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Micropit surfaces designed for accelerating osteogenic differentiation of murine mesenchymal stem cells via enhancing focal adhesion and actin polymerization

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ABSTRACT

Recent reports demonstrate that enhanced focal adhesion (FA) between cells and the extracellular matrix (ECM) and intracellular actin polymerization (AP) upregulates cellular functions such as proliferation, stem-cell fate and differentiation. Purposed to accelerate osteogenic differentiation, enhancement of FAs and AP of cells was induced by adding a tailor-made micropit (tMP, 3 × 3 μm^2) with different heights (2 or 4 μm). The tMP surface was examined for its differentiation efficiency using mouse mesenchymal stem cells, C3H10T1/2. Though the cell spreading area was not affected by the surface topography, cells on the tMP substrates had enhanced FAs which were significantly confined inside the micropits, increased actin polymerization and traction forces, and osteogenic differentiation. Further experiments with Y-27632 and Blebbistatin, which specifically regulate FA or AP functions, demonstrated that the tMP-induced acceleration of osteogenic differentiation was caused by the rho-associated, coiled-coil containing protein kinase (ROCK) and nonmuscle myosin II (NM II), which are key molecules of the RhoA/ROCK signaling pathway. The tMP is applicable as an osteo-active substrate for the instructive bone cell differentiation and population.

1. Introduction

As the demand for repairing degenerative tissues is becoming high with the aging population, there is a growing need for biofunctional materials that can induce stem cells into demanded cell types with reliable differentiation capability [1–3]. Although a number of biomaterials have been addressed for bone or musculoskeletal tissue engineering and regenerative medicine, there are still difficulties to secure consistent products due to the lack of osteoinductive effects and poor fabrication methods [4,5]. Stem cell fate and differentiation is regulated by several signal pathways initiated from specific kinds of stimulation in the microenvironment [6–8]. Among the numerous environmental cues, the physical or topographical properties of the extracellular matrix (ECM) to which cells adhere to is considered an indispensible parameter for cell fate and differentiation [9–13]. A growing number of reports have shown that functionally modified surfaces or particles on the biomaterials prompt specific cell behaviors such as cell adhesion, migration, proliferation and differentiation [14–18].

In this study, we fabricated a functional surface for cell culture that can easily enhance the osteogenic differentiation of mesenchymal stem cells (MSCs) for bone regeneration. The topography, geometry, and mechanical properties of the substrate can affect the FA and AP, which activates pathways that lead to osteogenic differentiation [9,19–22]. We designed a focal adhesion (FA)- and actin polymerization (AP)-enhancing tailor-made micropit (tMP, 3 × 3 μm) to test the effect of surface properties on the osteogenic differentiation of murine MSCs. Since the empirical average size of the FA on flat surfaces is small (<2 μm^2), we believe that the tMP area (9 μm^2) is sufficient to determine the FA size if the FAs are aggregated on the tMP [23].

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With the hypothesis that the tMP surface increases FAs and APs, and consequently upregulate the osteogenic differentiation (Fig. 1a), we initiated the fabrication of the tMP surfaces. We first determined if tMP surfaces enhance the FA and AP through imaging, and then evaluated the effect on osteogenic differentiation compared to those on a flat surface using quantitative RT-PCR, Western blotting, and immunostaining of key markers of osteogenesis. Finally, we further investigated the underlying mechanism by which the tMP influenced the osteogenic differentiation.

2. Materials and methods

2.1. Micropit substrates preparation

Using a silicon wafer mold, micropit substrates were prepared by curing a monomer solution with a crosslinking agent (10:1, w/w) in a 60 °C oven for 2 h. The resulting substrate was oxygen plasma treated for over 1 min and coated with fibronectin (Fn, 5 μg/ml, Sigma), an ECM protein, for 1 h at room temperature to improve the adhesive affinity between the cells and substrate.

2.2. Cell culture

Murine MSCs, C3H10T1/2, were used for the experiments. Cell were seeded on the Fn-coated substrates with Dulbecco's modified Eagle's Medium (DMEM, Gibco Invitrogen) with 4 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin (Invitrogen), 10% fetal bovine serum (FBS, Invitrogen). Cells were seeded on the tMP surfaces and flat substrate in the growth medium with osteogenic inducing factors (50 μg/ml L-ascorbic acid, 10 mM glycerophosphate and 100 nM dexamethasone).

2.3. Immunostaining

After washing with PBS, cells on the substrates were fixed in 3.7% paraformaldehyde for 15 min at room temperature and washed 3 times with PBS. The fixed cells were permeabilized with 0.2% Triton-X 100 for 3 min. After blocking with 1% bovine serum albumin (BSA, Sigma) for 15 min, cells were incubated with rhodamine phalloidin (Molecular Probes) and monoclonal anti–vinculin antibody (Sigma) or rabbit polyclonal anti-osteocalcin (Santa Cruz) as primary antibodies for 1 h. After incubation, cells were washed 3 times with PBS (10 min per wash), followed by the 1 h incubation with Alexa 488 conjugated rabbit anti-mouse antibody (Molecular Probes) or Alexa 488 conjugated goat anti-rabbit antibody (Molecular Probes) or 4′,6-diamidino-2-phenylindole (DAPI, Sigma). After washing 3 times with PBS, cells were mounted on a cover glass using a mounting agent (Vectashield, Vector Laboratories). Cells were observed with a laser scanning microscope (LSM 510 Meta CLSM, Zeiss) with a suitable objective lens such as Plan Neofluar 10× objective lens (Zeiss) and α Plan–FLUAR 100× objective lens (Zeiss). Fluorescence images were captured by a digital CCD camera (C7780–10, Hamamatsu Photonics) and analyzed by the Aquacosmos software tool (Hamamatsu Photonics) and ImageJ (NIH).

2.4. Field effect scanning electron microscope (FE-SEM)

Cells were fixed with 3% glutaraldehyde (Sigma) in PBS for 1 h. Cells were washed 3 times with 0.1 M sodium cacodylate at 4 °C and then post-fixed in 2% osmium tetroxide for 1 h at 4 °C. For dehydration, the cells were incubated in a graded series of ethanol (70, 80, 90, 95, and 100%) for 30 min each, and then coated with platinum before inspection. Cells were observed with FE-SEM (JEOL, JSM-7500F) at 1 kV.

2.5. Quantitative real time RT-PCR analysis

Total RNA was isolated from the cells on each substrate by using Trizol (Invitrogen) according to the manufacturer's protocol. Reverse transcription reactions for cDNA were executed using PrimeScript RT reagent Kit (TaKaRa) with following primers: GAPDH: forward 5′-AAATGGTGAAGGTCGGTGTG-3′, and reverse 5′-TGAAGGGGTCGTTGATGG-3′; ALP: forward 5′-AGTTACTGGCGACAGCAAGC-3′, and reverse 5′-GAGTGGTGTTGCATC GCG-3′; COL1: forward 5′-GCGAAGGCAACAGTCGCT-3′, and reverse 5′-CTTGGTG GTTTTGTATTCGATGAC-3′; OCN: forward 5′-TGAGGACCCTCTCTCTGCTC-3′, and reverse

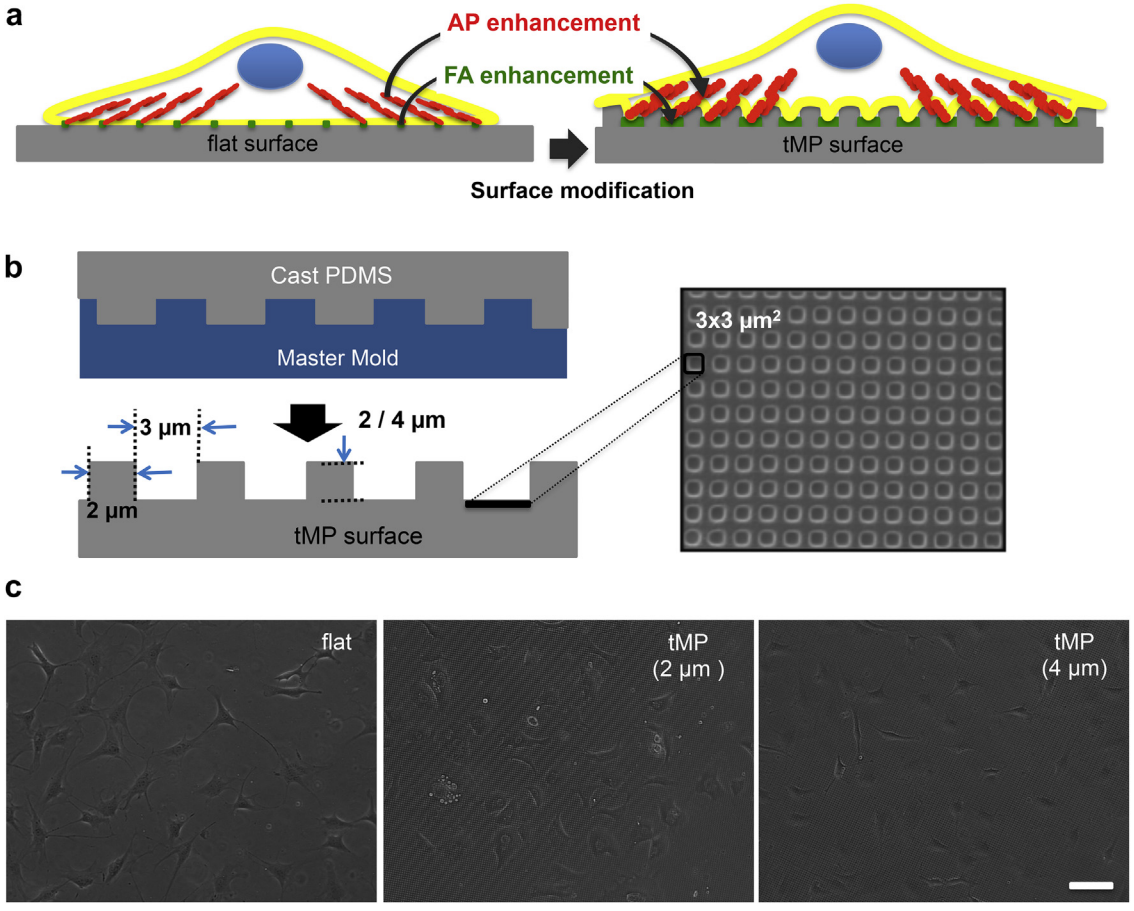

Fig. 1. (a) Schematic showing the strategy of the tMP bioactive surfaces which are intended to enhance focal adhesion (FA) and actin polymerization (AP) by the surface modification with rational parameters and (b) schematic showing preparation of the tMP surfaces which are intended to enhance FA and actin activation/polymerization and (c) phase contrast images of cells cultured on the prepared substrates 24 h after seeding (scale bar, 80 μm).