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Oct4-Induced Pluripotency in Adult Neural Stem Cells

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SUMMARY

The four transcription factors Oct4, Sox2, Klf4, and c-Myc can induce pluripotency in mouse and human fibroblasts. We previously described direct reprogramming of adult mouse neural stem cells (NSCs) by Oct4 and either Klf4 or c-Myc. NSCs endogenously express Sox2, c-Myc, and Klf4 as well as several intermediate reprogramming markers. Here we report that exogenous expression of the germline-specific transcription factor Oct4 is sufficient to generate pluripotent stem cells from adult mouse NSCs. These one-factor induced pluripotent stem cells (1F iPS) are similar to embryonic stem cells in vitro and in vivo. Not only can these cells can be efficiently differentiated into NSCs, cardiomyocytes, and germ cells in vitro, but they are also capable of teratoma formation and germline transmission in vivo. Our results demonstrate that Oct4 is required and sufficient to directly reprogram NSCs to pluripotency.

INTRODUCTION

For centuries, embryologists have been intrigued by the distinction between germ cells (GCs) and soma (McLaren, 1981; Pesce et al., 1998; Weismann, 1892). Any attempt to establish such a distinction eventually needs to address the question, which genetic program specifically defines the pluripotent founder population that gives rise to all cell lineages of the body as well as the GC lineage and ensures the flow of genetic information from one generation to the next. One way of establishing a distinction between germline and soma is to determine the regulatory factors expressed in both the pluripotent cells and stem cells of the GC lineage but not in somatic cells. Interestingly, the genetic programs of pluripotent cells and GC stem cells exhibit intriguing similarities. Several key factors required for pluripotency are also expressed in primordial germ and spermatogonial stem cells. However, when it comes to comparing them to the repertoire of factors in the soma, very few actually stand out. Based on its expression profile, the transcription factor Oct4 was from the beginning considered to be a key regulator during mouse embryogenesis. Oct4 is expressed in the pluripotent cells of an embryo and in cell lines derived thereof, as well as in the GC lineage. Embryonic stem cells (ESCs) derived from preimplantation embryos can easily integrate into the germline. This has previously been shown for mouse preimplantation embryos (Boiani and Schöler, 2005). In contrast, Oct4 is downregulated in the three somatic lineages, but only during gametogenesis around the initiation of male and female meiosis (Pesce et al., 1998). Oct4 is re-expressed in unfertilized oocytes after birth and Oct4 protein can be detected until the final stages of oocyte maturation.

Loss of Oct4 function early in development causes cells of the preimplantation embryo to acquire a trophectodermal fate (Nichols et al., 1998), whereas loss in the GC lineage leads to apoptosis of primordial germ cells (PGCs) (Kehler et al., 2004). These studies of the developing embryo were complemented by studies of ESCs in which the Oct4 function was abolished (Niwa et al., 2000). ESCs are especially interesting in this context, as they are derived from the inner cell mass of the blastocyst and thus represent a developmental stage following the establishment of the pluripotent founder population. While loss of Oct4 function results in differentiation into trophectodermal cells, overexpression of Oct4 in ESC leads to differentiation along the mesodermal and primitive endodermal lineages.

The early developmental phenotype suggests that Oct4 is part of a regulatory system that maintains pluripotency. A precise level of Oct4 is required to maintain pluripotency in ES cells, which is in agreement with this hypothesis. The fact that stem cells of the GC lineage are unipotent despite expressing Oct4, albeit at lower levels, shows that its mere presence

does not result in pluripotency. However, we hypothesize that the presence of Oct4 augments the induction of pluripotency. As shown for PGCs, Oct4-expressing cells can give rise to pluripotent cells (termed embryonic germ cells) when cultured in a simple cocktail of three growth factors (Matsui et al., 1992; Resnick et al., 1992).

Recently, Yamanaka and colleagues have shown that the genetic program of somatic cells can be induced to acquire pluripotency by overexpression of specific transcription factors. Initially, generation of pluripotent cells was achieved by ectopic expression of four factors (Oct4, Sox2, Klf4, and c-Myc) from mouse and human somatic cells (Lowry et al., 2008; Maherali et al., 2007; Meissner et al., 2007; Okita et al., 2007, 2008; Park et al., 2008; Stadtfeld et al., 2008; Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007; Yu et al., 2007). These ESC-like induced pluripotent stem (iPS) cells have characteristics similar to ESCs.

The molecular mechanisms underlying reprogramming are still far from being completely understood, which is largely due to several variables (number of exogenous factors required, heterogeneity of reprogramming target cells) within the system. We attempted to simplify this scenario by choosing NSCs as a cell model for reprogramming with different combinations of transcription factors (Kim et al., 2008). The combination of the specific factors needed to obtain iPS cells varies, though Oct4 can apparently not be replaced by other factors. Recently, we demonstrated that Oct4 and Klf4 are sufficient to induce pluripotency in NSCs (Kim et al., 2008). By omitting Klf4, we have established conditions that demonstrate that Oct4 is not only essential but also sufficient to induce pluripotency in NSCs. Our study shows for the first time that just one transcription factor can convert somatic cells into pluripotent cells, which can differentiate into derivatives of all three germ layers and into functional GCs.

RESULTS

Neural Stem Cells Endogenously Express AP and SSEA-1

In our previous study we utilized Oct4-GFP expression as a reprogramming marker, and we observed that the time required for reprogramming was affected by both the number (4, 3, or 2) and the composition of the pluripotency-inducing factors (Oct4, Sox2, Klf4, and c-Myc) (Kim et al., 2008). Other studies examined the reprogramming timing in more detail by reprogramming mouse fibroblasts using the doxycycline (dox)-inducible system (Brambrink et al., 2008; Wernig et al., 2007). These studies demonstrated the sequential expression of marker genes, such as alkaline phosphatase (*AP*), stage-specific embryonic antigen-1 (*SSEA-1*), and *Oct4* or *Nanog*, during the reprogramming process. Thus, expression of *SSEA-1* represents an intermediate stage, which precedes expression of *Oct4* or *Nanog* and which in turn is required before a cell can be fully reprogrammed to a pluripotent state. Interestingly, NSCs endogenously express SSEA-1 and also show AP activity (Figure S1 available online) (Capela and Temple, 2002; Peh et al., 2008). Following infection of NSCs with the four factors (4F), the cells give rise to GFP^+ cells on day 4 and GFP^+ colonies on day 7 (Kim et al., 2008). In contrast to NSCs, GFP^+ cells are first observed in MEFs on day 7 post-infection of 4F (Okita et al., 2007). We therefore considered the possibility that NSCs might represent a more advanced stage in the reprogramming process and that terminally differentiated cells have to go through a stage like this in order to subsequently acquire a pluripotent state. In this scenario, expression of AP and SSEA-1 in NSCs marks a quasi-intermediate state, during which NSCs are reprogrammed earlier and more efficiently than MEFs.

iPS Cells Generated from Adult Neural Stem Cells with Transduction of *Oct4* at the Molecular Level Are Similar to Embryonic Stem Cells

In our previous study, NSCs could be reprogrammed with three-factor (3F) and two-factor (2F) combinations (Kim et al., 2008). Moreover, in the 2F combinations c-Myc and Klf4 could be replaced with one another, although c-Myc was less efficient than Klf4 in inducing the formation of GFP^+ colonies (3–4 weeks, instead of 2–3 weeks). Next, we sought to define conditions in which NSCs can be reprogrammed by transduction of *Oct4* alone. As time has been an issue when using various factor combinations, we prolonged the culturing of *Oct4*-infected cells. In the current study, we succeeded in generating three one-factor (1F) iPS clones (clones 2, 3, and 4) from five GFP^+ colonies of *Oct4*-infected NSCs within 4–5 weeks in culture (Figure 1A). The iPS cells expressed Oct4-GFP, SSEA-1, and AP and were morphologically indistinguishable from mouse ESCs under mouse ESC culture conditions (Figure 1B). The estimated reprogramming efficiency for the one-factor approach can be calculated as 0.014%, which is 10-fold lower than our two-factor approach (Kim et al., 2008) and similar to reprogramming MEFs with four factors (Okita et al., 2007).

We next characterized the 1F iPS at the molecular level. All three clones expressed typical ESC marker genes (Figure 2A) and the endogenous genes *Oct4*, *Sox2*, *c-Myc*, and *Klf4*, similar to mouse ESCs (Figure 2B). The *Oct4* transgene was completely silenced after passage 5 (Figure S2). To determine the number of viral *Oct4* transgene integrations, we performed Southern blot analysis on ESCs, 1F, 2F, and 4F iPS cells. The 1F iPS clones displayed 2 and 5 integrations (Figure S3A), while 2F or 4F iPS cell displayed 7 integrations (Figure S3B). We performed Southern blot analysis from three independent subclones of clone 2 (Figure S3B). The same integration pattern confirmed that no crosscontamination occurred during the expansion of these subclones. Thus, 1F iPS could be generated with less viral integrations of the *Oct4* transgene than 2F or 4F iPS cells. We also confirmed integration of the *Oct4* transgene by polymerase chain reaction (PCR): all 1F iPS clones contained only the *Oct4* transgene (Figure S4).

We next performed DNA methylation analysis to assess the epigenetic status of the *Oct4*, *Sox2*, and *Nanog* promoters with DNA isolated from ESCs, NSCs, 2F iPS cells, and 1F iPS clones (Figure 2C). *Sox2* promoter maintains the demethylated status in all populations (ESCs: 10%, 2F iPS: 3%, 1F iPS: 5%, NSCs: 3%, NSC 1F: 3%). *Oct4* and *Nanog* promoters were highly demethylated in ESCs (13%, 9%) and 2F iPS cells (13%, 8%) and retained partial methylation in 1F iPS (31%, 27%). 1F iPS-derived NSCs

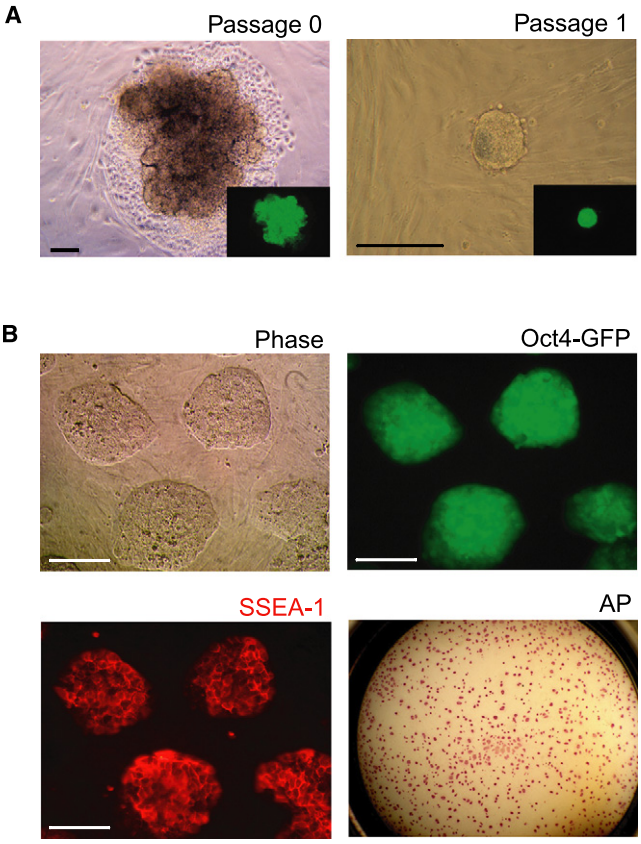

Figure 1. Generation of One-Factor iPS Cells from Adult NSCs by Retroviral Transduction of *Oct4*

(A) Morphology and *Oct4* promoter-driven GFP (Oct4-GFP) expression (inset) in reprogrammed one-factor (1F) iPS without feeders (passage 0) on day 35 post-infection and 1F iPS (clone 2) grown on feeders (passage 1). Scale bars, 100 μm.

(B) Phase contrast image showing ESC-like morphology (upper left) of 1F iPS (clone 2) on feeders. Colonies express Oct4-GFP (green, upper right) and stained positive for SSEA-1 (red, lower left) and for AP (lower right). Abbreviations: SSEA-1, stage-specific embryonic antigen-1; AP, alkaline phosphatase. Scale bars, 200 μm.

(NSC 1F) were highly methylated at those loci (68%, 85%), similar to donor NSCs (70%, 80%). The methylation data suggest slight differences between 1F iPS and ESCs. Scatter plots of the global gene expression profiles obtained from cDNA microarrays demonstrated that 1F iPS exhibit a distribution pattern of gene expression comparable to ESCs, hence completely different from NSCs (Figures 2D and 2E).

1F iPS Can Be Differentiated into Three Germ Layers including NSCs, Cardiomyocytes, and GCs In Vitro

To investigate the developmental potential of 1F iPS in vitro, we determined whether 1F iPS could differentiate into three germ layers by embryonic body (EB) differentiation. EBs derived from all three 1F iPS clones expressed markers of the three germ layers including *GATA-4* (endoderm), *Brachyury* (mesoderm), and *MAP2* (ectoderm) as determined by RT-PCR (Figure S5A).

Next, we attempted to determine whether 1F iPS could differentiate into lineage-committed populations of NSCs, cardiomyocytes, or PGCs. We derived NSCs from 1F iPS in serum-free adherent monolayer cultures according to a published protocol (Ying et al., 2003). Interestingly, these NSC 1F were very similar to the donor NSCs. NSC 1F can maintain their self-renewal capacity, even after repeated passaging. RT-PCR analysis revealed NSC-specific gene expression similar to donor NSCs but no expression of pluripotency markers such as *Oct4* or *Nanog* (Figure 3A). NSC 1F appeared highly homogenous, and immunocytochemical staining confirmed the uniform expression of the NSC marker Nestin (Figure 3B). Moreover, these NSC 1F were capable of differentiating into neurons, oligodendrocytes, and astrocytes after prolonged time in culture (passage 20) (Figures 3B and S6). The modal chromosome number of 40 was maintained throughout reprogramming and derivation of NSCs (Figure 3C). Our results demonstrate that 1F iPS can be differentiated efficiently into multipotent NSCs that have self-renewing capacity with global gene expression profiles similar to donor NSCs (Figure 3D).

During EB differentiation, we observed PECAM-positive cells (a marker of mature endothelial cells) with vessel-like structures and beating α-actinin-positive cardiomyocytes (Figures 4A and 4B). Intracellular recordings from the beating areas demonstrated both atrial- (Figure 4C, upper) and ventricular-like (Figure 4C, lower) cardiac action potentials. Chronotropic regulation was found to be intact (Figure 4D), indicating the presence of functional cardiomyocytes.

Previous reports have demonstrated the in vitro generation of GCs from murine ESCs (Geijsen et al., 2004; Hübner et al., 2003). To further verify the pluripotency of 1F iPS, we attempted to differentiate 1F iPS into GCs in vitro. Putative GC cultures showed meiotic competence as demonstrated by expression of Sycp3 protein (Figures 5A and 5B) and were FACS sorted for GFP-positive (GFP^+) or GFP-negative (GFP^-) cells representative of early and late postmigratory stages, respectively. Cell fractions were analyzed using quantitative real-time PCR. The GC character of in vitro differentiated 1F iPS was shown by the upregulation of the specific markers *Blimp1*, *Stella*, *Fragilis*, and *Oct4* (Figure 5C). Importantly, in vitro differentiated cells showed expression of *Gdf9*, an oocyte-specific marker, suggesting that 1F iPS, although male, could differentiate into oogonia-like cells as has been shown for ESCs (Hübner et al., 2003). These results demonstrate that 1F iPS can develop even in vitro into GCs. The capacity of 1F iPS to differentiate into lineage-committed cell populations of different germ layers validates their pluripotent status.

1F iPS Cells Have an In Vivo Developmental Potential Similar to Embryonic Stem Cells

To investigate the in vivo developmental potential of 1F iPS, we performed teratoma formation and chimeras-contribution assays. First, we determined that 1F iPS were capable of forming teratomas upon subcutaneous inoculation into nude mice. The teratomas contained tissues of all three germ layers including neural rosette (ectoderm), cuboidal epithelium (endoderm), and muscle (mesoderm) (Figures 6A and S5B). These data reveal that 1F iPS possess multilineage potential in vivo.

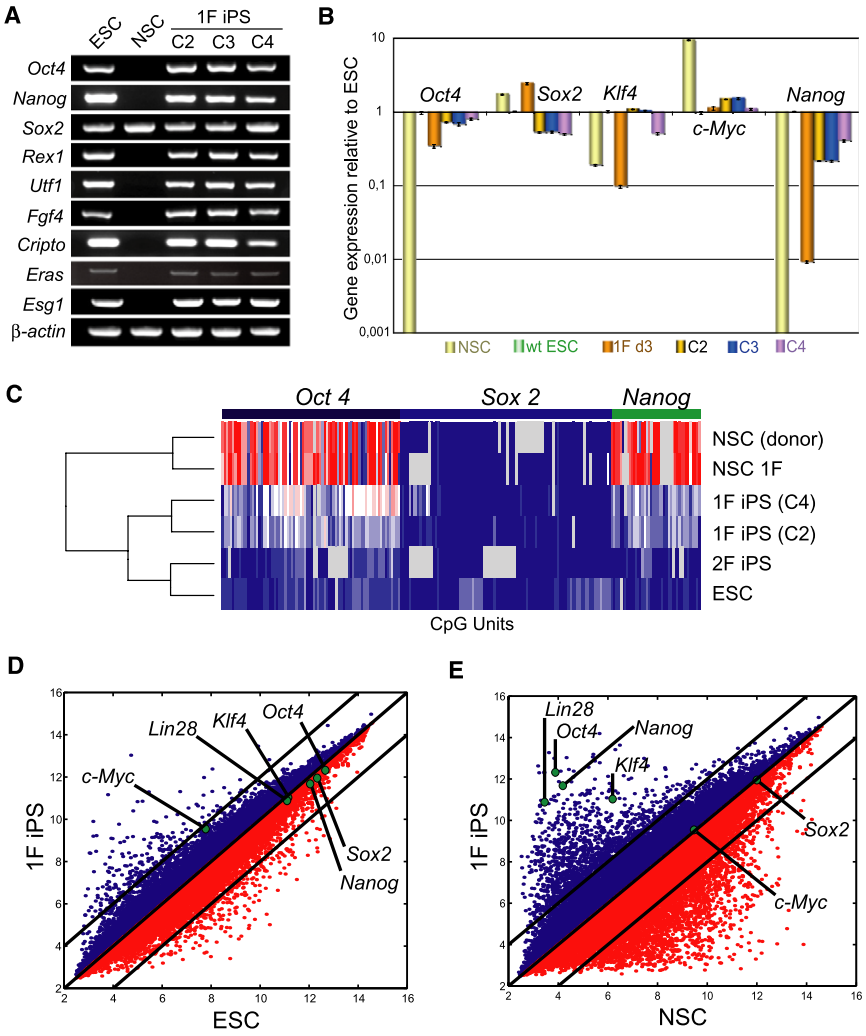

Figure 2. Characterization of 1F iPS

(A) RT-PCR analysis of pluripotency marker expression in 1F iPS clones (C2, C3, and C4) and ESCs (positive control), as well as NSCs (negative control). Primers are specific for transcripts from the respective endogenous locus. β-actin was used as loading control.

(B) Quantitative PCR analysis of endogenous expression of the four factors in 1F iPS clones (C2, C3, and C4). RNA levels were determined by quantitative real-time PCR using primers specific for endogenous transcripts. Endogenous relative expression levels of 1F iPS on day 3 (whole population) and passage 5 (clones 2, 3, and 4) were compared with those in ESCs. Transcript levels were normalized to β-actin levels. Shown are the averages with standard deviations of three independent experiments.

(C) Analysis of the DNA methylation pattern of the *Oct4*, *Sox2*, and *Nanog* promoters in NSCs and NSC 1F, as well as in 1F iPS (clones 4 and 2), 2F iPS cells (clone F-4), and ESCs. The red fragments indicate methylated CpG dinucleotides, whereas the blue fragments indicate unmethylated CpG dinucleotides. Missing values are represented in gray color.

(D and E) Scatter plots of the global gene expression patterns comparing 1F iPS (clone 2) with ESCs (D) and 1F iPS (clone 2) with NSCs (E) by cDNA microarrays. Black lines indicate 2-fold changes in gene expression levels. Up- and down-regulated genes in 1F iPS compared with ESCs or NSCs are shown in blue or red, respectively. The positions of the pluripotency genes *Oct4*, *Nanog*, *Sox2*, *c-Myc*, *Klf4*, and *Lin28* are shown in green.

To further assess their developmental potential, 1F iPS were microinjected into mouse blastocysts (Table S1). After transfer of the injected blastocysts into pseudopregnant recipients, we observed by means of Oct4-GFP expression chimeric embryo and germline contribution to the genital ridge of a 13 day post-coitum (dpc) embryo (Figure 6B). Histological analysis revealed that 1F iPS contributed to the development of various organs, as shown by β-galactosidase staining (Figure 6C). We furthermore obtained two adult chimeras from 1F iPS (clone 2) as determined by coat color and PCR genotyping for the presence of the *GFP* allele, the *lacZ* allele, and the *pMX-Oct4* transgene (Figures 6D and 6E, Table S1). In addition, we observed Oct4-GFP expression in the gonads of adult chimeras, which clearly demonstrates germline contribution (Figure 6F). Chimeric mice were mated in order to verify germline transmission and two pups obtained thereof showed derivation from 1F iPS, as revealed by genotyping for Oct4-GFP and viral *Oct4* transgene (Figure 6G). All these data demonstrate that 1F iPS in vivo possess developmental potential comparable to ESCs.

DISCUSSION

This study has several implications. (1) Oct4 alone can induce pluripotency in mouse adult NSCs. 1F iPS could be differentiated into lineage-committed populations including GCs and germline transmission. (2) iPS cells can be generated without the oncogenic factors c-Myc and Klf4. Since reactivation of the c-Myc virus may cause tumor development (Okita et al., 2007), iPS cells have been generated without infection of the *c-Myc* oncogene (Huangfu et al., 2008; Nakagawa et al., 2008; Wernig et al., 2008). However, Klf4, the remaining oncogenic factor, might cause tumor formation in offspring. (3) Reducing the number of factors decreases the chance of retroviral insertional mutagenesis. Analysis of previously described iPS cells revealed up to 20 retroviral integrations for all four factors (Aoi et al., 2008; Wernig et al., 2007). In this study, we demonstrate that 1F iPS contain five integrations of only the *Oct4* transgene (Figure S3). (4) The starting cell population of NSCs, which endogenously express Sox2, c-Myc, and Klf4 as well as AP and SSEA-1, are a unique source for studying the mechanisms

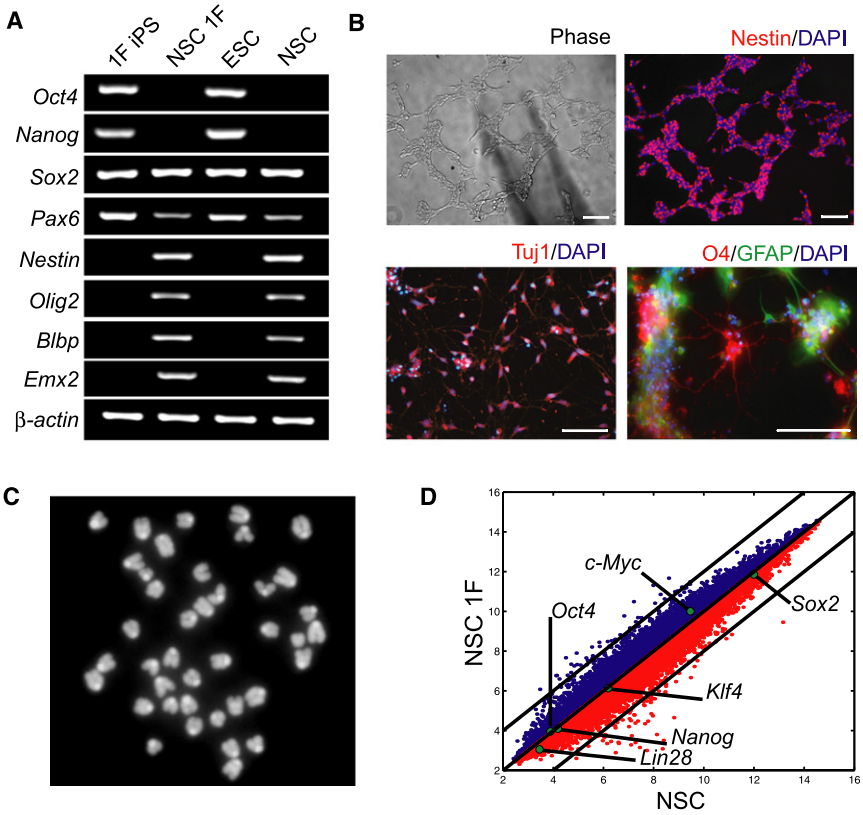

Figure 3. Differentiation of 1F iPS into NSCs

(A) RT-PCR analysis showing expression of NSC marker genes from NSC 1F. β-actin was used as loading control.

(B) Morphology (upper left) and Nestin expression (upper right) of NSC 1F (passage 20). NSC 1F differentiated into neurons (Tuj1), oligodendrocytes (O4), and astrocytes (GFAP). Nuclei were counterstained with DAPI (blue). Scale bars, 100 μm.

(C) Karyotype of NSC 1F. Metaphase spreads of undifferentiated NSC 1F with a normal set of 40 mouse chromosomes are shown.

(D) Scatter plot of the global gene expression comparing donor NSCs with NSC 1F by cDNA microarrays. Black lines indicate 2-fold changes in gene expression levels. Up- and downregulated genes in NSC 1F cells compared with NSCs are shown in blue or red, respectively.

of iPS cell generation since they can be reprogrammed by one, two, three, or four factors.

Strikingly, Oct4 alone is sufficient to induce pluripotency in NSCs, which demonstrates its crucial role in the process of reprogramming and supports our hypothesis that NSCs represent an intermediate state between differentiated and pluripotent cells. 1F iPS have the pluripotency to differentiate into all three germ layers, as demonstrated by in vitro and in vivo analysis. They can give rise to multipotential NSCs, cardiomyocytes with cardiac action potential and chronotropic regulation, and GCs as well as germline transmission. This study sheds light on the mechanisms involved in reprogramming somatic cells to a pluripotent state. Future studies will show whether other sources of neural stem or progenitor cell populations such as mouse or human bone marrow-derived mesenchymal stem cells (Hermann et al., 2004; Lee et al., 2003; Fu et al., 2008) or dental pulp can be reprogrammed to iPS cells and whether expression of Oct4 can be induced by nonretroviral

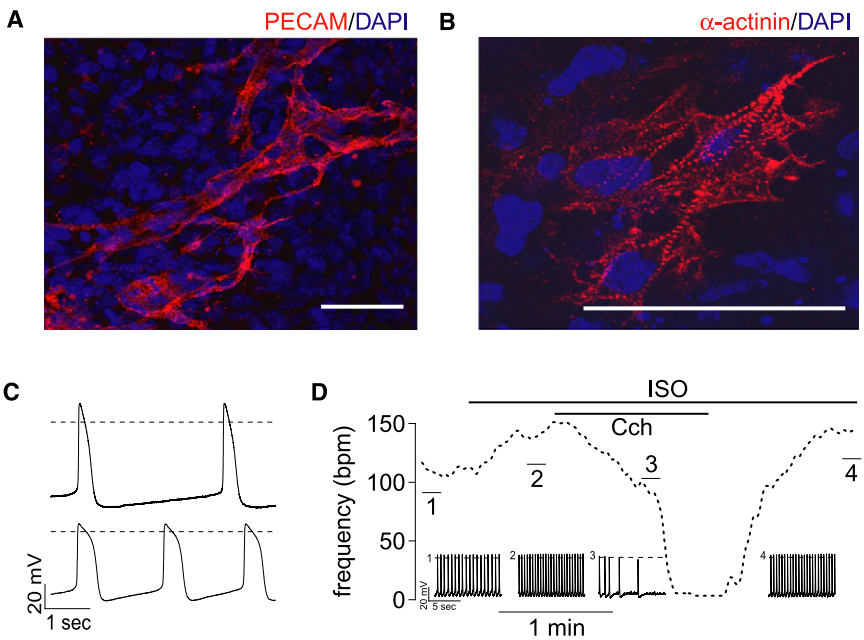

Figure 4. Differentiation of 1F iPS into Cardiomyocytes

In vitro differentiation of endothelial cell and cardiomyocytes through formation of embryoid bodies (EBs). (A) Clustered PECAM-positive (left, red) endothelial cells and (B) α-actinin-positive (right, red) cross-striated cardiomyocytes are shown. Nuclei were counterstained with Hoechst dye (blue). Scale bar, 50 μm.

(C) Atrial- (upper) and ventricular- (lower) like action potentials recorded from cardiomyocytes within a 7+11 EB.

(D) Frequency of beating is enhanced by isoprenaline (ISO, 0.1 μM) and slowed down to a complete halt by the additional application of charbachol (CCh, 10 μM). The CCh effect could be reversed by washout (bottom inserts: original traces from time points 1–4). Horizontal dashed lines indicate 0 mV lines.

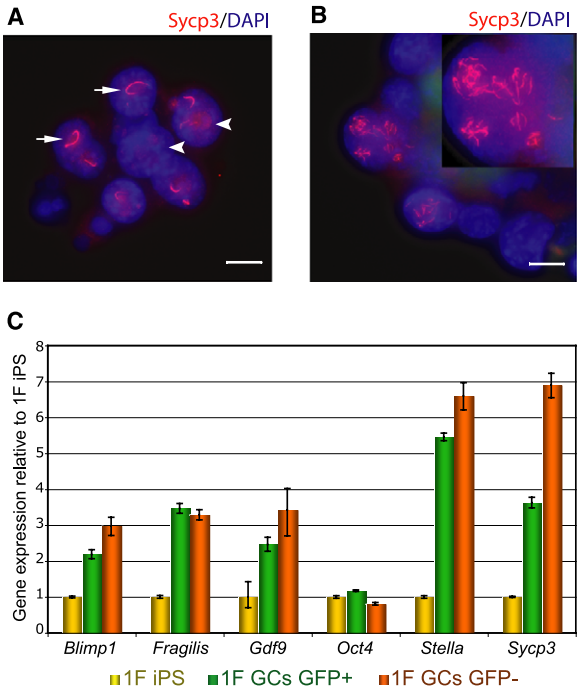

Figure 5. Characterization of 1F iPS In Vitro Differentiated GCs by Immunohistochemistry for SYCP3 and Gene Expression Analysis

(A) Sycp3 accumulates in the nucleolar regions of putative germ cell (GC) nuclei (arrowheads) and subsequently forms fibers (arrows), indicative of preleptotene stage of prophase I.

(B) Organization of Sycp3 fibers in pachytene stage GCs. Note that entry of meiosis is accompanied by a decrease of Oct4-GFP expression. Insert depicts a higher magnification of a stained cell.

(C) Gene expression analysis of FACS-sorted cell populations of in vitro derived GCs. GFP-positive cells (green) demonstrate upregulation of germ-specific markers and represent cells of an early postmigratory stage. GFP^- cells (orange) depict cells of a later stage of meiotic prophase I (B), characterized by a 2-fold higher Sycp3 expression when compared to the GFP^+ subpopulation. Scale bars, 10 μm. Transcript levels were normalized to β-actin levels. Shown are the average values with standard deviations of three independent experiments.

means (Okita et al., 2008; Stadtfeld et al., 2008), a prerequisite for the generation of iPS cells of therapeutic value.

EXPERIMENTAL PROCEDURES

Derivation of Adult NSCs and NSC 1F

Adult NSCs were isolated directly from the whole brain of OG2/Rosa26 transgenic mice and NSC 1F derived from 1F iPS. These cells were cultured in neural expansion medium as previously described (Conti et al., 2005; Kim et al., 2008).

Generation of 1F iPS

The pMX-based retroviral vector encoding the mouse cDNA of Oct4 (Takahashi and Yamanaka, 2006) was cotransfected with packaging-defective helper plasmids into 293 cells using Fugene 6 transfection reagent (Roche). Forty-eight hours later, virus supernatants were collected as previously described (Zaehres and Daley, 2006). NSCs derived from OG2/Rosa26 transgenic mice were seeded at a density of 5×10^4 cells per 6-well plate and incubated with virus-containing supernatants for Oct4 supplemented with 6 μg/ml protamine sulfate (Sigma) for 24 hr. Transduction efficiencies were calculated with pMX-GFP control virus. Cells were replated in fresh neural expansion medium. Three days after infection, the cells were further subcultured on irradiated MEFs in ESC medium containing LIF without any further selection. Oct4-GFP-positive colonies were mechanically isolated, and individual cells were dissociated and subsequently replated onto MEFs. iPS cells and ESCs were grown on irradiated MEFs and in ESC medium (DMEM supplemented with 15% FBS, nonessential amino acids, L-glutamine, penicillin/streptomycin, β-mercaptoethanol, and 1000 U/ml leukemia inhibitory factor [LIF]). The colonies were isolated for expansion.

qRT-PCR Analysis

Total RNA was extracted from cells using the MiniRNeasy Kit (QIAGEN GmbH; http://www.qiagen.com) according to the manufacturer's instructions. Complementary DNA synthesis was performed with the High Capacity cDNA Archive Kit (Applied Biosystems GmbH; http://www.appliedbiosystems.com) following the manufacturer's instructions with a downscaled reaction volume of 20 μl. Transcript levels were determined using the ABI PRISM Sequence Detection System 7900HT (Applied BioSystems) and the ready-to-use 5′-nuclease Assays-on-Demand. For each real-time amplification, the template was equivalent to 5 ng of total RNA. Measurements were performed in triplicate; a reverse-transcription-negative blank of each sample and a no-template blank served as negative controls. Amplification curves and gene expression were normalized to the housekeeping gene *βAct*, used as an internal standard. Primers and probes are listed in the Supplemental Experimental Procedures.

SSEA-1 and AP Staining

SSEA-1 and AP staining was performed with the ES Cell Characterization Kit (Chemicon) according to the manufacturer's protocol.

Teratoma Formation

iPS cells and NSCs (1.5×10^6 cells/mouse) were injected subcutaneously into the dorsal flank of nude mice. Four weeks after the injection, teratomas that had formed were fixed overnight in 4% PFA and embedded in paraffin. Sections were stained with hematoxylin and eosin dyes.

Chimera Formation

Four- to five-week-old female mice (B6C3F1) were induced to superovulation (7.5 I.U. PMSG administration followed, 48 hr after, by 7.5 I.U. hCG administration via intraperitoneal injection) and mated with CD1. Blastocysts were collected at day 3.5 after vaginal plug check and flushed in FHM medium containing 0.1% PVP. Blasocysts were then extensively washed in FHM medium and cultured in KSOM medium 0.2% BSA (KSOM-BSA) in the incubator (37°C, 5% CO_2 in air) until iPS cell injection.

Forty to fifty iPS colonies were selected and picked under a stereomicroscope based on the colony shape and morphology, washed in PBS, and then transferred into a drop of 0.05% Trypsin in order to obtain a single cell suspension. Single cells were then transferred into the micromanipulation chamber in a drop of FHM medium 0.1% PVP 0.2% BSA. Groups of 12 to 15 cells were injected into each single blastocyst. Injected embryos were then transferred into a drop of KSOM-BSA and cultured overnight at 37°C 5% CO_2 in air. The following day chimeric blastocysts were transplanted into 2.5 dpc pseudopregnant CD1 recipient females.

Genotyping of iPS Cells and Chimeras

Genotyping was performed on genomic DNA isolated from iPS cells, ESCs, and NSCs and on mouse tails, lysed by digestion at 55°C in extraction buffer (100 mM EDTA, 50 mM TRIS-HCl, 100 mM NaCl, 1% SDS, and 1.0 mg/ml proteinase K). DNA was precipitated by adding isopropanol, washed twice in 70% ethanol (v/v), and resuspended in TE (pH 8.0). After proteinase K inactivation at 75°C for 15 min, PCR was carried out with the following conditions: 94°C 30 s (1 cycle); 94°C 10 s, 57°C 30 s, 72°C 30 s (40 cycles); 72°C 5 min.

Primer Sequences for Viral-Specific qRT–PCR, Genotyping, and In Vitro Differentiation

Quantitative real-time PCR, genotyping of iPS cells, and in vitro differentiation were performed using the primers as described (Kim et al., 2008).

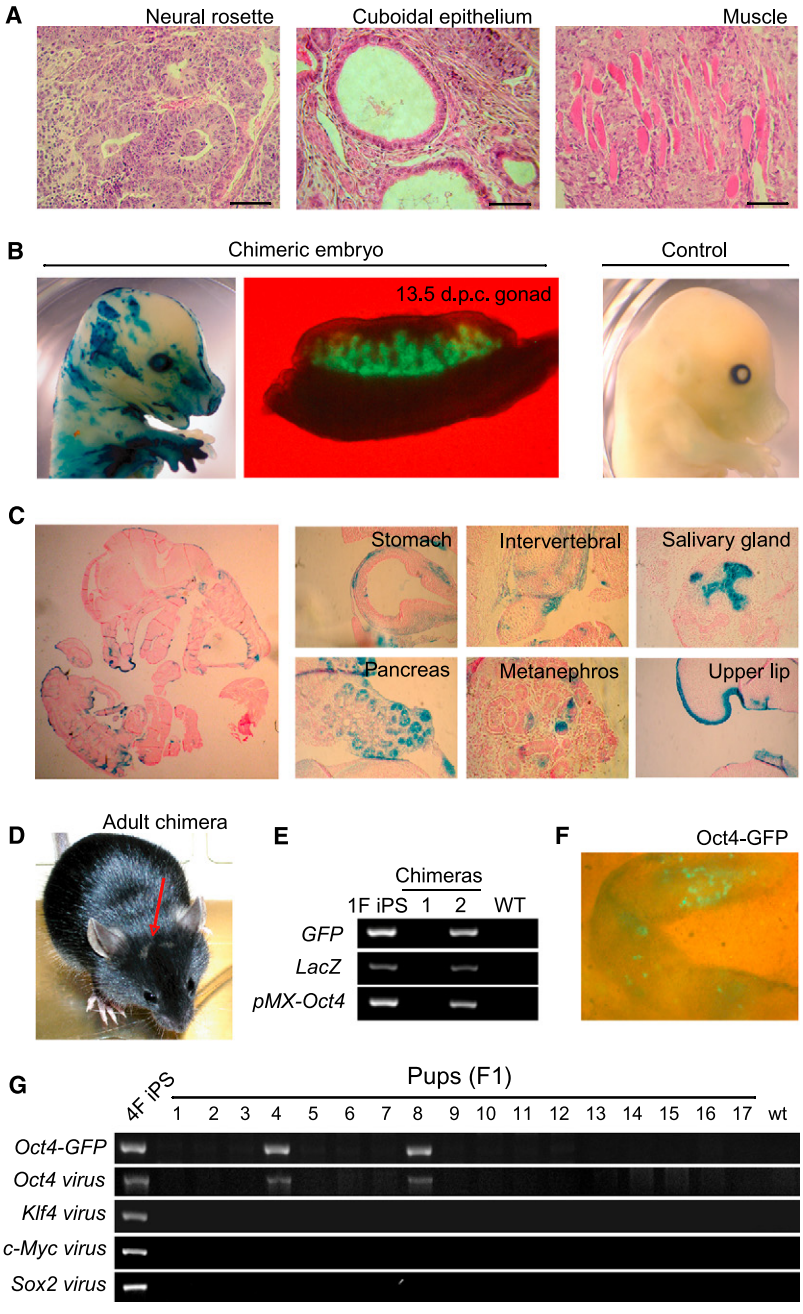

Figure 6. In Vivo Developmental Potential of 1F iPS

(A) Teratomas of 1F iPS (clone 2) contain all three embryonic germ layers: neural rosettes (ectoderm), epithelium (endoderm), and muscle (mesoderm). Hematoxylin and eosin-stained sections of teratomas derived in a nude mouse host from 1F iPS (clone 2) after 4 weeks are shown. Scale bars, 100 μm.

(B) Germline contribution of 1F iPS in chimeric embryos. A whole-mount 13.5 dpc chimeric embryo was stained with X-gal solution (left) and the fetal gonad was examined for expression of Oct4-GFP (middle) using a fluorescence microscope and control (right).

(C) Histological analysis of contribution of 1F iPS (clone 2) in whole chimeric embryo (left) to stomach, intervertebral, salivary glands, pancreas, metaphors, and upper lip.

(D) Chimeric mouse generated by 1F iPS (clone 2). The red arrow indicates the chimerism originating from 1F iPS.

(E) PCR genotyping of chimeras derived from 1F iPS (clone 2). PCR analysis was performed to genotype chimeric males mated with CD1 females and demonstrates the presence of GFP (top panel), *lacZ* allele (middle panel), and *pMX-Oct4* transgene (bottom panel).

(F) Germline contribution of 1F iPS in adult chimera gonads. Oct4-GFP-positive cells from 1F iPS in tescular tubules are shown.

(G) Germline transmission of 1F iPS, as judged from Oct4-GFP expression and the presence of the Oct4 viral transgene in two pups of the F1 generation.

Microarray Experiments

The gene expression profiles were obtained from ESC, 2F iPS, 1F iPS, NSC, and NSC 1F using the Mouse Genome 430 2.0 GeneChip arrays (Affymetrix). In the case of the ESC, NSC, and two samples of the 2F iPS, we used our previous data sets (Kim et al., 2008). In the case of an additional 2F iPS and triplicates of 1F iPS and NSC 1F, we performed new microarrays. Briefly, 1μ total RNA was subjected to probe preparation and cRNA was hybridized. Arrays were scanned using an Affymetrix GCS3000 device and images were analyzed using the GCOS software. Normalization was calculated with RMA algorithm (Irizarry et al., 2003) implemented in R-Bioconductor.

Characterization of NSC 1F Cells

NSC 1F cells were characterized by RT-PCR, karyotyping, in vitro differentiation, and immunocytochemistry. Additional experimental details are available in the Supplemental Experimental Procedures.

Cardiomyocyte Differentiation of 1F iPS

EBs were generated from 1F iPS with the hanging drop method for 2 days and subsequent culturing for another 3 days in DMEM + 20% FCS (Invitrogen) (Kolossov et al., 1998). Differentiated EBs were fixed with 4% paraformaldehyde and stained with antibodies against α-sarcomeric-actinin (1:400, Sigma), PECAM (1:800, PharMingen), and appropriate Cy3- or Cy5-conjugated secondary antibodies (1:400-1:1000, Dianova). Nuclei were stained with Hoechst dye (blue). Samples were imaged using a Zeiss AxioImager microscope equipped with an ApoTome and AxioCam MRm; images were acquired with the Zeiss software AxioVision. For RT-PCR, Oct4-GFP cells were isolated by FACS analysis and used for in vitro differentiation of EBs in hanging drops in ESC medium without LIF. After 3 days, EBs were plated onto gelatine-coated 4-well dishes for an additional 10 days. PCR was performed for 35 cycles for all marker genes.

Action potential recordings were performed with sharp electrodes (50–100 MΩ, filled with 3M KCl) impaled into beating areas of EBs and a BA-03X amplifier (NPI electronic) at 1 kHz sampling rate. Chronotropic regulation of cardiomyocytes was tested by applying first Isoprenaline (0.1 μM) and subsequently carbachol (10 μM). Beating frequency was calculated from beat to beat intervals with a 5 s running average. All physiological recordings were performed at 36°C ± 1°C. Recording solution (in mM): NaCl 140, KCl 5.4, $CaCl_2$ 2, $MgCl_2$ 1, glucose 10, HEPES 10, pH 7.4.

In Vitro Derivation of GCs

In vitro derivation of GCs from 1F iPS was performed according to Hübner et al. (2003), Hübner et al. (2006), and our unpublished data. Putative GC cultures were FACS sorted for GFP^+ and GFP^- cells. Cell fractions were analyzed using quantitative real-time PCR for GC markers.

Immunohistochemistry for SYCP3 was performed using the spreading technique described in Peters et al. (1997). Primary anti-SYCP3 (1:500; Abcam)

was incubated overnight on slides at room temperature. After washing in blocking solution, secondary fluorescent antibody (1:1000; Alexa 568; Molecular Probes) was incubated for 1 hr at room temperature and slides were mounted in DAPI containing mounting medium (Vectashield; Vector Laboratories Inc.).

Southern Blot Analysis

BamHI digested genomic DNA from 4F iPS, 2F (OK) iPS, 1F iPS, and ESC was separated on a 0.8% agarose gel and transferred to Biodyne B nylon membrane (PALL Life Sciences). DNA was hybridized with a ^{32}P-labeled fragment of Oct4 (PstI/HindIII fragment of MX-mOct4) using the DecaLabel DNA Labeling Kit (Fermentas). Labeled Lambda HindIII digested DNA served as a marker.

Methylation Analysis

Genomic DNA sodium bisulfite conversion was performed using EZ-96 DNA Methylation Kit (Zymo Research, Orange County, CA). The manufacturer's protocol was followed using 1 mg of genomic DNA and the alternative conversion protocol (a two temperature DNA denaturation).

Sequenom's MassARRAY platform was used to perform quantitative methylation analysis. This system utilizes matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry in combination with RNA base-specific cleavage (MassCLEAVE). A detectable pattern is then analyzed for methylation status. PCR primers were designed using EpiDESIGNER (http://www.epidesigner.com). When it was feasible, amplicons were designed to cover CpG islands in the same region as the 5′UTR. For each reverse primer an additional T7 promoter tag for in vivo transcription was added, as well as a 10 mer tag on the forward primer to adjust for melting temperature differences. The MassCLEAVE biochemistry was performed as previously described (Ehrich et al., 2005). Mass spectra were acquired using a MassARRAY Compact MALDI-TOF (Sequenom, San Diego, CA) and spectra's methylation ratios were generated by the Epityper software v1.0 (Sequenom).

ACCESSION NUMBERS

The microarray data are available from the GEO (Gene Expression Omnibus) website under accession number GSE12499.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)00071-3.

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