OsteoArthritis and Cartilage (2005) **13,** 527–536
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doi:10.1016/j.joca.2005.02.006

Osteoarthritis and Cartilage

International Cartilage Repair Society

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BMP-2 induces the expression of chondrocyte-specific genes in bovine synovium-derived progenitor cells cultured in three-dimensional alginate hydrogel

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Summary

Objective: According to recent reports, the synovial membrane may contain mesenchymal stem cells with the potential to differentiate into chondrocytes under appropriate conditions. In order to assess the usefulness of synovium-derived progenitor cells for the purposes of cartilage tissue engineering, we explored their requirements for the expression of chondrocyte-specific genes after expansion *in vitro*.

Design: Mesenchymal progenitor cells were isolated from the synovial membranes of bovine shoulder joints and expanded in two-dimensions on plastic surfaces. They were then seeded either as micromass cultures or as single cells within alginate gels, which were cultured in serum-free medium. Under these three-dimensional conditions, chondrogenesis is known to be supported and maintained. Cell cultures were exposed either to bone morphogenetic protein-2 (BMP-2) or to isoforms of transforming growth factor-β (TGF-β). The levels of mRNA for Sox9, collagen types I and II and aggrecan were determined by RT-PCR.

Results: When transferred to alginate gel cultures, the fibroblast-like synovial cells assumed a rounded form. BMP-2, but not isoforms of TGF-β, stimulated, in a dose-dependent manner, the production of messenger RNAs (mRNAs) for Sox9, type II collagen and aggrecan. Under optimal conditions, the expression levels of cartilage-specific genes were comparable to those within cultured articular cartilage chondrocytes. However, in contrast to cultured articular cartilage chondrocytes, synovial cells exposed to BMP-2 continued to express the mRNA for α1(I) collagen.

Conclusions: This study demonstrates that bovine synovium-derived mesenchymal progenitor cells can be induced to express chondrocyte-specific genes. However, the differentiation process is not complete under the chosen conditions. The stimulation conditions required for full transformation must now be delineated.
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Key words: Synoviocytes, Chondrogenesis, BMP-2, Differentiation.

Introduction

Because terminally differentiated adult cells are of limited use for tissue engineering, many researchers have tried to isolate multipotent progenitor cells, which have been considered promising. Multipotent progenitor cells from adult bone marrow have the potential to differentiate into specific somatic cells[1]. Since then, many researchers have utilized stem cells from several adult tissues for various applications[2–6].

For articular cartilage repair, different kinds of grafting methods, such as the use of osteochondral and periosteal tissue and implantation of autologous chondrocytes, have been used in clinical practice[7]. Unfortunately, there are no methods as of yet that produce consistent and successful results[8]. In recent years, chondrogenic progenitor cells that might be used for cartilage repair have been identified in various adult tissues: bone marrow[1,6], periosteum[9], fat[10], and synovial membrane[11–13]. By treating these cells in 3D culture with suitable agents, such as dexamethasone, transforming growth factor-β (TGF-β) and bone morphogenic proteins (BMPs), activation of chondrogenesis has been demonstrated[14]. In the ideal environment, the progenitor cells assume chondrocyte-like characteristics, such as a rounded shape, and synthesize glycosaminoglycans (GAGs), collagen type II and aggrecan, which are typical extracellular matrix components of hyaline cartilage.

One of the very promising tissue sources for chondrogenic progenitor cells to induce articular cartilage repair[12,15] seems to be synovium[13,15]. Synovial tissue contains various cell types, such as the pseudoepithelial lining cells (types A and B)[16], those within the connective tissue at subepithelial and perivascular locations, and endothelial cells (in blood capillaries)[17]. Moreover, it is known from clinical pathology that primary chondromatous tumors can form within this tissue[18], which further illustrates the potential of some subpopulations of synovial cells to transform into chondrocytes and to form cartilage-like tissue. In addition, the example of rheumatoid pannus shows that synovial membrane contains chondrocyte-like cells[19]. Experimentally, synovial cells can be induced to migrate into partial-thickness articular cartilage defects, therein to proliferate and subsequently to deposit a scar-like tissue[12] and differentiate fully into chondrocytes following appropriate stimulation[15,20]. There are some *in-vitro* experiments described in the literature that show the chondrogenic activity of progenitor cells isolated from synovial membranes. Such cells undergo

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Received 30 June 2004; revision accepted 6 February 2005.

chondrogenic differentiation when cultured on BMP-coated plates[13]. It has also been reported that explants from rabbit synovial tissues started to express collagen type II if treated with TGF-β1 *in vitro*[11]. Recently, progenitor cells which have chondrogenic, myogenic and adipogenic potential were identified in adult human synovial membrane[21].

The quality and nature of the culturing conditions adopted are of key importance in the induction of chondrogenic differentiation. It is well known, for example, that if chondrocytes are cultured under 2D conditions, they start to dedifferentiate and to lose their chondrogenic phenotype and activity[22]. However, when chondrocytes are cultured under 3D conditions, they readily recover a chondrogenic phenotype and metabolic activity following a redifferentiation process[23]. Therefore, 3D culture conditions are essential to maintain the chondrogenic potential[24]. And for chondrocytes or chondrogenic progenitor cells, particularly alginate hydrogel has previously been identified to maintain the chondrogenic phenotype[9,11].

In the present study, we cultured and expanded synovial cells under 2D conditions for the selection of chondrogenic precursor cells, and subsequently cultured these under 3D conditions in a defined serum-free medium[25]. The goal was to establish thoroughly defined culture conditions that allow the synovial progenitor cells to assume a gene-expression profile which resembles that of articular chondrocytes. For the initiation of the process of chondrogenic differentiation, various culture conditions and growth factors were examined. For 3D cultures, alginate hydrogel was used and as growth factors TGF-βs and BMP-2 were tested. The expression profile of chondrocyte-specific genes was monitored by real-time PCR, and the accumulation of GAGs was also measured. Cellular morphology and the distribution of fibrillar collagen were visualized by histological examination. All parameters were quantitatively compared to those obtained from articular chondrocytes isolated from the same joint, and cultured under the same conditions.

Methods

PREPARATION OF SYNOVIAL TISSUE AND CELL CULTURE

Pieces of synovial membrane were obtained aseptically from the shoulder joints of 2- to 4-year-old adult cows within 24 h of death. Tissue samples were washed with phosphate-buffered saline (PBS) [pH 7.4] containing gentamycin (50 μg/ml) and incubated in high-glucose Dulbecco's modified Eagle's medium (DMEM) [Life Technologies, Basel, Switzerland] containing 0.35 mM proline and 50 μg/ml gentamycin, for 3 h. Then, tissues were minced using a razor blade and digested with 1% pronase (Roche Diagnostics, Basel, Switzerland) in the medium for 1 h and with 0.14% collagenase (Life Technologies, Basel, Switzerland) overnight at 37 °C. Cells were washed with PBS and subsequentially filtered through 120 μm and 20 μm nylon filters (Millipore AG, Volketswil, Switzerland) to remove the undigested tissues. Cells were resuspended for expansion and selection in high-glucose DMEM with 10% fetal bovine serum (FBS) [Sigma, St Louis, USA], 0.35 mM proline, 20 μg/ml ascorbate, 1 mM cysteine, 1 mM pyruvate and 50 μg/ml gentamycin[21,26].

Isolated cells were cultured in 100 mm cell culture plates (Falcon, Bedford, MA, USA) at 37 °C in an incubator with 5% CO_2. The culture medium was replaced every 2–3 days. After 7–10 days, confluent cells were washed with PBS and trypsinized with trypsin-ethylenediamine tetra-acetic acid (EDTA) solution (0.25% trypsin, 1 mM EDTA; Life Technologies). Cells were replated in culture dishes at a 1:3 dilution rate to obtain the appropriate subculture cell system.

MICROMASS CULTURE

For passage one, cells from these subcultures were trypsinized and resuspended in culture medium to a concentration of 2×10^7 cells/ml. Twenty microliters of cell suspension were deposited per well in 24-well cell culture plates (Falcon, Bedford, MA, USA) and incubated for 4 h at 37 °C in the cell culture incubator for adherence of the cells. Then, 1 ml per well of serum-free medium [high-glucose DMEM with 0.35 mM proline, 50 μg/ml gentamycin, and 0.01% bovine serum albumin (BSA)][25] was added containing 20 ng/ml of one of the following growth factors: TGF-β1, TGF-β2, TGF-β3 (Sigma, St. Louis, USA) or BMP-2 (kindly supplied by Wyeth, Massachusetts, USA). Respectively, control cells were cultured in the same medium without growth factors. Cells were cultured at 37 °C and 5% CO_2 for 1 week with a change of medium every 2 days. Cells were fixed with 2.5% paraformaldehyde and washed with PBS. For alcian blue staining, cells were incubated with alcian blue solution (1% alcian blue 8GX in 3% acetic acid, pH 2.5) for 1 h. After staining, cells were washed with PBS and subjected to optical examination.

THREE-DIMENSIONAL CELL CULTURE IN ALGINATE DISKS

For cell culture under 3D conditions, alginate disks were prepared by following the protocol described by Wong *et al.*[27]. Briefly, 2% alginate solutions were prepared by mixing 1% Keltone LV alginate (Kelko Co., San Diego, CA, USA) and 1% Alginate LGV (Pronova Biomedical, Norway) in 0.9% NaCl. Cells from passage one were pelleted and suspended in alginate at a concentration of 4×10^6 cells/ml. Alginate solution containing cells was injected into an ethanol-sterilized, custom-designed mold encompassing a 22 mm diameter × 2 mm cylindrical space. Alginate was polymerized for 30 min by placing the mold in a gelation solution containing 50 mM $CaCl_2$ and 0.9% NaCl. Gels were cut with sterile dermal punches (outer diameter 6 mm, Stiefel Laboratorium AG, Winterthur, Switzerland) to create a small alginate disk (5.5 mm in diameter × 2 mm in height).

Alginate disks were cultured in high-glucose DMEM containing 1% ITS + Premix (Collaborative Biomedical Products), insulin (6.25 μg/ml), transferrin (6.25 μg/ml), selenous acid (6.25 μg/ml), linoleic acid (5.35 μg/ml), BSA (37.5 μg/ml), pyruvate (1 mM) and ascorbate (37.5 μg/ml)[14,25]. For BMP-2 treatment, different concentrations of BMP-2 were added from a 20 μg/ml stock solution to the culture medium, which was changed every 2–3 days. A possible enhancement of the chondrogenic effect by dexamethosone (10^{-8} M) was also investigated.

CELL HARVEST AND MEASUREMENT OF GAGs

After the desired culture period, cells in the alginate disks were harvested by solubilizing the alginate gels in 55 mM sodium citrate in 0.9% NaCl for 45 min at 37 °C. Cells were collected by centrifugation at 3500 rpm for 10 min. For measurement of glycosaminoglycan (GAG) content, cell pellets and supernatants were incubated with papain solution (0.56 mg/ml) in 50 mM sodium phosphate with 10 mM EDTA and 2 mM L-cysteine (pH 6.5) overnight at 65 °C. After cooling to room temperature, 20 μl of digested solutions were incubated with 400 μl of color reagent from the Blyscan kit

(Biocolor, Belfast, UK) for 1 h with shaking. The samples were centrifuged at 13,000 rpm for 10 min. After removing the supernatants, pellets containing the GAG-color reagent complex were dissolved in 400 μl of dissociation reagent from the kit by incubating at 37 °C for 1 h. Optical density at 590 nm was measured using a microtiter plate reader. The GAG contents were calculated from a standard curve prepared using purified chondroitin sulfate in the kit.

RNA EXTRACTION AND REVERSE TRANSCRIPTION REACTION

Alginate disks were solubilized with sodium citrate/NaCl buffer and cells were collected by centrifugation as described above. After washing the cells with PBS once, RNA was extracted using an RNA extraction kit (Qiagen, Basel, Switzerland). The extracted RNA was dissolved in 50 μl of RNase-free distilled water and stored at −80 °C until use. cDNA was synthesized from 500 ng of RNA using TaqMan Reverse Transcription Reagents (Applied Biosystems, Seraing, Belgium).

DESIGN OF PRIMERS AND PROBES FOR REAL-TIME PCR

Primers and probes for bovine type I collagen [α1(I)], type II collagen (α1(II)), aggrecan, and Sox9 were designed with the Primer Express software (Perkin−Elmer Applied Biosystems, Foster City, CA, USA). As an internal control, 18s rRNA primer and probe sets were used, which are available from the company. The sequence of the specific bovine genes was downloaded from the Gene Bank database. In the center of each probe sequence, an exon-exon junction was placed to avoid non-specific fluorescent emission derived from contaminating genomic DNA. The nucleotide sequences of the primers and probes are shown in Table I. Primers were purchased from Microsynth (Balgach, Switzerland). The probes were labeled with carboxyfluorescein (FAM) at the 5′ end and with *N*,*N*,*N*′,*N*′-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3′ end. They were obtained from Perkin-Elmer Applied Biosystems (Seraing, Belgium). The concentrations of the primers and probes were used following the company's User Bulletin No. 2; for primers: 900 nM, for probes: 300 nM.

REAL-TIME QUANTITATIVE PCR

Quantification of mRNAs was performed by real-time quantitative PCR with a Perkin-Elmer ABI Prism 7700 sequence detection system. PCR was performed with AmpliTaq Gold polymerase (Perkin-Elmer ABI) using 20 ng of cDNA per reaction. The cycle threshold (C_t) values for 18s rRNA and the mRNAs of interest were compared and calculated by sequence detector software (Perkin-Elmer ABI). Relative transcript levels were calculated as $x = 2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = \Delta E - \Delta C$ and $\Delta E = C_{t\,\mathrm{experimental}} - C_{t\,18s\,\mathrm{rRNA}}$; $\Delta C = C_{t\,\mathrm{control}} - C_{t\,18s\,\mathrm{rRNA}}$. For comparison, the C_t-value of the non-treated sample was used as a normalized control in most of the experiments.

HISTOLOGY AND IMMUNOHISTOCHEMISTRY

Immediately after culture, alginate disks were fixed in 4% paraformaldehyde buffered with 0.1 M sodium cacodylate (pH 7.4) containing 10 mM $CaCl_2$ for 4 h at room temperature and then washed overnight at 4 °C in 0.1 M cacodylate buffer (pH 7.4) containing 50 mM $BaCl_2$. The disks were dehydrated through ethanol and xylene, embedded in paraffin and 5-μm sections were prepared.

For histological evaluation, sections were stained with Toluidine Blue. Additional sections were used for the immunohistochemical analysis with antibodies against type II collagen.

Paraffin-embedded sections were deparaffinized using xylene and rehydrated in ethane solutions. Endogenous peroxidase activity was blocked by a 20-min incubation in 1% H_2O_2 in methanol. After one 5-min wash with Tris buffered saline (TBS), the sections were pretreated with 20 mU/ml chondroitinase ABC (Sigma, St Louis, MO, USA) in TBS buffer for 1 h at room temperature. Residual enzyme was removed with one 5-min wash with TBS, and 1% BSA was placed on the section for 15 min to block non-specific background staining.

Slides were incubated with primary antibodies [rabbit antibody against bovine collagen type II (Biodesign, Saco, ME, USA)], diluted 1:100 in TBS containing 1% bovine albumin (Serva, Heidelberg, Germany), for 1 h at room temperature. Slides were then washed three times with TBS, incubated at room temperature for 45 min with horseradish-peroxidase-conjugated secondary antibody [goat antibody to rabbit IgG (Biodesign, Saco, ME, USA)], diluted 1:100 in TBS/1% BSA. For positive and negative controls, normal bovine articular cartilage was used. Negative control sections did not receive the primary antibody. All incubations were performed in humidified chambers. Peroxidase activity

Table I
Primers and probes used for real-time PCR. Primers and probes with FAM/TAMRA are represented

Gene		Sequence	Amplicon size	Accession no.
Type I col*	Forward	ACATGCCGAGACTTGAGACTCA	86	AB008683
	Probe	CCACCCAGA(G/A)TGGAGCAGCGGTTACTACTG		
	Reverse	GCATCCATAGTACATCCTTGGTTAGG		
Type II col*	Forward	AGCAGGTTCACATATACCGTTCTG	73	X02420
	Probe	CCGGT(A/G)TGTTTCGTGCAGCCATCCT		
	Reverse	CGATCATAGTCTTGCCCCACTT		
Aggrecan*	Forward	CAGCCAGGCCACCCTAGAG	74	U76615
	Probe	ATGGAACACGATGCCTTT(C/T)ACCACGA		
	Reverse	GGGTGTAGCGCGTGGAGAT		
Sox9*	Forward	ACGCCGAGCTCAGCAAGA	71	AF278708
	Probe	CGTTCAG(A/C)AGTCTCCAGAGCTTGCCCA		
	Reverse	CACGAACGGCCGCTTCT		

*Mixtures were made based on mismatches between the bovine and human sequences.

was visualized using 3-amino-9-ethylcarbazole (Sigma, St. Louis, MO, USA) as substrate. Sections were counterstained with Mayer's hematoxylin.

STATISTICAL ANALYSIS

Each experiment was repeated 3 to 4 times. On each separate occasion, the cells were derived from a different animal. Hence, in the statistical analysis, the *n*-value (3 or 4) for each group relates to different animals. The significance of differences between experimental groups (i.e., $P < 0.05$) was estimated by applying ANOVA.

Results

SYNOVIUM-DERIVED PROGENITOR CELLS CULTURED UNDER 2D AND 3D CONDITIONS

After isolation from the synovium of adult bovine joints, cells were cultured on plastic dishes in 10% serum-containing medium for expansion. At passage one (cultured for 1 week), RNA was extracted from a portion of the cells as a 2D culture control. The remaining cells were cast within alginate disks for 3D culture and cultured for up to 4 weeks in medium containing 10% serum. Expression of chondrocyte-specific genes, aggrecan and collagen type II, was examined by real-time PCR. The activity of these two genes was found to be induced with time in 3D culture compared to the cells remaining attached to culture plates (Fig. 1). This indicates that synovium-derived progenitor cells have a chondrogenic potential when cultured under 3D conditions. The expression of aggrecan mRNA reached a plateau at 2 weeks and kept almost the same level up to the 4th week of culture (Fig. 1). In contrast, the collagen type II message did not reach a plateau after a 4-week culture period (Fig. 1). Since the culture medium contained 10% serum, the effect of individually added growth factors could not be monitored clearly under this culture condition. Therefore, in the following experiments, the culture medium was changed to a serum-free, defined medium, and growth factors known to induce chondrogenic activity were then introduced.

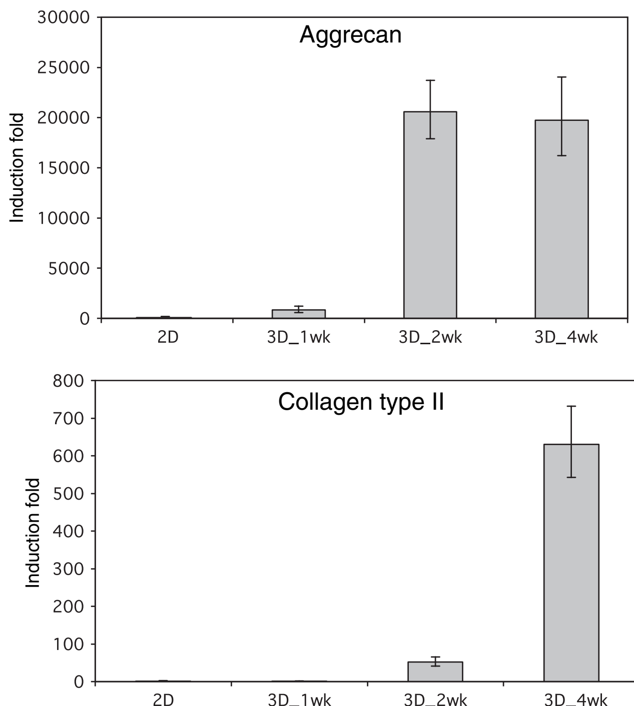

Fig. 1. Comparison of gene expression by synovium-derived progenitor cells cultured under 2D and 3D conditions. Bovine synovial cells from passage one were collected and used as a 2D control while the remaining cells were cultured in alginate disks for up to 4 weeks in DMEM containing 10% FBS. The expression levels of collagen type II and aggrecan mRNA were measured by real-time PCR ($n = 3$, error bars: SD). The values were normalized to 2D control samples. Abbreviations used: WK = week; D = dimension.

MICROMASS CULTURE OF SYNOVIUM-DERIVED PROGENITOR CELLS

An alternative method to grow chondrogenic precursor cells under 3D conditions is the micromass culture [9,14,28]. High concentrations of cells (400,000 per 20 μl drop) were plated and then overlaid with serum-free medium containing different growth factors: TGF-β1, TGF-β2, TGF-β3, and BMP-2 (each at 20 ng/ml). After 1 week in culture, the gene-expression activities of aggrecan and collagen type II were monitored using real-time PCR [Fig. 2(A)]. Cells treated with BMP-2 expressed the highest levels of aggrecan and collagen type II mRNA. TGF-β1-treated cells expressed the highest level of aggrecan mRNA among the TGF-β's. Interestingly, the expression level of collagen type II mRNA in the TGF-β-treated cells was lower than that in untreated control samples, indicating that TGF-β's inhibit the expression of this chondrocyte-specific gene in synovium-derived progenitor cells. The relative induction of aggrecan mRNA was much higher than that of collagen type II in the micromass culture system. All the samples treated with growth factors stained intensely with alcian blue (GAG synthesis) [Fig. 2(B)], there being no obvious differences between these. Dexamethasone (10^{-8} M) inhibited not only the serum-dependent chondrogenic effect [control, Fig. 2(A)] but also the BMP-2-induced response (Fig. 3). Hence, this agent was excluded from subsequent experiments.

DOSE-DEPENDENT EFFECTS OF BMP-2 ON GENE EXPRESSION BY THE SYNOVIUM-DERIVED PROGENITOR CELLS

Because BMP-2 showed the highest activity among the growth factors, it was selected as a chondrogenic agent in the rest of the experiments using alginate cultures. Dosage-dependency of BMP-2 effects was examined by measuring the expression level of chondrocyte-specific genes (Fig. 4). Synovium-derived precursor cells, as well as chondrocytes, cultured in alginate disks, were kept in serum-free medium with different concentrations of BMP-2 (0, 10, 50, and 200 ng/ml). After 1 week in culture, these cells were collected and real-time PCR was performed. The expression level of Sox9, a transcriptional activator of the aggrecan and collagen type II genes, was steadily increased by BMP-2 up to 50 ng/ml, where it reached a plateau. The maximal induction was approximately 10 times greater than in untreated samples. The maximal expression level of Sox9 mRNA in synovium-derived progenitor cells was roughly 4 times lower than that in chondrocytes cultured under the same condition (data not shown). Even though Sox9 expression reached a plateau at 50 ng/ml of BMP-2, aggrecan and collagen type II mRNA levels continued to increase over the concentration range tested (Fig. 4). When comparing synovium-derived

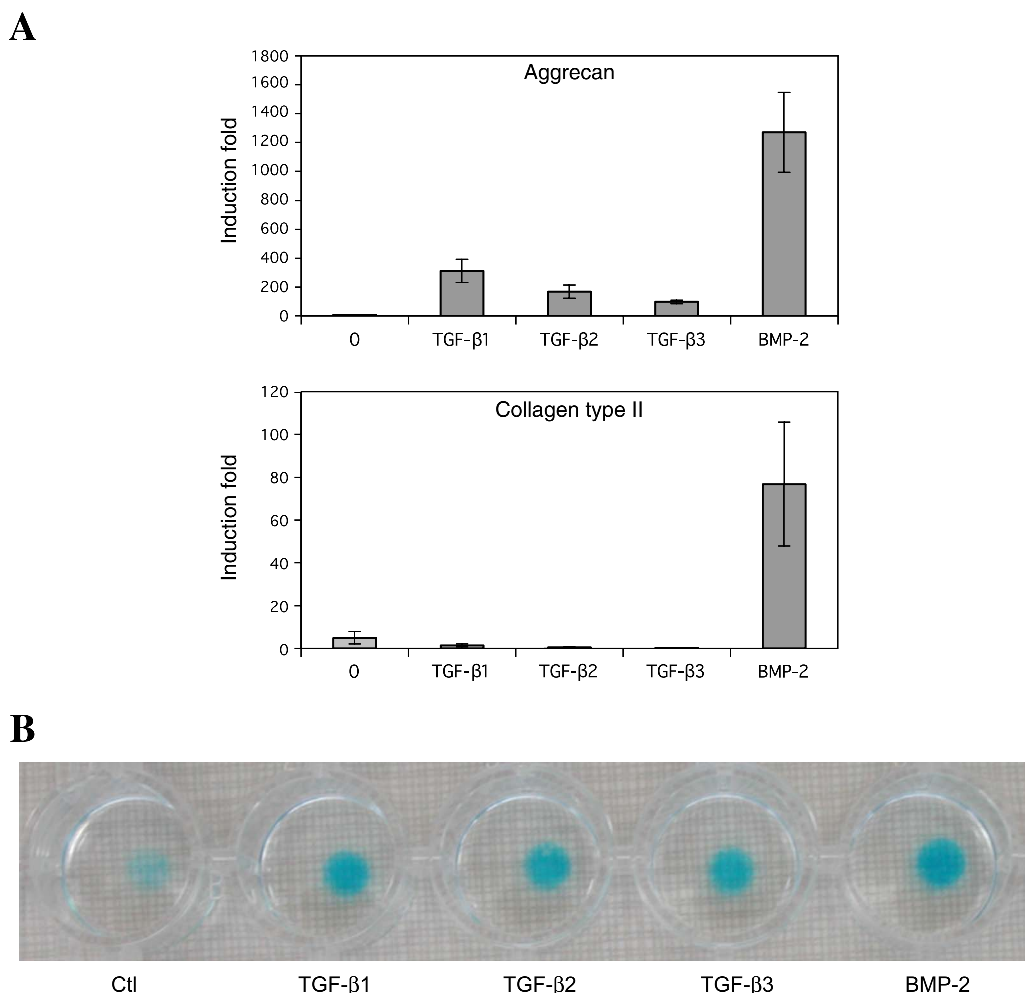

Fig. 2. (A) Collagen type II and aggrecan gene expression upon treatment of synovium-derived progenitor cells with different growth factors under micromass culture conditions. Cells from synovial membrane were seeded in 24-well culture plates at high density (2×10^7 cells/ml). Cell culturing was performed in serum-free medium with different growth factors (TGF-β1, -β2, -β3 and BMP-2) at 20 ng/ml. After 1 week, RNA from cells was extracted and gene-expression levels were analyzed by real-time PCR ($n = 3$, error bars: SD). Values were normalized to non-treated samples. (B) Alcian blue staining of micromass cultures grown in the presence of different growth factors for 1 week.

progenitor cells with chondrocytes, the maximal expression levels were two times lower for aggrecan and 30 times lower for collagen type II (not shown).

mRNA RATIO OF COLLAGENS TYPE II/TYPE I

For additional comparison, chondrocytes and synovium-derived progenitor cells were cultured in alginate disks for 2 weeks in serum-free medium. Collagen type I expression was measured and the expression ratio between collagen type I and type II was compared by means of real-time PCR (Fig. 5). In the chondrocytes, the expression of collagen type II mRNA was much higher than that of collagen type I, demonstrating a ratio of col II/col I of 1700:1. In contrast, the expression of collagen type I was much higher than that of collagen type II mRNA in the synovium-derived progenitor cells. When the latter cells were treated with BMP-2, the ratio of col II/col I increased continuously with the concentration of BMP-2. However, even at the highest concentration of BMP-2 (200 ng/ml), the ratio was still much lower (approximately 1:20) compared to that of the chondrocytes.

GAG CONTENT MEASUREMENTS

Cells were cultured in the alginate disks for 2 and 4 weeks in the serum-free medium condition with or without 50 ng of BMP-2, and the GAG synthesis was measured (Fig. 6). Among 2-week cultures, there was little difference between control and BMP-2-treated samples. However, a significant difference was seen in the 4-week cultured samples. The GAG content in the BMP-2-treated samples was approximately 2.3 fold higher than in the control.

CELL MORPHOLOGY AND IMMUNOHISTOCHEMISTRY

Synovium-derived progenitor cells in alginate disks had a rounded form (Fig. 7). Moreover, immunohistochemistry showed that synovial cells incubated with 200 ng/ml BMP-2

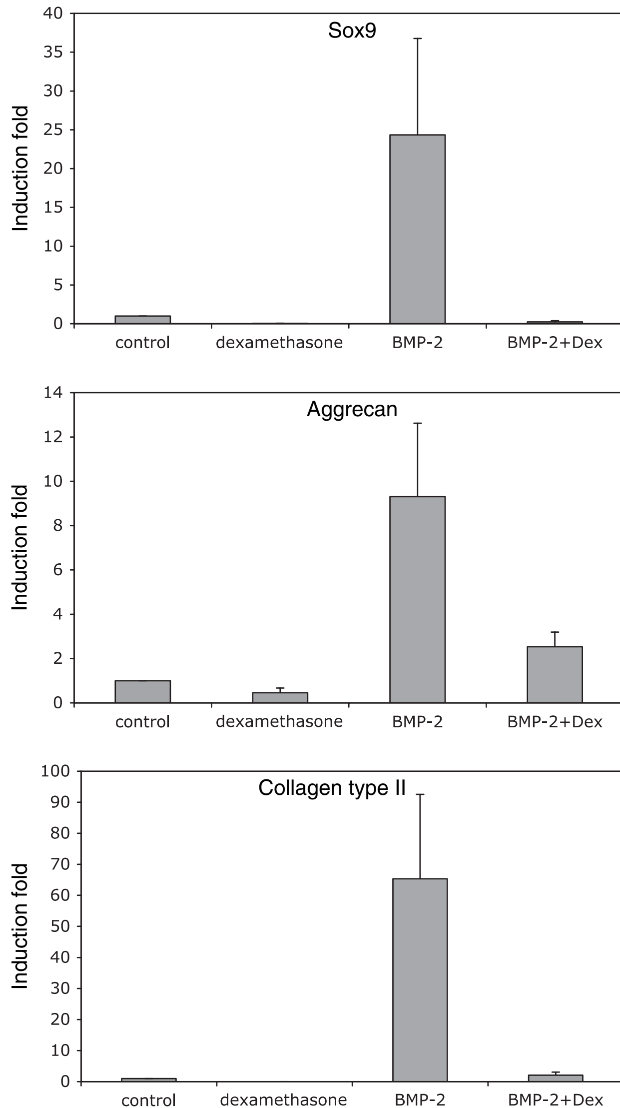

Fig. 3. mRNA expression levels for Sox9 (A), aggrecan (B) and collagen type II (C) in the absence and presence of dexamethasone (10^{-8} M). The data reveal this agent to have a profound inhibitory effect on each of these activities, both in control cultures (serum alone) and in cultures stimulated with BMP-2 (50 ng/ml). Incubation period: 1 week. Mean values (±SD) for three experiments are represented.

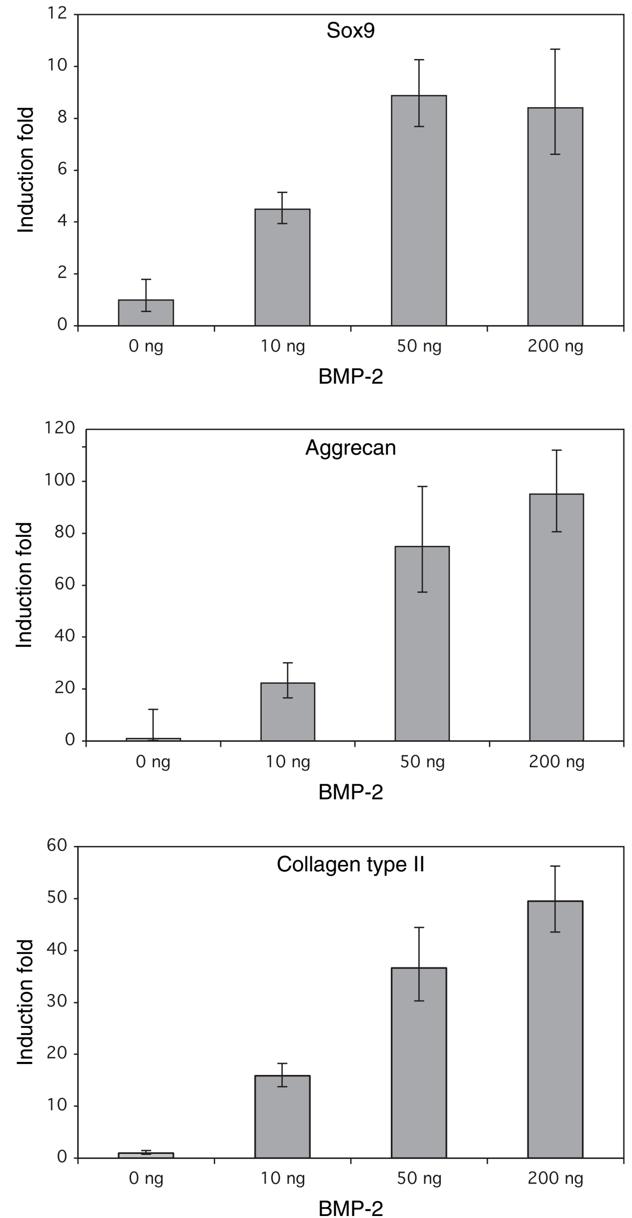

Fig. 4. Dose-dependent effects of BMP-2 on gene expression by synovium-derived progenitor cells in 3D culture. Synovium-derived progenitor cells in alginate disks were cultured for 1 week with different concentrations of BMP-2: 0, 10, 50, and 200 ng/ml. The mRNA levels of chondrocyte-specific genes, i.e., Sox9, aggrecan, and collagen type II were monitored by real-time PCR ($n = 4$, error bars: SD). The values were normalized to untreated samples under the same conditions.

expressed collagen type II protein (strong staining reaction), whereas cells cultured in the absence of BMP-2 exhibited little collagen II staining (Fig. 8).

Discussion

One of the advantages of using synovium as a cell source for cartilage repair is that this tissue has the potential of self-regeneration after removal from synovial joints. Moreover, it is well known from joint development studies and ontogenetic processes that the precursors for articular cartilage, meniscus and synovial tissue within the joint originate from the same precursor cell pool[29]. It thus may be advantageous to recruit cells for repair from this (ontogenetically) early chondrogenic precursor cell pool, a remaining portion of which seems to persist in the adult organism as a fraction of the heterogenous cell population within the synovial tissues. These chondrogenic precursor cells seem to preserve a lifelong high potential for proliferation and for chondrogenic differentiation activities. Moreover, they may retain the capacity to transform into joint-specific chondrocytes[21,30] and other mesodermally derived differentiated cells[26].

In this study, we present evidence that synovium-derived progenitor cells cultured under 3D conditions and treated with BMP-2 exhibit chondrogenic differentiation activities by

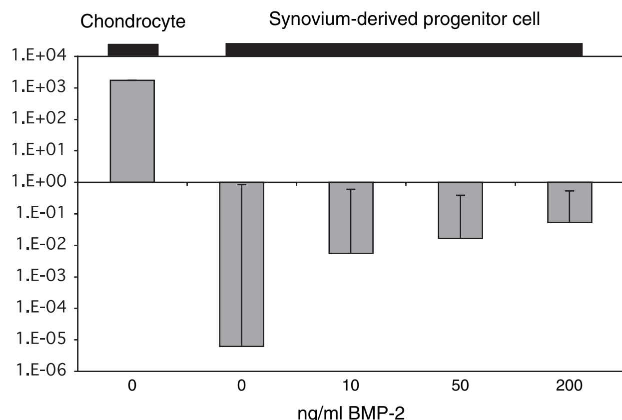

Fig. 5. Expression ratio of collagen type II to collagen type I in chondrocytes and synovium-derived progenitor cells, respectively. Chondrocytes and synovium-derived cells treated with different concentrations of BMP-2 (0, 10, 50, and 200 ng/ml) were cultured in alginate disks in serum-free medium for 2 weeks. The expression levels of collagen type II and type α_1(I) mRNAs were determined by real-time PCR. The relative ratio Col II/Col I was calculated by comparing the C_t (cycle of threshold) of the samples ($n = 3$, error bars: SD).

expressing chondrocyte-specific genes such as Sox9, collagen type II and aggrecan. Additionally, these cells begin to accumulate GAG and to express collagen type II protein when treated with BMP-2.

Interestingly, the expression of collagen type II was suppressed when these progenitor cells were treated with TGF-β's as compared to non-treated control samples, whereas cells treated with BMP-2 showed higher transcriptional activation for collagen type II [except in the presence of dexamethasone, which inhibited this effect to a measurable degree (Fig. 3)]. These results contradict those of Nishimura *et al.*[11], who reported higher expression of collagen type II in rabbit synovial cells treated with TGF-β1. These investigators used tissue explants and cell aggregates for culturing (derived from another species); they did not deal with isolated individual cells under 3D culturing conditions. Similarly in a study by De Bari *et al.*[21], synovial chondrogenic precursor cells of human origin were not cultured individually under 3D conditions. Nevertheless, the experimental set-up adopted by the latter authors corresponded more closely to our own, particularly with respect to the micromass culturing conditions. It is noteworthy that the reactivity of synovial chondrogenic progenitor cells differ significantly between species, with that for the human population being more akin to that for cells of bovine than of rabbit origin. Moreover, under explant conditions in the presence of an extracellular matrix and of other cell types, cell reactivity and responsiveness to signaling substances most likely differ greatly.

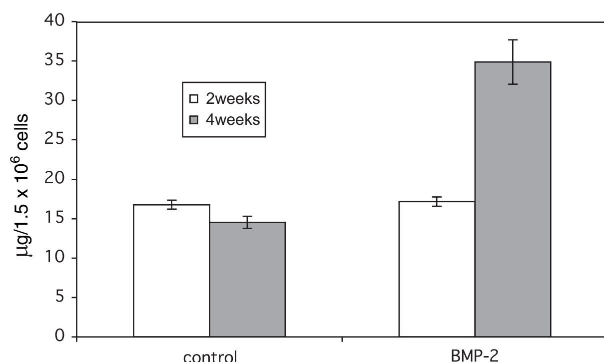

Fig. 6. Measurement of GAG contents in synovial cell cultures. Synovium-derived progenitor cells were cultured in alginate disks in serum-free medium with or without BMP-2 (50 ng/ml) for up to 4 weeks. GAG contents were measured using the Blyscan kit. The amount of GAG was calculated using a standard curve based on pure chondroitin sulfate ($n = 3$, error bars: SD).

Since BMP-2 demonstrated the highest chondrogenic activity in our experiments, this growth factor was thus used for the rest of the experiments in this study. There is much evidence in the literature that BMP-2 promotes chondrogenesis by various mechanisms: mesenchymal chondrogenesis[2,31], periosteal callus formation[32], and modulation of chondrocyte proliferation[33]. Moreover, BMP-2 is known to induce the formation not only of new cartilage, but also of bone tissue[34], thus demonstrating a true capability of BMP-2 to induce both chondrogenic and osteogenic pathways in appropriate precursor cells under specific biomechanical microenvironmental conditions. In this light, one of the important aspects of our experiments has been the induction of Sox9 expression by BMP-2. Sox9 is a transcriptional activator that binds to the promoters and activates transcription of collagen type II and aggrecan[35,36]. Induction of Sox9 by BMP-2 in synovium-derived progenitor cells showed that these cells have indeed a chondrogenic potential and are predetermined to become chondrocytes. Over-expression of Sox9 has been reported to be associated with the expression of chondrocyte-specific genes such as collagen type II and aggrecan, which both play important roles in the chondrogenic differentiation process[31].

The described dosaging experiments revealed that the expression of Sox9 mRNA reached a maximum at 50 ng/ml BMP-2 and remained constant up to 200 ng/ml. In contrast, the expression of collagen type II and aggrecan still had not achieved plateau values and thus may be further stimulated at higher concentrations of BMP-2. This showed that the expression levels of collagen type II and aggrecan mRNA do not depend exclusively on the expression of Sox9. One of the reasons may be that the expression of collagen type II and aggrecan are regulated by at least two different pathways. Sox9 or other transcriptional factors, induced by BMP-2, may increase the expression of collagen-specific extracellular matrix genes indirectly by binding to the respective promoters as a *trans*-acting element[31]. In addition to this, the BMP-2-signaling pathway may lead directly to over-expression via Smad signaling[37] and via an enhancer sequence on the collagen type II promoter. However, most likely other activation mechanisms are involved when we consider and compare the gene-expression levels of synovium-derived progenitor cells with those of chondrocytes. Even though mRNA expression levels of cartilage-specific genes were much higher in BMP-2-treated compared to non-treated synovial cells, they were still considerably lower than those in chondrocytes. The mRNA level of Sox9 in BMP-2-treated synovial cells was 4 times lower than the corresponding expression levels in chondrocytes, that of aggrecan was 2 times lower, and that of collagen type II was 30 times lower. BMP-2-treatment alone of synovial cells was thus not sufficient to attain gene activity levels comparable with chondrocytes *in vitro*.

When we looked at the post-translational cell activities, the GAG levels after a 4-week culture period indicated that measurable amounts appeared in the extracellular matrix

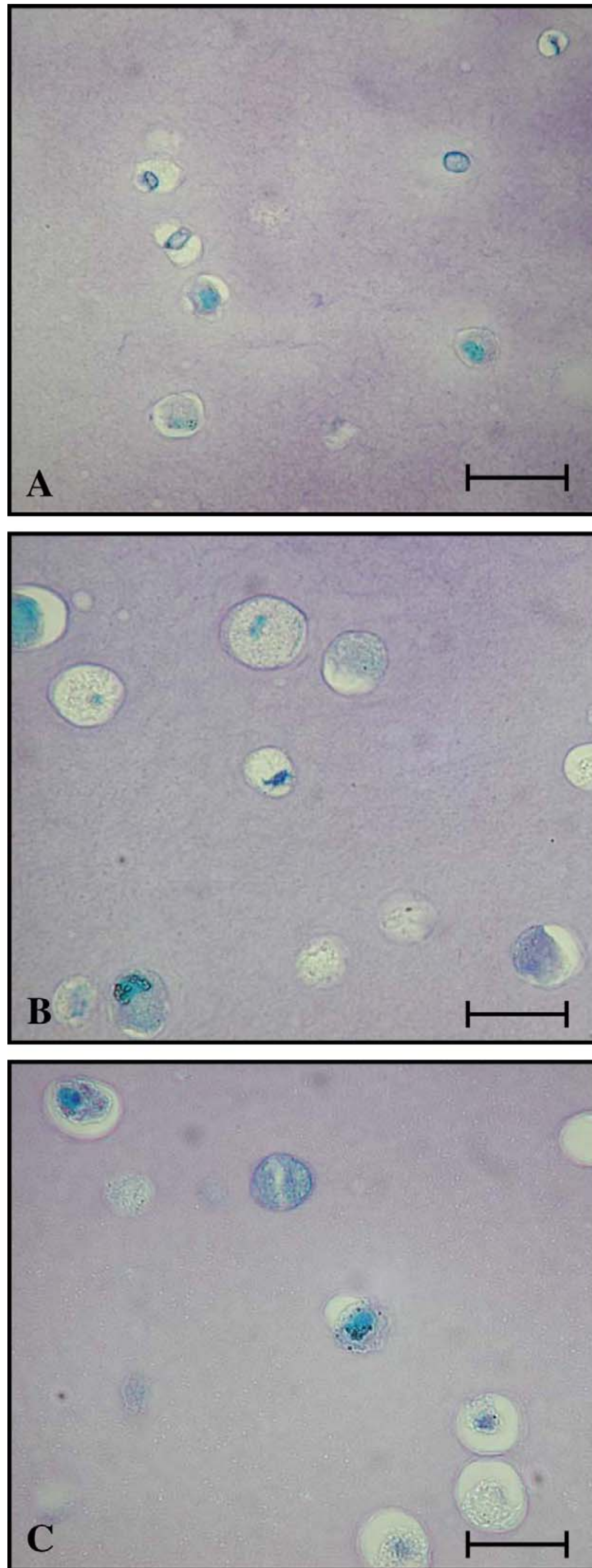

Fig. 7. Light micrographs illustrating synovium-derived chondrogenic precursor cells cultured for 4 weeks in control medium (A), in medium containing 50 ng/ml BMP-2 (B), and in medium containing 200 ng/ml BMP-2 (C). Under all three conditions, the chondrogenic precursor cells show a transformation into chondrocyte-like cells with lacuna formation. Under the control conditions, the cell population is of a relatively smaller size and cells frequently remain collapsed within the lacunae formed within the alginate material. The lacunar rims remain faintly stained or unstained with Toluidine Blue O. In the cell cultures supplemented with BMP-2, the cells grow to significantly larger sizes and volumes, and the population differentiates into a hypertrophic type of chondrocyte. The cells more rarely remain collapsed within the lacunae and the lacunar rims of the alginate generally stain intensely with Toluidine Blue O. However, the staining intensity varies considerably throughout the tissue blocks. The number of cells per volume (cellularity) under all three conditions appears very similar. Toluidine Blue O staining, semi-thin sections (1 μm). Bars = 20 μm.

only after a very long time, even though aggrecan mRNA was over-expressed from the beginning of the culturing period. In contrast, the cellular morphology showed that cells in the alginate disks rounded up to a chondrocytic phenotype and started to express the collagen type II protein after 1 week in culture when treated with BMP-2 (as evidenced by the immunohistochemistry experiments).

Attempts to engineer cartilage tissue *in vitro* from stem cells aim at the generation of a hyaline extracellular matrix. The major organic components of the extracellular cartilage matrix are aggrecan and collagen type II[38]. In particular, collagen type II is the major component in the cartilage among the different fibrillar and fibril-associated collagens, implying that collagen type II should be the major protein produced by the cells. In this study, it was found that chondrocytes express the collagen type II mRNA at a ratio of 1700:1 with type I collagen mRNA. However, synovium-derived progenitor cells in alginate without treatment with BMP-2 showed a ratio of 6×10^{-6}. When cells were treated with 200 ng/ml BMP-2, the expression ratio increased to 0.053. Even though this ratio was thus significantly improved, it still is too low compared to that of chondrocytes. It will thus be necessary not only to further improve the expression levels of collagen type II from synovium-derived precursor cells, but also to inhibit the expression activities for collagen type I (as was suggested by the use of interferon[39]).

Bovine synovium-derived chondrogenic precursor cells showed differential responses to different growth factors and it can be assumed that these cells are predetermined to the chondrogenic differentiation pathway when compared to other types of stem cells. Even though BMP-2, a potent chondrogenic agent for synovium-derived progenitor cells, can turn on the chondrogenic pathway in the appropriate chondrogenic precursor cell pool, these cells still express fibroblastic characteristics under *in-vitro* conditions. Turning off the fibroblastic characteristics will be one of the main future aims of *in-vitro* cartilage engineering concepts. The synovium-derived progenitor cells cultured within alginate discs clearly rounded up (Fig. 7) and increased in size. This differentiation process could proceed further, with fully-fledged hypertrophic chondrocytes being ultimately formed. And these terminally differentiated chondrocytes could, of course, begin to mineralize their extracellular matrix. Obviously, this process would not be desired in the context of tissue engineering, and future efforts must therefore be made to arrest chondrocyte differentiation during an early hypertrophic phase. However, the precise extent of the hypertrophic differentiation achieved in the present study was not ascertained. Nevertheless, this report suggests that synovial cells may be a possible and useful source of chondrogenic progenitor cells for future tissue engineering applications.

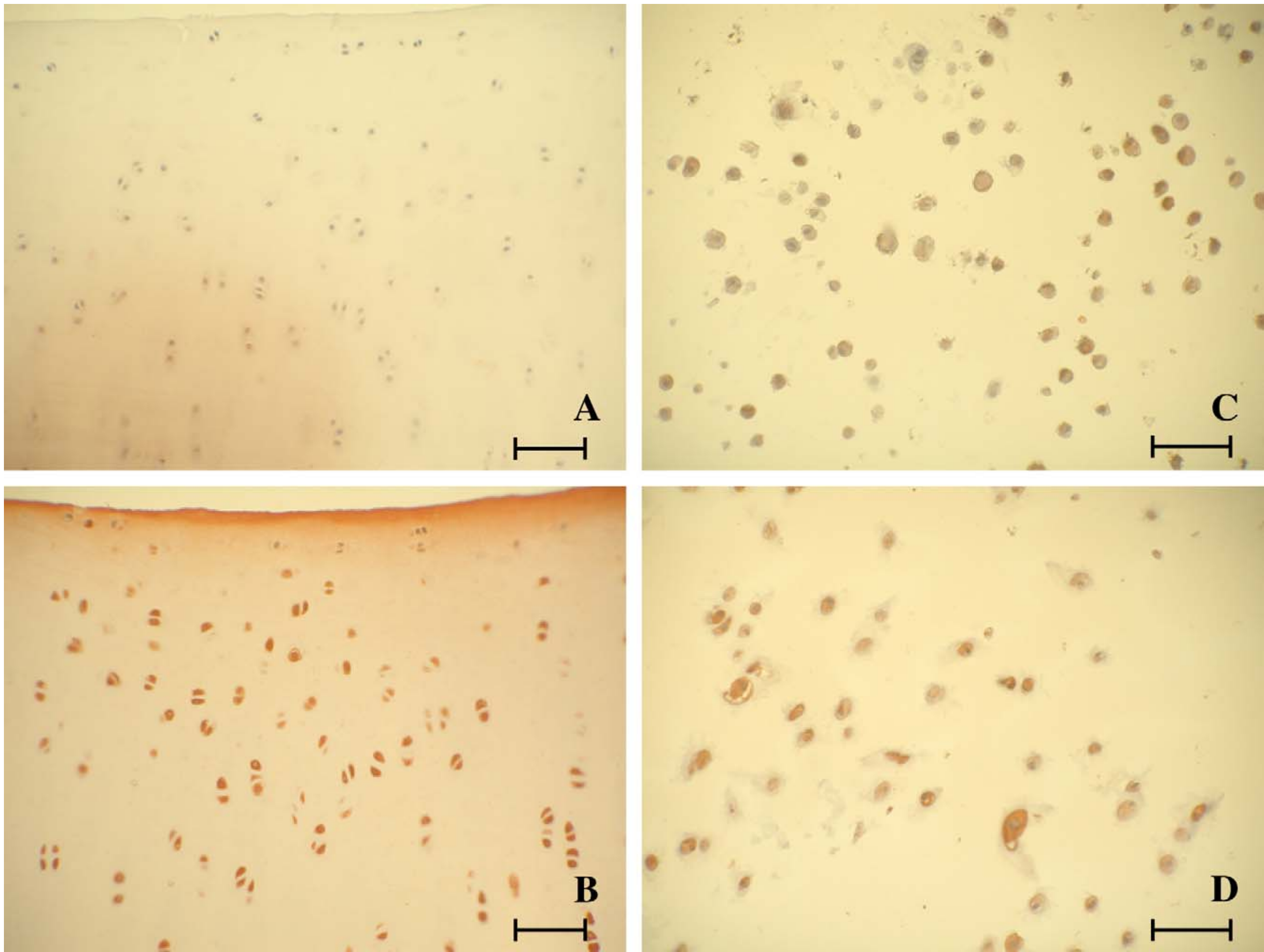

Fig. 8. Immunohistochemistry for collagen type II. Synovial cells were cultured in alginate disks with or without BMP-2 for 2 weeks. Cells were then fixed and processed for paraffin section production (control tissue used: adult bovine articular cartilage). Paraffin sections (5 μm) were incubated with antibody against bovine collagen type II. Secondary antibody tagged with horseradish peroxidase was used for visualization. (A) Cartilage control tissue without primary antibody. Negative staining result. (B) Cartilage control tissue with a primary antibody against collagen II. Positive staining reaction of chondrocytes. C + D: Synovial cells incubated in the absence (C) and in the presence (D) of BMP-2 (200 ng/ml) and treated with primary antibodies against collagen II. Both sections show scattered positive chondrocyte staining. The intensity of the staining appears somewhat higher in the BMP-2-treated group than in the untreated group. Bars = 100 μm.

Acknowledgement

This project was supported by the Swiss National Science Foundation, Bern, Switzerland, and by the Novartis Foundation, Basel, Switzerland (grants to EBH).

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