Tissue Engineering

DOI: 10.1089/ten.TEA.2021.0105

Title Page

The synovium of human osteoarthritic joints retains its chondrogenic potential irrespective of age

Ernst B. Hunziker[1], Nahoko Shintani[2], Miroslav Haspl[3], Kurt Lippuner[2], Esther Vögelin[4], Marius JB Keel[5]

[1]Departments of Osteoporosis and Orthopaedic Surgery, Inselspital Bern University Hospital, Bern, Switzerland

[2]Department of Osteoporosis, Inselspital Bern University Hospital, Bern, Switzerland

[3]Department of Orthopaedic Surgery, School of Medicine, University of Zagreb, Zagreb, Croatia

[4]Departments of Plastic and Hand Surgery, Inselspital Bern University Hospital, Bern, Switzerland

[5]Department of Orthopaedic Surgery, Inselspital Bern University Hospital, Bern, Switzerland

* Correspondence to:

Ernst B. Hunziker, MD, PhD

Departments of Osteoporosis and Orthopaedic Surgery, Inselspital Bern

University Hospital, Bern, Switzerland

Freiburgstrasse 3, 3010 Bern, Switzerland

Phone: +41 79 444 65 51

E-mail: ernst.hunziker@dbmr.unibe.ch

Authors` information

Ernst B. Hunziker, MD, PhD.

ernst.hunziker@dbmr.unibe.ch

Departments of Osteoporosis and Orthopaedic Surgery, Inselspital Bern University Hospital, Freiburgstrasse 3, CH-3010 Bern, Switzerland

Tel: +41 79 444 65 51

Nahoko Shintani, PhD

nahoko.shintani@ana.unibe.ch

Department of Osteoporosis, Inselspital Bern University Hospital, Freiburgstrasse 3, CH-3010 Bern, Switzerland

Tel: +41 79 306 73 36

Miroslav Haspl, MD

miroslav.haspl@akromion.hr

Department of Orthopaedic Surgery, School of Medicine, University of Zagreb, Salata 3, 10000 Zagreb, Croatia

Tel: +385 49 587 487

Kurt Lippuner, MD

kurt.lippuner@insel.ch

Department of Osteoporosis, Inselspital Bern University Hospital, Freiburgstrasse 3, CH-3010 Bern, Switzerland

Tel: +41 31 632 31 28

Esther Vögelin, MD

esther.voegelin@insel.ch

Departments of Plastic and Hand Surgery, Inselspital Bern University Hospital, Freiburgstrasse 3, CH-3010 Bern, Switzerland

Bern, Switzerland

Tel: +41 632 35 34

Marius JB Keel. MD

marius.keel@insel.ch

Department of Orthopaedic Surgery, Inselspital Bern University Hospital, Freiburgstrasse 3, CH-3010 Bern, Switzerland

Switzerland

Tel: +41 79 778 01 59

Location of Study: Inselspital Bern University Hospital Laboratories

Tissue Engineering
The synovium of human osteoarthritic joints retains its chondrogenic potential irrespective of age (DOI: 10.1089/ten.TEA.2021.0105)

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 human osteoarthritic patients compromises the chondrogenic potential of their knee-joint synovium and the structural and metabolic stability of the transformed tissue.

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 experimental group; total number of donors: 64). Synovial biopsies were induced *in vitro* to undergo chondrogenesis by exposure to either bone morphogenetic protein-2 (BMP-2) alone, transforming growth factor-ß1 (TGF-ß1) alone, or a combination of the two growth factors, for up to 6 weeks. The differentiated explants were evaluated morphologically and morphometrically 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-β1 combination, i.e. to a comparable level with each of the two stimulation protocols and within both age categories. The BMP-2/TGF-β1combination protocol resulted in chondrocytes of a physiological size for normal human articular cartilage, unlike the BMP-2 alone stimulation that resulted in cell sizes of terminal hypertrophy. The stable 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 cartilage matrix. The gene-expression levels of the catabolic markers remained extremely low. The data also confirmed the usefulness of experimental in vitro studies with bovine synovial tissue as a paradigm for human synovial investigations.

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.

Key Words

Human synovium, osteoarthritis, age, chondrogenesis, BMP-2, TGF-β1

Running Head

Chondrogenesis of human synovium in osteoarthritis

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 human osteoarthritic patients compromises the chondrogenic potential of their knee-joint synovium and the structural and metabolic stability of the transformed tissue.

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 experimental group; total number of donors: 64). Synovial biopsies were induced *in vitro* to undergo chondrogenesis by exposure to either bone morphogenetic protein-2 (BMP-2) alone, transforming growth factor-ß1 (TGF-ß1) alone, or a combination of the two growth factors, for up to 6 weeks. The differentiated explants were evaluated morphologically and morphometrically 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-β1 combination, i.e. to a comparable level with each of the two stimulation protocols and within both age categories. The BMP-2/TGF-β1combination protocol resulted in chondrocytes of a physiological size for normal human articular cartilage, unlike the BMP-2 alone stimulation that resulted in cell sizes of terminal hypertrophy ($p < 0.0001$). The stable 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 cartilage matrix. The gene-expression levels of the catabolic markers remained extremely low. The data also confirmed the usefulness of experimental in vitro studies with bovine synovial tissue as a paradigm for human synovial investigations.

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.

Key Words

Synovium, osteoarthritis, age, chondrogenesis, BMP-2, TGF-β1

Running Head

Chondrogenesis of synovium in osteoarthritis

Tissue Engineering
The synovium of human osteoarthritic joints retains its chondrogenic potential irrespective of age (DOI: 10.1089/ten.TEA.2021.0105)

Impact Statement

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. the chondrogenic potential of the human synovium is not compromised by ageing. These findings imply that the autologous synovium represents an adequate tissue source for the repair of articular cartilage in clinical practice by tissue engineering approaches in human patients suffering from osteoarthritis, independent of the patient`s age.

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. 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[1]. Ultimately therefore, the entire layer is implicated and destroyed[2,3]. Although several treatment strategies are currently investigated to promote the repair of articular cartilage, such as the microfracturing technique [4], the grafting of osteochondral tissue [5] or the implantation of autologous chondrocytes[6], the tissue thereby formed is biologically and mechanically grossly inferior to native articular cartilage[7].

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 [8]. Although MSCs are usually drawn from the bone marrow and fat tissue, the synovium is likewise a rich source of such cells exhibiting a very high chondrogenic potential [9-11]. Also anatomically, the proximity of the synovium to the layer of articular cartilage would render it more a suitable source of MSCs for the *in-situ* engineering of this tissue.

We had developed *in vitro* a suitable stimulation protocol for the formation of a hyaline-type of cartilage tissue from both isolated human synovial cells [12] and bovine synovial explants [13-15]. Specifically this protocol is able to arrest also the chondrocyte downstream differentiation into terminal cell hypertrophy and to prevent the associated intercellular matrix mineralization [16].

Synovial MSCs isolated from the knee joints of human osteoarthritic (OA) patients of various ages have shown chondrogenic potential [10,12]. On the other hand, the chondrogenic potential of synovial explants derived from OA synovium is still unknown, and in particular the possible modulating influence of the presence of a number of other cells, such as fibroblasts, endothelial cells, inflammatory cells etc as well as the extracellular matrix, that may act also as a reservoir of cytokines etc[17-19]. Furthermore, although many researchers have reported that the donor`s age affects the differentiation

potential of MSCs derived from bone marrow [20-22], but most likely not the synovium-derived ones [10,11,23], this is still unknown for synovial tissue explants.

The question thus arises whether the synovial explants derived from elderly osteoarthritis 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.

Materials and Methods

Explants of synovial tissue were obtained from osteoarthritic (OA) patients who were allocated to two age categories (54 - 65 years and 66 - 86 years). The choice of 65 years was arbitrarily chosen, but based on the findings that the highest numbers of OA cases occur at the ages of 55 – 64 years, and mild OA peaks around 65 and then decreases again [24] ; however, the general prevalence of OA increases almost linearly with age [25 24].

Donor exclusion criteria applied were chemotherapy, any systemic disease (such as diabetes, autoimmune diseases etc.), septic arthritis, intraarticular use of steroids, viscosuplementation etc.) or long-term pain therapy. After the explants were cultured for up to 6 weeks with BMP-2 or TGF-β1, or with a combination of BMP-2/TGF-β1(in order to prevent terminal differentiation to chondrocyte hypertrophy and matrix mineralization[16]), the differentiated tissue of each category was then analyzed for its cartilage properties morphologically (chondrocyte/lacunae formation) and histochemically (volume fraction of metachromasia), as well as immunohistochemically (Type-II collagen); and also the expression profiles of anabolic and catabolic marker genes were investigated.

Tissue preparation, growth factors and culturing

Synovial tissue was obtained (surgical waste material) from the knee joints (medial and lateral femoral condyle areas) of osteoarthritis patients (grades 3 or 4 of OA; 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-β1alone, or with a combination of BMP-2/TGF-β1. 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 (and a thickness of 1 - 2 mm, i.e. including the intimal and subintimal tissue layers) were prepared and sandwiched between two layers of agarose for culturing as previously described [13,14]. 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-β1 (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 donors 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). Owing to the limited availability of the donor material, the explants derived from the group of younger patients were cultured for only 4 weeks. Thus the age-goup comparison was restricted to the 4 week time duration of culturing.

Morphology, morphometry, histochemistry and immunohistochemistry

At the end of each culturing period (2, 4 or 6 weeks), a portion of the specimens were processed for morphology, morphometry of cell sizes, quantification of metachromasia (sulfated proteoglycans; internal normalization being performed by referring to the staining intensity of non-OA articular cartilage areas of the same joint) after staining with Toluidine Blue O, and for the immunhistochemical demonstration of type-II collagen.

The volume fraction of metachromasia was determined from the light micrographs by the point-counting technique, which was applied in accordance with stereological principles [26,27]. The mean cell volumes were estimated using the point sampled intercept method [28] and systematic random sampling strategies[26].

For the immunhistochemical demonstration of type-II collagen, the sections were first exposed to hyaluronidase, then to a type-II collagen antibody (clone CII C1; Hybridoma Bank, Iowa City, IA, USA). Immunoreactivity was enhanced by applying first the avidin-biotin-peroxidase complex (Vector Laboratories) and then biotinyl tyramide (Perkin Elmer, Waltham, MA, USA). Cell nuclei were counterstained with haematoxylin. The sections were evaluated and photographed in a Nikon Eclipse E1000 light microscope; for details see [14,16].

Isolation of RNA, reverse transcription and real-time PCR analysis

At the end of each culturing period samples 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) -1ß, -4 and -6, a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS-4), cyclooxygenase-2 (Cox-2), inducible nitric oxide synthase (*i*NOS), matrix metallopeptidase-13 (MMP-13) and tumor necrosis factor alpha (TNF-α)]; for details (and abbreviations): see Tables 1, 2 and 3.

The total amount of RNA was isolated using the RNeasy Micro Kit (Qiagen, Basel, Switzerland) in accordance with the manufacture'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). For methodological details of cDNA processeing see [14,16]; for use of Primers, Probes and final concentrations: see Tables 1 and 2.

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 levels of mRNA were quantified relative to those for 18S rRNA using the comparative cycle-threshold method [12]. For these calculations, the gene-expression levels in unstimulated synovial explants served as the basis for the comparison [29]

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 for morphological, 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.

Morphology, histochemistry, immunhistochemistry, 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/TGF-ß1, underwent chondrogenic differentiation, as evidenced by the intense metachromatic staining of the extracellular, and manifested the widespread appearance of pericellular lacunae – a characteristic feature of differentiated chondrocytes that have been chemically fixed in aldehyde for light microscopy [30] (Figure 1). The intensity of metachromasia increased as a function of culturing time. At each juncture, the distribution of metachromasia over the sections was inhomogeneous. Synovial explants that had been exposed to TGF-ß1 alone manifested only weak metachromasia. Immunoreactivity for type-II collagen (Figure 2) likewise reflected the findings described above for the metachromatic staining.

The histomorphometric quantification of the volume fraction of metachromasia (Figure 3A) confirmed the time-dependency of chondrogenesis induced by BMP-2 alone or by a combination of BMP-2/TGF-ß1. At each time-point, the volume fraction of metachromasia appeared greater after exposure to the BMP-2/TGF-ß1 combinations than to BMP-2 alone. Although such a temporal increase in this parameter appears graphically apparent (Figure 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-ß1groups, 4 weeks vs. 6 weeks)]. Moreover, the individual time-point values show a high inter-individual variability (coefficients of error (CE) ranging between 0.21 - 1.0). Figure 3B shows that the volume fractions of metachromasia were also on comparable levels when comparing 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-ß1groups, young vs. old)]. In the TGF-ß1- 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 Figure 4 shows an impressive scatter of results and confirms the age-independency of the quantitative metachromasia. The correlation analyses for the BMP-2 patient materials and for the BMP-2/TGF-ß1 treated groups showed a negative correlation between age and volume fraction [R^2 = 0.15 (for BMP-2 stimulation), 0.02 (for TGF-ß1 stimulation) and 0.05 (for BMP-2/TGF-ß1 stimulation)] (Figure 4). The graph also illustrates also the high variability of results within each age group. The final cell volumes achieved after 6 weeks are illustrated in Figure 5. The mean final cell volume in the BMP-2 group was 15452 µm^3 (CE 1.03%, n=754), and in the BMP-2/TGF-ß1group 1736 µm^3 (CE 1.1%, n=1064).

Gene-expression evaluation

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

Among anabolic genes, c*ollagen types II, X and XI, aggrecan and Sox9* were elevated after stimulation, irrespective of the nature of growth factors used (Figure 6). The expression levels of these genes generally peaked at the 4-week juncture. *COMP* and *lubricin* were up-regulated after stimulation with TGF-ß1 alone or BMP-2/TGF-ß1, but not with BMP-2 alone. *Alkaline phosphatase* was up-regulated after stimulation with BMP-2 alone or BMP-2/TGF-ß1, but not with TGF-ß1 alone. *Type-I collagen* level was slightly raised only after stimulation with TGF-ß1. *Osteocalcin* levels were not changed, irrespective of the type of growth factor applied.

The levels of catabolic genes were barely changed during culturing (Figure 7). Only the levels of *ADAMTS-4* (after stimulation with TGF-ß1), *COX-2* (after stimulation with BMP-2/TGF-ß1) and *i*NOS (after stimulation with BMP-2 or BMP-2/TGF-ß1) 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-ß1.

The change of gene expression after stimulation was compared between younger and older patients at the 4-week-juncture (Figure 7); and due to limited availability of donor materials from the young age group, this comparison was not performed at the 6 week juncture. 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-ß1, *type-X collagen* and *sox9* were elevated significantly higher in older patients than in younger patients. After stimulation with BMP-2/TGF-ß1, *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-1ß* was significantly higher in older patients than in younger patients, but only 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 [31] and experimental conditions [13,32], 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 [14,33,34]. 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 cartilage tissue after stimulation with appropriate growth factors [13,14,16]. 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 cartilage lesions in a clinical setting[35].

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* [20,21]. Consequently, the MSCs of elderly donors are considered to be unsuitable for the purposes of tissue engineering [36]. Also the influence of donor age on the chondrogenic potential of MSCs derived from various tissue sources had been addressed by several research groups [37,38], 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 of MSCs in regenerative medicine, a clarification of this issue is important, particularly in the fields of orthopaedics and rheumatology [39] , since patients who would qualify for the instigation of an autologous MSC-based cartilage-repair strategy, would be primarily patients with circumscribed articular cartilage defects, such as after trauma (may occur at any age), osteochondritis dissecans, a focal form of OA, etc.

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*) [12], a finding that was reconfirmed by other researchers [40,41] 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 [42,43]. These phenomena most likely would affect repair cartilage formation from synovial tissues when originating from such diseased joints; to clarify these potentially adverse issues was the purpose of the present study, using synovial explants.

Tissue Engineering
The synovium of human osteoarthritic joints retains its chondrogenic potential irrespective of age (DOI: 10.1089/ten.TEA.2021.0105)

As evidenced by the formation of chondrocytes with lacunae and a metachromatic matrix, BMP-2 alone, and the BMP-2/TGF-β1 combination, induced the chondrogenic differentiation of the synovial explants to the greatest and to similar degrees. Thus TGF-β1 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 [16]) . Given that the peak volume fraction of metachromasia was approximately 9-fold lower after stimulation with TGF-β1 alone than after exposure to either BMP-2 alone or to the BMP-2/TGF-β1 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, a time-dependent increase in this parameter was nonetheless graphically apparent (Figure 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 were compared at the 4-week juncture, no differences were revealed. And when this histomorphometric parameter was displayed as a function of an individual's age, the correlation coefficients were very low. Thus the general differentiation potential of synovial explants originating from human patients suffering from OA was not impaired as a function of donor age. This finding confirms a previous report of de Bari et al[10] using isolated human synovial cells. 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[13,14,16]. And also the chondrocyte sizes attained were of similar dimensions, but resulted in a more physiological size using the combination of BMP-2/TGF-beta1 rather then BMP-2 alone. This finding also confirms the usefulness of the bovine tissue in an in vitro model to simulate human tissue biology (in the sense of the 3R philosophy [44]).The osteoarthritic process thus seems not to affect the chondrogenic differentiation potential of the synovium.

The analysis of the gene-expression levels of key anabolic markers of chondrogenesis permits a more discriminative evaluation of the induced differentiation process into cartilage 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 . Only after exposure to the BMP-2/TGF-β1 combination a significant difference between the two age categories was revealed, with slightly higher levels in the group of older patients.

Type-II collagen is a characteristic component of cartilage 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. 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[20,36], as well as with perichondrial-derived cells[45] or with periosteum tissue flaps[38], this apparently does not apply for synovial tissue, as found here, nor for isolated synovial-derived MSCs, as previously found [10].Quantification of the immunhistochemical data was not performed since this method is intrinsically associated with such a high variance between blocks and sections that it does not provide sufficient solid grounds (only intrinsic control referencing principles for each case could provide some semi-quantitative indications).

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. Since TGF-β1 was found to suppress the terminal hypertrophic differentiation of chondrocytes [16], 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-β1 alone, or the BMP-2/TGF-β1 combination, the values being higher in the group of older osteoarthritic patients than in the group of younger ones. This finding is in correlation to findings relating to normal articular chondrocytes and osteoarthritic ones from OA patients [46,47] and with data from animal models [48]; interestingly it was also observed upon differentiation of synovial tissue from osteoarthritic patients.

Alkaline Phosphatase is likewise a marker of terminal chondrocyte hypertrophy and matrix mineralization [49]. In both age categories of patients, its gene-expression levels were lowest (barely above baseline values) after stimulation with TGF-β1 alone, and lower after exposure to the BMP-2/TGF-β1 combination than after treatment with BMP-2 alone.

Type-XI collagen is a marker of cartilage-specific collagen fibril formation since co-expressed with collagen type-II [50]. The gene expression levels of type-XI collagen were highest after exposure to either BMP-2 alone or the BMP-2/TGF-β1 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.

The gene-expression levels of aggrecan were higher after stimulation with either BMP-2 alone or the BMP-2/TGF-β1 combination than after exposure to TGF-β1 alone. These data accord with the histomorphometric findings for metachromasia. An age-related difference was revealed only after stimulation with the BMP-2/TGF-β1 combination, the values being higher in the group of older osteoarthritic patients than in the group of younger ones.

The gene-expression levels of COMP [51] and Lubricin [52-54], 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-β1 alone or the BMP-2/TGF-β1 combination, and lowest after exposure to BMP-2 alone. 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-β1 combination, the values being higher in the group of older osteoarthritic patients than in the group of younger ones [55]; this possibly may be related to TGF-β1effects [56]. No significant age-related differences in the gene-expression levels of lubricin were observed.

The gene expression levels of matrilin-1, which is a marker of cartilage tissue organization, and that may play a role in the development of osteoarthritis [57], lay below the limits of detection in all instances. Hence, in the newly formed cartilaginous tissue, anabolic processes override catabolic activity [58].

Sox9 is a transcription factor, which acts as a DNA-binding protein during chondrogenic differentiation. Irrespective of the stimulation protocol used, the gene-expression levels of Sox-9 peaked – as expected – at the 4-week juncture [59]. 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 [60]. Significant age-related differences in the gene-expression levels of Sox9 were revealed after stimulation with either TGF-β1 or the BMP-2/TGF-β1 combination, the values being higher in the group of older osteoarthritic patients than in the group of younger ones.

The gene activities for the catabolic markers [61], which included inflammatory factors [62 63] [IL-1β, IL-4 (not detected), IL-6, TNF-α (not detected), COX-2 and *i*NOS] and matrix proteases (MMP-13, ADAMTS-4) were generally expressed at very low levels. As for the anabolic markers, the peak levels were generally attained at the 4-week juncture. No consistent trend in favor of anyone particular stimulation protocol was observed, with the exception of IL-1β. And no significant age-related differences in the gene-expression levels of the catabolic markers were observed. In the case of IL-1β, the values were slightly higher in the group of older osteoarthritic patients. The finding that the eight catabolic-marker genes were expressed at very low or even non-detectable (IL-4 and TNF-α) levels indicates that the production of a cartilage matrix (attested by the stable histochemical, immunhistochemical and morphological results) was positively balanced against degradative processes [62]. 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.

The authors are fully aware that the gene expression activities are an indication only of possible posttranslational activities that need to be substantiated by demonstrating the presence/absence of such activities for each gene investigated [64]. Such additional investigations can now be performed on a more targeted level with this information now provided. Given the observed stability of the neoformed cartilage tissue structure the

outcome of an extensive posttranslational investigation can be expected to be most likely of a positive correlation to the observed gene activities.

Acknowledgements

Funding. This study was supported by SNF grant : 320000-118205 (to MJB Keel and E Vögelin) and the Foundation of the Clinic for Osteoporosis, Inselspital, Bern, Switzerland (to EB Hunziker). - The study sponsors had no influence on the study design, collection, analysis and interpretation of data nor on the writing of the manuscript or the decision where to submit the manuscript for publication.

References

1. Beris AE, Lykissas MG, Papageorgiou CD, Georgoulis AD. Advances in articular cartilage repair. *Injury.* Nov 2005;36 Suppl 4:S14-23.

2. Hada S, Kaneko H, Sadatsuki R, et al. The degeneration and destruction of femoral articular cartilage shows a greater degree of deterioration than that of the tibial and patellar articular cartilage in early stage knee osteoarthritis: a cross-sectional study. *Osteoarthritis Cartilage.* Oct 2014;22(10):1583-1589.

3. Park JY, Kim BH, Ro DH, Lee MC, Han HS. Characteristic location and rapid progression of medial femoral condylar chondral lesions accompanying medial meniscus posterior root tear. *Knee.* Jun 2019;26(3):673-678.

4. Steadman JR, Rodkey WG, Briggs KK, Rodrigo JJ. [The microfracture technic in the management of complete cartilage defects in the knee joint]. *Orthopade.* Jan 1999;28(1):26-32.

5. Chimutengwende-Gordon M, Donaldson J, Bentley G. Current solutions for the treatment of chronic articular cartilage defects in the knee. *EFORT Open Rev.* Mar 2020;5(3):156-163.

6. Brittberg M. Autologous chondrocyte implantation--technique and long-term follow-up. *Injury.* Apr 2008;39 Suppl 1:S40-49.

7. Chahla J, Piuzzi NS, Mitchell JJ, et al. Intra-Articular Cellular Therapy for Osteoarthritis and Focal Cartilage Defects of the Knee: A Systematic Review of the Literature and Study Quality Analysis. *J Bone Joint Surg Am.* Sep 21 2016;98(18):1511-1521.

8. Richter W. Mesenchymal stem cells and cartilage in situ regeneration. *J Intern Med.* Oct 2009;266(4):390-405.

9. Hunziker EB, Rosenberg LC. Repair of partial-thickness defects in articular cartilage: cell recruitment from the synovial membrane. *J Bone Joint Surg Am.* May 1996;78(5):721-733.

10. De Bari C, Dell'Accio F, Tylzanowski P, Luyten FP. Multipotent mesenchymal stem cells from adult human synovial membrane. *Arthritis Rheum.* Aug 2001;44(8):1928-1942.

11. Sakaguchi Y, Sekiya I, Yagishita K, Muneta T. Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source. *Arthritis Rheum.* Aug 2005;52(8):2521-2529.

12. Kurth T, Hedbom E, Shintani N, et al. Chondrogenic potential of human synovial mesenchymal stem cells in alginate. *Osteoarthritis Cartilage.* Oct 2007;15(10):1178-1189.

13. Shintani N, Hunziker EB. Chondrogenic differentiation of bovine synovium: bone morphogenetic proteins 2 and 7 and transforming growth factor beta1 induce the formation of different types of cartilaginous tissue. *Arthritis Rheum.* Jun 2007;56(6):1869-1879.

14. Shintani N, Hunziker EB. Differential effects of dexamethasone on the chondrogenesis of mesenchymal stromal cells: Influence of microenvironment, tissue origin and growth factor. *Eur Cell Mater.* 2011;22:302-320.

15. Hunziker EB, Lippuner K, Keel MJ, Shintani N. Novel organ-slice culturing system to simulate meniscal repair: Proof of concept using a synovium-based pool of meniscoprogenitor cells. *J Orthop Res.* Sep 2016;34(9):1588-1596.

16. Shintani N, Siebenrock KA, Hunziker EB. TGF-ß1 Enhances the BMP-2-Induced Chondrogenesis of Bovine Synovial Explants and Arrests Downstream Differentiation at an Early Stage of Hypertrophy. *PLoS One.* 2013;8(1):e53086.

17. Primorac D, Molnar V, Rod E, et al. Knee Osteoarthritis: A Review of Pathogenesis and State-Of-The-Art Non-Operative Therapeutic Considerations. *Genes (Basel).* Jul 26 2020;11(8).

18. Bartolotti I, Roseti L, Petretta M, Grigolo B, Desando G. A Roadmap of In Vitro Models in Osteoarthritis: A Focus on Their Biological Relevance in Regenerative Medicine. *J Clin Med.* Apr 28 2021;10(9).

19. Theeuwes WF, van den Bosch MHJ, Thurlings RM, Blom AB, van Lent P. The role of inflammation in mesenchymal stromal cell therapy in osteoarthritis, perspectives for post-traumatic osteoarthritis: a review. *Rheumatology (Oxford).* Mar 2 2021;60(3):1042-1053.

20. Huibregtse BA, Johnstone B, Goldberg VM, Caplan AI. Effect of age and sampling site on the chondro-osteogenic potential of rabbit marrow-derived mesenchymal progenitor cells. *J Orthop Res.* Jan 2000;18(1):18-24.

21. Erickson IE, van Veen SC, Sengupta S, Kestle SR, Mauck RL. Cartilage matrix formation by bovine mesenchymal stem cells in three-dimensional culture is age-dependent. *Clin Orthop Relat Res.* Oct 2011;469(10):2744-2753.

22. Fafian-Labora J, Fernandez-Pernas P, Fuentes I, et al. Influence of age on rat bone-marrow mesenchymal stem cells potential. *Sci Rep.* Nov 19 2015;5:16765.

23. Sasaki A, Mizuno M, Ozeki N, et al. Canine mesenchymal stem cells from synovium have a higher chondrogenic potential than those from infrapatellar fat pad, adipose tissue, and bone marrow. *PLoS One.* 2018;13(8):e0202922.

24. Roberts J, Burch TA. Osteoarthritis prevalence in adults by age, sex, race, and geographic area. *Vital Health Stat 11.* Jun 1966(15):1-27.

25. Shane Anderson A, Loeser RF. Why is osteoarthritis an age-related disease? *Best Pract Res Clin Rheumatol.* Feb 2010;24(1):15-26.

26. Gundersen HJ, Jensen EB. The efficiency of systematic sampling in stereology and its prediction. *J Microsc.* Sep 1987;147(Pt 3):229-263.

27. Gundersen HJ, Bendtsen TF, Korbo L, et al. Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. *APMIS.* May 1988;96(5):379-394.

28. Gundersen HJ, Jensen EB. Stereological estimation of the volume-weighted mean volume of arbitrary particles observed on random sections. *J Microsc.* May 1985;138(Pt 2):127-142.

29. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* Dec 2001;25(4):402-408.

30. Hunziker EB, Lippuner K, Shintani N. How best to preserve and reveal the structural intricacies of cartilaginous tissue. *Matrix Biol.* Oct 2014;39:33-43.

31. Maurice H, Crone M, Watt I. Synovial chondromatosis. *J Bone Joint Surg Br.* Nov 1988;70(5):807-811.

32. Elford PR, Graeber M, Ohtsu H, et al. Induction of swelling, synovial hyperplasia and cartilage proteoglycan loss upon intra-articular injection of transforming growth factor beta-2 in the rabbit. *Cytokine.* May 1992;4(3):232-238.

33. Park Y, Sugimoto M, Watrin A, Chiquet M, Hunziker EB. BMP-2 induces the expression of chondrocyte-specific genes in bovine synovium-derived progenitor cells cultured in three-dimensional alginate hydrogel. *Osteoarthritis Cartilage.* Jun 2005;13(6):527-536.

34. Shintani N, Kurth T, Hunziker EB. Expression of cartilage-related genes in bovine synovial tissue. *J Orthop Res.* Jun 2007;25(6):813-819.

35. Fan J, Varshney RR, Ren L, Cai D, Wang DA. Synovium-derived mesenchymal stem cells: a new cell source for musculoskeletal regeneration. *Tissue Eng Part B Rev.* Mar 2009;15(1):75-86.

36. Kassem M. Stem cells: potential therapy for age-related diseases. *Ann N Y Acad Sci.* May 2006;1067:436-442.

37. Palm K, Salin-Nordstrom T, Levesque MF, Neuman T. Fetal and adult human CNS stem cells have similar molecular characteristics and developmental potential. *Brain Res Mol Brain Res.* May 31 2000;78(1-2):192-195.

38. O'Driscoll SW, Saris DB, Ito Y, Fitzimmons JS. The chondrogenic potential of periosteum decreases with age. *J Orthop Res.* Jan 2001;19(1):95-103.

39. Kubosch EJ, Lang G, Furst D, et al. The Potential for Synovium-derived Stem Cells in Cartilage Repair. *Curr Stem Cell Res Ther.* Feb 23 2018;13(3):174-184.

40. Nagase T, Muneta T, Ju YJ, et al. Analysis of the chondrogenic potential of human synovial stem cells according to harvest site and culture parameters in knees with medial compartment osteoarthritis. *Arthritis Rheum.* May 2008;58(5):1389-1398.

41. Kurose R, Ichinohe S, Tajima G, et al. Characterization of human synovial fluid cells of 26 patients with osteoarthritis knee for cartilage repair therapy. *Int J Rheum Dis.* Feb 1 2010;13(1):68-74.

42. Aurich M, Squires GR, Reiner A, et al. Differential matrix degradation and turnover in early cartilage lesions of human knee and ankle joints. *Arthritis Rheum.* Jan 2005;52(1):112-119.

43. Hardingham T, Bayliss M. Proteoglycans of articular cartilage: changes in aging and in joint disease. *Semin Arthritis Rheum.* Dec 1990;20(3 Suppl 1):12-33.

44. Tornqvist E, Annas A, Granath B, Jalkesten E, Cotgreave I, Oberg M. Strategic focus on 3R principles reveals major reductions in the use of animals in pharmaceutical toxicity testing. *PLoS One.* 2014;9(7):e101638.

45. Morihara T, Harwood F, Goomer R, Hirasawa Y, Amiel D. Tissue-engineered repair of osteochondral defects: effects of the age of donor cells and host tissue. *Tissue Eng.* Dec 2002;8(6):921-929.

46. Fuerst M, Niggemeyer O, Lammers L, Schafer F, Lohmann C, Ruther W. Articular cartilage mineralization in osteoarthritis of the hip. *BMC Musculoskelet Disord.* Dec 29 2009;10:166.

47. Fuerst M, Bertrand J, Lammers L, et al. Calcification of articular cartilage in human osteoarthritis. *Arthritis Rheum.* Sep 2009;60(9):2694-2703.

48. Miller LM, Novatt JT, Hamerman D, Carlson CS. Alterations in mineral composition observed in osteoarthritic joints of cynomolgus monkeys. *Bone.* Aug 2004;35(2):498-506.

49. Iwamoto M, Sato K, Nakashima K, Shimazu A, Kato Y. Hypertrophy and calcification of rabbit permanent chondrocytes in pelleted cultures: synthesis of alkaline phosphatase and 1,25-dihydroxycholecalciferol receptor. *Dev Biol.* Dec 1989;136(2):500-507.

50. Eyre DR. The collagens of articular cartilage. *Semin Arthritis Rheum.* Dec 1991;21(3 Suppl 2):2-11.

51. Niki Y, Takeuchi T, Nakayama M, et al. Clinical significance of cartilage biomarkers for monitoring structural joint damage in rheumatoid arthritis patients treated with anti-TNF therapy. *PLoS One.* 2012;7(5):e37447.

52. Zhang D, Johnson LJ, Hsu HP, Spector M. Cartilaginous deposits in subchondral bone in regions of exposed bone in osteoarthritis of the human knee: histomorphometric study of PRG4 distribution in osteoarthritic cartilage. *J Orthop Res.* Jul 2007;25(7):873-883.

53. Catterall JB, Hsueh MF, Stabler TV, et al. Protein modification by deamidation indicates variations in joint extracellular matrix turnover. *J Biol Chem.* Feb 10 2012;287(7):4640-4651.

54. Watkins AR, Reesink HL. Lubricin in experimental and naturally occurring osteoarthritis: a systematic review. *Osteoarthritis Cartilage.* Oct 2020;28(10):1303-1315.

55. Fernandes FA, Pucinelli ML, da Silva NP, Feldman D. Serum cartilage oligomeric matrix protein (COMP) levels in knee osteoarthritis in a Brazilian population: clinical and radiological correlation. *Scand J Rheumatol.* May-Jun 2007;36(3):211-215.

56. Chua SD, Jr., Messier SP, Legault C, Lenz ME, Thonar EJ, Loeser RF. Effect of an exercise and dietary intervention on serum biomarkers in overweight and obese adults with osteoarthritis of the knee. *Osteoarthritis Cartilage.* Sep 2008;16(9):1047-1053.

57. Li P, Fleischhauer L, Nicolae C, et al. Mice Lacking the Matrilin Family of Extracellular Matrix Proteins Develop Mild Skeletal Abnormalities and Are Susceptible to Age-Associated Osteoarthritis. *Int J Mol Sci.* Jan 19 2020;21(2).

58. Goldring MB. Update on the biology of the chondrocyte and new approaches to treating cartilage diseases. *Best Pract Res Clin Rheumatol.* Oct 2006;20(5):1003-1025.

59. Kulyk WM, Franklin JL, Hoffman LM. Sox9 expression during chondrogenesis in micromass cultures of embryonic limb mesenchyme. *Exp Cell Res.* Mar 15 2000;255(2):327-332.

60. DeLise AM, Fischer L, Tuan RS. Cellular interactions and signaling in cartilage development. *Osteoarthritis Cartilage.* Sep 2000;8(5):309-334.

61. Shi S, Mercer S, Eckert GJ, Trippel SB. Regulation of articular chondrocyte catabolic genes by growth factor interaction. *J Cell Biochem.* Feb 27 2019.

62. Lee AS, Ellman MB, Yan D, et al. A current review of molecular mechanisms regarding osteoarthritis and pain. *Gene.* Sep 25 2013;527(2):440-447.

63. Malfait AM. Osteoarthritis year in review 2015: biology. *Osteoarthritis Cartilage.* Jan 2016;24(1):21-26.

64. Huang LF, Lin KH, He SL, et al. Multiple Patterns of Regulation and Overexpression of a Ribonuclease-Like Pathogenesis-Related Protein Gene, OsPR10a, Conferring Disease Resistance in Rice and Arabidopsis. *PLoS One.* 2016;11(6):e0156414.

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.

Figure Legends

Fig.1

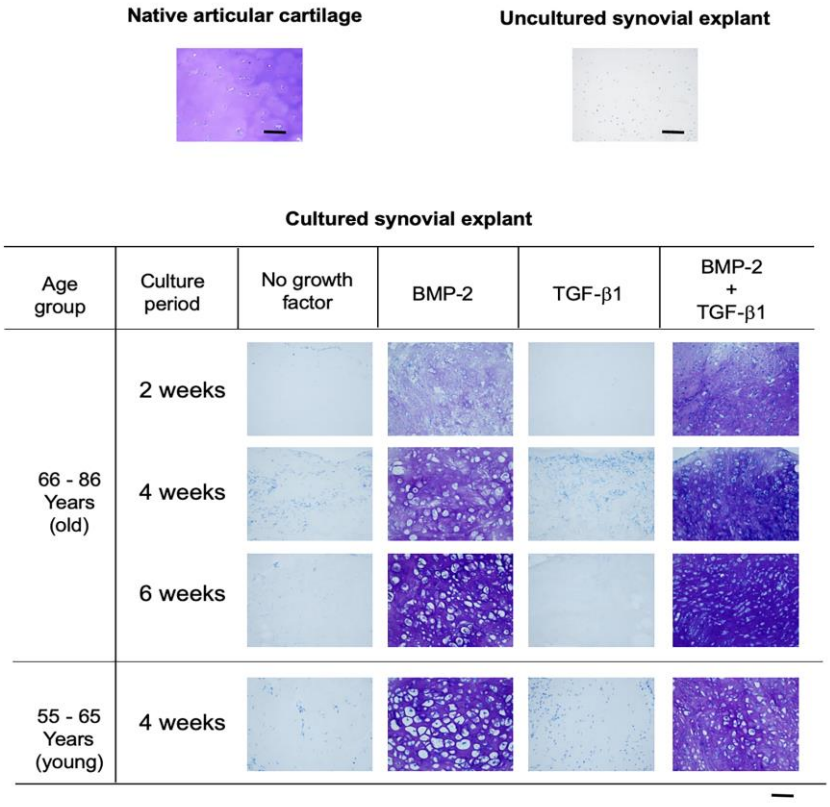

Fig. 1

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-β1 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-β1 combination manifested a temporal increase in the intensity of metachromatic staining, whereas those that had been exposed to TGF-β1 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. Bar = 100 micrometers.

Fig.2

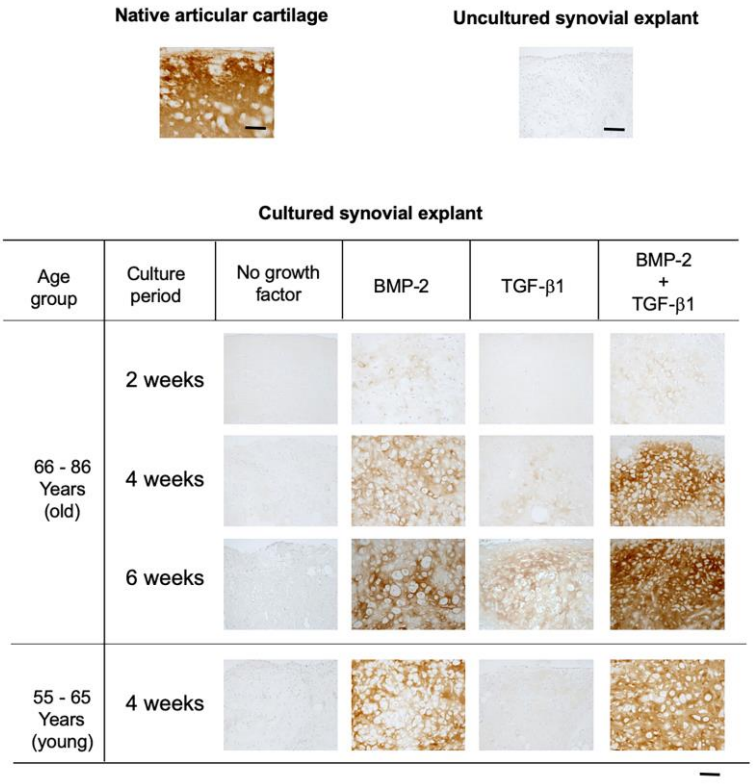

Fig. 2

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-β1, 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-β1 combination manifested a temporal increase in the intensity of immunostaining for type-II collagen, whereas those who had been exposed to

TGF-β1 exhibited only weak immunoreactivity at each juncture. Bar = 100 micrometers.

Tissue Engineering
The synovium of human osteoarthritic joints retains its chondrogenic potential irrespective of age (DOI: 10.1089/ten.TEA.2021.0105)

Fig.3

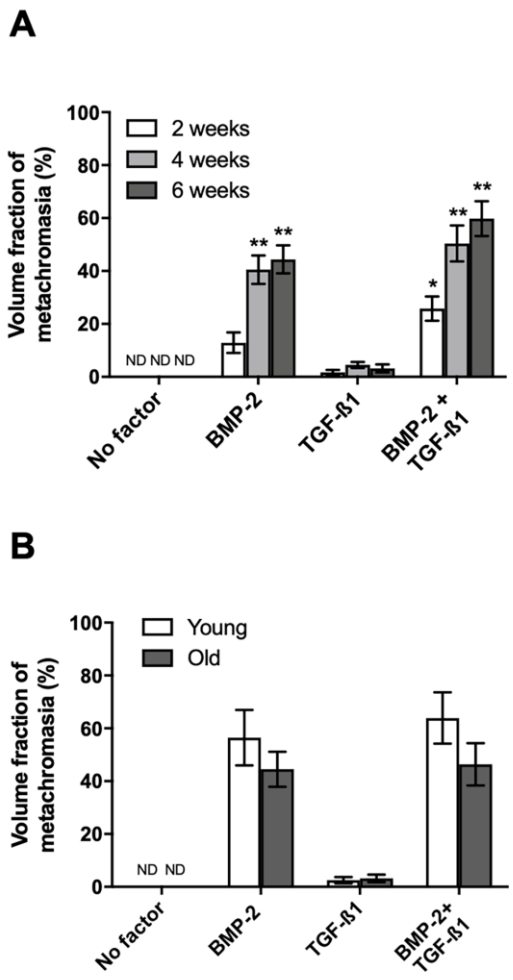

Fig. 3

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-β1 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-β1 combination, somewhat lower after exposure to BMP-2 alone, and by far the lowest after treatment with TGF-β1 alone. After exposure to either BMP-2 alone or the BMP-2/TGF-β1 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 ± 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-β1 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 ± SEM are represented (55 - 65 years: *n*= 9; 66 - 86 years: *n*=7). ND: not detected. Please note that the age-comparison is limited in this study to the 4-week culturing groups.

Tissue Engineering
The synovium of human osteoarthritic joints retains its chondrogenic potential irrespective of age (DOI: 10.1089/ten.TEA.2021.0105)

Fig.4

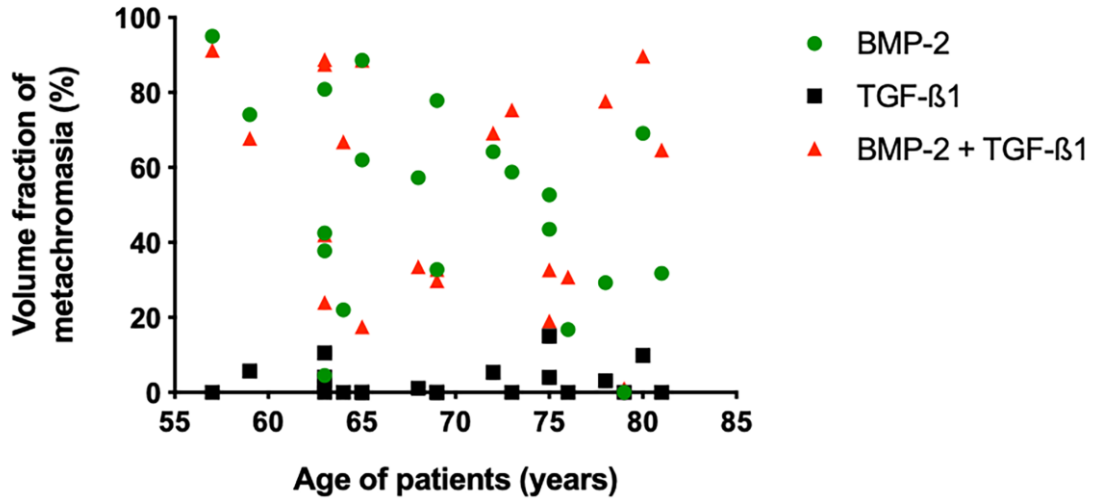

Fig. 4

Volume fraction of metachromasia within the synovial explants of each of the osteoarthritic patients, four weeks after exposure to either BM-2 alone, TGF-β1 alone, or a combination of the two growth factors, expressed as a function of the individual's age. A large data scatter can be identified, and no correlation with the age of the donors.

Tissue Engineering
The synovium of human osteoarthritic joints retains its chondrogenic potential irrespective of age (DOI: 10.1089/ten.TEA.2021.0105)

Fig.5

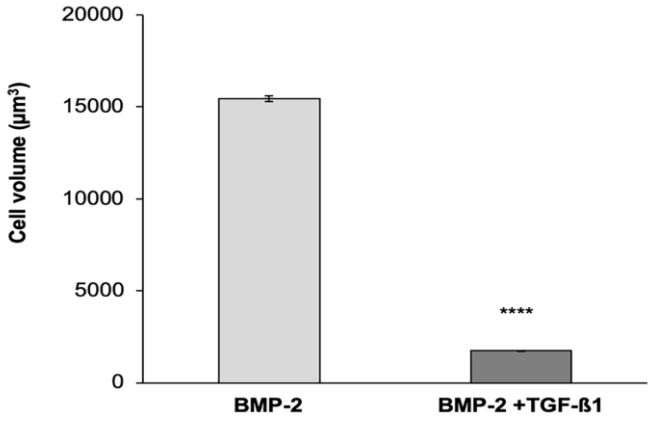

Fig. 5

Mean chondrocyte cell volumes achieved at the end of 6 weeks culturing periods for the groups stimulated by BMP-2 alone or by the BMP-2/ TGF-β1mixture. The final cell volumes achieved in these two groups (pooled data statistics) are significantly different from each other: ****: $p < 0.0001$). The ones attained by the BMP-2/ TGF-β1mixture correspond to the normal chondrocyte cell volume persisting throughout life in the adult human articular cartilage (radial zone) [62], whereas that of the BMP-2 generated chondrocyte volume relates in size to the terminal chondrocyte cell size achieved for the generation of maximal growth rate activities in growth plates for longitudinal bone growth[63].

Tissue Engineering
The synovium of human osteoarthritic joints retains its chondrogenic potential irrespective of age (DOI: 10.1089/ten.TEA.2021.0105)

Fig.6

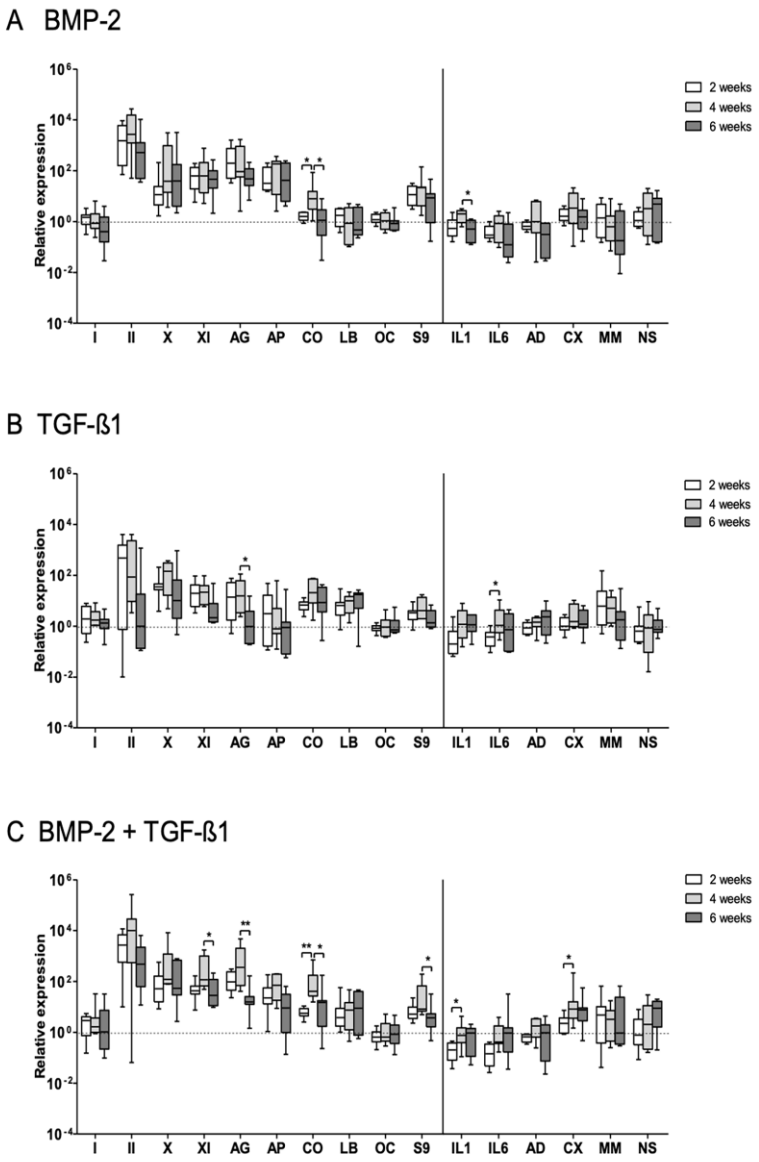

Fig. 6

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-β1 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*.

Tissue Engineering
The synovium of human osteoarthritic joints retains its chondrogenic potential irrespective of age (DOI: 10.1089/ten.TEA.2021.0105)

Fig.7

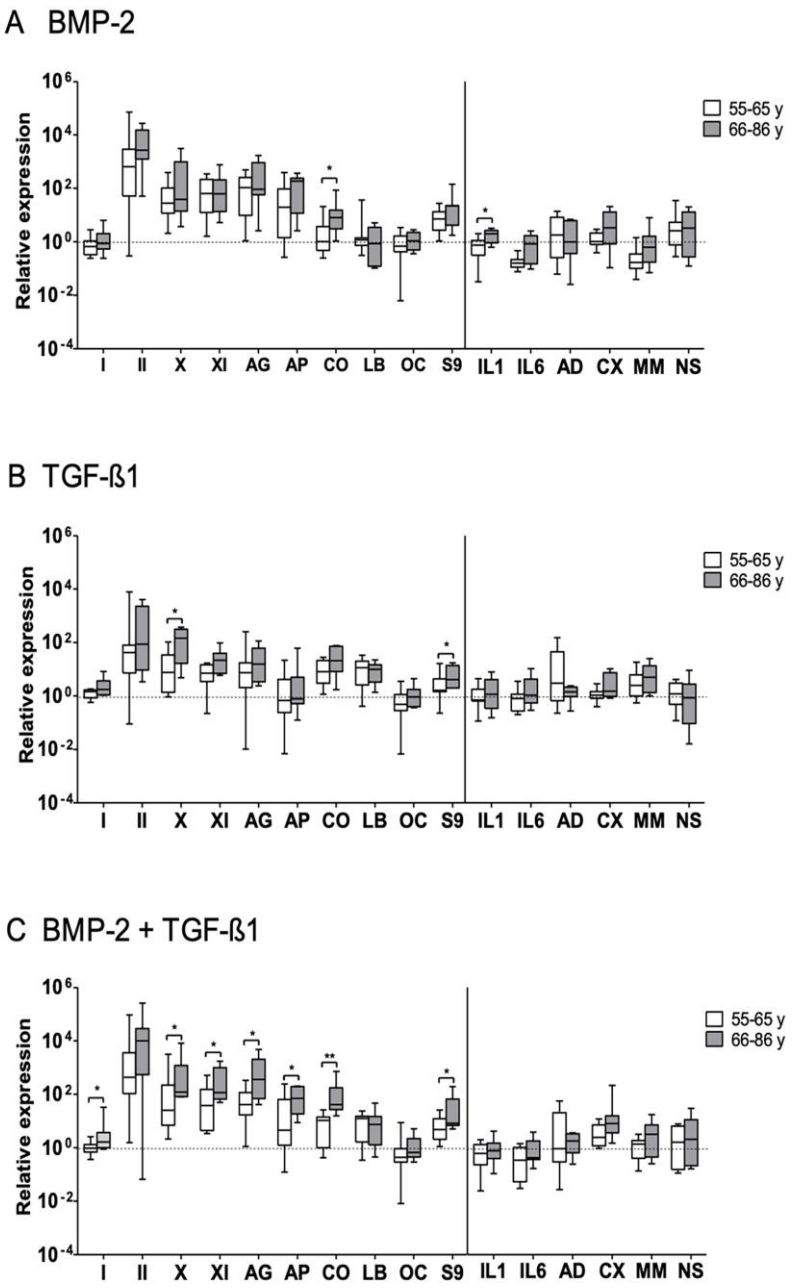

Fig. 7

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-β1 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 by *: $p < 0.05$; **: $p < 0.01$. 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:*i*NOS.

Tissue Engineering
The synovium of human osteoarthritic joints retains its chondrogenic potential irrespective of age (DOI: 10.1089/ten.TEA.2021.0105)

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	GGCAATAGCAGGTTCACGTACA	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	

Tissue Engineering
The synovium of human osteoarthritic joints retains its chondrogenic potential irrespective of age (DOI: 10.1089/ten.TEA.2021.0105)

Tissue Engineering
The synovium of human osteoarthritic joints retains its chondrogenic potential irrespective of age (DOI: 10.1089/ten.TEA.2021.0105)

Aggrecan			
Forward	CTACCGCTGCGAGGTGATG	900	74
Probe	ATGGAACACGATGCCTTT(C/T)ACCACGA	100	
Reverse	TCGAGGGTGTAGCGTGTAGAGA	900	
Alkaline phosphatase			
Forward	CAACAACTACCAGGCGCAGTC	50	84
Probe	CTGCGCCACGAGACCCACGG	100	
Reverse	GGGCCCTTGGAGAAGACG	50	
COMP			
Forward	CCAGAAGAACGACGACCAGAA	300	128
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	

Tissue Engineering
The synovium of human osteoarthritic joints retains its chondrogenic potential irrespective of age (DOI: 10.1089/ten.TEA.2021.0105)

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	CCCATGGACTGGGTTCCTCGCTACA	100	
Reverse	TGGCAGGTGAGTTTGCACTG	300	
MMP-13			
Forward	TTGTTGCTGCGCATGAGTTC	300	104
Probe	TCCAAGGACCCTGGAGCACTCATGTTT	100	
Reverse	AAAGTGGCTTTTGCCGGTGTAG	50	
IL-1β			
Forward	AATCCCCAGCCCTTTTGTTG	300	85
Probe	ACCTCTCCTACTCACTTAAAGCCCGCCTGA	100	
Reverse	AAATGTGGCCGTGGTTTCTG	300	
IL-4			
Forward	TTTGTCCACGGACACAAGTGC	900	124
Probe	TCACAGAGCAGAAGACTCTGTGCACCGA	100	
Reverse	TCTTGGAGGCAGCAAAGATGTC	900	

Tissue Engineering
The synovium of human osteoarthritic joints retains its chondrogenic potential irrespective of age (DOI: 10.1089/ten.TEA.2021.0105)

IL-6			
Forward	CCTGACCCAACCACAAATGC	300	146
Probe	TCTGCGCAGCTTTAAGGAGTTCCTGCA	100	
Reverse	CCATGCTACATTTGCCGAAGAG	300	
*i*Nos			
Forward	TGCTGATGCGCAAGACAATG	300	79
Probe	CGAGTCAGAGTCACCATCCTCTTTGCGA	100	
Reverse	AGCGCCTCTGATTTTCCTGTC	300	
TNF-α			
Forward	TTTGGGATCATTGCCCTGTG	900	130
Probe	AACATCCAACCTTCCCAAACGCCTCC	100	
Reverse	GCCCCCAATTCTCTTTTTGAGC	900	
Cox-2			
Forward	TGGAAGCCAAGCACTTTTGG	50	90
Probe	TGGGTTTTCAAATCATCAACACTGCCTCA	100	
Reverse	GCCCTTCACGTTATTGCAGATG	50	
18s rRNA			

Forward	CGGCTACCACATCCAAGGAA	900	187
Probe	TGCTGGCACCAGACTTGCCCTC	100	
Reverse	GCTGGAATTACCGCGGCT	50	

Tissue Engineering
The synovium of human osteoarthritic joints retains its chondrogenic potential irrespective of age (DOI: 10.1089/ten.TEA.2021.0105)

Table 3.

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 metallopeptidase-13

PBS: phosphate buffered saline

RT-PCR: real-time polymerase chain reaction

18S rRNA: 18S ribosomal ribonucleic acid

T: Thymine

TNF-α: tumor necrosis factor alpha

TGF-ß1: transforming growth factor beta 1