Depth-Dependent Properties of a Tri-layered Hyaluronic Acid Hydrogel Construct with Zonal Co-culture of Chondrocytes and MSCs

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Introduction

Biomimetic design in cartilage tissue engineering is a challenge given the complexity of the native tissue. While chondrocytes (CHs) can be a source for tissue engineering, mesenchymal stem cells (MSCs) are considered a promising alternative as they can undergo chondrogenesis in a variety of 3D contexts.[1] However, MSCs are multipotent and tend towards hypertrophy when removed from a pro-chondrogenic environment (e.g., in vivo implantation). To address this issue, recent studies have investigated co-culture of articular CHs with MSCs. CHs appear to enhance the initial efficiency of MSC chondrogenesis, as well as limit hypertrophic changes in some instances.[2] While these findings are intriguing, articular cartilage also has a unique mechanical and biochemical depth-dependence. A number of studies have shown that zonal CHs seeded in layered hydrogels can produce depth-dependent heterogeneity, suggesting they maintain their identity in 3D culture.[3] Recently, we have shown that the zonal CH populations retained their native production levels and influenced MSC fate decisions in hyaluronic acid (HA) hydrogel co-cultures.[4] In this study, we bring these various ideas together with the creation of a tri-layered HA construct containing zonal CHs in each layer co-cultured with MSCs, and evaluate the depth-dependent properties of these constructs.

Methods

MSCs (P3) and zonal articular CHs were isolated from juvenile bovine knees (Fig. 1). Full-thickness cartilage plugs were excised from the femoral condyle and divided to three layers to obtain CHs from the superficial (SCH; top 100 μm), middle (MCH; top half of remaining cartilage), and deep (DCH) zone (bottom half of remaining cartilage). CHs were isolated by collagenase digestion and expanded through passage 4. MSCs only or mixed cell populations (MSC:CH ratio = 4:1) were encapsulated at 60×10^6 cells/mL in 1% w/v HA hydrogel (Lifecore Biomedical).[5] Tri-layered constructs were created by exposing the first layer (DCH-MSC) of cell-laden HA solution to UV light for 2 minutes, followed by polymerization of the second layer (MCH-MSC) for 2 minutes, and finally adding the third layer (SCH-MMSC) with completion of polymerization for another 7 minutes. Constructs (Ø4.75 × 3.5 mm) were cultured in a defined medium containing 10ng/mL TGF-β3, with media changed thrice weekly, and constructs turned regularly to improve growth through the depth. Cell viability, distribution, and proliferation of mixed populations of MSCs (blue), superficial (red), middle (purple), and deep (green) zone chondrocytes were followed using CellTracker (Molecular Probes). Bulk compressive properties were assessed via unconfined compression,[6] and local compressive properties[7] were determined using a custom microscope compression device and texture correlation.[8] Glycosaminoglycan (GAG) and hydroxyproline

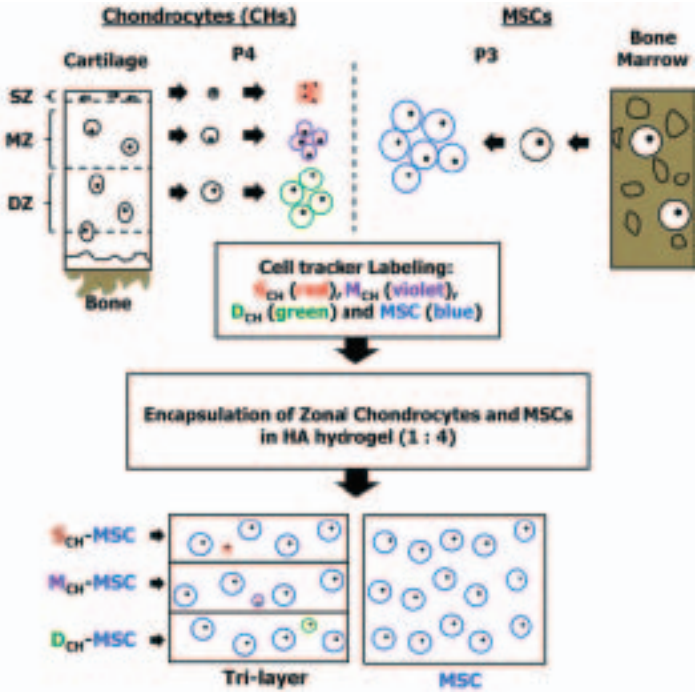

Figure 1. Stratified Construct with Co-culture of Zonal Chondrocytes and MSCs.

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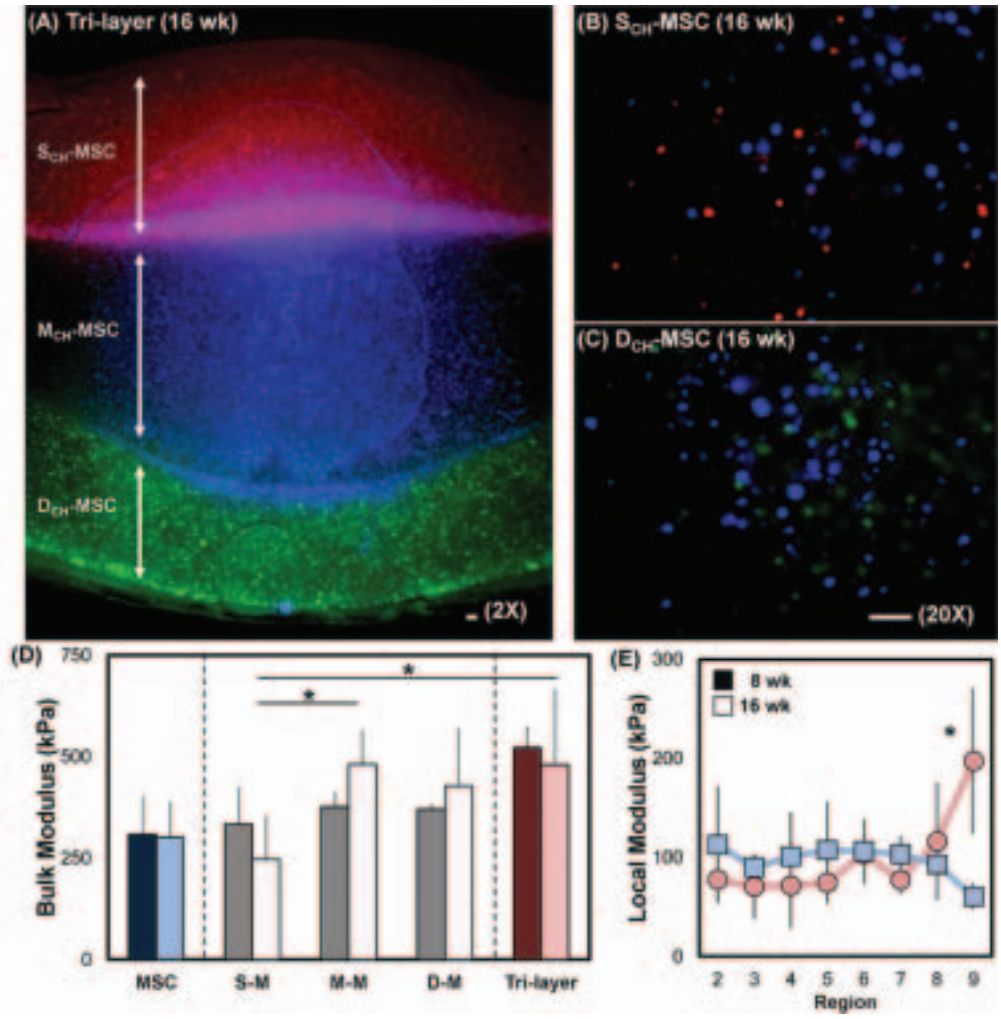

Figure 2A-E. Distinct zonal morphology of tri-layered construct and mechanical and biochemical properties at 8 and 16 weeks. (A) Visualization of zonal CHs co-cultured with MSCs in a tri-layered HA hydrogel construct; SCH (red), DCH (green) and MSCs (blue) (scale bar: 100 μm). Zoomed in images of (B) SCH-MSC (20x) and (C) DCH-MSC (20x). (D) Bulk modulus (kPa), where darker bars indicate 8 weeks and lighter bars indicate 16 weeks. (E) Local modulus (kPa) in MSC-only (pink) and tri-layer (blue) constructs, where region 9 is the deep zone ($n=4$; $p<0.05$).

contents were determined. Paraffin embedded sections (8μm) were stained with Alcian Blue for proteoglycans (PG) and by immunohistochemistry for collagen (COL) type I, II, X and chondroitin sulfate (CS). Significance was determined by two-way ANOVA with Tukey's post hoc tests ($p<0.05$).

Results

MSC and CH subpopulations were viable over the 16 week culture period, with each cell population well mixed within its appropriate layer (Fig. 2A-C). Bulk modulus and GAG content of mixed cell population constructs depended on the zonal origin of CHs; the lowest production was in constructs from the SCH-MSC group, and the highest production in MCH-MSC and DCH-MSC groups (Fig. 2D). The bulk modulus and GAG content of the tri-layered construct was 69% and 32% higher, respectively, than MSC-only constructs at 8 weeks, and 59% and 9% higher at 16 weeks. Further, the local modulus of the tri-layered constructs showed depth-dependent properties (72.6 kPa in region 2 and 197 kPa in region 9), while the MSC-only modulus was homogenous through the depth (Fig. 2E). The local modulus of tri-layered constructs in region 9 (DCH-MSC) was three times higher (197 kPa; $p=0.01$) than that of MSC-only (60 kPa). Both tri-layered and MSC-only constructs showed dense COL II and CS deposition at 8 weeks, with the tri-layered construct showing a clear interface between the layers, and intense COL II staining (Fig. 3).

Discussion

Here, we investigated the influence of co-culture of zonal articular CHs with bovine MSCs in 3D HA hydrogels, and created a tri-layered construct with mixed cell subpopulations in each layer to mimic the depth-dependent properties of articular cartilage. SCH-MSC produced constructs with the lowest compressive properties and GAG content (240 kPa; 3.2% WW), while MCH- and DCH-MSCs produced constructs with the highest bulk properties (>400 kPa; >4 %). Importantly, these differences were apparent with CH comprising only 20% of the starting cell population. When the single-layered constructs with mixed cell subpopulations were combined into one stratified construct, overall construct properties increased. The tri-layered construct retained a distinct cellular organization and produced constructs with depth-dependent properties, while MSC-only constructs showed no depth-dependence. The local modulus of the DCH-MSC layer in the tri-layered construct was ~2.5 times greater ($p=0.01$) than that of SCH-MSC constructs. Taken together, these results demonstrate that a layer-by-layer fabrication scheme, including co-cultures of zone-specific articular CHs, can reproduce the depth dependent characteristics of native cartilage. Future work will investigate how the individual cells within each layer communicate with one another and with adjacent layers, and scale this technology to produce constructs of anatomic relevance for cartilage repair applications.

Significance

Biomimetic design in cartilage tissue engineering is challenging. Formation of tri-layered engineered cartilage using zonal CHs co-cultured with MSCs holds promise for the repair of focal defects. This study is the first to demonstrate co-cultures of zonal articular CHs with MSCs to produce

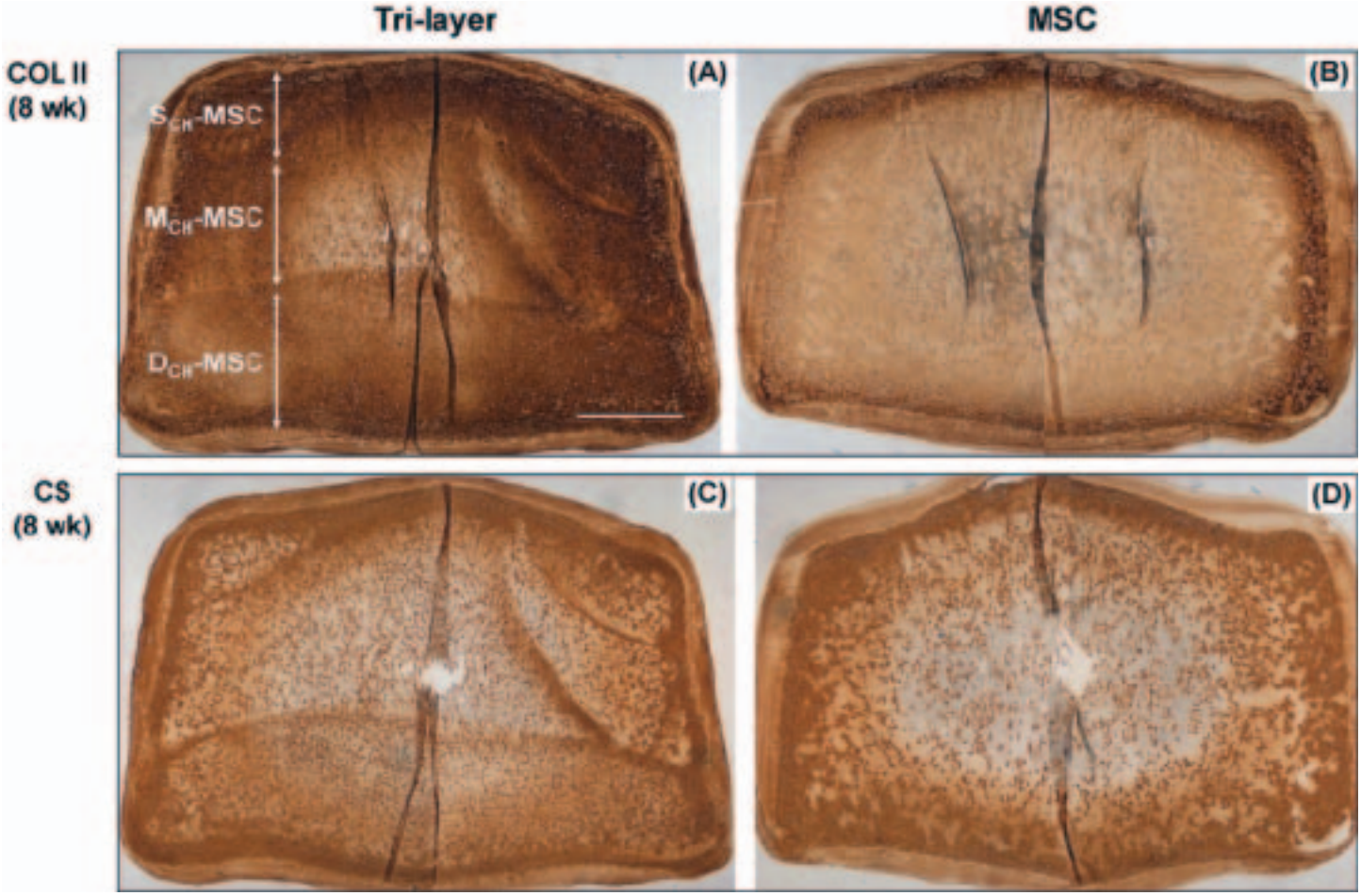

Figure 3A-D. Immunohistochemical analysis of tri-layered (A and C) and single layer (MSC only; B and D) constructs. Type II collagen (A-B) and chondroitin sulfate (C-D) staining on day 56 (scale bar: 1mm).

a tri-layered construct with depth dependent cellular and mechanical heterogeneity.

Acknowledgements

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