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Application Note

Generation and Characterization of Mouse Induced Pluripotent Stem Cells: Reprogramming of Oct4-neo MEFs with the Doxycycline-Inducible Mouse Transcription Factor Lentivirus

Authors: Chenmei Luo[1], Kevin Yi[1], Brad Hamilton[1,2]

[1]Stemgent, Inc., One Kendall Square, Suite B6201, Cambridge, MA 02139

[2]Corresponding Author

Sigma Products: ST000021, ST070007, ST070008, ST070009, ST070010

Summary

Here we demonstrate the reprogramming of Oct4-neo MEFs to an ES cell-like state using VSV-G-pseudotyped lentiviruses that are capable of infecting both dividing and non-dividing cells from many mammalian species. Doxycycline-inducible expression of the mouse transcription factors Oct4, Sox2, Klf4 and c-Myc, along with the constitutive expression of reverse tetracycline transcriptional activator, is sufficient to reprogram Oct4-neo reporter MEFs to iPS cells. Additionally, we demonstrate 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. 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

Reprogramming, the process by which induced pluripotent stem (iPS) cells are generated, is the conversion or "reprogramming" of adult somatic cells to an embryonic stem (ES) cell-like state. Using a defined set of transcription factors and cell culture conditions, Shinya Yamanaka 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 embryonic fibroblasts (MEFs)1. 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 types[2,3].

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 germline chimeras. 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[4,5,6]. However, iPS cells have a distinct advantage over human ES cells 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 therapies by eliminating the potential for immune rejection of non-autologous, transplanted cells[7].

The ability to reprogram adult somatic cells to the iPS cell state allows researchers to evaluate the utility of ES-like cells in a variety of applications. For example, reprogramming techniques have been used to evaluate the cellular mechanisms of iPS cell formation and to generate iPS cells from diseased and non-diseased patient-specific tissues. Stemgent scientific advisory board member Rudolph Jaenisch and his colleagues at the Whitehead Institute for Biomedical Research at M.I.T., developed the Dox-inducible, lentivirus-mediated expression system to regulate the expression of virally transduced genes[2,3]. Stemgent has developed several products to bring this technology to stem cell researchers. In this paper, we demonstrate that the iPSC Generation Dox Inducible Mouse TF Lentivirus Set: OKSM + RG (Stemgent Cat. No. 00-0021 or Cat. No. 00-0024) is able to reprogram Oct4-neo MEFs (Stemgent Cat. No. 08-0014 or included in Cat. No. 00-0024) into iPS cells.

Results

The lentiviruses contained within the iPSC Generation Dox Inducible Mouse TF Lentivirus Set: OKSM + RG (Stemgent Cat. No. 00-0021 or Cat. No. 00-0024) were used to infect Oct4-neo MEFs (Stemgent Cat. No. 08-0014 or included in Cat. No. 00-0024) to demonstrate (1) Dox-regulated expression of the mouse transcription factors, (2) lentivirus-mediated transduction efficiency, and (3) use in the reprogramming of MEFs to iPS cells.

In this experiment, the entire volume of lentiviral transduction medium (containing Oct4, Klf4, Sox2, c-Myc, and rtTA lentiviruses) was used to transduce each well of a 6-well plate seeded with 2 x 10^5 cells per well of Oct4-neo MEFs. After 24 hours the lentiviral transduction medium was removed and the cells were split into 4-well plates to evaluate gene expression by immunocytochemistry (ICC) and a 6-well plate for reprogramming efficiency determination and iPS cell colony selection. After a 48-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 these lentiviruses are capable of infecting Oct4-neo MEFs. Transduction efficiencies of 36%, 18%, 31% and 33% were achieved for the Oct4, Sox2, Klf4, and c-Myc transcription factors, respectively.

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Results, Continued

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 identification of iPS cell colonies 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 four 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 iPS colonies that can be manually picked and passaged for expansion.

Figure 1

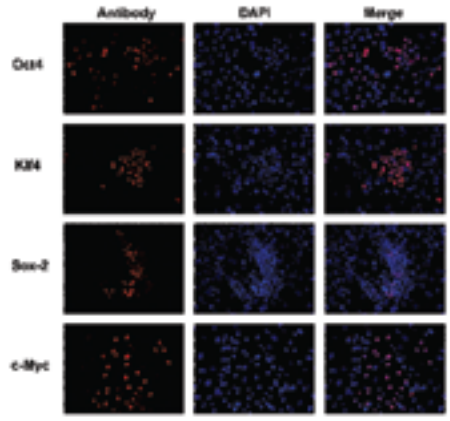

Figure 1. ICC Analysis 48 Hours Post-Dox Induction to Monitor Transduction Efficiency of the Lentiviruses in the iPSC Generation Dox Inducible Mouse TF Lentivirus Set: OKSM + RG. Oct4-neo MEFs were transduced with each of the four factors of the iPSC Dox Inducible Mouse TF Lentivirus Set. The left column 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 (100X magnification).

Four days of G418 antibiotic selection (starting on day 12 after Dox induction), removal of Dox and G418 from the growth medium at day 16, and the subsequent culture expansion for several days after Dox and G418 removal enabled the selection of iPS cell colonies that were proliferating independently of virally introduced transcription factor expression. The expression of the neomycin resistance gene from the endogenous Oct4 locus confirmed that the Oct4-neo MEFs were being reprogrammed to generate iPS cells. On day 22, fourteen iPS colonies were identified in the three wells of re-plated and Dox-induced MEFs (1.5×10^5 total cells). These colonies were manually picked and passaged to a 24-well plate for expansion. Nine out of the 14 colonies expanded and several colonies were passaged 1:8 into a 4-well plate format for pluripotency analysis. The generation of nine independently expanding iPS cell lines from 150,000 re-plated cells resulted in a reprogramming efficiency of 0.01%. A significantly higher reprogramming efficiency of 1% is calculated when considering the initial transduction efficiencies of the four transcription factors. Subsequent passage of the isolated colonies resulted in the generation of iPS cell colonies that uniformly expressed Alkaline Phosphatase (AP), SSEA-1, Oct4, and Nanog pluripotency markers **(Figure 3)**.

Figure 2

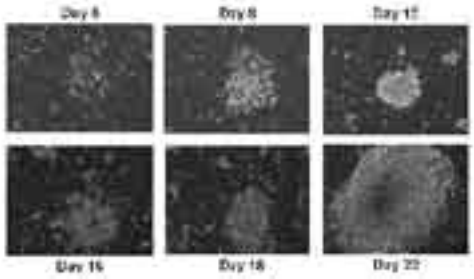

Figure 2. Cell Morphologies during the Cellular Reprogramming of Oct4-neo MEFs with the iPSC Generation Dox Inducible Mouse TF Lentivirus Set: OKSM + RG. Each of the colonies was imaged at the indicated days following Dox induction. Days 5, 8, and 12 show the progression of iPS cell colony generation. The day 16 imaged colony is representative of iPS cell colony morphologies seen after 4 days of G418 selection. Day 18 and day 22 imaged colonies are representative of colonies established after Dox and G418 have been removed from the growth medium.

Figure 3

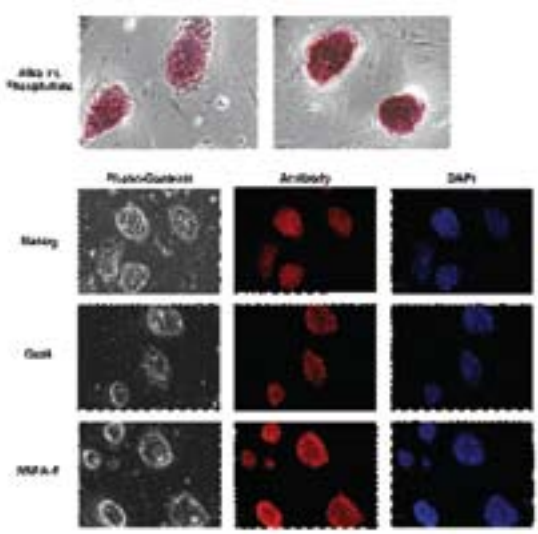

Figure 3. Analysis of iPS Cell Colonies Generated Using the iPSC Generation Dox Inducible Mouse TF Lentivirus Set: OKSM + RG. Oct4-neo MEFs were transduced with the four Dox-inducible lentiviruses, Oct4, Klf4, Sox2, and c-Myc, as well as a constitutively expressing rtTA lentivirus. Expression of the transcription factors was induced by adding Dox to initiate the reprogramming process. After selection with G418, the emergent colonies were manually isolated and passaged for further characterization. (Top Panel) Phase contrast microscopy of AP stained iPS cell colonies (200X magnification). (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 antibodies, respectively, and the right column represents DAPI-stained cells to visualize the nucleus.

These results demonstrate that the iPSC Generation Dox Inducible Mouse TF Lentivirus Set: OKSM + RG (Stemgent Cat. No. 00-0021 or Cat. No. 00-0024) are capable of infecting Oct4-neo MEFs (Stemgent Cat. No. 08-0014 or included in Cat. No. 00-0024) with transcription factor specific lentiviruses that enable Dox-inducible expression, thereby initiating the reprogramming process and transforming the Oct4-neo MEFs to iPS cells.

Mouse, Dox-inducible, OKSM

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Results, Continued

Experimental Procedures

Materials

- Oct4-neo MEFs
  (Stemgent Cat. No. 08-0014 or included in Cat. No. 00-0024)
- iPSC Generation Dox Inducible Mouse TF Lentivirus Set: OSKM
  (Stemgent Cat. No. 00-0021) or the Stemgent® Mouse iPSC Generation Kit II (Stemgent Cat. No. 00-0024)
- Stemolecule™ Doxycycline hyclate
  (Stemgent Cat. No. 04-0016 or included in Cat. No. 00-0024)
- 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)

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

4 ml of combined reprogramming factor lentiviruses combined with 5 ng of rtTA lentivirus per well; two wells were transduced using this protocol:

- 1 ml mOct4-Lentivirus
- 1 ml mKlf4-Lentivirus
- 1 ml mSox2-Lentivirus
- 1 ml mc-Myc-Lentivirus
- 5 ng of rtTA Lentivirus

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 107 leukemia inhibitory factor (LIF).

Preparation of Doxycycline

2 mg/ml in ddH_2O and filter sterilized.

Seeding MEFs for Reprogramming (Day 1)

Oct4-neo MEFs (P2) were plated in two 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 of MEF Medium overnight at 5% CO^2 and 37°C to reach the desired cell density of 50 to 80% confluency.

Viral Transduction (Day 2)

MEF Medium was removed and the Lentiviral Transduction Medium was added to each well of cells to be reprogrammed. After ensuring that the transduction medium was evenly distributed (by gentle rocking of the cell culture dish), the cells were incubated overnight at 37°C and 5% CO^2.

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% CO^2. (Note: any remaining transduced cells that are not re-plated can be frozen in liquid nitrogen using standard cryo-preservation techniques for future analysis.)

5 x 10^4 cells per well of a 6-well plate; 4 wells total with 2 ml of MEF growth medium per well.

2 x 10^4 cells per well in a 4-well plate; 3 plates total with 0.5 ml of MEF growth medium per well.

Dox-Induced Reprogramming (Day 0)

1. Fresh mES/iPS Medium [Dox (-)] was prepared and supplemented with Dox at 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; 3 wells mES/iPS Medium Dox (+) and 1 well mES/iPS Medium Dox (-).
   b. Determining Transduction Efficiency by ICC: 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% CO^2.

Medium Changes (Day 2 and beyond)

1. 6-well plate: the mES/iPS Medium Dox (+) and Dox (-) was replaced every 48 hours.
2. 4-well plates: no changes of media needed. Forty-eight 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 or included in Cat. No. 00-0024). 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

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Anti-c-Myc (follow manufacturer's recommendations)

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% CO_2 and monitored daily for iPS cell colony growth.
4. Individual wells that exhibited colony expansion and iPS cell 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.
- ICC: Pluripotency Analysis of Manually Selected and Passaged iPS Cell Colonies

The ICC staining procedure was performed as instructed in the ICC general protocol (Stemgent Cat. No. 00-0016 or included in Cat. No. 00-0024). Antibodies were diluted as described below:

- Anti-SSEA-1: diluted 1:100
- Anti-Nanog: diluted 1:100
- Anti-Oct4: diluted 1:400

AP Staining of iPS Cell Colonies

AP staining was performed as instructed.

ICC: Pluripotency Analysis of Manually Selected and Passaged iPS Cell Colonies

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

- Anti-SSEA-1: diluted 1:100
- Anti-Nanog: diluted 1:100
- Anti-Oct4: diluted 1:400

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