Generation of iPS Cells from Oct4-Neo Reporter Embryonic Fibroblasts

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SUMMARY
Reprogramming is the process of converting somatic cells to a pluripotent state known as the induced pluripotent stem (iPS) cell. This process remains highly inefficient and extensive time and experience are required to identify iPS cell colonies for isolation and expansion. To facilitate the study of the reprogramming process, Stemgent® has developed the Oct4-neo MEF (P2) cell line. These mouse embryonic fibroblasts (MEFs) have a knocked-in neomycin gene under the control of the endogenous Oct3/4 promoter. Oct4-neo MEFs tested free of common mouse viruses, bacteria, fungus and microplasma. As an application test, Oct4-neo MEFs were reprogrammed with a four transcription factor retrovirus set. iPS cells expressing the endogenous Oct4 protein were selected and identified by their ability to survive in medium containing neomycin (G418). The iPS cell colonies generated displayed typical mouse embryonic stem (ES) cell characteristics.

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.

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. However, the reprogramming process is still extremely insufficient. Even after reprogramming, extensive labor and experience are needed for the picking and identification of reprogrammed iPS colonies.

To facilitate the study of the reprogramming process, genetically modified reporter mouse cell lines were developed to provide tools to monitor endogenous pluripotent gene expression, and assist with the identification and isolation of iPS cells. In 2007, Rudolph Jaenisch and colleagues generated an Oct4-neo knock-in transgenic mouse cell line harboring an IRES-GFPneo fusion cassette downstream of exon 5 of Oct4 gene. The Stemgent cell line Oct4-neo MEF (P2) (Cat. No. 08-0014) was developed from these Oct4-neo knock-in mice. Here we show that this cell line is free from contamination and can be reprogrammed into iPS cells with high efficiency. After reprogramming, the resulting iPS cell colonies were exposed to G418 (neomycin). Fully reprogrammed iPS cells were resistant to G418, while cells that had not completed the reprogramming process were eliminated from the culture. After G418 selection, iPS cell colonies expressed the typical pluripotency markers alkaline phosphatase (AP), Oct4, and SSEA-1.

RESULTS
Oct4-neo MEFs were isolated from a timed pregnant mouse (day 13.5 post-conception). The cells were amplified and frozen at passage 2. Samples of the cryopreserved MEFs were sent to Wuxi AppTex for a full panel bioburden test for fungi and bacteria. Non-contamination tests of microplasma and common mouse viruses were done by Research Animal Diagnostic Laboratories (IMPACT I-mouse).

To test their ability to be reprogrammed into iPS cells, the Oct4-neo MEFs were co-transduced with ecotropic retroviruses expressing the mouse transcription factors Oct4, Sox2, Klf4, and c-Myc. Briefly, Oct4-neo MEFs were thawed and cultured for three days. The day before reprogramming, MEFs were passaged onto Matrigel™ coated 6-well plates. For the next two days, freshly packaged ecotropic retroviruses were added to the MEFs. On the third day, the MEF culture medium was changed to mouse ES cell culture medium. Reprogrammed cell colonies were observed as early as day 3 post-transduction, and reached a maximum in number around day 14. Figure 1 shows the reprogrammed colonies observed on day 5 post-transduction.
To verify the pluripotency of the iPSC cell colonies, AP staining was performed on passage 2 iPSC cell colonies. All colonies exhibited strong and uniform positive AP staining. Immunocytochemistry (ICC) staining was also performed to verify the expression of typical pluripotency markers. All of iPSC cell colonies showed high expression levels of Oct4 and SSEA-1 (Figure 3 and Figure 4).

Figure 1. Oct4-neo MEFs before and after transduction. Oct4-neo MEFs were plated on Matrigel coated plates and transduced with Oct4, Sox2, Klf4, and c-Myc ecotropic retroviruses. A) Oct4-neo MEFs before transduction. B) Oct4-neo MEFs 5 days post-transduction.

To test the Oct4-neo reporter activity during reprogramming, G418 selection was performed on the transduced MEFs four days post-transduction. Cell death was observed starting two days after the addition of G418. After eight days of selection, most of the unreprogrammed cells had been eliminated from the culture. Reprogramming efficiency was calculated based on the iPSC cell colonies established after G418 selection. The reprogramming efficiency was approximately 0.03%; comparable with what has been previously reported.

At day 14 post-transduction, iPSC cell colonies established from G418 selection were picked and passed onto pre-seeded DR4 MEF feeder cells. All of the iPSC cell colonies picked formed typical ES cell like colonies (Figure 2). iPSC cell cultures exhibited normal morphology after multiple passages (data not shown).

Figure 2. iPSC cell colonies at P1 and P2. Oct4-neo MEFs were transduced, exposed to G418 selection, picked and passaged. A) P1 iPSC cell colony and B) P2 iPSC cell colony show typical iPSC cell colony morphology.

Figure 3. AP staining of iPSC cell colonies at P2. Oct4-neo MEFs were transduced, exposed to G418 selection, picked and passaged. P2 iPSC cell colonies show strong AP staining (20X).

Figure 4. ICC of iPSC cell colonies P2. Oct4-neo MEFs were transduced, exposed to G418 selection, picked and passaged. P2 colonies were tested for A) Oct4 expression and B) SSEA-1 expression (20X).
EXPERIMENTAL PROCEDURES

MEF Derivation
A timed pregnant (13.5 days post conception) Oct4-neo knock-in mouse (The Jackson Laboratory, Strain name: B6;129S4-Pou5f1tm1Jae/J, Stock number: 008204) was sacrificed. After removal of the head and internal organs, the carves of the embryos were minced and trypsinized. The cells were seeded on MEF medium (450 ml DMEM supplemented with 50 ml ES qualified FBS, 5 ml non-essential amino acids, and 0.9 ml β mercaptoethanol) and passaged every 2 to 3 days. Passage 2 MEFs were frozen in DMEM supplemented with 20% ES qualified FBS and 10% DMSO.

Preparation of Oct4-neo MEFs for Reprogramming
One vial of Oct4-neo MEFs was thawed in a T25 flask in MEF medium. The cells were trypsinized and re-seed on Matrigel-coated 6-well plates at a density of 0.8 x 10^5 cells per well and incubated for 2 days.

Viral Transduction
The viruses were produced as previously described. Briefly, Plat-E cells were seeded at 2 x 10^6 cells per 6 cm dish in Plat-E medium. The next day, FuGENE® HD was used to transfect 3.3 µg of each retroviral vector DNA (pMXs-Oct4, pMXs-Sox2, pMXs-Klf4 and pMXs-cMyc) to the Plat-E cells. 48 and 72 hours post-transfection, the supernatant was collected and filtered, and fresh Plat-E medium was added to the cells. Each of the four retroviral supernatants from the 48 hour collection were mixed together at a 1:1:1:1 ratio and added to the Oct4-neo MEFs. The next day, the transduction was repeated with the 72 hour supernatant collection.

Medium Changes

Twenty-four hours after the last transduction, medium was changed to mouse ES cell medium (425 ml Knockout™ DMEM supplemented with 75 ml ES qualified FBS, 5 ml non-essential amino acids, 0.9 ml β mercaptoethanol, and 1,000 U/ml LIF). Transduced cells were incubated until the medium began to change to a yellow color, then medium was replaced every 2 to 3 days.

G418 Selection
300 µg/ml of G418 was added to the mouse ES cell medium beginning day 4 post-transduction.

Expansion of iPS Cell Colonies
iPS cell colonies were picked and passaged around day 14 post transduction. Briefly, DR4 MEF feeder cells were plated onto gelatin-coated 24 well plates and incubated overnight. The next day, the DR4 MEFs were washed twice with PBS and then 1 ml of mouse ES cell medium was added to each well. iPS cell colonies were picked and trypsinized in a 96-well plate. Trypsinization was stopped by adding mouse ES cell medium. Cells were then transferred onto the pre-seeded DR4 MEF feeders.

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

AP Staining of 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).
REFERENCES