Brief Report

Generation of Induced Pluripotent Stem Cells Using Recombinant Proteins

Hongyan Zhou,[1] Shili Wu,[4,7] Jin Young Joo,[5,7] Saiyong Zhu,[1] Dong Wook Han,[5] Tongxiang Lin,[1] Sunia Trauger,[2,3] Geoffery Bien,[4] Susan Yao,[4] Yong Zhu,[4] Gary Siuzdak,[2,3] Hans R. Schöler,[5] Lingxun Duan,[6] and Sheng Ding[1,*]
[1]Department of Chemistry
[2]Department of Molecular Biology
[3]Center for Mass Spectrometry
The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA
[4]ProteomTech, Inc., 3505 Cadillac Avenue, Suite F7, Costa Mesa, CA 92626, USA
[5]Department of Cell and Developmental Biology, Max Planck Institute for Molecular Biomedicine, Röntgenstrasse 20, Münster 48149, Germany
[6]LD Biopharma Inc., Sandown Way, San Diego, CA 92130, USA
[7]These authors contributed equally to this work
*Correspondence: sding@scripps.edu
DOI 10.1016/j.stem.2009.04.005

Groundbreaking work demonstrated that ectopic expression of four transcription factors, *Oct4*, *Klf4*, *Sox2*, and c-*Myc*, could reprogram murine somatic cells to induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006), and human iPSCs were subsequently generated using similar genetic manipulation (Takahashi et al., 2007; Yu et al., 2007). To address the safety issues arose from harboring integrated exogenous sequences in the target cell genome, a number of modified genetic methods have been developed and produced iPSCs with potentially reduced risks (for discussion, see Yamanaka, 2009, and references therein). However, all of the methods developed to date still involve the use of genetic materials and thus the potential for unexpected genetic modifications by the exogenous sequences in the target cells. Here we report generation of protein-induced pluripotent stem cells (piPSCs) from murine embryonic fibroblasts using recombinant cell-penetrating reprogramming proteins. We demonstrated that such piPSCs can long-term self-renew and are pluripotent in vitro and in vivo.

One possible way to avoid introducing exogenous genetic modifications to target cells would be to deliver the reprogramming proteins directly into cells, rather than relying on the transcription from delivered genes. Previous studies have demonstrated that various proteins can be delivered into cells in vitro and in vivo by conjugating them with a short peptide that mediates protein transduction, such as HIV tat and poly-arginine (Inoue et al., 2006; Michiue et al., 2005; Wadia and Dowdy, 2002). In addition, various solubilization and refolding techniques for processing inclusion body proteins expressed in *E. coli* to bioactive proteins have been developed to allow facile and large-scale production of therapeutic proteins (Lafevre-Bernt et al., 2008). To generate recombinant proteins that can penetrate across the plasma membrane of somatic cells, we designed and fused a poly-arginine (i.e., 11R) protein transduction domain to the C terminus of four reprogramming factors: *Oct4*, *Sox2*, *Klf4*, and c-*Myc* (see Figure S1A online). These proteins were expressed in *E. coli* in inclusion bodies, which were then solubilized, refolded, and further purified (Figure S1B). The protein identities were confirmed by mass spectrometry and western blot analysis (Figure S1C). To test the cell permeability and stability of the proteins, we treated mouse embryonic fibroblast (MEF) cells with the recombinant proteins at various concentrations by adding them to the cell culture media for 6–72 hr and examining cell morphology and protein presence by immunocytochemistry. We found that the purified 11R-tagged recombinant transcription factors readily entered cells at concentrations of 0.5–8 µg/ml within 6 hr and could translocate into nucleus (Figure S1D). In addition, we found that the transduced proteins appeared to be stable inside cells for up to 48 hr (Figure S1D).

We then employed this simple protein transduction protocol to reprogram OG2/Oct4-GFP reporter MEF cells. Because reprogramming through the iPSC mechanism/process typically requires sustained activity of reprogramming proteins for 7–10 days, we devised a strategy that involved treating the cells in four cycles. In each cycle the fibroblasts (initially seeded at the density of 5×10^4 cells/well in a six-well plate) were first treated overnight with the recombinant reprogramming proteins (i.e., Oct4-11R, Sox2-11R, Klf4-11R, and c-Myc-11R) at 8 µg/ml in the mESC growth media supplemented with or without 1 mM valproic acid (VPA), a HDAC inhibitor that can significantly improve reprogramming efficiency (Huangfu et al., 2008b), followed by changing to the same media without the recombinant reprogramming proteins and VPA, and culturing for additional 36 hr before the next cycle of the treatment. After completing four repeated protein transductions of reprogramming proteins, the treated cells were transferred onto irradiated MEF feeder cells and simply kept in mESC growth media until colonies emerged around day 30–35 (Figure 1A). We obtained three GFP+ colonies per 5×10^4 cells when they were transduced with four proteins and treated with VPA, and one GFP+ colony per 5×10^4 cells when they were transduced with only three proteins (i.e., Oct4-11R, Sox2-11R, and Klf4-11R) and treated with VPA. However, we did not obtain stable GFP+ piPSC colonies by transducing the three or four reprogramming proteins only for the same period of time, although GFP-negative cell colonies were observed. Those GFP-negative cell colonies stained positive with ALP, an early pluripotency marker, suggesting they might be partially reprogrammed cells. The initial GFP+ colonies were subsequently passaged under conventional mESC growth conditions to yield piPSCs and were characterized further.

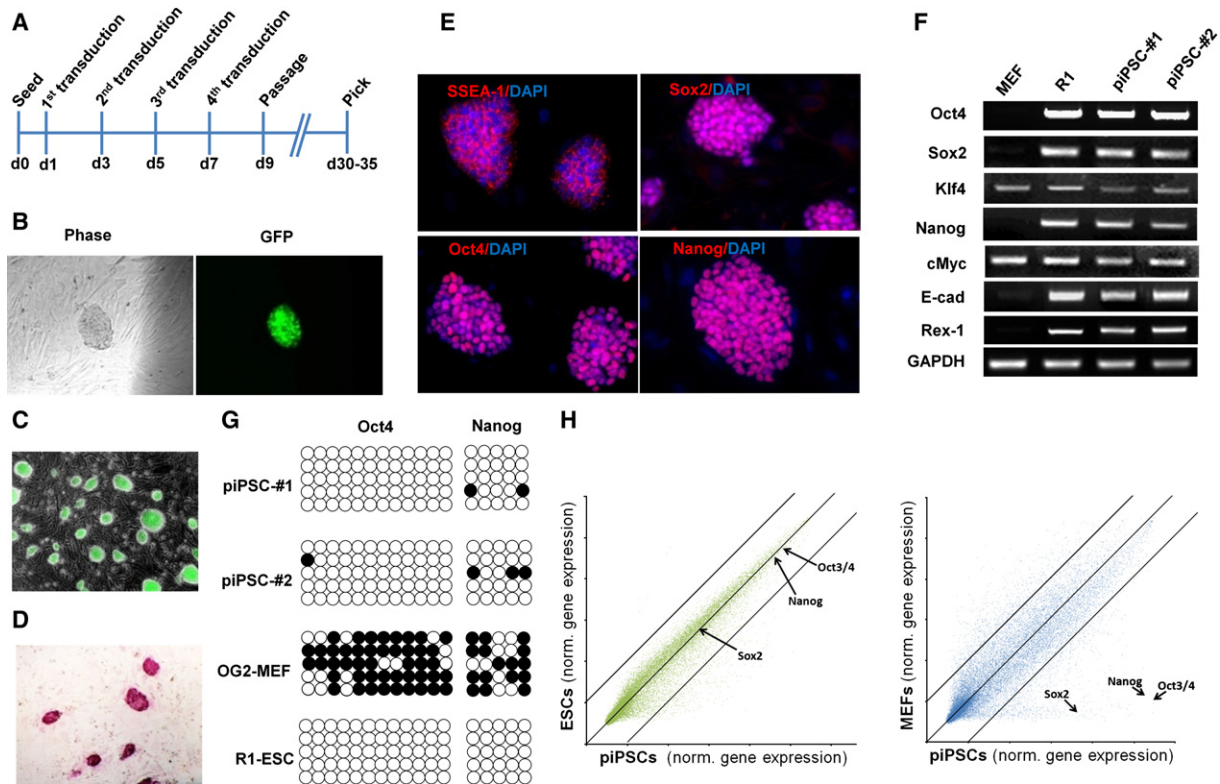The generated murine piPSCs have been stably and homogenously expanded

Figure 1. Generation of Protein-Induced Pluripotent Stem Cells by Recombinant Reprogramming Proteins
(A) Timeline of protein-induced pluripotent stem cell (piPSC) generation.
(B) Oct4-GFP+ piPSC colonies were initially observed around day 30–35. Representative phase contrast image (left) and fluorescence image (right) are shown.
(C) Oct4-GFP+ piPSCs sustain long-term and homogenous self-renewal under conventional mESC growth condition.
(D) The long-term expanded piPSCs grow as compact and domed colonies that express strong ALP, a typical pluripotency marker.
(E) piPSCs express other typical pluripotency markers, examined by immunofluorescence, including SSEA-1 (red), Sox2 (red), Oct4 (red), and Nanog (red). DAPI staining was performed to visualize the nuclei (blue), and the images were merged.
(F) RT-PCR analysis of endogenous pluripotency gene expression in piPSCs.
(G) Methylation analysis of *Oct4* and *Nanog* promoters by bisulfite genomic sequencing. Open and closed circles indicate unmethylated and methylated CpGs, respectively.
(H) Scatter plots comparing global gene expression patterns between piPSCs with murine ESCs, and between piPSCs and OG2-MEFs. The positions of the pluripotency genes *Oct4*, *Nanog*, and *Sox2* are shown by arrows. Black lines indicate the linear equivalent and two-fold changes in gene expression levels between the samples.

for over 30 passages and are morphologically indistinguishable from classic mESCs, forming compact domed small colonies (Figure 1C). They express typical pluripotency markers by immunocytochemistry and staining, including ALP (Figure 1D), Oct4, Nanog, Sox2, and SSEA1 (Figure 1E). RT-PCR analysis confirmed endogenous gene expression of these and additional pluripotency genes (Figure 1F). A single cell survival assay also demonstrated that piPSCs clonally expand efficiently as Oct4-positive colonies in feeder-free and N2/B27-chemically defined conditions (Figure S2A). Bisulfite genomic sequencing analyses of the *Oct4* and *Nanog* promoters revealed that both were demethylated in piPSCs as in mESCs, while in MEFs they were hypermethylated (Figure 1G). This result provides

further evidence for reactivation of the pluripotency transcription program in the piPSCs. Global gene expression analysis of piPSCs, OG2-MEFs, and mESCs showed that piPSCs are distinct from OG2-MEFs (Pearson correlation value: 0.895) and most similar to mESCs (Pearson correlation value: 0.969) (Figure 1H), consistent with previous reports.

To examine the developmental potential of piPSCs, standard in vitro differentiation using embryoid bodies (EBs) or monolayer chemically defined stepwise differentiation, and in vivo chimerism assays were performed. piPSCs could efficiently form EBs in suspension and differentiate into cells in the three primary germ layers, including endoderm derivatives (cells expressing AFP, Sox17, GATA4, or FoxA2; pancreatic cells [Pdx1]; and hepatic cells [Albumin]),

mesoderm derivatives (cells expressing Brachyury and mature beating cardiomyocytes [CT3 and MHC; Movie S1]), and ectoderm derivatives (neural [Sox1, Pax6] and characteristic mature neuronal [βIII-tubulin, MAP2ab] cells) (Figures 2A, 2B, and S2B). Most importantly, such piPSCs could efficiently incorporate into the inner cell mass of a blastocyst following aggregation with an eight-cell embryo and led to high-level chimerism with apparent germline contribution in vivo after the aggregated embryos were transplanted into mice, as confirmed by GFP genotyping in multiple three germ layer tissues of E13.5 fetuses (Figures 2D and S2C) and observation of Oct4-GFP+ cells in the gonad tissue in 3 out of 17 fetuses (Figure 2C). These in vitro and in vivo characterizations collectively confirm that the purified cell-penetrating
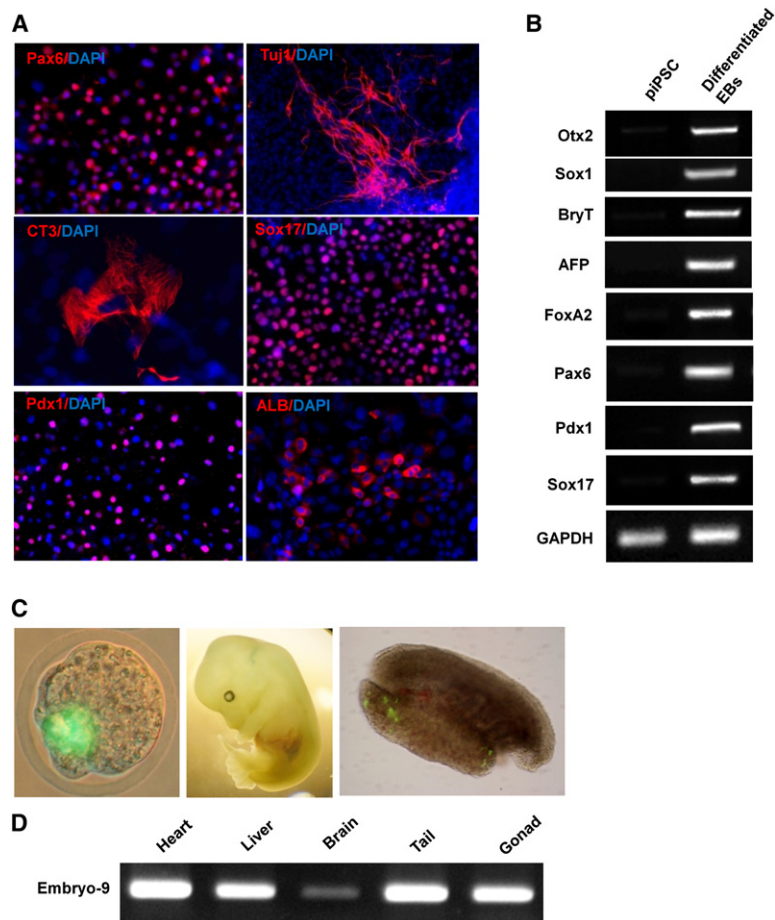
Figure 2. In Vitro and In Vivo Pluripotency of piPSCs

(A) piPSCs can effectively differentiate in vitro into cells in the three germ layers, including neural progenitor cells (Pax6^{+}), characteristic neurons (TUJ1^{+}), mature cardiomyocytes (CT3^{+}), definitive endoderm cells (Sox17^{+}), pancreatic cells (Pdx1^{+}), and hepatic cells (ALB[+]). Images were merged with DAPI (blue) staining.

(B) RT-PCR analysis of in vitro differentiation of piPSCs.

(C) piPSCs incorporate into the ICM of the blastocytes after aggregation with eight-cell embryos (left). Chimeric fetuses (13.5 dpc, middle) were obtained after transfer of the piPSC aggregated embryos into pseudopregnant mice. piPSCs contributed to the germline cells (Oct4-GFP positive) in isolated genital ridge tissue from chimeric fetuses (found in 3 out of 17 fetuses, right).

(D) GFP genotyping confirmed piPSC contribution to multiple three germ layer tissues in chimeric fetuses, including heart, liver, brain, tail, and gonad tissues. A representative genomic PCR of GFP was shown for embryo 9 that also contains piPSC germline contribution.

recombinant reprogramming proteins are sufficient to reprogram MEFs to become piPSCs, which are molecularly, morphologically, and functionally similar to conventional mESCs.

iPSCs (and especially patient-specific ones), which are similar to ESCs but are much easier to create and, in the case of human cells, less controversial, present unprecedented opportunities for biomedical research and clinical applications. Realization of the promise of iPSCs will require improved methods of directed differentiation for generating homogenous populations of lineage-specific cell types

as well as elimination of the risks and drawbacks associated with the current iPSC protocol, including genetic manipulation, and the low-efficiency/slow kinetics of induction. Recent advances in using various genetic approaches have addressed some of those iPSC challenges, including using nonintegrating adenoviruses to deliver reprogramming genes (Stadtfeld et al., 2008), transient transfection of reprogramming plasmids (Okita et al., 2008), a *piggyBac* transposition system (Woltjen et al., 2009; Kaji et al., 2009), Cre-excisable viruses (Soldner et al., 2009), and oriP/EBNA1-based

episomal expression system (Yu et al., 2009). In addition, strategies of exploiting endogenous gene expression in certain cell types also allowed easier reprogramming and/or fewer required exogenous genes (Shi et al., 2008b; Aasen et al., 2008; Kim et al., 2008). Moreover, small molecules have been identified that enhance reprogramming efficiency and replace certain reprogramming factors (Shi et al., 2008a, 2008b; Li et al., 2009; Huangfu et al., 2008a, 2008b). However, all of those methods have yet to produce iPSCs without the use of any genetic material. Our present study is the first to demonstrate that somatic cells (i.e., murine fibroblasts) can be fully reprogrammed into pluripotent stem cells by direct delivery of recombinant reprogramming proteins. This protein transduction method represents a significant advance in generating iPSCs and has several major advantages over previous iPSC methods. First, it effectively eliminates any risk of modifying the target cell genome by exogenous genetic sequences, which are associated with all previous iPSC methods, and consequently offers a method for generating safer iPSCs. Second, the protein transduction method provides a substantially simpler and faster approach than the currently most advanced genetic method, which requires time-consuming sequential selection of potentially integration-free iPSCs. And finally, given the robustness and wide availability of large-scale recombinant protein production, our demonstrated completely chemically defined reprogramming regimen could potentially enable broader and more economical application of reprogramming methodology.

REFERENCES

Aasen, T., Raya, A., Barrero, M.J., Garreta, E., Consiglio, A., Gonzalez, F., Vassena, R., Bilic, J., Pekarik, V., Tiscornia, G., et al. (2008). Nat. Biotechnol. *26*, 1276–1284.

Huangfu, D., Maehr, R., Guo, W., Eijkelenboom, A., Snitow, M., Chen, A.E., and Melton, D.A. (2008a). Nat. Biotechnol. *26*, 795–797.

Huangfu, D., Osafune, K., Maehr, R., Guo, W., Eijkelenboom, A., Chen, S., Muhlestein, W., and Melton, D.A. (2008b). Nat. Biotechnol. *26*, 1269–1275.

Inoue, M., Tomizawa, K., Matsushita, M., Lu, Y.F., Yokoyama, T., Yanai, H., Takashima, A., Kumon, H., and Matsui, H. (2006). Eur. Urol. *49*, 161–168.

Kaji, K., Norrby, K., Paca, A., Mileikovsky, M., Mohseni, P., and Woltjen, K. (2009). Nature *458*, 771–775.

Kim, J.B., Zaehres, H., Wu, G., Gentile, L., Ko, K., Sebastiano, V., Arauzo-Bravo, M.J., Ruau, D., Han, D.W., Zenke, M., et al. (2008). Nature *454*, 646–650.

Lafevre-Bernt, M., Wu, S., and Lin, X. (2008). Mol. Cancer Ther. *7*, 1420–1429.

Li, W., Wei, W., Zhu, S., Zhu, J., Shi, Y., Lin, T., Hao, E., Hayek, A., Deng, H., and Ding, S. (2009). Cell Stem Cell *4*, 16–19.

Michiue, H., Tomizawa, K., Wei, F.Y., Matsushita, M., Lu, Y.F., Ichikawa, T., Tamiya, T., Date, I., and Matsui, H. (2005). J. Biol. Chem. *280*, 8285–8289.

Okita, K., Nakagawa, M., Hyenjong, H., Ichisaka, T., and Yamanaka, S. (2008). Science *322*, 949–953.

Shi, Y., Desponts, C., Do, J.T., Hahm, H.S., Scholer, H.R., and Ding, S. (2008a). Cell Stem Cell *3*, 568–574.

Shi, Y., Do, J.T., Desponts, C., Hahm, H.S., Scholer, H.R., and Ding, S. (2008b). Cell Stem Cell *2*, 525–528.

Soldner, F., Hockemeyer, D., Beard, C., Gao, Q., Bell, G.W., Cook, E.G., Hargus, G., Blak, A., Cooper, O., Mitalipova, M., et al. (2009). Cell *136*, 964–977.

Stadtfeld, M., Nagaya, M., Utikal, J., Weir, G., and Hochedlinger, K. (2008). Science *322*, 945–949.

Takahashi, K., and Yamanaka, S. (2006). Cell *126*, 663–676.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007). Cell *131*, 861–872.

Wadia, J.S., and Dowdy, S.F. (2002). Curr. Opin. Biotechnol. *13*, 52–56.

Woltjen, K., Michael, I.P., Mohseni, P., Desai, R., Mileikovsky, M., Hamalainen, R., Cowling, R., Wang, W., Liu, P., Gertsenstein, M., et al. (2009). Nature *458*, 766–770.

Yamanaka, S. (2009). Cell *137*, 13–17.

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). Science *318*, 1917–1920.

Yu, J., Hu, K., Smuga-Otto, K., Tian, S., Stewart, R., Slukvin, I.I., and Thomson, J.A. (2009). Science. Published online March 26, 2009. 10.1126/science.1172482.