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Original Article

Calcitonin gene-related peptide stimulates BMP-2 expression and the differentiation of human osteoblast-like cells *in vitro*

Gang TIAN, Gang ZHANG, Ying-hui TAN*

Department of Stomatology, Xinqiao Hospital Affiliated to the Third Military Medicine College, Chongqing 400037, China

Aim: To investigate whether bone morphogenic protein-2 (BMP-2) expression was involved in calcitonin gene-related peptide (CGRP)-induced osteogenesis in human osteoblast-like cells *in vitro*.
Methods: MG-63 osteogenic human osteosarcoma cells were treated with CGRP (10^{-8} mol/L) for 48 h. Cell cycle phases were determined using flow cytometry assay. The protein levels of BMP-2, ALP, Osteocalcin, Colla1, CREB, and pCREB were measured with Western blotting, while the mRNA level of BMP-2 was measured with qR-T PCR. The expression of ALP in MG-63 cells was also studied using immunofluorescence staining. The level of cAMP was measured with ELISA assay.
Results: CGRP treatment significantly stimulated proliferation of MG-63 cells, and increased the expression of BMP-2 and the osteogenic proteins ALP, Osteocalcin and Colla1. Pretreatment with the BMP signaling inhibitor Noggin (100 ng/mL) did not affect CGRP-stimulated proliferation and BMP-2 expression, but abolished the CGRP-induced increases of the osteogenic proteins ALP, Osteocalcin and Colla1. Furthermore, CGRP treatment markedly increased cAMP level in MG-63 cells, whereas pretreatment with the cAMP pathway inhibitor H89 (5 μmol/L) abolished the CGRP-induced increases of cAMP level and BMP-2 expression.
Conclusion: In MG-63 cells, the BMP pathway is involved in CGRP-induced osteogenic differentiation but not in proliferation, whereas the cAMP/pCREB pathway is involved in the expression of BMP-2.

Keywords: c calcitonin gene-related peptide; Noggin; H89; MG-63 human osteosarcoma cell; osteogenesis; bone morphogenic protein; cAMP/pCREB pathway

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Introduction

Sensory nerve activation has a centrally mediated but poorly understood action on bone. The relative importance and interactions between autonomic, sensory, and peripheral nervous system actions on bone mass are not clear in healthy individuals and even less so in pathologic states. Understanding how the central nervous system integrates homeostatic signals with the regulation of bone homeostasis is an exciting research area.

Experimental evidence suggests that the nervous system is involved in bone remodeling. In response to fracture or other trauma, peripheral peptidergic neurons can influence osteoclast formation through the production of several neuropeptides. Calcitonin gene-related peptide (CGRP), a 37-residue peptide generated in specific neurons by alternative splicing of the calcitonin gene, is an important neuropeptide expressed in nerve fibers during bone development and repair[1, 2].

Numerous *in vivo* studies have suggested that CGRP is associated with bone development, metabolism and repair. *In vitro* studies have demonstrated that CGRP stimulates osteoblast proliferation, differentiation and maturation in both osteoblast cell lines and bone marrow mesenchyme stromal cells. CGRP and its receptors have been identified in the $[Ca^{2+}]_i$ (intracellular $Ca2^+$)[3], cAMP (cyclic adenosine monophosphate)[4], PKC (protein kinase C)[5], MAPK (mitogen-activated protein kinase)[2, 6], and ERK (extracellular signal-regulated protein kinase) signal transduction pathways[7].

Although CGRP is a recognized neuromodulator of osteoblast cell signaling, its mechanism of action in osteogenesis remains elusive. *In vivo* studies have shown that CGRP regulates osteoblast differentiation by 1) CGRP receptors localized on the osteoblast surface and 2) immune responses via many other factors in callus, such as cytokines, growth factors and the bone matrix itself. CGRP influences bone remodeling through the release of diverse growth factors, such as IGF-1[8, 9], TNF[10, 11], IL-6, and IL-1[10, 11]. However, the role of

* To whom correspondence should be addressed.
E-mail tanyhoms@yahoo.cn
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bone morphogenetic proteins (BMPs) in the regulatory mechanism of CGRP on osteoblasts has not been systematically studied.

BMPs are multi-functional growth factors that belong to the transforming growth factor beta (TGF-β) superfamily. The functions of BMPs in embryonic development and postnatal and adult animals have been extensively studied in recent years. Among the BMPs, BMP-2 has very strong osteoinductive activity. Both *in vivo* and *in vitro* studies have shown that BMP-2 is an essential regulator during the induction of osteogenic differentiation and bone formation[12-15]. Bone marrow mesenchyme stromal cells (BM-MSCs) effectively support bone formation via autocrine and paracrine functions when selectively facilitating the delivery and bioavailability of BMP-2[16]. BMP-2 promotes the proliferation of pre-osteoblast cells, induces the osteogenic or chondrogenic differentiation of mesenchymal cells[17, 18], and improves the osteogenic phenotype and capacity of stem cells through increased ALP activity and osteocalcin mRNA expression. Signal transduction studies have revealed that the Smad and P38MAPK pathways are immediately downstream of BMP receptors and that they play central roles in BMP signal transduction[4, 19–21].

To date, studies of the relationship between BMPs and CGRP are limited. Previous studies suggest that BMPs 2, 4, or 6 stimulate CGRP expression in 60% of DRG neurons[22]. While CGRP promotes the production of BMP-2 in pulp cells[23, 24], BMP-2 is also associated with the spatial and temporal regeneration of CGRP-positive nerve fibers in ectopic bone formation[25]. Neuropeptides and receptors such as CGRP play important roles in the regulation of bone remodeling[1], and CGRP induces BMP-2 expression in bone tissues[23, 26]. We hypothesized that BMP-2 expression is involved in CGRP-induced osteogenesis.

Materials and methods

Drugs

Human CGRP, noggin (BMP signaling antagonist), and H89 were purchased from Sigma-Aldrich (St Louis, MO, USA). A cAMP ELISA kit was purchased from R&D Systems. Polyclonal rabbit anti-pCREB and anti-CREB antibodies were purchased from New England Biolabs (Ipswich, MA, USA). CGRP was dissolved in distilled water to a stock concentration of 100 mmol/L and stored in 100-mL aliquots at -70 °C.

MG-63 cell culture and CGRP treatment

Low-passage MG-63 osteogenic human osteosarcoma cells were obtained from the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China) and maintained in Gibco RPMI-1640 medium with 10% FBS (Life Tech, Foster City, CA, USA). Cells were normally plated at a density of 5×10^5 cells/cm^2 and cultivated in humidified 5% CO_2 at 37 °C. Cultures were never allowed to become confluent. After 24 h in fresh RPMI-1640, cells were treated with RPMI-1640 with 10% FBS and the CGRP mixture at a final concentration of 10^{-8} mol/L. CGRP treatment lasted for 48 h, and RPMI-1640 with 10% FBS was used as a control[27, 28]. For the assays described below, cells were grown on sterile 1-mm-thick, 23-mm-diameter circular coverslips. All assays were performed in PBS.

Cell cycle flow cytometry assay

After the 48-h CGRP treatment, cells were harvested with Gibco Trypsin-EDTA (Life Tech) and fixed with ice-cold 70% ethanol at 4 °C for 30 min. After washing with PBS, cells were incubated with 1 mg/mL RNase A (Sigma-Aldrich) at 37 °C for 30 min. Cells were collected by centrifugation at 2000 rounds per minute for 5 min and then stained with a DNA staining solution (250 μL) for 3 min in the dark at room temperature (25 °C). The nuclear DNA content of cells was measured at high speed with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). The distribution of cell cycle phases was determined by Modfit software (BD Biosciences).

Western blotting

Western blots were performed as described previously. Briefly, MG-63 cells were washed and lysed in cold solution of 50 mmol/L Tris-HCl (pH 7.4), 10 mmol/L EDTA, 4.3 mol/L urea, and 1% Triton X-100. Protein samples were subjected to SDS-polyacrylamide (10%) gel electrophoresis and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% BSA in Tris-buffered saline with Tween 20 (TBS-T) for 2 h at room temperature (25 °C). The blots were incubated at 4 °C overnight with primary antibodies against BMP-2, ALP, Osteocalcin, CREB, pCREB, β-actin (Epitomics, Burlingame, CA, USA), and ColIa1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in TBS-T. After washing and incubation with the goat anti-rat HRP-conjugated secondary antibodies (Sigma), the blots were visualized with an ECL kit. β-Actin protein expression was used as an internal control.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated and converted to cDNA. According to the manufacturer's protocols, the cDNA was PCR-amplified on a 7500/7500 Fast Real-Time PCR System (Life Tech, Foster, CA, USA) with 0.5 mmol/L BMP-2 primers (Forward: 5′-ACCCGCTGTCTTCTAGCGT-3′; Reverse: 5′-TTTCAGGCCGAACATGCTGAG-3′; 180-bp product). The RT reaction included 1 μg of total RNA and was performed at 35 °C for 15 min followed by 85 °C for 5 s; the cDNA was then stored at 4 °C temporarily. The PCR reactions were performed in a GeneAmp PCR System 9600 thermal cycler (Life Tech) in triplicate with the following conditions: 95 °C for 30 s followed by 40 cycles of denaturation at 95 °C for 6 s, extension at 60 °C for 20 s, and single signal acquisition for 10 s. GAPDH was used as an internal control.

Immunofluorescence staining

MG-63 cells were fixed with 4% paraformaldehyde in PBS for 15 min and permeabilized with 0.1% Triton X-100 for 15 min at room temperature. After washing in PBS, specific binding sites were blocked with 5% BSA in PBS for 1 h at 4 °C. The cells were incubated with primary (human ALP; eBioscience,

San Diego, CA, USA) and secondary (FITC-conjugated goat anti-rat antibody; Santa Cruz Biotechnology) antibodies for 1 h at room temperature and stained with DAPI (Santa Cruz Biotechnology). The cells were visualized with a TCS SP2 confocal microscope (Leica, Tokyo, Japan).

cAMP assay

ELISAs were performed for quantification of cAMP. After CGRP treatment for 48 h, cells were harvested, and cAMP was quantified with a cAMP ELISA kit (R&D Systems) following the manufacturer's protocol.

Statistical analysis

Data were expressed as mean±SD. Statistical comparisons of the results were performed using analysis of variance. Significant differences (P<0.05) between the mean values of control and test groups were analyzed by Dunnett's test.

Results

CGRP induces the proliferation and differentiation of MG-63 cells

CGRP promotes MG-63 cell proliferation

As shown in Figure 1A and 1B, flow cytometry-based DNA content analysis indicated that the percentage of cells at S/G_2 phase was significantly higher in CGRP-treated cells than in the control group. These data suggested that CGRP promoted MG-63 cell proliferation.

CGRP enhances the osteoblast differentiation of MG-63 cells

After 48 h of CGRP treatment, we assessed the protein expression levels of ALP, Colla1 and Osteocalcin (OCN) by Western blotting, and ALP expression was also measured by immunofluorescent staining of MG-63 cells. CGRP significantly induced ALP (Figure 2A and 2B), Osteocalcin (Figure 2D), and Colla1 (Figure 2C) expression (P<0.05), suggesting that CGRP induced osteogenesis in MG-63 cells.

CGRP induces MG-63 cell differentiation but not proliferation through a BMP-2 dependent pathway

BMP pathway is not involved in the CGRP-induced proliferation of MG-63 cells

BMP-2 expression in osteoblasts enhances osteogenic differentiation *in vitro* and bone formation[29]. To determine the role of the BMP pathway in CGRP-induced MG-63 cell proliferation, cells were pretreated with 100 ng/mL Noggin, a soluble

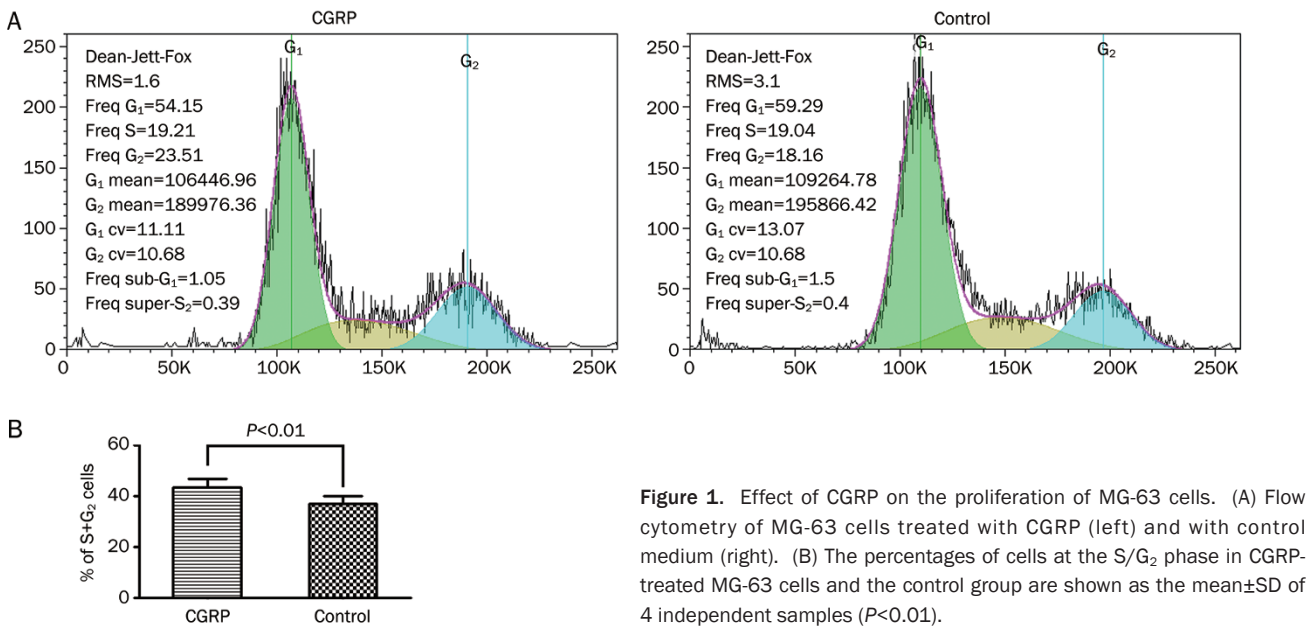

Figure 1. Effect of CGRP on the proliferation of MG-63 cells. (A) Flow cytometry of MG-63 cells treated with CGRP (left) and with control medium (right). (B) The percentages of cells at the S/G_2 phase in CGRP-treated MG-63 cells and the control group are shown as the mean±SD of 4 independent samples (P<0.01).

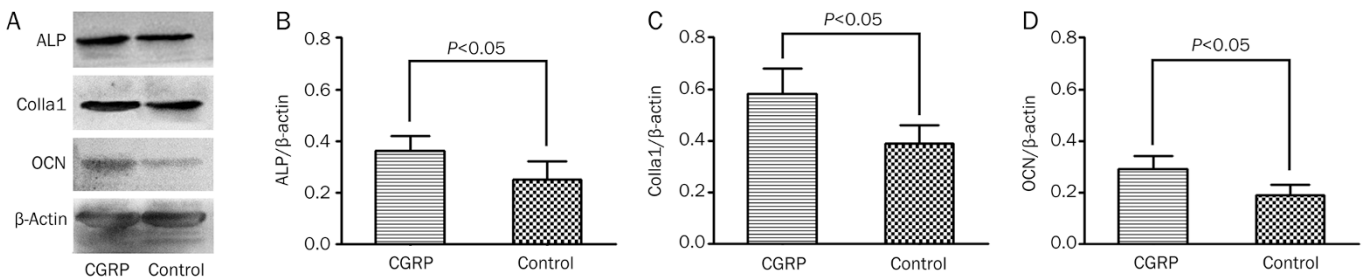

Figure 2. Western blot analysis for the expression of several osteogenic proteins including ALP, Colla1, and OCN in the CGRP-treated MG-63 cells. β-Actin was used as an internal control. (B–D) Western blots were quantified via densitometry scanning using NIH Image software and plotted in histograms representing mean±SD. Data were obtained from 4 independent experiments.

inhibitor of BMP signaling, for 30 min and then incubated with 10^{-8} mol/L CGRP for 48 h. The proportion of cells in various stages of the cell cycle was examined by flow cytometry. Figure 3A and 3B shows that Noggin pretreatment did not significantly alter the percentage of cells at the S/G_2 phase in the CGRP-treated group. However, qRT-PCR (Figure 4A) showed that BMP-2 mRNA increased after stimulation with CGRP, regardless of Noggin pretreatment. Thus, CGRP increased BMP-2 expression, but the CGRP-induced proliferation of MG-63 cells was not mediated by the BMP-2 pathway.

BMP pathway is involved in the CGRP-induced osteogenic differentiation of MG-63 cells

To determine whether the BMP pathway was involved in CGRP-induced osteogenic differentiation of MG-63 cells, the cells were pretreated with Noggin and incubated with 10^{-8} mol/L CGRP for 48 h. Cells were examined for ALP expression by anti-ALP (green) immunofluorescence staining (Figure 5), and the induced BMP-2 and osteogenic proteins were quantified by Western blot (Figure 4B, 4C, and Figure 6). As shown in Figure 4C, BMP-2 expression was not significantly different between control and Noggin-treated groups or between CGRP- and CGRP/Noggin-treated groups, indicating that inactivation of the BMP-2 pathway did not alter the expression of BMP-2. This result also suggests that the expression of CGRP-induced BMP-2 protein does not require activation of the BMP pathway.

In contrast, Noggin significantly decreased the expression of osteogenic proteins (ALP, ColIa1, and OCN) in CGRP-treated MG-63 cells compared with the CGRP-treated group (Figure 6B–6D). Blocking the BMP-2 pathway decreased the expression of differentiation-related proteins, indicating that BMP pathway activation leads to CGRP mediated-osteogenic differentiation in MG-63 cells.

CGRP upregulates BMP-2 expression by cAMP/pCREB signaling in MG-63 cells

cAMP/PCREB activation induces CREB phosphorylation, leading to the initiation of protein transcription. To investigate whether the cAMP/PCREB pathway was responsible

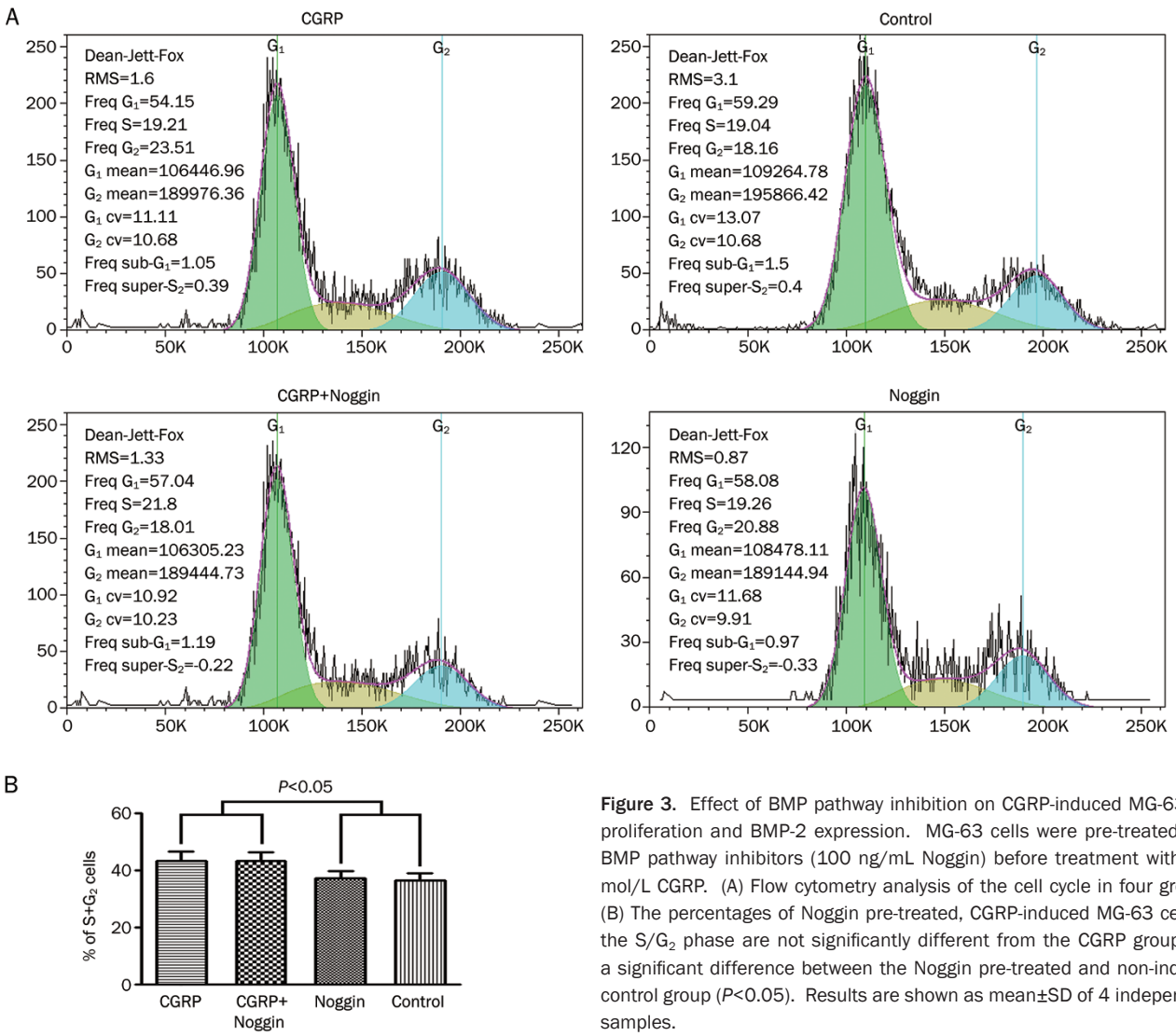

Figure 3. Effect of BMP pathway inhibition on CGRP-induced MG-63 cell proliferation and BMP-2 expression. MG-63 cells were pre-treated with BMP pathway inhibitors (100 ng/mL Noggin) before treatment with 10^{-8} mol/L CGRP. (A) Flow cytometry analysis of the cell cycle in four groups. (B) The percentages of Noggin pre-treated, CGRP-induced MG-63 cells at the S/G_2 phase are not significantly different from the CGRP group, but a significant difference between the Noggin pre-treated and non-induced control group (P<0.05). Results are shown as mean±SD of 4 independent samples.

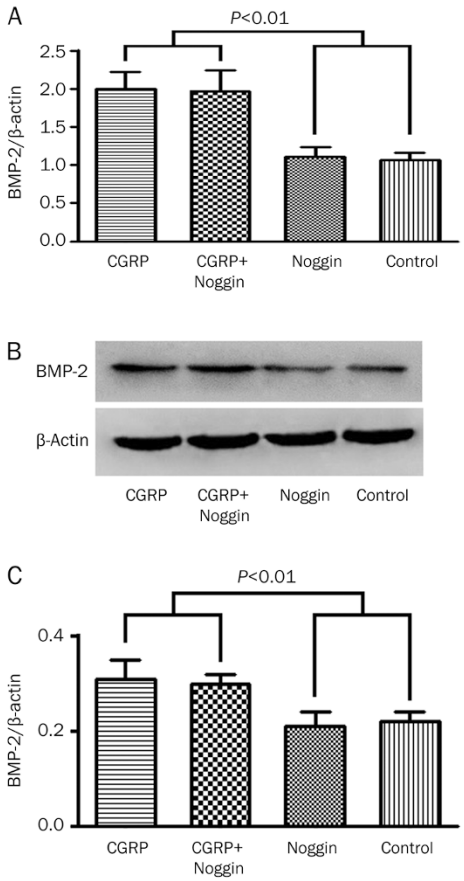

Figure 4. Expression of BMP-2 in 4 groups of MG-63 cells. (A) Densitometry of the RT-qPCR products normalized to GAPDH. BMP-2 expression was increased in both CGRP and CGRP+Noggin groups, with significant differences when compared with the Noggin and control groups (P<0.01). (B) Western blot for BMP expression in the different treatment groups; β-actin was used as an internal control. (C) Western blots were quantified via densitometry scanning using NIH Image software and plotted in histograms representing 4 mean±SD. Data were obtained from 4 independent experiments.

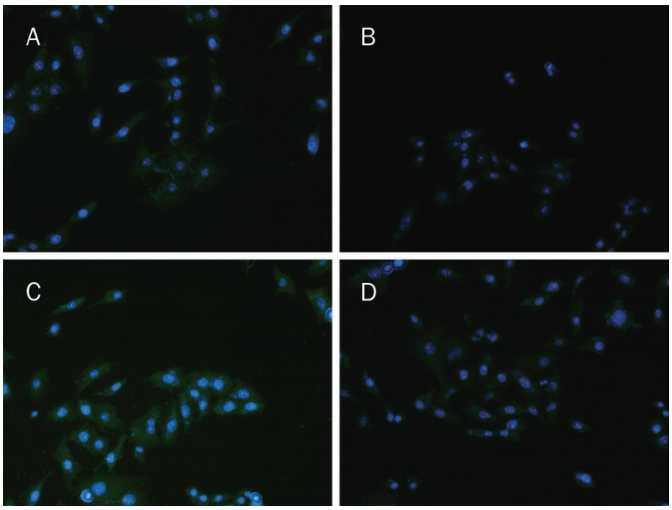

Figure 5. Immunofluorescence staining of ALP (green) in control (A), Noggin- (B), CGRP- (C), and CGRP+Noggin- (D) treated MG-63 cells (DAPI nuclear staining, blue). Magnification, 100×.

for CGRP-induced BMP-2 expression, MG-63 cells were pretreated with H-89, a selective inhibitor of cAMP-dependent protein kinase A, for 30 min and then exposed to CGRP for 48 h. We examined the levels of cAMP by ELISA, phosphorylated-CREB (p-CREB) and total CREB by Western blot, and BMP-2 by qRT-PCR and Western blot. As shown in Figure 7A, cAMP levels in CGRP-treated MG-63 cells were significantly higher than that in the control cells. Pretreatment with H-89 did not significantly influence the cAMP level in CGRP-treated cells, suggesting that CGRP stimulated the expression of cAMP.

In addition, CGRP significantly upregulated the expression of BMP-2 mRNA and protein (Figure 7B, 7C, and 7D). H-89 inhibited the CGRP-induced upregulation of BMP-2 mRNA and protein (Figure 7B and 7D), indicating that cAMP-dependent phosphorylation might be involved in CGRP-induced BMP-2 expression. Furthermore, pCREB but not CREB was increased by CGRP and decreased by H-89 (Figure 7E, 7F, and 7G). Altogether, these results suggested that CGRP upregulated BMP-2 expression through cAMP/PCREB signaling in MG-63 cells.

Discussion

CGRP, a member of the calcitonin peptide family, has been recognized in both *in vitro* and *in vivo* studies as a neurotransmitter involved in the osteoblast proliferation and bone remodeling[30]. However, the potential mechanisms of CGRP during osteoblast proliferation and differentiation have not been widely investigated. In this study, we determined how CGRP influences MG-63 cell proliferation and differentiation. We found that the CGRP-induced osteoblast cell proliferation was not BMP pathway-dependent. However, CGRP-induced differentiation-specific markers (ALP, collagen I and OCN) required activation of the BMP pathway. We also found that CGRP increased the mRNA and protein levels of BMP-2 in MG-63 cells. Furthermore, we demonstrated that the cAMP/pCREB pathway was involved in CGRP-induced BMP-2 expression. As expected, CGRP-induced cAMP synthesis enhanced CREB phosphorylation, resulting in increased expression of BMP-2.

The regulation of metabolism and fracture healing of bone tissue by the nervous system is closely related to the regulation of CGRP in osteoblasts. CGPR stimulates osteoblast proliferation and differentiation both *in vivo* and *in vitro*. However, these data are inconsistent in different cell lines. In the study of neural bone physiology, the human osteosarcoma cell line MG-63 is often used as an osteoblast-like cell model due to its relatively low degree of differentiation[31]. Additionally, the CGRP receptor can be detected on the MG-63 cell surface, and the downstream signal pathway is relatively well known[3, 32]. Our previous study demonstrated that the osteoblast-specific expression of ALP, ColIa1, and OCN in MG-63 cells increased

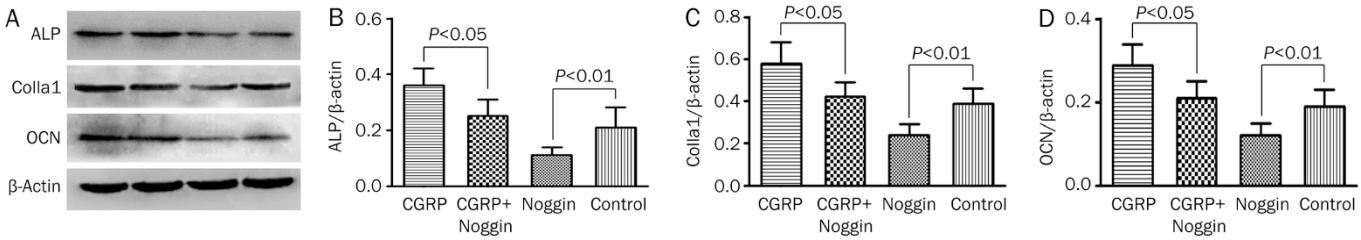

Figure 6. The effect of the BMP pathway on CGRP-induced MG-63 cell differentiation. MG-63 cells were pre-treated with the BMP pathway inhibitor Noggin (100 ng/mL) before treatment with 10^{-8} mol/L CGRP. (A) Western blot for BMP-2 and osteogenic proteins ALP, Colla1, and OCN in the different treatment groups. β-Actin was used as an internal control. (B-D) Western blots were quantified via densitometry scanning using NIH Image software, and the data were plotted in histograms showing mean±SD. Data were obtained from 4 independent experiments. The expression of osteogenic proteins including ALP, Colla1, and OCN decreased in both CGRP+Noggin and Noggin groups, with significant differences when CGRP *vs* CGRP+Noggin and Noggin *vs* control were compared ($P<0.05$).

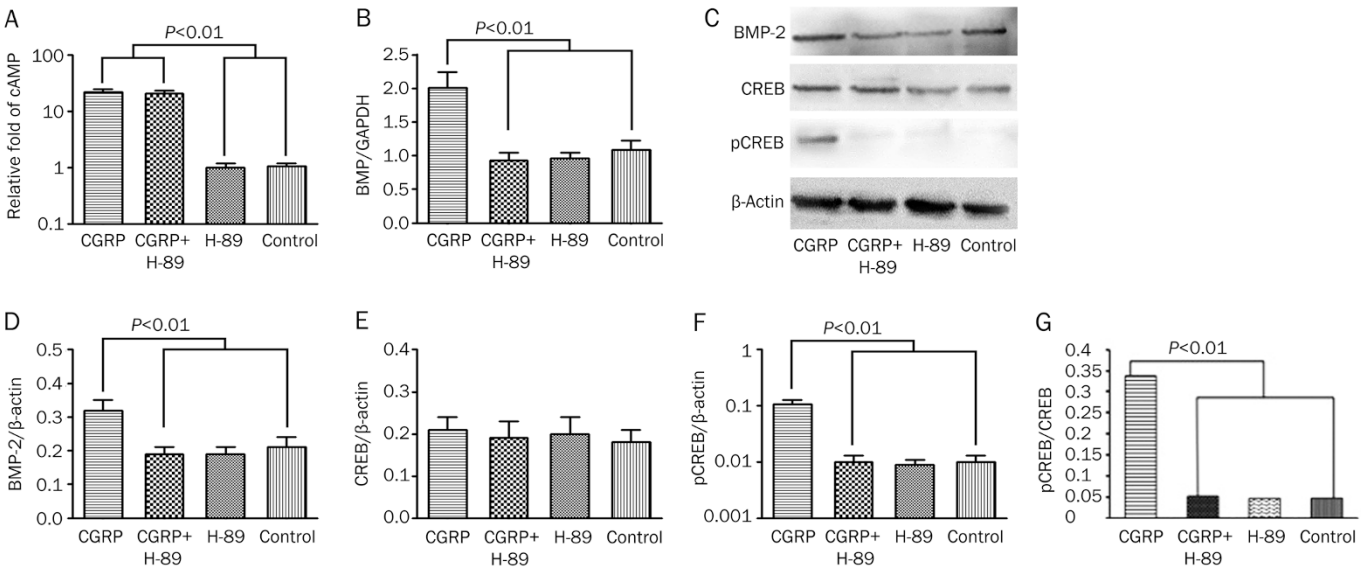

Figure 7. cAMP/CREB pathway involvement in CGRP-induced BMP-2 expression. MG-63 cells were divided into the following four treatment groups: 10^{-8} mol/L CGRP, 10^{-8} mol/L CGRP+5 μmol/L H-89, 5 μmol/L H-89 and control. (A) ELISA of cAMP concentrations. (B) qRT-PCR analysis of BMP-2 mRNA in the total RNA isolated from MG-63 cells. GAPDH was used as an internal control. (C) Western blot for BMP-2, CREB, and p-CREB in MG-63 cells. β-Actin was the internal control. The Western blot results were quantified and plotted in histograms representing mean±SD. Data were obtained from 4 independent experiments. (D) The expression of BMP-2 was significantly increased in the CGRP-treated group compared with the other groups ($P<0.01$). (E) The expression of CREB did not differ in 4 groups. (F) The expression of phosphorylated CREB (pCREB) was significantly increased in the CGRP treated group and was inhibited in H-89 pretreated group ($P<0.01$). (G) The ratio of pCREB/CREB in CGRP treated group was significantly increased.

with the concentration of CGRP. Thus, we selected MG-63 instead of other osteoblast cell lines as our model in this study.

In our previous studies, we found a time- and dose-dependence during the CGRP-stimulated differentiation and proliferation of MG-63[27, 28]. Of various CGRP concentrations (10^{-7} 10^{-9}, and 10^{-10} mol/L) and exposure times tested, CGRP treatment at 10^{-8} mol/L for 24–48 h was most effective in stimulating proliferation and differentiation of MG-63. We used these conditions in the current study to guarantee the positive effect of CGRP.

Our previous MTT assay also indicated that after 24–72 h the proliferation capacity was significantly higher in the CGRP-treated group than that in controls. However, propidium iodide staining indicated that more cells died after 48 h of CGRP incubation. Previous inconsistent results without time points were not reliable[2]. Thus, a delayed detection time point might erroneously suggest that CGRP does not affect osteoblast proliferation.

BMP signaling is involved in the maturation of stem cells into chondrocytes, osteoblasts, and osteocytes[33] and plays essential roles in early vertebrate embryogenesis events including limb development[34], stimulation of collagen synthesis[35], alkaline phosphatase activity[36], osteocalcin expression in chondroblasts/osteoblasts and other unrelated bone formation processes. A number of studies have confirmed that activation of osteoblastic CGRP receptors results in enhanced

osteoblast proliferation[8], and CGRP also increases the synthesis of growth factors and cytokines [including IGF-1 (insulin-like growth factor)], collagen synthesis and bone formation *in vivo*[1]. However, the signaling pathways by which CGRP exerts its osteogenic effects in osteoblasts are unclear.

In this study, we employed MG-63, the human osteoblast-like cell line, to investigate the pathway activated in response to CGRP-induced cell proliferation and differentiation. We proved that CGRP promoted MG-63 cell entry to the S/G_2 cell cycle phase and stimulated the synthesis of osteogenic proteins and differentiation markers including ALP, ColIa1 and OCN. Consistent with previous studies, our data suggested that CGRP promoted the proliferation and differentiation of osteoblasts. Furthermore, we demonstrated that the BMP pathway inhibitor Noggin significantly influenced the expression of ALP, ColIa1 and OCN but had no effect on CGRP-induced cell proliferation. However, Noggin did not inhibit CGRP-induced BMP-2 expression in MG-63. This result indicated that the BMP signaling pathway was not activated during CGRP-dependent osteoblast proliferation but was activated during CGPR-dependent osteoblast differentiation. Based on these findings, we hypothesize that the CGRP-promoted synthesis of BMP-2 may be dependent on a non-BMP route.

BMP-2 is a critical autocrine and paracrine growth factor that directs osteoblast differentiation and bone formation[37–39]. BMP-2 expression in osteoblasts promotes cell maturation and differentiation[40]. As we and others have shown, CGRP increases BMP-2 expression in osteoblasts[41].

Increasing intracellular cAMP and calcium leads to changes in cell morphology and function[42, 43], but the effect of cAMP signaling on CGRP-dependent BMP-2 expression remains unclear. To examine these events, we examined the synthesis of BMP-2 mRNA and protein after pretreatment with the cAMP pathway inhibitor H-89. CGRP increased BMP-2 mRNA and protein expression in MG-63 cells, but these effects were significantly inhibited by H-89, suggesting that activation of the cAMP pathway was required for CGRP-induced BMP-2 synthesis. We further determined that phosphorylated CERB played an essential role in this effect. Our data showed that CGRP induced BMP-2 expression via phosphorylated CREB and increased cAMP synthesis.

In summary, our findings confirm that CGRP increases proliferation and osteogenic differentiation in osteoblasts *in vitro*. Furthermore, CGRP-induced differentiation and proliferation of osteoblasts does not depend on the same BMP pathway. There may be another pathway besides the BMP pathway that contributes to CGRP-induced proliferation. We also provide compelling evidence for a mechanism by which CGRP promotes the BMP-2 expression in MG-63 cells through direct transactivation of cAMP/PCREB signaling. These data strongly support the hypothesis that cAMP pathway-dependent BMP-2 synthesis is important for osteogenesis.

Acknowledgements

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Author contribution

Ying-hui TAN designed the study; Gang TIAN and Gang ZHANG performed the experiments; and Gang TIAN analyzed the data and prepared the manuscript.

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