Highly Efficient Reprogramming and Characterization of Induced Pluripotent Stem Cells by Using a Microwell Array

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Reprogramming of human somatic cells to induced pluripotent stem cells (iPSCs) enables the possibility of generating patient-specific cells. However, the low efficiency issue associated with iPSCs generation has limited iPSCs usage in research and clinical applications. In this study, we developed a high efficiency system to generate iPSCs by using a polydimethylsiloxane stencil. This device could be applied to the localization and reprogramming of human fibroblasts. Herein, a well-defined culture system based on a stencil, which supported efficient reprogramming of fibroblasts into iPSCs with 2–4 fold increase in efficacy over conventional methods, is presented. Subsequently, we prepared a multiple analysis system, which used a micro-patterned scissile microarray to characterize iPSCs. The results showed that iPSCs could be cultured into micro-patterns in a precisely controlled manner on the scissile poly(ethylene terephthalate) sheet, which was cut into pieces for subsequent analyses, indicating that this method allows multiple analyses to establish iPSC pluripotency in the same sample. Our approach provides a simple, cost-effective, but highly efficient system for the generation and characterization of iPSCs, and will serve as a powerful tool for establishing patient- and disease-specific pluripotent stem cells.

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INTRODUCTION

In 2006, Takahashi et al. [1,2] directly reprogrammed somatic cells into induced pluripotent stem cells (iPSCs) that resemble embryonic stem cells (ESCs) in morphology, gene expression, epigenetic status, and *in vitro* differentiation. Like ESCs, iPSCs present a potential for therapies, as disease models, or in drug screening [3–6]. iPSCs present clear advantages. They can be produced from adult cells, avoiding the controversial need for a human embryo, and they can be derived from people with

diseases to create models or even therapies based on a specific individual's genetic make-up. iPSCs generated using the retroviral transduction protocols described by the Yamanaka group contain transgenes that are known as 4-defined factors (Oct3/4, Sox2, Klf4, and c-Myc) [1,2]. However, the reprogramming process is time-consuming and highly inefficient, as only 0.002% of cells are successfully reprogrammed [1]. To improve the reprogramming efficiency, several studies reported iPSC derivation by using reprogramming enhancing molecules [7,8]. Of note, many of these molecules have pleiotropic effects that could result in transient or permanent epigenetic or genetic alterations [9,10]. Thus, an efficient reprogramming method without these alterations is needed to achieve the widespread derivation of disease-specific iPSCs from humans with inherited or degenerative diseases.

A micro-patterned cell culture system can deliver cell patterns with well-controlled sizes, shapes, and positions in high uniformity, providing a useful tool for *in vitro* cell research [11,12]. Various microfabrication technologies such as microcontact

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printing [13], inkjet printing [14], photolithography [15] and stencilling [16,17] have been adopted to localize the cell in controlled manner. The stencilling uses a thin membrane with perforated patterns as cells are locally cultured through the perforated patterns of the stencil.

The goal of our study was to develop a micro-patterning system for efficient iPSCs derivation and subsequent characterization. To this end, we used a polydimethylsiloxane (PDMS) stencil for human skin fibroblast reprogramming. Considering that the thick stencil can provide physical space, similar to microwell array, to contain fluid such as reprogramming factors, this approach allowed us to efficiently introduce the 4-defined reprogramming factors into fibroblasts. In addition, we applied the PDMS stencil with the scissile poly(ethylene terephthalate) (PET) sheet for the characterization of iPSCs, which enables quick and efficient multi-analysis of a sample. Our results indicate that cell patterning by using a PDMS stencil could be useful in the generation and characterization of iPSCs, without the need for purchasing expensive equipment, and that these cells could be useful tools for generating disease-specific iPSCs, understanding the pathogenesis of a disease, and testing patient-specific therapies.

MATERIALS AND METHODS

Master mold fabrication

The overall size of the stencil was 37×37 mm in order to fit into a 60 mm culture dish. The size of perforated patterns was 5 mm in diameter. The stencil was built by PDMS (Sylgard 184, Dow Corning, Midland, MI, USA) elastomer casting from a microfabricated master mold or by direct perforation of PDMS films by biopsy punches.

The microfabricated master mold was fabricated by photolithography using Norland optical adhesives 63 (NOA 63, Norland Company, Cranbury, NJ, USA). Photomasks of the master mold was designed using AutoCAD (Autodesk, USA). A modified procedure of thick mold preparation was adopted from the previous report [18]. Two slide glasses were placed as spacers on a glass substrate in order to provide space for the photopolymer solution. The surface of the glass substrate was modified into hydrophilic and cleaned by using an oxygen plasma treatment (HARRICK PLASMA, USA). PET sheet and cover glass were placed on top of the spacers. Only surface of glass substrate was plasma treated in order to assure the attachment of the NOA 63 pattern on to the glass substrate. The space between the spacers was filled with the photopolymer solution by the capillary effect. The thickness of the spacer was 2 mm. A photomask was placed on cover glass, and the photopolymer solution was cured by UV exposure. After exposure, non-

modified photopolymer was removed by washing with acetone. Nitrogen gas was used to dry the mold after washing.

Fabrication of PDMS ultra-thick stencil

PDMS elastomer was replicated from the master mold of NOA 63 by casting with the gas blowing [19]. PDMS prepolymer was prepared by mixing Sylgard 184 silicone elastomer base and curing agent at a 10:1 ratio (Sylgard 184 kit, Dow Corning, Midland, MI, USA). The mixture was degassed in vacuum desiccators for 60 minutes in order to remove bubbles created during mixing and then poured onto the NOA 63 master mold. The poured PDMS prepolymer was swept with a doctor blade, set at a gap height of 3 mm, and was spread on the master glass. After spreading, the residual layer of PDMS prepolymer sitting on the top of master mold structures could be removed by gas flow of nitrogen injected through the air knife system resulting in perforated patterns of stencil. The PDMS prepolymer was then cured in an oven at 65°C for 60 minutes. Finally, the PDMS ultra-thick stencil replica was released from the NOA 63 master mold.

Production and infection of vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors

To establish iPSCs, VSV-G-pseudotyped retroviral supernatants were obtained from a 293 GPG system by using reported procedures [20]. Virus-containing medium was collected, filtered, and concentrated by ultracentrifugation at 50000 g for 2 hours and resuspended in phosphate-buffered saline (PBS, Life Technologies, Carlsbad, CA, USA). Viral titers were measured by serial dilution on 293T cells followed by flow cytometry analysis after 48 hours. The viral titer used ranged between $2-5 \times 10^9$ plaque forming units per milliliter.

Cell isolation and culture

Human skin fibroblasts (GM05399, Coriell Institute, Camden, NJ, USA) from healthy males were cultured in standard culture medium containing DMEM supplemented with 100 IU/mL penicillin-streptomycin and 10% fetal bovine serum (DMEM-FBS, Life Technologies, Waltham, MA, USA) and maintained at 37°C in a 5% CO₂ incubator. Cells were allowed to expand to 80–90% confluence before passaging with 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA, Life Technologies, Waltham, MA, USA).

For mouse embryonic fibroblasts (MEFs) isolation, we used day 13.5 embryos. After the removal of the head, visceral tissues, and gonads, the remaining bodies were washed and dissociated with 0.25% trypsin-EDTA (Sigma-Aldrich, Saint Louis, MI, USA). Ten-million cells were plated on each gelatin-coated 100-mm dish and incubated at 37°C with 5% CO₂. The next day,

floating cells were removed by washing with PBS. In this study, MEFs were used within passage 4 to avoid replicative senescence.

Established iPSCs and ESCs were maintained on mitomycin C-treated MEF in complete ES medium composed of DMEM (Sigma-Aldrich) supplemented with 20% knockout serum replacement, 5 ng/mL recombinant human basic FGF (Peprotech, Rocky Hill, NJ, USA), 20 mM HEPES buffer (pH 7.3), 0.1 mM 2-mercaptoethanol, 0.1 mM non-essential amino acids, 2 mM L-glutamine, and 100 U/mL penicillin/streptomycin (All chemicals were from Life Technologies unless otherwise stated).

Reprogramming of human skin fibroblasts

iPSCs were established from the human skin fibroblasts as previously described [21], with slight modifications. Briefly, fibroblasts were seeded at 3×10^5 cells in 60 mm dishes or 2×10^3 cells per well in microstencil coated with gelatin. On day 1, the VSV-G pseudotyped retroviral vector system carrying OCT3/4, SOX2, KLF4, and c-Myc was added to fibroblast cultures. On day 2, cells were subjected to the same transduction procedures and harvested 24 hours later. Transduced cells were re-plated on MEF layers in 60 mm or 100 mm culture dishes containing the fibroblast medium. The next day, the medium was changed to complete ES medium with 0.5 mM valproic acid (Sigma-Aldrich) and changed every other day thereafter. After 21 days, ES-like colonies appeared and were picked up to be reseeded on new MEF feeder cells. Cloned ES-like colonies were subjected to further analysis.

Preparation of scissile microarray

A scissile microarray was prepared by combining the stencil and a flexible polymer sheet as a culture plate. Two hundred and fifty micrometer-thick PET sheet was used as a culture plate (culture sheet). In order to make the culture sheet easily scissile, the polymer sheet was mechanically perforated in dashed lines shape by using a rotary cutter. The prepared PDMS stencil was placed on the culture sheet and cells were then cultured on them. After cell culture, the stencil was removed from the PET culture sheet leaving cell arrays on the PET sheet. The PET sheet was cut into parts. Each part was then processed further for cell analysis. The flexible and scissile microarray allowed us to separate the arrays of cells that are prepared in the common culture conditions for various subsequent processes for biological analysis.

Alkaline phosphatase and immunocytochemical staining

AP staining was performed using an ES-alkaline phosphatase detection kit (Chemicon, Temecula, CA, USA) according to the

manufacturer's recommendations. For the immunocytochemical detection of NANOG, cells were fixed with 4% paraformaldehyde in PBS for 15 minutes, washed with PBS, and incubated with 0.5% Triton-X 100 (Sigma-Aldrich) in PBS for 10 minutes. Cells were incubated with an anti-NANOG (rabbit, 1:1000, Abcam, Cambridge, UK, ab21624) in 1% PBS-BSA overnight at 4°C, followed by incubation with a secondary biotin-conjugated anti-rabbit antibody (Vector Laboratories, Burlingame, CA, USA, 1:2000) for an additional 2 hours at room temperature. Cells were then incubated with streptavidin-HRP (Vector Laboratories, 1:1000) and a DAB substrate kit for peroxidase (SK-4100, Vector Laboratories) was used to develop the staining. Immunofluorescence staining was performed according to previously described procedures [22]. The following antibodies were used: anti-SSEA4, TRA-1-60, and TRA-1-81 (mouse, 1:100, Chemicon, MAB4304, MAB4360, and MAB4381). The cells were analyzed with a laser scanning confocal microscope equipped with Fluoview SV1000 imaging software (Olympus FV1000, Olympus, Tokyo, Japan) or with an Olympus BX51 microscope (Olympus).

Quantitative real-time PCR

The RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) was used for RNA extraction from cell lysates. cDNA was synthesized from 5 µg of total RNA by using a commercially available kit from Clontech (Mountain View, CA, USA). Quantitative real-time PCR was performed using a Corbett research RG-6000 real-time PCR instrument and a one-step program: 95°C, 10 minutes; 95°C, 10 seconds; 58°C, 15 seconds; and 72°C, 20 seconds for 40 cycles. The primers used are described in Supplementary Table 1 (in the online-only Data Supplement).

Karyotyping

Karyotype analysis was conducted using standard chromosome analysis protocols. Briefly, iPSCs were cultured with daily change of medium and were treated with colcemid solution before collection. After hypotonic treatment, the cell suspension was fixed, placed on slides, and stained with Giemsa solution.

Teratoma formation and histological analysis

Established iPSCs were prepared at 1×10^7 cells/mL in PBS. Suspended cells ($1-3 \times 10^6$) were injected into the testes of anesthetized male SCID Beige mice (Charles River Laboratories, Wilmington, MA, USA). Eight weeks after transplantation, mice were euthanized with Avertin (250 mg/kg ip) and tumors were dissected. Tumor samples were fixed in 10% formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin. Mouse studies were approved by the Kyungpook National University Institutional Animal Care and Use Committee.

Statistical analysis

More than two groups were compared to each other. A one way ANOVA was used, followed by a Tukey's HSD test. All statistical analyses were performed using SPSS statistical software (IBM, Armonk, NY, USA). $p < 0.05$ was considered significant.

RESULTS

Fabrication of the PDMS stencil

The stencil was fabricated by spin-coating PDMS prepolymer onto a master mold, followed by blowing nitrogen gas onto the master mold surface to remove thin residual layers of PDMS from the top of the master mold patterns (Fig. 1A). The pattern of the PDMS stencil was a circle with a diameter of 5 mm. Finally, the PDMS stencil was released from the master mold (Fig. 1B). Figure 2A (see bottom box) shows the image of the released PDMS stencil with a well diameter of 5 mm in the 60 mm culture dish.

Highly efficient reprogramming of human skin fibroblasts by using the PDMS stencil

Our initial efforts focused on developing a simplified approach for the efficient derivation of iPSCs from human skin fibroblasts by using a PDMS stencil. To assess the potential ad-

vantages of this approach, the PDMS stencil method for reprogramming to pluripotency was compared with the conventional method. Fibroblasts were seeded at 3×10^5 cells in 60 mm dishes or 2×10^3 cells per well and transduced with retroviral vectors encoding the four Yamanaka factors (OCT3/4, SOX2, KLF4, and c-Myc) as follows: 20 μ L of each retrovirus on conventional dish; 0.133, 0.665, or 1.33 μ L of each retrovirus on well (Fig. 2A). The designation "1X(0.133 μ L/well)" indicates the same number of virus particles per cell when compared to the conventional method. Figure 2B depicts the experimental process used to generate iPSCs by using the PDMS stencil. One day before transduction, fibroblasts were seeded in each well of the PDMS stencil. On day 0, various concentrations of 4-defined retroviral vectors were added to each well. On day 2, the PDMS stencil was removed to provide enough space for proliferation of the transduced fibroblasts. After 4 days, transduced cells were re-plated on inactivated MEF feeder layers in 60 mm plate. Finally, 21 days following the initial infection, we either fixed/stained or manually picked the iPSC colonies obtained for further culture and characterization. Reprogramming efficiency was defined as the number of AP positive colonies per 30000 transduced cells seeded on MEFs. Compared to the control, reprogramming efficiencies were increased by using the PDMS stencil with the same number of vector particles per cell (1X; 0.133 μ L/well; Fig. 3A). The highest efficiency

Figure 1. (A) Schematic process for stencil fabrication. (B) Photographic image of the fabricated mold and stencil. NOA 63: Norland optical adhesives, PDMS: polydimethylsiloxane.

was observed when transduced with 5X(0.665 $\mu\text{L}/\text{well}$) of 4-defined retroviral vectors. Similar reprogramming experiments performed using NANOG immunostaining to evaluate

pluripotent colony formation showed comparable results (Fig. 3B). The conventional culture plate-based reprogramming process required large numbers of fibroblasts and retroviral

Figure 2. (A) Schematic experimental strategy used to reprogram human skin fibroblasts. Fibroblasts were seeded on a conventional culture dish and PDMS stencil and transduced with the four Yamanaka factors. (B) Schematic diagram of the reprogramming protocol used. The plated fibroblasts were transduced with retroviral vectors and re-plated into 60 mm dishes containing the feeder layer cells. The transduced cells were cultured for 2–3 weeks. The medium was replaced every 2 days. PDMS: polydimethylsiloxane, MEF: mouse embryonic fibroblast, ESC: embryonic stem cell.

vector preparations (3×10^5 cells transduced with 20 μL of each viral suspension in 60 mm culture dish). However, with the PDMS stencil method, the reprogramming process required only small amounts of cells and vectors and the iPSCs generation efficiency was increased compared to that of the conventional method. This may be because wells provide spatial control of cells and 4-defined viral vectors. Accordingly, this condition may enable the even distribution and transduction of 4-de-

fined viral vectors to the cells.

Characterization of iPSC line by using the micro-patterned scissile microarray

Our intent was also to develop a simple analysis system by using the microwell array to facilitate characterization of iPSCs. The conventional analysis system requires many experimental steps, a large number of iPSCs, and large amounts of reagents to

Figure 3. Highly efficient human skin fibroblasts reprogramming by using the PDMS stencil. The emergence of colonies was monitored and pluripotency marker expression was analyzed. AP (A) and NANOG (B) expression of reprogrammed cells cultured in a 60 mm dish, at day 21. The reprogramming efficiency using the stencil culture device with different amounts of transduced retroviral vectors ($n=3-4$ per group). * $p<0.05$, ** $p<0.01$. PDMS: polydimethylsiloxane, AP: alkaline phosphatase.

evaluate pluripotency, which limits high-throughput analysis. A microwell array-based method for characterization of iPSCs was developed to solve these problems. Thus, in this study, we used an inexpensive and simple micro-patterned scissile mi-

croarray as a high-throughput screening tool for the assessment of the pluripotent status of iPSCs that were established using stencil. A scissile microarray was prepared by combining the stencil and a scissile PET sheet as a culture plate (Fig. 4A). On

Figure 4. Characterization of iPSC line by using a micro-patterned scissile microarray. (A) A schematic illustration of the micro-patterned scissile microarray. A schematic process and timeline for characterization of iPSCs by using the micro-patterned scissile microarray. (B) Established iPSCs showed ESC-like morphology (Phase) and expressed pluripotent stem cell markers AP, SSEA-4, TRA-1-60 and TRA-1-81. Scale bar: 200 μ m. PDMS: polydimethylsiloxane, PET: poly(ethylene terephthalate), AP: alkaline phosphatase, iPSCs: induced pluripotent stem cells, ESC: embryonic stem cell.

day 0, the micro-patterned scissile microarray was coated with 0.1% gelatin. After 1 day of incubation, MEFs were seeded on the gelatin-coated microarray. On day 2, iPSCs were seeded on cell layers of mitomycin C-treated MEFs. On day 5, stencils were peeled-off and PET sheets, on which iPSCs were cultured, were cut for multiple analyses. As shown in Figure 4B, iPSCs cultured on PET sheet revealed similar morphology to ESCs and expressed pluripotent markers, including AP, SSEA-4, Tra-1-60, and Tra-1-81. These analyses can be achieved using the iPSCs cultured in a micro-patterned scissile microarray. These results indicate that the micro-patterned microarray allowed the acquisition of iPSCs into separated micro-patterns. Additionally, the scissile PET sheets allowed staining with different markers to assess the pluripotency of iPSCs that were cultured from the same environment. The isolated cell patterning and the separate analyses of the cell patterns allowed a more efficient analysis from a small quantity of cells, which is important when us-

ing rare samples.

To further assess whether iPSCs had been reprogrammed to pluripotency, we analyzed several pluripotency markers at the mRNA level by quantitative real-time PCR (Fig. 5A). The expression of several stem cell markers such as ZFP42, NANOG, DNMT3B, and DPPA4 was indistinguishable from human ESCs. In addition, karyotype analysis revealed that iPSCs retained a normal chromosomal integrity (Fig. 5B). We also tested the developmental potential of the generated iPSCs. The pluripotency of iPSCs was assessed by differentiation into the three embryonic germ layers *in vivo*, by teratoma formation. Injection of iPSCs into SCID mice resulted in the formation of teratomas containing tissues derived from all three embryonic germ layers (Fig. 5C). Together, these data demonstrate that iPSCs, generated using a scissile microarray, were morphologically, phenotypically, and functionally identical to pluripotent stem cells.

Figure 5. Characterization of iPSCs. (A) Quantitative real-time PCR assays for ESC-specific gene ZFP42, NANOG, DNMT3B, and DPPA4 indicates an increased expression in iPSCs relative to the parent (n=4 per group). (B) Normal karyotype of iPSCs. (C) *In vivo* differentiation assay by teratoma formation. Representative series of hematoxylin-eosin stained sections from a formalin fixed teratoma produced from iPSCs. They formed mature, cystic teratomas with tissues representing all three embryonic germ layers including: columnar epithelium (endoderm), cartilage (mesoderm), and pigmented epithelium (ectoderm). Scale bar: 50 μ m. *** p <0.001. ESCs: embryonic stem cells, iPSCs: induced pluripotent stem cells.

DISCUSSION

iPSC technology demonstrates that differentiated cells can be dedifferentiated to a pluripotent state by introducing only a few genes [1,2]. However, protocols for generating iPSCs are inefficient and slow. Since the first report by Takahashi et al. [1,2] describing iPSC derivation, on the focus has been on improving the efficiency of iPSC generation. In this study, we devised a simple, low-cost, and highly efficient method using a PDMS stencil, which supports robust iPSC derivation and characterization. Our results show that human fibroblast cultured in stencil based culture system can be reprogrammed to pluripotency with 5-fold higher efficiency compared with the conventional method. This might be because stencil provide an even distribution and transduction of 4-defined viral vectors to the fibroblasts. Furthermore, we showed that the stencil based culture system can also be used to characterize established iPSCs. Micro-patterned scissile microarray allowed the separation of iPSCs in micro-patterns and the scissile PET sheets allowed staining for different pluripotency markers. Thus, it is conceivable that the isolated cell patterning and the separate analyses of the cell patterns allow a more efficient multi-analysis using small quantities of cells and reagents.

Our stencil based culture system provides several important advantages compared with other conventional approaches: 1) By using the stencil, the reprogramming process required small amounts of cells and retroviral vectors, which is more important when using rare samples. 2) With the optimized reprogramming conditions, our approach provides a robust reprogramming efficiency of the fibroblasts compared to the conventional method, without the use of reprogramming enhancing molecules [7,8] that could induce epigenetic and genetic alterations. 3) Our system also provides a multiple analysis tool with micro-patterned scissile PET sheet to efficiently characterize human iPSCs. This method allowed us to observe the expression of different pluripotency markers in a single iPSCs culture. This technique presents a strong potential for a broad range of biological applications and provides a very valuable solution for assays requiring cells that are difficult to grow in large amounts.

Taken together, this newly introduced stencil based culture system is a simple, effective, and cheap method for the generation and characterization of iPSCs. Therefore, this approach represents a potentially important step toward the derivation of disease-specific iPSCs for the modeling of “diseases in a dish,” screening new drug compounds, and developing new therapies.

Our results demonstrate that a stencil can support the efficient reprogramming of human skin fibroblasts as well as their characterization with multiple analysis system in a single iPSCs culture. We believe that the optimized stencil culture system

provides a robust and cost-effective platform for the generation of human iPSCs for both research purposes and clinical applications.

Supplementary Materials

The online-only Data Supplement is available with this article at <https://doi.org/10.1007/s13770-016-0015-0>.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

There are no animal experiments carried out for this article.

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