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TAT κ Fusion Protein of OCT-3/4 and KLF-4: Stable Mixed Population Cell Lines Capable of Delivering Fusion Proteins to Target Cells

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Abstract

A rapidly growing body of evidence has shown that enforced expression of a small panel of genes, including Oct-3/4, KLF4, Sox2 and c-Myc, can induce the reprogramming of previously differentiated cells, to generate induced Pluripotent Stem Cells (iPSCs). However, the generation of iPSCs by genetic modifications raises the risk of malignant transformation. Therefore, efficient *in vitro* reprogramming of differentiated cells, without irreversible genetic modification is highly desirable. The improved *trans*-activator of transcription *kappa* (TATκ), a synthetic TAT-HIV, confers the ability to deliver several proteins into target cells, thus, making it a potential alternative delivery mechanism for transcription factors or therapeutic agents. Using Green Fluorescence Protein (GFP) and Apoptin as model proteins, we have recently described a strategy to generate cell lines that secrete proteins carrying a modified HIV-TAT Protein Transduction Domain (PTD) for subsequent protein transduction mediated uptake by target cells. Using this strategy we have generated 293T cells secreting the pluripotent factors Oct-3/4 or KLF4 in fusion with TATκ. Oct-3/4 and KLF4 were detected in the culture medium of the transduced 293T cell and uptake of Oct-3/4 by haematopoietic cell lines JURKAT and FDCP-1 was confirmed by Western blot analysis. KLF4 was present in the culture medium of the producer 293T cell but we are still unable to demonstrate uptake by target cells. Based on results obtained, we hope that these stable mix population cell lines can play an important role in generation of iPSCs for therapeutic purposes.

Keywords: TATκ; Protein Transduction Domain (PTD); iPSC; Transcription Factors; Producer Cells

Introduction

Viral transduction approach has been shown to be a more efficient strategy to generate iPSCs from human haematopoietic or fibroblast cells as compared to protein transduction. However, the efficiency of this strategy is marred by safety concerns especially in the clinical setting. There are various other strategies to minimise the permanent genetic modification of cell-based therapies have successfully generated iPSCs in both human and mouse system. These include adenoviral and direct plasmid transfection has been successfully used to generate iPSCs in the mouse system [1-3]. Also, piggyback transposon vectors [4,5], and most recently, the use of non-integrating episomal vectors [6-8], have confirmed the reprogramming of iPSCs from either human or mouse fibroblasts.

Recently, a number of studies have shown strategies to generate mouse iPSCs without viral vectors [1,2,9]. These studies show that genomic integration of viral vectors is not essential for successful reprogramming of iPSCs. This is achievable with episomal plasmids, excisable transposons, adeno or sendai viruses, mRNA, transient plasmid transfection, or recombinant proteins [3]. Another recently published data demonstrated the generation of iPSCs from murine embryonic fibroblasts using recombinant cell penetrating-reprogramming fusion proteins [10]. This was done by conjugating the pluripotent factors to a poly-arginine protein transduction domain, expressed in *E. coli*, solubilised, refolded and further purified. These recombinant proteins were delivered directly into culture media of murine embryonic fibroblasts at defined concentrations. The iPSCs that were generated using the direct delivery of recombinant proteins were pluripotent both *in vitro* and *in vivo* for more than 30 passages [10].

The potential of TAT to deliver therapeutic proteins for treatment of cancer has been widely studied. Although many studies have proven the uptake of TAT fusion proteins by target cells, the trans-activation potential of endogenous cellular genes by the 11 amino acids (YGRKKRRQRRR) remains unclear. A few studies have shown that the TAT peptide is energy-dependent, requiring a temperature above 4°C and ATP [11]. PTD was demonstrated to internalize without depending on a specific primary sequence; hence, it is receptor-independent. [12,13]. Other models have also emerged to explain PTD uptake into target cells by the perpendicular insertion of amphipathic peptides, electrostatic interaction or endocytosis.

Full-length expression of TAT protein stimulates the growth of Kaposi's sarcoma-derived cells [14], and TAT transgenic mice develop Kaposi's sarcoma [15]. However, there is no reported data on the TAT peptide used to be of any risk/danger of inducing Kaposi's sarcoma. In our studies, we used TATκ (YARKAARQARA), which was generated by introducing mutations to destroy the two furin cleavage sites that were present within the original TAT peptide. Furin, a human gene also known as PACE (*Paired basic Amino acid Cleaving Enzyme*), belongs to the subtilisin-like proprotein convertase family. Furin is enriched in the Golgi apparatus but can translocate between the trans-Golgi network

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and the cell surface [16]. The presence of these Furin sites in the TAT peptide resulted in the TAT peptide being cleaved from fusion proteins that are secreted via the constitutive pathway [17]. Therefore, the secreted protein will not be able to enter target cells and is trapped in the culture supernatant. In order to avoid this problem from occurring, the amino acid sequences RQRR and RKKR of TAT were modified by replacing five Ala residues within the peptide (Figure 1).

To enable the efficient transduction of transcription factors such as HoxB4, Sox2, KLF4, Bcl-2, OCT-3/4 or possibly others, we established a producer cell line for *in vivo* secretion of these factors. The feasibility of this producer cell to secrete TATκ fusion proteins have been demonstrated by the same group using a model system secreting TATκ-GFP as a protein marker fused to apoptin [17]. The secreted TATκ-GFP fused to Apoptin was able to transduce into human osteosarcoma cell line (Saos-2) and induce apoptosis [17]. Data obtained showed improved TATκ-GFP secretion compared to TAT-GFP by producer cells, correlating the presence of furin sites to reduced secreted protein. In this current study, we generated stable producer cell lines that continuously secrete TATκ fusion proteins that can be delivered into target cells. This provides a platform for us to generate iPSCs without genetic modification.

Materials and Methods

Cell lines

Human Embryo Kidney (HEK) 293T cell line was cultured in DMEM (Dulbecos's Modified Eagle Medium) (Sigma Aldrich) supplemented with 10% (v/v) heat-inactivated FCS (Feotal Calf Serum) (PAA, Laboratories), 100 µg/mL penicillin-streptomycin (Sigma Aldrich). These cells were used to screen for their suitability as 'factor producing cells' or 'feeder cells' for secretion of chimeric proteins containing a Protein Transduction Domain (PTD). A murine Factor-Dependent Progenitor Cell (FDC-P1), and immortalized line of human T lymphocyte cells (JURKAT) were cultured in RPMI 1640 (Roswell Park Memorial Institute) (Sigma Aldrich) supplemented with 10% heat-inactivated FCS, 100 µg/mL penicillin-streptomycin. 0.01µg/mL of Interleukin-3 (IL-3) (R&D Systems) was also added to FDC-P1 culture media. Mouse embryo fibroblasts (NIH3T3) were cultured in DMEM, supplemented with 10% (v/v) heat-inactivated NCS (neonate calf serum), 100 µg/mL penicillin-streptomycin (Sigma Aldrich).

Constructs

Full-length of human stem cell transcription factors KLF4, and Oct-3/4 complementary DNA (cDNA) with TATκ sequences were cloned into pSecTag2B (Invitrogen) which contains a murine Ig κ-chain V-J2-C signal peptide enabling the secretion of the encoded proteins (Figure 2). In order to select the stable clones after transfection, the pSecTag2 vector was further modified by replacing the zeosin selectable marker gene with puromycin. These plasmids were named spTATκ-KLF4, and spTATκ-Oct-3/4.

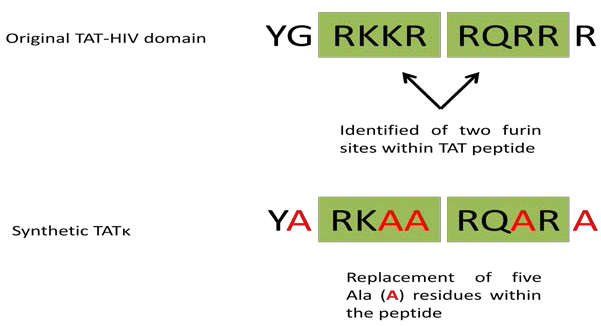

Figure 1: Comparison of TAT-HIV and TATκ peptides. The black-coloured letters indicate the replacement of the original amino acid in the original TAT-HIV domain. The green box within the TAT peptide indicates the presence of two furin sites. *A=Alanine; G=Glysine; K=Lysine; Q=Glutamine; R=Arginine; Y=Tyrosine.*

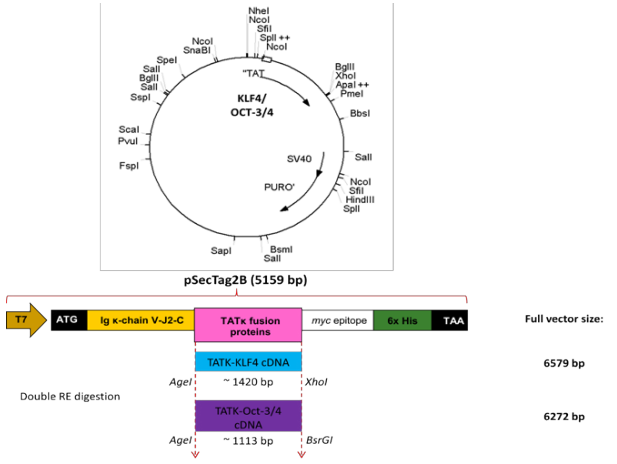

Figure 2: Schematic representation of pSecTag2B vector. The full-length of human OCT-3/4 and KLF4 genes were individually fused to the TATκ sequences and cloned into pSecTag2B vector backbone. The generated plasmid constructs encode the indicated fusion proteins.

Transient transfection

A standard calcium phosphate ($Ca\text{-}PO_4$) co-precipitation transfection protocol was used as described elsewhere [18]. Briefly, a total of 1 million 293T cells were seeded in 10mL complete growth medium in 100 mm diameter culture-dishes (polystyrene coated, Greiner Bio-One). The transfection was carried out the following day with 1 ml of DNA $Ca\text{-}PO_4$ co-precipitation mixture containing 20 µg of plasmid DNA per culture dish, 0.5 M $CaCl_2$ and 2X HEBS (HEPES Buffered Saline) at pH 6.7. The transfection medium was left to stand for 30 minutes at room temperature to form a fine opalescent precipitation. The DNA $Ca\text{-}PO_4$ co-precipitation mixture was added drop wise to the surface of the media containing the cells (50-60% confluent by the time of transfection). The culture dish was gently swirled to mix the media evenly and incubated at 37°C with 5% CO_2 for 72 hours. The culture medium was replaced with fresh complete growth medium the following day. Approximately 72 hours post-transfection, cells were harvested to determine TATκ fusion protein expression by either FACS or Western blot analysis.

Stable transfection

The same protocol used in transient transfection was employed for establishment of stable TATκ fusion protein producing cell lines. In summary, 72 hours post-transfection, the transfected cells were selected with puromycin (2.5 µg/mL). Selection media with puromycin was changed every 3-4 days and resistant mixed population or clones were picked after 3-4 weeks.

To determine the TATκ fusion protein expression in each selected mixed population or clones, 30,000 cells per mL were seeded in 6 well plates with complete growth medium with the addition of puromycin (2.5µg/mL). After 72 hours, cells were harvested and used for flow cytometry and Western blot analysis.

To confirm TATκ fusion protein secretion, culture mediums from the selected mixed population or clones were used for TCA precipitation or vivaspin filter column and analysed by Western blot.

Western blot analysis

For cell lysates, approximately 3 x 10^6 of cell pellets were washed twice in DPBS (Dulbecco's Phosphate Buffered Saline) and dissolved in 100 µL of lysis buffer containing 20mM HEPES, 50mM Sodium Chloride ($NaCl_2$), 2% Nonidet P40, 0.5% Sodium deoxycholate, 0.2% Sodium Dodecyl Sulfate (SDS), 1mM Ethylene Glycol Tetra-acetic Acid (EGTA), 1mM Sodium orthovanadate, 10mM Sodium fluoride (NaF), 1mM Phenyl Methyl Sulphonyl Fluoride (PMSF) and 10µL protease inhibitor cocktail (Sigma Aldrich). Following 10 minute incubation on ice, samples were centrifuged for 10 minutes at 10,000 x g at 4°C. Cell lysates were stored at -20°C.

For culture medium containing the secreted TATκ fusion proteins, the vivaspin column filtration method was used. 10 mL of culture medium was collected and filtered using a 0.45 µM syringe filter. The filtered medium was loaded into vivaspin column tube and centrifuged at 3,900 x g for 30 minutes (at 4°C) or until a final volume of 0.5 mL (20-fold concentration) was reached. For SDS-PAGE, 20 µL of concentrated culture medium was lysed with 2X Laemmli Sample Buffer (LSB) and incubated at 95°C for 5-10 minutes before loading onto 10% SDS-polyacrylamide gel.

Gel electrophoresis was carried out using 10% SDS-polyacrylamide gradient gels (SDS-PAGE) and transferred to Hybond ECL nitrocellulose membranes (GE Healthcare). The following primary antibodies were used: anti-α-Tubulin monoclonal antibody, anti-GKLF polyclonal antibody, and anti-Oct-3/4 polyclonal antibody; and the secondary antibodies were used; horseradish peroxidase anti-mouse IgG (Sigma) and horseradish peroxidase anti-rabbit polyclonal (Santa Cruz Biotech.). The immunoreactive bands were detected by enhanced chemiluminescence (ECL plus; GE Healthcare). The films were developed using a Photon Imaging system SRX-101A (Konica Minolta Medical & Graphic Inc.).

Transduction of target cells

A total of 100,000 293T (wild type for negative control) or stable producer cells were seeded into 6 transwell permeable support plates (Corning) and grown overnight or until 50-70% confluent. For stable producer cells, the media was refreshed with addition of 5 µM AzaC, one day prior to transduction. On the day of the transduction, 100,000 FDC-P1 or JURKAT cells were seeded into 24 mm transwells (0.4 µM pore size) to minimize direct contact with producer cells (Figure 3). Valproic acid (final concentration 1mM) was added to the cell suspension to increase the negative-charge of the target cells and aid the uptake of highly positively-charged fusion proteins. Both target and producer cells were further co-cultured for 24 hours.

To determine the uptake of TATκ fusion proteins in the FDC-P1 or JURKAT cells, the cells were pelleted, washed twice, followed by an acid wash of 2.0 M glycine-HCL (pH2.2) to remove any protein attached to the outer surface of the cells. The cells were either re-suspended in 300 µL DPBS (Dulbecco's Phosphate-buffered saline) for Western blot analysis. FDC-P1 or JURKAT cells that were co-cultured with wild type 293T cells were used as negative controls.

Results

Transient expression and secretion of TATκ fusion proteins

Oct-3/4 and KLF4 protein fused to TATκ domain were successfully expressed and secreted in 293T cells (Figure 4). Western blot analysis of pTATκ-Oct-3/4 transfected 293T cell line showed a prominent band at molecular weight ~47 kDa, indicating the intracellular expression of Oct-3/4 protein (Figure 4a). The intracellular expression of Oct-3/4 was greater in the cytoplasm compared to the nuclear extract of the transfected cell lysates. In addition, the same molecular weight band (~47 kDa) was also detected in the concentrated culture medium of the transfected 293T cell line, indicating the secretion of Oct-3/4 protein. Similar results were obtained for pTATκ-KLF4 transfected 293T cells. A prominent band of ~60 kDa, size of the TATκ-KLF4 protein, was observed in both cell lysate and concentrated culture medium (Figure 4b). This indicates expression and secretion of the TATκ-KLF4 protein.

Establishment of cell lines expressing TATκ fusion proteins

All transfected 293T cell lines were further treated with 2.5 µg/mL puromycin to select for stable mixed populations of cells. These stable mixed populations were observed between 3 to 4 weeks after treatment

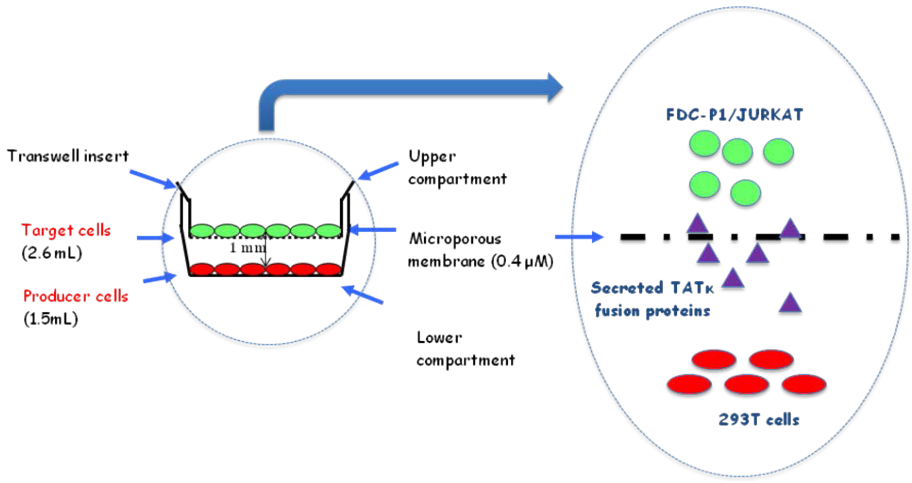

Figure 3: The transwell culture system (in-direct co-culture). Producer cells were cultured at the bottom of the transwell (lower compartment) and the target cells in the transwell insert (upper compartment) of the well. The upper and lower compartment of the transwell was separated by 0.4 µM microporous membrane to minimise direct contact between feeder cells and target cells.

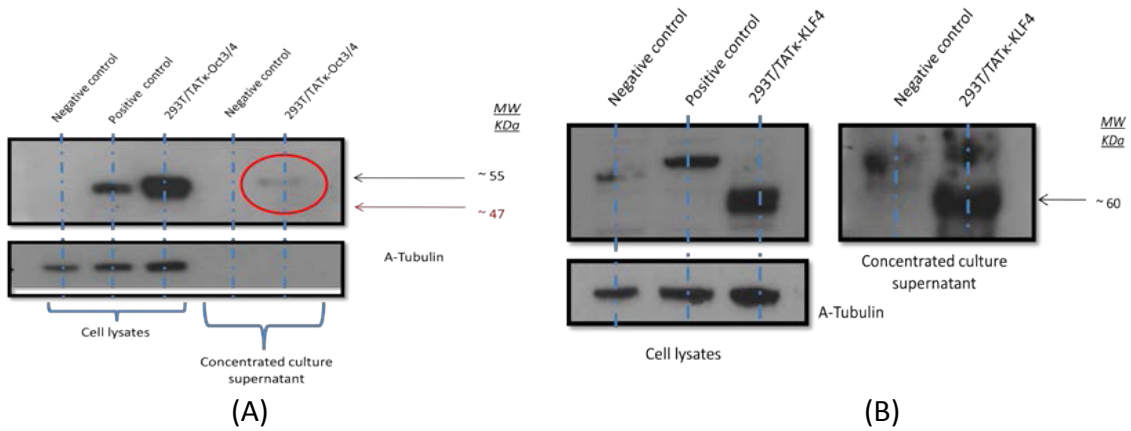

Figure 4: Western blot analysis of TATκ-Oct-3/4 and TATκ-KLF4 transfected 293T cells. Intracellular expression (cell lysates) and secretion of TATκ fusion proteins in the culture medium, 3 days after transfection of 293T cells with either TATκ-Oct-3/4 or pTATκ-KLF4 plasmid DNA. (a) TATκ-Oct-3/4 protein (~47 KDa), and (b) TATκ-KLF4 protein (~60 KDa). Cell lysates and concentrated culture medium from untransfected 293T cells were used as negative controls. Commercially available mouse embryonal carcinoma (F9) and mouse testis extract cell lysates were used as positive controls. An anti-α-Tubulin antibody was used to detect α-Tubulin (~ 55 KDa), which was used as a loading control for each sample. Similar results were obtained in three independent experiments.

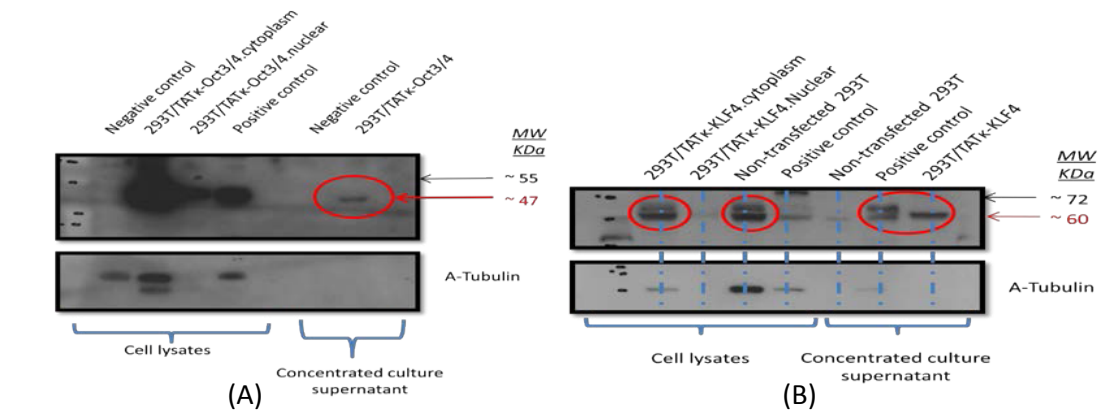

Figure 5: TATκ fusion protein expression and secretion in stable mixed populations of 293T cells by Western blot analysis. Intracellular expression (cell lysates) and secretion of (a) TATκ-OCT-3/4 (~60 KDa), and (b) TATκ-KLF4 proteins in the concentrated culture medium after 4 weeks of puromycin treatment (2.5 μg/mL). Cell lysates and concentrated culture medium from untransfected 293T were used as negative control. Commercially available mouse embryonal carcinoma (F9) and mouse testis extract cell lysates were used as positive controls. An anti-α-Tubulin antibody was used to detect α-Tubulin (~ 55 KDa), which was used as a loading control for each sample.

with puromycin. The intracellular expression and secretion of the TATκ-OCT-3/4 and TATκ-KLF4 proteins in stable mixed populations were further confirmed by Western blot analysis (Figure 5). A prominent band was observed in the concentrated culture medium of the stable mixed population of pTATκ-OCT-3/4 (Figure 5a), and pTATκ-KLF4 (Figure 5b).

Secreted TATκ-OCT-3/4 Protein Show Very Weak Biological Activities

In order to investigate the ability of the secreted TATκ fusion proteins to be taken up by target cells, correctly refolded and retains biological activity, a luciferase activity analysis was used. 6xO/S-Luc+ luciferase reporter plasmid (developed by Dr. Lisa Dailey from New York University) was used to investigate the biological activities of TATκ-Oct-3/4 and TATκ-Sox2 proteins. These secreted proteins are nuclear-bound and will specifically bind to 6 x repetitions of a short Oct-3/4 and Sox2 cDNA sequences within the vector. The FGF promoter (located downstream of 6xO/S sequences) will be activated and drives the luciferase expression upon binding of either Oct-3/4 or Sox2 or a combination of both proteins at the specific binding site. If this occurs, the complete binding of both proteins will enhance the expression of the luciferase gene.

In this study, we investigated: (i) direct transfection of pTATκ-Oct-3/4 and pTATκ-Sox2 DNA plasmids into 6xO/S-Luc+ transfected 293T (control study), and (ii) addition of secreted and concentrated TATκ-Oct-3/4 protein to 6xO/S-Luc+ transfected 293T cell cultures. 293T cells were co-transfected with 6xO/S-Luc+ luciferase reporter plasmid along with pTATκ-Oct-3/4 or pTATκ-Sox2 expression vectors independently or with both plasmids. 293T cells that were transfected with 6xO/S-Luc+ luciferase reporter plasmid, alone were used as a negative control. Relative Luciferase Activity (RLA) for each treatment was normalised to the negative control at 24 hours post-transfection. The expression of the Luc+ gene in the 6XO/S-Luc+ transfected 293T cells was low. However, 6xO/S-Luc+ was specifically activated by either Oct-3/4 or Sox2 protein expression ($p<0.05$) (Figuer 6). The luc+ expression was substantially higher (18-fold) when Oct-3/4 and Sox2

proteins were expressed together compared to control. This control study shows the specificity of the luciferase activity analysis.

In the subsequent studies, the 6xO/S-Luc+ luciferase reporter plasmid was used to evaluate the biological activities of the secreted TATκ-Oct-3/4. In order to further corroborate the data obtained via the luciferase activity analysis, the secreted and concentrated TATκ-OCT-3/4 protein was examined by Western blot analysis prior to luciferase analysis (Figure 7). Addition of the concentrated TATκ-Oct-3/4 protein to the 6xO/S-Luc+ transfected 293T cells for 3 hours resulted in a marginal increase of 1.2-fold in luciferase activity ($p>0.05$) (Figure 8).

TATκ-Oct-3/4 Protein Uptake by FDC-P1 and JURKAT Cell Lines

To evaluate the ability of secreted TATκ-OCT-3/4 protein to be taken up by target cells, a direct co-culture system in transwell plates was used to eliminate direct contact between producer and target cells. Target cells were added into transwells 72 hours after the transfection of producer cells with the plasmid DNA. The producer cells were confirmed for protein secretion prior to the transduction of target cells (data not shown).

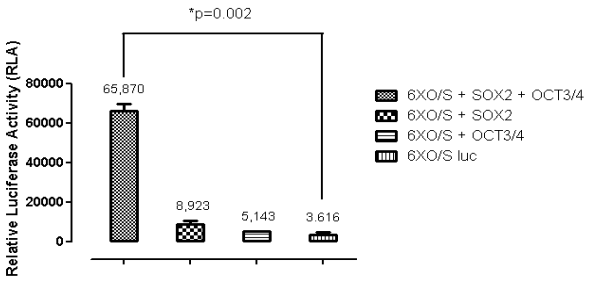

Figure 6: Luciferase expression in 293T cells co-transfected with pTATκ-Oct-3/4 or pTATκ-Sox2 and 6xO/S pGL3. 293T cells were co-transfected with 2.0 µg 6XO/S-Luc luciferase reporter plasmid (containing OCT3/4 and SOX2 binding sites) and either 2.0 µg OCT3/4 or 2.0 µg SOX2; or 1.0 µg OCT3/4 and 1.0 µg SOX2 expression vectors. Un-transfected cells were treated as negative controls. 24 hours after the transfection, cells were lysed with 1X Glo Lysis Buffer (Promega) and incubated for 10 minutes at room temperature. Luciferase activity was measured with the ONE-Glo™ Luciferase Assay system (Promega) and BioTek EL808 Microplate Luminometer detection system, according to the manufacturer's protocol. Data shown are the mean ± SD of Relative Luciferase Activity (RLA) from 3 independent experiments.

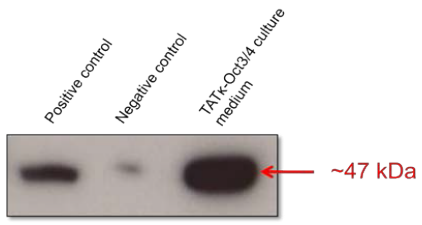

Figure 7: Validation of TATκ-OCT-3/4 secreted proteins in concentrated culture medium by Western blot analysis. The 'factor producing cells' were grown till 80-90% confluent in complete growth media (supplemented with 10% heat-inactivated FBS). The media was replaced with 10mL of serum free-media and incubated for a further 24 hours. The media containing TATκ-OCT-3/4 protein was concentrated using Vivaspin column (20 x concentrations). The presence of TATκ-Oct3/4 protein was detected using rabbit polyclonal antibody. The culture medium from wild-type 293T cells was used as a negative control, while a commercially obtained mouse embryonal carcinoma (F9) cell lysate was used as positive control.

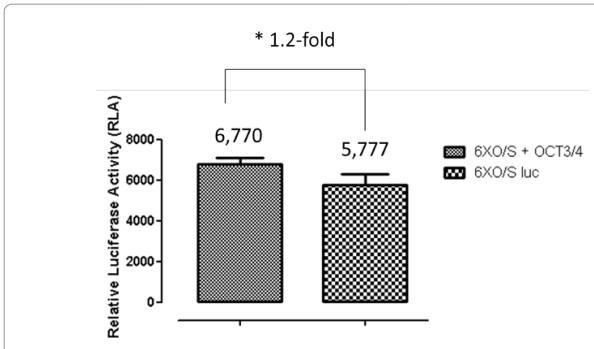

Figure 8: Analysis of luciferase induction by secreted TATκ-OCT-3/4 protein in 6xO/S pGL3 transfected 293T cells. 293T was transfected with 2.0 µg 6XO/S-Luc luciferase reporter plasmid (containing OCT3/4 and SOX2 binding sites). 24 hours after the transfection, concentrated culture medium containing the secreted proteins; TATκ-OCT3/4 was added to the culture media and incubated for 3 hours. Cells were harvested and lysed with 1X Glo Lysis Buffer (Promega) and incubated for 10 min at room temperature. Luciferase activities were measured with a ONE-Glo™ Luciferase Assay system (Promega) and BioTek EL808 Microplate Luminometer detection system, according to the manufacturer's protocol. Data shown are the mean ± SD of Relative Luciferase activity (RLA) from 3 independent experiments.

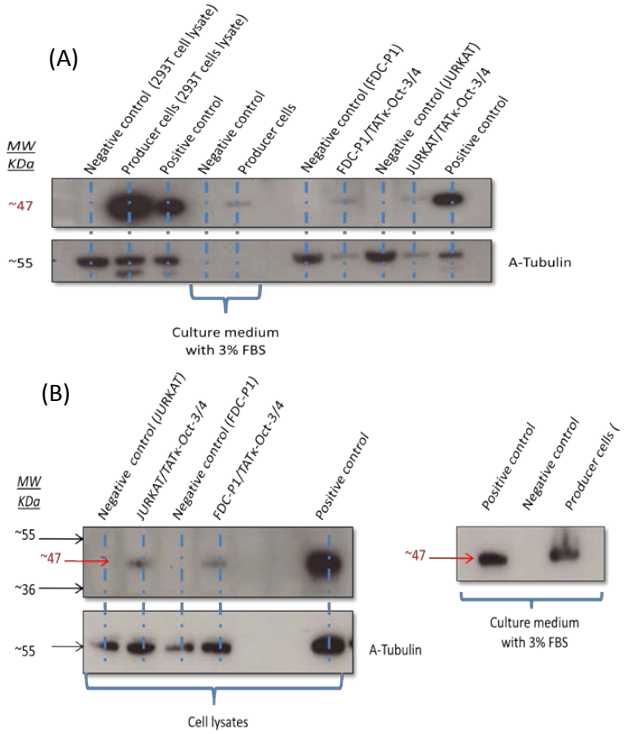

Figure 9: Oct3/4 protein transduction analysis. A total of $1x10^5$ target cells (JURKAT, FDC-P1 and primary mouse bone marrow cells) were added separately to 2.5 mL of culture medium containing secreted TATκ-Oct3/4 protein (supplemented with 3% heat-inactivated FBS). The cells were further incubated for 3 hours and lysed for Western blot analysis. Bands of the correct size were detected in both JURKAT and FDC-P1 cells, indicating the uptake of TATκ-Oct3/4 protein at (a) 24 hours, and (b) 48 hours after protein transduction. For negative control, target cells were incubated with culture medium of the untransfected 293T cells. A commercially obtained mouse embryonal carcinoma (F9) cell lysate was used as a positive control. The secretion of spTATκ-Oct3/4 protein in cultured medium by 293T producer cells was also confirmed as shown in lane 8. α-Tubulin (~ 55 KDa) was used to detect a tubulin protein which was used as a loading control for each sample.

Uptake of TATκ-Oct-3/4 protein was detected in both JURKAT and FDC-P1 cells 24 hours transduction with producer cells secreting TATκ-Oct-3/4 protein as confirmed by Western blot analysis (Figure 9a). Similar results were observed in the second experiment, after 48 hours transduction (Figure 9b). These bands indicated the uptake of TATκ-Oct-3/4 into the targeted cell lines. Although the stable producer cell was able to secrete TATκ-KLF4 protein there was no uptake of the TATκ-KLF4 protein into targeted cell lines as measured by Western blot analysis (data not shown).

Discussion

Oct-3/4 and KLF4 protein fused to TATκ domain were successfully expressed and secreted in 293T cells. Similar results were reported, using HEK293 (parental 293 cells, not SV40 transformed) to express and secrete Oct-3/4, KLF4, Sox2 and c-Myc proteins [2]. The SV40 gene sequences that lie within the transgene allow the episomal replication of transfected plasmids thus extending the expression of the desired gene products. A stable HEK 293 cell line that expresses each of the four human reprogramming factors (Oct-3/4, Sox2, KLF4 and c-Myc) fused with 9R (a synthetic TAT domain) and myc tag were generated [2]. They also demonstrated efficient intracellular translocation of each protein in newborn fibroblasts (HNFs) that were treated with cell extracts from HEK 293. This occurs mostly in the nucleus although some remained in the cytoplasm.

Currently, very few studies have reported the establishment of pluripotent factor secreting cell lines. There are only a handful of peer-reviewed literatures that describes successful secretion of pluripotent factors (KLF4, Oct-3/4, Sox2 and c-Myc) in transiently transfected HEK293 (293) cells [2]. In the present study, the establishment of TATκ-KLF4 and TATκ-OCT-3/4 secreting 293T cells was achieved using a similar approach to that in Kim's study. This is further expanded by selecting for stable mixed populations of transfected 293T cell line with puromycin.

Data presented here demonstrates that 6xO/S-Luc reporter gene was relatively activated by TATκ-Oct-3/4. Similar data was reported, whereby the RLA was slightly higher when both proteins were added together. [9] However, the luciferase expression was very weak relative to experiments using the expression plasmids. The possible explanations for these observations are; (1) the secreted TATκ fusion proteins might be degraded, (2) the secreted protein has lost TATκ domain, thus could not enter target cells, (3) TATκ-Sox2 might not have refolded efficiently and after being taken up by the target cells, it is not biologically functional, and (4) secreted proteins may accumulate in the cytoplasm instead of the nucleus. Unfortunately, limited data is available on the biological activities of the secreted Sox2 and Oct-3/4 proteins. In addition, the biological activity of KLF4 protein could not be investigated since there were no appropriate functional assays available as of time of press.

TATκ-Oct-3/4 was able to efficiently penetrate into FDC-P1 and JURKAT cells 24 hours post-transduction. However, the signal was relatively weak, suggesting low uptake of the Oct-3/4 protein. Using a similar approach to secrete pluripotent factors, a total extract of Oct-3/4 fused to a 9 arginine (9R) domain can efficiently penetrates into Human Newborn Fibroblast (HNF) cells within 8 hours incubation [2]. A few studies have demonstrated the successful uptake of pluripotent factors fused to different transduction domains. These studies have utilised the bacterial expression systems to produce the recombinant proteins, which were then solubilised, refolded and further purified [9]. Oct-3/4 fused to the original TAT domain (TAT-Oct-3/4 protein) could be taken up efficiently into human fibroblast IMR90 cells [9].

Despite the successful uptake of TATκ-Oct-3/4 protein into FDC-P1 and JURKAT cells, a number of experiments using transient secretion of TATκ-KLF4 protein failed to show the protein uptake even though the protein was readily secreted in the culture medium. This was probably due to loss of the TATκ domain by the KLF4 protein or due to protein degradation. Alternatively, the TATκ-KLF4 protein may be permeable to some, but not all cells. Although some studies have shown the successful uptake of KLF4 protein fused to different protein transduction domains, it is believed that the ability of the recombinant proteins to penetrate into target cells differs from one protein to another [19].

In conclusion, this study reports the establishment of a cell line that is able to continuously express TATκ-OCT-3/4 and TATκ-KLF4. This provides an alternative method to generate induced pluripotent Stem Cells (iPSCs) without the dangers and risks involved in viral transduction. The protein transduction system described here has several advantages over integrating viral system; (1) they effectively eliminate the potential risks associated with chromosomal integrations, (2) they are simpler and faster approach without the need for sequential selection of potentially integration-free transduced cells, and (3) the continues expression of pluripotency factors by producer cell lines in culture. We have shown the uptake of these factors by hematopoietic stem cell lines but the efficiency of these proteins in reverting differentiated cells to iPSCs must be further investigated. We hope that with these studies and future work, this method will provide a platform for generation of safe iPSCs for use in clinical transplants and potential delivery mechanisms for therapeutics.

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