Published in final edited form as:

Atherosclerosis. 2008 August ; 199(2): 271–277. doi:10.1016/j.atherosclerosis.2007.11.031.

BMP-2 Promotes Phosphate Uptake, Phenotypic Modulation, and Calcification of Human Vascular Smooth Muscle Cells

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Abstract

Vascular calcification is associated with increased risk of cardiovascular events that are the most common cause of death in patients with end-stage renal disease. Clinical and experimental studies indicate that hyperphosphatemia is a risk factor for vascular calcification and cardiovascular mortality in these patients. Our previous studies demonstrated that phosphate transport through the type III sodium-dependent phosphate cotransporter, Pit-1, was necessary for phosphate-induced calcification and osteochondrogenic phenotypic change in cultured human smooth muscle cells (SMC). BMP-2 is a potent osteogenic protein required for osteoblast differentiation and bone formation that has been implicated in vascular calcification. In the present study, we have examined the effects of BMP-2 on human SMC calcification *in vitro*. We found that treatment of SMC with BMP-2 enhanced elevated phosphate-induced calcification, but did not induce calcification under normal phosphate conditions. mRNAs for BMP receptors, including ALK2, ALK3, ALK6, BMPR-II, ActR-IIA and ActR-IIB were all detected in human SMCs. Mechanistically, BMP-2 dose-dependently stimulated phosphate uptake in SMC (200 ng/ml BMP-2 vs vehicle: 13.94 vs 7.09 nmol/30 min/mg protein, respectively). Real-time PCR and Western blot revealed the upregulation of Pit-1 mRNA and protein levels, respectively, by BMP-2. More importantly, inhibition of phosphate uptake by a competitive inhibitor of sodium-dependent phosphate cotransport, phosphonoformic acid, abrogated BMP-2-induced calcification. These results indicate that phosphate transport *via* Pit-1 is crucial in BMP-2-regulated SMC calcification. In addition, BMP-2 induced Runx2 and inhibited SM22 expression, indicating that it promotes osteogenic phenotype transition in these cells. Thus, BMP-2 may promote vascular calcification via increased phosphate uptake and induction of osteogenic phenotype modulation in SMC.

Keywords

BMP-2; sodium-dependent phosphate cotransporter; Pit-1; vascular calcification

1. Introduction

Vascular calcification is highly prevalent in the patients with atherosclerosis, diabetes and end-stage renal disease (ESRD). Vascular calcification is associated with increased risk of cardiovascular events that are the most common cause of death in patients with ESRD [1].

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Clinical studies have shown that hyperphosphatemia is an important contributor to vascular calcification and cardiovascular mortality in ESRD patients [1]. Elevated phosphate and calcium x phosphate product are significant predictors of cardiovascular mortality in hemodialysis patients, and the control of serum phosphate by dietary phosphate restriction or phosphate binders is now recognized as an important method to prevent arterial calcification in these patients [1]. Consistent with the clinical observations, mice with targeted-deletions of either *klotho*, a gene that encodes a membrane protein, or *FGF23*, a gene encoding a phosphaturic hormone, develop hyperphosphatemia as well as aortic calcification [2, 3]. These studies underscore the importance of phosphate in the process of vascular calcification.

Vascular calcification often presents with elements that mimic osteogenesis morphologically and biochemically. Several molecules that normally regulate osteoblast differentiation and bone formation have been found in calcifying vessels and valves, including the osteogenic transcription factor Runx2, osteopontin, bone sialoprotein, osteocalcin, type I collagen and BMP-2 [4–6]. In addition, our previous studies as well as those of others have shown that elevated phosphate induces SMC calcification and osteogenic phenotypic modulation, as evidenced by induction of Runx2 and inhibition of SMC lineage markers, SM22 and SM α -actin [6, 7]. More recently, we demonstrated that inhibition of phosphate uptake by RNA silencing of the sodium-dependent phosphate cotransporter, Pit-1, blocked phosphate-induced calcification as well as phenotypic transition in human SMC [8]. These studies suggest that vascular calcification and bone formation may share common regulatory mechanisms.

BMP-2 is an important molecule in the regulation of bone formation as well as vascular calcification. BMP-2 was originally identified as a protein that induced ectopic bone formation. In bone, BMP-2 promotes osteoblast differentiation and mineralization. Blockage of BMP-2 action by the antagonist, noggin, inhibits osteoblast differentiation and bone formation *in vivo* and *in vitro* [9]. Interruption of the receptor signal by targeted-deletion of BMP type I receptor results in reduced mineralization and bone mass [10]. Recently, several studies have suggested that BMP-2 may also be important in vascular calcification under pathological conditions. BMP-2 expression was found in calcified human atherosclerotic lesions [4]. BMP-2 was also expressed in calcified arteries of *LDLR*^{-/-} mice fed a high-fat diet [11]. In addition, treatment of calcifying vascular cells or bovine smooth muscle cells *in vitro* with BMP-2 resulted in enhanced calcification [12, 13]. Finally, mice with targeted-deletion of Smad6, an inhibitory Smad in the BMP2 signaling cascade, present with vascular calcification [14]. These studies suggest an important role of BMP-2 in regulation of vascular calcification.

In the present studies, we examined the mechanism by which BMP-2 promotes SMC calcification *in vitro*. We report that BMP-2 dose-dependently enhanced phosphate-induced calcification in human SMC. BMP-2 effects on calcification could be explained by its ability to increase phosphate uptake by up-regulation of Pit-1, previously shown to be required for calcification in this system [8], as well as its ability to induce osteogenic phenotype transition in SMC. These studies are the first to show that BMP-2 induces phosphate uptake and Pit-1 expression in vascular SMC.

2. Methods

2.1. Cell culture

Human immortalized aortic SMC were described previously [8]. Human primary aortic SMC were obtained from Clonetics Corporation (Palo Alto, CA). SMC phenotype of these cells were characterized by the expression of SM22 (data not shown). Cells were routinely

cultured in growth medium containing Delbecco's Modified Eagle's Medium (DMEM) supplemented 15% FBS, 100 U/mL of penicillin and 100 µg/mL of streptomycin.

2.2. Reverse transcription PCR and real-time quantitative PCR

Total RNA was isolated from cultured SMC using RNeasy kit from Qiagen (Chatsworth, CA). Reverse transcription was performed using Omniscript Reverse Transcriptase from Qiagen. The primers used for PCR amplification were: 1) Human ALK2 forward 5'-TCAGGAAGTGGCTCTGGTCT-3' and reverse 5'-CGTTTCCCTGAACCATGACT-3', the amplified fragment corresponds to base pairs 568–747 of human ALK2. 2) Human ALK3 forward 5'-TGATG TGCCCTTGAATACCA -3' and reverse primer 5'-ATTCTTCCACGATCCCTCCT -3', the amplified fragment corresponds to base pairs 1182 – 1357 of human ALK3. 3) Human ALK6 forward 5'-AAATGTGGGCACCAAGAAAG -3' and reverse 5'-ACAGGCAACCCAGAGTCATC -3', the PCR product is 171 base pairs and corresponds to position 27 – 197 of human ALK6. 4) Human BMPR-II forward 5'-GGACGCATGGAATATTGCT -3' and reverse primer 5'-ATCTCGATGGGAAATTGCAG -3', which corresponds to base pairs 811 – 999 of the human BMPR-II NHA. 5) Human ActR-IIA forward 5'-TTTCCGGAGATGGAAGTCAC -3' and reverse primer 5'-ACAGGAGGGTAGGCCATCTT -3', which corresponds to base pairs 349 – 512 of the human ActR-IIA. 6) Human ActR-IIB forward 5'-GAAGATGAGGCCACCATT -3' and reverse primer 5'-GACAGAGGTCACCAGGGAAA -3', which corresponds to base pairs 1302 – 1500 of the human ActR-IIB. The identities of the amplifications as human BMP receptors were confirmed by DNA sequence analysis.

Levels of human Pit-1, Runx2 and SM22 mRNAs were determined by quantitative real-time PCR performed with TaqMan PCR reagents kits using the ABI Prim 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The primers and probes for Pit-1, Runx2 and SM22 have been described previously [8]. Quantification of gene expression was calculated by the standard curve method according to the manufacturer's protocol and normalized to 18S rRNA.

2.3. Western blot analysis

Pit-1 protein levels were detected by anti-Pit-1 antibody as described previously [8].

2.4. Phosphate uptake assay

Phosphate uptake assay was described previously [8]. In brief, phosphate uptake was performed in Earle's Buffered Salt Solution (EBSS) containing $\text{H}_3^{32}\text{PO}_4$ (PerkinElmer Life Science, Inc. Boston, MA) at 37°C for 30 minutes. After rinsing with cold EBSS for three times, the cell layer was solubilized with 1 ml of the solution containing 0.1N NaOH and 0.1% SDS. The radioactivity of cell lysates was counted by LS 6500 Scintillation Counter (Beckman, Fullerton, CA). Sodium-dependent phosphate uptake was determined by subtracting uptake in the presence of EBSS containing choline from uptake in EBSS containing sodium. Uptake values were normalized by the protein content of the cell culture.

2.5. Calcification assay

Human recombinant BMP-2 (R&D Systems, Minneapolis, MN) was added every 2 days during the treatment period. SMC calcification was induced by incubation with calcifying medium (growth medium supplemented with $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ to various final concentrations of phosphate) for the indicated time frames. Calcium deposited in the extracellular matrix of the cell was extracted with 0.6N HCl overnight. After removing the HCl supernatant, the remaining cell layers on the plates were solubilized with lysis buffer

containing 0.1N NaOH and 0.1% SDS. Calcium contents in the HCl supernatant were determined by the *O*-cresolphthalein complexone method and normalized by protein concentration of the same well of the culture.

2.6. Statistical analysis

Results are expressed as mean $\pm$ SD. Significance between groups was determined by ANOVA, *P*-values less than 0.05 were considered significant.

3. Results

3.1. Expression of BMP receptors in human SMC

BMP-2 has been previously observed to enhance phosphate-induced calcification of bovine SMC [13]. Therefore, we first confirmed that human SMC contained BMP receptors, and that BMP-2 could enhance phosphate-induced calcification in these cells. BMPs initiate their actions by binding to transmembrane serine/threonine receptors, belonging to TGF- β /BMP receptor superfamily. The receptors have been classified as type I and type II receptors. At least three members of each type have been determined to bind to BMPs. Type I receptors include ALK2, ALK3 and ALK6 and type II receptors include BMPR-II, ActR-IIA and ActR-IIB. Binding to both type I and type II receptors are required for activation of BMPs signals. BMP-2 preferentially binds to ALK3, ALK6 and BMPR-II [16]. In order to determine the profile of BMP receptors expressed in human SMC, RT-PCR was performed using primers specific for each type I and type II BMP receptor. As shown in Fig 1, strong bands were obtained using primers for ALK2, ALK3, ALK6 and BMPR-II, weak bands were amplified using primers for ActR-IIA and ActR-IIB. Sequence analysis of these amplified fragments confirmed their identities as human BMP receptors. These results indicated that both type I and type II BMP receptors are expressed in cultured human SMC.

3.2. BMP-2 enhances phosphate-induced human SMC calcification

Next, confluent human SMC were treated with various concentrations of phosphate in the presence or absence of 200 ng/ml of human recombinant BMP-2 for 10 days. The concentrations of BMP-2 used were chosen based on previous study [13] and the potency of commercial preparations as indicated by the manufacturers. As shown in Fig 2A, calcification analysis indicated that BMP-2 increased matrix mineralization in the presence of various elevated phosphate concentrations (2.0, 2.3 and 2.6 mM) compared with vehicle. However, no mineralization was observed in SMC treated with BMP-2 under normal phosphate conditions (1.4 mM). To examine temporal effects of BMP-2 on calcification, SMC were incubated with 2.2 mM phosphate for 8 or 12 day. Fig 2B shows that a slight but significantly increased calcification was observed after 8 day treatment. The enhanced calcification by BMP-2 was more pronounced following 12 day treatment (BMP-2 vs vehicle: 176 vs 98 μ g Ca/mg protein). These results suggest that BMP-2-treated SMC are more susceptible to calcification than untreated cells, and that elevated phosphate is necessary for BMP-2 stimulatory effect on calcification, consistent with studies in bovine SMC cells [13].

3.3. BMP-2 increases phosphate uptake in human SMC

Since the effect of BMP2 on human SMC calcification was only observed under elevated phosphate conditions, we hypothesized that one way this factor might function was by modulating phosphate entry into the cell. Our previous studies indicated that phosphate uptake via sodium-dependent phosphate cotransporter, Pit-1, was required for calcification induction in human SMC, and the level of Pit-1 on the cell surface controlled SMC susceptibility to calcification [8]. Thus, we examined the effect of BMP-2 on phosphate

uptake in human SMC. Cells were incubated with various concentrations of BMP-2 or vehicle for 6 hour and phosphate uptake assays were performed as described in the methods. As shown in Fig 3A, BMP-2 treatment caused a dose-dependent increase in phosphate uptake (200 ng/ml BMP-2 vs vehicle: 14.6 vs 7.5 nmol/30 min/mg protein). SMC were incubated in 200 ng/ml of BMP-2 for 0.5, 2, 6 and 16 hours, uptake assay revealed that the stimulatory effect of phosphate uptake by BMP-2 was detected as early as at 2 hours following BMP-2 treatment and reached a maximal value after 6 hours (Fig 3B). These results indicate that BMP-2 causes a dose- and time-dependent increase of phosphate uptake in SMC.

3.4. Up-regulation of Pit-1 mRNA and protein by BMP-2

Phosphate uptake is mediated by sodium-dependent phosphate cotransporters. We previously identified that the type III sodium-dependent phosphate cotransporter, Pit-1, was a major transporter of phosphate in human SMC [8, 15]. To determine the expression levels of Pit-1 following BMP-2 treatment, human SMC were treated with 200 ng/ml of BMP-2 for 2 hours, total RNA was isolated for detection of expression levels of Pit-1 by real-time PCR. As shown in Fig 4A, BMP-2-treated SMC showed a 2 fold increase of Pit-1 mRNA levels compared with vehicle-treated cells. In contrast, mRNA levels of Pit-2, the other sodium-dependent phosphate cotransporter expressed in human SMC, were not increased (data not shown). The protein levels of Pit-1 were increased comparably following BMP-2 treatment for 12 hours (Fig 4B and C).

3.5. BMP-2-stimulated calcification is Pit-1 dependent

In order to further determine the role of phosphate uptake *via* Pit-1 in BMP-2-stimulated calcification, SMC were incubated with 0.5 mM of phosphonoformic acid (PFA), a competitive inhibitor of sodium-dependent phosphate cotransport, in the presence of BMP-2 or vehicle control for 10 days. Fig 5 shows that PFA blocked BMP-2-induced calcification (PFA vs vehicle: 14.58 vs 142.82 μ g Ca/mg protein), suggesting that Pit-1 is a key regulator for BMP-2-stimulated SMC calcification.

3.6. Induction of osteochondrogenic phenotype by BMP-2 in human SMC

We and others previously showed that SMC calcification occurs concomitant with an osteochondrogenic phenotype change, as evidenced by induction of the osteochondrogenic marker Runx2 and reduction of smooth muscle lineage marker, SM22 [6, 7]. To better understand the mechanism by which BMP-2 increases phosphate-induced calcification, we measured the mRNA levels of Runx2 and SM22 by real-time PCR in human primary SMC, which is a more suitable model for the study of phenotype change. Total RNA was isolated from SMC treated with 200 ng/ml of BMP-2 for 2 days. As shown in Fig 6, BMP-2 treatment caused a two-fold increase in Runx 2 expression, indicating the osteogenic effect of BMP-2 on SMC. In contrast, SM22 expression was substantially reduced by BMP-2. These results suggest that BMP-2 induces osteogenic phenotype change in human SMC, in addition to promoting phosphate uptake.

4. Discussion

The idea that BMP-2 might regulate vascular calcification was first posited by Demer et al [4] who identified BMP-2 expression in human atherosclerotic lesions. Studies by Bostrom et al further suggested that matrix gla protein (MGP), a protein expressed at high levels in the artery wall, normally inhibits arterial calcification by binding and blocking BMP-2 interaction with its receptors [17]. Indeed, MGP deficient mice die between 4 and 6 weeks of age due to severe arterial calcification and aortic rupture, potentially due to unopposed BMP-2 activity [18]. Towler et al established a hyperlipidemic and diabetic mouse model

for vascular calcification and provided evidence that a BMP-2-Msx2-Wnt signal pathway is involved in vascular calcification under these conditions [11]. Finally, Chen et al found significantly higher BMP2 levels in uremic serum compared to normal serum, and demonstrated that BMP-2 could induce calcification of phosphate-treated bovine smooth muscle cells [13]. Thus, these studies all point to an important role for BMP-2 in regulating vascular calcification. However, the mechanisms by which BMP2 regulates vascular calcification remain to be elucidated.

In the present studies, we have investigated potential mechanisms whereby BMP-2 enhances vascular calcification in cultured human SMC. We found that BMP-2 treatment enhanced elevated phosphate-induced calcification in human SMC, but did not promote calcification under normal phosphate conditions, indicating that the action of BMP-2 was dependent on the presence of elevated phosphate. We previously reported that elevated phosphate-induced SMC calcification was dependent on the activity of the sodium dependent phosphate cotransporter, Pit-1. Therefore, we examined the effect of BMP-2 on phosphate uptake. We found that BMP-2 increased phosphate uptake in human SMC *via* upregulation of the type III sodium-dependent phosphate cotransporter, Pit-1. Blockage of phosphate uptake by PFA abrogated BMP-2-stimulated calcification, indicating the important role of phosphate uptake via Pit-1 in BMP-2's procalcifying actions. Mechanistically, BMP-2 promoted osteochondrogenic phenotype transition of SMC, as evidenced by an increase of Runx2 (osteochondrogenic marker) and a decrease of SM22 (SMC marker) expression. Our results are the first to demonstrate that increased phosphate uptake and upregulation of Pit-1 expression are important mechanisms by which BMP-2 may regulate vascular calcification. Our studies provide a novel mechanism of how osteotropic factors, such as BMP-2, regulate vascular calcification and further support the notion that osteogenesis and vascular calcification might share common regulatory mechanisms. In addition, the findings further confirm our previous studies indicating that phosphate uptake through Pit-1 is crucial for vascular calcification *in vitro* [8].

The mechanisms underlining BMP-2-upregulated Pit-1 expression remains to be determined. Multiple signaling pathways may involve in BMP-2 effects. Csiszar et al showed that ERK and PKC play important roles in BMP-2-induced proinflammatory phenotype in endothelial cells [19]. In addition, JNK and PI3-kinase are also involved in BMP-2-upregulated Pit-1 expression [20, 21].

Many cell types express BMP-2, such as osteoblasts, chondrocytes, T-cells, endothelial cells and vascular SMC [9, 13, 22–24]. BMP-2 promotes mineralization of adjacent cells in a paracrine manner. For instance, BMP-2 released from T-cells and endothelial cells induces osteogenic differentiation and mineralization of adjacent cells, such as mesenchymal stromal cells or SMC [23, 24]. BMP-2 induction in the vasculature is regulated by many factors, including inflammation, oxidative stress and hyperglycemia [11, 22, 25]. Among these BMP-2 inducing factors, inflammation plays more important role in the regulation of vascular calcification. The proinflammatory cytokine, tumor necrosis factor- α (TNF- α), induces calcification in calcifying vascular cells [26]. In addition, endothelial cells are major targets of proinflammatory cytokines such as TNF- α and interleukin-1 β (IL-1 β) and these proinflammatory cytokines regulate BMP-2 expression in pathological conditions. For example, studies by Csiszar et al showed that TNF- α increased BMP-2 expression in endothelial cells [19, 27]. Thus, under inflammatory conditions, BMP-2 released from endothelial cells may act on adjacent SMC, induce osteochondrogenic differentiation and result in calcification in the arterial tree. Consistent with this notion, serum levels of TNF- α or other cytokines are associated with common complications such as vascular calcification in patients with ESRD [28] and elevated serum levels of BMP-2 have been found in uremic

patients [29]. Thus, interruption of BMP-2 signal may be a potentially therapeutic target for intervention of vascular calcification.

Phosphate uptake is a saturable, carrier-mediated process. Phosphate uptake by osteoblasts occurs concomitant with mineralization [30]. Studies by Yoshiko et al demonstrated that phosphate uptake *via* type III sodium-dependent phosphate cotransporter Pit-1, but not Pit-2, plays a crucial role in the regulation of bone mineralization both *in vivo* and *in vitro* [31]. Many osteotropic factors regulate phosphate uptake are associated with osteogenic differentiation and mineralization in osteoblasts. Upregulation of Pit-1 may be a common mechanism by which osteotropic factors, such as BMP-2, TGF- β and PDGF, promote biomineralization [20, 21, 32]. Consistent with these studies, we previously reported that phosphate uptake *via* Pit-1 was also required for SMC calcification, suggesting a crucial role of Pit-1 in osteogenic differentiation and mineralization of SMC [8]. Here we demonstrate a novel mechanism in which BMP-2 upregulates Pit-1, whose expression is required for SMC calcification, and promotes vascular calcification. Interestingly, while our studies were in progress, Suzuki et al reported that BMP-2-induced phosphate uptake and mineralization in osteoblast-like cells also required Pit-1 expression [20]. Thus, it is likely that upregulation of Pit-1 and enhanced phosphate uptake are common mechanisms by which BMP-2 promotes bone formation as well as vascular calcification.

In summary, we found that BMP-2 treatment resulted in phenotype change and elevated phosphate-induced mineralization in human SMC culture. The effects of BMP-2 are likely mediated by upregulation of Pit-1 expression and phosphate uptake. This study has provided a novel mechanism underlying procalcifying action of BMP-2 on human SMC.

Acknowledgments

This work was supported by grants HL-62329 and HL-081785 from the National Institutes of Health.

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Fig. 1.

Expression of BMP receptors in human SMC. Total RNA was isolated from human immortalized SMC and cDNA synthesis was performed using Omniscript Reverse Transcriptase (Qiagen). Identical amounts of cDNA were used for PCR reactions with primers specific to ALK2, ALK3, ALK6, BMPR-II, ActR-II-A and ActR-II-B.

Fig. 2. BMP-2 enhances phosphate-induced SMC calcification. Confluent human immortalized SMC were incubated with various concentrations of phosphate in the presence or absence of 200 ng/ml of human recombinant BMP-2 for 10 days (A) or 2.2 mM phosphate in the presence or absence of 200 ng/ml of human recombinant BMP-2 for the indicated days (B). Calcium content of the extracellular matrix is given as mean $\pm$ S.D. (n = 3). * P < 0.05.

Fig. 3. BMP-2 increases phosphate uptake in human immortalized SMC. (A). Human SMC were seeded in 24-well plates 1 day prior to the uptake assay. After treatment with different concentrations of BMP-2 for 6 hrs, phosphate uptake assays were performed by incubation of SMC with EBSS containing $H_3^{32}PO_4$ for 30 min. (B). Human SMC were treated with 200 ng/ml of BMP-2 for the indicated times, and the uptake assays were carried out as described in A. Uptake values were normalized to cellular protein content. The results are presented as mean $\pm$ S.D. (n = 3). *Significant increase compared with vehicle ($P < 0.05$).

Fig. 4. BMP-2 upregulates Pit-1 mRNA and protein levels. (A) Human immortalized SMC were treated with 200 ng/ml of BMP-2 for 2 hrs, total RNA was isolated and Pit-1 mRNA levels were determined by quantitative real-time PCR. Pit-1 mRNA levels were normalized to 18S rRNA levels. Data are expressed as means $\pm$ SD (n=3). * P < 0.05. (B) Human immortalized SMC were treated with 200 ng/ml of BMP-2 for 12 hrs, protein lysates were prepared and Pit-1 and β -tubulin were detected by immunoblot analysis. (C) Pit-1 protein levels were quantified and normalized to β -tubulin levels. Data are presented as the percentage of SMC treated with vehicle.

Figure 5. Phosphonoformic acid blocks BMP-2 induced calcification. Human immortalized SMC were incubated with 200 ng/ml of human recombinant BMP-2 in the presence of 0.5 mM of PFA for 10 days. Calcium content of the extracellular matrix is given as mean $\pm$ S.D. (n = 3). * P < 0.05.

Fig. 6. BMP-2 induces osteogenic phenotype in human SMC. Human primary SMC were treated with 200 ng/ml of human recombinant BMP-2 for 2 days. Total RNA was isolated and human Runx2 (A) and SM22 (B) mRNA levels were quantitated using real-time PCR, and normalized to 18S rRNA levels. $P < 0.05$