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Generation of mouse–human chimeric embryos

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Naive human pluripotent stem cells (hPSCs) can be used to generate mature human cells of all three germ layers in mouse–human chimeric embryos. Here, we describe a protocol for generating mouse–human chimeric embryos by injecting naive hPSCs converted from the primed state. Primed hPSCs are treated with a mammalian target of rapamycin inhibitor (Torin1) for 3 h and dissociated to single cells, which are plated on mouse embryonic fibroblasts in 2iLI medium, a condition essentially the same for culturing mouse embryonic stem cells. After 3–4 d, bright, dome-shaped colonies with mouse embryonic stem cell morphology are passaged in 2iLI medium. Established naive hPSCs are injected into mouse blastocysts, which produce E17.5 mouse embryos containing 0.1–4.0% human cells as quantified by next-generation sequencing of 18S ribosomal DNA amplicons. The protocol is suitable for studying the development of hPSCs in mouse embryos and may facilitate the generation of human cells, tissues and organs in animals.

Introduction

Mammalian development from a fertilized egg to a newborn follows a deterministic process prescribed by the genome. The pluripotent stem cells (PSCs) in the inner cell mass of a blastocyst generate all cells of the newborn. These PSCs in the preimplantation blastocyst, which are epitomized in vitro by mouse embryonic stem cells (mESCs), are in the naive state of pluripotency. In contrast, PSCs in the epiblast of a postimplantation embryo, which are represented in vitro by mouse epiblast stem cells (mEpiSCs)[1] or human embryonic stem cells (hESCs)[2], are in the primed state of pluripotency[3]. Injection of mESCs into a mouse blastocyst generates chimeric mice that show full developmental competency of the injected mESCs, including their ability to generate germ cells capable of reproduction[4]. However, injection of primed PSCs, such as mEpiSCs[1] or hESCs[5], into mouse blastocysts fails to generate chimeras. While primed mEpiSCs can be converted to mESC-like cells[6], chimera-competent mESC-like cells are generated only when E-cadherin is overexpressed in the mEpiSC[7]. Whether human PSCs can be converted from the primed state to the naive state has been under intense study for the past decade.

Naive mouse PSCs, such as mESCs and mouse induced pluripotent stem cells (iPSCs), form bright, refractive, dome-shaped colonies. They are maintained in an undifferentiated state indefinitely in 2iL medium containing leukemia inhibitory factor (LIF) and two inhibitors (2i): PD0325901 (a MEK inhibitor) and CHIR99021 (a glycogen synthase kinase-3 (GSK3) inhibitor)[8]. The 2iL medium also includes N2 and B27 supplements, which have an undisclosed amount of insulin and other components[8]. Primed PSCs, such as mEpiSCs, hESCs and human iPSCs, form large and flat colonies that can be maintained in indefinite self-renewal in hESC medium containing basic fibroblast growth factor (bFGF) and knockout serum replacement (KOSR). Previous studies have successfully utilized 2iL medium in combination with various small-molecule compounds to convert hPSCs from primed to naive state[9–14]. However, the resulting naive hPSCs do not generate robust chimerism in mouse embryos[15,16]. It is unclear whether the constant presence of various inhibitors beyond 2i in these methods confines the naive hPSCs to a state quite different from that of mESCs and thus may compromise their ability to form robust chimeras. In our recent study, we use a transient (3 h) inhibition of mammalian target of rapamycin (mTOR) to convert hPSCs from the primed to the naive state in a defined medium containing 2i, LIF and insulin (termed 2iLI)[17]. When our naive hPSCs are injected into mouse blastocysts, mature human cells of all three germ layers, including enucleated red blood cells, liver cells and ocular cells, are found in mouse embryos at E17.5.

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Using next-generation sequencing (NGS) of 18S ribosomal DNA (rDNA) amplicons, we find that the mouse–human chimeras contain 0.14–4.06% human cells[17].

In this protocol, we describe how to convert hPSCs, including hESCs and human iPSCs, from the primed to the naive state using a transient treatment of the mTOR inhibitor Torin1 in 2iLI medium, which is essentially the same medium used to maintain mESCs. We also describe the standard procedures used in our core facility to inject naive hPSCs into mouse blastocysts and to transfer the injected mouse blastocysts to pseudopregnant mice. Finally, we describe how to quantify the percentage of human DNA in genomic DNA isolated from E17.5 mouse embryos using NGS of 18S rDNA amplicons in our core facility.

Development of the protocol

Since 2010, several studies have shown that overexpression of Yamanaka factors in primed state hPSCs converts them to a naive-like state that can be maintained in the 2iL condition in a transgene-dependent manner[18–21]. Because naive pluripotency in these cells requires continuous expression of transgenes under the control of doxycycline, they are not tested for chimera competency. Subsequent experiments replace doxycycline-induced overexpression of Yamanaka factors with various combinations of chemicals and growth factors in addition to 2iL to maintain naive pluripotency[9–13,22]. Additional inhibitors and growth factors in these studies include either (1) c-Jun N-terminal kinase inhibitor SP600125, p38 inhibitor SB203580, protein kinase C inhibitor Gö6983, transforming growth factor β1 and bFGF[9]; (2) bone morphogenetic protein type I receptor inhibitor dorsomorphin[10]; (3) protein kinase C inhibitor Gö6983[22]; (4) GSK3β inhibitor IM-12, B-RAF inhibitor SB590885, Src inhibitor WH-4-023, Rho-kinase (ROCK) inhibitor Y-27632, activin A and bFGF[11]; (5) histone deacetylase inhibitors sodium butyrate and suberoylanilide hydroxamic acid, plus bFGF[12]; or (6) GSK3β inhibitor CHIR99021, bFGF and activin A[13]. Transcriptomic analyses suggest that some of these naive hPSCs share more similarities than others to cells in human blastocysts[17,23]. When injected into mouse or pig blastocysts, naive hPSCs made with methods (1), (4) or (6) generate very limited chimerism in early embryos[9,11,13,24]. Using various combinations of inhibitors that are quite different from the 2iL condition, expanded PSCs are generated from mouse, pig and human PSCs[14,25,26]. These inhibitors range from compounds that block histamine receptors and PARP1 (poly(ADP-ribose) polymerase 1)[14] to compounds that block Src kinase, c-Jun N-terminal kinase, p38 kinase, mitogen-activated protein kinase and tankyrases[25], along with activin and vitamin C[26]. The resulting expanded PSCs generate not only embryonic cells but also extraembryonic cells when they are injected into mouse blastocysts. However, the incorporation of human cells into mouse embryos is very modest and involves unknown cell types[14,26].

These previous studies suggest that chronic culture of naive hPSCs in compounds or growth factors in addition to 2iL does not support robust chimerism in mouse embryos. It seems plausible that the additional chemicals and/or growth factors may confine naive hPSCs to a state not quite compatible with codevelopment in mouse blastocysts. Thus, we seek to convert hPSCs from the primed state to the naive state by a transient treatment, the effects of which can be maintained afterward in the canonical 2iL condition. Transcription factor E3 (TFE3), critically involved in pluripotency[27] and nutrient sensing[28,29], is localized in the cytoplasm in primed hPSCs[9,21] but resides in the nucleus in naive mESCs[27] and naive hPSCs[9,21]. We find that a transient (3 h) inhibition of mTOR by Torin1 or rapamycin, which induces the translocation of TFE3 from the cytoplasm to the nucleus, converts hPSCs from the primed state to the naive state. The resulting naive hPSCs are maintained in the 2iLI medium and support the robust generation of mouse–human chimeric embryos[17]. The fully defined 2iLI medium (containing 2i, human LIF and human insulin) is very similar to the 2iL medium, which contains undisclosed amounts of insulin and other components from N2 and B27 supplements.

An accurate and robust method to quantify human cells in chimeras is essential to assessing how well injected naive hPSCs codevelop in a mouse embryo. An ideal method should have high sensitivity and specificity in detecting human cells in a vast number of mouse cells while maintaining the ability to handle samples generated by different laboratories on different platforms in a highly reproducible fashion. Previous studies use real-time quantitative PCR (qPCR) to measure a human-specific region in human mitochondria DNA (hmtDNA), which is normalized against the ultra-conserved noncoding region shared between human and mouse nuclear DNA[14,24,30]. The high copy number of hmtDNA allows the detection of human DNA diluted 1:10^5 in mouse DNA. However, there are three major caveats associated with this method. First, copy numbers of mitochondrial DNA

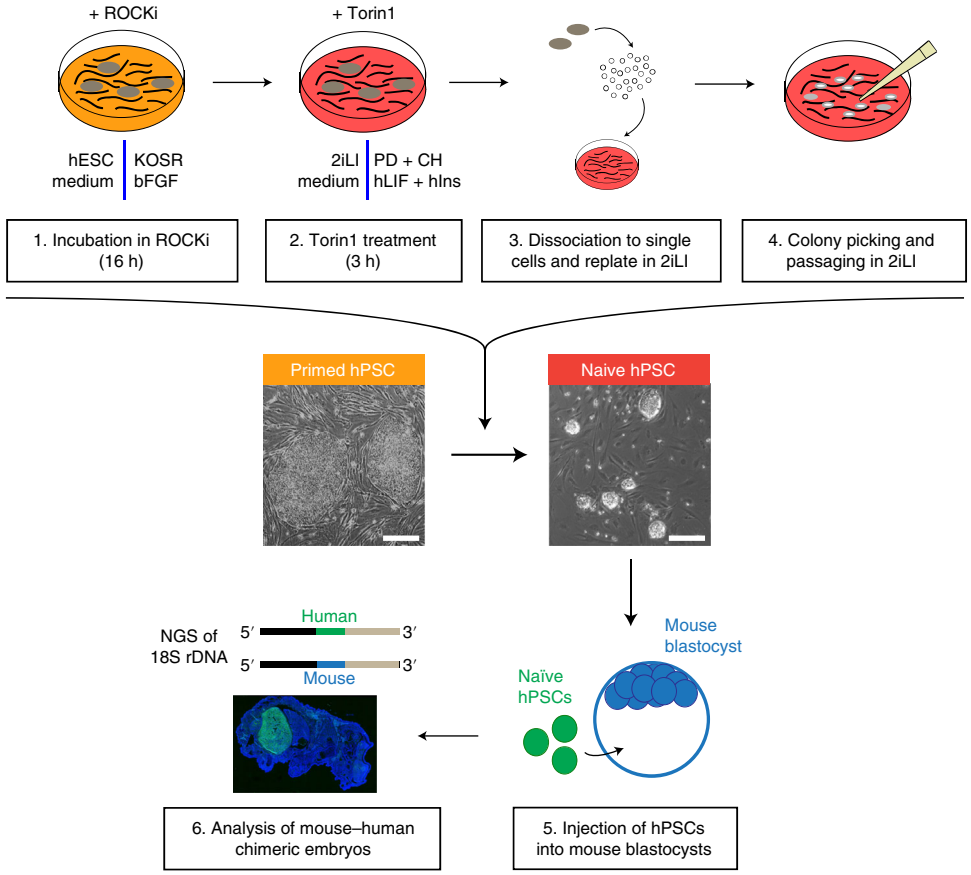

Fig. 1 | Overview of the protocol. A scheme depicting the conversion of hPSCs from the primed state to the naive state (Steps 1–32 of the main procedure), injection of naive hPSCs into mouse blastocysts (Steps 33–70 of the main procedure) and quantification of human cells in mouse–human chimeric embryos (Steps 71–120 of the main procedure). Primed hPSCs are incubated overnight with ROCK inhibitor (1), then treated with Torin1 for 3 h (2) and dissociated into single cells to be replated (3). Colonies with mESC morphology emerge in 3–4 d and are passaged manually (4). Established naive hPSCs are injected into mouse blastocysts, which are transferred to pseudopregnant mice to generate mouse–human chimeric embryos (5). Genomic DNA from the chimeras is analyzed by NGS of 18S rDNA amplicons to quantify the percentage of human DNA in total DNA (6). Scale bars, 250 μm. *CH*, CHIR99021; *hIns*, human insulin; *hLIF*, human leukemia inhibitory factor; *KOSR*, knockout serum replacement; *PD*, PD0325901; *ROCKi*, ROCK inhibitor.

vary substantially in different cell types and under different metabolic conditions. Thus, the amount of hmtDNA normalized against the single-copy ultra-conserved noncoding region may not accurately reflect the proportion of human cells in mouse–human chimeric embryos, which contain human and mouse cells with a great range of mitochondrial DNA per cell. Second, the specificity of PCR reactions, which are driven by the binding of a primer with a target, deteriorates when a small number of hmtDNA targets are vastly outnumbered by mouse mitochondrial and nuclear DNA that contains numerous but slightly imperfect matches. Many lower-affinity targets in mouse DNA compete against the interaction of the primers with a few high-affinity targets in hmtDNA. Third, uneven extraction efficiency and degradation rates in the much smaller, circular mitochondrial DNA versus the much longer, linear nuclear DNA in samples collected in different conditions, e.g., batches, storage time, fixation, extraction methods, etc., may have unforeseen effects on the quantification.

In light of these issues, we have developed a new method to accurately and reliably quantify human DNA in mouse–human chimeras using NGS of amplicons of 18S rDNA[17], which has a high copy number and is well conserved between mouse and human[31]. The V3 variable region of 18S rDNA contains a segment flanked by identical sequences in mouse and human[32]. In the middle of the segment, there are 8 bp mismatches between mouse and human sequences (Figs. 1 and 7a). Thus, we can amplify this segment from mouse and human genomic DNA at the same efficiency, regardless of their relative abundances. The PCR products can be accurately quantified by NGS counting of mouse

or human reads because the 8 bp mismatches enable unambiguous sequence identification while providing good tolerance for potential PCR or sequencing errors. The method is compatible with different tissue preparation, DNA extraction and DNA storage methods. It can even be used in archived, partially degraded DNA samples, as long as the degradation is not sequence specific. The specificity of this method is very high within its linear quantification range, down to 1:1,000 dilution of human DNA standard in mouse DNA standard. On NGS sequencing platforms, a multiplexing design allows the method to analyze many samples in one sequencing run.

Application of the method

The ability of this method to generate large quantities of mature human cells of all three germ layers in mouse–human chimeric embryos may enable many applications previously considered impossible. One type of application is to study human development in mouse–human chimeric embryos. It is impractical to study human development invasively to understand molecular and cellular mechanisms that go awry in many human developmental disorders. Using our method, one can now track the development of naive hPSCs in mouse–human chimeric embryos in real time, amenable to an array of genetic and/or pharmacological manipulations. For example, one can label naive hPSCs by inserting GFP in a hemoglobin gene to study the development of human red blood cells (hRBCs) in mouse–human chimeras. Another type of application is to generate humanized mouse models to study many human diseases more effectively. For example, it would be possible to generate even more hRBCs using blastocyst complementation, which allows normal PSCs from one species to reconstitute an organ niche vacated by a genetic mutation in a blastocyst of another species[33–35]. Mice with a substantial amount of hRBCs would be very useful to study diseases that specifically affect hRBCs, such as malaria or sickle cell diseases. If naive hPSCs are able to generate substantial amounts of mature human cells in other species (e.g., pigs), it would be possible to make human tissues or even human organs in chimeric animals using blastocyst complementation. Further development along this line of research may generate human organs suitable for transplantation and thus solve the problem of organ shortage. Ultimately, a better understanding of how human cells develop and grow in chimeras may enable the generation of human cells, tissues and organs in an artificial system without the need for animals.

Comparison with other methods

All previous methods that generate naive hPSCs use various media that contain small-molecule compounds and growth factors beyond the 2iL condition used to maintain mESCs[9–13,22]. Because of this, these naive hPSCs do not generate a substantial amount of human cells when they are injected into mouse blastocysts[9,11,13,24]. Our method inhibits mTOR only transiently for 3 h to induce the nuclear translocation of TFE3 to drive the expression of genes critical for maintaining naive pluripotency[27,36]. Treated cells are dissociated into single cells, which are selected in 2iLI medium. Converted cells survive and proliferate in 2iLI medium, while unconverted cells either die or differentiate[17]. Because our method generates naive hPSCs that are maintained in essentially the same condition for culturing mESCs, these naive hPSCs behave like mESCs when they are injected into mouse blastocysts. This leads to production of mature human cells of all three germ layers, accounting for up to 4% of cells in the chimeras[17]. Our method distinguishes itself from others by unifying the culture conditions for naive hPSCs and mESCs. The NGS-based method that we have developed to quantify the percentage of human cells in chimeric embryos[17] is more accurate and reliable than the qPCR-based method that measures the amount of hmtDNA relative to ultra-conserved noncoding region[14,24,30]. Our quantification method is based on NGS counting of mouse or human reads of the 18S rDNA amplicons, which are amplified using primers recognizing both mouse and human sequences equally. The qPCR-based method is affected by the unknown variations of mtDNA copy numbers, overabundance of lower affinity targets in mouse DNA, and differential extraction and degradation of mtDNA versus genomic DNA.

Experimental design

As illustrated in the experimental schematic, primed state hPSCs (including hESCs and human iPSCs) (phase-contrast image in Fig. 1) are first incubated with ROCK inhibitor Y-27632 (10 μM) overnight (16 h) in hESC medium containing KOSR and bFGF (Fig. 1, Step 1). The following morning, cells are incubated for 3 h in the 2iLI medium containing Torin1 (10 μM) or rapamycin (10 μM) (Fig. 1, Step 2) to transiently inhibit mTOR, which induces primed-to-naive conversion[17].

We find that either Torin1 or rapamycin induces the conversion, but we have not tested other mTOR inhibitors. The 2iLI medium is essentially the same as the 2iL medium used to culture mESCs. There are only two differences that substantially enhance primed-to-naive conversion[17]. One is to use human insulin (18 μg/ml) instead of the N2 and B27 supplements in the 2iL medium[8], which contain undisclosed amounts of insulin and other components. The second difference is to lower glucose concentration from 21.25 mM in the mESC medium to the physiological range (5 mM). We routinely use physiological O_2 level (5%) to culture all stem cells, which substantially enhances the conversion compared with using atmospheric O_2 level (21%)[17]. After 3 h of Torin1 (or rapamycin) treatment in 2iLI, hPSCs are trypsinized into single cells, which are plated on mouse embryonic fibroblast (MEF) feeders in fresh 2iLI medium (Fig. 1, Step 3). The medium is changed daily, and dome-shaped colonies emerge after 4 d. Some of the colonies are very bright under a phase-contrast microscope. Cells in these colonies have a high nucleus-to-cytoplasm ratio. We pick these colonies manually and dissociate them into single cells, which are replated on MEF cells in 2iLI medium (Fig. 1, Step 4). The process is repeated for about ten passages to generate a naive hPSC line that contains very few differentiated cells and exhibits rapid proliferation (phase-contrast image in Fig. 1).

The established naive hPSCs can be labeled with lentiviruses expressing GFP. We pick GFP^+ colonies and passage them a few times to generate naive hPSC cultures with 100% GFP^+ colonies. These GFP^+ naive hPSCs are dissociated into single cells, which are injected into mouse blastocysts (Fig. 1, Step 5). Injected blastocysts are transferred to pseudopregnant mice to carry the gestation up to E17.5. Mouse embryos at different developmental stages are retrieved and analyzed by fluorescence imaging of tissue sections or by NGS quantification of genomic DNA isolated from the chimeras (Fig. 1, Step 6). Fluorescence-based detection and quantification methods, regardless of whether they are based on GFP (or its variants) or a fluorophore-conjugated antibody, rely on signal-to-noise ratio, which can be compromised by autofluorescence in tissue sections. Antibody-based quantification methods, such as immunostaining or flow cytometry, rely on antibody–antigen recognition, which can be inundated by lower-affinity targets in more numerous mouse cells. Thus, we have designed a quantification method that is independent of signal-to-noise ratio or molecular recognition.

We choose 18S rDNA because its high copy numbers substantially facilitate detection. The human and mouse segments in the V3 region have identical sequences at both ends. This enables unbiased amplification of both human and mouse segments. The 8 bp sequence difference in the middle of the human and mouse segments allows accurate and reliable quantification by NGS counting of human or mouse reads. Using barcodes in the primers, multiplexed samples are pooled, purified, quantified and spiked-in with high-complexity library PhiX to enable successful cluster registration during sequencing. After NGS, raw reads are attributed to each sample by bioinformatics demultiplexing according to the barcode sequences used for each sample, then mapped against human and mouse reference V3 sequences. The percentage of human reads in the sum of human and mouse reads is used to calculate the percentage of human DNA in total genomic DNA. Because 18S rDNA has different copy numbers in different human individuals and different mouse strains[37], we use the same method to generate a standard curve using genomic DNA from the same naive hPSCs serially diluted in genomic DNA from the same mouse strain used to produce blastocysts (C57BL/6).

Expertise needed to implement the protocol

Prior expertise in culturing hPSCs and mESCs is required to successfully convert primed hPSCs to the naive state. All cell culture equipment and reagents are standard and can be purchased. Injections of naive hPSCs into mouse blastocysts and transfers of injected mouse blastocysts to pseudopregnant mice need to be performed by trained specialists, very often in a core facility that provides routine injections of mESCs to mouse blastocysts. Our injections and transfers are conducted by the Gene Targeting and Transgenic Resource at Roswell Park Comprehensive Cancer Center. Two specialists in the facility work together to inject 72 mouse blastocysts with naive hPSCs in ~2 h and transfer the injected 72 embryos into four pseudopregnant CD-1 female mice within another 2 h, with 18 blastocysts deposited into the oviduct of each mouse. NGS and bioinformatics analyses need to be conducted by trained specialists, often in a core facility. In our case, they are performed by the University at Buffalo Genomics & Bioinformatics Core Facility.

Limitations

Many factors, some unknown, may influence the successful implementation of this protocol. It is crucial to maintain hPSCs in an undifferentiated and genomically stable state, which means

restricting the culture time to a maximum of 2–3 months. A new vial of low-passage hPSCs should be thawed and used when the passage number of hPSCs reaches 30–40. We have used this protocol to convert five hPSC lines to a naive state. They include three hESC lines (H1, H9 and RUES2) and two hiPSC lines (C005 and N004)[17]. Upon receipt from vendor at around passage 26, the hESCs are passaged five times before conversion to the naive state. The hiPSCs are generated from fibroblasts grown out of skin biopsy in a process that takes ~20 passages. Conversion to the naive state occurs around passage 20, counting from the initial culture of biopsy.

Chronic low-dose UV exposure in a cell culture room should be removed by using yellow lamps in cell culture hoods and installing covers to block UV light from fluorescent bulbs on the ceiling. All hPSCs should be cultured at the physiological O_2 level of 5%, instead of at the atmospheric O_2 level of 21%. Routine testing of cells for mycoplasma contamination by PCR should be implemented to ensure the absence of mycoplasma in the entire laboratory. A face mask should be worn in the cell culture room to reduce the possibility of spreading mycoplasma from the human respiratory tract[38]. Injection of naive hPSCs to blastocysts and transfer of injected blastocysts to pseudopregnant mice should be conducted by experienced personnel who have a track record of routine and successful generation of mouse–mouse chimeras when injecting mESCs to mouse blastocysts. The incorporation of naive hPSCs into mouse embryos may be stochastic, and the degree of chimerism can be highly variable. Our experience shows that it should not substantially reduce the average number of embryos produced, in comparison with typical rounds of injecting mESCs into mouse blastocysts by the same personnel under the same setting[17]. Detection of human 18S rDNA in chimeras is more reliable when using genomic DNA freshly isolated from embryos instead of cryostat sections from fixed and embedded embryos[17]. DNA crosslinking by the fixative and DNA degradation in the embedding and storage process adversely affect the quantification of human 18S rDNA. The unknown interactions between injected human cells and their progenies with the mouse embryo are a critical factor that determines the level of chimerism. It may be possible to improve chimerism by using mouse embryos defective in making an organ, as demonstrated in mouse–rat[33] and rat–mouse chimeras[34]. This protocol will help researchers make further improvements in the generation of interspecies chimeras.

Materials

Biological materials

- Mice: E13.5 embryos from timed pregnant Hsd:NSA (CF-1) mice (Envigo, order code 033) are used to produce MEFs according to the published method[39]. C57BL/6J mice (Jackson Laboratory, stock number 000664) are used to produce blastocysts. Hsd:ICR (CD-1) mice (Envigo, order code 030) are used to generate pseudopregnant mice for gestation carriage of injected mouse blastocysts **! CAUTION** The care and use of animals, the use of human pluripotent stem cells and the use of human subjects (if applicable) should be approved by the relevant institutional ethics review committees. Our animal experiments are approved by the Institutional Animal Care and Use Committees of Roswell Park Comprehensive Cancer Center and University at Buffalo. The University at Buffalo/Roswell Park Comprehensive Cancer Center Stem Cell Research Oversight Committee has approved all experiments on hPSCs in the study. The University at Buffalo Institutional Review Board has determined that the use of human cells in the study is not human subject research **! CAUTION** All agents injected or applied to mice must be United States Pharmacopeia grade. Controlled substances used in anesthesia and pain management for surgery are obtained from the Laboratory Animal Shared Resource of Roswell Park Comprehensive Cancer Center by personnel approved to handle these agents.
- hPSCs: any well-characterized lines of hESCs or human iPSCs can be converted to the naive state **▲ CRITICAL** All cells in the laboratory should be routinely tested for mycoplasma contamination using PCR[40]. It is good practice to wear a face mask and minimize talking in the cell culture room to reduce the spread of mycoplasma, which can be released from the respiratory tract of asymptomatic carriers[38] **! CAUTION** In cell culture rooms, we have eliminated all sources of UV radiation, which causes DNA damage in cells. Fluorescent light bulbs in cell culture hoods are replaced with GE covRguard Gold lamps (GE, cat. no. 25768). Ceiling covers (ErgoMart, cat. no. F885) are installed to block UV emissions from fluorescent lights on the ceiling. We confirm UV elimination using a digital UVA/UVB meter (General Tools, cat. no. UV513AB), which shows 0 $\mu W/cm^2$ readings in the cell culture hood and the entire cell culture room.

Reagents

- Dulbecco's PBS (DPBS) with no Ca^{2+} and no Mg^{2+} (Thermo Fisher Scientific, cat. no. 14190-144)
- 2-Mercaptoethanol, 55 mM (Thermo Fisher Scientific, cat. no. 21985-023)
- Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific, cat. no. 11965-092)
- DMEM/F12 (Thermo Fisher Scientific, cat. no. 11330-032)
- KOSR (Thermo Fisher Scientific, cat. no. 10828-028)
- SILAC Advanced DMEM/F12 Flex (Thermo Fisher Scientific, cat. no. A24943-01)
- Neurobasal A Medium (Thermo Fisher Scientific, cat. no. A24775-01)
- [+] D-Glucose solution 200 g/L (Thermo Fisher Scientific, cat. no. A24940-01)
- L-Glutamine 200 mM (Thermo Fisher Scientific, cat. no. 25030-081)
- Minimum Essential Medium nonessential amino acid solution (MEM NEAA; Thermo Fisher Scientific, cat. no. 11140-050)
- Penicillin–streptomycin solution (Thermo Fisher Scientific, cat. no. 15140-122)
- BSA solution, 30% (Millipore-Sigma, cat. no. A9576)
- Human insulin solution (Millipore-Sigma, cat. no. I9278)
- TrypLE Express (Thermo Fisher Scientific, cat. no. 12604013)
- Dispase (STEMCELL Technologies, cat. no. 07913)
- Human bFGF (Peprotech, cat. no. 100-18B)
- Human LIF (Peprotech, cat. no. 300-05)
- PD0325901 (Tocris, cat. no. 4192)
- CHIR99021 (Tocris, cat. no. 4423)
- Torin1 (Tocris, cat. no. 4247)
- Rapamycin (Tocris, cat. no. 1292)
- Y-27632 (Tocris, cat. no. 1254)
- Mitomycin C (Millipore-Sigma, cat. no. M4287)
- FBS (Thermo Fisher Scientific, cat. no. 10437-028)
- Cell freezing medium FreSR-S (STEMCELL Technologies, cat. no. 05859)
- Dimethyl sulfoxide (DMSO; Millipore-Sigma, cat. no. D2650)
- UltraPure phenol:chloroform:isoamyl alcohol, 25:24:1 vol/vol (Thermo Fisher Scientific, cat. no. 15593049)
- HotStarTaq DNA polymerase (Qiagen, cat. no. 203203)
- QIAquick PCR Purification Kit (Qiagen, cat no. 28104)
- QIAamp DNA Mini Kit (Qiagen, cat no. 51304)
- Proteinase K from *Tritirachium album* (Millipore-Sigma, cat. no. P6556)
- KAPA HiFi HotStart ReadyMix (Roche, cat. no. KR0370)
- KAPA library quantification kit KK4824 (Roche, cat. no. 07960140001)
- Illumina Nextera XT indexes (Illumina, cat. no. FC-131-2001)
- PhiX Sequencing Control v3 (Illumina, cat. no. FC-110-3001)
- 300 cycle NextSeq 500 kit (Illumina, cat. no. 20024905)
- AMPure beads (Beckman Coulter, cat. no. A63882)
- Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, cat. no. Q32851)
- UltraPure 1 M Tris-HCI, pH 8.0 (Thermo Fisher Scientific, cat. no. 15568025)
- Nuclease-free water (Thermo Fisher Scientific, cat. no. AM9937)
- EB buffer (Qiagen, cat. no. 19086)
- 10 mM Tris-HCL with 0.05% Tween 20, pH 8.0 (Fisher Scientific, cat. no. NC1255337)
- Six-well cell culture plates (Falcon, cat. no. 353046)
- 12-well cell culture plates (Falcon, cat. no. 353225)
- 15 ml conical tubes (Corning, cat. no. 430828)
- 50 ml conical tubes (Corning, cat. no. 430790)
- Pregnant mare serum (PROSPEC, cat. no. hor-272-a)
- Human chorionic gonadotropin (PROSPEC, cat. no. hor-250-a)
- KSOM Mouse Embryo Medium (Millipore-Sigma, cat. no. MR-106-D)
- FHM Mouse Embryo Medium (Millipore-Sigma, cat. no. MR-024-D)
- Pasteur pipette, long tip (VWR, cat. no. 14673-043)
- Ketamine (Patterson Veterinary, cat. no. 078036637)
- Xylazine (Patterson Veterinary, cat. no. 078932121)
- Surgical scissors (Fine Science Tools, cat. no. 14090-09)

- Metal hub needle, 33 gauge, 0.5 inch length, point style 3 (Hamilton, cat. no. 7747-01)
- 4-0 violet polyglycolic acid braided absorbable suture (Stoelting, cat. no. 50496)
- EZ Clip Kit for wound closures (Stoelting, cat. no. 59020)
- Baby Dieffenbach Serrefine clamp (Biomedical Research Instruments, cat. no. 34-2800)
- Bupivacaine (Marcaine) (Covetrus, cat. no. 054893)
- Buprenorphine (Patterson Veterinary, cat. no. 078925235)
- Hank's balanced salt solution (HBSS; Thermo Fisher, cat. no. 14025092)

Primers (The underlined part binds to the segment of the V3 region in Fig. 7a; the rest are NGS barcode attachment sites.)

- NGS-18S-F: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGCTAATACATGCCGACGGG
- NGS-18S-R: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCTAGAGTCACCAAAGCCGC

Equipment

- Multigas incubator, 37 °C, 5% CO_2, 5% O_2 (Sanyo, model MCO-18M)
- CO_2 incubator for mouse embryos, 37 °C, 5% (Thermo Scientific, Heracell VIOS 160i)
- Biological safety cabinet (BSC; Baker, model SG603A-HE)
- Inverted microscope (Olympus, cat. no. CKX41)
- Eclipse TS100 inverted microscope (Nikon) (for cell culture and blastocyst injections)
- SMZ-10A stereozoom microscope (Nikon) with a dual-boom fiber optic ringlight attached with fiberoptic cord illuminated with Fiber-Lite High-Intensity Illuminator Series 180 (Dolan Jenner Industries)
- Microforge with SMZ-2B microscope (Nikon)
- Qubit fluorometer (Thermo Fisher Scientific)
- NanoDrop spectrophotometer (Thermo Fisher Scientific)
- Fragment analyzer system (Agilent)
- C1000 touch screen thermocycler (BioRad)
- C1000 thermocycler equipped with the CFX96 real-time PCR module (BioRad)
- NextSeq 500 (Illumina)
- Beckman Coulter Allegra X-22R centrifuge
- Heraeus Pico 21 microcentrifuge

Reagent setup

Either Torin1 or rapamycin is dissolved in DMSO to make 1 mM stock solutions. These stock solutions are stored at −20 °C for up to 6 months.

PD0325901 and CHIR99021 are dissolved in DMSO to make 1 mM and 3 mM stock solutions, respectively, which are stored at −20 °C for up to 6 months.

Y-27632 is dissolved in water to make a 10 mM stock solution that is stored at −20 °C for up to 6 months.

To prepare stock concentrations of human LIF (0.1 mg/ml) and bFGF (40 μg/ml), all contents in a vial from the vendor are dissolved in an appropriate volume of cold (4 °C) DPBS containing 0.1% BSA. The solutions are prepared into aliquots on ice and stored at −80 °C for up to 6 months. Aliquots are thawed in a 4 °C refrigerator prior to use. The compositions of 2iLI and hESC medium are listed in the two tables below. Stock concentrations are indicated in parentheses in the tables.

Recipe for making 2iLI medium (50 ml)

Component	Volume (ml)
SILAC Advanced DMEM/F12 Flex	23.7
Neurobasal A	23.7
[+] D-Glucose (200 g/L)	0.225
Human LIF (0.1 mg/ml)	0.01
L-Glutamine (200 mM)	0.25
MEM NEAA (100×)	0.5
Penicillin–streptomycin (100×)	0.5

Table continued

(continued)

Recipe for making 2iLI medium (50 ml)

Component	Volume (ml)
BSA (30%)	0.833
PD0325901 (1 mM)	0.05
CHIR99021 (3 mM)	0.05
Human insulin (9 mg/ml)	0.1
2-Mercaptoethanol (55 mM)	0.1

Recipe for making hESC medium (50 ml)

Component	Volume (ml)
DMEM/F12	38.41
KOSR	10
L-Glutamine (200 mM)	0.5
MEM NEAA (100×)	0.5
Penicillin–streptomycin (100×)	0.5
Human bFGF (40 μg/ml)	0.005
2-Mercaptoethanol (55 mM)	0.091

Software

- Bcl2fastq (Illumina, https://support.illumina.com/sequencing/sequencing_software/bcl2fastq-conversion-software.html)
- FastQC (Babraham Bioinformatics, https://www.bioinformatics.babraham.ac.uk/projects/fastqc/)
- MultiQC (https://multiqc.info/)[41]
- Cutadapt (https://cutadapt.readthedocs.io/en/stable/)[42]
- Vsearch (https://github.com/torognes/vsearch)[43]

Procedure

Converting hPSCs from the primed state to the naive state ● Timing 4–5 d

▲ **CRITICAL** All centrifugation steps to spin down cells are carried out in a Beckman Coulter Allegra X-22R centrifuge equipped with an SX4250 rotor. All hPSCs are maintained in a multigas incubator at 37 °C in 5% CO_2 and 5% O_2.

▲ **CRITICAL** Culturing hPSCs in 5% O_2 is very important for maintaining pluripotency and primed-to-naive conversion. Atmospheric O_2 level (21%) substantially reduces conversion efficiency compared with physiological O_2 level (5%)[17].

▲ **CRITICAL** Mitotically inactivated MEF cells (either by mitomycin C or gamma irradiation) need to be plated at least 1 d prior to their use as feeders. Before MEF cells are used, they need to be washed three times in an appropriate hPSC basal medium to ensure that hPSCs plated on the MEF cells subsequently are not differentiated by residual serum.

1 **Pre-Torin1/rapamycin treatment (day −1):** maintain primed hPSCs on mitomycin C-treated MEF feeders (2–3 × 10^4/cm^2) in hESC medium (see 'Reagent setup'). The following steps apply to one well of hPSCs in a six-well plate cultured to 70–80% confluency. Medium is changed daily, and hPSCs are passaged every 6–7 d using 1 mg/mL dispase. (Note: we have not tested the conversion of hPSCs maintained in feeder-free systems).

2 To prepare for Step 9 on the next day, plate mitomycin C-treated MEF feeders at 5–6 × 10^4/cm^2 in one well of a six-well plate.

▲ **CRITICAL STEP** MEF density should be increased in comparison with that in Step 1 to support the optimal growth of naive hPSCs, which have a much smaller colony size.

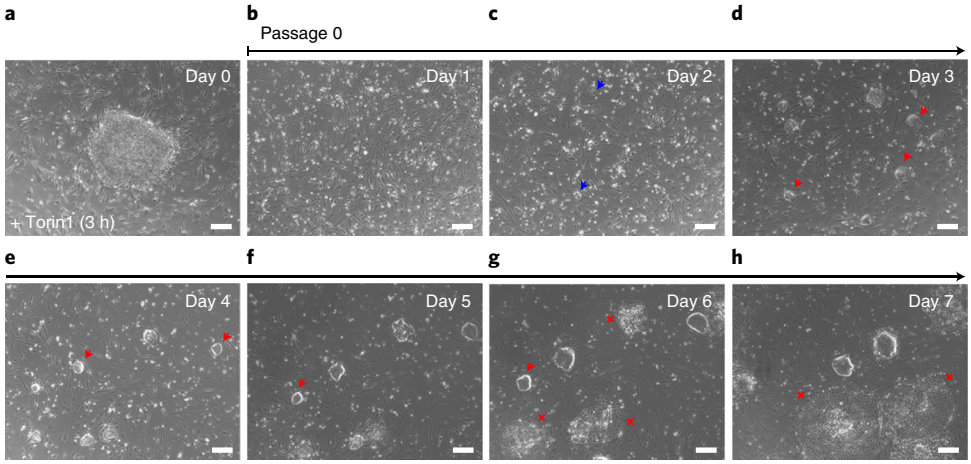

Fig. 2 | Growth of mESC-like colonies after hPSCs are treated with Torin1. a, On day 0, primed hPSC colonies grown to an intermediate size are treated with 10 μM Torin1 for 3 h and trypsinized to single cells for replating on MEF (passage 0). **b**–**h**, Single hPSCs are not easily identifiable on MEF feeders on day 1 (**b**), start to form visible colonies (indicated by blue arrows) on day 2 (**c**), and become dome-shaped colonies on day 3 (**d**), which grow increasingly larger on days 4–7 (**e**–**h**). Day 3–4 is the best time for picking ideal colonies as indicated by red arrows. On day 5 and beyond, more colonies will progressively differentiate (indicated by red ×). Scale bars, 250 μm.

3 Incubate hPSCs overnight in fresh hESC medium supplemented with 10 μM Y-27632 (Fig. 1, Step 1). ROCKi improves the survival of hPSCs when they are dissociated into single cells.
▲ **CRITICAL STEP** We achieve optimal primed-to-naive conversion when primed hPSC colonies are grown to half of the size at which they are normally passaged. This should occur 3–4 d after plating primed hPSCs onto new feeders (Fig. 2a).

4 **Torin1/rapamycin treatment (day 0):** thaw either Torin1 or rapamycin (1 mM stock) at room temperature (20–22 °C), and dilute 1:100 in 2iLI medium. Filtrate the solution through a 0.22 μm filter inside a BSC. Prewarm the solution by incubating briefly in a 37 °C water bath. Most of our conversions use Torin1, but rapamycin also works.
▲ **CRITICAL STEP** Do not filtrate 1 mM stock because undiluted DMSO damages the filter.
▲ **CRITICAL STEP** Freshly made 2iLI can be stored at 4 °C and should be used within 1 month. During this time, the color of 2iLI may change from light yellow to light pink. We find that it does not substantially affect the culture of naive hPSCs.

5 Wash hPSCs twice with DPBS. Aspirate and treat hPSCs with prewarmed 2iLI containing 10 μM Torin1 (or rapamycin) for 3 h at 37 °C in 5% CO_2 and 5% O_2.
▲ **CRITICAL STEP** If the hPSC colonies are fairly big (e.g., when cultured for more than 5 d after passaging), Torin1 (or rapamycin) may cause cell death in the center of colonies.

6 Aspirate medium. Apply TrypLE (1 ml/well for six-well plate) for 5 min at 37 °C. During incubation, prepare 9 ml prewarmed 2iLI in a 15 ml conical tube.

7 Add 3 ml of prewarmed 2iLI to the cells, and triturate cell suspension by pipetting up and down four to six times with a 10 ml serological pipette. Transfer contents into a 15 ml conical tube to obtain a total volume of 10 ml (9 ml 2iLI medium plus 1 ml of TrypLE originally used to dissociate cells).

8 Centrifuge at 200*g* for 5 min at room temperature.

9 Wash the new MEF cells from Step 2 three times with 2iLI to remove the remaining serum from the MEF medium.

10 Aspirate medium from Step 8, and resuspend the pellet with 1 ml of prewarmed 2iLI containing 10 μM Y-27632.

11 Count cell numbers in the hPSC suspension and replate the dissociated hPSCs onto new MEF cells from Step 9 in 2iLI containing 10 μM Y-27632.
▲ **CRITICAL STEP** We recommend seeding 4,000 dissociated cells onto the feeders in one well of a six-well plate, and seed four such wells to generate sufficient numbers of ideal colonies to establish a line of naive hPSCs for freezing. Plating 4,000 cells per well ensures that colonies are well dispersed, which prevents differentiation and facilitates colony picking later on. In a six-well plate, one well of hPSCs should yield over 100,000 cells after trypsinization. Therefore, the hPSC suspension must be diluted accordingly with 2iLI containing 10 μM Y-27632 prior to plating.

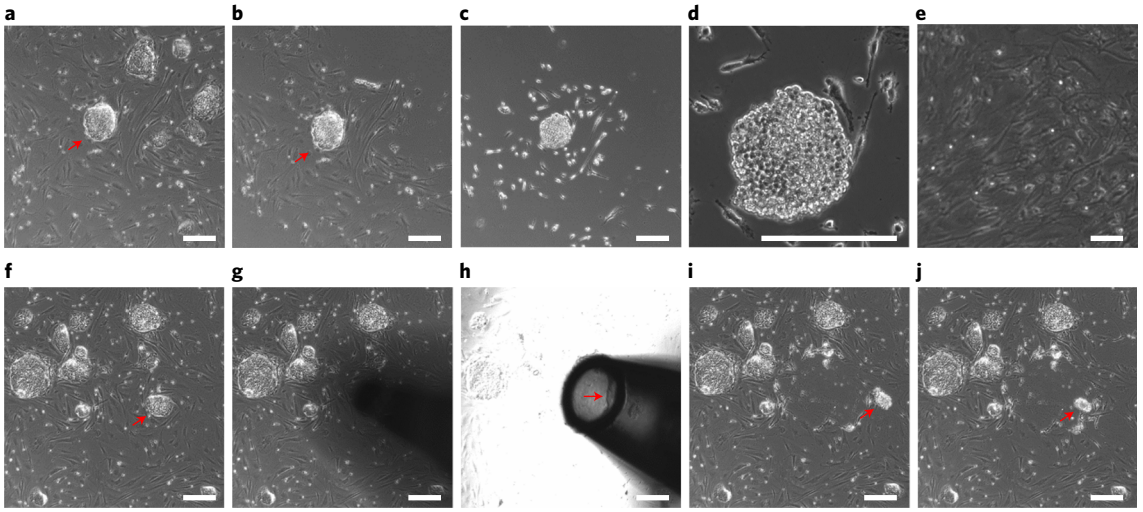

Fig. 3 | Two methods to passage naive hPSC colonies. a–e, Step 15A: this method should be used for passages 1–10 when there are only a few ideal colonies. Identify bright, dome-shaped colonies as indicated by the red arrow (**a**). With a pipette tip, scrape away all surrounding differentiated colonies, which will now float in the medium. Aspirate the medium to remove these differentiated colonies (**b**). It is unnecessary to remove the surrounding MEF cells. Incubate the cells in 1 ml TrypLE for 2–3 min in the incubator to dissociate all colonies with mESC-like morphology into single cells (**c**). Higher magnification of a dissociated colony shows that cells are loosely attached (**d**). Replace TrypLE with 2iLI containing ROCKi and triturate with a pipette (10 ml serological pipette or a P1000 pipette tip) to fully dissociate colony into single cells, which are replated on new MEF cells (**e**). **f–j**, Step 15B: this method should be used after passage 10 when there are many colonies with mESC-like morphology. Identify bright, dome-shaped colonies as indicated by the red arrow (**f**). Carefully position the pipette tip to the left of the colony (**g**). Gently glide the pipette tip to the right to dislodge the colony, which is indicated by the red arrow and is below the pipette tip (**h**). The colony is almost completely dislodged and is anchored through its right side (**i**). Gently glide the pipette tip to completely dislodge the colony, which is now floating in medium to be picked up (**j**). Repeat (**f–j**) for other ideal colonies in the well. Collect the medium containing the picked colonies and transfer to 15 ml conical tube for trypsinization into single cells. Scale bars, 250 μm.

12 **Day 1–2:** change the medium daily with fresh 2iLI medium
▲ **CRITICAL STEP** Do *not* add Torin1 or Y-27632 to the medium.

Passaging naive hPSCs ● Timing 1 d

13 **Day 3–4:** within 3–4 d after Torin1/rapamycin treatment, dome-shaped colonies should appear (Fig. 2b–e). These colonies grow bigger, and some of them differentiate with time (Fig. 2f–h). Pick as many colonies as possible, starting from day 3 after treatment, to ensure the establishment of a naive hPSC line.
▲ **CRITICAL** The ideal colonies to pick are those that are dome-shaped, bright, large and undifferentiated, for example, the one indicated by the red arrow in Fig. 3a. Although picking one such colony can generate many colonies and eventually establish a line, it would take far longer to reach the point where one can freeze down high-quality naive hPSCs. Thus, we recommend picking as many such colonies as possible to speed up the process.
? TROUBLESHOOTING

14 Wipe down an inverted microscope with 70% ethanol. We use Olympus CKX41 to pick colonies under the brightfield setting and Nikon Eclipse TS100 to pick fluorescently labeled colonies. Place the inverted microscope under a BSC in a position where the sample stage and culture vessel are behind the BSC sash. The lamp and the condenser unit should be inside the BSC, while the eyepiece should be outside.

15 Perform cell passaging using option A for the first 10 passages and option B for passages 11 and beyond.
▲ **CRITICAL** For both methods, the cell pellet must be resuspended into single cells prior to seeding onto new feeders. If clumps of cells remain, trypsinization time needs to be increased. Excessive pipetting (more than ten times) will not further dissociate cells and will more likely cause cell death.
(A) **Early passaging (passages 1–10)**
(i) Identify colonies for passaging based on characteristics described in Step 13. Remove all the unwanted surrounding colonies with a sterile pipette, leaving only the desired colonies (Fig. 3a–e).

▲ CRITICAL STEP Incomplete removal of unwanted cells will delay the eventual establishment of a stable line of naive hPSCs. Only naive hPSCs survive passaging in the 2iLI medium; primed hPSCs either die or differentiate in the 2iLI medium[17].

(ii) Aspirate the medium that now contains unwanted floating colonies.

(iii) Add 1 ml of TrypLE, and incubate the plate in the incubator at 37 °C for 2–3 min. Because of the short incubation time, cells within the colony will become visibly dissociated when observed under a microscope; however, the colony will remain attached to the plate (Fig. 3c,d).

(iv) Aspirate TrypLE and add 2iLI supplemented with 10 μM Y-27632. Pipette medium up and down using a 10 ml serological pipette or a P1000 pipette tip to fully dissociate cells into single cells.

(v) Transfer the cell suspension onto new MEF feeders ($5–6 \times 10^4/cm^2$) in one well of a 6-well plate or a well in a 12-well plate if there are fewer colonies.

(B) **Late passaging (passages 11 and beyond)**

(i) To manually pick the colony under the microscope, use a sterile pipette tip to detach the colony by gently gliding the tip toward the edges of the colony. Do not touch the center of the colony (Fig. 3f–j). Without changing the medium, continue picking other colonies with the same pipette tip.

(ii) Collect the medium, which now contains the floating colonies, and transfer it to a 15 ml conical tube. Centrifuge at 200*g* for 5 min at room temperature.

(iii) Aspirate the medium. Resuspend the pellet with 1 ml of TrypLE by pipetting up and down using a 10 ml serological pipette or a P1000 pipette tip. Incubate in a 37 °C water bath for 2–3 min.
▲ CRITICAL STEP Because of its small size, the pellet may not be visible. Nevertheless, one should target the bottom of the conical tube where the pellet is located after centrifugation. Add 9 ml of prewarmed 2iLI medium, and centrifuge at 200*g* for 5 min at room temperature.

(iv) Aspirate the medium and resuspend the pellet with 2iLI supplemented with 10 μM Y-27632. Transfer cell suspension onto new MEF feeders ($5–6 \times 10^4/cm^2$) in one well of a 6-well plate or one well of a 12-well plate if there are fewer cells.
▲ CRITICAL STEP For both methods, the cell pellet must be resuspended into single cells prior to seeding onto new feeders. If clumps of cells remain, trypsinization time needs to be increased. Excessive pipetting (more than ten times) will not further dissociate cells and will more likely cause cell death.
▲ CRITICAL STEP In option A, trypsinizing five large colonies or ten small colonies should generate >60% confluency in the subsequent passage. In option B, one should pick at least 25 colonies to achieve >60% confluency in the subsequent passage.
? TROUBLESHOOTING

16 Culture cells in an incubator at 37 °C with 5% CO_2 and 5% O_2. Replace medium with fresh 2iLI daily.

17 Passage cells every 3–4 d. It is expected that some differentiated colonies will appear. Use the approaches outlined in Step 15B to passage ideal colonies with mESC-like morphology.
▲ CRITICAL STEP During early passages, clonal expansion is slow, and the number of colonies with mESC-like morphology is low. Because cell survival using the early passaging method (Step 15A) is better than with the late passaging method (Step 15B), we recommend using option A during early passages (e.g., P1–P10) with a split ratio of 1:1. In later passages (e.g., P11 and onwards), hPSCs are stabilized in the naive state, producing fewer differentiated colonies after each passage (Fig. 4d). The colony number rises dramatically, allowing the split ratio to increase accordingly. As the colony number increases, it becomes tedious and difficult to locate and remove every differentiated cell in the well using the early passaging method (option A). At this point, the late passaging method (option B) is preferable over the early passaging method (option A) because, by selecting only ideal colonies with mESC-like morphology, it ensures that differentiated cells are not carried over to the next passage.

Cryopreserving naive hPSCs ● Timing 30 min

▲ CRITICAL For naive hPSCs grown in six-well plates, one well of colonies at 70–80% confluency can be frozen in one cryovial.

18 Aspirate medium.

19 Wash culture well with DPBS.

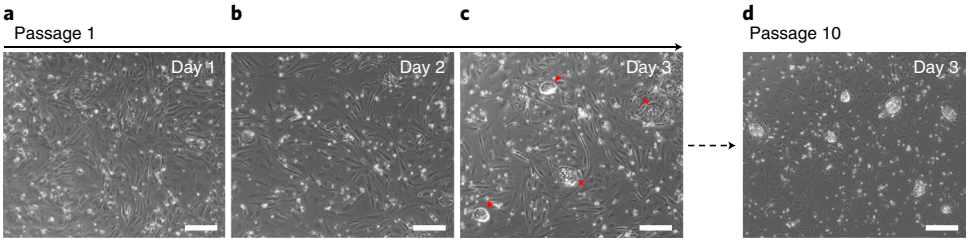

Fig. 4 | Continuous passaging of naive hPSCs in 2iLI. a–c, After the first passage (P1) with Step 15A, replated single cells on day 1 (**a**) will form small colonies on day 2 (**b**). On day 3, some ideal colonies (indicated by red arrows) can be passaged by removing differentiated colonies (marked by red ×) (**c**) using Step 15A again. It is possible to see the number of ideal colonies decrease during the first few passages, but repeated passaging with this method will stabilize and then increase the number and size of ideal colonies while reducing differentiated colonies. **d**, Rapidly growing naive hPSC colonies with very few differentiated colonies will be seen around passage 10. Scale bars, 250 μm.

20 Aspirate and add 1 ml of TrypLE. Incubate in the 37 °C incubator for 3 min.
21 From a 15 ml conical tube containing 9 ml of prewarmed 2iLI, transfer 3 ml to a well.
22 Pipette up and down to triturate cell suspension. Transfer the contents back to the 15 ml conical tube to bring the total volume to 10 ml.
23 Centrifuge at 200*g* for 5 min at room temperature.
24 Aspirate medium and resuspend pellet in 1 ml of cell freezing medium (FreSR-S). Freezing medium should be stored in a 4 °C refrigerator and can be used immediately once taken out.
25 Transfer the 1 ml cell suspension to a cryovial.
26 Place the cryovial in a Mr. Frosty container already at room temperature, and store the container in a −80 °C freezer overnight.
▲ **CRITICAL STEP** Do not leave cryovial in a Mr. Frosty container in −80 °C freezer for >1 d.
27 Transfer the cryovial from the −80 °C freezer to a liquid nitrogen tank.

Thawing naive hPSCs ● Timing 30 min

28 Prepare a 15 ml conical tube filled with 9 ml 2iLI supplemented with 10 μM Y-27632. Prewarm it in a 37 °C water bath.
29 Remove a cryovial from a liquid nitrogen tank, and thaw in a 37 °C water bath, swirling the cryovial occasionally. Once a small ice pellet remains, remove the vial from the water bath. Carefully wipe it down with 70% ethanol before transferring to a BSC.
30 Using a 2 ml cell culture pipette, transfer the entire contents of the cryovial dropwise into the 9 ml medium in the 15 ml conical tube.
▲ **CRITICAL STEP** Thawed cells should be handled gently. Minimize pipetting, and avoid making air bubbles.
31 Centrifuge at 200*g* for 5 min at room temperature.
32 Aspirate medium and resuspend pellet in prewarmed 2iLI supplemented with 10 μM Y-27632. Seed the cell suspension onto new MEF feeders (5–6 × 10^4/cm^2) in one well of a six-well plate.

Obtaining mouse blastocysts ● Timing 6 d

▲ **CRITICAL** Steps 33–70 require specialized training and are best done by experienced staff, usually in a core facility with a successful track record of generating mouse–mouse chimeras on a routine basis.
33 **Day 1:** super-ovulate 20 C57BL/6J females (age 3 weeks old) via i.p. injections of 5 IU pregnant mare serum, followed by i.p. injections of 5 IU human chorionic gonadotropin 46–48 h later.
34 **Day 3:** mate these female C57BL/6J mice with C57BL/6J male mice at 1:1 pairing. Check for vaginal plugs the next morning, which is designated as 0.5 d post coitum (dpc).
35 **Day 6:** harvest morula from plugged females at 2.5 dpc (in the morning) by flushing the oviduct of each female mouse using FHM mouse embryo medium loaded in a 1 cc syringe with a 33 gauge blunt end Hamilton metal hub needle (0.5 inch length, point style 3).
36 Collect the morulae, and wash them with KSOM embryo culture medium plus amino acids. Culture the morulae in KSOM plus amino acids under sterile paraffin oil overnight in a humidified incubator at 37 °C with 5% CO_2.

37 **Day 7:** assess the embryos the next morning. About 60–70% of the morulae develop into blastocysts. We usually obtain ~100 mouse blastocysts from the 20 super-ovulated female mice. Discard blastocysts that are already hatched.

Preparing single naive hPSCs for injection ● Timing 1.5 h

38 **Day 7:** wash naive hPSCs at 60–80% confluency in two wells of a six-well plate with 2 ml DPBS for each well.

39 Add 1 ml TrypLE per well, and incubate at 37 °C for 2 min.

40 Resuspend the cells with 10 ml of 2iLI medium, and transfer the cells to a 15 ml conical tube.

41 Gently pipette the cells four to five times to fully dissociate them to single cells.

42 Centrifuge the tube at 200*g* for 5 min.

43 Resuspend the cells with 10 ml 2iLI medium (containing 10 μM Y-27632), and place the cell suspension in a 10 cm dish precoated with 0.1% porcine gelatin. Incubate the dish for 45 min in a humidified incubator at 37 °C and 5% CO_2. MEF cells will attach to the gelatin layer, while dissociated naive hPSCs will float in the medium or will lightly attach.

44 Gently aspirate the cell suspension, wash the plate gently three times and centrifuge it at 200*g* for 5 min.

45 Resuspend naive hPSCs in 0.5 mL of 2iLI medium (containing 10 μM Y-27632) in a 15 ml conical tube. This step concentrates dissociated cells in a small volume. Keep the tube on ice throughout the injection process.

Injecting naive hPSCs into mouse blastocysts ● Timing 2 h

46 **Day 7:** place 300–400 μL of 2iLI medium (containing 10 μM Y-27632) onto a glass slide, and overlay the medium with sterile paraffin oil.

47 Using a mouth pipette with a pulled glass pipette or a P200 pipette, place a small amount of the cell suspension in the drop of medium under paraffin oil on the glass slide, which is placed on an inverted microscope with micromanipulators for holding and injecting mouse blastocysts.

48 Remove mouse blastocysts from the incubator, wash them through 2iLI medium (containing 10 μM Y-27632) and place the mouse blastocysts in the same drop of medium under paraffin oil on the slide on the injection stage.

49 Assess dissociated naive hPSCs for size and morphology. Choose round, bright human cells for injection.

50 Load five to seven human cells into the injection pipette (Fig. 5a). Hold a mouse blastocyst with a flame-polished holding pipette, and pierce the blastocyst with the injection pipette to deposit the five to seven human cells into the blastocoel cavity (Fig. 5b). After the injection pipette is withdrawn from the mouse blastocyst, injected human cells (as indicated by the red arrow) can be seen in the mouse blastocoel cavity (Fig. 5c). The trophoblast layer will partially detach from the zona pellucida (Fig. 5c).

51 After injecting 18 mouse blastocysts, remove them from the stage and wash them with KSOM medium plus amino acids. Return injected mouse blastocysts to the incubator until the surgical transfer of embryos, which takes place 1–2 h after blastocyst injection. Prior to embryo transfer, human cells (as indicated by the red arrow) are mixed up with mouse cells in the blastocyst, and the trophoblast layer reattaches to the zona pellucida (Fig. 5d).

▲ **CRITICAL STEP** A total of 72 mouse blastocysts are injected in four batches by two experienced staff members within ~2 h to minimize the exposure of mouse blastocysts outside the incubator.

Transferring injected mouse blastocysts to pseudopregnant mice ● Timing 2 h for embryo transfer

▲ **CRITICAL** The surgery is performed in a BSC, cleaned with 75% ethanol solution. Surgical instruments are sterilized in an autoclave.

52 **Day 7:** over the flame of a Bunsen burner, heat and pull the tip of a 9 inch glass Pasteur pipette into a thin needle ~0.1 mm in diameter. Cut the tip with a diamond pencil, and flame-polish the tip on the heated Tungsten filament in a Nikon microforge (Fig. 6a).

53 Assemble a surgical transfer mouth pipette (Fig. 6b) by inserting the flame-polished glass pipette into a piece of Tygon tubing, which is plugged with a cotton filter on the distal end to prevent accidental aspiration of the medium. The green adapter after the cotton filter connects to a 1.5–2 foot piece of rubber tubing, which is linked to a mouthpiece to control the flow rate of medium containing mouse embryos at the pulled end of the glass pipette.

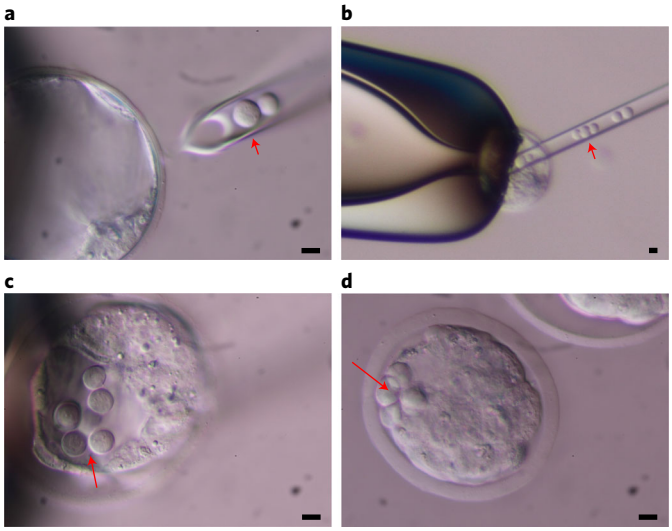

Fig. 5 | Injecting naive hPSCs into mouse blastocysts. a, A mouse blastocyst (3.5 dpc) held by a flame-polished holding pipette, next to an injection pipette loaded with a few dissociated naive hPSCs (indicated by the red arrow) (400× magnification). **b**, Injection of dissociated naive hPSCs (indicated by the red arrow) into the blastocoel cavity of a mouse blastocyst held by a flame-polished holding pipette (100 × magnification). **c**, A mouse blastocyst immediately after injection, showing the five to seven injected naive hPSCs (indicated by the red arrow) inside the blastocoel cavity (400× magnification). The trophoblast layer shows a partial detachment from the zona pellucida. **d**, A mouse blastocyst ~1 h after injection, before surgical transfer into a pseudopregnant mouse (400× magnification). Injected human cells (indicated by the red arrow) are mixed with mouse cells. The trophoblast layer reattaches to the zona pellucida. Scale bars, 10 μm.

54 The day before the surgical transfer of the injected blastocysts, CD-1 females are mated 1:1 with vasectomized CD-1 males. These males are surgically vasectomized by cautery of the vas deferens. Their sterility is confirmed by test mating with female mice. In the morning of embryo transfers, CD-1 females are separated and checked for the presence of vaginal plugs. Plugged females (0.5 dpc) are separated from unplugged females, which are reused for other experiments. We only use female mice weighing <25 g and <5 months of age to maximize the success of gestational carriage.

55 Bring two plugged females into a sterile surgical environment (from bioBubble), where they are anesthetized using i.p. injections of ketamine (20 mg/ml) and xylazine (2 mg/ml) solutions at the dose of 100 mg/kg body weight for ketamine and 10 mg/kg body weight for xylazine. Two females are prepped for surgery together, but surgeries are done individually.

56 Once the mice are completely anesthetized, as indicated by diminished pedal reflex, as well as slow and light respiratory rate, shave the surgical area (between the dorsal and ventral side caudal to the 13th rib) and wash with betadine. Rinse the area with 75% ethanol three times.

57 Place eye ointment over the eyes to prevent drying under the bright light.

58 Using sterile technique, make a 1 cm dorsal incision in the skin caudal to the 13th rib with 8.5 cm hardened fine straight surgical scissors.

59 The ovary is located underneath a fat pad under the dorsal muscle. Cut the dorsal muscle, grasp the fat pad and gently pull through the incision of the muscle wall and skin. This exposes the ovary, oviduct and anterior end of the uterus. Clamp the fat pad with a baby Dieffenbach Serrefine clamp to hold the organs in place.

60 Check that the bursa can be visualized. It is a membranous pouch that houses the ovaries and most of the oviduct. The distal portion of the oviduct, which contains the ampullae, is located outside of the bursa. Check this region for swelling, which indicates that the female mouse has been copulated with a vasectomized male mouse the night before and is now 'pseudopregnant'.
▲ **CRITICAL STEP** If the ampulla is not swollen, do not use this female.

61 Hold the short piece of oviduct with forceps. Use a 30 gauge needle to penetrate the oviduct proximal to the bursa but distal to the ampullae.

62 Move injected mouse blastocysts from the incubator (Step 51) onto a stereomicroscope with a thermoplate (set at 37 °C). Use the surgical transfer mouth pipette to wash the mouse embryos once with FHM medium. Aspirate a small amount of FHM into the pipette, and make several bubbles in it. Load nine injected blastocysts into the long tip of the flame-polished glass pipette (Fig. 6c).

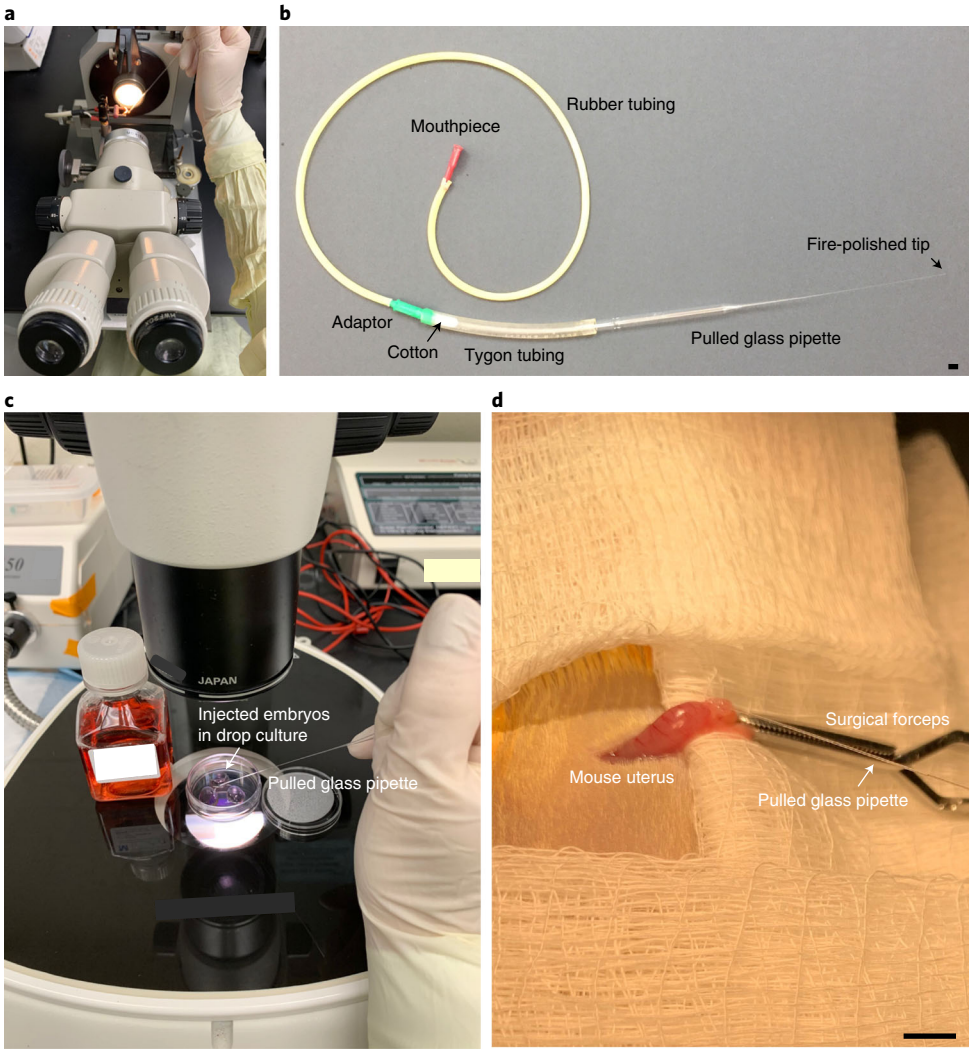

Fig. 6 | Equipment and tools needed to perform embryo transfers. a, After a 9 inch glass Pasteur pipette is pulled over an open Bunsen burner flame and nicked with a diamond pencil, the tip is flame-polished on the heated Tungsten filament in the Nikon microforge. **b**, A surgical transfer mouth pipette is assembled by inserting the flame-polished glass pipette into a piece of Tygon tubing, which is plugged with a cotton filter to prevent accidental aspiration of the medium. The green adapter connects the Tygon tubing to a 1.5–2 foot piece of rubber tubing, which is linked to a mouthpiece to control the flow rate of liquid in the glass pipette. **c**, Injected mouse blastocysts are taken from the incubator and washed with FHM medium before being loaded into the long tip of the flame-polished glass pipette. **d**, The surgical transfer setup showing the mouse uterus, oviduct, ovary (covered in the bursa) and fat pad. The fat pad is held with a Serrefine clamp to maintain the ovary in place. A hole is made in the oviduct, and the glass pipette containing injected mouse blastocysts is inserted into the hole. With a gentle blow delivered through the mouthpiece of the surgical transfer assembly, mouse blastocysts in the glass pipette are deposited into the ampulla of the oviduct, which is marked by striations. Scale bars, 0.5 cm.

63 Insert the glass pipette with the nine embryos into the opening created by a needle in the oviduct (Fig. 6d). Use the mouthpiece to gently blow the nine embryos into the oviduct.
▲ **CRITICAL STEP** Once backloaded bubbles appear in the ampulla, this indicates that all of the blastocysts have been deposited. Alternatively, the bursa of the ovary can be torn to expose the infundibulum, the opening of the oviduct. The embryos are transferred into the infundibulum with the flame-polished pipette. This is the less preferred method because it is more invasive.

64 After the transfer of embryos, replace organs into the body cavity. Suture the body wall with 4.0 vicryl thread using three equidistantly placed stitches that are double knotted.

65 Close skin incision with one or two Autoclip wound clips, which can be removed after 7–10 d.

66 Apply 0.1–0.2 cc of marcaine to wound site after closing the incision to manage topical pain.

67 Steps 58–66 are repeated to transfer nine embryos into the other oviduct. A total of 18 injected mouse blastocysts are transferred to a pseudopregnant mouse.

68 Inject the mouse with 0.5 ml warm sterile saline subcutaneously for rehydration and a single dose of buprenorphine (0.01 mg/ml) for analgesia.
69 Place the mouse in a prewarmed cage on a platform set to body temperature (37 °C). Provide food, water and Nestlets in the cage.
70 Monitor mice until they are ambulatory. Give buprenorphine every 8–12 h for the first 48 h and then as needed upon reassessment. Check mice at least once daily after surgery. They should stay in the warmed cage at least until mobile.

DNA extraction from mouse–human chimeric embryos ● Timing 2 d

▲ CRITICAL To avoid potential contaminations in tandem rounds of PCR required in NGS, a pre-PCR amplification area should be set up in a separate room with dedicated reagents and tools for retrieval and dissection of chimeric embryos and the first round of PCR that amplifies the 18S rDNA V3 regions. All steps after PCR amplification should be conducted in a post-PCR area in a separate room with dedicated reagents and tools. Face masks and gloves should be worn at all times. This is particularly important if the NGS experiments, which include two additional rounds of PCR, are done in the same laboratory. We isolate and dissect chimeric embryos in a building separate from where the animals are housed (Steps 71–74). Our NGS experiments are performed in another building by a core facility that implements standard operating procedures against PCR contaminations (Steps 103–117).

71 **Day 1:** euthanize a gestation carrier mouse at a desired stage of pregnancy by cervical dislocation.
72 Use 75% ethanol to disinfect the abdominal area of the mouse.
73 Open the abdominal wall with scissors.
74 Take out the entire uterus, and place it in a 10 cm dish filled with prechilled HBSS on ice.
? TROUBLESHOOTING
75 Under a dissection microscope, use scissors to cut the uterus into small segments, each containing one conceptus.
76 Transfer a uterus segment into a new 6 cm dish filled with prechilled HBSS on ice.
77 Use ophthalmic scissors and fine forceps to carefully open the uterine wall. Remove uterus, placenta tissue and amniotic membrane.
78 For mouse embryos later than E14.5, use a new blade to cut the embryo into two halves sagittally. Use one half for extraction of genomic DNA; use the other half for fixation and staining.
79 Chop isolated embryo into small pieces (<5 mm), then transfer all pieces into a 15 ml conical tube with a Pasteur pipette.
80 Centrifuge tubes at 200*g* for 5 min at 4 °C, then aspirate supernatant.
81 In an Eppendorf tube, add 700 µL Genomic DNA Extraction buffer for every 100 mg tissue. Incubate and rotate the tube at 50 °C overnight. The Genomic DNA Extraction buffer contains 100 mM Tris-HCl, pH 8.0–8.5; 5 mM EDTA; 0.2% SDS; and 200 mM NaCl. Stock solution of proteinase K (20 mg/ml) is added to achieve a final concentration of 250 µg/ml prior to use.
82 **Day 2:** add 1 volume of UltraPure phenol:chloroform:isoamyl alcohol (25:24:1, vol/vol), and mix well by rigorous shaking. Incubate for 5 min at room temperature.
83 Centrifuge at 14,000*g* in a tabletop microcentrifuge for 10 min at room temperature.
84 Carefully transfer the upper aqueous layer to a new Eppendorf tube without disturbing the middle and lower layers.
85 Add 0.7 volume of isopropanol. Mix well by inverting the tube a few times, and incubate for 10 min at room temperature.
86 Centrifuge at 14,000*g* in a tabletop microcentrifuge for 20 min at room temperature.
87 Remove supernatant. Wash pellet twice with 70% ethanol, then air-dry the pellet.
88 Dissolve the pellet in TE buffer. Use 100 µL of TE buffer for every 50 mg tissue used.
89 Measure the concentration of the genomic DNA with NanoDrop. To avoid repeated freeze–thaw cycles, genomic DNA should be prepared into aliquots and stored at −20 °C. For experiments, aliquots should be diluted with TE buffer to a final concentration of 100 ng/µL

Preparation of human and mouse genomic DNA standards ● Timing 10–14 d

▲ CRITICAL To ensure accuracy of quantification, standards must be prepared from freshly purified human genomic DNA from the same human cell line and mouse genomic DNA from the same strain used to generate blastocysts.

90 **Day 1:** to remove MEF cells, transfer the original, genetically identical primed hPSCs to mTeSR1/Matrigel feeder-free system, and passage at least twice.

91 **Day 10:** after two passages, which should take 10–14 d, dissociate the primed hPSCs with TrypLE for 3 min and collect 1×10^6 cells.
92 Collect 25 mg of genetically matched (C57BL/6 in this case) mouse tissue (e.g., brain or liver tissue).
93 Use QIAamp DNA Mini Kit to extract genomic DNA from human cells in Step 91 and mouse tissue in Step 92.
94 Dilute extracted human and mouse genomic DNA to 100 ng/μL using TE buffer.
95 To make 1/10 human/mouse genomic DNA standard, add 10 μL of human genomic DNA (100 ng/μL) to 90 μL mouse genomic DNA (100 ng/μL). Mix well by pipetting.
96 Serially dilute the 1/10 standard in appropriate volumes of mouse genomic DNA solution (100 ng/μL) to generate 1/100, 1/1,000, and 1/10,000 standards.

PCR amplification of the 18S rDNA V3 regions in mouse and human ● Timing 1 d

▲ **CRITICAL** For every batch of NGS, standards (1/10–1/10,000), pure mouse genomic DNA (negative control), pure human genomic DNA (positive control), and water (empty control) are processed in parallel with genomic DNA samples isolated from chimeras to control for technical variations. Each biological sample is run in triplicate. PCR should be conducted in a designated area with dedicated reagents, supplies and pipettes.

▲ **CRITICAL** Sequences of the NGS-18S-F and NGS-18S-R primers contain NGS barcode attachment sites and the 5′- or 3′-end sequences of the segment in the human and mouse V3 region of the 18S rDNA (Fig. 7a). The identical sequence of human and mouse in the primer-binding sites ensures unbiased amplification of both human and mouse segments, regardless of their abundances. We have verified the reference sequences for human and mouse in Fig. 7a by Sanger sequencing. The accession numbers for mouse and human 18S rDNA are MN537869.1 and K03432.1, respectively.

97 **Day 1:** calculate the total volume of each PCR reagent needed for all reactions as listed in the Table below. Prepare the PCR master mix by mixing 1.1× calculated volume of each component to compensate for pipetting loss. The use of DNA polymerase with proofreading activity is unnecessary because the divergence between human and mouse amplicons is big enough for NGS data to unambiguously assign species origin, even if PCR error occurs at a regular rate (<1/1,000).

Components of PCR Master Mix (for one sample)

Component	Volume (μL)
Nuclease-free water	40.75
10× buffer	5
10 mM dNTP	1
10 μM NGS-18S-F	1
10 μM NGS-18S-R	1
HotStarTaq DNA polymerase	0.25

98 Prepare aliquots of 49 μL PCR master mix for each reaction into 0.2 ml PCR tubes or 96-well PCR plate. Add 1 μL of the sample (100 ng) to corresponding tubes, and mix well by pipetting.
99 Run the following PCR program in a thermocycler with lid heated to 105 °C.

Thermocycling conditions for PCR amplification of the 18 S rDNA V3 regions

Step	Temperature	Duration	Number of cycles
1	95 °C	5 min	1
2	95 °C	30 s	40 cycles of Steps 2–4
3	58 °C	30 s	

Table continued

(continued)

Thermocycling conditions for PCR amplification of the 18 S rDNA V3 regions

Step	Temperature	Duration	Number of cycles
4	72 °C	1 min	
5	72 °C	5 min	1
6	4 °C	Hold	1

100 Run 5 μL PCR products from each reaction in 1.5% agarose gel to confirm successful PCR amplification. There should be a single band at 200 bp.

101 Purify the rest of the PCR products from each reaction using QIAquick PCR Purification Kit.

102 Measure the concentration of purified PCR products using Qubit and High Sense DNA quantification kit. Adjust the concentrations to 10 ng/μL.

Preparation and sequencing of NGS libraries ● Timing 1–2 d for preparation and 2 d for sequencing

▲ CRITICAL These procedures (Steps 103–117) are usually carried out in a core facility that routinely handles NGS and bioinformatics data analysis because they require specialized equipment, expertise and

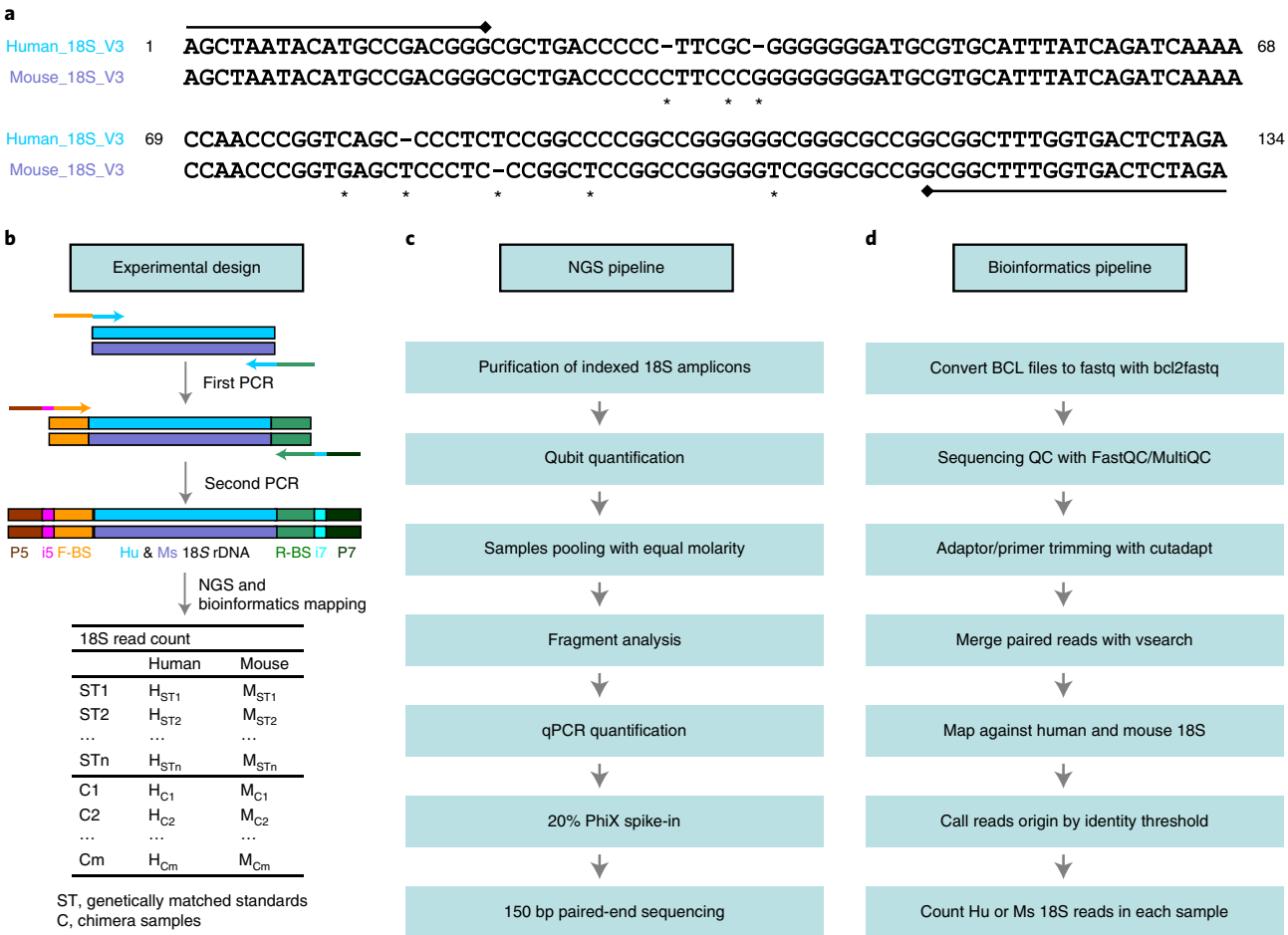

Fig. 7 | NGS quantification of human DNA in mouse–human chimeric embryos. a, Sequence alignment of the human and mouse 18S rDNA V3 regions used in NGS. Mismatches are marked with asterisks. Primer binding sites are marked with a solid line with a diamond end to indicate direction. **b**, The general design of 18S NGS quantification strategy, including unbiased amplification of the human and mouse V3 regions, sample multiplexing and design of controls and standards. **c**, A technical pipeline of NGS library preparation from barcoded amplicons. **d**, A bioinformatics pipeline converting raw sequencing data to read counts of human or mouse amplicons.

software. Products from the first PCR reaction are amplified in the second PCR to add barcodes for multiplexing many samples (Fig. 7b). The products of the second PCR are processed through the NGS pipeline (Fig. 7c). Sequencing results are processed in the Bioinformatics pipeline (Fig. 7d) to generate counts of human or mouse reads.

103 **Day 1:** prepare the second PCR reaction as detailed in the table below. Document the Illumina Nextera XT indexes used for each sample.

Setting up the second PCR reaction

Component	Amount
500s index	5 µL
700s index	5 µL
Kapa HiFi Hotstart Ready Mix	25 µL
Purified PCR product (Step 102)	5–15 ng
Nuclease-free water	Complete to 50 µL

104 Preprogram a thermal cycler with the following program: AMPLICON LIB. Set the lid temperature to 105 °C. We use a BioRad C1000 touch screen thermocycler, but any thermocycler can be used. Some optimization may be needed if using a different thermocycler. The number of cycles is dependent on the total PCR product concentration used for barcode addition. Refer to the tables below for the relevant parameters:

PCR parameters for AMPLICON LIB

Cycle no.	Temperature	Duration	No. of cycles
1	95 °C	∞	PREHEAT HOLD
2	95 °C	3 min	1
3	95 °C	30 s	Depend on input, see the table below for cycle steps 3–5
4	55 °C	30 s	
5	72 °C	30 s	
6	72 °C	5 min	1

Determining cycle parameters

Input (ng)	Cycle nos	Approximate time for PCR run
7	5	20 min
10	4	20 min
15	3	20 min

105 Clean second PCR product with 1.1× AMPure beads by following these steps:

▲ **CRITICAL STEP** AMPure beads are stored at 4 °C. An aliquot should be moved to room temperature at least 30 min prior to following the amplicon clean-up steps.

- Vortex an aliquot of AMPure beads for 30 s to resuspend them fully. Beads need to be fully resuspended to accurately remove contaminating nucleotides and primers.
- Add 56 µL of AMPure Beads to each 50 µL PCR sample. Pipette up and down 15 times to mix the solution completely.
- Incubate for 5 min at room temperature.
- Prepare 80% ethanol for the bead wash solution. Any molecular biology grade 100% ethanol can be used. Prepare enough solution for 500 µL per sample. Use nuclease-free water for dilution. For 96 samples, prepare 50 mL of bead wash solution. Store the bead wash solution at room temperature until used.

- Place the plate on magnetic stand until the solution appears clear (~5 min).
- While keeping the plate on the magnet, aspirate and discard 100 µL of supernatant. It is very important not to disturb the beads.
- Add 200 µL of 80% ethanol, and incubate for 30 s at room temperature.
- Remove and discard ethanol wash.
- Repeat wash with 80% ethanol for a total of two washes. Make sure all ethanol is removed. Use a P20 pipette to remove residual ethanol.
- Air-dry the plate on the magnetic stand for 5 min at room temperature. Do not over-dry the beads as this will reduce the concentration of eluted DNA.
- Remove plate from the magnet, and add 27.5 µL EB buffer. Pipette up and down 15 times to fully resuspend beads.
- Incubate the bead/EB buffer mix for 2 min at room temperature.
- Place the plate on a magnetic stand for 2 min.
- Remove 25 µL of the cleared solution, and transfer to a new plate. Do not disturb the beads. If there is bead carryover, return the solution to the magnet and repeat incubation for 2 min to ensure beads remain in the tube.
- Seal the plate with sealing tape. Store indexed amplicon plate at −20 °C until ready for further processing.

106 Pool purified second PCR products of all samples with equal molarity, and adjust the concentration of pool to 4 nM (amplicon size 274 bp) with a total volume of at least 100 µL in EB buffer.

107 Run the Agilent Fragment Analyzer Standard Sense Assay to check the quality of the pool.

108 Determine pooled library concentration using Kapa Biosystems Universal qPCR system by following these steps:

- Dilute the pooled amplicon libraries 1:1,000 in 10 mM Tris-HCl with 0.05% Tween 20 (pH 8.0). After 5 min incubation at room temperature, dilute the 1:1,000 sample 1:20 (5 µL 1:1,000 in 95 µL buffer, final concentration 1:20,000) in 10 mM Tris-HCl with 0.05% Tween 20 (pH 8.0). Store diluted samples on ice.
- In parallel, serially dilute 10 nM PhiX v3 (Illumina FC-110-3001) in 10 mM Tris-HCl with 0.05% Tween 20 (pH 8.0) 1:1,000 and then 1:20. This will be used as a control for nM correction.
- Set up qPCR using a hard-shell PCR plate on a 96-well ice block. Add 12 µL Kapa master mix (premixed 2× enzyme and 10× primer master mix according to manufacturer protocol), 4 µL nuclease-free water, and 4 µL each supplied standard or 4 µL individual diluted sample in triplicate (total reaction volume 20 µL). Spin down the plate at 280*g* for 1 min at 4 °C to remove bubbles.
- Load plate and run the following qPCR protocol on a BioRad C1000 equipped with the CFX96 real-time PCR module.

qPCR conditions to quantify pooled library concentration

Step	Temperature	Duration	No. of cycles
1	95 °C	30 s	35 cycles for Steps 1 and 2
2	60 °C	45 s	
3	65 °C to 95 °C	10 min	Melt curve, increments of 0.5 °C/s

- Export the run data to a run folder, and analyze the data using BioRad CFX Maestro software (v1.0). Determine the final concentration of the sample in nM. If the sample is >4 nM, dilute the pooled library to 4 nM.

109 **Day 2:** Perform PhiX spike-in and sequencing on NextSeq 500 (150 paired-end targeting at least 100,000 reads per sample) by following these steps:

- Thaw NextSeq reagent cartridge, flowcell and buffer HT1 following the user manual.
- Prepare a fresh dilution of NaOH for denaturation of the pooled amplicon library. Mix 45 µL nuclease-free water and 5 µL 2.0 N NaOH to make up 50 µL 0.2 N NaOH. Store at room temperature. The dilution must be used within 8 h.

- Prepare a fresh dilution of Tris-HCl, pH 7.0. Mix 40 µL nuclease-free water and 10 µL 1 M Tris-HCl, pH 7.0, to make up 50 µL of 200 mM Tris-HCl. Store at room temperature to be used that day.
- Denature the pooled amplicon libraries. Mix 5 µL of 4 nM pooled library and 5 µL of the freshly diluted 0.2 N NaOH in a low-bind microcentrifuge tube, vortex briefly and spin for 1 minute at 280*g*.
- Incubate at room temperature for 5 min.
- Add 5 µL of the 200 mM Tris-HCl, pH 7.0, dilution to the denatured library pool. Vortex briefly and spin for 1 min. at 280*g*.
- Add 985 µL of the prechilled HT1 buffer to the tube of denatured libraries. Vortex briefly and spin 1 for min. at 280*g*. This will result in a final concentration of 20 pM for the denatured library. Place the 20 pM library on ice until ready to proceed to the final dilution.
- Mix 78 µL 20 pM denatured library and 962 µL prechilled HT1 to make 1,040 µL.
- Add 260 µL of 1.8 pM PhiX (previously denatured and diluted according to the loading manual) to the 1,040 µL of denatured library to make 1,300 µL. This mixture has a final concentration of 1.2 pM of library and 0.36 pM of PhiX. Adding PhiX in the sample allows for low-complexity libraries to be efficiently sequenced on the instrument.

110 Load 1,300 µL of the denatured library + PhiX to the thawed NextSeq 300 cycle reagent cartridge. Pierce the foil in reservoir #10 (labeled 'load library here') in the cartridge. Immediately set up the NextSeq run following the on-board instructions.

Bioinformatics analysis of NGS sequencing results ● Timing 1 d

111 **Day 1:** Convert the per-cycle basecall (BCL) files generated by the Illumina NextSeq to per-read FASTQ files using bcl2fastq using default parameters.

112 Run FastQC and Multiqc to check the quality of the sequencing. Reject and resequence samples with quality scores (from multiQC) of the first 134 bp <30.

113 Trim forward and reverse barcode attachment sites using cutadapt using the adapter CTGTCTCTTATACACATCTCCGAGCCCACGAGAC for the forward read and CTGTCTCTTA TACACATCTGACGCTGCCGACGA for the reverse read.

114 Merge paired reads using fastq mergepairs function in vsearch. We usually see a merge rate >95% for each sample.

115 Filter the merged reads based on the number of expected errors. Merged reads with more than one expected error should be removed. We usually see >99% of the merged reads passing filter.

116 Align each merged read against human and mouse 18S reference sequences, and classify reads as either human or mouse using vsearch –usearch_global, with identity threshold 0.99.

The reference sequence of human 18S rDNA amplicon is AGCTAATACATGCC GACGGGCGCTGACCCCCTTCGCGGGGGGGATGCGTGCATTTATCAGATCAAAACCAAC CCGGTCAGCCCCTCTCCGGCCCCGGCCGGGGGGCGGGCGCCGGCGGCTTTGGTGACT CTAGA.

The reference sequence of mouse 18 *S* rDNA amplicon is AGCTAATACATGCCGAC GGGCGCTGACCCCCCTTCCCGGGGGGGGATGCGTGCATTTATCAGATCAAAACCAACC CGGTGAGCTCCCTCCCGGCTCCGGCCGGGGGTCGGGCGCCGGCGGCTTTGGTGACT CTAGA.

117 Output results into an OTU-style data table with columns for each of the samples and rows for human and mouse.

118 Calculate human reads abundance in each sample by the formula: number of human reads / (number of human reads + number of mouse reads).

? TROUBLESHOOTING

119 Run Student's *t*-test on standard samples against pure mouse genomic DNA samples. The standard sample with the lowest human DNA abundance showing significance defines the lower quantification limit.

120 Run linear regression analysis on human reads abundance versus human DNA abundance in the standards. If R^2 of this linear model is >0.9, then calculate the human DNA abundance of each chimera sample using this linear model. Otherwise, human DNA abundance in chimera samples should be calculated by linear interpolation using the two closest standards. Quantification range should be limited to the range covered by the standard curve.

Troubleshooting

Troubleshooting advice can be found in Table 1.

Table 1 | Troubleshooting table

Step	Problem	Possible reason	Solution
13	No colony with mESC-like morphology	Not using high-quality primed hPSCs	Use primed hESCs from established sources, such as RUES2, H1 or H9, which we have tested. *Do not inject* H1 or H9 into mouse embryos. It is not allowed per MTA
		Too much differentiation in primed hPSCs	Primed hPSCs need to be cultured in conditions as close to perfect as possible. Use O_2 at 5% instead of 21%
		Primed hPSCs have too many mutations	Eliminate UV in workflow. Check genomic stability by microarray or karyotyping
		Primed hPSCs passaged too many times	Use low-passage hESCs obtained from reliable sources
		Mycoplasma contamination	Ensure a mycoplasma-free workflow by wearing a face mask, minimizing talking in the culture room and checking for mycoplasma routinely
13, 15	Naive hPSC line cannot be established because of excessive differentiation	Too many colonies and too much differentiation	Colonies cannot grow too close to each other. Adjust the split ratio to reduce confluency to <80%. Cells need to be seeded as single cells during passaging. Verify this when counting cells using a hemocytometer. If cell clumping persists, increase the incubation time with TrypLE
		Poor MEF quality or low density	Use high-quality MEF feeders at a density of 5–6 × 10^4/cm^2
		Picking the wrong types of colonies	Only select colonies that are bright and dome-shaped
		Picking colonies too slowly	For one culture vessel, do not spend >10 min picking clones. Need practices to be quick
		CHIR99021 concentration may not be optimal. This may occur when converting primed iPSCs generated with integrating methods	As shown for naive N004 line[17], vary CHIR99021 concentrations (e.g., 0.1 μM to 6 μM) to find the optimal concentration that produces the highest percentage of NANOG$^+$ cells among hNA$^+$ cells
15	Number of colonies keep decreasing in consecutive passages	Not enough colonies were picked	Pick more colonies, and lower the split ratio
		Cells were damaged during passaging	Optimize TrypLE incubation time, which should be long enough to make single cells, but short enough for cells to survive well. Use option A (Step 15) to increase survival
74	No or very few mouse embryos produced at E10.5 through E17.5	Primed hPSCs not fully converted	Passage the hPSCs in 2iLI for 20 passages. If not naive, hPSCs cannot sustain in this condition Check growth rate, clonal efficiency, mitochondrial respiration, etc., to ensure that hPSCs are naive
		Naive hPSCs accumulate too many mutations in culture	Eliminate UV radiation in the entire process
			Check genomic stability by microarray-based methods or karyotyping
			Inject naive hPSCs around passage 20
		Problems in injecting naive hPSCs	Finish injections within 2 h to reduce embryo exposure outside an incubator. Injections need to be done by experts with a good track record
		Problems in embryo transfer	Finish embryo transfers within 2 h to minimize embryo exposure and surgery time. Embryo transfers need to be done by experts with proven records
118	No human cells in mouse embryos as demonstrated by PCR of GFP or human-specific DNA, but normal numbers of mouse embryos obtained	hPSCs not naive	Passage the hPSCs in 2iLI for 20 passages. If not naive, hPSCs cannot sustain in this condition Check growth rate, clonal efficiency, mitochondrial respiration, etc., to ensure that hPSCs are naive

Table continued

Table 1 (continued)

Step	Problem	Possible reason	Solution
		Naive hPSCs with too many mutations	Eliminate UV radiation in the entire process
			Check genomic stability by microarray-based methods or karyotyping
			Inject naive hPSCs around passage 20
		Naive hPSCs contaminated with mycoplasma	Ensure a mycoplasma-free workflow by wearing a face mask, minimizing talking in the culture room and checking for mycoplasma routinely
		Problems in handling naive hPSCs during injections	Dissociate naive hPSCs with TrypLE for 2 min to single cells. Inject only five to seven round and bright single cells
		MEF feeders not removed completely from naive hPSCs	Make sure MEF cells are attached to the gelatinized 10 cm dish in Step 43 by examining the plate under a microscope. Inject only round and bright single cells like those in Fig. 5a
	Percentage of human DNA <0.1%	Problems in embryo retrieval and DNA extraction	DNA needs to be extracted from freshly retrieved mouse embryos. Do not use fixatives
		Not trying enough injection rounds	Try at least five rounds of injections
		Development of human cells may take longer	Seek approval to retrieve embryos at E17.5, instead of an earlier time
	Presence of human reads in negative controls	Amplicon contamination	Use separate areas, reagents and tools for embryo dissection and DNA extraction, and PCR. Let a core facility handle NGS. Most core facilities already have standard operating procedures to prevent contaminations in tandem PCRs

Timing

Note that MEF cells should be plated at least 1 d prior to all steps using MEF cells. It is recommended to use MEF cells within 2 d from the time they were seeded.
Steps 1–12, converting hPSCs from the primed state to the naive state: 4–5 d from Torin1 (or rapamycin) treatment to the first passage
Steps 13–17, passaging naive hPSC: 1 d
Steps 18–27, cryopreserving naive hPSC: 30 min
Steps 28–32, thawing naive hPSC: 30 min
Steps 33–37, obtaining mouse blastocysts: 6 d
Steps 38–45, preparing single naive hPSCs for injection: 1.5 h
Steps 46–51, injecting naive hPSCs into mouse blastocysts: 2 h
Steps 52–70, transferring injected mouse blastocysts to pseudopregnant mice: 2 h
Steps 71–89, DNA extraction from chimeric embryos: 2 d
Steps 90–96, adapting primed hPSCs to feeder-free culture and extracting genomic DNA: 10–14 d
Steps 97–102, 18S PCR: 1 d
Steps 103–109, NGS library preparation: 1–2 d
Step 110, sequencing: 2 d (depending on the schedule and turn-over time in sequencing core facility)
Steps 111–120, bioinformatics analysis: 1 d

Anticipated results

With the conversion protocol, many bright and dome-shaped colonies resembling mESCs should be seen on days 3–5 (Fig. 2). There are some differentiated colonies, particularly with prolonged culture without passaging to days 6 and 7 (Fig. 2), but there should not be any colonies with primed hPSC morphology. Unconverted primed hPSCs die or differentiate in the 2iLI medium[17]. The presence of these nonnaive cells makes it necessary to pick ideal colonies, which is commonly done in the derivation of mESC lines from mouse blastocysts[44], and in the generation of iPSCs from human[45] or mouse[46] somatic cells. The first few passages of naive hPSCs can be difficult and yield only a few bright and dome-shaped colonies. Option A (Step 15) should be used because its clonal efficiency

(31.0 ± 1.1 %) is much higher than that of option B (Step 15; 4.6 ± 0.3 %). The much lower clonal efficiency of option B may be caused by the damages incurred in picking a colony. We speculate that it may take several passages for converted cells to stabilize their epigenetic state in the 2iLI medium. It becomes increasingly easy from passage 10 onwards when option B should be used to quickly pick many bright and dome-shaped colonies. With option B, fewer and fewer differentiated cells are seen with additional passaging. As there are so many clones at this stage, we can sacrifice clonal efficiency to generate a very high percentage of ideal colonies in the culture. Due to their substantially higher growth rates, naive hPSCs should be passaged every 3–4 d, unlike primed hPSCs, which require passaging every 6–7 d. Naive hPSCs also have much higher clonal efficiency than primed hPSCs and need to be passaged as single cells using TrypLE. Naive hPSCs have cellular properties that are highly similar to mESCs, showing robust mitochondrial respiration, hypomethylation and hypohydroxymethylation of genomic DNA, and reactivation of X-inactivated genes in female naive hPSCs[17]. Transcriptomic analyses, e.g., by principal component or clustering, should show substantial similarities to other naive hPSCs and cells from human blastocysts, but not their primed parental cells[17].

When naive hPSCs produced with this protocol are injected into mouse blastocysts, substantial amounts of human cells of all three germ layers should be found in mouse embryos at E17.5[17]. Human cells can be readily located in earlier mouse embryos, but it might be more difficult to ascertain what types of cells they are, because the developmental pace of human cells in a mouse embryo is unclear. The identification of mature human cells, such as enucleated red blood cells, liver cells and ocular cells[17], in E17.5 mouse embryos suggests that the development of naive hPSCs is substantially accelerated. It should be possible to identify many other types of human cells in the chimeric embryos using immunostaining or cell sorting, which can provide some information on the relative abundance of human cells. One can use PCR to detect GFP or other genetic labels of naive hPSCs or examine the presence of individual-specific human DNA by DNA fingerprinting[17]. Using qPCR, one can estimate the amount of hmtDNA in the chimera. This protocol provides a method to definitively quantify the amount of human DNA in the chimera by NGS counting of the number of human and mouse 18S rDNA amplicons. The abundance of human DNA can have substantial variations depending on many factors, such as the nature of the naive hPSCs, technical expertise in blastocyst injection and embryo transfer, and experimental variations. Our results show a range of 0.14–4.06% in one batch of 14 embryos at E17.5[17].

Reporting Summary

Further information is available in the Nature Research Reporting Summary linked to this article.

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Author contributions

B.Z. and H.L. contributed equally to the study. B.Z. developed the conversion protocol based on the initial method of Z.H. H.L. developed the method to analyze chimeric embryos by NGS. H.J. contributed to the analyses of naive hPSCs and chimeric embryos. A.B.S. performed blastocyst injections and embryo transfers. B.J.M. and D.A.Y. performed bioinformatics analysis of NGS data. J.F. conceived and supervised the study. All authors contributed to writing the paper.

Competing interests

J.F. is a cofounder of Vitropy, LLC and ASDDR, LLC, and has a patent application regarding this work (no. 16/346534). All other authors declare no competing interests.

Additional information

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Data collection	All software are listed in the Materials section (page 17 of the manuscript).
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Sample size	One cell line was used in the protocol to illustrate the process of converting primed hPSCs to the naive state.
Data exclusions	No data was excluded.
Replication	The protocol has been applied to several other cell lines in https://advances.sciencemag.org/content/6/20/eaaz0298
Randomization	Cells were evenly plated on multiwell plates. There was no randomization as the cells show no obvious variation between wells.
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Methods

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Antibodies used	Information on antibody is provided in Supplementary Table S7 in https://advances.sciencemag.org/content/6/20/eaaz0298
Validation	The antibodies have been validated by vendors and by our own experiments.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	hESC lines H1 and H9 were from WiCell. RUES2 were from Rockefeller University. Human iPSCs C005 and N004 were made by us and are published before.
Authentication	All hESCs were directly purchased from reputable vendors. Our own iPSCs have been authenticated in previous publications cited.
Mycoplasma contamination	All cells were tested regularly for mycoplasma contamination by PCR. No mycoplasma was detected.
Commonly misidentified lines (See ICLAC register)	Not applicable.

Animals and other organisms

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Laboratory animals	E13.5 embryos from timed pregnant Hsd:NSA (CF-1) mice (Envigo, order code 033) were used to make MEF cells. Blastocysts were from C57BL/6J mice (Jackson Laboratory, stock number 000664). Injected blastocysts were transferred to foster mice (Hsd:ICR (CD-1) (Envigo, order code 030).
Wild animals	Not applicable.
Field-collected samples	Not applicable.
Ethics oversight	Our animal experiments are approved by the Institutional Animal Care and Use Committees of Roswell Park Comprehensive Cancer Center and University at Buffalo. The University at Buffalo/Roswell Park Comprehensive Cancer Center Stem Cell Research Oversight (SCRO) Committee has approved all experiments on hPSCs in the study. The University at Buffalo Institutional Review Board has determined that the use of human cells in the study is not human subject research.

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