

# The Synovium of Human Osteoarthritic Joints Retains Its Chondrogenic Potential Irrespective of Age

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## Research article

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# Abstract

The autologous synovium is a potential tissue source for local induction of chondrogenesis by tissue engineering approaches to repair articular cartilage defects such as they occur in osteoarthritis. It was the aim of the present study to ascertain whether the aging of osteoarthritic patients compromises the chondrogenic potential of their knee-joint synovium.

The patients were allocated to one of the following two age categories: 54 - 65 years and 66 - 86 years (n = 7-11 donors per time point and per experimental group). Synovial biopsies were induced *in vitro* to undergo chondrogenesis by exposing them to either bone morphogenetic protein-2 (BMP-2) alone, transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) alone, or a combination of the two growth factors for up to 6 weeks. The differentiated explants were evaluated histomorphometrically for the volume fraction of metachromasia (sulfated proteoglycans), immunohistochemically for type-II collagen, and for the gene-expression levels of anabolic chondrogenic markers as well as catabolic factors by a real-time polymerase-chain-reaction (RT-PCR) analysis.

Quantitative metachromasia revealed that chondrogenic differentiation of human synovial explants was induced to the greatest degree by either BMP-2 alone or the BMP-2/TGF- $\beta$ 1 combination, i.e. to a comparable level with each of the two stimulation protocols and within both age categories. The gene-expression levels of the anabolic chondrogenic markers confirmed the superiority of these two stimulation protocols and demonstrated the hyaline-like qualities of the generated cartilaginous matrix. The gene-expression levels of the catabolic markers were extremely low.

Our data reveal the chondrogenic potential of the human knee-joint synovium of osteoarthritic patients to be uncompromised by ageing and catabolic processes. The potential of synovium-based clinical engineering (repair) of cartilage tissue using autologous synovium may thus not be reduced by the age of the human patient.

## Introduction

Osteoarthritis (OA) is one of the diseases for which there currently exists no effective, biologically-based therapeutic strategy to reconstitute the damaged layer of articular cartilage [1]. During the early stages of osteoarthritis, cartilage damage is confined to discrete structural lesions. However, owing to the intrinsically poor healing capacity of cartilaginous tissue, the lesioning process, once initiated, cannot be arrested by spontaneous repair [2]. Ultimately therefore, the entire layer is implicated and destroyed [3, 4]. Although several treatment strategies are currently investigated to promote the repair of articular cartilage, such as the microfracturing technique [5], the grafting of osteochondral tissue [6] or the implantation of autologous chondrocytes [7], the tissue thereby formed is biologically and mechanically grossly inferior to native articular cartilage [8].

For the engineering of cartilage tissue *in vitro* and *in vivo*, multipotential stromal cells (MSCs) are often preferred to committed chondrogenic cells, since their differentiation can be induced under more

physiological stimulation conditions [9]. Although MSCs are usually drawn from the bone marrow, the synovium is likewise a rich source of such cells exhibiting a very high chondrogenic potential [10–12]. Also anatomically, the proximity of the synovium to the layer of articular cartilage would render it a suitable source of MSCs for the *in-situ* engineering of this tissue. Indeed, we have indirect evidence in preclinical studies that under appropriate stimulation conditions, synovial cells can be induced to migrate into a neighboring articular defect, to proliferate therein, and then to differentiate into chondrocytes that lay down a hyaline-type of cartilage tissue [10, 13]. We had developed *in vitro* a suitable stimulation protocol for the formation of a hyaline-type of cartilage tissue from both isolated synovial cells [14] and synovial explants [15–17]. This protocol was recently optimized to yield articular cartilage-like tissue under growth factor based stimulation conditions. Specifically this protocol is also able to arrest the cellular chondrocytic downstream differentiation at the appropriate stage of cell size (similar size to that of adult articular cartilage chondrocytes), and thus prevent the continuation of cellular differentiation into terminal cell hypertrophy and the associated intercellular matrix mineralization [18].

In clinical practice, indications for the instigation of a cartilage-repair strategy are usually coupled with a process of pathological degeneration (rather than with acute traumatic injuries), most commonly with osteoarthritis. Synovial MSCs isolated from the knee joints of osteoarthritic (OA) patients of various ages have shown chondrogenic potential [11, 14]. On the other hand, the chondrogenic potential of synovial explants derived from OA patients is still unknown. The chondrogenic potential of synovial explants derived from OA synovium, which often is also associated with inflammation, could be different from that of isolated synovial MSCs since synovial explants contain not only MSCs but also other cells, such as fibroblasts and endothelial cells as well as extracellular matrix, the latter acting as a reservoir of cytokines. Furthermore, although many researchers have reported that the donor's age affects the differentiation potential of MSCs derived from bone marrow [19–21], but most likely not the synovium-derived ones [11, 12, 22], this is still unknown for synovial tissue explants.

The question thus arises whether the synovial explants derived from elderly osteoarthritic patients retain their chondrogenic potential, or if this potential is deteriorating with age. It was the aim of the present study to address this question. For this purpose, explants of synovial tissue were obtained from osteoarthritic patients who were allocated to two age categories (54–65 years and 66–86 years). After the explants were cultured for up to 6 weeks with BMP-2 or TGF- $\beta$ 1, or with a combination of BMP-2 and TGF- $\beta$ 1 (in order to prevent terminal differentiation to chondrocyte hypertrophy and matrix mineralization [18]), the differentiated tissue of each category was then analyzed for its cartilaginous properties histochemically (volume fraction of metachromasia after staining for sulfated proteoglycans with Toluidine Blue O), immunohistochemically (for Type-II collagen) and by the expression profiles of anabolic and catabolic marker genes.

## Materials And Methods

### Tissue preparation, growth factors and culturing

Synovial tissue was obtained (surgical waste material) from the knee joints of osteoarthritic patients (aged 54–86 years) who were undergoing total knee replacement surgery. A total number of 64 patients served as donors. Informed consent and local ethical commission approval were obtained. Synovial explant differentiation was induced by BMP-2 alone, TGF- $\beta$ 1 alone, or with a combination of BMP-2 and TGF- $\beta$ 1. Negative control groups with an absence of growth factors were also established. In the gene expression analysis (see below) this control group is not shown separately in the Results section since used for the ratio computation of the gene activity levels.

## Details Of Tissue Preparation And Culturing

Quadratic pieces of tissue with a side length of approximately 2 mm were prepared and sandwiched between two layers of agarose gel [upper layer: 0.5% (0.75 ml); lower layer : 1.0% (0.25 ml)], which was diluted with Dulbecco's Modified Eagle Medium [DMEM (Sigma-Aldrich, St. Louis, MO, USA)], as previously described [15, 16]. The explants (maintained in 24-well plastic plates) were bathed with 1 ml of high-glucose DMEM (Invitrogen, Carlsbad, CA, USA) containing 1% ITS-Premix (BD Biosciences, San Diego, CA, USA), 0.35 mM proline (Sigma-Aldrich), gentamicin (50 mg/ml) (Invitrogen) and ascorbic acid 2-phosphate (25 mg/ml) (Sigma-Aldrich). They were incubated at 37°C in an atmosphere containing 5% CO<sub>2</sub>. To induce their chondrogenic differentiation, the synovial explants were exposed to either BMP-2 alone (2000 ng/ml) (InductOs®, Pfizer/Medtronic, Minneapolis, MN, USA), TGF- $\beta$ 1 (10 ng/ml) (Peprotech, Rocky Hill, NJ, USA), or a combination of the two growth factors (at the aforementioned concentrations) for 2, 4 or 6 weeks [n = 7 to 11 explants (donors) per experimental group]; from the total of 64 patients we were able to culture on the average 3 to 4 explants per experimental group and per donor (i.e. 12–16 explants totally per donor). Culture media were changed every two days. Owing to the limited availability of the donor material, the explants derived from the group of younger patients were cultured for only 4 weeks.

## Histomorphometry, histochemistry and immunohistochemistry

At the end of the culturing period (2, 4 or 6 weeks), a portion of the specimens were processed for the histomorphometric quantification of metachromasia (sulfated proteoglycans) after staining with Toluidine Blue O and for the immunohistochemical demonstration of type-II collagen.

For these investigations the synovial explants were chemically fixed with a 2% solution of paraformaldehyde in phosphate buffered saline [PBS (pH 7.4)] for 2 hours at ambient temperature, rinsed three times with PBS, dehydrated in ethanol, and embedded in paraffin. 5- $\mu$ m-thick sections were prepared from the embedded material [15]. For the histomorphometric quantification of metachromasia, the sections were stained with 1% Toluidine Blue O (pH 2.5) for 5 minutes and then mounted in Entellan® (Merck, Darmstadt, Germany) prior to photography at a final magnification of 40x in a Nikon Eclipse E1000 light microscope (Nikon, Tokyo, Japan). The volume fraction of metachromasia was determined

from the light micrographs by the point-counting technique, which was applied in accordance with stereological principles [23, 24].

For the immunohistochemical demonstration of type-II collagen, the sections were first exposed to hyaluronidase [H-6254; Sigma-Aldrich (1 mg/ml of sodium acetate buffer, pH 5.5)] for 30 minutes at 37°C to activate the antigen. After rinsing with PBS for 5 minutes, they were incubated first with blocking buffer (1.5% equine serum in 3% skimmed milk) for 30 minutes at ambient temperature, and then with the avidin-biotin complex for 15 minutes, likewise at ambient temperature. Thereafter the sections were exposed to the primary antibody against type-II collagen (clone CII C1; Hybridoma Bank, Iowa City, IA, USA (diluted 1:25,000 in PBS containing 3% skimmed milk) for 1 hour at 37°C in a humid chamber. After rinsing, the sections were exposed to the secondary antibody (VECSTATIN ABC Kit, Vector Laboratories, Burlingame, CA, USA) for 30 minutes at ambient temperature. Endogenous peroxidase activity was blocked with hydrogen peroxide. Immunoreactivity was enhanced by applying first the avidin-biotin-peroxidase complex (Vector Laboratories) and then biotinyl tyramide (Perkin Elmer, Waltham, MA, USA). To visualize immunoreactivity, the sections were reacted with diaminobenzidine (DAB Kit, Vector Laboratories). Cell nuclei were counterstained with haematoxylin. The sections were evaluated and photographed in a Nikon Eclipse E1000 light microscope [16, 18].

## **Isolation Of Rna, Reverse Transcription And Real-time Pcr Analysis**

At the end of the culturing period (2, 4 or 6 weeks), the portion of the samples that were not used for histomorphometric and immunohistochemical evaluations were subjected to an RT-PCR analysis to determine the gene-expression levels of key anabolic cartilaginous markers [collagen types I, II, X and XI, aggrecan, alkaline phosphatase, cartilage oligomeric matrix protein (COMP), lubricin, matrilin-1, osteocalcin and Sox-9] and of a panel of catabolic factors [interleukins (IL) -1 $\beta$ , -4 and -6, a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS-4), cyclooxygenase-2 (Cox-2), inducible nitric oxide synthase (iNOS), matrix metalloproteinase-13 (MMP-13) and tumor necrosis factor alpha (TNF- $\alpha$ )]; for details (and abbreviations): see Tables 1, 2 and 3.

Table 1  
Primers and probes used for real-time PCR (anabolic factors)

Gene	Sequence	Final conc. (nM)	Amplicon size
Type-I collagen			
Forward	CATGCCGTGACTTGAGACTCA	50	86
Probe	CCACCCAGAGTGGAGCAG(T/C)GGTTACTACTG	100	
Reverse	GCATCCATAGTGCATCCTTGGT	900	
Type-II collagen			
Forward	GGCAATAGCAGGTTACGTACA	900	79
Probe	CCGGT(A/G)TGTTTCGTGCAGCCATCCT	100	
Reverse	CGATAACAGTCTTGCCCCACTT	300	
Type-X collagen			
Forward	AGGCTTCAGGGAGTGCCATC	300	82
Probe	GACCAGGTGTGGCTCCAGCTTCCC	100	
Reverse	AGGCCATTTGACTCGGCATT	900	
Type-XI collagen			
Forward	CTGCAGGTGCAGAGGGAAGA	300	102
Probe	GGGAAGCAGGTGCAGAAGGTCCTCCT	100	
Reverse	CAGGTCCCTGAGGACCGACT	900	
Aggrecan			
Forward	CTACCGCTGCGAGGTGATG	900	74
Probe	ATGGAACACGATGCCTTT(C/T)ACCACGA	100	
Reverse	TCGAGGGTGTAGCGTGTAGAGA	900	
Alkaline phosphatase			
Forward	CAACAACCTACCAGGCGCAGTC	50	84
Probe	CTGCGCCACGAGACCCACGG	100	
Reverse	GGGCCCTTGGAGAAGACG	50	
COMP			
Forward	CCAGAAGAACGACGACCAGAA	300	128

Gene	Sequence	Final conc. (nM)	Amplicon size
Probe	ACGGCGACCGGATCCGCAA	100	
Reverse	TCTGATCTGAGTTGGGCACCTT	900	
Matrilin-1			
Forward	AAGGTGGGCATTGTCTTCACTG	900	132
Probe	ATGTTTGCTGTGGGTGTGGGCAATG	100	
Reverse	TTCCCTCAGCTCATCCTCCAC	300	
Lubricin			
Forward	ATCCCACATCACCACCATCTTC	300	92
Probe	CCACCTTCAGGAGCATCTCAAACCATCA	100	
Reverse	TGGTTTGGGTGAACGTTTGG	300	
Osteocalcin			
Forward	CGGTGCAGAGTCCAGCAAAG	50	101
Probe	TCCAAGCAGGAGGGCAGCGAGG	100	
Reverse	GGGCTCCCAGCCATTGATAC	300	
Sox9			
Forward	ACGCCGAGCTCAGCAAGA	900	71
Probe	CGTTCAG(A/G)AGTCTCCAGAGCTTGCCCA	100	
Reverse	CACGAAGGGCCGCTTCT	300	

Table 2  
Primers and probes used for real-time PCR (catabolic factors and 18s rRNA)

Gene	Sequence	Final conc. (nM)	Amplicon size
ADAMTS4			
Forward	GAGCAGTGTGCTGCCTACAACC	300	119
Probe	CCCATGGACTGGGTTCCCTCGCTACA	100	
Reverse	TGGCAGGTGAGTTTGCACTG	300	
MMP-13			
Forward	TTGTTGCTGCGCATGAGTTC	300	104
Probe	TCCAAGGACCCTGGAGCACTCATGTTT	100	
Reverse	AAAGTGGCTTTTGCCGGTGTAG	50	
IL-1 $\beta$			
Forward	AATCCCAGCCCTTTTGTTG	300	85
Probe	ACCTCTCCTACTCACTTAAAGCCCGCCTGA	100	
Reverse	AAATGTGGCCGTGGTTTCTG	300	
IL-4			
Forward	TTTGTCCACGGACACAAGTGC	900	124
Probe	TCACAGAGCAGAAGACTCTGTGCACCGA	100	
Reverse	TCTTGGAGGCAGCAAAGATGTC	900	
IL-6			
Forward	CCTGACCCAACCACAAATGC	300	146
Probe	TCTGCGCAGCTTTAAGGAGTTCCTGCA	100	
Reverse	CCATGCTACATTTGCCGAAGAG	300	
/Nos			
Forward	TGCTGATGCGCAAGACAATG	300	79
Probe	CGAGTCAGAGTCACCATCCTCTTTGCGA	100	
Reverse	AGCGCCTCTGATTTTCCTGTC	300	
TNF- $\alpha$			
Forward	TTTGGGATCATTGCCCTGTG	900	130
Probe	AACATCCAACCTTCCCAAACGCCTCC	100	

Gene	Sequence	Final conc. (nM)	Amplicon size
Reverse	GCCCCCAATTCTCTTTTTGAGC	900	
Cox-2			
Forward	TGGAAGCCAAGCACTTTTGG	50	90
Probe	TGGGTTTTCAAATCATCAACTGCCTCA	100	
Reverse	GCCCTTCACGTTATTGCAGATG	50	
18s rRNA			
Forward	CGGCTACCACATCCAAGGAA	900	187
Probe	TGCTGGCACCAGACTTGCCCTC	100	
Reverse	GCTGGAATTACCGCGGCT	50	

The total amount of RNA was isolated using the RNeasy Micro Kit (Qiagen, Basel, Switzerland) in accordance with the manufacturer's standard protocol, which included a deoxyribonuclease-catalyzed digestion step. The concentrations of mRNA were measured in a Nano Drop spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). The samples of mRNA were stored at -70°C and then subjected to reverse transcription (200-ng aliquots) using an ImProm-II- Reverse Transcription (Promega, Madison, WI, USA), in accordance with the manufacturer's instructions. Samples of cDNA were diluted 1:10 in nuclease-free water and stored at -20° C. 1-ng aliquots of cDNA were employed for the relative quantification of RNA by an RT-PCR analysis using an ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and 96-well PCR plates (Thermo Fisher Scientific, Rockford, IL, USA). The sequences of the primers and probes and their concentrations are given in Table 1 and 2. The probes were labelled with 6-carboxy-fluorescein at the 5' end and with Eclipse→ Dark Quencher at the 3' end. Primer and Probes were used at the given final concentrations (Tables 1 and 2) in a volume of 25 µl. In preparation for the RT-PCR analysis, 25-µl aliquots of the primers, probes, cDNA and qPCR MasterMix Plus (Eurogentec, Seraing, Belgium) were subjected to an initial 10-minute denaturation step at 95°C, followed by 45 cycles of a 15-second denaturation at 95°C and a 60-second extension at 60°C. The reactivity of the primers and probes was confirmed using samples of cDNA that had been prepared from human cartilage or the stimulated human synovial explants. The reactivity of the primer and probe for IL-4 was confirmed using cDNA that had been prepared from human blood. The levels of mRNA were quantified relative to those for 18S rRNA using the comparative cycle-threshold method [14]. For these calculations, the gene-expression levels in unstimulated synovial explants served as the basis for the comparison [25].

## Statistical Analyses

All statistical analyses were conducted using Prism 8 (version 8.4.3; GraphPad Software, San Diego, CA, USA). For volume fractions of metachromasia, comparisons between two sets of data were statistically

evaluated by unpaired *t*-test. Comparisons between multiple groups were evaluated by one-way ANOVA and then by implementing Dunette's multiple comparison test. For gene expressions, comparisons between two sets of data were statistically evaluated by the Mann-Whitney test. Comparisons between multiple groups were evaluated by Kruskal-Wallis test and then by implementing Dunn's post test. The correlation between the age of patients and the volume fraction of metachromasia was determined using R square values calculated by a liner regression.

## Results

### *Donors and sampling.*

From the total number of 64 patients participating in this study we were able to obtain per donor on the average 3 to 4 explants per experimental group for culturing and/or histomorphometrical, histochemical or gene-expression analyses, i.e. 12–16 explants totally per donor. The total duration of time over which sampling was performed was 18 months.

### *Histology, histochemistry, immunohistochemistry, morphometry.*

Negative control groups (absence of a growth factors) did not show any signs of chondrogenic differentiation (Figs. 1 and 2). Those that had been exposed to BMP-2 alone or a combination of BMP-2 and TGF- $\beta$ 1, underwent chondrogenic differentiation, as evidenced by the intense metachromatic staining properties of the extracellular matrix – indicative of the presence of sulfated glycosaminoglycans in abundance – and manifested the widespread appearance of pericellular lacunae – a characteristic feature of chondrocytes that have been chemically fixed in aldehyde for light microscopy [26] (Fig. 1). The intensity of metachromasia increased as a function of culturing time. At each juncture, the distribution of metachromasia over the sections was inhomogenous. Synovial explants that had been exposed to TGF- $\beta$ 1 alone manifested only weak metachromasia. Immunoreactivity for type-II collagen (Fig. 2) likewise reflected the findings described above for the metachromatic staining properties of the tissue.

The histomorphometric quantification of the volume fraction of metachromasia (Fig. 3A) confirmed the time-dependency of chondrogenesis induced by BMP-2 alone or by a combination of BMP-2 and TGF- $\beta$ 1. At each time-point, the volume fraction of metachromasia appeared greater after exposure to the BMP-2/TGF- $\beta$ 1 combinations than to BMP-2 alone. Although such a temporal increase in this parameter appears graphically apparent (Fig. 3A), a comparable level of metachromasia was attained between these two groups already after 4 weeks of exposure to the growth factors [ $p = 0.78$  (comparison between the BMP-2 groups, 4 weeks vs. 6 weeks) and  $p = 0.6$  (BMP-2/TGF- $\beta$ 1 groups, 4 weeks vs. 6 weeks)]. Moreover, the individual time-point values show a high inter-individual variability (coefficients of error ranging between 0.21–1.0). Figure 3B shows that the volume fractions of metachromasia were also on comparable levels when comparing the explant differentiation results of the young and the old age groups with each other [ $p = 0.33$  (BMP-2 groups, young vs. old) and  $p = 0.09$  (BMP-2/TGF- $\beta$ 1 groups, young vs. old)]. In the TGF- $\beta$ 1- only group the degree of metachromasia attained was clearly lower (e.g. in

the young group:  $p = 0.0005$  vs. BMP-2; in the old group:  $p = 0.002$  vs. BMP-2). The illustration of the data distribution for all patients in Fig. 4 confirms the age-independency of the quantitative metachromasia results. The correlation analyses for the BMP-2 patient materials and for the BMP-2/TGF- $\beta$ 1 treated groups showed a negative correlation between age and volume fraction [ $R^2 = 0.1534$  (for BMP-2 stimulation), 0.021 (for TGF- $\beta$ 1 stimulation) and 0.0512 (for BMP-2/TGF- $\beta$ 1 stimulation)] (Fig. 4). The graph also illustrates the large scatter of the results, i.e. the high variability within each age group.

### *Gene-expression evaluation*

The gene-expression levels were temporally monitored after culturing periods of 2, 4 and 6 weeks. With the exception of *matrilin-1*, *IL-4* and *TNF- $\alpha$* , the mRNA levels of which lay below the limits of detection, the activities of each of the investigated genes could be quantified (Fig. 5).

Among anabolic genes, *collagen types II, X and XI*, *aggrecan* and *Sox9* were elevated after stimulation, irrespective of the nature of growth factors used (Fig. 5). The expression levels of these genes generally peaked at the 4-week juncture. *COMP* and *lubricin* were up-regulated after stimulation with TGF- $\beta$ 1 alone or BMP-2/TGF- $\beta$ 1, but not with BMP-2 alone. *Alkaline phosphatase* was up-regulated after stimulation with BMP-2 alone or BMP-2/TGF- $\beta$ 1, but not with TGF- $\beta$ 1 alone. *Type-I collagen* level was slightly raised only after stimulation with TGF- $\beta$ 1. *Osteocalcin* levels were not changed, irrespective of the type of growth factor applied.

The levels of catabolic genes were barely changed during culturing (Fig. 5). Only the levels of *ADAMTS-4* (after stimulation with TGF- $\beta$ 1), *COX-2* (after stimulation with BMP-2/TGF- $\beta$ 1) and *iNOS* (after stimulation with BMP-2 or BMP-2/TGF- $\beta$ 1) were slightly elevated (< 10-fold) time-dependently. On the other hand, the levels of *IL-1* and *IL-6* were down-regulated, especially at the 2-week juncture after stimulation with BMP-2/TGF- $\beta$ 1.

The change of gene expression after stimulation was compared between younger and older patients at the 4-week-juncture (Fig. 6). The differences between these two age groups were mainly observed among anabolic genes depending upon the stimulation condition. After stimulation with BMP-2, *COMP* was elevated significantly higher in older patients than in younger patients. After stimulation with TGF- $\beta$ 1, *type-X collagen* and *sox9* were elevated significantly higher in older patients than in younger patients. After stimulation with BMP-2/TGF- $\beta$ 1, *collagen types I, X and XI*, *aggrecan*, *COMP* and *sox9* were elevated significantly higher in older patients than in younger patients. Among catabolic genes, only *IL-1 $\beta$*  was significantly higher in older patients than in younger patients after stimulation with BMP-2 alone.

## **Discussion**

Compared with isolated MSCs, the most important advantage of synovial explants is that the physiological scaffold [extracellular matrix (ECM)] is present around the cells and is provided by the synovial tissue itself. Such scaffold might be more conducive to chondrogenic differentiation than other, non-joint associated scaffolds (or if ECM is absent). Indeed, the synovial tissue is known to differentiate

into cartilaginous tissue under both clinicopathological [27] and experimental conditions [15, 28], resulting in the formation of cartilaginous tumors and cartilage-bone-like tissues. Furthermore, our previous studies have shown that bovine synovial explants are able to form more abundant cartilaginous matrix than isolated, alginate-cultured and aggregate-cultured synovial MSCs [29–31]. These findings suggest that a system using synovial explants, which can obviate the need for cell isolation and cell preculturing, could be one of the most promising strategies for the repair of articular-cartilage lesions.

We had shown previously that bovine synovial explants of healthy animals are able to differentiate into cartilaginous tissue after stimulation with appropriate growth factors [15, 16, 18]. In the present study, we demonstrated that synovial explants derived from OA patients have a chondrogenic potential, and maintain this capacity irrespective of age. Given the age-independent high potential of human synovial tissue it may thus be possible to exploit this potential for the repair of cartilaginous lesions in a clinical setting [32].

The activity and the differentiation potential of MSCs of various origins were found to decline with ageing and with the number of passages *in vitro* [19, 20]. Consequently, the MSCs of elderly donors are considered to be unsuitable for the purposes of tissue engineering [33]. Also the influence of donor age on the chondrogenic potential of MSCs derived from various tissue sources had been addressed by several research groups [34, 35], and the data are conflicting. One possible reason for the discrepant findings might be that MSCs may not be considered as a “universal” population subject to the same process of ageing. The ageing of MSCs may be a tissue-specific phenomenon, or at least a process that is differentially influenced by their origin. Given the great potential value and widespread use of MSCs in regenerative medicine, a clarification of this issue is important, particularly in the fields of orthopaedics and rheumatology, since patients who would qualify for the instigation of an autologous, MSC-based cartilage-repair strategy, would be primarily elderly ones suffering from osteoarthritis.

In a previous study, we demonstrated that synovial cells derived from the knee joints of osteoarthritic patients could be induced to differentiate into cartilage-producing chondrocytes (*in vitro*) [14], a finding that was reconfirmed by other researchers [36, 37] on similar grounds. However, the influence of donor age on the process of chondrogenic differentiation was not addressed, nor the issue if the generally shifted balance of cell metabolism to catabolic activities of chondrocytes in osteoarthritic joints [38, 39]. These phenomena most likely would affect repair cartilage formation from synovial tissues when originating from such diseased joints; to clarify these potentially adverse issues in tissue engineering of diseased joint cartilage in human patients when using synovial cells and tissue sources of the same joint was the purpose of the present study, using synovial explants.

As evidenced by the deposition of sulfated glycosaminoglycans (volume fraction of metachromasia), BMP-2 alone, and the BMP-2/TGF- $\beta$ 1 combination, induced the chondrogenic differentiation of the synovial explants to the greatest and to similar degrees (no significant difference between the values) (Figures.3A & 3B). Thus TGF- $\beta$ 1 in a combined use with BMP-2 exerted no synergistic/enhancing effect in the human synovial tissue (of OA-joints) differentiation (unlike in synovial tissue of normal bovine joints

of young adult animals [18]). Given that the peak volume fraction of metachromasia was approximately 9-fold lower after stimulation with TGF- $\beta$ 1 alone than after exposure to either BMP-2 alone or to the BMP-2/TGF- $\beta$ 1 combination, this is not a surprising finding, and it was, moreover, supported by the immunohistochemical staining profiles for type-II collagen. Although temporal differences in the volume fraction of metachromasia were not significant (owing to high inter-individual variability), a time-dependent increase in this parameter was nonetheless graphically apparent (Fig. 3A), thereby indicating that the chondrogenic activity of the synovial MSCs could be sustained for at least 6 weeks *in vitro*. When the volume fractions of metachromasia at the 4-week juncture for the two age categories of osteoarthritic patients were compared, no differences were revealed (Fig. 3B). And when this histomorphometric parameter was displayed as a function of an individual's age, the correlation coefficients were very low (Fig. 4). Thus the general differentiation potential of synovial explants originating from human patients suffering from OA was not impaired as a function of donor age. Moreover the achieved degree of tissue transformation into cartilage-like tissue of the synovial explants was found to be of the same order of magnitude as that encountered in synovial tissue originating from healthy young adult bovine sources [15, 16, 18]. The osteoarthritic process thus seems not to affect the differentiation potential of the synovial tissue in human patients.

The analysis of the gene-expression levels of key anabolic markers of chondrogenesis permits a more discriminative evaluation of the induced differentiation process into cartilaginous tissue.

Type-I collagen is not a marker of chondrogenesis, but a characteristic component of fibrous tissues, and the presence of high levels of its mRNA in repair cartilage is an indication that it is deficient in hyaline-like qualities. Irrespective of the stimulation protocol, the gene-expression levels of type-I collagen were extremely low and bordering on baseline values (Fig. 5). Only after exposure to the BMP-2/TGF- $\beta$ 1 combination a significant difference between the two age categories was revealed, with slightly higher levels in the group of older patients (Fig. 6).

Type-II collagen is a characteristic component of cartilaginous tissues, and, irrespective of the stimulation protocol, its gene-expression levels were higher than for any other marker. However, no age-related differences were revealed (Fig. 6). This is somewhat surprising since on the basis of data in the literature relating to bone marrow derived MSCs and their declining proliferation and differentiation potential with increasing age[19, 33], as well as with perichondrial-derived cells[40] or with periosteum tissue flaps[35], this apparently does not apply for synovial tissue, as found here, nor for isolated synovial-derived MSCs, as previously found [11].

Type-X collagen is a marker of terminal chondrocyte hypertrophy, and high mRNA levels indicate that the extracellular matrix is undergoing calcification, which is undesirable. Irrespective of the stimulation protocol, the gene-expression levels of type-X collagen were elevated, but they were still lower than those of type-II collagen. In the group of younger patients the levels were lower after stimulation with TGF- $\beta$ 1 alone, than after exposure to either BMP-2 alone or the BMP-2/TGF- $\beta$ 1 combination (Fig. 6). Since TGF- $\beta$ 1 was found to suppress the terminal hypertrophic differentiation of chondrocytes [18], this finding is not

surprising. Significant age-related differences in the gene-expression levels of type-X collagen were revealed after stimulation with either TGF- $\beta$ 1 alone, or the BMP-2/TGF- $\beta$ 1 combination, the values being higher in the group of older osteoarthritic patients than in the group of younger ones. This phenomenon is in correlation to findings in relation to articular cartilage and osteoarthritic cartilage cells in OA patients [41, 42] and animal models [43], and interestingly is also observed upon differentiation of synovial tissue from osteoarthritic patients; an explanation, however, could not be identified in the literature for this phenomenon. However, given the data relating to the alkaline phosphatase gene activities (see next paragraph) the phenomenon may not be of a generalized metabolic implication but rather a gene-specific activity of type-X collagen gene, without posttranslational activity effects, and thus without functional effects.

Alkaline Phosphatase is likewise a marker of terminal chondrocyte hypertrophy and matrix mineralization [44]. In both age categories of patients, its gene-expression levels were lowest (barely above baseline values) after stimulation with TGF- $\beta$ 1 alone – which accords with the known suppressive effect of this growth factor on terminal hypertrophy –, and lower after exposure to the BMP-2/TGF- $\beta$ 1 combination than after treatment with BMP-2 alone. An age-difference was revealed only after stimulation with the BMP-2/TGF- $\beta$ 1 combination, the values being higher in the group of older osteoarthritic patients than in the group of younger ones.

Type-XI collagen is a marker of cartilage-specific collagen fibril formation since co-expressed with collagen type-II [45]. The gene expression levels of type-XI collagen were highest after exposure to either BMP-2 alone or the BMP-2/TGF- $\beta$ 1 combination. In the latter case, a significant age-related difference was revealed, the values being higher in the group of older osteoarthritic patients than in the group of younger ones. Type-XI collagen gene activities were thus stimulated by both stimulation protocols i.e. by BMP-2 alone or by the BMP-2/TGF- $\beta$ 1 combination, and this was indeed expected given the histological differentiation data obtained and presented above. However the reasons for the differences obtained respecting the different degrees of activation remained unclear.

The gene-expression levels of aggrecan were higher after stimulation with either BMP-2 alone or the BMP-2/TGF- $\beta$ 1 combination than after exposure to TGF- $\beta$ 1 alone. These data accord with the histomorphometric findings for the deposition of sulfated glycosaminoglycans. An age-related difference was revealed only after stimulation with the BMP-2/TGF- $\beta$ 1 combination, the values being higher in the group of older osteoarthritic patients than in the group of younger ones. This data accord with the observations described above relating to type-II collagen gene activities, i.e. the independence of age of the synovial tissue differentiation activity potential.

The gene-expression levels of COMP [46] and Lubricin [47–49], which are markers of the articular-cartilage layer as a whole and of its superficial zone and of synovial fluid, were highest after stimulation with either TGF- $\beta$ 1 alone or the BMP-2/TGF- $\beta$ 1 combination, and lowest after exposure to BMP-2 alone. Indeed, using the latter stimulation protocol, the levels of lubricin were not raised above baseline values. This differs to reported findings relating to muscle-derived MSCs following their chondrogenic

differentiation [50]. Significant age-related differences in the gene-expression levels of COMP were revealed after stimulation with either BMP-2 alone or the BMP-2/TGF- $\beta$ 1 combination, the values being higher in the group of older osteoarthritic patients than in the group of younger ones [51]; this possibly may be related to TGF- $\beta$ 1 effects [52]. No significant age-related differences in the gene-expression levels of lubricin were disclosed. Irrespective of the stimulation protocol applied, the gene-expression levels of osteocalcin, which is a marker of chondrocytic hypertrophy and mineralization [53], were not raised above baseline values. This finding confirms that the process of chondrogenic differentiation was not proceeding to terminal chondrocyte hypertrophy and matrix mineralization [18] within the time period investigated; however, after longer time periods of culturing these unwanted effects are expected to occur in the absence of TGF- $\beta$ 1 [18, 42, 44, 53].

The gene expression levels of matrilin-1, which is a marker of tissue degradation [54], lay below the limits of detection in all instances. Hence, in the newly formed cartilaginous tissue, anabolic processes override catabolic activity [55].

Sox9 is a transcription factor, which acts as a DNA-binding protein during chondrogenic differentiation. Its mRNA levels are believed to peak during the mid-phase of chondrogenesis [56]. Irrespective of the stimulation protocol used, the gene-expression levels of Sox-9 peaked – as expected – at the 4-week juncture. The same temporal pattern of gene-expression was observed for type-X collagen, aggrecan and COMP, which accords with the current opinion that their induction occurs via Sox9 regulated pathways [57]. The peak-gene expression levels of Sox9 were higher after stimulation with either BMP-2 alone, or the BMP-2/TGF- $\beta$ 1 combination, than after exposure to TGF- $\beta$ 1 alone, which accords with the histomorphometric findings, namely, that the chondrogenic differentiation of synovial explants occurred to a greater degree after stimulation with either BMP-2 alone or the BMP-2/TGF- $\beta$ 1 combination than after exposure to TGF- $\beta$ 1. Significant age-related differences in the gene-expression levels of Sox9 were revealed after stimulation with either TGF- $\beta$ 1 or the BMP-2/TGF- $\beta$ 1 combination, the values being higher in the group of older osteoarthritic patients than in the group of younger ones.

Compared to the genes for the anabolic markers, those for the catabolic ones [58], which included inflammatory factors [59, 60] [IL-1 $\beta$ , IL-4 (not detected), IL-6, TNF- $\alpha$  (not detected), COX-2 and iNOS] and matrix proteases (MMP-13, ADAMTS-4) were generally expressed at very low levels. As for the anabolic markers, the peak levels were usually attained at the 4-week juncture (Fig. 6). No consistent trend in favor of anyone particular stimulation protocol was observed. But, generally speaking, lower peak levels (the desired results) were achieved in the absence of TGF- $\beta$ 1 (i.e. with BMP-2 alone) than in its presence (TGF- $\beta$ 1, or a combination of BMP-2/TGF- $\beta$ 1). With the exception of IL-1 $\beta$ , no significant age-related differences in the gene-expression levels of the catabolic markers were observed. In the case of IL-1 $\beta$ , the values were slightly higher in the group of older osteoarthritic patients than in the group of younger ones. The finding that the eight catabolic-marker genes were expressed at very low or even non-detectable (IL-4 and TNF- $\alpha$ ) levels indicates that the production of a cartilaginous matrix (attested by the histochemical and immunohistochemical observations, and substantiated by the gene-expression levels of the anabolic markers) was positively balanced against degradative processes [59]. The finding that five of the six

detected catabolic marker genes were expressed at similar levels in the older and the younger individuals indicates that the state of positive equilibrium between anabolic and catabolic processes was not compromised by ageing.

## Conclusion

Our data reveal that in younger and older age groups alike, synovial explants from osteoarthritic joints can be equally well induced to undergo chondrogenesis *in vitro*; i.e. synovial MSCs are not compromised by ageing and that the chondrogenic differentiation process can be sustained for minimally 6 weeks without the onset of matrix degradation in osteoarthritic patients. And as evidenced by the very low gene-expression levels of osteocalcin, there were no indications that the process of chondrogenesis proceeded to osteogenesis during the 6-week culturing period. Notably, the gene-expression levels of all six catabolic markers investigated were very low, and for five of these, no age-related differences were revealed. These findings imply that the autologous synovium can be used in patients suffering from osteoarthritis independent of their age for the clinical engineering of repair cartilage.

## List Of Abbreviations

ADAMTS-4: a disintegrin and metalloproteinase with thrombospondin motifs 4

A: Adenine

BMP-2: bone morphogenetic protein 2

cDNA: complementary desoxyribonucleic acid

COMP: cartilage oligomeric matrix protein

COX-2: cyclooxygenase-2

C: Cytosine

DMEM: Dulbecco's Modified Eagle Medium

G: Guanine

IL: interleukin

iNOS: inducible nitric oxide synthase

mRNA : messenger ribonucleic acid

MMP-13: matrix metalloproteinase-13

PBS: phosphate buffered saline

RT-PCR: real-time polymerase chain reaction

18S rRNA: 18S ribosomal ribonucleic acid

T: Thymine

TNF- $\alpha$ : tumor necrosis factor alpha

TGF- $\beta$ 1: transforming growth factor beta 1

## **Declarations**

### **Ethical Approval and Consent to participate**

Informed consent was obtained from the individual patients to donate their surgical waste material for the present study. An approval by the local ethical commission was also obtained.

### **Consent for publication**

All authors/coauthors agree with the text/content of this manuscript and to submit it for publication in this Journal.

### **Availability of supportive data**

All data and supportive data of this study are available on an unrestrictive basis.

### **Competing interests**

The authors do not have any kind of a competing interest and do not have any conflict of interest.

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### **Authors` Contributions**

Ernst B. Hunziker: Conception, Design, Planning, Supervision, Data Analysis, Drafting and  
writing of manuscript, Administration

Nahoko Shintani: Data Analysis, Lab work, Collection and Assembly of Data, Statistics,  
Critical Revision

Miroslav Haspel: Study Design, Technical and Logistical Support, Critical

Revision, Provision of Study Materials

Kurt Lippuner: Analysis and Interpretation of Data, Text Editing, Critical Revision, Final

Approval, Obtaining Funding

Esther Vögelin: Design, Analysis and Interpretation of Data, Text Editing, Critical Revision,

Obtaining Funding, Final Approval

Marius JB Keel: Provision of Study Materials, Design, Analysis and Interpretation of Data,

Text Editing, Critical Revision, Obtaining Funding, Final Approval

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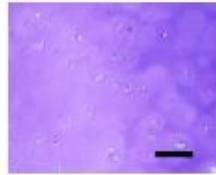
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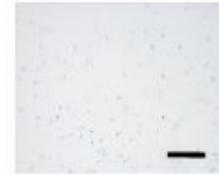
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## Figures

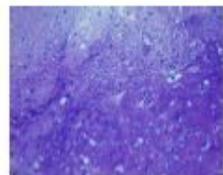
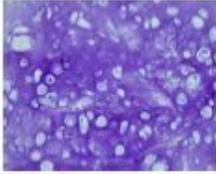
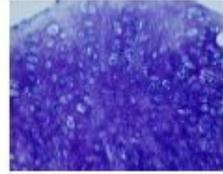
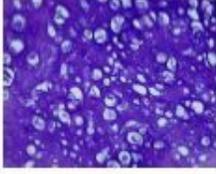
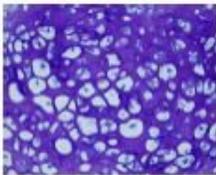
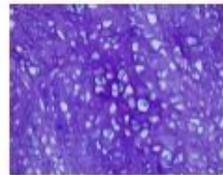
**Native articular cartilage**



**Uncultured synovial explant**



**Cultured synovial explant**

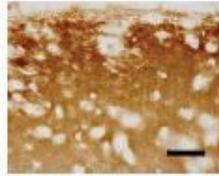
Age group	Culture period	No growth factor	BMP-2	TGF- $\beta$ 1	BMP-2 + TGF- $\beta$ 1
66 - 86 Years (old)	2 weeks				
	4 weeks				
	6 weeks				
55 - 65 Years (young)	4 weeks				

**Figure 1**

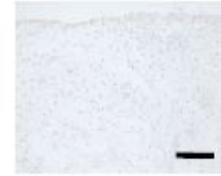
Light micrographs of sections through synovial explants in the two age categories of osteoarthritic patients, 2, 4 or 6 weeks after exposure to either BMP-2 alone, TGF- $\beta$ 1 alone, or a combination of the two growth factors, and staining with Toluidine Blue O. Untreated synovial explants and native articular cartilage served as negative and positive controls, respectively. Synovial explants that had been stimulated with either BMP-2 alone, or the BMP-2/TGF- $\beta$ 1 combination manifested a temporal increase in

the intensity of metachromatic staining, whereas those that had been exposed to TGF- $\beta$ 1 alone exhibited only weak metachromasia at each juncture. The development of metachromasia was accompanied by the widespread appearance of pericellular lacunae, which are a characteristic feature of chondrocytes.

**Native articular cartilage**



**Uncultured synovial explant**



**Cultured synovial explant**

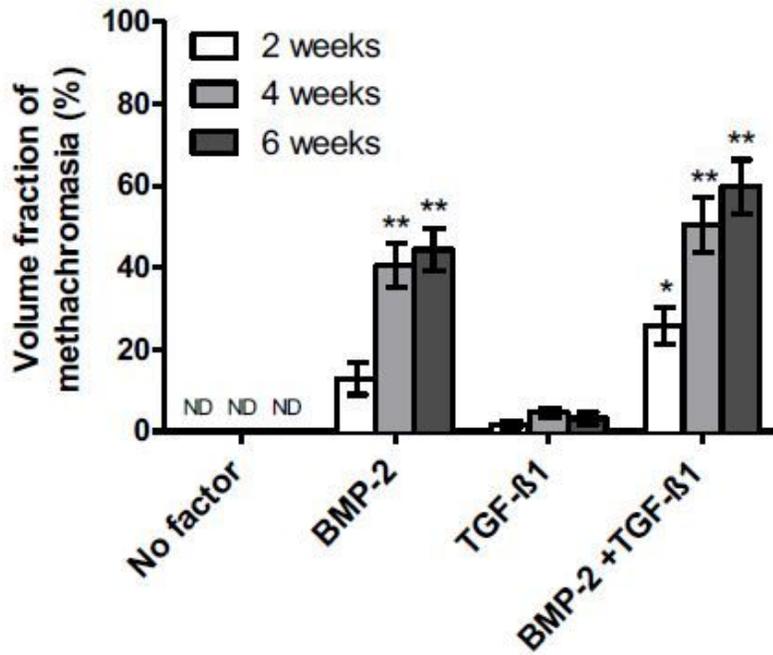
Age group	Culture period	No growth factor	BMP-2	TGF- $\beta$ 1	BMP-2 + TGF- $\beta$ 1
66 - 86 Years (old)	2 weeks				
	4 weeks				
	6 weeks				
55 - 65 Years (young)	4 weeks				

**Figure 2**

Light micrographs of sections through synovial explants in the two age categories of osteoarthritic patients, 2, 4 or 6 weeks after exposure to either BMP-2 alone, TGF- $\beta$ 1, or a combination of the two growth

factors, and immunostaining for type-II collagen. Untreated synovial explants and native articular cartilage served as negative and positive controls, respectively. Synovial explants that had been stimulated with either BMP-2 alone or the BMP-2/TGF- $\beta$ 1 combination manifested a temporal increase in the intensity of immunostaining for type-II collagen, whereas those who had been exposed to TGF- $\beta$ 1 exhibited only weak immunoreactivity at each juncture.

**A**



**B**

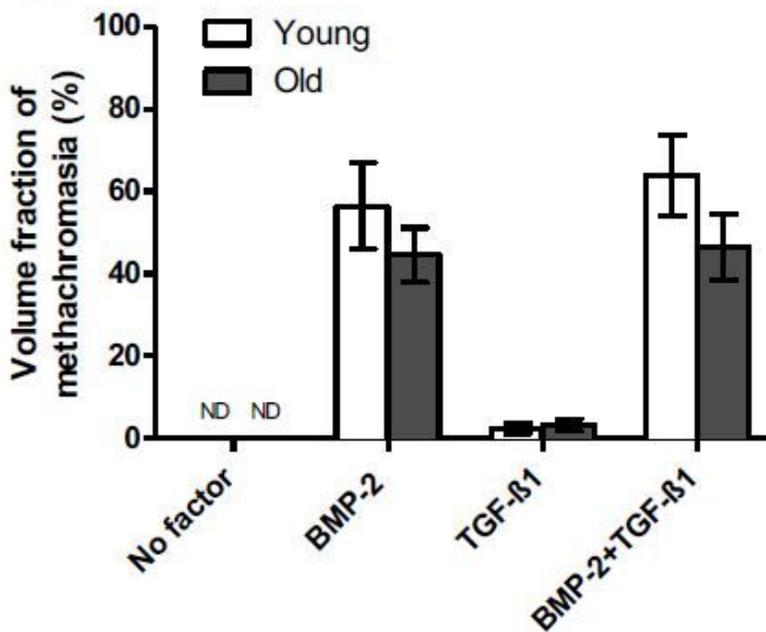


Figure 3

A: Temporal changes in the volume fraction of metachromasia within synovial explants that had been derived from the group of older osteoarthritic patients (66 - 86 years) after exposure to either BMP-2 alone, TGF- $\beta$ 1 alone, or a combination of the two growth factors. At each time point, the volume fraction of metachromasia was highest after stimulation with the BMP-2/TGF- $\beta$ 1 combination, somewhat lower after exposure to BMP-2 alone, and by far the lowest after treatment with TGF- $\beta$ 1 alone. After exposure to either BMP-2 alone or the BMP-2/TGF- $\beta$ 1 combination, the volume fraction of metachromasia appeared to increase as function of culturing time. However, owing to the high inter-individual variability (coefficient of variance: 50 - 90%), the differences were not statistically significant. Mean values  $\pm$  SEM are represented. (The n-values at the 2, 4- and 6- week junctures were 7, 8 and 7, respectively.) ND: not detected.\*:  $p < 0.05$ , \*\*:  $p < 0.01$  vs. No factor. B: Volume fractions of metachromasia within the synovial explants of the two age categories of osteoarthritic patients (55 - 65 years and 66 - 86 years), four weeks after exposure to either BMP-2 alone, TGF- $\beta$ 1 alone, or a combination of the two growth factors. Irrespective of the stimulation protocol, the volume fractions of metachromasia were similar in the two age categories of osteoarthritic patients ( $p > 0.05$ ). Mean values  $\pm$  SEM are represented (55 - 65 years:  $n=9$ ; 66 - 86 years:  $n=7$ ). ND: not detected.

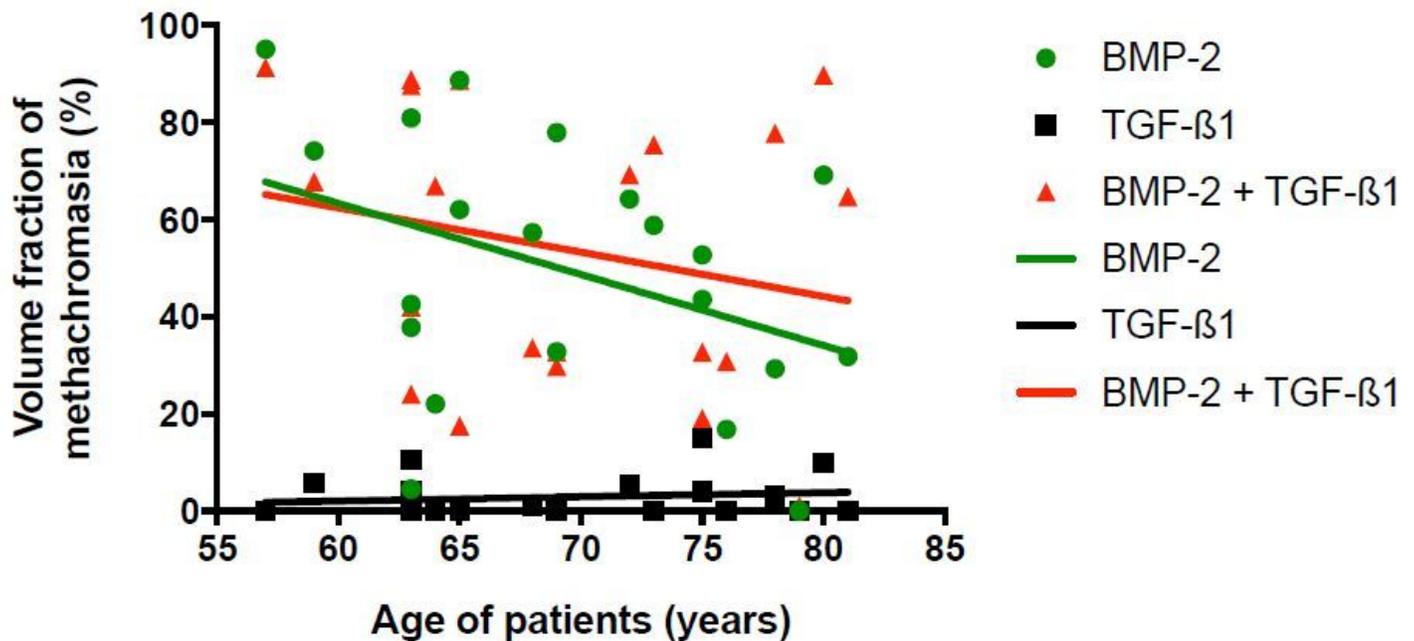
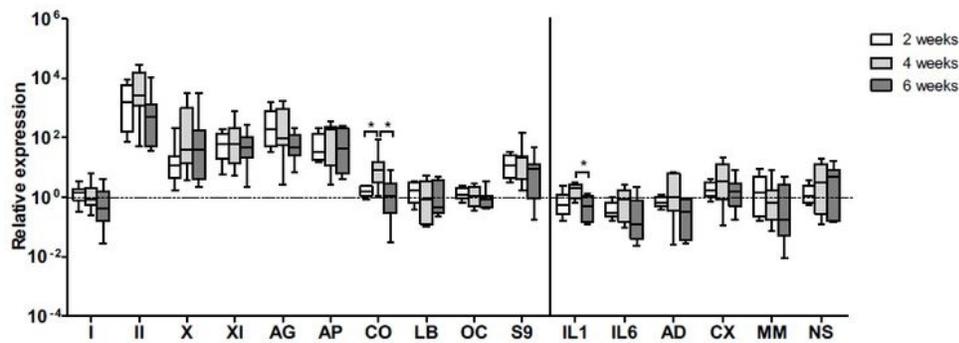


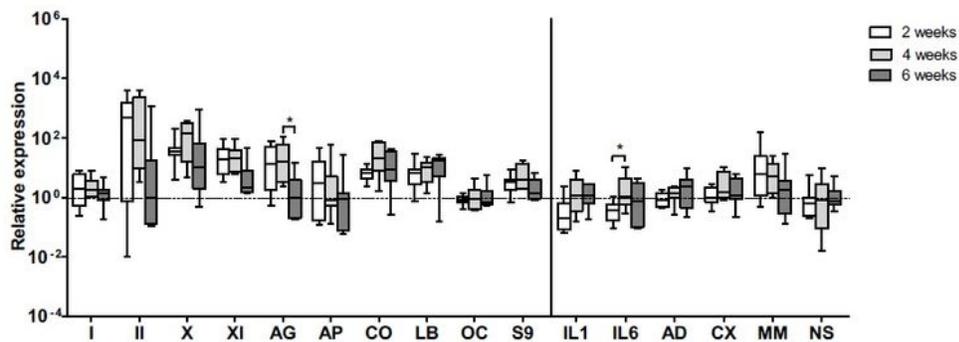
Figure 4

Volume fraction of metachromasia within the synovial explants of each of the osteoarthritic patients, four weeks after exposure to either BM-2 alone, TGF- $\beta$ 1 alone, or a combination of the two growth factors, expressed as a function of the individual's age. The regression curve yielded very low correlation coefficients, thereby indicating that the volume fraction of metachromasia was not influenced by the age of the osteoarthritic patient. BMP-2 alone:  $y = -1.5629x + 154.95$  ( $R^2 = 0.10919$ ); TGF- $\beta$ 1 alone:  $y = 1.0866x + 130.74$  ( $R^2 = 0.0819$ ); BMP-2/TGF- $\beta$ 1:  $y = 0.0894x - 2.819$  ( $R^2 = 0.0246$ ).

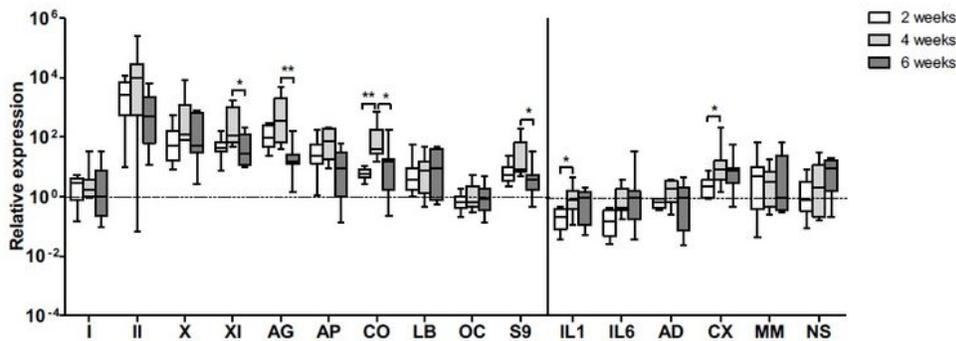
### A BMP-2



### B TGF- $\beta$ 1



### C BMP-2 + TGF- $\beta$ 1

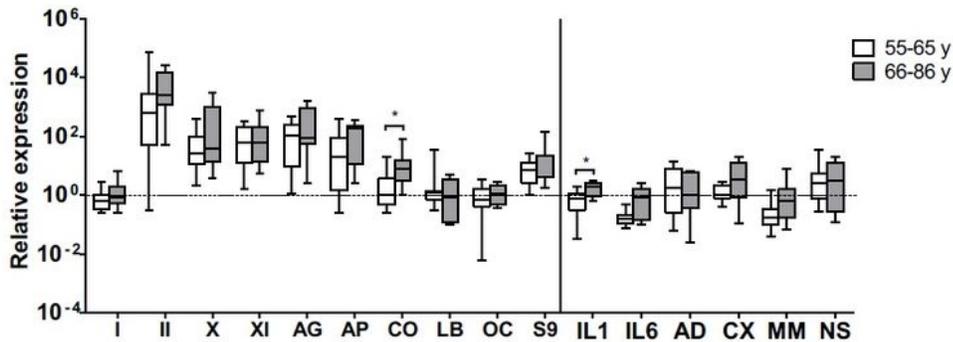


## Figure 5

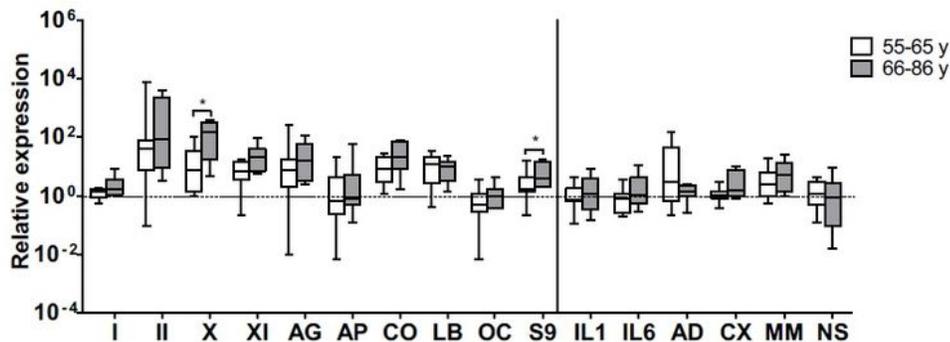
Temporal changes in the gene expression levels of the indicated anabolic and catabolic markers (to the left and to the right of the dividing vertical line, respectively) within the synovial explants of the group of older patients (66 - 86 years) after stimulation with either BMP-2 alone, TGF- $\beta$ 1 alone, or a combination of the two growth factors. The values are presented as box plots, in which the lines within the box represent the median, the box represents the 25th to 75th percentile, and the whiskers represent the maximum and

minimum values (the n-values at the 2, 4- and 6- week junctures were 8, 8 and 7 respectively.) Differences with p-values smaller than 0.05 between the time-points for each stimulation protocol are indicated: \*: p < 0.05; \*\*: p < 0.01. Key to abbreviations: I:Type-I collagen; II:Type-II collagen; X:Type-X collagen; XI:Type-XI collagen; AG:aggrecan; AP:Alkaline Phosphatase; CO:COMP; LB:Lubricin; OC:Osteocalcin; S9:Sox-9; IL1:IL-1; IL6:IL-6; AD:ADAMTS-4; CX:Cox-2; MM:MMP-13; NS:iNOS.

### A BMP-2



### B TGF-β1



### C BMP-2 + TGF-β1

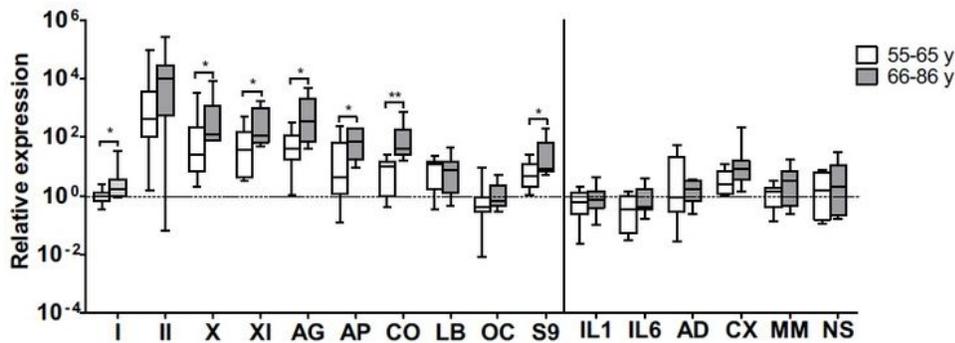


Figure 6

Comparison of changes in the gene-expression levels of the indicated anabolic and catabolic markers (to the left and to the right of the dividing vertical line, respectively) within the synovial explants of the age groups of osteoarthritic patients, 4 weeks after stimulation with either BMP-2 alone, TGF- $\beta$ 1 alone, or a combination of the two growth factors. The values are presented as box plots, in which the lines within the box represent the median, the box represents the 25th to 75th percentile, and the whiskers represent the maximum and minimum values (55 - 65 years: n = 11; 66 - 86 years: n = 8). P-value differences between the time-points for each stimulation protocol are indicated: \*: p < 0.05; \*\*: p < 0.01. Key to abbreviations: I:Type-I collagen; II:Type-II collagen; X:Type-X collagen; XI:Type-XI collagen; AG:aggrecan; AP:Alkaline Phosphatase; CO:COMP; LB:Lubricin; OC:Osteocalcin; S9:Sox-9; IL1:IL-1; IL6:IL-6; AD:ADAMTS-4; CX:Cox-2; MM:MMP-13; NS:iNOS.