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Establishment of a reporter system to monitor silencing status in induced pluripotent stem cell lines

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ABSTRACT

Induced pluripotent stem (iPS) cells have proven to be an effective technology in regenerative medicine; however, the low efficiency of reprogramming is a major obstacle to the successful generation of iPS cell lines. One of the most important characteristics of a high-quality iPS cell line is the inactivation of transgenes driven by a retrovirus-derived long terminal repeat promoter. In this study, we established a novel marker system containing three kinds of proteins: secreted-type luciferase (MetLuc), copepod *Pontellina plumata* green fluorescent protein (copGFP), and an antibiotic-resistant gene product (Neo^r). The introduction of MetLuc–copGFP–Neo^r in mouse embryonic fibroblasts (MEFs) allowed us to monitor the reporter expression changes as an indicator of the state of silencing during reprogramming. Transformation of iPS cells induced a remarkable reduction in reporter activity, indicating that the retroviral silencing was detected successfully. Our system enables us to monitor the silencing status of transgenes and to efficiently select iPS cell lines that can be used for further applications.

Reprogramming of human and mouse fibroblasts to generate induced pluripotent stem (iPS)[1] cell lines has been achieved by the expression of only four transcription factors: Oct4, Sox2, Klf4, and c-Myc. These were subsequently referred to as the "four factors" [1–4]. Such iPS cell lines have major potential in the study and therapy of human diseases. This is because they are capable of self-renewal and can give rise to all three primary germ layers: ectoderm, mesoderm, and endoderm. Thus, they are very similar to embryonic stem (ES) cells. However, several fundamental questions of the biology of iPS cells are still unresolved. For example, the mechanisms of genomic reprogramming with the four factors are poorly understood. During this process, continuous expression of the four exogenous transcription factors causes epigenetic changes in the genome, leading to the reactivation of endogenous stem cell-related genes [5]. This results in a dramatic change from a terminally differentiated cell into a stem cell [6–8]. However, the efficiency of this reprogramming process is low; only a few cells can change into iPS cells successfully. It is not clear why the efficiency of this process is so poor. Several studies indicated that iPS cells might be established by an incidental unknown cellular reaction, but this hypothesis on the reprogramming process is still under discussion [9–12].

Because of the low efficiency of iPS cell generation, there are large biological variations in iPS cells compared with ES cells [13,14]. For example, some iPS cells are fully reprogrammed, but some are not. One of the indicators of reprogramming is the silencing of introduced transgenes driven by the retrovirus-derived long terminal repeat (LTR) promoter. In general, these are transcriptionally silenced at a high frequency in "superior" iPS cells during the reprogramming process [2]. As supporting evidence that retroviral silencing is a good indicator of successful reprogramming, Ramos-Mejia and coworkers reported that iPS cells show chromosome abnormalities when the transgene is not well silenced [15]. Furthermore, iPS cells exhibit longer and more stable passages when the retroviral transgene is silenced at an early passage number [16]. The quality of iPS and ES cells is mainly determined by chromosome analysis and/or morphology of the cells, which requires much effort and technical skill.

In this study, we designed a reporter cassette containing three types of markers: MetLuc [17], copepod *Pontellina plumata* green

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[1] *Abbreviations used:* iPS, induced pluripotent stem; ES, embryonic stem; LTR, long terminal repeat; MetLuc, secreted-type luciferase; copGFP, copepod *Pontellina plumata* green fluorescent protein; Neo^r, neomycin-resistant protein; MCS, multiple cloning site; MEF, mouse embryonic fibroblast; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; PEI, polyethylenimine; DIC, differential interference contrast; AP, alkaline phosphatase; PCR, polymerase chain reaction; PBST, PBS with Tween 20; DAPI, 4',6-diamidino-2-phenylindole; SSEA, stage-specific embryonic antigen; cDNA, complementary DNA; RT, reverse transcription; mRNA, messenger RNA; qRT–PCR, quantitative real-time PCR; ETn, early transposon.

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fluorescent protein (copGFP), and neomycin-resistant protein (Neo^r). This system can detect the remaining expression of transduced genes using only the conditioned medium from iPS cell cultures. It offers an effective system for selecting suitable iPS cell lines for further applications.

Materials and methods

Plasmid construction

The reporter gene cassette encoding MetLuc, copGFP, and Neo^r was chemically synthesized and cloned into the multiple cloning site of pJ204 plasmids (more detailed information is available in Supplementary Document 1 of the online supplementary material). This 2347-bp cassette was inserted into the multiple cloning site (MCS) of the pMYs retroviral vector at the *Bam*HI and *Xho*I restriction sites [18]. The STEMCCA–loxP lentiviral vector was kindly provided by Gustavo Mostoslavsky (Boston University School of Medicine) [19].

Cell culture

293T cells and mouse embryonic fibroblasts (MEFs) were cultured in MEF medium: Dulbecco's modified Eagle's medium (DMEM, cat. no. 08459-35, Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (cat. no. 12483-020, Invitrogen, Carlsbad, CA, USA) and 100× Antibiotic–Antimycotic Mixed Solution (cat. no. 02892-54, Nacalai Tesque). Infected MEFs were selected for 9 days with DMEM containing 5% fetal bovine serum and 1.2 mg ml^{-1} G418. Mouse iPS cells were cultured on MEF feeder cells in mouse iPSC medium: DMEM with 15% Knockout Serum Replacement (cat. no. 10828-028, Invitrogen), 0.22 mM 2-mercaptoethanol (cat. no. 21438-82, Nacalai Tesque), 100× MEM Nonessential Amino Acids Solution (cat. no. 139-15651, Wako Pure Chemical Industries, Osaka, Japan), 100× Antibiotic–Antimycotic Mixed Solution, and 1000× Leukemia Inhibitory Factor (human, recombinant, culture supernatant, cat. no. 125-05603, Wako Pure Chemical Industries). From the end of the infection to selecting colonies, low-molecular-weight compounds—1.5 μM CHIR99021 (cat. no. 163-24001, Wako Pure Chemical Industries), 0.5 μM PD0325901 (cat. no. 13034, Cayman Chemical, Ann Arbor, MI, USA), and 0.5 μM thiazovivin (cat. no. 04-0017, Stemgent, Cambridge, MA, USA)—were also added to the mouse iPSC medium as reprogramming enhancers [20]. The human iPS cell line was maintained on MEF feeder cells in human iPSC medium: DMEM/F12 GlutaMAX (cat. no. 10565-018, Invitrogen) with 15% Knockout Serum Replacement, 0.22 mM 2-mercaptoethanol, 100× MEM Nonessential Amino Acids Solution, 100× Antibiotic–Antimycotic Mixed Solution, and 4 ng ml^{-1} basic fibroblast growth factor. A human iPS cell line (201B7) was provided by the RIKEN BioResource Center through the Project for Realization of Regenerative Medicine and the National BioResource Project of the Ministry of Education, Culture, Sports, Science, and Technology (MEXT, Japan) [21]. MEFs were isolated from the embryonic tissues of C57BL/6JJcl mice (CLEA Japan, Tokyo, Japan) at embryo day 13.5. For feeder cell preparation, confluent MEFs were treated with mitomycin C (cat. no. M0503-2G, Sigma–Aldrich, St. Louis, MO, USA) at a concentration of 10 μg ml^{-1} for 2.25 h and washed twice in phosphate-buffered saline (PBS). Feeder cells were seeded at a density of 2×10^6 cells per plate. The cells used in these experiments were all incubated at 37 °C under 5% CO_2. All cells were trypsinized using 0.05% trypsin (1:10 dilution, cat. no. 35556-44, Nacalai Tesque). Animal experiments and related activities were approved by the Center for Laboratory Animal Research at Tohoku University.

Production of retroviral reporter gene cassette and infection

293T cells were initially plated at a density of 3×10^6 cells per 100-mm dish for viral production. They were transfected with 3 μg of reporter and packaging plasmids (pCL-10A1, IMGENEX, San Diego, CA, USA) complexed with 18 μg of polyethylenimine (PEI, cat. no. 23966, Polysciences, Warrington, PA, USA). After transfection for 48 h, the culture medium was replaced with new medium containing 8.9 μM forskolin. After an additional incubation for 48 h, the supernatant containing the virus was collected and filtered using a 0.45-μm syringe filter unit (cat. no. 17598, Sartorius, Göttingen, Germany) to remove cell debris. Target MEFs were seeded at a density of 1×10^5 cells per well of a 6-well plate. Virus infection was carried out for 48 h with 6 μg of polybrene (cat. no. 17736-44, Nacalai Tesque).

Production of STEMCCA lentiviral vector and infection

The 293T cells were used as retrovirus producer cells with transient transfection of the packaging plasmid and STEMCCA–loxP lentivirus. The detailed method for the transfection and packaging was described in the previous section. Cells in a 100-mm dish were transfected with 2 μg of STEMCCA–loxP and 4 μg of the packaging plasmids (pCAG-HIVgp and pCMV-VSV-G-RSV-Rev, RIKEN BioResource Center), each complexed with 18 μg of PEI. For determining viral concentration, the solution was placed overnight at 4 °C for precipitation after the addition of a 4× virus concentration (32% polyethylene glycol [PEG] 6000, 0.4 M NaCl, and 40 mM Hepes). The virus pellet was obtained by centrifuging at 3500 rpm for 1 h. The virus pellet was suspended in cell culture medium (DMEM + 10% fetal bovine serum) and infected into the target cells with polybrene. The cell culture medium was replaced with mouse iPSC medium after infection.

Microscopy

Cell morphology was evaluated using differential interference contrast (DIC) microscopy with a Nikon Eclipse TS100 microscope fitted with a Nikon digital camera (DS-Fi1, Nikon, Tokyo, Japan).

AP staining

Cells were fixed with 4% paraformaldehyde and then incubated with alkaline phosphatase (AP) staining solution at 37 °C for 10–15 min. The staining solution contains 0.6 mg ml^{-1} Fast Red TR Salt (hemi [zinc chloride] salt, cat. no. F8764, Sigma–Aldrich), 0.1 mg ml^{-1} naphthol phosphate (cat. no. 23821-24, Nacalai Tesque), 0.7 mM *N,N*-dimethylformamide, 0.2 mM $MgCl_2$, and 0.1 mM Tris–HCl (pH 8.5). Positive staining was detected as red deposits stained by Fast Red.

Genomic PCR

Genomic DNA was extracted from cells grown to confluence in 6-well plates with the TaKaRa FastPure DNA Kit (cat. no. 9191, TaKaRa Bio, Shiga, Japan) according to the manufacturer's protocol. Polymerase chain reaction (PCR) was carried out in a mixture containing 2× PCR buffer of KOD-FX, 0.4 mM dNTP, 0.5 U KOD-FX (cat. no. KFX-101, Toyobo, Osaka, Japan), and 0.3 μM of each primer. The sequences of primers are listed in Supplemental Table 1 of the online supplementary material. The PCR was performed under the following conditions: 50 cycles of 10 s denaturing at 98 °C, 30 s of annealing at 60 °C, and 1 min of extension at 68 °C for STEMCCA–loxP amplification; 30 cycles of 10 s denaturing at 98 °C, 30 s of annealing at 60 °C, and 30 s of extension at 68 °C for MetLuc–copGFP–Neo^r amplification. PCR products were