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Effects of substrate stiffness on adipogenic and osteogenic differentiation of human mesenchymal stem cells

Wen Zhao [a,b,1], Xiaowei Li [c,1], Xiaoyan Liu [c], Ning Zhang [c,d,e], Xuejun Wen [c,d,f,g,h,*]

[a] Department of Orthopedic Surgery, The General Hospital of Chinese People's Liberation Army (301 hospital), Beijing, 100853, PR China
[b] Department of Orthopedic Surgery; Beijing Aerospace General Hospital, Beijing, 100076, PR China
[c] Clemson -MUSC Bioengineering Program, Department of Bioengineering, Clemson University, Charleston, SC 29425, USA
[d] Department of Regenerative Medicine and Cell Biology, Medical University of South Carolina, Charleston, SC 29425, USA
[e] Department of Microbiology and Immunology, Medical University of South Carolina, Charleston, SC 29425, USA
[f] Department of Orthopedic Surgery, Medical University of South Carolina, Charleston, SC 29425, USA
[g] East Hospital, Tongji University School of Medicine, Shanghai, 200120, PR China
[h] The Institute for Biomedical Engineering and Nano Science, Tongji University School of Medicine, Shanghai, 200092, PR China

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ABSTRACT

Substrate mechanical properties, in addition to biochemical signals, have been shown to modulate cell phenotype. In this study, we inspected the effects of substrate stiffness on human mesenchymal stem cells (hMSCs) derived from adult human bone marrow differentiation into adipogenic and osteogenic cells. A chemically modified extracellular matrix derived and highly biocompatible hydrogel, based on thiol functionalized hyaluronic acid (HA-SH) and thiol functionalized recombinant human gelatin (Gtn-SH), which can be crosslinked by poly (ethylene glycol) tetra-acrylate (PEGTA), was used as a model system. The stiffness of the hydrogel was controlled by adjusting the crosslinking density. Human bone marrow MSCs were cultured on the hydrogels with different stiffness under adipogenic and osteogenic conditions. Oil Red O staining and F-actin staining were applied to assess the change of cell morphologies under adipogenic and osteogenic differentiation, respectively. Gene expression of cells was determined with reverse transcription polymerase chain reaction (RT-PCR) as a function of hydrogel stiffness. Results support the hypothesis that adipogenic and osteogenic differentiation of hMSCs are inclined to occur on substrate with stiffness similar to their *in vivo* microenvironments.

1. Introduction

Stem cell niche is defined as a specialized microenvironment required for stem cells to retain their "stemness" [1]. It is characterized by a battery of niche proteins including both soluble factors and adhesive and membrane components associated with supporting cells. The ability of stem cell niche to maintain stem cell function is dependent upon niche physico-chemical properties such as cellular compositions and biochemical as well as mechanical properties of the microenvironments. By regulating the delicate balance between differentiation-inducing and -inhibiting factors, the properties of the niche dictate the balance between self-renewal and differentiation/stem cell fate specifications [2,3]. In particular, substrate biomechanics [4] as well as the topography of the microenvironment [5] may modulate stem cell viability, proliferation, differentiation, and 3-dimensional (3D) organization in a way similar to biochemical signals. It has been shown that substrates of varying stiffness may lead to dramatic changes in stem cell lineage specifications [6]. For example, human mesenchymal stem cells (hMSCs) cultured on collagen-coated polyacrylamide gels differentiated to neuronal, muscle, and bone cells as a function of increasing hydrogel stiffness [6], suggesting the potential of substrate mechanical properties in the directed differentiation of stem cells toward specific lineages. Therefore, creating engineered analogies to recapitulate the central characteristics of natural stem cell niche in the tissue using biomaterials with tunable mechanical properties may present a powerful tool in manipulating stem cell function, as well as controlling the fate and trajectory of stem cell differentiation.

Toward this end, biomaterials have been rationally designed and engineered to mimic native stem cell niche. Among them, hydrogels represent the major category of materials attributing to their biomimetic physiochemical properties to stem cell niche [6–8], such as tailorable stiffness/softness, and high water content, which allow high permeability for oxygen, nutrients, and metabolites to support cell survival, growth, and tissue regeneration. Further, injectable, biodegradable hydrogels would allow coincident releases of biomolecules loaded in the hydrogels, fill in irregular shape of the tissue defect sites where stem cell therapy is intended, and facilitate gel–host tissue integration for functional tissue regeneration. Upon gelation, hydrogels become

* Corresponding author at: Clemson–MUSC Bioengineering Program, Department of Bioengineering, Clemson University, Charleston, SC 29425, USA. Tel.: +1 843 876 2395; fax: +1 843 792 0664.
E-mail address: xjwen@clemson.edu (X. Wen).
[1] Contributed equally to this work.

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elastic materials with many tissue-like properties. Using different synthesis schemes and by varying the amount of crosslinkers, hydrogels with adjustable biochemical and biomechanical properties can be produced. In this study, thiol-modified hyaluronan (HA-SH) and thiol-modified gelatin (Gtn-SH) were used as a model hydrogel system, which can be crosslinked by poly(ethylene glycol) tetra-acrylate (PEGTA). This material offered a flexible biochemical composition, biomechanical compliance, and simplicity of use. This series of hydrogels can provide sustained release of biologically active soluble factors, such as hepatocyte growth factor (HGF), for over three weeks *in vitro* [9]. In addition, HGF released from the hydrogel attracted hMSCs to migrate into the hydrogels following the HGF gradient established by the HGF-loaded hydrogels [9]. By varying the amount of PEGTA crosslinker, the stiffness of the hydrogel and the gelation rate can be readily tuned. The stiffness of the hydrogel can be tuned from 1 Pa (soft) to 4 kPa (rigid) with magnitude increments, which span the stiffness of native brain (0.1–1 kPa) and adipose (1–10 kPa) tissue [10,11].

Human mesenchymal stem cell (hMSC), a type of adult stem cells that may be derived from bone marrow, cord blood, synovial membrane, and so on, possesses broad differentiation capacity. In addition to their lack of tumorigenicity *in vivo* [12], studies [13–15] have documented the ability of hMSCs to differentiate into mesodermal and non-mesodermal lineages including neurogenic, myogenic, adipogenic, osteogenic, and chondrogenic lineages, suggesting great clinical potential of these stem cells for therapy. Within this broad spectrum of potential fates, adipogenic and osteogenic lineages are the two primary lineages that modulate normal bone homeostasis as it pertains to the function of bone marrow. Intricate pathways that regulate the delicate balance between adipogenic and osteogenic differentiation of MSCs exist [16–19]. While biochemical cues such as soluble factors, and adhesive molecules and other biochemical cues are critical for the differentiation induction of MSCs into osteogenic [20,21], adipogenic [22], and chondrogenic [23,24] lineages, the role of a surrounding material environment is equally important. Like other types of adult stem cells, hMSCs may circulate away from their niche, dive into circulation, home to different tissues and organs, and differentiate within a range of tissue microenvironments to contribute to tissue regeneration and homeostasis. Their differentiation is highly sensitive to the environmental elasticity [6].

To elucidate the effect of substrate biomechanical properties on stem cell differentiation, we have cultured hMSCs on the surface of hydrogels of different stiffness. Our objective was to interrogate the role of biomechanical microenvironment on stem cell lineage specifications. Adipogenic and osteogenic differentiation will be examined as two model lineages and compared. The information derived from the commitment to these two lineages will help to design appropriate materials for the re-establishment of natural bone and bone marrow structures. Our data have indicated that hydrogels with varying biomechanical properties influence the fate specifications of hMSCs along both adipogenic and osteogenic pathways, and yet they have greater impact on osteogenic lineage specification. These results provide insights into the roles of substrate biomechanical properties on fate specifications of hMSCs and validate the hydrogels of tunable mechanical properties as candidate materials to create specified niches for stem cells.

2. Materials and methods

2.1. Materials

Thiol functionalized hyaluronic acid (HA-SH) solution (Glycosan BioSystems Inc., Salt Lake City, UT) and thiol functionalized human recombinant gelatin (Gtn-SH) solution were prepared in sterile deionized distilled water under aseptic conditions. Poly (ethylene glycol) tetra-acrylate (MW, 10 kDa, PEGTA) was obtained from Creative PEGWorks (Winston Salem, NC). Nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT–BCIP) kit was purchased from Sigma Aldrich (St. Louis, MO). Alexa Fluor-488 phalloidin and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) were obtained from Molecular Probes (Eugene, OR). All other chemical reagents were purchased from Sigma Aldrich (St. Louis, MO).

2.2. Preparation of hydrogel substrates

HA-SH solution and Gtn-SH solution were prepared in sterile deionized distilled water under aseptic conditions. A 5% (w/v) PEGTA stock solution was prepared by dissolving PEGTA powder in phosphate buffered saline (PBS). Two volumes of HA-SH solution and two volumes of Gtn-SH solution were then mixed with one volume of PEGTA solution with a range of crosslink densities to form hydrogels with different stiffness.

For rheological study, hydrogel solutions with varying PEGTA concentrations (5%, 2.5%, 1.75%, 1%, 0.5%, 0.25%, and 0) were mixed on the steel plate geometry and inspected by oscillatory shear rheometry immediately. As substrate for hMSC culture, hydrogel solutions with different crosslink concentrations (2.5%, 1.75%, and 0.25%) were added to the wells of a 48-well culture plate and cured for overnight to stabilize PEGTA mediated crosslinking.

2.3. Oscillatory shear rheometry of hydrogels

An ARG2 rheometer (TA Instruments Inc.) with standard steel parallel-plate geometry of 25 mm diameter was used for the rheological characterization of all hydrogel samples. The test methods employed were oscillatory time sweep, frequency sweep and stress sweep. The time sweep was performed to monitor the *in situ* gelation of the hydrogel solutions at 37 °C. The test, which was operated at constant frequency (1 Hz) and strain (5%) and terminated after 60 min, recorded the temporal evolution of shear storage modulus, G′ and the shear loss modulus, G″. The stress sweep was set up by holding the temperature at 37 °C and constant frequency (1 Hz) while increasing the stress level from 1 to 10 Pa. The applied range of 1–10 Pa was found to be safe-for-use from a prior experiment where we determined the linear viscoelastic region (LVR) profiles of the hydrogels by shearing them until structure breakdown. We also subjected hydrogels to a frequency sweep at 50% of their respective ultimate stress levels. At this fixed shear stress and temperature (37 °C), the oscillatory frequency was increased from 0.1 to 100 Hz and the G′ was recorded [25].

2.4. Cell culture

Human bone marrow-derived mesenchymal stem cells (hMSCs) were obtained from Lonza (Allendale, NJ). Human MSCs were maintained in growth media (GM) (Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS), 0.3 mg/mL glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin; PT-3001, Lonza). Human MSCs were used for the study before passage 3. Human MSCs were seeded on the hydrogels at 3000 cells/cm^2 and cultured for 21 days in a 37 °C, 5% CO_2, and 95% humidity incubator. For adipogenic differentiation, hMSCs were exposed to three cycles of adipogenic induction media (AIM, 0.5 mM isobutylmethylxanthine, 1 μM dexamethasone, 10 μg/mL insulin, and 200 μM indomethacin; PT-3102B, Lonza) for 4 days and then adipogenic maintenance media (AMM, 10 μg/mL insulin; PT-3102A, Lonza) for 3 days. Osteogenic induction was initiated using osteogenic induction media (OIM, 100 nM dexamethasone, 10 mM β-glycerophosphate, and 50 μM ascorbic acid-2-phosphate; PT-3002, Lonza) for 21 days of cultivation. Following the *in vitro* cultivation period, hMSCs were evaluated for their extent of osteogenesis or adipogenesis for all conditions.

2.5. Oil Red O staining

Morphological change of the cells cultured with adipogenic medium was evaluated by Oil Red O staining. Adipogenesis was indicated by the accumulation of neutral lipid vacuoles. The staining was performed at