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Reprogramming human fibroblasts using HIV-1 TAT recombinant proteins OCT4, SOX2, KLF4 and c-MYC

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Abstract

It has been shown that human and murine fibroblasts can be reprogrammed by ectopic expression of transcription factors using viral vectors. For the purpose of human therapeutic applications, the integration of viral transgenes into the genome is unlikely to be accepted. We therefore produced recombinant transcription factor proteins in *E. coli* (OCT4, SOX2, c-MYC and KLF4) carrying the cell penetrating TAT domain from HIV1. The purified proteins were able to enter into mammalian cells when added to tissue culture medium but appeared not to translocate to the nucleus. Further investigation indicated that most of the protein was tied up in the endosomes and was unavailable for reprogramming. Once this problem has been solved it seems likely that protein reprogramming will be the method of choice for clinical applications.

Keywords

Induced pluripotent stem cells (iPSCs); TAT peptide; Reprogramming; Transcription factors; Gene expression

Introduction

The ability to generate autologous, pluripotent stem cells from skin fibroblasts, by relatively simple reprogramming steps, is a major scientific advance which will have a great impact in many areas of basic research and applied clinical medicine. In the first reports of reprogramming in the mouse, [1, 2] *Oct4*, *Sox2*, *Klf4* and *c-Myc* were used. Unfortunately,

~20% of mice derived after blastocyst transfer developed tumors in which *c-Myc* was re-activated. This group has now succeeded in reprogramming mouse and human fibroblasts using the same factors, but without *c-Myc*, and such iPS derived mice no longer showed tumor formation [3]. Yu et al. [4] also reported that reprogramming human cells did not require the use of *c-MYC*. Instead, *OCT4*, *SOX2*, *NANOG* and *LIN28* were able to efficiently produce iPS cells from adult human fibroblasts.

In a remarkable demonstration of the potential clinical application of iPS cells Hanna et al. [5] used a humanized sickle cell anemia mouse model, to show that mice can be rescued after transplantation with hematopoietic progenitors obtained in vitro from autologous iPS cells. It is highly unlikely, however, that any reprogramming protocol using viral vectors, even in the absence of *c-MYC*, will be acceptable for use in patients. In any reprogrammed cell line there are multiple viral integrations some of which could lead to inactivation of normal genes or activation of potential oncogenes. It is of utmost importance that alternative reprogramming protocols without virus be developed.

Due to the relatively low efficiency of generating iPS cells (<0.01%), several authors have investigated the possibility of increasing reprogramming efficiency using various drugs. Mali et al. [6] show that the use of the SV40 large T antigen (T) increases efficiency 23–70 fold when used in conjunction with the four transcription factors. Hangfu et al. [7] report that DNA methyltransferase and histone deacetylase (HDAC) inhibitors. In particular, valproic acid (VPA), was shown to improve reprogramming efficiency by approximately 100-fold. This enabled reprogramming to be carried out using only two factors—*OCT4* and *SOX2*. Shi et al. [8] report increased efficiencies using BIX-01294 (BIX)-G9a histone methyltransferase inhibitor and PD0325901 (MEK inhibitor) enabling reprogramming to be carried out using only *OCT4* and *KLF4*. This would seem to indicate that only one factor—*OCT4*—is actually essential for reprogramming.

Recent publications show that it is possible to reprogram mouse cells, albeit inefficiently, using DNA vectors but without apparent integration of the DNA into the recipient nucleus [9, 10]. Stadtfeld et al. used non-integrating adenoviral vectors whereas Okita et al. used repeated transfection of cells with plasmid vectors containing the reprogramming genes. Although this is a good first step, adenovirus is known to undergo integration in a small number of cells and repeated DNA transfection can also lead to DNA integration. Such integration events might be very difficult to detect. Thus, we still believe that reprogramming without using DNA is preferable.

Normally the hydrophobic nature of the lipid bilayer of the cell membrane makes it impossible for most proteins to cross the membrane. An exception to this is a family of small cationic peptides, termed protein transduction domains (PTD), which allow large, biologically active proteins to directly penetrate and accumulate within the cell [11–13]. The most common amongst these are derived from the Antennapedia (Antp), Herpes simplex (Vp22) and the HIV transactivator (TAT) proteins, (reviewed in [14]).

The TAT protein transduction domain (PTD) appears to hold the most clinical potential. It was derived from amino acids 47–57 of the HIV TAT protein after it was shown that full length TAT protein could be taken up by cells and activates transcription of the viral genome [12]. TAT has been used to deliver large (~110 kD), active proteins into the cells of live mice, and TAT fusion proteins and peptides have been used to treat mouse models of cancer, inflammation and other diseases [14]. Although the exact mechanism of TAT and other PTDs is not fully understood, it is thought to occur by way of macropinocytosis, a specialized form of endocytosis [15].

We therefore proposed to test the hypothesis that recombinant reprogramming proteins, carrying the TAT cell penetrating motif, will be able to enter somatic cells and reprogram them without any virus being involved. If this can be achieved, it will make reprogramming in any species, especially Human, that much more valuable as a potential clinical tool for tissue engineering and regenerative medicine.

Materials and methods

cDNA cloning and plasmids construction

The cDNA of human transcription factors *SOX2*, *OCT4*, *KLF4* and *c-MYC* were PCR amplified, using *Pfu* DNA polymerase (Clontech, USA), plasmids containing these genes (Clone ID: 2823424, 40125986, 5111134, 6012670, respectively, Open Biosystems, USA). A His6 tag (for affinity purification) and a 9 amino acid (RKKRRQRRR) membrane penetrating domain (MPD) from HIV TAT protein were added using at the N terminus using modified sense primers as shown below (Fig. 1).

KLF4-F: 5 -

ATAGCCCGGGCCGCAAGAAGCGCAGACAGCGCCGTCGAGGAGGCGGTGGG
ATGGCTGTCAGCGACGCGCTG-3 (GenBank: BC030811/0299 23);

KLF4-R: 5 -ATAGCGGCGCTTAA AAATGCCTC TTCATGTGTAAG-3
(GenBank: BC 030811/029923);

c-MYC-F: 5 -

ATAGCCCGGGCCGCAAGAAGCGCAGACAGCGCCGTCGAGGAGGCGGTGGG
CTGGATTTTTTTCGGGTAGTGG-3 (GenBank: BC058901);

c-MYC-R: 5 -ATAGCGGCGCTTACGCACAAGAGTTCCGTAG-3 (GenBank:
BC058901);

OCT4-F:5 -

ATAGCCCGGGCCGCAAGAAGCGCAGACAGCGCCGTCGAGGAGGCGGTGGG
ATGGCGGGACACCTGGCTTCG-3 (GenBank: BC117435);

OCT4-R: 5 -ATAGCGGCGCTCAGTTTGAATGCATGGGAGAGC-3 (GenBank:
BC117435);

SOX2-F: 5 -

ATAGCCCGGGCCGCAAGAAGCGCAGACAGCGCCGTCGAGGAGGCGGTGGG
ATGTACAACATGATGGGAGCG-3 (GenBank: BC013923);

SOX2-R: 5 -ATAGCGGCGCTCACATGTGTGAGAGGGGCAG-3 (GenBank:
BC013923).

The PCR products were then subcloned into pCR4-TOPO vector (Invitrogen). After sequence verification, they were restricted with *Sma*I and *Not*I followed by subcloning into the *Sma*I and *Not*I sites of the bacterial expression vector pProEx-HTa (Invitrogen). The resulting clones were then verified again by sequencing.

Expression and purification of fusion proteins

E. coli C41 or Rosetta strain (Invitrogen, USA) were transformed with plasmids encoding the *TAT-SOX2*, *TAT-OCT4*, *TAT-KLF4* and *TAT-c-MYC* genes were grown overnight at 37°C in LB broth supplemented with 100 µg/ml Ampicillin. The overnight cultures were then diluted 50-fold with fresh LB media and cultured at 37°C while shaking at 200 rpm until an OD 600 = 0.5 was reached. Protein expression was induced by the addition of IPTG to a final concentration of 1.0 mM for 4 h at 30°C. To prepare the denatured fusion proteins,

the induced cells were harvested and lysed by sonication in lysis buffer (50 mM HEPES, 8 M Urea, 10 mM imidazole, pH 8.0, for TAT-KLF4; 20 mM Tris-Cl, 8 M Urea, 10 mM imidazole, pH 8.0, for TAT-OCT4, TATA-SOX2 and TAT-c-MYC). After removal of the cell debris by centrifugation, 1 ml of a 50% Ni-NTA slurry was added to 4 ml lysate and mixed by shaking for 15–60 min at room temperature. The lysate resin mixture was carefully loaded into an empty column and washed twice with 4 ml wash buffer (lysis buffer with 20 mM imidazole, pH 8.0). Finally, proteins were eluted using an elution buffer (lysis buffer with 250 mM imidazole, pH 8.0).

Desalting through PD-10 gel filtration column

Econo-Pac®10DG columns (BIO-RAD,) were used for desalting. After equilibrating the column with 20 ml of DMEM, 3.0 ml purified fusion protein was added to the column. 1 ml fractions were eluted using DMEM medium, and the protein concentrations in each fraction were quantitated by a densitometric analysis after separation by SDS-PAGE, using bovine serum albumin (BSA) as the standard. The purified desalted fusion proteins dissolved in DMEM were then aliquoted and stored at –80°C.

Luciferase analysis

HEK-293T/17 cells were co-transfected with 1.0 µg 6xO/S-Luc luciferase reporter plasmids (containing OCT4 and SOX2 binding sites) and 1.0 µg OCT4 or 1.0 µg SOX2, or 0.5 µg OCT4 and 0.5 µg SOX2 expression vectors. 1.0 µg 6xO/S-Luc and 1.0 µg TK-Luc reporter plasmids were used to confirm the promoter specificity and system sensitivity, respectively. Untransfected cells were treated as negative controls. After 24 h of transfection, cells were lysed with 1× Glo Lysis Buffer (Promega) and incubated for 5 min at room temperature. Luciferase activities were measured with a Steady-Glo Luciferase Assay system (Promega) and Veritas Microplate Luminometer detection system (Turner Biosystems, CA), according to the manufacturer's protocol.

293T cells were transfected with 6xO/S-Luc reporter plasmid then 24 h after transfection, 40 nM of TAT-OCT4 and TAT-SOX2 fusion proteins were added to the culture media and incubated for 3 h. Cells were harvested and analyzed by the Luciferase assay. Relative luciferase activity of each treatment is shown normalized to that of the 6xO/S-Luc control (given as 1.0).

Transduction of fusion proteins

Human fibroblast IMR90 cells were cultured in DMEM containing 10% fetal bovine serum (FBS), and antibiotics (100 mg/ml streptomycin, 100 U/ml penicillin) at 37°C. To evaluate the transduction ability of denatured recombinant TAT transcription factors, IMR90 cells were grown to 80% confluence in 12-well plates. Culture medium was replaced with serum-free DMEM containing denatured TAT fusion proteins at different concentrations (2.5, 5.0, 10.0, 20.0, 40.0 nM) for 8 and 10 h and the dose and time-dependent analysis of transduced proteins was analyzed by Western blotting.

To determine the intracellular localization of the fusion proteins, IMR90 cells were grown on glass coverslips in 12-well plates. 40 nM of TAT-SOX2, TAT-OCT4, TAT-KLF4, TAT-c-MYC were added to the serum-free culture media and incubated for 4 h at 37°C. Subsequently, cells were washed with PBS, followed by an acid wash of 0.2 M glycine-HCl, pH 2.2 to remove any proteins attached to the outer surface of the cells.

For Immunofluorescence staining, cells were fixed in 4% (v/v) formaldehyde in 1× PBS at room temperature for 30 min and permeabilized in 0.1% Triton X-100 in 1× PBS for 3 min at room temperature. For removal of nonspecific binding of the antibodies, cells were

incubated with DAKO protein block solution for 1 h at room temperature. After washing three times with 1× PBS, cells were incubated in the diluted specific primary antibody (Santa Cruz Biotechnology, USA) 1 h at room temperature or overnight at 4°C. After washing three times, fluorescence conjugated secondary antibody (Vector Laboratories, USA) was added and incubated for 1 h at room temperature in the dark. After washing three times with PBS, they were mounted with DAPI mounting medium (Vector Laboratories, USA). The immunoreactions were observed on fluorescence microscope (ZEISS, USA).

Western blot analysis

For western blot analysis, cells were lysed with a lysis buffer (125 mM Tris/HCl, pH 6.8, 2% SDS, 10%, v/v glycerol). Lysates were separated using 10% SDS-polyacrylamide Ready Gel precast gels (BIO-RAD, USA), electrotransferred to nitrocellulose membranes (Amersham Biosciences, Germany), and blocked with 5% (w/v) dried milk in 1× TBST. The membrane was probed with primary antibody (anti-His probe antibody: Sc-803; anti-GKLF (H-180) antibody: Sc-20691; anti-c-Myc antibody: Sc-40; anti-Oct3/4 antibody: Sc-5279; anti-Sox2 antibody: Sc-20088; Santa Cruz Biotechnology, USA) diluted 1:1,000, followed by incubation with Horseradish Peroxidase (HRP) conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (Thermo Fisher Scientific Inc., USA) diluted 1:1,000. The bound antibodies were visualized by enhanced chemiluminescence (Western Blotting Luminol Reagent: sc-2048, Santa Cruz Biotechnology, Inc.) and detected by an LAS-3000 imaging system.

Results

Identification of recombinant proteins by Coomassie blue staining and Western blot analysis

Expression constructs in pProEx-HTa/TAT-gene were transformed into *E. coli* C41 or Rosetta host. Cell lysates were analyzed by SDS-PAGE followed by Coomassie Blue staining to examine the expression of the recombinant proteins. After induction by IPTG, fusion proteins (arrowed) were expressed as a major component of the total host cell protein (Fig. 2a). The induced proteins were shown to react with a rabbit polyclonal antibody to 6xHis probe by Western blot analysis (Fig. 2b). All the four fusion proteins were then purified with Ni-NTA-agarose under denaturing conditions. As shown in Fig. 2c, all the fusion proteins were purified although there were some degradation products in each.

Denatured TAT recombinant proteins showed very weak biological activities

To investigate whether the denatured TAT fusion proteins could enter cells, become correctly refolded and retain biological activity, a luciferase activity analysis was used. First, HEK-293T/17 (293T) cells were co-transfected with 6xO/S-Luc luciferase reporter plasmid [16] along with OCT4 or SOX2 expression vectors independently or OCT4 and SOX2 combined. 6xO/S-Luc and TK-Luc reporter plasmids were used to confirm the promoter specificity and system sensitivity, respectively. Relative luciferase activity of each treatment shown was normalized to that of the 6xO/S-Luc control (given as 1.0). As shown in Fig. 3a, the 6xO/S-Luc was specifically activated by OCT4 and SOX2 protein expression. When OCT4 and SOX2 were expressed together, the Luciferase reporter was also significantly activated.

We then utilized the 6xO/S-Luc luciferase reporter plasmid to evaluate the biological activities of denatured TAT-OCT4 and TAT-SOX2 proteins. 40 nM of each fusion protein was added to the culture medium of 293T cells for 3 h. These cells had been transfected with 6xO/S-Luc reporter plasmid 24 h earlier. As shown in Fig. 3b denatured TAT-OCT4 and

TAT-SOX2 proteins together can activate 6xO/S-Luc luciferase reporter only very weakly compared to the expression plasmids.

TAT recombinant proteins accumulated in the exonuclear region

To evaluate the ability of denatured TAT fusion proteins to penetrate cells proteins were added to the culture media of human fibroblast IMR90 cells at different concentrations (2.5, 5.0, 10.0, 20.0, 40.0 nM) for 8 and 10 h. After cell lysis the dose-dependent and time-dependent analysis of transduced proteins were analyzed by Western blotting. As shown in Fig. 4 for TAT-KLF4 proteins, 5 and 10 nM were detectable in cells after 8 and 10 h incubation. At 40 nM proteins had an extra degraded band at 8 h incubation, but did not show up after 10 h incubation. For TAT-c-MYC proteins, 2.5 and 10 nM were detectable in cells after 8 and 10 h incubation but at 40 nM the protein had a strong extra degraded band at both 8 and 10 h incubation. For TAT-OCT4 proteins, 2.5 nM were weakly detectable in cells at 8 and 10 h incubation, and at 40 nM, proteins had a weak extra degraded band at 8 and 10 h incubation. Finally, for TAT-SOX2 proteins, 5 nM was detectable in cells after 8 and 10 h incubation. An extra degraded band could be detected at 20 and 5 nM at 8 and 10 h incubation, respectively. The levels of proteins in the cultured IMR90 cells increased with the amounts of fusion proteins added to the media. These data indicated that 40 nM was the highest concentration for reprogramming.

To determine the subcellular localization of the TAT fusion proteins that had penetrated into cells, 40 nM of each denatured fusion proteins was added to the culture media of IMR90 cells for 4 h and then analyzed using Immunofluorescence staining. As shown in Fig. 5, all four recombinant TAT transcription factors proteins (TAT-SOX2, TAT-OCT4, TAT-KLF4, and TAT-c-MYC) were successfully delivered into approximately 60% of the cells. However, virtually none of the protein had translocated to the nucleus and the vast majority appeared to be localized to discrete endosomal packages.

Discussion

In this study, we aimed to test the feasibility of reprogramming human fibroblast cells using recombinant transcription factor proteins carrying the TAT cell penetrating motif at the N terminus and expressed in *E. coli*. Although purification of the proteins left them denatured, there is good evidence that denatured proteins will refold and regain biological activity inside the cell. Jin et al. [17] demonstrated that the denatured TAT-CAT and 9Arg-CAT fusion proteins could be refolded into a biologically active conformation in mammalian cells. Parallel results were obtained from Park et al. [18] using a TAT-GFP fusion protein. Kwon et al. [19] provided evidence that denatured TAT-SOD could be correctly refolded in HeLa cells either by through molecular chaperones or by some form of spontaneous process.

The purified proteins were able to enter into mammalian cells when added to tissue culture medium and remained detectable for at least 10 h. However, a functional assay using the synthetic luciferase promoter (6xO/S-Luc) indicated that the biological activity of TAT-OCT4 and TAT-SOX2 was very low. In contrast, the activity of control expression plasmids was very high. When we investigated the intracellular localization of the recombinant proteins we saw that very little had translocated to the nucleus where we expected to find it. It seemed that the majority of the protein was localized in discrete cytoplasmic bundles leading us to conclude that it was still trapped within the endosomes.

The mechanism of TAT mediated transduction into cells is not completely understood. Previous findings suggest that the transduction of TAT occurs by way of macropinocytosis, a specialized form of endocytosis. It is a multistep process that involves binding of TAT to the cell surface, stimulation of macropinocytotic uptake of TAT, cargo into macropinosomes

and finally endosomal escape into the cytoplasm [15]. In order to enhance endosomal release we used different concentrations of chloroquine in the media but it did not help for our proteins (data not shown). Yoshikawa et al. [20] delivered TAT fused cargo into the nucleus using an endosome-disruptive peptide (hemagglutinin-2 subunit) and a nuclear localization signal. Thus, we synthesized the HA2-TAT peptide (GLFEAIEGFIENGWEGMIDGWYGYGRKKRRQRRR) and treated the cells with TAT-transcription factors along with HA2-Tat. Despite the fact that OCT4 and SOX2 have strong nuclear localization signals and even with the help of HA2-TAT, no appreciable endosomal release was seen.

Very recently, Zhou et al. have published successful reprogramming of mouse fibroblast cells using recombinant proteins. These authors used OCT4, SOX2, KLF4 and c-MYC fused to the poly-arginine cell penetrating motif rather than TAT. It was also fused to the C terminus rather than N terminus as in our case [21]. Another major difference that may have contributed to their success was the fact that the denatured proteins were refolded and repurified before use. In our case, we did not attempt any refolding which may be the reason the proteins became “stuck” in the endosomes whereas that of Zhou et al. translocated to the nucleus. Resolution of this problem should lead to effective cell reprogramming using recombinant TAT proteins.

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References

- Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. *Nature*. 2007; 448:313–317. doi:10.1038/nature05934. [PubMed: 17554338]
- Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K, Bernstein BE, Jaenisch R. In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature*. 2007; 448:318–324. doi:10.1038/nature05944. [PubMed: 17554336]
- Nakagawa M, Koyanagi M, Tanabe K, Takahashi K, Ichisaka T, Aoi T, Okita K, Mochiduki Y, Takizawa N, Yamanaka S. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat Biotechnol*. 2008; 26:101–106. doi:10.1038/nbt1374. [PubMed: 18059259]
- Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA. Induced pluripotent stem cell lines derived from human somatic cells. *Science*. 2007; 318:1917–1920. doi:10.1126/science.1151526. [PubMed: 18029452]
- Hanna J, Wernig M, Markoulaki S, Sun CW, Meissner A, Cassady JP, Beard C, Brambrink T, Wu LC, Townes TM, Jaenisch R. Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. *Science*. 2007; 318:1920–1923. doi:10.1126/science.1152092. [PubMed: 18063756]
- Mali P, Ye Z, Hommond HH, Yu X, Lin J, Chen G, Zou J, Cheng L. Improved efficiency and pace of generating induced pluripotent stem cells from human adult and fetal fibroblasts. *Stem Cells*. 2008; 26:1998–2005. doi:10.1634/stemcells.2008-0346. [PubMed: 18511599]
- Huangfu D, Maehr R, Guo W, Eijkelenboom A, Snitow M, Chen AE, Melton DA. Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. *Nat Biotechnol*. 2008; 26:795–797. doi:10.1038/nbt1418. [PubMed: 18568017]
- Shi Y, Do JT, Despons C, Hahm HS, Scholer HR, Ding S. A combined chemical and genetic approach for the generation of induced pluripotent stem cells. *Cell Stem Cell*. 2008; 2:525–528. doi:10.1016/j.stem.2008.05.011. [PubMed: 18522845]

9. Okita K, Nakagawa M, Hyenjong H, Ichisaka T, Yamanaka S. Generation of mouse induced pluripotent stem cells without viral vectors. *Science*. 2008; 322:949–953. doi:10.1126/science.1164270. [PubMed: 18845712]
10. Stadtfeld M, Nagaya M, Utikal J, Weir G, Hochedlinger K. Induced pluripotent stem cells generated without viral integration. *Science*. 2008; 322:945–949. doi:10.1126/science.1162494. [PubMed: 18818365]
11. Fawell S, Seery J, Daikh Y, Moore C, Chen LL, Pepinsky B, Barsoum J. Tat-mediated delivery of heterologous proteins into cells. *Proc Natl Acad Sci USA*. 1994; 91:664–668. doi:10.1073/pnas.91.2.664. [PubMed: 8290579]
12. Mann DA, Frankel AD. Endocytosis and targeting of exogenous HIV-1 Tat protein. *EMBO J*. 1991; 10:1733–1739. [PubMed: 2050110]
13. Vives E, Brodin P, Lebleu B. A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J Biol Chem*. 1997; 272:16010–16017. doi:10.1074/jbc.272.25.16010. [PubMed: 9188504]
14. Gump JM, Dowdy SF. TAT transduction: the molecular mechanism and therapeutic prospects. *Trends Mol Med*. 2007; 13:443–448. doi:10.1016/j.molmed.2007.08.002. [PubMed: 17913584]
15. Murriel CL, Dowdy SF. Influence of protein transduction domains on intracellular delivery of macromolecules. *Expert Opin Drug Deliv*. 2006; 3:739–746. doi:10.1517/17425247.3.6.739. [PubMed: 17076596]
16. Ambrosetti DC, Scholer HR, Dailey L, Basilico C. Modulation of the activity of multiple transcriptional activation domains by the DNA binding domains mediates the synergistic action of Sox2 and Oct-3 on the fibroblast growth factor-4 enhancer. *J Biol Chem*. 2000; 275:23387–23397. doi:10.1074/jbc.M000932200. [PubMed: 10801796]
17. Jin LH, Bahn JH, Eum WS, Kwon HY, Jang SH, Han KH, Kang TC, Won MH, Kang JH, Cho SW, Park J, Choi SY. Transduction of human catalase mediated by an HIV-1 TAT protein basic domain and arginine-rich peptides into mammalian cells. *Free Radic Biol Med*. 2001; 31:1509–1519. doi:10.1016/S0891-5849(01)00734-1. [PubMed: 11728823]
18. Park J, Kim KA, Ryu JY, Choi EY, Lee KS, Choi SY. Generation and characterization of cell-permeable green fluorescent protein mediated by the basic domain of human immunodeficiency virus type 1 Tat. *J Microbiol Biotechnol*. 2000; 10:797–804.
19. Kwon HY, Eum WS, Jang HW, Kang JH, Ryu J, Lee BR, Jin LH, Park J, Choi SY. Transduction of Cu, Zn-superoxide dismutase mediated by an HIV-1 Tat protein basic domain into mammalian cells. *FEBS Lett*. 2000; 485:163–167. doi:10.1016/S0014-5793(00)02215-8. [PubMed: 11094160]
20. Yoshikawa T, Sugita T, Mukai Y, Yamanada N, Nagano K, Nabeshi H, Yoshioka Y, Nakagawa S, Abe Y, Kamada H, Tsunoda S, Tsutsumi Y. Organelle-targeted delivery of biological macromolecules using the protein transduction domain: potential applications for Peptide aptamer delivery into the nucleus. *J Mol Biol*. 2008; 380:777–782. doi:10.1016/j.jmb.2008.05.047. [PubMed: 18571668]
21. Zhou H, Wu S, Joo JY, Zhu S, Han DW, Lin T, Trauger S, Bien G, Yao S, Zhu Y, Siuzdak G, Schöler HR, Duan L, Ding S. Generation of induced pluripotent stem cells using recombinant proteins. *Cell Stem Cell*. 2009; 4:381–384. doi:10.1016/j.stem.2009.04.005. [PubMed: 19398399]

Fig. 1.

Recombinant TAT-transcription factor design. (His)₆ was used for purification followed by the 9AA TAT cell penetrating motif

Fig. 2.

Generation and purification of TAT recombinant transcription factors. **a** Expression of recombinant transcription factors in *E. coli*. Liquid culture was induced at $OD_{600} \sim 0.5$ by 1 mM IPTG. The whole cell lysate was analyzed by 10% SDS-PAGE and stained with Coomassie Blue. Induction by IPTG produced an extra band (*arrowed*) in lane 2, 4, 6, 8 compared with lane 1, 3, 5, 7, respectively. Lane M, Pre-stained protein ladder; lane 1, 3, 5, 7, 0 h culture before IPTG induction; lane 2, 4, 6, 8, induced TAT-KLF4, TAT-c-MYC, TAT-OCT4, TAT-SOX2 fusion protein expression. **b** Western blot analysis of recombinant proteins whole cell lysate was separated by SDS-PAGE and analyzed by Western blot. Primary antibody: His Probe (H-15) antibody, sc-803. Lane 1, 3, 5, 7, 0 h culture before IPTG induction. Lane 2, the bigger band was TAT-Klf4, the lower one was the degraded band. Lane 4, TAT-c-Myc; Lane 6, TAT-Oct4; Lane 8, TAT-Sox2. **c** Analysis of purified fusion proteins. Proteins were analyzed by 10% SDS-PAGE and stained with Coomassie Blue. Lane M, Pre-stained protein ladder; Lane 1, purified TAT-Klf4; Lane 2, purified TAT-c-Myc; Lane 3, purified TAT-Oct4; Lane 4, purified TAT-Sox2

Fig. 3. Analysis of biological activity of recombinant TAT:SOX2 and TAT:OCT4 proteins. **a** HEK-293T/17 cells were co-transfected with 6xO/S-Luc luciferase reporter plasmids along with Oct4 or Sox2 or Oct4 and Sox2 expression vectors. 6xO/S-Luc and TK-Luc reporter plasmids were used to conform the promoter specificity and system sensitivity, respectively. Untransfected cells were treated as negative control. After 24 h of transfection, cells were harvested and luciferase assays were performed. Relative luciferase activity of each treatment is shown, which is normalized to that of 6xO/S-Luc control (given as 1.0). **b** HEK-293 cells were transfected with 6xO/S-Luc luciferase reporter plasmids. Negative control was untransfected cells. After 24 h of transfection, cells were incubated with 40 nM TAT-Oct4 and 40 nM TAT-Sox2 in serum-free medium for 3 h. Then harvested cells and performed luciferase assays. Relative luciferase activity of each treatment is shown, which is normalized to that of 6xO/S-Luc control (given as 1.0)

Fig. 4.

Dose-dependent and time-dependent analysis of transduced proteins. Various concentrations of TAT fusion proteins were added to IMR90 cells for 8 and 10 h, and the presence of transduced protein in the cells were analyzed by western blotting. Lane 1–5, IMR90 cells were treated with 2.5, 5.0, 10.0, 20.0, 40.0 nM TAT fusion proteins for 10 h; Lane 6–10, IMR90 cells were treated with 2.5, 5.0, 10.0, 20.0, 40.0 nM TAT fusion proteins for 8 h; Lane 11, untreated IMR90 cells

Fig. 5. Visualization of TAT fusion proteins transduced into IMR90 fibroblast cells by Immunofluorescence. 40 nM TAT fusion proteins were added to culture media of IMR90 cells. Four hours later, the cells were washed once with 0.2 M glycine-HCl, pH 2.2 to remove those proteins attached on the outer surface of the cells. Then cells were then examined by Immunofluorescence using specific antibodies. **a** Untreated, **b** treated with TAT-KLF4, **c** treated with TAT-c-MYC, **d** treated with TAT-OCT4 and **e** treated with TAT-SOX2. In all cases very little protein has translocated to the nucleus and remains bound to the endosomes in discreet packages. Scale bar: 20 μ m