

## Chondrogenic Differentiation of Bovine Synovium

### Bone Morphogenetic Proteins 2 and 7 and Transforming Growth Factor $\beta$ 1 Induce the Formation of Different Types of Cartilaginous Tissue

Nahoko Shintani and Ernst B. Hunziker

**Objective.** To compare the potential of bone morphogenetic proteins 2 and 7 (BMP-2 and BMP-7) and transforming growth factor  $\beta$ 1 (TGF $\beta$ 1) to effect the chondrogenic differentiation of synovial explants by analyzing the histologic, biochemical, and gene expression characteristics of the cartilaginous tissues formed.

**Methods.** Synovial explants derived from the metacarpal joints of calves were cultured in agarose. Initially, BMP-2 was used to evaluate the chondrogenic potential of the synovial explants under different culturing conditions. Under appropriate conditions, the chondrogenic effects of BMP-2, BMP-7, and TGF $\beta$ 1 were then compared. The differentiated tissue was characterized histologically, histomorphometrically, immunohistochemically, biochemically, and at the gene expression level.

**Results.** BMP-2 induced the chondrogenic differentiation of synovial explants in a dose- and time-dependent manner under serum- and dexamethasone-free conditions. The expression levels of cartilage-related genes increased in a time-dependent manner. BMP-7 was more potent than BMP-2 in inducing chondrogenesis, but the properties of the differentiated tissue were similar in each case. The type of cartilaginous tissue formed under the influence of TGF $\beta$ 1 differed in terms of both cell phenotype and gene expression profiles.

**Conclusion.** The 3 tested members of the TGF $\beta$  superfamily have different chondrogenic potentials and induce the formation of different types of cartilaginous tissue. To effect the full differentiation of synovial explants into a typically hyaline type of articular cartilage, further refinement of the stimulation conditions is required. This might be achieved by the simultaneous application of several growth factors.

Lesioning of the articular cartilage layer is a common consequence of sports injuries and accidents and is a characteristic feature of the osteoarthritis process. Structural lesions that are confined to the articular cartilage layer do not heal spontaneously, and a number of therapeutic strategies have been introduced to induce their repair (1). Unfortunately, none of the modalities currently implemented yields satisfactory results.

Because of their capacity for self-renewal, their potential for multidifferentiation, and their accessibility, mesenchymal stem cells (MSCs) are an attractive option in tissue-engineering approaches to cartilage repair. They have been identified in various adult tissues, such as bone marrow (2–4), periosteum (5–7), perichondrium (8), muscle (9), fat (10), and synovium (11–13). Since the synovium and articular cartilage develop from a common pool of mesenchymal precursor cells (14), the synovium would be a seemingly ideal source of MSCs for the repair of articular cartilage, and the tissue formed might also be more joint specific. MSCs originating from the synovium have a higher chondrogenic capacity than do those derived from bone marrow, periosteum, fat, or muscle (15,16). An additional advantage of using the synovium as a source of MSCs is that the tissue can regenerate after partial removal (17–19).

The high chondrogenic potential of synovial MSCs has been well established. Indeed, we have shown

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that when appropriately stimulated *in vivo*, these cells can be induced to migrate from the synovium into partial-thickness articular cartilage defects and therein to differentiate into chondrocytes (20,21). The chondrogenesis of synovial MSCs has also been induced *in vitro* (11–13,15,16,22), using transforming growth factor  $\beta$  (TGF $\beta$ ), bone morphogenetic protein 2 (BMP-2), or combinations of the 2 classes of agent. However, the characteristics of the cartilaginous tissues formed have not been analyzed in detail, and the stimulation conditions required to generate a typically hyaline type of cartilage have not been delineated.

Synovial MSCs can undergo chondrogenesis only if they are cultured under 3-dimensional conditions (22), and several systems have been developed. These include micromass (13) and pellet preparations (15,16), and culturing in alginate (22) or a collagenous gel (23). However, the physiologic scaffold afforded by synovial tissue itself might be more conducive to chondrogenic differentiation. Indeed, the synovium is known to transform into cartilaginous tissue under both clinicopathologic (24) and experimental (12,25) conditions. If a synovial explant system could be developed for the repair of articular cartilage lesions, then the need for cell isolation and preculturing would be obviated. And, most important, the physiologic microenvironment of the MSCs would be preserved.

In this study, we evaluated the potential of 3 different growth factors (BMP-2, BMP-7, and TGF $\beta$ 1) to induce the chondrogenic differentiation of MSCs within synovial explants. Each of these agents is known to have a chondrogenic effect on MSCs (11–13,22,26,27). To establish the optimal stimulation conditions for the formation of a hyaline type of articular cartilage, the differentiated tissue was characterized histologically and histomorphometrically (cell morphology and volume fraction of metachromasia after staining with toluidine blue), immunohistochemically (type II collagen [CII]), and biochemically (glycosaminoglycan [GAG] content). In addition, the expression levels of genes for cartilage-related proteins (collagens CI, CII, CIX, CX, and CXI, aggrecan, cartilage oligomeric matrix protein [COMP], and SOX9) were quantified using real-time polymerase chain reaction (PCR). The tested growth factors not only manifested different potentials for the chondrogenic differentiation of synovial MSCs *in situ*, but also induced the formation of cartilaginous tissue with different cell phenotypes and gene expression profiles.

## MATERIALS AND METHODS

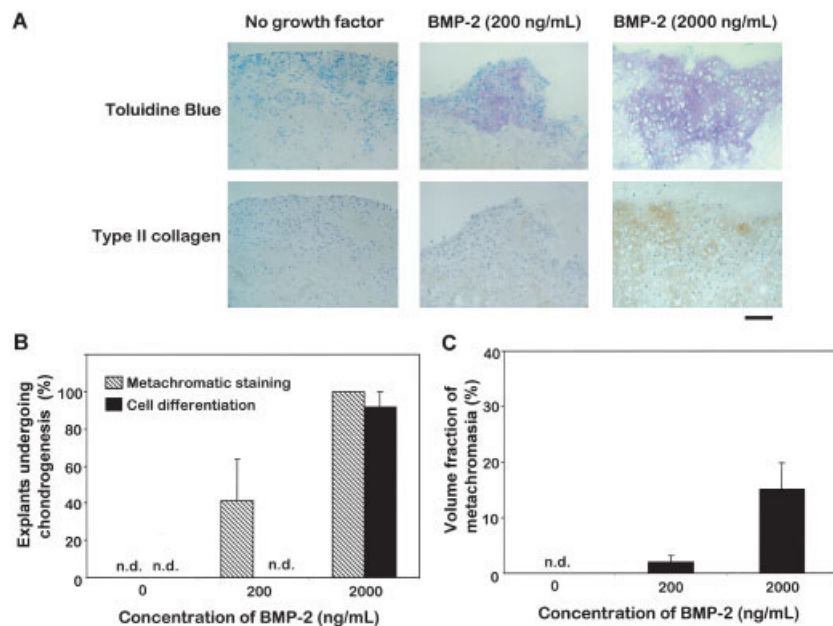
**Preparation of synovial explants.** Synovial tissue was harvested from the metacarpal joints of 3–5-month-old calves within 24 hours of slaughter. It was washed in cold Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Paisley, UK) and then cut into small pieces (~5 mm long by 1–2 mm wide) under sterile conditions.

**Tissue culturing.** Synovial explants were sandwiched between 2 layers of agarose to maintain them for an extended period and to prevent the outgrowth of chondroprogenitor cells (6,12). Since agarose impedes the diffusion of large molecules (28), culturing within this polysaccharide serves to establish nutritional and oxygen-tension conditions that are similar to those operating physiologically. Initially, each cavity of a 24-well, flat-bottomed tissue culture plate (Sarstedt, Newton, NC) was precoated with 250  $\mu$ l of 1% low-melt agarose (Bio-Rad, Richmond, CA). After gelation, 1 explant was introduced and covered with 750  $\mu$ l of 0.5% agarose, which was likewise allowed to gel. Each agarose-sandwiched explant was then covered with 1 ml of high-glucose DMEM containing 1% ITS+ Premix (BD Biosciences, Bedford, MA), 1 mM proline, 50  $\mu$ g/ml ascorbic acid, and 50  $\mu$ g/ml gentamicin. The medium was changed every 2 days. Fresh ascorbic acid was added daily to yield a final concentration of 25  $\mu$ g/ml.

BMP-2 has previously been shown to have a higher potential than TGF $\beta$  to induce the chondrogenic differentiation of alginate-cultured bovine synovial cells (22). Hence, this agent was selected to evaluate the chondrogenic potential of synovial explants and the temporal course of their differentiation. The synovial explants were cultured with recombinant human BMP-2 (a generous gift from Wyeth, Cambridge, MA) for 2 weeks (at 200 or 2,000 ng/ml), 4 weeks, or 6 weeks (both at 200 ng/ml) in the absence or presence of  $10^{-7}$  M dexamethasone (DEX; Sigma, St. Louis, MO). Initially, the explants were cultured in high-glucose DMEM containing 10% fetal bovine serum (FBS; Invitrogen) instead of 1% ITS+ Premix. But no chondrogenic differentiation took place under these conditions. In all subsequent experiments, explants were cultured in serum-free medium.

On the basis of data gleaned from this preliminary investigation, a culturing period of 6 weeks was chosen for the comparative experiments involving BMP-2, recombinant human BMP-7 (a generous gift from Stryker Biotech, Hopkinton, MA), and recombinant human TGF $\beta$ 1 (PeproTech, Rocky Hill, NJ). The tested concentration of each growth factor (200 ng/ml of BMP-2, 200 ng/ml of BMP-7, 10 ng/ml of TGF $\beta$ 1) was determined in screening experiments with micromass cultures (see below). The growth factors were introduced every 2 days when the medium was changed. Control cultures containing no growth factors were grown in parallel. All cultures were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

**Micromass cultures.** To determine the basic chondrogenic concentration range for each growth factor, synovial cells were grown in micromass cultures. They were isolated and expanded as previously described (22). The cells were suspended at a numerical density of  $2 \times 10^7$ /ml. Aliquots (10  $\mu$ l) of this suspension were transferred to 24-well plates and incubated for 4 hours at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. BMP-2 (at a final concentration of 10, 50,



**Figure 1.** Bone morphogenetic protein 2 (BMP-2)-induced chondrogenic differentiation of bovine synovial explants as a function of BMP-2 dose (200 or 2,000 ng/ml) after 2 weeks of culturing under serum-free conditions. **A**, Metachromasia of the extracellular matrix was revealed after staining with toluidine blue; immunoreactivity with type II collagen occurred only with the higher dose of BMP-2. Bar = 100  $\mu$ m. **B**, Rates of occurrence of metachromatic staining and cell differentiation within synovial explants. **C**, Volume fraction of metachromasia in synovial explants. Results in **B** and **C** are the mean and SEM of 3–4 explants per animal from 3 animals. nd = not detectable.

200, or 2,000 ng/ml), BMP-7 (at a final concentration of 10, 50, 200, or 2,000 ng/ml), or TGF $\beta$ 1 (at a final concentration of 2.5, 10, 50, or 200 ng/ml) was then added to each well with 1 ml of medium. Control cultures containing no growth factors were grown in parallel. All cultures were maintained for 3 or 4 days at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. The cells were then fixed with 4% formaldehyde in phosphate buffered saline (PBS; pH 7.4) for 30 minutes and stained overnight with 1% Alcian blue (in 3% acetic acid). After staining, the cells were rinsed with distilled water and inspected using an Olympus SZH-ILLD stereomicroscope (Olympus, Lake Success, NY).

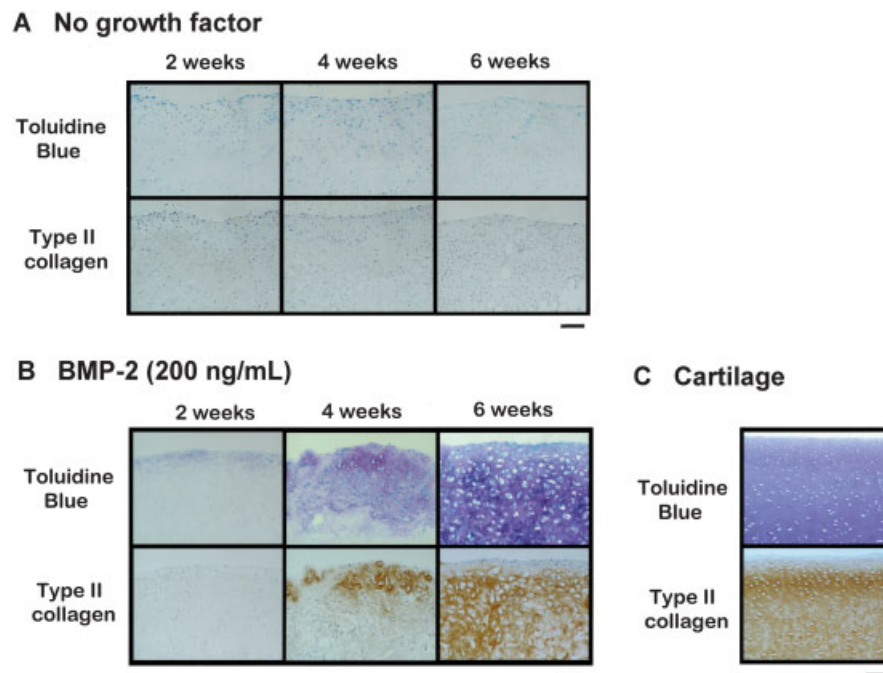
**Histologic and histomorphometric analysis.** After culturing, synovial explants were fixed with 2% formaldehyde in PBS (pH 7.4), embedded in paraffin, cut into 5- $\mu$ m-thick sections, and stained with 1% toluidine blue (pH 2.5). Evaluation of chondrogenesis was performed using an Olympus Vanox AH2 light microscope.

The degree of chondrogenic differentiation was quantified by determining the volume fraction of metachromasia on histologic sections, and the rate of occurrence (between explants) of tissue metachromasia and of synovial cell differentiation into a chondrocytic phenotype (with pericellular lacunae). The volume fraction of metachromasia was assessed morphometrically using standard stereologic methods (29), and was expressed as a percentage of the total tissue volume. Systematic random sampling protocols were implemented. The rate of occurrence of metachromasia and cell differentiation

was expressed as the number of positive explants relative to the total number of explants per experimental group.

**Immunohistochemical analysis of CII.** For the immunohistochemical analysis, 5- $\mu$ m-thick paraffin-embedded sections were used. These were deparaffinized and then treated with hyaluronidase (1 mg/ml of sodium acetate buffer) (Sigma) for 30 minutes at 37°C. The activity of nonspecific epitopes was blocked by exposure to 3% decreamed cows' milk and 1.5% horse serum (in PBS) for 30 minutes at ambient temperature. The sections were then incubated first with mouse anti-CII IgG (clone CII C1; Developmental Studies Hybridoma Bank, Iowa City, IA) diluted 1:25,000 in PBS containing 3% decreamed cows' milk, and then with biotinylated horse anti-mouse IgG. Endogenous peroxidase activity was blocked with H<sub>2</sub>O<sub>2</sub>, and the signal was enhanced with the avidin-biotin-peroxidase complex (Vector, Burlingame, CA). Cell nuclei were counterstained with Mayer's hemalum. Synovial explants cultured in the absence of a growth factor served as negative controls, and articular cartilage derived from the same joints served as positive controls.

**Measurement of tissue weight, total DNA, and GAG content.** The wet weight of synovial explants was determined before and after culturing. After culturing, the explants were digested (overnight at 60°C) with 0.1% papain and 0.1% proteinase K in 1 mM CaCl<sub>2</sub> and 10 mM Tris HCl (pH 8) (30). The DNA content of the digests was measured spectrofluorometrically using the high-sensitivity Quant-iT DNA assay kit (Molecular Probes, Eugene, OR) and expressed per



**Figure 2.** Chondrogenic differentiation of the bovine synovial explants. **A**, In the absence of bone morphogenetic protein 2 (BMP-2), the explants did not undergo differentiation. **B**, In the presence of BMP-2, differentiation was apparent after 4 weeks. **C**, Native (uncultivated) articular cartilage, derived from the same joint as the synovial tissue, served as a positive control. Bars = 100  $\mu$ m.

milligram of the preculture tissue wet weight. The GAG content of the digests was determined colorimetrically using dimethylene blue (Serva Electrophoresis, Heidelberg, Germany) (31). Chondroitin sulfate was used as a standard, and the GAG content was normalized to the total amount of DNA.

**RNA extraction, reverse transcription, and real-time PCR.** Prior to the gene expression analysis, each cultured explant was stored in RNAlater reagent (Qiagen, Hilden, Germany) at  $-70^{\circ}\text{C}$ . For calibration purposes, cartilage was collected from the metacarpal joints of 4 different animals. RNA was isolated and real-time PCR was performed as previously described (32). The samples were homogenized in QIAzol lysis reagent (Qiagen), and RNA was isolated using the RNeasy Micro Kit (Qiagen). RNA was reverse transcribed using the ImProm-II reverse transcription system (Promega, Madison, WI). Real-time PCR was performed using the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). Primers and probes for bovine CI, CII, CIX, CX, and CXI, aggrecan, COMP, SOX9, and 18S ribosomal RNA (rRNA) were generated as previously described (32). Gene expression was normalized to the level of 18S rRNA using the formula  $2^{-\Delta C_t}$ , where  $C_t$  is the threshold cycle and  $\Delta C_t = C_t$  (target gene)  $- C_t$  (18S rRNA). For each gene, the normalized messenger RNA (mRNA) level was calculated relative to that in articular cartilage using the formula  $2^{-\Delta\Delta C_t}$ , where  $\Delta\Delta C_t = \Delta C_t$  (synovial explant)  $- \Delta C_t$  (articular cartilage).

**Statistical analysis.** For each experiment, 3–4 explants per group were prepared from 1 animal. Each experiment was repeated 3–4 times using tissue derived from a different donor animal on each occasion. Mean  $\pm$  SEM values for 3–4 donors

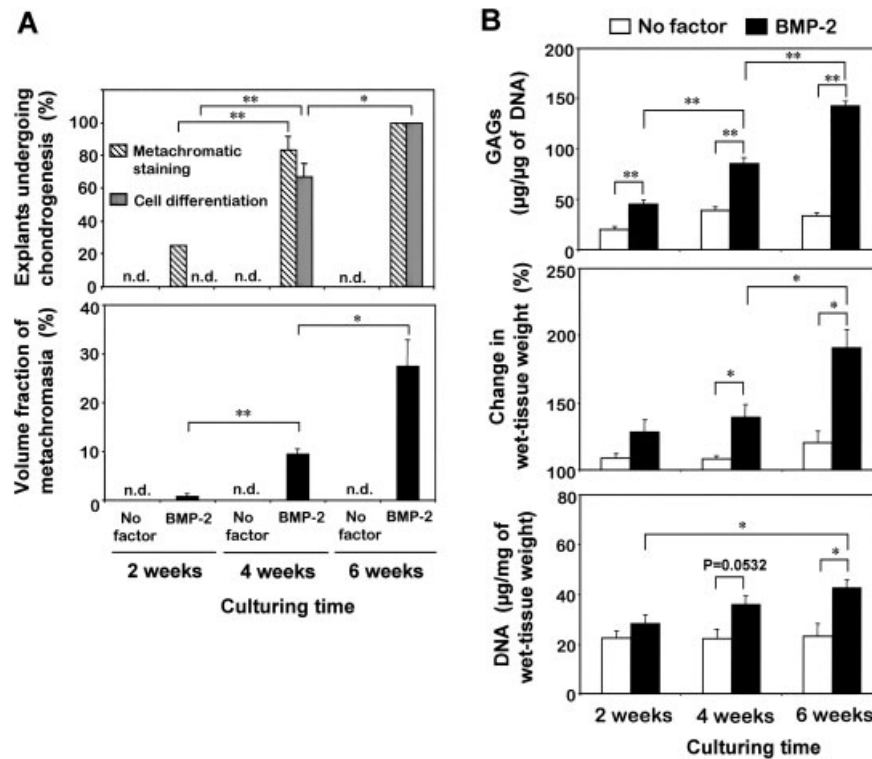
were calculated. The data were statistically evaluated by analysis of variance (ANOVA). Comparisons between 2 groups were made using the *t*-test, and comparisons between multiple groups were made using Dunnett's multiple comparison test. Statistical analyses were performed using SPSS software, version 11.0.4 (SPSS, Chicago, IL). *P* values less than 0.05 were considered significant.

## RESULTS

**Preliminary experiments with BMP-2. Dose-dependency of chondrogenic differentiation.** In an initial investigation, bovine synovial explants were cultured with different concentrations of BMP-2 (200, 1,000, or 10,000 ng/ml) for 2, 4, or 6 weeks in the presence of 10% FBS. Thirty explants, which were derived from 4 different animals, were used for these experiments. Chondrogenic differentiation did not occur in the presence of FBS. Hence, all subsequent experiments were conducted under serum-free conditions.

Synovial explants were then cultured with BMP-2 at concentrations of 200 ng/ml or 2,000 ng/ml for 2 weeks. At a dose of 200 ng/ml, metachromatic staining of the extracellular matrix was observed, but the synovial cells did not differentiate into a chondrocytic phenotype with lacunae (Figure 1A). The mean  $\pm$  SEM rate of occurrence of metachromasia was  $41.7 \pm 22.1\%$  (Figure





**Figure 3.** Time course of changes in the morphologic, histologic, and biochemical characteristics of bovine synovial explants cultured in the absence or presence of bone morphogenetic protein 2 (BMP-2). **A**, Rates of occurrence of metachromasia and cell differentiation, and volume fraction of metachromasia in the explants. **B**, Glycosaminoglycan (GAG) content, change in tissue wet weight, and DNA content over time. The tissue wet weight of each synovial explant was determined before and after culturing. The DNA content of each explant is expressed per milligram of tissue wet weight prior to culturing. Results are the mean and SEM of 3–4 explants per animal from 3 animals. \* =  $P < 0.05$ ; \*\* =  $P < 0.01$ . nd = not detectable.

1B). At a dose of 2,000 ng/ml, metachromasia occurred in all explants (at a higher volume fraction) (Figure 1C) and cell differentiation occurred in  $91.7 \pm 8.3\%$  of explants (Figure 1B).

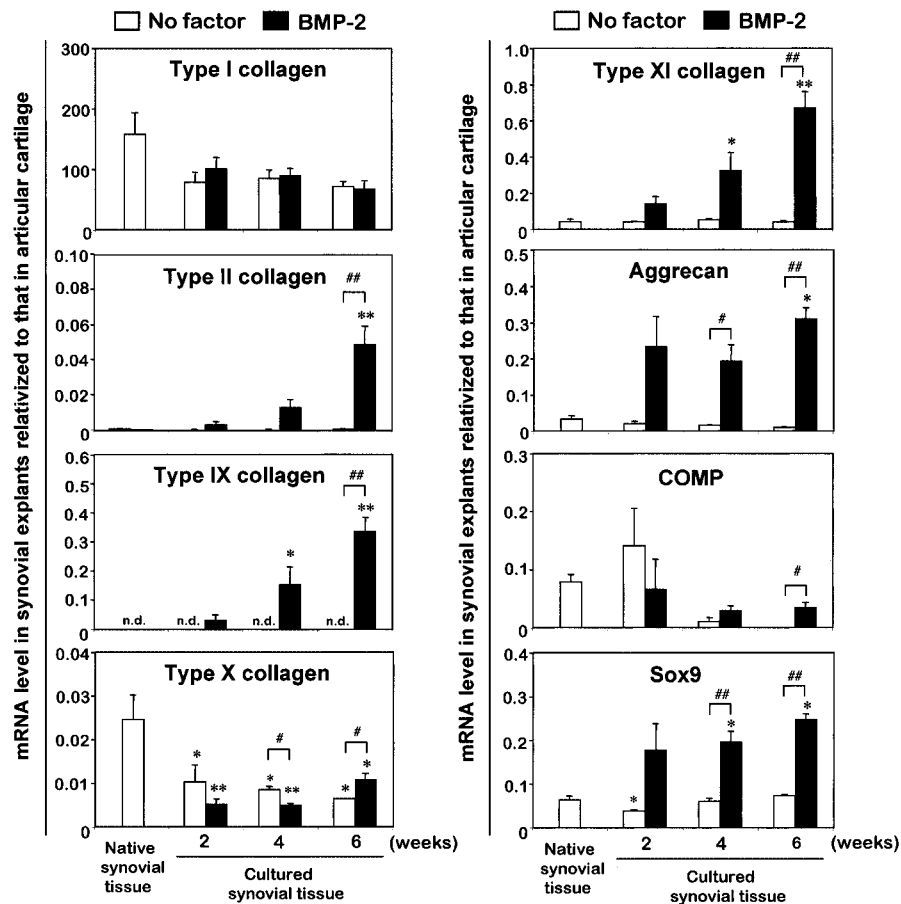
Immunohistochemical analysis of CII revealed its presence only at the higher dose of BMP-2 (Figure 1A). In some experiments, a possible additive effect of DEX ( $10^{-7}M$ ) was tested. However, this agent consistently inhibited the BMP-2–induced chondrogenic differentiation of synovial MSCs (data not shown).

**Time course of chondrogenic differentiation.** Synovial explants were cultured in the absence or presence of BMP-2 at 200 ng/ml for 2, 4, or 6 weeks. In the absence of BMP-2, the explants did not undergo chondrogenic differentiation, even when cultured for 6 weeks (Figure 2A). In the presence of this growth factor, metachromasia was already apparent after 2 weeks, and cell differentiation into a chondrocytic phenotype was apparent after 4 weeks (Figure 2B). Immunohistochemical analysis of CII revealed its presence after 4 weeks but not

after 2 weeks. In comparison with native articular cartilage chondrocytes (Figure 2C), the differentiated synovial cells were grossly hypertrophic after 6 weeks of culturing.

The temporal course of chondrogenic differentiation was quantified histomorphometrically and biochemically (Figure 3). The rate of occurrence of metachromasia and cell differentiation increased with culturing time and, by the sixth week, had attained levels of 100% (Figure 3A). The volume fraction of metachromasia likewise increased in a time-dependent manner, with a mean  $\pm$  SEM of  $27.4 \pm 5.4\%$  being achieved after 6 weeks (Figure 3A). The GAG content of synovial explants, as well as their DNA content and tissue wet weight, also increased with culturing time (Figure 3B).

**Time course of gene expression.** Synovial explants that had been cultured in the absence or presence of BMP-2 at 200 ng/ml for 2, 4, or 6 weeks were subjected to real-time PCR. The mRNA levels of genes for cartilage-related proteins (collagens CI, CII, CIX, CX,

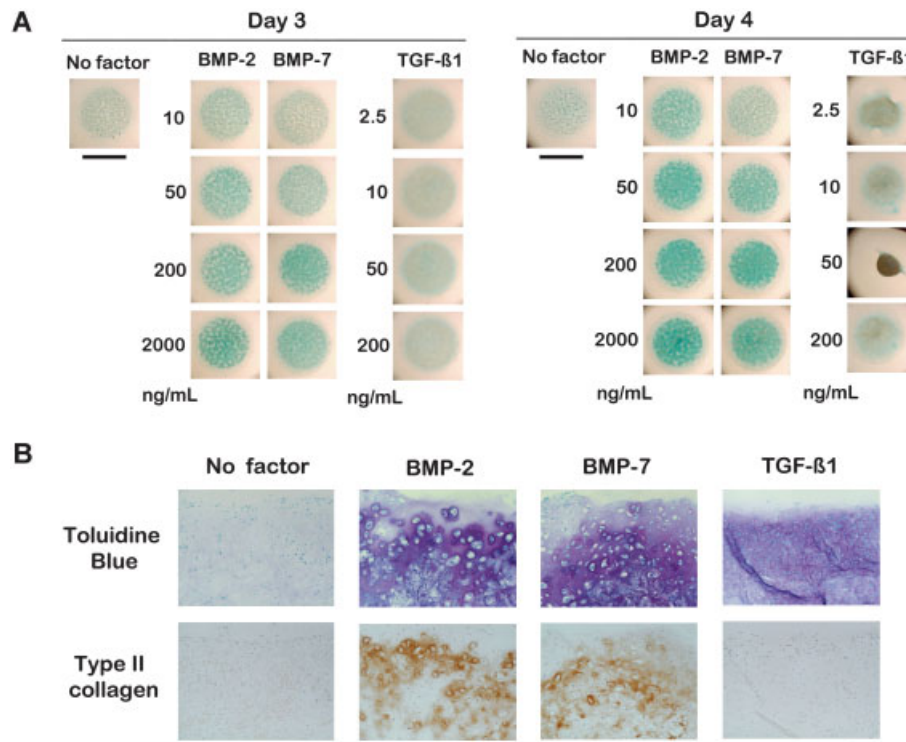


**Figure 4.** Changes in the mRNA levels of genes for cartilage-related proteins within bovine synovial explants cultured in the absence or presence of bone morphogenetic protein 2 (BMP-2). The mRNA levels in synovial explants were related to those in native articular cartilage derived from the same joint, after normalizing each value to that of 18S ribosomal RNA. Results are the mean and SEM of 4 explants per animal from 4 animals. # =  $P < 0.05$ ; ## =  $P < 0.01$ ; \* =  $P < 0.05$ ; \*\* =  $P < 0.01$  versus native synovial tissue. nd = not detectable; COMP = cartilage oligomeric matrix protein.

and CXI, aggrecan, COMP, and SOX9), which were related to those in native articular cartilage, are shown in Figure 4. In native (uncultivated) synovial tissue, the mRNA for CI was expressed at an ~160-fold higher level than in native articular cartilage. This level tended to decrease with culturing time, irrespective of whether BMP-2 was absent or present. But even at the 6-week juncture, the levels of mRNA (in the absence or presence of BMP-2) were still ~70-fold higher than in native articular cartilage. In the presence of BMP-2, the mRNA levels of CII, CIX, and CXI, aggrecan, and SOX9 increased with culturing time. The mRNA level of CX decreased between the second and fourth weeks of culturing, but increased between the fourth and sixth

weeks. At the 6-week juncture, the level was higher than that in the absence of BMP-2. The mRNA level of COMP decreased with time, but was still measurable after 6 weeks of culturing. In the absence of BMP-2, the mRNA level of COMP barely exceeded the detection threshold after 6 weeks of culturing.

**Comparison of the chondrogenic efficacies of BMP-2, BMP-7, and TGF $\beta$ 1.** To determine the appropriate chondrogenic induction doses of BMP-2, BMP-7, and TGF $\beta$ 1, isolated synovial cells were grown in micro-mass cultures and exposed to different concentrations of the 3 growth factors (for BMP-2, 10, 50, 200, and 2,000 ng/ml; for BMP-7, 10, 50, 200, and 2,000 ng/ml; for TGF $\beta$ 1, 2.5, 10, 50, and 200 ng/ml) for 3 or 4 days



**Figure 5.** Comparative chondrogenic efficacies of bone morphogenetic protein 2 (BMP-2), BMP-7, and transforming growth factor  $\beta$ 1 (TGF $\beta$ 1) on micromass cultures of bovine synovial cells. **A**, Alcian blue staining of micromass cultures that were grown for 3 days or 4 days in the absence or presence of BMP-2, BMP-7, or TGF $\beta$ 1. Bars = 3 mm. **B**, Immunohistochemical analysis of sectioned synovial explants that were cultured for 6 weeks in the absence or presence of BMP-2 (200 ng/ml), BMP-7 (200 ng/ml), or TGF $\beta$ 1 (10 ng/ml). At the end of the culturing period, metachromasia of the extracellular matrix after toluidine blue staining and immunoreactivity with type II collagen were determined. Bar = 100  $\mu$ m.

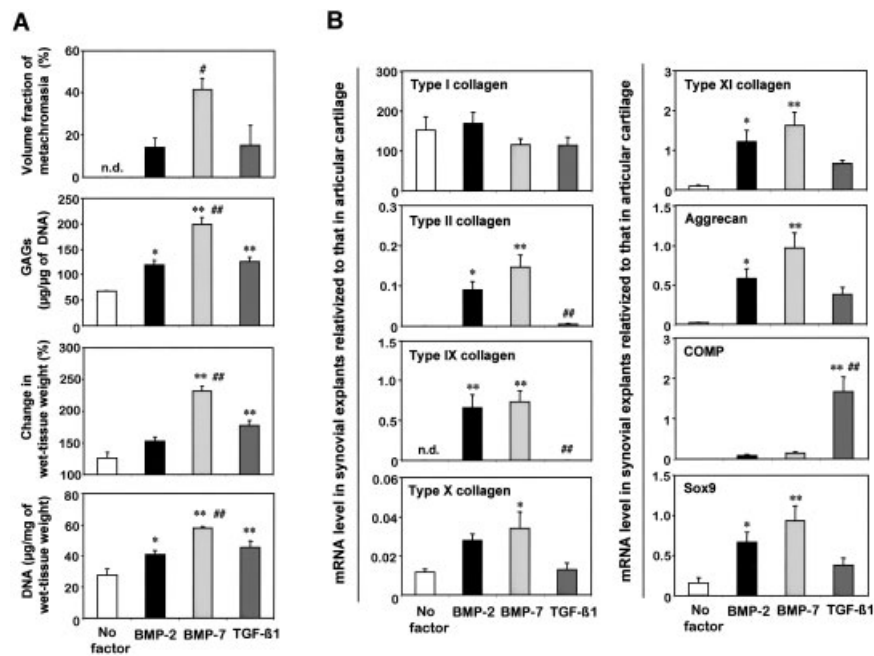
(Figure 5A). Cell aggregates that had been treated with BMP-2 manifested a dose-dependent increase in the intensity of staining with Alcian blue. Those that had been exposed to BMP-7 likewise exhibited a dose-dependent increase in staining intensity, but the reaction peaked at 200 ng/ml (BMP-7) instead of at 2,000 ng/ml (BMP-2). For both BMP-2 and BMP-7, the intensity of staining at each concentration was greater after 4 days than after 3 days of culturing. Cell aggregates that had been exposed to TGF $\beta$ 1 did not manifest a positive staining reaction with Alcian blue at any of the tested concentrations. Moreover, after 4 days of culturing, they began to shrink (at all concentrations). On the basis of these results, the minimal chondrogenic doses of BMP-2 and BMP-7 were set at 200 ng/ml. On the basis of previous studies (12,13,26), TGF $\beta$ 1 was used at a concentration of 10 ng/ml.

Synovial explants were cultured with the defined

doses of BMP-2 (200 ng/ml), BMP-7 (200 ng/ml), and TGF $\beta$ 1 (10 ng/ml) for 6 weeks. At the end of this period, metachromasia was observed within all explants that had been exposed to a growth factor, irrespective of its nature. BMP-2 and BMP-7, but not TGF $\beta$ 1, also induced the differentiation of synovial cells into a chondrocytic phenotype (with lacunae). Immunohistochemical analysis of CII revealed its presence after exposure to either BMP-2 or BMP-7, but not after treatment with TGF $\beta$ 1 (Figure 5B).

Among the 3 growth factors, BMP-7 induced the greatest increases in the volume fraction of metachromasia, GAG content, tissue wet weight, and total DNA (Figure 6A). For each of these parameters, the levels were significantly higher than those achieved using either BMP-2 or TGF $\beta$ 1, with there being no significant difference between the latter 2.

The effects of the 3 growth factors on the expres-



**Figure 6.** Comparative effects of bone morphogenetic protein 2 (BMP-2; 200 ng/ml), BMP-7 (200 ng/ml), and transforming growth factor  $\beta$ 1 (TGF $\beta$ 1; 10 ng/ml) on the histologic and biochemical characteristics of synovial explants, and on the mRNA levels of genes for cartilage-related proteins within the explants, after culturing for 6 weeks under serum-free conditions. **A**, Volume fraction of metachromasia, glycosaminoglycan (GAG) content, change in tissue wet weight, and DNA content. The tissue wet weight of each explant was determined before and after culturing. The DNA content of each explant is expressed per milligram of tissue wet weight prior to culturing. Results are the mean and SEM of 3–4 explants per animal from 3 animals. **B**, Expression levels of mRNA for cartilage-related protein genes. The mRNA levels in the explants were relativized to those in native articular cartilage derived from the same joint, after normalizing each value to that of 18S ribosomal RNA. Results are the mean and SEM of 4 explants per animal from 4 animals. \* =  $P < 0.05$  versus no factor; \*\* =  $P < 0.01$  versus no factor; # =  $P < 0.05$  versus BMP-2; ## =  $P < 0.01$  versus BMP-2. nd = not detectable; COMP = cartilageoligomeric matrix protein.

sion levels of cartilage-related genes are shown in Figure 6B. Neither BMP-2, BMP-7, nor TGF $\beta$ 1 elicited a significant increase in the mRNA level of CI. But both BMP-2 and BMP-7 induced significant increases in the mRNA levels of CII, CIX, CX (BMP-7 only), and CXI, aggrecan, and SOX9, and the levels tended to be higher (although not significantly) for BMP-7 than for BMP-2. TGF $\beta$ 1 induced no significant increases in the mRNA levels of CII, CIX, CX, and CXI, aggrecan, or SOX9. However, this agent elicited a substantial increase in the mRNA level of COMP, whereas neither BMP-2 nor BMP-7 did so.

## DISCUSSION

Synovial explants represent a potentially valuable system for the in situ culturing of MSCs that are destined

for clinical autotransplantation in the context of cartilage repair. To repair cartilage lesions in large human joints (e.g., knee, hip, or shoulder), tissue areas several square centimeters in area must be regenerated. The removal of sufficient quantities of synovium from the affected joint at the time of surgery would be a simple procedure with no deleterious consequences, since this tissue can undergo self-renewal (17–19). Furthermore, the use of synovial explants would obviate the need for cell isolation and preculturing and, most important, preserve the physiologic microenvironment of the MSCs. Nevertheless, the conditions under which even these explants are cultivated may have a profound influence on the capacity of the precursor cells to undergo chondrogenesis.

Initially, we investigated the culturing conditions



under which BMP-2, a representative member of the TGF $\beta$  superfamily, induced the chondrogenic differentiation of synovial explants. Interestingly, BMP-2 elicited this response only under serum-free conditions. And DEX, which has been reported to enhance the TGF $\beta$ 1-induced chondrogenic differentiation of pelleted bone marrow-derived MSCs (26), inhibited the BMP-2-induced chondrogenic differentiation of synovial explants. Since we have observed the same effect using alginate-cultured synovial cells of human origin (data not shown), the phenomenon is apparently not species specific. The different and often opposing effects elicited by DEX may be linked to the topographic origin of the MSCs and/or to the nature of their supporting matrix.

Under serum- and DEX-free conditions, BMP-2 elicited the chondrogenic differentiation of synovial explants: cell differentiation into a chondrocytic phenotype, expression of CII, and increased accumulation of GAGs in a dose- and time-dependent manner. The synovium is delimited from the joint cavity by a double or triple layer of lining cells (types A and B), which is supported by an underlying layer of loose vascularized connective tissue containing fibroblasts, macrophages, and adipocytes (33,34). Thus, compared with articular cartilage, synovial tissue produces large quantities of CI (35, 36). Indeed, the gene expression level of CI in native (uncultivated) synovial explants was 160-fold higher than that in native articular cartilage.

In addition to CI, native synovial explants expressed mRNA for CII, CX, and CXI, aggrecan, COMP, and SOX9, albeit at much lower levels than in native articular cartilage. The mRNA for CIX was not expressed in native synovial explants. After exposing synovial explants to BMP-2, the gene expression levels of CII and CXI, aggrecan, and SOX9 increased; the gene for CIX was also expressed. On the other hand, the gene expression level of CI decreased. These results indicate that BMP-2 does indeed induce a significant chondrogenic differentiation of synovial MSCs.

BMP-2 also acted on the gene expression levels of CX and COMP. CX is associated predominantly with the hypertrophic zones of epiphyseal and articular cartilage (37,38), and it serves as a marker for terminally differentiated chondrocytes. BMP-2 did not augment the expression level of the CX gene during the early stages of culturing (2 and 4 weeks), but it had done so by the 6-week juncture. Indeed, at this stage, the cells were grossly hypertrophic. However, staining with von Kossa's stain revealed no evidence of matrix mineralization (results not shown).

COMP was first purified from bovine cartilage

(39). It has also been identified in the vitreous membrane of the eye (40), tendons (41), vascular smooth muscle cells (42), and the synovium (43,44). In the present study, mRNA for COMP was detected in native synovial explants, but the expression level decreased with culturing time in the absence of a growth factor. BMP-2 was able to hinder this down-regulation.

Our findings relating to the chondrogenic effects of BMP-2 on synovial explants under serum- and DEX-free conditions served as a basis for the comparison between this growth factor, BMP-7, and TGF $\beta$ 1. Hitherto, the chondrogenic effects of BMP-7 on synovial MSCs have not been reported. On the basis of morphologic criteria, the cartilaginous tissues formed by the actions of BMP-2 and BMP-7 manifested similar characteristics, e.g., the differentiated cells exhibited a typically hypertrophic chondrocytic phenotype, were surrounded by lacunae, and were embedded within a metachromatically stained matrix. TGF $\beta$ 1, on the other hand, induced only a positive metachromatic staining reaction within the extracellular matrix. The cells did not assume a chondrocytic appearance, and they were not surrounded by obvious lacunae.

Unlike BMP-2 and BMP-7, TGF $\beta$ 1 failed to induce the expression of immunohistochemically detectable amounts of CII. Members of the TGF $\beta$  superfamily bind to 2 different groups of transmembranous serine/threonine kinase receptors (type I and type II) (45). BMP-2 and BMP-7 bind to the same type II receptors (BMP type II receptor/activin type IIA receptor) and to several common type I receptors (activin receptor-like kinase 3 [ALK-3] and ALK-6). TGF $\beta$ 1 binds to different type I and type II receptors (46). We speculate that the similarity of the chondrogenic responses induced by BMP-2 and BMP-7 reflects the commonality of their receptors, and that the different responses elicited by TGF $\beta$ 1 bespeak the occupation and activation of another set of receptors and the triggering of an alternative signaling pathway.

We also compared the effects of the 3 growth factors on the expression levels of cartilage-related genes within synovial explants. Consistent with the morphologic and histologic characteristics of the differentiated tissue, the gene expression profiles elicited by BMP-2 and BMP-7 were similar, but differed from those induced by TGF $\beta$ 1. The gene expression levels of CII, CIX, CX, and CXI, aggrecan, and SOX9 were lower in explants that had been exposed to TGF $\beta$ 1 than in those that had been treated with either BMP-2 or BMP-7. Indeed, the mRNA levels of CII and CIX were dramatically lower. In contrast, the gene expression level of

COMP was greatly augmented above that elicited in the presence of either BMP-2 or BMP-7. This effect of TGF $\beta$ 1 has also been observed in human synovial cells and chondrocytes (44). However, it is not known whether TGF $\beta$ 1 influences the expression of the COMP gene during the postnatal development of cartilage tissue. The biologic function of COMP is not well understood, but a role in the formation of collagen fibrils and in maintaining the extracellular collagenous network has been implicated (47,48).

Concerning the chondrogenic effects of TGF $\beta$  on synovial cells, conflicting results have been reported. TGF $\beta$ 1 induces the chondrogenesis of pelleted human synovial cells cultured in the presence of DEX (12), but TGF $\beta$ 3 does not (15). In our preliminary experiments involving bovine synovial cells grown under micromass conditions, TGF $\beta$ 1 induced chondrogenesis in both the absence and the presence of DEX. Conceivably, these 2 isoforms of TGF $\beta$  manifest different potentials for the chondrogenic differentiation of synovial cells, which may reflect different affinities for their receptors on the surface of these cells.

On the basis of our findings, BMP-2 and BMP-7 appear to have much higher chondrogenic potentials than TGF $\beta$ 1. However, long-term culturing in the presence of these 2 growth factors was associated with hypertrophic differentiation, which is a disadvantage.

Among the 3 growth factors, BMP-7 induced the formation of the largest volume of cartilaginous tissue. Even so, the volume fraction of metachromasia achieved after 6 weeks of culturing did not exceed  $41.6 \pm 5.1\%$  (mean  $\pm$  SEM). This limited response may be linked to a constraint of the culturing system itself, since cell and matrix differentiation tended to occur mainly in superficial regions of the explant, not in more centrally located ones. Furthermore, although neither BMP-2, BMP-7, nor TGF $\beta$ 1 promoted expression of the CI gene, the mRNA levels were not appreciably lowered after 6 weeks of culturing. Whether this persistent expression of the CI gene is confined to the undifferentiated central regions of the explants or also includes the superficial ones that have differentiated into cartilage has yet to be ascertained. But clearly, both the degree of tissue differentiation and the hyaline-like qualities of the cartilage formed must be improved.

In summary, we have demonstrated that BMP-2, BMP-7, and TGF $\beta$ 1 manifest different potentials for the chondrogenic differentiation of synovial explants and induce the formation of cartilaginous tissue with different characteristics. Neither BMP-2, BMP-7, nor TGF $\beta$ 1 alone is capable of inducing the complete differentiation

of synovial explants into a typical hyaline type of articular cartilage. Given that the physiologic formation of articular cartilage during skeletal development depends upon the concerted action of several growth factors (49,50), this finding is perhaps not surprising. To effect the full differentiation of synovial explants into hyaline articular cartilage, a further refinement of the stimulation conditions is needed, and this may be best achieved by the simultaneous application of several growth factors.

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## AUTHOR CONTRIBUTIONS

Dr. Shintani had full access to all of the data in this study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Study design.** Shintani, Hunziker.

**Acquisition of data.** Shintani.

**Analysis and interpretation of data.** Shintani, Hunziker.

**Manuscript preparation.** Shintani, Hunziker.

**Statistical analysis.** Shintani.

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