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Induced Pluripotent Stem Cell Lines Derived from Human Somatic Cells

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Somatic cell nuclear transfer allows trans-acting factors present in the mammalian oocyte to reprogram somatic cell nuclei to an undifferentiated state. We show that four factors (*OCT4*, *SOX2*, *NANOG*, and *LIN28*) are sufficient to reprogram human somatic cells to pluripotent stem cells that exhibit the essential characteristics of embryonic stem (ES) cells. These induced pluripotent human stem cells have normal karyotypes, express telomerase activity, express cell surface markers and genes that characterize human ES cells, and maintain the developmental potential to differentiate into advanced derivatives of all three primary germ layers. Such induced pluripotent human cell lines should be useful in the production of new disease models and in drug development, as well as for applications in transplantation medicine, once technical limitations (for example, mutation through viral integration) are eliminated.

Mammalian embryogenesis elaborates distinct developmental stages in a strict temporal order. Nonetheless, because development is dictated by epigenetic rather than genetic events, differentiation is, in principle, reversible. The cloning of Dolly demonstrated that nuclei from mammalian differentiated cells can be reprogrammed to an undifferentiated state by trans-acting factors present in the oocyte (1), and this discovery led to a search for factors that could mediate similar reprogramming without somatic cell nuclear transfer. Recently, four transcription factors (*Oct4*, *Sox2*, *c-myc*, and *Klf4*) were shown to be sufficient to reprogram mouse fibroblasts to undifferentiated, pluripotent stem cells [termed induced pluripotent stem (iPS) cells] (2–5). Reprogramming human cells by defined factors would allow the generation of patient-specific pluripotent cell lines without somatic cell nuclear transfer, but the observation that the expression of *c-Myc* causes death and differentiation of human ES cells suggests that combinations of factors lacking this gene are required to reprogram human cells (6). We demonstrate that *OCT4*,

SOX2, *NANOG*, and *LIN28* are sufficient to reprogram human somatic cells.

Human ES cells can reprogram myeloid precursors through cell fusion (7). To identify candidate reprogramming factors, we compiled a list of genes with enriched expression in human ES cells relative to that of myeloid precursors and prioritized the list based on known involvement in the establishment or maintenance of pluripotency (table S1). We then cloned these genes into a lentiviral vector (fig. S1) to screen for combinations of genes that could reprogram the differentiated derivatives of an *OCT4* knock-in human ES cell line generated through homologous recombination (8). In this cell line, the expression of neomycin phosphotransferase, which makes cells resistant to geneticin, is driven by an endogenous *OCT4* promoter, a gene that is highly expressed in pluripotent cells but not in differentiated cells. Thus, reprogramming events reactivating the *OCT4* promoter can be recovered by geneticin selection. The first combination of 14 genes that we selected (table S2) directed the reprogramming of adherent cells, which were derived from human

ES cell–derived CD45⁺ hematopoietic cells (7, 9), to geneticin-resistant (*OCT4*⁺) colonies with an ES cell morphology (fig. S2A) (10). These geneticin-resistant colonies expressed typical human ES cell–specific cell surface markers (fig. S2B) and formed teratomas when injected into immunocompromised severe combined immunodeficient–beige mice (fig. S2C).

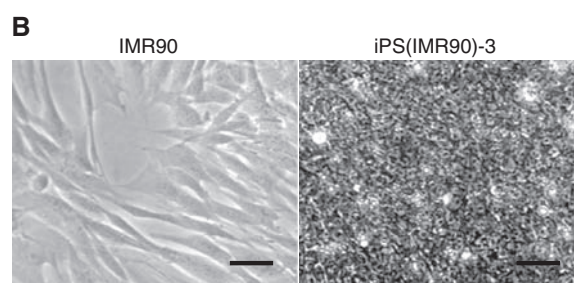
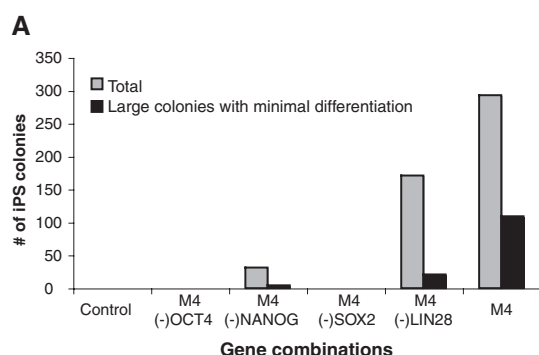
By testing subsets of the 14 initial genes, we identified a core set of 4 genes, *OCT4*, *SOX2*, *NANOG*, and *LIN28*, that were capable of reprogramming human ES cell–derived somatic cells with a mesenchymal phenotype (Fig. 1A and fig. S3). Removal of either *OCT4* or *SOX2* from the reprogramming mixture eliminated the appearance of geneticin-resistant (*OCT4*⁺) reprogrammed mesenchymal clones (Fig. 1A). *NANOG* showed a beneficial effect in clone recovery from human ES cell–derived mesenchymal cells but was not required for the initial appearance of such clones (Fig. 1A). These results are consistent with cell fusion–mediated reprogramming experiments, where overexpression of Nanog in mouse ES cells resulted in over a 200-fold increase in reprogramming efficiency (11). The expression of *NANOG* also improves the cloning efficiency of human ES cells (12) and thus could increase the survival rate of early reprogrammed cells. *LIN28* had a consistent but more modest effect on reprogrammed mesenchymal cell clone recovery (Fig. 1A).

We next tested whether *OCT4*, *SOX2*, *NANOG*, and *LIN28* are sufficient to reprogram

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Fig. 1. Optimization of human reprogramming gene combinations with mesenchymal cells derived from human *OCT4* knock-in H1 ES cells. (A) Effect of removal of individual genes from the M4 (*OCT4*, *NANOG*, *SOX2*, and *LIN28*) reprogramming mixture. Human iPS colonies were counted on day 15 after lentiviral transduction.



Control indicates no transduction or indicates that cells were transduced with *NANOG* only. In three independent experiments with different preparations of mesenchymal cells, individual removal of either *OCT4* or *SOX2* from reprogramming combinations eliminated the appearance of reprogrammed clones, whereas the individual removal of either *NANOG* or *LIN28* reduced the number of reprogrammed clones but did not eliminate such clones entirely.

The data presented are from one representative experiment. (B) Bright-field images of IMR90 fibroblasts (p18) and iPS(IMR90)-3 (p18+p18), where the first p18 refers to the passage number of IMR90 fibroblasts, and the second p18 means that the reprogrammed clones underwent 18 passages on irradiated mouse embryonic fibroblasts (MEFs). Scale bars, 50 μ m.

primary, genetically unmodified, diploid human fibroblasts. We initially chose IMR90 fetal fibroblasts, because these diploid human cells are being extensively characterized by the ENCODE Consortium (13), are readily available through the American Type Culture Collection [(ATCC), catalog number CCL-186], and have published

DNA fingerprints that allow confirmation of the origin of reprogrammed clones. IMR90 cells also proliferate robustly for more than 20 passages before undergoing senescence but grow slowly in human ES cell culture conditions, a difference that provides a proliferative advantage to reprogrammed clones and aids in their selection by

morphological criteria (e.g., compact colonies, high nucleus-to-cytoplasm ratios, and prominent nucleoli) alone (14, 15). IMR90 cells were transduced with a combination of *OCT4*, *SOX2*, *NANOG*, and *LIN28*. Colonies with a human ES cell morphology (iPS colonies) first became visible 12 days after transduction. On day 20, a

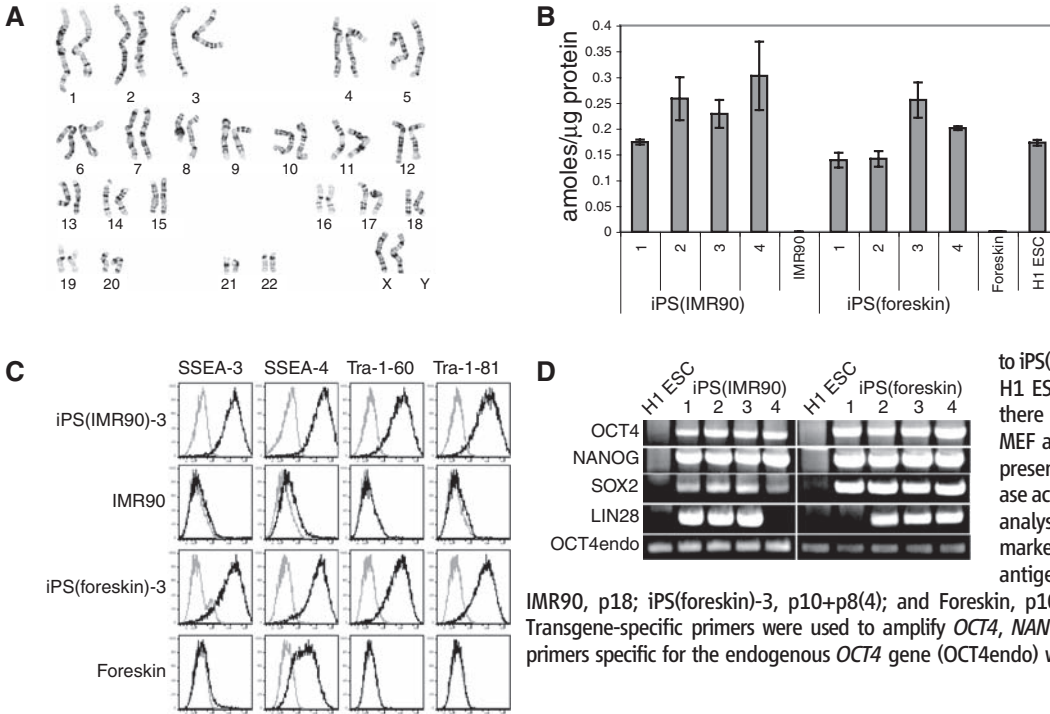


Fig. 2. Reprogramming of IMR90 fibroblasts. **(A)** G-banding chromosome analysis of iPS(IMR90)-3 (p18+p23). **(B)** Telomerase activity is shown for the following: iPS(IMR90)-1 to iPS(IMR90)-4, p18+p22(20) [where p18 refers to the passage number of IMR90 fibroblasts and where p22(20) means that the reprogrammed clones underwent 22 passages, with 2 on MEF and 20 on matrigel in human ES cell culture medium conditioned with MEF (CM)]; IMR90, p18; iPS(foreskin)-1 to iPS(foreskin)-4, p10+p9(5); Foreskin, p10; and H1 ESC (human H1 ES cells), p63(13) [where there was a total of 63 passages, with 50 on MEF and 13 on matrigel in CM]. The data are presented as mean \pm SD from three telomerase activity assays. **(C)** Flow cytometry expression analyses of human ES cell-specific cell surface markers. Gray line, isotype control; black line, antigen staining. iPS(IMR90)-3, p18+p5(3); IMR90, p18; iPS(foreskin)-3, p10+p8(4); and Foreskin, p10. **(D)** Provirus integration in iPS cells. Transgene-specific primers were used to amplify *OCT4*, *NANOG*, *SOX2*, and *LIN28* provirus, whereas primers specific for the endogenous *OCT4* gene (*OCT4*endo) were used as a positive control.

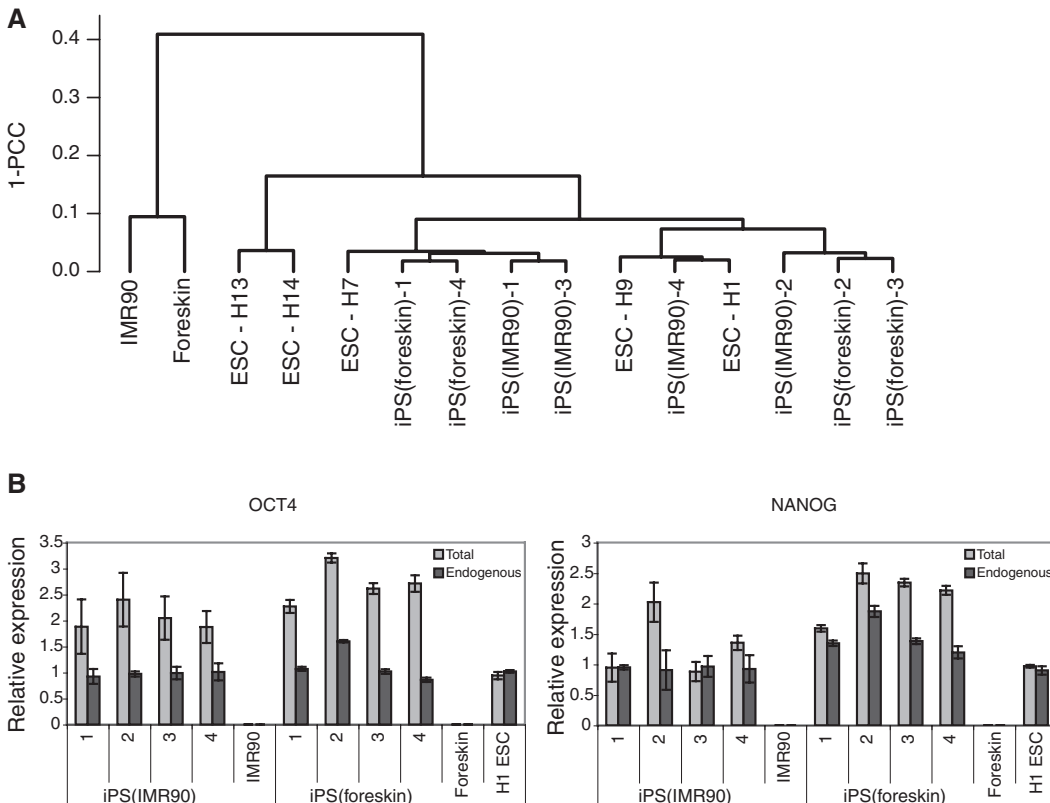


Fig. 3. Global gene expression analyses of iPS cells. **(A)** Pearson correlation analyses of global gene expression (47,759 transcripts) in iPS(IMR90) clones [p18+p6(4)], IMR90 fibroblasts (p19), iPS(foreskin) clones [p10+p7(3)], foreskin fibroblasts (p10), and five human ES cell lines: H1 [p42(12)], H7 [p73(3)], H9 [p50(5)], H13 [p43(5)], and H14 [p61(5)] ES cells (GEO accession number GSE9164). 1-PCC, Pearson correlation coefficient. **(B)** Quantitative reverse transcription-PCR analyses of *OCT4* and *NANOG* expression in iPS(IMR90) [p18+p6(4)] and iPS(foreskin) clones [p10+p7(3)]. IMR90, p19; foreskin fibroblasts, p10; and H1 ESC, p42(12). “Endogenous” indicates that primers were included in the 3’ untranslated region measure expression of the endogenous gene only, whereas “total” indicates that primers in coding regions measure expression of both the endogenous gene and the transgene if present. The data are presented as mean \pm SD from three telomerase activity assays.

total of 198 iPS colonies was visible from 0.9 million initial IMR90 cells, whereas no iPS colonies were observed in nontransduced controls. Forty-one iPS colonies were selected, 35 of which were successfully expanded for an additional 3 weeks. Four clones [iPS(IMR90)-1 to iPS(IMR90)-4] with minimal differentiation were selected for continued expansion and detailed analysis.

Each of the four iPS(IMR90) clones had a typical human ES cell morphology (Fig. 1B) and a normal karyotype at both 6 and 17 weeks of culture (Fig. 2A). Each iPS(IMR90) clone expressed telomerase activity (Fig. 2B) and the human ES cell-specific cell surface antigens SSEA-3, SSEA-4, Tra-1-60, and Tra-1-81 (Fig. 2C), whereas the parental IMR90 cells did not. Microarray analyses of gene expression of the four iPS(IMR90)

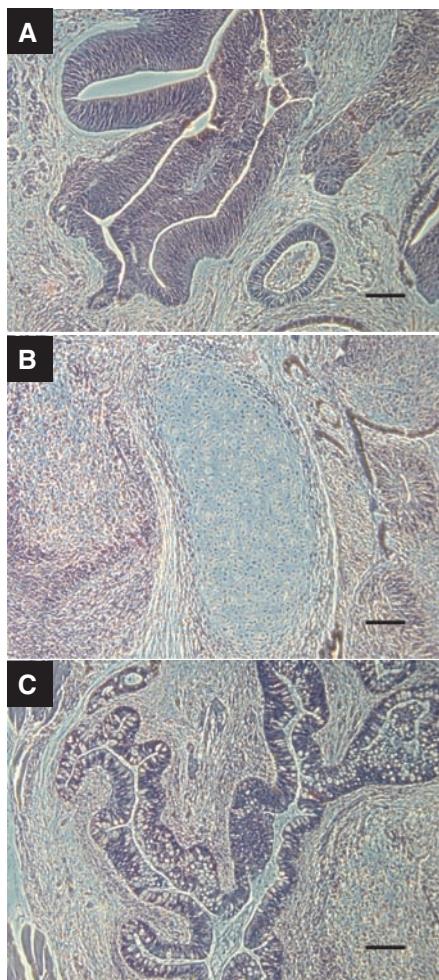


Fig. 4. Pluripotency of iPS(IMR90) cells. Hematoxylin and eosin staining of teratoma sections of iPS(IMR90)-1 (9 weeks after injection). Two six-well plates of iPS(IMR90)-1 cells on MEF (~60 to 70% confluent) were injected into the hind-limb muscle of two mice. Teratomas were obtained from all four iPS(IMR90) clones, injected both at 7 and 15 weeks after initial transduction. The two control mice, each of which were injected with ~12 million IMR90 (p19) fibroblasts, failed to form teratomas. (A) Neural tissue (ectoderm); (B) cartilage (mesoderm); (C) gut epithelium (endoderm). Scale bars, 0.1 mm.

clones confirmed a similarity to five human ES cell lines (H1, H7, H9, H13, and H14) and a dissimilarity to IMR90 cells (Fig. 3, table S3, and fig. S4). Although there was some variation in gene expression between different iPS(IMR90) clones (fig. S5), the variation was actually less than that between different human ES cell lines (Fig. 3A and table S3). For each of the iPS(IMR90) clones, the expression of the endogenous *OCT4* and *NANOG* was at levels similar to that of human ES cells, but the exogenous expression of these genes varied between clones and between genes (Fig. 3B). For *OCT4*, some expression from the transgene was detectable in all of the clones, but for *NANOG*, most of the clones demonstrated minimal exogenous expression, which suggests silencing of the transgene during reprogramming. Analyses of the methylation status of the *OCT4* promoter showed differential methylation between human ES cells and IMR90 cells (fig. S6). All four iPS(IMR90) clones exhibited a demethylation pattern similar to that of human ES cells and distinct from that of the parental IMR90 cells. Both embryoid body (fig. S7) and teratoma formation (Fig. 4) demonstrated that all four of the reprogrammed iPS(IMR90) clones had the developmental potential to give rise to differentiated derivatives of all three primary germ layers. DNA fingerprinting analyses [with short tandem repeat (STR) markers] confirmed that these iPS clones were derived from IMR90 cells and confirmed that they were not from the human ES cell lines that we have in the laboratory (table S4). The STR analysis published on the ATCC website for IMR90 cells used the same primer sets and confirms the identity of the IMR90 cells used for these experiments. The iPS(IMR90) clones were passed at the same ratio (1:6) and frequency (every 5 days) as human ES cells, had doubling times similar to that of the human H1 ES cell line assessed under the same conditions (table S5), and, as of this writing, have been in continuous culture for 22 weeks with no observed period of replicative crisis. Starting with an initial four wells of a six-well plate of iPS cells (with one clone per well, which is equivalent to about 1 million cells), after 4 weeks of additional culture, 40 total 10-cm dishes (representing about 350 million cells) of the four iPS(IMR90) clones were cryopreserved and confirmed to have normal karyotypes.

Because IMR90 cells are of fetal origin, we next examined the reprogramming of postnatal fibroblasts. Human newborn foreskin fibroblasts (ATCC, catalog number CRL-2097) were transduced with *OCT4*, *SOX2*, *NANOG*, and *LIN28*. From 0.6 million foreskin fibroblasts, we obtained 57 iPS colonies. No iPS colonies were observed in nontransduced controls. Twenty-seven out of 29 chosen colonies were successfully expanded for three passages, four of which [iPS(foreskin)-1 to iPS(foreskin)-4] were selected for continued expansion and analyses. DNA fingerprinting of the iPS(foreskin) clones matched the fingerprints for the parental fibroblast cell line published on the ATCC website (table S4).

Each of the four iPS(foreskin) clones had a human ES cell morphology (fig. S8A), had a normal karyotype (fig. S8B), and expressed telomerase, cell surface markers, and genes characteristic of human ES cells (Figs. 2 and 3 and fig. S5). Each of the four iPS(foreskin) clones proliferated robustly and, as of this writing, have been in continuous culture for 17 weeks. Each clone demonstrated multilineage differentiation both in embryoid bodies and teratomas (figs. S9 and S10); however, unlike the iPS(IMR90) clones, there was variation between the clones in the lineages, apparent in teratomas examined at 5 weeks. In particular, neural differentiation was common in teratomas from iPS(foreskin) clones 1 and 2 (fig. S9A) but was largely absent in teratomas from iPS(foreskin) clones 3 and 4. Instead, there were multiple foci of columnar epithelial cells reminiscent of primitive ectoderm (fig. S9D). This is consistent with the embryoid body data (fig. S10), where the increase in *PAX6* (a neural marker) in iPS(foreskin) clones 3 and 4 was minimal as compared with the other clones, a difference that correlated with a failure to downregulate *NANOG* and *OCT4*. A possible explanation for these differences is that specific integration sites in these clones allowed continued high expression of the lentiviral transgenes, partially blocking differentiation.

Polymerase chain reaction (PCR) for the four transgenes revealed that *OCT4*, *SOX2*, and *NANOG* were integrated into all four of the iPS(IMR90) clones and all four of the iPS(foreskin) clones but that *LIN28* was absent from one iPS(IMR90) clone [iPS(IMR90)-4] and from one iPS(foreskin) clone [iPS(foreskin)-1] (Fig. 2D). Thus, although *LIN28* can influence the frequency of reprogramming (Fig. 1A), these results confirm that it is not absolutely required for the initial reprogramming, nor is it subsequently required for the stable expansion of reprogrammed cells.

The human iPS cells described here meet the defining criteria that we originally proposed for human ES cells (14), with the notable exception that the iPS cells are not derived from embryos. Similar to human ES cells, human iPS cells should prove useful for studying the development and function of human tissues, for discovering and testing new drugs, and for transplantation medicine. For transplantation therapies based on these cells, with the exception of autoimmune diseases, patient-specific iPS cell lines should largely eliminate the concern of immune rejection. It is important to understand, however, that before the cells can be used in the clinic, additional work is required to avoid vectors that integrate into the genome, potentially introducing mutations at the insertion site. For drug development, human iPS cells should make it easier to generate panels of cell lines that more closely reflect the genetic diversity of a population and should make it possible to generate cell lines from individuals predisposed to specific diseases. Human ES cells remain controversial because their derivation

involves the destruction of human preimplantation embryos, and iPS cells remove this concern. However, further work is needed to determine whether human iPS cells differ in clinically important ways from ES cells.

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declare competing financial interests. J.A.T. owns stock, serves on the Board of Directors, and serves as Chief Scientific Officer of Cellular Dynamics International and Stem Cell Products. J.A.T. also serves as Scientific Director of the WiCell Research Institute. Microarray data have been deposited in the Gene Expression Omnibus (GEO) database (accession number GSE9164).

Supporting Online Material

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Materials and Methods
Figs. S1 to S10
Tables S1 to S7
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Treatment of Sickle Cell Anemia Mouse Model with iPS Cells Generated from Autologous Skin

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It has recently been demonstrated that mouse and human fibroblasts can be reprogrammed into an embryonic stem cell–like state by introducing combinations of four transcription factors. However, the therapeutic potential of such induced pluripotent stem (iPS) cells remained undefined. By using a humanized sickle cell anemia mouse model, we show that mice can be rescued after transplantation with hematopoietic progenitors obtained *in vitro* from autologous iPS cells. This was achieved after correction of the human sickle hemoglobin allele by gene-specific targeting. Our results provide proof of principle for using transcription factor–induced reprogramming combined with gene and cell therapy for disease treatment in mice. The problems associated with using retroviruses and oncogenes for reprogramming need to be resolved before iPS cells can be considered for human therapy.

A major goal of human therapy is to develop methods that allow treatment of patients afflicted with genetic and degenerative disorders with a ready supply of defined transplantable cells. This has raised great interest in embryonic stem (ES) cells, which have the potential to generate all cell types in culture (1). ES cell–based therapy, however, would be complicated by immune rejection due to immunological incompatibility between patient and donor cells. As a result, the concept of deriving genetically identical “customized” ES-like cells by somatic cell nuclear transfer (SCNT) using a donor cell from the patient was developed (2). This strategy was expected to eliminate the requirement for immune suppression (3), but technical and ethical complexities of SCNT impede the practical realization of “therapeutic cloning” (4).

In a recent series of studies, mouse and human fibroblasts were reprogrammed *in vitro* into pluri-

potent stem cell–like cells (termed “induced pluripotent stem cells,” or iPS) through retroviral transduction of combinations of transcription factors (5–9). This was achieved by selection for reprogrammed cells by reactivation of marked endogenous pluripotency genes Oct4 or Nanog or by subcloning of colonies based on morphological criteria (5–11). iPS cells derived from mouse and human fibroblasts are highly similar to ES cells by genetic, epigenetic, and developmental criteria. However, it remained to be determined whether mouse iPS cells obtained from adult fibroblasts can serve to restore physiological function of diseased tissues *in vivo*.

To gain insights into the therapeutic applicability of mouse iPS cells, we evaluated whether hematopoietic progenitors (HPs) could be derived from iPS cells *in vitro* for subsequent engraftment into adult recipients (12). Tail-tip fibroblasts were isolated from a 2-week-old Oct4-Neomycin knock-in mouse (8) and a 3-month-old genetically unmodified mouse. Cells were transduced with retroviruses encoding for Oct4, Sox2, Klf4, and c-Myc transcription factors (13). Neomycin was added 9 days after infection to fibroblasts derived from Oct4-neo mouse to select for cells that reactivated the endogenous Oct4 gene, a master regulator of pluripotency, and neomycin-

resistant colonies were picked on day 20. Transduced fibroblasts from genetically unmodified mice gave rise to colonies that were picked based on morphological criteria (10, 11). Ten out of 12 picked clones eventually generated cell lines with ES-like morphology that expressed ES cell markers AP, SSEA1, and Nanog. Lines designated as ITT026 and ITT4 iPS were randomly chosen from Oct4-Neo and unmodified donor cells, respectively, for further analysis (fig. S1).

Ectopic expression of homeodomain protein HoxB4 in differentiating ES cells has been shown to confer engraftment potential on *in vitro*–derived hematopoietic cells from ES cells grown in hematopoietic cytokines on the OP9 bone marrow stroma cell line, which has been shown to support hematopoietic differentiation (12, 14). Dissociated embryoid body (EB) differentiated cells generated from V6.5 ES cells and fibroblast-derived ITT026 and ITT4 iPS cells were infected with Moloney virus encoding green fluorescent protein (GFP)–tagged HoxB4 protein (12). Cells expressing CD41 and c-kit antigens (markers of early HPs), as well as markers for myeloid and erythroid differentiation, were detected at similar levels on cells differentiated from the ES and iPS lines (Fig. 1A and fig. S2). Moreover, methylcellulose colony formation assays showed that all samples formed a variety of immature and mature myeloid colonies at comparable frequencies (Fig. 1B and fig. S3). We next transplanted these *in vitro*–generated HPs into irradiated genetically identical adult C57black6/129Sv F1 recipient mice. HPs from both ES and iPS cells conveyed multilineage reconstitution of recipient mice, as determined by GFP content in peripheral blood for up to 20 weeks, and rescued the mice from lethal irradiation (Fig. 1C). Fluorescence-activated cell sorting (FACS) analysis showed predominant myeloid lineage formation from the transplanted progenitors (fig. S4), consistent with previous studies (12, 14, 15).

These experiments prompted us to evaluate the therapeutic potential of iPS cells derived from adult fibroblasts of mice afflicted with a genetic disorder of the hematopoietic system. The general therapeutic strategy applied involved (i) reprogramming of mutant donor fibroblasts into iPS cells, (ii) repair of the genetic defect through homologous recom-

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