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Exploring refined conditions for reprogramming cells by recombinant Oct4 protein

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ABSTRACT The generation of human induced pluripotent stem (iPS) cells would represent an appealing option for the derivation of pluripotent patient-specific cells, as no embryos or oocytes are required. However, crucial safety issues have to be addressed in order to create human iPS cells that are clinically useful, as the classical iPS technique involves permanent genetic manipulation that may result in tumor formation. Various experimental strategies have been suggested to accomplish transgene-free derivation of iPS cells, including the use of non-integrating viruses, site specific recombinases to excise transgenes after reprogramming, or RNA transfection. Protein transduction, i.e. the direct delivery of biologically active proteins into cells, has been employed to generate iPS cells but has been found to have very low efficiency. In fact, success of protein transduction is limited by poor stability and solubility of recombinant factors, as well as their poor endosomal release. We recently reported the generation of cell-permeant versions of Oct4 and Sox2 and showed that both can be delivered intracellularly as biologically active proteins. Here we explore conditions for enhanced protein stabilization and delivery into somatic cells. Employing optimized conditions, we demonstrate that Oct4 protein delivery can substitute for Oct4 virus, yielding iPS derivation efficacy comparable to a four virus transduction protocol. The number of colonies is strictly dependent on the dose and duration of cell-permeant Oct4 exposure. We expect our transduction system to reach a thus far unattained level of control over reprogramming activity, turning it into a valuable tool for both the analysis of the reprogramming mechanism and the derivation of transgene-free iPS cells.

KEY WORDS: *reprogramming, protein transduction, cell-penetrating peptide, factor-free iPS, Oct3/4, TAT*

Introduction

The identification of induced pluripotent stem (iPS) cells by Yamanaka and co-workers (Takahashi and Yamanaka, 2006) is about to revolutionize modern life science. They showed that viral transduction of a limited set of transcription factors is sufficient to reprogram somatic cells to a pluripotent, embryonic stem (ES) cell-like state. Human iPS cells would represent the most attractive option for the derivation of pluripotent patient specific cells as no embryos or oocytes are required for their generation. By this, they are a thus far unattainable source for regenerative medicine and disease modeling (Kiskinis and Eggan, 2010). However, crucial safety issues have to be addressed in order to generate human iPS cells that are clinically useful. The classical iPS technique involves permanent genetic manipulation that may result in tumor formation (Jalving and Schepers, 2009; Kane *et al.,* 2010).

Soon after identification of the viral reprogramming protocol in mouse cells (Takahashi and Yamanaka, 2006) and its adaptation to human cells (Takahashi *et al.*, 2007b, Yu *et al.*, 2007) the apparent unwanted side effects resulting from the viral integration were addressed by various strategies. Depending on the degree of genomic manipulation these can be divided into three classes (Fig. 1). Firstly, reprogramming can be achieved by techniques

Abbreviations used in this paper: CPP, cell-penetrating peptide; ES cell, embryonic stem cell; FCS, fetal calf serum; GFP, green fluorescent protein; iPS cell, induced pluripotent stem cell; MEF, murine embryonic fibroblast cells; NEAA, non-essential amino acids; SR, serum replacement.

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that in fact first integrate the reprogramming transgenes. After iPS formation, a second round of genetic manipulation is needed to remove the transgenes. The Cre/loxP recombination system has been successfully employed to remove reprogramming factors from iPS cells (Fig. 1A) (Soldner *et al.*, 2009; Sommer *et al.*, 2009). In fact, the groundbreaking work by Takahashi and Yamanaka had already incorporated loxP-flanked reprogramming genes, however, Cre-mediated deletion failed in this case. This presumably was due to the high number of integration events and unwanted interchromosomal recombination of loxP sites. The use of polycistronic constructs minimizes the integration events and thereby favors successful deletion. Intriguingly, it has been shown that the residual presence of integrated reprogramming transgenes indeed has an impact on the developmental potential of iPS cells (Soldner *et al.*, 2009; Sommer *et al.*, 2009). A direct comparison of iPS cell clones before and after adenoviral Cre mediated deletion revealed significant improvement of the 'cleaned cells' to undergo specific differentiation *in vitro* and *in vivo* (Sommer *et al.*, 2009). Notably, the Cre/loxP recombination mechanism entails a single loxP site, 34 bp in size, remaining in the locus. The PiggyBac transposon system (Fig. 1B), in contrast, is able to de-integrate without genomic traces and has recently been applied to generate iPS cells of mouse and human origin (Woltjen *et al.*, 2009; Yusa *et al.*, 2009; Ivics *et al.,* 2009). Therefore, this strategy represents an attractive alternative to the use of Cre/loxP recombination system. A general concern with transposons is, however, that these might re-integrate instantly into another locus. Hence, further laborious and cumbersome genetic methods are needed to identify and confirm transgene-free iPS clones. To date, the factor excision strategy, either mediated by Cre/loxP recombination or transposase, represents the best compromise resulting in high reprogramming efficiencies while involving a relatively less-invasive cellular manipulation. In this respect the application of cell-permeant Cre recombinase (Peitz *et al.*, 2002) (Nolden *et al.*, 2006) might be instrumental since this would circumvent a second round of transfection needed for the excision of the transgenes. Indeed, we have recently observed that Cre protein transduction is highly efficient in removing reprogramming transgenes from iPS cells (unpublished observations).

In contrast to the first group, which still involves genomic integration, the second group of factor free strategies is characterized by non-integrating genetic manipulation of cells. Since a minimal window of eight days of transgene overexpression is needed for iPS derivation the straightforward approach of transient transfection of target cells with conventional plasmids is

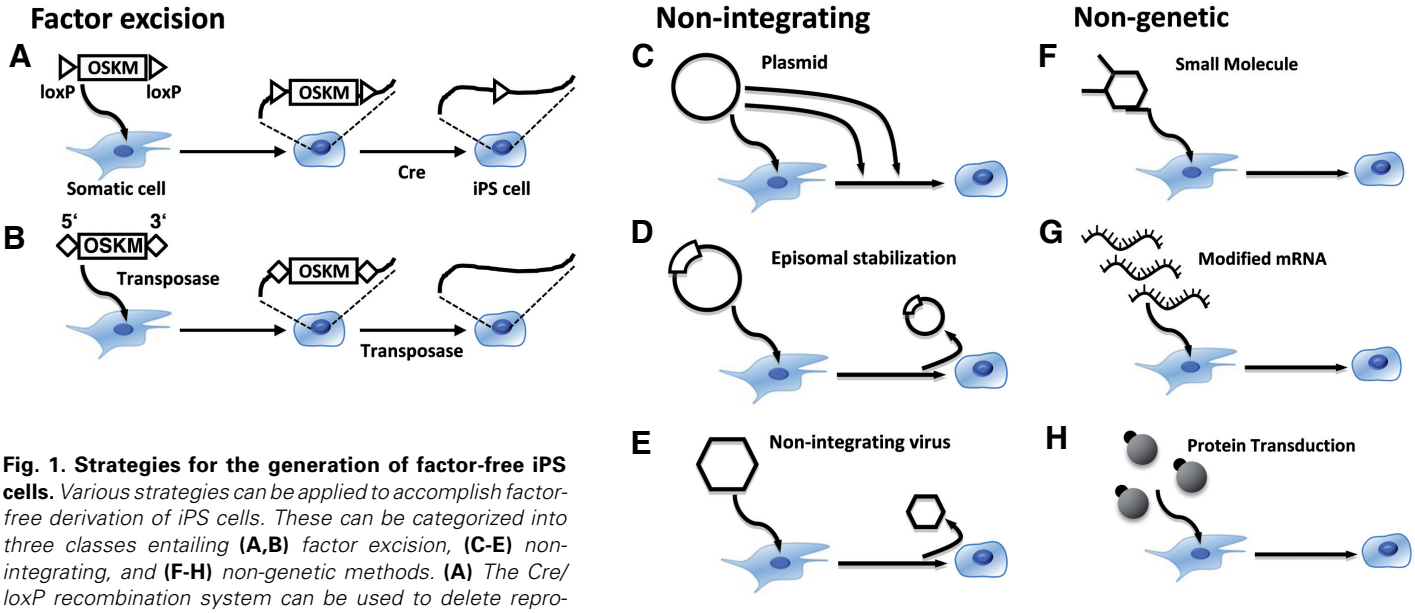

Fig. 1. Strategies for the generation of factor-free iPS cells. *Various strategies can be applied to accomplish factor-free derivation of iPS cells. These can be categorized into three classes entailing* **(A,B)** *factor excision,* **(C-E)** *non-integrating, and* **(F-H)** *non-genetic methods.* **(A)** *The Cre/loxP recombination system can be used to delete reprogramming transgenes. To that end, transgenes flanked by two site-specific recombination recognition sites (loxP), are integrated e.g. by viral transduction and iPS cells are derived. In a second step, Cre recombinase plasmid is transfected to 'clean' the generated iPS cells.* **(B)** *The PiggyBac transposase enables integration and subsequent deletion of transgenes from iPS cells. In contrast to the Cre/loxP system, PiggyBac transposase leaves no trace in the genome.* **(C)** *Repetitive transient transfection of plasmids allows the expression of reprogramming transgenes over a long period of time.* **(D)** *The viral oriP/EBNA1 system can be used for the episomal stabilization of transgene-carrying plasmids under selection pressure. After iPS cell derivation, removal of selection pressure results in the loss of episomes.* **(E)** *Viruses that do not integrate into the host genome, such as Adeno- and Sendai virus, have been used to derive iPS cells. Notably, non-integrating methods are usually less efficient than integrating strategies and must involve careful genetic analysis in order to confirm that genetic material did not integrate, since unwanted integration always occurs to a certain extent.* **(F)** *Small molecules that interfere with intracellular key signaling pathways may be used to induce pluripotency. Chemicals like histone-deacetylase inhibitors were identified enabling either the enhancement of iPS cell induction or the replacement of single reprogramming transgenes in a viral setting. Screening of compound libraries using appropriate cellular assays may identify new iPS modulators or replacers.* **(G)** *Repetitive lipofection of synthetic mRNA has been recently reported to yield iPS cells.* **(H)** *Protein transduction allows the direct delivery of proteins instead of gene delivery. The method involves engineering and purification of recombinant fusion proteins carrying so-called cell-penetrating peptides (CPP). Thus far, generation of iPS cells by protein delivery turned out to be very inefficient and not feasible with human adult fibroblasts. Further progress critically depends on the stabilization of the CPP fusion protein under cell culture conditions and the enhancement of endosomal release.*

insufficient. Repeated plasmid transfection (Fig. 1C) turned out to result in iPS colony formation albeit with a very low efficiency (Okita *et al.*, 2008). Transfection of minicircle vectors lacking bacterial DNA and thus exhibiting high transfection efficiency and long ectopic expression were reported to reprogram human adipose stem cells (Jia *et al.*, 2010). Another study exploited viral elements to episomally stabilize the reprogramming plasmids (Fig. 1D) (Yu *et al.*, 2009). Moreover, the transduction with viruses that do not integrate their genome into host cells was applied (Fig. 1E). Adenoviral vectors have been reported to reprogram mouse and human cells (Stadtfeld *et al.*, 2008; Zhou *et al.,* 2009). A Sendaivirus-based approach was recently reported to be successful in human iPS cell derivation (Fusaki *et al.*, 2009).

From the safety perspective the third group of factor-free iPS induction would represent the most favorable group of reprogramming strategies since it entails no genetic manipulation at all. Small molecules (Fig. 1F) that are able to translocate into cells and interfere with signaling molecules have been identified to either enhance the process of reprogramming (Huangfu *et al.*, 2008; Mikkelsen *et al.*, 2008) or replace (Huangfu *et al.*, 2008; Shi *et al.*, 2008) single viral factors (for review see Feng *et al.*, 2009). However, the search for small reprogramming molecules is largely empirical and a functional screening represents a significant hurdle to their identification since the validation of the cellular read-out is very complex. The direct delivery of either factor-encoding mRNA (Fig. 1G) or reprogramming proteins (Fig. 1H) represents a more rational approach to achieve non-genetic iPS derivation (Bosnali and Edenhofer, 2008; Zhou *et al.*, 2009; Warren *et al.,* 2010). Given the already acquired knowledge of reprogramming genes and the projected role of the corresponding proteins encoded within, here, the challenge is to deliver a cocktail of biologically active proteins into cells.

So-called cell-penetrating peptides (CPPs) or protein transduction domains (PTDs) were reported to confer cell permeability when linked to cargo molecules (for review see (Brooks *et al.*, 2005; Edenhofer, 2008; Gump and Dowdy, 2007). A highly basic CPP derived from the *human immunodeficiency virus type 1* (HIV-1) Tat (trans-activator of transcription) protein has mainly been applied in the literature based on the observation that the Tat protein is able to directly enter cells in culture and activate transcription of the viral genome (Frankel and Pabo, 1988; Green and Loewenstein, 1988). Over the past years a broad variety of molecules have been transduced into mammalian cells by direct delivery including oligonucleotides (Astriab-Fisher *et al.*, 2000), fluorescent dyes (Bolton *et al.*, 2000; Ho *et al.*, 2001), peptides (Dostmann *et al.*, 2000; Williams *et al.*, 1997), proteins (Bosnali and Edenhofer, 2008; Peitz *et al.*, 2002; Schwarze *et al.*, 1999), antibodies (Cohen-Saidon *et al.*, 2003; Heng and Cao, 2005),

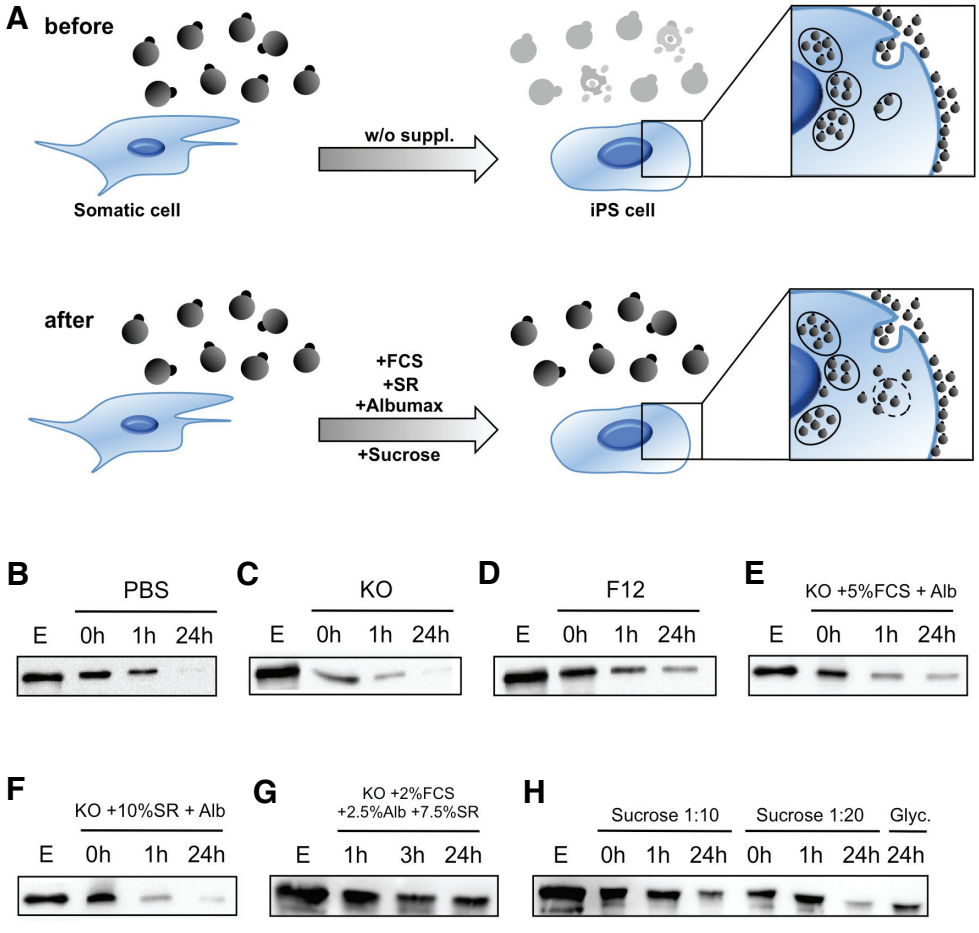

Fig. 2. Enhancing protein stability of recombinant cell-permeant Oct4 fusion protein. (A) *The stability of recombinant fusion protein as well as the endosomal release after cellular uptake represent limitations to the protein transduction technology. The capacity of media supplements like fetal calf serum (FCS), serum replacement (SR) and Albumax was analyzed to enhance the stability of recombinant cell-permeant Oct4 fusion protein Oct4-TAT. Depicted are Western blots of Oct4-TAT fusion protein-containing fractions dialyzed against* **(B)** *PBS,* **(C)** *KnockOut D-MEM,* **(D)** *D-MEM/F12,* **(E)** *KnockOut D-MEM supplemented with 5% FCS and 2.5% Albumax,* **(F)** *KnockOut D-MEM supplemented with 10% SR and 2.5% Albumax,* **(G)** *KnockOut D-MEM supplemented with 2% FCS, 7.5% SR and 2.5% Albumax (designated 'MT3' media) and incubated at 37°C for indicated periods of time.* **(H)** *Dialysis of Oct4-TAT protein against 20% sucrose results in concentration of recombinant factor. Dilutions of 1:10 and 1:20, respectively, into MT3 were incubated at 37°C for indicated periods of time and subsequently analyzed by immunoblotting. sucrose has been described to exert lysomotrophic activity thereby facilitating endosomal release. Western blot analysis was performed employing an anti-Oct4 antibody. E: elution fraction, i.e. protein fraction before dialysis; PBS: phosphate buffered saline; KO: KnockOut D-MEM; F12: D-MEM/F12; FCS: fetal calf serum; Alb: Albumax; SR: serum replacment; Glyc: glycerol stock.*

nanoparticles (Lewin *et al.*, 2000) or liposomes (Torchilin *et al.*, 2001). By this, various cellular processes have been successfully targeted by means of CPP-mediated protein transduction. For instance, manipulation of the cell cycle machinery has been accomplished by delivering biologically active p53 (Michiue *et al.*, 2005; Snyder *et al.*, 2005) and p27 (Nagahara *et al.*, 1998). Protein transduction technology has been used to generate cell-penetrating versions of developmentally relevant transcription factors such as HoxB4 (Krosl *et al.*, 2003), Pdx1 (Kwon *et al.*, 2005), Scl (Landry *et al.,* 2008), and Nkx2.2 (Stock *et al.*, 2010). These applications demonstrated the feasibility to manipulate the differentiation potential of stem cells without ectopic gene expression. We previously reported the derivation of cell-permeant versions of recombinant reprogramming factors Oct4 and Sox2 (Bosnali and Edenhofer, 2008). These proteins turned out to specifically bind DNA such as the Oct4/Sox2 combined element in the Nanog promoter and compensated the RNAi-induced loss-of-function of Oct4 and Sox2, respectively, by direct delivery into ES cells. More recently, Zhou *et al.* reported application of similar proteins for iPS derivation from mouse fibroblasts, albeit with an extremely low efficiency (Zhou *et al.*, 2009). The recently reported use of ES cell extracts to induce pluripotency in murine fibroblasts (Cho *et al.*, 2010) needs further investigation in order to demonstrate that it can be adapted to human cells as well.

In general, further development of protein transduction for cellular reprogramming, in particular its adaptation to reprogramming adult human fibroblasts, will greatly depend on overcoming the two major bottlenecks associated with protein transduction: stability of recombinant factors under cell culture conditions and endosomal release after cellular uptake. In this study we explore conditions for enhanced protein stabilization and delivery into somatic cells by systematically analyzing optimal culture and transduction conditions. We show that cell-permeant Oct4 protein can be stabilized by lipid-rich albumin supplements in serum replacement or low serum supplemented media. Employing optimized conditions for protein delivery, we demonstrate that Oct4 protein transduction is able to substitute for viral Oct4 delivery with an efficiency ranging in the same order of magnitude as a four virus approach.

Results

Improving protein stability of cell-permeant Oct4 fusion protein

The solubility as well as the stability of recombinant cell-permeant fusion proteins represent major challenges for the derivation of a robust and efficient protein transduction system (Fig. 2A). We have previously shown that Oct4 can be purified from *E.coli* as a TAT-modified cell-permeant version, designated Oct4-TAT. Oct4-TAT was shown to specifically bind to DNA and to compensate for the RNAi-induced loss of activity in ES cells (Bosnali and Edenhofer, 2008). Any long term application that would be necessary to induce pluripotency in somatic cells, however, is hampered by the limited stability of the recombinant cell-permeant Oct4 fusion protein under physiological, i.e. cell culture conditions. Thus, we set out to optimize conditions for Oct4 protein transduction in order to be able to use it in a reprogramming assay. First we analyzed the influence of various basal media on the stability of extracellular Oct4-TAT. Oct4 fusion protein was dialyzed either against PBS, KnockOut D-MEM or D-MEM/F12. Western blot analysis was employed to determine the stability of the Oct4 fusion protein. In both, PBS and KnockOut D-MEM, the recombinant protein is detectable after 1 hour of incubation at 37°C. However, almost no signal is observed after 24 hours (Fig. 2 B,C). Dialysis against D-MEM/F12 yielded a stabilized fraction of the Oct4 fusion protein as judged by Western blot analysis after 24 hours of incubation (Fig. 2D). Moreover, serum components have been shown to stabilize recombinant fusion proteins in cell culture (Peitz *et al.,* 2002) (Stock *et al.*, 2010). Thus, we aimed at further stabilizing the Oct4 fusion

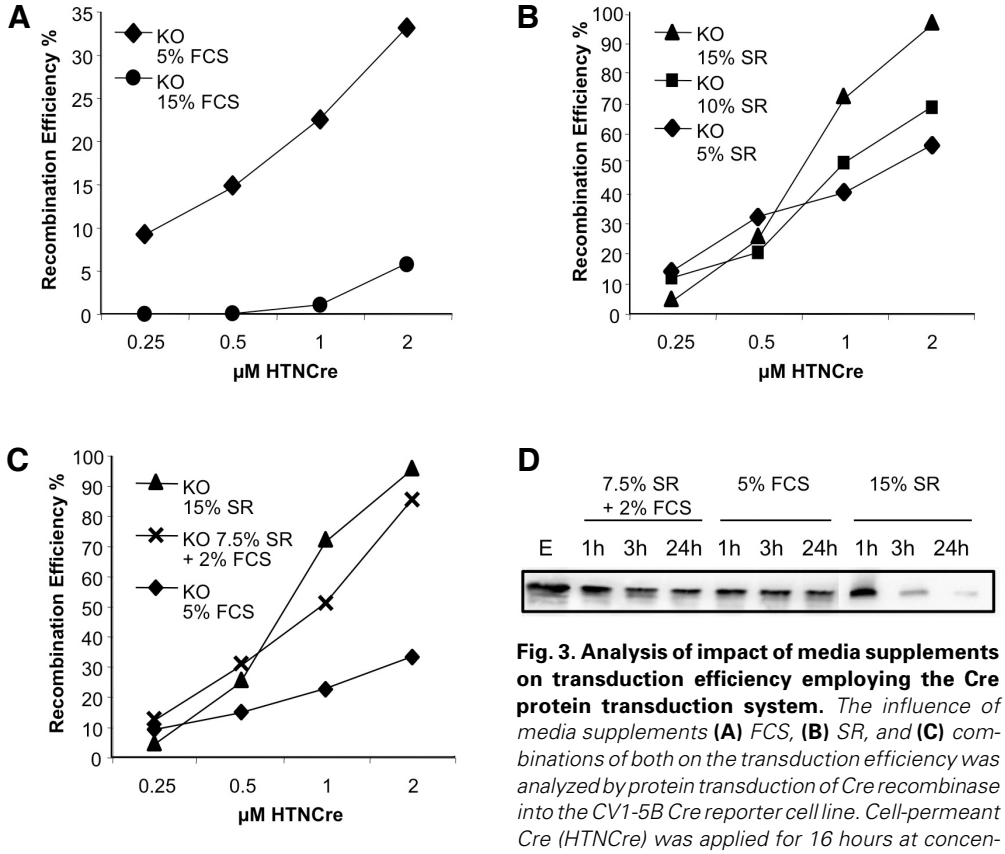

Fig. 3. Analysis of impact of media supplements on transduction efficiency employing the Cre protein transduction system. *The influence of media supplements* **(A)** *FCS,* **(B)** *SR, and* **(C)** *combinations of both on the transduction efficiency was analyzed by protein transduction of Cre recombinase into the CV1-5B Cre reporter cell line. Cell-permeant Cre (HTNCre) was applied for 16 hours at concentrations indicated. Cells were fixed and stained for β-galactosidase activity using X-Gal. Recombinase efficiency was determined by counting blue cells and setting them into relation to total cell numbers.* **(D)** *Protein stability of Oct4-TAT in different media. Oct4-TAT-containing media were supplemented as specified and incubated under cell culture conditions for 1, 3 and 24 hours. Western blot analysis was performed employing an anti-Oct4 antibody. Elution fraction (E) served as a control.*

protein by supplementing the KnockOut D-MEM media with either fetal calf serum (FCS) or serum replacement (SR) (Mansfield *et al.,* 2004) supplemented with Albumax. While FCS and SR alone turned out to exhibit only a minor effect on stabilization (Fig. 2 E,F) the combination of both resulted in significantly increased stability even after 24 hours (Fig. 2G).

Endosomal release represents a bottleneck for the intracellular delivery of translocated cell-permeant proteins. Several lysomotrophic agents including sucrose have been reported to interfere with endosomal integrity thereby facilitating endosomal release (Caron *et al.*, 2004). Moreover, we anticipated that sucrose might allow the concentration of Oct4-TAT during dialysis as has been formerly described for dialysis against glycerol buffer (Bosnali and Edenhofer, 2008). Hence, we dialyzed Oct4 fusion protein against 20% sucrose buffer and found that purified Oct4-TAT can be concentrated up to 500 μg/ml (Fig. 2H). This sucrose-based stock solution of Oct4-TAT allows storage at -20°C and can conveniently be used on demand by directly diluting into the media of choice.

Assessment of transduction capacity of various media using cell-permeant Cre

As a matter of fact, the ideal protein transduction medium has to fulfill three main criteria: the stabilization of recombinant protein, the support of protein/cell-interaction to promote intracellular delivery, and compatibility to cell type(s) to be transduced. In the particular case of cellular reprogramming the fact that the cell type changes, thereby considerably increasing the complexity of media requirements, has to be taken into account. After identifying optimal conditions for the stabilization of the recombinant Oct4 fusion protein, we thus set out to analyze the extent to which the stabilizing supplements interfere with both the protein transduction process as such as well as with cellular reprogramming. To address the first question we used the well-established transduction read-out system based on a cell-permeant version of the DNA recombinase Cre developed in our laboratory, referred to as HTNCre (Peitz *et al.*, 2002). A major feature of this protein transduction system is the fact that the efficiency of intracellular recombinase delivery can easily be quantified employing a Cre recombinase reporter line that has incorporated a Cre-dependent reporter gene. We used CV1-5B Cre reporter cells (Kellendonk *et al.,* 1996) that express β-Galactosidase only after Cre-mediated recombination. Using this read-out system we first tested the influence of FCS on the transduction efficiency (Fig. 3A). We used 5% and 15% FCS, respectively, together with increasing concentrations of HTNCre. 2μM of HTNCre in 5% FCS-supplemented media induced recombination in approx. 35% of cells, whereas only 5% were recombined in medium containing 15% FCS. Lower concentrations of HTNCre such as 0.5 μM still resulted in intracellular activity in about 15% of the cells in the presence of 5% FCS. However, the activity in cells treated with the same concentration in 15% FCS-supplemented medium was nearly undetectable, indicating a strong influence of FCS on the overall efficiency of protein transduction.

Next we assessed the influence of SR (Mansfield *et al.,* 2004) on transduction efficiency using the same experimental setting. It turned out that in contrast to FCS increasing concentrations of SR positively correlate with increasing transduction efficiency, at least in the case of higher Cre concentrations (Fig. 3B). This result might be due to the fact that SR has a less broad protein-stabilizing spectrum compared to FCS. Consequently high concentrations of HTNCre (above 0.5 μM) might not be stabilized well by low serum replacement contents and therefore show less efficient transduction. Thus, a well-defined balance between protein stability and protein delivery is more critical in the case of

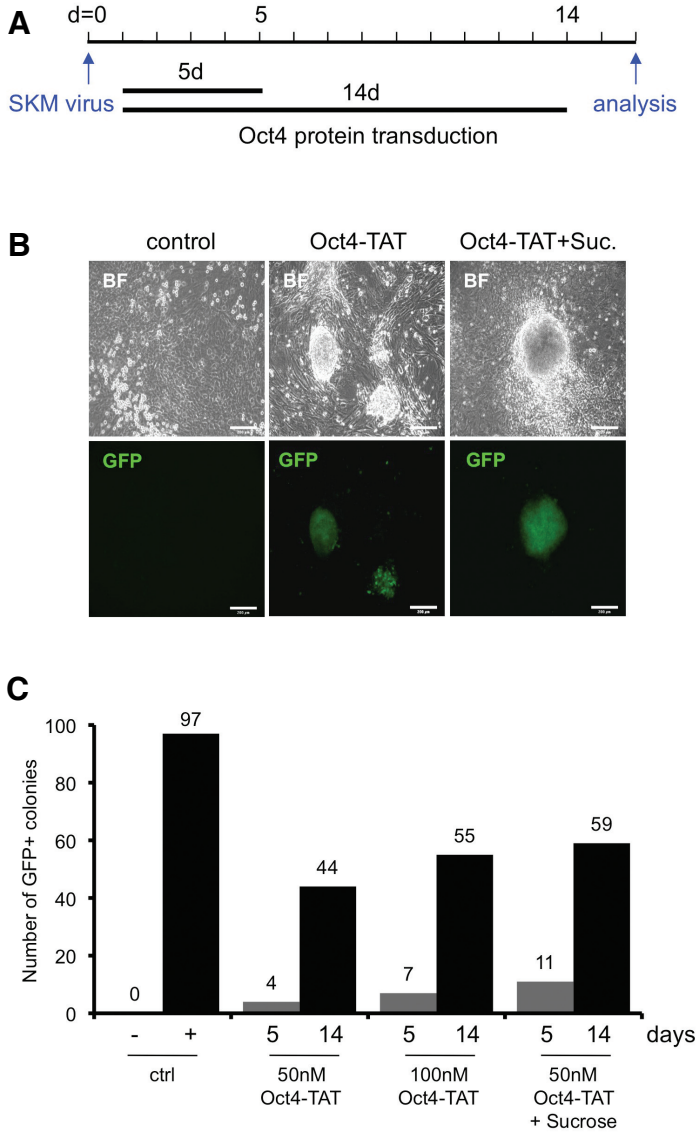

Fig. 4. Oct4 protein delivery substitutes for Oct4-encoding virus during reprogramming. (A) *Schematic presentation showing the timeline of the experimental setting. Oct4-GiP MEFs were infected with viruses encoding Sox2, Klf4 and c-Myc (SKM) at day 0. Starting at day 1 post infection (p.i.), cells were incubated with Oct4-TAT for 5 and 14 days, respectively, changing the Oct4-TAT-supplemented media daily.* **(B)** *Representative pictures of cells transduced with Oct4 protein (50nM) displaying phase contrast (upper row) and GFP channel (lower row) 16 days p.i. sucrose (2% final) was supplemented in one sample to enhance protein delivery (Oct4-TAT + Suc). Cells treated with medium only served as controls. Scale bar = 100μm* **(C)** *Quantification of GFP-positive colonies at day 16 p.i. demonstrating concentration and time-dependent effects of Oct4-TAT on the formation of GFP-positive colonies.*

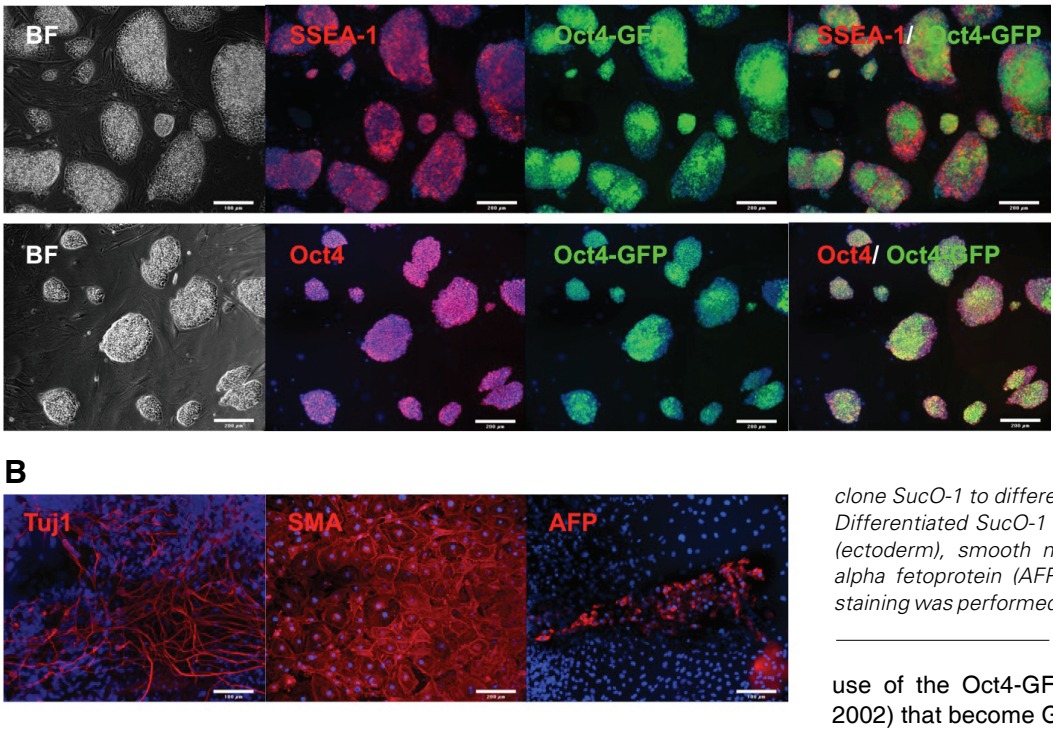

Fig. 5. Characterization of iPS clone derived from Oct4-TAT substitution. (A) *Immunostaining for surface and nuclear pluripotency markers for iPS clone SucO-1. The clone was generated from a substitution experiment in which Oct4-TAT-containing medium, derived from the sucrose stock (final conc. 2%), was applied for 5 days. The figure shows brightfield (BF), SSEA-1/ Oct4 immunostaining, Oct4-GFP transgene activity and overlay pictures, as indicated. DAPI co-staining was applied in every condition. Scale bar, 200 μM* **(B)** *Immunostaining confirming the potential Oct4-TAT-iPS clone SucO-1 to differentiate into all the germ layers in vitro. Differentiated SucO-1 cells were stained for beta-III-tubulin (ectoderm), smooth muscle actin (SMA, Mesoderm) and alpha fetoprotein (AFP, endoderm) as indicated. DAPI co-staining was performed in every condition. Scale bar, 100 μm.*

serum replacement.

Compared even to the low FCS (5%) containing medium, 15% SR supplemented medium exhibit a 2 to 3-fold higher transduction efficiency (Fig. 3 A,B). These data indicate that for intracellular protein delivery, SR is preferred over FCS. We then analyzed a medium supplemented with both FCS and SR. The content of serum components was tested on MEFs and murine ES cells using a medium supplemented with 5% FCS and 15% SR. Considering the FCS content to be critical, the percentage of FCS was reduced to the minimally tolerated concentration in a first step. Subsequently, the content of SR was adjusted likewise. By this, we identified 2% FCS and 7.5% SR (referred to as MT3 in the following) being minimally required to support growth of both cell types as well as maintaining ES cells in an undifferentiated state. Using the Cre reporter assay, MT3 medium showed a protein delivery capacity only slightly lower as compared to 15% SR-supplemented media; however, it was significantly higher than that of the 5%-FCS supplemented medium (Fig. 3C). Moreover, Western blot analysis employing all three media revealed that MT3 supports stability of recombinant Oct4-TAT protein similar to FCS-supplemented (5%) medium (Fig. 3D). In conclusion, these data demonstrate that beneficial effects of both supplements, FCS and SR, can be combined using a mixture of 2% FCS and 7.5% SR.

Cell-permeant Oct4 protein substitutes for Oct4-encoding viral vector

Next we assessed whether the optimized MT3 transduction medium would be able to fulfill the complex requirements needed to allow iPS generation from fibroblasts. Therefore we applied the classical four factor viral reprogramming paradigm and aimed at substituting the Oct4 virus by Oct4-TAT protein transduction employing MT3 conditions (Fig. 4A). As a target cell line we made use of the Oct4-GFP-transgenic MEFs (Ying *et al.*, 2002) that become GFP-positive upon reprogramming due to the reactivation of the Oct4 promoter region. We decided to test two concentrations of Oct4-TAT protein in MT3 medium and one condition in which Oct4-TAT was diluted into MT3 from the 20% sucrose storage buffer. On day one after infection, cells were transduced with Oct4-TAT either for 5 or 14 days (Fig. 4A). We changed the protein-supplemented media every day to ensure a continuous delivery of recombinant reprogramming factor. The viral transduction of the three factors Sox2, Klf4, and c-Myc (SKM) alone did not yield any GFP-positive colony whereas additional protein transduction of Oct4 resulted in numerous GFP-positive colonies (Fig. 4B). GFP-positive colonies were quantified 16 days post infection. We counted 97 GFP-positive colonies in control dishes containing cells infected with all four viruses, whereas no single GFP-positive colony was observed in samples with SKM infection. The application of Oct4-TAT protein to SKM-infected cells yielded GFP-positive colonies in a concentration and time-dependent manner (Fig. 4C). While treating SKM-infected cells with 100 nM Oct4-TAT protein for 5 days yielded rare GFP-positive colonies a continuous transduction of Oct4-TAT protein for 14 days increased the outcome more than 10-fold. Applying a higher concentration led to an enhanced formation of GFP-positive colonies, increasing numbers from 44 (50 nM) to 55 (100 nM). A further slight increase was detected in dishes where cells were transduced in the presence of sucrose. Under these optimized conditions the protein transduction of recombinant Oct4 in SKM-infected cells exhibits a reprogramming efficiency of about 60% in relation to the classical four factor (OSKM) virus protocol (Fig. 4C). To analyze whether protein substitution iPS cells could be expanded clonally and fulfill the criteria for pluripotency *in vitro*, iPS lines from all Oct4-TAT application conditions were generated. It turned out that even clones derived from short-term (5 days) and low (50 nM) Oct4-TAT application could readily be expanded and showed charac-

teristic pluripotency marker expression (Fig. 5A). The differentiation potential *in vitro* was addressed by spontaneous differentiation employing an embryoid body paradigm (Fig. 5B). The clones analyzed gave rise to all three germ layers in a percentage similar to ES cell controls.

Discussion

The direct delivery of recombinant transcription factors represents a highly attractive strategy to derive transgene-free iPS cells. However, the stability of recombinant proteins and the endosomal release represent major bottlenecks for achieving robust and efficient reprogramming by protein transduction. Here we set out to elaborate an optimized protocol for the delivery of cell-permeant Oct4 protein by focusing on media supplements. The ideal transduction medium has to fulfill three major criteria: stabilization of recombinant protein, support of protein/cell-interaction to promote intracellular delivery, and compatibility to the needs of the cell types to be transduced. Purified cell-permeant Oct4 protein displays limited solubility and/or stability under cell culture conditions (Bosnali and Edenhofer, 2008). We show that a combination of 2% FCS and 7.5% SR (designated as MT3) is beneficial to enhance the stability of Oct4 fusion protein in the medium. Employing a robustly quantifiable Cre protein transduction system we observed that FCS strongly reduces the cellular uptake. Here, supplementation of SR is preferred over FCS since it strongly increases transduction efficiency although it shows lower protein stabilizing capacity compared to FCS. However, SR is not well tolerated by the fibroblast target cells. Transduction of Cre protein demonstrated that a mixture of 2% FCS and 7.5% SR strongly minimizes the transduction-inhibiting effect of FCS while providing transduction efficiencies similar to SR-only media. Thus, MT3 media represents an optimal compromise for cultivation during protein-induced reprogramming of fibroblasts.

Employing these optimized conditions the transduction of recombinant Oct4-TAT in SKM-infected fibroblast cells resulted in a reprogramming efficiency of about 60% relative to the classical four factor (OSKM) virus protocol. Furthermore stable Oct4-TAT-iPS cell lines can readily be generated and show pluripotent characteristics in respect to marker expression and differentiation potential *in vitro*. These data demonstrate that protein transduction of Oct4-TAT can substitute for viral delivery with efficiency ranging in the same order of magnitude observed when using the four viruses. Previous studies indicated that cellular reprogramming by recombinant proteins exhibits a very low efficiency, i.e. 3 colonies out of 5 x 10^4 cells (Zhou *et al.,* 2009). Further investigations are needed to demonstrate that robust reprogramming of human adult fibroblast can be achieved employing recombinant proteins. In a non-genetic reprogramming context, we expect our transduction system to reach a thus far unattained level of control over reprogramming activity, turning it into a valuable tool for both the analysis of the reprogramming mechanism and the derivation of transgene-free iPS cells.

Materials and Methods

Protein expression and purification

pSESAME-Oct4 expression plasmid (Bosnali and Edenhofer, 2008) was transformed into *E. coli* BL21 (DE3) gold strain (Stratagene, La Jolla, USA) by heat shock at 30°C and incubated for 1h in SOC medium at 30°C. Transformed bacteria were inoculated overnight at 30°C with shaking at 140rpm in LB medium containing 50mg/mL carbenicillin. For protein expression the overnight culture was diluted 1:20 in TB medium (terrific broth)/50mg/mL ampicillin, 0.5% glucose and incubated at 37°C with shaking at 110 rpm until OD600 reached 1.5. Protein expression was induced by IPTG at a final concentration of 0.5mM. Cells were harvested by centrifugation and cell pellets were stored at -20°C.

For purification of His-tagged Oct4-TAT, cell pellets were thawed and resuspended in 20mL of lysis buffer (50mM Na2HPO4, 5mM Tris, pH 7.8, 500mM NaCl, 10mM imidazole) per 1 L of expression culture. Cells were lysed by application of 1 mg/mL lysozyme (Sigma, Deisenhofen, Germany), 10–15 U/mL Benzonase (Novagen, Darmstadt, Germany) and sonication. After centrifugation (17.200*g*, 20min) the cleared lysate was incubated with Ni-NTA agarose beads (Qiagen, Hilden, Germany) (1mL of slurry for 1L of bacterial expression culture) for 1h with rotation at 4°C. The slurry was packed into a column and washed with 8 column volumes of wash buffer (50mM Na2HPO4, 5mM Tris, pH 7.8, 500mM NaCl, and 90mM for Oct4-TAT). The protein was eluted with 3 column volumes of elution buffer (50mM Na2HPO4, 5mM Tris, pH 7.8, 500mM NaCl, 250mM imidazole).

Preparation of stock solution and transduction media

For preparation of stock solutions the purified Oct4-TAT fraction was transferred to a 20% sucrose/PBS buffer by dialysis. After flash-freezing, Oct4-TAT could be stored at -80°C and diluted into transduction media up to 1:5. Alternatively Oct4-TAT eluate fraction was dialyzed against D-MEM/F12 or Knockout D-MEM (Invitrogen) over night at 4°C. The next day the dialyzed fraction was diluted either to 50% or 25% with D-MEM/F12 or Kockout D-MEM and supplemented with either i) 5% FCS, 2.5% Albumax II (200mg/mL), 0.5% Insulin-Transferrine-Selenit (ITS, 100x, Invitrogen), 0.1mM non-essential amino acids (NEAA), 1mM sodium pyruvate, 2mM L-glutamine, 100 μM β-mercaptoethanol and 1000 U/mL LIF; or ii) 15% SR, 2.5% Albumax II (200mg/ml), 0.1 mM NEAA, 1mM sodium pyruvate, 2mM L-glutamine, 100 μM β-mercaptoethanol and 1000U/mL LIF; or iii) 2% FCS, 7.5% SR, 2.5% Albumax II (200mg/mL), 0.5% ITS, 0.1mM non-essential amino acids, 1mM sodium pyruvate, 2mM L-glutamine, 100 μM β-mercaptoethanol and 1000 U/mL LIF. The mixture, either generated by direct dialysis or diluted from the sucrose buffer, was pre-conditioned in a water bath for 1 h at 37°C and cleared by centrifugation (5 min at 2500 *g*) and sterile filtration. The protein concentration was determined by Bradford and/or dot blot assays.

Cell culture

Oct4-GiP MEFs (Ying *et al*., 2002) were cultured in high-glucose D-MEM (Invitrogen) with 10% FCS, 0.1mM NEAA, 1mM sodium pyruvate and 2mM L-glutamine. MEFs were trypsinized and split every 4 days and reseeded on tissue culture dishes coated with gelatin. For reprogramming assays MEFs were used to a maximum of passage 5. For culture maintenance, mouse ES and iPS cells were cultivated in KnockOut D-MEM (Invitrogen) with 15% SR, 0.1mM NEAA, 2mM glutamine, 100μM β-mercaptoethanol and 1000 U/mL LIF. Cells were splitted every 3 days and cultivated on irradiated feeder cells.

Retroviral infection and iPS induction

Plasmids of pMXs-Oct3/4 (positive control), pMXs-Sox2, pMXs-c-Myc and pMXs-Klf4 were obtained from ADDGENE (Takahashi and Yamanaka, 2006). The Retroviruses were generated by the Plat E packaging cell line as previously described (Takahashi *et al*., 2007a). Target cells were seeded at 10x10^4 cells per well in six-well plates. 24 hours after transfection the supernatant, comprising the viruses, was collected and filtered through a 0.45μm cellulose acetate filter. For substitution experiments and for negative controls Sox2, Klf4 and c-Myc were mixed in equal shares and supplemented with polybrene (Millipore) at a final concentra-

tion of 4 μg/mL. Positive controls additionally contained pMXs-Oct3/4 virus and were treated alike. Oct4-GiP cells were incubated with virus for 16 hours. Protein transduction experiments began after the virus-containing supernatant had been removed. After 5 days cells were split onto irradiated feeder cells. After 16 days cells were analyzed by fluorescence microscopy. For the purpose of generating stable iPS cell lines, cells were cultivated for 21 days under designated conditions. The colonies were then picked and expanded monoclonally.

In vitro differentiation

Cells were harvested by trypsination and transferred to petri dishes. Cells were grown in ES medium without the cytokine LIF for 3 days. The embryoid bodies generated were transferred to gelatine-coated tissue dishes afterwards and incubated for another 3 days. Immunostaining for the three germ layers was conducted with antibodies directed against Tuj1, smooth muscle actin and alpha fetoprotein.

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References

ASTRIAB-FISHER, A., SERGUEEV, D.S., FISHER, M., SHAW, B.R. and JULIANO, R.L. (2000). Antisense inhibition of P-glycoprotein expression using peptide-oligonucleotide conjugates. *Biochem Pharmacol* 60: 83-90.

BOLTON, S.J., JONES, D.N., DARKER, J.G., EGGLESTON, D.S., HUNTER, A.J. and WALSH, F.S. (2000). Cellular uptake and spread of the cell-permeable peptide penetratin in adult rat brain. *Eur J Neurosci* 12: 2847-2855.

BOSNALI, M. and EDENHOFER, F. (2008). Generation of transducible versions of transcription factors Oct4 and Sox2. *Biol Chem* 389: 851-861.

BROOKS, H., LEBLEU, B. and VIVES, E. (2005). Tat peptide-mediated cellular delivery: back to basics. *Adv Drug Deliv Rev* 57: 559-577.

CARON, N.J., QUENNEVILLE, S.P. and TREMBLAY, J.P. (2004). Endosome disruption enhances the functional nuclear delivery of Tat-fusion proteins. *Biochem Biophys Res Commun* 319: 12-20.

CHO, H.J., LEE, C.S., KWON, Y.W., PAEK, J.S., LEE, S.H., HUR, J., LEE, E.J., ROH, T.Y., CHU, I.S., LEEM, S.H. *et al.* (2010). Induction of pluripotent stem cells from adult somatic cells by protein-based reprogramming without genetic manipulation. *Blood* 116: 386-395.

COHEN-SAIDON, C., NECHUSHTAN, H., KAHLON, S., LIVNI, N., NISSIM, A. and RAZIN, E. (2003). A novel strategy using single-chain antibody to show the importance of Bcl-2 in mast cell survival. *Blood* 102: 2506-2512.

DOSTMANN, W.R., TAYLOR, M.S., NICKL, C.K., BRAYDEN, J.E., FRANK, R. and TEGGE, W.J. (2000). Highly specific, membrane-permeant peptide blockers of cGMP-dependent protein kinase Ialpha inhibit NO-induced cerebral dilation. *Proc Natl Acad Sci USA* 97: 14772-14777.

EDENHOFER, F. (2008). Protein transduction revisited: novel insights into the mechanism underlying intracellular delivery of proteins. *Curr Pharm Des* 14: 3628-3636.

FENG, B., NG, J.H., HENG, J.C. and NG, H.H. (2009). Molecules that promote or enhance reprogramming of somatic cells to induced pluripotent stem cells. *Cell Stem Cell* 4: 301-312.

FRANKEL, A.D. and PABO, C.O. (1988). Cellular uptake of the tat protein from human immunodeficiency virus. *Cell* 55: 1189-1193.

FUSAKI, N., BAN, H., NISHIYAMA, A., SAEKI, K. and HASEGAWA, M. (2009). Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. *Proc Jpn Acad Ser B Phys Biol Sci* 85: 348-362.

GREEN, M. and LOEWENSTEIN, P.M. (1988). Autonomous functional domains of chemically synthesized human immunodeficiency virus tat trans-activator protein. *Cell* 55: 1179-1188.

GUMP, J.M. and DOWDY, S.F. (2007). TAT transduction: the molecular mechanism and therapeutic prospects. *Trends Mol Med* 13: 443-448.

HENG, B.C. and CAO, T. (2005). Making cell-permeable antibodies (Transbody) through fusion of protein transduction domains (PTD) with single chain variable fragment (scFv) antibodies: potential advantages over antibodies expressed within the intracellular environment (Intrabody). *Med Hypotheses* 64: 1105-1108.

HO, A., SCHWARZE, S.R., MERMELSTEIN, S.J., WAKSMAN, G. and DOWDY, S.F. (2001). Synthetic protein transduction domains: enhanced transduction potential *in vitro* and in vivo. *Cancer Res* 61: 474-477.

HUANGFU, D., OSAFUNE, K., MAEHR, R., GUO, W., EIJKELENBOOM, A., CHEN, S., MUHLESTEIN, W. and MELTON, D.A. (2008). Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. *Nat Biotechnol* 26: 1269-1275.

IVICS Z, LI MA, MÁTÉS L, BOEKE JD, NAGY A, BRADLEY A, IZSVÁK Z. (2009) Transposon-mediated genome manipulation in vertebrates. *Nat. Methods* 6: 415-422.

JALVING, M. and SCHEPERS, H. (2009). Induced pluripotent stem cells: will they be safe? *Curr Opin Mol Ther* 11: 383-393.

JIA, F., WILSON, K.D., SUN, N., GUPTA, D.M., HUANG, M., LI, Z., PANETTA, N.J., CHEN, Z.Y., ROBBINS, R.C., KAY, M.A. *et al.* (2010). A nonviral minicircle vector for deriving human iPS cells. *Nat Methods* 7: 197-199.

KANE NM, NOWROUZI A, MUKHERJEE S, BLUNDELL MP, GREIG JA, LEE WK, HOUSLAY MD, MILLIGAN G, MOUNTFORD JC, VON KALLE C. *et al.* (2010). Lentivirus-mediated reprogramming of somatic cells in the absence of transgenic transcription factors. *Mol. Ther.* 18: 2139-2145.

KELLENDONK C, TRONCHE F, MONAGHAN AP, ANGRAND PO, STEWART F, SCHÜTZ G. (1996). Regulation of Cre recombinase activity by the synthetic steroid RU 486. *Nucl. Acids Res.* 24:1404-1411.

KISKINIS, E. and EGGAN, K. (2010). Progress toward the clinical application of patient-specific pluripotent stem cells. *J Clin Invest* 120: 51-59.

KROSL, J., AUSTIN, P., BESLU, N., KROON, E., HUMPHRIES, R.K. and SAUVAGEAU, G. (2003). *In vitro* expansion of hematopoietic stem cells by recombinant TAT-HOXB4 protein. *Nat Med* 9: 1428-1432.

KWON, Y.D., OH, S.K., KIM, H.S., KU, S.Y., KIM, S.H., CHOI, Y.M. and MOON, S.Y. (2005). Cellular manipulation of human embryonic stem cells by TAT-PDX1 protein transduction. *Mol Ther* 12: 28-32.

LANDRY JR, KINSTON S, KNEZEVIC K, DE BRUIJN MF, WILSON N, NOTTINGHAM WT, PEITZ M, EDENHOFER F, PIMANDA JE, OTTERSBACH K. *et al.* (2008). Runx genes are direct targets of Scl/Tal1 in the yolk sac and fetal liver. *Blood.* 111: 3005-3014.

LEWIN, M., CARLESSO, N., TUNG, C.H., TANG, X.W., CORY, D., SCADDEN, D.T. and WEISSLEDER, R. (2000). Tat peptide-derivatized magnetic nanoparticles allow *in vivo* tracking and recovery of progenitor cells. *Nat Biotechnol* 18: 410-414.

MANSFIELD, J.H., HARFE, B.D., NISSEN, R., OBENAUER, J., SRINEEL, J., CHAUDHURI, A., FARZAN-KASHANI, R., ZUKER, M., PASQUINELLI, A.E., RUVKUN, G. *et al.* (2004). MicroRNA-responsive 'sensor' transgenes uncover Hox-like and other developmentally regulated patterns of vertebrate microRNA expression. *Nat Genet* 36: 1079-1083.

MICHIUE, H., TOMIZAWA, K., MATSUSHITA, M., TAMIYA, T., LU, Y.F., ICHIKAWA, T., DATE, I. and MATSUI, H. (2005). Ubiquitination-resistant p53 protein transduction therapy facilitates anti-cancer effect on the growth of human malignant glioma cells. *FEBS Lett* 579: 3965-3969.

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.

NAGAHARA, H., VOCERO-AKBANI, A.M., SNYDER, E.L., HO, A., LATHAM, D.G., LISSY, N.A., BECKER-HAPAK, M., EZHEVSKY, S.A. and DOWDY, S.F.

(1998). Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27Kip1 induces cell migration. *Nat Med* 4: 1449-1452.

NOLDEN, L., EDENHOFER, F., HAUPT, S., KOCH, P., WUNDERLICH, F.T., SIEMEN, H. and BRUSTLE, O. (2006). Site-specific recombination in human embryonic stem cells induced by cell-permeant Cre recombinase. *Nat Methods* 3: 461-467.

OKITA, K., NAKAGAWA, M., HYENJONG, H., ICHISAKA, T. and YAMANAKA, S. (2008). Generation of mouse induced pluripotent stem cells without viral vectors. *Science* 322: 949-953.

PEITZ, M., PFANNKUCHE, K., RAJEWSKY, K. and EDENHOFER, F. (2002). Ability of the hydrophobic FGF and basic TAT peptides to promote cellular uptake of recombinant Cre recombinase: a tool for efficient genetic engineering of mammalian genomes. *Proc Natl Acad Sci USA* 99: 4489-4494.

SCHWARZE, S.R., HO, A., VOCERO-AKBANI, A. and DOWDY, S.F. (1999). *In vivo* protein transduction: delivery of a biologically active protein into the mouse. *Science* 285: 1569-1572.

SHI, Y., DESPONTS, C., DO, J.T., HAHM, H.S., SCHOLER, H.R. and DING, S. (2008). Induction of pluripotent stem cells from mouse embryonic fibroblasts by Oct4 and Klf4 with small-molecule compounds. *Cell Stem Cell* 3: 568-574.

SNYDER, E.L., SAENZ, C.C., DENICOURT, C., MEADE, B.R., CUI, X.S., KAPLAN, I.M. and DOWDY, S.F. (2005). Enhanced targeting and killing of tumor cells expressing the CXC chemokine receptor 4 by transducible anticancer peptides. *Cancer Res* 65: 10646-10650.

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). Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. *Cell* 136: 964-977.

SOMMER, C.A., SOMMER, A.G., LONGMIRE, T.A., CHRISTODOULOU, C., THOMAS, D.D., GOSTISSA, M., ALT, F.W., MURPHY, G.J., KOTTON, D.N. and MOSTOSLAVSKY, G. (2009). Excision of reprogramming transgenes improves the differentiation potential of iPS cells generated with a single excisable vector. *Stem Cells* 28: 64-74.

STADTFELD, M., NAGAYA, M., UTIKAL, J., WEIR, G. and HOCHEDLINGER, K. (2008). Induced pluripotent stem cells generated without viral integration. *Science* 322: 945-949.

STOCK, K., NOLDEN, L., EDENHOFER, F., QUANDEL, T. and BRUSTLE, O. (2010). Transcription factor-based modulation of neural stem cell differentiation using direct protein transduction. *Cell Mol Life Sci.* 67: 2439-2449.

TAKAHASHI, K., OKITA, K., NAKAGAWA, M. and YAMANAKA, S. (2007a). Induction of pluripotent stem cells from fibroblast cultures. *Nat Protoc* 2: 3081-9.

TAKAHASHI, K., TANABE, K., OHNUKI, M., NARITA, M., ICHISAKA, T., TOMODA, K. and YAMANAKA, S. (2007b). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131: 861-872.

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.

TORCHILIN, V.P., RAMMOHAN, R., WEISSIG, V. and LEVCHENKO, T.S. (2001). TAT peptide on the surface of liposomes affords their efficient intracellular delivery even at low temperature and in the presence of metabolic inhibitors. *Proc Natl Acad Sci USA* 98: 8786-8791.

WARREN L, MANOS PD, AHFELDT T, LOH YH, LI H, LAU F, EBINA W, MANDAL PK, SMITH ZD, MEISSNER A *et al.* (2010). Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell Stem Cell* 7: 618-630.

WILLIAMS, E.J., DUNICAN, D.J., GREEN, P.J., HOWELL, F.V., DEROSSI, D., WALSH, F.S. and DOHERTY, P. (1997). Selective inhibition of growth factor-stimulated mitogenesis by a cell-permeable Grb2-binding peptide. *J Biol Chem* 272: 22349-22354.

WOLTJEN, K., MICHAEL, I.P., MOHSENI, P., DESAI, R., MILEIKOVSKY, M., HAMALAINEN, R., COWLING, R., WANG, W., LIU, P., GERTSENSTEIN, M. *et al.* (2009). piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature* 458: 715-716.

YING, Q.L., NICHOLS, J., EVANS, E.P. and SMITH, A.G. (2002). Changing potency by spontaneous fusion. *Nature* 416: 545-548.

YU, J., HU, K., SMUGA-OTTO, K., TIAN, S., STEWART, R., SLUKVIN, II and THOMSON, J.A. (2009). Human induced pluripotent stem cells free of vector and transgene sequences. *Science* 324: 797-801.

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.

YUSA, K., RAD, R., TAKEDA, J. and BRADLEY, A. (2009). Generation of transgene-free induced pluripotent mouse stem cells by the piggyBac transposon. *Nat Methods* 6: 363-369.

ZHOU, W., FREED, C.R. (2009). Adenoviral gene delivery can reprogram human fibroblasts to induced pluripotent stem cells. *Stem Cells* 27: 2667-2674.

ZHOU, H., WU, S., JOO, J.Y., ZHU, S., HAN, D.W., LIN, T., TRAUGER, S., BIEN, G., YAO, S., ZHU, Y. *et al.* (2009). Generation of induced pluripotent stem cells using recombinant proteins. *Cell Stem Cell* 4: 381-384.

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