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BMP-5 expression increases during chondrocyte differentiation *in vivo* and *in vitro* and promotes proliferation and cartilage matrix synthesis in primary chondrocyte cultures

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Abstract

Bone morphogenetic proteins (BMPs) play pivotal roles in bone and cartilage growth and repair. Through phenotypes of *short ear* mice, which have BMP-5 mutations, a role for BMP-5 in some specific aspects of skeletogenesis and cartilage growth is known. This report examines BMP-5 expression in the growth plate and in differentiating cultures of primary chondrocytes, and the effects of addition of BMP-5 or its inhibition by anti-BMP-5 antibody in chondrocyte cultures. By laser capture microdissection and immunohistochemistry, we found that BMP-5 is expressed in proliferating zone chondrocytes and that the expression increases sharply with hypertrophic differentiation. A similar pattern was observed in differentiating cultures of primary chondrocytes, with BMP-5 expression increasing as cells differentiated, in contrast to other BMPs. BMP-5 added to cultures increased cell proliferation early in the culture period and also stimulated cartilage matrix synthesis. Also, BMP-5 addition to the cultures activated phosphorylation of Smad 1/5/8 and p38 MAP kinase and caused increased nuclear accumulation of phospho-Smads. Anti-BMP-5 antibody inhibited the endogenous BMP-5, reducing cell proliferation and phospho-Smad nuclear accumulation. Together, the results demonstrate that BMP-5 is normally an important regulator of chondrocyte proliferation and differentiation. Whether other BMPs may compensate in BMP-5 loss-of-function mutations is discussed.

Keywords

bone morphogenetic protein; endochondral ossification; bone growth; short ear mouse; growth plate

INTRODUCTION

The bone morphogenetic proteins (BMPs) are members of the TGFβ superfamily and were originally discovered as bone growth-promoting factors(Urist, 1965). Although their evolutionary appearance predates the skeleton, in vertebrates the BMPs are critical modulators of skeletogenesis and maintenance. BMP's have been actively investigated and developed as anabolic agents for bone and cartilage repair(Einhorn, 2003). BMP-5, along with −6, −7, −8a

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and −8b, is a member of BMP subfamily 60A, characterized by their high degree of amino acid sequence homology(Balemans and Van Hul, 2002).

BMPs constitute one of the more complex networks of growth regulatory molecules, with some 14 different ligands, multiple receptors, and intracellular and extracellular regulatory factors. Although BMP receptors are slightly less numerous than the ligands, they have additional complexity due to their hetero-tetramerization. They consist of 4 type I receptors (BMP receptors IA and IB, activin receptor type IA, and activin receptor-like kinase I) and 3 type II receptors (type II BMP receptor and types II and II B activin receptors). All are serine-threonine kinases. Ligands bind to tetramers of two type I and two type II receptors. Both types are required for bound BMPs to initiate signaling(Balemans and Van Hul, 2002; Cao and Chen, 2005). Upon ligand binding, signaling occurs via transphosphorylation of the type I receptor by the type II receptor in the complex. The type I receptor then phosphorylates receptor-regulated Smad proteins (R-Smads) which form oligomeric complexes and, along with cofactors called co-Smads, are translocated to the nucleus where they regulate gene expression. The intracellular activity of Smad proteins is suppressed by inhibitory Smad proteins, called I-Smads, which are in turn upregulated by BMP signaling, thereby forming an auto-regulatory loop (Miyazono et al., 2000). Modulators of BMP activity also abound in the extracellular environment, where antagonists bind BMPs and limit their effective concentrations, helping to regulate the precise levels of active BMPs. There is a long and growing list of such antagonists in vertebrates, including noggin, chordin, chordin-like, follistatin, and many others (Balemans and Van Hul, 2002). An additional layer of modulation is provided by pseudo-receptors that bind BMPs but lack kinase domains.

Despite the well-documented effectiveness of several members of the BMP family in promoting bone formation by osteoblasts, BMP-5 does not exhibit strong osteogenic potential in a variety of pre-osteoblast-like cell lines(Cheng et al., 2003). Several loss-of-function mutations which cause the short-ear (*se*) phenotype in mice, the first described in 1921(Lynch, 1921), have been mapped to the BMP-5 gene(Kingsley et al., 1992). In addition to shortened ear pinnae and specific, localized skeletal defects(Green and Green, 1942), these mice also exhibit strain-specific soft tissue abnormalities of variable penetrance affecting lung, kidney, intestine, bladder, uterus and liver, all tissues in which BMP-5 is expressed developmentally (King et al., 1994). Adult *se* mice also have a decreased ability to repair rib fractures(Green, 1958). The failure of the ear to grow normally has been attributed to impaired cartilage growth after birth. Also pertinent to postnatal cartilage growth, a detailed morphometric study revealed subtle disturbances in endochondral bone growth in the *se* mouse tibia, in which growth plate chondrocytes failed to undergo a normal age-related decrease in the duration of the proliferative phase and also had a slight increase in hypertrophic phase duration(Bailon-Plaza et al., 1999). These results, taken together, suggest a significant role for BMP-5 in cartilage, not only in site-specific endochondral condensation patterning, but also in endochondral bone growth and repair. Despite these indications, no systematic study of BMP-5 activity in differentiating chondrocytes has been reported.

In the present paper, we describe the expression and activities of BMP-5 in chondrocytes. We report, for the first time, that BMP-5 expression is significantly up-regulated in microdissected hypertrophic zone chondrocytes (HZ) when compared with those in the proliferating zone (PZ) of tibial growth plates from normal rats. Moreover, enhanced expression of BMP-5 is also evident in costochondral chondrocyte cultures, where it is associated with the accelerated synthesis of matrix and to a lesser extent mineralization. Importantly, BMP-5 has a dose-dependent effect on the proliferation of cartilage cells and causes downstream signaling, as indicated by the phosphorylation of both Smads and p38MAPK. Taken together, results from our study indicate that BMP-5 plays a major role in cartilaginous differentiation and likely also influences the growth of these cells.

MATERIALS AND METHODS

Primary chondrocyte culture

All experiments involving animals were done in accordance with the University of Massachusetts Medical School Institutional Animal Care and Use Committee. Costochondral chondrocytes were isolated from 3-day-old rat pups by sequential enzymatic digestion as detailed previously (Gartland et al., 2005). Following isolation, cells were grown in DMEM/F12 (1:1; Gibco, Grand Island, NY) supplemented with 10% FBS (Gibco), 1% antimycotic antibiotic solution (AAS; Sigma, St-Louis, MO), 0.2μg/mL sodium selenite (Sigma) and 10μg/mL of human transferrin (Gibco). Upon confluence, chondrocyte differentiation was induced by addition of bovine insulin (10μg/mL; Sigma) and decreasing serum concentration over a period of 17 days(Gartland et al., 2005).

In some experiments, 0.5–1 μg/mL of a goat BMP-5 polyclonal antibody (Biovision Research Products, Mountain View, CA) or various concentrations (1–100ng/mL) of recombinant human BMP-5, or 100 ng/mL of BMP-6 or BMP-7 (R&D Systems, Minneapolis, MN) were added 24 hours post plating and replaced at every cell medium change.

Cell proliferation

Cells were washed with Hank's balanced salt solution (HBSS, Gibco) and incubated in a sequence of three different enzymes for 15 minutes each: 0.5% hyaluronidase (Sigma), 0.2% collagenase P (Roche Diagnostics, Indianapolis, IN) and 0.25% trypsin (Gibco) to facilitate matrix digestion and cell release. The released chondrocytes were pelleted, resuspended in HBSS, and viable cells were counted using Trypan blue. To confirm cell count results, some wells were digested overnight at 50°C in a buffer composed of 10mM Tris-base, 100mM NaCl, 25mM EDTA, 0.5% SDS, 0.1mg/mL proteinase K (Sigma), 20 μg/mL DNase-free RNase (Sigma), pH 8.0. Genomic DNA was extracted with a mixture of phenol:chloroform:isoamyl alcohol (25:24:1, Sigma), precipitated with 7.5M ammonium acetate (Sigma) and ethanol. DNA concentration was measured spectrophotometrically.

Qualitative and quantitative assessment of glycosaminoglycan content and matrix mineralization

To determine glycosaminoglycan content of the cultures, cells were fixed in a solution of 3.7% paraformaldehyde and washed twice with double distilled water before overnight incubation at 4° with 0.5% Alcian blue (Fluka Chemika, Sigma Aldrich Chemie Gmbh, Steinheim,Germany) in 0.1N HCl solution. Cells were then rinsed five times in excess distilled water to remove unbound dye and allowed to dry for a minimum of 15 minutes.

To assess matrix mineralization, cells were fixed and rinsed as above, stained 20 minutes at room temperature with a solution of 40mM Alizarin Red (Sigma) in distilled water at pH 4.2 and rinsed four times with water. A final rinse in PBS was performed to remove any unbound stain. Plates were kept at −20° until quantitative assessment. To compare cartilage matrix synthesis (alcian blue) and mineralization (alizarin red), each plate was photographed on a light box with a digital camera using identical exposures, and the resulting images were subjected to densitometric analysis of individual wells using the Image J program (http://rsb.info.nih.gov/ij).

RNA isolation and real-time PCR

At different times of differentiation, RNA was extracted using TRIZOL reagent (InVitrogen, Carlsbad, CA) according to manufacturer's instructions except for the addition of coprecipitant (EMD Biosciences, San Diego, CA) to facilitate RNA precipitation. 1 μg of total RNA was subjected to reverse transcription using random hexamers and Superscript II reverse

transcriptase (InVitrogen). cDNA was used as template for real-time PCR reactions. Briefly, 1μL of template was mixed with 9μL of LightCycler FastStart DNA Master SYBR Green I mix (Roche Diagnostics, Indianapolis, IN) to which $MgCl_2$ and gene-specific forward and reverse PCR primers had been added to a final concentration of 2μM for $MgCl_2$ and 0.5μM for each primer. Intron-spanning primers were designed and are listed in Table 1. All RT-PCR reaction products were cloned into plasmids and sequenced to confirm their identities. Linearity of the real-time results was confirmed by including a serial dilution of the corresponding plasmid.

Laser microdissection

Tibiae from normal 4week-old rats were surgically removed, split longitudinally and cut transversly to shorten the bone shaft by removing the distal end. Both tibia halves were transferred to small plastic molds to which Tissue Freezing Medium (Triangle Biomedical Sciences) was added. Samples were frozen immediately on dry ice and stored at −80° until sectioning. Before each laser microdissection, embedded tibiae were removed from their molds, mounted onto stubs and serial sections (6 microns in thickness) were cut at −28° on a Hacker-Bright 5040 Cryostat using a stainless steel knife. CryoJane Tape Transfer System (Instrumedics, St-Louis, MO) was used to facilitate transfer and adherence of sections to 1X adhesive coated glass slides (Instrumedics). Sections were kept at −80 ° until processing. Frozen sections were fixed in pre-chilled 70% ethanol made up with diethyl pyrocarbonate (DEPC)-treated water for 30 seconds and quickly dipped in DEPC water before staining in RNase-free hematoxylin (Arcturus, Mountain View, CA) for 15 to 30 sec. Sections were dehydrated in a graded series of ethanol up to 100%, for 30sec to 1 min. each, followed by 2 xylene incubations of 5 min. each. Sections were then placed under vacuum in a dessicator over silica gel for at least 1hr before laser session.

Chondrocytes from the articular cartilage and from the growth plate were microdissected using a Pixcell 2 LCM unit (Arcturus, Mountain View, CA). Four to five sections were microdissected per each laser cap membrane. Sections from several rats were pooled together. Upon completion of dissection, the membrane containing microdissected material was immediately peeled off the cap, transferred to lysis buffer (Epicentre, Madison, WI) and stored at −80°C until extraction. Total RNA was extracted using the Array Pure Nano-scale RNA purification kit (Epicentre) according to the manufacturer's instructions except for inclusion of coprecipitant (EMD Biosciences) at the time of RNA precipitation. RNA concentration was measured by fluorescence spectrophotometry and a total of twenty nanograms of RNA was reverse transcribed using the Full Spectrum RNA Amplification kit (System Biosciences, Mountain View, CA). 1μL of the resulting cDNA served as template for real time PCR reactions as described above.

Immunoblotting

Primary chondrocytes were plated at a density of 5×10^5–1×10^6 cells in 10 cm^2 dish and cultured as described above. Upon confluence, cells were starved overnight in the same culture medium supplemented with 0.5% FBS. Total protein was extracted using lysis buffer: 150mM NaCl, 1% Nonidet-40 (Fluka Biochemika), 0.5% sodium deoxycholate (Sigma), 0.1% SDS (BioRad), 50mM Tris, pH 8.0) supplemented with a cocktail of protease inhibitors (Complete, Roche Diagnostics). Twenty micrograms of total protein was electrophoresed in 12% polyacrylamide denaturing gels and electrophoretically transferred to PVDF membrane. The membrane was blocked for 2 hours at room temperature with 5% non-fat dry milk in TTBS (160mM Tris-base, 0.15M NaCl, 25mM sodium azide, 0.1% (v/v) Tween-20 (Sigma), pH 7.5) and incubated overnight at 4° with the following antibodies diluted in TTBS containing 5% bovine serum albumin (Sigma): 1:1000 rabbit polyclonal phospho-Smad1-5-8 or 1:1000 rabbit monoclonal phospho p38 MAPK (both from Cell Signaling Technology, Danvers, MA). Membranes were

thoroughly washed in TTBS and further incubated with secondary antibody; goat anti-rabbit conjugated to alkaline phosphatase (AP) (BioRad) 1:3000 in 5% milk TTBS. Signal was detected using AP substrate Attoglow (Michigan Diagnostics, Troy, MI) and visualized by exposure to x-ray film. For detection of p38MAPK and Smad, membranes were stripped and reprobed with 1:1000 rabbit polyclonal p38 MAP kinase and 1:500 mouse monoclonal Smad 5 (both from Cell Signaling Technology) following the same procedure.

Immunohistochemistry

Tibiae were dissected from 2- and 4-week-old wild type rat pups of the *toothless* rat stock. Tissues were prepared for paraffin embedment and immunostained by the immunoperoxidase method as previously described(Yang, 2006). Polyclonal anti-BMP-5 primary antibody (Biovision) was used at 1:50 dilution.

Immunofluorescence

Primary chondrocytes were plated on coverslips at a density of 5×10^4 cells per well in 24 well plates and grown until confluence in the same medium as described above. Some wells were treated with 1μg/mL of anti-BMP-5 (BioVision) 24 h prior to cell fixation while others received 100ng/mL of recombinant human BMP-5 (R&D Systems) 1h before fixation. Cells were rinsed in PBS and fixed in 3.7% paraformaldehyde for 10 minutes. Following two washes with PBS, cells were permeabilized in 0.1% Triton-X (Sigma), then rinsed twice in PBS and incubated in PBS containing 1% bovine serum albumin (PBSA; Sigma) for a minimum of 15 minutes. Rabbit polyclonal phospho-Smad 1,5,8 and monoclonal phospho p38MAPkinase antibodies were diluted 1:50 in PBSA and incubated with fixed cells for 2h at 37°. Negative controls were incubated with PBSA alone. After four rinses in PBSA, coverslips were incubated with donkey anti-rabbit IgG conjugated to Alexa 488 fluorochrome (Molecular Probes, Eugene, OR) for 1 hour at 37°. After two washes in PBSA, coverslips were rinsed once in PBS plus 5μg/mL 4'–6-diamidino-2-phenylindole (DAPI) and 0.1%Triton X-100, once in PBS, and mounted on Antifade Gold (Molecular Probes). Images were obtained using Axiovision software version 4.1 with an Axioplan microscope equipped for epifluorescence with an Axiocam MRm camera and a 63X objective (Carl Zeiss, Oberochen, Germany). Exposure times for each color were identical for all experimental conditions.

Statistics

Data are expressed as mean +/− SEM. Comparisons between group means were done using one- or two-way ANOVA and in presence of main effect significance, Tukey multiple comparison post-hoc test was done. These statistical tests were performed using NCSS (Kaysville, UT). Some experimental results were analyzed by *t*-test after determining variances by *F*-tests using Microsoft Excel. $P<0.05$ was chosen as cutoff for significance.

RESULTS

BMP-5 expression during chondrocyte differentiation in vivo

Laser capture microdissection was carried out on nearly 100 frozen sections of proximal tibial growth plates from 5 normal, 4-week-old rats to compare RNA from proliferative (PZ) zone versus hypertrophic zone (HZ) cells. The microdissection accurately removed cells from the desired zones, as shown by the micrographs in Figure 1A and B. Evaluation of expression of osteoblast- and osteoclast-specific genes, such as osteocalcin, α1(I) collagen, and TRAP revealed negligible bone contamination of the microdissected samples (not shown). Real-time RT-PCR for BMP-5 showed that BMP-5 is expressed in PZ chondrocytes and that it increases roughly 6-fold in HZ cells (1C). Confirmatory immunohistochemistry experiments were done on tibiae and femora from 2- and 4-week-old rats and results from a 2-week-old tibia are shown

in Figure 1D–F. Immunostaining was essentially negative in the proliferating zone and increased dramatically as the cells became hypertrophic. Similar up-regulation was seen in hypertrophic chondrocytes in the secondary ossifications centers, and a few of these can be seen at the top of 1–E. Thus, BMP-5 mRNA and protein are differentially expressed in hypertrophic zone chondrocytes in vivo in growing long bones.

BMP expression in costochondral chondrocyte cultures

To investigate this phenomenon further, we used a previously described and well-characterized rat costochondral primary chondrocyte culture method (Gartland et al., 2005) in which differentiation is induced by decreasing serum and adding insulin. Figure 2 shows the results of real-time RT-PCR on RNA isolated over the time course of chondrocyte differentiation. As expected, type II collagen RNA decreases and type X collagen increases as cells move toward hypertrophy. Concomitantly, BMP-5 mRNA also increases. The day 17 value is significantly different from the day 0 and day 3 values ($P < 0.05$). Together, Figures 1 and 2 show that BMP-5 is expressed by primary chondrocytes and its expression increases during chondrocyte differentiation *in vivo* and *in vitro*. Investigating several other BMPs and receptors during the differentiation time course (Figure 3), we found that BMP-6 and -7 and BMPRII did not undergo significant changes over the culture. BMP-4 peaked at day 9 and then gradually fell (day 9 vs. day 17 *t*-test, $P < 0.05$) and BMPR1a gradually increased over the differentiation course, reaching significance on day 17 ($P < 0.02$).

BMP-5 and chondrocyte proliferation

Because BMPs may affect cell proliferation, we investigated the effect of inhibiting endogenous BMP-5 in primary chondrocyte cultures. Figure 4 shows the results from multiple wells in two independent experiments. The values shown were calculated as the per cent of the mean counts in untreated control wells on that day. When anti-BMP-5 was added to the differentiating chondrocyte cultures, it had a significant, anti-proliferative effect. ANOVA yielded a highly significant effect of antibody treatment ($P < 0.001$). For the individual culture time points, day 9 was significantly different from day 0 ($P < 0.05$). Releasing the cells for counting required enzymatic digestion of extracellular matrix, which increasingly accumulates over the culture period. We therefore also extracted total DNA in some experiments as an independent measure of cell proliferation. In all cases, the DNA and cell count data were in agreement (not shown). These results do not reflect cell die-off, since counts in all wells were increasing. Rather, the proliferation in anti-BMP5-treated wells lagged significantly behind untreated wells.

We also measured the effects of BMP-5 addition on chondrocyte proliferation. Recombinant BMP-5 was added to the medium at 1, 10, and 100 ng/mL with some wells left untreated for use as controls, and cell counts were performed at time points throughout the differentiation time course. The counts in treated wells were calculated as the per cent of the counts in untreated control wells at the same time points, and the results are shown in Figure 5. Cell counts were significantly increased in a dose-dependent manner by BMP-5 at 10 and 100 ng/mL, with the strongest effect seen roughly half way through the culture time course. The 100 ng dose of BMP-5 doubled the cell number as compared with controls on day 9.

Effects of BMP-5 on signal mediators, growth, and differentiation in cultured chondrocytes

The finding of increasing BMP-5 expression during chondrocyte differentiation in vivo and in vitro prompted us to determine if addition of BMP-5 to chondrocytes would affect differentiation and signaling events. We wished to establish whether signaling mechanisms previously demonstrated to be activated by BMP's in various cells and tissues were also stimulated by BMP-5 in chondrocytes. Addition of recombinant BMP-5 to primary chondrocytes caused phosphorylation of Smads in a time-dependent manner (Figure 6). Smad

phosphorylation was detectable by 15 minutes, increased markedly by 30 minutes, reached a peak at 6 hours and decreased thereafter. Phosphorylation of p38 MAPK was much less apparent, although it did increase slightly after 15 minutes and remained weakly positive for the 8-hour duration of the experiment. Thus, BMP-5 caused activation of the Smad, and to a lesser extent, of p38 MAPK, in these primary chondrocyte cultures. These results are consistent with other BMP's in other cell systems.

We next investigated the nuclear accumulation of p-Smads upon stimulation of primary chondrocytes by BMP-5, and the results are shown in Figure 7. Cells were grown to confluence and then treated with BMP-5 or with anti-BMP-5 antibody for 1 and 24 hours respectively, or were left untreated. The untreated control cultures consistently showed some relatively strongly labeled cells, often in clusters, indicating nuclear phospho-Smad 1/5/8, presumably due to endogenous BMP synthesis and signaling (7D). When BMP-5 was added, phospho-Smad nuclear labeling increased sharply (7D vs. 7H), indicating a strong cellular response to BMP-5. Conversely, when anti-BMP-5 was added to the cultures, the endogenous level of nuclear Smad seen in untreated cultures was greatly decreased (7D vs. 7F), indicating that the presence of nuclear p-Smads depends on endogenous BMP-5.

BMP-5 also had an effect on cartilage matrix synthesis and mineralization (Figure 8). This was measured by staining with alcian blue (8A, D) or alizarin red (8B, E) to stain proteoglycans or mineral, respectively. Digital photographs of the stained cultures were analyzed densitometrically. Addition of BMP-5 greatly accelerated matrix synthesis and, to a lesser extent, mineralization, particularly at the highest dose. Except for day 0, all the time points for cultures treated with 100 ng/mL of BMP-5 had significantly higher alcian blue staining than untreated controls. By day 9, the staining had already reached over twice the level that was achieved in the untreated wells at the experiment's end. The last 2 time points for the high-dose samples were roughly 5-fold higher than controls. The differences in mineralization were less pronounced, although at day 13 the treated wells had over twice the alizarin red staining of untreated controls. Note that this culture system uses no exogenous enhancers of mineralization, and so may underestimate the potentiation of mineral deposition(Gartland et al., 2005).

In addition to studying the effects of BMP-5, we also performed some culture experiments in which BMP-6 or 7 were added to wells. Figure 8C shows a typical result. BMP5 induced matrix synthesis, as indicated by strong alcian blue staining relative to the untreated control well shown, and BMP6 elicited a similar response. In contrast, BMP7 had no effect on cartilage synthesis. We found very similar effects of BMP6 on cell counts and mineralization compared to BMP5 (not shown), not surprising since BMP-5 and -6 are highly homologous and bind to the same receptors.

DISCUSSION

Results from our current study show that BMP-5 mRNA is expressed by chondrocytes at all stages of differentiation, but that its expression increases significantly during the maturation of growth plate chondrocytes *in vivo* as well as in differentiating primary chondrocyte cultures. BMP-5 protein was strongly expressed in vivo in growth plate hypertrophic zone chondrocytes (Figure 1). Consistent with these results, BMP-5 expression in the growth plate was reported previously in a detailed histochemical study of human fetal tissue and postnatal rats(Anderson et al., 2000), in which BMP-5 expression was lower in the proliferating zone cells and higher in hypertrophic zone cells. Although in our hands BMP-5 protein was not detected in PZ chondrocytes (Figure 1 E and F), we did detect its mRNA (1C). We surmise that the protein is also present, but at levels that were not high enough to be detected under the antibody conditions we used.

The present study demonstrates that BMP-5 exerts a proliferative effect and stimulates cartilage matrix synthesis. The importance of endogenous BMP-5 was shown by the anti-BMP-5 inhibition studies, which suppressed cell proliferation (Figure 4). The Smad and, to a lesser extent, the p38 MAP kinase pathways are involved in the chondrocytes' cellular response to BMP-5. Again, inhibition of endogenous BMP-5 with anti-BMP-5 antibody suppressed the nuclear accumulation of phospho-Smad (Figure 7). In addition, BMP-5 promoted, to a modest extent, the mineralization of cartilage matrix in this differentiation system. Cartilage mineralization is an essential step which *in vivo* marks the terminal stage of endochondral cartilage. The deposition of mineral renders cartilage able to be resorbed by osteoclasts, or "chondroclasts," and permits the vascular invasion of cartilage anlagen during the skeletal morphogenesis as well as on the metaphyseal side of the chondroosseous junction during endochondral bone growth. In our experimental system, BMP-5 expression increased in parallel with collagen type X expression, a hallmark of chondrocyte hypertrophy. The differentiation system used here does not employ exogenous stimulators of mineralization such as ascorbic acid or added phosphate sources(Gartland et al., 2005), instead relying on the inherent ability of the cells to deposit mineral available in the medium. It may well be, therefore, that in an augmented system a greater effect on mineralization would be seen; i.e., this study may actually underestimate the impact of BMP-5 on mineral deposition. In sum, it would appear from the present studies that BMP-5 is exerting effects both in driving the cell cycle to promote chondrocyte proliferation, and then, as its expression increases and cells become terminally differentiated, it is promoting matrix maturation.

The regulation of chondrocyte proliferation and differentiation, especially during embryogenesis and endochondral ossification, has been the subject of many investigations. Key among the regulatory pathways is the balance between parathyroid-related protein (PTH-rP) and Indian hedgehog (Ihh) in promoting proliferation and hypertrophy, respectively (Kronenberg, 2003; van der Eerden et al., 2003). Vortkamp and co-workers have shown that BMP signaling, with particular emphasis on the role of BMP-2, antagonizes fibroblast growth factor effects during chondrocyte differentiation to maintain the proper balance between proliferation and hypertrophy(Minina et al., 2002; Minina et al., 2001). *In vivo* studies in mice with constitutively active BMPR1a showed that BMPs regulate both the chondrogenic differentiation of precursors in condensing mesenchyme during pattern formation and the maintenance of the growth plate(Kobayashi et al., 2005). The work presented here shows that BMP-5 is differentially expressed during endochondral ossification *in vivo* and has a significant effect on primary chondrocyte proliferation, differentiation and matrix synthesis *in vitro*, indicating that it likely plays a previously underappreciated role in bone growth. It appears that BMP-5 is exerting proliferative influences during the time at which chondrocytes are in the cell cycle, and is also contributing to matrix synthesis and maturation as the cells cease dividing and become terminally differentiated. The regulatory sequences of the BMP-5 gene are of uncommon complexity, including some as distant as 270 kilobases from the transcription start site, that together direct its expression to specific locations and times during skeletal development(DiLeone et al., 2000; DiLeone et al., 1998). Evidently, at least some of these elements also direct its increasing expression during the differentiation of chondrocytes toward hypertrophy.

BMP5 up-regulation is clearly associated with terminal chondrocyte differentiation in vivo, but we also identified BMP-5 mRNA in PZ chondrocytes (Figure 1C). Although we did not see BMP-5 protein in the PZ of immuno-stained growth plates, it may be present at levels not detectable by this technique. In our culture system, BMP-5 had a significant proliferative effect, especially at relatively early time points. No culture system can faithfully reproduce the complex microanatomy of the growth plate in vivo, with many local and global regulatory factors all playing a role, including growth hormones, IGF's, hypoxia-induced transcription factors, and the yin-yang of PTH-rP and Ihh. It is clear from our results, however, that BMP5

at high levels can cause increases in the proliferative capacity of immature chondrocytes. Our finding, and that of others, that BMP-5 is low in PZ chondrocytes suggests that other signals dominate in stimulating chondrocyte proliferation in the growth plate in vivo.

The correlation of increasing expression of BMP-5 and BMPRIa suggests that the effects of BMP-5 during chondrocyte differentiation are likely mediated via a receptor complex that includes BMPRIa. This is not surprising, since many essential functions of BMPs are mediated via BMPR1a. Knockout of BMPR1a is embryonic-lethal in mice, resulting from a failure of gastrulation, which requires BMP-4(Mishina et al., 1995; Winnier et al., 1995). Conditional alleles have shown that BMPR1a is also required for postnatal cartilage maintenance(Rountree et al., 2004).

Given the striking effects of BMP-5 on chondrocytes *in vitro*, its increasing expression during their differentiation *in vivo*, and its complex developmental regulation, one might expect a dramatic and global cartilage and skeletal phenotype in loss-of-function mutants. This is not the case. The *se* mouse cartilage phenotype is subtle and site-specific. In particular, in a detailed morphometric analysis of *se* mouse growth plates, Bailon-Plaza et al. (Bailon-Plaza et al., 1999) showed that, over time, normal chondrocytes spent longer in the proliferative phase with age, increasing by two-thirds between the 5th and 9th postnatal weeks, while *se* mice showed no change in that parameter. There was also a slight increase in the hypertrophic cell number and duration in the *se* mice compared with wild type. Two explanations for this very mild phenotype are possible: either BMP-5 is not very important in the process or it is compensated by other BMP family members. Compensation is a well-known phenomenon first seen in some of the earliest knockout experiments undertaken, for example, the MyoD mouse. It wasn't until mice carrying mutations in MyoD and Myf-5 were crossed that the predicted lack of skeletal muscle was observed(Athanasou and Sabokbar, 1999; Rudnicki et al., 1993). One might reasonably infer that other BMPs are able to compensate for the loss of BMP-5 in *se* mice. That inference begs the question as to which BMP(s) compensate(s) for BMP-5 in the *se* mouse. The most likely candidate is BMP-6, which is the closest homologue of BMP-5, but which, unlike the increasing levels seen for BMP-5, we found to be expressed at fairly constant levels. When we performed identical experiments to those described here, but instead added recombinant BMP-6 to the cultures, we observed similar effects on cell proliferation and on matrix synthesis and mineralization (Figure 8C and data not shown). Future investigations should shed light on mechanisms and extent to which the effects of BMP-5 loss-of-function can be compensated for in skeletogenesis, growth, and repair.

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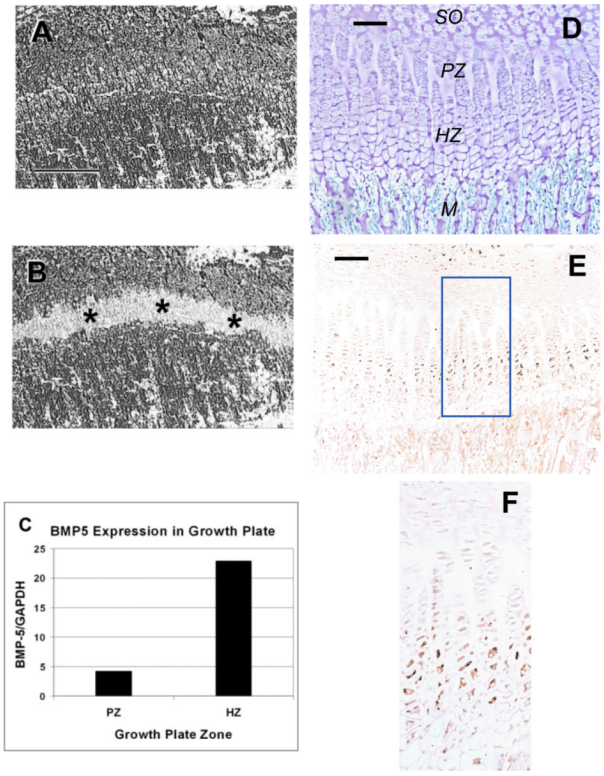

Figure 1. BMP-5 mRNA and protein expression in growth plate cartilage

Chondrocytes were captured from the growth plates of frozen sections of 4 week-old normal rat tibiae. Micrographs show proximal tibia frozen sections before (A) and after (B) laser capture of hypertrophic zone chondrocytes of a typical section. Asterisks (*) in B denote area dissected to obtain HZ RNA. C: Real-time PCR showed BMP-5 mRNA to be roughly 6-fold higher in hypertrophic zone chondrocytes (HZ) than in proliferative zone chondrocytes (PZ). 98 sections from 5 animals were pooled and 20 ng of total RNA was reverse transcribed and analyzed in duplicate. Data were normalized to GAPDH values and the means of two independent experiments are shown. D, E, F, show adjacent sections from a 2-week-old rat proximal tibia stained with toluidine blue (D) or labeled with anti-BMP5 antibody (E, F). Proliferating zone (PZ), hypertrophic zone (HZ), and metaphysis (M) are indicated, and some hypertrophic chondrocytes from the developing secondary ossification center (SO) are visible near the top. HZ chondrocytes are markedly more stained than PZ cells. Hypertrophic cells in the SO are also positive for BMP-5. Panel F is a higher magnification of the area in the rectangle in E. Bar in A = 500 μm. Bars in D, E = 100 μm.

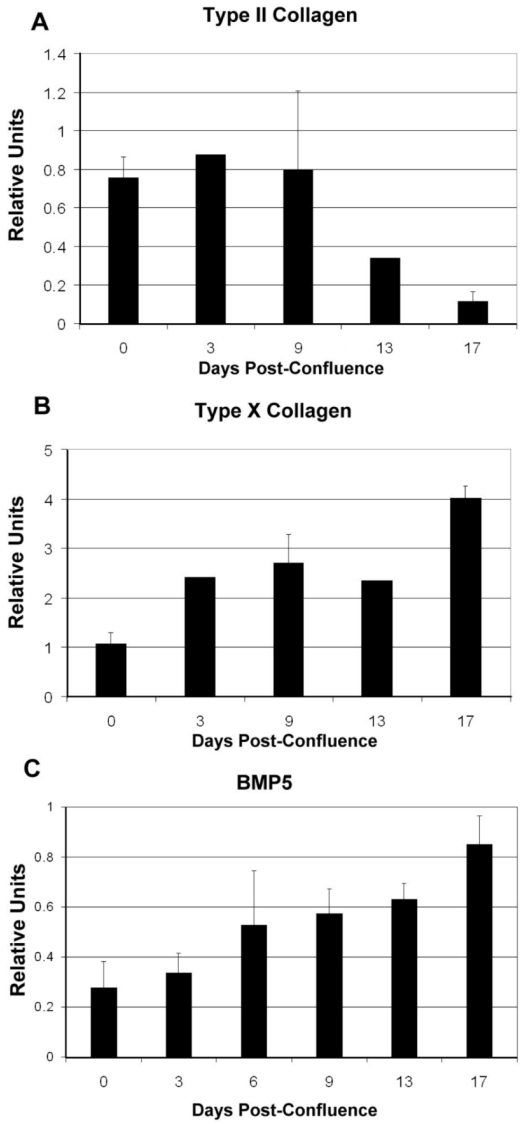

Figure 2. BMP-5 expression increases during chondrocyte differentiation

Primary costochondral chondrocytes from neonatal rats were cultured until confluence (day 0), at which time differentiation was initiated and RNA was isolated on the days indicated on the X-axis. Real-time PCR for type II (A) and type X collagen (B) confirm differentiation toward hypertrophy. BMP-5 expression increased throughout the culture period (C). Results are expressed as the ratio of the specific mRNA to GAPDH. Mean + 1 SEM is shown. Results are the means of at 4 to 6 independent experiments. By *t*-test, day 17 BMP-5 is significantly different from the day 0 and day 3 values ($P < 0.05$).

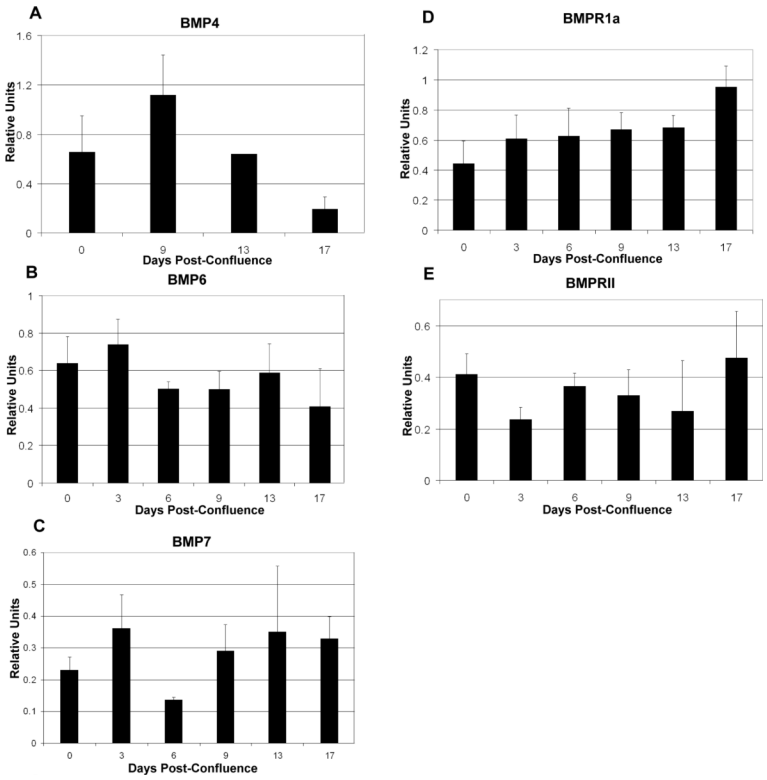

Figure 3. Expression of other BMPs and receptors during chondrocyte differentiation

Real-time PCR on RNA from primary chondrocytes shows that BMP-4 (A) rose then fell (day 9 vs. day 17, $P < 0.05$, others n. s.), whereas neither BMP-6 (B) nor 7 (C) show changes. BMPRIa (D) gradually increased (day 17 vs. day 0, $P < 0.02$), whereas BMPRII did not. Data are means + 1SEM of between 2 and 8 independent experiments with triplicate determinations in each.

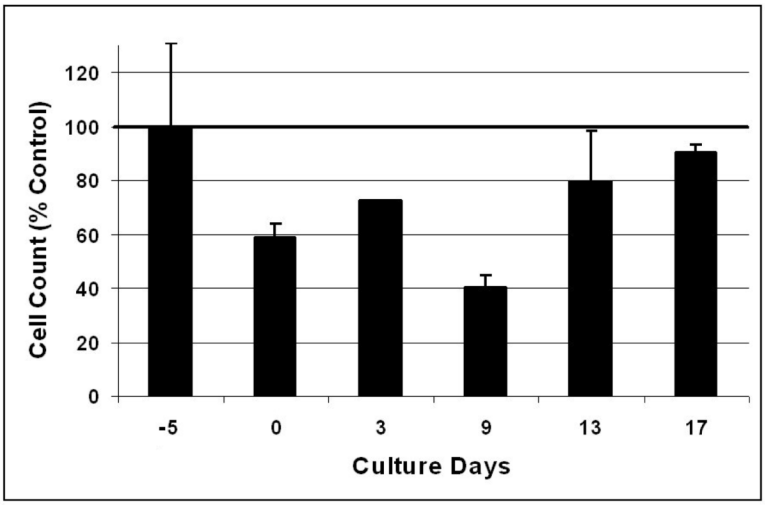

Figure 4. Anti-BMP-5 inhibits primary chondrocyte proliferation

Cells were plated (day -5), grown to confluence (day 0) and then induced to differentiate with low serum and insulin. On the days indicated, cells were enzymatically released and counted. Some wells were left as untreated controls and others were treated with anti-BMP-5 in the medium beginning on day -4 and replaced with every medium change. The bold line at 100% indicates the count in the untreated control wells on each day. Results shown are % of the untreated control wells on that day, e.g., on day 9, cell counts in treated wells were 40% of those in untreated controls wells. Mean + 1 s.e.m. ANOVA for effect of antibody treatment: $P < 0.001$; effect of time, not significant.

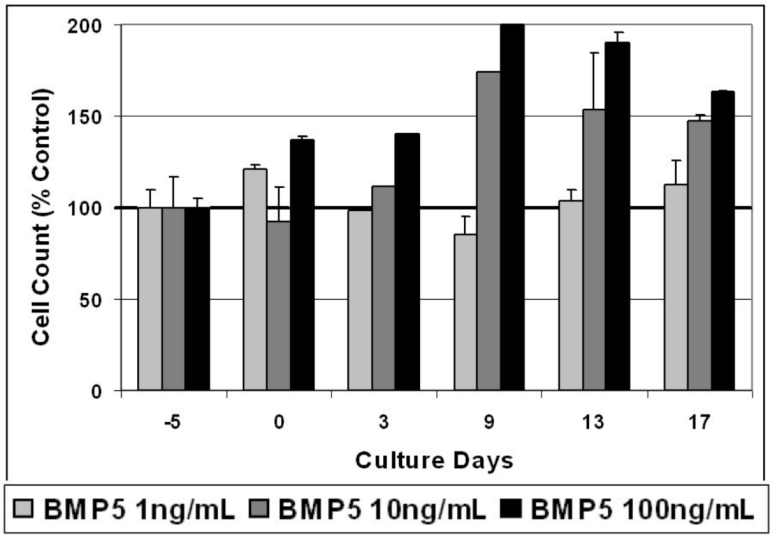

Figure 5. Effect of added BMP-5 on cell counts

Rat chondrocytes were grown in the presence of BMP-5 (1, 10 and 100 ng/mL) or were untreated controls. Day 0 = confluence. The bold line at 100% indicates the mean values for control wells on each day. At times indicated, cells were released by sequential enzyme digestion and counted, and the results normalized to untreated cells at the same time point (100%). Mean + SEM of 4–5 separate wells coming from two independent experiments are shown. Differences between group means were determined in relation to time or treatment using two-way ANOVA. Global effect of treatment, $p<0.00001$, global effect of time, $p<0.05$ and significant interaction $p<0.05$. BMP-5 at 10 and 100 ng/mL strongly stimulated proliferation.

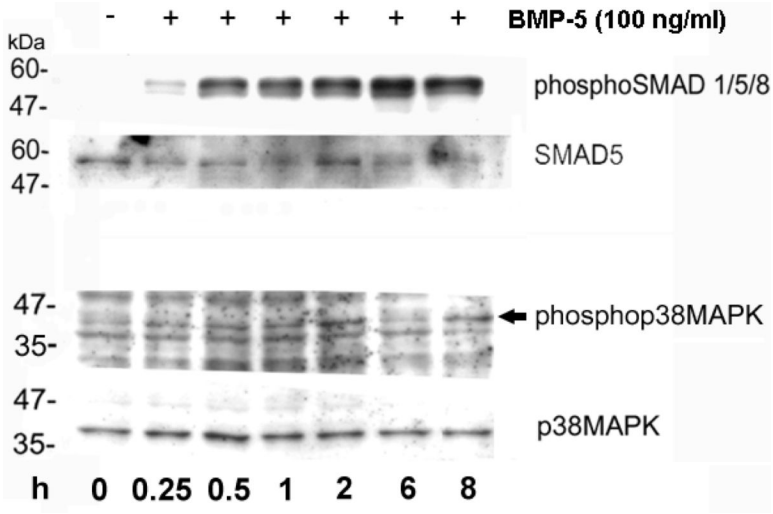

Figure 6. Phosphorylation of Smad 1/5/8 and p38MAPK by BMP-5 in primary rat chondrocytes

Chondrocytes were cultured to confluence and analyzed either untreated (C) or incubated with BMP-5 for the number of hours (h) indicated. Equal amounts of protein extract were subjected to SDS-PAGE and blotted for Smad (upper panels) or p38MAPK (lower panels). After phospho-specific antibody development, blots were stripped and re-probed with anti-Smad5 or anti-p38MAPK. Phosphorylation of Smad had begun by 15 minutes, was very strong by 30 minutes, and increased through the 6-hour time point. Phospho-p38 also rose slightly within 15 minutes, but after that, changes were minor, but phosphorylation persisted for the 8 hours of the experimental time course. Three replicate experiments produced essentially identical results.

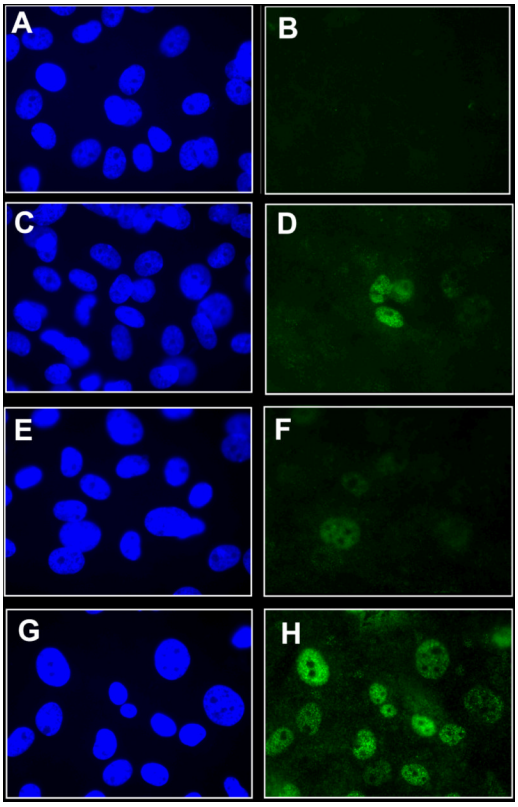

Figure 7. Phosphorylation and nuclear accumulation of Smad 1/5/8 are induced by BMP-5 and blocked by anti-BMP-5 antibody

Rat primary chondrocytes were cultured until confluence on coverslips in 24 well plates. A, C, E, and G show DAPI nuclear stain, B, D, F, H show the same fields labeled with anti-phospho-Smad (D, F, H) or negative control without primary antibody (B). Cells were untreated (A, B, C, D); treated with 1 μg/mL anti-BMP-5 antibody for 24 hours (E, F) or with 100 ng/mL of BMP-5 for 1 hour (G, H) before fixation. In untreated cells (D), we consistently observed some intensely stained clusters of cells, as shown, whereas anti-BMP5 treatment suppressed that endogenous activity (F). BMP5 treatment caused a marked increase in the number and intensity of nuclear phospho-Smad (H).

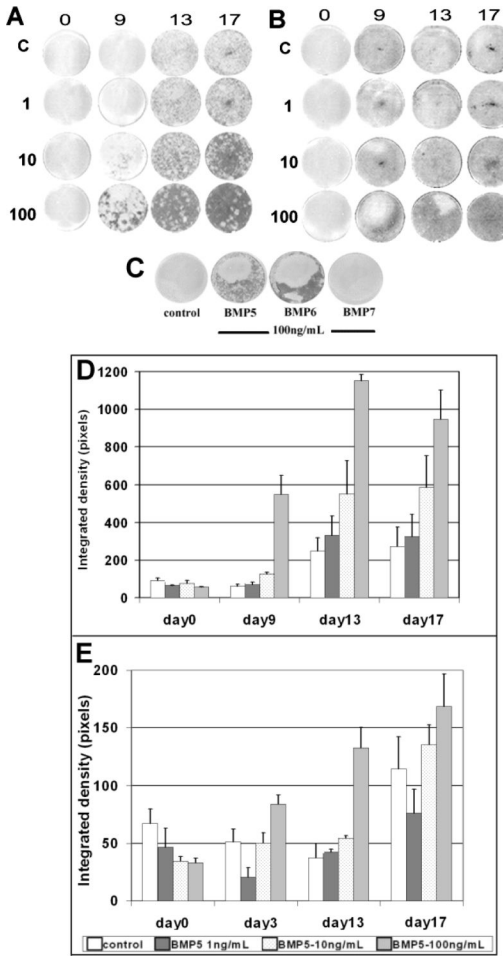

Figure 8. Effect of BMP-5 addition on matrix secretion and mineralization

Rat chondrocytes were grown in 24-well plates in the presence of BMP-5 (1, 10 and 100 ng/mL) or anti-BMP-5 (1μg/mL). At different days post-confluence, cells were fixed and stained with Alcian blue for cartilage matrix (A) or Alizarin Red for matrix mineral (B). Culture days post-confluence are indicated. Treatments of the cultures are indicated at the left. C= untreated control; 1, 10 and 100 indicate ng/mL of recombinant BMP-5 added to the cultures. Digital scans of the plates were analyzed quantitatively, and results are shown in D (alcian blue) and E (alizarin red). Means + 1 SEM are shown. *t*-tests for significance of alcian blue: Control versus 1 or 10 ng BMP-5: not significant at any time point. Panel C shows effect of 100 ng/mL of BMP-6 or BMP-7 treatment for 9 days after plating on alcian blue staining. Left-hand, untreated control well shows no cartilage synthesis, whereas both BMP-5 and -6 show strong staining, while BMP-7 had no effect. Analysis of data in D and E: Control versus 100 ng BMP-5: day 0 $P > 0.05$; day 9 $P < 0.0005$; day 13 $P < 0.0005$; day 17 $P < 0.005$. Tests for significance of alizarin red: control vs. 1 or 10 ng BMP-5 not significant at any point; Control vs. 100 ng BMP-5, day 13 $P < 0.005$; others not significant.

Table 1

Intron-spanning RT-PCR primers for the mRNAs indicated on left are shown, along with the expected base pair size of the PCR products. The resulting PCR products were cloned and sequenced to confirm identity.

mRNA	Primer	Product size
Col α1(X)	5'-CCTTTTGGTCCTGGTAACCC-3'	305 bp
	5'-GGTCATACTGGAGAGCCTGG-3'	
Col α1(II)	5'-GTCATACTGGAGAGCCTGG-3'	152 bp
	5'-GCTGACCTGACCTGATATTCAA-3'	
BMP-5	5'-TGGATTTATTGCGGTTTTGG-3'	270 bp
	5'-CACCTGATGTTTCCTGACCA-3'	
BMP-4	5'-GCTCTTCTTCCTCCTCCTCC-3'	153 bp
	5'-GCTCTGCTTTTCGTTTCTTCTT-3'	
BMP-6	5'-CGATTGCGACTCTGCTGTC-3'	216 bp
	5'-CTTCCCTCTCAATGCACACA-3'	
BMP-7	5'-TCTTCAGGATGACGTTGGAGCTGT-3'	249 bp
	5'-TCTTCAGGATGACGTTGGAGCTGT-3'	
BMPRIa	5'-GGTCAGCAATACAGCAACTACC-3'	160 bp
	5'-CCATTTCCAGCCCTACATCA-3'	
BMPRII	5'-GCGAGGAAGTCATCCCT-3'	352 bp
	5'-ATGGAACATACCGTTTTTGC-3'	