Cell Stem Cell

Brief Report

Reprogramming of Mouse and Human Cells to Pluripotency Using Mature MicroRNAs

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SUMMARY

Induced pluripotent stem cells (iPSCs) can be generated from differentiated human and mouse somatic cells using transcription factors such as Oct4, Sox2, Klf4, and c-Myc. It is possible to augment the reprogramming process with chemical compounds, but issues related to low reprogramming efficiencies and, with a number of protocols, residual vector sequences, remain to be resolved. We show here that it is possible to reprogram mouse and human cells to pluripotency by direct transfection of mature double-stranded microRNAs (miRNAs). Our approaches use a combination of mir-200c plus mir-302 s and mir-369 s family miRNAs. Because this reprogramming method does not require vector-based gene transfer, it holds significant potential for biomedical research and regenerative medicine.

Induced pluripotent stem cells (iPSCs) can be directly generated from fibroblast cultures by expression of only a few defined factors, such as Oct4, Sox2, Klf4, and c-Myc (Takahashi and Yamanaka, 2006). Since the initial description of this approach, numerous studies have described modifications of the original protocol using additions or alternatives to these factors, including small molecules, with the aim of improving the efficiency and/or moving it toward potential clinical application (for example, Huangfu et al., 2008a, 2008b; Yoshida et al., 2009; Judson et al., 2009; Esteban et al., 2010). Genomic modification also remains a concern, because viral vector-mediated transduction of reprogramming genes involves random insertions of exogenous sequences into the genome. Some recent studies have indicated that iPSCs can be obtained with virus-free, removable PiggyBac transposons or episomal systems (Okita et al., 2008; Kaji et al., 2009; Woltjen et al., 2009; Jia et al., 2010), but these approaches still use DNA constructs, so the possibility of genomic integration of introduced sequences remains. Sendai virus has been used as an alternative (for example, in Seki et al., 2010), because it has an RNA genome, but it is still a virus. Reprogramming using just protein or mRNA has also been reported, but the protocols involved are technically challenging (Kim et al., 2009; Zhou et al., 2009; Warren et al., 2010).

MicroRNAs (miRNAs) are well-characterized regulators of development and differentiation (Lee et al., 1993; Ruvkun, 2001). Recent reports have demonstrated that specific miRNAs are highly expressed in embryonic stem cells (ESCs) and play a critical role in the control of pluripotency-related genes (Houbaviy et al., 2003; Judson et al., 2009; Suh et al., 2004). To identify candidate miRNAs to test for reprogramming activity, we analyzed miRNA expression in mouse ESCs, mouse iPSCs, and adult mouse adipose stromal cells (mASCs). miRNAs that were expressed >2-fold more strongly in mouse iPSCs and ESCs relative to mASCs (Figure S1A available online) were used for subsequent transfection assays (Figure S1D). The transfection efficiency was assessed by fluorescence microscopy using Fluorophore-labeled miRNAs, and we determined that 75.1% ± 0.1% of cells were fluorescent positive (data not shown). We introduced the miRNAs into mASCs obtained from *Nanog* promoter-driven green fluorescent protein (GFP) reporter mice (Okita et al., 2007). Real-time RT-PCR analysis indicated that transfected miRNA levels decreased markedly 72 hr post-transfection, so the transduction method was optimized to include four transfections at 48 hr intervals (Figures S1B and S1C; Table S1A available online). Eight days after initial transfection, the cells were passaged and grown in ESC-maintaining medium. We were able to detect GFP expression on day 14 after the transfection of mir-200c, mir-302 s, and mir-369 s family miRNAs, and by day 15 we observed approximately five GFP-positive colonies from 5×10^4 cells (Figures 1A–1D), giving an apparent efficiency that is comparable to that seen with the original report of retrovirus-mediated transcription factor introduction (Takahashi and Yamanaka, 2006). These colonies were not seen in mock transfectants in which negative control miRNAs were introduced (data not shown). Our analysis showed that introduction of all three of these miRNA candidates into mASCs from *Nanog* reporter mice successfully generated GFP-positive colonies, whereas the transduction of just one or two factors in

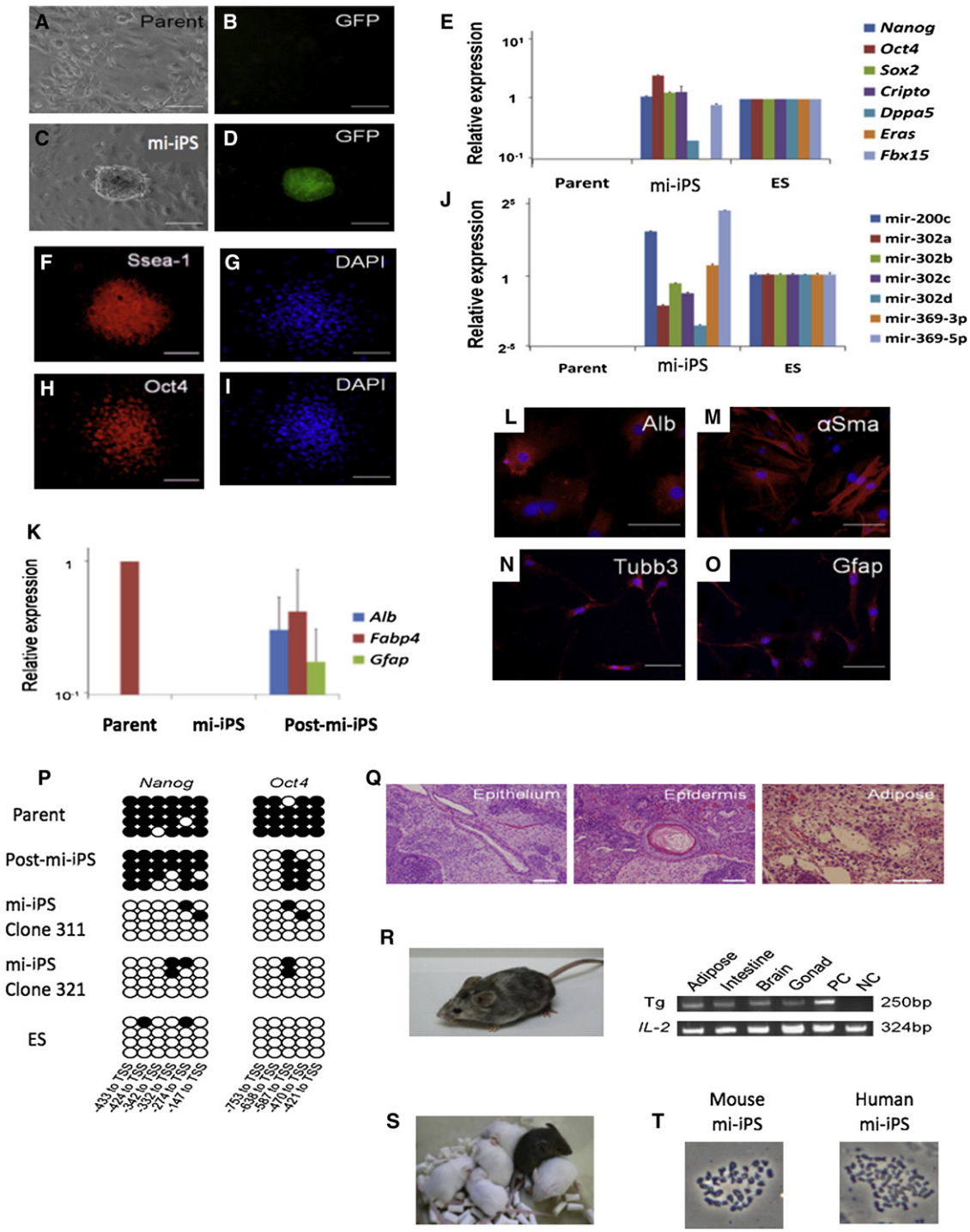

Figure 1. Induction of ES-like Cells from mASCs Using miRNAs

(A–D) Morphology of transfected mASCs. At day 15, colonies with defined margins and round shapes (C and D) that were distinct from parental cells (A and B) were observed. Brightfield (left) and fluorescence (right) images of parental and transfected cells are shown.

(E) The mi-iPSCs (clone 311) expressed undifferentiated ESC-marker genes. The expression of mRNA copies was normalized against *Gapdh* mRNA expression (mean ± SEM; n = 3).

(F–I) Immunocytochemistry revealed mi-iPSCs expressing Ssea-1 (F and G) and Oct4 (H and I).

(J) The expression of miRNA copies was normalized against U6 expression (mean ± SEM; n = 3).

(K–O) The differentiation of mi-iPSCs was induced by EB-like formation. The morphology of EB-like formation is shown in Figure S2D. (K) Real-time RT-PCR analysis verified the expression of differentiation markers, such as *Alb*, *Fabp4*, and *Gfap*. The expression of mRNA copies was normalized against *Gapdh* mRNA

any combination did not (experiments summarized in Figure S1D). We thus used all three candidates, mir-200c, mir-302 s, and mir-369 s, in subsequent studies. We refer to the reprogrammed cells generated using this approach as miRNA-induced pluripotent stem cells (mi-iPSCs).

Real-time RT-PCR (Table S1B) data revealed that on post-transfection day 30, mi-iPSCs expressed several genes characteristic of undifferentiated ESCs, such as *Nanog*, *Oct4*, *Sox2*, *Cripto*, *Dppa5*, and *Fbx15* (Figure 1E). The mi-iPSCs also expressed ESC-specific markers, such as Ssea-1 and Oct4 (International Stem Cell Initiative et al., 2007; Takahashi and Yamanaka, 2006) (Figures 1F–1I). In addition, greater mir-200c, mir-302 s, and mir-369 s expression was observed in mi-iPSCs than in the parental cells (Figure 1J). Expression profiling revealed the greater similarity of mi-iPSCs to mouse iPSCs (Takahashi and Yamanaka, 2006) and ESCs than mASCs (Figure S2E; accession number GSE28586). Karyotype analysis showed that 66.7% of the cells had a normal chromosome complement (40 mouse chromosomes in 8/12 cells) (Figure 1T).

To evaluate the differentiation capacity of mi-iPSCs, we examined the formation of embryoid bodies (EBs) in floating culture (Zhou et al., 2009) (Figure S2D). In suspension culture, mi-iPSCs formed ball-shaped EB-like structures. We transferred them to primary culture conditions (PCCs) consisting of gelatin-coated plates maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Attached cells, which we termed post-mi-iPSCs, were analyzed as described below and were compared with mi-iPS and parental cells. Twenty days after transfer to PCCs, the post-mi-iPSCs exhibited various morphological changes and expressed several genes characteristic of differentiation into all three germ layers. Real-time RT-PCR analysis confirmed *Alb* (endoderm), *Fabp4* (mesoderm), and *Gfap* (ectoderm) expression in post-mi-iPSCs (Figure 1K). The expression level of ES-like genes that were highly expressed in ESCs and mi-iPSCs was significantly reduced in post-mi-iPSCs (Figure S2A). Immunocytochemistry detected post-mi-iPSCs that were positive for Alb, αSma (mesoderm), Tubb3 (ectoderm), and Gfap (Figures 1L–1O and S2B). mir-200c and mir-302 s miRNA expression was lower in post-mi-iPSCs than in mi-iPSCs and ESCs (Figure S2C).

We next performed bisulfite genomic sequencing analyses to study the methylation status of cytosine guanine (CpG) dinucleotides in the promoter regions of pluripotency-associated genes, such as *Nanog* and *Oct4* (Table S1C; Zhou et al., 2009). The results revealed that the CpG dinucleotides of these gene promoters were less frequently methylated in mi-iPSCs and ESCs than in post-mi-iPSCs and parental cells (Figure 1P). These findings are consistent with reactivation of the promoter regions of immature status-related genes in mi-iPSCs.

To examine their in vivo differentiation properties, mouse mi-iPSCs were subcutaneously transplanted into the dorsal flanks of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. Teratoma formation was observed in mice 12 weeks postinjection (Figure 1Q). Histological analysis revealed that the tumors contained various tissues found in epithelial (endoderm), epidermal (ectoderm), and adipose (mesoderm) tissues. Furthermore, the injection of blastocysts with mi-iPSCs resulted in the generation of chimeric mice (Figure 1R and Table S1D). Genotyping (Table S1E) of the chimeric mice demonstrated the contribution of mi-iPSCs in intestinal, brain, adipose, and gonadal tissues, i.e., across all three germ layers. Crossing the chimeric mice resulted in the generation of progeny mice with the same coat color distribution as the original mi-iPSC parental strain, although the frequency of this occurrence was low (Figure 1S). Nevertheless, overall we have shown clearly that we can generate mouse mi-iPSCs that have acquired pluripotency via the introduction of synthesized miRNA from mASCs (Figure 1). We have also been successful in generating mi-iPSCs from MEFs, albeit with lower efficiency (one to five colonies from 5×10^5 cells) (data not shown).

To evaluate whether we can also reprogram human cells using this approach, we transfected mir-200c, mir-302 s, and mir-369 s into human ASCs (hASCs) and human dermal fibroblasts (HDFs) (results summarized in Table S2). Cells seeded at densities of 1×10^4 to 5×10^4 cells/well in 6-well culture plates were transfected with miRNAs through the same four-cycle protocol as for mouse cells at 48 hr intervals, and on day 8 the cells were harvested by trypsinization and transferred to ESC culture conditions according to methods described previously (Miyoshi et al., 2010). Twenty days after initial transfection, some colonies with sharp and defined margins and a different morphology from parental cells appeared (Figure 2A), at a frequency of two colonies from 1×10^5 adult human cells. We detected undifferentiated ESC marker gene expression in these colonies by immunocytochemistry (Figures 2B and 2C). Real-time RT-PCR revealed that these human mi-iPSCs expressed ES-like genes, including *NANOG*, *OCT4*, *SOX2*, and *LIN28* (Figure 2D). Using RT-PCR, we also found that hASC- and HDF-derived mi-iPSCs express high levels of mir-200c, mir-302 s, and mir-369 s (Figure 2E). The karyotype was predominantly normal (46 human chromosomes were observed in 10/12 cells [83.3%]; Figure 1T). To examine their differentiation

expression (mean ± SEM; n = 3). (L–O) Two weeks later, attached post-mi-iPSCs exhibited various morphologies, and immunocytochemistry confirmed the expression of Alb (L), αSma (M), Tubb3 (N), and Gfap (O) in these cells.

(P) *Nanog* and *Oct4* were not appreciably methylated in mi-iPSCs (clones 311 and 321), whereas the CpG dinucleotides of the regions were methylated in parental and post-mi-iPSCs (open and closed circles indicate unmethylated and methylated, respectively).

(Q) mi-iPSCs were subcutaneously transplanted into the dorsal flanks of NOD/SCID mice, and tumor formation was exhibited in various tissues as epithelial (endoderm), epidermal (ectoderm), and adipose (mesoderm) layers.

(R) Chimeric mice were gauged by coat color. Genotyping of the chimeric mice demonstrated that mi-iPSCs contributed to adipose, intestinal, brain, and gonadal tissues. Il-2 was used as an internal control. Tail tips of a *Nanog* reporter mouse and parental mouse were used as positive and negative control templates, respectively. Tg, transgene characteristic of *Nanog* reporter mice; PC, positive control; NC, negative control.

(S) Germline transmission. The crossing of chimeric mice (>70% BL6 hair color contribution, as shown in R) with Bulb/c mice resulted in the generation of black mice at a frequency identical to that observed in the original murine mi-iPSC strain (the frequency was less than 1/20).

(T) The karyotype of human and mouse mi-iPSCs. Forty-six chromosomes in human mi-iPSCs and forty chromosomes in mouse mi-iPSCs are shown.

See also Figures S1 and S2 and Tables S1 and S2. Bar = 100 μm; original magnification, × 200.

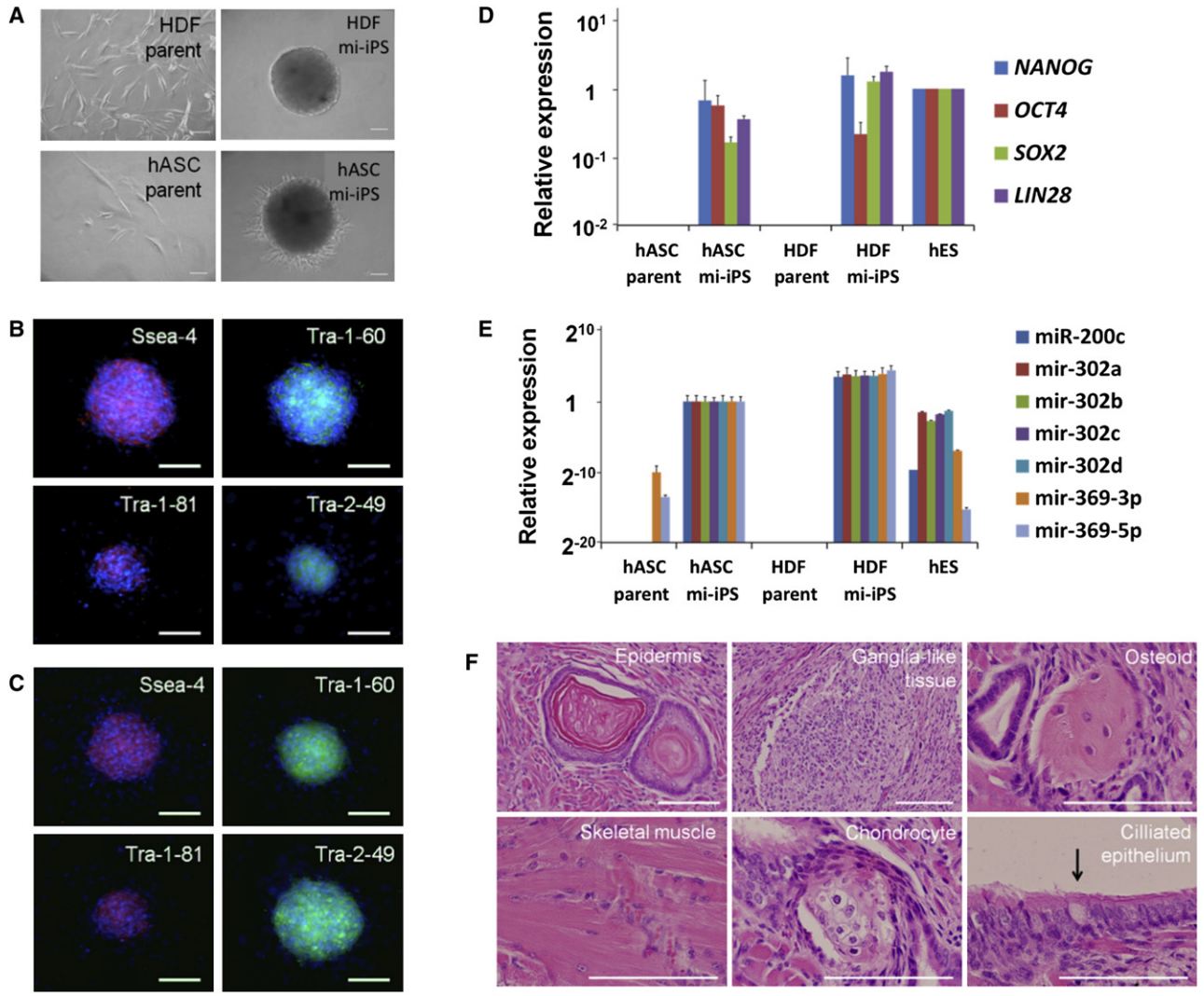

Figure 2. Pluripotency-Associated Gene Expression in mi-iPSCs from Human Somatic Cells

(A) At day 20, some colonies with sharp and defined margins appeared that were morphologically different from the parental hASCs and HDFs.

(B and C) Immunocytochemistry revealed mi-iPSCs from hASCs (B) and HDFs (C) expressing Ssea-4, Tra-1-60, Tra-1-81, and Tra-2-49.

(D) The mi-iPSCs from hASCs and HDFs highly expressed undifferentiated ESC-marker genes compared to the observed expression in their respective parental cells. The expression of mRNA copies was normalized against *GAPDH* mRNA expression (mean ± SEM; n = 3).

(E) Real-time RT-PCR analysis of miRNAs in parental mi-iPSCs. The expression of miRNA copies was normalized against *RNU48* expression, and the mean expression of hASC mi-iPSC was set to 1 for each gene (mean ± SEM; n = 3).

(F) mi-iPSCs (clone 3621A1) were transplanted subcutaneously into the dorsal flanks of NOD/SCID mice, and teratomas formed in various tissues and cells as epidermal, ganglia-like, osteoid, skeletal muscle, chondrocyte, and ciliated epithelial tissue (arrow, goblet cell).

See also Figure S2 and Tables S1 and S2. Bar = 100 μm; original magnification, × 200.

capacity, human mi-iPSCs were subcutaneously transplanted into the dorsal flanks of NOD/SCID mice. Teratoma formation was observed in mice 12 weeks postinjection (Figures 2F and S2F). Histological findings indicated that the tumors contained various tissues found in the epithelial (endoderm), epidermal (ectoderm), and adipose (mesoderm) tissues, representing all three germ layers. Overall, these findings suggest that direct transfection of mature miRNAs can also induce pluripotency in human somatic cells, and that these miRNA properties are conserved across species.

Our findings are consistent with previous analyses of miRNA function in pluripotent cells. Microarray analysis indicated that the promoter region of mir-302 s was conserved across species and driven by the transcription factor Oct4 (Marson et al., 2008). Members of the mir-302 s family were investigated as important key factors in the maintenance of ESC renewal and pluripotency as zygotic inhibitors of premature cell differentiation during early embryonic development (Rosa and Brivanlou, 2011). TargetScan (http://www.targetscan.org/) prediction for the mir-302 s family indicated that they inhibit the amine oxidase domain 1 (*Aof1*)

gene, which is associated with DNA methylation (Ciccone et al., 2009; Gregory et al., 2008). We found that *Aof1* was specifically repressed in mi-iPSCs, but not in parental cells (data not shown). During the course of reprogramming by defined factors, the morphology of somatic fibroblasts changed after cell-to-cell interactions, and iPSCs begin expressing E-cadherin, an epithelial cell marker highly expressed in ESCs (Gregory et al., 2008). These reports support our experimental results with mir-200c, which represses the epithelial-mesenchymal transition by inhibiting TGFβ signaling and is highly expressed in epithelial cells (Gregory et al., 2008). TargetScan prediction for mir-369 s also indicates that it inhibits *ZEB2*-related TGFβ signaling (Grimson et al., 2007) (data not shown). In our study, we could not generate GFP-positive colonies in mASCs from *Nanog* reporter mice using transfection of only one or two of these miRNAs. We concluded that all three of these factors are essential to generate PSCs from somatic cells. Other potential combinations of miRNAs that may also be able to generate pluripotent cells from somatic cells remain to be assessed.

Most of the reprogramming methods reported so far involve introduction of genetic materials and thus run the risk that exogenous vector sequences can be integrated into the host genome (Okita et al., 2008; Kaji et al., 2009; Woltjen et al., 2009; Jia et al., 2010). Very recently, highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency was reported (Anokye-Danso et al., 2011), but using integrating viral vectors and not direct transfection of mature miRNAs. Previous studies have also indicated that mir-302 s has reprogramming activity in human cells (Lin et al., 2011). Our study uses a different methodological approach, direct transfection of mature miRNAs, that has benefits from the perspective of potential clinical translation, although it does operate at considerably lower efficiency. We have successfully generated mi-iPSCs using this protocol more than six times. The resulting mi-iPSCs are subject to a reduced risk of mutations and tumorigenesis relative to most other protocols because mature miRNAs function without vectors or genomic integration. We also note that miRNA-based approaches are already under direct clinical investigation in a number of other therapeutic contexts. For example, a recent report demonstrated the utility and safety of therapy with miRNA in the treatment of hepatitis C (Lanford et al., 2010). We hope that mi-iPSC generation will eventually prove to be of significant benefit for both biochemical research and clinical regenerative medicine.

ACCESSION NUMBERS

The GenBank accession number for the expression profiling data reported in Figure S2E is GSE28586.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes Supplemental Experimental Procedures, two figures, and two tables and can be found with this article online at doi:10.1016/j.stem.2011.05.001.

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