

Derivation, expansion and differentiation of induced pluripotent stem cells in continuous suspension cultures

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We describe derivation of induced pluripotent stem cells (iPSCs) from terminally differentiated mouse cells in serum- and feeder-free stirred suspension cultures. Temporal analysis of global gene expression revealed high correlations between cells reprogrammed in suspension and cells reprogrammed in adhesion-dependent conditions. Suspension culture–reprogrammed iPSCs (SiPSCs) could be differentiated into all three germ layers *in vitro* and contributed to chimeric embryos *in vivo*. SiPSC generation allowed for efficient selection of reprogramming factor–expressing cells based on their differential survival and proliferation in suspension culture. Seamless integration of SiPSC reprogramming and directed differentiation enabled scalable production of beating cardiac cells in a continuous single cell– and small aggregate–based process. This method is an important step toward the development of robust PSC generation, expansion and differentiation technology.

The derivation of mouse and human iPSCs from terminally differentiated somatic cells has opened up new avenues to address important fundamental questions in developmental biology^{1–3}. iPSC technology also sparks hope for therapeutic advances by enabling the generation of high-quality disease models, derivation of individual-specific iPSC lines, improvements in the predictability of drug action or as a source of cells for regenerative medicine. One of the biggest challenges in the application of this technology has been the robustness of cell generation, including the ability to produce cells at scale^{4–6}.

Despite the rapid progress in the development of molecular tools for iPSC generation, the development of culture methods providing controlled microenvironments devoid of animal components at scale have lagged behind, primarily because of the dependence of PSCs on adhesion or aggregation for propagation^{7–11}. Current protocols typically involve reprogramming somatic cells

to pluripotency by serial passage under adherent culture conditions on feeder cells or on extracellular matrix components¹². These approaches risk contamination by pathogens, require separation of feeder cells from the cell type of interest, increase costs and are prone to variability. Both mouse and human embryonic stem cells (ESCs) can be maintained and expanded in a pluripotent state as floating aggregates in the absence of feeder cells^{13–16}. However, all suspension cultures reported to date require serial dissociation and reaggregation steps (manipulations that typically limit cell yields).

In this work we derived mouse iPSCs in a continuous adherence- and matrix-free suspension culture system. We took advantage of inducible secondary mouse embryonic fibroblasts (MEFs) to compare reprogramming in suspension culture to that in routine adherent culture. Gene expression analysis showed high correlation between the two processes with regard to hallmark reprogramming genes. Differentially expressed transcripts mainly belonged to gene products involved in interactions with extracellular matrix components or cell adhesion, suggesting that these proteins may not be critical for reprogramming. We directly differentiated primary SiPSCs to cardiac progenitor cells, demonstrating that somatic cells can be reprogrammed, expanded and differentiated in a continuous suspension culture. This system should prove useful for fundamental studies into iPSC reprogramming processes and for investigating the impact of media and the microenvironment on cellular reprogramming. It represents an important step toward the robust, scalable production of iPSC for a variety of applications.

RESULTS

Reprogramming in suspension culture

Fibroblast-like cells are typically anchorage-dependent and stop dividing and undergo apoptosis when shifted from adherent to suspension culture conditions. To quantitatively study the effect

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of reprogramming on fibroblast survival in suspension culture, we used secondary inducible fibroblasts derived from chimeric embryos generated from reprogrammed primary fibroblasts. These cells are a mixed population of wild-type (control) cells and GFP-expressing (reprogrammable) cells; the latter reprogram at high frequencies¹¹. These secondary fibroblasts started to proliferate when cultured in suspension in the presence, but not in the absence, of doxycycline (Fig. 1a). We observed an initial lag phase, where overall cell densities decreased, before cultures entered a phase of rapid cell division (Fig. 1a).

Secondary fibroblasts cultured in suspension exhibited increased annexin V staining during the first 4 d compared to adherent conditions (Fig. 1b). We assayed both adherent cells and suspension-cultured cells for active cell division by tracing incorporation of 5-ethynyl-2'-deoxyuridine (EdU) (Fig. 1c). In the case of adherent cells, both GFP⁺ (cells harboring the reprogramming factors) and GFP⁻ populations showed strong incorporation of EdU, indicating actively dividing cells, regardless of the presence of doxycycline (Fig. 1c). With increased culture time in the presence of doxycycline, GFP⁺ cells had an increased cell division rate compared to the GFP⁻ population. In contrast, suspension-cultured cells had substantially weaker incorporation of EdU into GFP⁻ cells in the presence and absence of doxycycline, indicating decreased proliferation rates for this subpopulation compared to adherent conditions (Fig. 1c). In the presence of doxycycline, a rapidly proliferating GFP⁺, EdU⁺ subpopulation emerged (Fig. 1c), indicating actively reprogramming cells in suspension culture. These data suggest that suspension culture preferentially supports cells undergoing reprogramming via differential survival and proliferation.

Doxycycline-induced cells grew in suspension culture as a mixture of aggregates and viable single cells, as confirmed by calcein acetoxymethyl ester (calcein-AM) staining (Fig. 1d).

The cells entered a rapid growth phase and showed sustained growth during later stages (after day 12) of suspension-culture reprogramming (Fig. 1e). Thus, secondary fibroblasts are capable of surviving and proliferating in suspension culture upon induction of reprogramming factors.

Serum-free suspension culture reprogramming to pluripotency

To assess whether secondary fibroblasts can be reprogrammed to pluripotency in suspension culture, we assayed for SSEA-1 expression over 15 d by flow cytometry (Fig. 2a). SSEA-1 expression was detectable as early as 2 d after doxycycline induction and reached >70% of total viable cells by day 15 of culture. Cells reprogramming in adherent conditions and in suspension cultures with or without serum exhibited strikingly similar SSEA-1 expression kinetics (Fig. 2b). We observed NANOG expression in a substantial proportion of the population 8 d after induction (Supplementary Fig. 1).

We assayed whether SiPSCs become independent of exogenous factor expression by removing doxycycline at day 15 after induction. Upon doxycycline removal, cells aggregated (Supplementary Fig. 2) and a trypsin-dissociation step had to be included to passage cells. Doxycycline-independent aggregates exhibited nuclear staining for the transcription factors OCT4, SOX2, KLF4, NANOG and membrane staining for SSEA-1, indicating stable expression of endogenous pluripotency factors (Fig. 2c). Doxycycline-independent SiPSCs expressing NANOG and SSEA-1 could also be obtained after more than 40 d in culture, indicating that suspension-culture reprogramming is suitable for long-term growth of pluripotent cells (Supplementary Fig. 3). Pluripotency factor expression was additionally confirmed by western blot analysis (Supplementary Fig. 4). Doxycycline-independent SiPSCs could differentiate into all three germ layers *in vitro* (Fig. 2d). Finally, we injected secondary fibroblast derived

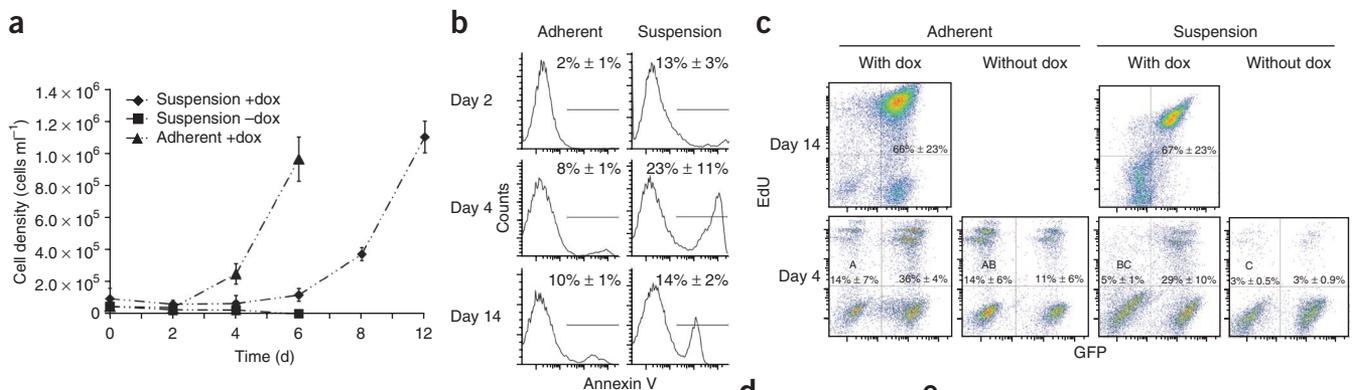
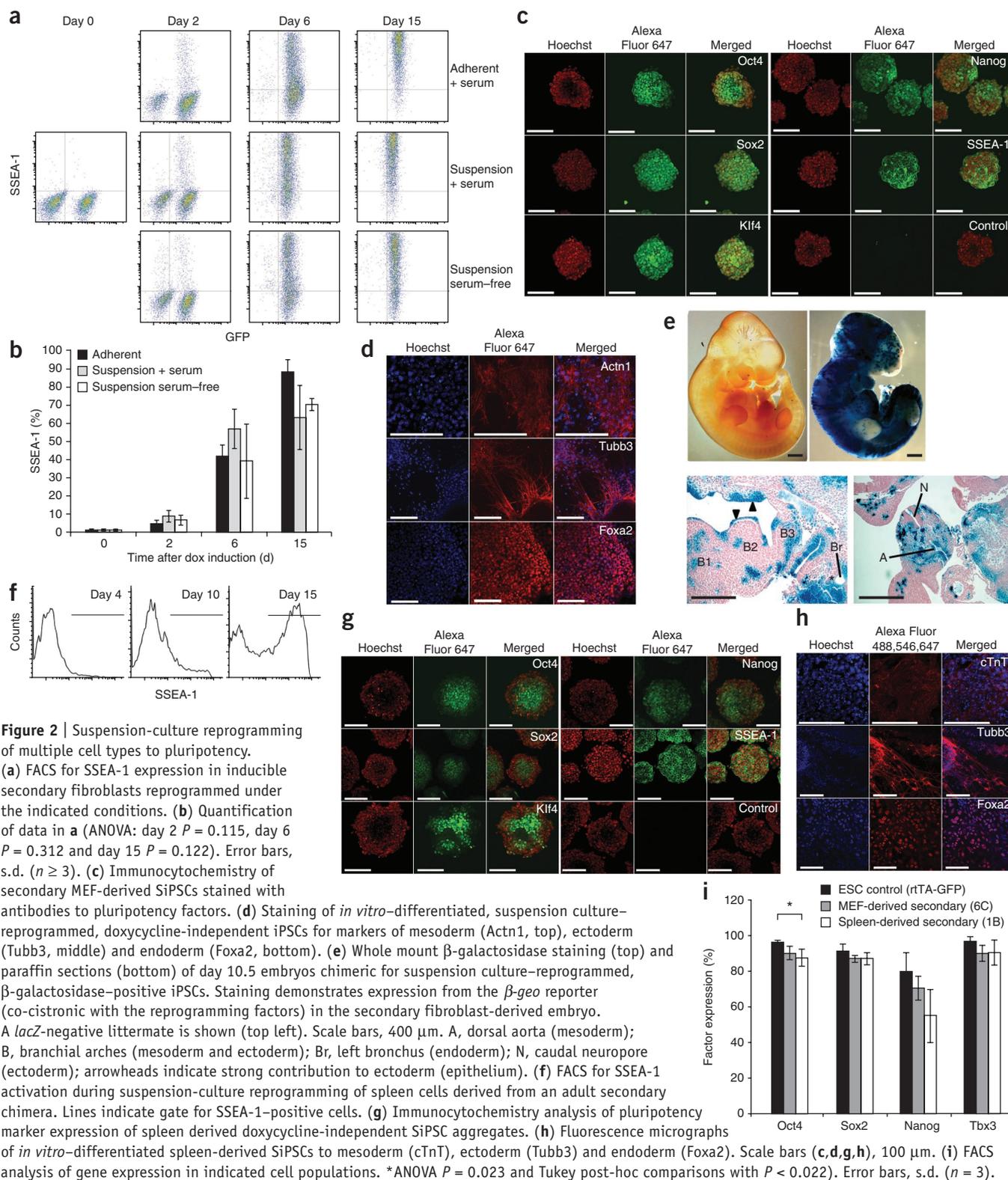


Figure 1 | Secondary fibroblasts inducibly expressing reprogramming factors survived and proliferated in suspension culture. (a) Growth kinetics of inducible secondary fibroblasts cultured under the indicated conditions. Dox, doxycycline. Error bars, s.d. ($n = 3$). (b) FACS of annexin V surface localization for secondary fibroblasts cultured under the indicated conditions. Values are means \pm s.d. ($n \geq 3$). (c) FACS of EdU incorporation by secondary fibroblasts cultured under the indicated conditions. Percentages of GFP⁻, EdU⁺ populations that do not have a letter in common were significantly different (ANOVA, $P = 0.003$ and Tukey post-hoc, $P = 0.0113$). Values are means \pm s.d. ($n = 5$). (d) Live/dead staining of secondary fibroblasts cultured in suspension in the presence of dox at day 6 of culture. EthD, ethidium homodimer-1 (dead); Ca-AM, calcein-AM (live). Scale bars, 100 μ m. (e) Growth kinetics of dox-induced secondary fibroblasts growing in mouse ESC medium from day 12 to day 20. Suspension cultures inoculated with day-12 cells (5×10^4 cells ml⁻¹) were serially expanded with 20-fold medium dilution every 3 d. Error bars, s.d. ($n = 3$).

SiPSCs into mouse blastocysts and observed chimerism and contribution to all three germ layers at embryonic day 10.5 by monitoring β -galactosidase activity (Fig. 2e).

To assay whether other cell types can be reprogrammed in suspension culture, we isolated spleen cells and T-cell progenitors from adult doxycycline-inducible secondary chimeric mice. Spleen-derived cells had robust SSEA-1 induction and became

doxycycline-independent after 15 d of exogenous factor expression (Fig. 2f). Spleen-derived SiPSCs stably expressed pluripotency factors, were capable of differentiating into all three germ layers *in vitro* and showed a pluripotency-related gene expression profile closely related to that of mouse ESC controls (Fig. 2g,h and Supplementary Fig. 5). We analyzed stable pluripotency factor expression by FACS in MEF- and spleen-derived SiPSCs.



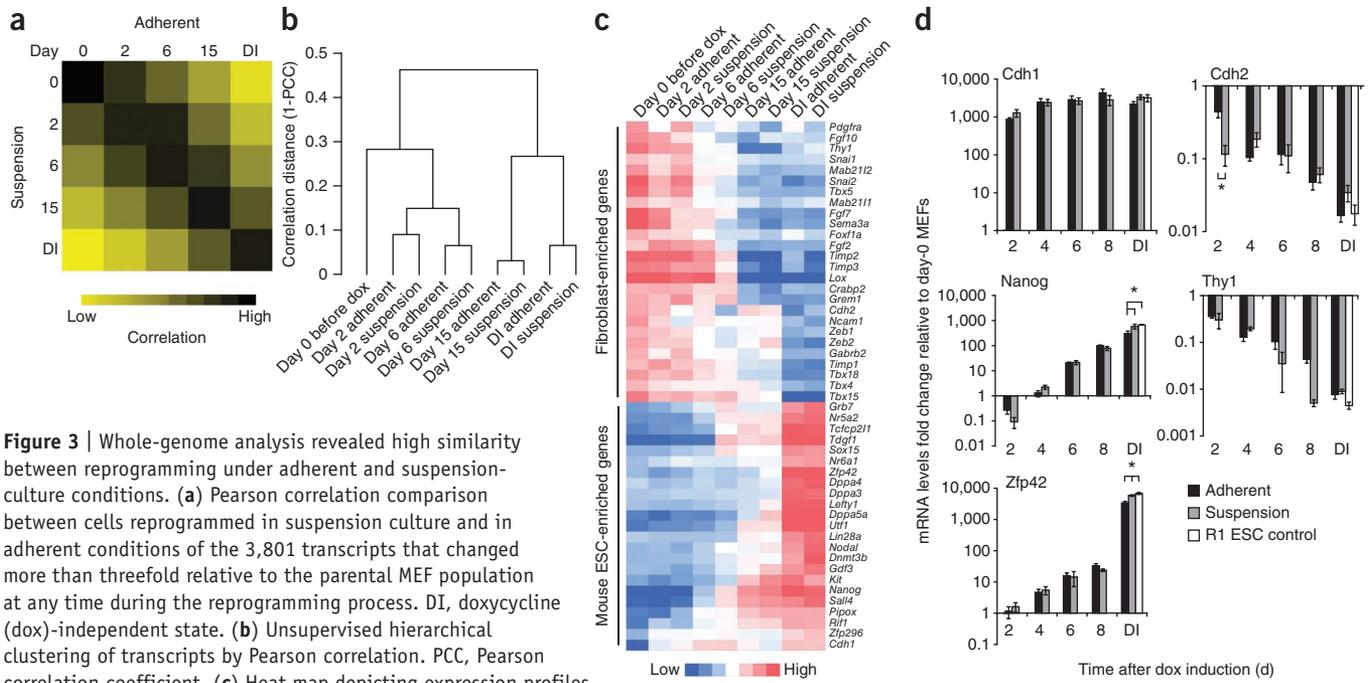


Figure 3 | Whole-genome analysis revealed high similarity between reprogramming under adherent and suspension-culture conditions. **(a)** Pearson correlation comparison between cells reprogrammed in suspension culture and in adherent conditions of the 3,801 transcripts that changed more than threefold relative to the parental MEF population at any time during the reprogramming process. DI, doxycycline (dox)-independent state. **(b)** Unsupervised hierarchical clustering of transcripts by Pearson correlation. PCC, Pearson correlation coefficient. **(c)** Heat map depicting expression profiles of subsets of selected fibroblast-enriched (top) and mouse ESC-enriched (bottom) genes during the reprogramming process under suspension and adherent conditions. **(d)** Expression profiles over the course of the reprogramming process for the indicated genes determined by quantitative PCR. Fold-changes relative to day 0 MEFs are depicted for adherent and suspension culture conditions. *ANOVA $P < 0.001$ and Tukey post-hoc with $P = 0.0014$ for each significant difference. Error bars, s.d. ($n = 3$).

We observed small variations in OCT4 expression for spleen-derived SiPSCs compared to *rtTA-GFP* control ESCs; MEF-derived SiPSCs did not reveal any notable differences (Fig. 2i). Purified T-cell progenitors could also be reprogrammed in suspension culture into doxycycline-independent SSEA-1- and NANOG-expressing cells (Supplementary Fig. 6). Thus, multiple mouse cell types (adult and embryonic as well as adherent and suspension-cultured cell types) can be reprogrammed in suspension culture.

Expression analysis of suspension-culture reprogramming

We collected samples of adherent and suspension-cultured cells undergoing reprogramming at different time points after doxycycline induction and analyzed them for expression of 22,348 transcripts. We selected genes that changed more than threefold at any of the four time points, in either adherent or suspension-culture conditions, relative to the uninduced MEF controls (3,801 transcripts) and examined Pearson correlation coefficients for all conditions. Cells cultured under adherent and suspension-culture conditions showed similar trends for Pearson correlation coefficients throughout the reprogramming process (Fig. 3a). Unsupervised hierarchical clustering analysis on the 3,801 genes that changed more than threefold during the reprogramming process suggested that similar molecular events occurred over the reprogramming process under the two conditions (Fig. 3b).

Fibroblast- and pluripotency-specific hallmark genes¹⁷ exhibited similar expression changes over the course of reprogramming for both adherent and suspension-culture conditions (Fig. 3c). Several of the analyzed pluripotency factors showed different expression levels in cells on day 15 under doxycycline and cells in the doxycycline-independent state (for both adherent cells and cells grown in suspension). This suggests that exogenous

factor expression is repressed to establish endogenous expression levels of pluripotency genes or that further selection steps lead to the establishment of doxycycline independent cultures with expression profiles similar to ESC.

We next selected factors that are known to be up- or down-regulated at different phases of the reprogramming process¹⁷ and followed expression levels of these factors in 2-d time intervals by quantitative real-time PCR. We observed early (*Cdh1*), intermediate (*Nanog*) and late (*Zfp1*) induction under adherent conditions (Fig. 3d), in accordance with results reported earlier¹⁷. Cells reprogrammed in suspension culture showed comparable induction profiles for these factors (Fig. 3d). Factors known to be downregulated during reprogramming, such as *Cdh2* and *Thy1*, were also downregulated during suspension-culture reprogramming (Fig. 3d). We observed small differences between adherent culture-generated and suspension culture-generated iPSC, and R1 (mouse ESC line) controls, for *Nanog* and *Zfp42* expression in stably reprogrammed cell populations (Fig. 3d). Overall the two reprogramming protocols did not show notable expression differences for the analyzed factors over the entire reprogramming process (with the exception of *Cdh2*, a cell-adhesion marker that was downregulated more quickly in suspension culture conditions compared to adherent conditions) (Fig. 3d).

Some genes, however, were differentially expressed between cells reprogrammed under suspension-culture and adherent conditions. To investigate these differences we selected genes with greater than twofold expression change between the two conditions at paired time points during the reprogramming process. Analysis of the differentially expressed genes by Gene Ontology (GO) enrichment analysis (Table 1) showed a considerable over-representation of GO terms associated with extracellular

Table 1 | GO term enrichment analysis

GO term	Input genes in GO term (%)	P value
Day 2		
Extracellular region	24.9	1.6×10^{-36}
Extracellular region part	14.3	8.5×10^{-28}
Extracellular space	8.9	8.4×10^{-16}
Cell adhesion	8.7	1.6×10^{-15}
Biological adhesion	8.7	1.9×10^{-15}
Extracellular matrix	6.6	5.3×10^{-15}
Proteinaceous extracellular matrix	5.9	1.6×10^{-12}
Insulin-like growth factor binding	1.6	1.6×10^{-10}
Calcium ion binding	9.6	3.1×10^{-10}
Pattern binding	3.2	9.8×10^{-10}
Day 6		
Extracellular region	27.1	3.0×10^{-27}
Extracellular region part	16.7	6.8×10^{-23}
Extracellular matrix	8.5	1.7×10^{-14}
Proteinaceous extracellular matrix	7.8	9.7×10^{-13}
Extracellular space	10.1	2.3×10^{-12}
Growth factor activity	4.0	2.2×10^{-7}
Response to wounding	6.1	6.0×10^{-7}
Positive regulation of cell-substrate adhesion	1.9	2.1×10^{-6}
Regulation of cell substrate adhesion	2.1	3.2×10^{-6}
Calcium ion binding	9.9	7.5×10^{-6}
Day 15		
Extracellular matrix binding	2.6	8.9×10^{-5}
Epithelial cell differentiation	3.7	9.9×10^{-4}
Regulation of smoothened signaling pathway	2.1	1.1×10^{-3}
Intermediate filament cytoskeleton organization	2.1	1.1×10^{-3}
Calcium ion binding	10	1.1×10^{-3}
Intermediate filament-based process	2.1	1.5×10^{-3}
Regulation of cell adhesion	3.2	1.8×10^{-3}
Embryonic organ development	4.7	1.8×10^{-3}
Positive regulation of cell-substrate adhesion	2.1	1.0×10^{-3}
Keratinization	2.1	2.2×10^{-3}
Doxycycline-independent		
Extracellular region	14.1	5.0×10^{-7}
Extracellular region part	8.5	7.3×10^{-7}
Basement membrane	2.3	7.5×10^{-6}
Proteinaceous extracellular matrix	4.2	3.7×10^{-5}
Extracellular matrix part	2.3	4.9×10^{-5}
Basolateral plasma membrane	2.8	6.0×10^{-5}
Extracellular matrix	4.2	6.1×10^{-5}
Regulation of cell proliferation	6.1	1.6×10^{-4}
Contractile fiber part	2.1	1.8×10^{-4}
Regulation of angiogenesis	1.6	3.2×10^{-4}

Analysis for genes that vary more than twofold between adherent and suspension-culture conditions. Terms are ordered according to their P values. Percentages correspond to fractions of differentially expressed genes in corresponding GO terms.

compartments, cell adhesion and extracellular matrix components (Table 1 and Supplementary Table 1). The absence of highly ranked enrichment terms for stem-cell maintenance, nuclear compartment and transcription factor binding suggests that differences are mainly centered around the cell environment and not on the transcriptional network. Our data suggest that reprogramming under suspension-culture conditions is similar to anchorage-dependent reprogramming for inducible secondary fibroblasts.

Primary cell reprogramming in suspension culture

We delivered doxycycline-inducible reprogramming factors (Supplementary Fig. 7) by lentiviral transduction into MEFs derived from mice with an *rtTA-IRES-GFP* cassette in the *Rosa26* (*Gt(ROSA)26Sor*) locus. Cultures had increasing percentages of SSEA-1⁺ cells and reached levels of >70% of total viable cells by day 14 (Fig. 4a). Similar to inducible secondary fibroblasts, reprogrammed primary SiPSCs formed compact aggregates when we withdrew doxycycline. Expression of pluripotency-associated transcription factors in aggregates was similar to that in ESC controls and surface staining for SSEA-1 was also similar (Fig. 4). We confirmed expression of OCT4, NANOG and DPPA3 by western blot (Supplementary Fig. 4). SiPSCs derived from primary fibroblasts could differentiate *in vitro* into all three germ layers (Fig. 4d). We also observed induction of SSEA-1 in *Myc*, *Klf4*, *Pou5f1* (also known as *Oct4*) and *Sox2* cassette (MKOS)-transduced adult fibroblasts during suspension-culture reprogramming (Supplementary Fig. 8), although this was delayed relative to observations in MEFs. Flow cytometry analysis of pluripotency factor expression demonstrated that whereas fibroblast-derived SiPSCs had slightly lower NANOG expression than that in *rtTA-GFP* ESC controls, we saw no considerable differences for OCT4, SOX2 and TBX3 (Fig. 4e). These studies confirm that primary embryonic and adult cells can be reprogrammed to pluripotency in suspension culture.

We compared the kinetics of fibroblast reprogramming under adherent and suspension culture conditions. SSEA-1⁺ cell populations exhibited comparable profiles over the reprogramming process, with moderately increased SSEA-1 induction kinetics for suspension culture conditions (Fig. 4f). Primary cells depended on the optimal delivery of the reprogramming factors and displayed slower reprogramming compared to secondary MEFs. We tested the effect of two different lentivirus doses on reprogramming kinetics. For the high virus dose (eight times more virus than the low virus dose), reprogramming was significantly faster over the first 7 d (low virus dose: slope = 4.64 ± 0.68 , $P = 0.002$; high virus dose: slope = 6.86 ± 0.56 , $P = 0.001$), suggesting that more of the fibroblasts were transduced with the factors and initiated reprogramming (Fig. 4f). Later in the process, however, the two curves converged, indicating that rapidly dividing cells undergoing reprogramming outcompeted non-reprogramming cells regardless of the initial virus dose.

Suspension-culture reprogramming and differentiation

We next subjected primary fibroblasts to reprogramming, expansion and differentiation in an integrated stirred suspension culture system (Fig. 5a). Transition to doxycycline independence and differentiation induction led to decreased growth rate and increased cell death; we also observed this phenomenon in adhesion cultures, although it was masked by the efficiency of re-adhesion upon passaging. We tested different conditions to optimize this transition step and measured cell survival after 3 d of culture in the absence of doxycycline (Fig. 5b). We observed the best cell survival upon transition to serum-free embryonic stem cell (SFES) medium with or without ROCK inhibitor. We shifted cells to doxycycline-free SFES medium from day 16 to day 19. The cultures began to form compact SiPSC aggregates during this transition period. Cell yields in culture 3 d after doxycycline

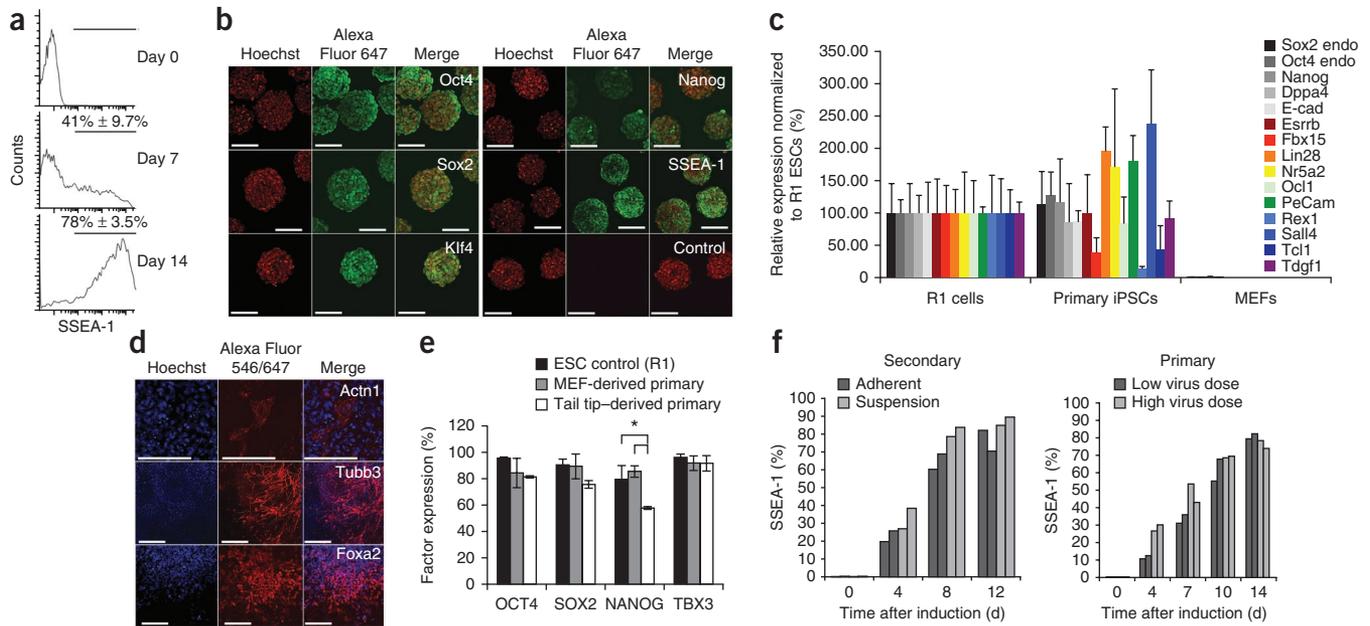


Figure 4 | Primary MEFs reprogrammed under suspension culture conditions. (a) FACS analysis of SSEA-1 activation in inducible reprogramming factor-transduced fibroblasts reprogrammed in suspension culture. Error, s.d. ($n = 3$). (b) Fluorescence micrographs of pluripotency marker expression in doxycycline-independent suspension culture-reprogrammed primary iPSCs. (c) Quantification of expression pluripotency genes in primary SiPSCs and parental MEFs. Values were normalized to control R1 ESCs. Error bars, s.d. ($n = 3$). Endo, endogenously expressed. (d) Micrographs show suspension culture-reprogrammed primary fibroblasts differentiated *in vitro* and stained for Actn1 (mesoderm), Tubb3 (ectoderm) and Foxa2 (endoderm). Scale bars, 100 μm (b,d). (e) FACS analysis of the indicated factor expression in MEF-derived and tail tip-derived primary SiPSCs. *ANOVA $P = 0.023$ and Tukey post-hoc $P < 0.022$. Error bars, s.d. ($n = 3$). (f) Kinetics of SSEA-1 activation in secondary fibroblasts under different conditions (left) and in primary fibroblasts transduced with different virus doses (right).

removal were on the order of 0.5–0.6 per input cell, suggesting that additional selection occurs during reprogramming during the transition to factor independence (Fig. 5b).

We next transitioned the cells (either dissociated or not) into serum-free medium for cardiac differentiation¹⁸. We obtained on the order of 40% FLK1⁺,PDGFR α ⁺ cells at the end of the process (Fig. 5c), comparable with differentiation results previously obtained for optimized conditions¹⁹. Dissociation of the aggregates before differentiation led to considerably higher frequencies of double-positive cell fractions but reduced

overall cell yields (Fig. 5c). We then differentiated the cardiac progenitor cells in suspension culture along the cardiac lineage. On day 29 after induction of fibroblast reprogramming, about 14% of the cells stained positive for the definitive cardiac-specific marker Tnnt2 (cTnT) (Fig. 5d) and formed aggregates that exhibited spontaneous beating (Supplementary Movie 1).

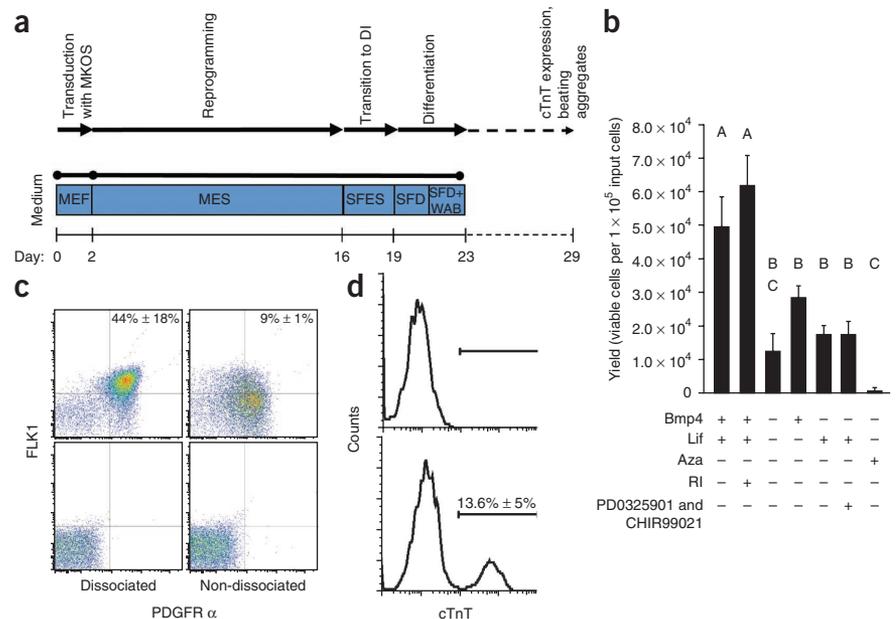


Figure 5 | Integrated derivation, expansion and differentiation of SiPSCs toward cardiac progenitors. (a) Schematic of the suspension culture-based reprogramming and differentiation process. DI, doxycycline (dox)-independent state. cTnT, cardiac troponin T; MES, mouse embryonic stem cell; SFES, serum-free embryonic stem cell; SFD, serum-free differentiation; WAB, Wnt3a, ActivinA and Bmp4. (b) Cell yields after transitioning SiPSCs from dox-containing mouse ESC medium to the indicated doxycycline-free medium at day 19 of culture. Conditions that do not have a letter in common are significantly different from each other (ANOVA $P = 0.001$ and Tukey post-hoc with $P = 0.0042$). Error bars, s.d. ($n = 3$). (c) FACS analysis for FLK1⁺,PDGFR α ⁺ cardiac progenitor cells at day 23 after virus transduction. After suspension-culture reprogramming for 16 d, doxycycline was removed and cultures were maintained for 3 d before cells were induced to differentiate toward cardiac progenitor cells^{18,19} either without dissociation (right, top) or after dissociation to single cells (left, top). Corresponding isotype controls are shown (bottom). Values are expressed as means \pm s.d. ($n \geq 3$). (d) FACS analysis of cTnT expression at day 29 after the initiation of reprogramming. Values are expressed as mean \pm s.d. ($n = 3$).



DISCUSSION

We demonstrated the derivation of iPSCs from somatic mouse cells in suspension cultures in the absence of serum, tissue culture substrates or supporting feeder-cell layers. Global gene expression profiles, expression of pluripotency markers and functional characteristics of suspension culture-reprogrammed cells were comparable to those in control cultures reprogrammed under conventional adherence-dependent conditions and in other published reports^{11,17}. Genes that were differentially expressed between suspension- and adherent-culture reprogramming were mainly associated with transcripts involved in cell adhesion and extracellular matrix interactions, suggesting that these processes may not be critical to transitioning cells toward pluripotency. Suspension culture-based reprogramming allows for continuous analysis of reprogramming in the absence of repeated cell passaging (adhesive selection) steps. Moreover, stringent control of environmental parameters in suspension culture systems might allow for the reduction of confounding factors introduced by static culture systems. Finally, compared to reprogramming under adherent conditions, suspension-culture reprogramming simplifies the process, reduces overall costs and should ultimately enable iPSC culture under conditions amenable to bioprocess engineering strategies (O₂ control, media perfusion and others).

Mouse and human ESCs have been previously shown to be amenable to expansion in microcarrier-free suspension cultures if appropriate conditions and signaling cues are provided^{13,16,20,21}. We demonstrated SiPSC derivation under scalable culture conditions, both in serum-containing and in defined serum-free medium. Reprogramming kinetics in adherent and suspension-culture conditions were comparable, and reprogrammed cells became independent of exogenous factor expression at the end of the processes. Commonly, suspension culture protocols for pluripotent stem cells involve repeated dissociation and reaggregation steps^{14,15}. However, fibroblasts transduced with reprogramming factors were amenable to reprogramming and expansion without repeated dissociation, in the presence of doxycycline. This allowed for improved scalability of the process and enabled us to continuously produce cardiac progenitors under stirred suspension-culture conditions, demonstrating the feasibility of generating desired target cells from mixed input populations.

Suspension-culture reprogramming using primary somatic cells led to non-uniform populations of SiPSCs. The absence of clonal selection can yield cultures with variegated transgene expression owing to positional effects. The presence of partially reprogrammed cells or incomplete transgene repression would be expected to impact the differentiation potential of such populations^{2,22-24}. For both primary and secondary systems, we focused in this study on transgene cassettes driven by doxycycline-responsive promoters²⁵. The advantage of inducible configurations is the efficient repression of the exogenous reprogramming factors upon removal of doxycycline. Incompletely reprogrammed cells undergo apoptosis and differentiation upon doxycycline withdrawal, increasing the frequency of generation of bona fide iPSCs compared to non-inducible configurations²³. With this setup, cells that survive and proliferate upon doxycycline removal exhibited typical ESC-like properties.

Translating this technology to human cells, as well as implementing high-density perfusion-based cell production systems, would overcome several of the key bottlenecks in current

pluripotent cell-mediated cell production⁴, accelerating the development of iPSC biology and technology.

METHODS

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

Accession codes. Gene Expression Omnibus: GSE35422.

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

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AUTHOR CONTRIBUTIONS

D.A.F. designed and performed most experiments. P.D.T. performed chimera experiments, part of quantitative PCR experiments and contributed to drafting the manuscript. H.S. performed cTnT differentiation experiments and did confocal imaging. R.P.B. performed western blot analysis and did part of the secondary MEF, spleen and adult fibroblast reprogramming experiments. N.S. did part of the secondary MEF and spleen reprogramming experiments. S.S. performed secondary DN1 blood reprogramming experiments. G.C. performed statistical analysis. A.N. helped to design the project. D.A.F. and P.W.Z. designed the project and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Cell maintenance and reprogramming. MEFs (inducible 6C, 1B lines)¹¹ and MEFs stably expressing the reverse tetracycline transactivator (rtTA) (*Rosa26 rtTA-IRES-GFP knock-in*)²⁶ were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) FBS (Gibco), 1 mM sodium pyruvate (Gibco), 2 mM Glutamax (Invitrogen) and 1% (v/v) penicillin and streptomycin (Invitrogen). Mouse fibroblasts were reprogrammed either in serum containing mouse ESC medium consisting of DMEM supplemented with 15% (v/v) FBS (Wisent), 0.1 mM β -mercaptoethanol (BME, Sigma), 1 mM sodium pyruvate, 0.1 mM MEM non-essential amino acid (NEAA, Gibco), 2 mM Glutamax, 1% (v/v) penicillin-streptomycin and 1,000 U ml⁻¹ of leukemia inhibitory factor (LIF) (Millipore) or in serum-free ESC (SFES) medium consisting of DMEM with F12 (Gibco) and Neurobasal (Gibco)-based medium supplemented with N2 (Gibco), B27 (Gibco), 0.05% (w/v) BSA, 2 mM Glutamax, 1% (v/v) penicillin and streptomycin, 1.5 \times 10⁻⁴ M monothioglycerol, 1000 U ml⁻¹ LIF, and 10 ng ml⁻¹ BMP4 (ref. 18). Mouse ESC lines (R1 and *rtTA-GFP*) were maintained in mouse ESC medium.

To reprogram in adherent conditions, secondary inducible 6C MEFs were plated in gelatin-coated six-well plates (Sarstedt) and induced with 1 μ g ml⁻¹ doxycycline (Sigma) the day after. Medium exchanges were carried out every 48 h during the entire reprogramming process. Primary mouse fibroblasts were transduced with respective viral preparations 24 h and 36 h after seeding. Culture medium was supplemented with doxycycline 24 h after the last viral transduction, and cells were reprogrammed following the same protocols as for secondary fibroblasts.

To reprogram in suspension conditions, secondary inducible 6C MEFs were trypsinized 8 h after doxycycline induction and seeded either into Sigmacote (Sigma)-treated spinner flasks (Integra Biosciences) at 0.5 \times 10⁵ to 1 \times 10⁵ cells ml⁻¹ or in low-cell-binding plates (Nunc). Primary mouse fibroblasts were transduced with viral preparations 24 h and 36 h after seeding. Cultures were supplemented with doxycycline 24 h after the last viral transduction. Eight to twelve hours after induction (doxycycline addition), cells were trypsinized and seeded into spinner flasks at 2 \times 10⁵ cells ml⁻¹. Culture volumes were between 30–50 ml, cultured with a constant stirring speed of 65 r.p.m. One-third of the culture medium was replaced every day. Spinner flasks were replaced every 6 d to prevent sticking of cells to vessel walls. To remove large aggregates from high-density cultures, cells were passed through 100- μ m cell strainers (BD Biosciences). All adherent cultures, spinner flasks and low-cell-binding plates were incubated in a humidified 5% (v/v) CO₂ air environment at 37 °C.

Suspension reprogramming of secondary chimera-derived spleen cells and T-cell progenitors. Spleen of secondary chimeric adult 1B mice was isolated, minced and cultured in minimal essential medium alpha (α MEM) (Gibco) containing 16.7% (v/v) FBS (HyClone), 1% (v/v) penicillin and streptomycin, 10 ng ml⁻¹ human IL-2 and 5 ng ml⁻¹ mouse granulocyte macrophage colony-stimulating factor (GM-CSF) (R&D Systems) to promote survival. The cells were seeded at 1 \times 10⁶ cells ml⁻¹ density in low-adhesion plates (Nunc) in the presence of doxycycline for 8 h. After 8 h, cells were seeded at 1 \times 10⁵ cells ml⁻¹ in spinner flasks in α MEM with cytokines and doxycycline as above or at a density of 5 \times 10⁴ cells per six-well plate on irradiated feeder MEFs. After 2 d,

the medium was replaced with fresh α MEM supplemented with 40% mouse ESC medium. Once the cells started forming aggregates (after 4 d), stirring was initiated in the spinner flasks at 65 r.p.m., and the medium was replaced to 100% ESC medium containing IL-2, GM-CSF and doxycycline. Blood cytokines were completely removed after 8 d. Doxycycline was removed after 15 d for expansion of spleen-derived SiPSCs.

For isolation of double-negative (DN1) progenitor T cells, the thymus of secondary chimeric adult 1B mice was isolated, minced and stained for CD4⁻CD8⁻CD25⁻CD44⁺ surface-marker expression. FACS-separated DN1-T cells were cultured in low-adhesion plates (Nunc) at 0.1 M ml⁻¹ in α MEM containing 16.7% (v/v) FBS (HyClone), 1% (v/v) penicillin and streptomycin, 50 ng ml⁻¹ mouse stem cell factor (SCF), 10 ng ml⁻¹ human FLT3L and 10 ng ml⁻¹ mouse IL-7 (R&D Systems) to promote survival and doxycycline (1 mg ml⁻¹) to initiate reprogramming. After 2 d, the medium was replaced with fresh α MEM supplemented with 40% (v/v) mouse ESC medium. After 4 d, the medium was exchanged with 100% mouse ESC medium containing SCF, FLT3L, IL-7 and doxycycline. After 6 d, reprogramming DN1 T cells started forming aggregates and stirring was initiated. ESC medium was replaced with half the concentration of blood cytokines, which were completely removed after 8 d. Doxycycline was removed after 15 d for expansion of DN1 cell-derived SiPSCs.

Integrated production of cardiac progenitors. *RtTA* MEFs were transduced in T75 flasks at day 0 and day 1. Cells were induced with doxycycline at day 2, and 8 h after induction were shifted to suspension conditions in spinner flasks. After day 10, cells were diluted to concentrations below 10⁶ cells ml⁻¹ to avoid medium depletion. At day 16, cells were shifted from mouse ESC medium with doxycycline to SFES medium without doxycycline and incubated in spinner flasks or in low-adherence 10-cm Petri dishes (Fisher) placed on an orbital shaker (65–75 r.p.m.). Day-19 cultures were passed through a 40- μ m strainer (BD Biosciences), and filter retentates were used for cardiac induction as described previously¹⁸. Input populations were either non-dissociated aggregates or single cells after trypsin dissociation. Differentiation to cTnT cells was performed by dissociating day-21 cultures and re-aggregating single cells as 100 cell aggregates in 400- μ m microwell inserts²⁷ in serum-free differentiation (SFD) medium¹⁸ supplemented with 10 ng ml⁻¹ BMP4 (R&D), 10 ng ml⁻¹ Activin A (R&D) and 5 ng ml⁻¹ VEGF (Sigma) for 3 d. On day 24, aggregates were transferred from microwells to 6-cm plates containing Stempro medium (Invitrogen) supplemented with 150 ng ml⁻¹ DKK1 (R&D), 5 ng ml⁻¹ VEGF, 10 ng ml⁻¹ bFGF (Peprotech) and 10 ng ml⁻¹ FGF10, and cultured for an additional 5 d and then analyzed by FACS.

Small molecules used were 5-azacytidine (3 μ M, Sigma), ROCK inhibitor (Y27632, 10 μ M, Sigma), PD0325901 (1 μ M, Stemgent), CHIR99021 (3 μ M, Stemgent). The 2i medium consisted of SFES medium without BMP4, supplemented with PD0325901 and CHIR99021.

Plasmid generation and virus production. pDFTET-Myc and pDFTET-Sox were generated as follows. The *cMyc* and *Sox2* open reading frames were PCR-amplified from retroviral backbones (Addgene) using primers *cMyc_fwd*, *cMyc_rev*, *Sox2_fwd* and *Sox2_rev* (all primers used are listed in **Supplementary Table 2**).

The amplified *cMyc* and *Sox2* fragments were subcloned by ligating the *NheI* and *NotI*-digested inserts into the corresponding sites of pCEP4 (Invitrogen). Fragments were excised from these intermediates by *NheI* and *XhoI* restriction and subsequently ligated into the corresponding sites of pMF351 (ref. 28) generating *PDFCMV-cMyc* and *PDFCMV-Sox2*. In a second step the *Tet* promoter (*PTET*) was amplified by PCR from *PB-TET-Myc*¹¹ using primers *PTET_fwd* and *PTET_rev*, restricted using *AscI* and *NheI* and subsequently ligated into the *AscI* and *NheI*-digested *PDFCMV-Myc* and *PDFCMV-Sox2* backbones.

PDFTET-KOS was generated by PCR-amplifying *KOS* from *PB-TET-MKOS*¹¹ using primers *Klf4_fwd* and *Sox2_rev*. The *KOS*-containing fragment was restricted using *NheI* and *NotI* and ligated into the corresponding sites of *PDFTET-Sox2*.

Viral particles were produced by transfection of HEK293 T cells with calcium phosphate–DNA precipitates. Medium was changed to Advanced DMEM (Gibco) supplemented with 1% (v/v) penicillin and streptomycin, 1% (v/v) Glutamax, 2% (v/v) FBS (Gibco) and 0.01 mM cholesterol 16 h after transfection. Supernatants were collected twice, 36 and 60 h after transfection, filtered through a 0.45- μ m filter, aliquoted and stored at -80°C . For standard transductions, 8 ml of MEF culture medium was mixed with 400 μ l of each viral supernatant and added to MEF cells grown in T75 tissue culture flasks in the presence of 8 $\mu\text{g ml}^{-1}$ Polybrene (Sigma).

Microarray analysis. Whole-genome expression analysis of cells reprogramming under adherent and suspension conditions was carried out by isolating total RNA at indicated time points using RNAeasy RNA isolation kits (Qiagen). Hybridizations were carried out on Affymetrix mouse Gene 1.0 ST arrays (one array per time point and condition) and normalized using RMA (Affymetrix expression console). Fold changes and Pearson correlation coefficients were calculated in Excel (Microsoft). Hierarchical clustering was performed using Cluster 3.0 (similarity metric was correlation (centered); clustering method was average linkage) and visualized by JavaTree software. GO term enrichment analysis was performed using the database for annotation, visualization and integrated discovery (DAVID) bioinformatics resource²⁹.

Quantitative PCR analysis. Total RNA was extracted from cells using Qiagen RNAeasy miniprep columns according to the manufacturer's protocol. Total RNA was used to generate cDNA using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Generated cDNA was mixed with respective primers and SYBR green mix (Roche, Sigma) and run on an Applied Biosystems 7900 HAT real-time PCR machine. Relative expression of described genes was determined by the delta-delta cycle threshold (Ct) method with the expression of *Gapdh* as an internal reference. Primer sequences are listed in **Supplementary Table 2**.

Differentiation protocols. Mesoderm and endoderm differentiation was carried out by dissociating iPSC and seeding in low-adhesion plates at a density of 1×10^6 cells per 10 ml in DMEM containing 15% (v/v) FBS, 1% (v/v) penicillin and streptomycin, 2 mM Glutamax, 0.1 mM BME and 0.1 mM NEAA. Cells were cultured for 4 d on an orbital shaker (65 r.p.m.) with medium exchange at day 2. After 4 d, suspension aggregates were seeded

on gelatin-coated tissue-culture plates and cultured for another 5 d before staining with antibodies.

Ectoderm differentiation was performed according to ref. 30. Briefly, iPSCs were trypsinized and plated at 5×10^5 cells per 10 ml in SFEB medium (Glasgow minimum essential medium (GMEM) supplemented with 5% (v/v) knockout serum replacement, 0.1 mM NEAA, 1 mM sodium pyruvate, 1% (v/v) penicillin and streptomycin and 0.1 mM BME). Cells were cultured for 3 d in low-adherence plates. At day 3 cells were re-fed by replacing 70% of the medium and cultured for another 2 d. Spheres were transferred intact to Matrigel-coated six-well plates and incubated for 5 d in N2B27 medium (DMEM with F12 and Neurobasal medium supplemented with B27, N2 supplements, 0.005% (w/v) BSA and 1 mM sodium pyruvate) before staining for TUBB3 expression.

Protein extraction and western blot analysis. Cell pellets were washed in chilled phosphate-buffered saline (PBS) and incubated in ice-cold lysis buffer containing freshly added protease inhibitors (Sigma). Lysates were cleared by centrifugation at 4°C for 15 min at 12,000g, and protein concentrations determined using a bicinchoninic acid assay (Thermo Scientific). For western blot analysis, 20 μg of total protein was size fractionated by SDS-PAGE on a TGXAnykD precast gel (BioRad), transferred to a Hybond nitrocellulose membrane (GE Healthcare) in transfer buffer containing 10% methanol. The membrane was probed with specific primary antibodies (**Supplementary Table 2**) and secondary horseradish peroxidase–conjugated antibodies (anti-mouse IgG-HRP, HAF007; anti-rabbit IgG, HAF008, R&D Systems). Antibody-protein complexes were detected using ECL-plus (GE Healthcare) on a GelDoc 2000 (BioRad).

Immunocytochemistry, flow cytometry and cell staining. Antibodies used for flow cytometry and immunostainings are listed in **Supplementary Table 3**. All surface stainings for flow cytometry were performed in the presence of 7AAD (Molecular Probes), and populations were gated on live cells. For cell sorting, cells isolated from the thymus were first blocked for 10 min on ice with mouse CD16/CD32 Fc block (clone 2.4G2, BD 553142). The cells were subsequently labeled with the conjugated antibodies above for 20 min on ice to sort for $\text{CD4}^{-}\text{CD8}^{-}\text{CD25}^{-}\text{CD44}^{+}$ DN1 T cells using a FACSAria flow cytometer (BD Biosciences). For intracellular staining, cells were fixed with PBS containing 4% formaldehyde and then permeabilized with methanol. Analysis was performed on FACSDiva (BD Biosciences) as well as FlowJo (Tree Star).

Immunocytochemistry stainings were performed by fixing cells in PBS containing 4% (v/v) formaldehyde. Cells were permeabilized in PBS containing 0.1% (v/v) Triton X-100 and subsequently blocked in PBS containing 10% (v/v) donkey serum. Samples were incubated with primary and secondary antibodies in PBS containing 1% (w/v) BSA and imaged using a confocal microscope (FV1000 laser scanning confocal; Olympus) with 5 μm optical sections. Images represent the z-stack projection of five to ten confocal optical sections. Annexin V (Invitrogen) staining was carried out according to the manufacturer's protocol. EdU cell proliferation assays were performed according to the manufacturer's protocol (Invitrogen). Calcein-AM and ethidium homodimer I staining (Sigma) was performed as indicated in the manufacturer's protocol.

Generation of chimeras. Doxycycline-independent suspension iPSCs were plated and collected as cell clumps of 8–15 cells from gelatinized dishes by gentle trypsinization. For diploid chimeras, 2.5 d post-coitum (d.p.c.) *Hsd:ICR(CD-1)* embryos were aggregated with iPSC clumps and cultured overnight at 37 °C in 5% (v/v) CO₂ in potassium simplex optimized medium (KSOM) (Millipore). Embryos were transferred into pseudopregnant recipient ICR females 24 h later. For β -galactosidase detection, pregnant dams were treated with doxycycline (1.5 mg ml⁻¹ doxycycline; 5% (w/v) sucrose in water) 20 h before dissection. β -galactosidase staining and sectioning were performed as described in ref. 11. The mice were housed in a pathogen-free environment and the care of the animals was in accordance with institutional guidelines (Toronto Centre for Phenogenomics).

Statistical analysis. Results were expressed as mean \pm s.d. from at least three independent replicates if not otherwise stated. Data were checked for normalcy using the Kolmogorov-Smirnov

test with $P = 0.05$. Multiple comparisons were performed using ANOVA with Tukey post-hoc comparisons to determine significant differences between groups ($P < 0.05$, two-tailed). Results were confirmed using non-parametric Mann-Whitney U tests. Calculations were performed using Minitab 16.2.1 (Minitab).

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