Generation of iPS Cells by Reprogramming Human Fibroblasts with a Four Transcription Factor, Dox-Inducible Lentivirus Set

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SUMMARY
The Stemgent® Dox Inducible Reprogramming Lentivirus Set: Human OKSM was used to reprogram BJ human fibroblast cells to an embryonic-like cell state known as the induced pluripotent stem (iPS) cell. This reprogramming set is comprised of four individual doxycycline (dox)-inducible human lentiviruses (Oct4, Sox2, Klf4, and c-Myc), as well as a lentivirus for the constitutive expression of reverse tetracycline transactivator (rtTA).

Here we demonstrate that the Dox Inducible Reprogramming Lentivirus Set: Human OKSM can be used to generate iPS cell colonies from human foreskin fibroblasts. Subsequently, we demonstrate effective lentiviral transduction and dox-inducible expression of the four transcription factors hOct4, hSox2, hKlf4, and hc-Myc. We also demonstrate successful reprogramming through morphology tracking of iPS cell colonies, and through confirmation of the expression of common pluripotency markers such as Oct4, Tra-1-60, Tra-1-81, Nanog, Rex1, SSEA-4, and alkaline phosphatase (AP).

INTRODUCTION
Reprogramming, the process by which induced pluripotent stem (iPS) cells are generated, is the conversion or “reprogramming” of adult somatic cells to an embryonic cell-like state. Using a defined set of transcription factors and cell culture conditions, Shinya Yamanaka and colleagues demonstrated that retrovirus-mediated delivery and expression of Oct4, Sox2, c-Myc, and Klf4 is capable of inducing pluripotency in mouse fibroblasts. This group later reported the successful reprogramming of human somatic cells into iPS cells using human versions of the same transcription factors. Subsequent reports have demonstrated the utility of the doxycycline (dox) inducible lentiviral delivery system for the generation of both primary and secondary iPS cells from a variety of other adult human and mouse somatic cell types.

iPS cells are similar to embryonic stem (ES) cells in morphology, proliferation, and the ability to induce teratoma formation. In mice, pluripotency of iPS cells has been fully demonstrated through the generation of germline chimeras and tetraploid complementation. Furthermore, both ES cells and iPS cells can be used as the pluripotent starting material for the generation of differentiated cells or tissues in regenerative medicine. However, iPS cells have a distinct advantage as they exhibit key properties of ES cells without the ethical dilemma of destroying an embryo to obtain the cells. Finally, 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.

The ability to reprogram adult somatic cells to iPS cells allows researchers to assess the utility of these cells in a variety of applications. For example, the study of iPS cells generated from diseased and normal patient-specific tissues allow researchers to investigate the cellular mechanisms behind the disease. These iPS cells can be evaluated for their ability to differentiate into a variety of somatic cells. The ability to reprogram adult somatic cells to the iPS cell state also enables the researcher to explore how and why differentiated cell types are able to return to the pluripotent cell state.

Stemgent’s Dox Inducible Reprogramming Lentivirus Set: Human OKSM (Cat. No. ST000036) and Dox Inducible Reprogramming Lentivirus Set: Human OKSM (conc) (ST000037) contain VSV-G-pseudotyped lentiviruses capable of transducing both dividing and non-dividing cells from many mammalian species, including mice and humans. This reprogramming set utilizes Tet-on™ technology where the post-transductional expression of rtTA combined with the addition of dox to the cell culture medium allows for the inducible expression of the human transcription factors Oct4, Sox2, Klf4, and c-Myc.

Here we show the Dox Inducible Reprogramming Lentivirus Set: Human OKSM is capable of expressing each of the four transcription factors Oct4, Sox2, Klf4, and c-Myc upon transduction into BJ human fibroblast (BJ) cells. When transduced and induced cells were expanded, they were shown to have similar morphology compared to ES cells; express...
the pluripotency markers Rex 1, SSEA-4, Nanog, Oct4, Tra-1-60, and Tra-1-81; and express alkaline phosphatase (AP). Taken together, these data demonstrate that the Dox Inducible Reprogramming Lentivirus Set: Human OKSM can be used to reprogram human somatic cells into iPS cells.

RESULTS
The Dox Inducible Reprogramming Lentivirus Set: Human OKSM was used to infect BJ Human Fibroblasts (BJ cells). Lentivirus mediated transduction efficiency was demonstrated by detecting transcription factor expression by immunocytochemistry (ICC) (Figure 1). Transduction efficiencies of 35%, 10%, 35%, and 35% were achieved for the Oct4, Sox2, Klf4, and c-Myc transcription factors, respectively, demonstrating that these lentiviruses are capable of efficiently expressing the four human transcription factors when infecting BJ cells.

The over-expression of the four human transcription factors correlated with observed cell colony morphology progressions that are indicative of the reprogramming process in human cells (Figure 2). This morphological progression occurred over the course of 25 days of dox treatment to generate ES cell-like colonies where individual constituent cells demonstrated both high nucleus to cytoplasm ratios and high phase contrast between individual cells. These colonies also demonstrated high phase contrast defined colony borders as well as some three dimensional colony growth (Figure 2d).

Changes continued to progress through the removal of dox on day 26 and resulted in the establishment of iPS cell colonies that endogenously expressed pluripotency genes for colony survival, such that the colonies no longer relied on ectopic transcription factor expression for maintenance and expansion. Dox-independent colonies were ready for expansion by day 32 (Figure 2e).

**Figure 2.** Representative cellular morphologies during the reprogramming of BJ cells. Each colony was imaged at the indicated timepoints following dox induction. a) Day 4: initial non-iPS cell colony b) Day 11: partially reprogrammed colony c) Day 14: partially reprogrammed colony d) Day 23: human iPS cell colony two days before dox removal e) Day 32: pickable human iPS cell colonies seven days after removal of dox f) Human iPS cell colonies six days after initial manual pick and passage to a 24-well plate (100X).
On day 33, twelve iPS cell colonies were manually picked and plated in 24-well plates for expansion. Based on the selection of iPS cell colonies from wells where dox had been withdrawn, a reprogramming efficiency of 0.008% was obtained. Subsequent passage of the isolated colonies resulted in the generation of iPS cell colonies that uniformly expressed AP (Figure 3) as well as the pluripotency markers SSEA-4, Nanog, Oct4, Rex1, Tra-1-60, and Tra-1-81 (Figure 4). These results demonstrate that the Dox Inducible Reprogramming Lentivirus Set: OKSM is suitable for the reprogramming of human fibroblasts to iPS cells.

**Figure 3.** AP analysis of iPS cell colonies. BJ cells were transduced with the Dox Inducible Reprogramming Lentivirus Set: OKSM. Expression of the four transcription factors was induced by adding dox to initiate the reprogramming process. The emergent colonies were manually isolated, passaged, and stained for AP activity.

**Figure 4.** Pluripotency analysis of iPS cell colonies. BJ cells were transduced with the Dox Inducible Reprogramming Lentivirus Set: OKSM. The emergent colonies were manually isolated (day 32), passaged, and stained for the expression of pluripotency markers. Left Panel: Phase contrast. Middle Panel: DAPI staining. Right Panel: ICC staining for pluripotency markers: Rex1 (red) and SSEA4 (green); Nanog (red) and Tra-1-60 (green); and Oct4 (red) and Tra-1-81 (green).

**EXPERIMENTAL PROCEDURES**

**Plating BJ Cells for Reprogramming (Day 1)**
BJ Human Fibroblasts (early passage, p6) (Cat. No. 08-0027) were plated at 1 x 10⁵ cells per well in 2 wells of a 6-well plate coated with 0.2% gelatin. The cells were incubated in 2 ml per well of BJ Growth Medium (450 ml EMEM supplemented with 50 ml FBS) overnight to reach the desired cell density of 50% to 80% confluency.

**Viral Transduction (Day 2)**
The next day 16.5 µl of each lentivirus in the Dox Inducible Lentivirus Reprogramming Set: Human OKSM (conc) (Cat. No. ST000037) were combined with 1 ml of rtTA lentivirus and 934 µl of BJ Medium. Medium was aspirated from one well of BJ cells and the lentivirus mixture was added. Plates were rocked to ensure even distribution of the medium then incubated overnight. The next day a second round of transduction was performed by aspirating the medium, replacing with freshly made lentiviral mixture, and incubating overnight.

**Re-plating of Transduced BJ Cells (Day 4)**
Twenty-four hours after the last transduction, cells were trypsinized, centrifuged, and plated in 6 and 4-well plates. To determine transduction efficiency, transduced BJ cells were plated in BJ Medium on 4-well gelatin coated plates at a density of 1 x 10⁶ to 2 x 10⁶ cells per well. For colony isolation and reprogramming, BJ cells were plated in BJ Medium on 6-well plates previously plated with irradiated CF-1 MEF feeder cells. The cells were then incubated overnight.
APPLICATION NOTE

Dox-induced Reprogramming (Day 0)

Fresh iPS Medium (450 ml Knockout™ DMEM supplemented with 45 ml Knockout Serum Replacement, 45 ml Plasmanate®, 4 ng/ml bFGF, 5 ml 100X non-essential amino acids, 5 ml penicillin (5,000 U/ml)-streptomycin (5,000 µg/ml), 5 ml 200 mM L-glutamine, and 0.5 ml of 55 mM β-mercaptoethanol) was supplemented with Stemolecule™ Doxycycline hyclate (Cat. No. 04-0016) at a final concentration of 2 µg/ml to generate Reprogramming Medium. For the transduced cells in the 4-well plates, medium was aspirated and Reprogramming Medium was added to 2 of the wells while iPS Medium was added to the other 2 wells. For the transduced cells in the 6-well plates, medium was aspirated and Reprogramming Medium was added to 4 of the wells while iPS Medium was added to 1 of the wells.

Fourty-eight hours after dox-induction, the cells in the 4-well plates were used to determine transduction efficiency by immunocytochemistry (ICC).

MEF Conditioned Medium

CF-1 gamma irradiated feeder MEFs were plated in a T-75 flask at a density of 3 x 10⁶ to 4 x 10⁶ cells per flask and incubated overnight. The next day, the medium was aspirated and 25 ml of fresh iPS Medium was added. Twenty-four hours later, the medium was collected, filtered, and stored at -20°C. 25 ml of fresh iPS Medium was added to the cells and they were incubated overnight. The process was repeated for a total of 7 collections (175 ml total). The aliquots were thawed, pooled, realiquoted, and stored at -20°C. When MEF Conditioned Medium was thawed for use, an additional 16 ng/ml of bFGF was added to the medium for a final bFGF concentration of 20 ng/ml.

Medium Changes

For the 6-well plate, medium was replaced every 24 hours up until day 7 post-dox induction. On day 8, medium was changed to MEF Conditioned Medium. MEF Conditioned Medium was supplemented with dox at a final concentration of 2 µg/ml for wells that contained Reprogramming Medium. MEF Conditioned Medium (with or without dox) was appropriately replaced every 24 hours until day 25.

On day 26, dox was removed from the medium for all wells. Medium was replaced every 24 hours until day 33 when colonies were picked and passaged for expansion.

Immunocytochemistry

ICC was performed using standard procedures (reference Protocol: Immunocytochemistry online at www.stemgent.com/support/protocols).

To determine transduction efficiency, ICC staining was performed for each of the four individual transcription factors (Oct4, Klf4, Sox2 and c-Myc) on both a dox (-) and a dox (+) well. Each 4-well plate had 2 wells of dox (+) and 2 wells of dox (-). One well of each dox condition was incubated with the primary and secondary antibodies, while one well of the dox (+) condition served as a negative control with only a secondary antibody incubation.

ICC was also performed on expanded iPS cell colonies to determine the expression of SSEA-4, Nanog, Tra-1-60, Tra-1-81, Rex1, and Oct4.

Expanding and Characterizing iPS Cell Colonies

Twelve colonies were manually picked and passaged into individual wells of a 24-well plate pre-coated with a feeder layer of gamma irradiated MEFs. The iPS Medium was changed daily. The 24-well plates were cultured for up to 8 days and monitored daily for iPS cell colony growth. Individual wells from the 24-well plate that demonstrated colony attachment and expansion were manually passaged 2 to 3 more times until enough iPS cell colonies could be passaged into four wells of a 4-well plate for pluripotency analysis. These plates were then used to analyze pluripotent marker expression by AP staining, as well as ICC detection of Tra-1-60, Tra-1-81, SSEA-4, Rex1, Nanog, and Oct4.

AP Staining of Manually Selected and Passaged iPS Cell Colonies

AP staining was performed using the Alkaline Phosphatase Staining Kit (Cat. No. 00-0009) (reference Protocol: Alkaline Phosphatase Staining Kit online at www.stemgent.com/support/protocols). AP staining was carried out on all four wells of the ICC plate after analysis and imaging.
REFERENCES


ADDITIONAL READING


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