Application Note

Generation and Characterization of Mouse Induced Pluripotent Stem Cells: Reprogramming of Oct4-neo MEFs with the Doxycycline-inducible Mouse 4F2A Polycistronic Lentivirus

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Sigma Products ST000043, ST070029

Summary

Here we demonstrate that the doxycycline-inducible expression of the mouse transcription factors Oct4, Klf4, Sox2, and c-Myc contained within a single 4F2A polycistronic mRNA is sufficient to reprogram Oct4-neo MEFs to iPS cells when the 4F2A polycistronic lentivirus is co-transduced with a reverse tetracycline transcriptional activator lentivirus. These VSV-G pseudotyped lentiviruses are capable of infecting both dividing and non-dividing cells from any mammalian species. Additionally, we show that G418 is useful for iPS cell colony selection for expansion as the Oct4-neo MEFs contain a neomycin resistance gene targeted to the endogenous Oct4 locus. Endogenous expression of the neomycin resistance gene from the Oct4 locus is a clear indication that the iPS cell colonies are indeed pluripotent and capable of self-renewal. Lastly, we demonstrate that the iPS cell colonies generated display typical ES cell-like morphology and express common mouse ES cell pluripotency markers such as SSEA-1, Oct4, Nanog, and alkaline phosphatase.

Introduction

Embryonic stem (ES) cells are pluripotent cells that can differentiate into the three primary germ layers, and as such, are important in many areas of research in the scientific and medical communities. However, ES cells are obtained by embryo destruction, posing a significant ethical dilemma. In 2006 the discovery by Shinya Yamahaka and colleagues that mouse embryonic fibroblasts (MEFs) could be reprogrammed into ES cell-like induced pluripotent stem (iPS) cells started a new era of stem cell research. Using a defined set of transcription factors and cell culture conditions, Takahashi et al. demonstrated that retrovirus-mediated delivery and expression of Oct4, Klf4, Sox2, and c-Myc is capable of inducing pluripotency (i.e. generating iPS cells) in mouse fibroblasts¹. 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 mouse somatic cell types²³. The Dox-inducible mouse 4F2A polycistronic lentivirus was developed in the lab of Stemgent scientific advisory board member Rudolf Jaenisch, M.D., at the Whitehead Institute at MIT. The single polycistronic lentiviral vector can effectively deliver the transcription factors Oct4, Sox2, Klf4, and c-Myc to the targeted cell type to be reprogrammed, while minimizing the number of proviral integrations required for successful reprogramming, thereby reducing the risk of insertional mutagenesis associated with lentiviral transductions. The polycistronic 4F2A lentivirus expresses the four mouse transcription factors contained within a single expression cassette under the control of the Dox-inducible tetO operator. The inclusion of three unique 2A peptide sequences (P2A, T2A, and E2A; one between each transcription factor) results in “ribosomal skipping”, which allows for the equivalent expression of all four transcription factors from a single vector⁴. The individual expression of these transcription factors, along with the expression of reverse tetracycline transcriptional activator (rtTA), has been shown to reprogram a variety of mouse cell types including fibroblasts, B cells and T cells to iPS cells⁴. iPS cells are similar to ES cells in morphology, proliferation and ability to induce teratoma formation. In mice, pluripotency of iPS cells has been fully demonstrated through the generation of adult mice by tetraploid complementation⁵⁶⁷. Furthermore, both ES cells and iPS cells can be used as the pluripotent starting material for the generation of differentiated cells or tissues for regenerative medicine applications⁵⁶⁷. However, iPS cells have a distinct advantage as they have the properties of ES cells without the ethical dilemma of destroying embryos to obtain them. 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 cells⁸.

Here we show that Sigma’s Stemgent iPSC Generation Lentivirus Dox Inducible Polycistronic Mouse TF Set: 4F2A + RG (Cat. No. ST000043) is able to reprogram Stemgent Oct4-neo MEFs. After viral transduction, cells expressed each of the four reprogramming factors, Oct4, Klf4, Sox2, and c-Myc, as shown by immunocytochemistry (ICC). G418 selected iPS cell colonies were manually picked and passaged to test for typical pluripotency marker expression. The isolated iPS cell colonies stained positive for alkaline phosphatase (AP) and expressed the SSEA-1, Nanog, and Oct4 pluripotency markers.
Results

Viral Infectious Unit Titer Determination

The Stemgent® iPSC Generation Lentivirus Dox Inducible Polycistronic Mouse TF Set: 4F2A + RG (Cat. No. ST000043) was used to infect Stemgent Oct4-neo MEFs to demonstrate (1) Dox-regulated expression of the mouse transcription factors, (2) m4F2A polycistronic lentivirus-mediated transduction efficiency, and (3) use of the m4F2A polycistronic lentivirus in the reprogramming of Oct4-neo MEFs to iPScells.

In this experiment, the entire volume of lentiviral transduction medium containing the 4F2A and rtTA lentiviruses was used to transduce each well of a 6-well plate of Oct4-neo MEFs seeded at a density of 2 x 10^5 cells per well. After 24 hours the lentiviral transduction medium was removed and the transduced cells were re-plated into both 4-well plates to evaluate transduction efficiency and a 6-well plate for reprogramming efficiency determination and iPSc colony selection. After a 72-hour Dox treatment, the inducible expression of the four mouse transcription factors was monitored by ICC. Each mouse transcription factor was inducibly expressed (Figure 1), demonstrating that the 4F2A lentivirus is capable of infecting Oct4-neo MEFs. A transduction efficiency of 35% was achieved for the Oct4, Klf4, Sox2, and c-Myc transcription factors.

The Dox-inducible over-expression of the four mouse transcription factors in the Oct4-neo MEFs correlated well with morphological changes indicative of the reprogramming process observed in the 6-well plate (Figure 2). This morphological transformation progressed over the course of the first 12 days of Dox induction to generate ES cell-like colonies with defined edges and three dimensional growth. Reporter MEFs utilizing the neomycin resistance gene targeted to the endogenous locus of Oct4 allow for the simple selection of iPSc colony to pick and expand for analysis. Endogenous expression from the Oct4 locus, independent of exogenous reprogramming factor expression, is a clear indication that the colonies generated are indeed pluripotent and capable of self-renewal. G418 antibiotic selection can be started as early as day 6 in the selection process by adding 400 µg/ml into the culture medium. However, it is recommended that the G418 be applied 10 to 12 days post-Dox induction. Maintenance of the cultures in the G418 for 4 days is sufficient to remove reporter MEFs that have not initiated the reprogramming process. Once the G418 is removed, allow several days for the recovery of iPSc colonies that can be manually picked and passaged for expansion.

Four days of G418 antibiotic selection (starting on day 12 after Dox induction), removal of Dox from the growth medium at day 16, and the subsequent culture expansion for 3 days after Dox and G418 removal enabled the selection of iPSc cell colonies. On day 19, thirty iPSc cell colonies were identified in the 6 wells of re-plated and Dox-induced MEFs (3.0 x 10^5 cells total). Twelve of the 30 colonies were manually picked and passaged to a 24-well plate for expansion. Nine out of the 12 colonies expanded and several colonies were passaged 1:8 into a 4-well plate format for pluripotency analysis. The generation of 30 iPSc cell colonies from 300,000 re-plated cells resulted in a reprogramming efficiency of 0.01%. A significantly higher reprogramming efficiency of 0.03% (based on 35% transduction efficiency from Figure 1, approximately 105,000 cells were capable of being reprogrammed; 30

Figure 1. ICC Analysis 72 Hours Post-Dox Induction to Monitor Transduction Efficiency of the Lentiviruses in the iPSC Generation Lentivirus Dox Inducible Polycistronic Mouse Set 4F2A + RG. Oct4-neo MEFs were co-transduced with the Dox-inducible 4F2A lentivirus and a constitutively expressing rtTA lentivirus. Expression of four transcription factors (Oct4, Klf4, Sox2, and c-Myc) as a single polycistronic mRNA, was induced by adding Dox to the culture medium. The left column represents Oct4-, Klf4-, Sox2-, or c-Myc-specific antibody staining; the middle column represents DAPI-stained nuclei; and the right column represents the merged images of the DAPI- and antibody-stained cells (200X magnification).

Figure 2. Cell Morphologies Observed during the Reprogramming of Oct4-neo MEFs with the iPSC Generation Lentivirus Dox Inducible Polycistronic Mouse TF Set: 4F2A + RG. Each of the colonies was imaged (200X magnification) in culture at the indicated days following Dox induction. Day 3, 5, and 10 shows the progression of iPSc colony generation. The day 17 imaged colony in representative of morphologies seen after 4 days of G418 selection. The day 19 morphology is representative of manually picked and passaged iPSc cell colonies remaining after 3 days of growth recovery in medium not containing G418 or Dox.
Results, Continued

Colonies generated from 105,000 cells equals 0.03%) is calculated when considering the initial transduction efficiencies of the four transcription factors.

Subsequent passage of the isolated and expanded colonies resulted in the generation of iPS cell colonies that uniformly expressed AP, SSEA-1, Oct4 and Nanog pluripotency markers (Figure 3), indicating that the colonies generated are indeed pluripotent and capable of self renewal.

These results demonstrate that the Stemgent® iPSC Generation Lentivirus Dox Inducible Polycistronic Mouse Set: 4F2A + RG (Cat. No. ST000043) is capable of infecting Oct4-neo MEFs to enable Dox-inducible reprogramming factor expression, thereby initiating the reprogramming process and transforming the Oct4-neo MEFs to iPS cells.

Experimental Procedures

Materials

- Stemgent Oct4-neo MEFs (P2) (Stemgent Cat. No. 08-0014)
- Stemolecule™ Doxycycline hyclate (Stemgent Cat. No. 04-0016)
- iPSC Generation Lentivirus Dox Inducible Polycistronic Mouse TF Set: 4F2A + RG (Sigma Cat. No. ST000043)
- Knockout™ DMEM (Invitrogen Cat. No. 10829-018)
- Antibiotic G418 (Sigma Cat. No. A1720)
- Stemgent Alkaline Phosphatase Staining Kit (Stemgent Cat. No. 00-0009)
- Affinity Purified anti-Mouse/Human Oct4 Antibody (Stemgent Cat. No. 09-0023)
- Affinity Purified anti-Mouse/Human Klf4 Antibody (Stemgent Cat. No. 09-0021)
- Stemgent Purified anti-Mouse/Human Sox2 Antibody (Stemgent Cat. No. 09-0024)
- Affinity Purified anti-Mouse/Human SSEA1 Antibody (Stemgent Cat. No. 09-0005)
- Affinity Purified anti-Mouse/Human Nanog Antibody (Stemgent Cat. No. 09-0020)

Figure 3. Pluripotency Analysis of iPS Cell Colonies Generated Using the iPSC Generation Lentivirus Dox Inducible Polycistronic Mouse TF Set: m4F2A + RG. Oct4-neo MEFs were co-transduced with the Dox-inducible m4F2A lentivirus and the constitutively expressing rtTA lentivirus. Expression of four transcription factors (Oct4, Sox2, Klf4, and c-Myc) was induced by adding Dox to initiate the reprogramming process. iPS cell colonies remaining after a 4 day treatment with G418 were manually isolated and passaged for further characterization. (Top Panel) Phase contrast microscopy of AP stained iPS cell colonies (100X and 200X magnification for left and right images, respectively). (Bottom Panel) Pluripotency marker analysis (200X magnification). The left column represents phase-contrast images; the middle column represents ICC staining for Nanog, Oct4, and SSEA-1, respectively; and the right column represents DAPI-stained cells to visualize the nucleus.
Experimental Procedures, Continued

Preparation of MEF Medium
450 ml DMEM supplemented with 10% FBS, 5 ml 100x non-essential amino acids, 5 ml penicillin (10,000 U/ml)-streptomycin (10,000 mg/ml), 5 ml of 200 mM L-glutamine, and 0.5 ml of 55 mM β-mercaptoethanol.

Preparation of Lentiviral Transduction Medium
25 ml of Dox-inducible mouse 4F2A lentivirus and 15 ng of rtTA lentivirus supplemented with MEF Medium to 2 ml per well; two wells were transduced using this protocol.

Preparation of mES/iPS Medium Dox (-)
450 ml Knockout™ DMEM supplemented with 15% ES cell-qualified fetal calf serum, 5 ml of 100x non-essential amino acids, 5 ml of penicillin (10,000 U/ml)-streptomycin (10,000 mg/ml), 5 ml of 200 mM L-glutamine, 0.5 ml of 55 mM β-mercaptoethanol, and 25 μl of 10’ leukemia inhibitory factor (LIF).

Preparation of Doxycycline
2 mg/ml in ddH2O and filter sterilized.

Seeding MEFs for Reprogramming (Day 1)
Oct4-neo MEFs (passage 2) were plated in 2 wells of a 6-well plate previously coated with 0.2% gelatin, at a density of 2 x 10^5 cells per well. Plated MEFs were incubated in 2 ml per well of MEF Medium overnight at 5% CO2 and 37°C to reach the desired cell density of 50% to 80% confluency.

Viral Transduction (Day 2)
MEF Medium was removed and Lentiviral Transduction Medium was added to each well of cells to be reprogrammed. After gentle rocking of the cell culture dish to ensure that the Lentiviral Transduction Medium was evenly distributed, the cells were incubated overnight at 37°C and 5% CO2.

Re-seeding of Transduced MEFs (Day 3)
Twenty to 24 hours post-transduction Lentiviral Transduction Medium was removed. The transduced MEFs were trypsinized and counted for re-plating on 0.2% gelatin coated plates in MEF Medium at the cell densities listed below. The re-plated and transduced MEFs were incubated overnight at 37°C and 5% CO2. (Note: any remaining transduced cells that are not re-plated can be frozen in liquid nitrogen using standard cryopreservation techniques for future analysis.)

Doxycycline-Induced Reprogramming (Day 0)
1. Fresh mES/iPS Medium Dox (-) was prepared and supplemented with Dox to a final concentration of 2 μg/ml to generate the mES/iPS Medium Dox (+).
2. MEF Medium was aspirated and replaced with either the mES/iPS Medium Dox (+) or Dox (-) as described below:
   a. Colony Isolation/Determining Reprogramming Efficiency: 2 ml per well in a 6-well plate; 6 wells mES/iPS Medium Dox (+).
   b. Determining Transduction Efficiency: 0.5 ml per well in each 4-well plate; 2 wells mES/iPS Medium Dox (+) and 2 wells mES/iPS Medium Dox (-) per plate (three 4-well plates).
3. Plates were incubated at 37°C and 5% CO2.

Medium Changes (Day 2 and beyond)
1. 6-well plate: the mES/iPS Medium Dox (+) was replaced every 48 hours until day 12. G418 (400 µg/ml) was then added to the mES/iPS Medium Dox (+). Medium with G418 was replaced every 24 hours until day 16 when all Dox (+) and G418 (+) wells were switched to mES/iPS Medium Dox (-). Medium was replaced every 48 hours for the remainder of the experiment.
2. 4-well plates: no changes of media needed. Seventy-two hours after Dox-induction, the 4-well plates were processed as described below to determine transduction efficiency by ICC.

ICC: Transduction Efficiency
The ICC staining procedure was performed as instructed in the ICC general protocol (Stemgent Cat. No. 00-0016). 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. Primary antibodies were diluted as follows:
   • Anti-Oct4 diluted 1:400
   • Anti-Klf4 diluted 1:200
   • Anti-Sox2 diluted 1:100
   • Anti-c-Myc (follow manufacturer’s recommendations)
Experimental Procedures, Continued

Identification and Expansion of iPS Colonies for Pluripotency Analysis

1. Colonies with iPS cell morphology were manually picked and expanded as follows:
   a. Using a sterile glass picking tool, the identified colony was gently separated from surrounding cells.
   b. Using the glass picking tool, each colony was gently detached from the tissue culture well.
   c. Using a 10 μl pipettor set at 10 μl, the detached colony was pipetted out of the 6-well plate and into an individual well of a 96-well plate (each well containing 15 μl of PBS).
   d. Each individually isolated iPS cell colony was trypsinized by adding 20 μl of 0.05% trypsin/EDTA to each well to dissociate the colony cells from one another.

2. Each dissociated colony was re-plated in mES/iPS Medium Dox (-) into individual wells of a 24-well plate pre-coated with gamma-irradiated feeder MEFs at a density of 5 x 10^4 cells per well. The mES/iPS Medium Dox (-) was changed daily.

3. The 24-well plates were incubated for up to 7 days in a tissue culture incubator at 37°C and 5% CO₂, and monitored daily for iPS cell colony growth.

4. Individual wells that exhibited colony expansion and iPS morphology independent of Dox were trypsinized and passaged 1:8 from the 24-well plate into two 4-well plates that were pre-seeded with gamma-irradiated feeder MEFs at a density of 5 x 10^4 cells per well. Wells were monitored for equivalent iPS cell colony growth and morphology and analyzed for pluripotency 6 days later.
   • AP Staining of iPS Cell Colonies
   • AP staining was performed as instructed.

Additional Reading


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