ARCHIVES OF ORAL BIOLOGY XXX (2010) XXX–XXX

available at www.sciencedirect.com

ScienceDirect

journal homepage: http://www.elsevier.com/locate/aob

Epidermal growth factor and bone morphogenetic proteins upregulate osteoblast proliferation and osteoblastic markers and inhibit bone nodule formation

Claude Laflamme, Sèverine Curt, Mahmoud Rouabhia *

Groupe de recherche en écologie buccale, Faculté de médecine dentaire, Pavillon de médecine dentaire, Université Laval, Québec, Canada G1K 7P4

ARTICLE INFO

Article history:
Accepted 21 June 2010

Keywords:
BMPs EGF
Osteoblasts
Bone nodules
Runx2
Alkaline phosphatase
Osteocalcin

ABSTRACT

Objective: The aim of this study was to investigate the *in vitro* osteogenic activity of EGF in association with bone morphogenetic proteins BMP2 and BMP7.
Methods: SaOS-2 (osteoblast-like cell line from human osteosarcoma) were cultured in the presence of EGF and BMPs for various culture periods to assess (a) cell proliferation by MTT assay, (b) Runx2, alkaline phosphatase (ALP) and osteocalcin (OC) mRNA expression using quantitative RT-PCR and ELISA, and (c) bone tissue mineralization using Alizarin Red staining.
Results: EGF alone was able to stimulate osteoblast growth in a time-dependent manner. When mixed with BMP2, BMP7, and their combination, EGF greatly promoted osteoblast growth, compared to the BMP- and EGF-stimulated cells, suggesting a possible synergistic effect between EGF and BMPs on osteoblast growth. Stimulation with EGF, EGF/BMP2, and EGF/BMP2/BMP7 for 7 days upregulated Runx2 mRNA expression by the osteoblasts. EGF downregulated ALP mRNA expression, which was recovered when the BMP2/BMP7 combination was added to the osteoblast culture. Tested on OC mRNA expression, EGF had no effect and inhibited the enhancing effect of BMP2 and BMP7 on osteocalcin expression. The bone mineralization assay showed that EGF reduced both the number and size of the bone nodules. This reducing effect was observable even in the presence of BMP2 and BMP7.
Conclusion: This study demonstrated that EGF may act in the early phase to promote osteoblast growth and specific marker expression rather than the late phase involving cell differentiation/mineralization.

1. Introduction

Reconstruction of craniofacial bone defects resulting from trauma or as a treatment for disease is an extremely challenging medical task. Despite the many clinical bone replacement approaches using a vast array of materials ranging from alloplastic materials to autogenous bone grafts, the ideal bone tissue for clinical applications is yet to be found. To overcome the limitations of current clinical procedures, bone-based tissue engineering *in vitro* and its use *in vivo* may show promise in providing an advanced and reliable therapeutic strategy for craniofacial tissue repair. *In vivo* bone regeneration involves numerous bioactive molecules found in circulating fluids and within bone matrices and readily

* *Corresponding author*. Tel.: +1 418 656 2131x16321; fax: +1 418 656 2861.
E-mail address: Mahmoud.Rouabhia@fmd.ulaval.ca (M. Rouabhia).
0003–9969/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.archoralbio.2010.06.010

available during bone modeling, remodelling, and healing processes in humans.[1]

Engineering bone tissue using osteoblasts can be achieved using natural or artificial scaffolds and various growth factors such as bone morphogenetic proteins (BMPs).[2] Part of the transforming growth factor-beta family, these secreted proteins are pleiotropic signalling molecules that play a critical role at various stages during the formation of a variety of tissues and organs, including bone.

More than 20 types of BMPs have been recognized, but only BMP2, BMP4, BMP6, BMP7, and BMP9 have been shown to display significant osteogenic properties.[3–5] BMPs are involved in several events during bone morphogenesis, including bone remodelling, bone formation, chondrogenesis, and mesenchymal cell infiltration and proliferation.[6,7] BMPs are also active as both homodimers and heterodimers.[8] Native BMPs are expressed by many cells in bone fracture sites undergoing healing,[9] which suggests that they have a local regulatory function during the bone repair process.

BMPs are capable of inducing the formation of bone tissue in ectopic sites and in critical-sized bone defects in several animal models.[10] BMP molecules appear to induce bone formation in a stepwise manner, with individual BMP molecules functioning at different stages of osteoblastic differentiation and osteogenesis.[3] Among these BMPs, BMP2 and BMP7 have been extensively studied leading to their approval for clinical use. Aside from spinal applications,[11] BMP2 has been approved for the treatment of open tibial fractures,[12] and BMP7 for the treatment of tibial non-unions[13] and with limited indications, for spinal fusion. Both growth factors are often used to stimulate bone and defect healing in the upper and lower extremities,[14,15] and also in craniofacial surgery.[16]

Several studies have shown that BMP2 induces many of the events necessary for both intramembranous and endochondrial ossification as well as for bone formation.[17] This molecule upregulates the genes associated with osteogenic differentiation and downregulates the genes associated with myogenic differentiation as early as the first 24 h of stimulation.[18] The osteoinductive effect of BMPs may be conjugated to some other growth factors, such as epidermal growth factor (EGF).

EGF is a key molecule in the regulation of cell growth and differentiation. Human EGF contains 53 amino acid residues and three intramolecular disulfide bonds.[19] EGF also interacts with EGF receptors located in the dental follicle and in alveolar bone, thereby implying their important role during tooth development.[20] High levels of EGF and EGF receptors have been detected in cells derived from healthy functional epithelium and periodontal ligament fibroblasts.[21]

EGF receptors decrease when cells differentiate into cell types capable of forming mineralized tissue, which suggests that these receptors may participate in the phenotype stabilization of periodontal ligament cells.[22] It has been shown that the interaction between mechanical stress and the EGF/EGF receptor system regulates periodontal ligament cells as a source of cementoblasts and osteoblasts.[22] This dual system also promotes osteogenic cell proliferation while suppressing cell differentiation by inhibiting osteoblast marker expression.[23] Overall data suggest the possible implication of EGF in bone remodelling and regeneration. Because BMPs are key players in bone remodelling/formation, and as EGF is an active molecule in alveolar bone and tooth development, we sought to investigate the effect of EGF on osteoblast growth, specific marker expression, and bone tissue mineralization, and subsequently, to gain knowledge on the possible interaction between EGF, BMP2, and BMP7 and their impact on bone tissue formation *in vitro*.

2. Materials and methods

2.1. SaOS-2 osteoblast cell cultures

SaOS-2 (osteoblast-like cell line from human osteosarcoma) were subcultured in 75 cm^2 culture flasks in Dulbecco's Modified Eagle's Medium with Ham's F-12 (Invitrogen, Burlington, ON, Canada) supplemented with 100 U/ml of penicillin and 100 U/ml of streptomicin (Schering, Pointe-Claire, QC, Canada), 250 μg/ml of amphotericin B (Sigma–Aldrich Canada Ltd., Oakville, ON, Canada) and 10% fetal bovine serum (FBS) (Gibco, Burlington, ON, Canada). The medium was changed three times a week. When the cultures reached 90% confluence, the cells were detached from the flasks using a 0.05% trypsin–0.1% EDTA solution, washed twice, resuspended in 10% FBS-supplemented DMEH medium, and used for subsequent experiments.

2.2. Effect of EGF and BMP stimulation on osteoblast growth

To investigate the effect of EGF with and without BMPs on osteoblast growth, cells were seeded into 12-well plates at a density of 2×10^4/well and cultured in a humid 5% CO_2 atmosphere at 37 °C. After 24 h, the culture medium was refreshed, and the three growth factors (EGF, BMP2, and BMP7) were introduced to the culture at 10 ng/ml. These concentrations were previously reported to be effective.[22,24]

The osteoblasts were incubated in a humid atmosphere containing 5% CO_2 at 37 °C for 3, 5, and 7 days. The culture medium was replaced every 24 h. Following each culture period, osteoblast growth was assessed by means of an MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide)] assay, an established method for the spectrophotometric measurement of cell proliferation as a function of mitochondrial activity in living cells.[24] Briefly, a stock solution (5 mg/ml) of MTT was prepared in PBS and added to each culture well at a final concentration of 1% (v/v). The various stimulated or unstimulated cultures were then incubated with MTT for 4 h at 37 °C. The supernatant was then removed, and the cultures were washed twice with PBS. Following the final wash, 2 ml of HCl in isopropanol (0.04 N) was added to each well, and the cultures were incubated again for 15 min at room temperature. Lastly, 200 μl (in triplicate) of the reaction mixture was transferred from each well to a 96-well flat-bottom plate. The absorbance was measured at 550 nm in an enzyme-linked immunosorbent assay (ELISA) reader (xMark microplate spectrophotometer, Bio-Rad Laboratories, Mississauga, ON, Canada). Results are reported as the means ± SD of six separate experiments.

Please cite this article in press as: Laflamme C, et al. Epidermal growth factor and bone morphogenetic proteins upregulate osteoblast proliferation and osteoblastic markers and inhibit bone nodule formation. *Arch Oral Biol* (2010), doi:10.1016/j.archoralbio.2010.06.010

2.3. Effect of EGF and BMPs on the Runx2, ALP, and OC mRNA expression by the stimulated and unstimulated osteoblasts

To investigate the effect of EGF with and without BMPs on the expression of osteoblast-specific markers, osteoblasts were seeded into 12-well plates at a density of 2×10^4/well and cultured in a humid 5% CO_2 atmosphere at 37 °C. After 24 h, the culture medium was refreshed, and 10 ng/ml of each growth factor (EGF, BMP2 and BMP7) were introduced to the cultures. The cultures were maintained for 7 and 14 days, with medium changing each day. Following each culture period (7 and 14 days), the osteoblasts were used to extract the total RNA from each condition.

2.3.1. RNA extraction and quantification

Total cellular RNA content was extracted using the Illustra RNAspin Mini (GE Health Care UK Ltd., Buckingham, UK), while RNA concentration, purity, and quality were determined by means of the Experion system and RNA StdSens analysis kit according to the instructions provided by the manufacturer (Bio-Rad, Hercules, CA, USA).

2.3.2. Quantitative RT-PCR

RNA (1 μg of each sample) was reverse transcripted into cDNA using Maloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen Life Technologies, Mississauga, ON, Canada) and random hexamers (Amersham Pharmacia Biotech Inc., Baie d'Urfé, QC, Canada). RT conditions were 10 min at 65 °C, 1 h at 37 °C, and 10 min at 65 °C. Quantitative PCR was carried out thereafter. The mRNA transcripts were measured using the Bio-Rad CFX96 real-time-PCR detection system and reactions were performed using a PCR supermix from Bio-Rad (iQ SYBR Green supermix). Primers (Table 1) were added to the reaction mix at a final concentration of 250 nM. Five μl of each cDNA sample were added to a 20 μl PCR mixture containing 12.5 μl of iQ SYBR Green supermix, 0.5 μl of specific primers (Runx2, ALP, OC, GAPDH) (Medicorp Inc., Montréal, QC, Canada), and 7 μl of RNase- and DNase-free water (MP Biomedicals, Solon, OH, USA). Each reaction was performed in a MyCycler™ Thermal Cycler (Bio-Rad). For the qPCR, the CT was automatically determined using the accompanying Bio-Rad CFX manager. The thermocycling conditions for ALP and OC were 5 min at 95 °C, followed by 45 cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C, with each reaction performed in triplicate. For the Runx-2, the thermocycling conditions were 3 min at 95 °C, followed by 45 cycles of 10 s at 95 °C, 10 s at 63 °C, and 30 s at 72 °C, with each reaction also performed in triplicate. The specificity of each primer pair was verified by the presence of a single melting temperature peak. GAPDH produced uniform expression levels varying by less than 0.5 CTs between sample conditions and was therefore used as a reference gene for this study.

2.4. Effect of EGF and BMPs on the osteocalcin secretion by the osteoblasts

In order to investigate the effect of EGF with and without BMPs on the secretion of osteocalcin by osteoblasts, cells were seeded into 12-well plates at a density of 2×10^4/well and cultured in a humid 5% CO_2 atmosphere at 37 °C. After 24 h, the culture medium was refreshed, and 10 ng/ml of each growth factor (EGF, BMP2 and BMP7) were introduced to the culture. The cells were maintained for 7 and 14 days with medium changing each day. Following each culture period, the supernatants were collected from each condition and used to quantify the levels of secreted osteocalcin. Measurements were taken of triplicate samples using an enzyme-linked immunosorbent assay of human osteocalcin (hOST) (Medicorp). The supernatants were filtered through 0.22-μm filters and used to quantify osteocalcin concentrations according to the manufacturer's instructions. The plates were read at 405 nm against the reference filter set at 650 nm and were analysed using the xMark microplate spectrophotometer (Bio-Rad). According to the manufacturer, the ELISA kit can detect less than 0.4 ng/ml of OC. Each assay was repeated four times, and the means ± SD were calculated and plotted.

2.5. Effect of EGF and BMPs on the alkaline phosphatase (ALP) activity secreted by the osteoblasts

The terminal differentiation of SaOS-2 cells was determined by tissue-nonspecific alkaline phosphatase activity (TNAP) using osteoblast cell lysates. To do so, osteoblast cells were seeded into 12-well plates at a density of 2×10^4/well and were cultured in a humid 5% CO_2 atmosphere at 37 °C. After 24 h, the culture medium was refreshed, and 10 ng/ml of each growth factor (EGF, BMP2 and BMP7) were introduced to the culture and maintained for 21 days, with medium changing each day. Following the culture period, the cells were washed with PBS, lysed using 1000 μl of Tris buffer (10 mM, pH 7.5, 0.1% Triton® X-100), and used to measure the ALP activity. Each lysate was centrifuged at 2000 rpm for 1 min and 10 μl of supernatant solution from each sample were combined with 10 μl of *p*-nitrophenyl phosphate at pH 10.3 with $MgCl_2$–diethanolamine buffer (*p*-NPP, Pierce, Rockford, IL, USA)

Table 1 – Primers sequences used for qRT-PCR.

Gene name	GeneBank no. #	Primers sequences	Product size (bp)
OST	NM_199173	Sense: 5′-TAGTGAAGAGACCCAGGCGC-3′ Antisense: 5′-CACAGTCCGGATTGAGCTCA-3′	107
ALP	NM_000478	Sense: 5′-GGGAACGAGGTCACCTCCAT-3′ Antisense: 5′-TCGTGGTGGTCACAATGCC-3′	72
Runx2	NM_001024630	Sense: 5′-AACCCACGAATGCACTATCCA-3′ Antisense: 5′-CGGACATACCGAGGGACATG-3′	75
GAPDH	NM_002046	Sense: 5′-GGTATCGTCGAAGGACTCATGAC-3′ Antisense: 5′-ATGCCAGTGAGCTTCCCGTTCAGC-3′	188

Please cite this article in press as: Laflamme C, et al. Epidermal growth factor and bone morphogenetic proteins upregulate osteoblast proliferation and osteoblastic markers and inhibit bone nodule formation. *Arch Oral Biol* (2010), doi:10.1016/j.archoralbio.2010.06.010

dispensed into 96-well plates. The samples were then incubated in the dark at room temperature for 30 min, after which time the reaction was halted by adding 5 μl of 0.2 mol/l of NaOH. Absorbance was recorded at 405 nm on an xMark microplate spectrophotometer (Bio-Rad). The enzyme units were one micromole of *p*-nitrophenolate released per minute per milligram of protein.[25]

2.6. *Qualitative Alizarin Red S and quantitative CPC extraction to assess the effect of EGF alone or EGF/BMPs on the mineral nodules*

We assessed the mineral formation of the osteoblasts under culture using Alizarin Red S (ARS) staining[26] at 21 days. Briefly, the medium was aspirated from the wells, and cells were rinsed twice with phosphate-buffered saline (PBS) and fixed with ice-cold 70% (v/v) ethanol for 1 h. Following removal of the ethanol, the cells were rinsed twice with deionized water and stained thereafter with 40 mM Alizarin Red S in deionized water (pH 4.2) for 10 min at room temperature. The Alizarin Red S solution was then removed by aspiration, and the cells were washed three times with sodium acetate buffer solution (pH 6.3). The mineral nodules were documented by photomicrography at random locations within each well. To quantify the ARS staining, the cells were rinsed with deionized water, washed in PBS while rocking for 15 min at room temperature, then destained for 15 min with 10% (w/v) cetylpyridinium chloride (CPC) in 10 mM sodium phosphate (pH 7.0). The extracted stain was transferred to a 96-well plate, and the concentration of Alizarin Red S in each sample was quantified by comparing the absorbance with that from Alizarin Red S standards. Absorbance was determined at 570 nm by means of the xMark microplate spectrophotometer (Bio-Rad).

2.7. *Statistical analysis*

Experimental values are presented as means ± SD. The statistical significance of differences between the control values and the test values was evaluated using a one-way ANOVA. *Posteriori* comparisons were done using Tukey's method. Normality and variance assumptions were verified using the Shapiro–Wilk test and the Brown and Forsythe test,

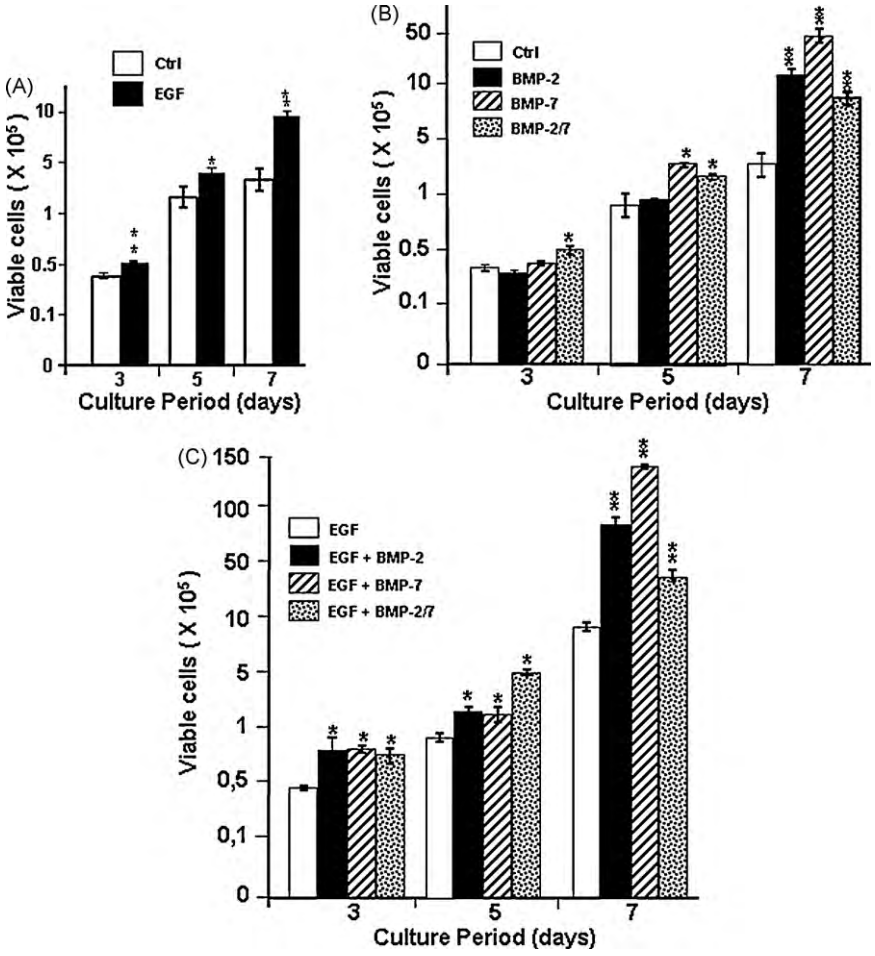

Fig. 1 – Effect of EGF with/without BMPs on osteoblast growth. Cells were cultured and incubated for 3, 5, and 7 days in a 5% CO_2 humid atmosphere at 37 °C. After 24 h, the cells were stimulated with EGF, BMP2, BMP7, and BMP2/BMP7 (all at 10 ng/ml). Thereafter, each culture was subjected to an MTT assay. Cell proliferation was assessed by means of absorbance measurements. The statistical difference was obtained by comparing the values obtained with (A) EGF alone compared to the control, (B) BMP2, BMP7, and BMP2/BMP7 compared to the control, and (C) EGF/BMPs compared to the control. Values are means ± SD of six separate experiments. Differences were considered significant at $p < 0.01$ (*) and $p < 0.001$ (**).

Please cite this article in press as: Laflamme C, et al. Epidermal growth factor and bone morphogenetic proteins upregulate osteoblast proliferation and osteoblastic markers and inhibit bone nodule formation. *Arch Oral Biol* (2010), doi:10.1016/j.archoralbio.2010.06.010

respectively. All of the assumptions were fulfilled. p values were declared significant at 0.05. The data were analysed using the SAS version 8.2 statistical package (SAS Institute Inc., Cary, NC, USA). Results were considered significant at <0.05.

3. Results

3.1. *EGF alone or with BMP2/BMP7 promoted osteoblast proliferation*

When used alone, EGF significantly enhanced osteoblast growth after 3 ($p < 0.001$), 5 ($p < 0.01$), and 7 ($p < 0.001$) days of culture (Fig. 1A). This effect was time-dependent. On the other hand, osteoblast proliferation was enhanced following stimulation with either BMP2 or BMP7. Indeed, BMP2 upregulated osteoblast growth after 7 days, while BMP7 promoted osteoblast growth after 5 and 7 days. A combination of BMP2 and BMP7 promoted osteoblast growth starting at 2 days post-stimulation. This effect was time-dependent (Fig. 1B).

As the separate use of EGF, BMP2, and BMP7 promoted osteoblast growth, we also examined their combined effect on osteoblast proliferation. As shown in Fig. 1C, osteoblast growth increased when the cells were stimulated with EGF/BMP2. This effect was significant and time-dependent at 5 and 7 days. Comparable results were obtained when the cells were stimulated with EGF/BMP7. Finally, the combination of EGF and both BMPs promoted greater osteoblast growth than did the EGF/BMP2 and EGF/BMP7 combinations. This effect was time-dependent (Fig. 1C).

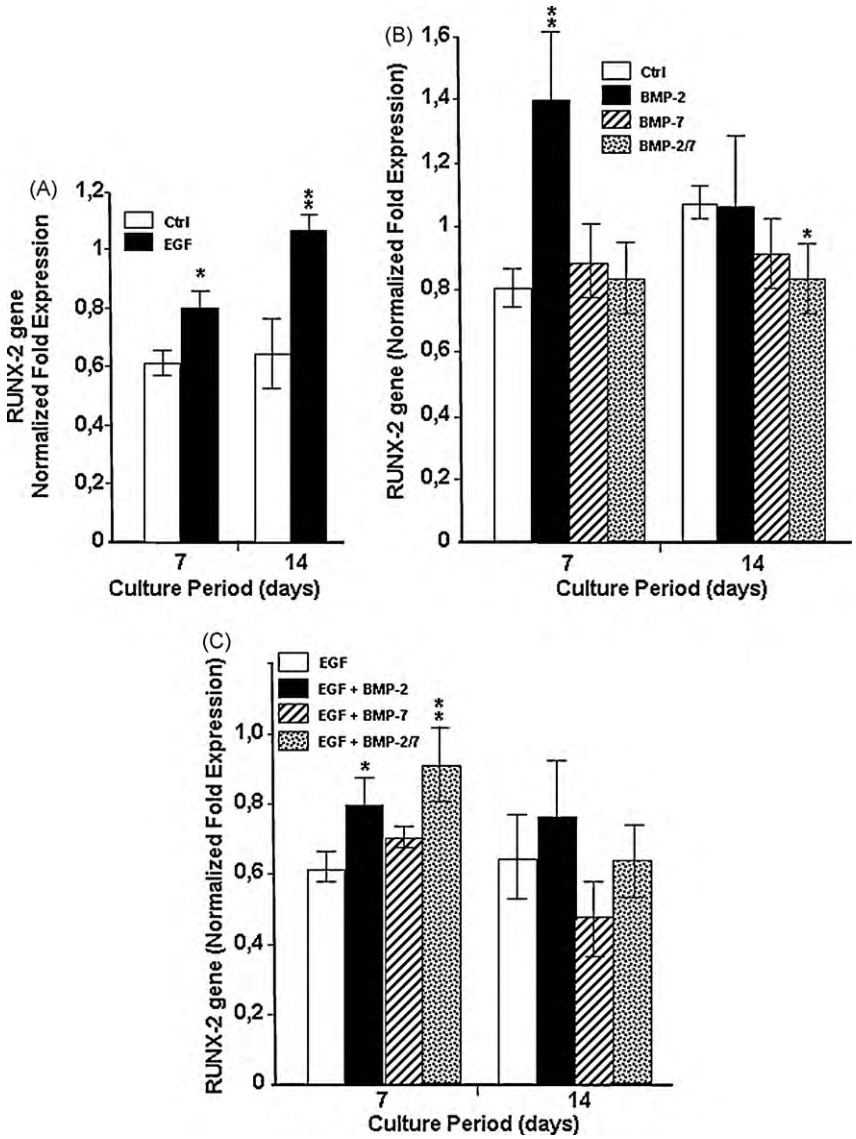

Fig. 2 – EGF/BMPs modulate Runx2 gene expression. Following osteoblast culture in the presence or in the absence of EGF, BMP2, BMP7, and BMP2/BMP7 with or without EGF for 7 and 14 days, total RNA were extracted using specific primers for Runx2 and GAPDH. Each reaction was performed in a thermal cycler and the CT was automatically determined using the accompanying CFX manager. The statistical difference was obtained by comparing the values obtained with (A) EGF alone compared to the control, (B) BMPs compared to the control, and (C) EGF/BMPs compared to the control. Values are means ± SD of three separate experiments. Differences were considered significant at $p < 0.01$ (*) and $p < 0.001$ (**).

Please cite this article in press as: Laflamme C, et al. Epidermal growth factor and bone morphogenetic proteins upregulate osteoblast proliferation and osteoblastic markers and inhibit bone nodule formation. *Arch Oral Biol* (2010), doi:10.1016/j.archoralbio.2010.06.010

3.2. EGF and BMPs modulated the Runx2 expression by the osteoblasts

As EGF was shown to upregulate osteoblast growth, we proceeded to investigate the expression of the osteoblastic marker Runx2. The qRT-PCR revealed (Fig. 2A) that Runx2 mRNA expression was significantly increased after 7 days ($p < 0.01$) and 14 days ($p < 0.001$) following osteoblast stimulation with 10 ng/ml of EGF. Fig. 2A also shows that the effect of EGF on Runx2 expression was time-dependent. When the osteoblasts were stimulated with the BMPs, only BMP2 managed to upregulate Runx2 mRNA expression after 7 days. No significant effect was obtained with BMP7 alone or in combination with BMP2. Only at 14 days post-stimulation did BMP2/BMP7 decrease Runx2 expression. The addition of EGF to the BMP-stimulated osteoblast cultures revealed (Fig. 2C) a significant increase of Runx2 mRNA expression. This significant effect was obtained when EGF was combined with BMP2 and BMP2/BMP7 after 7 days of stimulation.

3.3. EGF and BMPs modulated alkaline phosphatase expression and activity by the osteoblasts

To confirm the effect of EGF on bone engineering, we examined a second marker, ALP. As shown in Fig. 3A, EGF downregulated ALP mRNA expression, as ascertained by qRT-PCR. This effect was present after both 7 and 14 days of stimulation. BMP2 and BMP7 used separately or in combination upregulated ALP mRNA

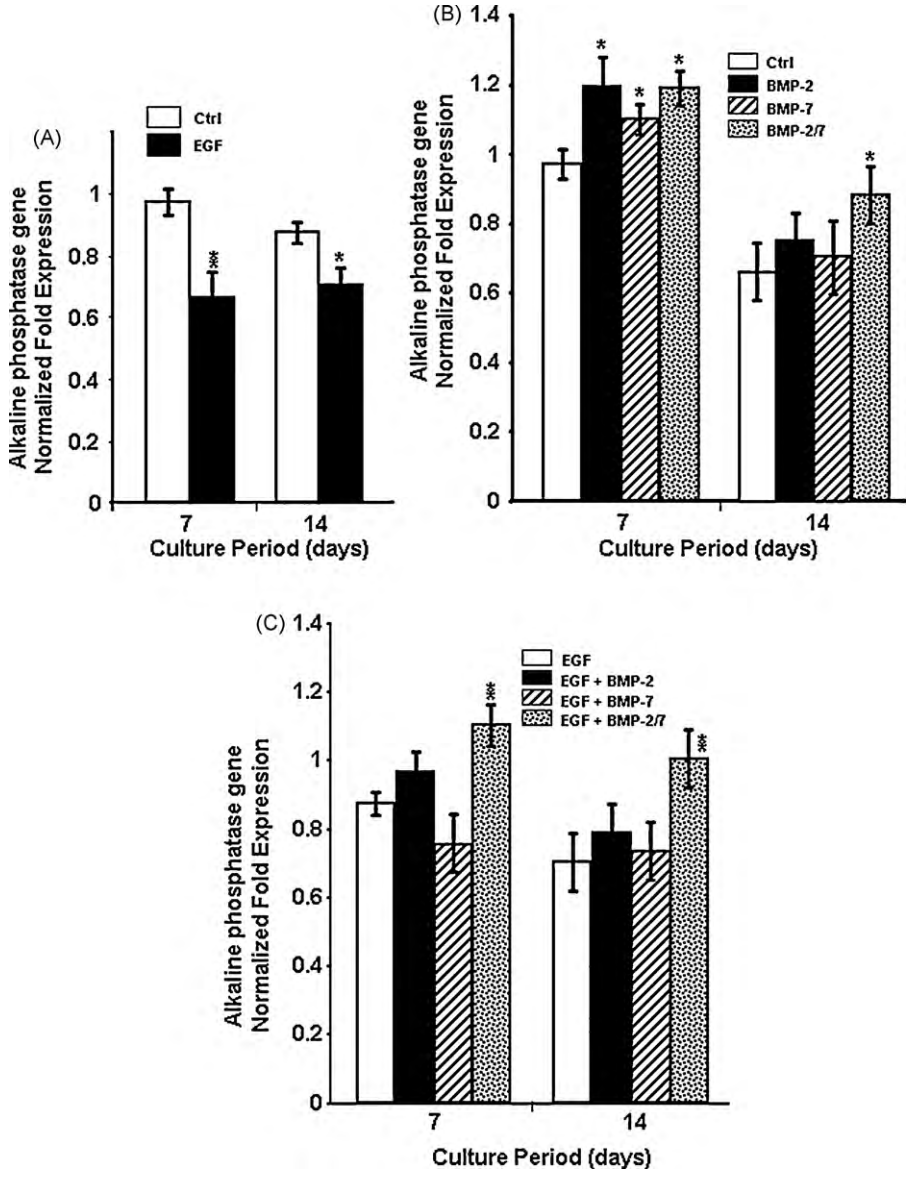

Fig. 3 – EGF/BMPs modulate alkaline phosphatase gene expression. Following osteoblast culture in the presence or in the absence of EGF, BMP2, BMP7, and BMP2/BMP7 with or without EGF for 7 and 14 days, total RNA were extracted using specific primers for ALP and GAPDH. Each reaction was performed in a thermal cycler and the CT was automatically determined using the accompanying CFX manager. The statistical difference was obtained by comparing the values obtained with (A) EGF alone compared to the control, (B) BMPs compared to the control, and (C) EGF/BMPs compared to the control. Values are means ± SD of three separate experiments. Differences were considered significant at $p < 0.01$ (*) and $p < 0.001$ (**).

Please cite this article in press as: Laflamme C, et al. Epidermal growth factor and bone morphogenetic proteins upregulate osteoblast proliferation and osteoblastic markers and inhibit bone nodule formation. *Arch Oral Biol* (2010), doi:10.1016/j.archoralbio.2010.06.010

expression by the osteoblasts. These effects were basically observed after 7 days of stimulation (Fig. 3B).

The presence of EGF in an osteoblast culture stimulated with BMP2 and/or BMP7 blocked the increase of ALP mRNA expression. However, EGF was not able to stop the upregulation of ALP mRNA expression obtained with BMP2/BMP7 stimulation (Fig. 3C). To confirm this effect, ALP activity was produced using tissue-nonspecific alkaline phosphatase activity (TNAP). As reported in Fig. 4A, EGF alone promoted TNAP activity, meaning that a greater ALP protein production was obtained when the osteoblasts were stimulated with EGF, compared to the control. When the osteoblast culture was stimulated with BMP7 for 7 days and BMP2 for 14 days, the resulting TNAP was either stable or had increased (Fig. 4B). Interestingly, when EGF was added at the same time as BMP2, BMP7, or BMP2/BMP7, the TNAP activity was significantly reduced in all of the tested conditions. These effects were observed after 7 and 14 days of stimulation.

3.4. *EGF and BMPs modulated osteocalcin expression and production by the osteoblasts*

Osteocalcin is a typical late osteoblast differentiation marker at the onset of mineralization, therefore we investigated osteocalcin mRNA expression following EGF stimulation. As shown in Fig. 5A, EGF alone had no effect on osteocalcin mRNA expression, while BMP2 and BMP7, used separately, enhanced this expression (Fig. 5B); this effect, however, was completely blocked when EGF was added to the BMPs (Fig. 5C). Protein production was measured by ELISA assay to confirm the effect

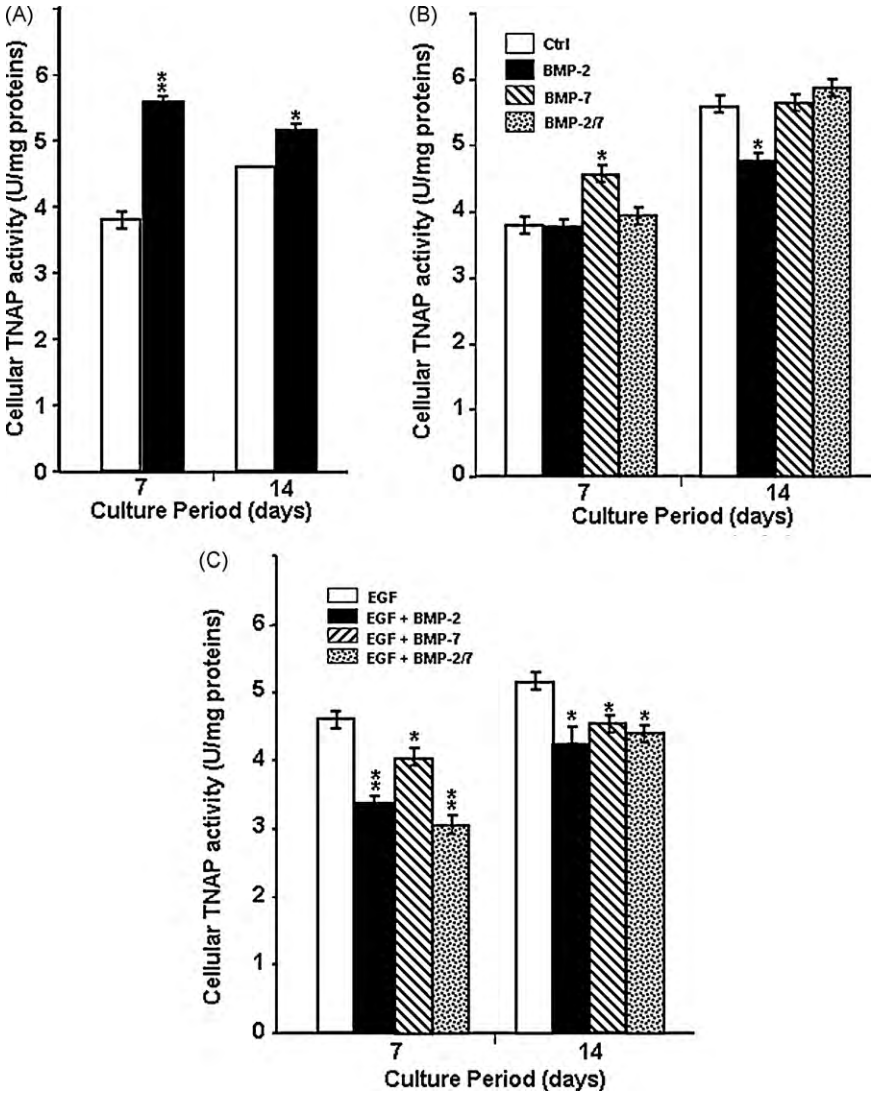

Fig. 4 – EGF/BMPs modulate osteocalcin gene expression. Following osteoblast culture in the presence or in the absence of EGF, BMP2, BMP7, and BMP2/BMP7 with or without EGF for 7 and 14 days, total RNA were extracted using specific primers for osteocalcin and GAPDH. Each reaction was performed in a thermal cycler and the CT was automatically determined using the accompanying CFX manager. The statistical difference was obtained by comparing the values obtained with (A) EGF alone compared to the control, (B) BMPs compared to the control, and (C) EGF/BMPs compared to the control. Values are means ± SD of three separate experiments. Differences were considered significant at $p < 0.01$ (*) and $p < 0.001$ (**).

Please cite this article in press as: Laflamme C, et al. Epidermal growth factor and bone morphogenetic proteins upregulate osteoblast proliferation and osteoblastic markers and inhibit bone nodule formation. *Arch Oral Biol* (2010), doi:10.1016/j.archoralbio.2010.06.010

of EGF on the osteocalcin. As shown in Fig. 6A, EGF slightly yet significantly increased osteocalcin secretion by the osteoblasts by 7 days of stimulation. Thus BMP2 and BMP7 used either alone or in combination, basically upregulated osteocalcin secretion after 7 days of stimulation (Fig. 6B). The simultaneous addition of EGF and BMPs to the osteoblast cultures significantly promoted osteocalcin secretion after 7 days of stimulation with BMP7 and BMP2/BMP7 (Fig. 6C).

3.5. *EGF inhibited osteoblast nodule formation*

Growth factors are known to affect osteoblast proliferation, differentiation, and mineralization. In this context, we investigated the effect of EGF on bone mineralization *in vitro* using the ARS staining assay. As shown in Fig. 7A, nodules were present after 21 days of culture with or without growth factors in the medium. In the EGF-stimulated osteoblast cultures, the nodules decreased in both number and size, compared to the nodules in the EGF-unstimulated cultures. However, unlike the unstimulated and EGF-stimulated cultures, many large dark-coloured nodules were present in the BMP-stimulated cultures.

The combination of EGF and BMPs resulted in EGF reducing the nodule formation, thereby suggesting its antagonistic effect on BMP2 and BMP7. To confirm these results, quantitative analyses were performed using a CPC assay. EGF reduced osteoblast mineralization and down regulated the mineralizing activities of BMP2 and BMP7 (Fig. 7B), which suggest that EGF is not required during the late phase of bone formation.

4. Discussion

Growth factors such as BMPs play a key role in bone regeneration through growth and differentiation,[1,3] whether secreted locally or used as stimulators. The most bioactive osteogenic growth factors (BMP2 and BMP7) have been shown to promote bone regeneration *in vitro*[27] and *in vivo*,[28] as well as

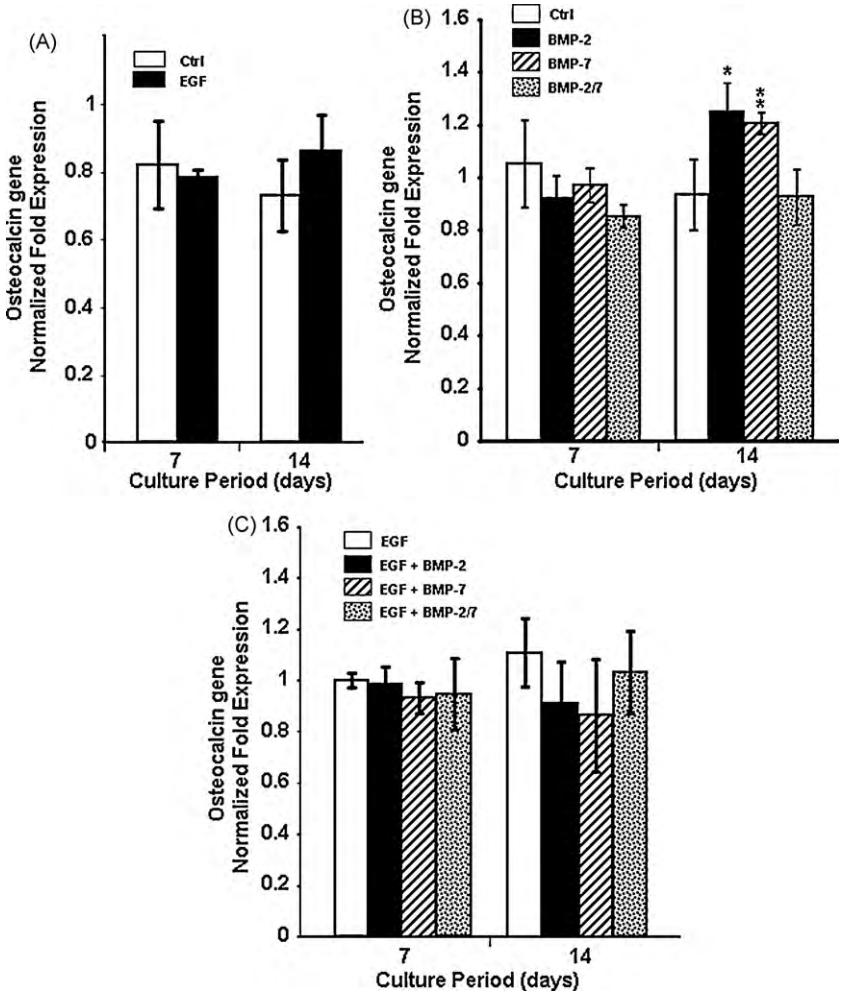

Fig. 5 – EGF/BMPs modulate osteocalcin protein production. Following stimulation with EGF, BMP2, BMP7, and BMP2/BMP7 with or without EGF, the cultured supernatants were collected and used to quantify the osteocalcin. The culture medium was filtered and an ELISA kit was used to determine the amount of secreted OC. The statistical difference was obtained by comparing the values obtained with (A) EGF alone compared to the control, (B) BMPs compared to the control, and (C) EGF/BMPs compared to the control. Values are means ± SD of four separate experiments. Differences were considered significant at $p < 0.01$ (*) and $p < 0.001$ (**).

Please cite this article in press as: Laflamme C, et al. Epidermal growth factor and bone morphogenetic proteins upregulate osteoblast proliferation and osteoblastic markers and inhibit bone nodule formation. *Arch Oral Biol* (2010), doi:10.1016/j.archoralbio.2010.06.010

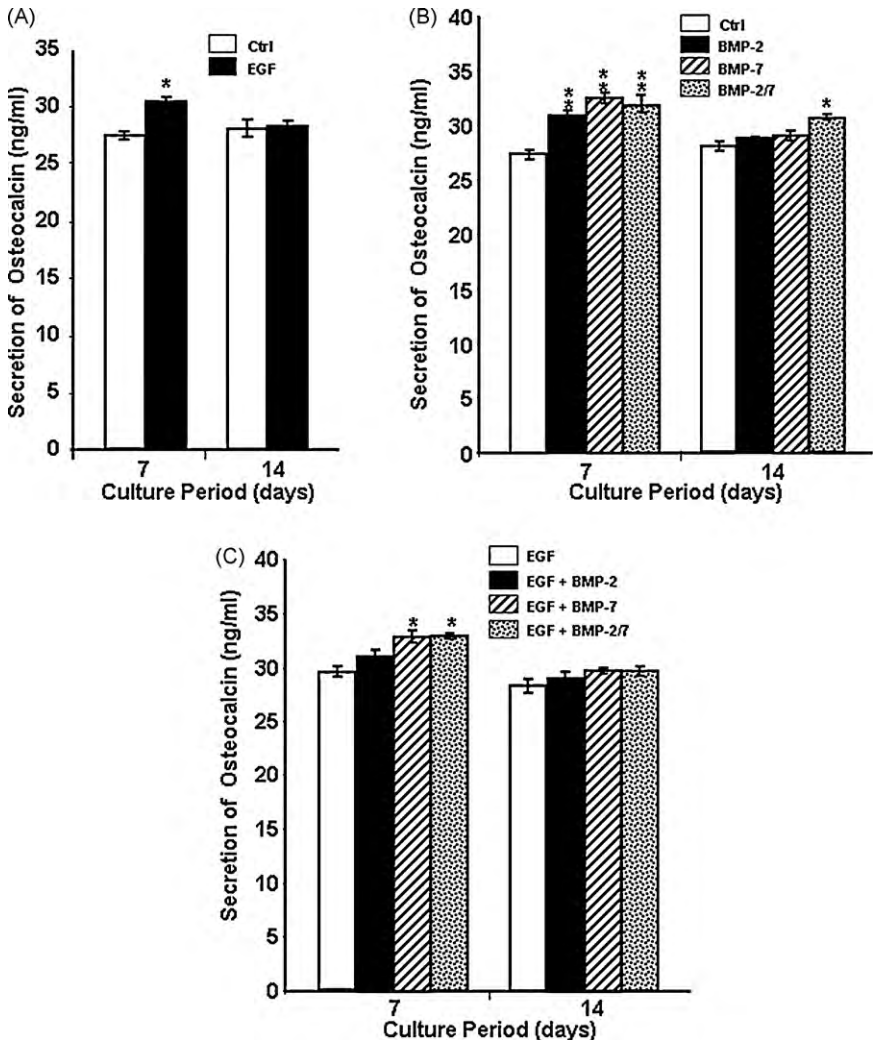

Fig. 6 – EGF/BMPs modulate alkaline phosphatase protein production. Following stimulation with EGF, BMP2, BMP7, and BMP2/BMP7 with or without EGF, the cell lysates were collected and used to quantify tissue-nonspecific alkaline phosphatase (TNAP). The lysates were assayed with p-NPP to determine the amount of secreted ALP. The statistical difference was obtained by comparing the values obtained with (A) EGF alone compared to the control, (B) BMPs compared to the control, and (C) EGF/BMPs compared to the control. Values are means ± SD of six separate experiments. Differences were considered significant at $p < 0.01$ (*) and $p < 0.001$ (**).

in clinical studies.[29] Another important growth factor, EGF, is known to be a potent mitogen for many cell types, such as keratinocytes and epithelial cells.[30] EGF may therefore have a possible effect on osteoblast growth and bone formation.

In our study, we found that EGF alone significantly promoted osteoblast proliferation, while EGF combined with BMP2 and BMP7 significantly upregulated this proliferation. The effect of EGF on osteoblast growth may thus occur through specific receptors that are expressed by the osteoblasts. Indeed, it has been reported that EGF binds to the EGF receptor, member of the tyrosine kinase super-family, similar to the BMP receptor found on cellular membranes of epithelial origin.[31] Through its receptor, EGF negatively affects osteoblast differentiation, which inversely correlates with the proliferation capacity.[32] This reflects the phenotype-stabilizing feature of growth-keeping cells at less differentiated stages with a higher potential for dividing and thus for regeneration.[33,34] A synergic or antergic relation between EGF and TGF-beta at various periods in epithelial cells was reported.[35] In our study, the efficacy of EGF on osteoblast proliferation confirms previous reports with human,[36] rat,[37] and ovine[38] bone precursor cells.

To further investigate the efficacy of EGF with or without BMPs on bone formation, we examined the bone-specific marker osteocalcin. This well-known bone marker is exclusively synthesized by mature osteoblasts and is an important regulator of bone development.[39] Osteocalcin expression contributes to the maturation of osteoblasts and to their early differentiation. Following stimulation with EGF, osteocalcin protein secretion by the osteoblasts increased significantly. The simultaneous presence of BMPs and EGF promoted osteocalcin expression, thus confirming the synergistic

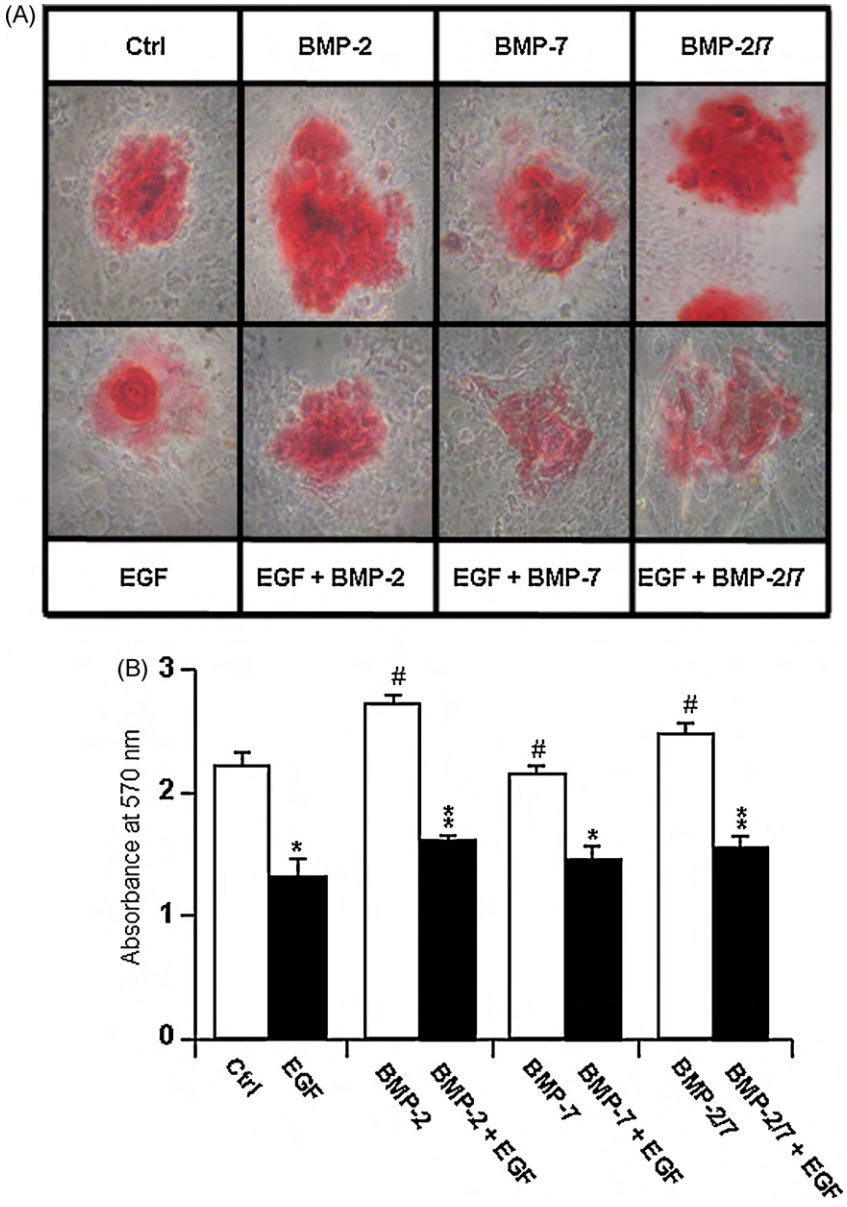

Fig. 7 – EGF decreases osteoblast mineralization. Following stimulation with EGF, BMP2, BMP7, and BMP2/BMP7 with or without EGF, ARS staining was performed from osteoblast cultures after 21 days. From the ARS staining, CPC was added to each sample of nodules for quantification obtained with a spectrophotometer reading. The levels of significance were obtained by comparing the values obtained with the EGF/BMP-stimulated cultures and those obtained with the untreated control cultures, as well as between values. Values are means ± SD of three separate experiments. Differences were considered significant at $p < 0.01$ (* or #) and $p < 0.001$ (or ##).**

interaction of EGF and BMPs on osteoblast growth and maturation. These findings are in agreement with those of previous studies on MG-63 human osteoblasts,[40] human multipotent adiposite-derived stem cells,[41] and MC3T3 osteoblasts,[23] showing that EGF promoted osteoblastic phenotype through osteocalcin expression. In our study, the opposite effect of EGF on osteocalcin mRNA expression and protein secretion may be explained by the late time points (7 and 14 days) used to investigate gene expression and protein production.

Osteocalcin expression by osteoblasts is under the control of runt-related factor-2 (Runx2),[39] a key osteogenesis regulator that promotes osteocalcin expression by osteoblasts during the maturation process.[41] Deleting the Runx2 gene leads to a total absence of osteoblast maturation, which signifies that no other transcription factor is capable of matching the regulatory function of Runx2 during differentiation.[42] We therefore investigated the effect of EGF with or without BMPs on Runx2 expression and found a significant upregulation of Runx2 expression by the osteoblasts following stimulation with EGF alone and in combination with BMP2 and BMP7. While previous studies have investigated the implication of growth factors, such as BMPs[43] and EGF,[44] on Runx2 expression by

osteoblasts, our study is the first to show a Runx2 modulation by EGF alone and in combination with BMPs, which suggests a collaborative pathway between BMP2, BMP7, and EGF. A similar collaboration pathway is described by Grasser et al.[45] showing that BMP6 upregulated EGF expression in animal and human osteoblasts. Thus a possible interaction between BMP2, BMP6, BMP7, and EGF may play an active role in the osteogenetic pathway following bone formation.

Because EGF modulated the expression of osteocalcin and Runx2, we thus examined ALP activity and found that EGF alone slightly promoted ALP expression and activity, yet tended basically to reduce ALP activity at a later period (2 weeks) when combined with BMPs. EGF evoked a biphasic response on ALP gene expression, which must be further investigated to better understand the signal pathway involving EGF during osteogenesis. Our findings are in agreement with others showing a moderate increase of ALP mRNA levels at the early stimulation period followed by a gradual decrease over subsequent weeks in human bone marrow stromal cells (BMSC).[46] Another study reported similar biphasic changes in mRNA levels during differentiation of human osteoblast cell lines, showing an increase in alkaline phosphatase with a stimulation of ascorbic acid or dexamethasone.[47] The increase/decrease pattern in TNAP activity for EGF may be explained by the peak of ALP activity for SaOS-2 on day 7 of culture which coincided with the initiation of mineralization. A decreased ALP expression may promote Runx2 gene expression, as these are related in the early stages of osteoblastic differentiation.[48]

Mineralization is the final phase in the bone formation process. This occurs through calcification at nucleation sites and nodules. These are believed to accumulate Ca^{2+} and inorganic phosphate that serve as nucleating agents for the formation of hydroxyapatite, the primary component of bone tissue.[1,3] We used an ARS assay to demonstrate that EGF alone and in combination with BMPs decreased nodule formation. Inversely, BMP2, BMP7, and BMP2/BMP7 all promoted nodule formation at 21 days, pointing to interference within the EGF/BMP2/BMP7 pathways in the mineralization process. This suggests that EGF plays an active role in bone formation during the early stages that include osteoblast proliferation and differentiation, even if this factor is not required and even undesired during the later phase of bone formation (mineralization). This hypothesis is supported by other studies[23,49] showing that EGF causes a dose-related inhibition of nodule formation, as well as a reduction in the size of mineralized nodules. Our overall findings point to the possible role of EGF as an osteogenic molecule in conjunction with BMP2 and BMP7 on bone formation. Thus the osteogenic growth factor family may be enriched with an additional one, namely, EGF. As reviewed by Fischer et al.[50] multiple growth factors are actively involved in osteogenesis. This includes BMP2, BMP4, BMP6, BMP7, and BMP9, with some other growth factors such as basic fibroblast growth factor, platelet-derived growth factor, and insulin-like growth factors. Consequently, further research will shed light on the mechanisms involved in this selective implication of EGF on early bone phase formation as well as the possible synergistic/antagonistic interaction between multiple growth factors and BMPs during bone formation.

In conclusion, we demonstrated that EGF, used either alone or in combination with BMP2 and BMP7, exerts a positive effect on osteoblast proliferation and protein (osteocalcin, Runx2, and ALP) expression and a negative effect on bone mineralization by inhibiting nodule formation. Additional studies will focus on the mechanisms underlying the bimodal phenomenon observed within the EGF signalling process in osteoblast proliferation/differentiation and not mineralization.

Acknowledgements

This study was supported by an operating grant from the ''Fonds Émile-Beaulieu, FMD''.

Funding: Fonds Émile-Beaulieu, FMD.

Conflict of interest: None declared.

Ethical approval: Not required.

REFERENCES

1. Salgado AJ, Coutinho OP, Reis RL. Bone tissue engineering: state of the art and future trends. *Macromol Biosci* 2004;**4**(8):743–65.
2. Geiger M, Li RH, Friess W. Collagen sponges for bone regeneration with rhBMP-2. *Adv Drug Deliv Rev* 2003;**55**(12):1613–29.
3. Wozney JM, Rosen V, Byrne M, Celeste AJ, Moutsatsos I, Wang EA. Growth factors influencing bone development. *J Cell Sci Suppl* 1990;**13**:149–56.
4. Celeste AJ, Iannazzi JA, Taylor RC, Hewick RM, Rosen V, Wang EA, et al. Identification of transforming growth factor beta family members present in bone-inductive protein purified from bovine bone. *Proc Natl Acad Sci USA* 1990;**87**:9843–7.
5. Cheng H, JiangW. Phillips FM, Haydon RC, Peng Y, Zhou L, et al. Osteogenic activity of the fourteen types of human bone morphogenetic proteins (BMPs). *J Bone Joint Surg Am* 2003;**85**:1544–52.
6. Nakagawa T, Tagawa T. Ultrastructural study of direct bone formation induced by BMPs-collagen complex implanted into an ectopic site. *Oral Dis* 2000;**6**(3):172–9.
7. Kato M, Toyoda H, Namikawa T, Hoshino M, Terai H, Miyamoto S, et al. Optimized use of a biodegradable polymer as a carrier material for the local delivery of recombinant human bone morphogenetic protein-2 (rhBMP-2). *Biomaterials* 2006;**27**(9):2035–41.
8. Israel DI, Nove J, Kerns KM, Kaufman RJ, Rosen V, Cox KA, et al. Heterodimeric bone morphogenetic proteins show enhanced activity in vitro and in vivo. *Growth Factors* 1996;**13**(3–4):291–300.
9. Onishi T, Ishidou Y, Nagamine T, Yone K, Imamura T, Kato M, et al. Distinct and overlapping patterns of localization of bone morphogenetic protein (BMP) family members and a BMP type II receptor during fracture healing in rats. *Bone* 1998;**22**(6):605–12.
10. Mont MA, Jones LC, Elias JJ, Inoue N, Yoon TR, Chao EY, et al. Strut-autografting with and without osteogenic protein-1: a preliminary study of a canine femoral head defect model. *J Bone Joint Surg Am* 2001;**83**:1013–22.
11. Benglis D, Wang MY, Levi AD. A comprehensive review of the safety profile of bone morphogenetic protein in spine surgery. *Neurosurgery* 2008;**62**:423–31.
12. Govender S, Csimma C, Genant HK, et al. Recombinant human bone morphogenetic protein-2 for treatment of open

tibial fractures: a prospective, controlled, randomized study of four hundred and fifty patients. *J Bone Joint Surg Am* 2002;**84**:2123–4.
13. Kanakaris NK, Calori GM, Verdonk R, Blokhuis TJ, Cherubino P, De Biase P, et al. Application of BMP-7 to tibial nonunions: a 3-year multicenter experience. *Injury* 2008;**39**:S83–90.
14. Dimitriou R, Dahabreh Z, Katsoulis E, Matthews SJ, Branfoot T, Giannoudis PV. Application of recombinant BMP-7 on persistent upper and lower limb non-unions. *Injury* 2005;**36**:S51–9.
15. Einhorn TA, Majeska RJ, Mohaideen A, Kagel EM, Bouxsein ML, Turek TJ, et al. A single percutaneous injection of recombinant human bone morphogenetic protein-2 accelerates fracture repair. *J Bone Joint Surg Am* 2003;**85**: 1425–35.
16. Smith DM, Cooper GM, Mooney MP, Marra KG, Losee JE. Bone morphogenetic protein 2 therapy for craniofacial surgery. *J Craniofac Surg* 2008;**19**:1244–59.
17. Kang Q, Sun MH, Cheng H, Peng Y, Montag AG, Deyrup AT, et al. Characterization of the distinct orthotopic bone-forming activity of 14 BMPs using recombinant adenovirus-mediated gene delivery. *Gene Ther* 2004;**11**:1312–20.
18. de Jong DS, Vaes BL, Dechering KJ, Feijen A, Hendriks JM, Wehrens R, et al. Identification of novel regulators associated with early-phase osteoblast differentiation. *J Bone Miner Res* 2004;**19**:947–58.
19. Barnham KJ, Torres AM, Alewood D, Alewood PF, Domagala T, Nice EC, et al. Role of the 6-20 disulfide bridge in the structure and activity of epidermal growth factor. *Protein Sci* 1998;**7**:1738–49.
20. Shroff B, Kashner JE, Keyser JD, Hebert C, Norris K. Epidermal growth factor and epidermal growth factor-receptor expression in the mouse dental follicle during tooth eruption. *Arch Oral Biol* 1996;**41**(6):613–7.
21. Tajima Y, Yokose S, Kashimata M, Hiramatsu M, Minami N, Utsumi N. Epidermal growth factor expression in junctional epithelium of rat gingiva. *J Periodontal Res* 1992;**27**:299–300.
22. Matsuda N, Morita N, Matsuda K, Watanabe M. Proliferation and differentiation of human osteoblastic cells associated with differential activation of MAP kinases in response to epidermal growth factor, in hipoxia and mechanical stress *in vitro*. *Biochem Biophys Res Commun* 1998;**249**:350–4.
23. Chien HH, Lin WL, Cho MI. Down-regulation of osteoblastic cell differentiation by epidermal growth factor receptor. *Calcif Tissue Int* 2000;**67**:141–50.
24. Laflamme C, Rouabhia M. Effect of BMP-2 and BMP-7 homodimers and a mixture of BMP-2/BMP-7 homodimers on osteoblast adhesion and growth following culture on a collagen scaffold. *Biomed Mater* 2008;**3**:1–10.
25. Thouverey C, Strzelecka-Kiliszek A, Balcerzak M, Buchet R, Pikula S. Matrix vesicles originate from apical membrane microvilli of mineralizing osteoblast-like Saos-2 cells. *J Cell Biochem* 2009;**106**(1):127–38.
26. Martino CF, Belchenko D, Ferguson V, Nielsen-Preiss S, Qi HJ. The effects of pulsed electromagnetic fields on the cellular activity of SAOS-2 cells. *Bioelectromagnetics* 2008;**29**:125–32.
27. Kim J, Kim IS, Cho TH, Lee KB, Hwang SJ, Tae G, et al. Bone regeneration using hyaluronic acid-based hydrogel with bone morphogenetic protein-2 and human mesenchymal stem cells. *Biomaterials* 2007;**28**:1830–7.
28. Jin D, Pei GX, Wang KH, Chen B, Qin Y. The regulatory effect of human bone morphogenetic protein 7 gene transfer on the proliferation and differentiation of rabbit bone marrow mesenchymal stem cells. *Zhongguo Yi Xue Ke Xue Bao* 2003;**25**:22–5.
29. Bessa PC, Casal M, Reis RL. Bone morphogenetic proteins in tissue engineering: the road from the laboratory to the clinic, part II (BMP delivery). *J Tissue Eng Regen Med* 2008;**2**: 81–96.
30. Kusumoto K, Parton A, Barnes D. Mitogen limitation and bone morphogenetic protein-4 promote neurogenesis in SFME cells, an EGF-dependent neural stem cell line. *In Vitro Cell Dev Biol Anim* 2009;**45**(1–2):55–61. Epub. 2008 December 5.
31. DeHaan AM, Wolters NM, Keller ET, Woods Ignatoski KM. EGFR ligand switch in late stage prostate cancer contributes to changes in cell signaling and bone remodeling. *Prostate* 2009;**69**:528–37.
32. Zhu J, Jia X, Xiao G, Kang Y, Partridge NC, Qin L. EGF-like ligands stimulate osteoclastogenesis by regulating expression of osteoclast regulatory factors by osteoblasts. *J Biol Chem* 2007;**282**(37):26656–65.
33. Sibilia M, Kroismayr R, Lichtenberger BM, Natarajan A, Hecking M, Holcmann M. The epidermal growth factor receptor: from development to tumorigenesis. *Differentiation* 2007;**75**:770–87.
34. Kretzschmar M, Doody J, Massagué J. Opposing BMP and EGF signaling pathways converge on the TGF-β family mediator Smad-1. *Nature* 1997;**389**:618–22.
35. Semlali A, Jacques E, Plante S, Biardel S, Milot J, Laviolette M, et al. TGF-beta suppresses EGF-induced MAPK signaling and proliferation in asthmatic epithelial cells. *Am J Respir Cell Mol Biol* 2008;**38**(2):202–8.
36. Gronthos S, Simmons PJ. The growth factor requirements of STRO-1-positive human bone marrow stromal precursors under serum-deprived conditions in vitro. *Blood* 1995;**85**(4):929–40.
37. Jiang Y, Vaessen B, Lenvik T, Blackstad M, Reyes M, Verfaille CM. Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. *Exp Hematol* 2002;**30**:896–904.
38. McCarty RS, Gronthos S, Zannettino AC, Foster BK, Xian CJ. Characterisation and development potential of ovine bone marrow derived mesenchymal stem cells. *J Cell Physiol* 2009;**219**:324–33.
39. Handschin AE, Egermann M, Trentz O, Wanner GA, Kock H-J, Zünd G, et al. CBFA-1 (Runx-2) and osteoclacin expression by human osteoblasts in heparin osteoporosis in vitro. *Clin Appl Thromb/Hemost* 2006;**12**(4):465–72.
40. Yarram SJ, Tasman C, Gidley J, Clare M, Sandy JR, Mansell JP. Epidermal growth factor and calcitriol synergistically induce osteoblast maturation. *Mol Cell Endocrinol* 2004;**220**:9–20.
41. Elabd C, Chiellini C, Massoudi A, Cochet O, Zaragosi LE, Trojani C, et al. Human adipose tissue-derived multipotent stem cells differentiate *in vitro* and *in vivo* into osteocyte-like cells. *Biochem Biophys Res Commun* 2007;**361**:342–8.
42. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, et al. Targeted disruption of Cbfa1 results in complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* 1997;**89**:755–64.
43. Philipp Seib F, Franke M, Jing D, Werner C, Bornhäuser M. Endogenous bone morphogenetic proteins in human bone marrow-derived multipotent mesenchymal stromal cells. *Eur J Cell Biol* 2009;**88**:257–71.
44. Omoteyama K, Takagi M. FGF8 regulates myogenesis and induces Runx2 expression and osteoblast differentiation in cultured cells. *J Cell Biochem* 2009;**106**:546–52.
45. Grasser WA, Orlic I, Borovecki F, Riccardi KA, Simic P, Vukicevic S, et al. BMP-6 exerts its osteoinductive effect through activation of IGF-I and EGF pathways. *Int Orthop* 2007;**31**(6):759–65.
46. Frank O, Heim M, Jakob M, Barbero A, Chafer D, Bendik I, et al. Real-time quantitative RT-PCR analysis of human bone marrow stromal cells during osteogenic differentiation in vitro. *J Cell Biochem* 2002;**85**(4):737–46.

Please cite this article in press as: Laflamme C, et al. Epidermal growth factor and bone morphogenetic proteins upregulate osteoblast proliferation and osteoblastic markers and inhibit bone nodule formation. *Arch Oral Biol* (2010), doi:10.1016/j.archoralbio.2010.06.010

47. Prince M, Banerjee C, Javed A, Green J, Lian JB, Stein GS, et al. Expression and regulation of Runx2/Cbfa1 and osteoblast phenotypic markers during the growth and differentiation of human osteoblasts. *J Cell Biochem* 2001;**80**(3):424–40.
48. Kotobuki N, Matsushima A, Kato Y, Kubo Y, Hirose M, Ohgushi H. Small interfering RNA of alkaline phosphatise inhibits matrix mineralization. *Cell Tissue Res* 2008;**332**: 279–88.
49. Antosz ME, Bellows CG, Aubin JE. Biphasic effects of epidermal growth factor on bone nodule formation by isolated rat calvaria cells in vitro. *J Bone Miner Res* 1987;**2**(5):385–93.
50. Fischer J, Kolk A, Wolfart S, Pautke C, Warnke PH, Plank C, et al. Future of local bone regeneration—protein versus gene therapy. *J Craniomaxillofac Surg* 2010. doi:10.1016/j.jcms.2010.03.016.

Please cite this article in press as: Laflamme C, et al. Epidermal growth factor and bone morphogenetic proteins upregulate osteoblast proliferation and osteoblastic markers and inhibit bone nodule formation. *Arch Oral Biol* (2010), doi:10.1016/j.archoralbio.2010.06.010