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Generation of induced pluripotent stem cells without genetic defects by small molecules

Hang-Soo Park [a], Insik Hwang [a], Kyung-Ah Choi [a], Hyesun Jeong [a], Ji-Yun Lee [b], Sunghoi Hong [a, *]

[a] School of Biosystem and Biomedical Science, College of Health Science, Korea University, Jeongneung-dong, Sungbuk-gu, Seoul 136-703, Republic of Korea
[b] Department of Pathology, College of Medicine, Korea University, Anam-dong, Sungbuk-gu, Seoul 136-701, Republic of Korea

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ABSTRACT

The generation of induced pluripotent stem cells (iPSCs) often causes genetic and epigenetic defects, which may limit their clinical applications. Here, we show that reprogramming in the presence of small molecules preserved the genomic stability of iPSCs by inhibiting DNA double-strand breaks (DSBs) and activating Zscan4 gene. Surprisingly, the small molecules protected normal karyotype by facilitating repair of the DSBs that occurred during the early reprogramming process and long-term culture of iPSCs. The stemness and cell growth of iPSCs(+) were normally sustained with high expression of pluripotency genes compared that of iPSCs(−). Moreover, small molecules maintained the differentiation potential of iPSCs(+) for the three germ layers, whereas it was lost in iPSCs(−). Our results demonstrate that the defined small molecules are potent factors for generation of high quality iPSCs with preservation of genomic integrity by facilitating the reprogramming process.

1. Introduction

The therapeutic potential of induced pluripotent stem cells (iPSCs) is very promising for regenerative medicine and cell therapy [1–4]. Similar to embryonic stem cells (ESCs), iPSCs can be differentiated into lineages of all three germ layers, including neurons, cardiac cells, and pancreatic beta cells [5–8]. However, recent evidence shows that iPSCs exhibit aberrant gene expression [9–13] and genomic abnormalities [14–19], which raises concerns about their safety in clinical applications.

While many studies have reported methods to improve the reprogramming efficiency and kinetics of iPSC generation by combinations of defined factors and various small molecules [20–25], the low efficiency and kinetics of iPSC generation and their genomic instability remain to be solved [17,26–29]. In particular, it is important to investigate whether small molecules can maintain genomic stability during *in vitro* reprogramming of somatic cells to iPSCs.

Indeed, several small molecules have been identified that are involved in epigenetic regulating inhibitors, cell-signalling antagonists or agonists, reactive oxygen species (ROS) factors, cell adhesion and survival regulating factors. Since this class of small molecules increased the reprogramming efficiency and kinetics, it is likely that treatment with these compounds would be beneficial to preserve genomic stability during reprogramming.

Recently, it has been reported that Zscan4 (zinc finger and SCAN domain containing 4) is specifically expressed in 2-cell stage embryos and ESCs [30,31], and is involved in the maintenance of genomic stability and the normal karyotype of ESCs [31]. Zscan4 promotes the efficiency and quality of iPSC generation, although it is only transiently activated for the initial few days during the early reprogramming process [32,33]. This finding strongly suggests that activation of Zscan4 may improve the quality of iPSCs during early reprogramming and long-term culture. However, it is not known whether small molecules could activate the endogeneous Zscan4 during *in vitro* reprogramming process and long-term culture.

In the current study, we tested this notion and found that certain small molecules were defined to preserve the normal karyotype by promoting repair of DNA double-strand breaks (DSBs), and significantly increase the expression of Zscan4 gene during *in vitro* reprogramming for iPSC generation. Therefore, we demonstrated that the small molecules are potent modulators in the generation of high quality iPSCs without any genetic defects during the *in vitro* reprogramming process.

* Corresponding author. School of Biosystem and Biomedical Science, College of Health Science, Korea University, San 1, Jeongneung-dong, Seongbuk-ku, Seoul 136-703, Republic of Korea. Tel.: +82 2 940 2816; fax: +82 2 940 2809.
E-mail address: shong21@korea.ac.kr (S. Hong).

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2. Materials and methods

2.1. Retrovirus production, titration and transductions

Retroviral vectors harbouring Oct4, Klf4, Sox2, and cMyc genes (pMXs-hOCT3/4, pMXs-hKLF4, pMXs-hSOX2, and pMXs-hc-MYC, respectively) were purchased from Addgene (Cambridge, MA). The vectors were introduced into the retrovirus packaging cell line 293 GPG by transient transfection with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). At 48 h post-transfection, the supernatants were harvested daily for 2 weeks and stored at −80 °C. To estimate the concentration of functional transducing units (TU/ml), FT-293 cells were transduced with serial dilutions of an aliquot of each vector preparation. After 72 h, cells were harvested, and analysed by a FACSCalibur (BD Biosciences). Based on these results, somatic cells were transduced three times (once a day) with the same final concentration of retroviral vectors (3.3×10^5 TU/ml; MOI = 2) in 500 μl of media in the presence of 4 μg/ml polybrene (Sigma). Media was changed on the next day.

2.2. Generation and culture of mouse iPSCs

To generate mouse iPSCs, 5×10^4 mouse tail tip fibroblasts were seeded in a 60-mm culture dish. The next day, the fibroblasts were infected with retroviruses carrying OKSM genes in the presence of polybrene (4 μg/ml) for 4–5 h at 37 °C with 5% CO_2. At one day post-transduction, the transduced cells were transferred to a well of a 6-well culture dish containing mitomycin C (Sigma, St. Louis, MO)-treated mouse embryonic fibroblasts as feeder cells. The transduced cells were cultured with ESC medium consisting of Dulbeco's modified minimal essential medium (DMEM) containing 10% horse serum (Sigma), 2 mM L-glutamine (GIBCO, Carlsbad, CA), 0.1 mM MEM NEAA (GIBCO), 10 mM β-mercaptoethanol (GIBCO), 500 U/ml LIF (Millipore, Billerica, MA), and penicillin/streptomycin (GIBCO). For small molecule treatments, the ESC medium was supplemented with 2 μM SB431542 (CAYMAN, Ann Arbor, MI), 0.5 μM PD0325901 (CAYMAN), 0.5 μM thiazovivin (Stemgent, Cambridge, MA), 200 μM ascorbic acid (Sigma), 100 μM valproic acid (Stemgent), 5 μM 5-Aza-2′-deoxycytidine (Sigma), and 10 μM CHIR99021 (CAYMAN). The medium was changed every day. ESC-like colonies were picked up at 5–7 days and expanded continuously. Colonies were passaged once every 3–4 days. One large colony was separated into 9–16 smaller colonies.

2.3. Differentiation and teratoma formation of mouse iPSCs

ESCs, iPSCs were differentiated as embryoid bodies (EBs) on nonadherant bacterial dishes for 4 days in EB culture medium consisting of DMEM supplemented with 10% foetal bovine serum (GIBCO), 2 mM L-glutamine, 0.1 mM MEM NEAA, 10 mM β-mercaptoethanol, and penicillin/streptomycin. The EBs were transferred to gelatin-coated dishes. After 24 h in culture, differentiation of EBs was initiated by replacing the medium with serum-free ITS medium for another 10 days, and the differentiated cells were characterized with markers and genes for three-germ layers. For teratoma formation, 1×10^6 iPSCs were gently mixed on ice with BD Matrigel™ Basement Membrane Matrix (BD Biosciences, San Jose, CA). The cells were then subcutaneously injected into immune-deficient BALB/c nu mice (Japan SLC, Hamamatsu, Japan). At 8–10 weeks post-injection, the teratomas were dissected out, rinsed once with 1 × PBS, and then fixed with 4% paraformaldehyde in 1 × PBS. The fixed teratomas were embedded in paraffin, sectioned, mounted on slides, and subjected to H&E staining.

2.4. AP staining and immunofluorescence

An Alkaline Phosphatase Staining Kit (Stemgent) was used for AP staining according to the manufacturer's instructions. For immunofluorescence, the cells were washed with 1× PBS, fixed with 4% paraformaldehyde in 1× PBS, and then incubated at 4 °C overnight with primary antibodies against the following markers. ESC markers Oct4 (1:200; Abcam, Cambridge, MA), Sox2 (1:300; Millipore), Nanog (1:1000; Millipore), and SSEA1 (1:300; Santa Cruz, Dallas, TX), ectodermal marker Tuj1 (1:1000; Covance, Princeton, NJ), endodermal marker AFP (1:100; R&D Systems, Minneapolis, MN), mesodermal marker desmin (1:100; Thermo, Marietta, OH), and DNA damage marker γH2AX (1:500; Millipore). Alexa Fluor 488 and 594-conjugated secondary antibodies (1:1000; Invitrogen) were used for visualization. Nuclei were counterstained with DAPI (1:10000).

2.5. RT-PCR and real-time RT-PCR

Total RNA was prepared using TRI reagent (MRS, Cincinnati, OH) following the manufacturer's instructions. Three micrograms of total RNA was reverse transcribed to cDNA using M-MLV reverse transcriptase (Roche, Penzberg, Upper Bavaria) and oligo(dT) primers. The cDNA was subjected to PCR in a 2720 thermal cycler (Applied Biosystems, Carlsbad, CA). PCR conditions were 5 min at 94 °C and then 25–30 cycles of 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C, followed by 5 min at 72 °C. Real-time RT-PCR was performed with the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA) with KAPA SYBR FAST ABI prism qPCR kit reagents (KAPA Biosystems, Woburn, MA). . PCR primers are listed in Suppl. Table 1. PCR products were electrophoresed on 1% agarose gels containing ethidium bromide.

2.6. PI staining and flow cytometric analysis

For cell cycle analysis, the cells were detached with trypsin-EDTA and collected by centrifugation. Approximately 1×10^6 cells were fixed and permeabilized with 70% ethanol at 4 °C. The fixed cells were treated with 2.0 mg/ml RNase A at 37 °C for 1 h. The cells were then treated with 50 μg/ml PI and analysed by a FACSCalibur (BD Biosciences).

2.7. Bisulphite genomic DNA sequencing

Genomic DNA was isolated from fibroblasts, ESCs, and iPSC lines using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). Bisulphite modification of 1 μg genomic DNA was performed with a CpGenome DNA modification kit (Millipore) following the manufacturer's instructions. Bisulphite-modified DNA was amplified by PCR in the promoter region of the NANOG gene using the following primers (F: 5′-GAT TTT GTA GGT GGG ATT AAT TGT GAA TTT -3′, R: 5′-ACC AAA AAA ACC CAC ACT CAT ATC AAT ATA-3′). PCR products were electrophoresed on 1% TAE-agarose gels and then extracted using a NecleoSpin® Gel and PCR Clean-up Kit (Macherey–Nagel, Düren, Germany). Extracted PCR products were cloned using a CloneJET™ PCR cloning kit (Thermo, Marietta, OH). Among transformed DH5α cell colonies, eight clones were randomly picked up and cultured in LB medium containing ampicillin. Plasmids were isolated using a Plus Plasmid Mini Kit (Nucleogen, GyengGi-do, Korea), sequenced, and analysed with BIQ analyzer software (Max Planck Institute, Saarbrucken, Germany).

2.8. Chromosomal analysis

iPSCs were cultured in ESC medium with or without small molecules. The cultured cells were arrested at metaphase by addition of 0.02 μg/ml colcemid (Biological Industries, Kibbutz Beit Haemek, Israel) to the culture medium for 1 h. The cells were then washed with 1× PBS and collected by trypsin-EDTA treatment. A hypotonic solution (0.8% sodium citrate) was added to the cells, followed by incubation for 30 min at 37 °C. The cells were fixed with Carnoy's fixative (3:1 methanol:glacial acetic acid) and then spread onto glass slides. The chromosomes were stained by conventional G banding. Twenty cells for each cell line were analysed and karyotyped with the CytoVision® system (Leica Biosystems). Mouse fibroblasts and J1 mouse ESCs were used as controls.

2.9. Western blot analysis

The cultured mouse fibroblasts and iPSCs were harvested and lysed with RIPA buffer (Thermo, Marietta, OH) with the addition of complete mini protease inhibitor cocktail (Roche, Penzberg, Upper Bavaria). Lysates were centrifuged for 10 min at 15,000 rpm at 4° C, and the supernatant was collected in a new tube. After adding an appropriate amount of 6X SDS-PAGE sample buffer, the cell extracts were incubated in a boiling water bath for 5 min. The samples were separated on a 12% SDS acrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked in 5% skimmed milk in PBST with 0.1% Tween 20 for 1 h and then incubated with a specific antibody in the same buffer in order to detect each protein. The anti-Rad50 (Milipore, Billerica, MA), anti-Rad51 (Santa Cruz, Dallas, TX), anti-gammaH2AX (Milipore, Billerica, MA), anti-actin antibodies (Santa Cruz, Dallas, TX) were used at a dilution of 1:500 in order to detect the proteins. HRP conjugated secondary antibodies (Thermo, Marietta, OH) were used at a dilution of 1:10000. The labelled proteins were detected by Clarity Western ECL Substrate (Bio-Rad, Hercules, CA).

2.10. Cell counting

Cell numbers were determined by counting 700–1000 cells per field at ×100 magnification under an EVOS FL fluorescence microscope (AMG, Bothell, WA) or confocal microscope (Olympus, Tokyo, Japan) using TissueQuest software (TissueGnostics, Vienna, Austria). Three visual fields were randomly selected and counted for each sample.

2.11. Statistical analysis

Results are presented as the mean ± S.D. Statistically significant differences were calculated using independent and paired Student's *t*-tests for unpaired and paired samples. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Selection of small molecules for the generation of iPSCs without genetic defects

To select the small molecules that would be beneficial for the generation of iPSCs preserving genomic integrity without DNA damage, mouse embryonic and tail tip fibroblasts were prepared and transduced with retroviruses carrying Oct4, Klf4, Sox2, and c-Myc genes (OKSM). We tested the inhibitors of various cell