Osteogenic differentiation of murine mesenchymal stem cells by combination of surface topography and uniaxial stress

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Received: 28 December 2019; Revised: 10 March 2020; Accepted: 19 May 2020

Abstract
Recent research has shown that enhanced focal adhesion between cells and extracellular matrix (ECM) and intracellular actin polymerization can accelerate cellular functions like proliferation and differentiation. It is a desirable and necessary technique to modulate cellular functions in the desired lineage in tissue engineering. Previously, we have shown that topographical effects of micropatterns on a cell culture substrate promoted osteogenic differentiation of mesenchymal stem cells (MSCs) without administration of osteogenic growth factors. In this study, bone marrow mesenchymal stem cells from rats were cultured with combined biophysical stimuli, such as surface topography of biomaterials and uniaxial tensile stress and induced osteogenic differentiation. We evaluated MSCs by assessment of alkaline phosphatase (ALP). We demonstrated that a polydimethylsiloxane substrate with microgroove patterns (2 μm of ridge thickness, 1 μm of depth and 1 μm of groove distance) could suppress osteogenic differentiation compared to a flat substrate. Changes of cell adhesion and shape were observed at microgroove substrates by immunofluorescence staining of focal adhesion and actin filaments. We showed that to apply the stretching force promoted differentiation at microgroove substrates but inhibited differentiation at the flat surface. Through this study, a more efficient method to control cellular fate is expected to be established for tissue regeneration in vitro.

Keywords: Mesenchymal stem cells, Osteoblast differentiation, Uniaxial tensile stress, Surface topography, Micropattern, Alkaline phosphatase, Focal adhesion

1. Introduction

In regenerative medicine applications, mesenchymal stem cells (MSC) have been studied and used because they can differentiate into various cell types including osteoblasts, chondrocytes, adipocytes and so on (Pittenger et al., 1999). While retaining their pluripotency, MSCs have the ability of self-renewal that can extend their population as well. Niches in bone marrow provide MSCs to maintain the stemness and deliver the cellular microenvironment signals. The external interaction and stimulation are important keys to modulate cell functions. Especially, bones in our body containing bone marrow are constantly physically stimulated and have several different tissue structures such as articular cartilage, cortical bone and spongy bone (Gao, 2006; Safadi et al., 2009). Differentiation into cells for each site occurs according to its biophysical properties; MSC keeps its stemness in a niche, differentiate into osteoblastic cell in the bone interface, and chondrocyte in the cartilage.

In tissue engineering, it has been considered that in vitro culture of MSCs can be controlled to differentiate into desired tissue cells by providing the mimicked surrounding environment (Pati et al., 2015; Ventre et al., 2012; Winer et al., 2008). To achieve in vitro control, the studies on MSC differentiation by biophysical stimuli have been conducted. Mechanical stimulation and biomaterial properties, including hydrostatic pressure, shear stress, tension, stiffness, and
surface topography have been applied to modulate cellular behaviors (Chen et al., 2017; Engler et al., 2006; Rutkovskiy et al., 2016). Qi et al found that tensile strain for a short-term can promote the proliferation of MSCs and activate several osteogenic differentiation markers like alkaline phosphatase and Cbfa1 (Qi et al., 2008). We also have reported that osteogenic differentiation could be promoted by surface topography of cell substrates that up-regulated RhoA/ROCK signaling pathway (Seo et al., 2011a, 2014). Thus, at the initial stage of cell lineage determination, it is important which kind and how much biophysical stimulation is given to MSC. Surface topography of the cell interface in the tissue is formed by various extracellular matrix compounds. In addition, there are continuous physical stimuli such as tensile stress due to movement. Therefore, these combined biophysical stimuli are expected to achieve the effective control of MSC differentiation in vitro. In this study, we created an in vitro culture environment that simply mimics the complex stimuli in vivo by applying micropatterns and uniaxial stretching to MSCs and examined its effect on osteoblast differentiation (Fig. 1 (A)). In this study, we examined early osteogenic differentiation of murine bone marrow-derived MSCs by microgroove patterned substrate and uniaxial tensile stress. We also qualitatively compared cell adhesion and morphology affecting by those stimuli. We elucidated the relationship between changes of cell morphology and MSC differentiation influenced by the combined stimuli.

Fig. 1 (A) A concept image in a simple form for *in vitro* culture of MSCs with surface topography and mechanical stress imitated to MSCs in the bone that are always influenced by their microenvironments such as ECM and mechanical stimuli. (B) Microgroove patterned substrate with 2 μm of ridge thickness (R), 1 μm of depth (Dp), and 1 μm of groove distance (G). B) SEM image of the fabricated PDMS substrates with groove (yellow arrow) and ridge (white arrow). Scale bar: 10 μm. (C) The image of the uniaxial stretching system with stepping motors.

**2. Materials and methods**

**2.1 Preparation of microgroove patterned substrates and scanning electron microscope observation**

When mimicking biophysical stimuli in bone in vivo to in vitro cell culture, we considered that surface topography and mechanical stress have important effects on cell differentiation. We reproduce this extracellular environment by a simple model as the cell culture substrate with microtopography and stretching.

The microgroove patterned substrates were fabricated as described previously in the modified design (Seo et al., 2014). The thickness of the microgrooves was the same as that of lattice in the previous study, but the micropattern was designed linearly to induce cell morphology different from cells in the flat surface. Briefly, polydimethylsiloxane (PDMS) solution with 10% of curing agent was cast in a pre-designed silicon mold at 60 degrees for 2 hours, of which Young’s modulus was approximately 1.7 MPa (Seo et al., 2013). After peeling off, the PDMS substrate was in oxygen plasma treatment and fibronectin incubation (1µg/cm²) for 1 hour at room temperature. As shown in Fig. 1(B), the PDMS substrate was coated by osmium gas and was observed by a high-resolution scanning electron microscope (SEM, JSM-7000F, JEOL).
2.2 Osteogenic induced culture of MSCs
MSC isolation was conducted as described previously (Helfrich et al., 2012). We obtained bone marrow-derived MSCs from femur of 6 to 10 week-old Sprague Dawley rats and incubated in α-minimum essential medium (α-MEM) with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100mg/ml streptomycin (Invitrogen) in a humidified atmosphere of 5% CO₂ at 37°C. At passage 3-10, MSCs were seeded on the fabricated substrates and cultured in osteogenic induction medium with 10mM β-glycerophosphate and 50 µg/ml ascorbic acid 2-phosphate.

2.3 Cell stretching system
Experimental conditions such as frequency, loading time and strain were determined from reference papers (Chen et al., 2008; Simmons et al., 2003). In order to realize the above conditions, we designed a new uniaxial tensile loading system (Fig. 1 (C)). The system was driven by two stepping motors controlled by stage controller (SIGMA KOKI, SHOT-202) and PC. It could provide constant stretching force to the stretching chambers, which was fully made of PDMS, the same materials of the substrate. We applied uniaxial tensile stress to MSCs with 3% strain and 1 Hz for 8 hours per day.

2.4 Alkaline phosphatase assay
For quantification of alkaline phosphatase (ALP) activity level and protein amount, MSCs were lysed by PBS with 50mM Tris/HCl, pH 7.6 and 0.1% Triton X-100. As follow the instructions of each manufacturer, ALP activity level and protein amount of the collected samples were assessed by LabAssay™ ALP kit (FUJIFILM Wako Pure Chemical Corporation) DC™ protein assay kit (Bio-Rad).

2.5 Immunofluorescence staining and quantitative analysis of cell adhesion
For immunofluorescence staining of focal adhesion and actin filaments, cultured MSCs were rinsed with PBS and fixed in 10% formalin neutral buffer solution for 15 min at room temperature. After removing fixation solution and rinsing with PBS, cells were incubated in 1% BSA/PBS for 45 min, primary antibody (anti-vinculin, V 9131, Sigma-Aldrich) solution for 60 min, secondary antibody (Alexa Fluor 488, A11029, Invitrogen) solution with rhodamine phalloidin (PHDR1, Cytoskeleton) for 60 min, and DAPI (40,6-diamidino-2-phenylindole, Dojindo) for 5 min at room temperature sequentially. The stained cells were observed with a laser scanning microscope (LSM 510 Meta CLSM, Zeiss) with a Plan Neofluar 10 x objective lens (Zeiss) and an α Plan-FLUAR 40 x objective lens (Zeiss) and the images were obtained in the resolution of 672*512 pixels (0.2 µm/pixel) with a digital CCD camera (C7780-10, Hamamatsu Photonics). In order to measure the size of focal adhesion and area of cell, fluorescence images of vinculin and actin staining were analyzed by ImageJ. Especially, the analysis of focal adhesion was conducted as follows the methods of Berginski et al., and Horzum et al. (Berginski et al., 2011; Horzum et al., 2014). Briefly, we converted the image to 8 bit and uploaded to the focal adhesion analysis server (URL: https://faas.bme.unc.edu/), picking up the stained vinculin area without noise as possible. Focal adhesion area was chosen from the marked area by comparison with the merged image in actin.

2.6 Statistical analysis
Two-sided Student’s t-test was used for statistical analysis. A P value of less than 0.5 was considered as a significant difference. The data represent means ± standard error.

3. Results
3.1 Evaluation of early osteogenic differentiation by ALP activity levels
In the early stage of differentiation, ALP enzyme activity is expressed high in osteoblast (Safadi et al., 2009). ALP activity level is one of useful indicators in verifying the differentiation process of MSCs as an early osteogenic differentiation marker. Fig. 2 (A) and (B) showed ALP activity levels and protein amount of MSCs respectively at each condition. Although there was no significant difference, the ALP activity at microgrooves without stretching was less than that of flat surface. After applying uniaxial tensile stress, the level dramatically decreased at the flat surface, even the protein amount increased, suggesting that differentiation was suppressed. However, in the case of microgroove substrates, the ALP activities increased regardless of the direction of stretching.
3.2 Observation of cell adhesion and shape by immunofluorescence staining

We performed immunofluorescence staining of MSCs at flat and microgroove patterned substrates to identify the focal adhesion sites and cytoskeleton. In the focal adhesion complex, vinculin is significantly integrated at the site of integrin-mediated adhesion and links to actin cytoskeletons (Levy et al., 2010). In Fig. 3, we showed the distribution of focal adhesion complex by staining vinculin and actin filaments at each substrate. The MSCs had focal adhesion and actins along the direction of the microgroove patterns. However, those at the flat substrate showed randomly.

3.3 Quantification analysis of cell area and focal adhesion

We assessed cell area, the size of focal adhesion in a single cell, and aspect ratio of a cell at each substrate based on the immunostaining images (Fig. 4). At day 2 of culture, the area of MSCs was larger in the flat surface without stretching than other groups, suggesting that the spread of cells might be suppressed by the grooves and stretching (Fig. 4 (A)). Also, the size of focal adhesion complex expressed in a single cell showed the largest value in the flat surface plane without stretching, indicating that cell adhesion might be disturbed by the grooves and stretching. Contrary, the cell aspect ratio was higher at microgrooves that caused cell elongation, even with stretching in parallel and perpendicular.
4. Discussion

In this study, we expect that more comprehensive methods could be provided in regulating cellular behaviors by culturing MSCs on the microgroove substrate and combining uniaxial stretching (Fig. 5). We assessed ALP activity level of MSCs to evaluate early osteoblast differentiation. MSCs determine their fate in vitro by the initial biophysical environment such as mechanical stresses (Ahmed et al., 2010; Huang and Ogawa, 2012; Yan et al., 2012) or modification of the cell-material interface to nano-/micro-patterns (Abagnale et al., 2015; Wilkinson et al., 2011). Moreover, in vitro culture, MSC differentiation is easily affected by the initially formed cell adhesion and cytoskeleton (Chen et al., 2011; Wang et al., 2016). It is particularly well applied for research involving osteoblast and osteocyte that are affected by sustained stimuli in vivo (Ahmed et al., 2010; Rutkovskiy et al., 2016; Wang et al., 2000).

In our previous research, the specific parameters of lattice patterns were proven to have a positive effect in promoting osteogenic differentiation by enhanced focal adhesion and actin polymerization (Seo et al., 2011a). Other studies have also shown similar opinion that changes of cytoskeleton and cell adhesion by the surface microtopography could enhance the osteogenic differentiation and the higher aspect ratio of cells, the more osteoblast differentiation down-regulated (Shin et al., 2011; Yao et al., 2013; Zhang et al., 2016). Similarly, our results of cell aspect ratio showed a possibility that the raise of aspect ratio by microgrooves may also down-regulated the osteogenic differentiation process of MSCs. By microgrooves, cells were elongated but reduced their size and decreased focal adhesion formation. As a result, it is thought that the cellular signaling for osteogenic differentiation by the decrease of intracellular myosin-actin tension could not be activated.

MSCs promote osteogenesis as myosin II increases cell tension (Mcbeath et al., 2004). The cell tension enhanced by increasing focal adhesion complex, which sensitively responds to microtopography and stress of cell interface (Alexandrova et al., 2008). Cells form focal adhesion complex for strong adhesion to cell surfaces and respond to

Fig. 4 Quantification analysis of (A) cell area, (B) size of focal adhesion in a single cell, (C) the proportion of focal adhesion possessed in a single cell and (D) aspect ratio of a cell on each substrate for 1 day without stretching, and 2 days with and without stretching. FA; focal adhesion. The data represents mean ± S.E. (n=15, * p ≤ 0.05, ** p ≤ 0.01).
extracellular stimulations via focal adhesion by transmitting signals into the cells. Therefore, we measured the cell area and the area of focal adhesion complex on each substrate. As a result, the tendency of cell area and focal adhesion area was changed according to the conditions of the substrate and stress. In other words, surface topography affects cell spreading, and cell adhesion, and thus can be interpreted as affecting osteogenic differentiation.

Rutkovskiy et al reviewed that stretching the cell substrates with low magnitude (~3%) could enhance osteoblast proliferation and increase the expression of osteogenic markers (Rutkovskiy et al., 2016). Oppositely, our results which applied 3% strain and 1 Hz stretching to MSCs showed the decrease of ALP activity. These phenomena might be related to the formation density of FA as shown in Fig. 4C. The results were inconsistent with the results on the flat surface by Rutkovskiy et al., but these might be due to differences in the cellular response between osteoblasts and MSCs.

We have previously reported, using osteoblasts, that the depth or spacing of lattice-like patterns controls osteogenic differentiation (Seo et al., 2011a, 2011b, 2014). In this study, we tried to differentiate MSCs into osteoblasts using a similar pattern, and found that MSCs did not promote the induction of bone differentiation. It was also found that external strain promotes differentiation induction of osteoblasts on the lattice structure. These studies suggest that simultaneous stimulation of extracellular matrix-like lattice structures and external physical stimuli may be a useful tool for inducing stem cell differentiation.

5. Conclusion

We cultured bone marrow-derived MSCs on microgroove substrates with uniaxial tensile strain and induced osteogenic differentiation. As a result of ALP activity assay, we demonstrated that microgroove substrates, which has 2 μm of ridge thickness, 1 μm of depth and 1 μm of groove distance suppressed osteogenic differentiation. This was accompanied by a decrease in focal adhesion and an increase in the aspect ratio. Parallel (blue arrows) and perpendicular stretching (red arrows) against the patterns enhanced early OD, although stretching at the flat substrate suppressed OD.

Fig. 5 A schematic image describing the different aspects of MSC behaviors on microgroove patterned substrate with uniaxial stretching. Microgroove patterns could inhibit osteogenic differentiation (OD) compared to flat substrate by decrease in focal adhesion (FA) and increase in the aspect ratio. Parallel (blue arrows) and perpendicular stretching (red arrows) against the patterns enhanced early OD, although stretching at the flat substrate suppressed OD.

Acknowledgement

SEM observation was conducted at Advanced Characterization Nanotechnology Platform of The University of...
Tokyo, supported by "Nanotechnology Platform" of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. The authors acknowledge the support for manipulating the confocal laser scanning microscope by Sakai Laboratory, Department of Chemical System Engineering, The University of Tokyo.

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