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Effect of cell density on adipogenic differentiation of mesenchymal stem cells

Hongxu Lu [a,b,1], Likun Guo [b,c,1], Michal J. Wozniak [b], Naoki Kawazoe [b,d], Tetsuya Tateishi [b], Xingdong Zhang [c], Guoping Chen [a,b,d,*]

[a] Graduate School of Pure and Applied Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8577, Japan
[b] Biomaterials Center, National Institute for Materials Science, 1-1 Namiki, Tsukuba, Ibaraki 305-0044, Japan
[c] National Engineering Research Center for Biomaterials, Sichuan University, 29 Wangjiang Road, Chengdu 610064, China
[d] International Center for Materials Nanoarchitectonics (MANA), National Institute for Materials Science, 1-1 Namiki, Tsukuba, Ibaraki 305-0044, Japan

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ABSTRACT

The effect of cell density on the adipogenic differentiation of human bone marrow-derived mesenchymal stem cells (MSCs) was investigated by using a patterning technique to induce the formation of a cell density gradient on a micropatterned surface. The adipogenic differentiation of MSCs at a density gradient from 5×10^3 to 3×10^4 cells/cm^2 was examined. Lipid vacuoles were observed at all cell densities after 1–3 weeks of culture in adipogenic differentiation medium although the lipid vacuoles were scarce at the low cell density and abundant at the high cell density. Real-time RT-PCR analysis showed that adipogenesis marker genes encoding peroxisome proliferator-activated receptor γ2 (PPARγ2), lipoprotein lipase (LPL), and fatty acid binding protein-4 (FABP4) were detected in the MSCs cultured at all cell densities. The results suggest that there was no apparent effect of cell density on the adipogenic differentiation of human MSCs.

Mesenchymal stem cells (MSCs) are a prospective source of cells for tissue engineering because they are relatively easy to obtain from a small aspirate of bone marrow and are multipotent, able to differentiate into different cell lineages such as osteoblasts, chondrocytes, adipose cells, and neural cells [1–3]. MSCs have been used for tissue engineering of various tissues such as cartilage [4], bone [5], muscle [6], tendon [7], ligament [8], and fat [9,10].

Manipulation of stem cell differentiation remains a great challenge in tissue engineering. Many factors affect the differentiation of mesenchymal stem cells. These include soluble growth factors and cytokines [11,12], mechanical stimuli [13], surface properties [14], and culture conditions [15]. Cell density has also been reported [16,17] as affecting cell functions such as proliferation and differentiation. McBeath et al. [18] has reported that human mesenchymal stem cells (MSCs) plated at low density have a high potential to become osteoblasts, whereas cells plated at high density have a propensity to become adipocytes. The effect of cell seeding density on differentiation remains controversial. Both et al. [19] and Colter et al. [20] have reported the effect of increased expansion of MSCs cultured at lower densities, respectively. However, the results of cell differentiation from the two groups are different. Both et al. indicated that the osteogenic differentiation potential of MSCs both in vitro and in vivo seems to decrease with repeated passages while Colter et al. reported that MSCs retained their multipotentiality for differentiation during expansion. For these studies, the effects of cell density on cell functions were compared by separately culturing cells at different cell densities. It is difficult to completely avoid the influence of other factors induced during separate cell culture from the results. It is easier to compare the effect of cell density on cell differentiation more directly if cells at various cell densities can be cultured simultaneously on a single surface. In this study, we used a patterning technique to prepare a micropatterned surface that allowed the formation of a cell density gradient on a single surface. A cell density gradient of human MSCs was formed when MSCs were cultured on a micropatterned surface, enabling direct comparison of the effect of cell density on adipogenic differentiation of MSCs on a single surface.

Materials and methods

Preparation of micropatterned surface. Azidophenyl-derivatized poly(vinyl alcohol) conjugate (AzPhPVA) was synthesized by coupling poly(vinyl alcohol) with 4-azidobenzoic acid as previously described [21]. DMSO solution containing 234.09 mg dicyclohexylcarbodiimide (2 mL) was added dropwise to 5 mL DMSO solution containing 185.37 mg 4-azidobenzoic acid under stirring at room temperature in the dark. Then, 2 mL of DMSO solution dissolving 16.84 mg 4-(1-pyrrolidinyl) pyridine was added dropwise to the

* Corresponding author. Address: Biomaterials Center, National Institute for Materials Science, 1-1 Namiki, Tsukuba, Ibaraki 305-0044, Japan. Fax: +81 29 860 4714.
E-mail address: Guoping.CHEN@nims.go.jp (G. Chen).
[1] These two authors are contributed equally to this work.

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reaction mixture under stirring. After 10 min, 8 mL of DMSO solution containing 100 mg of poly(vinyl alcohol) was added dropwise to the above reaction mixture under stirring in the dark, and the reaction was allowed to proceed overnight. After 24 h, dicyclohexylurea, which formed during the reaction, was filtered off. The filtrate was collected and purified by dialysis against Milli-Q water. After purification, the product was freeze-dried and stored at room temperature in the dark. The number of azidophenyl groups in the AzPhPVA was determined by ^{1}H NMR from the peak intensities of the azidophenyl protons at around 7 ppm, and those of the methylene and methylidyne protons of the polymer main chain at 1.5 and 3.9 ppm, respectively.

A polystyrene plate (2 × 2 cm) was cut from a cell culture polystyrene flask. The AzPhPVA was dissolved in water (200 μg/mL). The solution (100 μL) was placed on the polystyrene plate and air-dried at room temperature in the dark. The plate was covered with a patterned photomask and irradiated with ultraviolet light at an intensity of 5.0×10^3 μJ/cm^2 from a distance of 15 cm for 25 s. After irradiation, the plate was immersed in Milli-Q water and then sonicated to completely remove any unreacted polymer from the unirradiated areas. After complete washing, PVA-micropatterned surfaces were obtained.

Observation by scanning probe microscopy (SPM). An SPA-400 multi-function SPM unit (SII NanoTechnology Inc., Tokyo, Japan) connected to an SPI4000 controller was used for measurements in air and liquid in contact mode. The cantilevers used were OTR8 silicon nitride probes for measurement in air and OTR4 for measurement in liquid (Veeco Instruments, Santa Barbara, CA) having spring constants of 0.15 N/m and 0.02 N/m, respectively. All SPM measurements were made at room temperature. The polystyrene plates with the PVA-micropattern grafted surfaces were observed in a dry state in air and in a wet state in Milli-Q water. A 100 × 100 μm area of the sample was observed by SPM. The heights of the micropatterned PVA were measured from the SPM images. Nine spots from the topographic images of each kind of grafted pattern were used to measure the mean heights and standard deviations. The data were expressed as the average ± standard deviation of the nine spots.

Cell culture. Human bone marrow-derived MSCs (MSCs) were obtained from Osiris (Worthington Biochemical, Lakewood, NJ) at passage 2. The cells were seeded in T-75 culture flasks (Iwaki Glass, Tokyo, Japan) using proliferation medium purchased from Lonza (Walkersville, MD). The proliferation medium contained 440 mL MSC basal medium, 50 mL mesenchymal cell growth supplement, 10 mL 200 mM L-glutamine, and 0.5 mL penicillin/streptomycin mixture. After reaching confluence, the cells were further subcultured once and used at passage 4. The cells were collected by treatment with trypsin/EDTA solution and suspended in serum (control) medium at a density of 2.50×10^4 cells/mL. The serum medium was composed of Dulbecco's modified Eagle's medium (Sigma–Aldrich, St. Louis, MO) supplemented with 4500 mg/L glucose, 584 mg/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.1 mM nonessential amino acid, 0.4 mM proline, 50 mg/L ascorbic acid, and 10% fetal bovine serum (FBS). The micropatterned polystyrene plates were put in a cell culture dish and a glass cylinder (10 mm in both bore diameter and height) was placed over each PVA-micropatterned polystyrene plate. Cell suspension solution (0.157 mL/well) was added into the glass cylinder (initial cell density: 5.00×10^3 cells/cm^2). The MSCs were cultured in the serum medium for 3 days. To induce adipogenic differentiation, the MSCs were cultured in adipogenic differentiation medium [22] consisting of DMEM serum medium supplemented with 1 μM dexamethasone and 0.5 mM methyl-isobutylxanthine, insulin (10 μg/mL), and 100 μM indomethacin. The cells on the micropatterned surfaces were incubated in the serum medium or the serum control medium for 1, 2, and 3 weeks and used for cell staining. Alternatively, for analyzing gene expression, the MSCs were seeded into 6-well cell culture plates at respective densities of 5.00×10^3, 1.00×10^4, 2.00×10^4, and 3.00×10^4 cells/cm^2 and cultured in the adipogenic differentiation medium or the serum control medium for 1, 2, and 3 weeks.

Oil Red O staining. The cultured cells were rinsed with phosphate buffer saline (PBS) three times, fixed with 4% paraformaldehyde for 1 h at 4 °C, and then stained with fresh Oil Red O solution for 2 h. The Oil Red O solution was prepared by mixing three parts stock solution (0.5% in isopropanol; Sigma) with two parts water and filtering through a 0.2-μm filter. The stained cells were washed three times with PBS to remove any background Oil Red O stain. Photomicrographs were captured by a optical microscope with a DP-70 CCD camera (Olympus, Tokyo, Japan).

RNA isolation and quantitative real-time RT-PCR analysis. After culture for 1 and 3 weeks in the 6-well cell-culture plates, the cells were washed with PBS three times, and 1 mL of Isogen reagent (Nippon Gene, Toyama, Japan) was added to each well. Total RNA was extracted following the manufacturer's protocol. Total RNA (1 μg) was reversely transcribed into cDNA using a random hexamer primer (Applied Biosystems, Foster City, CA) in a 20 μl reaction. An aliquot (1 μl) of 10-times diluted reaction solution was used for each 25 μl real-time PCR reaction together with 300 nM forward and reverse primers, 150 nM probes, and qPCR MasterMix (Eurogentec, Seraing, Belgium). Taqman® probes and primer pairs for fatty acid binding protein-4 (FABP4, assay identification number Hs00609791_m1) [23], lipoprotein lipase (LPL, assay identification number Hs00173425_m1) [23], and peroxisome proliferator-activated receptor γ2 (PPARγ2, assay identification number Hs01115510_m1), which are assay-on-demand gene expression products, were obtained from Applied Biosystems. Real-time quantitative RT-PCR analysis was performed using a housekeeping gene GAPDH and 18S ribosomal RNA, as previously described. After an initial incubation step of 2 min at 50 °C and denaturation for 10 min at 95 °C, 40 cycles of PCR (95 °C for 15 s, 60 °C for 1 min) were performed. Reactions were performed in triplicate. 18S ribosomal RNA levels were used as endogenous controls and gene expression levels relative to GAPDH were calculated using the comparative Ct method. To calculate the means and standard deviations, three samples under each condition were measured. A one-way analysis of variance (ANOVA) with Tukey's post hoc test for multiple comparisons was used for statistical analysis. A value of $p < 0.05$ was considered statistically significant.

Results and discussion

PVA-micropatterned surface

Poly(vinyl alcohol) was micropatterned on polystyrene plates cut from cell culture flasks. First, photoreactive AzPhPVA was synthesized by coupling poly(vinyl alcohol) with 4-azidobenzoic acid. The introduction of photoreactive azido groups in AzPhPVA was confirmed by the appearance of peaks assigned to the phenylazido proton around 7 ppm in the ^{1}H NMR spectrum. The percentage of the hydroxyl groups in the PVA coupled with the azidophenyl groups was 2.1%. Subsequently, the photoreactive AzPhPVA was micropatterned on polystyrene plates by photolithography (Fig. 1A). An aqueous solution of AzPhPVA was eluted on a polystyrene plate and air-dried in the dark. The cast plate was covered with a photomask and photoirradiated. The photomask pattern was composed of alternate UV-transparent and UV-nontransparent stripes (Fig. 1B). The UV-nontransparent stripes were 200 μm wide and the UV-transparent stripes had gradient widths from 20 to 1000 μm. AzPhPVA in the irradiated areas should be intermolecularly and intramolecularly crosslinked and grafted to the poly-