Requirement of a bone morphogenetic protein for the maintenance and stimulation of osteoblast differentiation

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Summary. The requirement of a bone morphogenetic protein for the maintenance and stimulation of an osteoblast phenotype was examined using mouse MC3T3-E1 cell cultures. Cells expressed BMP-4 mRNA, which correlated with the stimulation of the osteoblast phenotype. The addition of a BMP-4 specific antibody reduced bone nodules, suggesting that BMP-4 is required for the osteogenic activity of osteoblasts in an autocrine manner. Exogenously added BMP-7 gradually decreased the expression of BMP-4 with a concurrent stimulation of the osteoblast phenotype. Exogenous BMP-7 can therefore substitute for endogenously produced BMP-4 acting as a paracrine factor on osteoblasts. The addition of 17β estradiol decreased BMP-4 expression but initiated synthesis of BMP-6 mRNA, an endocrine signal for osteoblasts, which also substituted for the lack of endogenous BMP-4, as evidenced by normal bone nodule formation. The addition of dexamethasone and parathyroid hormone did not affect the BMP-4 expression but induced transcripts for BMP-2 and BMP-3, respectively, suggesting that their effects on bone can be in part achieved via the BMP signaling. These experiments support the requirement of a BMP for osteoblast differentiation and function, demonstrating for the first time that a BMP can functionally substitute for another BMP in an autocrine/paracrine manner or mediate a response to an endocrine action on osteoblasts.

Introduction

Bone formation and bone resorption are regulated by local and systemic factors acting in concert to maintain skeletal bone mass. Bone morphogenetic proteins (BMPs) are growth and differentiation factors originally isolated from the bone matrix based on their ability to induce new bone formation in vivo (Reddi, 1998). A single recombinant BMP with an appropriate carrier is sufficient to restore lost bone in post fetal life by recapitulating the cellular events that are involved in the formation of embryonic bone in rodents, large mammals, and humans (Martinovic et al., 2002). BMPs have been shown to influence the recruitment, proliferation, and differentiation of osteoblast progenitors and to maintain phenotype markers in mature osteoblasts (Martinovic et al., 2004). The alignment of protein sequences in the cysteine domains of BMPs reveals striking sequence similarities and differences among the family members. When compared with other BMPs, BMP-7 is most closely related by sequence to BMP-5 and BMP-6 with 88% and 87% amino acid sequence identities, respectively, in the cysteine rich C-terminus domain. BMP-7 is more distantly related to BMP-2 and BMP-4, having 60% and 58% identities, respectively. GDF-5, another BMP that has been extensively evaluated, has even less similarity, showing a 51% identity. BMP-3, on the other hand, is not osteoinductive and, in fact, is more distant, having a 42% identity (Martinovic et al., 2002).

According to the one proposed model (Stein and Lian, 1993), the maturation of the osteoblast phenotype in osteoblast primary cultures occurs via three consecutive phases: proliferation, extracellular matrix maturation, and mineralization. These include the synthesis of type I collagen, the expression of alkaline phosphatase (ALP),
the secretion of osteocalcin and the production of a mineralized matrix. The expression of these phenotypic markers can be modulated by systemic factors.

Previous studies have shown that BMPs are capable of enhancing the expression of markers that are characteristic of the osteoblast phenotype in mesenchymal progenitors (Asahina et al., 1993, 1996; Ju et al., 2000), bone marrow stromal cells (Yamaguchi et al., 1996; Abe et al., 2000a), C2C12 myoblast cells (Yeh et al., 2002; Katagiri et al., 2002), and C3H10T1/2 mouse embryoid-derived mesenchymal cells (Kaps et al., 1998; Bachner et al., 1998; Spinella-Jaegle et al., 2001), MC3T3-E1 mouse calvaria-derived osteoblasts (Vukicevic et al., 1990; Spinella-Jaegle et al., 2001; Xiao et al., 2002), and rat calvaria-derived primary osteoblast cells (Vukicevic et al., 1989; Hughes et al., 1995; Chen et al., 1997; Hino et al., 1999). However, little is known regarding the temporal distribution of BMPs during the differentiation of osteoblasts.

In another in vitro model, the preosteoblastic cell line KS483 synthesized various BMPs and their receptors throughout the differentiation process. Inhibition of their activity, as in our study, resulted in a decreased mineralized nodule area independent of the phase of osteoblast differentiation, confirming that BMPs are essential for bone formation. Moreover, exogenous BMP-4 additionally stimulated KS483 cell differentiation. It was not tested whether the addition of BMP-4 influenced the expression of other BMP family members (van der Horst et al., 2002). The authors found that autocrine BMP signaling is not only required for the initiation of differentiation but also during matrix maturation and mineralization (van der Horst et al., 2002). In fetal rat calvarial cells, the expression of various BMPs has been detected, with BMP-2 and BMP-7 mRNA unchanged, with BMP-4, -5 and -6 gradually increasing during the differentiation process (Harris et al., 1994; Yeh et al., 2000).

In order to determine the expression level and profile of BMP molecules during the differentiation process in vitro in comparison with known osteoblastic markers, we used MC3T3-E1 cells at different stages of the differentiation process. In parallel, we explored the effect of BMP-7 (OP-1), dexamethasone, the parathyroid hormone, and 17β-estradiol on the expression levels of investigated genes and their functions. The results indicate that MC3T3-E1 cells produce BMP-4, whose expression decreases during normal differentiation in vitro, and which is both necessary and sufficient for osteoblastic differentiation and bone matrix deposition. Moreover, a functional redundancy exists between BMP-7 and BMP-4 in which BMP-7 can substitute for BMP-4 in an autocrine and paracrine manner or mediate a response to an endocrine action of osteoblasts.

Materials and Methods

Cell culture

The mouse osteoblast-like MC3T3-E1 cell line, derived from newborn mouse calvaria, was routinely cultured in a modified medium (αMEM, Sigma; Gaithersburg, MA, USA) supplemented with 10% fetal bovine serum (FCS, Gibco BRL; Grand Island, NY, USA) and penicillin/streptomycin (10 U/0.01 mg/ml, Sigma). For experiments, cells were cultured under standard conditions in 15 cm dishes, 6 or 96 well plates (Becton Dickinson; Franklin Lakes, NJ, USA) or 8 well chamber slides (Nunc; Naperville, IL USA) until confluence. β-glycerophosphate (5 mM, Sigma) and ascorbic acid (50 μg/ml, Sigma) were added when the cells reached confluence. Parallel cultures were treated with 20 ng/ml BMP-7 (OP-1, Creative BioMolecules; Hopkinton, MA USA), dexamethasone, or the parathyroid hormone (10−8 M, Sigma) for 12 h and 72 h for each time point (days 0, 3, 7, 12 and 17 following confluence) in a medium with a reduced amount of serum (1%). In a separate experiment, cells were grown in the medium without phenol red (Gibco BRL) with 10% charcoal treated FCS (Sigma) until confluence. The media were then replaced by media containing 0.1% bovine serum albumin (BSA) and different concentrations of 17β-estradiol (10−11 to 10−7 M; Sigma), and the cells were further incubated for 48 or 72 h.

RT-PCR analysis

At designated time points (days 0, 3, 7, 12 and 17 following confluence, and 12, 48 or 72 h after treatment), total RNA was extracted with TRIzol (Gibco BRL) and cDNA was synthesized from 4 μg of total RNA with Superscript II Rnase H-Reverse Transcriptase as indicated by the manufacturer (Gibco BRL). After initial denaturation at 94 °C for 5 min, thirty-two to forty cycles of amplification were completed by denaturation for 40s at 94 °C, annealing at temperatures specified for each pair of primers for 40s, and extension for 60s at 72 °C. Samples were amplified with AmpliTaq DNA polymerase (Perkin Elmer Roche; New Jersey, USA) using the following primers: GAPDH (5′ ACC ACA GTC CAT GCC ATC AC, 3′ TCC ACC ACC CTG TTG CTG TA); BMP-2 (5′ CAG AGA CCC ACC CCC ACG A, 3′ CTG TTT GTG TTG GGC TTG AC); BMP-3 (5′ GTG GGA GGA GAG AAA GCC TT, 3′ AAC TGG CAC GCT CCA CAG GA); BMP-4 (5′ TCC CTG GTA ACC GAA TGC T, 3′ GGG GCT TCA TAA CCT CAT AA); BMP-5 (5′ TCT GTG TGT CCT CAC AGA C, 3′ GAT TTA TTG CGG TTT TGA TT); BMP-6 (5′ CAG GAG CAT CAG CAC AGA
GA, 3’ ATG TGT GCG TTG AGT GGG AA); BMP-7 (5’ GCA GAG CAT CAA CCC CAA GT, 3’ GGA CAG AGA TGG CTT TCG AGA C); alkaline phosphatase (5’ TGA GAC CCA CCG TGA AGA AGA C, 3’ AGC TTG GCA ACC CTG GGT AGA C); collagen type I (5’ GAC CGA TGG ATTCCA GTT CGA G, 3’ ACC AGA TGG GGA TGG AGG GAG T); osteopontin (5’ GTG AAC TCG GAT GAA TCT GAC G, 3’ CTT GTC CTC ATG GCT GTG AAA C) and osteocalcin (5’ TTG GCC CAG ACC TAG CAG ACA C, 3’ AGG ATC AAG TCC CGG AGA GCA G).

Reactions were performed in a GeneAmp 4800 thermal cycler (Perkin Elmer Cetus; Emeryville, CA, USA) for 32–40 cycles. To compare the relative quantity of the RT-PCR reactions, the transcription level of GAPDH, a “housekeeping” gene, was used as a control. Reactions without cDNA were used as a negative control and kidney cDNA as a positive control. Results were visualized by gel electrophoresis in 1% agarose (Seakem GTG, Bioproducts, Rockland, MA, USA) in a TAE buffer (TRIS HCl, acetic acid, EDTA, pH 8.0) and stained with ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA). Reactions were repeated at least twice.

**Immunostaining**

Cells were seeded in 8-well chamber-slides (Nunc) at a concentration of 12.5×10^3/ml (5×10^3/well) and incubated in α-MEM containing antibiotics and 10% FCS overnight. At confluence, β-glycerophosphate and ascorbic acid were added to the medium with 1% FCS, with or without BMP-7 (20 ng/ml), dexamethasone (10^-8 M), the parathyroid hormone (10^-8 M), or 17β-estradiol (10^-9 M). Cultures were incubated for 72 h and then fixed in 4% paraformaldehyde in PBS for 5 min, washed three times in PBS, immersed in methanol for 1 min, treated with 3% hydrogen peroxide for 5 min, and washed in PBS. Immunostaining was performed using a primary BMP-4 specific polyclonal antibody (Creative BioMolecules) raised in rabbits, with a biotinylated secondary antibody against rabbit IgG, and horseradish peroxidase-conjugated streptavidin.

**Osteocalcin radioimmunoassay**

Osteocalcin levels in the conditioned media were measured by a radioimmunoassay using a goat anti-mouse osteocalcin antibody (Biomedical Technologies; Stoughton, MA, USA). Medium samples were collected just before cells were homogenized for RNA isolation and stored at -20°C until analyzed.

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**Fig. 1.** A: Expression of BMPs and osteoblast differentiation markers in MC3T3-E1 cells during the differentiation process. Cells were cultured and analyzed as described in Methods. Controls represent samples isolated from cells cultivated for 17 days in a medium containing 10% FCS with or without β-glycerophosphate and ascorbic acid (-βG), p = positive control, n = negative control; ALP = alkaline phosphatase, Col I = collagen type I, OP = osteopontin, OC = osteocalcin. The size of the PCR product is indicated by a number on the left side. B: Effects of BMP-7 protein and BMP-4 antibody on bone nodule formation in MC3T3-E1 osteoblasts. Cells were treated with a polyclonal BMP-4 antibody (Ab), Herpes simplex antibody (Hs Ab), or BMP-7 protein. Data are presented as mean ± SEM. *P<0.05, ANOVA.
**Bone nodule formation assay**

The function of endogenous BMP-4 mRNA and exogenously added BMP-7 was also investigated in bone nodule formation experiments using a BMP-4 polyclonal antibody (5 µg/ml, Santa Cruz Biotechnology; Santa Cruz, CA, USA), BMP-6 antibody against the N-terminal domain (500 ng/ml; Santa Cruz Biotechnology), BMP-7 protein (OP-1, 20 ng/ml, Creative Biomolecules), dexamethasone, the parathyroid hormone, or 17β-estradiol (10⁻⁹ M) applied daily starting from day 5 until day 28 following confluence. An irrelevant and unrelated protein (herpes simplex virus glycoprotein D) was used in control experiments. The mineralization assay was performed as reported previously by Franceschi et al. (1994). Briefly, MC3T3-E1 cells were seeded in 24-well tissue culture plates at a density of 5 × 10⁴ cells/cm² and maintained in α-MEM containing 10% FCS with antibiotics overnight. On the following day, media were removed and cells were treated with α-MEM containing 10% FCS and 50 mg/ml ascorbic acid or vehicle control. At day 8, 4.5 mM inorganic phosphate was added to induce mineral nodule formation. On day 28, the von Kossa stain was used to determine the mineralized matrix formation and density of nodules quantified. The experiments were performed three times.

**Statistical methods**

Data were analyzed by Student's t-test or by one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test. P < 0.05 was considered significant. All data are presented as the mean ± SEM of three cultures.

**Results**

**Endogenous BMP-4 is required for osteoblast differentiation**

The expression of several osteoblastic markers in MC 3T3-E1 cells observed from day 0 to day 17 (Fig. 1A) resembled a pattern of gene transcription observed in
other osteoblast culture systems (Yamaguchi et al., 2000). Alkaline phosphatase expression was detected as early as when cells reached the confluency and then gradually increased in time with progression towards the late mineralization phase (Fig. 1A). The expression of collagen type I reached maximal values on day 7 of culture. Osteopontin expression increased towards day 7, was suppressed on day 12, and was lower on day 17. As expected, osteocalcin gene transcripts were detected later on days 12 and 17 following confluence (Fig. 1A).

Among the BMPs examined, BMP-4 mRNA and protein were expressed and attained a maximum level at day 3 following confluence—prior to the onset of differentiation, and then gradually decreased in time (Fig. 1A, 3B). The expression of other members of the BMP family was not detectable. Cells cultured with a BMP-4 specific polyclonal antibody from day 5 on following confluence showed a reduction in the number
of bone nodules formed on day 28, as compared with cells grown in absence of the BMP-4 specific antibody, suggesting an important role for BMP-4 in the function of MC3T3-E1 osteoblasts (Fig. 1B, 2).

**Exogenous BMP-7 can substitute for endogenous BMP-4**

When BMP-7 was added exogenously to the cultures at days 0, 3, 7, 12 or 17 for 12 h or 72 h, a complete inhibition of the BMP-4 gene expression (Fig. 4) and reduction in protein synthesis was observed (Fig. 3C). It appears that BMP-7 treatment inhibited the expression of BMP-4 regardless of the duration of exposure. However, the number of bone nodules increased (Fig. 1B, 5). This suggests that exogenous BMP-7 can functionally substitute for BMP-4 during the differentiation and maturation of osteoblastic MC3T3-E1 cells. The addition of BMP-7 did not, however, affect alkaline phosphatase, osteopontin, or collagen type I expression during the course of cell differentiation. On the contrary, exogenous BMP-7 increased the expression of osteocalcin mRNA on day 7 and then throughout the mineralization phase (Fig. 4). The stimulation of osteocalcin production was further confirmed in conditioned media using a mouse osteocalcin specific radioimmunoassay (Fig. 6A,B). Osteocalcin mRNA expression was increased both at 12 h or 72 h following the addition of BMP-7 (Fig. 4), while the osteocalcin protein was increased only in cultures examined at 72 h following BMP-7 addition (Fig. 6B).

**17β-Estradiol suppresses BMP-4 expression and induces mRNA for BMP-6**

To further explore the influence of an endocrine agent in this system, MC3T3-E1 cultures were treated with 17β-estradiol, which—like BMP-7 at 12 and 72 h following treatment—reduced the BMP-4 mRNA (data not shown) and the protein expression (Fig. 3F). Surprisingly, evaluation of the expression of other BMP-family members following treatment with various doses of 17β-estradiol revealed that only BMP-6 gene transcripts were induced (Fig. 7A). In parallel, estrogen treatment enhanced the expression of osteopontin, osteocalcin, and collagen type I mRNA during osteoblast differentiation and maturation (Fig. 7B) as well as bone nodule formation by MC3T3-E1 cells (Fig. 5). When a BMP-6 antibody was added parallel to 17β-estradiol, fewer bone nodules were found, suggesting that 17β-estradiol, at least partially, exerts its function on osteoblasts via the activation of BMP-6 (Fig. 8). The stimulation of osteocalcin gene expression by 17β-estradiol was further
confirmed in the conditioned media, showing a higher production of osteocalcin protein in cultures treated during the initiation of osteoblastic differentiation and throughout the maturation phase (Fig. 9).

**Dexamethasone induces expression of BMP-2**

Unlike BMP-7 and 17β-estradiol, the addition of dexamethasone to MC3T3-E1 osteoblasts did not reduce the BMP-4 gene and protein expression (Fig. 10, 3D). However, at 12 h of dexamethasone treatment, a transient

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**Fig. 6.** Osteocalcin (OC) protein synthesized by MC3T3-E1 cells in vitro. Cells were grown as described in Methods. BMP-7 was added to the medium at designated times for 12 h (A) and 72 h (B). Conditioned media were collected and analyzed using a specific OC radioimmunoassay. Data are presented as mean ± SEM. * P<0.05, Student’s t-test.

**Fig. 7. A:** Expression of BMP-6 in MC3T3-E1 cells treated with different concentrations of 17β-estradiol (10^{-11} to 10^{-7} M). Cells were cultured and analyzed as described in Methods. **B:** Expression of osteoblastic differentiation markers in MC3T3-E1 cells treated with 17β-estradiol (10^{-9} M). Col I = collagen type I, OP = osteopontin, OC = osteocalcin. The size of the PCR product is indicated by a number on the left side.
induction of the BMP-2 gene expression was observed. The expression of alkaline phosphatase, collagen type I, or osteopontin did not change (Fig. 10). However, the osteocalcin gene expression was markedly enhanced following both 12 h and 72 h of dexamethasone treatment. The protein content also increased from day 7 on in the conditioned media of treated cells (Fig. 11), resulting in the formation of more bone nodules, as evaluated at 28 days of culture (Fig. 5).

**Parathyroid hormone induces expression of BMP-2 and BMP-3**

Like dexamethasone, the parathyroid hormone did not influence the BMP-4 gene or protein expression in MC3T3-E1 cells (Fig. 12, 3E). In parallel to the expression of BMP-4 gene, cells treated with PTH for 12h during the first 7 days expressed mRNA of BMP-2 and BMP-3 (Fig. 12). Although the expression of alkaline phosphatase, collagen type I, osteopontin, or osteocalcin did not change (Fig. 12), the number of bone nodules formed was increased at day 28 as compared with the
Fig. 10. Expression of BMPs and osteoblast differentiation markers in MC3T3-E1 cells treated with dexamethasone (10^{-9} M). Cells were cultured and gene expression analyzed as described in Methods. ALP = alkaline phosphatase, Col I = collagen type I, OP = osteopontin, OC = osteocalcin. The size of the PCR product is indicated by a number on the left side.

control cultures (Fig. 5). However, the osteocalcin protein was increased in the conditioned media from day 7 on (Fig. 13B).

**Discussion**

Results of this study show that the BMP-4 gene and protein in preosteoblastic MC3T3-E1 cells are required for in vitro bone nodule formation. Both exogenous BMP-7 and 17β-estradiol suppress the BMP-4 gene transcription but maintain the process of MC3T3-E1 differentiation and even increase the number of bone nodules formed. While BMP-7 can substitute for BMP-4 in a paracrine manner, 17β-estradiol influences the bone nodule formation, at least in part, via a mechanism involving the synthesis of BMP-6 mRNA and protein. The expression of various components of the BMP signaling pathway in MC 3T3-E1 cells has been demonstrated earlier although the temporal expression of BMPs during the differentiation process has not been studied (Namiki et al., 1997; Wada et al, 1998; Funaba et al., 2001).

**Fig. II.** Osteocalcin protein synthesized by MC3T3-E1 cells treated with dexamethasone (10^{-9} M) for 12 h (A) or 72 h (B). Data are presented as mean ± SEM. * P < 0.05, Student’s t-test.

*The role of BMPs in osteoblast differentiation and bone formation*

In our study, MC3T3-E1 cells synthesized and produced only BMP-4, which was both required and sufficient for osteoblastic differentiation into mature cells forming bone nodules in vitro. In contrast to prior observations (Chen et al., 1997; Suzawa et al., 1999; Perreira et al., 2000), after a short increase in mRNA expression for BMP-4 (within first 72 h), a progressive down-regulation of BMP-4 transcripts was observed in MC3T3-E1 cells in correlation with an up-regulation of genes expressed...
Fig. 12. Expression of BMPs and osteoblast differentiation markers in MC3T3-E1 cells treated with the parathyroid hormone (10^{-9} M). Cells were cultured and gene expression analyzed as described in Methods. ALP = alkaline phosphatase, Coll = collagen type I, OP = osteopontin, OC = osteocalcin. The size of the PCR product is indicated by a number on the left side.

Fig. 13. Osteocalcin protein synthesized by MC3T3-E1 cells treated with the parathyroid hormone (10^{-9} M) for 12 h (A) or 72 h (B). Data are presented as mean ± SEM. * P < 0.05, Student’s t-test.

by differentiated osteoblasts. As shown earlier (ten Dijke et al., 1994), during the entire differentiation period, MC3T3-E1 cells constitutively expressed specific BMP receptors type I required for the differentiation capability of these cells (Namiki et al., 1997; Akiyama et al., 1997; Chen et al., 1998; Fujii et al., 1999), suggesting that signaling through the BMP pathway is a prerequisite for progression of the osteoblastic differentiation process towards the formation of mineralized nodules.

Regulation of BMP expression and function

Although it has been reported that BMPs can bind to different matrix components (Paralkar et al., 1990; Vukicevic et al., 1993; Franceschi, 1999) and that BMP-4 synthesized by MC3T3-E1 cells is stored in the extracellular matrix (Suzawa et al., 1999), it remains to be explored if its availability is regulated at the expression level by the presence of other growth factors (Chen and Fry, 1999). Results of our study suggest that the BMP gene expression profile in MC3T3-E1 cells during the differentiation process can be modulated by systemic hormones like estradiol, dexamethasone, and the parathyroid hormone. Furthermore, mechanisms of autoregulation were shown for several BMP molecules (Harris et al., 1995; Perreira et al., 2000) as well as regulation by closely related members of the BMP family in different in vitro systems (Chen et al., 1997; Honda et al., 1997). We here demonstrate that another BMP family member can successfully replace the function of BMP-4 in MC3T3-E1 cells without changing differentiation marker expression levels. It has been previously shown
that BMP-7 acts on early stage mesenchymal progenitor cells to induce chondroblastic differentiation (Asahina et al., 1996) and that it can induce differentiation along elements of the endochondral ossification pathway according to the stage and potential of the target cell. In this study, BMP-7 inhibited the synthesis of endogenous BMP-4 and enhanced the mature osteoblastic phenotype, suggesting a regulatory effect and possible overlapping roles for these molecules in the MC3T3-E1 cell system. It has also been shown earlier that BMP-4 and BMP-7 share the highest affinity for the same type I receptors, preferentially binding to ALK-6 (ten Dijke et al., 1994).

A BMP redundancy such as in this study has been previously shown for different BMPs in preclinical and clinical studies in healing critical size bone defects, delayed non-unions, and spinal fusions (Friedlender et al., 2001; Cook and Rueger, 2002). Although different BMPs have a restricted spatial and temporal distribution during fracture callus formation, the addition of one BMP may not perturb healing by changing the expression pattern of other members which appear during callus formation (Einhorn, 2003) and bone elongation in the mouse (Cho et al., 2002), rat (Sato et al., 1999), and human (Kloen et al., 2003).

Hormonal regulation of BMP expression

Dexamethasone treatment in this study induced the expression of several BMPs to promote the earliest phases of osteoblast differentiation. Although the stimulation of osteoblastic differentiation by glucocorticoids has been previously demonstrated, the mechanism of this enhancement is not clear. Glucocorticoids at physiological concentrations increase the bone nodule size and number, as has been demonstrated in rat calvarial cultures (Bellows et al., 1987). They further initiate osteoblastic differentiation in bone marrow stromal cell cultures (Cheng et al., 1994; Rickard et al., 1998) and act synergistically with different BMPs to increase their alkaline phosphatase activity or mineralized nodule formation (Takuwa et al., 1991; Boden et al., 1996; Rickard et al., 1998; Abe et al., 2000b). In this study, dexamethasone selectively induced the expression of BMP-2 during the early differentiation phase of MC3T3-E1 cells, which was in contrast with its deleterious effect on bone in vivo (Canalis, 2003). This may be due to a restricted cell response to glucocorticoids, such as the absence of BMP inhibitors including bone specific inhibitor sclerostin (SOST) (van Bezooijen et al., 2004).

Whether PTH acts in vivo—at least in part—via inducing BMPs in bone is not known and should be further explored. Its strong bone inducing effect when given daily in small amounts resulted in a new drug for treating severe osteoporosis in postmenopausal women (Black et al., 2003). We have recently shown that PTH, at least in part, exerts its bone anabolic activity via a BMP-related signalling pathway (Simic et al., 2006a).

Estrogen has a direct effect on the proliferation, differentiation, and protein synthesis of osteoblastic or bone marrow cells (Majeska et al., 1994; Robinson et al., 1997; Yamaguchi and Matsui, 1997; Balica et al., 1997; Qu et al., 1998), and some of the skeletal effects of estrogen may be modulated by the increased production of BMP-6 by osteoblasts (Rickard et al., 1994). In this study, 17β-estradiol also inhibited the expression of BMP-4 but induced the synthesis of BMP-6, which was also sufficient for the differentiation and deposition of mineralized bone nodules. Moreover, with 17β-estradiol treatment, the expression profile of specific BMP receptors by MC3T3-E1 cells changes. Besides ALK-3 and ALK-6, MC3T3-E1 cells treated with 17β-estradiol expressed the ALK-2 receptor, a specific BMP type I receptor reported to be preferentially used by BMP-6 in osteoblasts (Ebisawa et al., 1999). Recently, we showed that BMP-6 given systemically to aged ovariectomized rats has a strong bone anabolic effect and is not synergistic with estradiol (Simic et al., in press). Similarly, estrogen is not efficacious in restoring the trabecular bone of the peripheral skeleton in mice with BMP-6 gene knock-out, suggesting a collaborative effect of both estradiol and BMP-6 signaling in restoring bone (Simic et al., 2006b), which together support the in vitro results presented in this study.

Taken together, the results of the present study suggest that a single BMP, triggering a differentiation cascade, can be both required and sufficient for normal osteoblastic differentiation and extracellular matrix deposition in vitro, after which the preosteoblastic cells attain the mature osteoblastic phenotype. However, the suppression of BMP-4 by autoregulation or by another BMP suggests a necessary negative feedback to regulate BMP activity in the bone. Different local and systemic growth factors and hormones with the ability to modulate BMP gene expression can exert their effects on bone tissue. The multiple control levels described for this protein family emphasize its keyrole for bone morphogenetic proteins in osteoblastic differentiation and the maintenance of the bone tissue metabolism and homeostasis.

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References


