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Production of Lentiviral Vector with Polycistronic Transcripts for Reprogramming of Mouse Fibroblast Cells

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ABSTRACT

iPS cells were originally generated using monocistronic retroviral vectors carrying the Yamanaka factors 'OSKM'. The development of a polycistronic viral vector with OSKM linked by 2A peptides has simplified reprogramming procedure and reduced the risk of multiple proviral integrations and insertional mutagenesis. In this study, we demonstrated the production of the polycistronic lentiviral vector encoding OSKM in a single cassette without a reporter gene or drug-based selection system. Syncytia formations were clearly seen following the co-transfection of a lentiviral plasmid construct with the structural and packaging plasmids. The virion was collected at 48 hours post-transfection. Afterwards, the viral titers were measured by the expression of Sox2 protein from transduced HT1080 cells. Subsequently, Oct4 expression was successfully detected in mouse fibroblasts in the range of 5, 10 and 20 MOIs with expression of 90.7%, 97.5% and 98%, respectively. The results obtained from this study could be used as a model for the production of OSKM lentiviral vector for newcomers to cellular reprogramming research.

Keywords: 2A peptides, iPS cells, lentivirus production, OSKM, polycistronic vectors

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INTRODUCTION

The first iPS cells were generated from Oct4, Sox2, Klf4, and c-Myc transcription factors or 'OSKM', which were delivered by monocistronic retroviral vectors (Takahashi & Yamanaka, 2006). Following this, interest in research into iPS cells spread rapidly, and a

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number of strategies have been developed to eliminate the random transgene integration into the genome that may lead to accidental activation of proto-oncogene (Lai et al., 2011; Nordin et al., 2011). In order to reduce the risk, a polycistronic vector was developed, through which OSKM genes can be delivered in one reprogramming cassette. The mRNA of each gene is translated independently to generate separate proteins for cellular conversion (Kaji et al., 2009; Sommer et al., 2009). This strategy is able to convert mouse and human fibroblasts into iPS cells, albeit at low conversion efficiency (Carey et al., 2008). This obstacle can be resolved by the insertion of an internal ribosome entry site (IRES) in combination with multiple promoters. Unfortunately, the use of IRES-based polycistronic vectors is limited due to the substantially lower levels of downstream cistron expression (Chang et al., 2009; Gonzalez et al., 2009).

To overcome this constraint, a polycistronic vector containing 2A 'self-cleaving' peptides from foot-and-mouth disease virus (FMDV) linking the OSKM was generated to express a sufficient level of OSKM for cellular reprogramming (Carey et al., 2008; Kim et al., 2011). In addition, the 2A 'self-cleaving' polycistronic vector system could express the four proteins in near equimolar amounts *in vivo* (Szymczak et al., 2004). As yet, successful production of a polycistronic vector separated by 2A sequences without a reporter gene or drug selection in a serum-free medium has not been reported.

An HIV-based vector has been the preferred strategy for *in-vivo* gene transfer. The genome of the HIV lentiviral vector construct has been engineered to enhance its biosafety and expression profiles (Jurgens et al., 2001; Zufferey & Dull, 1998). Two of the most interesting features of this vector are its ability to transduce proliferating and non-dividing cells and to integrate its own genome into the host genome, an action that may lead to prolonged expression of the transgene (Hotta et al., 2009; Kutner, Zhang, & Reiser, 2009). InvivoGen has developed the LENTI-Smart™ lentiviral system equipped with two essential packaging plasmids, pLV-iVSV-G and pLV-HELP containing *gag*, *pol*, *rev* and *tat*, that supply structural and replication proteins *in trans* for complete virion production. These plasmids are pre-complexed with LyoVec™ transfection reagent forming lyophilizate. In addition, the transfer vector genome encoding the Yamanaka factors, pLV-OSKM, was also designed in a single polycistronic transcript separated by the 'self-cleaving' peptides.

In our study, a polycistronic lentiviral vector from InvivoGen encoding the Yamanaka factors was produced in a serum-free environment, concentrated and then evaluated for transduction efficiency by analysing the expression of the transcription factor genes. The Multiplicity of Infection (MOI) was determined using tail-tip fibroblast infection of an adult C57BL/6 mouse.

MATERIALS AND METHODS

Cell Lines and Mouse Cell Culture

Human embryonic kidney (293FT) and human fibrosarcoma (HT1080) cell lines were purchased from Invitrogen and American Type Culture Collection (ATCC), respectively. The C57BL/J mouse strain was purchased from Jackson Laboratory. The tail-tip fibroblasts mouse cells were isolated as previously described (Takahashi & Yamanaka, 2006). The experimental procedure

was approved by the Animal Care and Use Committee (ACUC) of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM/FPSK/PADS/BR-UUH/00427). Cell lines 293FT and HT1080 were cultured in the DMEM medium, whereas the mouse cells were cultured in the DMEM/F12 medium. The medium was supplemented with 10% FBS, 1× non-essential amino acid, 2 mM GlutaMAX® and 1 mM MEM sodium pyruvate. Five hundred microgram of Geneticin® was used to maintain the sterility of the 293FT cells. All the products were purchased from Invitrogen.

Lentivirus Particle Production

In a T-75 flask, 1×10^7 293FT cells were suspended in 12 ml of a medium supplemented with KnockOut® Serum Replacement (SKR) (Invitrogen). The cells were co-transfected with 12 µg polycistronic plasmid encoding murine Oct4, Sox2, Klf4 and c-Myc (pLV-OSKM) and the LENTI-Smart™ (InvivoGen) structural and packaging plasmids. After 12 hours of co-transfection, the medium was discarded, and replaced with 12 ml of fresh DMEM medium. Virus particles in the supernatant were harvested over a period of 48 h, centrifuged, filtered through low protein binding 0.4 µM filters (Millipore), concentrated at 650×g for 30 min using Amicon Utra 100 kDa filter (Millipore) and stored at -80°C in aliquots. The culture medium used was antibiotic free, while the transfection was performed in a suspension cell state.

Flow Cytometry Titration Method

Virus titration was performed using the HT1080 suspension cell state transduction procedure. The virus solution was diluted to 10^{-1}, 10^{-5} and 10^{-6} in a complete DMEM medium containing 8 µg/ml polybrene (Sigma-Aldrich). The cells (1×10^5) were transduced with the diluted virus on a 48-well gelatin coated plate at a final volume of 200 µl. Upon 4 h of incubation, the transduction medium was discarded and replaced with 500 µl of fresh culture medium, after washing with 1X PBS. At 72 h post-transduction, the positive population of Sox2-transduced cells was assessed using flow cytometry. The data were converted into numerical value using the following formula: (F x C/V) x D, where F = frequency of $Sox2^+$ cells (percentage obtained divided by 100); C = total number of cells in the well at the time of transduction; V = volume of inocula in ml; D = LV dilution (Ngai et al., 2012a).

Lentiviral-OSKM Transduction of Mouse Fibroblasts

Mouse cells (1×10^5) were transduced with 5, 10 and 20 MOI of the virus in 1 ml complete DMEM medium containing 8 µg/ml polybrene (Sigma-Aldrich) in a 12-well gelatin-coated plate. At 12 h post-transduction, the medium was aspirated and replaced with 2 ml of mouse reprogramming medium (KnockOut™ DMEM supplemented with 15% KnockOut™ Serum Replacement (SKR), nonessential amino acids 0.1 mM, Gluta-MAX™ 2 mM and 2-mercaptoethanol 1×) (Invitrogen). The percentage of Oct4-expressing cells was measured by flow cytometry and immunochemistry at day 3 post-transduction.

RT-PCR Analyses

Total RNA was harvested from the transduced HT1080 cells using RNeasy® plus kit (Qiagen). The RNA was then subjected to cDNA synthesis using the QuantiTect® reverse transcription kit (Qiagen). Complementary DNA was synthesised from nearly 1 µg of total RNA using a random primer and the reverse transcription system (Qiagen). PCR was carried out with GoTaq® flexi buffer final concentration (1×), $MgCl_2$ solution (1.5 mM), dNTP (0.2 mM), forward/reverse primers (1 µM) and GoTaq® DNA polymerase (1.25u). These products were purchased from Promega. A sample DNA (250 ng) was also used. The synthesised cDNA was amplified by 35 cycles of PCR, at 55 to 65°C annealing temperature.

The primer sequences for exogenous mouse marker genes are: Oct4 (1024 bp: F,C CCGCTAGCCTGCCTTCTCCCTCCTGTG); Oct4 (1024 bp: R,CGCGGATCCACTG TCTAGAGTTTGAATGCATGG), Sox2 (996 bp: F,CCCGCTAGCAGGTCCCATGT ATAACATGATGGA), Sox2 (996 bp: R,CGCGGATCCCAGAGAGAAGTTCGTGG CTCT), c-Myc (1436 bp: F,CCCGCTAGCGGTGACGTGGAGGAGAATCC), c-Myc (1436 bp: R, CGCGGATCCGTACTGCTAGCTTTATGCACCAGA), β Actin (150 bp: F,CCTGTCAGCAATGCCTGGGT), β Actin (150 bp: R, CCAGCCTTCCTTCTTGGGTA).

Detection of Exogenous Protein Expression

Cells were collected, gently fixed and permeabilised using Cytofix/Cytoperm (BD™). The cells were stained with primary antibodies on ice, Oct4 (1.1) mouse IgG_{2b} (mouse, monoclonal), rabbit anti mouse/human Sox2 (Stemgent, USA) or relevant isotype controls for 45 min. After washing, the cells were stained with secondary antibodies, anti-mouse IgG_{2b}- Alexa (Fluor®488) and anti-rabbit Alexa (Fluor®488) on ice (Molecular, Probes UAS) for 30 min. The stained cells were subsequently analysed using a flow cytometer (FC500, BD Biosciences). For immunocytochemistry analysis, the transduced cells were fixed with 4% paraformaldehyde and permeabilised with 0.1% Triton X -100. This was followed by washing three times with 1X PBS and incubation with blocking buffer (10% goat serum and 0.3% BSA) (Sigma-Aldrich). The cells were incubated overnight at 4°C with Oct4 mouse IgG_{2b} (mouse, monoclonal- Santa Cruz, Bio) and Klf4 (mouse anti-mouse/human- Stemgent, Cambridge) primary antibodies. Next, the cells were incubated with the secondary antibodies, anti-mouse Alexa Fluor®IgG_{2b}, anti-rabbit Alexa Fluor®488 and anti-mouse Cy™3 (Molecular Probes and Stemgent, USA) at room temperature. Stained cells were mounted in DAPI (4, 6-diamidino-2-phenylindole) for 10 min at room temperature and washed three times with 1X PBS before nuclear visualisation using Olympus fluorescence microscope.

Statistical Analysis

One-way ANOVA was performed to determine the statistical significance between different serial dilutions using SPSS (Version 20.0, Chicago: SPPS Inc). The value $p<0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

Syncytia Formation

As shown in Schematic 1, the four-defined mouse cellular reprogramming factors Oct4, Sox2, Klf4 and c-Myc were separated by three different types of self-cleaving 2A peptide. This report describes the production and assessment of a polycistronic lentiviral vector carrying OSKM linked with 2A peptides. To produce the virus, 293FT producer cells were co-transfected with a lentiviral vector plasmid (pLV-OSKM) and the packaging/structural plasmids (LENTI-Smart™ components). The culture-cell medium was devoid of serum during transfection in order to increase viral titer as suggested in previous studies (Ansorge et al., 2009; Broussau et al., 2008; Kuroda et al., 2011; Kuroda et al., 2009; Reiser, 2000). In addition, it has been reported that the influence of serum in the culture media can be cytotoxic as the serum interferes with the physiological state of the transduced cells (Reiser, 2000). Of note, we observed small clumps in the culture due to spontaneous breakdown of the cell membranes by the transfection reagent (data not shown). The 293FT cell line was derived from the HEK-293 cells after insertion with a plasmid (pCMVSPORT6TAg.neo) encoding the simian virus 40 (SV40) large T antigen driven by an internal cytomegalovirus (CMV) promoter (Naldini et al., 1996). The SV40 T antigen was used to induce transient expression of proteins on the helper plasmid through the incorporation of viral vector replication signals (Hotta et al., 2009).

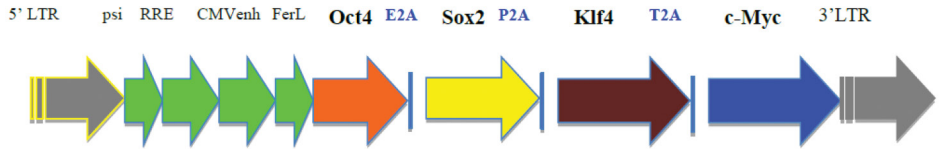


Figure 1. Schematic diagram of vector illustrating the polycistronic lentiviral backbone encoding mouse reprogramming factors Oct4, Sox2, Klf4 and c-Myc linked by 3 different types of 2A peptides, E2A (equine rhinitis A virus), P2A (porcine teschovirs-1) and T2A (*Thosea asigna* virus). Abbreviations: LTR: long terminal repeat, psi: packaging signal, RRE: rev response element, CMVenh: human cytomegalovirus enhancer/promoter, FerL: ferritin light promoter

Syncytia of fused 293FT cells were seen (Figure 2) within 24 h post-transfection due to the expression of the VSV-G envelope (Kutner, Zhang, & Resiser, 2009; Papapetrou & Sadelain, 2011). After the host 293FT genome was transiently transfected, the viral structural proteins and replication enzymes were transcribed, resulting in the production of viral particles containing the vector genome (Malim et al., 1989; Buchschacher & Wong-Staal, 2000). Subsequently, the *psi* (Ψ) packaging signal allowed packaging of viral vector RNAs into virus capsids, which bud from the producer cells together with the VSV-G glycoprotein on the cell surface into the culture supernatant (Gama-Norton et al., 2001; Buchschacher & Wong-Staal, 2000).

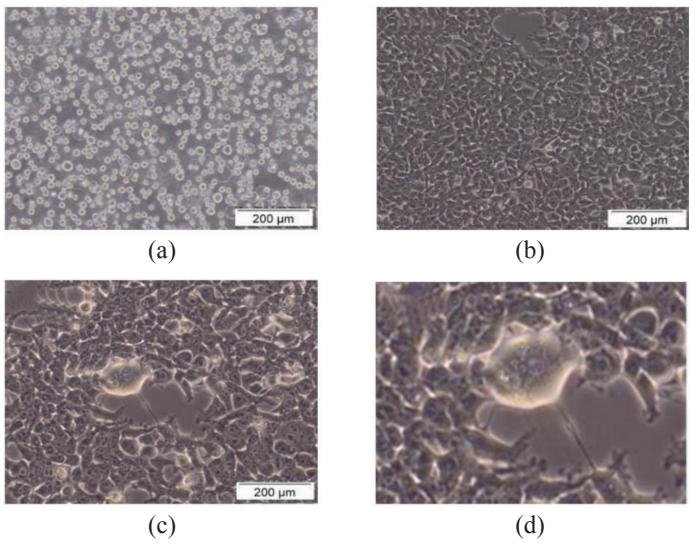


Figure 2. Co-transfection of 293FT cells with pLV-OSKM and LENTI-Smart structural and packaging plusmids. (a) 293FT suspended cell during co-transfection, (b) healthy 293FT cells, (c) fused, and large multinucleated 293FT cells at 24 h post transfection. (d) A zoomed in fused, giant cell from the original photo (c). The scale bar is 200 µm

LV-OSKM Titration

Generally, a high titer of virus was obtained when the lentivirus was collected at day 3 post-transfection (Ngai et al., 2012b). Therefore, the virion was harvested from the supernatant medium at day 3 post-transfection. It was noteworthy that 239FT cells need to be handled carefully due to their weak attachment to the surface of culture plates upon transfection (Dick et al., 2011; Tiscornia, Singer, & Verma, 2006).

Concentrating the lentiviral vector using ultracentrifugation permits higher titer of pseudotyped viral particles and better MOI (Hotta et al., 2009; Ichim et al., 2011). Hence, we concentrated virus particles using Amicon Ultra 100 kDa filter, which is a well-established technique.

To measure the viral titer, HT1080 cells were transduced with the diluted virus and flow cytometry analysis was utilised to determine the proportion of $Sox2^+$ cells at day 3 post-transduction. This indicated the efficiency of transduction when proteins are recognised by the specific antibodies (Dull et al., 1998). The cells transduced with virus solution at 10^{-1} and 10^{-5} showed a significantly high percentage of $Sox2^+$ expression (73.6% and 37.4%, respectively) compared with non-transduced cells and the 'rabbit IgG' isotype (Figure 3). Meanwhile, the cells transduced with virus diluted at 10^{-6} showed 27.5% of Sox2 expression. To avoid a multiple-copy number of integration, the infectivity of the diluted virus has to be between 5 and 30% (Papapetrou et al., 2011). Therefore, the virus diluted at 10^{-6} was chosen in this study for the lentiviral titer calculation. The Sox2 expression level of the transduced sample was statistically significant compared with the control ($p<0.001$).

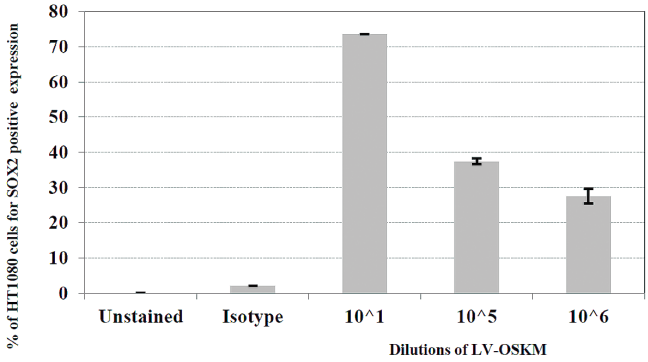


Figure 3. Titration of LV-OSKM. HT1080 cells were transduced with diluted LV-OSKM and harvested at 72 hr post-transduction. Sox2 expression was detected using flow cytometry. The cells were stained with rabbit IgG as Isotype control. Samples were prepared in duplicate and results are shown as mean ± SD. Total event of 10,000 was acquired

To complement the results obtained from cells transduced with 10^{-6} diluted virus, the expression of exogenous genes was assessed by RT-PCR. The exogenous genes (Oct4, Sox2 and c-Myc) were clearly expressed in the 10^{-6} diluted virus. The pLV-OSKM plasmid (as positive control) was used to validate the amplification process (Figure 4).

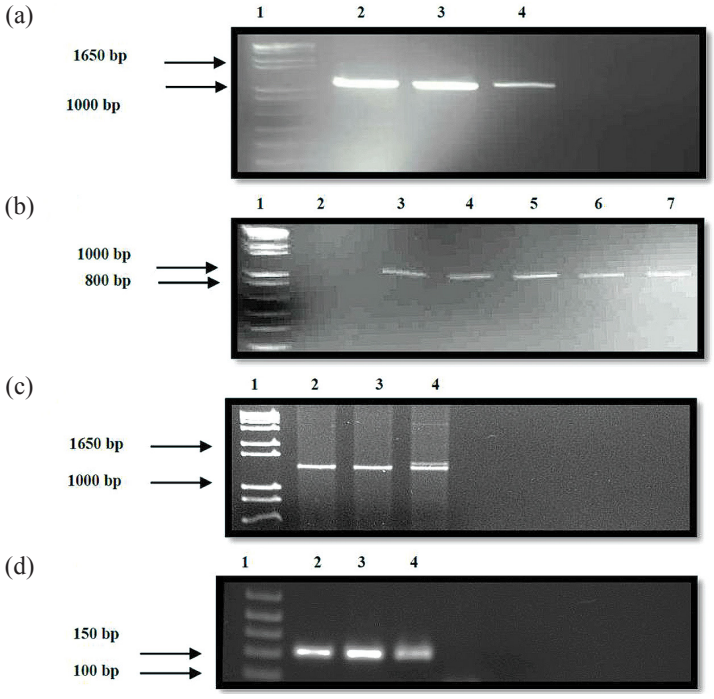


Figure 4. Exogenous gene expression of Oct4, Sox2 and c-Myc from HT1080 cells transduced with 10^{-6} diluted virus analyzed by RT-PCR. Lanes 1 in panel (a), (b) and (c) show a DNA ladder (Tracklt® 1 kb plus). Panel (a) represents Oct4 expression; Lane 2 shows pLV-OSKM used as a positive control to validate amplification of DNA fragments of 1024 bp (Oct4), lanes 3 and 4 show Oct4 expression from 2 independent samples. Panel (b) represents Sox2 expression; lanes 3 and 4 are the pLV-OSKM positive control that gave rise to an amplified DNA fragment of 995 bp (Sox2), lanes 5, 6 and 7 show Sox2 expression from 2 independent samples. Panel (c) represents c-Myc (1317 bp) and pLV-OSKM, which served as the positive control (lane 2), transduced samples (lanes 3-4). None of these genes was expressed in non-transduced HT1080 cells (as a negative control) as shown in Panel (a- lane 5), (b- lane 2), and (c- lane 5). Panel (d) lane1: 50 bp DNA ladder from GeneDriex®, while lanes 2, 3, and 4 indicate β-actin expression (150 bp)

To further authenticate the results, we detected the cells expressing Klf4 by immunocytochemistry. A high expression level of Klf4 was observed in cell nuclei transduced with 10^{-6} diluted virus (Figure 5). Klf4 is considered a major player in regulating cell growth (Garrett-Sinha et al., 1996; Katz et al., 2005). It was noteworthy that our initial attempt was to use the average value of Oct4 expression from HT1080 transduced cells to measure the viral titer. Surprisingly, there was a high percentage of control cells that had Oct4 expression. It is known that Oct4 has two isoforms: Oct4-A and Oct4-B (Araki et al., 2009; Smith et al., 2010). This could be the confounding factor for the unexpected results. To overcome the false positive expression, a mouse IgG_{2b}-specific antibody was chosen for Oct4-A specific detection.

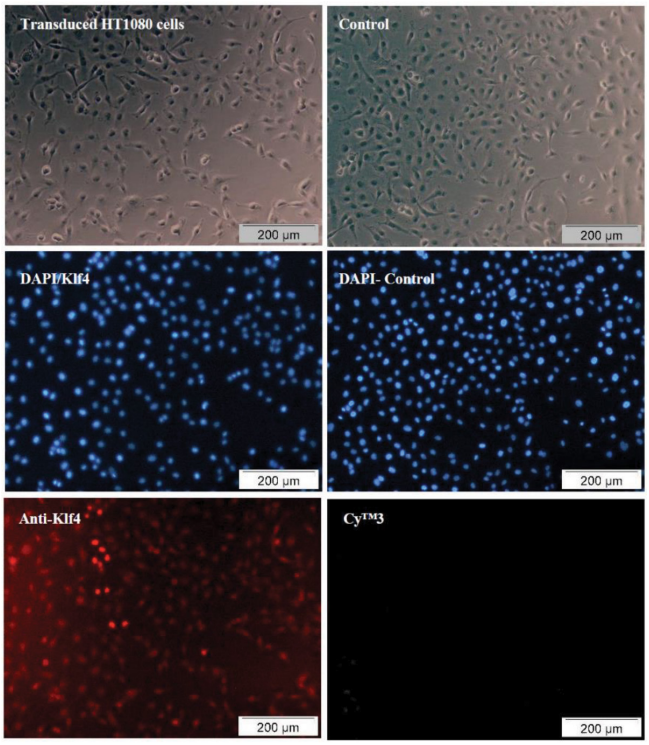


Figure 5. Expression of Klf4 in HT1080 cells transduced with 10^{-6} diluted virus. Phase-contrast and immunocytochemistry images show DAPI-stained nuclei. High expression of Klf4 in HT1080 cell nuclei following transduction with virus diluted at 10^{-6} at day 3 post-transduction. Cells were treated with Klf4 antibody at 1:200 dilution followed by a secondary Cy™3 conjugated antibody (red). Cy™3 was used as a negative control. The scale bar is 200 µm

Mouse Fibroblasts Transduction

We next sought to optimise the MOI for LV-OSKM transduced mouse fibroblasts. The cells were transduced with different MOIs (5, 10 and 20) of LV-OSKM in a serum-free medium to increase the conversion efficiency (Okada, Oka, & Yoneda, 2010). The desired MOI was

calculated using the lowest concentration of the LV-OSKM that considerably yielded the highest percentage of transduced target cells based on the expression of Oct4, which serves as a faithful indicator for the acquisition of pluripotency.

At 48 h post-transduction, the cells seemed smaller and appeared to proliferate faster compared with the non-transduced cells (data not shown). These dynamic morphological changes could be due to the induction of cell proliferation genes, histone modifications and silencing of the somatic genes expression following the expression of the transduced exogenous genes (Araki et al., 2009; Smith et al., 2010).

Expression of Oct4 protein was examined using a flow cytometer at 72 h post-transduction. Histogram results of untransduced mouse cells showed auto-fluorescence (2.5%), while the cells transduced with different MOIs of virus showed apparent Oct4 expression of 90.7% (MOI 5), 97.5% (MOI 10) and 98% (MOI 20) (Figure 6). Immunochemistry analysis was also performed and it revealed positive Oct4 markers in the nuclei of cells transduced with virus at MOI 5, although some cells showed faint cytoplasmic Oct4 protein immunoreactivity (Figure 7). In this study, the results showed that the expression level of Oct4 in cells transduced with virus at MOI 5 was favourable for reprogramming (Carey et al., 2008).

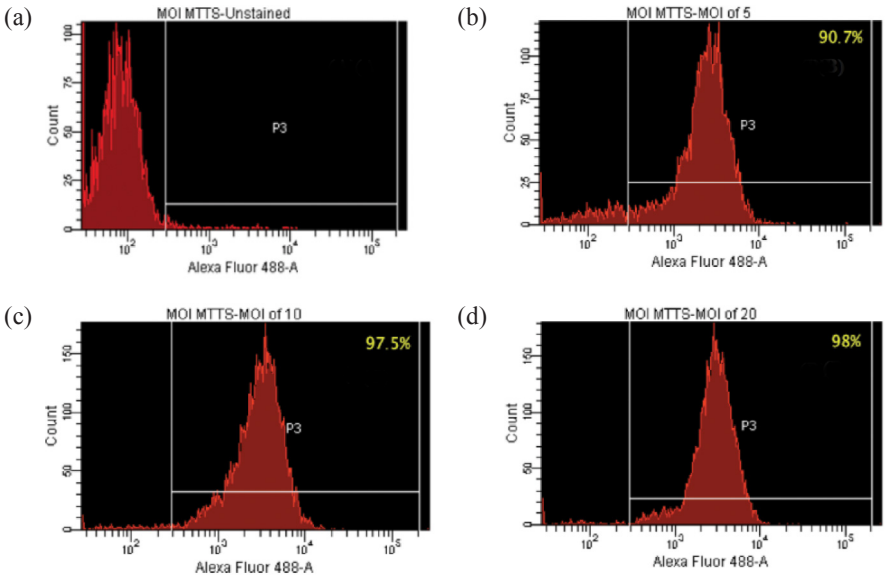
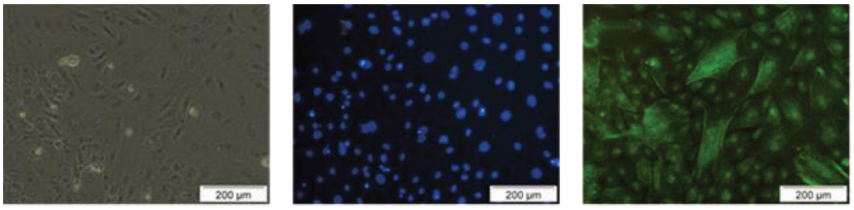


Figure 6. Representative flow cytometry histograms of transduced mouse fibroblasts at day 3 post-transduction with increasing MOI of LV-OSKM. As shown in (a), the peak was gated for negative cells (non-transduced). In contrast, peaks shifted to the right when cells transduced with virus at MOI 5 (b), 10 (c), and 20 (d) were positive for Oct4. Total event of 10,000 was acquired. Data indicated no significant difference, when 5, 10 and 20 MOI of virus was used for transduction. Hence, the lowest MOI of virus producing high expression level was selected. In this study, the most optimal MOI was 5



(a) (b) (c)

Figure 7. Immunocytochemical analysis of Oct4 expression. (a) Bright field view of mouse fibroblast cells transduced with virus at MOI 5. (b) Cells treated with DAPI staining to visualize nuclei. (c) Immunocytochemical analysis revealed high level of Oct4 in cell nuclei. Cells were incubated with Oct4 antibody (1:1000 dilution) followed by an Alex-Fluor®488 IgG_{2b} conjugated antibody (green). The scale bar is 200 µm

The expression of c-Myc was also detected at MOI 5 (result not shown). It is known as 'double-edged sword' because the low c-Myc expression was sufficient for cellular reprogramming (Sridharan et al., 2009); however, the over-expression of c-Myc could lead to tumorigenicity (Gonzalez et al., 2009; Nakagawa et al., 2007). Therefore, it is crucial to regulate its expression to achieve the targeted effect without inducing tumorigenesis.

CONCLUSION

This report described the production and assessment of polycistronic lentiviral vector encoding OSKM in a single cassette devoid of a reporter gene or drug-based selection system in a serum-free medium. The level of the OSKM expression mediated by the vector could fulfil the requirement for cellular reprogramming.

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