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Resource

A High-Efficiency System for the Generation and Study of Human Induced Pluripotent Stem Cells

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SUMMARY

Direct reprogramming of human fibroblasts to a pluripotent state has been achieved through ectopic expression of the transcription factors OCT4, SOX2, and either cMYC and KLF4 or NANOG and LIN28. Little is known, however, about the mechanisms by which reprogramming occurs, which is in part limited by the low efficiency of conversion. To this end, we sought to create a doxycycline-inducible lentiviral system to convert primary human fibroblasts and keratinocytes into human induced pluripotent stem cells (hiPSCs). hiPSCs generated with this system were molecularly and functionally similar to human embryonic stem cells (hESCs), demonstrated by gene expression profiles, DNA methylation status, and differentiation potential. While expression of the viral transgenes was required for several weeks in fibroblasts, we found that 10 days was sufficient for the reprogramming of keratinocytes. Using our inducible system, we developed a strategy to induce hiPSC formation at high frequency. Upon addition of doxycycline to hiPSC-derived differentiated cells, we obtained "secondary" hiPSCs at a frequency at least 100-fold greater than the initial conversion. The ability to reprogram cells at high efficiency provides a unique platform to dissect the underlying molecular and biochemical processes that accompany nuclear reprogramming.

INTRODUCTION

While human fibroblasts and a multitude of mouse somatic cell types can be reprogrammed to pluripotency by ectopic expression of transcription factors (Takahashi and Yamanaka, 2006; Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007; Takahashi et al., 2007; Yu et al., 2007; Lowry et al., 2008; Aoi et al., 2008; Hanna et al., 2008; Stadtfeld et al., 2008a), the conversion is highly inefficient (~0.01%), making it difficult to examine the underlying molecular events. Further, all hiPSCs to date have been generated with retroviruses and noninducible lentiviruses, both of which are inefficiently silenced and maintain transgene expression. Another limitation in the generation of hiPSCs is the efficiency of viral gene transduction. While the fraction of cells infected by each individual factor and combinations of factors have not been addressed, only a small proportion of cells likely receive all factors. To overcome these limitations, we have established an inducible lentiviral system to generate "secondary" pluripotent cells, in which hiPSC clones are differentiated in vitro to yield fibroblast-like cells that harbor the inducible viral transgenes required for reprogramming. Because these cells maintain the same viral integrations that mediated the initial conversion to hiPSCs, this system bypasses the need for direct viral infection and produces a population of cells that can inducibly and homogeneously express the reprogramming factors. Such a system provides a powerful tool for mechanistic analysis, chemical and genetic screening for factors that enhance or block reprogramming, and the optimization of hiPSC derivation methods.

RESULTS

cDNAs encoding human OCT4, SOX2, cMYC, KLF4, and NANOG were cloned into doxycycline (Dox)-inducible lentiviral vectors as previously described (Stadtfeld et al., 2008b). In addition, a reverse tetracycline transactivator (rtTA) driven by the ubiquitin promoter was cloned into a lentiviral vector. To generate hiPSCs, we infected neonatal foreskin fibroblasts (BJ) and keratinocytes with lentiviruses containing the rtTA and either four (OCT4, SOX2, cMYC, and KLF4; for fibroblasts only) or five reprogramming factors (4 + NANOG; both fibroblasts and keratinocytes) according to the scheme in Figure 1A. Following infection, cells were plated onto mouse embryonic fibroblast feeder cells (MEFs) under hESC conditions and induced with Dox. From the fibroblast cultures, non-hESC-like colonies emerged approximately 2 weeks after the addition of Dox, as previously observed (Takahashi et al., 2007). These colonies contained only Oct4 and Myc integrations and could not be

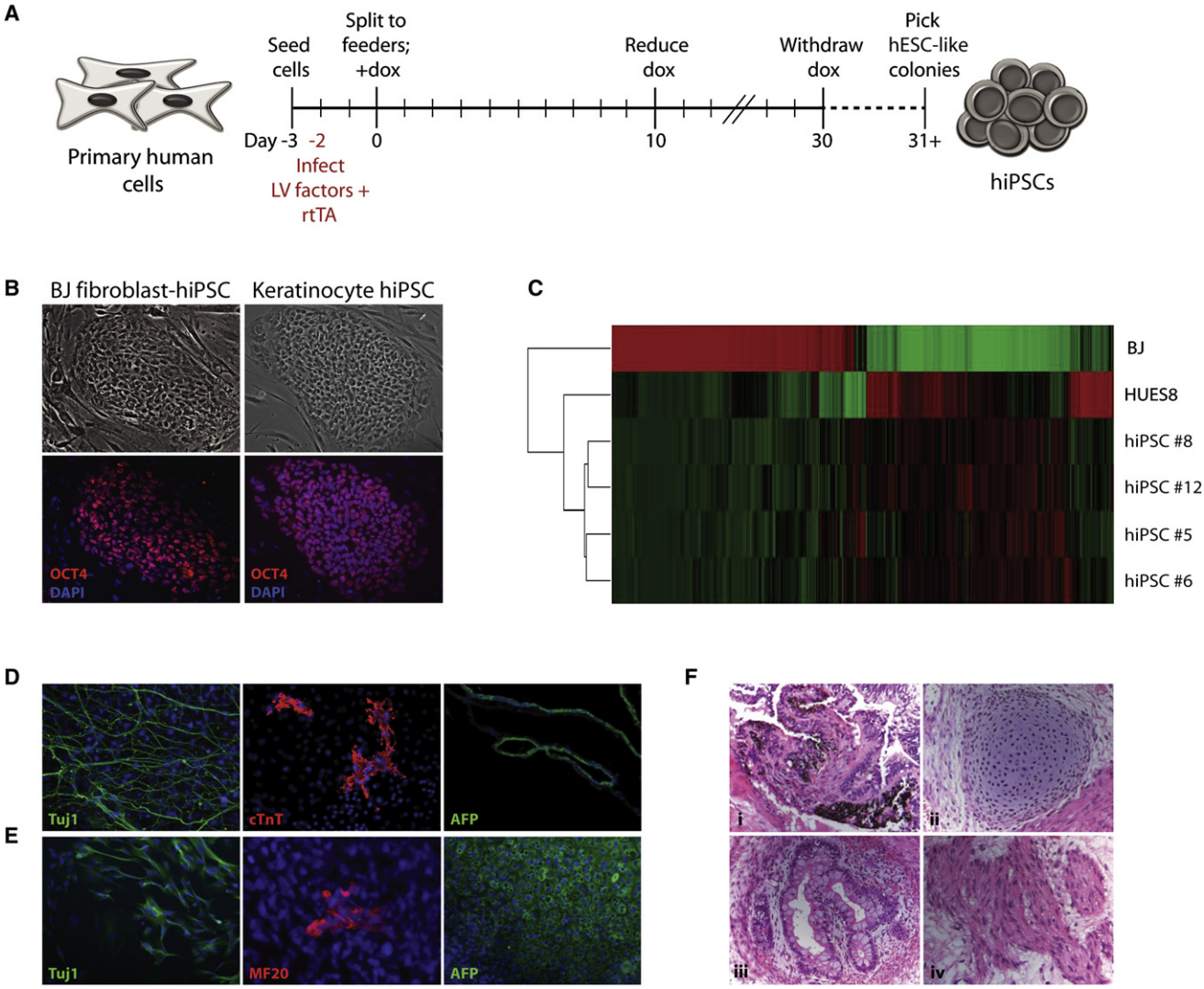

Figure 1. Generation of hiPSCs Using Inducible Lentiviruses

(A) Experimental scheme for the generation of hiPSCs. Primary human fibroblasts and keratinocytes were infected with separate lentiviruses (LV) containing a constitutively active rtTA and Dox-inducible reprogramming factors. After infection, cells were seeded to feeders, and Dox was applied for 30 days. hiPSC clones were picked based on hESC-like morphology and Dox-independent growth.

(B) Morphology and marker expression in hiPSC colonies. Dox-independent fibroblast- and keratinocyte-derived hiPSCs express OCT4 protein.

(C) Microarray analysis of gene expression in hiPSCs. Genes with greater than 2-fold expression level between HUES8 hESCs and BJ fibroblasts were analyzed. Shown are BJ fibroblasts, HUES8 hESCs, and BJ fibroblast-derived hiPSC clones.

(D) In vitro differentiation of fibroblast-derived hiPSCs into lineages from all three germ layers. Immunostaining for (i) Tuj1 (neuronal), (ii) cardiac troponin T (cTnT; cardiac muscle), and (iii) alpha-fetoprotein (AFP; epithelial, early endodermal).

(E) In vitro differentiation of keratinocyte-derived hiPSCs into lineages from all three germ layers. Immunostaining for (i) Tuj1, (ii) skeletal muscle (MF20), and (iii) alpha-fetoprotein.

(F) Hematoxylin and eosin stain of teratomas generated from fibroblast-derived hiPSCs. Differentiated structures from all three germ layers were present. (i) Pigmented epithelium (ectoderm), (ii) cartilage (mesoderm), (iii) gut-like epithelium (endoderm), and (iv) muscle (mesoderm).

expanded in the absence of Dox (data not shown). After 30 days of culture, colonies that resembled hESCs were observed, noted by a high nucleus-to-cytoplasmic ratio, prominent nucleoli, and well-defined phase-bright borders. All hESC-like colonies expressed the hESC-specific surface antigen Tra-1-81 (data not shown) and could be expanded in the absence of Dox. Colonies that did not resemble hESCs did not express Tra-1-81 and could not be passaged independent of Dox. Of ~2.5 × 10^5 infected fibroblasts seeded, four iPS colonies were obtained from each condition (four- or five-factor), representing a frequency of ~0.002%. From the keratinocyte cultures, large non-ES-like colonies appeared within 1 week; within 3 weeks, hESC-like colonies appeared and could be passaged in the absence of Dox. Of ~3 × 10^5 cells seeded, seven colonies emerged, similar to the frequency observed for hiPSC derivation from fibroblasts (~0.002%).

hiPSC colonies stained positive for OCT4 protein and the hESC-specific surface antigen Tra-1-81 (Figure 1B and data not shown). Further, these cells showed expression of pluripotency genes from the endogenous loci and lacked expression of the viral transgenes (see Figure S1A available online). To assess whether hiPSCs were molecularly similar to hESCs, we examined promoter methylation and performed global transcriptional analysis. The NANOG and OCT4 promoters in fibroblast-derived hiPSCs were demethylated to a similar extent as in hESCs, in contrast to the highly methylated promoters in BJ fibroblasts (Figure S1B), thus demonstrating epigenetic reprogramming in hiPSCs. Global analysis of gene expression in fibroblast-derived hiPSCs, hESCs, and fibroblasts was conducted by microarray through comparison of differentially expressed genes between fibroblasts and hESCs (Figure 1C), indicating that hiPSCs had repressed the fibroblast program of gene expression and reactivated an embryonic program of transcription.

Pluripotency of both fibroblast- and keratinocyte-derived hiPSCs was examined in vitro through embryoid body (EB) formation. After 7 days in suspension culture, EBs were explanted and gave rise to well-defined neuronal outgrowths and beating cardiomyocyte structures (data not shown). Immunofluorescence analysis confirmed the presence of neurons, cardiomyocytes, skeletal muscle cells, and epithelial structures (Figures 1D and 1E), thus demonstrating multilineage differentiation. As a more stringent test of pluripotency, fibroblast-derived hiPSCs cells were injected either subcutaneously or under the kidney capsule of immunodeficient SCID mice to assay for teratoma formation. Tumors were recovered after 10 weeks and contained well-defined structures arising from all three embryonic germ layers, including pigmented cells, cartilage, skeletal muscle, and gut-like epithelium (Figure 1F). These results indicate that hiPSCs generated with an inducible system strongly resemble hESCs and fulfill all criteria for pluripotency.

Noting that keratinocyte-derived hiPSC colonies appeared faster than fibroblast-derived hiPSCs, we sought to determine the minimum amount of time required to convert keratinocytes to hiPSCs. To test this, keratinocytes were infected with rtTA and five factors (OCT4, SOX2, cMYC, KLF4, and NANOG), and Dox was withdrawn at different time points throughout the reprogramming process. The number of hESC-like colonies was counted at day 30 and plotted against the day of Dox withdrawal (Figure S1C). hESC-like colonies first appeared after 18 days when Dox had been withdrawn after 10 days. The frequency of reprogramming appeared to decline with the length of Dox exposure, which may reflect unfavorable culture conditions at later time points or adverse effects of continued transgene expression.

To establish the system of "secondary" hiPSCs, we differentiated several fibroblast-derived hiPSC clones to fibroblast-like cells in vitro according to the scheme in Figure 2A. hiPSC colonies were placed in suspension culture for 1 week, and the resulting EBs were then plated to adherent conditions. Outgrowths of fibroblast-like cells were picked and passaged a minimum of three times prior to experimental manipulation to ensure that no residual pluripotent cells were present. Quantitative RT-PCR analysis confirmed a lack of pluripotency gene expression in these populations (Figure S2A).

We assessed the ability of hiPSC-derived cells to generate "secondary" hiPSCs through Dox addition and transfer to hiPSC derivation conditions. Fibroblast-like cells derived from two hiPSC clones demonstrated reprogramming in the presence, but not absence, of Dox (Figure 2B). The frequency of conversion ranged from 1% to 3%. Reinduction of viral transgenes in hiPSC-derived fibroblasts clones was also assessed by quantitative RT-PCR (Figure S2B), demonstrating a correlation between factor reactivation and the ability of the clone to produce secondary hiPSCs. Clones that gave rise to secondary hiPSCs showed reactivation of all factors (BJ hiPSCs #11 and #12), while those that did not lacked re-expression of a factor (BJ hiPSC #5) or re-expressed the factors at levels that are likely not permissive for the induction of pluripotency (BJ hiPSC #8).

Secondary hiPSCs were molecularly and functionally similar to primary hiPSCs and hESCs. They stained positive for OCT4 and Tra-1-81 (Figure 2C), had a similar gene expression profile to hESCs (Figure 2D), and demonstrated pluripotency in vitro, generating cell types from all three embryonic germ layers (Figure 2E).

To determine the temporal requirement of transgene expression for secondary hiPSC formation, we seeded 10^4 fibroblast-like cells per time point under hiPSC derivation conditions and withdrew Dox daily from days 4–19. The final number of Tra-1-81+ hESC-like colonies was counted at day 25 (Figure 2F). hiPSC colonies began to appear after withdrawal of Dox at day 6 (one colony), and the number of colonies increased with the time of Dox exposure, reaching a maximum after withdrawal at 16 days (frequency of ~2%). This increase in frequency with length of Dox exposure has also been reported in the reprogramming of mouse cells (Wernig et al., 2008).

Transformed granular colonies did not appear during secondary hiPSC induction, in contrast to the early colonies that appeared during primary induction with direct viral infection. The lack of these background colonies coupled with the high efficiency of the secondary system enabled us to monitor the progression of colonies during reprogramming. We tracked individual colonies on a daily basis with different time points of Dox withdrawal (Figure S3) and observed that induced cells transited through non-ES-like structures prior to acquiring a hiPSC phenotype. Not all colonies developed fully to hiPSCs; those that did not undergo successful reprogramming began to regress 2 days after Dox withdrawal and ultimately formed fibroblast-like structures. The colonies that successfully generated hiPSCs also showed some regression after withdrawal of Dox; however, hESC-like outgrowths gradually appeared, which could be expanded into stable hiPSC lines.

DISCUSSION

Here we have described the use of an inducible lentiviral system for the reprogramming of human somatic cells. Using this system, we converted neonatal foreskin fibroblasts and keratinocytes to a pluripotent state molecularly and functionally similar to hESCs. This system also enabled us to establish the temporal requirement of factor expression for cells undergoing reprogramming. While fibroblasts relied on transgene expression for several weeks, keratinocytes required only 10 days of factor expression to revert to a state that was poised to become pluripotent. It is unknown why keratinocytes are more amenable to reprogramming. Keratinocytes, like hESCs, represent an epithelial

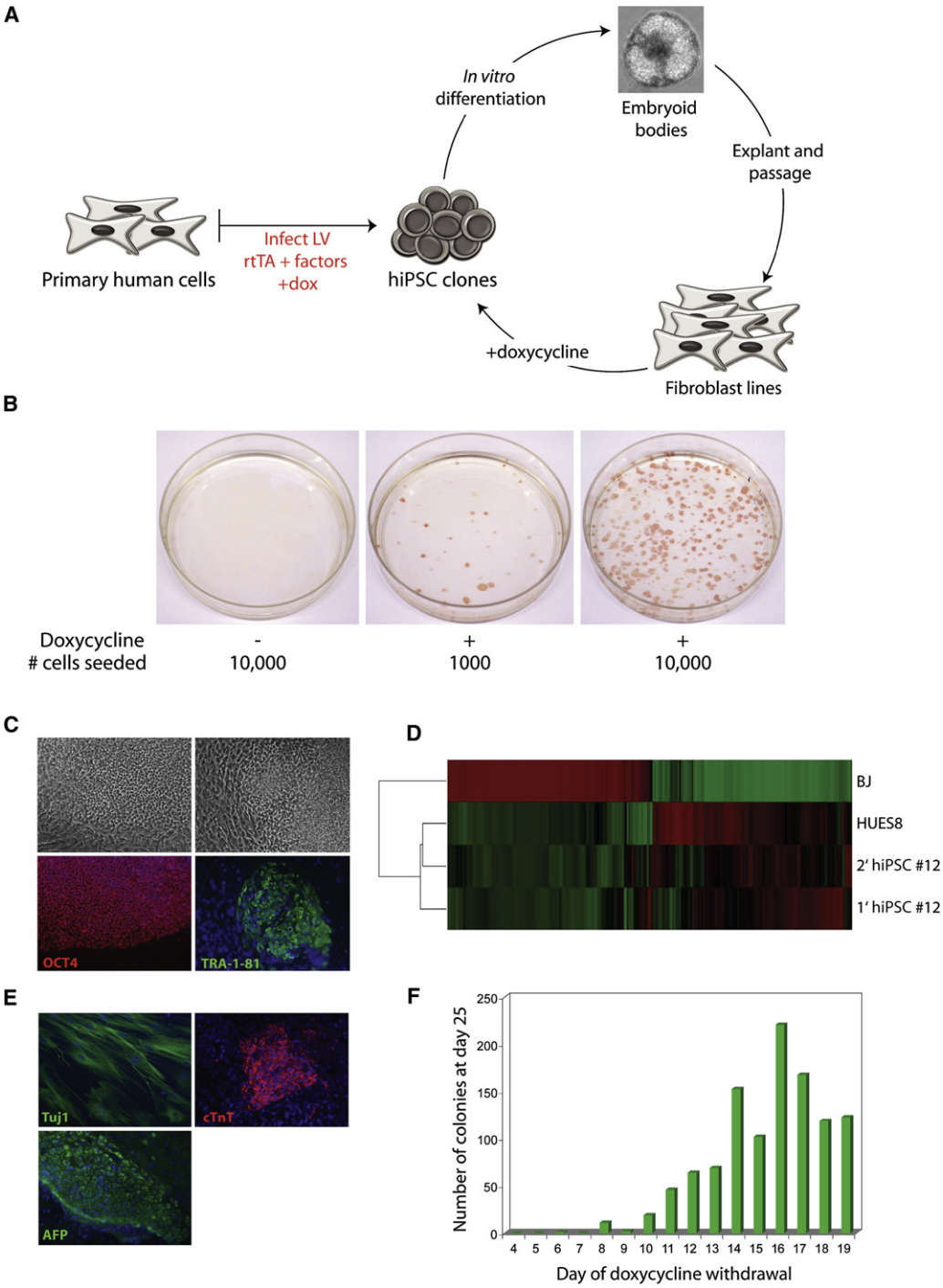

Figure 2. Generation of Secondary hiPSCs

(A) Experimental scheme depicting the generation of secondary hiPSCs. hiPSCs were differentiated in vitro as embryoid bodies for 7 days, then plated to adherent conditions. Fibroblast-like colonies were picked and expanded for at least three passages prior to undergoing reinduction by Dox.

(B) Alkaline phosphatase staining of reprogrammable cells grown in the absence or presence of Dox. Dox was withdrawn at day 21, and colonies were stained and counted at day 30.

(C) Morphology and expression of OCT4 and Tra-1-81 in Dox-independent secondary hiPSCs.

(D) Microarray analysis of gene expression between BJ fibroblasts, HUES8 hESCs, primary fibroblast-derived hiPSCs, and a resulting secondary hiPSC clone. Shown are genes with >2-fold expression value between BJ fibroblasts and HUES8 hESCs.

(E) In vitro differentiation of secondary hiPSCs into lineages from all three germ layers. Immunostaining for (i) Tuj1, (ii) cardiac troponin T, and (iii) alpha-fetoprotein.

(F) Temporal requirement of factor expression in hiPSC-derived fibroblast-like cells. 10^4 cells were plated per time point, and Dox was withdrawn daily from days 4–19. The hESC-like colonies that expressed Tra-1-81 were counted at day 25.

cell type and in contrast to fibroblasts may not need to undergo a mesenchymal-epithelial transition during reprogramming (Yang and Weinberg, 2008). Differences in reprogrammability between fibroblasts and keratinocytes may also be explained by differences in the cell-cycle status or viral infectivity. Moreover, keratinocytes express much higher levels of endogenous MYC and KLF4 than fibroblasts (Figure S1A), which may accelerate their conversion to hiPSCs. The fast kinetics of reprogramming observed for keratinocytes suggests that these cells would be useful for the development and optimization of methods to reprogram cells by transient delivery of factors.

Controlled expression of the reprogramming factors provided an inherent selection strategy by eliminating cells that were dependent upon viral transgene expression and conferring a growth advantage to fully reprogrammed cells that were Dox independent. This is in contrast to the constitutive retro- and lentiviral systems that have so far been used to reprogram human cells, where the viral transgenes maintain expression in the hiPSC state. The persistence of viral gene expression could have deleterious effects in clinical applications such as in vitro disease modeling; for example, the overexpression of Oct4 or Sox2 has been shown to promote the differentiation of mouse ES cells (Niwa et al., 2000; Kopp et al., 2008), suggesting that their continued expression during in vitro differentiation of hiPSCs may bias the resulting cell fate.

The extremely low efficiency of reprogramming led us to develop a system of "secondary" pluripotent cells in which we could reprogram hiPSC-derived differentiated cells at a high frequency. The >100-fold increase we observed was likely attributed to the ability to reactivate all factors within a given cell, thus enabling efficient reprogramming. The kinetics of this process were faster than that of primary fibroblasts but similar to keratinocytes, with the highest efficiency occurring after 16 days of factor expression. In vitro-derived fibroblasts appeared to be more amenable to reprogramming than primary fibroblasts, as previously observed (Yu et al., 2007; Park et al., 2008), which may reflect a lack of exposure to potent differentiation cues in vitro. However, four lines of evidence suggest that the reprogramming of hiPSC-derived differentiated cells is representative of the process that occurs in primary cells: (1) hiPSC-derived fibroblast-like cells lacked detectable expression of key pluripotency genes, (2) no colonies formed in the absence of Dox, (3) clones that did not reactivate all factors could not successfully form secondary hiPSCs, and (4) visual tracking of colonies demonstrated that cells transit through a non-hESC-like state prior to becoming bona fide hiPSC colonies. These data collectively support the use of a secondary system as a platform for mechanistic dissection of the reprogramming process.

Despite the fact that viral transgenes were reactivated in most of the hiPSC-derived cells, the frequency of reprogramming remained quite low, ranging from 1% to 3%. The basis for the low efficiency is poorly understood but may reflect a multitude of factors such as the starting cell type, cell-cycle status and the ability to undergo replication, variability in factor reactivation, and other stochastic events. Epigenetic events have been implicated in the reprogramming process; for example, it has recently been shown that valproic acid, which acts primarily as a histone deacetylase inhibitor, can enhance the efficiency of reprogramming (Huangfu et al., 2008). Also, DNA methylation and the activation of differentiation pathways have been shown to impede the reprogramming of mouse fibroblasts (Mikkelsen et al., 2008). However, these events account only for a small proportion of cells, and it will be interesting to further define the limiting factors that constrain the efficiency of reprogramming.

The secondary system of hiPSCs presents an attractive model for which to study the molecular events that underlie the reversion of human somatic cells to a pluripotent state. By providing a homogeneous population of cells that harbor all the viral transgenes, the cultures are not subject to the background of cells that do not receive all factors, allowing proper analysis of the reprogramming process. The secondary system will enable chemical and genetic screening efforts to identify key molecular constituents of reprogramming, as well as important obstacles in this process, and will ultimately lend itself as a powerful tool in the development and optimization of methods to produce hiPSCs.

EXPERIMENTAL PROCEDURES

Virus Production

Vectors were constructed as previously described (Stadtfeld et al., 2008b). To generate virus, 293T cells were transfected at 30% confluence using FuGENE 6 reagent (Roche). For a 10 cm plate, 560 μL DMEM, 27 μL FuGENE, and 12 μg DNA (4:3:2 vector:Δ8.9:vsv-g) were used. Virus was harvested over 3 days and concentrated 300-fold. For a standard infection in a 35 mm dish (~10^5 cells), 10 μL rtTA + 5 μL factors (OCT4, SOX2, KLF4, and NANOG) + 2 μL cMYC was used in an overnight infection supplemented with 6 μg/mL polybrene.

Cell Culture and hiPSC Generation

Fibroblasts were grown in DMEM with 10% FBS, nonessential amino acids, glutamine, and β-mercaptoethanol; keratinocytes were cultured on collagen IV in keratinocyte serum-free medium and growth supplement (Invitrogen). Human ES and iPS cells were cultured as previously described (Cowan et al., 2004). hiPSCs were generated as follows: day 0, to 1 μg/mL Dox in hESC media + 2% defined FBS (GIBCO) for fibroblasts, 1% FBS for keratinocytes; day 2, hESC + 1 μg/mL Dox + 1% FBS (fibroblasts) or hESC + Dox (keratinocytes); day 4, hESC + 1 μg/mL Dox; day 10, hESC + 0.5 μg/mL Dox (continued until colonies appeared).

Differentiation

For in vitro differentiation, hiPSC colonies were mechanically picked and placed in suspension culture with fibroblast media. After 1 week, embryoid bodies were plated to adherent conditions with gelatin. For teratoma formation, ~10^7 hiPSCs were pelleted and injected into SCID mice, either subcutaneously above the dorsal flank or underneath the kidney capsule. Tumors were harvested after 10–12 weeks and processed for histological analysis.

Bisulfite Sequencing

Genomic DNA was converted using the Epitect bisulfite kit (QIAGEN). OCT4 and NANOG promoters were PCR amplified using primers listed in Table S2. PCR products were cloned into TOPO vectors and sequenced.

Immunostaining

Immunostaining was performed using the following antibodies: α-Oct4 (sc-8628, Santa Cruz Biotech), α-Tra-1-81 (MAB4381, Millipore), α-β-III tubulin (T2200, Sigma), α-cardiac troponin T (clone 13-11, Neomarkers), α-myosin heavy chain (clone MF20, Developmental Studies Hybridoma Bank), and α-AFP (sc-15375, Santa Cruz Biotech).

qPCR

RNA was extracted by using a QIAGEN RNeasy kit, then converted to cDNA with the Superscript III First-Strand synthesis system (Invitrogen) using oligo-dT primers. qRT-PCRs were carried out using Brilliant II SYBR Green mix (Stratagene) and run on a Stratagene MXPro400. Reactions were carried out in duplicate with −RT controls, and data were analyzed using the delta-delta Ct method. Primer sequences are listed in Table S2.

Whole-Genome Expression Analysis

Total RNA was isolated using an RNeasy kit (QIAGEN). Samples were processed as independent triplicates. RNA probes for microarray hybridization were prepared and hybridized to Affymetrix HGU133 plus two oligonucleotide microarrays. Data were extracted and analyzed using the Rosetta Resolver system. During importation, the data were subjected to background correction, intrachip normalization, and the Rosetta Resolver Affymetrix GeneChip error model (Weng et al., 2006). For the generation of intensity plots, genes that showed greater than a 2-fold difference in expression value ($p < 0.01$) in HUES8 hESCs and BJ fibroblasts were noted (19,663 probes) and their expression analyzed. A hierarchical clustering was performed.

ACCESSION NUMBERS

Microarray data have been deposited under Gene Expression Omnibus (GEO) accession number GSE12390.

SUPPLEMENTAL DATA

The Supplemental Data include three figures and two tables and can be found with this article online at http://www.cellstemcell.com/cgi/content/full/3/3/340/DC1/.

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REFERENCES

Aoi, T., Yae, K., Nakagawa, M., Ichisaka, T., Okita, K., Takahashi, K., Chiba, T., and Yamanaka, S. (2008). Generation of pluripotent stem cells from adult mouse liver and stomach cells. Science *321*, 699–701. Published online February 14, 2008. 10.1126/science.1154884.

Cowan, C.A., Klimanskaya, I., McMahon, J., Atienza, J., Witmyer, J., Zucker, J.P., Wang, S., Morton, C.C., McMahon, A.P., Powers, D., and Melton, D.A. (2004). Derivation of embryonic stem-cell lines from human blastocysts. N. Engl. J. Med. *350*, 1353–1356.

Hanna, J., Markoulaki, S., Schorderet, P., Carey, B.W., Beard, C., Wernig, M., Creyghton, M.P., Steine, E.J., Cassady, J.P., Foreman, R., et al. (2008). Direct reprogramming of terminally differentiated mature B lymphocytes to pluripotency. Cell *133*, 250–264.

Huangfu, D., Maehr, R., Guo, W., Eijkelenboom, A., Snitow, M., Chen, A.E., and Melton, D.A. (2008). Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. Nat. Biotechnol. *26*, 795–797.

Kopp, J.L., Ormsbee, B.D., Desler, M., and Rizzino, A. (2008). Small increases in the level of sox2 trigger the differentiation of mouse embryonic stem cells. Stem Cells *26*, 903–911.

Lowry, W.E., Richter, L., Yachechko, R., Pyle, A.D., Tchieu, J., Sridharan, R., Clark, A.T., and Plath, K. (2008). Generation of human induced pluripotent stem cells from dermal fibroblasts. Proc. Natl. Acad. Sci. USA *105*, 2883–2888.

Maherali, N., Sridharan, R., Xie, W., Utikal, J., Eminli, S., Arnold, K., Stadtfeld, M., Yachechko, R., Tchieu, J., Jaenisch, R., et al. (2007). Directly reprogrammed fibroblasts show global epigenetic reprogramming and widespread tissue contribution. Cell Stem Cell *1*, 55–70.

Mikkelsen, T.S., Hanna, J., Zhang, X., Ku, M., Wernig, M., Schorderet, P., Bernstein, B.E., Jaenisch, R., Lander, E.S., and Meissner, A. (2008). Dissecting direct reprogramming through integrative genomic analysis. Nature *454*, 49–55.

Niwa, H., Miyazaki, J., and Smith, A.G. (2000). Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat. Genet. *24*, 372–376.

Okita, K., Ichisaka, T., and Yamanaka, S. (2007). Generation of germline-competent induced pluripotent stem cells. Nature *448*, 313–317.

Park, I.H., Zhao, R., West, J.A., Yabuuchi, A., Huo, H., Ince, T.A., Lerou, P.H., Lensch, M.W., and Daley, G.Q. (2008). Reprogramming of human somatic cells to pluripotency with defined factors. Nature *451*, 141–146.

Stadtfeld, M., Maherali, N., Breault, D.T., and Hochedlinger, K. (2008a). Defining molecular cornerstones during fibroblast to iPS cell reprogramming in mouse. Cell Stem Cell *2*, 230–240.

Stadtfeld, M., Brennand, K., and Hochedlinger, K. (2008b). Reprogramming of pancreatic β cells into induced pluripotent stem cells. Curr. Biol. *18*, 890–894.

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell *126*, 663–676.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell *131*, 861–872.

Weng, L., Dai, H., Zhan, Y., He, Y., Stepaniants, S.B., and Bassett, D.E. (2006). Rosetta error model for gene expression analysis. Bioinformatics *22*, 1111–1121.

Wernig, M., Meissner, A., Foreman, R., Brambrink, T., Ku, M., Hochedlinger, K., Bernstein, B.E., and Jaenisch, R. (2007). In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature *448*, 318–324.

Wernig, M., Lengner, C.J., Hanna, J., Lodato, M.A., Steine, E.J., Foreman, R., Staerk, J., Markoulaki, S., and Jaenisch, R. (2008). A drug-inducible transgenic system for direct reprogramming of multiple somatic cell types. Nat. Biotechnol. *26*, 916–924. Published online July 1, 2008. 10.1038/nbt1483.

Yang, J., and Weinberg, R.A. (2008). Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. Dev. Cell *14*, 818–829.

Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R., et al. (2007). Induced pluripotent stem cell lines derived from human somatic cells. Science *318*, 1917–1920.