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Reprogramming of mouse somatic cells into pluripotent stem-like cells using a combination of small molecules

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ABSTRACT

Somatic cells can be reprogrammed to generate induced pluripotent stem cells (iPSCs) by overexpression of four transcription factors, Oct4, Klf4, Sox2, and c-Myc. However, exogenous expression of pluripotency factors raised concerns for clinical applications. Here, we show that iPS-like cells (iPSLCs) were generated from mouse somatic cells in two steps with small molecule compounds. In the first step, stable intermediate cells were generated from mouse astrocytes by Bmi1. These cells called induced epiblast stem cell (EpiSC)-like cells (iEpiSCLCs) are similar to EpiSCs in terms of expression of specific markers, epigenetic state, and ability to differentiate into three germ layers. In the second step, treatment with MEK/ERK and GSK3 pathway inhibitors in the presence of leukemia inhibitory factor resulted in conversion of iEpiSCLCs into iPSLCs that were similar to mESCs, suggesting that Bmi1 is sufficient to reprogram astrocytes to partially reprogrammed pluripotency. Next, Bmi1 function was replaced with Shh activators (oxysterol and purmorphamine), which demonstrating that combinations of small molecules can compensate for reprogramming factors and are sufficient to directly reprogram mouse somatic cells into iPSLCs. The chemically induced pluripotent stem cell-like cells (ciPSLCs) showed similar gene expression profiles, epigenetic status, and differentiation potentials to mESCs.

1. Introduction

Somatic cells have been reprogrammed into induced pluripotent stem cells (iPSCs) that closely resemble embryonic stem cells (ESCs) by the introduction of a defined set of transcription factors (Oct4, Sox2, Klf4 and c-Myc (OSKM) [1]. Over the past few years, various approaches which enhance reprogramming efficiency and/or replace reprogramming factors have successfully been developed to create iPSCs [2–6]. For example, CHIR99021 and PD0325901 enhance the completion and efficiency of the reprogramming process [7]. BIX01294 together with BayK8644 compensate for viral transduction of reprogramming factors and improve reprogramming efficiency [8]. Even though safety issue still needs to be addressed for small molecule-based reprogramming, it can overcome some limitations of transcription factor-based reprogramming methods. Therefore, identification of small molecules to substitute for reprogramming factors would provide alternative approaches to move toward a more efficient and safe reprogramming process [6]. Recently, Oct4 was replaced by small molecule which make possible to generate iPSCs only with small molecules combination in mouse somatic cells [9,10].

Mouse embryonic stem cells (mESCs) and post-implantation epiblast stem cells (EpiSCs) represent two phases in the ontogeny of pluripotent stem cells. Both mESCs and EpiSCs exhibit

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pluripotency; however, EpiSCs, unlike mESCs, readily form teratomas, but rarely form chimeras [11,12]. Thus, the pluripotent state of mESCs is termed 'naïve' and that of EpiSCs is termed 'primed' [13]. The development of ground state culture conditions utilizing small molecule inhibitors of MEK/ERK and GSK3 signaling (2i) has enhanced the generation of both mESCs and iPSCs. Furthermore, mESCs can be induced to differentiate into EpiSCs by exposure to activin A and bFGF, but the reverse transition requires transfection with *Klf4* or other reprogramming factors [14,15]. Recent studies indicate that the transcription factors OSKM can not only generate iPSCs under mESC culture conditions, but also induce the formation of EpiSCs (termed induced EpiSCs or iEpiSCs) under EpiSC culture conditions [1,16]. These results demonstrate that a simple modification of the extrinsic signaling conditions can determine whether overexpression of OSKM will give rise to iPSCs or iEpiSCs. Furthermore, OSKM also termed reprogramming factors, can result in the efficient trans-differentiation of fibroblasts into functional neural stem/progenitor cells or cardiomyocytes provided that appropriate culture conditions are used [17,18]. Thus, the culture environment in transcription factor-mediated reprogramming determines the fate of the reprogrammed cell.

This study aimed to find small molecules that can replace and/or enhance expression of exogenous reprogramming factors and enable the generation of iPS-like cells (iPSLC) from somatic cells in chemically defined conditions. We first established stable induced epiblast stem cell-like cells (iEpiSCLCs) during dedifferentiation of mouse astrocytes by *Bmi1*, which is not only essential for self-renewal of stem cells but is also required for the cellular reprogramming process itself [19–25]. Using these stable iEpiSCLCs, we identified combinations of two small molecules that can enable reprogramming of iEpiSCLCs into iPSLCs. Next, we reprogrammed mouse somatic cells into iPSLCs using the same two small molecules in combination with activators of the Shh signaling pathway [22,24]. This study highlights the usefulness of our phenotypic screening approach in identifying small molecules that can effectively compensate for reprogramming factors.

2. Materials and methods

2.1. Cell culture

Astrocytes were isolated from the forebrain of individual embryonic (E13.5–18.5), neonatal (1–5 days old), or adult (CF1 strain; 10–12 weeks old) mice, as described previously [26], and cultured in DMEM (Life Technologies, Gaithersburg, MD, USA) containing 10% FBS (Life Technologies), 2 mM L-glutamine, and 3 g/l D-glucose. The dedifferentiation process was observed by following the morphological changes as flat polygonal cells changed into bipolar cells and spherical aggregates in the final culture [22,27]. Mouse fetal NSCs were isolated from the subventricular zone of the brain of E13.5 embryos as described previously [22,27]. Primary astrocytes were plated at a clonal density of 2500–5000 cells/cm^2 in NSC culture medium. NSC/iEpiSCLC medium consisted of DMEM/F-12 (1:1) containing N_2 (Life Technologies), penicillin/streptomycin (Cambrex Bioscience, Walkersville, MD, USA), 10 ng/ml bFGF (R&D Systems, Minneapolis, MN, USA), with/without 10 ng/ml EGF (R&D Systems), respectively. Primary NSCs and *Bmi1*-expressing neurospheres were passaged by dissociation of the spheres into single cells using trituration through a fire polished pipette [22,27]. Floating cells were cultured as suspensions in untreated 60 mm diameter plates (BD Biosciences, Franklin Lakes, NJ, USA) at densities of 1–3 $\times$ 10^5 cells/plate in NSC medium. Cells were maintained in this medium and the growth factor was replaced daily. The expansion protocol was repeated every 3–4 days, as previously described [23,26,28]. For secondary sphere formation assays, single cells from spheres were plated at a density of 100 cells/well in 12-well plates and the number of single cell-derived spheres was counted after 14 days [23]. Six of these independently derived cultures were studied in the dedifferentiation experiments.

MEFs were isolated from the uteri of pregnant mice (E13.5). Embryos were washed with phosphate-buffered saline (PBS) and the head, heart, and spinal cord were dissected from the isolated embryos. The remainder of the embryo was minced using scissors and forceps, treated with trypsin/EDTA solution, and incubated at 37 °C for 2–3 min. After incubation, the sample was briefly triturated to produce a single cell suspension. Cells from each embryo were cultured in a T75 flask with fresh medium at 37 °C and in 5% CO_2. MEFs collected within the first three passages were used to avoid replicative senescence.

For PM2i-iPSLC, OM2i-iPSLC generation, MEFs (2 $\times$ 10^5) are seeded in six-well plate before induction. At day 1, medium is replaced in NSC/iEpiSCLC medium containing purmorphamine (0.5, 1 μM; Calbiochem), or 25-hydroxycholesterol (oxysterol; 0.1, 0.5 μM; Sigma) and incubated for 3 days. Next, transfer the cells to uncoated culture plate and form sphere for 7 days and dissociate carefully. Single cells were seeded in gelatin coated plate with 2i/LIF condition and incubated for 7 days. Finally, cells were seeded on MEF feeder with conventional mESC condition. mESC like colonies were observed at 7 days after seeding on feeder.

2.2. Retroviral transduction

Astrocytes were infected with a retrovirus produced from the PT67 amphotropic packaging cell line (Clontech, Palo Alto, CA, USA) transfected with retroviral vectors [22,23], using Lipofectamine 2000 (Life Technologies), according to the manufacturer's protocol. Astrocytes plated 24 h earlier at a density of 1 $\times$ 10^6 cells/10 cm dish were transduced by reseeding them with pre-filtered (0.45 μm) retroviral supernatant containing 6 μg/ml polybrene (Sigma–Aldrich, St Louis, MO, USA). This step was repeated twice.

2.3. Flow cytometry

Astrocytes, mESCs, and iEpiSCLCs were trypsinized and 1 $\times$ 10^6 cells were incubated with anti-SSEA1 antibody (EMD Millipore, Billerica, MA, USA) at 4 °C for 1 h, and resuspended in 100 μl of Cy3-labeled secondary antibody (1:200, Jackson Immunoresearch). The cells were then washed with PBS containing 1% FBS and resuspended in fixative solution for FACS analysis. Immunoglobulin G and Cy3-labeled secondary antibodies (1:200, Jackson Immunoresearch) were used to determine nonspecific signals. Cells to be probed for internal markers such as Oct4 were fixed with 0.1% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for 10 min and permeabilized with 90% methanol for 30 min on ice. Fixed cells (1–5 $\times$ 10^5) were probed for 1 h with a 1:10 dilution of anti-Oct4 antibody or isotype control antibody diluted in PBS containing 1% FBS and analyzed using a FACS Calibur flow cytometer. The data were analyzed with the Cell Quest 3.0 software (BD Biosciences). All treatments were performed in triplicate.

2.4. RT-PCR and qRT-PCR

Total RNA was extracted using the Trizol reagent (Life Technologies) and used to synthesize cDNA using an RT premix (Bioneer, Daejeon, South Korea) and oligo-dT (Life Technologies), according to the manufacturer's instructions. For qRT-PCR, first-strand cDNA was generated using 500 ng RNA, RT premix (Bioneer), and oligo-dT (Life Technologies) using the SYBR supermix (Bio-Rad, Hercules, CA, USA). Subsequent PCRs were performed in a final volume of 20 μl containing 1 μl of cDNA and 1 μl of 10 pM primers in PCR-Premix (Bioneer). Primer sequences and the reaction conditions used in this study are listed in Supplementary Table S4. For each sample, the ratio of the mRNA level to that of the *GAPDH* mRNA was calculated. At least three independent samples were analyzed.

2.5. Western blot analysis

Total protein was extracted using RIPA buffer containing a protease inhibitor cocktail (Roche Molecular Diagnostics, Pleasanton, CA, USA). The proteins were separated by SDS-PAGE on a 4–12% gradient-precast gel and transferred onto a PVDF membrane (EMD Millipore). The membrane was incubated with the indicated primary antibody (Supplementary Table S5) followed by HRP-conjugated secondary antibodies against mouse, rabbit, or goat immunoglobulin G. The secondary antibodies were detected with the SuperSignal West Pico Kit (Thermo Fisher Scientific, Rockford, IL, USA).

2.6. Alkaline phosphatase (AP) staining and immunofluorescence analysis

AP staining was performed using the Alkaline Phosphatase Detection Kit (EMD Millipore) according to the manufacturer's instructions. Immunofluorescence analysis was performed as previously described [23]. The primary antibodies used in this study are listed in Supplementary Table S5.

2.7. Microarray analysis

A microarray analysis was performed using the Mouse II Genome 430 2.0 GeneChip arrays (Affymetrix, Santa Clara, CA, USA) essentially as described previously [29]. The experiment was performed in triplicate for mESCs, astrocytes, un-induced cells (*Bmi1*-expressing astrocytes), and iEpiSCLCs. Normalization was calculated with the RMA algorithm26 and implemented in the Bioconductor software (Bioconductor, Seattle, WA, USA).

2.8. Bisulfite genomic sequencing analysis

A Genomic DNA Purification Kit (Promega, Fitchburg, WI, USA) and EpiTect Bisulfite Kit (Qiagen, Hilden, Germany) were used for the isolation and sodium bisulfite conversion of genomic DNA from MEFs, mESCs, un-induced cells, and iEpiSCLCs. Treated DNA was amplified and cloned using the pGEM-T Easy vector (Promega) and sequenced using the T7 forward and SP6 reverse primers. The

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