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# Generation of Mouse Induced Pluripotent Stem Cells by Protein Transduction

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Somatic cell reprogramming has generated enormous interest after the first report by Yamanaka and his coworkers in 2006 on the generation of induced pluripotent stem cells (iPSCs) from mouse fibroblasts. Here we report the generation of stable iPSCs from mouse fibroblasts by recombinant protein transduction (Klf4, Oct4, Sox2, and c-Myc), a procedure designed to circumvent the risks caused by integration of exogenous sequences in the target cell genome associated with gene delivery systems. The recombinant proteins were fused in the frame to the glutathione-S-transferase tag for affinity purification and to the transactivator transcription-nuclear localization signal polypeptide to facilitate membrane penetration and nuclear localization. We performed the reprogramming procedure on embryonic fibroblasts from inbred (C57BL6) and outbred (ICR) mouse strains. The cells were treated with purified proteins four times, at 48-h intervals, and cultured on mitomycin C treated mouse embryonic fibroblast (MEF) cells in complete embryonic stem cell (ESC) medium until colonies formed. The iPSCs generated from the outbred fibroblasts exhibited similar morphology and growth properties to ESCs and were sustained in an undifferentiated state for more than 20 passages. The cells were checked for pluripotency-related markers (Oct4, Sox2, Klf4, cMyc, Nanog) by immunocytochemistry and by reverse transcription–polymerase chain reaction. The protein iPSCs (piPSCs) formed embryoid bodies and subsequently differentiated towards all three germ layer lineages. Importantly, the piPSCs could incorporate into the blastocyst and led to variable degrees of chimerism in newborn mice. These data show that recombinant purified cell-penetrating proteins are capable of reprogramming MEFs to iPSCs. We also demonstrated that the cells of the generated cell line satisfied all the requirements of *bona fide* mouse ESCs: form round colonies with defined boundaries; have a tendency to attach together with high nuclear/cytoplasmic ratio; express key pluripotency markers; and are capable of *in vitro* differentiation into ecto-, endo-, and mesoderm, and *in vivo* chimera formation.

## Introduction

Somatic cells can be reprogrammed to induced pluripotent stem cells (iPSCs) by the introduction of pluripotency related transcriptional factors OCT4, KLF4, SOX2, and cMYC, as first reported by Yamanaka and his coworkers.[1] Recently, there have been significant advances in iPSC technology, with iPSCs being generated in a broad range of species, including human,[1–4] mouse,[1,5–7] rat,[8] pig,[9,10] sheep,[11,12] and rabbit,[13] and from a range of cell types, including fibroblasts,[14] terminally differentiated lymphocytes[15] and other blood cells, stomach and liver cells,[16] neural progenitors,[17] keratinocytes,[18] melanocytes,[19] and pancreatic β cells.[20]

A large number of these iPSC lines were generated by retroviral transduction, which is a highly efficient and reproducible method. However, in this process, small sequences of the retroviral vector are integrated in the host genome along with the genes coding for reprogramming factors. As a result, these permanent insertions potentially increase the risk of tumor formation.[6,21]

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To overcome the unwanted side effects resulting from viral integration, a number of methods have been developed to produce iPSC with potentially reduced risks:

(1) nonviral reprogramming, such as the PiggyBac[22,23] and Sleeping Beauty[24] transposon systems;
(2) nonintegrating genetic manipulation of cells, including repeated plasmid transfection,[25] transfection with minicircle DNA vectors,[25–28] reprogramming with episomal vectors carrying the reprogramming factors,[29] or the treatment of cells with nonintegrating viruses, like Adeno-[30–34] and Sendai virus[35,36]; and
(3) nongenetic treatments, including small molecules translocating into the cells and interfering with different signaling pathways,[37–41] direct delivery of synthetic mRNA,[42,43] or reprogramming proteins.[44–46]

The disadvantage of the nonviral and nonintegrative methods is their very low reprogramming efficiency compared to viral transduction. Furthermore, the instability of some of the nonintegrative systems (mRNA and plasmids) means that multiple treatments are required, making the entire reprogramming procedure laborious.[47]

Proteins can be delivered into cells both *in vivo* and *in vitro* if they are fused in frame to cell-penetrating peptides (CPP) or protein transduction domains.[48] The advantage of reprogramming by protein transduction is that no genetic integration occurs; thus, allowing the generation of exogene-free iPSC lines, which is an important safety advantage for human therapy. However, the reprogramming efficiency of purified protein transduction is very low (<0.05% of the cells treated), which might be caused by the cellular uptake mechanisms active in the process of internalization of the CPP harboring proteins.

The aim of this study was to generate murine iPSCs by purified recombinant protein transduction using a novel approach that entirely replaces the gene delivery systems. This strategy differs from other protein transduction systems[44–46] as the four transcriptional factors (Oct4, Klf4, Sox2, and cMyc) were fused to the transactivator transcription (TAT) sequence, to the nuclear localization signal (NLS), and to the glutathione-S-transferase (GST) sequence. The TAT sequence is derived from the human immunodeficiency virus type 1 (HIV1) and it facilitates cell penetration of the recombinant proteins.[49] The NLS sequence promotes nuclear localization,[50] potentially decreasing the quantity of proteins trapped in organelles. Finally, the GST sequence is used for affinity purification.

## Materials and Methods

### Experimental procedures

All chemicals were from Sigma-Aldrich (St. Louis, MO). The cell culture media and supplements were purchased from Invitrogen Life Technologies (Carlsbad, CA), unless otherwise specified. The protocols for animal care and handling were approved by the Animal Experiments Committee of ABC (Godollo, Hungary) and the Animal Health Authorities.

For more details, see Supplementary Materials and Methods section (Supplementary Data are available online at www.liebertpub.com/tec).

## Results

### Expression and purification of recombinant reprogramming proteins

To generate the recombinant reprogramming proteins Oct4, Klf4, Sox2, and c-Myc, we constructed the GST-tagged recombinant transcriptional factors harboring a short peptide sequence of the HIV trans-activator of Transcription protein (TAT), and the NLS to direct the proteins into the nucleus (Fig. 1A). The two sequences were separated by a short linker to assure the flexibility of the protein structure.

All recombinant reprogramming proteins were produced in *Escherichia coli* BL21 (DE3) bacteria, affinity purified, dialyzed against phosphate-buffered saline, and sterilized by filtration, and the aliquots were stored at −80°C. The purified proteins were visualized by SDS-PAGE and SimplyBlue Safe Stain (Fig. 1B–F).

To verify the stability of the proteins after delivery, mouse embryonic fibroblasts (MEFs) were transduced with 2 μg individual recombinant protein (Oct4, cMyc, Klf4 or Sox2). The cells were fixed 12 and 24 h after transduction, and probed for the presence of recombinant proteins. A small population of cells showed positive staining and proper nuclear localization of the proteins (Fig. 1G and data not shown).

### Generation of PSCs by protein transduction

To generate iPSCs from mouse somatic cells, the cells were subjected to four protein transduction cycles at 48-h intervals (Fig. 2A), as described earlier.[44,45] On day 9, the cells were gently dissociated and transferred in mitomycin C-treated feeder coated 10 cm dishes and cultured until ES-like colonies appeared (around day 40). The colonies were picked and expanded for further characterization. The iPSC line lost the morphology characteristics of fibroblast cells (Fig. 2B, left panel) and its morphology and growth dynamics (Fig. 2B, middle panel) resembled natural, *bona fide* mouse embryonic stem cells (mESCs) (Fig. 2B, right panel), forming tight round colonies, with large nucleus, and characterized by high nucleus-to-cytoplasm ratio. To verify the undifferentiated state of the generated piPSC line, H1 (piPS-H1), we first assessed alkaline phosphatase (ALP) activity. The piPSC-H1 formed typical ES-like colonies and exhibited positive staining for ALP (Fig. 2C, left panel). We were able to culture the cells for more than 20 passages and the colonies remained undifferentiated.

To increase the reprogramming efficacy, we also performed experiments al low $O_2$ level. The numbers of ES-like colonies obtained under hypoxic conditions were higher compared to the number of colonies obtained under atmospheric conditions.

### Verification of pluripotency gene expression of the new piPSCs

For a more profound evaluation, we performed immunocytochemical staining of the cells for the major pluripotency markers SSEA1, Oct4, Klf4, Nanog, and Sox2 (Fig. 3A). The piPS-H1 line uniformly expressed the nuclear Oct4, Klf4, Nanog, and Sox2 and a high proportion of cells showed positive staining for the membrane marker SSEA1, similar to the control ESC line (Supplementary Fig. S1).

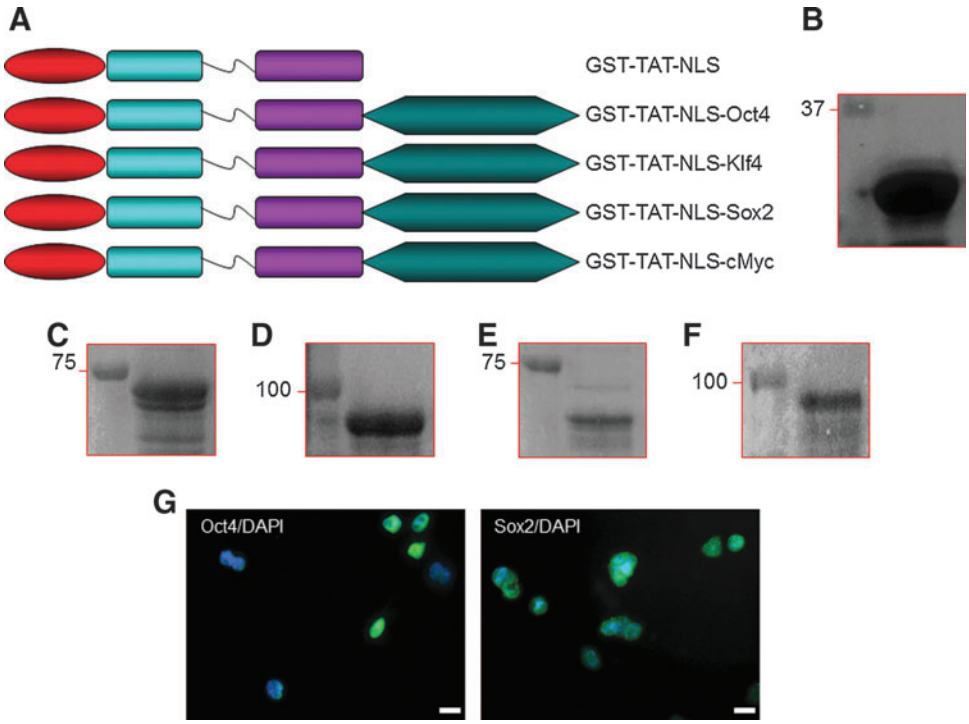


**FIG. 1.** Mouse recombinant transcriptional factors. **(A)** Schematic representation of glutathione-S-transferase (GST) tagged fusion protein. Mouse transcriptional factors were fused to the N terminus of the GST tag followed by the transactivator transcription-nuclear localization (TAT-NLS) polypeptide sequence to facilitate membrane penetration and nuclear localization. **(B-F**) Recombinant proteins were overexpressed in bacteria. The protein expression was induced by IPTG. The proteins were affinity purified using glutathione-Sepharose 4B, dialyzed against phosphate-buffered saline (PBS). The purified proteins were concentrated and sterilized by filtration, and stored in PBS at −80°C. The proteins were visualized by SDS-PAGE and SimplyBlue Safe Stain: GST **(B)**, Oct4 **(C)**, Klf4 **(D)**, Sox2 **(E)**, and cMyc **(F)**. **(G)** The stability of transduced recombinant Oct4 and Sox2 proteins. Twenty-four hours after transduction, mouse fibroblasts were fixed and probed for the presence of proteins. Scale bar: 100 μm. Color images available online at www.liebertpub.com/tec

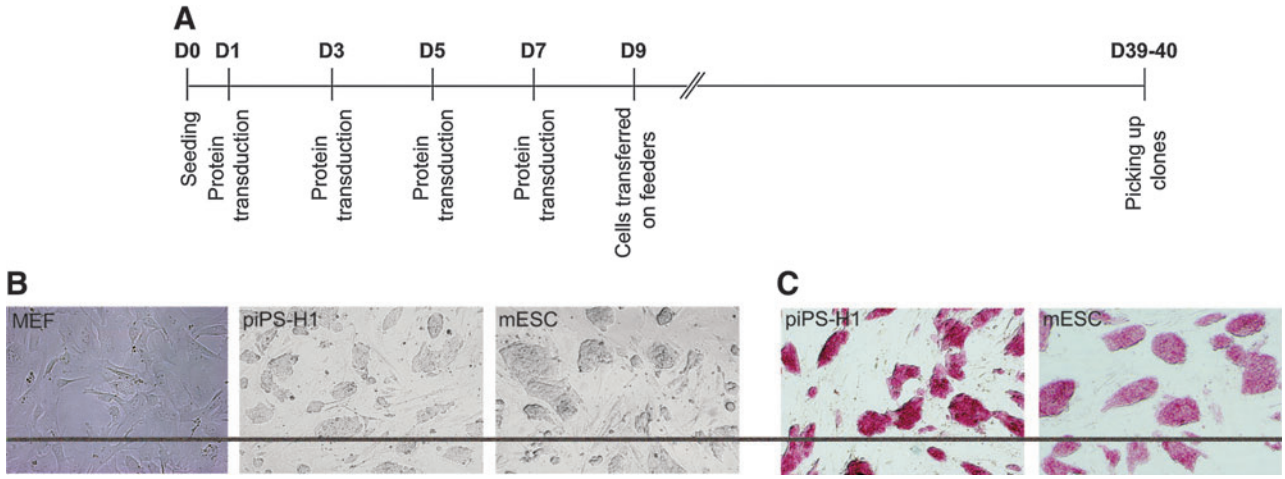


**FIG. 2.** Generation of protein induced pluripotent stem cells (piPSCs). **(A)** Transduction strategy: On day 0 (D0) $5 \times 10^4$ cells were seeded in one well of a six-well plate. Twenty-four hour later the cells were treated with 8 μg/mL purified protein cocktail (Oct4 + Klf4 + Sox2 + cMyc) or purified GST as control in embryonic stem cell medium (EM). After 12 h incubation with the proteins, the transduction medium was changed to normal EM. The protein treatment was repeated four times at 48-h intervals. On day 9 (D9) the cells were transferred into mouse embryonic fibroblast (MEF)-coated dishes and cultured further until embryonic stem cell (ESC)-like colonies appeared. The EM was refreshed every other day. **(B)** Morphology of MEF, iPS, and ESCs: The morphology of the iPSCs, piPS-H1, (middle panel) during reprogramming changed from fibroblast (left panel) morphology and progressively resembled the morphology observed for the control mouse ESCs (mESCs) (right panel). **(C)** Alkaline phosphatase (ALP) staining: The expended piPSCs (left panel) grew in compact colonies similar to the *bona fide* mESCs (right panel) and stained positive for ALP. Color images available online at www.liebertpub.com/tec

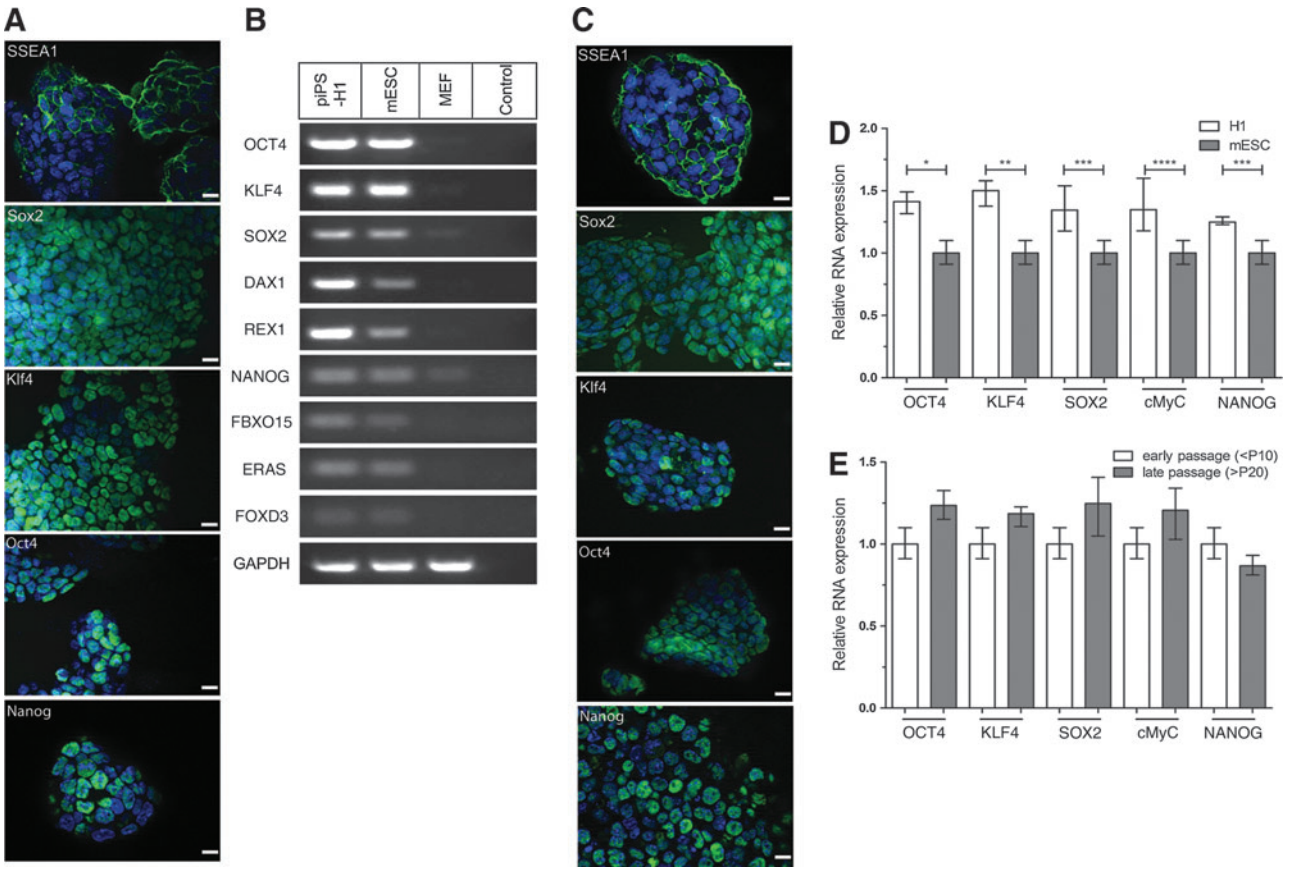


**FIG. 3.** Characterization of piPSCs. **(A)** Immunostaining of piPSCs**:** At low passage number, the piPS-H1 line was positive for all major pluripotency markers (SSEA1, Oct4, Sox2, Klf4, Nanog) by immunostaining. To visualize the nuclei, the cells were counterstained with DAPI. Scale bar: 100 μm. **(B)** Determination of pluripotency markers by reverse transcription–polymerase chain reaction (RT-PCR): Total RNA was isolated from cultured cells with the RNEasy Kit (Qiagen). One microgram RNA was transcribed to cDNA using SuperScript III Reverse Transcriptase (Invitrogen). RT-PCRs were performed using GoTaq Hot Start Green Master Mix (Promega). **(C)** Verification of pluripotency marker expression at higher passage number ($p > 20$) by immunostaining: After long-term culture of the piPS-H1 line, immunostaining was used to assess expression of the pluripotency markers SSEA1, Oct4, Sox2 and Klf4. **(D)** Relative RNA expression of the major five pluripotency markers for the piPS-H1 line was determined by quantitative real time polymerase chain reaction (qRT-PCR). The housekeeping gene GAPDH was used as reference. **(E)** The relative RNA expression was not changed during long term culture. Data presented as mean ± SEM and included at least three independent experiments. The difference was considered significant for a $p \leq 0.05$. *$p \leq 0.05$; **$p < 0.001$; ***$p < 0.02$; ****$p < 0.01$. Color images available online at www.liebertpub.com/tec

We also performed reverse-transcription polymerase chain reaction analysis for an extended set of pluripotency markers: OCT4, KLF4, SOX2, NANOG, FOXD3, E-RAS, FBXO15, REX1, and DAX1. The piPS-H1 line expressed all pluripotency markers similar to the control mESC (Fig. 3B) line.

Therefore, the derived piPS-H1 line displayed all features specific to a PSC, based on morphology, growth rate, and pluripotency marker expression.

To assess the stability of the piPS-H1 line, we verified the pluripotency marker expression by immunostaining over long-term culture ($p > 20$, generally the passage number was between 22–25). Even after 4–5 weeks' culture, the piPS-H1 cells still expressed the nuclear pluripotency markers Oct4, Sox2, Nanog, and Klf4 and the membrane marker SSEA1 (Fig. 3C).

The relative expression of the pluripotency marker genes was determined for the piPS-H1 and the mESC lines using quantitative real time polymerase chain reaction (qRT-PCR). The housekeeping gene, GAPDH, was used as a reference. The expression of all five pluripotency markers was slightly higher for the piPS-H1 line compared to the control mESCs (Fig. 3D).

To determine whether long-term culture affected the gene expression of the five major pluripotency markers, we compared the RNA expression level of the piPS-H1 line at early (between passages 8–10) and later passages (between passages 22–25), respectively (Fig. 3E). No significant differences in gene expression could be detected.

### In vitro *differentiation ability of piPSCs*

*In vitro* differentiation assays were performed to assess the *in vitro* developmental potential of the piPS-H1 line. Similar to the control ESC line, the piPSC line was able to form embryoid bodies (EBs) (Fig. 4A) under feeder-free culture conditions and in the absence of leukemia inhibitory factor (LIF) (Fig. 4B).

To determine whether the piPS-H1 line could form cell types of the three germ layers, EBs generated by hanging drop technology were plated onto gelatin-coated plates and

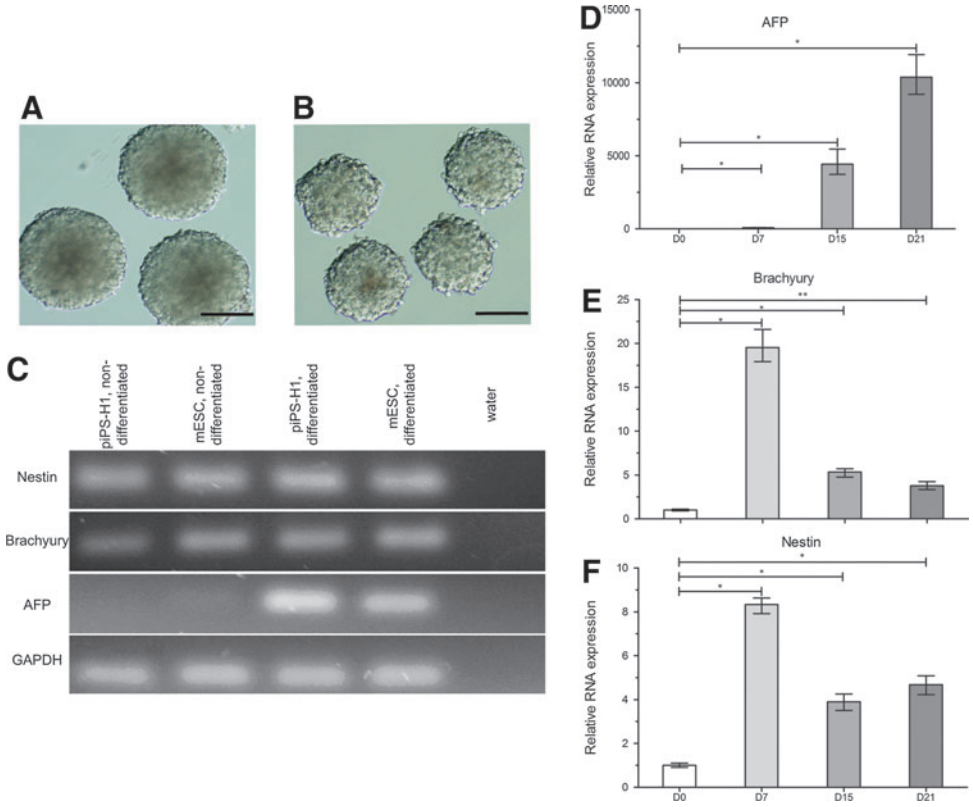


**FIG. 4.** *In vitro* differentiation of piPS-H1 line. **(A, B)** Embryoid body (EB) formation: In feeder-free conditions both piPSC **(A)** and control mESC **(B)** formed EBs. Scale bar: 250 μm. **(C)** Spontaneously differentiated and nondifferentiated iPSCs and control mESCs were collected on day 7 of differentiation and total RNA was isolated; RNA was transcribed to cDNA and the expression of germ layer markers was verified by RT-PCR analysis. **(D–F)** Relative RNA expression of the three germ layer markers detected during the spontaneous differentiation of piPS-H1 line. Data presented as mean ± SEM and included at least three independent experiments. The difference was considered significant for a $p \leq 0.05$. *$p < 0.001$; **$p < 0.03$. Color images available online at www.liebertpub.com/tec

cultured in ESC medium (EM) in the absence of LIF for a further 7 days. The cells were collected and total RNA was isolated and transcribed to cDNA. The expression of the three germ layer markers was verified by reverse-transcription PCR (Fig. 4C) and the relative RNA expression level was determined by qRT-PCR (Fig. 4D–F). As NESTIN and BRACHYURY T were also detected in the nondifferentiated cells, we checked the rate of up-regulation of these markers during differentiation. For BRACHYURY T (Fig. 4E) and NESTIN (Fig. 4F), the relative RNA expression was highest on D7 of differentiation, followed by a progressive decline. For α-fetoprotein (Fig. 4D), the relative RNA expression increased continuously until D21.

Because we plan to use the piPS-H1 line for drug selection and for modeling human diseases in animal models, we investigated the cardiac and neural differentiation ability of these cells. The differentiation assays were performed at both early (between passages 8–10, Figs. 5 and 6) and later passages (between passages 22–25, data not shown) and no significant differences could be detected.

Cardiac differentiation. After EB formation (day 2), each individual EB was plated onto a single, gelatin coated well of a 24-well plate in EM in the absence of LIF. Two days after replating, 24/24 plated EBs were attached to the gelatin coated surface. On day 6, 22/24 plated EBs started to beat; on

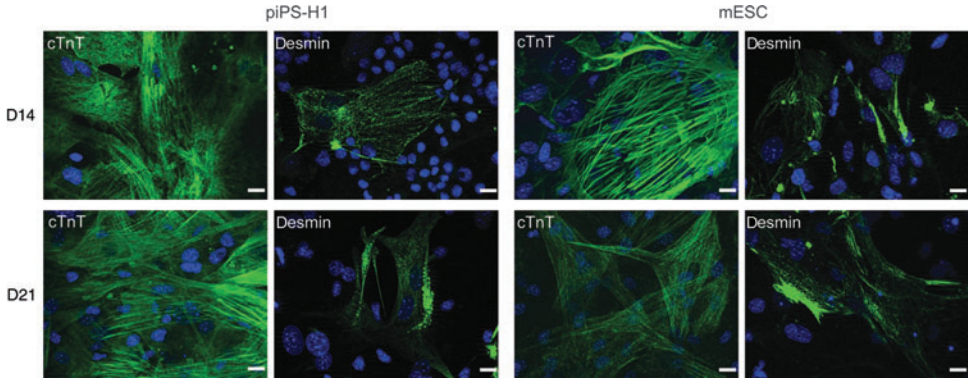


**FIG. 5.** Cardiac differentiation of piPSC and mESC. *In vitro* differentiation of piPSCs towards cardiac lineages: The EBs obtained by hanging drop method were plated on day 2 of differentiation on gelatin coated coverslips and cultured further until day 14 and 21 of differentiation. The EBs were stained for Desmin, a mesoderm marker, and for mature cardiomyocyte marker, cardiac Troponin T (cTnT). Similar to the control ESCs (right panels), the new piPSC-derived cells (left panels) started to beat on day 6 and was 98% on day 7. Scale bar 100 μm. Color images available online at www.liebertpub.com/tec

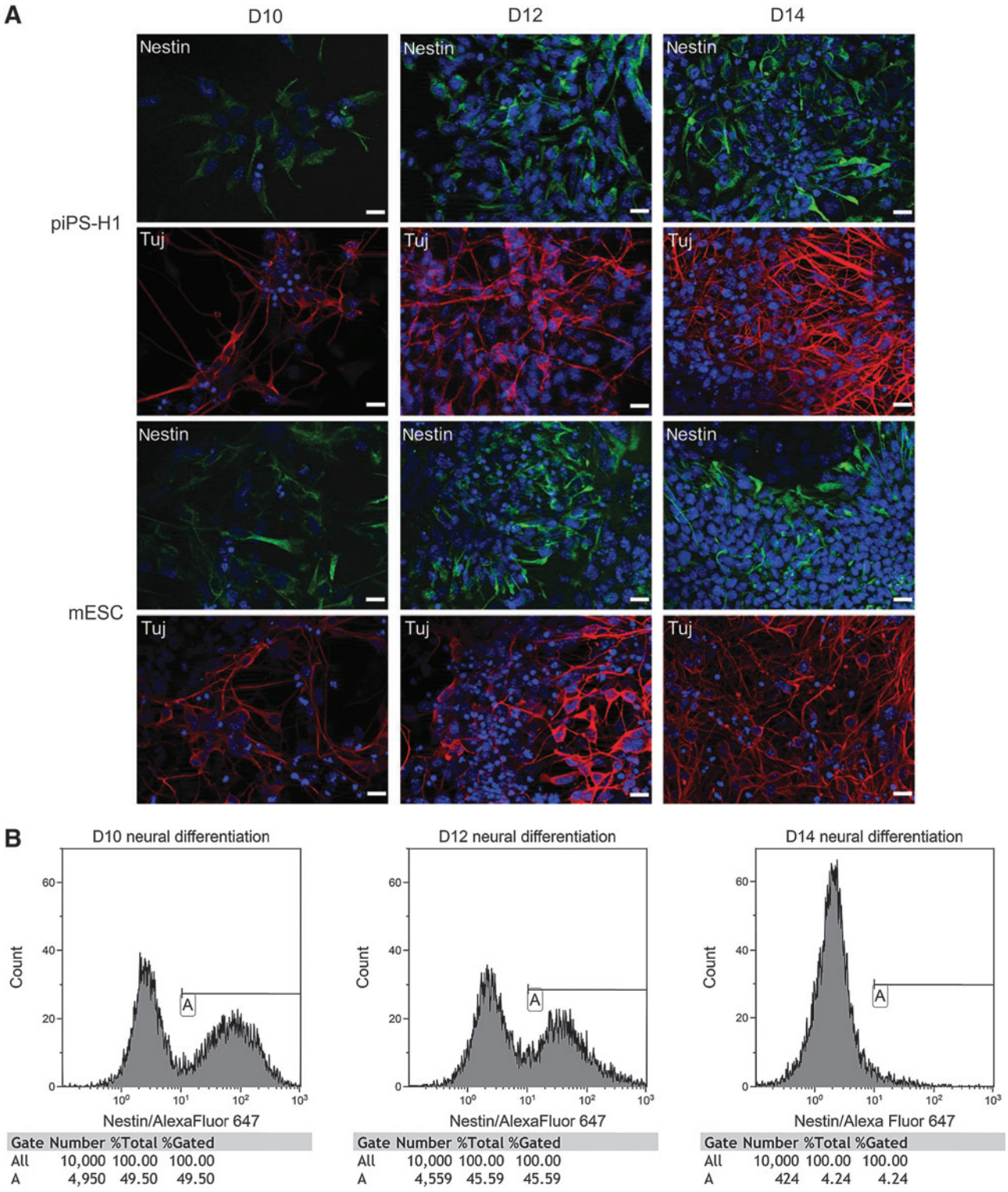


**FIG. 6.** *In vitro* differentiation of the piPSCs towards neural lineages. **(A)** The cells were cultured in suspension for 8 days for EB formation. On day 8, the EBs were trypsinized and seeded on poly-ornithine/laminin coated cover-slips and differentiated further. The cells were probed for ectodermal markers on day 10, 12, and 14. The cells were Nestin- (characteristic marker for neural progenitor cells [NPCs]) and III Tubulin- (Tuj 1; neuronal marker) positive. Scale bar 100 μm. **(B)** Flow cytometry analysis of nestin positive NPCs. Color images available online at www.liebertpub.com/tec

day 7, all 24 EBs were beating, similar to the control mESC line. The EBs were cultured further, until day 14 and 21, and then stained for cardiac troponin T, a mature cardiomyocyte marker, and Desmin, a mesoderm marker. Both the piPSC and control mESC lines were strongly positively stained for these markers (Fig. 5).

Neural differentiation. The EBs generated by culturing the cells in suspension for 8 days in bacterial (low attachment) plates coated with 2-hydroxyethyl methacrylate, were gently dissociated and replated on a poly-ornithin/laminin coated surface in medium enriched with N2 culture supplement and basic fibroblast growth factor. The medium was changed the following day, and from day 10 the cells were cultured in medium enriched with N2 and B27 supplements. Two days after replating (day 10 of differentiation), the cells started to develop neuronal morphology, and to form a 2D neural network. The cells were positive for Nestin, the neural progenitor cell (NPC) marker, and for neural class III β-Tubulin (Tuj1), a neuron marker (Fig. 6A), similar to the marker patterns in the control cell line. This experiment showed that piPSCs are able to generate NPCs and differentiate further into matured neurons through EB formation in cell culture. We quantified the amount of Nestin positive NPCs by flow cytometry (Fig. 6B). On D10, 49–50% of the analyzed cells were nestin positive. This ratio gradually decreased, and by D14 only 4–5% of the cells were nestin positive.

### *Chimera formation*

The most important characteristic of veritable PSCs is the ability to incorporate into the developing embryo. To assess the ability of our piPS-H1 line (passage 8–9) to form chimeras, individual cells were injected into F1 (B6D2) hybrid blastocysts. The adult mice that developed from these injected embryos exhibited different degrees of chimerism, demonstrated by the white coat color due to the piPS-H1 cells on the black coat color background of the host embryo genotype (Fig. 7).

The results indicate that the piPS-H1 line can develop and differentiate *in vivo* to generate adult chimeric mice.

## Discussion

The aim of this study was to reprogram somatic cells using a different approach to those published previously.[44,45] During the preparation of this manuscript, another group used an approach similar to ours to generate human iPSCs (46). However, that group used multiple treatments to generate hiPSCs, transducing the cells daily with the purified protein cocktail over a period of 17 days. In contrast, in our experimental design the cells were treated only four times, at 48-h intervals.

The process of endocytosis, the major uptake mechanism of the bioactive macromolecules carrying CPPs, is influenced by numerous factors, including experimental conditions and physicochemical properties of CPPs and their cargoes.[51,52] Endocytosed biomolecules are often trapped in cell organelles (vesicles, lysosomes, and endosomes), and contributed to protein downregulation and elimination of misfolded proteins. However, the escape of these proteins from the organelles is essential for maintaining their biological activity. By using endosome disruptive peptides, triggered by endosomal acidification, all or at least the majority of the trapped proteins can escape. The presence of the NLS, or some other peptides facilitate the cytosolic escape of the trapped proteins; thus, preventing their degradation. Recently, several membrane destabilizing peptides have been derived,[53] including the HA2 peptide from human influenza virus, which both improves the cytosolic delivery of biological active molecules, and also enhances the cytotoxic activity.[54]

Our recombinant proteins were fused in frame at the N terminus to the TAT peptide sequence formed by positively charged amino acids, with high arginine content, and to the NLS sequence, which had a double role of supporting the nuclear localization of the proteins, while minimizing the endosomal/lysosomal trapping and ultimately the degradation of the cargo.

The reprogramming experiments were carried out with mouse fibroblasts from two different genetic backgrounds (inbred [C57BL6] and outbred [ICR]). We were able to fully reprogram fibroblasts from the outbred but not from the inbred background. A possible explanation is that modifier genes can act differently in different genetic backgrounds. Phenotype penetrance is often influenced by genetic background, and the modulation of a given phenotype can be diverse, as exemplified in the case of several mouse models of human disease.[55] iPSC generation can also be dependent on genetic background and may differ from one strain to the other as shown by our team[24] using nonviral integrating vectors. The other factor that may influence reprogramming is the efficacy of protein transduction in inbred versus outbred strains.

Because the reprogramming efficacy of mouse and human somatic cells has been reported to be improved under hypoxic conditions,[56] we performed the experiments at both low (5%) and atmospheric $O_2$ levels. Indeed, the number of ES-like colonies obtained for both genetic backgrounds increased considerably when the reprogramming was performed under low $O_2$ conditions (24 colonies for ICR and 18 for C57Bl6) compared to atmospheric $O_2$ level conditions (8 colonies for ICR and 6 colonies for C57BL6). However, in spite of the high number of colonies obtained under hypoxic conditions, we couldn't achieve full reprogramming of the

**FIG. 7.** Chimera formation. Adult chimeras obtained by blastocyst injection of piPS-H1 cells. Color images available online at www.liebertpub.com/tec

genetic background, as the picked colonies flattened and stopped multiplying after two to three passages. The efficacy may also be influenced by the physico-chemical properties of the purified recombinant proteins in the culture medium. It has been reported that purified Oct4 has a limited solubility and/or stability under certain cell culture conditions[57] and that the combination of 2% fetal calf serum and 7.5% serum replacement increased the stability of Oct4[58] and Sox2[59] fusion protein in the medium. The efficacy of reprogramming is also affected by the protein concentration and the frequency of the treatment.[46] In several reprogramming systems, improvement was achieved by the addition of certain small molecules.[39,40] In view of these findings, further optimization of the process to increase the reprogramming rate may be possible.

The piPS-H1 line demonstrated similar morphology and growth dynamics to the control ESCs, while qRT-PCR results revealed a slightly increased relative RNA expression level of all five pluripotency markers in the piPS-H1 line. OCT4 together with SOX2 and NANOG are important in maintaining pluripotency and self-renewal, and it was shown recently that a defined level of OCT4 is required.[60] Once pluripotency is established, the OCT4 level can decrease up to sevenfold without losing the self-renewal properties in stem cells. As these genes are acting together, the upregulation of one may result in an increased gene expression for the others.

For the directed cardiac differentiation, we couldn't detect any significant difference from the day when the EBs started to beat, as 24 out of 24 plated EBs formed by piPSC-H1 were beating on day 7, similar to the mESC control. However, there was a significant difference in neural fate commitment of the control mESCs and the piPS-H1 cell line, with 80% of the control cells (data not shown), but only 40–50% of the piPS-H1 cells, positive by flow cytometry for the neural precursor marker Nestin on day 10 (Fig. 6B). It has been shown that ESC clones vary in their ability to differentiate into neurons.[61] The transcriptome analysis of these undifferentiated ESC sublines also indicated differences in gene expression even though all clones expressed pluripotency markers. If there are substantial differences in the differentiation ability of sublines generated from the same parental ESC line, then it is possible that the differences in the neural differentiation capability observed between the ESCs and iPSCs might be "corrected" with generating and selecting subclones of the H1 line.

Importantly, the piPSC-H1 cells could be incorporated into blastocysts and form chimeras, proving that the cell line could contribute to the development of the embryo proper. We established this through coat color chimerism, a well-accepted method. However, it is worth noting that cell lines may exhibit distinct developmental potentials *in vitro* or *in vivo*, and that one line may contribute to the development of a given organ but not to others. Although we obtained chimeras, we did not obtain germline transmission of the piPS-H1 cell genetics for these animals; thus, the extent of reprogramming of these cells is not entirely clear.

We have also generated miPSCs in our laboratory using the non–viral reprogramming Sleeping Beauty transposon system[24] and a Cre recombinase excisable lentiviral system. Although the iPSC lines generated with these two methods performed better *in vivo* resulting in live born germline chimeras, their *in vitro* cardiac and neural differentiation ability remained below the capacity observed for the piPS-H1 line generated by protein transduction (data not shown).

In summary, we describe a noninvasive, transgene-free, protein-mediated approach to reprogramming somatic cells. This approach eliminates the potential risks associated with the use of viruses, DNA transfection, and potentially harmful chemicals and in the future could provide a safe source of patient-specific cells for regenerative medicine. Further experiments are now needed to increase the efficiency and reproducibility of this approach, including its application in human cell reprogramming, to make this iPSC generation method more attractive as a method of choice.

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## Disclosure Statement

No competing financial interests exist.

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