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Resource

Efficient Generation of Human iPSCs by a Synthetic Self-Replicative RNA

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SUMMARY

The generation of human induced pluripotent stem cells (iPSCs) holds great promise for the development of regenerative medicine therapies to treat a wide range of human diseases. However, the generation of iPSCs in the absence of integrative DNA vectors remains problematic. Here, we report a simple, highly reproducible RNA-based iPSC generation approach that utilizes a single, synthetic self-replicating VEE-RF RNA replicon that expresses four reprogramming factors (*OCT4*, *KLF4*, and *SOX2*, with *c-MYC* or *GLIS1*) at consistent high levels prior to regulated RNA degradation. A single VEE-RF RNA transfection into newborn or adult human fibroblasts resulted in efficient generation of iPSCs with all the hallmarks of stem cells, including cell surface markers, global gene expression profiles, and in vivo pluripotency, to differentiate into all three germ layers. The VEE-RF RNA-based approach has broad applicability for the generation of iPSCs for ultimate use in human stem cell therapies in regenerative medicine.

INTRODUCTION

The generation of human induced pluripotent stem cells (hiPSCs) by retroviral expression of four reprogramming factors opened the potential for regenerative medicine therapies based on patient-specific, personalized stem cells (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007). However, the insertional mutagenic potential of retroviruses combined with the potential for latent reprogramming factor gene activation, especially *c-MYC*, all but eliminates integrative DNA-based approaches for use in regenerative medicine therapies (Okita and Yamanaka, 2011; González et al., 2011; Hussein et al., 2011; Ben-David and Benvenisty, 2011). Several methods based on DNA, RNA, miRNAs, and proteins have been developed to generate integration-free iPSCs, and the advantages and disadvantages have been discussed elsewhere (González et al., 2011; Hussein et al., 2011; Mochiduki and Okita, 2012; Okita and Yamanaka, 2011). Of all these methods, RNA-based iPSC approaches using Sendai virus (Fusaki et al., 2009), miRNAs, and mRNA transfection (Warren et al., 2010) avoid potential integration problems associated with DNA-based approaches and at this point in time appear inherently safer methods for future clinical applications.

Expression of pluripotent factors by infection with Sendai virus, a negative-sense, single-stranded RNA virus that does not go through a DNA intermediate, offers a highly efficient iPS approach in the absence of concerns for integration into the genome (Fusaki et al., 2009; Ban et al., 2011). However, due to persistent Sendai virus replication in iPSC clones, this approach requires a negative selection step followed by one or more recloning steps from the single-cell level to isolate virus-free iPSCs. A temperature-sensitive mutant of Sendai virus is a successful alternative method to remove the virus (Ban et al., 2011), though it requires a higher biosafety due to production of infectious virus particles. One of the more-promising non-DNA-based iPS approaches involves transfection of four individual RF mRNAs generated by in vitro transcription (Warren et al., 2010, 2012; Angel and Yanik, 2010). Recent work has shown that activation of the innate immune system enhances the overall efficiency of iPSC generation by repeated mRNA transfection (Lee et al., 2012). However, due to the rapid degradation of reprogramming factor mRNAs, this approach requires repetitive daily transfection of four individual mRNAs into the same target cells over the 14-day reprogramming period. Although both Sendai virus and mRNA transfection approaches have been shown to generate iPSCs, there remains a significant need for a simple, highly reproducible, non-DNA-based approach to generate hiPSCs.

To develop an RNA-based iPSC generation strategy, we focused our efforts on an approach that (1) utilizes a single RNA species capable of self-replicating for a limited number of cell divisions, thereby reducing the number of transfections; (2) is capable of encoding at least four reprogramming factor open reading frames (ORFs); (3) consistently expresses all reprogramming factor genes at high threshold levels over multiple

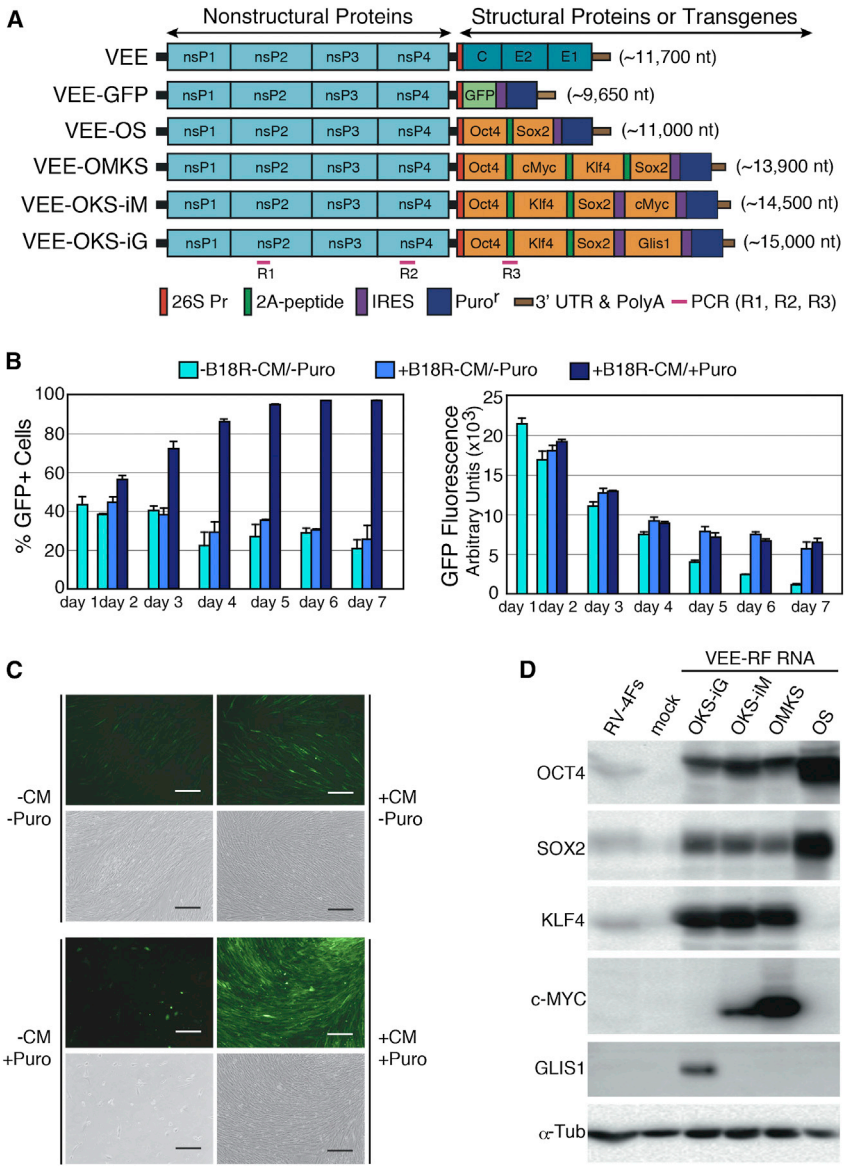

Figure 1. Construction and Persistence of Synthetic VEE-RF RNA Replicons in Primary Human Fibroblasts

(A) Schematic of VEE-RF RNA replicons. 5′ end nsP1–nsP4, nonstructural proteins 1–4; 3′ end C, E2, and E1, structural proteins. Location of 26S internal promoter, ribosome-shifting 2A peptide, IRES sequence, puromycin (Puro)-resistance gene, and PCR detection of replicon as indicated.

(B) B18R-CM conditioned media and puromycin selection are required for the persistence of VEE-GFP RNA over 7 days. HFF cells were transfected on day 0 with VEE-GFP RNA and treated as indicated. GFP fluorescence of GFP-positive cell population was measured by flow cytometry. Error bars represent the SD of duplicate samples from representative experiments.

(C) B18R-CM and puromycin are required for retention of VEE-GFP RNA. Photographs of GFP expression on day 7 as indicated. Scale bars, 200 μm.

(D) Immunoblot analysis of VEE RNA-expressed reprogramming factors expressed in HFF cells on day 1 versus retrovirus (RV-4Fs: OCT4, SOX2, KLF4, c-MYC) expression.

See also Figure S1.

cellular divisions; and (4) can be selectively retained and degraded in a controlled fashion. To ectopically express all four reprogramming factors, we modified a noninfectious (non-packaging), self-replicating Venezuelan equine encephalitis (VEE) virus RNA replicon (Kinney et al., 1989; Petrakova et al., 2005) that is currently being investigated as an expression platform for vaccine development (Davis et al., 2002; Durbin and Whitehead, 2010). The VEE replicon is a positive-sense, single-stranded RNA that mimics cellular mRNA with a 5′ cap and poly(A) tail and does not utilize a DNA intermediate, so there is no potential for genomic integration (Kinney et al., 1989). Here, we report on the generation of hiPSCs by a single transfection of a self-replicating VEE RNA species that expresses four reprogramming factor ORFs (OCT4, KLF4, SOX2, with either c-MYC or GLIS1). VEE-reprogramming factor (VEE-RF) RNA generated hiPSCs that were free of VEE RNA and had all the hallmarks of human stem cells (expression of embryonic stem cell [ESC] markers, global gene expression, and differentiation in vivo into all three germ lineages). The VEE-RF RNA can also be selectively retained or removed from cells. The non-DNA and nonintegrating, self-replicating VEE RNA approach has the potential to simplify the generation of hiPSCs for use in disease cell-modeling studies and eventual cell therapy applications.

RESULTS

Development of Self-Replicative VEE RNA to Express Reprogramming Factors

To develop a single RNA iPSC generation approach, we focused on a polycistronic, self-replicative RNA system that would consistently express the reprogramming factors over multiple cellular divisions. We modified a noninfectious, self-replicating VEE RNA (Petrakova et al., 2005). VEE RNA is a positive-stranded RNA that encodes four nonstructural replication complex proteins (nsPs) as a single ORF in the 5′ end of the RNA that is separated from the viral structural protein ORFs in the 3′ end (Figure 1A). Petrakova et al. showed the ability to express exogenous proteins by replacing the 3′ structural protein ORFs with GFP (Petrakova et al., 2005). To evaluate the VEE RNA replicon in primary human fibroblasts, we replaced the 3′ ORF with GFP, followed by an internal ribosomal entry site (IRES) (Pelletier and Sonenberg, 1988) and a Puromycin-resistance gene (Puro^r) (Figure 1A). Current SP6 or T7 RNA in vitro transcription kits can transcribe RNAs in excess of 25 kb in length (Schelle and Thiel, 2007). VEE-GFP RNA was produced using either SP6 or T7 RNA polymerases from a standard in vitro transcription kit followed by 5′ capping,

and poly(A) tail addition resulting in a high-yield, full-length 11,500 nt RNA transcript. In our hands, both SP6 and T7 RNA polymerases readily produced high-yield in vitro transcripts in excess of 14,000 nt (Figure S1A available online).

Exposure of cells to single-stranded VEE RNA induces a strong interferon (IFN)-α/β innate immune response. To mitigate the innate immune response to VEE-GFP RNA, we utilized B18R protein from Western vaccinia virus that binds to and neutralizes type I IFNs (Alcamí et al., 2000). We compared GFP expression in primary human foreskin fibroblasts (HFFs) transfected with VEE-GFP RNA alone or cotransfected with B18R mRNA. Consistent with induction of a strong innate immune response to cells exposed to single-stranded RNA, in the absence of B18R, we observed little-to-no GFP expression 1 day after transfection (Figure S1B). In contrast, cotransfection of VEE-GFP RNA replicon with B18R mRNA resulted in high levels of GFP expression in HFFs (Figure S1B), showing that B18R is required for efficient expression of proteins from the VEE RNA replicon.

The generation of iPSCs requires consistent, high-level expression of reprogramming factors for >7 days; therefore, we examined the persistence of the VEE-GFP RNA replicon in human primary fibroblasts over 7 days. To continuously suppress the innate immune response over several weeks while avoiding daily transfection of B18R mRNA, we prepared conditioned media harvested from human fibroblasts expressing B18R protein (B18R-CM) (Figures S1C and S1D). HFFs were cotransfected with VEE-GFP RNA replicon and B18R mRNA (3:1 ratio) on day 0, then cultured in the presence or absence of 20% B18R-CM plus/minus puromycin on day 1 (Figure 1B). Puromycin selection in the presence of B18R-CM resulted in a >90% GFP-positive population, whereas puromycin selection in the absence of B18R-CM resulted in <1% viable GFP cells (Figures 1B and 1C). We also observed that the level of GFP expression in the presence of B18R-CM gradually decreased from day 1 to day 4 but then remained steady out to day 7. In contrast, the level of GFP expression in the absence of B18R-CM continuously dropped to <10% intensity (Figure 1B). VEE GFP replicon persistence was dose dependent on B18R-CM (Figures S1E and S1F). We note the persistence of high levels of GFP expression from VEE-GFP RNA-treated fibroblasts for over a month when continuously cultured in the presence of B18R-CM and puromycin (data not shown). Taken together, these results showed both the necessity of B18R protein to overcome the VEE RNA-induced innate immune response and also demonstrated the ability to selectively retain or degrade the VEE RNA replicon from cells by exposure to or withdrawal from B18R-CM.

Generation of iPSCs by VEE RNA Replicon

We next engineered the VEE RNA replicon 3′ ORF to encode four reprogramming factors (*OCT4*, *KLF4*, *SOX2*, with *c-MYC* or *GLIS1*), which avoids the potential genomic instability induced by *c-MYC* (Nakagawa et al., 2008; Maekawa et al., 2011). We generated and compared several VEE-RNA construct configurations (Figure 1A) using the following nomenclature: VEE-OMKS = *OCT4*, *c-MYC*, *KLF4*, *SOX2* separated by internal ribosomal skipping 2A peptides (Szymczak et al., 2004) followed by an IRES and Puro^r ORF; VEE-OKS-iM = *OCT4*, *KLF4*, *SOX2* separated by 2A peptides followed by an IRES then *c-MYC* and a second IRES and Puro^r ORF; and VEE-OKS-iG = *OCT4*, *KLF4*, *SOX2* separated by 2A peptides followed by IRES then *GLIS1* and a second IRES and Puro^r ORF (Figure 1A). Similar to the VEE-GFP RNA protocol, VEE-RF RNAs were produced by SP6 or T7 in vitro transcription, 5′ capping, and poly(A) tail addition resulting in full-length VEE-OKS-iM RNA, VEE-OMKS RNA, and VEE-OKS-iG RNA (Figure S1A). Transfection of various VEE-RF RNA replicons with B18R mRNA into human fibroblasts resulted in high levels of expression of all four reprogramming factors that exceeded reprogramming factor expression levels from retroviruses on day 1 and day 10 (Figures 1D and S2B). Together, these observations demonstrated the ability to express four reprogramming factors and a Puro^r gene from a single, synthetic VEE-RF RNA replicon in primary human cells, while utilizing B18R to block the innate immune response.

To develop a VEE-RF RNA replicon-based iPSC protocol, we evaluated several parameters, including number and timing of VEE-RF RNA transfections, selection for VEE-RF RNA replicon retention by puromycin, and the genetic organization of the VEE-RF RNA replicon (Figures 1A, 2A, and S2). Human HFFs or BJ fibroblasts were cotransfected with VEE-RF RNA replicons and B18R mRNA on day 1, then cultured in the presence of 20% B18R-CM plus puromycin. Based on expression results of VEE-RF RNAs (Figures 1D and S2), we initially compared iPSC generation from either twice (days 1 and 3) or five-times (days 1, 3, 5, 7, and 9) transfections of VEE-OMKS RNA or VEE-OSK-iM RNA. Strikingly, alkaline phosphatase (AP)-positive staining iPS colonies were only generated from VEE-OKS-iM RNA in both BJ and HFFs, and no iPSC colonies were observed from VEE-OMKS RNA (Figure 2B). One significant difference between the two RNA vectors is the relative level of c-MYC expression to the other reprogramming factors with lower c-MYC levels expressed from the VEE-OKS-iM RNA, where c-MYC is the last ORF with a 5′ IRES, and higher levels of c-MYC expressed from the VEE-OMKS RNA non-iPS-generating replicon, where c-MYC is the second ORF and utilizes a 2A ribosome-skipping peptide (Figures 1A, 1D, and S2B). Moreover, we noted an inverse c-MYC expression sensitivity to generating iPSCs using retroviral vectors, where high c-MYC levels were correlated with a decreased number of iPSC colonies. Using VEE-OKS-iM RNA, we further optimized conditions for iPSC generation and found that B18R-CM was required until the appearance of iPSC colonies on feeding culture, whereas puromycin could be removed at the point of plating onto feeder cultures (Figure S2C).

To avoid the potential for genomic instability induced by c-*MYC*, we also generated a related VEE-OKS-iG RNA construct that substituted GLIS1 for c-*MYC* (Figure 1A). Initially, we found that several transfections of either VEE-OKS-iM RNA or VEE-OKS-iG RNA in the presence of B18R-CM and puromycin selection over the first 7 days resulted in the highest generation of AP-positive colonies (Figures 2C and 2D; Table S1). Starting with one well of a 6-well format (4×10^5 cells/well), we generally observed >100 iPS colonies per starting well from both HFFs and BJ fibroblasts when using three (or more) VEE-RF RNA transfections (Table S1). Although iPSCs were generated in the absence of puromycin selection, we observed a substantially reduced efficiency. To further refine the approach, we changed the type of media used during the first 7 days after transfection to either Advanced-DME or Pluriton, both of which resulted in

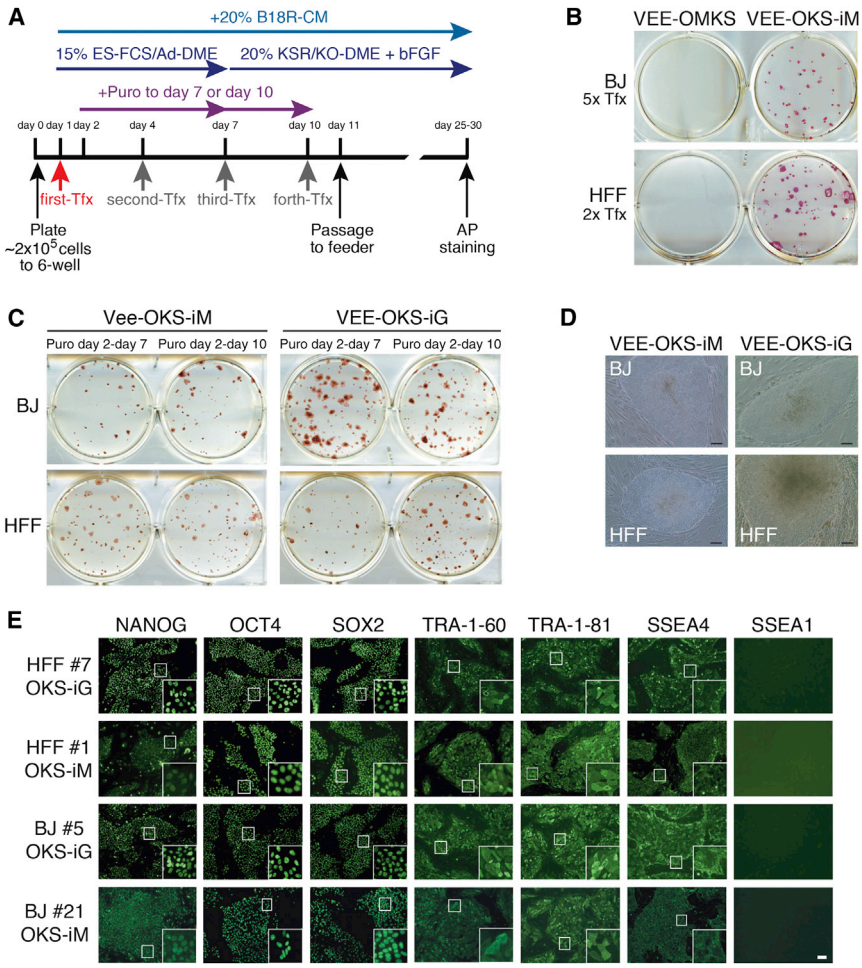

Figure 2. Generation of iPSCs by VEE-RF RNA

(A) Schematic of epigenetic VEE-RF RNA iPSC generation protocol. Human fibroblasts were plated on day 0 and cotransfected (Tfx) with VEE-RF RNA replicon plus B18R mRNA on day 1 (confluent, ~4 × 10^5 cells) and treated with puromycin until day 7 (or 10) as indicated. Cells were cultured in B18R-CM until iPSC colonies were isolated on day 25 (to 30).

(B) iPSC colonies stained with AP were generated with VEE-OKS-iM RNA, but not VEE-OMKS RNA. Transfection was performed on days 1 and 3 (2× Tfx), or 1, 3, 5, 7 and 9 (5× Tfx).

(C) AP staining of iPSC colonies generated from BJ or HFFs from day 1, 4, 7, and 10 transfection protocol as indicated.

(D) Typical images of iPSC colonies on day 26 by VEE-OKS-iM RNA and day 22 for VEE-OKS-iG RNA from BJ or HFFs fibroblasts as indicated. Scale bars, 100 μm.

(E) Immunohistochemistry staining of pluripotent ES marker genes in isolated iPSC clones generated as indicated. Similar results obtained for 26 additional iPSC clones (30 clones total). Scale bar, 100 μm; insets, 10× amplification.

See also Figures S2, S3, and S4.

a large number of AP-positive colonies from a single transfection of either VEE-OKS-iM RNA or VEE-OKS-iG RNA in BJ or HFFs (Table 1). Moreover, a single transfection of VEE-OKS-iM RNA or VEE-OKS-iG RNA produced from either SP6 or T7 RNA polymerases into human adult cells of normal human dermal fibroblasts (NHDF-c) (aged 50) and human dermal fibroblasts (HDFs) (aged 58) generated AP-positive colonies with a characteristic iPSC morphology (Table 1). Thus, we refined the methodology to generate iPSCs from a single transfection of the VEE-RF RNA replicon into both newborn and adult human fibroblasts.

Characterization of iPSC Clones

We mechanically isolated >100 iPSC colonies from multiple independent VEE-OKS-iM RNA and VEE-OKS-iG RNA protocols and had a >95% success rate for the ability of isolated iPS clones to continuously divide and retain a human embryonic stem cell (hESC) morphology. Of the >100 iPS morphology-like clones isolated, we analyzed 30 clones for expression of stem cell markers by immunofluorescence. All 30 VEE-RF RNA iPS clones analyzed (6× HFF VEE-OKS-iM clones, 12× BJ VEE-OKS-iM clones, 6× HFF VEE-OKS-iG clones, 6× BJ VEE-OKS-iG clones) showed strong nuclear staining of endogenous OCT4, SOX2, and NANOG and strong cell surface staining of SSEA4, TRA-1-60, and TRA-1-81, with negative staining of SSEA1 (Figure 2E). In addition, we examined six clones from HDF human adult fibroblasts and three clones from NHDF-c human adult fibroblasts by immunofluorescence and found that all clones expressed TRA-1-60, TRA-1-81, SSEA4, and NANOG but did not express SSEA1 (Figures S3 and S4).

Continuous exposure to B18R-CM was essential for both retention of the VEE-RF RNA replicon and iPSC generation (Figures 1B and S2C). Withdrawal of B18R-CM from iPS culture medium resulted in the elimination of the VEE-RF RNA replicon. To confirm the complete loss of VEE-RF-RNA replicons, we developed a highly sensitive and specific PCR protocol capable of detecting <10 fg of the VEE-RF-RNA replicon (Figure S5A). RT-PCR analysis of isolated RNAs showed that all iPSC clones had lost the VEE-RF-RNA replicon by passage 8, whereas most clones lost the RNA replicon in passage 5 or 6 (Figure 3; Table S2). Geuking et al. (2009) reported that nonretroviral RNA viruses under extreme conditions can recombine with endogenous retrotransposon genetic elements and result in a reverse transcription into DNA followed by genomic integration. Therefore, we examined VEE-RF iPSC clones for the presence of DNA copies. However, consistent with an RNA-only vector that does not go through a DNA intermediate, we did not detect any genomic integrations of VEE-RF by genomic PCR analysis or by Southern blot analysis (Figures S5B–S5E). A consistent concern for iPS generation protocols is the generation of aneuploid or tetraploid iPSC clones (Yu et al., 2007). By flow cytometry DNA analysis, we observed several tetraploid iPSC clones generated from VEE-RF OKS-iM RNA, but no tetraploid colonies were detected from VEE-RF OKS-iG RNA (data not shown).

Table 1. Generation of iPSCs by Single Transfection of VEE-RF RNA Replicon

RNA Replicon	Cell	Age	Tfx Day	Media	No. of AP+ Colonies/ Starting Well
SP6-OKS-iM	BJ	newborn	1	Pluriton	89
T7-OKS-iM	BJ	newborn	1	Pluriton	181
SP6-OKS-iM	BJ	newborn	1	Ad-DME	16
T7-OKS-iM	BJ	newborn	1	Ad-DME	54
SP6-OKS-iG	BJ	newborn	1	Pluriton	173
T7-OKS-iG	BJ	newborn	1	Pluriton	167
SP6-OKS-iG	BJ	newborn	1	Ad-DME	60
T7-OKS-iG	BJ	newborn	1	Ad-DME	55
SP6-OKS-iM	HFF	newborn	1	Pluriton	245
T7-OKS-iM	HFF	newborn	1	Pluriton	169
SP6-OKS-iM	HFF	newborn	1	Ad-DME	757
T7-OKS-iM	HFF	newborn	1	Ad-DME	422
T7-OKS-iG	HFF	newborn	1	Ad-DME	51
SP6-OKS-iM	NHDF-c	adult	1	Ad-DME	59
T7-OKS-iM	NHDF-c	adult	1	Ad-DME	5
SP6-OKS-iG	NHDF-c	adult	1	Ad-DME	31
T7-OKS-iG	NHDF-c	adult	1	Ad-DME	44
SP6-OKS-iM	HDF	adult	1	Ad-DME	8
T7-OKS-iM	HDF	adult	1	Ad-DME	4
Retroviruses O, K, S, M	BJ	newborn	–	Ad-DME	117
Retroviruses O, K, S, M	HFF	newborn	–	Ad-DME	294
Retroviruses O, K, S, M	NHDF-c	adult	–	Ad-DME	22

VEE-RF RNAs were generated with SP6 or T7 RNA polymerase as indicated. HFFs (male, newborn) or BJ (male, newborn) newborn fibroblasts or NHDF-c (female, age 50) or HDF (female, age 58) adult fibroblasts were cotransfected once with VEE-RF RNA replicon and B18R mRNA on day 1. Cells were selected with puromycin from days 2 to 10. Pluriton medium was present on days 1–10, and Advanced-DME (Ad-DME) was present on days 1–7, then changed to ES medium. All cultures were passaged to STO feeder cells and cultured in ES medium on day 10. Cells received daily 20% B18R-CM conditioned medium. iPSC colonies were stained with AP on day 24, and the number of colonies is indicated based on the starting well.
See also Table S1.

However, karyotype analysis of four independent iPSC clones generated from both OKS-iM and OKS-iG VEE-RF RNA replicons that showed normal DNA content by flow cytometry contained normal diploid karyotypes (Figure S6).

To further characterize the established iPSC clones, we examined expression of hES marker genes by qRT-PCR. Consistent with expression levels in human HUES9 ESCs, iPSC clones generated from both parental BJ and HFFs with either the VEE-OKS-iM RNA or VEE-OKS-iG RNA protocol expressed robust levels of endogenous *OCT4*, *SOX2*, *NANOG*, *LIN28*, *TDGF1*, *DNMT3B*, and *TERT*, in contrast to low or no expression levels in starting parental BJ and HFFs (Figure 4A). Likewise, VEE-OKS-iM RNA or VEE-OKS-iG RNA-generated iPS clones from human adult HDFs and NHDF-c fibroblasts also expressed *OCT4*, *SOX2*, *NANOG*, *LIN28*, *TDGF1*, *DNMT3B*, and *TERT* by qRT-PCR (Figures S3 and S4). A hallmark of induced pluripotency is reduced DNA methylation of CpG dinucleotides in the *OCT4* and *NANOG* promoter regions (González et al., 2011). Bisulfite genomic sequencing of both the *OCT4* and *NANOG* promoter regions showed extensive demethylation in iPSC clones compared to parental fibroblasts (Figure 4B). To investigate mRNA expression profiles in iPSC clones, we performed whole-genome RNA sequencing (RNA-seq). All four iPSC clones analyzed by RNA-seq showed unsupervised hierarchical clustering and expression signatures characteristic of human HUES9 ESCs that were highly divergent from parental human fibroblasts (Figures 4C and 4D). Together, these results demonstrate that VEE-RF RNA replicon-generated iPSCs have all of the expression profile hallmarks of hESCs.

Differentiation of VEE-RNA Replicon-Generated iPSC Clones

Finally, we tested the pluripotency of VEE-RF RNA replicon-generated hiPSC clones to differentiate in vitro and in vivo. First, we differentiated iPSCs in vitro into cardiomyocytes per the method of Yang et al. (2008). Four independent iPSC clones derived from either the OKS-iM or OKS-iG VEE-RF replicons (HFF OKS-iM #1, BJ OKS-iM #2 and #21, BJ OKS-iG #5) were treated with activin A, bone morphogenetic protein 4 (BMP4), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and dickkopf homolog 1 (DKK1) in serum-free media. Differentiated embryoid bodies (EBs) from all four iPS clones began spontaneously contracting at day 7 and were stably contracting on day 15 (Movie S1). Using antibodies against cTNT and α-actinin (Figure 5A), immunohistochemistry revealed that all four differentiated iPS EBs were positive for cardiomyocyte markers. These data combined with the spontaneously contracting EBs confirm the ability of VEE-RNA replicon-generated iPSC clones to differentiate into cardiomyocytes.

To test for in vivo pluripotency to differentiate into cells of all three germ layers, human VEE-RF RNA iPSC clones were injected into immunodeficient mice to generate differentiated teratomas. H&E of sections from two independent iPSC clones contained representative cell types of all three germ layers (ectoderm, endoderm, and mesoderm) that are spread throughout the sections (Figure 5B). Immunohistochemistry staining of four additional, independent iPSC-derived teratomas was positive for ectoderm markers AE1/AE3 (cytokeratin), NF-1 (neuronal cells), and GFAP (neuronal cells), mesoderm marker Desmin (muscle cells), and endoderm marker AFP (primitive and definitive endoderm) (Figure 5C). Collectively, these observations confirm the ability of both VEE-OKS-iM and VEE-OKS-iG RNA replicons to efficiently generate pluripotent hiPSCs.

DISCUSSION

The generation of iPSCs has great potential for the development of personalized stem cell therapies (Okita and Yamanaka, 2011; González et al., 2011; Hussein et al., 2011). Here, we devised a simple, straightforward and highly reproducible RNA-based approach to generate hiPSCs by a single transfection of a synthetic, polycistronic self-replicating VEE-RF RNA replicon that expresses four independent reprogramming factors plus

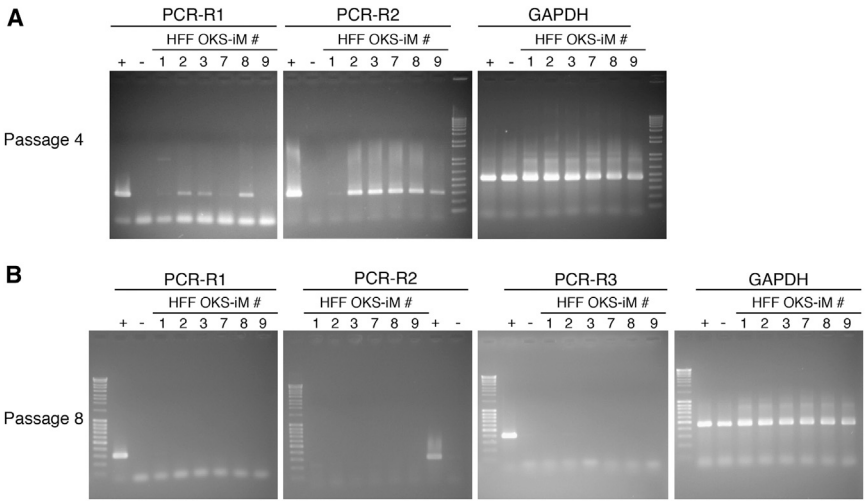

Figure 3. RT-PCR Analysis for Persistent VEE-RF RNA Replicon in iPSC Clones

(A and B) RT-PCR of HFF-OKS-iM iPSC clones from total RNA prepared from passage 4 (A) and passage 8 (B), as indicated. +, positive control, total RNA was prepared from 1 day after transfection of OKS-iM-RNA replicon. −, negative control, total RNA was prepared from mock-transfected HFFs.

See also Table S2.

a selectable marker (Puro^r) for positive selection of the RNA replicon. We found that both VEE-OKS-iM RNA and VEE-OKS-iG RNA replicons efficiently and consistently generated iPSCs from newborn and adult human fibroblasts that acquired full pluripotency by rigorous in vivo biological and molecular criterion that paralleled hESCs. By expressing four reprogramming factors at consistent levels and ratios over time in the same cell combined with replication of the VEE-RF RNA for a limited number of multiple cell generations, the VEE-RF RNA approach solves both of the major inefficiency problems associated with generating iPSCs by repetitive daily transfections of four individual RF mRNAs.

Consistently maintaining the ratio of reprogramming factors to each other appears to be critically important for efficiently generating iPSCs. As an example, we note that expression of too much c-MYC relative to the other factors by the VEE-OMKS RNA replicon dramatically inhibited iPSC generation, whereas the lower c-MYC ratio expressing VEE-OKS-iM RNA replicon was highly efficient at generating iPSCs. In contrast to simultaneously transfecting multiple mRNAs for each factor into cells, having the fixed factor ratio built into the single VEE-RF replicon RNA consistently results in the appropriate reprogramming factor expression levels and ratios. The generation of the VEE-RF-RNA transcript utilizes a standard SP6 or T7 RNA polymerase in vitro transcription kit that does not require special conditions and thereby further simplifies the approach for broad use. Although previously restricted to generating limited transcript lengths, current in vitro RNA transcription kits now exceed 25 kb in length (Schelle and Thiel, 2007), significantly longer than the ~15 kb length of the VEE-RNA replicons generated here. The ability to generate very long in vitro transcripts allowed us to generate VEE-RNA replicons with four pluripotent factor ORFs plus a puromycin selection gene (five ORFs).

Activation of the innate immune system has recently been reported to enhance iPSC generation (Lee et al., 2012). However, in our hands, inhibiting the innate immune response by B18R exposure was critical for increased efficiency of iPSC generation. We speculate that exposure of VEE-RF RNA-harboring cells to B18R-CM may sufficiently decrease the innate immune response to allow for VEE-RF RNA persistence, while retaining a low-level innate immune response to stimulate iPSC generation. Although we generated the data presented here by a fixed protocol of B18R mRNA cotransfection on day 1 followed by addition of B18R-conditioned media, we note in subsequent studies that addition of B18R condition media during the transfection, in lieu of B18R mRNA cotransfection, followed by daily addition of B18R-CM also sufficed to suppress the innate immune response and generated iPSCs.

The VEE-RF RNA approach is an ectopic hit-and-run approach that does not utilize a DNA intermediate, and therefore, there is no opportunity for integrative mutation that can occur with DNA vector-based types of iPSC approaches. Moreover, the timing of VEE-RF RNA replicon loss by degradation can be regulated by B18R-CM withdrawal from the media. Here, we used a combination of four reprogramming factors (*OCT4*, *KLF4*, and *SOX2*, with *c-MYC* or *GLIS1*) to reproduce the retrovirus iPSC generation. However, many factors to enhance reprogramming or generate safer iPSCs have now been reported to generate iPSCs (González et al., 2011; Hussein et al., 2011; Ben-David and Benvenisty, 2011; Mochiduki and Okita, 2012). Therefore, we note that the VEE-RF RNA approach has the versatility to be engineered to express alternative reprogramming factor combinations and/or insertion of additional reprogramming factor ORFs into the VEE RNA backbone for refining iPSC generation from specific cell types or for use in driving transdifferentiation. In summary, the non-DNA-based, self-replicating VEE-RF RNA approach has broad applicability for the efficient generation of hiPSCs for use in disease cell-modeling studies and eventual human cell therapy-regenerative medicine applications.

EXPERIMENTAL PROCEDURES

Experimental Oversight

All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of University of California, San Diego (protocol number S01103). Studies using human ESCs and hiPSCs were approved by the IRB/ESCRO committee at University of California, San Diego (project number 071552ZF).

Cells

BJ foreskin fibroblasts and STO cell line were obtained from ATCC, primary HFFs were kindly obtained from M. Haas (UCSD), and HUES-9 hESC line was from D. Melton (HMS). Adult donor NHDF-c and HDFs were obtained from PromoCell (c-12302) and Cell Applications (106-05a), respectively. BJ, HFFs, NHDF-c, HDFs, and STO were cultured in DMEM containing 10% FBS, MEM nonessential amino acids (NEAAs), pyruvate, penicillin, and

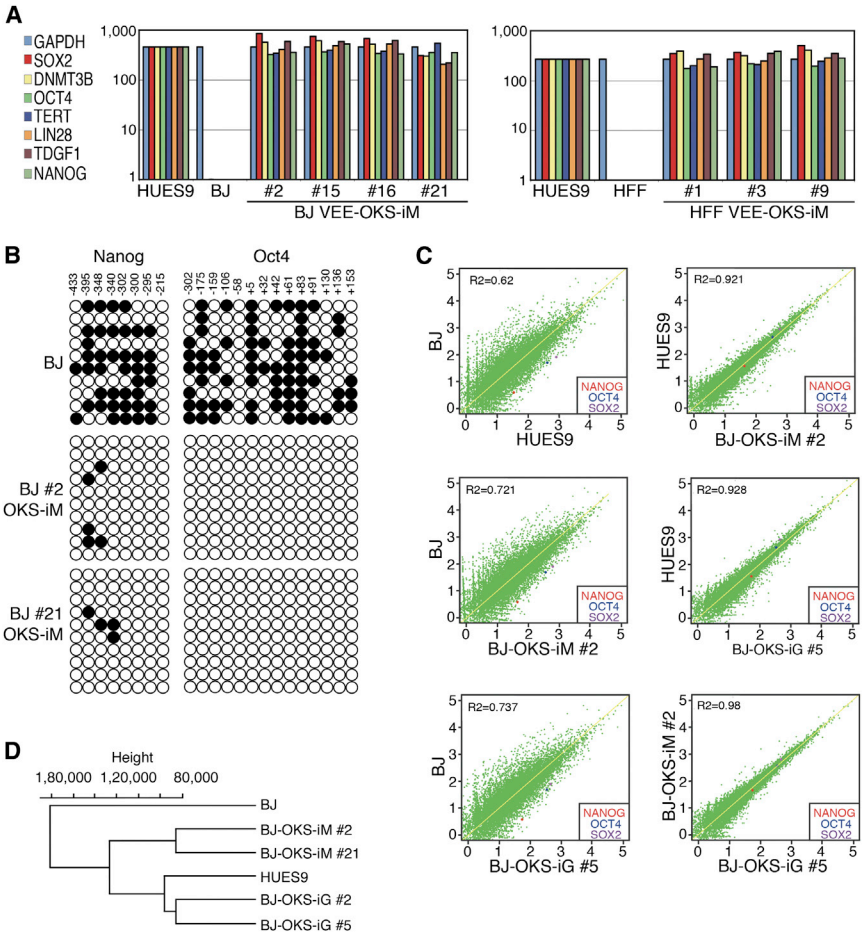

Figure 4. Characterization of VEE-RF RNA iPSC Clones

(A) Expression of ES maker genes by qRT-RCR analysis from indicated BJ and HFF VEE-RF RNA iPSC clones. Error bars represent the SD of triplicate samples.

(B) DNA methylation analysis of *NANOG* and *OCT4* promoter regions. Solid circle represents methylated; open circle is demethylated. Numbers indicate CpG position relative to transcription start site.

(C) Genome-wide mRNA sequence profile scatterplot analysis of BJ-OKS-iM #2 and BJ-OKS-iG #5 compared to parental human BJ fibroblasts and human HUES9 ESCs with pluripotency NANOG, OCT4, and SOX2 indicated.

(D) Unsupervised hierarchical dendrogram of genome-wide RNA sequence analysis showing clustering of four independent iPSC clones with HUES9 compared to BJ fibroblasts.

See also Figures S3, S4, S6, and Table S1.

streptomycin. HUES-9 and iPSCs were cultured with ES culture medium in Knockout D-MEM containing 20% Knockout SR, GlutaMAX, NEAA, 2-mercaptoethanol, penicillin, streptomycin, and bFGF (10 ng/ml). STO feeder cells were prepared by mitomycin C treatment (10 μg/ml; Sigma-Aldrich). Matrigel (BD Biosciences)-coated wells and conditioned medium of STO feeder cells were used for feeder-free culture.

Plasmid Construction

GFP/Pac genes and partial 3′ UTR in p5′VEE/S/GFP/Pac (VEE backbone plasmid; a kind gift from I. Frolov) were deleted with XbaI/MfeI digestion and then introduced the multiple cloning sites (Table S3): IRES and Puro^r. This vector was renamed as pVEE-IRES-Puro. Multicistronic expression of RFs with viral 2A peptide sequences (Table S3) and IRES was constructed in pBluescript SK+ vector and then cloned into pVEE-IRES-Puro to generate VEE-OS, VEE-OMKS, VEE-OKS-iM, VEE-OKS-iG plasmids. To generate RNA with T7 RNA polymerase, SP6 promoter (ATTTAGGTGACACTATAG) was replaced to T7 promoter (TAATACGACTCACTATAG) by PCR (Table S3) using the SacI/BstZ17I fragment of VEE vector as a template (SP6 promoter is located next to the SacI site). *B18R* (D01019) was obtained from Addgene and cloned into pTNT vector (Promega) for mRNA synthesis.

RNA Synthesis

VEE plasmids were linearized with MluI digest and used as templates for RNA synthesis. The synthesis of RNA replicon was performed with the RiboMAX Large Scale RNA Production System-SP6 or T7 (Promega) kit. After the DNase treatment, the synthesized RNA was purified by ammonium acetate precipitation (2.5 M). For the 5′ capping of RNA, we used ScriptCap m7G Capping System and ScriptCap 2′-O-Methyltransferase (Epicenter, currently available from CELLSCRIPT) to produce cap 1-capped RNA, which proceeds 100% efficiency of capping. After the 5′ capping of RNA, RNA was purified by ammonium acetate precipitation, and then additional poly(A) tail (~150 bases) was added by Poly(A) Polymerase (Epicenter, currently available from CELLSCRIPT). RNA was purified, resuspended in the RNA Storage Solution (Ambion) at 1 mg/ml concentration, and stored at −80°C. B18R mRNA was synthesized using the same replicon RNA protocol except for using 25% modified nucleotides (pseudouridine and 5-methyl-cytidine; TriLink BioTechnologies).

Preparation of B18R Conditioned Medium

B18R-CM (conditioned medium) was generated by B18R mRNA (1 μg for one well of a 6-well plate) transfection into HFFs with Lipofectamine 2000 (Invitrogen). After 3 hr, medium was changed to Advanced DMEM containing 15% FCS (ESC qualified; Millipore) or ES culture medium. Medium was collected on the next day, filtrated, and diluted with fresh culture medium to 20% final concentration.

iPSC Generation by Replicon Transfection

BJ and HFFs were passaged to a 6-well plate on day 0 and cultured to 90%–100% confluency (4×10^5 cells/well) on day 1. A total of 1 μg RNA mixture (3:1 ratio VEE RNA to B18R mRNA) was transfected with Lipofectamine 2000. After 3 hr, transfection medium was changed to the Advanced DMEM (Invitrogen) containing 15% FCS (ESC qualified). ES culture medium was used from day 7. One day after the final transfection, cells were passaged to STO feeder with several dilutions. ES medium containing B18R-CM was changed every day until iPSC colonies were generated. Colonies were mechanically picked for isolation of clones or subjected to AP staining with the Alkaline Phosphatase Detection Kit (Millipore) or manually prepared AP-staining solution containing 1 mg/ml of FastRed TR (Sigma-Aldrich) and 0.4 mg/ml of 1-Naphthyl phosphate (Sigma-Aldrich) in AP buffer (100 mM Tris, 100 mM NaCl, and 50 mM $MgCl_2$ [pH 9.5]).

Optimization of iPSC Generation with One-Time Transfection of Replicon

Cells were passaged on a gelatin-coated 6-well plate on day 0 and cultured to 90%–100% confluency on day 1. To minimize the IFN response, cells were started to treat with 20% B18R-CM 20 min before transfection. A total of 1 μg RNA mixture (3:1 ratio VEE RNA to B18R mRNA for SP6 VEE RNA, or 1:1 ratio for T7 VEE RNA) was transfected with Lipofectamine 2000. After 3 hr, transfection medium was changed to the Advanced DMEM or Pluriton medium (Stemgent) containing 20% B18R-CM. On day 7, Advanced DMEM

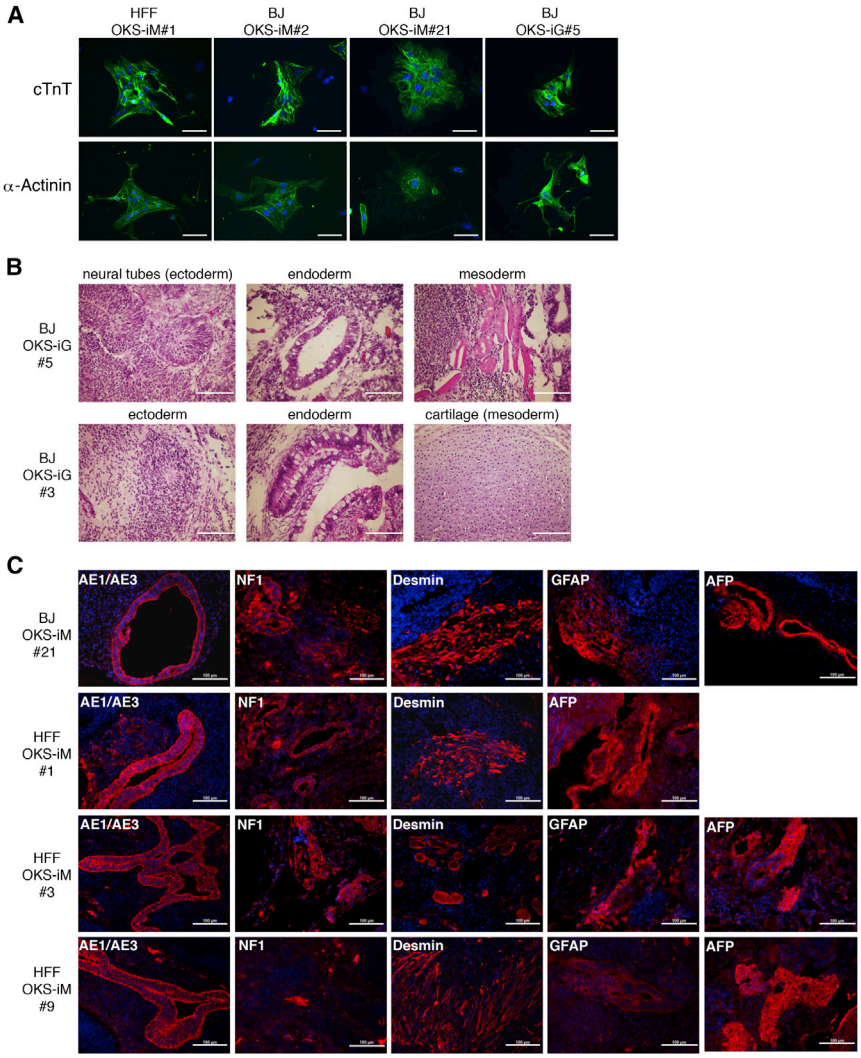

Figure 5. Differentiation Assays of VEE-RF RNA iPSC Clones

(A) VEE-RF RNA iPS clones were differentiated into cardiomyocytes as described in the Experimental Procedures. Contractile EBs were recorded (see Movie S1) and then dissociated and replated onto slides for immunofluorescence staining with mouse anti-Cardiac Troponin or mouse anti-α-actinin, Anti-Mouse IgG Alexa Fluor 488, and DAPI. Scale bars, 50 μm.

(B) Teratoma formation of VEE-RF RNA iPS clones in nude mice. H&E; Scale bars, 100 μm.

(C) Immunohistochemistry staining of VEE-RF RNA iPSC clone teratomas in nude mice. AE1/AE3 (cytokeratin), NF-1 (neuronal cells), and GFAP (neuronal cells) were used for markers of ectoderm, Desmin (muscle cells) was used for a marker of mesoderm, and AFP (primitive and definitive endoderm) was used for a marker of endoderm. Scale bars, 100 μm.

See also Movie S1 and Table S1.

was replaced to ES culture medium. Puromycin (0.8 μg/ml) was added from day 2 to 10. Cells were passaged onto STO feeder cells on day 10 and cultured in ES culture medium. B18R-CM was supplied every day until iPSC colonies were generated.

qRT-PCR

Total RNAs from feeder-free culture of iPSC clones, HUES-9, BJ, and HFFs were isolated with RNeasy mini kit. TaqMan RT-PCRs were carried out using RNA-to-Ct one-step reaction (Applied Biosystems). Primers and probes were obtained from AB TaqMan Gene Expression Assay catalog (GAPDH, Hs99999905_m1; POU5F1 Hs03005111_g1; Sox2 Hs01053049_s1; DNMT3B Hs00171876_m1; TERT Hs00972656_m1; Lin28 Hs00702808_s1; Nanog Hs02387400_g1; TDGF1 Hs02339499_g1). Data were analyzed on the 7300 real-time PCR system using the delta-delta Ct method.

Bisulfite Genomic Sequencing

Conversion of unmethylated cytosines into uracil of genomic DNA was performed with EZ DNA Methylation-Gold Kit (Zymo Research). The promoter region of OCT4 or NANOG was amplified by PCR (Table S3), cloned into the T vector, and then sequenced.

Immunoblotting

Cells were lysed with 2 × RIPA buffer containing 0.3 M NaCl, 80 mM Tris-HCl (pH7.5), 0.4% SDS, 2% Triton X-100, 2% sodium deoxycholate, 100 μg/ml phenylmethanesulfonylfluoride (PMSF), aprotinin (5 μg/ml), and leupeptin (5 μg/ml). Equal amounts of proteins (around 20 μg) were used for 9% SDS-PAGE and electroblotted onto a nitrocellulose membrane. Membranes were incubated with primary antibodies overnight at 4°C after blocking with 4% milk in PBS-T (0.05% Tween 20) and then incubated with horseradish peroxidase-conjugated anti-rabbit, goat, or mouse IgG (Santa Cruz Biotechnology). Protein bands were visualized using the ECL reagent (SuperSignal West Pico; Thermo Scientific).

Antibodies

Antibodies used in this research are as follows: anti-OCT4 (sc-9081), anti-KLF4 (sc-20691), anti-GLIS1 (sc-67584), anti-c-MYC (sc-42), TRA-1-60 (sc-21705), SSEA1 (sc-21702), SSEA4 (sc-21704), anti-mouse (sc-2005), anti-rabbit (sc-2004), and anti-goat (sc-2020) from Santa Cruz; anti-SOX2 (AF2018) and anti-NANOG (AF1997) from R&D Systems; TRA-1-81 (09-0011) from Stemgent; AE1/AE3 (RB-9010P0), Desmin (MS-376-S0), AFP (RB-365), and GFAP (RB-087) from LabVision; NF-1 (NB-300-155) from Novus Biologicals; anti-α-actinin (A7811) from Sigma-Aldrich; anti-Cardiac Troponin T (MS-295-P0) from Thermo Scientific; and Alexa Flour 488 anti-mouse (A11001) for cardiomyocytes analysis, Alexa Fluor 488 anti-mouse (A11029), Alexa Fluor 488 anti-rabbit (A11034), and Alexa Fluor 488 anti-goat (A11055) for immunostaining of iPS clones from Life Technologies.

RNA-Seq

Total RNAs were isolated with the RNeasy Mini Kit (QIAGEN), and cDNA libraries of each cell were synthesized and analyzed as described before by Fox-Walsh et al. (2011).

Cardiomyocyte Differentiation

hiPSCs were differentiated into cardiomyocytes as previously described with minor modifications (Yang et al., 2008). Briefly, iPSCs were treated with activin A, BMP4, bFGF, VEGF, and DKK1 in serum-free media at normoxia and video recorded on day 15. At day 16, spontaneously contractile EBs were dissociated, replated onto glass slides, fixed in 4% formaldehyde, and immunostained with mouse anti-α-actinin (Sigma-Aldrich; #A7811) or anti-Cardiac Troponin T (Thermo Scientific; #MS-295-P0) that were detected with Anti-Mouse IgG Alexa Fluor 488 (Life Technologies; #A11001).

Teratoma Formation

iPSC clones were intramuscularly or subcutaneously injected into the hindlimb muscles or dorsal flank of nude mice (approximately 10 cm dish-cultured cells for one shot of injection). After 5–8 weeks of injection, tumors were dissected and fixed with 4% paraformaldehyde, embedded into paraffin, and sectioned for H&E or immunostaining.

ACCESSION NUMBERS

RNA-seq data have been submitted and can be accessed by the Gene Expression Omnibus (GEO) accession number GSE38265.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, three tables, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2013.06.001.

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