome information currently allows the rapid development of gene targeting cassettes, by providing detailed sequence information (red helices). Typical targeting cassettes consist of a gene promoter (p) which directs expression of a given therapeutic gene (gene). The targeting cassette also contains an antibiotic selection marker (sel) under the control of a secondary promoter (p), which is expressed in iPS cells. The selection marker allows the identification of clones that have incorporated the targeting cassette into their genomic DNA (yellow arrowheads). An analysis of 5′ and 3′ regions surrounding the targeted locus identifies the clones in which homologous targeting has occurred. In order to remove unnecessary gene sequences after targeting, recombinase systems such as Cre have been utilized for removing DNA sequences between target (loxP, lp) sites. After analysis of specific gene loci using HTS (for quality assurance), selected iPS clones can be expanded and differentiated to lineage-specific stem cells or other somatic derivatives for cellular transplant into patients. (**b**) Research. The development of iPS cell lines enables research in several critically important areas of human biology. The derivation of iPS cell lines from cells containing rare somatic mutations (1) will be extremely helpful in improving our understanding of the molecular basis of human disease. Another critically important issue relates to the molecular mechanisms directing pluri- to mutipotential stem cell development. (2) A third area, not permissible under current regulations, is the analysis of early human cellular differentiation using animal/human hybrids (cellular chimeras or "celmeras"). (3) The introduction of small numbers of tagged human iPS cells (blue) into developing murine blastocysts could allow researchers to examine the establishment of primary germ lineages, and possibly organogenesis. Such research must be considered with care, because improper protocol control could favor the development of new human or animal pathogens. Additional potential applications from celmera generation are indicated.

integration into the target gene, "hit-and-run" strategies utilizing Cre/loxP or similar methods could be employed (**Figure 2**). Immunologic concerns could potentially be abrogated in iPS cells, given their potential patient-specific nature and the fact that they are free of xenogenic (viral) proteins.

STEM CELLS: REGULATORY AND ETHICAL CONSIDERATIONS

Laws governing stem cell research in the United States presently prohibit utilization of federal research funds for experimentation that results in the destruction of human embryos (federal funds can be employed for research on human ES cell lines created prior to 2001).[25] In Canada and the United Kingdom, a more permissive approach has generally been adopted, allowing federal research funds to be utilized for basic research into human stem cells.[26] While the development of iPS cell techniques will undoubtedly stimulate much further discussion and legislation, this procedure removes what, for many, has been a primary objection toward the development of new human stem cell lines. Indeed, iPS techniques remove the genesis of stem cell lineages from the arena of human reproduction/embryology, into the realm of regenerative medicine. This will likely have a profound impact upon scientific oversight requirements regulating iPS cell experimentation. Some crucial applications of iPS cells will likely remain associated with human reproduction/embryology, such as mechanisms of stem cell differentiation, because of the potential requirement of human/animal (mouse) hybrids to investigate these processes. This kind of chimera (animal/human stem cell) is not presently approved under federal guidelines in the United States, United Kingdom, and Canada but has been discussed scientifically for several years.[27] Recent (UK) rulings allowing animal/human hybrids relate to the construction of "cybrids" (**Figure 1**), or the introduction of animal genes into human embryos,[28] and prohibits development of such embryos beyond 14 days.[29] Therefore, while the introduction of human iPS cells into developing murine blastocysts could provide important new insights into mechanisms of *in vivo* human development, regulations concerning such experimentation will need to be developed and evaluated with care.

CONCLUDING REMARKS

Historically, therapeutic stem cell applications have focused upon the *in vitro* production of sufficient quantities of specific cell types, tissues, or organoids for implantation, or upon direct *in vivo* applications of stem cells to specific sites.[30] iPS cells promise to assist these endeavors and accelerate the analysis of lineage relationships between ES cells, multipotential tissue progenitors, and differentiated somatic cells. The production of specific cell types and tissues *in vitro* is presently limited by our incomplete knowledge of the developmental signaling patterns regulating these events. Similarly, failures to reproduce normal cellular differentiation *in vivo* following stem cell implantation may reflect aberrant local signaling, given that high local concentrations of stem cells have been shown to alter cell communication.[31] An alternative approach, which may prove useful in the near term, would be to implant very small numbers of tissue-specific progenitors into damaged tissue. The aim would be to more closely replicate natural conditions during tissue repair, with the target tissue itself providing the needed signaling instructions to replace aged and damaged cells. In order to promote such efforts, future research will need to focus upon the signaling mechanisms that regulate the differentiation of pluripotent cells to various tissue-specific multipotential progenitors. Finally, there has been significant discussion over the past decade of the potential therapeutic benefit of generating primary human neurons using SCNT (and now iPS) to address neural injuries. While neural differentiation and development remain critically important areas of basic research, the immediate therapeutic benefits derived from neural–iPS investigations are likely to be pharmacologic/toxicologic in nature rather than in the field of neuroregenerative medicine. This is because, for large regions of the central nervous system, appropriate neural function is a result of both proper cellular differentiation/organization and a specific pattern of neural connectivity. Even when efficient strategies for the genesis of appropriate neural subtypes are determined, it is unclear how such cells will recapitulate proper patterns of neural connectivity. While some central nervous system regions can exhibit significant functional plasticity, this is not a general property of the system.[32] Clearly, if transplanted cells fail to recapitulate appropriate patterns of synaptic response, it will be difficult to rationalize the benefits of such "therapeutic" interventions. Therefore, the central nervous system may represent the ultimate boundary for cellular replacement.

ACKNOWLEDGMENTS

J.T.H. is a professor at the University of Toronto, working on mechanisms of neural injury and repair in the mammalian central nervous system.

CONFLICT OF INTEREST

The author declared no conflict of interest.

1. Krause, D.S. *et al.* Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell* **105**, 369–377 (2001).
2. Zwaginga, J.J. & Doevendans, P. Stem cell-derived angiogenic/vasculogenic cells: possible therapies for tissue repair and tissue engineering. *Clin. Exp. Pharmacol. Physiol.* **30**, 900–908 (2003).
3. Campbell, K.H. *et al.* Somatic cell nuclear transfer: past, present and future perspectives. *Theriogenology* **68** (suppl. 1), S214–S231 (2007).
4. Collas, P. & Taranger, C.K. Epigenetic reprogramming of nuclei using cell extracts. *Stem Cell Rev.* **2**, 309–317 (2006).
5. Tada, M., Tada, T., Lefebvre, L., Barton, S.C. & Surani, M.A. Embryonic germ cells induce epigenetic reprogramming of somatic nucleus in hybrid cells. *EMBO J.* **16**, 6510–6520 (1997).
6. Campbell, K.H., McWhir, J., Ritchie, W.A. & Wilmut, I. Sheep cloned by nuclear transfer from a cultured cell line. *Nature* **380**, 64–66 (1996).
7. Cibelli, J.B. *et al.* Cloned transgenic calves produced from nonquiescent fetal fibroblasts. *Science* **280**, 1256–1258 (1998).
8. Meng, L., Ely, J.J., Stouffer, R.L. & Wolf, D.P. Rhesus monkeys produced by nuclear transfer. *Biol. Reprod.* **57**, 454–459 (1997).
9. Onishi, A. *et al.* Pig cloning by microinjection of fetal fibroblast nuclei. *Science* **289**, 1188–1190 (2000).
10. Baguisi, A. *et al.* Production of goats by somatic cell nuclear transfer. *Nat. Biotechnol.* **17**, 456–461 (1999).
11. Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663–676 (2006).

12. Okita, K., Ichisaka, T. & Yamanaka, S. Generation of germline-competent induced pluripotent stem cells. *Nature* **448**, 313–317 (2007).
13. Wernig, M. *et al. In vitro* reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature* **448**, 318–324 (2007).
14. Tanaka, M., Hadjantonakis, A.K. & Nagy, A. Aggregation chimeras. Combining ES cells, diploid and tetraploid embryos. *Methods Mol. Biol.* **158**, 135–154 (2001).
15. Meissner, A., Wernig, M. & Jaenisch, R. Direct reprogramming of genetically unmodified fibroblasts into pluripotent stem cells. *Nat. Biotechnol.* **25**, 1177–1181 (2007).
16. Takahashi, K. *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861–872 (2007).
17. Yu, J. *et al.* Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**, 1917–1920 (2007).
18. White, J. & Dalton, S. Cell cycle control of embryonic stem cells. *Stem Cell Rev.* **1**, 131–138 (2005).
19. Nakagawa, M. *et al.* Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat. Biotechnol.* **26**, 101–106 (2007).
20. Clayton, E., Doupé, D.P., Klein, A.M., Winton, D.J., Simons, B.D. & Jones, P.H. A single type of progenitor cell maintains normal epidermis. *Nature* **446**, 185–189 (2007).
21. Hanna, J. *et al.* Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. *Science* **318**, 1920–1923 (2007).
22. Ertl, H.C. Challenges of immune responses in gene replacement therapy. *IDrugs* **8**, 736–738 (2005).
23. Wu, X. & Burgess, S.M. Integration target site selection for retroviruses and transposable elements. *Cell Mol. Life Sci.* **61**, 2588–2596 (2004).
24. Misra, R.P. & Duncan, S.A. Gene targeting in the mouse: advances in introduction of transgenes into the genome by homologous recombination. *Endocrine* **19**, 229–238 (2002).
25. Little, M., Hall, W. & Orlandi, A. Delivering on the promise of human stem-cell research. What are the real barriers? *EMBO Rep.* **7**, 1188–1192 (2006).
26. Caulfield, T., Ogbogu, U. & Isasi, R.M. Informed consent in embryonic stem cell research: are we following basic principles? *CMAJ* **176**, 1722–1725 (2007).
27. Karpowicz, P., Cohen, C.B. & van der Kooy, D. It is ethical to transplant human stem cells into nonhuman embryos. *Nat. Med.* **10**, 331–335 (2004).
28. Animal-human hybrid-embryo research. *Lancet* **370**, 909 (2007).
29. Daley, G.Q. *et al.* Ethics. The ISSCR guidelines for human embryonic stem cell research. *Science* **315**, 603–604 (2007).
30. Mimeault, M., Hauke, R. & Batra, S.K. Stem cells: a revolution in therapeutics-recent advances in stem cell biology and their therapeutic applications in regenerative medicine and cancer therapies. *Clin. Pharmacol. Ther.* **82**, 252–264 (2007).
31. Zandstra, P.W., Le, H.V., Daley, G.Q., Griffith, L.G. & Lauffenburger, D.A. Leukemia inhibitory factor (LIF) concentration modulates embryonic stem cell self-renewal and differentiation independently of proliferation. *Biotechnol. Bioeng.* **69**, 607–617 (2000).
32. Chen, R., Cohen, L.G. & Hallett, M. Nervous system reorganization following injury. *Neuroscience* **111**, 761–773 (2002).