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Short Article

Reprogramming of Human Fibroblasts to Pluripotency with Lineage Specifiers

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SUMMARY

Since the initial discovery that *OCT4*, *SOX2*, *KLF4*, and *c-MYC* overexpression sufficed for the induction of pluripotency in somatic cells, methodologies replacing the original factors have enhanced our understanding of the reprogramming process. However, unlike in mouse, *OCT4* has not been replaced successfully during reprogramming of human cells. Here we report on a strategy to accomplish this replacement. Through a combination of transcriptome and bioinformatic analysis we have identified factors previously characterized as being lineage specifiers that are able to replace *OCT4* and *SOX2* in the reprogramming of human fibroblasts. Our results show that it is possible to replace *OCT4* and *SOX2* simultaneously with alternative lineage specifiers in the reprogramming of human cells. At a broader level, they also support a model in which counteracting lineage specification networks underlies the induction of pluripotency.

INTRODUCTION

Induced pluripotent stem cells (iPSCs) can be generated by forced expression of transcription factors (TFs) commonly enriched in embryonic stem cells (ESCs). Accordingly, it has been generally assumed that such factors are specific to the pluripotent state and they are referred to as "pluripotency factors." However, identification of a specific gene signature defining pluripotent identity remains elusive and pluripotency is routinely evaluated by functional differentiation assays rather than mere marker expression. Pluripotency does not seem to represent a discrete cellular entity but rather a functional state elicited by a balance between opposite differentiation forces (Loh and Lim, 2011; Zipori, 2004) (Figure 1A). In support of this hypothesis, *OCT4* and *SOX2* have been shown to counteract for the expression of lineage specification genes (Loh and Lim, 2011; Thomson et al., 2011; Wang et al., 2012). If the pluripotent state does in fact represent a balance between counteracting differentiation forces, it might be possible to achieve reprogramming by replacing the "core" pluripotency factors in the reprogramming cocktail with downstream genes related to lineage specification or additional counteracting factors potentially expressed in ESCs. Indeed, reprogramming can be accomplished in the absence of *SOX2* in mouse and human cells, as endogenous *SOX2* levels in neural progenitor cells (NPCs) can suffice for *OCT4*-driven reprogramming into iPSCs (Kim et al., 2009a, 2009b, 2008). Similarly, exogenous *OCT4* expression can be dispensable for the reprogramming of mouse cells when substituted by the nuclear receptor Nr2a5 (Heng et al., 2010) or by E-cadherin expression (Redmer et al., 2011). However, identification of molecules able to substitute for *OCT4* in the reprogramming of human cells has remained elusive.

Interestingly, recent reports have indicated that *OCT4* plays an essential role in the establishment of primitive endoderm (Frum et al., 2013). Two additional reports indicated that precise levels of *OCT4* govern transition through different pluripotent states and differentiation into embryonic lineages (Karwacki-Neisius et al., 2013; Radzisheuskaya et al., 2013). These observations demonstrate a role for *OCT4* in differentiation apart from its well-known functions in pluripotent cells. Similarly, other reprogramming factors are expressed in cells other than pluripotent stem cells and associated with lineage specification (Loh and Lim, 2011; Sarkar and Hochedlinger, 2013; Suzuki et al., 2006; Wang et al., 2012). Together, all these data support the idea that the current definitions of "pluripotency factors" and "lineage markers/specifiers" are not necessarily mutually exclusive.

Here we report on the identification of several factors that, although traditionally related to lineage specification, also allow for the replacement of *SOX2* and of *OCT4* in the reprogramming of human fibroblasts to iPSCs. Our results shed new light on the molecular determinants of reprogramming and support the notion that pluripotency represents a functional cellular state achieved by the fine-tuned balance of opposing differentiation forces.

RESULTS

Human Pluripotent Cells Express Markers Related to Differentiation and Linage Specification

We have previously demonstrated that mouse ESCs (mESCs) display a dynamic equilibrium in the expression of the early mesendodermal marker *T* (Suzuki et al., 2006) while maintaining an

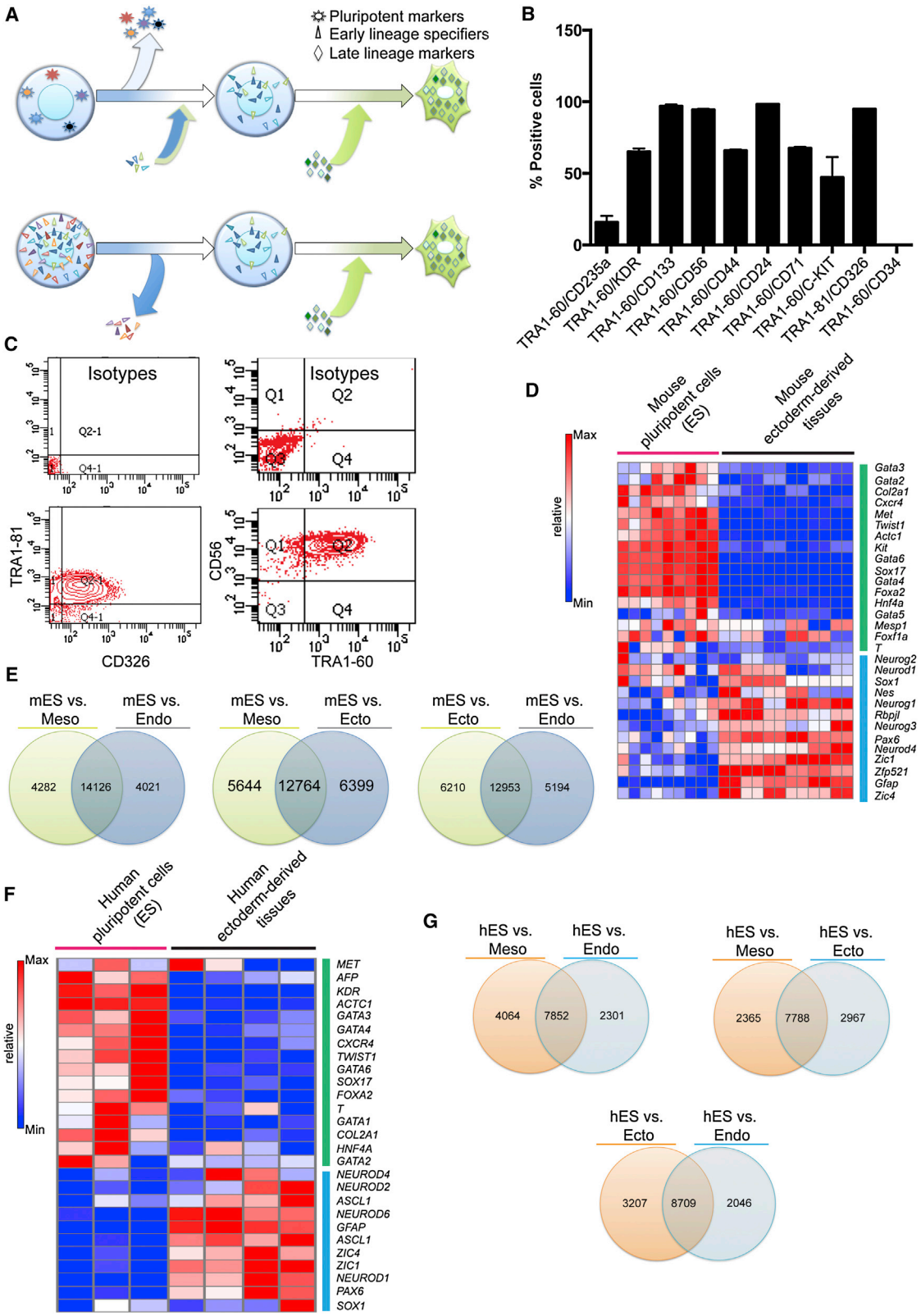

Figure 1. Undifferentiated PSCs Express Genes Related to Lineage Specification

(A) Schematic representation of the different models exemplifying PSC state and differentiation. Upper panels: PSCs are characterized by the expression of "specific" pluripotency markers. Differentiation induces the downregulation of pluripotent markers accompanied by upregulation of early lineage specifiers and ultimately the expression of lineage-specific markers. Bottom panels: PSCs express markers typical of different lineages alongside pluripotency-related ones.

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undifferentiated pluripotent state. In order to extend our previous observations we decided to evaluate the protein expression levels of a number of different lineage markers. We particularly focused on mesendodermal gene expression, as *OCT4* has been described to regulate the mesendodermal lineage in pluripotent cells (Loh and Lim, 2011; Thomson et al., 2011; Wang et al., 2012) and be necessary for the establishment of primitive endoderm and efficient differentiation (Frum et al., 2013; Karwacki-Neisius et al., 2013; Radzisheuskaya et al., 2013). To this end, we monitored lineage-related protein expression in parallel to the expression of pluripotency markers generally viewed as characteristic of pluripotent cells (Chan et al., 2009). Our results confirmed that different subpopulations of pluripotent cells coexpress pluripotency markers with so-called lineage markers including CD56, CD71, CD235a, CD326, CD24, CD133, *KDR*, and *KIT* (Figures 1B and 1C), in line with the notion that pluripotent stem cells (PSCs) may not represent a "blank cellular entity" (Zipori, 2004).

To start addressing whether TFs related to lineage specification could potentially be used for the reprogramming of human somatic cells to iPSCs, we devised a strategy to identify expression of lineage markers based on microarray analysis. We reasoned that two-pair microarray comparisons of tissues derived from each of the major three germ layers could highlight expression of markers typical of other lineages, even if they are not very highly expressed, in ESCs. Three major comparisons were analyzed for both human and mouse cells using existing array data sets: ectodermal derivatives relative to undifferentiated ESCs with the aim of highlighting expression of potential mesendodermal markers in ESCs; mesodermal derivatives relative to undifferentiated ESCs with the aim of assessing expression of ectodermal and endodermal markers; and endodermal derivatives relative to undifferentiated ESCs for manifesting the expression of mesoderm and ectoderm markers. This approach enabled us to generate three different gene data sets in which lineage markers expressed in ESCs were highlighted (Figures 1D–1G and Tables S1, S2, S3, and S4, available online). Interestingly, the resulting "mesendoderm enriched" data sets highlighted genes present in both mouse and human PSCs. The expression of genes typically associated with lineage specification in PSCs, even though at low levels, together with reports of the expression of traditional pluripotency factors in differentiated lineages (Kurian et al., 2013; Loh and Lim, 2011; Suzuki et al., 2006; Wang et al., 2012), further indicated that there is overlap between pluripotency and lineage marker expression.

GATA3 Replaces *OCT4* for Reprogramming Human Fibroblasts

We decided to focus our attention on the GATA family of TFs as they can regulate transcription by acting as "pioneer TFs" in a similar way to that recently described for the Yamanaka factors (Soufi et al., 2012; Zaret and Carroll, 2011). Among these *GATA3* is involved, with *CDX2*, in the specification of trophoblast and in ESC differentiation toward mesendodermal lineages (Home et al., 2009; Ralston et al., 2010; Thomson et al., 2011). Additionally, a previous study showed that increased *GATA3* expression in ESCs results in broad transcriptome changes leading to the upregulation of mesendodermal genes, thus resembling the role of *OCT4* in lineage specification (Nishiyama et al., 2009). Most interestingly, the balance between *OCT4* and *Cdx2* expression shifts cell fate during preimplantation development (Niwa et al., 2005). Together, these observations highlight the role that fine-tuned balancing of gene expression programs play in lineage specification and pluripotency maintenance (Niwa et al., 2005; Wang et al., 2012) and suggest that GATA factors might potentially contribute to the reprogramming of human somatic cells to iPSCs by replacing *OCT4* (Soufi et al., 2012; Zaret and Carroll, 2011).

To investigate the activity of GATA proteins in reprogramming, we subjected human fibroblasts to reprogramming experiments with several construct combinations in the presence or absence of different GATA family members (*GATA3*, *GATA6*, and *GATA4*). We decided to pursue a strategy involving a range of vector constructions, and thus expression approaches, because the relative levels of the reprogramming factors have been shown to play an important role during iPSC generation and contribute to the overall quality of pluripotent cells (Carey et al., 2011; Karwacki-Neisius et al., 2013). As part of that strategy, we made use of VP16 transactivation domains constructed in different combinations and positions to enhance the activity of different expressed factors (Wang et al., 2011). Upon overexpression in human fibroblasts, we observed iPSC colonies only in combinations including *GATA3-VP16* and not other GATA family members to replace *OCT4* (Figure 2A). Pluripotency marker expression was upregulated at both the RNA and protein levels (Figures 2B and 2C). The iPSC colonies generated stained positive for alkaline phosphatase as well as for the pluripotency markers TRA-1-60, TRA-1-81, SSEA3/4, OCT4, SOX2, and NANOG, indicating the pluripotent nature of the cells (Figures 2A and 2B). Genomic DNA PCR analysis for transgene sequences confirmed that the analyzed colonies contained exogenous *GATA3* integrated into their genome (Figure 2D). Importantly, promoter methylation analysis confirmed demethylation of the *GATA3* promoter not only when *GATA3* was used for reprogramming but also in iPSC lines generated by the traditional Yamanaka factors (*OCT4*, *SOX2*, *KLF4* and *c-MYC*, hereafter referred to as *OSKM*) (Figure 2E).

As shown in Figure 2, all of the iPSC lines generated demonstrated in vitro differentiation toward derivatives of the three major germ layers, teratoma formation upon in vivo transplantation (Figure 2F), and an appropriate response to BMP4-induced differentiation (Figures S1A and S1B, available online). Additionally, karyotype analysis demonstrated correct genomic content and the lack of major deletions or duplications (Figure 2G). Once

Upon differentiation, pluripotent marker expression is downregulated alongside unrelated lineage specifiers. Downregulation of certain lineage specifiers disrupts the balance defining PSCs and leads to differentiation toward lineages specified by the remaining molecules.
(B) Percentage of cells double-positive for TRA1-60 or TRA1-81 and different lineage-related surface markers.
(C) Representative flow cytometry plots depicting expression of lineage markers in undifferentiated PSCs.
(D–G) Comparative microarray analysis highlighting the expression level of mesendodermal genes (green) and ectodermal genes (blue) in murine (D and E) and human ESCs (F and G). Venn diagrams depict the number of common gene probes upregulated for murine (E) and human cells (G).
Data are represented as mean ± SD. See also Tables S1, S2, S3, and S4.

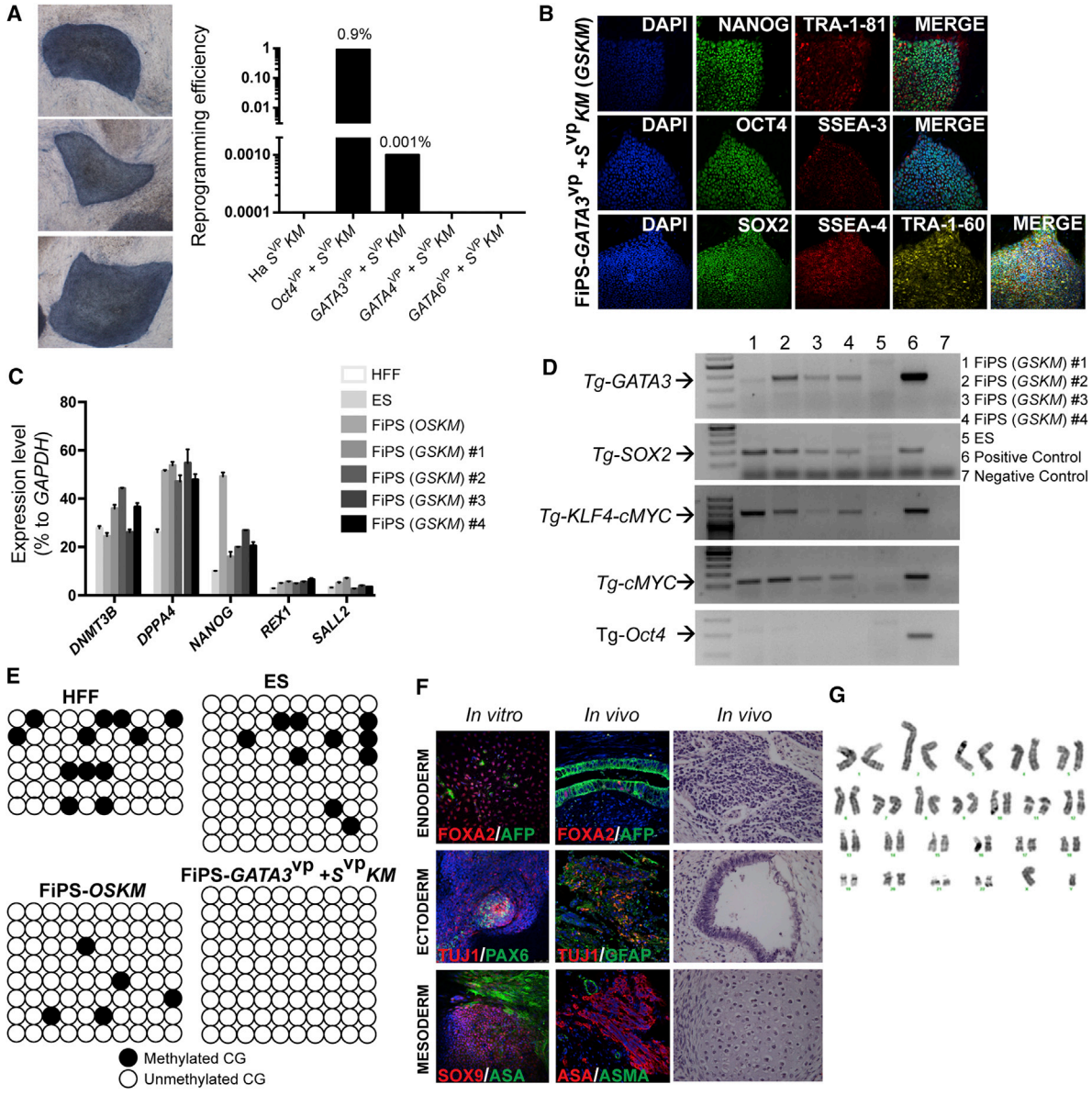


Figure 2. Generation of iPSCs by Replacement of *OCT4* with the Mesendodermal Lineage Specifier *GATA3*

(A) Representative pictures of alkaline phosphatase-positive pre-iPSC colonies generated by replacing *OCT4* with *GATA3*. On the bottom are shown reprogramming efficiencies, based on TRA1-60 expression (Chan et al., 2009), achieved by different methodologies.

(B) Representative immunofluorescence pictures demonstrating pluripotent marker expression in *GATA3*-reprogrammed iPSCs.

(C) mRNA expression level of different pluripotent-related genes.

(D) Genomic DNA PCR demonstrating integration of the exogenous genes.

(E) Methylation analysis of the *GATA3* promoter in the indicated cell types.

(F) *GSKM*-iPSCs are able to differentiate into derivatives of the three germ layers in vitro and in vivo.

(G) Representative karyotype analysis of *GSKM*-iPSCs.

Data are represented as mean ± SD.

See also Figure S1.

the pluripotent nature of the generated iPSCs was confirmed we sought to further characterize and investigate the potential differences between iPSCs generated by different reprogramming factor combinations. Genome-wide transcription analysis demonstrated two well-defined and separated clusters, a pluripotent cluster in which *GATA3*-generated iPSCs were indistinguishable from other iPSCs and ESCs, and an independent cluster containing the initial somatic human fibroblasts (Figure S1C). Together, our results demonstrate that *GATA3-VP16* is sufficient for the functional replacement of *OCT4* during the reprogramming of human somatic cells to iPSCs and that *GATA3*-iPSCs appear to be indistinguishable from other hPSCs.

GATA3 Overexpression Induces Endogenous *OCT4* Expression during Reprogramming and Its Downregulation Compromises PSC Viability

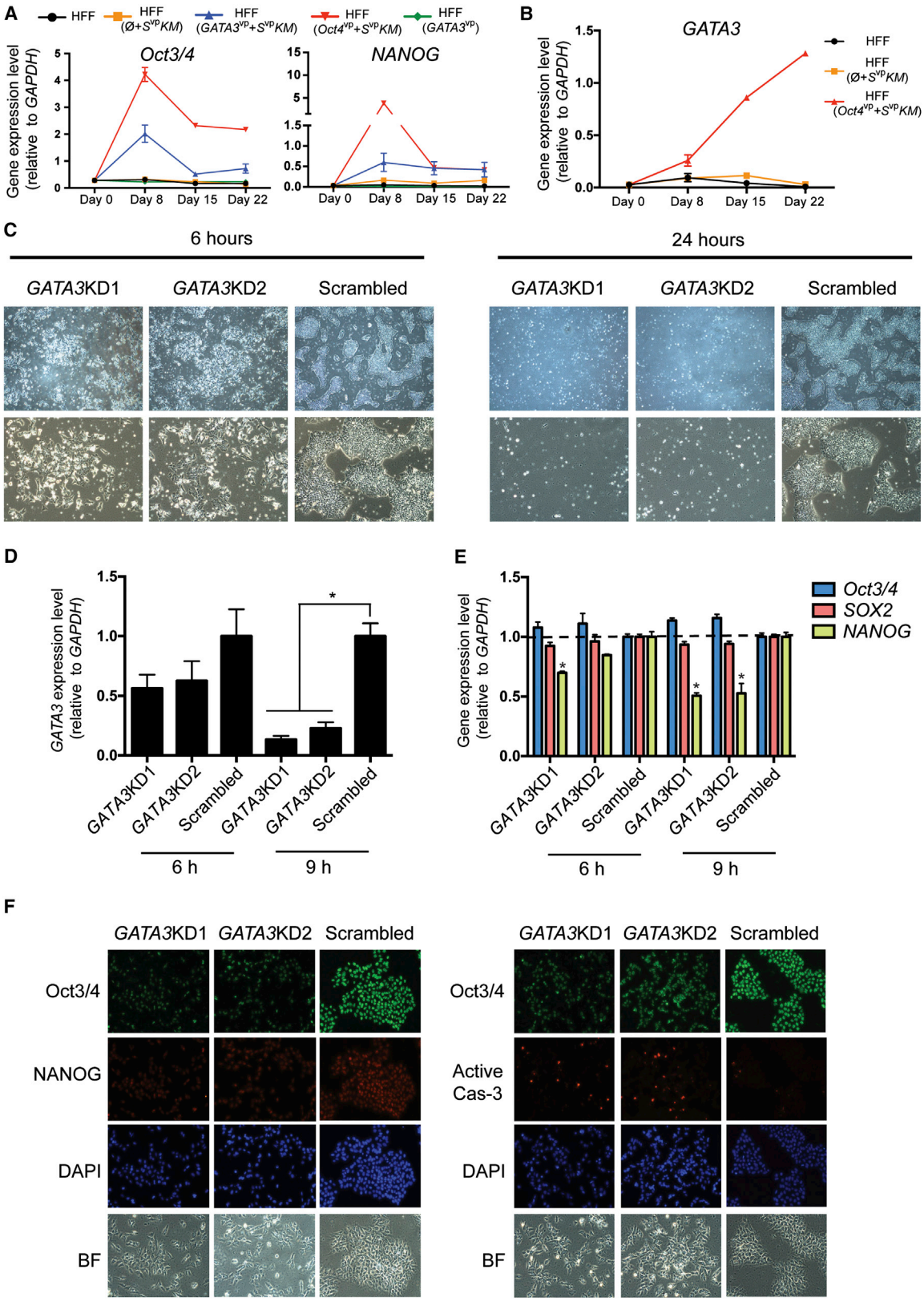
Next we investigated the potential mechanisms by which *GATA3* could replace *OCT4* in these reprogramming experiments. Infection of human fibroblasts with different TF combinations demonstrated significant upregulation of endogenous *OCT4* expression when *GATA3-VP16* was combined with other Yamanaka factors (Figure 3A). Interestingly, upregulation of endogenous *OCT4* and *NANOG* was less pronounced than that observed upon *OSKM* overexpression, potentially explaining the reduced reprogramming efficiencies observed (Figure 3A) (Carey et al., 2011). In support of the role of *GATA3* during reprogramming, endogenous *GATA3* upregulation was observed when *OCT4* was employed in combination with *KLF4*, *c-MYC*, and *SOX2* (Figure 3B). As *GATA3* overexpression in ESCs results in upregulation of mesendodermal gene expression (Nishiyama et al., 2009), we also investigated the consequences of using an inverse approach, i.e., reducing GATA3 expression in PSCs. After *GATA3* knockdown, PSCs displayed aberrant colony morphology and then cell death after 3 days (Figures 3C and 3D). During the early events after knockdown, before substantial cell death was observed, *GATA3* downregulation resulted in reduced expression of *SOX2* and *NANOG* while *OCT4* levels remained unchanged (Figures 3E and 3F). These results suggest an intricate connection between *OCT4* and *GATA3* expression during reprogramming of human cells and identify a critical role for appropriate levels of *GATA3* in the maintenance of the human pluripotent state. Our findings are in accordance with previous reports indicating that disturbance of the appropriate levels of the core pluripotent machinery or *GATA3* overexpression results in the loss of pluripotency (Nishiyama et al., 2009).

A Seesaw Model Allows for the Reprogramming of Human Fibroblasts

Using a similar logic, we investigated whether early ectodermal lineage specifiers might similarly permit reprogramming to iPSCs by replacing *SOX2*. We evaluated the role of different TFs related to early ectodermal commitment including *PAX6*, *OTX2*, *RBPJ*, *ASCL1*, *ZIC2*, *ZNF521*, *FOXD5*, and *HESX1*. Overexpression of *ZIC2*, *ZNF521*, *ASCL1*, *HESX1*, and *FOXD5* in human fibroblasts alongside *OCT4*, *KLF4*, and *c-MYC* resulted in the appearance of iPSC-like colonies albeit with low efficiency (0.0008%). Further validation demonstrated pluripotent marker expression and pluripotent differentiation potential toward derivatives of the three germ layers (Figures 4A, 4B, and S2A), together confirming the pluripotent nature of these lines. Next we evaluated each factor individually and, although with a very low efficiency (0.0004%), in our experimental conditions we observed that *ZNF521* alone could replace *SOX2* and generate iPSCs expressing the hallmarks of pluripotency (Figures 4A, 4B, and S2A).

Based on these results, it seemed possible that other members of the *SOX* gene family might also serve as a replacement for *SOX2*. As expected, combination of *SOX1*, *SOX3*, *RBPJ*, *OTX2*, and *PAX6* with *OCT4*, *KLF4*, and *c-MYC* resulted in the appearance of reprogrammed colonies (data not shown). Similar to a previously report in mice (Nakagawa et al., 2008), individual overexpression of *SOX1* or *SOX3*, alongside *OCT4*, *KLF4*, and *c-MYC*, sufficed in the generation of human iPSCs with an efficiency of 0.01% and 0.004%, respectively (Figures S2B and S2C). Interestingly, *SOX1* and *SOX3* overexpression in human fibroblasts resulted in the upregulation of endogenous *SOX2* expression (Figure S2D). Likewise, murine *Zfp521*, an ortholog of human *ZNF521*, has been reported to promote neural differentiation in ESCs by acting upstream of *SOX* family members, such as *Sox3* and *Sox1*, as well as other TFs involved in the formation of ectodermal lineages (Kamiya et al., 2011). This finding suggests that *ZNF521* overexpression might also upregulate endogenous SOX-family members and thus facilitate reprogramming in an analogous way to that described for NPCs (Kim et al., 2009a, 2009b, 2008). RNA analysis of *ZNF521*-infected fibroblasts confirmed the significant and rapid upregulation of endogenous *SOX2* expression (Figure S2D). All of the iPSCs generated in the absence of *SOX2* demonstrated a gene expression profile closely resembling that of other PSC lines including ESCs and *OSKM*-derived iPSCs (Figure S2E).

Considering both of these sets of results regarding individual replacement of *OCT4* and *SOX2*, we wondered whether, in accordance with a model in which counteracting lineage specification pathways promote pluripotency (Loh and Lim, 2011; Zipori, 2004), simultaneous replacement of *OCT4* and *SOX2* with genes characteristic of opposing lineages could suffice for the reprogramming of human fibroblasts into iPSCs. To avoid potential compensatory effects resulting from similarities between the different proteins in the SOX family, we decided to focus our attention on replacement of *SOX2* by ectodermal-related genes other than *SOX1* and *SOX3*. Additionally, because replacement of *SOX2* by *ZNF521* resulted in iPSC generation at very low efficiencies, we speculated that *ZNF521* alone might not posses sufficient counteracting force to balance the effect of *GATA3-VP16* on mesendodermal specification. We therefore evaluated reprogramming with *GATA3-VP16* in the presence of three different genes related to ectodermal specification *(ZNF521*, *OTX2*, and *PAX6)* alongside *KLF4* and c-*Myc* expression. As shown in Figure 4, dual replacement of *OCT4* and *SOX2* with mesendoderm-related and ectoderm-related genes, respectively (Figure 4C), resulted in the appearance of colonies (0.0002%) displaying typical ESC characteristics (Figures 4D and 4E), including the expression of TRA1-81, NANOG, and TRA1-60, a recognized surrogate of pluripotency in human reprogramming experiments (Chan et al., 2009) (Figures 4E and 4F). Upon spontaneous differentiation, the generated iPSCs were able to form well-defined embryoid bodies (EBs) and immunofluorescence analysis demonstrated the expression of markers typical of the three germ layers (Figures 4D and 4F). Next, we subjected these iPSCs to directed differentiation experiments. As shown in Figure 4G, iPSCs generated in the absence of *OCT4* and *SOX2* were able to give rise to hepatocyte-like cells and neurons. Methylation analysis further demonstrated efficient demethylation of the *GATA3* promoter, suggesting reprogramming to an iPSC state (Figures 2E and 4H). Lastly, in vivo teratoma formation assays further demonstrated differentiation toward derivatives of the three germ layers (Figure 4I). Together, our results indicate that cells reprogrammed by simultaneous replacement of *OCT4* and *SOX2* were indeed pluripotent.



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DISCUSSION

We have identified a set of factors that are able to replace the core pluripotency factors *OCT4* and *SOX2* for the derivation of human iPSCs. These results provide a proof-of-concept for the dispensability of *OCT4* for the acquisition of pluripotency in human cells and establish lineage-related genes as important players on the road to pluripotency. Indeed, the fact that factors involved in the specification of two major counteracting lineages, mesendoderm and ectoderm, allowed for the reprogramming into iPSCs sheds new light on the role that "lineage specifiers" play in pluripotent cells and the delicate transcriptional balance governing the pluripotent state. These observations are in good agreement with the reported role of *OCT4* and *SOX2* in mesendoderm and ectodermal specification as well as the expression of so-called pluripotent genes in cell types other than PSCs. At a general level, they also underscore the idea that there is significant overlap between the concepts "pluripotency factors" and "lineage specifiers" and that individual factors can play multiple roles depending on the specific circumstances involved.

Together, our observations indicate that reprogramming to pluripotency, whether accomplished by the traditional Yamanaka factors or alternative combinations, might be due to the equilibrium of counteracting differentiation forces as opposed to the specification of a discrete PSC cellular entity by PSC-specific factors (Loh and Lim, 2011; Zipori, 2004). In support of these observations, during the preparation of this manuscript an elegant study by Deng and colleagues reported similar results for reprogramming of mouse cells (Shu et al., 2013). Our findings support the idea that a "seesaw model" also applies to the reprogramming of human cells, although with certain differences. The fact that not only *Gata3* but also other GATA family members could reprogram mouse cells in the absence of *OCT4* indicates that human and mouse cells might have different requirements in terms of lineage specification forces and in the balance required for achieving pluripotency. It also again highlights the importance of adequate gene expression stoichiometry in defining an iPSC state (Carey et al., 2011; Karwacki-Neisius et al., 2013). Indeed, mouse cells have been previously shown to generate iPSCs while the same factors, and even chemical compound screenings, have failed to reprogram human cells to iPSCs (Xu et al., 2008). In addition, *GATA3* knockdown in ESCs resulted in massive cell death, rather than ectodermal differentiation, whereas its overexpression led to mesendoderm specification as expected (Nishiyama et al., 2009). A potential explanation might imply a differential role for gene networks in the maintenance, as opposed to the acquisition, of pluripotent properties. Alternatively, and similarly to what has been recently described for *OCT4*, small differences in PSC gene expression may result in different phenotypic responses (Karwacki-Neisius et al., 2013; Radzisheuskaya et al., 2013).

Together, our results show that *OCT4* is not indispensable for human iPSC generation and shed new light on the molecular mechanisms underlying reprogramming and pluripotency. The identification of reprogramming activity for factors typically thought to be involved in differentiation further highlights the possibility that *OCT4* and *SOX2* might act as "lineage specifiers" for the acquisition and maintenance of pluripotency and reopens a long-standing debate on the nature of the pluripotent state (Loh and Lim, 2011; Zipori, 2004). Further supporting a "seesaw model," chemical inhibition of TGFβ signaling, which is activated during mesendodermal specification during development (Sakaki-Yumoto et al., 2013), can also functionally replace *Sox2* during reprogramming (Ichida et al., 2009). Further investigation related to the "seesaw model" could include computational modeling of the relative "weight" of each opposing "lineage specification" side, plus comprehensive high-throughput screening, to identify additional factors other than *GATA3* that can replace *OCT4*, and thus potentially contribute to further refinement of stoichiometry toward generating higher-quality iPSCs (Carey et al., 2011). Our results also open up the opportunity for the identification and design of small molecules targeting reprogramming factors other than *OCT4*, which might result in alternative approaches for the generation of human iPSCs with clinical potential.

EXPERIMENTAL PROCEDURES

Induced Pluripotent Stem Cell Generation and Subculture

Human fibroblasts were obtained by foreskin biopsies after signed informed consent of the donors and with the approval of the Institutional Review Board of the CMRB. For the generation of human iPS cells, primary human foreskin fibroblasts (HFF) were infected with an equal ratio of retroviruses for each tested combination by spinfection of the cells at 1,850 rpm for 1 hr at 32°C in the presence of polybrene (4 μg/ml). After two serial infections, cells were passaged onto fresh irradiated mouse embryonic fibroblasts (iMEFs) and switched to hES medium. For the derivation of hiPS cells lines, iPS-like colonies were manually picked and maintained on fresh iMEF feeder layers for five passages before being transferred onto Matrigel/mTesR1 conditions. To assess reprogramming, we first evaluated alkaline phosphatase positivity. To further calculate the efficiency of reprogramming, we plated the same number of cells on iMEFs after the infection and calculated the ratio of TRA1-60+ (TRA1-60+) colonies, the best described surrogate of pluripotent reprogramming, respective to the initial number of plated cells (Chan et al., 2009).

GATA3 Knockdown Experiment

Human ES cells (H1) were infected with lentiviral particles coding for GATA3-shRNA in the presence of 8 μg/ml polybrene. Two days after infection, cells were treated with puromycin (2 μg/ml).

Immunofluorescence and AP Analyses

Briefly, cells were washed thrice with PBS and fixed using 4% PFA in 1× PBS for 12 min and then washed three times in PBS. For tissue analysis, injected

Figure 3. *GATA3* Downregulation Leads to Cell Death in Human ESCs

(A) *GSKM* overexpression results in the upregulation of pluripotent genes during the reprogramming of human fibroblasts to iPSCs.
(B) *OSKM* overexpression results in the upregulation of *GATA3* during the reprogramming of human fibroblasts to iPSCs.
(C) Two days after infection with two different shRNAs against *GATA3* (KD1 and KD2) and subsequent Puromycin selection for 6 and 24 hr treated iPSC colonies were microscopically analyzed. In (C), representative bright field pictures show the disassembling of iPSC colonies demonstrating compromised PSC viability.
(D) *GATA3* mRNA levels after Puromycin selection for the indicated time points.
(E) *GATA3* knockdown results in the downregulation of *SOX2* and *NANOG* prior to cell death.
(F) Immunofluorescence analysis demonstrating aberrant colony morphology and initiation of cell death (Caspase-3) upon *GATA3* knockdown in ESCs.
Data are represented as mean ± SD. *$p < 0.05$.

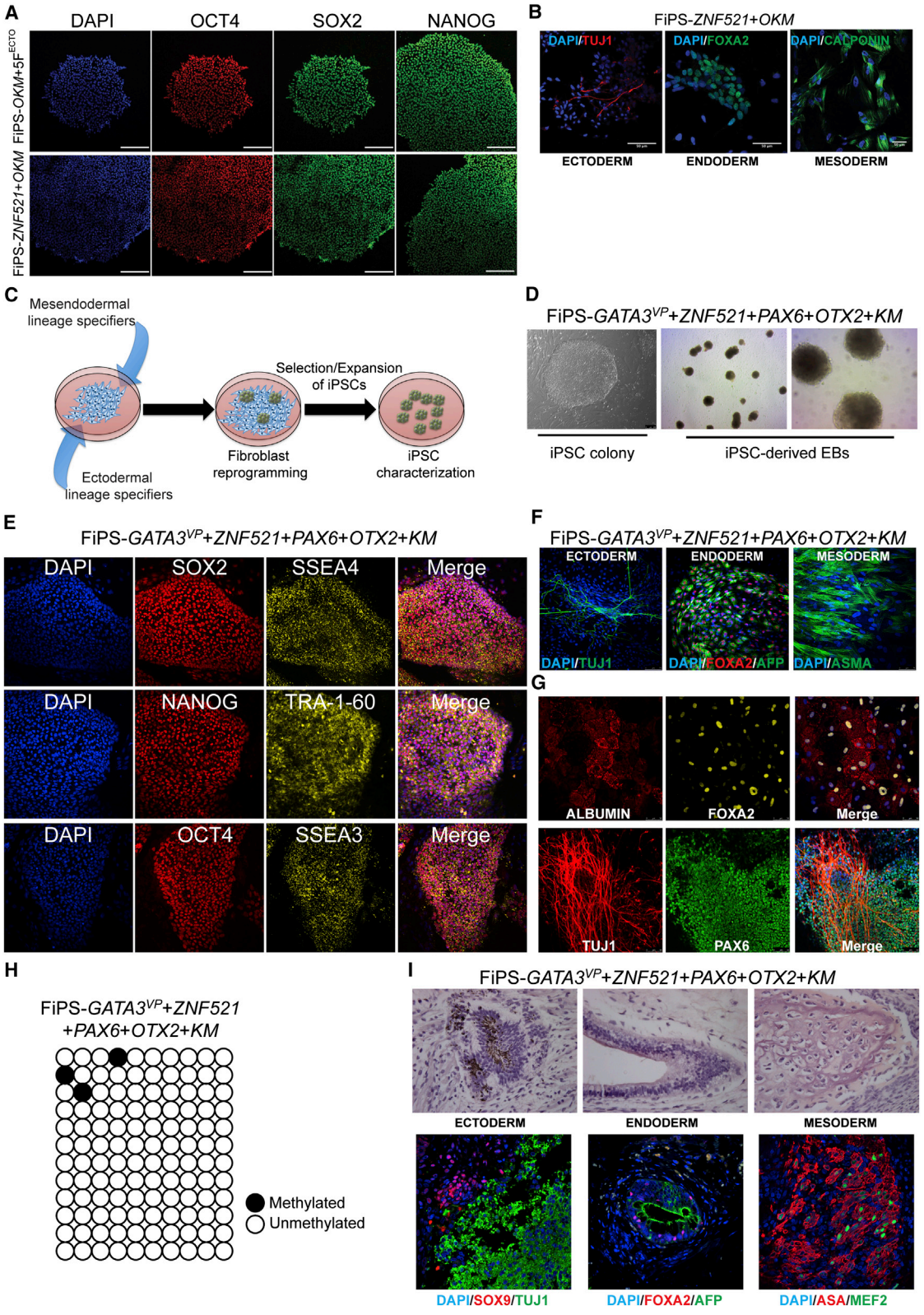

Figure 4. Counteracting Differentiation Forces Allow for Human iPSC Reprogramming

(A) Immunofluorescence analysis demonstrating pluripotent marker expression in different iPSCs generated by replacing *SOX2* with ectodermal lineage specifiers (ect-iPSCs).

(B) ect-iPSCs demonstrate pluripotent differentiation potential.

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testes were harvested and fixed overnight in a 4% PFA solution before being processed for paraffin sectioning. Cells and tissue sections were blocked and permeabilized for 1 hr at RT with 5% BSA/5% appropriate serum/1× PBS in the presence of 0.1% Triton X-100. Subsequently, cells and tissue sections were incubated with the indicated primary antibody either for 1 hr at RT or overnight at 4°C. Cells and tissue sections were then washed thrice with 1× PBS and incubated for 1 hr at RT with the respective secondary antibodies and 20 min with DAPI. Cells and tissue sections were washed thrice with 1× PBS before analysis. For alkaline phosphatase staining, direct enzymatic activity was analyzed using an Alkaline Phosphatase Blue/Red Membrane substrate solution kit (Sigma) according to the manufacturer's guidelines. Cells and tissue sections were analyzed by using an Olympus 1X51 upright microscope equipped with epifluorescence and TRITC, FITC, and DAPI filters. Confocal image acquisition was performed using a Zeiss LSM 780 laser-scanning microscope (Carl Zeiss Jena) or a Leica SP5 confocal microscope.

High Resolution, G-Banded Karyotype

Karyotype analysis was performed on 85% confluent iPS cells growing on Matrigel. Cells were treated with colcemid at 20 ng/ml, followed by a 45 min incubation at 37°C. Upon trypsinization, the cells were treated with Carnoy's fixative solution at −20°C prior to analysis with the software Cytovision (Applied Imaging).

Teratoma Assay

Severe combined immune-deficient-Beige male mice (n = 2 animal/iPS clone), ~8 weeks old, were injected with iPSCs (1 million for each injection site, approximately) subcutaneously in the testicular parenchyma. All procedures involving animals were approved by the Institutional Animal Ethical Board, and the protocols were approved by the Conselleria De Salut of Cataluña. Mice were sacrificed 8 weeks after the injections or when a tumor was detected by palpation, whichever came first. Teratoma formation was assessed by immunofluorescence techniques.

Statistical Evaluation

Statistical analyses were performed by using standard unpaired Student's t test (two-tailed, 95% confidence intervals) with Welch's correction using the SPSS/PC + statistics 11.0 software (SPSS, Inc.). All data are presented as mean ± standard deviation and represent a minimum of two independent experiments with at least two technical duplicates.

ACCESSION NUMBERS

Data sets for gene expression microarray analysis performed on the new iPS lines presented in the manuscript are available on the Gene Expression Omnibus (Gse48275).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2013.06.019.

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(C) Schematic representation of reprogramming experiments in which ectodermal- and mesendodermal-related genes have simultaneously replaced *SOX2* and *OCT4*, respectively.
(D) Representative bright-field pictures of iPSC colonies generated by *SOX2* and *OCT4* replacement and their differentiated EBs.
(E) Immunofluorescence analysis demonstrating pluripotent marker expression in different iPSCs generated by replacing *SOX2* and *OCT4*.
(F) iPSCs generated by replacing *SOX2* and *OCT4* demonstrate in vitro pluripotent differentiation potential.
(G) iPSCs generated by replacing *SOX2* and *OCT4* differentiated to hepatocyte-like (upper panels) and neuronal cells (lower panels).
(H) Methylation analysis of the *GATA3* promoter in the indicated iPSC line.
(I) iPSCs generated by replacing *SOX2* and *OCT4* are able to form well-defined teratomas containing derivatives of the three germ layers in vivo.
See also Figure S2.

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