**Hydrogel Composition Regulates Chondrogenesis by Mesenchymal Stem Cells and Endochondral Ossification in Engineered Cartilaginous Interfacial Tissues**

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**Statement of Purpose:** Developing proper biomaterials that promote stem cell chondrogenesis is not only critical for articular cartilage repair, but for regeneration of cartilaginous interfacial tissues such as the epiphyseal growth plate and tendon and ligament insertion site (enthesis). These consist of spatially discrete but proximate populations of chondrocytes at distinct states of differentiation, including reserve/polymorphic, proliferative, and hypertrophic cells. A material that supports the proper progression of chondrocytes through their differentiation states is needed to regenerate these tissues. Here we evaluate the effect of two photocrosslinkable hydrogel formulations on stem cell chondrogenesis and progression of differentiation state using in vitro cultures and a murine subcutaneous implant model. The PGH hydrogel was composed methacrylated gelatin, methacrylated polyethylene-glycol(PEG), and methacrylated heparin, and mimics the collagenous and glycosaminoglycan (GAG) composition of cartilage. The GEL hydrogel was composed of methacrylated gelatin alone and served as a control and reference to works developing gelatin based hydrogels.

**Methods:** *Fabrication:* Polymers (bovine type B gelatin, MW=45,000; PEG, MW=4000; intestinal mucosa sodium heparin, MW=15,000, Sigma Aldrich) were modified in-house. 10% w/v hydrogel solutions were prepared in HBSS, adding 0.005% w/v initiator (lithium phenyl-2,4,6-trimethylbenzoyl phosphinate), and photopolymerizing with 2.5 J/cm2/mm UV-A.

*Evaluation of the effect of the hydrogels on hMSC chondrogenesis*: human mesenchymal stem cells (hMSC) (RoosterBio Inc.) were encapsulated at 30 million/mL in cylindrical constructs (3mm x 5mm diam), implanted in immunocompromised mice and grown for up to 8 weeks (n=7/time point).

*Evaluation of the effect of the hydrogels on progression of chondrocyte states:* three different chick chondrocyte populations(proliferative=Z1,prehypertrophic=Z2,hypertrophic=Z3)were encapsulated at 30 million/mL in three distinct layers of the cylindrical constructs and implanted (n=5). Chick chondrocytes were isolated from separate regions of chick embryo sternae.

*Analysis:* hMSC implants were analyzed for tissue composition with serial histology, GAG content, and gene expression with real time polymerase chain reaction (RT-PCR) for collagen type IIα1 (COL2), collagen type Xα1 (COL10), bone sialoprotein-II (BSP), and aggrecan (AG). Chick chondrocyte implants were analyzed with histology including immunohistochemistry. To determine the mechanism driving hydrogel effects, we further investigated differentiation state progression using in vitro cultures where we added inhibitors of matrix metalloproteinases (GM6001 ) and Rho-associated protein kinase (Y27632) to the constructs with chick chondrocytes (n=3). Samples were collected after one week for histological and GAG analysis.

**Results:** Regarding hMSCs constructs, the PGH hydrogel promoted only chondrogenic differentiation by hMSCs whereas GEL promoted direct osteogenic and chondrogenic differentiation by hMSCs (FIG.1). RT-PCR confirmed that PGH inhibited osteogenic differentiation, with PGH showing significantly higher Col2 and AG while very low levels of BSP compared to GEL. Regarding chick chondrocyte constructs, the GEL hydrogel showed decreased GAG accumulation and accelerated chondrocyte progression to hypertrophy compared to PGH constructs as evidenced by COL10 immunostaining and mineralization (FIG 2). The PGH hydrogel augmented maintenance of chick sternal chondrocytes in a GAG producing state while promoting timely development of the hypertrophic phenotype as indicated by immunostaining of COL10. Regarding the mechanism of hydrogel effect (inhibitor study), GAG staining and normalized GAG content increased in both PGH and GEL constructs when treated with MMP inhibitor. ROCK inhibitor showed no significant effect.

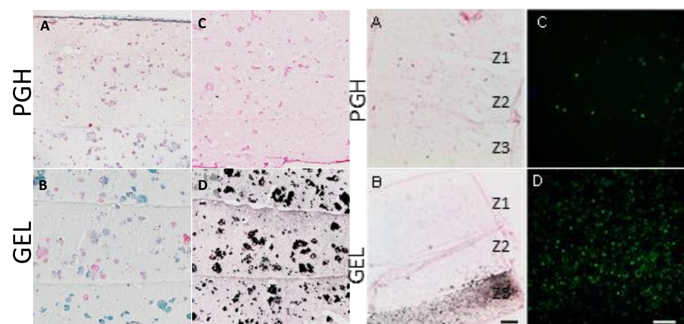


FIG 1: hMSC implants at wk8. A,B, hematoxylin, safranin-O, fast green. C,D, Von Kossa. Scale bar=100μm

FIG 2: Chick chondrocyte implants. A-B, wk8, Von Kossa. C-D, COL10 of Z2 at wk1. Black scale bar=500μm.White scale bar=200μm

**Conclusions:** The PGH hydrogel formulation augmented maintenance of chondrocytes in a GAG producing state and prevented osteogenesis of hMSCs. Chondrocytes respond to matrix molecules and their degradation products, with collagen fragments promoting hypertrophy (1). The decreased gelatin content in PGH relative to GEL hydrogels likely decreases the amount of collagenous fragments produced via endogenous MMP activity, and thereby the rate of osteogenesis and hypertrophy. Thus the PGH hydrogel is a promising candidate for physeal engineering because it supports endochondral ossification while inhibiting direct osteogenesis by progenitor cells, and because it supports progression of chondrocytes through their differentiation states.

**References:** 1.Gauci, S. J. Modulating chondrocyte hypertrophy in growth plate and osteoarthritic cartilage. JMNI. 8 (4), 308-310 (2008).