

Induced pluripotent stem cells from highly endangered species

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For some highly endangered species there are too few reproductively capable animals to maintain adequate genetic diversity, and extraordinary measures are necessary to prevent extinction. We report generation of induced pluripotent stem cells (iPSCs) from two endangered species: a primate, the drill, *Mandrillus leucophaeus* and the nearly extinct northern white rhinoceros, *Ceratotherium simum cottoni*. iPSCs may eventually facilitate reintroduction of genetic material into breeding populations.

Induced pluripotent stem cells (iPSCs) are generated from somatic cells by direct molecular reprogramming and, as are embryonic stem cells, are capable of unlimited expansion and differentiation into multiple cell types. iPSCs have been generated from somatic cells of humans^{1,2} and research animals such as mouse³ and rhesus monkey⁴. We adapted iPSC technology to reprogram rare animal species, in an effort to use this technology to preserve genetic material of endangered species. The San Diego Zoo Institute for Conservation Research maintains an extensive collection of frozen fibroblast cultures, called the Frozen Zoo, comprised of viable cells of over 8,600 individual vertebrates of ~800 species. We report here to our knowledge the first generation of iPSCs from the endangered primate, the drill *Mandrillus leucophaeus*, and from the nearly extinct northern white rhinoceros, *Ceratotherium simum cottoni*.

The drill is one of Africa's most endangered mammals. In the wild, drills are found only in small areas of Nigeria, Southwestern Cameroon and Equatorial Guinea⁵. The number of drills is declining as a result of illegal bush meat commerce and habitat destruction. Populations of drills in North American zoos are derived from a small number of founders, which risks excessive inbreeding, raising concerns for population sustainability. In addition, disorders such as diabetes compromise population growth and management⁶. The northern white rhinoceros is hunted for its horns and is on the

verge of extinction. An estimated population of 2,230 individuals in 1960 has been reduced to only seven living individuals today⁷. Two wild-born northern white rhinoceroses currently reside in the San Diego Zoo Safari Park. The other five (one wild-born and four born in captivity) were kept until recently at the Zoo Dvůr Králové in the Czech Republic. In an effort to save the species, four rhinoceroses with reproductive potential were relocated from the Zoo Dvůr Králové to the Ol Pejeta Conservancy in Kenya in 2009. However, efforts to encourage natural reproduction have so far been unsuccessful, and the last birth of a northern white rhinoceros was in 2000. Because of the critical status of both the drill and northern white rhinoceros populations, we chose these species for reprogramming to iPSCs that might aid conservation efforts.

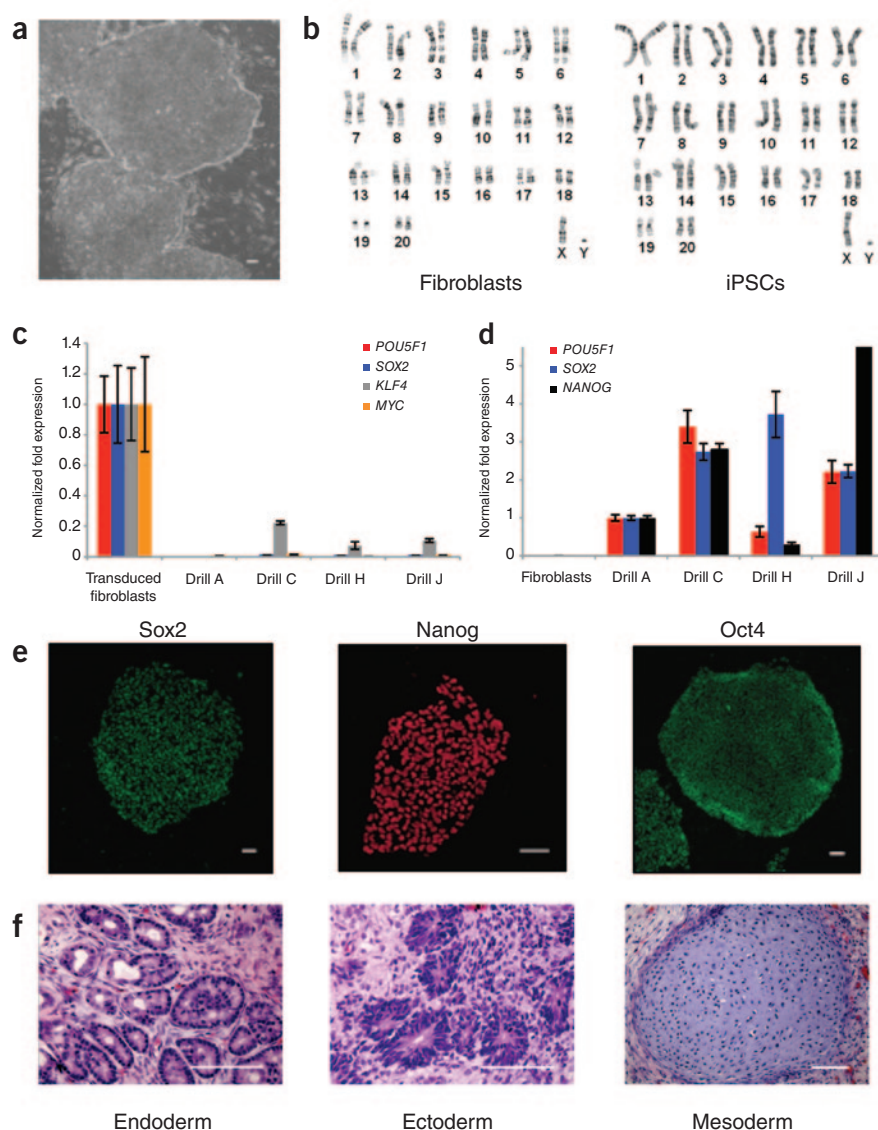
To reprogram the primate cells, we used retroviral vectors carrying human sequences of the reprogramming factors *POU5F1* (also known as *OCT4*), *SOX2*, *KLF4* and *MYC* (also known as *cMYC*) on a cryopreserved fibroblast cell line from a male drill (named Loon) sampled at 15 years of age (now dead). We generated four iPSC lines (named drill A C, H and J) from the fibroblasts (Fig. 1). We initially selected these lines by their unique morphology that is characteristic of embryonic stem cells and iPSCs derived from other species (Fig. 1a). Drill iPSCs were karyotypically normal, as determined by comparison with the source fibroblasts (Online Methods, Fig. 1b and **Supplementary Fig. 1**). Genomic PCR revealed that the drill iPSCs carried the exogenous sequences for the reprogramming genes (**Supplementary Fig. 2**). Because we found that northern white rhinoceros fibroblasts could not be transduced with amphotrophic retroviruses, we used retro-VSV.G viruses (**Supplementary Fig. 3**) carrying the same four reprogramming factors, on a cryopreserved fibroblast cell line initiated from the youngest living northern white rhinoceros, a female named Fatu, born in 2000 and sampled at ten years of age (currently residing in the Conservatory in Kenya). We generated three rhinoceros iPSC lines (northern white rhinoceros (NWR) A, B and C) from this cell line (Fig. 2). We also selected these lines by their characteristic morphology (Fig. 2a). NWR iPSCs were karyotypically normal, as determined by comparison with the source fibroblasts (Fig. 2b and **Supplementary Fig. 4**). Genomic PCR revealed that the three reprogrammed NWR cell lines carried the exogenous sequences for the reprogramming genes *POU5F1*, *SOX2* and *MYC*, but not *KLF4* (**Supplementary Fig. 5**). This observation suggests that *KLF4* is not essential to the reprogramming process in the northern white rhinoceros.

Two critical characteristics of fully reprogrammed iPSCs are silencing of the integrated exogenous sequences and the induction of endogenous genes associated with pluripotency. Quantitative reverse transcription-PCR for the four reprogramming factor

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Figure 1 | Characterization of drill iPSCs.

(a) Phase-contrast micrograph of representative drill iPSCs. (b) Karyotypes of drill fibroblasts and drill iPSC line G (passage 10). (c) Quantitative PCR analysis of exogenous reprogramming factors *POU5F1*, *SOX2*, *KLF4* and *MYC* in drill iPSC lines and in fibroblasts collected 10 d after transduction with viruses encoding the exogenous transcription factors. Expression was normalized to *GAPDH* levels. (d) Normalized expression of endogenous *POU5F1*, *SOX2* and *NANOG* analyzed in drill iPSC lines and fibroblasts by quantitative PCR. (e) Micrographs show representative immunocytochemical staining of drill iPSCs with antibodies to the indicated pluripotency markers (all four drill iPSC lines had the same immunocytochemical marker profile). (f) Hematoxylin and eosin staining of teratoma derived from drill iPSC line J. Endoderm lineage is represented by seromucinous gland, mesoderm lineage by cartilage and ectoderm by neural rosettes. All scale bars, 100 μ m.



human transcripts showed that the exogenous genes were silenced in drill iPSC lines (Fig. 1c) and NWR iPSC lines (Fig. 2c) and endogenous pluripotency-associated genes, *POU5F1*, *SOX2* and *NANOG*, were activated in the drill iPSC lines (Fig. 1d). Drill and NWR iPSCs had additional characteristics of pluripotent stem cells. Drill iPSCs had alkaline phosphatase activity (Supplementary Fig. 6) and were immunopositive for Oct4, Sox2 and Nanog (Fig. 1e). Flow cytometry analysis of two of the lines showed that more than 80% of the drill iPSCs were Nanog-positive and more than 65% were Oct4-positive (Supplementary Fig. 7). Immunocytochemistry analysis showed that endogenous pluripotency-associated genes were also activated in the NWR iPSC lines (Fig. 2d), and more than 85% of the NWR cells (two lines) were Nanog-positive and 75% were Oct4-positive by flow cytometry (Supplementary Fig. 8). Glycomic profiling⁸ showed that the drill and NWR iPSCs clustered with pluripotent stem cell lines from other species, and were very different from differentiated tissues and the fibroblasts used for reprogramming (Supplementary Fig. 9).

Both drill and NWR iPSCs formed embryoid bodies *in vitro* that differentiated into cells expressing markers of the three germ layers: ectoderm, mesoderm and endoderm, as determined by immunocytochemistry and gene expression analyses for the drill (Supplementary Fig. 10) and by immunocytochemistry analysis for the rhinoceros (Fig. 2e). We confirmed tri-lineage differentiation potential of all drill iPSC lines *in vivo* by the observation of teratomas in CB17-Prkdc^{scid} (JAX database identifier) severely combined immunodeficient mice. Upon histological inspection of the teratomas, we observed seromucinous gland, cartilage and neural rosettes, which demonstrated the differentiation potential into endoderm, mesoderm and ectoderm, respectively (Online Methods and Fig. 1f). Sequencing of the mitochondrial gene encoding cytochrome *b* confirmed that the teratomas were of drill origin (Supplementary Fig. 11).

The iPSC lines we generated are to our knowledge the first reported for any endangered species and the first iPSCs derived from cells preserved in the Frozen Zoo. The Frozen Zoo of the San Diego Zoo Institute for Conservation Research was established in 1972 to preserve cells from animals for ongoing and future efforts to assist in the survival of species that are in danger of extinction. In many cases, the cryopreserved cells are fibroblasts; based on our success with drill and northern white rhinoceros fibroblasts, it seems likely that iPSCs can be generated from other endangered species, which could be useful for their assisted reproduction and species-specific cell therapy.

Although mouse iPSCs have been generated from human reprogramming factors, we did not anticipate that the rhinoceros cells would respond to human factors. But cloning of species-specific reprogramming factors requires material such as embryos or embryonic stem cells that express the appropriate genes, and obtaining such material from endangered species is not possible. Furthermore, genomes of neither of the two species whose cells we reprogrammed had been sequenced. We initially set out to construct reprogramming vectors from related animals, the rhesus monkey for the drill and

Figure 2 | Characterization of northern white rhinoceros iPSCs.

(a) Phase-contrast micrograph of northern white rhinoceros iPSCs. (b) Karyotype of northern white rhinoceros fibroblasts and iPSCs (line C, passage 4). (c) Quantitative reverse-transcription-PCR analysis of the exogenous sequences for the human reprogramming factors in northern white rhinoceros iPSC lines. (d) Micrographs show immunocytochemical staining of northern white rhinoceros iPSC lines with antibodies to the indicated pluripotency markers. (e) Micrographs show immunocytochemistry analysis for markers of all three germ layers in northern white rhinoceros embryoid bodies. SMA, smooth muscle actin. All scale bars, 100 μ m.

the horse for the rhinoceros, but then observed that the human sequences were effective, albeit at a very low efficiency (Online Methods). Similarly, we found that some, but not all, human-specific antibodies and primers gave positive results in the nonhuman cells.

Because they are self-renewing and pluripotent, iPSCs from endangered species have several possible applications⁹. The iPSCs we produced are potentially useful for developing therapeutic applications for captive animals that suffer from diabetes and other degenerative diseases. For nearly extinct species such as the northern white rhinoceros, iPSCs might be a means to rescue the species from extinction: preserving the genomes of individual animals as pluripotent stem cells opens the possibility of producing iPSC-derived germ cells, which could be used in conjunction with assisted reproduction efforts to increase the size and diversity of the population. Generation of germ cells from pluripotent stem cells is an active area of research^{10,11} and progress reported in the development of assisted reproductive technologies for related Old World monkeys^{12–15} and rhinoceros¹⁶ indicates that advanced reproductive technologies may be available for these rare species in the future. Substantial challenges remain, and dedicated efforts will be necessary to integrate reprogramming technology into the aggressive concerted efforts to conserve endangered species, but our success in generating iPSCs offers hope that these cells may be a means to help rescue species on the verge of extinction.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturemethods/>.

Note: Supplementary information is available on the Nature Methods website.

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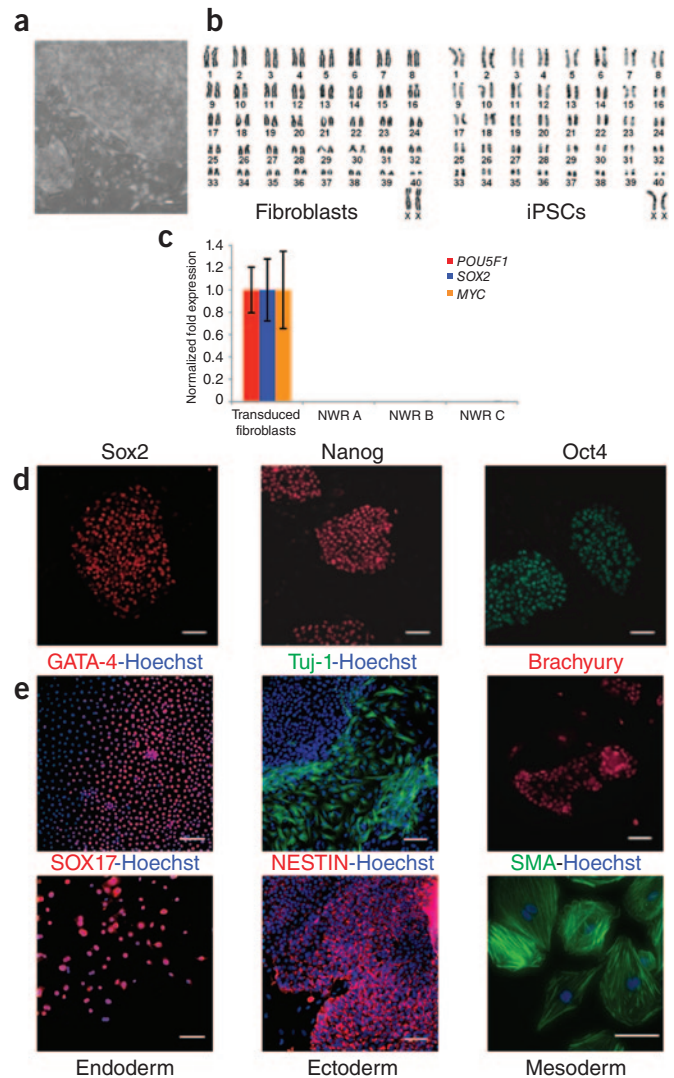
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AUTHOR CONTRIBUTIONS

J.F.L., O.A.R. and I.F.B.-N. conceived the study. I.F.B.-N. designed and performed the experiments. S.C.M. assisted with tissue-culture work. O.A.R., M.L.H. and S.J.C. generated the fibroblast cell lines and karyotyped the cells. H.T.T., I.G. and T.R.L. assisted with teratoma formation. Y.-C.W. performed glycomic profiling. L.C.L. contributed to experiment design. I.F.B.-N. and J.F.L. wrote the manuscript with input from all authors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.



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ONLINE METHODS

Generating endangered species fibroblast cell lines. Fibroblast cells were established using tissue dissociation with 0.5% (wt/vol) collagenase on a 4-mm skin biopsy. Fibroblast cultures at passage 2 were cryopreserved and accessioned into the Frozen Zoo at the Zoological Society of San Diego. Cell line 7078 was initiated on 25 February 1994 from a 15-year-old male drill, *M. leucophaeus*. This drill named Loon was born on 16 September 1979 and died on 17 June 2003. The fibroblast cell line was submitted to the Integrated Primate Biomaterials and Information Resource (7078-3207; PR#325) supported by a US National Science Foundation grant (BCS0094993). Cell line 17626 was initiated in January 2010, from a 10-year-old female northern white rhinoceros, *C. simum cottoni*. This northern white rhinoceros named Fatu was born 29 June 2000 and is alive as of 29 August 2011.

Cell culture. The retroviral packaging cell line Phoenix Amphi (Orbigen) was cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% (vol/vol) FBS. We cultured 293FT cells (Life Technologies) in DMEM supplemented with 10% FBS, 0.1 mM non-essential amino acids (NEAA), 2 mM GlutaMAX (Life Technologies), 1 mM sodium pyruvate and 500 $\mu\text{g ml}^{-1}$ G418. *M. leucophaeus* (drill) fibroblasts at passage 6–8 were cultured in Alpha MEM supplemented with 10% FBS and 2 mM GlutaMAX. Northern white rhinoceros fibroblasts at passage 6–8 were cultured in 1:1 mixture of Alpha MEM (supplemented with 10% FBS and 2 mM GlutaMAX) and Clonetic's Fibroblast Growth medium (Lonza). Drill and northern white rhinoceros iPSCs were cultured in iPSC growth medium consisting of DMEM-F12 1:1 (Life Technologies) supplemented with 20% (vol/vol) Knockout Serum Replacement (Life Technologies), 2 mM GlutaMAX, 0.1 mM NEAA, insulin-transferrin-selenium G (100 \times , diluted 1:100), 0.1 M 2-mercaptoethanol and 12 ng ml^{-1} of human recombinant fibroblast growth factor (basic FGF; Stemgent). Drill and northern white rhinoceros iPSCs were expanded by mechanical passaging and cryopreserved using methods developed for human iPSCs.

Virus production and generation of iPSCs. The vectors pMXs encoding human *POU5F1*, pMXs encoding human *SOX2*, pMXs encoding human *KLF4* and pMXs encoding human *MYC* were obtained from Addgene (plasmids 17217, 17218, 17219 and 17220, respectively). For retrovirus production, Phoenix Amphi cells (Orbigen) were seeded onto dishes coated with poly(D-lysine) (Sigma) (5.5×10^6 cells per 100 mm dish). Twenty-four hours later the cells were transfected with 20 μg of pMXs encoding human *POU5F1*, *SOX2*, *KLF4* or *MYC* using Lipofectamine 2000 (Life Technologies), following the manufacturer's instructions. For retrovirus production with the VSV.G protein, 293FT cells (Life Technologies) were seeded onto poly(D-lysine)-coated dishes (5.5×10^6 cells per 100 mm dish). Twenty-four hours later the cells were transfected with 10 μg of pMXs vector, 10 μg of *MSCV-gag-pol* (Addgene plasmid 14887) and 10 μg of *MSCV-VSV.G* (Addgene plasmid 14888) using Lipofectamine 2000, following the manufacturer's instructions.

On the day of transduction, 10 ml of virus-containing medium was collected from each dish and filtered through a 0.45- μm polyvinylidene fluoride (PVDF) filter. Polybrene (10 $\mu\text{g ml}^{-1}$) was added, and 2 ml of each retrovirus were applied to drill 7078 fibroblasts that were seeded 24 h before, at 10^5 cells per 35-mm dish.

Cells were transduced twice, with virus collected 48 h and 72 h after transfection. Twenty-four hours after the second round of transduction, transduced cells were fed with fibroblast medium and were allowed to grow for another 2 d. Then, drill cells were collected and seeded onto irradiated mouse embryo fibroblast (MEF) layers at 10^5 cells per 100-mm dish, supplemented with fibroblast medium. The medium was changed 24 h later to iPSC medium supplemented with 10 μM ROCK inhibitor (Y427632, Stemgent) and 0.5 mM valproic acid (Stemgent). iPSC medium supplemented with valproic acid was changed every 2 d for 14 d. After 14 d, cells were grown in iPSC medium only. The first drill iPSC colony (drill A) was isolated 30 d after transduction, with reprogramming efficiency of 0.0003%. The other three iPSC lines (drill C, H and J) were isolated 21 d, 32 d and 44 d after transduction, respectively, with reprogramming efficiency of 0.001%. Northern white rhinoceros 17626 fibroblasts were seeded at 5×10^4 cells per 35-mm dish, and were transduced 24 h later with 2 ml of each retro-VSV.G virus. The transduced cells were treated as described above for drill transduced fibroblasts. Northern white rhinoceros iPSC lines were isolated 27 d (NWR lines A and B) and 34 d (NWR C) after transduction, with reprogramming efficiency of 0.0006%. In each reprogramming experiment we transduced human dermal fibroblast cells (ScienCell) in parallel for a quality control for the reprogramming process and iPSC characterization. Efficiency of reprogramming of the human fibroblasts was 0.01–0.05%.

Karyotyping. Metaphase chromosomes were obtained from either fibroblasts or iPSCs following the standard protocol for monolayer cultures¹⁷, followed by G-banding of the mitotic cell samples¹⁵. Chromosomes were identified and numbered according to previous reports for *M. leucophaeus*¹⁴ and *C. simum cottoni*¹⁸. Karyotypes were prepared using the Genetix CytoVision imaging system. Drill iPSC lines drill A, C, H and J were karyotyped at passage 16, 10, 9 and 18, respectively. Northern white rhinoceros iPSC lines NWR A, B and C were karyotyped at passage 4.

Immunocytochemistry. Cells were fixed in 4% (wt/vol) paraformaldehyde for 15 min, washed with PBS (pH 7.2), and blocked in 2% (wt/vol) BSA in PBS, 0.1% (vol/vol) Triton X-100, 2% (wt/vol) low-fat milk for 20 min at room temperature (20–25 °C). Cells were then incubated overnight at 4 °C with antibodies to human proteins diluted in blocking solution. After washing with PBS, cells were incubated for 90 min at room temperature with secondary antibodies diluted in blocking solution and were stained with 1 $\mu\text{g ml}^{-1}$ Hoechst 33258 (Acros Organics) to stain nuclei.

Primary antibodies used were: Oct4 (1:100, Santa Cruz, sc-5279), Sox2 (1:100, Santa Cruz, sc-17320), Nanog (1:50, Santa Cruz, sc-33759), Tuj-1 (b-tubulin; 1:1,500, Convance, MRB-435P), smooth muscle actin (SMA, 1:4,000, Chemicon, CBL171), Sox17 (1:100, R&D Systems, AF1924), Nestin (1:10,000, Chemicon, AB5922), Brachyury (1:500, Santa Cruz, sc-H-210), GATA4 (1:100, SC-1237). Secondary antibodies used were: DyeLight549-labeled donkey antibody to rabbit immunoglobulin gamma (IgG), DyeLight549-labeled donkey antibody to mouse IgG, DyeLight488-labeled donkey antibody to mouse IgG, DyeLight488-labeled donkey antibody to rabbit IgG, DyeLight549-labeled donkey antibody to goat IgG (all 1:200; Jackson ImmunoResearch Laboratories).

Flow cytometry analysis for quantification of pluripotent stem cell-associated marker expression. Cells were collected (passage 9 (drill C), passage 20 (drill H), passage 5 (NWR B), and passage 6 (NWR C)) and fixed with 4% paraformaldehyde for 15 min, washed with PBS and incubated for 1 h in either blocking solution alone (0.1% Tween 20 and 0.25% (wt/vol) BSA in PBS) or blocking solution containing the primary antibody to Oct4 (1:100, Santa Cruz, sc-5279) or to Nanog (1:50, Santa Cruz, sc-33759). Cells were then washed with PBS and incubated for 1 h in blocking solution containing the respective secondary antibody. Cells were then washed with PBS and resuspended in buffer (1 mM EDTA, 25 mM HEPES (pH 7.0) and 1% FBS in PBS) and kept in the dark at 4 °C until the day of analysis. Flow cytometry was performed with a BD FACSCalibur Flow Cytometer. The results were analyzed using FlowJo software.

Reverse transcriptase-PCR and genomic PCR. Total RNA was extracted using mirVana kit (Ambion) according to the manufacturer's instructions. Removal of genomic DNA followed by reverse transcription of the RNA was performed with the Quantitect RT kit (Qiagen). Genomic DNA was extracted from cells with DNeasy Blood and Tissue Kit (Qiagen). PCR was performed on genomic DNA using 2× ReddyMix PCR Master Mix (Thermo Scientific). Quantitative PCR was performed on either cDNA or genomic DNA using SYBR Green PCR Master Mix (Life Technologies) in CFX96 Real-Time system (C1000 Thermal Cycle, Bio-Rad). Gene expression levels were normalized to *GAPDH* levels. Primer sequences are available in **Supplementary Table 1**.

Copy-number assay. The copy number assay was done on genomic DNA following the manufacturer's instructions, using copy-number variation (CNV) TaqMan assays (Life Technologies) for *POU5F1* (also known as *OCT4*), *SOX2*, *KLF4* and *MYC*.

Glycomic profiling of proteins isolated from cultured cells. The proteins of cultured pluripotent and nonpluripotent cells were isolated using CellLytic MEM protein extraction kit (Sigma-Aldrich), and the manufacturer's protocol with minor modifications⁸. In brief, hydrophobic phase containing hydrophobic proteins was obtained from cells by homogenization in 300 µl of lysis and separation buffer. The proteins were labeled with 50 µg of Alexa Fluor 555 succinimidyl ester (Life Technologies) and purified using Zeba desalt spin columns (7,000 Da molecular weight cutoff; Thermo

Scientific). The concentration of the purified proteins was adjusted into 500 ng ml⁻¹ with Probing solution (GPBiosciences) to produce chip-ready samples. The LecChip lectin microarray chips were incubated with 100 µl of the chip-ready samples at 4 °C for 16 h. Hierarchical clustering analysis to determine the similarity of glycoprotein compositions among cells was performed using US National Institute on Aging Array Analysis Tool (<http://lgsun.grc.nia.nih.gov/ANOVA/index.html>). Additional details and results of glycomic analysis of iPSCs are available in ref. 8.

Differentiation. iPSC lines were mechanically cut and placed in ultra-low attachment plates in iPSC medium without bFGF. The medium was changed every 2 d. After a week in culture, embryoid bodies were transferred onto gelatin-coated plates, and cultured for another week in differentiation medium containing DMEM, 20% FBS, 0.1 mM NEAA and 2 mM GlutaMAX. Embryoid bodies were then fixed and stained with antibodies as described above.

Teratoma formation and histological evaluation. iPSCs were collected by STEMPro EZPassage tool (Life Technologies), collected into tubes, counted and then centrifuged. Cells were then resuspended in a 1:1 mixture of DMEM-F12 and Matrigel (BD Biosciences) and placed on ice. One million cells were injected into the right testis of a CB-17-Prkdc^{scid} mouse (Jackson Laboratories). Six to eight weeks after injection, tumors were dissected, weighed and fixed with PBS containing 4% paraformaldehyde. The specimens were sectioned and stained with hematoxylin and eosin. The sections were microscopically evaluated by a histopathologist. All animal work was conducted under the approval of the Institutional Care and Use Committee of The Scripps Research Institute. Genomic DNA was extracted from a teratoma using genomic DNA extraction buffer containing (20 mM Tris-HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA, 0.5% SDS and 1 mg ml⁻¹ proteinase K). The mitochondrial gene cytochrome *b* (*cytb*) was amplified using primers L14841 and H15149. Primer sequences are available in **Supplementary Table 1**. The PCR products were sequenced and identified by comparison with GenBank sequences. Drill iPSCs and the teratoma both contained drill cytochrome *b*, and the human cells had human sequence.

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