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Application Note

Generation of Induced Pluripotent Stem Cells by Reprogramming Human Fibroblasts with a Human TF Lentivirus Set

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Sigma Products ST000005, ST070012, ST070013, ST070016, ST070017

Summary

We demonstrate the generation of iPS cells from human somatic cells using lentivirus-mediated delivery of the human factors Oct4, Sox2, Nanog, and Lin28. Viral vectors have been widely used to deliver transcription factors into mammalian somatic cells. The iPSC Generation Human TF Lentivirus Set: OSLN utilizes a VSV-G pseudotyped lentivirus system that is capable of transducing both dividing and non-dividing cells from many mammalian species, including mouse and human. Here we show that co-transduction of the viruses from this set induces reprogramming in human foreskin fibroblast BJ cells grown on a mouse embryonic fibroblast feeder layer. Pluripotency of the reprogrammed cells was confirmed by the presence of ES cell-specific markers. Our study demonstrates the utility of the iPSC Generation Human TF Lentivirus Set: OSLN for the generation of iPS cells from human somatic cells.

Introduction

Reprogramming is the process by which cells are converted from a differentiated state to a pluripotent state. In 2006, using a defined set of transcription factors and cell culture conditions, Shinya Yamanaka and colleagues first demonstrated that retrovirus-mediated delivery and expression of Oct4, Sox2, c-Myc, and Klf4 is capable of inducing the pluripotent state in mouse fibroblasts[1]. The same group reported the successful reprogramming of human somatic cells into induced pluripotent stem (iPS) cells using human versions of the same transcription factors delivered by retroviral vectors[2]. Additionally, James Thomson reported that the lentivirus-mediated co-expression of another set of factors (Oct4, Sox2, Nanog, and Lin28) was capable of reprogramming human somatic cells into iPS cells[3].

iPS cells are similar to embryonic stem (ES) cells in morphology, proliferation, and ability to differentiate into all the tissue types of the body. In mice, pluripotency of iPS cells has been fully demonstrated through the generation of adult mice by tetraploid complementation.[8,9,10] Pluripotency of human iPS cells has been proven by formation of mature teratomas in mice[2,3]. Human iPS cells have a distinct advantage over ES cells as they exhibit key properties of ES cells without the ethical dilemma of destroying an embryo to obtain the cells.

Additionally, iPS cells generated from patients will provide a powerful research tool to investigate the mechanisms underlying genetic diseases. The generation of patient-specific iPS cells circumvents an important roadblock to personalized regenerative medicine therapies by eliminating the potential for immune rejection of non-autologous transplanted cells.

Here we demonstrate that the iPSC Generation Human TF Lentivirus Set: OSLN (Sigma® Cat. No. ST000005) is capable of expressing each of the following four factors: Oct4, Sox2, Nanog, and Lin28. Also, co-transduction of the four factors can reprogram human somatic cells into iPS cells that display the pluripotency markers characteristic of ES cells.

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Results

Viral Infectious Unit Titer Determination

The iPSC Generation Human TF Lentivirus Set: OSLN (Sigma Cat. No. ST000005) contains four viruses carrying human Oct4, Sox2, Nanog, and Lin28. Viral titer was determined by p24 (lentivirus capsid protein) concentration. The p24 values for each of the viruses used in this reprogramming experiment are listed in the table below. To calculate the infectious titer for each virus, we first determined the transduction unit (TU) titer of the GFP lentivirus that was used as a manufacturing process control for the human transcription factor virus production. For this batch of production, the TU titer of the control GFP virus was 2.72×10^7 TU/ml. The co-efficiency value was 1.49×10^5 TU/ng. Therefore, the TU titer for each virus is the p24 concentration times the co-efficiency value (see table).

Lentivirus	p24 (ng/ml)	TU Titer (TU/ml)
hOct4	6.64	0.99×10^6
hSox2	68.8	1.02×10^7
hNanog	82.8	1.23×10^7
hLin28	68.8	1.02×10^7

Protein Expression Validation

To demonstrate that each virus expressed the transcription factor it encoded, we infected HEK293-AD cells with each of the four human transcription factor viruses and the ectopically expressed proteins were detected by immunocytochemistry (ICC). In this experiment, 2×10^4 HEK293-AD cells were seeded for transduction. For hOct4, hSox2, hNanog, and hLin28 viruses, 200 µl, 20 µl, 20 µl and 20 µl were added to the cells, respectively, to reach a multiplicity of infection (M.O.I.) of 10. Seventy two hours after the transduction, the ectopically expressed proteins were detected using their corresponding antibodies (**Figure 1**). ICC staining for hOct4, hSox2, and hNanog was localized in the nucleus as indicated by the overlap with the DAPI stain for the cell nuclei, while the signal for hLin28 was present primarily in the cytoplasm (**Figure 1**). This showed the correct cellular compartment localization for each ectopically expressed protein.

Reprogramming of BJ Cells

To demonstrate the ability of the iPSC Generation Human TF Lentivirus Set: OSLN (Sigma Cat. No. ST000005) to induce reprogramming in somatic cells, human foreskin fibroblast cells (BJ cells) were co-transduced with the set of viruses. The transduction was performed in one well of a 6-well plate seeded with 1×10^5 BJ cells. In consideration of the low TU titer for the hOct4 virus, the viral transduction was carried out at an M.O.I. of 5. Therefore, 500 µl of hOct4 virus and 50 µl of the other viruses were used. The next day, the cells were passaged into 3 wells of a 6-well plate pre-seeded CF-1 MEF feeder cells. Morphological changes were observed as early as day 4 post-transduction. The elongated cells started to round up and form clusters sporadically in the well (**Figure 2**). The cluster of cells became more tightly packed at day 17 (**Figure 2**). Colonies displaying the appropriate morphology were manually picked at day 25 and cultured on CF-1 MEF

Figure 1

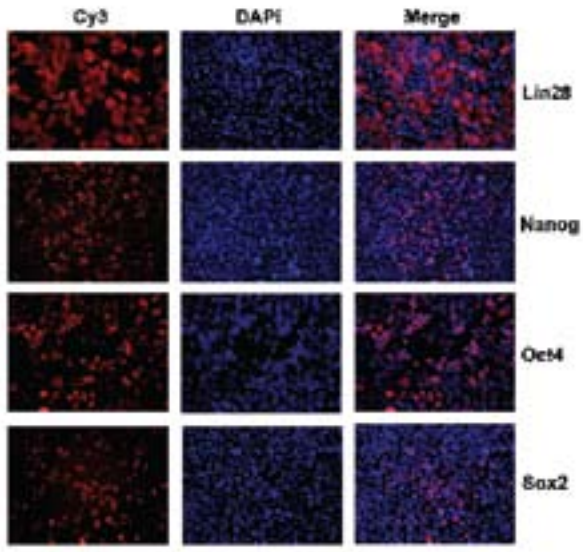

Figure 1. ICC Detection of Proteins Ectopically Expressed by Human TF Lentiviruses
HEK293-AD cells were transduced with the indicated lentiviruses (Sox2, Oct4, Nanog, and Lin28) at an M.O.I. of 10. The cells were fixed 72 hours post-transduction, stained with the corresponding antibodies, and visualized by Cy3-conjugated secondary antibodies. Cell nuclei were counter-stained with DAPI.

Figure 2

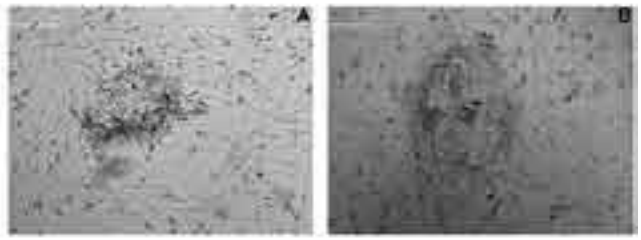

Figure 2. iPS Cell Colony Formation post Viral Transduction
Bright field images of a typical iPS cell colony formed at (A) 4 days and (B) 17 days post-transduction.

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Results, Continued

feeder cells. Recent studies have shown that Rho-associated kinase (ROCK) inhibitors can enhance the survival and cloning efficiency of dissociated human ES cells[4,5]. To facilitate the iPS cell colony formation after reprogramming, we used small molecule Y27632 at 10 mM for the initial overnight seeding during each passage. iPS cell colonies with good morphology (i.e. tightly packed, flat and with clear edges) were observed after three sequential rounds of colony picking and passaging (**Figure 3**).

iPS Cell Characterization

To further characterize the isolated iPS cell colonies, we looked for the presence of common pluripotency markers expressed in ES cells. The colonies exhibited strong alkaline phosphatase (AP) activity (**Figure 3**). Additionally, ICC was performed on the iPS cell colonies with the pluripotency marker-specific cell surface antibodies TRA-1-81, TRA-1-60, SSEA-4, SSEA-3, and SSEA-1 as well as the nuclear markers Oct4, Sox2, and Nanog. The isolated iPS cell colonies were positive for all markers except SSEA-1, which is not present in undifferentiated human ES cells (**Figure 4**). The ICC results showed that the iPS cells exhibited the appropriate pluripotency marker expression pattern, demonstrating that these iPS cells closely resemble undifferentiated human ES cells.

Conclusion and Discussion

We demonstrated that the iPSC Generation Human TF Lentivirus Set: OSLN (Sigma Cat. No. ST000005) is able to reprogram human fibroblast cells into iPS cells. Since different cell types may have different reprogramming efficiencies, several factors should be taken into consideration when planning your reprogramming experiment. First and foremost, you may need to modify the active virus-to-target cell ratio (M.O.I.) during the primary transduction step to achieve optimum transduction efficiency. Second, the growth condition of the target cells can impact reprogramming. Healthy and proliferative cells are more amenable to reprogramming. Third, when modifying the protocol for different cell numbers, it is recommended that target cell numbers are adjusted proportionally to the surface area of the culture dish. Lastly, applying ROCK inhibitors such as Y27632 should be considered to help ensure successful reprogramming as recent studies have demonstrated its utility in enhancing human ES colony survival[4,5]. While no reprogramming method can guarantee reprogramming of any and all cell types, the iPSC Generation Human TF Lentivirus Set: OSLN (Sigma Cat. No. ST000005) was functionally validated in BJ cells to ensure that all researchers will be able to generate human iPS cells.

Figure 3

Figure 3. Human iPS Cell Colonies are Positive for AP Staining
The images show three different colonies stained after 3 passages.

Figure 4

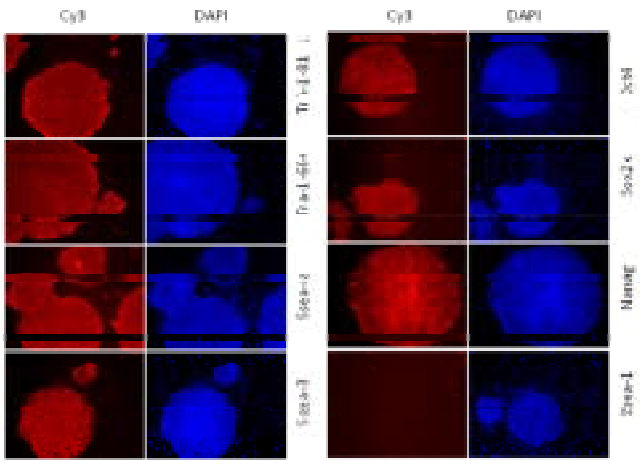

Figure 4. Pluripotency Marker Detection for Human iPS Cells
Human iPS cells express high level of the following ES cell specific surface markers: Tra-1-81, Tra-1-60, SSEA-4, and SSEA-3, and the following nuclear markers: Nanog, Sox2, and Oct4. As expected, human iPS cells were negative for SSEA-1.

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Experimental Procedures

Materials

- Stemgent® iPSC Generation Human TF Lentivirus Set: OSLN (Sigma Cat. No. ST000005)
- Knockout™ Serum Replacement (Invitrogen Cat. No. 10828-028)
- Stemfactor™ Fibroblast Growth Factor-basic (human recombinant) (Stemgent Cat. No. 03-0002)
- Stemolecule Y27632 (Stemgent Cat. No. 04-0012)
- Affinity Purified anti-Human TRA-1-81 antibody (Stemgent Cat. No. 09-0011)
- Affinity Purified anti-Human TRA-1-60 antibody (Stemgent Cat. No. 09-0010)
- Affinity Purified anti-Human SSEA-4 antibody (Stemgent Cat. No. 09-0006)
- Affinity Purified anti-Human SSEA-3 antibody (Stemgent Cat. No. 09-0014)
- Affinity Purified anti-Mouse/Human SSEA-1 antibody (Stemgent Cat. No. 09-0005)
- Stemgent Alkaline Phosphatase Staining Kit (Stemgent Cat. No. 00-0009)

Preparation of BJ Cell Growth Medium

450 ml EMEM was supplemented with 50ml ES-qualified FBS, 5ml 10 mM non-essential amino acids, 5 ml penicillin (10,000 U/ml)-streptomycin (10,000 µg/ml), 5 ml 200 mM L-glutamine, and 0.9 ml 55 mM β-mercaptoethanol.

Preparation of Human ES/iPS Cell Medium

400 ml DMEM/F12 was supplemented with 100 ml Knockout Serum Replacement, 5 ml 10 mM non-essential amino acids, 5 ml 200 mM L-glutamine, 0.9 ml 55 mM β-mercaptoethanol, and 10ng/ml human recombinant bFGF.

Preparation of CF-1 Medium

450 ml DMEM was supplemented with 50ml ES-qualified FBS and 5 ml 10mM non-essential amino acids.

Preparation of CF-1 MEF Plates

CF-1 cells were seeded in a 6-well plate and incubated overnight at 37°C and 5% CO_2.

MEF Conditioned Medium

1. CF-1 MEF feeder cells were seeded at 2 × 10^5 cells per well of a 6-well plate in CF-1 Medium.
2. After an overnight incubation at 37°C and 5% CO_2, the medium was changed to Human ES/iPS Cell Medium.
3. Every 24 hours for 4 days, the supernatant was collected, centrifuged, and filtered through a 0.22 µm filter.
4. Before adding the supernatant to the cells, the supernatant was supplemented with 50 ng/ml bFGF.

Seeding BJ Cells

BJ cells (P6) were seeded at a density of 1 x 10^5 cells in one well of a 6-well plate. The cells were cultured in 2 ml of BJ Cell Growth Medium overnight at 37°C and 5% CO_2.

Viral Transduction

The next day, medium was removed and 1.35 ml of BJ Cell Growth Medium supplemented with 6 µg/ml of polybreen, 500 µl hOct4-lentivirus, 50 µl hSox2-lentivirus, 50 µl hNanog-lentivirus, and 50 µl hLin28-lentivirus was added. After ensuring that the medium was distributed evenly by gentle rocking of the cell culture dish, the cells were incubated overnight at 37°C and 5% CO_2.

Passaging of Transduced BJ Cells

Twenty four hours post-transduction, the cells were trypsinized, centrifuged at 200 x g for 5 minutes, re-suspended in BJ Cell Growth Medium, and re-plated in 3 wells of a 6-well CF-1 MEF plate. These cells were incubated overnight at 37°C and 5% CO_2.

Media Changes

Twenty four hours after re-seeding, BJ Cell Growth Medium was replaced with Human ES/iPS Cell Medium. The medium was changed every day for the first 7 days. After 7 days, medium was transitioned to MEF Conditioned Medium.

iPS Colony Selection and Passaging

All ES cell-like colonies were selected and re-seeded in Human ES/iPS Cell Medium supplemented with 10 µM Stemolecule Y27632 on CF-1 MEF Plates. Human ES/iPS Cell Medium was changed every day for 7 days, then medium was changed to MEF Conditioned Medium. Passaging of cells continued until they showed typical human ES cell morphology.

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Experimental Procedures, Continued

ICC: Pluripotency Analysis

The ICC staining procedure was performed as instructed in the ICC general protocol (Stemgent Cat. No. 00-0016). Antibodies were diluted as described below:

Primary Antibodies:

- Anti-Tra-1-81 diluted 1:100
- Anti-Tra-1-60 diluted 1:100
- Anti-SSEA-4 diluted 1:100
- Anti-SSEA-3 diluted 1:100
- Anti-SSEA-1 diluted 1:100
- Anti-Oct3/4 diluted 1:100 as per manufacturer's instructions
- Anti-Sox2 diluted 1:100 as per manufacturer's instructions
- Anti-Nanog diluted 1:100 as per manufacturer's instructions
- Anti-Lin28 diluted 1:100 as per manufacturer's instructions

Secondary Antibodies:

- Goat anti-Mouse IgM, Cy3 conjugate diluted 1:200 as per manufacturer's instructions
- Goat anti-Mouse IgG, Cy3 conjugate diluted 1:200 as per manufacturer's instructions
- Goat anti-Rat IgM, Cy3 conjugate diluted 1:200 as per manufacturer's instructions
- Goat anti-Rabbit IgG, Cy3 conjugate diluted 1:200 as per manufacturer's instructions

AP Staining

AP staining was performed following the recommended protocol provided in the kit.

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