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Issue: *Skeletal Biology and Medicine***iPS cell technology in regenerative medicine****Christopher J. Lengner**

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The promise of treating human genetic and degenerative diseases through the application of pluripotent cell-based tissue engineering and regenerative medicine has come significantly closer to realization since the isolation of human embryonic stem (ES) cells. While the study of ES cells has greatly increased our fundamental understanding of pluripotency, technical and ethical limitations have been seemingly insurmountable impediments to the application of these cells in the clinic. The recent discovery that somatic mammalian cells can be epigenetically reprogrammed to a pluripotent state through the exogenous expression of the transcription factors OCT4, SOX2, KLF4, and c-MYC has yielded a new cell type for potential application in regenerative medicine, the induced pluripotent stem (iPS) cell. Here we discuss how advances in iPS cell technology have led to the generation of patient-specific cell lines that can potentially be used to model human diseases and ultimately act as therapeutic agents.

**Keywords:** regenerative medicine; stem cells; epigenetic reprogramming; iPS cells

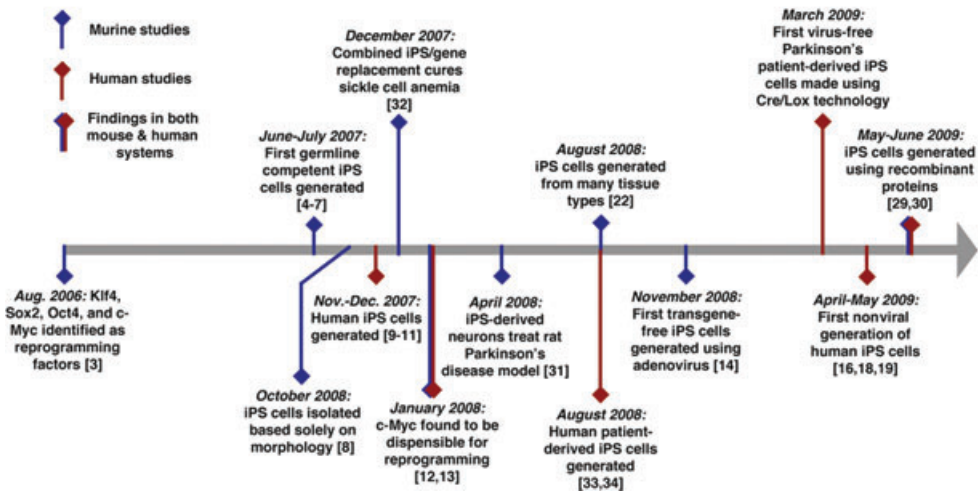
**From embryonic stem to induced pluripotent stem cells**

Since their isolation, embryonic stem cells have been regarded as the gold standard for potential use in cell-based regenerative medicine because of their pluripotent nature, that is, the ability to self-renew indefinitely in culture while retaining the capacity to differentiate into any cell type in the body.<sup>1</sup> Despite these remarkable qualities, ES cell-based therapy suffers from several limitations that have thus far prevented its application in treating human disease. The fact that ES cells can only be derived from early-stage embryos precludes the establishment of autologous cell lines for patients, and immune rejection hinders the use of nonautologous ES cell lines. The advent of mammalian somatic cell nuclear transfer (SCNT) seemed to offer solutions to overcoming these roadblocks. SCNT, or nuclear cloning, involves the transfer of a nucleus derived from a somatic cell into an enucleated oocyte, after which undefined factors in the ooplasm act to reverse the epigenetic state of the somatic donor cell to that of a pluripotent embryonic cell. This cloned cell is then allowed to develop until the blastocyst stage, at which point nuclear transfer-derived ES (NT-ES) cells can be isolated from the inner cell mass. These

NT-ES cells would then act as autologous donor cells for therapy. In principle, this strategy is feasible and has been demonstrated in a mouse model of immunodeficiency in which the generation of NT-ES cells followed by correction of the disease-causing genetic mutation and subsequent differentiation into and transplantation of the therapeutically relevant effector cells resulted in restoration of immune function in the diseased mouse.<sup>2</sup> While these experiments clearly demonstrate the potential for such a strategy, SCNT has not yet been successfully performed in humans.

The other major impediment to ES- and NT-ES-based therapies in humans involves the moral and ethical dilemma surrounding the requisite blastocyst destruction and oocyte donation necessary to generate patient-specific pluripotent stem cell lines. While technical hurdles could surely be overcome to make human SCNT successful, moral and ethical questions may not be so easily resolved. It is these limitations that have driven researchers to understand the mechanisms governing pluripotency and to experimentally impose the gene expression program underlying pluripotency upon the somatic genome. In 2006, a research group led by Dr. Shinya Yamanaka identified four transcription factors—Klf4, Sox2, Oct4, and c-Myc (hereafter

## Direct Epigenetic Reprogramming: The first three years



**Figure 1.** Recent advances in pluripotent stem cell-based technology.

referred to as KSOM)—that when introduced into mouse fibroblasts through retroviral transduction would lead to the formation of pluripotent clones that were selected for their ability to reactivate the nonessential Oct4 downstream target gene, *Fbx15* (Figure 1).<sup>3</sup> These first-generation induced pluripotent stem (iPS) cells exhibited partial demethylation and reactivation of the canonical pluripotency-governing genes *Oct4* and *Nanog*, but could not give rise to viable, germline-competent chimeras upon injection into blastocysts.

### Advances in iPS technology

Less than one year after the Yamanaka group's initial report, fully pluripotent iPS cells were generated simply by increasing the stringency of the selection strategy and selecting for reactivation of the master pluripotency regulators *Oct4* and *Nanog* themselves.<sup>4-7</sup> These new, second-generation iPS cells had fully demethylated *Oct4* and *Nanog* promoters and could contribute to the formation of germline-competent chimeras upon blastocyst injection, demonstrating their true pluripotency. Interestingly, these cells could not form viable mice by tetraploid complementation, an assay in which ES or iPS cells are injected into tetraploid blastocysts, resulting in embryos derived entirely from the injected cells, while extraembryonic tissues are derived

from the host blastocyst. Whether this failure is due to effects of the randomly integrated retroviral vectors used for reprogramming or represents a more fundamental defect in the developmental potency of iPS cells remains an open question. Shortly thereafter it was shown that pluripotent iPS cells could be identified solely on the basis of their morphology, eliminating the need for genetically modified reporter genes<sup>8</sup> and paving the way for isolation of iPS cells derived from human fibroblasts.<sup>9-11</sup> The finding that direct epigenetic reprogramming with the KSOM factors (or, in the case of Yu *et al.*, a combination of OCT4, SOX2, NANOG, and LIN28) can be applied to human cells represents the breaking of a species barrier that SCNT has yet to overcome.

One significant concern regarding the therapeutic value of iPS cells is the presence of proviral integrations harboring known oncogenes, particularly c-Myc, as well as Oct4 and Klf4. Indeed, chimeras derived from iPS cells are highly prone to tumors exhibiting c-Myc reactivation.<sup>7</sup> Subsequent studies fortuitously revealed that c-Myc was dispensable for iPS generation from fibroblast target cells (although iPS formation occurred with reduced efficiency), and chimeric mice derived from three-factor iPS cells (Oct4, Sox2, and Klf4) did not exhibit tumor formation as their four-factor predecessors had.<sup>12,13</sup> Despite the nonessential role of c-Myc in the reprogramming process, the potential for insertional

mutagenesis and the oncogenic properties of the other reprogramming factors has prompted several groups to undertake direct epigenetic reprogramming approaches using either nonviral methods, or retrospectively eliminate proviral integrations after iPS cell generation.

## Toward virus-free iPS cells

In working toward eliminating the potential for insertional mutagenesis during direct reprogramming, two fundamental strategies have been followed: the use of nonintegrating genetic elements or excision of genetic elements after iPS formation, and reprogramming without the use of genetic elements using small molecules or cytokines. In the fall of 2008, Stadtfeld *et al.* demonstrated successful reprogramming of murine hepatocytes with nonintegrating adenoviral vectors encoding KSOM, albeit with extremely low efficiency and with frequent tetraploidy in the resulting iPS cultures.<sup>14</sup> One month later, Okita *et al.* were able to eliminate the use of viral vectors altogether and reprogram murine embryonic fibroblasts by transient transfection of two plasmids encoding the four factors (Oct4, Sox2, and Klf4 in one plasmid, c-Myc in the other), yielding iPS cells free of transgenic integration.<sup>15</sup>

Reprogramming of human cells through transient introduction of the KSOM factors posed a greater challenge on account of the longer exposure required to generate human iPS cells. In 2009, Yu *et al.* reported successful iPS cell formation from human fibroblasts using self-replicating Epstein-Barr virus-derived episomal vectors encoding not only KSOM, but also the RNA binding protein LIN28, NANOG, and the SV40LT.<sup>16</sup> These transient approaches to reprogramming are all highly inefficient and frequently result in foreign DNA insertions in the genome, thus demanding rigorous screening after iPS clones are isolated to insure that integration-free lines are established. Using slightly different approaches, several other groups generated human iPS cells using integrating vectors that could subsequently be removed from the genome using *piggy-BAC* retrotransposition.<sup>17–19</sup> These studies employed polycistronic vectors harboring the four factors that could be integrated into the genome when cotransfected with a transposase. Once iPS cell lines were obtained, the vectors could be efficiently

excised from the genome by a second round of exposure to transposase. Another approach involved the use of traditional lentiviral vectors encoding the KSOM factors and carrying a LoxP site in the viral LTR that, upon insertion into the genome, becomes duplicated and allows for excision of the expression cassette, leaving behind only a single LoxP site in the genome.<sup>20</sup> Of importance, this study generated iPS cell lines from fibroblasts derived from patients with Parkinson's disease, making them the first patient-specific iPS lines free of viral integrations.

The second major approach to generating virus-free iPS cell lines employs the use of small molecules or recombinant proteins. Numerous studies have demonstrated improved reprogramming efficiency and/or kinetics using various compounds or proteins that effect cellular processes such as apoptosis, proliferation, chromatin modification, extracellular signaling cascades, and DNA methylation (reviewed by Feng *et al.*<sup>21</sup>). While the aforementioned studies provide valuable insight into the molecular mechanisms underlying the reprogramming process, the efficiencies currently achieved using the KSOM factors alone are sufficient to generate autologous iPS cell lines from cultures of patient-derived somatic fibroblasts (on the order of 0.001 [0.1%]). We therefore focus solely on studies aimed at the replacement of KSOM factors that have traditionally been delivered via genetic elements rather than on those aiming to increase reprogramming efficiency or kinetics. It is also of particular importance that KSOM factor replacement in reprogramming be assessed in adult dermal fibroblasts or keratinocytes, as these are the primary cell types readily accessible from patients.

In order to begin to establish large-scale chemical screens for reprogramming factor replacement, Wernig *et al.* initially generated lentiviral vectors in which the expression of the KSOM factors is under control of the tetracycline-responsive promoter (Tet-O).<sup>22</sup> By using these viruses to transduce fibroblasts harboring the reverse tetracycline transactivator (rtTA), iPS cell lines could be generated by the addition of doxycycline (dox) to the cultures, with kinetics very similar to those observed with direct infection using retroviral vectors.<sup>23</sup> Once iPS cell lines are established, dox was simply removed from the cultures and chimeric mice were generated by injection of the stable iPS cells into blastocysts. These chimeric mice were therefore

composed of iPS-derived cells harboring identical viral insertion sites and host blastocyst-derived cells. Such genetically identical iPS-derived somatic cells were termed “secondary” cells and could be isolated from chimeric tissues by virtue of a constitutively expressed puromycin resistance cassette at the ROSA26 locus. Re-exposure of secondary somatic cells to dox resulted in efficient reprogramming (up to 4%) in all cell types examined,<sup>22</sup> suggesting that KSOM-mediated reprogramming is not limited by the cell of origin. Because these mouse lines carried only one or two lentiviral integrations for each of the KSOM factors, the proviruses could be segregated in all possible combinations of K, S, O, and M simply by outbreeding to wild-type mice. This resulted in a “library” of mice as well as somatic cells derived from these mice that carry various combinations of three factors under dox-inducible control as well as a pluripotency reporter gene such as *Nanog-GFP* or *Nanog-neomycinR*. Ultimately, this library of three-factor somatic cells provides a screening platform in which the cell lines can be used to interrogate chemical libraries for compounds capable of replacing the fourth, missing factor.<sup>24</sup>

Several studies have recently made significant progress in replacing the KSOM factors with small molecules. Using an unbiased approach, Lyssiotis *et al.* screened a chemical library using SOM-infected fibroblasts for molecules that activate the endogenous *Nanog* locus (via a *Nanog-Luciferase* knockin) and identified one molecule, kenpaullone, that was able to replace Klf4.<sup>25</sup> Of interest, successful reprogramming in the absence of Klf4 was dependent on the presence of c-Myc, which is otherwise dispensable in KSO-mediated reprogramming. This suggests that kenpaullone does not replace the activity of Klf4 but might achieve reprogramming through activation of an alternative pathway.

Other studies have prospectively identified molecules that aid in reprogramming based on their known function. The G9a histone methyltransferase inhibitor BIX-01294 is able to replace Sox2, leading to successful iPS cell generation from fibroblasts using only Oct4 and Klf4.<sup>26</sup> This finding is of particular interest as G9a is known to play a central role in silencing the *Oct4* locus during early embryonic development and differentiation of ES cells,<sup>27</sup> suggesting that inhibition of *Oct4* repression in the presence of high exogenous Oct4 levels is sufficient to overcome the absence of Sox2. Sim-

ilarly, Huangfu *et al.* observed that the use of the histone deacetylase inhibitor valproic acid (VPA) not only increased reprogramming efficiency in the presence of KSOM, but was ultimately able to replace Klf4 and c-Myc, yielding full iPS clones with an Oct4-Sox2-VPA combination.<sup>28</sup> Taken together these findings indicate that chromatin remodeling acts as a rate-limiting factor in the reprogramming process. Whether molecules found to replace individual KSOM factors in these studies will ultimately be able to successfully mediate epigenetic reprogramming when used in combination has not yet been established.

Most recently, two studies demonstrated successful reprogramming of fibroblasts using recombinant KSOM proteins. Zhou *et al.* reprogrammed mouse fibroblasts with KSOM proteins tagged with a polyarginine protein transduction domain added repeatedly to the culture media along with VPA.<sup>29</sup> Similarly, Kim *et al.* were able to successfully reprogram human fibroblasts using cell lysates from HEK293 cells overexpressing KSOM proteins tagged with another highly basic peptide sequence derived from the HIV-TAT protein.<sup>30</sup> While this approach is relatively inefficient (around 0.001%), it is still reasonable to generate autologous iPS cell lines. Protein transduction also seems the most feasible approach for translation into a clinical setting on account of the complete absence of genetic elements, obviating the need to perform time-consuming and costly procedures to ensure the absence of integrations common to other approaches.

## Therapeutic potential of iPS cells

With an eye toward potential clinical application, the efficacy of iPS cells for several therapeutic applications has recently been tested using mouse and rat models of human disease. Areas of immediate interest for iPS-based cell therapy include neurodegenerative diseases that have been difficult to model and treat with small-molecule-based therapies and are increasingly affecting an aging population, as well as diseases in which *in vitro* differentiation and delivery protocols have been established with traditional ES cells. Wernig *et al.* demonstrated that iPS cells could be sequentially differentiated first into neural precursor cells, and then into dopaminergic neurons *in vitro* (with approximately 5% of neuronal cells generating tyrosine hydroxylase-positive dopaminergic

neurons).<sup>31</sup> Furthermore, upon transplantation of these dopaminergic neurons into the brain of a rat Parkinson's disease model, tyrosine hydroxylase-positive iPS-derived cells were found to have engrafted, expressed dopamine transporters, and ultimately alleviated behavioral symptoms. In another series of experiments, Hanna *et al.* treated a mouse model of sickle cell anemia using a targeted gene-correction approach.<sup>32</sup> Here, iPS cell lines were generated from a mouse strain expressing a human sickle hemoglobin allele using the KSOM factors, with a floxed c-Myc expression cassette allowing for deletion of the oncogene upon stable iPS generation. The resulting iPS lines then had the sickle hemoglobin allele replaced by a wild-type allele using standard gene-targeting techniques. These corrected iPS lines were subsequently differentiated into hematopoietic progenitor cells and transplanted into the affected donor mice, resulting in restoration of the functional hemoglobin protein in the bloodstream and restoring disease parameters such as red blood cell count, hematocrit, weight, and breath rate to normal or near-normal values. These studies offer a proof of principle for using iPS cell-based therapies for treatment of neurodegenerative and genetic disorders. Since the lines used in these experiments harbor numerous proviral integrations, the long-term safety of such iPS-derived cells remains unknown; however, when strategies such as these are combined with virus-free reprogramming and proper *in vitro* differentiation protocols to avoid teratoma formation resulting from the presence of undifferentiated iPS cells in the transplant, safe and long-lasting therapeutic effects should be achievable.

Several groups have also begun the generation of patient-specific human iPS cell lines. Park *et al.* generated a library of patient-derived iPS lines from numerous disorders including Huntington's disease, juvenile diabetes mellitus, Down syndrome, muscular dystrophy, and several others.<sup>33</sup> Of particular interest are iPS cells derived from patients suffering from neurodegenerative diseases.<sup>20,33–35</sup> These iPS lines can be differentiated *in vitro* into the affected neuronal cell type, generating for the first time a model for idiopathic neurodegenerative disorders which can be screened in culture for the onset, cell autonomy, and contribution of environmental factors to the phenotype. Ultimately, if human neurodegenerative phenotypes can be recapit-

ulated in iPS cell-derived culture models, these cells could be screened using chemical libraries to identify molecules that can arrest or even reverse the progression of these disorders. Most recently, Raya *et al.* generated iPS cells from Fanconi's anemia (FA) patient-derived fibroblasts first infected with lentiviral vectors expressing FANCA or FANCD2 (proteins known to alleviate the FA phenotype).<sup>36</sup> These "corrected" iPS lines were demonstrated to have re-established a functional FA pathway, indicating that iPS generation combined with traditional gene therapy may be a viable approach for treating this disorder.

## Conclusions

Since the identification of the four reprogramming factors in the fall of 2006, the iPS cell field has experienced dramatic progress in such a short period of time that the possibility of patient-customized regenerative medicine promised by somatic cell nuclear transfer may now be realized by iPS cells. While nuclear transfer offered extraordinary insight into the process of epigenetic reprogramming, progress was slow because of the extreme technical demands, inefficiency, and cost of the technique compounded by the moral and ethical concerns previously discussed. This resulted in advances coming from a very small number of highly specialized labs. The speed at which the iPS field has advanced can be directly ascribed to the ease and cost-efficiency of KSOM-based reprogramming that has enabled many labs across the globe to use this technique to study epigenetic reprogramming and optimize therapeutic approaches. The accelerated pace of iPS research now more than ever necessitates the establishment of standard practices and procedures in order to control genetic and epigenetic stability of iPS cell lines for potential use in human therapies. With a coordinated and cooperative effort, iPS technology could, for the first time, make autologous pluripotent cell-based regenerative medicine a reality.

## Conflicts of interest

The author declares no conflicts of interest.

## References

1. Evans, M.J. & M.H. Kaufman. 1981. Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**, 154–156.

2. Rideout, W.M., 3rd, K. Hochedlinger, M. Kyba, *et al.* 2002. Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy. *Cell* **109**: 17–27.
3. Takahashi, K. & S. Yamanaka. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**: 663–676.
4. Wernig, M. *et al.* 2007. In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature* **448**: 318–324.
5. Takahashi, K., K. Okita, M. Nakagawa & S. Yamanaka. 2007. Induction of pluripotent stem cells from fibroblast cultures. *Nat. Protoc.* **2**: 3081–3089.
6. Maherali, N. *et al.* 2007. Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. *Cell Stem Cell* **1**: 55–70.
7. Okita, K., T. Ichisaka & S. Yamanaka. 2007. Generation of germline-competent induced pluripotent stem cells. *Nature* **448**: 313–317.
8. Meissner, A., M. Wernig & R. Jaenisch. 2007. Direct reprogramming of genetically unmodified fibroblasts into pluripotent stem cells. *Nat. Biotechnol.* **25**: 1177–1181.
9. Park, I.H. *et al.* 2008. Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* **451**: 141–146.
10. Yu, J. *et al.* 2007. Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**: 1917–1920.
11. Takahashi, K. *et al.* 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**: 861–872.
12. Wernig, M., A. Meissner, J.P. Cassady & R. Jaenisch. 2008. c-Myc is dispensable for direct reprogramming of mouse fibroblasts. *Cell Stem Cell* **2**: 10–12.
13. Nakagawa, M. *et al.* 2008. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat. Biotechnol.* **26**: 101–106.
14. Stadtfeld, M., M. Nagaya, J. Utikal, *et al.* 2008. Induced pluripotent stem cells generated without viral integration. *Science* **322**: 945–949.
15. Okita, K., M. Nakagawa, H. Hyenjong, *et al.* 2008. Generation of mouse induced pluripotent stem cells without viral vectors. *Science* **322**: 949–953.
16. Yu, J. *et al.* 2009. Human induced pluripotent stem cells free of vector and transgene sequences. *Science* **324**: 797–801.
17. Yusa, K., R. Rad, J. Takeda & A. Bradley. 2009. Generation of transgene-free induced pluripotent mouse stem cells by the piggyBac transposon. *Nat. Methods* **6**: 363–369.
18. Woltjen, K. *et al.* 2009. piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature* **458**: 766–770.
19. Kaji, K. *et al.* 2009. Virus-free induction of pluripotency and subsequent excision of reprogramming factors. *Nature* **458**: 771–775.
20. Soldner, F. *et al.* 2009. Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. *Cell* **136**: 964–977.
21. Feng, B., J.H. Ng, J.C. Heng & H.H. Ng. 2009. Molecules that promote or enhance reprogramming of somatic cells to induced pluripotent stem cells. *Cell Stem Cell* **4**: 301–312.
22. Wernig, M. *et al.* 2008. A drug-inducible transgenic system for direct reprogramming of multiple somatic cell types. *Nat. Biotechnol.* **26**: 916–924.
23. Brambrink, T. *et al.* 2008. Sequential expression of pluripotency markers during direct reprogramming of mouse somatic cells. *Cell Stem Cell* **2**: 151–159.
24. Markoulaki, S. *et al.* 2009. Transgenic mice with defined combinations of drug-inducible reprogramming factors. *Nat. Biotechnol.* **27**: 169–171.
25. Lyssiotis, C.A. *et al.* 2009. Reprogramming of murine fibroblasts to induced pluripotent stem cells with chemical complementation of Klf4. *Proc. Natl. Acad. Sci. USA* **106**(22): 8912–8917.
26. Shi, Y. *et al.* 2008. Induction of pluripotent stem cells from mouse embryonic fibroblasts by Oct4 and Klf4 with small-molecule compounds. *Cell Stem Cell* **3**: 568–574.
27. Feldman, N. *et al.* 2006. G9a-mediated irreversible epigenetic inactivation of Oct-3/4 during early embryogenesis. *Nat. Cell Biol.* **8**: 188–194.
28. Huangfu, D. *et al.* 2008. Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. *Nat. Biotechnol.* **26**: 1269–1275.
29. Zhou, H. *et al.* 2009. Generation of induced pluripotent stem cells using recombinant proteins. *Cell Stem Cell* **4**: 381–384.
30. Kim, D. *et al.* 2009. Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. *Cell Stem Cell* **4**: 472–476.
31. Wernig, M. *et al.* 2008. Neurons derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease. *Proc. Natl. Acad. Sci. USA* **105**: 5856–5861.
32. Hanna, J. *et al.* 2007. Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. *Science* **318**: 1920–1923.

33. Park, I.H. *et al.* 2008. Disease-specific induced pluripotent stem cells. *Cell* **134**: 877–886.
34. Dimos, J.T. *et al.* 2008. Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. *Science* **321**: 1218–1221.
35. Ebert, A.D. *et al.* 2009. Induced pluripotent stem cells from a spinal muscular atrophy patient. *Nature* **457**: 277–280.
36. Raya, A. *et al.* 2009. Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells. *Nature* **460**(7251): 53–59.