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Engineering articular cartilage with spatially-varying matrix composition and mechanical properties from a single stem cell population using a multi-layered hydrogel

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ABSTRACT

Despite significant advances in stem cell differentiation and tissue engineering, directing progenitor cells into three-dimensionally (3D) organized, native-like complex structures with spatially-varying mechanical properties and extra-cellular matrix (ECM) composition has not yet been achieved. The key innovations needed to achieve this would involve methods for directing a single stem cell population into multiple, spatially distinct phenotypes or lineages within a 3D scaffold structure. We have previously shown that specific combinations of natural and synthetic biomaterials can direct marrow-derived stem cells (MSC) into varying phenotypes of chondrocytes that resemble cells from the superficial, transitional, and deep zones of articular cartilage. In this current study, we demonstrate that layer-by-layer organization of these specific biomaterial compositions creates 3D niches that allow a single MSC population to differentiate into zone-specific chondrocytes and organize into a complex tissue structure. Our results indicate that a three-layer polyethylene glycol (PEG)-based hydrogel with chondroitin sulfate (CS) and matrix metalloproteinase-sensitive peptides (MMP-pep) incorporated into the top layer (superficial zone, PEG:CS:MMP-pep), CS incorporated into the middle layer (transitional zone, PEG:CS) and hyaluronic acid incorporated in the bottom layer (deep zone, PEG:HA), creates native-like articular cartilage with spatially-varying mechanical and biochemical properties. Specifically, collagen II levels decreased gradually from the superficial to the deep zone, while collagen X and proteoglycan levels increased, leading to an increasing gradient of compressive modulus from the superficial to the deep zone. We conclude that spatially-varying biomaterial compositions within single 3D scaffolds can stimulate efficient regeneration of multi-layered complex tissues from a single stem cell population.

1. Introduction

In the past decade, significant advances have been made in regenerating various tissue structures from stem and progenitor cells. Most of these efforts so far have focused on engineering homogenous tissues with bulk properties similar to their native counterpart [1–7]. However, most mammalian tissues are complex, both structurally and functionally, and possess spatially-varying biochemical compositions and mechanical properties. A model example is articular cartilage, which has been widely studied in regenerative medicine. Anatomically and functionally, articular cartilage consists of four, spatially distinct regions; the superficial, transitional, deep, and calcified zones [8]. Each zone is characterized by specific extra-cellular matrix (ECM) compositions, mechanical properties and cellular organization.

Overall, the cartilage ECM is primarily composed of type II collagen and glycosaminoglycans (GAGs) whose relative concentrations vary spatially from the superficial to the deep zone leading to varying mechanical properties [9]. The superficial zone contains high levels of collagen II and low levels of GAG [10]. The transitional zone has lower collagen II content while the GAG concentration increases [11]. The deep zone contains the highest concentration of GAGs and the lowest level of collagen II fibers [12]. Finally, the calcified cartilage zone has high levels of collagen X and integrates the cartilage to the subchondral bone [10–12]. The mechanical properties of articular cartilage are sensitive to this ECM composition since compressive forces experienced by the tissue stimulates GAG synthesis and ECM remodeling [13,14]. Specifically, the compressive modulus increases significantly from the articular surface to the deep zone and is dictated by the varying composition and structural organization of the ECM molecules [9]. Fig. 1A

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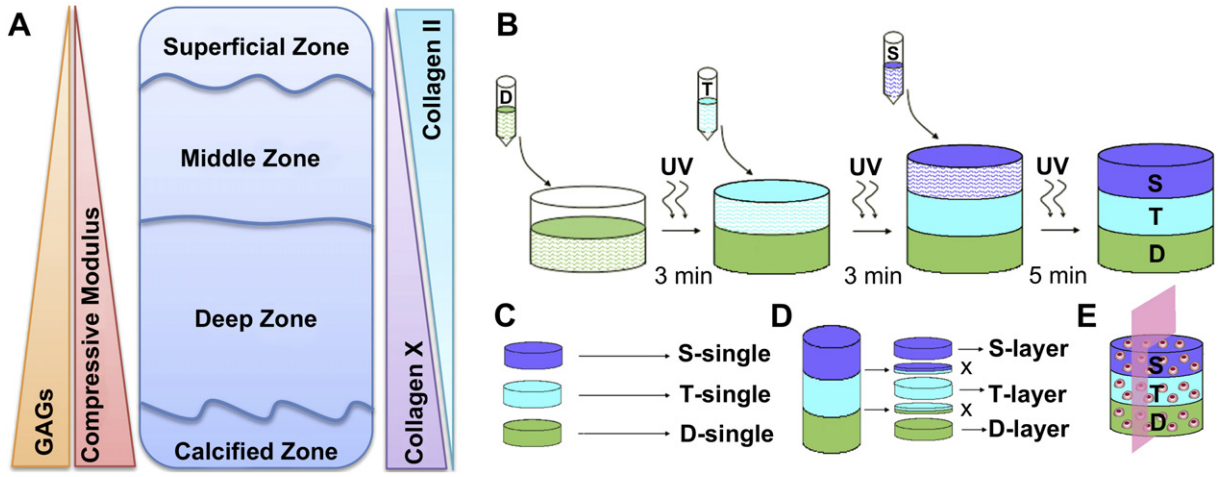

Fig. 1. (A) Schematic of articular cartilage anatomy illustrating the trend of increasing GAG content, compressive modulus and collagen X content as well as the decreasing trend of collagen II content from the superficial to the deep zone. (B) MLHC fabrication with three distinctive layers, each corresponding to the superficial, transitional, and deep zones of articular cartilage. (C) Single layer hydrogels of same biomaterial compositions were used as controls. Individual layers were fabricated using 100 μL of the layer-specific biomaterial, UV polymerized for 10 min. (D) To analyze the MLHCs, at 2, 4 and 6 weeks each layer was carefully separated. The separated layers were analyzed individually. (E) Schematic of a cell laden, composite multi-layer hydrogel showing plane of section for all microscopic analyses.

summarizes this spatially-varying native articular cartilage anatomy illustrating the trend of increasing GAG content, compressive modulus and collagen X content and the decreasing trend of collagen II content from the superficial to the deep zone.

Since the properties of articular cartilage or any complex tissue for that matter, are dictated by its spatially-varying structural organization, it is important to reproduce the native architecture and function when attempting to generate tissue substitutes from stem cells. The classical tissue engineering approach of creating homogenous tissue replacements for articular cartilage has failed to achieve widespread clinical effectiveness because the bulk properties of the homogenous tissue substitutes do not mimic native tissue function. Few recent studies have attempted to mimic the spatial organization [15,16] of articular cartilage, but only using zone-specific chondrocytes isolated from donor animal cartilage. Concepts that allow generation of three-dimensional (3D), zonally-organized native-like articular cartilage, starting from a single stem cell population, have not been reported. The key innovation necessary for this would involve methods to direct a single stem cell population into multiple, spatially distinct phenotypes within a single 3D structure.

We have previously demonstrated that specific material compositions can be used to direct mesenchymal stem cell differentiation into specific types of chondrocytes [17]. Specifically, PEG:CS:MMP-pep, PEG:CS, and PEG:HA hydrogels produced chondrocytes with properties similar to cells from the superficial, transitional and deep zones respectively [17]. Building on these findings, here we studied whether pre-designed multi-layered hydrogel constructs (MLHC) (Fig. 1B), with spatially-varying biomaterial compositions, are able to direct the simultaneous differentiation of encapsulated mouse bone-marrow derived D1 mesenchymal stem cells (BMSCs) [17,18], into organized zones of articular cartilage. Although we use articular cartilage as a model, this approach can be applied to engineer other complex tissues such as skin, blood vessels, osteochondral junctions etc.

2. Materials and methods

2.1. Materials

D1 mouse bone marrow progenitor cells were purchased from ATCC (Manassas, VA). Poly(ethylene glycol) dimethacrylate (MW ~ 3400) was purchased from Laysan Bio (Arab, AL). Chondroitin sulfate A and chondroitinase ABC was obtained from Sigma–Aldrich (St. Louis, MO). Hyaluronic acid and hyaluronidase type IV was purchased from Fluka (Sigma–Aldrich, St. Louis, MO). The MMP-sensitive peptide (MMP-pep) was synthesized and modified in the ICMB core facility (Austin, TX). Collagenase type III was purchased from Worthington Biochemical (Lakewood, NJ). ITS + 1 supplement was purchased from Sigma–Aldrich (St. Louis, MO). TGF-β1 was purchased from Peprotech (Rocky Hill, NJ). The primers for collagen type II, type X, type I and GAPDH were purchased from SA Biosciences (Frederick, MD).

2.2. Synthesis and modification of scaffold materials

MMP-pep (QPQGLAK: Gln-Pro-Gln-Gly-Leu-Ala-Lys) was synthesized using an automatic peptide synthesizer (Protein Technologies, Inc. Symphony Quartet) as previously described [17]. MMP-pep was modified by adding acryloyl groups to the two reactive amine groups of the peptide as previously described [17]. Chondroitin Sulfate A (CS) was acrylated using methods adopted from Li et al. [19] with slight modifications as previously described [17]. Hyaluronic acid (HA) was acrylated following a protocol adopted from Leach et al. [20] as previously described [17].

2.3. Fabrication of multi-layer hydrogels with spatially-varying materials & MSC culture

D1 bone marrow progenitor cells [18] were encapsulated at a cell density of 20 million cells/mL within MLHCs. The polymer solutions were syringe-filtered before mixing with the cells. To fabricate the multi-layered hydrogel we used specific biomaterial compositions based on the results of our previous findings [17]. Briefly, the superficial zone composition consists of 9% (w/v) PEG, 9% (w/v) CS and 2% (w/v) MMP-pep, while the transitional zone composition consists of 10% (w/v) PEG, and 10% (w/v) CS. Lastly, the deep zone composition consists of 19% (w/v) PEG and 1% (w/v) HA. We started from the bottom and first polymerized 100 μL of PEG:HA-MSC mixture under UV for 3 min to represent the deep zone then we added 100 μL of PEG:CS-MSC mixture on top of the partially polymerized bottom layer and polymerized it under UV for an additional 3 min to represent the transitional zone. Finally, for the superficial zone we used 100 μL of PEG:CS:MMP-pep and fully polymerized the entire hydrogel for 5 min. A schematic of the layered hydrogel fabrication is shown in Fig. 1B. This multi-layered scaffold was cultured in serum free chondrogenic media containing 1% penicillin/streptomycin, 10 nM Dexamethasone, 50 μg/mL ascorbic acid-2-phosphate, 40 μg/mL L-proline, 5 mL ITS + 1, and 10 ng/mL TGF-β1 for 2, 4 and 6 weeks in a 12-well plate. The media was changed every other day.

2.4. RNA isolation and RT-PCR analyses of the multi-layered constructs

Chondrogenesis within the multi-layered hydrogel constructs were determined by gene expression of collagen II, X and I at 2, 4, and 6 week time points. MSC-encapsulated single layer hydrogels (Fig. 1C), as described previously [17], were used as controls. The hydrogel matrices were removed from culture and the gene expressions of encapsulated cells were analyzed. The hydrogels were first sliced with a razor blade to separate the layers and the interface was used to determine the modulus (Fig. 1D). The individual layers were placed into individual 2 mL