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A newly identified mechanism involved in regulation of human mesenchymal stem cells by fibrous substrate stiffness

Huihua Yuan [a,b], Yaxian Zhou [b,c], Ming-Song Lee [b], Yanzhong Zhang [a,*], Wan-Ju Li [b,c,*]

[a] College of Chemistry, Chemical Engineering & Biotechnology, Donghua University, Shanghai, China
[b] Department of Orthopedics and Rehabilitation, University of Wisconsin-Madison, Madison, WI, USA
[c] Department of Biomedical Engineering, University of Wisconsin-Madison, Madison, WI, USA

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ABSTRACT

Stiffness of biomaterial substrates plays a critical role in regulation of cell behavior. Although the effect of substrate stiffness on cell behavior has been extensively studied, molecular mechanisms of regulation rather than those involving cytoskeletal activities still remain elusive. In this study, we fabricated aligned ultrafine fibers and treated the fiber with different annealing temperatures to produce fibrous substrates with different stiffness. Human mesenchymal stem cells (hMSCs) were then cultured on these fibrous substrates. Our results showed that annealing treatment did not change the diameter of electrospun fibers but increased their polymer crystallinity and mechanical properties. The mRNA expression of *RUNX2* was upregulated while the mRNA expression of *scleraxis* was downregulated in response to an increase in substrate stiffness, suggesting that increased stiffness favorably drives hMSCs into the osteogenic lineage. With subsequent induction of osteogenic differentiation, osteogenesis of hMSCs on stiffer substrates was increased compared to that of the cells on control substrates. Cells on stiffer substrates increasingly activated AKT and YAP and upregulated transcript expression of YAP target genes compared to those on control substrates, and inhibition of AKT led to decreased expression of YAP and RUNX2. Furthermore, macrophage migration inhibitory factor (MIF) was increasingly produced by the cell on stiffer substrates, and knocking down MIF by siRNA resulted in decreased AKT phosphorylation. Taken together, we hereby demonstrate that simply using the annealing approach can manipulate stiffness of an aligned fibrous substrate without altering the material chemistry, and substrate stiffness dictates hMSC differentiation through the MIF-mediated AKT/YAP/RUNX2 pathway.

Statement of Significance

Stiffness of biomaterial substrates plays a critical role in regulation of cell behavior. Although the effect of substrate stiffness on cell behavior has been extensively studied, molecular mechanisms of regulation rather than those involving cytoskeletal activities still remain elusive. In this manuscript, we report our new findings that simply using the annealing approach can manipulate stiffness of an aligned fibrous substrate without altering the material chemistry, and substrate stiffness dictates human mesenchymal stem cell (hMSC) differentiation through the macrophage migration inhibitory factor-mediated AKT/YAP/RUNX2 pathway. The findings are novel and interesting because we have identified a new mechanism rather than those involving cytoskeleton activity, by which substrate stiffness regulates hMSC behavior.

1. Introduction

Mesenchymal stem cells (MSCs) are capable of differentiating into a variety of connective tissue cell types, such as osteoblasts, chondrocytes, adipocytes, myoblasts [1]. It has been reported that the extracellular matrix (ECM) delivers biochemical and biophysical signals to direct MSC differentiation [2–5]. Among the biophysical signals, ECM stiffness plays an important role in modulating

* Corresponding authors at: College of Chemistry, Chemical Engineering & Biotechnology, Donghua University, 2999 North Renmin Road, Shanghai 201620, China (Y.Z. Zhang). Department of Orthopedics and Rehabilitation, University of Wisconsin-Madison, 1111 Highland Avenue, WIMR 5051, Madison, WI 53705-2275, USA (W.-J. Li).

E-mail addresses: yzzhang@dhu.edu.cn (Y.Z. Zhang), li@ortho.wisc.edu (W.-J. Li).

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stem cell differentiation and functions [6,7]. It has been demonstrated that MSCs cultured on substrates with stiffness mimicking that of brain, muscle, or bone tissue preferentially become neurons, muscles, or osteoblasts, respectively [7]. Moreover, the early mesendoderm differentiation and terminal osteogenic differentiation of embryonic stem cells (ESCs) were both enhanced when ESCs were cultured on a stiff polydimethylsiloxane substrate compared to a soft one [8]. Hence, a deeper understanding of how stem cells respond to matrix stiffness is crucial for developing viable strategies to improve the efficiency of stem cell differentiation.

Recently, electrospun ultrafine fibers have attracted great interest in constructing biomimetic scaffolds for tissue engineering [9–11]. Previous studies have demonstrated that cell adhesion, proliferation, and differentiation can be regulated by the polymer chemistry and structural architecture of electrospun fibers [12–14]. However, there are few reports focusing on how the stiffness of electrospun fibers regulates MSC differentiation. Jiang et al. altered the stiffness of pullulan/dextran nanofibers by differential *in situ* crosslinking during electrospinning and they found stiff nanofibers promoted MSC neuronal differentiation compared to soft ones [15]. However, their approach of using different crosslinkers to change substrate stiffness also unavoidably alters chemistry of the material. It is therefore difficult to conclude if substrate stiffness, material chemistry, or both is the cause to regulation of cell response in their study. With that, it is important to have a setup capable of decoupling substrate stiffness from material chemistry for studying the effect of substrate stiffness on cell response. Recently, Baker et al. have used UV to modulate the stiffness of electrospun methacrylated dextran fibers without altering the fiber chemistry. They demonstrated that lower fiber stiffness permitted active cellular forces to recruit nearby fibers, dynamically increasing ligand density at the cell surface and promoting the formation of focal adhesions and related signaling [16].

The pro-inflammatory cytokine macrophage migration inhibitory factor (MIF) is produced by several cell types, including monocytes, macrophages, vascular smooth muscle cells, and cardiomyocytes [17–19]. Macrophage migration inhibitory factor plays multiple roles in mediating inflammation, apoptosis, autophagy, and carbohydrate metabolism [20–23]. It has also been shown that MIF production can be modulated in response to arterial stiffness [24]. Recent studies have shown that MIF regulates AKT activity in various types of cells to affect their behavior [25–28]. Our group has previously demonstrated that endogenous MIF produced in hMSCs under hypoxia activates AKT signaling to delay the progression of cellular senescence [28]. While a previous study has shown that a substrate with higher stiffness enhances osteogenic differentiation of MSCs through activation of AKT signaling [29], it is unclear whether MIF is involved in the regulatory mechanism. Moreover, yes-associated protein (YAP) is a transcription factor that is known to play a role in stiffness-mediated cell activities [30], which suggests that YAP may be a downstream molecule of the AKT signaling pathway.

In this study, we hypothesized that stiff substrates increase the expression of MIF in hMSCs, which in turn regulates AKT/YAP signaling to direct differentiation of hMSCs. To test the hypothesis, aligned fibrous substrates were fabricated by stable jet electrospinning (SJES), and then subjected to annealing treatment to alter the substrate stiffness. In addition to determining the effect of substrate stiffness on regulation of hMSC behavior, we were particularly interested in finding out whether the regulation is mediated through the MIF-mediated AKT/YAP pathway. We aimed to identify a new mechanism, rather than cytoskeleton-mediated regulation, of how hMSC activities are modulated by substrate stiffness.

2. Materials and methods

2.1. Electrospun aligned ultrafine fibers and annealing treatment

Aligned PLLA ultrafine fibers were electrospun as described in our pervious study [31]. Briefly, poly (ethylene oxide) (PEO, Mw ~5,000,000, Sigma-Aldrich, 1 w/v%)-doped poly(L-lactic acid) (PLLA, Mw 100,000, Polysciences, 5 w/v%) in 2,2,2-trifluoroethanol (TFE, Sigma-Aldrich) was prepared and then electrospun onto a rotatory shaft at the rotating speed of 1000 rpm to produce aligned ultrafine fibers. Electrospun aligned fibrous mats were placed in a vacuum oven for about 24 h to remove any residual organic solvent before further use. Annealing of fibers was conducted by first treating fibrous mats with the temperature at 65 °C (65PLLA) or 75 °C (75PLLA) under mechanical tension for 3 h and then allowing it to cool down under room temperature. Fibrous mats without annealing treatment were used as an untreated control (PLLA).

2.2. Characterization of annealing-treated aligned ultrafine fibers

Morphology of electrospun fibrous mats was analyzed by scanning electron microscopy (SEM, TM-1000, Hitachi) at an accelerating voltage of 8–10 kV. Prior to imaging, samples were sputter-coated with gold for 50 s. The average diameter of electrospun fibers was determined by measuring fibers shown in the SEM images using the ImageJ software. For each sample, an average of 50 ultrafine fibers was counted.

X-ray diffractometer (D/Max-2550 PC, Rigaku, Japan) was used to determine the X-ray diffraction (XRD) pattern of as-electrospun PLLA fibers with Cu Kα radiation in the range of 2θ around 5°~35°. To determine the surface roughness of fibrous substrates, atomic force microscopy (AFM, Dimension FastScan Bio, Bruker, Germany) was used to scan the surface of specimens.

Mechanical properties of substrates were determined using a tensile testing machine (H5K-S, Hounsfield, UK) equipped with a 50 N load cell. Rectangular-shaped specimens (50 mm × 10 mm × 0.10–0.15 mm) were stretched at a constant cross-head speed of 10 mm/min. Five specimens were tested for each type of samples. In addition to the analysis of tensile properties, the surface stiffness of fibrous substrates was determined by measuring mechanical properties of individual fibers subjected to nanoindentation using AFM. Spherical contact tips were applied in contact mode to image and indent fibers to determine Young's modulus based on the Hertz model.

2.3. Human MSC isolation and culture

With approval from the Institutional Review Board at the University of Wisconsin-Madison, bone marrow-derived hMSCs were harvested from femoral heads of patients undergoing total hip arthroplasty. Human MSCs were isolated following a previously described protocol. Briefly, after being curetted from the interior of femoral head and neck, whole bone marrow was mixed with Dulbecco's modified Eagle medium (DMEM; Gibco, Carlsbad, CA, USA). A syringe with an 18-gauge needle was used to filter out bone debris from the bone marrow/DMEM mixture. The collected medium was then centrifuged at 1000 rpm for 5 min. After removing the supernatant, the resulting cell pellet was reconstituted using 25 mL of Hank's Balanced Salt Solution (Invitrogen, Carlsbad, CA, USA), and then slowly added into a 50-mL conical tube containing 20 mL of Ficoll solution (GE Health, Pittsburgh, PA, USA). After centrifugation at 500 g for 30 min, mononuclear cells were collected and plated in cell culture flasks with culture medium composed of low-glucose DMEM, 10% fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA, USA) and antibiotics. The cells