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# BMP activation and Wnt-signalling affect biochemistry and functional biomechanical properties of cartilage tissue engineering constructs

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SUMMARY

*Objectives:* Bone morphogenetic protein (BMP-) and Wnt-signalling play crucial roles in cartilage homeostasis. Our objective was to investigate whether activation of the BMP-pathway or stimulation of Wnt-signalling cascades effectively enhances cartilage-specific extracellular matrix (ECM) accumulation and functional biomechanical parameters of chondrocyte-seeded tissue engineering (TE)-constructs.

*Design:* Articular chondrocytes were cultured in collagen-type-I/III-matrices over 6 weeks to create a biomechanical standard curve. Effects of stimulation with 100 ng/mL BMP-4/-7 heterodimer or 10 mM lithium chloride (LiCl) on ECM-deposition was quantified and characterized histologically. Biomechanical parameters were determined by the *Very Low Rubber Hardness* (VLRH) method and under confined compression stress relaxation.

*Results:* BMP-4/-7 treatment resulted in stronger collagen type-II staining and significantly enhanced glycosaminoglycan (GAG) deposition (3.2-fold; $^*P < 0.01$) correlating with improved hardness (~1.7-fold; $^*P = 0.001$) reaching 83% of native cartilage values after 28 days, a value not reached before 9 weeks without stimulation. LiCl treatment enhanced VLRH slightly, but significantly (~1.3-fold; $^*P = 0.016$) with a trend to more ECM-deposition. BMP-4/-7 treatment significantly enhanced the E Modulus (105.7 ± 34.1 kPa; $^*P = 0.000001$) compared to controls (8.0 ± 4.2 kPa). Poisson's ratio was significantly improved by BMP-4/-7 treatment (0.0703 ± 0.0409; $^*P = 0.013$) vs controls (0.0432 ± 0.0284) and a significantly lower permeability (5.8 ± 2.1056 × $10^{-14}$ m4/N.s; $^*P = 0.00001$) was detected compared to untreated scaffolds (4.4 ± 3.1289 × $10^{-13}$ m4/N.s).

*Conclusions:* While Wnt-activation is less effective, BMP-4/-7 heterodimer stimulation approximated native cartilage features in less than 50% of standard culture time representing a promising strategy for functional cartilage TE to improve biomechanical parameters of engineered cartilage.



## Introduction

As a highly specialized tissue, articular cartilage protects underlying bone from destructive forces mainly caused by the specific composition of the extracellular matrix (ECM), which consists of a highly ordered framework of several types of collagens and proteoglycan[1]. Tensile strength of the tissue is attributed predominantly to collagen type II, while proteoglycans contribute mainly to compressive properties in two ways: a bulk of proteoglycan aggregates are immobilized within the collagen fibre network and, associated with the negative charge of the molecules, proteoglycans bind water which leads to a swelling-pressure[2].

Damage of native tissue as consequence of trauma or due to a degenerative joint disease such as osteoarthritis (OA)[3], is related to a loss of the healthy ECM structure that generally leads to a deficit in functional biomechanical characteristics. In particular, in chondral defects, where the lesion is located within articular cartilage, progenitor cells from blood and bone marrow cannot enter the damaged region to contribute to repair owing to a lack of vascularization. Additionally, resident chondrocytes are unable to migrate into the defect and, therefore, the lesion is not filled with repair tissue and remains permanently[4,5]. To overcome this problem, tissue engineering (TE) approaches based on carrier materials seeded with chondrocytes are currently under investigation in order to enhance

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biochemical and biomechanical characteristics of *in vitro* engineered cartilage. Improved biomechanical properties of TE-constructs were achieved by increasing the ECM-deposition as a result of a prolonged culture period[6–8], enhanced cell seeding density[7], physical[9] or mechanical stimulation during culture[7,10,11]. Additionally, the combination of growth factor treatment and dynamic loading in chondrocyte-seeded TE-constructs revealed a synergistic effect on functional properties[12]. However, the unique functional biomechanical characteristics of articular cartilage still have to be reached[6,13].

A number of biological factors affecting important signalling pathways of cartilage ECM-deposition have been shown to enhance functional properties of chondrocyte-seeded TE-constructs. Members of the transforming growth factor (TGF)-β superfamily such as bone morphogenetic protein (BMP) homodimers are promising inducers of cartilage matrix synthesis[14,15]. Interestingly, heterodimeric BMP ligands have been demonstrated to display a higher activity compared to their homodimeric counterparts[16]. While homodimers form symmetric molecules binding to a restricted set of BMP type I-receptors with high affinity, BMP heterodimers can form asymmetric ligands with broader BMP-receptor binding resulting in enhanced signalling activity[17,18]. Of the BMP homodimers, BMP-4 and BMP-7 have been identified as potent stimulators of articular chondrocyte anabolism in monolayer and 3D culture[19–21]. Additionally, BMP-4 and BMP-7 target distinct BMP type I-receptors: while BMP-4 possesses a high affinity to activin-like-kinase (ALK)-3 and ALK-6 receptors, BMP-7 preferentially binds ALK-2[22,23]. Stimulation of ectopic bone formation with BMP-4/-7 heterodimer in rats resulted in superior bone formation in comparison with BMP homodimer[24]. In line, murine embryonic fibroblasts, overexpressing either BMP-2, BMP-4, BMP-7 homodimer, respectively, or the combination of two different BMPs before ectopic transplantation into mice, revealed that the combination of BMP-4/-7 resulted in two- to threefold more bone[25]. The activity of BMP heterodimers on cartilage ECM-production by chondrocytes remained so far, however, enigmatic.

Another essential pathway involved in chondrogenesis and joint formation is the Wnt-signalling cascade[26,27], which regulates chondrocyte proliferation, differentiation and maintenance of the cell phenotype[28,29]. Canonical Wnt-signalling is mediated by β-catenin which in the presence of lithium chloride (LiCl) accumulates in the cytoplasm and then translocates into the nucleus. There, β-catenin forms a complex with transcription factors to activate the transcription of target genes. Stimulation of Wnt-signalling by means of the Wnt agonist LiCl in an undifferentiated mesenchymal stem cell line stimulated expression of chondrogenic marker genes like COL2 and aggrecan[30]. Others demonstrated elevated proteoglycan levels after LiCl treatment of mesenchymal stromal cells during chondrogenic differentiation[31]. Nevertheless it is still unclear whether Wnt-activation affects ECM-deposition by chondrocytes in a TE-matrix.

A variety of methods to describe functional properties of cartilage and TE-constructs have been identified. Indentation[32,33] and confined compression[34–37] are established methods to study mechanical properties of tissue due to the simplicity of loading configurations and the ability to achieve the desired boundary conditions. We have furthermore recently identified a new sensitive method to evaluate mechanical properties of *in vitro* generated cartilage constructs[38]. This indentation test, following the *Very Low Rubber Hardness* (VLRH) principle, was originally designed to analyse soft materials like silicone[39] and may represent a sensitive system detecting consequences of ECM stimulation in cartilage TE-constructs in terms of absolute hardness values.

While BMP- and Wnt-signalling act through well-studied pathways, surprisingly, it is still not known whether their activation either by a BMP heterodimer or by LiCl, respectively, affects biomechanical properties of chondrocyte-seeded articular cartilage TE-constructs. Therefore, the objective of this study was to analyse whether activation of BMP- or Wnt-signalling is effective to enhance biomechanical properties of chondrocyte-seeded collagen matrix-based TE-constructs holding potential to further improve therapeutic strategies for cartilage repair.

Articular chondrocytes were cultured in a collagen carrier in the presence of BMP-4/-7 heterodimer or the Wnt agonist LiCl in chondrogenic medium for 4–6 weeks. Following factor treatment, a detailed characterization of biochemical and biomechanical parameters of TE-constructs was performed relating to the properties of control cultures and native cartilage tissue.

## Materials and methods

### *Cell isolation and cultivation*

Articular chondrocytes were isolated from porcine knee joints ($n = 5$ donors). Rinsed cartilage was cut into small pieces, digested overnight with collagenase B (Roche) and hyaluronidase (Sigma–Aldrich), as described[14,40]. Chondrocytes were filtered through a 50 μm nylon mesh washed in PBS and seeded at 8500 cells/cm$^2$. Chondrocytes were expanded for two to three population doublings (PD) in DMEM (low-glucose) with 10% foetal calf serum (FCS) (Biochrom), 10 U/mL penicillin, 100 mg/mL streptomycin (Biochrom) at 37°C and 6% $CO_2$. Medium was replaced twice a week.

### *Cartilage TE-constructs*

Chondrocytes were harvested and suspended in chondrogenic medium (high-glucose DMEM with 0.1 mM dexamethasone, 0.17 mM ascorbic acid-2 phosphate, 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL sodium selenite, 1 mM sodium pyruvate, 0.35 mM proline, 1.25 mg/mL bovine serum albumin (BSA), 100 U/mL penicillin, 100 mg/mL streptomycin) with 10 ng/mL TGFβ-1 (Miltenyi Biotech) at $5 \times 10^7$ cells/mL. Collagen type I/III matrices (Ø 5 mm, 1.5 mm height; Matricel) were seeded with 30 μL cell suspension following the manufacturer's drop-on protocol ($1.5 \times 10^6$ cells/construct). Cells were allowed to attach for 2 h until 2 mL of chondrogenic medium or chondrogenic medium containing 100 ng/mL BMP-4/-7 (R & D Systems Inc.) or 10 mM LiCl (Sigma–Aldrich) were added. Five constructs per donor and group were cultured in independent experiments for 0, 7, 21, 28 and 42 days with medium changed three times a week. Thickness at termination of culture was measured at the centre point by a needle probe method[41]. A custom MATLAB® procedure (MATLAB®, Linux version 2011b, Mathworks Inc.) was used to evaluate the results.

### *Histology*

Samples were fixed in 1 mL 4% paraformaldehyde dehydrated using a graded alcohol series and embedded in paraffin. For the detection of proteoglycans, 5 μm sections were stained with safranin O (0.2% in 1% acetic acid, Chroma) and Certistain fast green (0.04% in 0.2% acetic acid, Merck) using a standard procedure.

Immunohistology for collagen type II was performed as described[42]. Briefly, deparaffinised and rehydrated sections were treated with 4 mg/mL hyaluronidase in PBS pH 5.5 (Sigma–Aldrich) and 1 mg/mL pronase (Roche). Non-specific background was blocked with 5% BSA and sections were incubated with anti-human collagen type II antibody (1:1000, clone II-4C11, ICN Biomedicals) followed by biotinylated goat anti-mouse antibody (1:500, Dianova), and streptavidin–alkaline phosphatase (Dako) and fast red detection (Sigma–Aldrich).

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