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The effects of interactive mechanical and biochemical niche signaling on osteogenic differentiation of adipose-derived stem cells using combinatorial hydrogels

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ABSTRACT

Stem cells reside in a multi-factorial environment containing biochemical and mechanical signals. Changing biochemical signals in most scaffolds often leads to simultaneous changes in mechanical properties, which make it difficult to elucidate the complex interplay between niche cues. Combinatorial studies on cell–material interactions have emerged as a tool to facilitate analyses of stem cell responses to various niche cues, but most studies to date have been performed on two-dimensional environments. Here we developed three-dimensional combinatorial hydrogels with independent control of biochemical and mechanical properties to facilitate analysis of interactive biochemical and mechanical signaling on adipose-derived stem cell osteogenesis in three dimensions. Our results suggest that scaffold biochemical and mechanical signals synergize only at specific combinations to promote bone differentiation. Leading compositions were identified to have intermediate stiffness (~55 kPa) and low concentration of fibronectin (10 μg ml^{-1}), which led to an increase in osteocalcin gene expression of over 130-fold. Our results suggest that scaffolds with independently tunable niche cues could provide a powerful tool for conducting mechanistic studies to decipher how complex niche cues regulate stem cell fate in three dimensions, and facilitate rapid identification of optimal niche cues that promote desirable cellular processes or tissue regeneration.

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1. Introduction

Stem cells hold great promise for tissue regeneration due to their ability to self-renew and differentiate into specific tissue types. However, a long-standing bottleneck in the stem-cell-based approach to tissue regeneration is the lack of understanding of how stem cell fate is regulated in three dimensions. Stem cells reside in a highly complex niche in vivo where a variety of microenvironmental cues form an intertwined signaling regulatory network that maintains stem cell fate and function [1,2]. Both biochemical cues such as soluble growth factors and cytokines, extracellular matrix (ECM) molecules, as well as mechanical cues such as intrinsic matrix stiffness and extrinsic forces, are crucial regulators of stem cell fate [3]. Extensive studies have been performed to investigate how stem cells respond to individual types of microenvironmental cues. However, how the complex interplay among niche cues collectively influence stem cell fate and function remains largely unknown. Recently, combinatorial screening of stem cell interactions with material libraries has emerged as a novel approach to achieve rapid identification of optimal niche signals with reduced materials and costs [4–8]. These combinatorial screening studies showed that the stem cells respond to interactive niche signals in a non-linear manner, highlighting the importance of examining the response of stem cells to interactive niche signals in a systematic manner to elucidate how they collectively regulate stem cell fate in vivo [4,5].

In conventional three-dimensional (3-D) culture systems, various microenvironmental cues are often intertwined and cannot be individually controlled. For instance, type I collagen is widely used for tissue engineering applications, but increasing its concentration to increase biochemical ligand density will also lead to simultaneous change in the mechanical stiffness of the matrix

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[9,10]. Given the complex interplay among different microenvironmental cues, a 3-D culture system that allows for the independent control of individual microenvironmental cues would help elucidate how stem cells respond to interactive niche signals in three dimensions. Recent combinatorial screening studies on stem cell–niche interactions have shed light on stem cell–microenvironmental cue interactions. However, most work to date were conducted on two-dimensional (2-D) surfaces [4,6,7], while the architecture of the stem cell niche in vivo is three dimensional. Cells in the body reside in a 3-D environment and previous work has highlighted that cell behavior in three dimensions may vary significantly from cell behavior in two dimensions [11–13]. While 3-D combinatorial platforms have been developed to examine the effects of interactive biochemical cues on stem cell adhesion and differentiation [5,8], how biochemical cues interact with mechanical cues to regulate stem cell fate remains largely unknown. Recent studies have revealed the critical role of mechanical cues in directing stem cell differentiation [14]. In particular, matrix stiffness together with adhesion-ligand presentations has been shown to regulate stem cell fate in three dimensions [15,16]. However, platforms that allow the examination of stem cell responses to interactive biochemical and mechanical cues remain lacking.

The goal of this study is to develop novel 3-D combinatorial hydrogels with independent control of biochemical and mechanical properties to facilitate the analysis of interactive niche signaling on stem cell osteogenesis in three dimensions. We hypothesized that biochemical and mechanical cues of the microenvironment interact in a non-linear manner in regulating stem cell osteogenesis in three dimensions. To vary the mechanical properties of the hydrogels, poly(ethylene glycol) diacrylate (PEGDA) with varying molecular weight or concentration was used [17–19]. To vary the biochemical cues within the combinatorial hydrogels, different amounts of fibronectin (FN) and laminin (LN) were incorporated into the hydrogel network due to their importance in cell signaling and cell adhesion [5,20–25]. Type I collagen, the most abundant protein found in bone extracellular matrix, was included at a constant concentration to facilitate cell adhesion. We chose to examine the osteogenesis of adipose-derived stem cells (ADSCs) due to their relative abundance, ease of isolation and potential to differentiate into bone lineage [26,27]. ADSCs were encapsulated in combinatorial hydrogels for 21 days and outcomes were examined using gene expression, biochemical assays and immuonfluorescence staining. In addition, to examine the role of matrix stiffness on ADSC osteogenesis, blebbistatin, a small molecule inhibitor for non-muscle myosin II, was also added to the ADSC culture in combinatorial hydrogels and the gene expression of non-muscle myosin II isoforms (IIA and IIB) and osteocalcin were quantified.

2. Materials and methods

2.1. Cell culture

Human adipose-derived stem cells (hADSCs) were isolated from excised human adipose tissue from informed and consenting patients following procedures as previously described [26]. HADSCs were subcultured upon 90% confluence until passage 5 before use for all experiments in growth medium consisting of Dulbecco's minimal essential medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco), 100 units ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin.

2.2. Combinatorial hydrogels

To generate combinatorial hydrogels with independently tunable biochemical and mechanical properties, both synthetic and extracellular-matrix-derived natural polymers were used. To tune the mechanical properties of hydrogels, synthetic polymer poly(ethylene glycol)-diacrylate (PEGDA) with different molecular weights (3400 or 5000) were prepared in sterile Dulbecco's phosphate-buffered saline (dPBS) and used at a final concentration of 10%, 15%, and 20% (w/v) containing photoinitiator Igracure D2959 (0.05% (w/v)) (Ciba Specialty Chemicals, Tarrytown, NY, USA). Collagen I (3.0 mg ml^{-1}, BD Biosciences, San Jose, CA, USA) was incorporated in all hydrogel compositions and mixed with PEGDA solution to reach a final concentration of 1 mg ml^{-1}, which provided adhesion sites for hADSCs to facilitate mechanosensing. To tune the biochemical properties of hydrogels, fibronectin (FN) (10 and 25 μg ml^{-1}) and laminin (LM) (10, 50 and 100 μg ml^{-1}) were interspersed in the hydrogel at different final concentrations. Specifically, FN and LN stock solutions were added to the collagen I solution prior to gelation such that they were physically entrapped and interdispersed in the hydrogel network after gelation. PEGDA hydrogels with collagen I but no FN and LM were included as control. Passage 6 hADSCs were homogeneously suspended in the hydrogel solutions with varying compositions at a cell density of 15 million ml^{-1}. The cell–hydrogel mixture (50 μl) was loaded into 96-well plates and exposed to UV light (365 nm, 4 mW cm^{-2}) for 5 min to induce photocrosslinking of the PEGDA network. The hydrogels were further incubated at 37 °C for 1 h to induce collagen I gelation. The resulting hydrogel microarrays consisted of 42 different compositions in total (Table 1) and all experiments were conducted in triplicates. All cell-laden hydrogels were then transferred to 24-well culture plates and cultured in the presence of osteogenic medium. Cell viability in all 42 hydrogel compositions was assessed 24 h post-encapsulation using LIVE/DEAD Cell Viability Assay Kit (Molecular Probes) with calcein-AM and ethidium homodimer following the manufacturer's protocol.

2.3. In vitro cultivation

All hydrogel samples were cultured at 37 °C in 5% CO_2 in 2 ml of osteogenic medium for 21 days. Osteogenic medium consists of high-glucose Dulbecco's modified Eagle's medium (DMEM; GIBCO), 100 nM dexamethasone (Sigma), 50 mg ml^{-1} ascorbic acid 2-phosphate (Sigma), 10 mM β-glycerophosphate (Sigma), 10% fetal bovine serum (Gibco), 100 units ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin (Gibco). Medium was changed three times per week.

2.4. Mechanical testing

Unconfined compression tests were conducted using an Instron 5944 materials testing system (Instron Corporation, Norwood, MA) fitted with a 10 N load cell (Interface Inc., Scottsdale, AZ, USA). The test set-up consisted of custom-made aluminum compression platens lined with PTFE to minimize friction. Acellular hydrogels were fabricated and equilibrated in PBS solution for 24 h at room temperature prior to mechanical testing. Before each test, specimen diameter (~6 mm) and thickness (~3 mm) were measured. All tests were conducted in PBS solution at room temperature. A preload of ~5 mN was applied to ensure the hydrogel surface was in full contact with the upper platen. The upper platen was then lowered at a rate of 1% strain s^{-1} to a maximum strain of 15%. The resulting stress–strain curves were fitted with a second-order polynomial and the compressive tangent moduli were determined at strain values of 15%.

2.5. Calcium assay

Lyophilized hydrogel samples were homogenized in 0.5 M HCl and vigorously vortexed for 16 h at 4 °C. The supernatant was

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