

Epigenetic Reprogramming of the Germ Cell Nuclear Factor Gene is Required for Proper Differentiation of Induced Pluripotent Cells

HONGRAN WANG,^a XIAOHONG WANG,^{a,b} XUEPING XU,^a THOMAS P. ZWAKA,^a AUSTIN J. COONEY^a

^aDepartment of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas, USA; ^bStem cell center, Texas Heart Institute, Houston, Texas, USA

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ABSTRACT

Somatic cells have been reprogrammed into induced pluripotent stem (iPS) cells that recapitulate the pluripotent nature of embryonic stem (ES) cells. Reduced pluripotency and variable differentiation capacities have hampered progress with this technology for applications in regeneration medicine. We have previously shown that germ cell nuclear factor (*Gcnf*) is required for the repression of pluripotency genes during ES cell differentiation and embryonic development. Here we report that iPS cell lines, in which the *Gcnf* gene was properly reprogrammed, allowing expression of *Gcnf*, repress pluripotency genes during subsequent differentiation. In contrast, iPS clones in which the *Gcnf* gene was not reprogrammed maintained pluripotency gene expression during differentiation and

did not differentiate properly either in vivo or in vitro. These mal-reprogrammed cells recapitulated the phenotype of *Gcnf* knockout (*Gcnf*^{-/-}) ES cells. Reintroduction of *Gcnf* into either the *Gcnf* negative iPS cells or the *Gcnf*^{-/-} ES cells rescued repression of *Oct4* during differentiation. Our findings establish a key role for *Gcnf* as a regulator of iPS cell pluripotency gene expression. It also demonstrates that reactivation of the *Gcnf* gene may serve as a marker to distinguish completely reprogrammed iPS cells from incompletely pluripotent cells, which would make therapeutic use of iPS cells safer and more practical as it would reduce the oncogenic potential of iPS cells. STEM CELLS 2013;31:2659–2666

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Overexpression of a defined set of transcription factors suffices to reprogram somatic cells into induced pluripotent stem (iPS) cells, which have been derived from various tissues, including embryonic fibroblasts and adult tail-tip fibroblasts, hepatocytes, gastric epithelial cells, pancreatic cells, neural cell and B lymphocytes in the mouse [1–5], and skin fibroblasts, keratinocytes, and peripheral blood cells in the human [6–8]. iPS cells have enormous therapeutic potential because they can be derived from not only from normal but also patient-specific cells [9, 10]. There are significant similarities between ES cells and iPS cells, including indistinguishable global histone modification and gene expression patterns [11, 12]. Despite these similarities, there is emerging evidence of differences between ES cells and iPS cells and even between different iPS colonies. For example, differences in mRNA and microRNA (miRNA) expression [13–15], as well as in DNA methylation patterns [16–18], have been reported between ES cells and iPS cells. These observations indicate that transcription factor-mediated reprogramming can result in abnormalities in subsequent iPS cells, which could impede their therapeutic utility [16].

Pluripotency genes, which are required to maintain ES and iPS cell self-renewal, function in part through inhibition of somatic gene expression [19]. Thus, pluripotency genes are repressed and/or silenced in a timely manner during differentiation to ensure the expression of lineage determinants, which in turn allows differentiation into the three germ layers and their derivatives [19, 20]. It has been demonstrated that pluripotency gene expression persists in some differentiated iPS cell clones, while in others they are silenced as normal [1]. Germ cell nuclear factor (*Gcnf*; NR6a1), a transcriptional repressor and an orphan member of the nuclear receptor gene family, is required for the repression and silencing of pluripotency genes such as *Oct4* and *Nanog* in vitro and in vivo [21–23]. Loss of *Gcnf* function in *Gcnf* knockout (*Gcnf*^{-/-}) mice results in embryonic lethality by E10.5 [24]. Importantly, there is loss of normal repression of the *Oct4* gene in somatic cells after gastrulation, where it is generally silenced. Likewise *Gcnf*^{-/-} ES cells maintain *Oct4* expression during differentiation upon retinoic acid (RA) treatment, which impedes full differentiation of these cells [24]. Whether *Gcnf* plays a similar pivotal role in iPS cell differentiation is unknown.

Here we report that the *Gcnf* gene, which is silenced in fibroblasts, can be reactivated during somatic cell

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Correspondence: Austin J. Cooney, Ph.D., Department of Molecular and Cellular Biology, Baylor College of Medicine, One Baylor plaza, Houston, Texas 77030, USA. Telephone: +1-713-798-6250; Fax: +1-713-790-1275; e-mail: acooney@bcm.edu Received July 25, 2012; accepted for publication February 8, 2013; first published online in STEM CELLS EXPRESS March 14, 2013. © AlphaMed Press 1066-5099/2013/\$30.00/0 doi: 10.1002/stem.1367

reprogramming using *Oct4*, *Sox2*, *c-Myc*, and *Klf4*, however, the *Gcnf* gene is not reprogrammed in all iPS colonies. *Oct4* expression was repressed in those clones in which *Gcnf* expression was reactivated during subsequent differentiation with RA. In contrast, in the clones within which *Gcnf* was still silenced *Oct4* expression was maintained during differentiation. Maintained *Oct4* expression after differentiation affects the pluripotency of iPS cells, leading to poor differentiation in vitro, aberrant expression of differentiation genes, as well as failure to produce chimeras. Reintroduction of *Gcnf* rescues *Oct4* repression during differentiation. Thus, reprogramming of the *Gcnf* gene is an important variable affecting quality that should be considered during iPS formation.

MATERIALS AND METHODS

Generation of iPS Cells

pMXs-base retroviral vectors *Oct4*, *Sox2*, *Klf4*, and *C-myc* (Addgene, catalog #s 13366, 13367, 13370, and 13375) [1] were introduced into each 10 cm plate with Plat-E cells, respectively, using Fugene 6 transfection reagent (Roche, Basel, Switzerland, <http://www.roche-applied-science.com>, catalog no.11814443001) according to the manufacturer's recommendations. One day before infection, mouse embryonic fibroblasts (MEFs) were seeded at 8×10^5 cells per 10 cm gelatin-coated plate. After 24 hours of transfection, virus-containing supernatants derived from these Plat-E cultures were filtered through a 0.45-mm cellulose acetate filter and supplemented with 4 mg/mL polybrene. Target cells were incubated in the virus/polybrene-containing supernatants overnight. MEFs were infected twice. After 24 hours of infection, the media were replaced with 10 mL fresh ES cell medium. No selection markers were used in this experiment, thus iPS cells were generated in the absence of antibiotics [25]. Three weeks later, alkaline phosphatase (ALP) staining (Vector Laboratories, Burlingame, CA, <http://www.vectorlabs.com>, Catalog no SK-5100) was carried out to evaluate reprogramming efficiency, and colonies were picked based on a morphology of characteristic ES colonies and green fluorescent protein (GFP) positive. To establish stable iPS cell lines, single iPS cell colonies were each picked into one well of a 24-well plate. iPS colonies in which the viral ectopic genes (*Oct4*, *Sox2*, *c-Myc*, and *Klf4*) were silenced were used in the experiments described here.

Teratoma Formation and Histological Analysis

The iPS clones that expressed *Gcnf* at day 1.5 of differentiation were designated *Gcnf*^{on}, while the iPS clones that did not express *Gcnf* were designated *Gcnf*^{off}. 1×10^6 wild-type (wt) ES cells, *Gcnf*^{-/-} ES cells, *Gcnf*^{on} iPS cells, and *Gcnf*^{off} iPS cells were injected into the rear leg muscle of 4–6-week-old severe combined immunodeficient (SCID) mice. Four weeks later, tumors were surgically dissected from the mice. Samples were fixed in 10% formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin.

Chimera Generation and Germ Line Transmission

To determine the ability of *Gcnf*^{off} iPS cells to contribute to adult chimeras, *Gcnf*^{on} iPS cells or *Gcnf*^{off} iPS cells were injected into the blastocysts of C57 Albino white genetic background at passage 10. Chimeric blastocysts were subsequently transferred to day 2.5 pseudopregnant recipient CD-1 females, and the percentage of chimera contribution was estimated by scoring the level of coat color pigmentation. To evaluate the capacity for germ line transmission of iPS cells,

chimeric males were bred with C57BL/6 females. Pups were identified by coat pigmentation and genotype after birth.

iPS Cell Differentiation Assays

Differentiation of iPS cells and ES cells was induced by leukemia inhibitory factor (LIF) withdrawal and addition of presence of 1 μ M RA. The differentiating ES cells or iPS cells were collected over the course of several days, and the expression of pluripotent genes and germ layer markers was analyzed by regular reverse transcription polymerase chain reaction (RT-PCR) or quantitative RT-PCR (Q-RT-PCR) with SYBR (Qiagen, Hilden, Germany, <http://www1.qiagen.com>, catalog no. 204056). Primers used in this article are described in Supporting Information Table S1. Protein samples were analyzed by Western blot. For embryoid body (EB) formation, the iPS cells and ES cells were dispersed into single cells using trypsin/EDTA and were plated into bacterial grade Petri dishes. Both ES cell and iPS cells aggregated into EBs for 4 days, and iPS cell EBs were plated on to gelatin-coated dishes for differentiation for another 4 days. The differentiated cells were identified by the cellular morphology.

PCR Analysis

Total RNA was prepared using the Trizol reagent (Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>, catalog no. 15596-018) and quantified by NanoDrop™ 1000 Spectrophotometer v3.7 (Thermo Fisher Scientific, Waltham, MA, <http://www.thermofisher.com/global/en/home.asp>). RNA samples were reverse transcribed to complementary DNA (cDNA) using SuperScript III First-Strand Synthesis System (Invitrogen, Catalog no. 18080-051). PCR primers are described in Supporting Information Table S1.

Western Analysis

Whole cell lysates were subjected to Western analyses. Antibodies against mouse Nanog (Abcam, Cambridge, U.K., <http://www.abcam.com>, ab80892), Sox2 (Millipore, Billerica, MA, <http://www.millipore.com>, ab5603), Oct4 (Santa Cruz, Santa Cruz, CA, <http://www.scbt.com>, catalog no. sc-5279), and Gcnf were used at 1:1,000 dilution. β -Actin (Sigma, St. Louis, MO, <http://www.sigmaaldrich.com/united-states.html>, catalog no. A1978) was used at a 1:5,000 dilution. The secondary antibodies, goat anti-mouse IgG-HRP (horse radish peroxidase) (Santa Cruz, catalog no. sc-2055) and goat anti-rabbit IgG-HRP (Santa Cruz, catalog no. sc-2004) were used at a 1:2,000 dilution. HRP activity was detected by a chemiluminescence using Pierce ECL Western Blotting Substrate kit (Thermo scientific, catalog no. 32106). The emitted light was detected by photographic film.

DNA CpG Methylation Analysis

Bisulfite treatment was performed [18] using the EZ DNA Methylation Kit (Zymo research, Irvine, CA, www.zymoresearch.com/, Catalog no. D5001) according to the manufacturer's instructions. CpG methylation was analyzed within the *Oct4* promoter in *Gcnf*^{on} (m5 iPSCs), *Gcnf*^{off} (m8 iPSCs), and MEF cells. PCR primers are: forward: CTGAAAATCACCAC-CACC; reverse: CTAATAATGAGCCTTTCC. Amplified PCR products were cloned into pCR2.1-TOPO vector (Invitrogen, catalog no. K2040-01). Ten randomly selected clones were sequenced with the M13 forward and M13 reverse primers.

Gcnf Rescue

A full-length mouse *Gcnf* cDNA was obtained from reverse transcribed PCR products generated from d1.5 differentiated ES cell mRNA. The resulting cDNA was cloned into the Gateway/Topo TA vector (Invitrogen, catalog no. K250020) and

then was recombined into the destination retroviral plasmid with the mscv promoter by Gateway LR Clonase (Invitrogen, catalog no. 11791-020). For packaging the virus, 1.5×10^6 BOSC cells were plated in six well of plates [10% fetal bovine serum (FBS) Dulbecco's modified Eagle's medium] and were cultured overnight. The mscv-*Gcnf* and mscv-*GFP* plasmids along with the packaging plasmid pDuo were introduced into each 6 cm plate with BOSC cells, respectively, using Fugene 6 transfection reagent (Roche, catalog no.11814443001) according to the manufacturer's recommendations. After 24 hours of transduction, virus-containing supernatants derived from these BOSC cell cultures were filtered through a 0.45 mm cellulose acetate filter and supplemented with 4 mg/ml polybrene. 3×10^5 target cells were seeded into 24-well plates that were incubated with 0.5 mL of the virus/polybrene-containing supernatants overnight and infected twice. After 24 hours of infection, the media were replaced with 0.5 mL fresh ES cell medium. The transfected cells were selected for 2 weeks with 1 μ g/mL of puromycin after 3 days of transfection. The selected cells were used for the described analysis.

RESULTS

The Degree of *Gcnf* Reprogramming Determines the Level of *Oct4* Repression During iPS Cell Differentiation

Mouse iPS cells were generated by virally introducing four factors: *Oct4*, *Sox2*, *c-Myc*, and *Klf4* [1] into *Oct4*-GFP MEF (Supporting Information Fig. S1A). The selected iPS colonies were positive for Oct4-GFP fluorescence (Supporting Informa-

tion Fig. S1B) and ALP staining (Supporting Information Fig. S1C, S1D). We induced iPS cell differentiation by treatment with RA for 6 days. wt and *Gcnf*^{-/-} ES cells were used as controls. The highest levels of *Gcnf* expression are induced at 1.5 days of RA differentiation in wt ES cells [21]. Thus, *Gcnf* expression was analyzed at 1.5 days of RA treatment by RT-PCR and Q-RT-PCR in nine different iPS clones that had been selected based on optimal ES cell morphology [25], GFP positive, and silencing of the viral genes (*Oct4*, *Sox2*, *c-Myc*, and *Klf4*) (Supporting Information Fig. S1E, S1F, S1G, S1H). Unexpectedly, we observed *Gcnf* expression in only four of the iPS clones (44.4%) (Fig. 1A). The remaining five iPS clones (55.6%) did not express *Gcnf*, suggesting that the *Gcnf* gene had not been properly reprogrammed during the process of iPS cell formation. The iPS clones that expressed *Gcnf* at day 1.5 of differentiation were designated *Gcnf*^{on}, while the iPS clones that did not express *Gcnf* were designated *Gcnf*^{off}.

Oct4 expression was detected in undifferentiated ES cells (d0) and at 3 days of RA-induced differentiation (d3) (Fig. 1A). *Oct4* expression was repressed by day 3 of RA treatment in the four *Gcnf*^{on} iPS clones. In contrast, the five *Gcnf* negative (*Gcnf*^{off}) clones maintained *Oct4* expression at day 3 of differentiation. Confirmation of this finding with Q-RT-PCR showed that the levels of *Oct4* at day 3 of differentiation inversely correlated with the level of induction of *Gcnf* at day 1.5 (Fig. 1B). Silencing of pluripotency gene expression is necessary for normal differentiation to proceed so that lineage determinants can be properly expressed.

Two cell lines were selected to further characterize the importance of reactivation of *Gcnf* expression during iPS cell formation. The m8 iPS clone was observed to be a typical *Gcnf*^{off} cell, whereas the m5 iPS clone was a typical *Gcnf*^{on} cell.

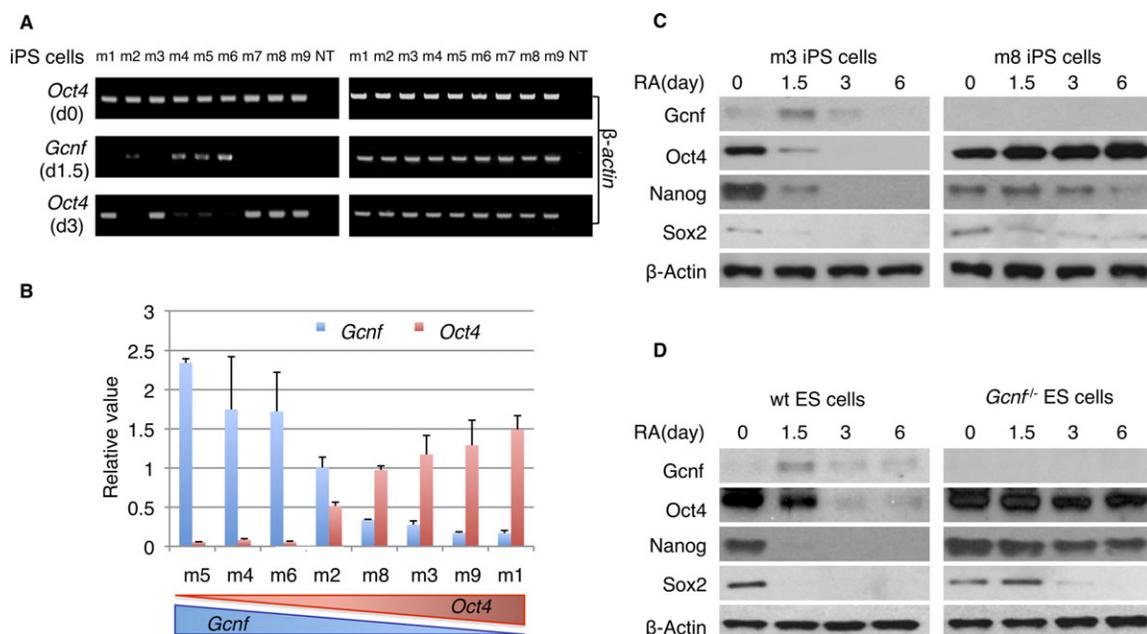


Figure 1. Repression of *Oct4* expression is dependent on induced *Gcnf* expression during iPS cell differentiation. (A): Using reverse transcription polymerase chain reaction (RT-PCR), *Oct4* was detected in individual undifferentiated iPS clones and in differentiated iPS cells treated with RA for 3 days; *Gcnf* expression was detected at day 1.5 of RA-induced differentiation. β -Actin served as a loading and integrity control (B). Quantitative RT-PCR was used to quantify the mRNA expression level of *Oct4* at day 3 of RA-induced differentiation and *Gcnf* at 1.5 days of differentiation and compared with undifferentiated cells, respectively. (C): Protein levels of *Gcnf*, *Oct4*, *Nanog*, *Sox2*, and control β -actin were detected by Western analysis in undifferentiated iPS cells (day 0) and differentiated cells treated with RA at days 1.5, 3, and 6 in *Gcnf*^{on} iPS cells (m3) and *Gcnf*^{off} iPS cells (m8). (D): Protein levels for *Gcnf*, *Oct4*, *Nanog*, *Sox2*, and control β -actin were detected by Western analysis in undifferentiated wt and *Gcnf*^{-/-} ES cells (day 0) and differentiated wt and *Gcnf*^{-/-} ES cells treated with RA at days 1.5, 3, and 6. Abbreviations: ES, embryonic stem cells; *Gcnf*, germ cell nuclear factor; iPS, induced pluripotent stem cell; RA, retinoic acid; wt, wild type.

Western analysis showed that the *Gcnf* and *Oct4*, *Nanog* and *Sox2* expression patterns in *Gcnf^{on}* and *Gcnf^{off}* clones are similar to that of their genetic counterparts, wt and *Gcnf^{-/-}* ES cells, respectively. *Oct4*, *Nanog*, and *Sox2* expression in wt ES cells and *Gcnf^{on}* iPS cells is silenced after differentiation, as a result of *Gcnf* induction in both cell lines (Fig. 1C, 1D). In contrast, *Gcnf* was undetectable in *Gcnf^{-/-}* ES cells and *Gcnf^{off}* iPS cells, and *Oct4*, *Nanog*, and *Sox2* expression was maintained even at day 6 of differentiation (Fig. 1C, 1D). Thus, *Gcnf^{off}* iPS cells cannot efficiently repress *Oct4*, *Nanog*, and *Sox2* expression during differentiation because of the lack of *Gcnf*. The *Gcnf^{off}* iPS cells behaved just like the *Gcnf^{-/-}* ES cells and thus represent an epigenetic knock out (KO) of *Gcnf*, equivalent to the genetic KO. The level of *Oct4* repression is dependent on the level of *Gcnf* induction during differentiation.

Aberrant *Gcnf^{off}* iPS Cell Differentiation In Vitro

To investigate the effect of the level of *Gcnf* expression on iPS cell pluripotency and the capacity for differentiation into the three germ layers in vitro, a standard differentiation protocol was adopted using monolayer culture or EB formation and stepwise differentiation [1, 21]. First, these cell lines were induced to differentiate by withdrawal of LIF and the addition of RA. The ability of these two iPS cell lines to differentiate into derivatives of the three different embryonic germ layers was characterized by analyzing marker gene expression. Expression of the endoderm markers, *Afp* and *FoxA2* increased during *Gcnf^{on}* iPS cell and wt ES cell differ-

entiation [26]. In contrast, *Afp* and *FoxA2* expression was undetectable in differentiated *Gcnf^{off}* iPS cells and *Gcnf^{-/-}* ES cells (Fig. 2A, 2B). Induction of *FoxA2* requires *FoxD3*, which binds to a response element located in the *FoxA2* promoter [27]. *Oct4* and *FoxD3* bind identical regulatory sequences, implying that *Oct4* likely competes with *FoxD3* for DNA binding on the *FoxA2* promoter. The loss of *FoxA2* induction in *Gcnf^{off}* clones is likely due to maintained *Oct4* expression. To further investigate whether the loss induction of endoderm gene expression correlated to *Gcnf* expression, we used Q-RT-PCR to detect *Sox17* expression. The results showed that *Sox17* expression increased rapidly during RA-induced differentiation in wt ES cells and *Gcnf^{on}* iPS cells (m4 and m5 iPS cells), in contrast *Sox17* was barely induced in *Gcnf^{-/-}* ES cells and *Gcnf^{off}* iPS cells (m3 and m8 iPS cells) (Fig. 2C). We also found that the level of *Sox17* gene expression positively correlated with the level of *Gcnf* induction at 1.5 days of differentiation (Fig. 2D).

The expression of the mesoderm markers *Pax6*, *gata1*, and the ectoderm markers, β -*Tubulin III* and *Nestin* were also determined by RT-PCR. *Gata6* expression showed no significant differences between the *Gcnf^{off}* and *Gcnf^{on}* iPS and ES cell lines. *Pax6* expression gradually increased in both m5 iPS cells (*Gcnf^{on}*) and wt ES cells, opposite to what was observed in both m8 iPS (*Gcnf^{off}*) cells and *Gcnf^{-/-}* ES cells during differentiation (Fig. 2A, 2B). *Nestin* expression displayed no significant difference between the different cell lines. Expression of β -*Tubulin III* was detected in all four

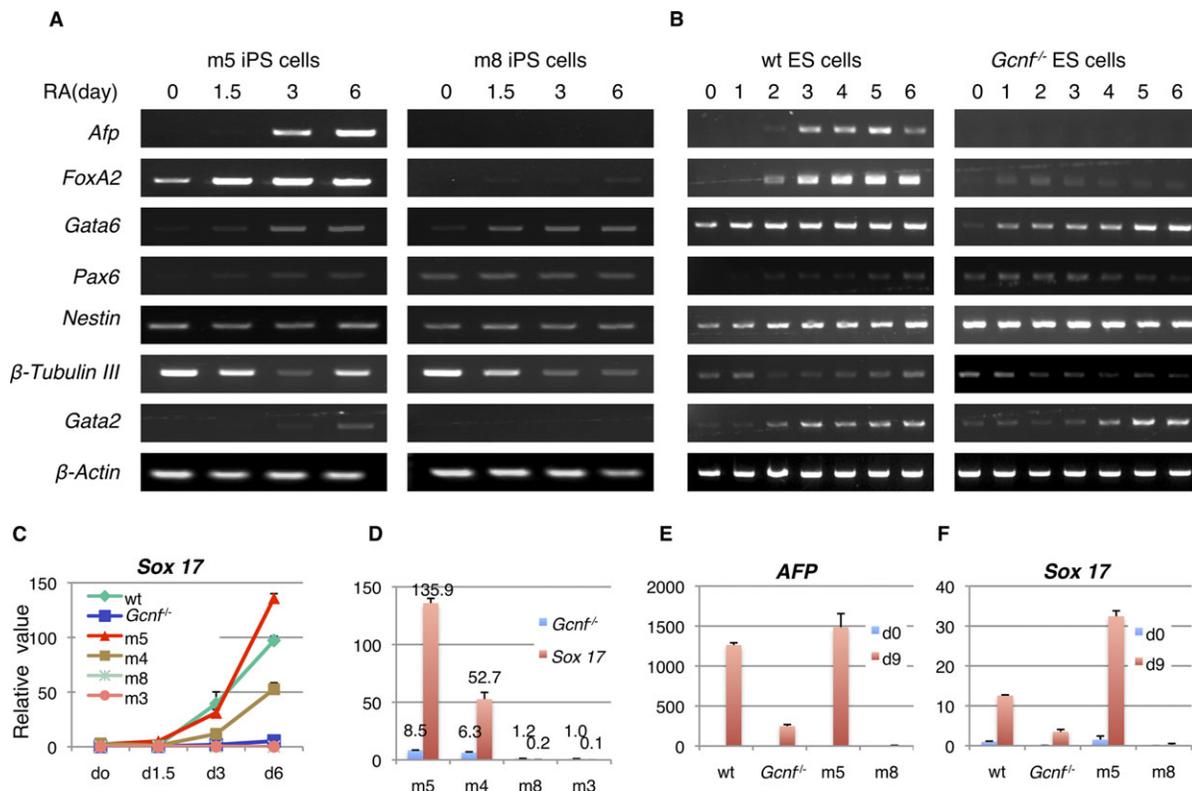


Figure 2. Loss of *Gcnf* reprogramming results in defective activation of endoderm genes during iPS cell differentiation with RA treatment in vitro. Endoderm markers (*Afp* and *FoxA2*), mesoderm markers (*Gata6* and *Pax6*), ectoderm markers (*Nestin* and β -*Tubulin III*) were detected by reverse transcription polymerase chain reaction (RT-PCR) in (A) *Gcnf^{on}* (m5) and *Gcnf^{off}* (m8) iPS cells, (B) wt and *Gcnf^{-/-}* cells during RA-induced differentiation. (C): The mRNA levels of *Sox17* were detected by quantitative RT-PCR (Q-RT-PCR) in wt ES cells, *Gcnf^{-/-}* ES cells, *Gcnf^{on}* iPS cells, and *Gcnf^{off}* iPS cells. (D): Relationship of *Gcnf* and *Sox17* mRNA levels, *Gcnf* was detected at 1.5 days of differentiation, and *Sox17* was detected at 6 days of differentiation. (E): Comparison of *Afp* mRNA levels in EBs at d0 and d9 of differentiation *Afp* mRNA was detected by Q-RT-PCR. (F): Comparison of *Sox17* mRNA levels in EBs at d0 and d9 of differentiation *Sox17* mRNA was detected by Q-RT-PCR. Abbreviations: ES, embryonic stem cell; iPS, induced pluripotent stem cell; RA, retinoic acid; wt, wild type.

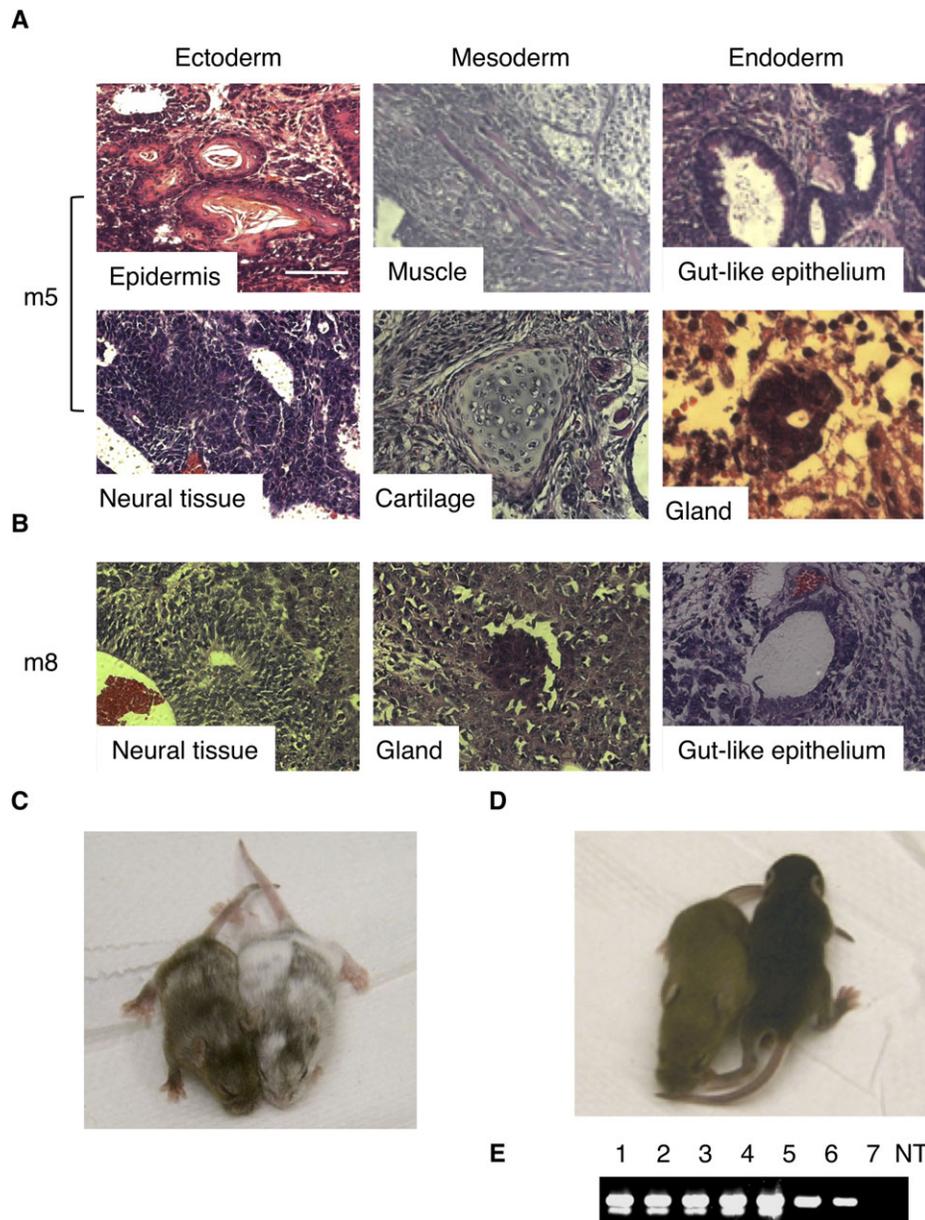


Figure 3. Assessment of pluripotency of induced pluripotent stem (iPS) Cells in vivo. (A): *Gcnf^{on}* iPS cells were injected into the rear legs of severe combined immunodeficient mice. Three weeks later, teratomas were collected and histologic H & E staining was used to determine the differentiation into cell types distinctive of the three germ layers. (B): Teratomas from *Gcnf^{off}* iPS cells were analyzed by H & E staining. (C): Chimera generation from *Gcnf^{on}* iPS cells by injection into albino C57 mouse blastocysts. No chimeras were generated from *Gcnf^{off}* (m8) iPS cell injections. (D): Mice generated from germ line transmission from *Gcnf^{on}* iPS cell derived chimeric mice. (E): Mice with germ line transmission were confirmed by polymerase chain reaction genotyping for the *Oct4 GFP* reporter. Scale bars = 50 μ m.

undifferentiated cell types analyzed and decreased to the lowest level at day 3 of differentiation. Subsequently, the expression of β -Tubulin gradually began to increase in m5 iPS cells (*Gcnf^{on}*) and wt ES cells. In contrast, the m8 iPS cells (*Gcnf^{off}*) and *Gcnf^{-/-}* ES cells exhibited no induction of β -Tubulin III expression at later stages of differentiation (Fig. 2A, 2B), affirming the deficiency reported in neural cell development attributed to the loss of Gcnf [28, 29].

EB formation demonstrates the capacity of pluripotent cells to generate many cell types. To analyze the ability of *Gcnf^{off}* iPS cells to form EBs, the m5 and m8 iPS cells, as well as wt and *Gcnf^{-/-}* ES cells, were dispersed as single cells in Petri dishes. After 4 days, all four cell lines formed EBs and their morphology did not display significant differences between the

different cell types (Supporting Information Fig. S2A). After plating the EBs onto gelatin-coated tissue culture plates, the m5 iPS cells (*Gcnf^{on}*) showed extensive differentiation in contrast to the m8 cells (*Gcnf^{off}*), which differentiated into cells exhibiting a fibroblast-like morphology, instead of extensive differentiation (Supporting Information Fig. S2B).

In order to verify that endoderm genes are also not activated during EB formation in *Gcnf^{-/-}* and *Gcnf^{off}* iPS cells, the expression of the markers *Sox17* and *Afp* were tested by Q-RT-PCR at day 9 of EB differentiation. The results showed that *Sox 17* and *Afp* were activated in *Gcnf^{on}* iPS cells (m5) and wt ES cells indicative of the formation of endoderm, however, *Sox 17* and *Afp* expression was not induced in *Gcnf^{off}* or *Gcnf^{-/-}* EBs (Fig. 2E, 2F).

Gcnf^{off} iPS Cell Differentiation In Vivo

The differentiation capability of *Gcnf*^{off} iPS cells was assessed in vivo via teratoma formation. The m5 and m8 iPS cells, as well as control wt and *Gcnf*^{-/-} ES cells, were injected into SCID mice. All four cell types gave rise to teratomas. Histological analysis showed that well-differentiated teratomas with components of all three germ layers were found both in m5 iPS and in wt ES cell teratomas (Fig. 3A), whereas extensive differentiation derivatives of the three germ layers was not observed in m8 iPS derived teratomas (Fig. 3B). *Gcnf*^{-/-} ES cells differentiated slightly better than the m8 (*Gcnf*^{off}) iPS cells, but not better than wt ES cells or m5 (*Gcnf*^{on}) iPS cells (Supporting Information Fig. S3). This result indicates that loss of *Gcnf* affected the capacity of the cells to differentiate into all cell types in vivo.

Loss of Chimeric Potential in *Gcnf*^{off} iPS Cells

Chimerism and germ line transmission, which are the most stringent assays for developmental potential and pluripotency of ES or iPS cells [30, 31], was also used to further evaluate the differentiation capacity of the iPS clones in vivo. The experimental schedule is diagrammed in Supporting Information Figure S4. The m8 (*Gcnf*^{off}) iPS cells were unable to give rise to chimeras after blastocyst injection, whereas m5 (*Gcnf*^{on}) iPS cells were able to generate live postnatal animals with high coat color chimerism (Fig. 3C). A chimeric male mouse was mated with albino C57/BL6 females in order to test for germ line transmission and true pluripotency. Germ line transmission was achieved from a m5 chimera (Fig. 3D), which was confirmed by PCR genotyping for the *Oct4-GFP* reporter contained in the original reprogrammed MEFs (Fig. 3E) and coat color, indicating that *Gcnf*^{on} iPS cells (m5) were competent for germ line transmission (Fig. 3D). These results indicate that *Gcnf*^{on} iPS cells are pluripotent, and loss of *Gcnf* expression through improper reprogramming leads to loss of iPS cell pluripotency.

Epigenetic Changes in the *Gcnf* Gene Promoter During Reprogramming

Reprogramming of somatic cells requires removal of epigenetic modifications on chromatin that are laid down during differentiation and developmental processes, allowing reactivation of pluripotency genes that are necessary for maintaining self-renewal and pluripotency of iPS cells. One type of epigenetic modification is DNA methylation of CpG dinucleotides, a hallmark of silenced genes [13, 18]. However, somatic cell reprogramming does not completely demethylate CpG dinucleotides of genes that are epigenetically silenced during differentiation because iPS cells display greater levels of methylation than the ES cells [11]. The DNA methylation status at CpG dinucleotides correlates with gene expression. To determine the DNA methylation status of the *Gcnf* gene, bisulfite sequencing was performed to determine the extent of CpG methylation at a CpG island located between the *Gcnf* translational start site and 66 bp upstream (Fig. 4A). This region of the *Gcnf* gene is hypermethylated in the MEFs, which were reprogrammed to form the iPS cells (Fig. 4B). As expected, the m5 (*Gcnf*^{on}) iPS cells were predominantly demethylated in this region in the undifferentiated state indicative of reprogramming and then remethylated during RA-induced differentiation (day 6) as *Gcnf* is transiently induced (Fig. 4B). This is consistent with epigenetic remodeling of the *Gcnf* promoter after retroviral infection. In contrast, m8 (*Gcnf*^{off}) cells were predominantly methylated at the *Gcnf* CpG island in the undifferentiated and differentiated states, which is indicative of a failure to reprogram this locus (Fig. 4B). The methylation of the *Gcnf* promoter region in m8

clones correlates with the failure to induce *Gcnf* expression during iPS cell differentiation. Thus, during somatic cell reprogramming, epigenetic characteristics of iPS cells exhibit diversity among different iPS cell lines [11]. Interestingly, the m8 iPS cells represent an epigenetic KO of the *Gcnf* gene that phenocopies the genetic *Gcnf* KO in many ways.

Gcnf Rescues the Repression of *Oct4* in *Gcnf*^{off} iPS Cells

In order to determine if the loss of *Oct4* repression in the *Gcnf*^{off} iPS cells and *Gcnf*^{-/-} ES cells can be rescued, mscv-*Gcnf* retrovirus (Supporting Information Fig. S5A) and mscv-*GFP* control retrovirus (Supporting Information Fig. S5B) were transfected into the *Gcnf*^{off} iPS cells and *Gcnf*^{-/-} ES cells. After puromycin selection from transfected *Gcnf*^{off} iPS cells, M8 #1 and M8 #2 cell lines were used to analyze the expression of *Gcnf* and *Oct4* during RA-induced differentiation. *Gcnf* was expressed in both M8 #1 and M8 #2 cell lines, concomitantly *Oct4* was repressed at day 3 of differentiation. In contrast, nontransfected *Gcnf*^{off} iPS cells and the control GFP-transfected iPS cells both maintained *Oct4* expression at day 3 because no *Gcnf* expression was detectable (Fig. 4C). The same results were observed after *Gcnf* was transfected into *Gcnf*^{-/-} ES cells (Fig. 4D). Thus, reintroduction of *Gcnf* can rescue the repression of *Oct4* in the epigenetic KO of *Gcnf* m8 iPS cells. This result is the first demonstration in a gain-of-function experiment that *Gcnf* is required for inhibition of *Oct4* expression during pluripotent cell differentiation.

DISCUSSION

Our study demonstrates that reactivation of the silenced *Gcnf* gene during somatic cell reprogramming is required to maintain optimum pluripotency of iPS cells. Not all of the iPS clones selected reactivated the *Gcnf* gene. Only *Gcnf*^{on} iPS clones can efficiently inhibit *Oct4* expression after differentiation. Although the *Gcnf*^{off} iPS cells behave like self-renewing pluripotent cells in undifferentiated conditions, that is, have the ability to form EBs and teratomas, and to differentiate into three germ layers, the capacity of differentiation into all cell types is significantly decreased similar to *Gcnf*^{-/-} ES cells. However, like wt ES cells, the *Gcnf*^{on} iPS cells can differentiate into the three germ layers in vitro and in vivo and give rise to chimeras capable of germ line transmission.

Gcnf, as a silencer of pluripotency genes, represses not only *Oct4* and *Nanog* [21] but also *Cripto* [32], and the level of *Oct4* expression ultimately affects the direction ES cell differentiation [33]. During somatic cell reprogramming there is variable reactivation of the silenced *Gcnf* gene in iPS clones. The iPS clones that have demethylated the *Gcnf* gene can reactivate *Gcnf* expression during subsequent iPS cell differentiation (Fig. 4E). In contrast iPS clones in which the *Gcnf* gene was not demethylated *Gcnf* expression is not induced by RA and thus *Oct4* expression is maintained during differentiation (Fig. 4F) [21]. Aberrant epigenetics often occur between different iPS clones during somatic cell reprogramming, which will affect the degree of pluripotency observed after differentiation [11]. This is an important finding because aberrant epigenetic reprogramming of the *Gcnf* gene was observed in nearly 50% of the clones analyzed. Maintenance of the epigenetic silencing of *Gcnf* appears to have a selective advantage during iPS formation as stable pluripotency gene expression is maintained. The aberrant reprogramming of the *Gcnf* gene during iPS formation can account for “good looking” iPS colonies that are not functional because of stable

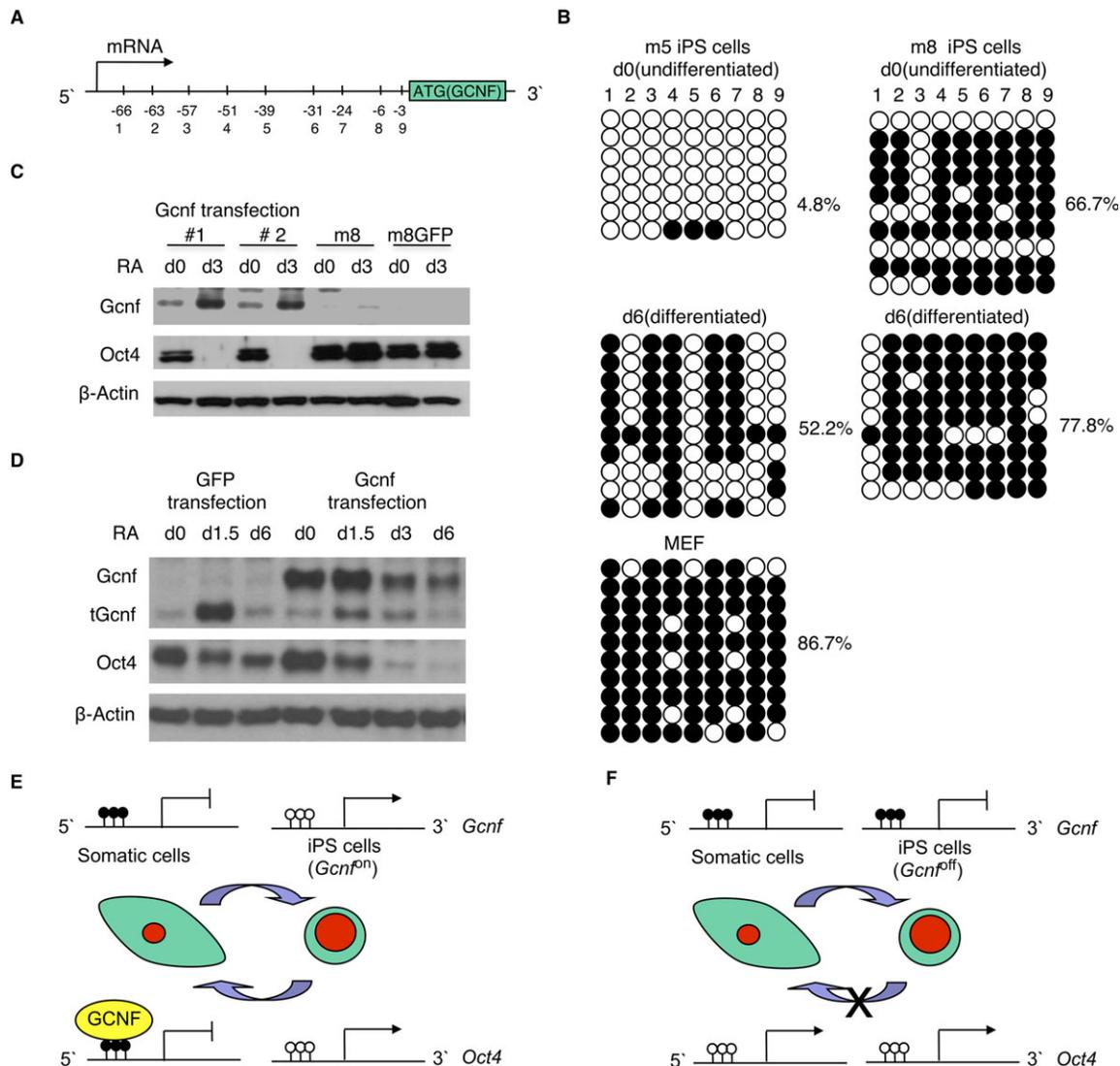


Figure 4. Analysis of the DNA methylation status of the *Gcnf* promoter, and rescue of the m8 iPS cells. **(A):** The CpG island located in the *Gcnf* gene is close to the ATG and downstream of the transcriptional start site. **(B):** Methylation status of the *Gcnf* CpG island was determined by bisulfite sequencing in undifferentiated and differentiated iPS cells in both *Gcnf*^{m5} (m5) and *Gcnf*^{m8} (m8) iPS cells, as well as the starting MEFs used for the reprogramming. White circles represent unmethylated CpG dinucleotides; black circles represent methylated CpG dinucleotides. **(C):** Oct4 repression was rescued in *Gcnf*^{m8} (m8) iPS cells after retroviral re-expression of Gcnf. **(D):** Oct4 repression was rescued in *Gcnf*^{-/-} ES cells after retroviral re-expression of Gcnf. The lower band was that of a truncated form of Gcnf (tGcnf) produced in this *Gcnf* mutant ES cell model. **(E):** Model showing that in good iPS cells DNA methylation of the *Gcnf* promoter has been removed during reprogramming allowing expression of Gcnf and repression of Oct4 during differentiation. **(F):** Model showing that in bad iPS cells DNA methylation of the *Gcnf* promoter has not been removed during reprogramming thus preventing expression of Gcnf and maintenance of Oct4 expression during subsequent iPS cell differentiation. White lollipop represents unmethylated CpG dinucleotides; black lollipop represents methylated CpG dinucleotides. Abbreviations: GFP, green fluorescent protein; Gcnf, germ cell nuclear factor; iPS, induced pluripotent stem cell; MEF, mouse embryonic fibroblast; RA, retinoic acid.

pluripotency gene expression [1]. However, stable maintenance of pluripotency gene expression comes at an expense, as these cells do not differentiate correctly.

Since the first patient-specific iPS cell lines have been developed [9, 10], the derivation of patient tissue-specific cell types have been demonstrated, such as motoneurons [9]; thus the safety and quality of iPS cells is considered important and a limitation to their therapeutic application. Significant reprogramming variability in iPS cells compared to both ES cells and other iPS cells has been observed, especially differential iPS cell DNA methylation [34, 35]. The methylation status of the *Gcnf* gene should be considered one of the markers of high-quality and safe iPS cells, as it is the silencer of pluripotency

genes. In addition, as an orphan member of the nuclear receptor gene family of ligand-activated transcription factors, these findings identify Gcnf as a small molecule target for the manipulation of iPS reprogramming. Based on the results an antagonist that inhibits *Gcnf* transcriptional repression function should promote iPS formation in a stable but reversible manner.

CONCLUSION

Somatic cell reprogramming is a complicated stochastic process with variable degrees of rewriting the epigenetic code of

important regulatory genes, which can subsequently affect iPS cell self-renew or differentiation. Our results show that correct reprogramming of the *Gcnf* gene is indispensable for the ability of iPS cells to differentiate correctly. Only those iPS clones where *Gcnf* expression was reactivated have the potential to differentiate into all three germ layers in vivo and in vitro. Clones that do not reprogram the *Gcnf* gene maintain stable expression of pluripotency factors such as Oct4 and Nanog in the face of strong differentiation cues. The epigenetic status of the *Gcnf* gene should be considered one of the markers of high-quality and safe iPS cells, as it is a repressor of pluripotency genes. The ability to rescue this defect by virally re-expressing *Gcnf* in these cells establishes *Gcnf* as a target to manipulate pluripotency gene expression. This is significant as *Gcnf* is an orphan member of the nuclear receptor gene family and thus has the potential to be manipulated by small molecules.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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