# Cartilage Organoid Production and Fusion Using Human Articular Chondroprogenitor Cells

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INTRODUCTION: Regeneration of damaged articular cartilage using autologous articular chondrocytes (ACs) is a clinical procedure. However, their 2D expansion is time-consuming and causes dedifferentiation which in turn can lead to fibrocartilaginous repair tissue. Recently, the use of spinner flasks (SFs) has shown to be an efficient method to create cartilage organoids where ACs can proliferate without dedifferentiating[1]. These organoids could be used in cartilage repair strategies or personalized models. However, notochordal cell-derived matrix (NCM), a porcine matrix additive, is needed for the ACs to aggregate[1], limiting the application of the organoids. Furthermore, the number of organoids is limited by the number of chondrocytes obtained during a biopsy. To solve this problem, articular chondroprogenitor cells (ACPCs) have been suggested. These cells can differentiate towards the chondrogenic lineage even after more than 30 passages, where both BMP-9 and TGF-β1 with dexamethasone have been studied to stimulate the ACPCs to undergo this differentiation during pellet culture[2,3]. Therefore, the goal of this study was to create cartilage organoids using ACPCs with and without NCM that are similar to the AC cartilage organoids. Furthermore, the ability of these ACPC organoids without NCM to fuse into a cartilage-like tissue has been investigated to show the tissue repair capabilities of the organoids.
METHODS: Human ACPCs and ACs were harvested from redundant articular cartilage tissue of TKA patients (n=3 or 4, groups A & B or C & AC control, respectively) at the Máxima Medical Centre (approved by a relevant medical ethics review committee, METC #N16.148) and expanded until P4[4]. Three protocols were investigated for their potential to create ACPC organoids using SFs: two differentiation protocols with NCM (groups A and B) and one without NCM (group C) (Table 1). These ACPC organoids were compared to the control AC organoids with NCM[1]. After 14 days, the organoids were harvested, the projected area was measured, and preserved for further analysis. Fusion was evaluated after a 28-day culture, by seeding a layer of organoids from group C and AC organoids in collagen-coated transwells covering the bottom. Further analysis of the organoids and fusions consisted of biochemical assays (for glycosaminoglycans (GAGs) and DNA), histology (picrosirius red and alcian blue), and immunohistochemistry (Collagen I, II, and VI, Ki67, and SOX9). Quantitative data were statistically analyzed with Kruskal-Wallis tests with Dunn's multiple post hoc comparisons and unpaired t-tests for the organoids and fusions, respectively.
RESULTS: Preliminary results (n=1 or 2; not all immunostains) showed that the projected areas of the ACPC organoids were not significantly different to the AC control, however, variance was large and group B tended to be 50% of group A (Figure 1A). Even though group C aggregated without NCM, this group contained more GAGs per DNA compared to the AC control (99.3±52.2 µg GAG/µg DNA and 34.2±15.8 µg GAG/µg DNA, respectively) (Figure 1B). Collagen type II was made in organoids from all groups, even in group C without NCM (Figure 1C-F). However, some collagen type I was present in all ACPCs organoids, especially in group C, while the AC control organoids showed no collagen type I (results not shown). Both group C and AC organoids fused into disc-shaped tissues, with organoids being recognizable on top. There was no difference in GAG per DNA between both groups (Figure 2A). Histology showed that the fusion between organoids consisted of GAGs and collagen (results not shown). Even though the organoids from group C originally contained collagen type I, only collagen type II was seen in the fusion areas in both groups (Figure 2B and C).
DISCUSSION: The results showed that it is possible to produce cartilage organoids from ACPCs with NCM that are not too dissimilar to AC organoids. Besides the size, analysis did not show differences between groups A and B, suggesting that the attempted differentiation of ACPCs in the first four days is effective in both pellets[2,3] and in SFs cultures, respectively. Moreover, ACPC organoids without porcine NCM were successfully produced, increasing application possibilities. However, these group C organoids showed differences to the AC control organoids. The area of the group C organoids was less consistent, there was substantial collagen type I present, and the GAG content was significantly higher. Despite their differences, the group C organoids and the AC organoids were able to fuse in a similar way to form hyaline cartilage. The presented method could be improved by optimizing SF culture conditions, as the ACPCs seem to produce more collagen type I in this mechanically stimulated culture than in the static fusion culture. Optimization of the ACPC organoids without NCM could lead to a quantitative and qualitative improvement of the regenerative treatment options for cartilage defects.

SIGNIFICANCE/CLINICAL RELEVANCE: The present study showed that cartilage organoids can be produced by using progenitor cells which are able to fuse into a larger cartilage tissue. This could be applied in cartilage repair strategies or (personalized) organ-on-a-chip models.

REFERENCES: [1]Crispim J.F. et al., *Acta Biomater*. 2021 [2]Morgan B.J. et al., *Stem Cells Dev.* 2020 [3]Rikkers M. et al., *Cartilage* 2021 [4]Schmidt S. et al., *Int. J. Mol. Sci.* 2020

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IMAGES AND TABLES:
Table 1: The supplements added to the standard medium[1,4] during 2D expansion of the ACPCs and the organoid cultures.

| | Group A | Group B | Group C |
|---|---|---|---|
| 2D expansion P3 to P4 | + 10 ng/ml BMP-9 | + 10 ng/ml BMP-9 | + 5 ng/ml bFGF[4] |
| Differentiation or aggregation day 0 to 4 | SF culture (50.000 cells/ml) + 10 ng/ml TGF-β1 + 0.1 µM dexamethasone | Pellet culture (250.000 cells/pellet) + 10 ng/ml TGF-β1 + 0.1 µM dexamethasone | SF culture (50.000 cells/ml) |
| SF culture day 4 to 14 | + NCM | 50.000 dissociated cells/ml + NCM | + 100 ng/ml BMP-9 |

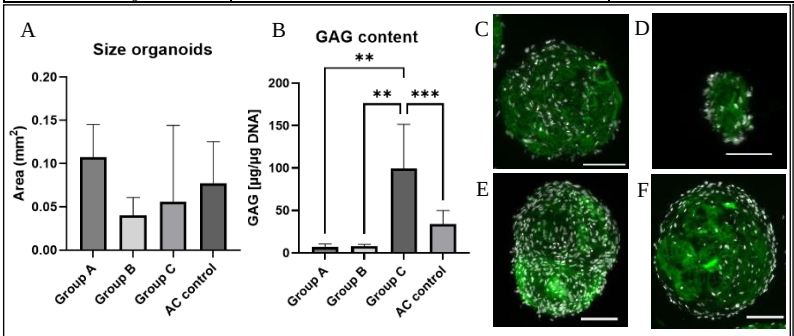


Figure 1: Size, biochemical, and immunohistochemical analysis of the organoids. A) The projected area of the organoids of group A-C and the AC control as calculated with ImageJ. B) GAG content normalized against DNA content for the organoids of group A-C and the AC control. C) Collagen type II (green) and the cells (white) in an organoid from group A, D) from group B, E) from group C, F) and from the AC control. ***p<0.001 and **p<0.01. Scale bar = 100 µm.

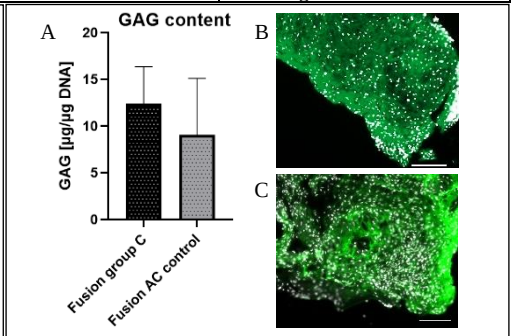


Figure 2: Biochemical and immunohistochemical analysis of the fusions of group C and AC control. A) GAG content normalized against DNA content B) Collagen type II (green) and the cells (white) in the fusion of group C, C) and in the fusion of the AC control organoids. Scale bar = 100 µm.