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FAST TRACK

Bone Morphogenetic Protein-2 Enhances Osterix Gene Expression in Chondrocytes

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Abstract Osterix is a recently identified zinc-finger-containing transcription factor, which is required for skeletogenesis as no bone formation was observed in osterix-deficient mice. Osterix was first cloned as a gene whose expression was enhanced by BMP in C2C12 cells. As BMP induces ectopic bone formation in vivo via a pathway reminiscent to endochondral bone formation, BMP may also regulate osterix gene expression in chondrocytes. However, no information was available regarding the BMP actions on osterix gene expression in chondrocytes. We therefore examined the effects of BMP-2 on osterix gene expression in chondrocytes in culture. RT-PCR analysis indicated that osterix mRNA was expressed in the primary cultures of chondrocytes derived from mouse rib cartilage. The treatment with BMP-2 enhanced the levels of osterix transcripts within 24 h and the enhancement was still observed at 48 h based on RT-PCR analysis. This BMP effect was specific to this cytokine, as TGF-β did not alter osterix gene expression. BMP effects on the osterix mRNA levels were also confirmed by Northern blot analysis. The enhancing effect of BMP on osterix gene expression was observed in a dose-dependent manner starting at 200 ng/ml. The BMP enhancement of the osterix gene expression in chondrocytes was blocked in the presence of a protein synthesis inhibitor, cycloheximide, while it was still observed in the presence of 5,6-dichloro-1-β D-ribofuranosylbenzimidazol (DRB) suggesting the involvement of post-transcriptional events, which require new protein synthesis. These results indicated that osterix gene is expressed in the primary cultures of chondrocytes and its expression is under the control of BMP-2. J. Cell. Biochem. 88: 1077–1083, 2003.

Key words: chondrocytes; BMP; osterix; gene expression

Determination of the cell fate by transcription factors is under the control of cytokines and hormones, which coordinately regulate bone formation in the body [Lee et al., 2000; Murakami et al., 2000]. Among these cytokines and hormones, BMP has been known to be a potent inducer of bone formation via its action to stimulate differentiation of mesenchymal cells to become osteoblastic cells [Katagiri et al., 1994]. BMP was first described by Urist for its activity to induce ectopic bone formation [Urist, 1965, 2002], and subsequently genes encoding BMPs were cloned by Wozney et al. [1988]. In the experiments using either purified BMP proteins or recombinant BMP proteins, ectopic bone formation can be observed in a manner reminiscent to endochondral bone formation. Thus, BMP activates differentiation of mesenchymal cells into chondrocytes and also subsequently promotes their differentiation into osteoblasts in vivo. In terms of in vitro cell data, BMP has been reported to accelerate differentiation of osteoblasts in monolayer cultures [Rickard et al., 1994] and chondrocytes in micromass cultures [Atkinson et al., 1997]. In other experiments, BMP-2 was observed to enhance hypertrophic chondrocyte differentiation [Venezian et al., 1998] and stimulate expression

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of genes encoding Ihh, PTHrP, type X collagen, and osteocalcin, which are the phenotypic markers of these cells [Kameda et al., 2000].

BMP activation of genes encoding phenotypic markers of chondrocytes suggests that BMP would enhance the expression of certain sets of transcription factors, which are in charge to regulate the expression of the downstream phenotypic genes. One of such transcription factors, which are under the control of BMP, is osterix [Nakashima et al., 2002]. Osterix is a transcription factor containing a zinc finger domain at its C-terminal end [Nakashima et al., 2002]. Osterix was identified based on the subtractive screening of the genes whose expression was enhanced by the treatment with BMP-2 in C2C12 cells [Nakashima et al., 2002]. Knockout experiment indicated that osterix is prerequisite for osteoblast differentiation as mice lacking osterix fail to develop bone in their body [Nakashima et al., 2002]. The phenotypes observed in these osterix knockout mice were similar to those in Cbfa1 knockout mice [Komori et al., 1997]. However in the osterix knockout mice Cbfa1 gene was expressed without major alterations while no osterix expression was observed in Cbfa1 knockout mice, suggesting that osterix lies downstream to Cbfa1 [Komori et al., 1997].

Since BMP-2 induces bone formation in ectopic sites through the endochondral bone formation, we examined whether osterix expression in chondrocytes is also regulated by BMP-2. We found that osterix gene was expressed in murine rib cartilage-derived chondrocytes in culture and BMP-2 treatment enhanced the expression of osterix in these cells.

MATERIALS AND METHODS

Cell Cultures

Chondrocytes were isolated from rib cartilage of 1-day-old ICR mice as described previously [Lefebvre et al., 1994]. Briefly, cartilaginous rib cages were pre-incubated in 3 mg/ml collagenase (Sigma, St. Louis, MO) in DMEM (GIBCO BRL, NY) for 45 min at 37°C and rinsed with PBS. They were further incubated in 3 mg/ml collagenase in DMEM at 37°C for 5 h and undigested parts were discarded. Chondrocytes were collected and rinsed with medium twice before they were seeded at 1.0×10^5 cells/cm^2 in tissue culture plates and the cells were grown in DMEM supplemented with antibiotics (100 U/ml penicillin G sodium, 0.25 μg/ml amphotericin B, and 100 μg/ml streptomycin sulfate) and 10% FBS (GIBCO BRL). These chondrocytes in cultures were treated with 200, 400, and 800 ng/ml human recombinant BMP-2 or 5 ng/ml TGF-β for the indicated periods of time. In some experiments, these chondrocytes were treated with BMP in the presence of cycloheximide or 5,6-dichloro-1-β D-ribofuranosylbenzimidazol (DRB).

RNA Extraction and RT-PCR

Total cellular RNA was prepared according to the acid guanidium thiocyanate-phenol-chloroform method [Chomczynski and Sacchi, 1987]. Reverse-transcriptase polymerase chain reaction (RT-PCR) was performed to investigate the gene expression of osterix. RT-PCR was performed using GeneAmp RNA PCR kit and the primers generated based on the published murine sequences [Nakashima et al., 2002]. Primers for osterix were 5′-(214)-CTGGGGAAAGGAGGCACAAAGAAG-(238)-3′ and 5′-(687)-GGGTTAAGGGGAGCAAAGTCAGAT-(663)-3′. Primers for GAPDH were 5′-(550)-ACCACAGTCCATGCCATCAC-(569)-3′ and 5′-(1001)-TCCACCACCCTGTTGCTGTA-(982)-3′. Primers for osteopontin were 5′-CTGGCTTTGGAACTTGCTTGAC-3′ and 5′-CGACGATGATGACGATGATGAT-3′. Primers for alkaline phosphatase (ALP) were 5′-CCTCTGGTGGCATCTCGTTAT-3′ and 5′-ATTGCCCTGAAACTCCAAAACC-3′. First experiments were conducted to determine optimal cycle numbers of RT-PCR within the linear range for each of the primer sets. The GeneAmp PCR System 9700 (PE Applied Biosystems, Foster City, CA) was used for gene amplification. The PCR-conditions were as follows. RT reaction was performed at 65°C for 10 min and at 37°C for 60 min. PCR was performed for 30 s at 94°C, 40 s at 60°C, 50 s at 72°C. PCR products were electrophoresed and quantitated using a densitometer. The values of the osterix bands were normalized against those of glyceraldehyde phosphate dehydrogenase (GAPDH) bands. The PCR cycles for osterix and GAPDH were 29 cycles and 19 cycles, respectively.

Northern Blot Analysis

Aliquots of 20 μg total RNA were loaded in each lane and were electrophoresed in agarose gels (1% agarose, 20 mmol/L MOPS, 0.5 mmol/L EDTA, 0.66 mol/L formaldehyde). The RNA was

transferred to nylon filters (Gene screen; NEN Research Products, Boston, MA) and the filters were prehybridized for 2 days at room temperature. Mouse osterix cDNA (473 bp fragment corresponding to the nucleotide at 214–687) was obtained by RT-PCR based on the published sequence reported by Nakashima et al. [2002]. The probes were labeled with BcaBEST random primer labeling kit (Takara Shuzo Co., Ltd, Tokyo, Japan) and alpha^{32}P-dCTP. Hybridization was performed at 42°C for 24 h in a fresh hybridization buffer containing 2×10^6 c.p.m./ml of the labeled probe. Filters were rinsed in $1 \times$ SSC, 0.1% SDS three times at room temperature and in $0.5 \times$ SSC, 0.1% SDS twice at 60°C, and were exposed to X-ray film using intensifying screens at −80°C for several days. The bands in the Northern blot autoradiograms were quantified using a densitometer and the values were normalized against those of the 28S bands in the corresponding lanes. The normalized values were used to calculate the levels of fold induction.

Statistical Analysis

Statistical significance of the data was evaluated based on Mann–Whitney's *U*-test and *P* values less than 0.05 was considered to be significant. Data were expressed as the mean ± SEM.

RESULT

We first examined the effect of BMP treatment on the expression of osterix in the primary cultures of murine rib cartilage-derived chondrocytes. Based on the RT-PCR analysis, osterix mRNA was detected in the primary cultures of chondrocytes as a 473 bp product (Fig. 1). Treatment with BMP enhanced osterix gene expression levels about 2.4-fold after the normalization against GAPDH (Fig. 1). This effect of BMP was specific to BMP since TGF-β, which possesses a structure similar to BMP, did not enhance osterix gene expression in these chondrocytes (Fig. 1). This is not simply due to the inability of the chondrocytes in culture to respond to TGF-β since TGF-β treatment enhanced the expression of osteopontin mRNA level in the same cells (Fig. 1). BMP-2 treatment, on the other hand suppressed the osteopontin gene expression. GAPDH mRNA levels were not altered by any of the treatment, thus serving as an internal control.

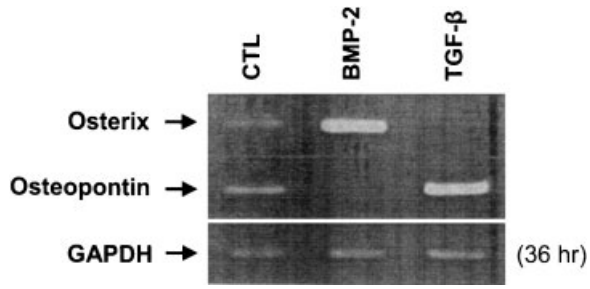

Fig. 1. Expression of osterix mRNA and its regulation by BMP. Primary cultures of mouse rib chondrocytes were treated for 36 h with 400 ng/ml BMP-2, 5 ng/ml TGF-β, or vehicle (CTL). Total RNA was extracted and subject to RT-PCR analysis as described in Materials and Methods.

The time course profile of the BMP effect on the expression of osterix mRNA in the primary cultures of chondrocytes was further examined based on the RT-PCR analysis. As shown in Figure 2A, BMP-2 treatment enhanced the expression levels of osterix within 24 h and this enhancement was, though slightly reduced, still observed at 48 h of the BMP-2 treatment in these cells (Fig. 2C). We further tested whether expression of osterix in the primary cultures of chondrocytes could be detectable by Northern blot analysis. As shown in Figure 2B, Northern blot analysis indicated only very faint band of osterix mRNA in the cultures of these primary rib chondrocytes. BMP-2 treatment enhanced the levels of 3.2 kb osterix mRNA band. 28S bands were shown to indicate similar levels of RNA loading.

In order to see whether BMP effects on the expression of osterix mRNA in the primary cultures of chondrocytes were dependent on the doses of the cytokine, the chondrocytes were treated with BMP-2 at the concentrations ranging from 200 ng/ml to 800 ng/ml. RT-PCR analysis again indicated that a band of osterix and this band intensity was enhanced starting at 200 ng/ml. The effect was observed in a dose-dependent manner as BMP further enhanced osterix mRNA levels up to 800 ng/ml (Fig. 3A). Northern blot examination on the dose dependence profile of the BMP-2 effects on osterix gene expression in these chondrocytes indicated that the effect was hard to be observed at 200 ng/ml BMP-2 while it was detectable at 400 and further at 800 ng/ml (Fig. 3B).

To address the mode of BMP-2 actions to enhance osterix gene expression in the primary cultures of chondrocytes, the cells were treated with BMP-2 in the absence or presence of a

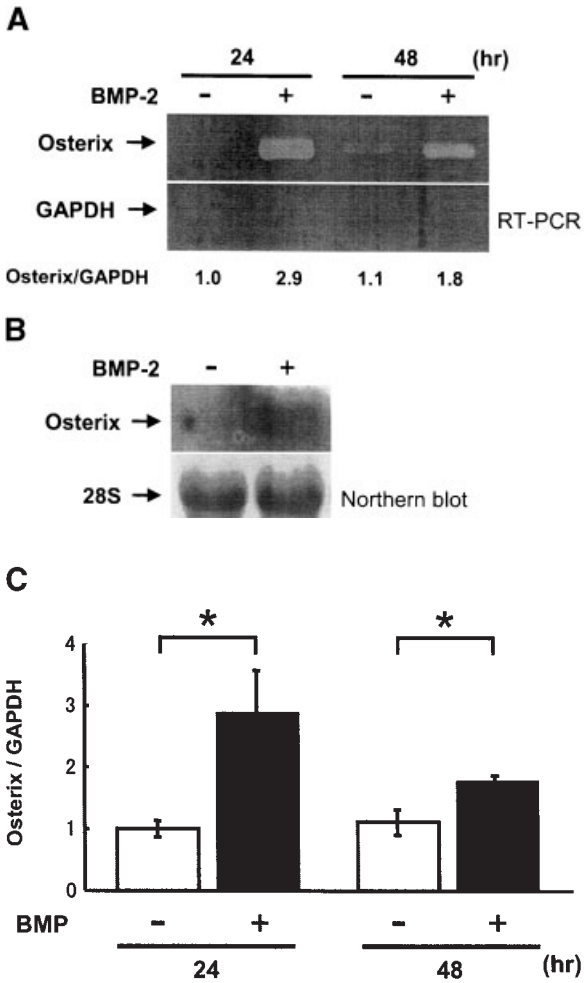

Fig. 2. Time course of the BMP-2 effects on osterix mRNA expression in the primary cultures of rib chondrocytes. **A**: The levels of osterix mRNA expression in the primary mouse rib chondrocytes were examined after 24 and 48 h treatment with 400 ng/ml BMP-2. Osterix and GAPDH mRNA levels were estimated based on semi-quantitative RT-PCR as described in Materials and Methods. **B**: Northern blot analysis was conducted to detect osterix transcripts as described in Materials and Methods using the total RNA of the chondrocytes treated with BMP-2 (+) or vehicle (−) for 24 h. **C**: Quantification of the data shown in (B). Asterisks indicate that the difference was statistically significant ($P < 0.05$) using three independent cultures per group.

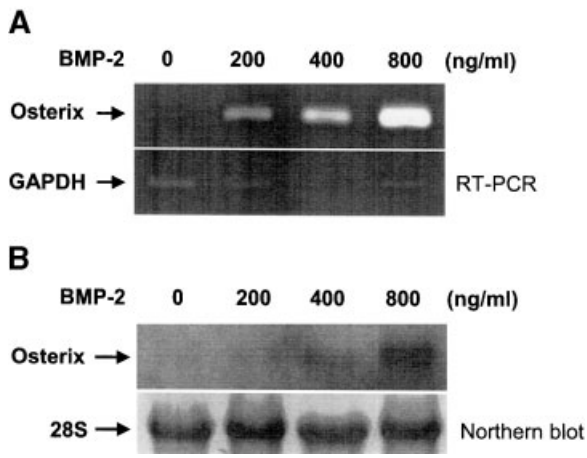

Fig. 3. Dose-dependence of the BMP-2 on osterix mRNA expression in primary chondrocytes. **A**: Primary chondrocytes were treated for 48 h with BMP-2 at the indicated concentrations of BMP-2 raging from 200 to 800 ng/ml. Total RNA was extracted and semi-quantitative RT-PCR was conducted to estimate the levels of osterix mRNA. **B**: Expression levels of osterix mRNA were examined by Northern blot analysis.

protein synthesis inhibitor, cycloheximide. As shown in Figure 4, in the presence of the protein synthesis inhibitor, BMP-2 treatment no longer enhanced osterix gene expression indicating that new protein synthesis was necessary to enhance osterix gene expression by BMP-2. Further examinations to test whether BMP-2 treatment modulates osterix gene expression via transcriptional events, the cells were treated with BMP-2 in the presence of a transcription inhibitor, dichloro-ribofuranosylbenzimidazole (DRB). DRB treatment slightly reduced the basal level of osterix gene expression. However, the treatment with BMP-2 still enhanced the levels of osterix gene expression suggesting

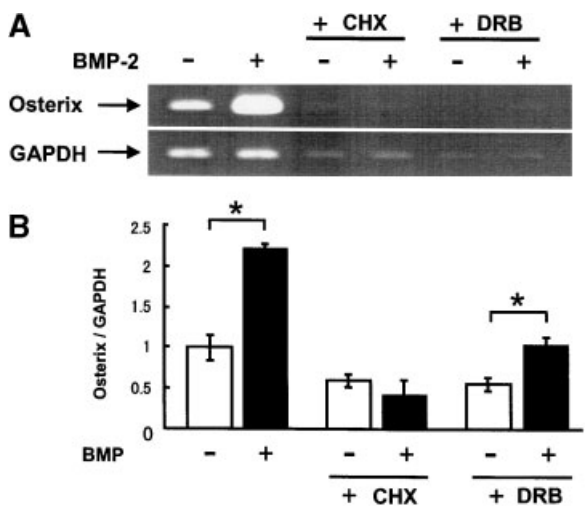

Fig. 4. Modulation by cycloheximide and DRB of BMP-2-induced enhancement of osterix mRNA expression in chondrocytes. **A**: Primary chondrocytes were treated for 24 h with or without 400 ng/ml BMP-2 in the presence or absence of 5 μg/ml cycloheximide (CHX) or 10 μg/ml DRB (dichlororibofuranosyl benzimidazole). Semi-quantitative RT-PCR analysis was conducted as described in Materials and Methods. **B**: Quantification of the data shown in (A) was conducted and osterix mRNA levels were normalized against those of GAPDH. Three independent chondrocyte cultures were used per each group. Asterisks indicate that the data were statistically different ($P < 0.05$).

that the BMP-2 regulated osterix gene expression at least in part via post-transcriptional event.

To examine whether BMP enhancement of osterix mRNA expression could be in parallel to phenotypic gene expression, we investigated the ALP gene expression. ALP mRNA was expressed in primary cultures of rib chondrocytes and its expression levels were enhanced by the treatment with BMP-2 similarly to the BMP-2 enhancement on osterix gene expression. In addition, TGF-β treatment did not enhance the levels of ALP mRNA expression (Fig. 5A). BMP-2 enhanced expression of ALP mRNA within 24 h and the levels of ALP gene expression were still enhanced at 48 h of the BMP-2 treatment (Fig. 5B).

DISCUSSION

In this report, we observed the expression of osterix mRNA in the primary cultures of rib cartilage-derived chondrocytes. Osterix gene expression was enhanced by the treatment with BMP-2. BMPs induce cartilage formation when these cytokines were implanted in the subcutaneous tissue or muscle. This step precedes the BMP-2 induction of bone formation in these ectopic sites. In fact BMP-2 induces genes encoding cartilage phenotype related proteins such as Sox 9 [Zehentner et al., 1999], type II collagen [Majumdar et al., 2001], typeX collagen [Leboy et al., 1997], aggrecan [Stewart et al., 2000], and link protein [Denker et al., 1999]. Since osterix gene was identified as the one being induced by the treatment with BMP-2 in mesenchymal stem cell like C2C12, this gene could be involved in the endochondral bone formation pathway induced by BMP-2. Thus, BMP-2 may not only enhance osterix gene expression to induce bone formation but also to induce cartilage formation prior to bone formation.

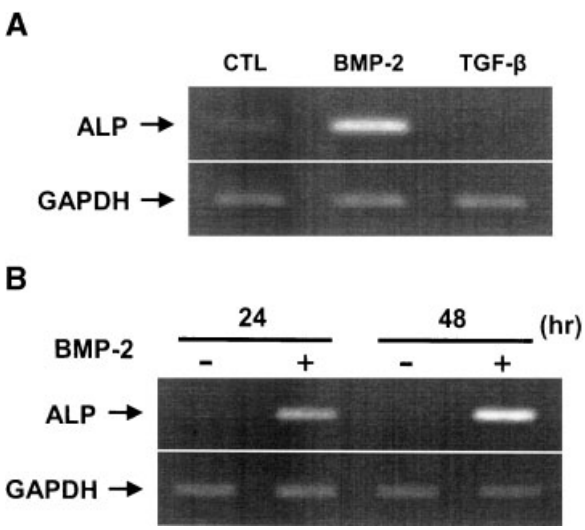

Fig. 5. Expression of ALP mRNA and its regulation by BMP. **A**: Primary cultures of mouse rib chondrocytes were treated for 36 h with 400 ng/ml BMP-2, 5 ng/ml TGF-β, or vehicle (CTL). Total RNA was extracted and subject to RT-PCR analysis as described in Materials and Methods. **B**: The levels of ALP mRNA expression in the primary mouse rib chondrocytes were examined after 24 and 48 h treatment with 400 ng/ml BMP-2. ALP and GAPDH mRNA levels were estimated based on semi-quantitative RT-PCR as described in Materials and Methods.

Although osterix gene knockout mice, which do not form bony skeleton, still exhibit cartilaginous skeletal formation, similar superficial phenotypes were observed in Cbfa1 knockout mice. In Cbfa1 knockout mice, bony skeleton was not observed and cartilage was present to form most of the cartilaginous skeletons [Komori et al., 1997]. However, further examinations revealed that Cbfa1 is necessary for the normal development of cartilage [Inada et al., 1999]. It is possible that osterix gene is also required for the development of cartilaginous tissues. Since chondrocytes are expressing BMP ligands and BMP receptors [Enomoto-Iwamoto et al., 1998], osterix gene expression in chondrocytes would be regulated in autocrine or paracrine manners. Thus, our observations on the BMP-2 enhancement of osterix gene expression in the primary cultures of rib chondrocytes suggest that BMP could regulate osterix gene expression in an autocrine manner in these cells.

Osterix gene expression was not observed in Cbfa1 knockout mice while osterix gene knockout mice still expressed Cbfa1 gene, indicating that osterix lies downstream to cbfa1 [Nakashima et al., 2002]. Our observations that BMP-2 is controlling osterix gene expression added a notion that BMP-2 promotes normal chondrogenesis by acting more than two steps in terms of the transcription factors required for the development of skeletons, i.e., Cbfa1 and osterix. As osterix is both direct and indirect (via Cbfa1) target of BMP, it is still possible that other as yet identified transcription factors may still be in the signaling line.

TGF-β has been implicated in chondrogenesis based on the previous publications [Johnstone et al., 1998]. It is intriguing that osterix gene

expression was not regulated by the TGF-β treatment in the primary cultures of rib chondrocytes in our experiments. Thus, TGF-β alone may not be effective to promote chondrocytes. Recent observations suggest that TGF-β treatment in combination with BMP could enhance efficiently the differentiation of chondrocytes [Sekiya et al., 2001]. This indicated that the pathway of the BMP-2 action to enhance osterix would be required for the chondrocyte differentiation in the presence of TGF-β. BMP rather than TGF-β would be more critical for the development of cartilaginous tissues and cells.

Analysis of the mode of BMP actions indicated that new protein synthesis is required for the BMP-2 actions to enhance the levels of osterix mRNA in the primary cultures of chondrocytes. BMP-2 has been shown to require new protein synthesis to enhance gene expression in osteoblastic cells and chondrocytes. For instance, IGF and activin A gene expression is regulated in such a way [Canalis and Gabbitas, 1994; Kearns and Demay, 2000]. In addition, BMP-2 also has been shown to enhance gene expression post-transcriptionally [Yeh et al., 2001]. Examples of these types of regulation would include the regulation of Id (inhibitor of differentiation) and PAI-1 by BMP [Ogata et al., 1993; Yeh et al., 2001]. In the presence of DRB, BMP-2 still enhanced the level of osterix gene expression suggesting that post-transcriptional events are at least in part involved in the regulation of BMP-2. Such post-transcriptional regulation has been observed in several genes related to extracellular matrix. Type I collagen and fibronectin genes have been shown to be regulated post-transcriptionally by TGF-β [Overall et al., 1991]. Mechanisms of such post-transcriptional events have not been well elucidated in any system. Stabilizer proteins have been suggested to regulate mRNA half-life in a few cases, but responsible molecules in most of the cases are still not known. BMP-2 actions on the regulation of osterix gene expression in the primary cultures of chondrocytes would be similarly regulated though molecular profile could be hard to be identified.

In conclusion we found that osterix gene expression could be detected in the primary cultures of rib chondrocytes and BMP-2 treatment clearly enhanced osterix gene expressions in these cells via at least in part post-transcriptional events in a manner independent from new protein synthesis.

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