Influence of the Direction of Growth Stimulation on Collagen Orientation in Articular Cartilage

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INTRODUCTION: Articular cartilage exhibits a distinct structural arrangement of collagen fibers, extending upright from the underlying bone, through the deep cartilage zone, arching over and lying flat in the superficial zone. This arcade-like organization was originally described by Benninghoff [1] and plays a crucial role in the functional properties of cartilage. Consequently, it is key for cartilage tissue engineering to replicate this distinct collagen architecture that enables proper functionality [2]. Although it is recognized that the fiber organization transitions from an isotropic arrangement after birth to the anisotropic architecture in adult cartilage, the mechanism driving these structural changes are not well understood. Previous research found that the apophyseal neoformation of cartilage during postnatal development, similar to the growth governed by the growth plate, attributes to this specific alignment in cartilage [3]. This approach would suggest that localized and directional stimulation of growth is important and could result in the fiber alignment perpendicular to the surface. Therefore, this study aims to investigate the influence of the direction of growth stimulation to achieve fiber alignment similar to that of the deep zones of native cartilage.
METHODS: Chondrocytes were isolated from bovine metacarpal articular cartilage (n=5) and cultured in spinner flasks for 12 days, supplemented with porcine derived notochordal cell matrix (NCM) to induce cartilage organoid formation [4]. Cell culture inserts with a polyethylenterephthalate (PET; Ø 6mm) membrane were coated with 0.125 mg/mL rat tail collagen type I. Organoids were transferred to the inserts to cover the full membrane and were cultured for 49 days in this double-compartment platform. After 7 days of initial attachment and fusion, culture medium was supplemented with 10 ng/mL human transforming growth factor β1 (TGF-β1) in the upper compartment for the top-down (TD) condition, and in the lower compartment for the bottom-up (BU) condition. On days 7 and 49 constructs were evaluated for dry weight, sulfated glycosaminoglycan (GAG), hydroxyproline (HYP), and DNA content. Moreover, sections were stained with alcian blue and picrosirius red to visualize the distribution of GAG and collagen, respectively. Collagen fiber organization was evaluated with polarized light microscopy (PLM). Orientation of alignment was quantified using a fiber-tracking image analysis tool and the proportionality between fiber alignment in the angular range of 70° to 105° versus other orientations of alignment was determined [5]. Data were statistically analyzed using one-way ANOVA (statistical significance was set to $p<0.05$) and a Tukey's multiple comparison post-hoc test to compare between timepoints and groups.
RESULTS: Over the 49 days of culture, substantial tissue growth was visible for both TD and BU groups, with a significant increase for dry weight, GAG and collagen content (Fig. 1). GAG content showed a 6-fold increase for both groups, whereas the collagen content only increased two-fold. No significant differences were found between groups. These findings were supported by the alcian blue and picrosirius red staining, with both groups showing GAG and collagen rich organoids loosely on top and new matrix bridging the organoids in the deepest layers, forming disc shaped tissues (Fig. 2C). In addition to matrix formation between the organoids, areas of neo-tissue formation were visible in the BU group underneath the organoids of the deepest layers (Fig. 2A). Immunohistochemistry analysis showed the presence of collagen type II in all constructs after 49 days, with little collagen type I present (not shown). The PLM images were focused on these areas of new tissue, indicating fiber alignment to be perpendicular to the surface, predominantly for the BU constructs (Fig. 2B). Quantification of the fiber alignment in the PLM images of the dense lower organoid layers was performed showing the proportionality of perpendicularly aligned fibers versus other orientations. These results revealed an increase in fiber alignment of the perpendicular orientations with 59±9% for the BU-group compared to 30±12% for the TD-group (Fig. 3). No significant differences in alignment were found in the organoid layers before supplementation of TGF-β1 on day 7 or in the loose organoids on top after 49 days.
DISCUSSION: The results showed that the stimulation of tissue growth with TGF-β1 supplementation was established for both BU and TD groups, with hyaline cartilage-like tissue formation in the deepest layers. Although it was shown that similar amount of growth was obtained, the manner of growth was different between groups with neo-tissue formation underneath the organoids when stimulated from below. These areas of new tissue in the BU group showed increased collagen type II fiber alignment perpendicular to the surface and along the direction of growth, like in the deep zones of native articular cartilage. Although it is possible that the observed oriented collagen fibers are simply stretched into the growth direction, this is highly unlikely because collagen in the organoids mostly derived from NCM is non-fibrillar and perpendicular fiber orientation was only observed in newly formed cartilage below the organoids in the BU group. Therefore, fiber alignment perpendicular to the surface is most likely derived from fibers that were newly deposited in an aligned manner during growth. Optimization of native cartilage-like fiber alignment using directional stimulation of growth could lead to improvements in functional tissue engineering grafts in the future.

SIGNIFICANCE/CLINICAL RELEVANCE: This study shows that the direction of cartilage growth can guide the creation of aligned collagen fibers in tissue engineered constructs. Such a mechanism could be exploited to achieve the native fiber organization in these constructs to overcome challenges of mechanical failure in current cartilage repair strategies. It may also have general implications for tissue engineering of other collagen rich tissues.

REFERENCES: [1] Benninghoff A., *Z Zelforsch Mikrosk Anat.*, 1925 [2] Nagel T, Kelly DJ *Tissue Eng Part A.* 2013 [3] Hunziker EB et al., *Osteoartritis and Cartilage*, 2007 [4] Crispim JF et al., *Acta Biomater.* 2021. [5] Dittmar R et al., *Global Spine J.*, 2016

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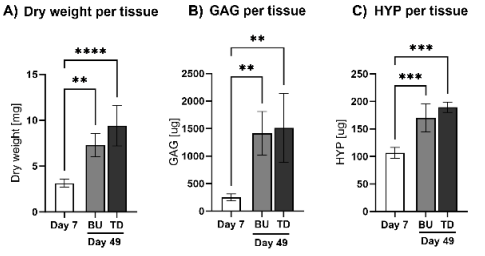

Figure 1: A) Dry weight, B) GAG and C) HYP content per tissue. **p<0.005 and ***p<0.0005

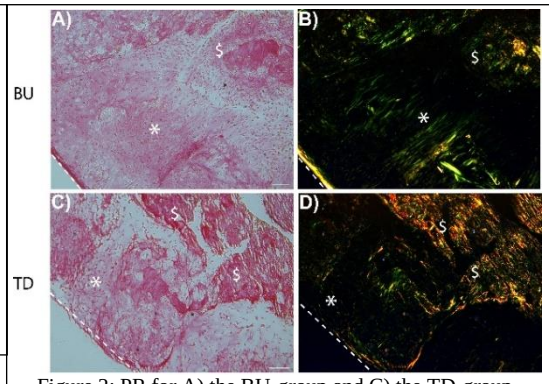

Figure 2: PR for A) the BU-group and C) the TD-group, with corresponding PLM images of B) the BU-group and D) the TD-group. Samples are oriented in 45°. Dotted line represents the bottom of the samples, * neo-tissue formation, $ organoid structures. Scale bar = 100µm.

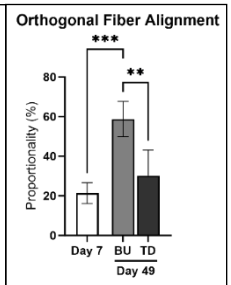

Figure 3: The proportionality of perpendicular fiber alignment versus other orientations. **p<0.005 and ***p<0.0005