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Chronic Exposure of Bone Morphogenetic Protein-2 Favors Chondrogenic Expression in Human Articular Chondrocytes Amplified in Monolayer Cultures

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ABSTRACT

Articular cartilage is a specialized connective tissue containing chondrocytes embedded in a network of extracellular macromolecules such as type II collagen and presents poor capacity to self-repair. Autologous chondrocyte transplantation (ACT) is worldwide used for treatment of focal damage to articular cartilage. However, dedifferentiation of chondrocytes occurs during the long term culture necessary for mass cell production. The aim of this study was to investigate if addition of bone morphogenetic protein (BMP)-2, a strong inducer of chondrogenic expression, to human chondrocytes immediately after their isolation from cartilage, could help to maintain their chondrogenic phenotype in long-term culture conditions. Human articular chondrocytes were cultured according to the procedure used for ACT. Real-time PCR and Western blotting were performed to evaluate the cellular phenotype. Exogenous BMP-2 dramatically improves the chondrogenic character of knee articular chondrocytes amplified over two passages, as assessed by the BMP-2 stimulation on type II procollagen expression and synthesis. This study reveals that BMP-2 could potentially serve as a therapeutic agent for supporting the chondrogenic phenotype of human articular chondrocytes expanded in the conditions generally used for ACT. J. Cell. Biochem. 111: 1642–1651, 2010.

KEY WORDS: AUTOLOGOUS CHONDROCYTE TRANSPLANTATION; BONE MORPHOGENETIC PROTEIN-2; TYPE II COLLAGEN

Cartilage lesions have limited capacity for self-repair, often leading to osteoarthritis. Autologous Chondrocyte Transplantation (ACT) was the first application of cell therapy to orthopedic surgery [Brittberg et al., 1994] and is now widely used with various modifications for focal damage to articular cartilage treatment. Chondrocytes are isolated from a biopsy specimen, amplified for about 4–5 weeks in monolayer. The cell suspension is then transplanted onto the cartilage defect under a sealed cover. However, during their multiplication in culture through repeated passaging, chondrocytes loose their ability to produce cartilaginous macromolecules such as type II collagen and begin producing type I collagen, a well-known process called dedifferentiation.

Bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily that regulate a wide range of developmental processes, and control the differentiation of several musculoskeletal tissues including bone and cartilage. BMP-2 and -7 are already clinically used for bone regeneration [Geesink et al., 1999; Groeneveld and Burger, 2000; Friedlaender et al., 2001; Boden et al., 2002; Baskin

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et al., 2003; Burkus et al., 2003; Granjeiro et al., 2005]. Besides, several BMPs, including BMP-2, -4, -6, -7, -9, and -13, stimulate cartilage matrix macromolecules synthesis by articular chondrocytes [Flechtenmacher et al., 1996; Gooch et al., 2002; Blunk et al., 2003; Bobacz et al., 2003; Lin et al., 2008], revealing their potential to promote cartilage repair. In this context, we recently showed that BMP-2 can reactivate the program of chondrogenic expression in dedifferentiated human nasal chondrocytes [Hautier et al., 2008]. However, a progressive loss of chondrogenic responsiveness to BMP-2 was observed after passaging [Hautier et al., 2008]. This prompted us to investigate if addition of BMP-2 to human chondrocytes, immediately after their isolation, could help to maintain their chondrogenic potential in long-term culture conditions used for mass cell production necessary for ACT. Since chondrocytes from different origin may behave differently in term of gene expression or protein synthesis in response to growth factors, we used here human chondrocytes isolated from knee joint. Chondrogenic expression was evaluated at the gene and protein level.

MATERIALS AND METHODS

ANTIBODIES

Polyclonal rabbit antibodies to human type II collagen were from Novotec (Ref. 20211), and to Sox9 from Millipore (Ref AB5535). Other antibodies were as follows: anti-actin, Sigma (A5060); Cy2-conjugated secondary antibodies, Jackson ImmunoResearch Laboratories; alkaline phosphatase-conjugated anti-rabbit IgG, Bio-Rad (170-6518).

CELL CULTURE

Primary cultures of human chondrocytes were prepared from macroscopically healthy zones of osteoarthritic knee joints obtained from 14 donors (age range: 51–79, average: 69 years) undergoing total knee replacement. The study was carried out in full accordance with local ethics guidelines and cartilage samples were collected after obtaining written informed consent of the donors. Chondrocytes were extracted as previously described [Hautier et al., 2008]. Briefly, cartilage small slices were sequentially digested with 0.2% trypsin (Sigma) followed by 0.06% bacterial collagenase A (Roche Applied Science) then seeded at a density of 1.5×10^4 cells/cm^2 in culture medium consisting of DMEM/Ham's F12 (Gibco-Invitrogen) supplemented with 10% Newborn Calf Serum (NCS) (Hyclone), 50 μg/ml streptomycin (Panpharma) and 2 μg/ml amphotericin B (Bristol Myers Squibb). At this stage, cells were designated P0. At the end of each passage, cells were detached with trypsin-ethylenediamine tetraacetic acid (Sigma) and replated at the same density for two other passages (P1–P2). Dose–response experiments were performed with chondrocytes obtained from donors 1–4, of which cells were passaged every 6 days. Cells were not confluent at these time points. Cells obtained from donors 5 to 14 were allowed to reach confluence before trypsinization (10–15 days for P0, 7–10 days for P1 and P2), to mimic the cell amplification procedure used in our clinical trial of ACT at Lyon Sud Hospital (Centre Hospitalier Lyon Sud, Hospices Civils de Lyon, CHLS, HCL) [Piperno et al., 2005]. Culture medium containing or not BMP-2 was replaced three times a week.

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNAs from cultured cells were extracted and Reverse Transcribed as previously described [Valcourt et al., 2002].

Conventional PCR amplifications using 10 μl RT aliquots were performed in a PCR Thermal Cycler MyCycler^{TM} (Bio-Rad) as previously described [Valcourt et al., 2003]. Different type II isoforms transcripts were amplified using primers designed as previously described [Hautier et al., 2008] (Table I). *Glyceraldehyde-3-phosphate dehydrogenase* (GAPDH) was used for gene expression normalization. Amplicons were purified with Nucleospin Extract II (Macherey-Nagel) and subcloned in pCRII-TOPO (Invitrogen) for subsequent sequencing. To reveal potential secondary structures, amplicons were incubated with Mung Bean Nuclease (New England Biolabs) according to manufacturer's instructions, to cleave potential single strand hairpin loops.

Real-time PCR amplifications were performed as previously described [Hautier et al., 2008] (Table II). *GAPDH* C_t value was substracted from the target sequence C_t value to obtain ΔC_t. The level of expression was then calculated as $2^{-\Delta Ct}$ and expressed as the mean of triplicate samples.

IMMUNOFLUORESCENCE

Cell cultures were treated as previously described [Valcourt et al., 2003]. Incubation with type II collagen antibodies was followed by incubation with Cy2-conjugated secondary antibodies. Coverslips were mounted for observation by epifluorescence with an axioplan 2 Zeiss microscope equiped with a CoolSNAP Fx camera (Roper Scientific). Image acquisition was achieved with Metaview software (Universal Imaging).

TABLE I. Oligonucleotide Primers Used for the Conventional PCR Analyses

Gene	Primers	Strand	Product size (bp)	T (°C)	PCR cycles	Refs.
COL2A1 (total form)	TGGAGAAACCATCAATGGTGG	+	481	52	30	NM_001844
	GATTGGAAAGTACTTGGGTCC	−				
COL2A1 (IIA and IIB forms)	GCCCCGCGGTGAGCCATGATT	+	IIA: 475	53	30	NM_001844
	CTCCATCTCTGCCACGAGGTC	−	IIB: 268			
GAPDH	ATCACTGCCACCCAGAAGAC	+	443	57	25	Valcourt et al. [1999]
	ATGAGGTCCACCACCCTGTT	−				

Primers are presented in a 5′–3′ orientation, with that for the forward strand (+) being above that for the reverse strand (−). The amplicon size is indicated, together with the annealing temperature and the number of PCR cycles used for each primer set. The source of the databank used for designing the primers is presented as an accession number. When primers have been used in other studies, the references are indicated.

TABLE II. Oligonucleotide Primers Used for the Real-Time PCR Analyses

Gene	Primers	Strand	Product size (bp)	Refs.
COL2A1 (total form)	GGCAATAGCAGGTTCACGTACA	+	79	Martin et al. [2001]
	CGATAACAGTCTTGCCCCACTT	−		
AGGRECAN	TCGAGGACAGCGAGGCC	+	85	Martin et al. [2001]
	TCGAGGGTGTAGCGTGTAGAGA	−		
SOX9	ACGCCGAGCTCAGCAAGA	+	71	Park et al. [2005]
	CACGAACGGCCGCTTCT	−		
OSTEOCALCIN	TCACACTCCTCGCCCTATTGG	+	83	NM_199173
	TTCGTCCTCCCGTCGCTCCAT	−		
COL1A1	CAGCCGCTTCACCTACAGC	+	83	Martin et al. [2001]
	TTTTGTATTCAATCACTGTCTTGCC	−		
COL10A1	CAAGGCACCATCTCCAGGAA	+	70	Martin et al. [2001]
	AAAGGGTATTTGTGGCAGCATATT	−		
GAPDH	ATGGGGAAGGTGAAGGTCG	+	70	Martin et al. [2001]
	TAAAAGCAGCCCTGGTGACC	−		

Primers are presented in a 5′–3′ orientation, with that for the forward strand (+) being above that for the reverse strand (−). The product size generated by reverse transcription and PCR amplification of the mRNA is indicated. The source of the data bank used for designing the primers is presented as an accession number. When primers have been designed and used in other studies, the references are indicated.

WESTERN-BLOT ANALYSIS

Cells were processed as described previously [Valcourt et al., 2003]. Equivalent amounts of proteins were separated on 4–12% gradient SDS–polyacrylamide gels. After transfer, membranes were probed with antibodies to collagen II, washed, and incubated with alkaline phosphatase-conjugated IgG. After multiple washes, bound antibodies were detected on X-ray films using a Immun-star AP chemiluminescent substrate (Bio-Rad). Membranes were re-probed with antibodies to actin after striping (Re-Blot Plus Strong, Chemicon).

STATISTICAL ANALYSIS

Because of important inter-individual differences in the transcripts expression level, qPCR experiments data showing relative mRNA levels were first reported as column scatter graphs (Figs. 2–4, left panels). Then BMP-2 induction effect on gene expression was calculated in comparison with P0 chondrocytes analyzed 36 h after their seeding (reference value = 1) (Figs. 2–4, right panels). In this case, induction factors were reported as means ± SEM and analyzed with Mann–Whitney *U*-test ($P < 0.05$ was considered as significant). The SEM represents the standard deviation resulting from the analysis of donors 5–12 and is represented as error bar in log form in Figures 2–4, right panels.

RESULTS

EFFECT OF BMP-2 ON GENE EXPRESSION IN HACS EXPANDED IN MONOLAYER CULTURES

When freshly isolated human articular chondrocytes (HACs) were examined by immunofluorescence, they were all positively stained for type II collagen with variable intensities of labeling, attesting their differentiated character (Fig. 1). In a first series of experiments aimed to examine BMP-2 dose–response, cells were expanded for 6 days at each passage with 10% NCS supplemented with 0, 25, 50,

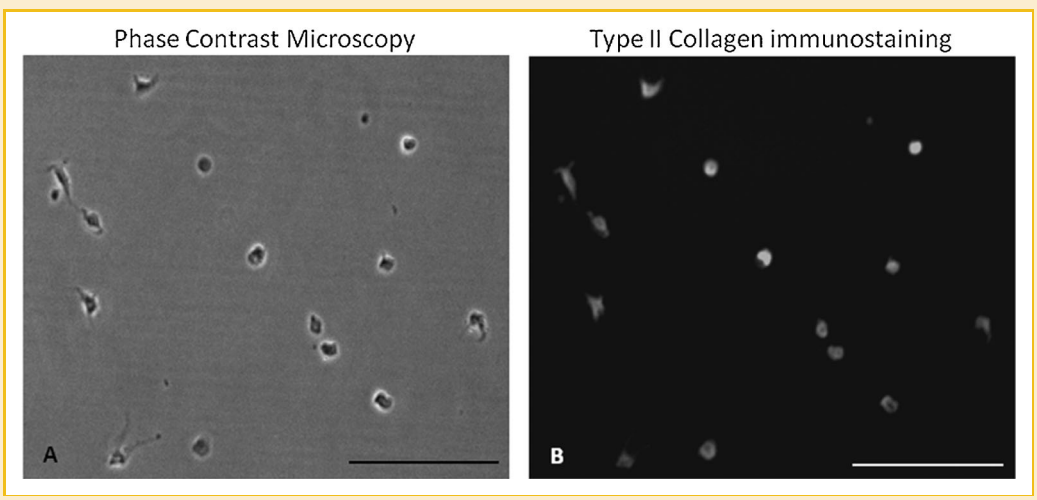

Fig. 1. Characterization of human articular chondrocytes. A: Morphology of P0 chondrocytes seeded on glass coverslip for 36 h. Note that most cells show a rounded shape. B: Immunostaining of the P0 chondrocytes seen in (A), using an anti-type II collagen antibody. These views were obtained from donor 14 and are representative of chondrocytes isolated from donors 1–14. Scale bar: 100 μm.

100, or 200 ng/ml BMP-2. Cells were not confluent at these time points and these culture conditions were chosen to examine the effect of BMP-2 independently of the one of high cell density already known to favor expression of the chondrocyte phenotype. Beside *COL2A1* and *AGGRECAN* coding for the two major proteins of cartilage, we also monitored the expression of *SOX9*, coding for a transcription factor essential for *COL2A1* and *AGGRECAN* expression regulation [Lefebvre et al., 1997]. From P0 to P2, serial expansion resulted in progressive loss of *COL2A1* in control cultures whereas *AGGRECAN* and *SOX9* expressions remained relatively stable. Interestingly, these expressions were increased to a higher level in the presence of BMP-2, from P0 to P2, with a dose-dependent effect (data not shown). We further analyzed BMP-2 responsiveness of HACs by treating the cell cultures with the selected concentrations of 100 or 200 ng/ml of BMP-2. In addition, at every passage, cells were allowed to reach confluence to mimic at best the cell amplification procedure used in our clinical trial of ACT at Lyon Sud Hospital. First, gene expression analysis of separately expanded HACs isolated from eight donors was performed to quantify the relative abundance of the transcripts mentioned above. Again, serial expansion from P0 to P2 resulted in a progressive loss of *COL2A1* mRNA expression in control cultures whereas *AGGRECAN* and *SOX9* levels remained stable. These expressions were increased to a higher level in the presence of the two different BMP-2 concentrations, globally with a dose-dependent effect (Fig. 2). More precisely, when the levels of these transcripts in P2

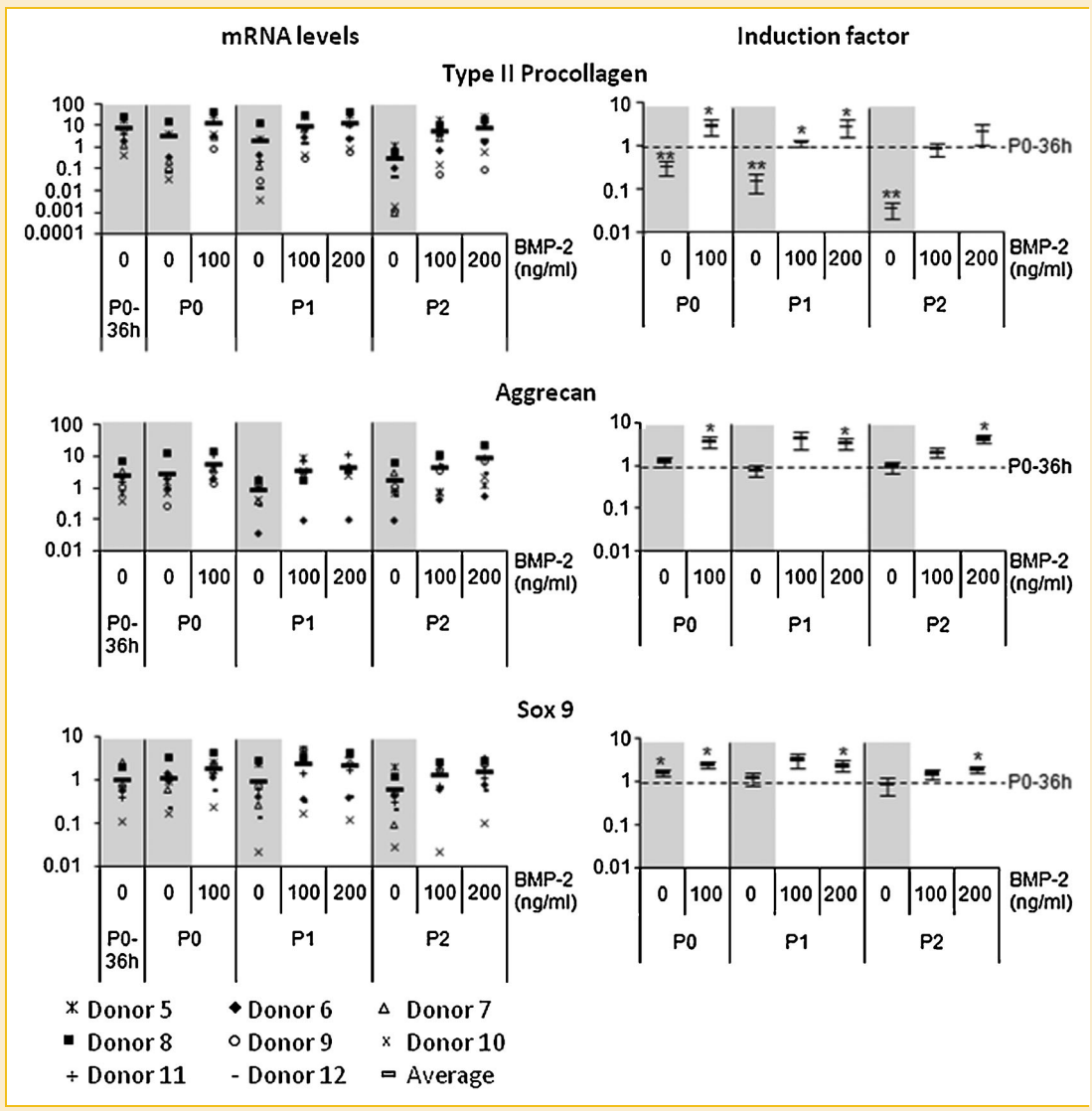

Fig. 2. BMP-2 stimulates expression of marker genes for the articular chondrocyte phenotype. Real-time PCR analyses were performed to measure levels of mRNA and the corresponding gene products are indicated. Chondrocytes were obtained from donors 5 to 12 and grown separately until confluence in the presence of 10% NCS (gray background) or of 10% NCS supplemented with 100 or 200 ng/ml BMP-2, as indicated. On the left are shown mRNA levels in P0–P2 chondrocytes. On the right, BMP-2 induction effect is expressed relative to P0 chondrocytes 36 h after their isolation (reference value = 1). In this case, values of donors 5–12 were pooled and induction factors are reported as means (horizontal bars) ± SEM. P0-36 h: P0 chondrocytes cultured for 36 h in the presence of 10% NCS only. $^{*}P < 0.05$, $^{**}P < 0.01$. The cDNA amount obtained for each marker was normalized to the amount of GAPDH cDNA.

cultures were compared to those of P0 chondrocytes 36 h after their isolation, the average expression level in P2 chondrocytes treated with 200 ng/ml BMP-2 remained higher than the average level observed in P0–36 h chondrocytes (two times higher for COL2A1 and SOX9 and four times higher for AGGRECAN, see Fig. 2). BMP-2 is known to enhance not only chondrogenic differentiation, but also hypertrophic maturation of chondrocytes and osteogenic differentiation in several cell types, which is a critical point for cartilage cell therapy. Therefore we monitored expression of genes coding for type X collagen (*COL10A1*), a characteristic marker of hypertrophic chondrocytes [Schmid and Linsenmayer, 1985] and for osteocalcin (*OSTEOCALCIN*), a bone-specific marker [Hauschka et al., 1989]. Expressions of *COL10A1* and *OSTEOCALCIN* were barely, if not detected in the cultures and BMP-2 showed modest modulatory effect on these gene's expression (Fig. 3). Regarding *COL1A1*, coding for type I procollagen α1 chain, subculturing led to *COL1A1* expression increase, a well-known phenomenom associated with chondrocyte dedifferentiation in vitro. Addition of BMP-2 had no effect on this expression in P0 chondrocytes and a weak stimulatory effect in P1 and P2 chondrocytes (about 1.5-fold in the presence of 200 ng/ml BMP-2 when compared to untreated chondrocytes, see Fig. 3). Importantly, the ratio of type II/type I procollagen mRNA levels, a differentiation index for chondrocytes [Marlovits et al., 2004], now widely used to monitor the differentiation status of

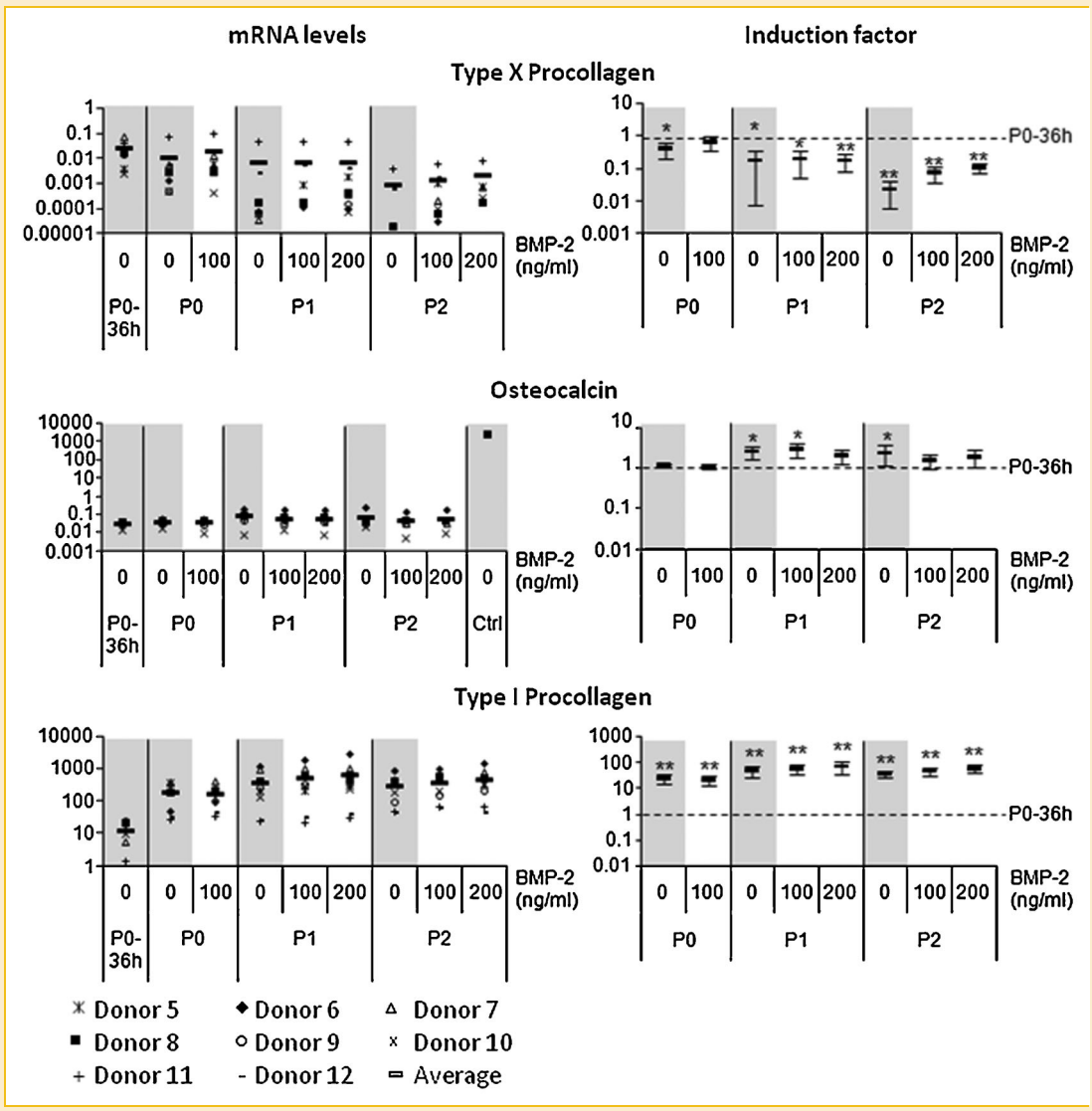

Fig. 3. BMP-2 weakly affects non-characteristic chondrogenic gene expression. Real-time PCR analyses were performed to measure levels of mRNA and the corresponding gene products are indicated. Type X procollagen, osteocalcin and type I procollagen are markers of the hypertrophic chondrocytes, osteoblasts, and dedifferentiated chondrocytes, respectively. Chondrocytes were obtained from donors 5 to 12 and grown separately until confluence in the presence of 10% NCS (gray background) or of 10% NCS supplemented with 100 or 200 ng/ml BMP-2, as indicated. On the left are shown mRNA levels in P0–P2 chondrocytes. Osteocalcin mRNA expression in human osteoblastic cells is used as a reference value (Control: Ctrl). On the right, BMP-2 induction effect is expressed relative to P0 chondrocytes 36 h after their isolation (reference value = 1). In this case, values of donors 5–12 were pooled and induction factors are reported as means (horizontal bars) ± SEM. P0–36 h: P0 chondrocytes cultured for 36 h in the presence of 10% NCS only. $^{*}P < 0.05$, $^{**}P < 0.01$. The cDNA amount obtained for each marker was normalized to the amount of GAPDH cDNA.

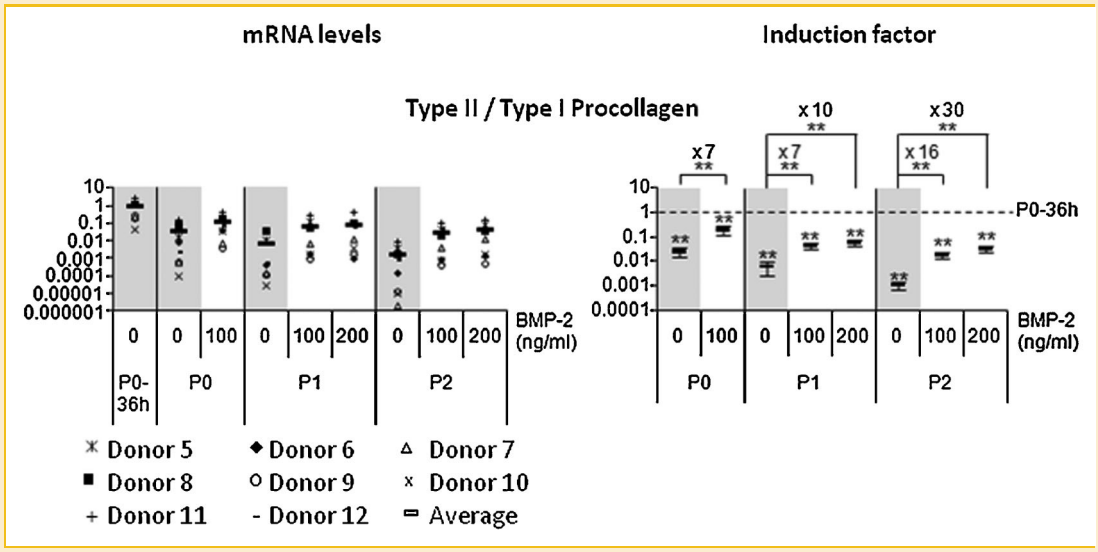

Fig. 4. BMP-2 favors the ratio of type II/type I procollagen gene expression in human articular chondrocytes expanded in monolayer. On the left, the ratio of type II/type I mRNA level is expressed using values of type II procollagen and type I procollagen mRNA levels as reported in Figures 2 and 3, respectively. On the right, BMP-2 induction effect on this ratio is expressed relative to P0 chondrocytes 36 h after their isolation (reference value = 1). In this case, values of ratios of type II/type I mRNA levels of donors 5–12 were pooled and induction factors are reported as means (horizontal bars) ± SEM. In addition and for each passage, the fold increase of the induction factors in the presence of BMP-2, relative to the untreated cultures, are also indicated. P0–36 h: P0 chondrocytes cultured for 36 h in the presence of 10% NCS only. $^{**}P < 0.01$.

human chondrocytes in the diverse ACT protocols, was on average significantly higher in the presence of BMP-2, from P0 to P2 (Fig. 4). Taken together, these first data showed that maintaining subcultures to confluence in the presence of BMP-2 prove to be a beneficial factor for the chondrogenic status of HACs expanded in vitro.

In addition, we analyzed more precisely BMP-2 effect on alternatively spliced isoforms of *COL2A1* mRNAs. This alternative splicing is developmentally regulated during chondrogenesis and reflects the status of chondrocyte differentiation. Exon 2 can be included or excluded from mature mRNA producing either type IIA procollagen (with exon 2) in chondroprogenitor cells or type IIB procollagen (without exon 2) in differentiated chondrocytes [Ryan and Sandell, 1990]. Very recently, expression of two other alternatively spliced *COL2A1* isoforms (IIC and IID) have been revealed during chondrogenic differentiation of bone marrow-derived mesenchymal stem cells (MSCs) [McAlinden et al., 2008]. In this latter study, steady-state levels of IIC mRNA during chondrogenic differentiation of MSCs were very low whereas the IID transcript was shown to be co-expressed with the IIA isoform during in vitro differentiation of human MSCs and to be about one-third as abundant as the IIA isoform. Although the role of the IID isoform remains to be determined, it represents an early marker of chondrogenic conversion of MSCs [McAlinden et al., 2008]. Firstly, by using primers spanning exons 1–7 and conventional RT-PCR to follow expression of the IIA and IIB isoforms, we found that type IIB mRNA was the only spliced variant expressed in human articular cartilage samples, as expected from well-differentiated chondrocytes (data not shown). However, once the chondrocytes were plated on culture dishes and subsequently subcultured, we observed two bands at the position where IIA was expected to migrate in addition to the band corresponding to IIB. No band corresponding to IIC was detected (Fig. 5 and supplemental Fig. 1A). Using cloning and sequencing approaches, we confirmed that the lower band corresponds to type IIB cDNA sequence, while each of the two upper bands corresponds to a mixture of both type IIA and IID cDNA sequences (sequencing data not shown). Regarding the fact that only three additional bases sign the difference between IIA and IID sequences [McAlinden et al., 2008], the upper doublet might well represent a conformational heteroduplex PCR product, as already suggested in a study of temporal differences in expression of *COL2A1* alternative isoforms during chondrogenesis of MSC in vitro [McAlinden et al., 2008]. To prove the existence of such an heteroduplex, we used the Mung Bean Nuclease which is a single-strand-specific nuclease, also able to degrade hairpin loops. After Mung Bean Nuclease digestion of the PCR products, the lower band, corresponding to IIB, was not affected by the digestion, whereas the upper doublet band was reduced to a single band (supplemental Fig. 1B), which is in support of the existence of an IIA/IID heteroduplex containing either mismatched IIA and IID cDNA strands with single-stranded extensions, or a hairpin fold or other secondary structure. Although BMP-2 stimulated global type II procollagen expression in accordance with our real time PCR results, there was no obvious BMP-2 effect on the IIA, IID, and IIB isoforms relative expressions (Fig. 5 and supplemental Fig. 1A).

The responsiveness of the chondrocytes to BMP-2 could also be observed at the morphological level for the cells expressing the highest levels of chondrogenic markers, like those obtained from donor 5: cells cultured in the presence of serum alone became fibroblastic whereas those treated with BMP-2 became polygonal, a morphological feature often seen with embryonic chondrocytes showing high chondrogenic expression [Mallein-Gerin and Olsen, 1993]. This morphological difference was particularly marked in P1 chondrocytes (Fig. 6). It is known for a long time that, in monolayer culture, differentiated chondrocytes display a rounded or polygonal

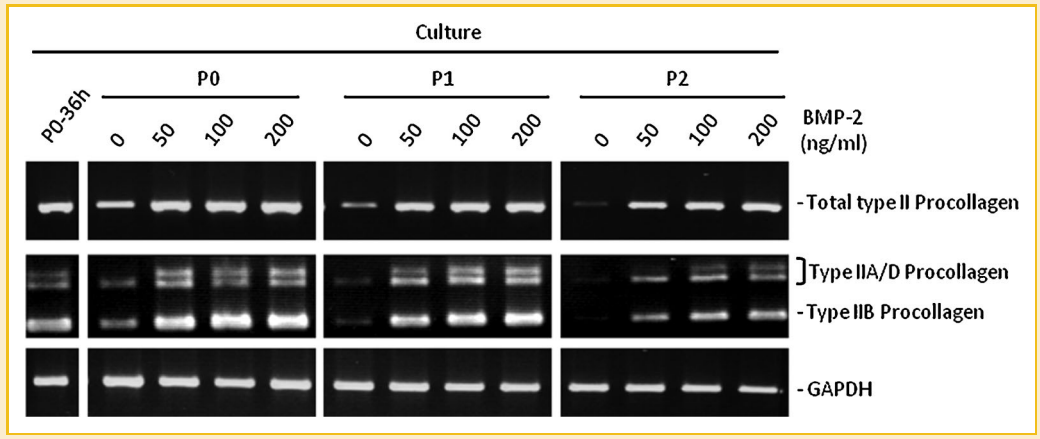

Fig. 5. BMP-2 stimulates expression of the alternatively spliced IIA/IID and IIB isoforms of the type II procollagen gene. mRNA expressions of total type II procollagen and types IIA/D and IIB procollagen isoforms obtained from donor 10. At each passage, cells were cultured until confluence in the presence of 10% NCS only (0) or of 10% NCS supplemented with 50–200 ng/ml BMP-2, as indicated. The conditions used for the conventional PCR analyses shown here were designed to detect either total type II procollagen expression (on the top) or expression of the IIA/IID and IIB isoforms (at the bottom). P0-36 h: P0 chondrocytes cultured for 36 h in the presence of 10% NCS only. At the bottom, GAPDH expression is shown as a loading control.

morphology whereas dedifferentiated chondrocytes look fibroblastic. However, the underlying mechanisms have just started to be unraveled at the molecular level. A recent study has demonstrated that dedifferentiation into a fibroblast morphology results from a profound induction of RhoA protein and stress fibers [Kumar and Lassar, 2009]. Very importantly, RhoA signaling and actin polymerization have been shown to block the function of Sox9 [Kumar and Lassar, 2009]. Thus it is tempting to speculate that in our study BMP-2 prevents dedifferentiation of HACs to fibroblast-like cells by, at least in part, controlling actin organization.

BMP-2 SUSTAINS TYPE II COLLAGEN SYNTHESIS IN HACS EXPANDED IN MONOLAYER CULTURES

We next evaluated the chondrogenic effect of BMP-2 at the protein level. Western-blotting analysis revealed that BMP-2 favored type II collagen synthesis in BMP-2-treated chondrocytes compared to untreated cells, from P0 to P2 and in a dose-dependent manner (Fig. 7). Importantly, the level of type II collagen synthesis in P2 chondrocytes treated with 200 ng/ml BMP-2 remained close to the one observed in freshly isolated P0 chondrocytes (Fig. 7). This result indicated that BMP-2 was able to maintain type II collagen synthesis during expansion of HACs in vitro and was in concordance with the profile of *COL2A1* mRNA expression obtained by real-time PCR (Fig. 2).

DISCUSSION

The success of ACT correlates with the capacity of transplanted chondrocytes to produce a cartilaginous matrix. In this view, we evaluated the ability of HACs to maintain their chondrogenic properties by adding BMP-2 during their amplification in vitro. The focus on BMP-2 to support cartilage repair is based on recent research on its role in articular cartilage development, homeostasis, and repair. For instance, genetic disruption of the BMP receptor IA in mouse results in postnatal articular cartilage degeneration [Rountree et al., 2004]. Besides, BMP-2 is increased following articular

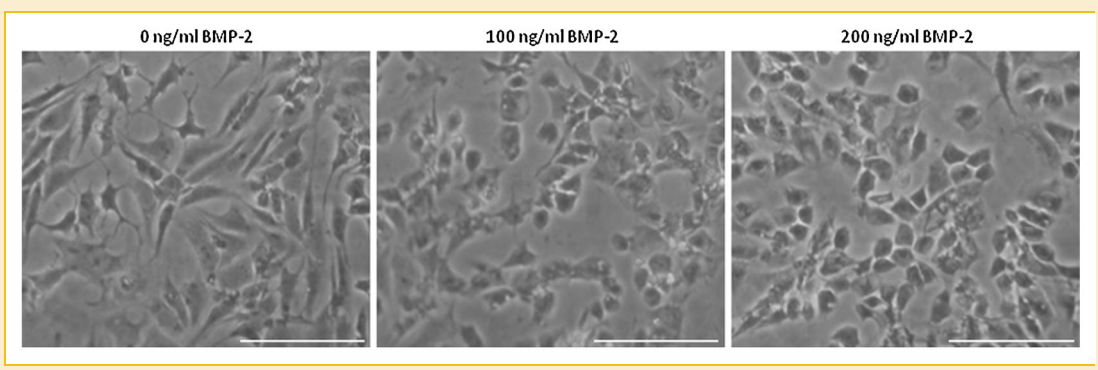

Fig. 6. Effect of BMP-2 on the shape of human chondrocytes expanded in monolayer. Morphology of P1 chondrocytes isolated from donor 5 and continuously cultured in the presence of 10% NCS only (0 ng/ml BMP-2) or of 10% NCS supplemented with 100 or 200 ng/ml BMP-2, as indicated. Note that the presence of BMP-2 supports polygonal morphology of the cells in a dose-dependent manner. Scale bar: 100 μm.

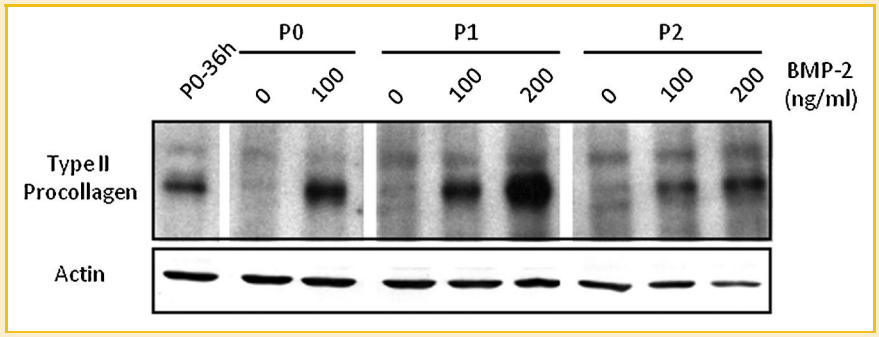

Fig. 7. BMP-2 sustains type II procollagen synthesis in human articular chondrocytes expanded in monolayer. Western-blotting analysis of type II procollagen synthesis in P0–P2 chondrocytes obtained from donor 14. At each passage, cells were cultured until confluence in the presence of 10% NCS only (0) or supplemented with 100 or 200 ng/ml BMP-2, as indicated. At the bottom, immunoblotting for actin is shown as a loading control. This result is representative of chondrocytes isolated from three donars.

cartilage damage and is also up-regulated by cytokines that provoke cartilage degradation in osteoarthritis [Fukui et al., 2003, 2006], suggesting therefore a role in homeostasis and repair [Dell'Accio et al., 2006]. Recently, we showed that BMP-2 can stimulate chondrogenic expression in dedifferentiated human nasal chondrocytes, but a progressive decrease in BMP-2 responsiveness was observed after subculture [Hautier et al., 2008]. Thus, the experiments presented in this study were carried out to determine whether culture of HACs in the continuous presence of BMP-2 right from the isolation time could improve maintenance of the chondrocytic phenotype.

Our results indicate that human chondrocytes grown under Good Manufacturing Practice (French Health Agency Afssaps no. TCG/07/M/001) conditions suitable for ACT are responsive to BMP-2. Our data are in agreement with previous reports showing that exogenous BMP-2 prevents or delays dedifferentiation of equine or bovine articular chondrocytes cultured in monolayer in the presence of 10% bovine serum [Sailor et al., 1996; Stewart et al., 2000]. However, our data contrasts with the report of Grunder et al. [2004] showing no significant improvement of the chondrocytic phenotype through addition of 100 ng/ml BMP-2 on HACs expanded in monolayer in the presence of 10% human serum. In this latter study, freshly isolated chondrocytes (P0) were first expanded in monolayer for 7–10 days to reach confluence without addition of exogenous growth factor, then BMP-2 was added in P1 and P2 subcultures for 2 weeks [Grunder et al., 2004]. Thus, difference in the nature of serum and/or in the duration of BMP-2 treatment could explain this discrepancy. In this line, a recent study has shown a biphasic effect of BMP-2 in long-term cultures (8 weeks) of rabbit articular chondrocytes [Krawczak et al., 2009]. When added to the cultures grown with 15% bovine serum, BMP-2 enhances chondrogenic expression and deposition of cartilage-specific molecules for up to 3 weeks, then stimulates cartilage-degradative enzymes and alters cartilage matrix deposition [Krawczak et al., 2009]. Most likely, the effects of BMP-2 on chondrocyte monolayer cultures are dependent on the specifics of the culture conditions and cellular model systems.

In cellular models of the present study, chronic exposure of BMP-2 favors chondrogenic expression, at the gene and protein level. The clear dose effect of BMP-2 observed in the cultures grown for 6 days or to confluence confirms that BMP-2 was effective in both of our culture conditions. Moreover, the differential effects of BMP-2 on chondrogenic and non-chondrogenic gene expression reveals a beneficial use of BMP-2 in already existing ACT protocols since, importantly, there was no sign of hypertrophic maturation or osteogenic induction in our cultures. Besides, the level of *COL1A1* expression triggered by cell amplification was very modestly enhanced by BMP-2. These results are consistent with the absence of BMP-2 effect on *COL10A1*, *OSTEOCALCIN*, and *COL1A2* expressions noted in bovine or equine articular chondrocytes cultured in monolayer [Sailor et al., 1996; Stewart et al., 2000]. They are also in line with our previous study where the chondrogenic potential of BMP-2 was challenged after dedifferentiation of human nasal chondrocytes and where no sign of hypertrophic maturation, osteogenic induction and stimulation of *COL1A1* expression was observed [Hautier et al., 2008]. Very importantly, in comparison with this latter study, the present study reveals that addition of exogenous BMP-2 better sustains the chondrogenic potential of human chondrocytes in long-term culture when added immediately after cells isolation not only at the mRNA but also at the protein level. This last feature is important with regard of ACT where functional chondrocytes are needed to build a cartilage ECM.

Besides, our study reveals for the first time that HACs expanded in monolayer express the alternatively spliced IID isoform of *COL2A1*, described until now only in MSCs undergoing chondrogenic differentiation [McAlinden et al., 2008]. The exact role of this particular isoform is not yet unraveled but it is interesting to note that chondrogenic differentiation of human MSCs indicated a switch in the expression of the IIA, IIB, IIC, and IID isoforms, showing predominantly IIC initially, when IIB is less abundant, followed by IIA/D and IIB [McAlinden et al., 2008]. In this regard, our conventional PCR analysis showing here the presence of IIA/IIB/IID isoforms from P0 to P2 chondrocytes indicates that the chondrogenic character of the cells is maintained during their amplification. Since our conventional PCR was not quantitative, it is difficult to judge in this study if there was an influence of BMP-2 on the ratio of these isoforms with time in culture.

Sox transcription factors are most likely other important downstream mediators of BMP-2 signaling in the context of the study presented here. In a previous study designed to evaluate the chondrogenic potential of BMP-2 on dedifferentiated human

nasal chondrocytes, we showed that BMP-2 acts by enhancing DNA-binding of Sox transcription factors to the cartilage-specific enhancer of the *COL2A1* gene [Hautier et al., 2008]. Here, we did not examine the effect of BMP-2 on DNA-binding of the Sox factors but our Western-blot analysis has revealed induction of Sox9 protein in response to BMP-2 (data not shown). In the same vein, it is interesting to note that induction of chondrogenesis by addition of BMP-2 in the pluripotent mesenchymal cell line C3H10T1/2 was accompanied by induction of Sox6 protein [Fernandez-Lloris et al., 2003]. More precisely, higher expression of Sox6 triggered by BMP-2 was found to correlate with higher Sox6 function, such as higher binding of Sox6 to the *COL2A1* enhancer [Fernandez-Lloris et al., 2003]. Altogether, these observations support the view where Sox transcription factors regulate *COL2A1* expression in response to BMP-2 in HACs.

Although it has already been shown that BMP-2 can act as a chondrogenic factor, this study is the first to reveal that BMP-2 could serve as a therapeutic agent for the support of HACs expanded in conditions generally used for ACT. Importantly, the level of *COL1A1* expression was not particularly enhanced by BMP-2 treatment and this result is in accordance with the study of Stewart et al. [2000], in which BMP-2 does not affect the expression of another procollagen gene (*COL1A2*) in equine articular chondrocytes cultured in monolayer. Thus, the next challenge is to turn off the expression of type I procollagen in this context of cartilage therapy. The use of three-dimensional (3D) matrices might be helpful, since a 3D environment is known to favor redifferentiation of dedifferentiated human chondrocytes. For instance, encapsulation of dedifferentiated HACs in alginate beads helps to restore the differentiated phenotype while reducing expression of type I procollagen [Bonaventure et al., 1994; Murphy and Sambanis, 2001; Murphy and Polak, 2004]. Following this line, it is now widely accepted by the health agencies that survey ACT in several countries, that the method remains perfectible by using growth factors and biomaterials, particularly to extend the technique to developing osteoarthritic lesions. Thus, we will pursue our efforts to evaluate the potential of BMP-2 as therapeutic agent for cartilage repair in combination with collagen-based biomaterials. We have shown recently that collagen sponges represent a suitable material for tissue engineering of cartilage [Ronziere et al., 2005; Cortial et al., 2006; Freyria et al., 2009]. In this context, it is important to note that BMP-2 and collagen sponges have already been approved for clinical use.

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