Induction of Stem Cell Like Cells from Mouse Embryonic Fibroblast by Short-Term Shear Stress and Vitamin C

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Abstract: Induced pluripotent stem cells (iPSCs) are a good medicine source because of their potential to differentiate into various tissues or cells. However, traditionally, iPSCs made by specific transgenes and virus vectors are not appropriate for clinical use because of safety concerns and risk of tumor development. The goal of this research was to develop an alternative method for reprogramming, using small molecules and external stimuli. Two groups were established: short-term shear stress (STSS) under suspension culture and a combination of short-term shear stress and vitamin C (SSVC) under suspension culture. For STSS, the pipetting was carried out for cells twice per day for 2 min for 14 days in the embryonic stem cell (ES) medium. In the case of SSVC, the procedure was the same as for STSS however, its ES medium included 10 μM of vitamin C. After 14 days, all spheroids were picked and checked for pluripotency by ALP (alkaline phosphatase) assay and immunocytochemistry. Both groups partially showed the characteristics of stem cells but data demonstrated that the spheroids under shear stress and vitamin C had improved stem cell-like properties. This research showed the possibility of external stimuli and small molecules to reprogram the somatic cells without the use of transgenes.

Keywords: mouse embryonic fibroblast; reprogramming; sphere formation; shear stress; vitamin C

1. Introduction

In 1957, Waddington suggested the concept of an “epigenetic landscape” which is now involved in reprogramming [1]. In 1962, Gurdon replaced the UV-treated nucleus of a frog egg with a differentiated nucleus which proved that cells could be reprogrammed [2]. Nearly 45 years after Gurdon’s report, Yamanaka first generated induced pluripotent stem cells (iPSCs) from mouse and human somatic cells using Oct4, Sox2, Klf4, and c-Myc transgenes (OSKM) [3,4]. Developing iPSCs was a ground-breaking event in cancer therapeutic research and the field of regenerative medicine because of the recapitulated pluripotency with potential to differentiate into various cells or tissues [5,6]. However, low efficiency of iPSC generation and safety issues involved with the use of viruses or transgenes poses limitations for clinical translation [6]. Thus, many researchers have worked to improve the efficiency and to develop low-risk methods for generating iPSCs
such as using specific proteins, small molecules, and virus-free carriers [7–14]. Some researchers have applied external stimuli to induce iPSCs. Low-frequency electromagnetic fields could support the generation of iPSCs from somatic cells [15]. A graphene-based substrate resulted in increasing the efficiency of iPSCs by promoting the mesenchymal to epithelial transition (MET) process [16]. It also has been reported that shear stress maintains and strengthens the stemness of stem cells [17]. As a small molecule, vitamin C improved iPSC generation from mouse and human somatic cells with transgenes [18]. Vitamin C treatment downregulated the genes involved in differentiation while upregulating the ones related to pluripotency. It was indicated that vitamin C inhibited mESC differentiation and maintained pluripotency [19].

In this study, we induced stem cell-like cells from mouse embryonic fibroblasts (MEF) using external stimuli such as short-term shear stress (STSS) or a combination of short-term shear stress and vitamin C (SSVC) under suspension culture without using any transgene. Figure 1 showed the schematic diagram of the methods. For short-term shear stress, we chose pipetting of culture media. To induce pluripotent stem cells, this method has never been used before. After generating shear stress and treatment with vitamin C to MEF, we checked pluripotency by ALP (alkaline phosphatase) assay, immunocytochemistry, and Western blotting. Finally, we checked the differentiation ability of spheroids with induced pluripotency.

![Figure 1. The schematic diagram for induction method of pluripotent stem cell-like cells. (a) Short-term shear stress method (STSS). (b) Short-term shear stress and vitamin C method (SSVC).](image)

2. Materials and Methods

2.1. MEF Isolation and Cell Culture

Pregnant mice of strain ICR (E13.5) were dissected and embryos were separated by uterine horns for isolating MEF (SNU-120409-3-1). The embryos were washed with 70% ethanol (Duksan, Welgene, Korea) and Dulbecco’s phosphate-buffered saline (DPBS) without Ca$^{2+}$ and Mg$^{2+}$ (Welgene, Kyunggi, Korea). After removing the head and rear organs, the remnants of the embryos were minced by a sterile razor blade. Chopped remnants were treated with 0.05% trypsin-EDTA (Welgene, Korea) including 100 units of DNase (Promega, Madison, WI, USA) and incubated for 15 min at 37 °C. Transferred into MEF medium, comprised of Dulbecco’s Modified Eagle’s Medium (DMEM) (Welgene, Korea), 10% fetal bovine serum (FBS) (Gibco, Gaithersburg, MD, USA), 10 μM L-glutamine (Sigma-aldrich, St. Louis, MO, USA), 1% antibiotic antymycotic solution, and 1% non-essential amino acids solution (NEAA) (Gibco, USA), for inactivating trypsin-EDTA. The solution including MEF was centrifuged. The supernatant was removed and transferred
to a 0.2% gelatin (Sigma-aldrich, USA) coated T-75 flask with fresh MEF medium at 37 °C and in 5% CO₂. The fibroblasts of passage 0 were split until passage 2.

2.2. Shear Stress and Vitamin C Treatment

MEF cells of passage 2 were seeded at an initial cell density of 1 × 10⁶ in a 60 mm non-coated petri dish (SPL, Gyeonggi-do, Korea) or at 4 × 10⁶ in a 150 mm petri dish (SPL, Korea) at D0. The cells were cultured in the embryonic stem cell (ES) medium. The cells were stimulated with 1 mL pipet (Eppendorf, Hamburg, Germany) and 1 ml pipet tips (Axygen, Union City, CA, USA) for 2 min (~55–60 times) twice a day for 14 days in dark room. A time gap of minimum 5 h was maintained between the first and the second stimulation. After 14 days of incubation, the spheroids were picked for subsequent experiments. In the SSVC method, a similar procedure was performed except that the ES medium additionally included 10 µM of L-ascorbic acid (Sigma-aldrich, USA). After the addition of vitamin C to ES medium, cells were subjected to shear stress in a dark room continually for 14 days.

2.3. Alkaline Phosphatase (ALP) Staining Assay

ALP staining was performed using Alkaline Phosphatase Blue Membrane Substrate Solution (Sigma-aldrich, USA) according to the manufacturer’s instructions. The MEF spheroids were observed by Nikon ECLIPSE Ti with DS- U3 (Nikon, Tokyo, Japan) and NIS-Elements AR. 4.20 program.

2.4. Immunocytochemistry (ICC)

MEF spheroids were picked at D15 and transferred onto a 4-well plate (Nunc, Rochester, NY, USA) coated with hESC-qualified matrigel (BD Biosciences, San Jose, CA, USA) at 37 °C for 2 h. Spheroids attached on a 4-well plate were fixed with 4% paraformaldehyde (Sigma-Aldrich, USA) for 15 min, and then washed twice with DPBS. The cells in spheroids were permeabilized with DPBS including 0.2% Tween 20 (Amresco, Solon, OH, USA) for 10 min at room temperature. After washing twice with DPBS, the spheroids were incubated with 10% bovine serum albumin (BSA) (Bio basic Canada Inc., Markham, ON, Canada) for 1 h at room temperature for blocking nonspecific binding. MEF spheroids were then incubated with each of the primary antibodies diluted in 10% BSA solution at 4 °C overnight. The primary antibodies used at 1:20 dilution were anti-Oct4 (Santa Cruz biotechnology, Santa Cruz, CA, USA), and anti-SSEA-1 (Abcam, Cambridge, MA, USA), and those used at 1:200 dilutions were anti-Neuronal Class III b-Tubulin (TUJ1) (Covance, Princeton, NJ, USA), anti-Sarcomeric Alpha Actinin (Abcam, USA) and anti-alpha 1 fetoprotein (NOVUS Biologicals, Salem, MA, USA). The spheroids were rinsed twice with DPBS and incubated with secondary antibodies diluted in 10% BSA solution at room temperature for 2 h. The secondary antibodies of Alexa Fluor 488 goat anti-mouse IgG (Life Technologies, Carlsbad, CA, USA) or Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies, USA) used for pluripotent markers were diluted at 1:50 and those for differentiation markers at 1:400. The MEF spheroids were then washed twice with DPBS and 2 mg/mL of 4’,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, USA) was added for nuclear staining. After 10 min of incubation at room temperature and washing once with DPBS, the spheroids were placed on a slide glass and mounted using aqua poly/mount (Polysciences Inc., Warrington, PA, USA). Images of Oct4 and SSEA-1 stained spheroids were acquired by LSM710 (Carl Zeiss, Oberkochen, Germany) with Zen 2009 LE, and images of three germ layers stained in vitro were obtained by an ECLIPSE Ti (Nikon, Japan) with Zyla sCMOS (Andor Technology, Belfast, UK) camera and NIS-Elements AR. 4.20 program.

2.5. Western Blotting

For isolating protein, MEF cells were treated with 1 X RIPA lysis buffer (Millipore, Burlington, MA, USA) containing phosphatase (GenDEPOT, Katy, TX, USA) and protease inhibitor cocktail solution (GenDEPOT, USA) for 20 min on ice. After centrifugation for
20 min at 4 °C, proteins in the supernatant were measured by Pierce TM BCA protein assay kit (Thermo scientific, Waltham, MA, USA). Total proteins and Xpert 2 prestained protein marker (GenDEPOT, USA) were loaded onto a 4–12% Bis-Tris gel (Invitrogen, Carlsbad, CA, USA) and run for 35 min in XCell SureLock® Mini-Cell (Invitrogen, USA). The separated proteins were transferred to a nitrocellulose membrane (Invitrogen, USA) using the iBlot Dry Blotting System (Invitrogen, USA) for 7 min. The membranes were blocked with DPBS (Welgene, Korea) containing 5% of blotting grade blocker (Bio-Rad, USA) and 0.1% Tween 20 (Amresco, USA) for 1 h. The membranes were then incubated with primary antibodies (at a 1:1000 dilution) of Oct4 (Santa Cruz, USA), and SSEA-1 (Abcam, USA) diluted in skim milk at 4 °C for overnight. The membranes were washed twice and placed in skim milk solution containing secondary antibody (at a 1:2000 dilution) of anti-mouse horseradish peroxidase (Life Technologies) for 2 h at room temperature. Enhanced chemiluminescence (ECL) chemiluminescent Substrate Reagent (Life technologies) was spread on the membrane and observed using the Chemidoc (Bio-rad, Hercules, CA, USA).

2.6. Differentiation to Three Germ Layers In Vitro

For neuronal differentiation, MEF spheroids were transferred on a matrigel-coated plate and cultured in a neuronal differentiation medium containing DMEM (Welgene, Korea) and L-glutamine with daily medium change. To differentiate into cardiomyocytes, MEF spheroids were placed on a 0.2% gelatin-coated plate and cultured in ES medium, supplemented with 100 µM L-ascorbic acid without LIF. The medium was changed every two days. MEF spheroids were cultured in ES medium without LIF to differentiate into endoderm. The medium was changed every 2 days.

2.7. Statistical Aata Analysis

All data were replicated three times and analyzed using one-way analysis of variance (ANOVA) test using Originlab version 9. P values less than 0.05 were considered significant.

3. Results and Discussion

It is already documented that shear stress conditions and vitamin C aid in transgene-integrated generation of iPSCs. However, we alternatively induced stem cell-like cells using external stimuli without integrating OSKM genes required for iPSC induction. Figure 1 provides the schematic illustration of the induction methods used in this study for generating stem cell-like cells in suspension culture. We used an electronic pipette to produce short-term shear stress (STSS) and compared its results to induce spheroid formation from MEF cells with the group that was subjected to shear stress together with vitamin C (SSVC) treatment. Subsequently, the spheroids were characterized for stemness and potential to differentiate into different cell types. Figure 2a and Figure S1 show the morphology of spheroids induced by STSS and SSVC from MEF. The area of spheroids was increased over time in both of the treatments by STSS and SSVC. After picking each spheroid, they were measured for length and area. The spheroids of the SSVC group tended to be longer and larger than that of the STSS group (Figure 2b,c), suggesting that SSVC has high efficiency for generating cells per spheroid. Alkaline phosphatase (ALP) staining was performed to identify the early marker of iPSC (Figure 2d). The MEF spheroids cultured under SSVC responded stronger with ALP solution than that of the STSS group. These data showed that the SSVC technique was more likely to form spheroids than STSS technique, as well as increase the possibility of reprogramming. Thus, we draw a hypothesis that MEF spheroids, which have been subjected to shear stress, gain potential for cellular reprogramming and that it is further enhanced with the addition of vitamin C.
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Figure 2. Comparative induction of MEF (mouse embryonic fibroblasts) into stem cell-like cells by STSS and SSVC methods in comparison to the control MEF. (a) Morphology of induced spheroids (scale bars = 200 µm). (b) Average area and (c) average length of spheroids show SSVC-generated spheroids to be significantly larger and longer than the STSS ones. (error bars represent standard deviation, *p < 0.05 was considered to be statistically significant, n = 3). (d) ALP (alkaline phosphatase) staining of spheroids after 14 days (scale bars = 100 µm).

To identify pluripotent characteristics of MEF spheroids, we analyzed the expression of Oct4 and SSEA-1 by immunocytochemistry (ICC) (Figure 3a,b). Both the SSTS- and SSVC-treated groups showed the presence of pluripotency markers in comparison to negligible expression in the MEF control group. These results suggest that both groups have properties of stem cells as indicated by the ALP analysis results. However, no significant difference was observed between the two groups in ICC. For quantification of expressed pluripotent markers, Western blot assay was performed (Figure 3c). The Western blot results clearly determine the increased expression level of Oct4 and SSEA-1 in the SSVC experimental group compared to the STSS group, suggesting that addition of vitamin C to shear-stress-induced cells, as in the SSVC method, enhances the expression of pluripotent stem cell markers more than the SSTC method.

Finally, we determined the differentiation potential of MEF spheroids induced by SSVC method (Figure 4). To validate the differentiation capability, Neuronal Class III b-Tubulin (TuJ1), which is a neuronal marker of the ectoderm, sarcomeric alpha-actinin, which is a cardiomyocyte marker of the mesoderm, and alpha 1 fetoprotein, which is one of the endoderm markers, were used. The SSVC-induced spheroids were observed to be partially differentiated into neuron, mesoderm, and endoderm. These results showed that shear stress and vitamin C together induced partial reprogramming.

In summary, we showed that the SSVC method enhances spheroid formation and expression of pluripotent stem cell markers more than the STSS method. In other words, shear stress and vitamin C caused a synergistic effect in inducing stem cell-like cells. However, we observed that only a small fraction of the spheroids induced by SSVC differentiated into specific cells. Therefore, we conclude that spheroids formed by various external stimuli were stem cell-like cells, not induced pluripotent stem cells, and could suggest the possibility of direct conversion from the fibroblast.
significant, than the STSS and control MEF group (error bars represent standard deviation, *p < 0.05 was considered to be statistically significant, n = 3).

Figure 3. Comparison of pluripotency markers for STSS- and SSVC-generated spheroids. (a) Fluorescence images of SSEA-1-expressing spheroidal cells. (b) Fluorescence images of Oct4-expressing Scheme 20 µm. (c) Western blot analysis determined that SSVC experimental group showed significantly higher expression of Oct4 and SSEA-1 pluripotency markers than the STSS and control MEF group (error bars represent standard deviation, *p < 0.05 was considered to be statistically significant, n = 3).

Figure 4. In vitro differentiation potential of SSVC-treated cells into three germ layers (scale bars = 100 µm).

4. Conclusions
The objective of this research was to develop an alternative method to induce pluripotency in MEF using short-term shear stress and vitamin C without the need for transgene integration. We observed that shear stress and vitamin C influenced cell reprogramming, but it was impossible to reprogram into a pluripotent stem cell state completely. This research showed the possibility that methods using external stimuli and small molecules without transgenes can reprogram somatic cells into variously differentiated cells. In future, we intend to improve the methods using external stimuli for cellular reprogramming.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-3417/11/4/1941/s1, Figure S1: Morphology of differentiated cells.
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Conflicts of Interest: The authors declare no conflict of interest.

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