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The combined effects of matrix stiffness and growth factor immobilization on the bioactivity and differentiation capabilities of adipose-derived stem cells

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ABSTRACT

Biomaterial designs are increasingly incorporating multiple instructive signals to induce a desired cell response. However, many approaches do not allow orthogonal manipulation of immobilized growth factor signals and matrix stiffness. Further, few methods support patterning of biomolecular signals across a biomaterial in a spatially-selective manner. Here, we report a sequential approach employing carbodiimide crosslinking and benzophenone photoimmobilization chemistries to orthogonally modify the stiffness and immobilized growth factor content of a model collagen-GAG (CG) biomaterial. We subsequently examined the singular and combined effects of bone morphogenetic protein (BMP-2), platelet derived growth factor (PDGF-BB), and CG membrane stiffness on the bioactivity and osteogenic/adipogenic lineage-specific gene expression of adipose derived stem cells, an increasingly popular cell source for regenerative medicine studies. We found that the stiffest substrates direct osteogenic lineage commitment of ASCs regardless of the presence or absence of growth factors, while softer substrates require biochemical cues to direct cell fate. We subsequently describe the use of this approach to create overlapping patterns of growth factors across a single substrate. These results highlight the need for versatile approaches to selectively manipulate the biomaterial microenvironment to identify synergies between biochemical and mechanical cues for a range of regenerative medicine applications.

1. Introduction

The development of biomaterial tools for a range of tissue engineering applications increasingly relies on the coordinated presentation of multiple instructive signals. These efforts are often inspired by the native extracellular matrix (ECM), where cells receive cues from an assortment of solution phase [1–9] and substrate-supported stimuli [10–15]. The ECM is a complex biomolecular network secreted by cells that serves as a mechanical and structural support within which biomolecular cues can be presented in spatially [16–19] and temporally [20,21] defined manners. Given the regulatory role played by the ECM in a multitude of important processes such as tissue development [22] and repair [19,23], it is not surprising biomaterials platforms are increasingly trying to regulate the coordinated presentation of multiple classes of ECM-inspired signals. However, untangling the interactions between mechanical and biochemical cues is often complicated. New approaches are needed to both elucidate the effects of and then recapitulate the functional properties of such complicated in vivo microenvironments.

In the context of musculoskeletal regeneration, a wide range of studies have concentrated on coordinated presentation of biomolecular signals. Efforts initially focused on presenting a bioactive dose of a given single factors (tenogenesis: IGF-1 [3,24,25], PDGF [9], GDF-5 [26], bFGF [7,27,28]; osteogenesis: BMP-2 [29,30]; angiogenesis: VEGF [31,32]). Our previous work using collagen-GAG biomaterials demonstrated enhanced tenocyte chemotaxis (via IGF-1) and proliferation (via PDGF-BB) in a dose-dependent

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manner [3]. Moving towards more complex tissue injuries, Thomopoulos et al. demonstrated single factor (BMP-2) supplementation can promote healing at the tendon–bone interface [30]. More recently, efforts have begun to explore the potential of coordinated presentation of multiple cues [1,2,6,8]. Borselli et al. showed a synergistic response in muscle cells with combined delivery of angiogenic and myogenic growth factors [1]. We previously reported advantages of coordinated growth factor presentation for promoting phenotypic stability and proliferation [6], but also found unintended consequences of multi-factor supplementation in the context of MSC-guided regenerative medicine applications [33]. There are often trade-offs in stem cell proliferation and differentiation, which suggests that multiple cues may need to work in tandem to elicit the desired response. Notably, Tan et al. demonstrated that GDF-5 induced tenogenic differentiation in MSCs without negatively impacting proliferation [26], while other studies have looked at multiple cues to promote proliferation (e.g. PDGF) and tenogenesis simultaneously [9,34].

While solution phase supplementation in the media is the most straightforward method to provide instructive biomolecular cues in vitro, it is limited by diffusion and a lack of spatial localization. Inspired by the biomolecular tethering to the native ECM, recent efforts have suggested that immobilized growth factors can enhance bioactivity. A range of methods (e.g., carbodiimide crosslinking [12,29,35], biotin-avidin linkages [36], and 'click' chemistries [37]) have recently been reported for covalently attaching growth factors to collagen-based scaffolds. However, these approaches did not include the ability to control the spatial distribution of these biomolecules. In addition, the same crosslinking techniques which are used to immobilize growth factors can also increase biomaterial stiffness. However, biomaterial mechanical properties can have both direct and indirect effects on cell, and particularly MSC, response. The mechanical properties of the matrix are also known to play profound roles impacting cell fate, with a range of stem cells showing particular sensitivity to mechanical stiffness [38–43], making it potentially difficult to assess the individual impact of matrix-immobilized growth factors.

Increasing evidence suggests that mechanical and biomolecular signals may act in a more coordinated manner. Recently Allen et al. reported that matrix stiffness differentially primed the TGFβ signaling pathway in the context of MSC-chondrogenesis [44], suggesting the mechanical stiffness of the environment impacts how sensitive a cell may be to an exogenous factor. Further, Zouani et al. and Tan et al. both demonstrated different types of coordinated responses for MSCs to matrix stiffness and BMP-2 presentation [40,45]. Notably, Zouani et al. demonstrated a minimum stiffness (3.5 kPa) for MSCs to be responsive to BMP-2 with no synergy between matrix stiffness and BMP-2 dose [40], while Tan et al. reported a synergetic effect of matrix stiffness and BMP-2 [45]; however in this case, matrix stiffness was modified via hydrogel density, which likely significantly altered the microstructural cues presented to the cells. Both of these studies support the contention that MSCs integrate multiple extrinsic signals in the context of fate decisions [46–48]. However, these reports also motivate the development of new classes of biomaterials able to orthogonally modify matrix structure, stiffness, and biomolecule incorporation, as well as approaches that are amenable to spatial control over biomolecule incorporation, in order to better control stem cell differentiation and proliferation. Such platforms offer unique potential for the field of tissue engineering and regenerative medicine to more efficiently and effectively utilize structural, mechanical, and biomolecular signals that have yet to be fully realized.

Herein we report a strategy that allows for independent manipulation of the mechanical properties and spatially controlled presentation of biomolecular cues using a collagen-GAG (CG) biomaterial platform. CG biomaterials have been used for a range of soft (e.g., skin, peripheral nerve) [49–51] and hard/musculoskeletal (e.g., bone, cartilage, tendon) [52–54] tissue engineering applications, making them an attractive target for technologies to selectively incorporate exogenous biomolecular signals. We have recently demonstrated approaches to orthogonally modify the microstructural [3] and mechanical [55] properties of, as well as approaches to transiently [20] or covalently [6,56] modify growth factor presentation within these CG biomaterials. Here we build on a previously reported benzophenone (BP) photolithography approach to spatially control immobilization of biomolecules to a CG biomaterial [57]. In this work, we explore the integration of a separate crosslinking approach to allow orthogonal manipulation of matrix stiffness of the density of immobilized biomolecular signals (BMP-2, PDGF-BB). We subsequently describe the individual and combined impacts of matrix mechanical (elastic modulus) cues and immobilized BMP-2/PDGF-BB growth factors on adipose-derived mesenchymal stem cell (ASC) bioactivity. This combined technological approach provides an enabling platform to map synergies between multiple biochemical and mechanical cues across a single biomaterial platform for a range of tissue engineering applications.

2. Materials and methods

All reagents were purchased from Sigma–Aldrich (St. Louis, MO) unless otherwise noted. Phosphate buffered saline buffer (PBS) was reconstituted and pH adjusted to 7.4.

2.1. Preparation of CG membranes

CG membranes were prepared via a previously described evaporative process [58,59]. Briefly, a CG suspension was prepared from type I collagen (1.0% w/v) isolated from bovine Achilles tendon and chondroitin sulfate (0.1% w/v) derived from shark cartilage in 0.05 M acetic acid. The suspension was homogenized at 4 °C to prevent collagen gelatinization during mixing [49]. The CG suspension was degassed, pipetted into a petri dish, and allowed to evaporate under ambient conditions to produce a film. Circular membrane specimens (8 mm dia.) were cut using a biopsy punch (Integra-Miltex, York, PA) and stored in a desiccator until use.

2.2. Chemical crosslinking of CG membranes to modulate stiffness

Prior to use, CG membranes were hydrated in ethanol followed by PBS. They were subsequently crosslinked using carbodiimide chemistry [35,55] for 1 h in a solution of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (NHS) at molar ratios of 1:1:5, 5:2:5, 5:20.7:1, 5:2:1 EDC:NHS:COOH where COOH represents the amount of collagen in the scaffold [35,55]. A control group of membranes were not crosslinked (NX). After crosslinking, membranes were rinsed and stored in PBS until further use.

2.3. Covalent immobilization of growth factors via benzophenone photolithography

Benzophenone (BP) was immobilized to the CG membrane using a previously described approach [57,60]. Briefly, benzophenone-4-isothiocyanate was synthesized as previously reported [61] and dissolved in dimethyl formamide (DMF) to a final concentration of 20 mM. To that solution, *N,N*-diisopropylethylamine was added to a final concentration of 0.5 M. CG membranes were submerged in this solution and allowed to react for 48 h protected from light. Membranes were then rinsed in DMF, ethanol, and PBS to remove unreacted benzophenone reagent, and subsequently stored in PBS at 4 °C in the dark until use [57].

2.4. Biomolecular photoimmobilization

Stock solutions of bone morphogenetic protein 2 (BMP-2, R&D Systems, Minneapolis, MN) and platelet derived growth factor BB (PDGF-BB, R&D Systems) were prepared according to the manufacturer's instructions and stored at −20 °C until use. In preparation for patterning, CG membranes were soaked in a solution containing 5 μg/mL protein (PDGF-BB or BMP-2) in PBS. Membranes were subsequently transferred to a glass slide, covered with a glass coverslip, and exposed to 20 mW/cm^2 of ~365 nm light provided by a beam of an argon ion laser (Coherent Innova 90–4 with UV optics, Laser Innovations, Santa Paula, CA) that had been expanded and homogenized using refractive beam shaping optics (π-Shaper, Molecular Technologies, Berlin, Germany). Membranes were exposed for a defined time (1 or 5 min). After UV exposure, membranes were rinsed in a solution of 0.2% pluronic F-127 in PBS. For patterning multiple proteins, membranes were further rinsed in PBS then soaked in the second protein solution and subsequently irradiated as described above. After patterning, membranes were stored in PBS at 4 °C until use.