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Simultaneous engagement of mechanical stretching and surface pattern promotes cardiomyogenic differentiation of human mesenchymal stem cells

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It has been widely recognized and proved that biophysical factors for mimicking in vivo conditions should be also considered to have stem cells differentiated into desired cell type in vitro along with biochemical factors. Biophysical factors include substrate and biomechanical conditions. This study focused on the effect of biomimetic mechanical stretching along with changes in substrate topography to influence on cardiomyogenic differentiation of human mesenchymal stem cells (hMSCs). Elastic micropatterned substrates were made to mimic the geometric conditions surrounding cells in vivo. To mimic biomechanical conditions due to beating of the heart, mechanical stretching was applied parallel to the direction of the pattern (10% elongation, 0.5 Hz, 4 h/day). Suberoylanilide hydroxamic acid (SAHA) was used as a biochemical factor. The micropatterned substrate was found more effective in the alignment of cytoskeleton and cardiomyogenic differentiation compared with flat substrate. Significantly higher expression levels of related markers [GATA binding protein 4 (GATA4), troponin I, troponin T, natriuretic peptide A (NPPA)] were observed when mechanical stretching was engaged on micropatterned substrate. In addition, 4 days of mechanical stretching was associated with higher levels of expression than 2 days of stretching. These results indicate that simultaneous engagement of biomimetic environment such as substrate pattern and mechanical stimuli effectively promotes the cardiomyogenic differentiation of hMSCs in vitro. The suggested method which tried to mimic in vivo microenvironment would provide systematic investigation to control cardiomyogenic differentiation of hMSCs.

[**Key words:** Cardiomyogenic differentiation; Human mesenchymal stem cells; Mechanical stretching; Micropatterned substrate; Suberoylanilide hydroxamic acid]

Stem cell therapy can be used in cases of damage to the myocardium due to cardiovascular diseases, such as myocardial infarction (1,2). There are three main sources of stem cells: embryonic stem cells (ESCs), adult stem cells (ASCs), and induced pluripotent stem cells (iPSCs). Although ESCs and/or iPSCs have greater potency than ASCs, a number of problems still remain to be resolved, including ethical issues with regard to ESCs and lack of fully established protocols for modulating iPSCs (3,4). Mesenchymal stem cells (MSCs) are multipotent stromal adult stem cells that have been widely investigated (5). Moreover, various protocols are available for modulating their differentiation into cells of various lineages (5–7). They are known to be useful in stem cell-based therapies, as they evoke less immune problems through suppression of T-cell activities (8). However, despite a number of previous investigations, clinically useful methods for inducing the differentiation of human mesenchymal stem cells (hMSCs) into cardiomyocytes have to be developed. Typical methods reported to date have adopted various growth factors, cytokines, or co-culture techniques (9–12). However, the differentiation ratio of MSCs into cardiomyocyte is still very low and their potential clinical benefits for stem cell therapy have not been established yet. Moreover, the reproducibility of differentiation results is still unsatisfactory even with similar protocols and is highly dependent on the investigator. This suggests that more effective approach is needed to induce MSC to cardiomyogenic differentiation.

To overcome these disadvantages of utilizing only biochemical reagents, there have been recent studies regarding use of microphysical environments to mimic the in vivo conditions (12–14). The in vivo microphysical environment is comprised of mechanical components due to daily activities or physiological activities as well as matrices where the cells reside. Therefore, the effects of various mechanical stimuli and type of substrate have been examined (15–18). Huang et al. (19) confirmed the possibility of modulating the differentiation of MSCs into cardiomyocytes by adjusting the magnitude and frequency of mechanical stimulation. Park et al. (20) reported that the physical microenvironment can modulate stem cell fate. The results of these investigations of mechanical stimulation and substrate properties suggest that appropriate combinations of these two factors would be effective at regulating the differentiation of these cells.

Cardiomyocytes in human body are continuously exposed to specific mechanical and geometric conditions with tension due to the heartbeat and the presence of fibers in cardiac tissues (21,22).

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However, there have been few studies considering these factors. Moreover, most studies were performed with rat MSCs, and they did not simultaneously include these two mechanical and geometric factors.

Therefore, the present study adopted micropatterned substrate to investigate the effect of topographical cues on morphology, alignment, and cardiomyogenic differentiation of hMSCs. Then we engaged mechanical stretching to the selected substrate based on the results obtained. Meanwhile, a biochemical reagent, one of the widely used reagents in cardiomyogenic differentiation, was used for all experiments.

MATERIALS AND METHODS

Preparation of cells hMSCs (Lonza, Basel, Switzerland) were cultured in Dulbecco's Modified Eagle's Medium Low Glucose (DMEM-LG; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Hyclone, Logan, UT, USA) at 37°C in a humidified 5% CO_2 incubator. The medium was replaced every 3 days after seeding. Subconfluent cells were subcultured using trypsin/EDTA (Gibco), and hMSCs at the fourth passage were used in subsequent experiments.

Fabrication of substrates Polydimethylsiloxane (PDMS), one of the most widely used silicone-based polymer, was used to fabricate elastic substrates (23,24). Micropatterned substrates were fabricated based on silicon wafers manufactured using photolithography and deep reactive ion etch (DRIE) techniques. The wafer surface patterns contained groove × ridge widths of 20 × 20 μm and 20 × 10 μm, with a groove depth of 3 μm. Silicone wafers were coated with a mixture composed of Sylgard 184 silicone elastomer and curing agent (Dow Corning, Midland, MI, USA) at a ratio of 10:1 using a spin coater (PC8; Karl Süss, RC8, Germany), and subsequently cured on a hot plate at 120°C for 20 min. A substrate without a pattern was also made for use as a control. The fabricated substrate was sterilized using 70% ethanol and deionized water followed by ultraviolet (UV) exposure for 1 h. Finally, the substrate surface was coated with a mixture of 12.5 mg/L fibronectin (Sigma) and 0.02% gelatin in PBS to promote cell adhesion.

Inducing differentiation To investigate the effect of substrate pattern, hMSCs were seeded on to the three types of substrate in DMEM-LG supplemented with 10% FBS and 1% penicillin/streptomycin. At confluence, they were exposed to 1 μM suberoylanilide hydroxamic acid (SAHA; Sigma, St. Louis, MO, USA). After 24 h of incubation, the medium was replaced by fresh medium containing 1 μM SAHA. The medium was then replaced with fresh medium every 3 days. The schedule of the related experiment is shown in Fig. 1B.

To investigate the effect of mechanical stretching on micropatterned substrate, hMSCs were seeded onto selected patterned substrate in DMEM-LG supplemented with 10% FBS and 1% penicillin/streptomycin and then engaged mechanical stretching with 1 μM SAHA. A bioreactor (ACBT-200; Anycasting Inc., Seoul, Korea) was used for mechanical stretching of substrates (4 cm wide × 8 cm in length) where hMSCs were seeded at 1×10^4 cells/cm^2. They were cultured in a humidified 5% CO_2 incubator at 37°C for 4 days to ensure cell adhesion to the micropatterned substrate before stretching. The stretching pattern was 10% elongation at 0.5 Hz for 4 h/day. Several experimental groups were set based on the types of stimulus: (i) no stimulus as a control (NS); (ii) mechanical stretching for 2 days (S_D2); and (iii) mechanical stretching for 4 days (S_D4). The schedule of the related experiment is shown in Fig. 2C.

Real-time PCR analysis GATA binding protein 4 (GATA4), troponin I, troponin T, natriuretic peptide A (NPPA), and β-actin mRNA levels were analyzed by real-time polymerase chain reaction (PCR) (Table 1). Total cellular RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). A high-capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA) was used for reverse transcription of RNA into 0.5 μg/20 μL of cDNA. For real-time PCR analysis, 10 ng of cDNA was used as the template in each well. The cDNA was amplified using Power SYBR Green PCR Master Mix (Applied Biosystems). Reaction mixtures contained 2 × PCR Master Mix, forward primer, reverse primer, cDNA template, and deionized water. After initial denaturation for 10 min at 95°C, PCR was performed for 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, followed by melting curve analysis. Expression levels were calculated using the $^{\Delta\Delta}$CT method.

Staining of actin filaments To examine the cytoskeletal arrangements of hMSCs, actin filament orientation was observed using rhodamine phalloidin staining (Sigma). The cells were fixed with 4% paraformaldehyde (Sigma) in phosphate buffered saline (PBS) for 10 min, followed by permeabilization with 0.2% Triton X-100 (USB, Cleveland, OH, USA) in PBS for 10 min. They were then incubated with 1% bovine serum albumin (BSA; Sigma) in PBS for 30 min at room temperature to block nonspecific binding and reacted with rhodamine phalloidin in PBS (1:50) for 20 min in the dark, and nuclei were stained with Hoechst 33258 (Invitrogen, Grand Island, NY, USA). The stained cells were observed using a confocal laser scanning microscope (LSM 510 META; Carl Zeiss, Jena, Germany). The degree of actin filament orientation was quantified using a MATLAB (version R2012; MathWorks, Natick, MA, USA).

Immunofluorescence staining Cardiomyogenic differentiation of hMSCs was also assessed by fluorescence staining and confocal microscopy. The cells on the substrate were fixed with 4% paraformaldehyde (Sigma) and permeabilized in 0.1% Triton X-100 (USB) for 10 min. They were then washed twice with PBS and incubated with 1% BSA (Sigma) in PBS for 30 min at room temperature to block nonspecific binding. Next, the cells were labeled with antibodies specific to the indicated cardiomyogenic markers. The presence of the cardiomyogenic marker GATA4 was verified using a mouse anti-GATA4 monoclonal antibody (1:100; Santa Cruz, Dallas, TX, USA), and that of Nkx2.5 was determined with a mouse anti-Nkx2.5 monoclonal antibody (1:200; Abcam, Cambridge, UK). Both GATA4 and Nkx2.5 were then visualized with an Alexa Fluor 488 goat anti-rabbit secondary antibody (1:200; Molecular Probes, Eugene, OR, USA). Incubations with both primary and secondary antibodies were performed at room temperature for 1 h. Finally, the nuclei were stained with Hoechst 33258 (Invitrogen). Images were obtained with a confocal microscope (Carl Zeiss). Expression levels of the genes of interest were calculated utilizing MATLAB (version R2012; MathWorks).

Statistical analysis One-way analysis of variance (ANOVA) was performed using SPSS (Version 10.0K; SPSS Inc., Chicago, IL, USA). When ANOVA indicated a significant difference among groups, the difference was then evaluated using the least-significant difference test. All data are presented as the means ± standard deviation (SD), and $p < 0.05$ was considered to indicate statistical significance.

RESULTS AND DISCUSSION

Choice of chemical stimulus among the three common agents (5-azacitidine, SAHA, and trichostatin A) Based on previous studies (25–29), three potential chemical factors were tested: 5-azacytidine, SAHA, and trichostatin A (TSA). We examined the effects of three candidate agents for cardiomyogenic differentiation. Potential differentiation media were added to 6-well plates that had been seeded with hMSCs. The expression of related markers was measured on day 28 of culture and are shown in Fig. S1. 5-Azacytidine significantly enhanced the expression of GATA4, troponin T, and NPPA compare to the non-treated cells. Also, the expression levels of GATA4, troponin I, and NPPA were significantly higher in the hMSCs induced with SAHA than those in the non-treated cells. Therefore, the effect of 5-Azacytidine and SAHA looked comparable. On the other hand, TSA was found relatively ineffective in the present study. Although 5-Azacytidine, a DNA methylation inhibitor, has been widely used for cardiomyogenic differentiation, the mechanism by which it induces differentiation has not been elucidated (29). Also, its effect is known to be not specific (30). Moreover, it has been proved not enough to induce mature functional cardiomyocyte from MSCs for clinical application (28). Meanwhile histone acetylation has recently been shown to be involved in the regulation of gene expression and cell differentiation (31). Histone deacetylases (HDACs) inhibitor increase the level of histone acetylation, leading to enhanced cell differentiation. Specifically, HDACs inhibitor like SAHA have been widely studied in relation to cardiomyocyte differentiation (29,32). Also, downregulation of class I HDACs is known to be effective at differentiation of rat MSCs into cardiomyocytes (33). Therefore, SAHA was selected as an inducer for cardiomyogenic differentiation of MSCs in this study.

Effects of the substrate pattern on cellular morphology and cytoskeletal arrangement Two types of micropatterned substrate were successfully fabricated, as shown in Fig. 1A. Previous studies used groove depths of 3 and 5 μm for differentiation into smooth muscle- and osteoblast-like cells, respectively (34–37). It is known that the heart muscle matrix mainly consisted of three types of fibers. The average diameter of each type is known as several micrometers, ~1 μm, and 20–100 nm, respectively (38–40). Therefore, we used a depth of 3 μm rather than 5 μm in the present study. The width of groove was 10 or 20 μm. For substrate pattern selection, the changes in cellular morphology were examined in addition to F-actin staining during culture. No

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