Biomaterials 34 (2013) 7257–7268

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Synergistic effects of nanotopography and co-culture with endothelial cells on osteogenesis of mesenchymal stem cells

Jangho Kim [a,1], Hong Nam Kim [b,1], Ki-Taek Lim [a], Yeonju Kim [d], Shambhavi Pandey [a], Pankaj Garg [a], Yun-Hoon Choung [c], Pill-Hoon Choung [d,***], Kahp-Yang Suh [b,**], Jong Hoon Chung [a,e,*]

[a] Department of Biosystems & Biomaterials Science and Engineering, Seoul National University, Seoul 151-742, Republic of Korea
[b] Division of WCU Multiscale Mechanical Design, School of Mechanical and Aerospace Engineering, Seoul National University, Seoul 151-742, Republic of Korea
[c] Department of Otolaryngology, Ajou University School of Medicine, Suwon 443-721, Republic of Korea
[d] Tooth Bioengineering National Research Lab, Department of Oral and Maxillofacial Surgery, School of Dentistry, Seoul National University, Seoul 110-749, Republic of Korea
[e] Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul 151-742, Republic of Korea

ARTICLE INFO

Article history:
Received 11 April 2013
Accepted 14 June 2013
Available online 5 July 2013

Keywords:
Nanotopography
Osteogenesis
Mesenchymal stem cells
Endothelial cells
Co-culture

ABSTRACT

Inspired by the aligned nanostructures and co-existence of vascular cells and stem cells in human cancellous bone, we quantitatively investigated the relative contributions of nanotopography and co-culture with human umbilical endothelial cells (HUVECs) to the osteogenesis of human mesenchymal stem cells (hMSCs). Although both nanotopography and co-culture independently enhanced the osteogenesis of hMSCs, osteogenesis was further enhanced by the two factors in combination, indicating the importance of synergistic cues in stem cell engineering. Interestingly, nanotopography provided a larger relative contribution to the osteogenesis of hMSCs than did co-culture with HUVECs. Furthermore, the osteogenesis of hMSCs was also affected by the density of parallel nanogrooves, exhibiting a maximum at a 1:3 spacing ratio, as defined as the ratio of ridge width to groove width. Analysis of (i) biochemical soluble factors, (ii) hMSC-substrate interaction and (iii) hMSC-HUVEC interaction suggests that (ii) and (iii) play a crucial role in mediating osteogenic phenotypes.

1. Introduction

Bone defects are still highly common pathological problems in the world, requiring approximately 2.2 million orthopedic procedures worldwide every year [1]. In spite of the availability of surgical treatments using alloplastic materials or autologous and allogeneic tissues, the regeneration of large bone defects remains one of the most critical orthopedic diseases [2,3]. Stem cell-based bone tissue engineering provides a strategy for creating new functional bone constructs by combining stem cells with scaffolds [2]. Specifically, the engineering of functional bone constructs using stem cells and scaffolds may offer new opportunities not only for the repair of orthopedic diseases but also for improvements in the fundamental understanding of the development and regeneration of bone pathologies. Therefore, an important step in this strategy is to develop an effective platform to improve stem cell functions, especially osteogenesis, for bone regeneration [2–5].

Stem cells are located within an instructive and tissue-specific niche, containing complex and controlled biochemical mixtures of soluble and insoluble factors and surrounded by the extracellular microenvironment [6,7]. In the native environment in cancellous bone, stem cells [usually mesenchymal stem cells (MSCs)] physically interact with unique topographical cues at the nanometer length scale, suggesting that nanotopography may play an important role in regulating stem cell function [2]. For example, it has been reported that nanopits or nanotubes can promote osteogenesis of MSCs [8,9]. Although these studies have provided important insights into nanotopography as an enabling tool for advanced stem cell-based bone tissue engineering, further progress

* Corresponding author. Department of Biosystems & Biomaterials Science and Engineering, Seoul National University, Seoul, 151-742, Republic of Korea. Tel./fax: +82 2 880 4601.
** Corresponding author. Tel.: +82 2 880 9103; fax: +82 2 880 8302.
*** Corresponding author. Tel.: +82 10 3300-3604; fax: +82 2 740 8717.
E-mail addresses: choungph@snu.ac.kr (P.-H. Choung), sky4u@snu.ac.kr (K.-Y. Suh), jchung@snu.ac.kr (J.H. Chung).
[1] J. Kim and H. N. Kim contributed equally to this work.

0142-9612/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2013.06.029

is required for the recapitulation of the natural nanotopography observed in the native bone environment. Accordingly, considerable benefits may be derived from improving stem cell function with a combination of bio-inspired nanotopography fabricated from the aspect of native bone structures and the traditional soluble signaling molecules (e.g., differentiation-inducing chemicals).

In addition, it has been recognized that the cells adjacent to a stem cell niche would also be important regulators of the fate and functions of stem cells through extracellular communication via direct or indirect cell–cell interactions [7,10,11]. Because bone is a highly vascularized tissue, it can be inferred that bone cells or stem cells must inevitably interact with vascular cells [12]. It has recently been reported that the osteogenesis of human MSCs (hMSCs) was greatly enhanced by co-culture with human umbilical vein endothelial cells (HUVECs) in two-dimensional cultures in standard cell culture dishes [12,13]. These studies showed that the extent of cell–cell communication between hMSCs and HUVECs in a co-culture system in combination with secreted cytokines, such as bone morphogenetic proteins (BMPs) and endothelial growth factor (VEGF), might be able to enhance the osteogenesis of hMSCs.

Guided by these considerations, we propose a rational design for engineering platforms for stem cell-based bone tissue engineering using the combination of tissue-mimetic nanotopography and co-culture with endothelial cells. We hypothesized that the bone tissue-like matrix nanotopography and endothelial cell co-culture would provide a more physiologically relevant microenvironment, which could synergistically regulate the structure and functions of stem cells. To address this challenge, we prepared precisely defined bone-mimetic nanotopography using ultraviolet (UV)-assisted capillary force lithography (CFL) [14] and investigated how this platform synergistically influenced the function of hMSCs with the goal of promoting a more osteogenic phenotype.

2. Materials and methods

2.1. Preparation and observation of ex vivo human bone tissue

Bone tissue was obtained from patients during chronic otitis media surgeries under the approval of the Institutional Review Board of the Ajou University School of Medicine (Suwon, Korea). The bone tissue was fixed overnight with a solution containing 2% glutaraldehyde, 0.1 M sodium cacodylate, and 3 mM calcium chloride (pH 7.4) at 4 °C. The bone tissue was rinsed three times with PBS. The specimen was perfused with 1% osmium tetroxide and placed on a tissue rotator for 30 min. The sample was then rinsed in PBS three times. The tissue was serially dehydrated in 50%, 70%, 90%, 95%, and 100% of acetone. Each specimen was treated with hexamethyldisilazane (HMDS), air dried, and placed on a stub for sputter-coating with gold. The tissue was then observed with a field-emission scanning electron microscope (FESEM; JEOL, JSM-5410LV, Japan).

2.2. Design and fabrication of nanopatterned matrix as a bone tissue-like substratum

The bone tissue-like matrix nanotopography was fabricated by UV-assisted capillary molding technique with polyurethane acrylate (PUA) [14,15]. Regularly-spaced nanogrooves with the width of 550 nm and three different gaps of 550, 1650 and 2750 nm (spacing ratio: 1:1, 1:3 and 1:5, respectively) were replicated from the pre-fabricated silicon masters over a large area of 25 × 25 mm^2. The silicon masters had been prepared by standard photolithography and dry etching. In the replication step, a UV-curable PUA precursor (Minuta Tech., South Korea) was drop-dispensed onto the master and brought into contact with a 100 μm-thick polyethylene terephthalate (PET) film (SKC Inc., South Korea) as a backing plane. After subsequent irradiation of UV for few tens of seconds, a negative PUA replica was formed on the PET film. Then the same replication process was performed onto a cleaned cover slip using the replicated PUA pattern as a mold. The flat and patterned surfaces were generated on the same cover slip in order to maintain the same experimental conditions. The fabricated samples were coated with gold and imaged by a FESEM (JEOL, JSM-5410LV) at an accelerating voltage of 2 kV.

2.3. Isolation and culturing of hMSCs

Adipose tissues were isolated from the patients undergoing ear surgeries under sufficient informed consent at the Ajou University School of Medicine (Suwon, Korea). The experimental protocol was approved by the Institutional Review Board at the same university. Tissues were washed with PBS and digested with 100 Unit/mL collagenase type I (Sigma–Aldrich, St. Louis, MO, USA) with low glucose Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA) and incubated for 8 h to lyse the adipose tissues. The stromal fraction was collected by centrifugation and then passed through a cell strainer (100 μm size) to remove any large cell clumps and particles. For cell culture and expansion of adipose-derived hMSCs, cells were grown in low glucose DMEM with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco, Milan, Italy) at 37 °C in a 5% CO_2 atmosphere. All cells used in this study were at passage 3 or 4.

2.4. Culturing of HUVECs

HUVECs were purchased from Lonza (Walkersville, USA) and cultured in endothelial growth medium with 2% FBS, 0.4% fibroblast growth factor, 0.04% hydrocortisone, 0.1% insulin-like growth factor, 0.1% ascorbic acid, 0.1% heparin, and 0.1% gentamicin (Gibco, Milan, Italy) at 37 °C in a 5% CO_2 atmosphere. All cells used in this study were at passage 3 or 4.

2.5. Culturing of cells seeded on substrata

hMSCs and HUVECs were cultured with 1:1 ratio of hMSCs:HUVECs on nanopatterned and flat substrata (unpatterned) in normal media (DMEM with 10% FBS and 1% antibiotics) or osteogenic differentiation media (100 nM dexamethsone, 50 μM ascorbic acid, and 10 mM glycerol 2-phosphate in normal media). The medium was changed twice or three times a week. To visualize cell types, the hMSCs and HUVECs were stained with the lipophilic fluorescent dyes Vybrant-DiO and DiD (Invitrogen, USA), respectively, before seeding cells on the substrata.

2.6. Quantification of cell morphology and orientation

For the quantitative analysis of the morphology and orientation of hMSCs and HUVECs on the substrata, the images of the cells obtained by fluorescence microscopy (Zeiss, Germany), and analyzed using a custom written MATLAB script. Ten fluorescent images with 50–100 cells were used to quantify the cell morphology and orientation.

2.7. Imaging cell morphology using FESEM

hMSCs and HUVECs (0.5×10^4 cells/sample, respectively) were cultured for 14 h on the substrata. Cells adhered onto the sample surfaces were fixed with modified Karnovsky's fixative consisting of 2% paraformaldehyde and 2% glutaraldehyde (Sigma–Aldrich) in a 0.05 M sodium cacodylate buffer (Sigma–Aldrich) for 4 h. The samples were washed with 0.05 M sodium cacodylate buffer 3 times for 10 min and fixed with 1% osmium tetroxide (Sigma–Aldrich). The samples were then washed with distilled water and dehydrated with graded concentrations (50, 70, 80, 90, and 100% v/v) of ethanol. Then, the samples were treated with hexamethyldisilazane (Sigma–Aldrich) for 15 min. Finally, the samples were coated with gold prior to cell shape observation with FESEM (JEOL, JSM-5410LV, Japan).

2.8. Immunofluorescence staining

Adhered cells on samples were fixed with a 4% paraformaldehyde solution (Sigma–Aldrich, Milwaukee, WI, USA) for 20 min, permeabilized with 0.2% Triton X-100 (Sigma–Aldrich, WI, Milwaukee, USA) for 15 min, and stained with TRITC-conjugated phalloidin (Millipore, Billerica, MA, USA) and 4, 6-diamidino-2-phenylindole (DAPI; Millipore, Billerica, MA, USA) for 1 h. Focal adhesions (FAs) were also stained with a monoclonal anti-vinculin antibody (1:100; Millipore, Billerica, MA, USA) and a FITC-conjugated goat anti-mouse secondary antibody (1:500; Millipore, Billerica, MA, USA). Images of the stained cells were taken using a fluorescence microscope (Zeiss, Germany). For the quantitative analysis of the nuclear elongation factor [NEF = (major axis)/(minor axis) of nucleus] and FA sizes of hMSCs on the substrata, the images obtained by fluorescence microscopy were analyzed using the custom written MATLAB script.

2.9. Western blot analysis

Total cellular protein was extracted by RIPA lysis buffer (62.5 mM Tris-HCL, 2% SDS, 10% glycero, pH 7.5) with added proteinase inhibitor cocktail (Invitrogen, USA). Cell lysates were incubated on ice for 30 min and then centrifuged at 13,000 rpm for 30 min at 4 °C. Supernatant (protein lysate) was collected and protein concentration was determined by a micro bicinchoninic acid (BCA) Protein Assay Kit (Bio-rad, Hercules, Calif). 25 μg aliquots of the cell lysates were separated by 8% SDS-PAGE under reducing conditions. Separated proteins were transferred to a PVDF membrane (Millipore, Corporation, Bedford, MA, USA) at 30 V for 1 h. After blocking with 5% skim milk in PBST, the membrane were incubated overnight in primary antibody at 4 °C. Primary antibody was purchased from the company (Integrin β1 (Santa Cruz, Biotechnology, Santa Cruz, CA); Connexin 43 (Cell Signaling Technology, Beverly, MA); Osteopontin (Abcam, Cambridge, MA, USA)). After washing, the membranes