Autocrine effects of neuromedin B stimulate the proliferation of rat primary osteoblasts

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Abstract

Neuromedin B (NMB) is a mammalian bombesin-like peptide that regulates exocrine/endocrine secretion, smooth muscle contraction, body temperature, and the proliferation of some cell types. Here, we show that mRNA encoding Nmb and its receptor (Nmbr) are expressed in rat bone tissue. Immunohistochemical analysis demonstrated that NMB and NMBR colocalize in osteoblasts, epiphyseal chondrocytes, and proliferative chondrocytes of growth plates from mouse hind limbs. Then, we investigated the effect of NMB on the proliferation of rat primary cultured osteoblasts. Proliferation assays and 5-bromo-2-deoxyuridine incorporation assays demonstrated that NMB augments the cell number and enhances DNA synthesis in osteoblasts. Pretreatment with the NMBR antagonist BIM23127 inhibited NMB-induced cell proliferation and DNA synthesis. Western blot analysis showed that NMB activates ERK1/2 MAPK signaling in osteoblasts. Pretreatment with the MAPK/ERK kinase inhibitor U0126 attenuated NMB-induced cell proliferation and DNA synthesis. We also investigated the effects of molecules that contribute to osteoblast proliferation and differentiation on Nmb expression in osteoblasts. Real-time PCR analysis demonstrated that 17β-estradiol (E2) and transforming growth factor β1 increase and decrease Nmb mRNA expression levels respectively. Finally, proliferation assays revealed that the NMBR antagonist BIM23127 suppresses E2-induced osteoblast proliferation. These results suggest that NMB/NMBR signaling plays an autocrine or paracrine role in osteoblast proliferation and contributes to the regulation of bone formation.

Key Words
- Neuromedin B
- NMB and its receptor
- Osteoblasts
- Proliferation
- Estradiol

Introduction

Originally isolated from porcine spinal cord, neuromedin B (NMB) is a member of the bombesin-like peptide family, which also includes gastrin-releasing peptide (GRP) in mammals (Minamino et al., 1983, Ohki-Hamazaki 2000). NMB has been detected in rat gastrointestinal tissues, spinal cord, pancreas, pituitary gland, and several brain
NMB binds to its receptor (NMBR) – expressed in mouse brain, esophagus, intestine, testis, and uterus – to regulate exocrine and endocrine secretion, smooth muscle contraction, feeding, blood glucose levels, and body temperature (Minamino et al. 1983, Ohki-Hamazaki 2000).

NMB also acts as a proliferative growth factor for cancer cell lines derived from human breast, colon, lung, ovary, pancreas, and prostate tissues (Giacchetti et al. 1990, Bartholdi et al. 1998, Siegfried et al. 1999, Sun et al. 2000, Burghardt et al. 2001, Matusiak et al. 2005). We recently demonstrated that both Nmb and Nmbr are expressed in mouse ATDC5 chondrogenic cells, which proliferate in response to NMB (Saito et al. 2012).

Bone tissue is primarily made via two mechanisms. During endochondral bone formation in long bones and vertebrae, a fibrocartilaginous template is replaced by calcified bone in a chondrocyte-dependent process. On the other hand, during intramembranous bone formation in calvaria, chondrocytes are not involved and bone is laid down directly by osteoblasts derived from mesenchymal stem cells in the bone marrow, periosteum, and soft tissues. Osteoblasts develop via a series of choreographed steps, including proliferation, extracellular matrix maturation, and mineralization; each step is characterized by gene expression profiles that define the particular osteoblast phenotype (Aubin 1998). Bone is continually remodeled via bone resorption and formation – processes controlled by local and systemic factors that regulate osteoblast and osteoclast differentiation, proliferation, and function.

Bone morphogenetic protein 2 (BMP2) induces osteoblast differentiation, and transforming growth factor β1 (TGFβ1) and 17β-estradiol (E2) are involved in differentiation and proliferation of both osteoblast and osteoclast (Ernest et al. 1988, Antosz et al. 1989, Selvamurugan et al. 2007). The role of NMB in the osteoblastic regulation of bone formation has not been characterized so far.

In this study, we used RT-PCR and immunohistochemical analyses to assess the expression of NMB and NMBR in rat bone tissue. We also examined the effects of NMB on osteoblast proliferation and intracellular signaling, as well as the effects of regulators of bone formation on Nmb expression in osteoblasts.

Materials and methods

Immunohistochemistry

Adult BALB/c mice (Charles River Japan, Kanagawa, Japan) were anesthetized with pentobarbital (50 mg/kg, i.p.) and perfused with saline, followed by 10% buffered formalin. The joints of the fixed hind limbs were decalcified in 1% formic acid for 7 days, dehydrated, and embedded in paraffin. Paraffin sections (6-μm thick) were used for immunostaining as described previously (Nakamachi et al. 2008). Paraffin sections were incubated in 0.3% H2O2 and blocked with 5% normal horse serum for 1 h. Subsequently, sections were incubated overnight at 4 °C with rabbit anti-NMB antibody (antibody no. 104-5; 1:800; Minamino et al. 1984) or rabbit anti-NMBr antibody (Acris Antibodies GmbH, Herford, Germany; 1:200; Matusiak et al. 2005). Primary antibodies were visualized with avidin–biotin complexes and the chromogen DAB (Vector Laboratories, Burlingame, CA, USA). For double immunofluorescence staining, paraffin sections were blocked in 5% normal horse serum and incubated overnight at 4 °C with combinations of primary antibodies, including rabbit anti-NMB antibody (1:800), rabbit anti-NMBr antibody (1:200), and mouse anti-osteocalcin antibody (Biomedical Technologies, Stoughton, MA, USA; 1:500) as an osteoblast marker (Rosati et al. 1994, Sharma et al. 2006). Anti-NMB and anti-NMBr antibodies were detected using Alexa 546-labeled goat anti-rabbit IgG (Invitrogen; 1:800), whereas anti-osteocalcin antibody was detected using Alexa 488-labeled goat anti-mouse IgG (Invitrogen; 1:800) in 90-min incubations at room temperature. Sections were incubated for 5 min with 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI; Roche Diagnostics; 1:10 000) as a nuclear stain and imaged under a microscope (Carl Zeiss, Jena, Germany). The specificity of the immunolabeling was confirmed by incubating the sections in blocking buffer without primary antibodies.

Primary osteoblast cultures and treatment

Primary cultured osteoblasts were prepared from neonatal calvaria of 1-day-old Wistar rats. Fifteen calvaria were excised and all adhering soft tissue was removed. Calvaria were cut into pieces and subjected to six sequential 20-min digestions with 2 mg/ml collagenase type II (Worthington Biochemical Corporation, Freehold, NJ, USA), 0.5 mg/ml trypsin, and 4 mM EDTA (Biken, Osaka, Japan) at room temperature as described previously (Hino et al. 1999). Single-cell suspensions were recovered from each digestion and numbered as populations 1–6 based on the digestion number. Cells from populations 4–6 were pooled and cultured. Cells were then seeded at a density of 1.8×10⁴ cells/cm² on 10 cm dishes in a-modified essential medium supplemented with 10% fetal bovine serum.

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Sigma–Aldrich). The cells were treated with BMP2 (PeproTech EC, London, UK), E2 (Sigma–Aldrich), or TGFβ1 (PeproTech EC).

RT-PCR and real-time PCR

After decapitation of 5-week-old Wistar rats, the tissues (bone, brain, heart, lung, liver, kidney, spleen, jejunum, ileum, and colon) were rapidly removed and stored at −70 °C. Total RNA from the tissues and cells was prepared as described previously (Saito et al. 2012). PCRs were performed using Taq polymerase (Applied Biosystems) with a sense primer (5'-CTCCGTGGTCTCCTGCTTT-3') and an antisense primer (5'-GCTTTCTTTTCGAGGAGGAT-3') specific for Nmb (GenBank accession number NM_001109149); a sense primer (5'-CCGTTTGCTCTTTACCTG-3') and an antisense primer (5'-TTCTGTGTGTGCTATGT-3') specific for Nmbr (GenBank accession number NM_012799); or a sense primer (5'-CTCGGTGGTCCTCCTGCTTT-3') and an antisense primer (5'-GCTTTCTTTTCGAGGAGGAT-3') specific for glyceraldehyde-3-phosphate dehydrogenase (Gapdh; GenBank accession number NM_017008) as a control. The reaction conditions were as follows: denaturation at 94 °C for 1 min; annealing at 58.8 °C for Nmb, 61 °C for Nmbr, or 58 °C for Gapdh for 1 min; and extension at 72 °C for 1 min (38 cycles for Nmb, 35 cycles for Nmbr, and 25 cycles for Gapdh). The expression level of Nmb mRNA was determined by real-time PCRs, which were performed at 95 °C for 15 s and 60 °C for 30 s (50 cycles for Nmb and 40 cycles for Gapdh) using SYBR Premix Ex Taq II (Takara Bio, Inc., Kyoto, Japan) with a sense primer (5'-CCTGCTCTGTGCTTTGTTTCCTG-3') and an antisense primer (5'-GAATCTTGCTTTGGCGTGCTG-3') specific for Nmb or a sense primer (5'-CGGCAAGTTCAGGCCCCACA-3') and an antisense primer (5'-AAGACGCGCATGACTCCACCGA-3') specific for Gapdh on a thermal cycler dice real-time system TP800 (Takara Bio, Inc.).

Alkaline phosphatase assay and alizarin red staining

To induce differentiation and mineralization, cells were incubated at a density of 3 × 10^4 cells/well in 24-well plates and cultured in differentiation medium (α-modified essential medium containing 0.1% BSA, 5 mM β-glycerophosphate, and 50 mg/ml ascorbic acid) with or without NMB. For alkaline phosphatase (ALP) assay, after 7 and 14 days of the culture, cells were washed once with PBS and sonicated in 0.02% Triton X-100. After centrifugation at 13 000 g for 5 min, the ALP activity of the supernatant was measured with a Lab Assay ALP kit (Wako, Osaka, Japan) according to the manufacturer’s protocol. For alizarin red staining, after 21 days of the culture, cells were washed twice with PBS, fixed with 100% ethanol, and

Figure 1
Distribution of Nmb and Nmbr mRNA in rats. RNA was isolated from rat tissues and analyzed in RT-PCRs. The following tissues were examined:

bone, brain, heart, lung, liver, kidney, spleen, jejunum, ileum, and colon.

Figure 2
Immunostaining of NMB and NMBR in osteoblasts of mouse hind limb joints. (A, B and C) Immunostaining results for NMB in joints are shown at low (A) and high (C) magnifications. (A) Box indicates areas enlarged in (C). No signal was detected when joints were incubated without primary antibodies (B). Arrows denote examples of NMB-positive osteoblasts (C). Immunostaining results for NMBR in joints at high magnification. Arrows mark examples of NMBR-positive osteoblasts (D). Full colour version of this figure available via http://dx.doi.org/10.1530/JOE-12-0488.
stained with 1% solution of Alizarin red S (Sigma–Aldrich) as described previously (Hino et al. 1999).

**Proliferation and 5-bromo-2′-deoxyuridine incorporation assays**

Cells were seeded at 3000 cells/well in 96-well plates and grown for 24 h. The cells were starved for 24 h before treatment with different concentrations of NMB (Peptide Institute, Osaka, Japan) and E2 in starvation medium for 24 h. The NMBR antagonist BIM23127 (Bachem, Torrance, CA, USA) and MEK inhibitor U0126 (Calbiochem, San Diego, CA, USA) were added 1 h before NMB treatment. Cell proliferation was evaluated in 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) proliferation and 5-bromo-2′-deoxyuridine (BrdU) incorporation assays using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) and a cell proliferation ELISA for BrdU (Roche Diagnostics) respectively according to the manufacturers’ protocols. When treating cells with E2, the samples were starved in phenol red-free DMEM (Sigma–Aldrich), because phenol red interferes with estrogenic signaling.

**Western blot analysis**

Cells were grown to 80% confluence on 35 mm dishes and then starved for 24 h before treatment with 10 nM NMB. At the indicated time points, the cells were lysed in buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS (Sigma–Aldrich) and centrifuged at 13 000 g for 15 min. Protein concentrations in the resulting supernatant were determined using the Bradford method (Bradford 1976). Ten micrograms of each lysate were subjected to 10% SDS/PAGE and transferred to PVDF membranes. The resulting membranes were subjected to immunoblotting with rabbit anti-phospho-ERK or anti-total-ERK antibodies (Cell Signaling Technology, Beverly, MA, USA; 1:1000). After incubation with HRP-conjugated goat anti-rabbit IgG (Cell Signaling Technology; 1:1000), proteins were detected using enhanced chemiluminescence western blotting reagents (Amersham) according to the manufacturer’s protocol.

![Figure 3](http://joe.endocrinology-journals.org/C209)

**Figure 3**

Expression of NMB and NMBR in osteoblasts. (A, B and C) Double immunostaining of mouse hind limb joints was performed using antibodies specific for NMB (red) and the osteoblast marker osteocalcin (OC; green). Nuclei were counterstained with DAPI (blue). (D, E and F) Double immunostaining of joints was performed using antibodies specific for NMBR (red) and the osteoblast marker OC (green). Nuclei were counterstained with DAPI (blue). (G) RNA was isolated and used in PCRs to examine Nmb and Nmbr expression in rat bone tissue and osteoblasts. The numbers in brackets indicate the predicted sizes of the PCR products in base pairs, which were confirmed using DNA sequencing. Full colour version of this figure available via [http://dx.doi.org/10.1530/JOE-12-0488](http://dx.doi.org/10.1530/JOE-12-0488).
Statistical analysis

Data are expressed as means ± S.E.M. Statistical analyses were performed using Student’s t-tests. P values < 0.05 were defined as statistically significant.

Results

NMB and NMBR in bone tissue

We examined Nmb and Nmbr mRNA expression in rat bone tissue. RT-PCR analysis demonstrated that Nmb expression was detected in all examined tissues and in bone was as abundant as in brain, whereas Nmbr was restrictively expressed in bone, kidney, jejunum, ileum, and colon tissues among peripheral tissues, and its expression in bone was next to that in brain (Fig. 1). Immunohistochemical study of mouse hind limb tissues using specific antibodies detected NMB and NMBR proteins in lining osteoblasts of the medullary cavity in the articular cartilage (Fig. 2A, B, C and D). When we performed double immunostaining using mouse hind limb tissue and antibodies specific for NMB, NMBR, and the osteoblast marker osteocalcin (Fig. 3A, B, C, D, E and F), NMB and NMBR colocalized with osteocalcin. Incidentally, immunostaining the limb tissue detected NMB and NMBR in round chondrocytes in the articular cartilage (Supplementary Figure 1B and D, see section on supplementary data given at the end of this article). In epiphyseal growth plates of the limb tissue, NMB and NMBR were detected in proliferative chondrocytes (Supplementary Figure 1C and E).

NMB induces osteoblast proliferation

To elucidate the physiological roles of NMB in osteoblasts, we assessed the effects of NMB on the differentiation and proliferation of rat primary osteoblasts. At first, we verified mRNA expressions of Nmb and Nmbr in rat primary osteoblasts by RT-PCR analysis (Fig. 3G). Incidentally, mRNA expression of Gprr was detected in rat osteoblasts but that of Gpr was not, although GRP shows high homology with NMB (Ohki-Hamazaki 2000; data not shown).

Regarding differentiation of rat primary osteoblasts, we assessed the effects of NMB on ALP activity by ALP assay and evaluated bone nodule formation using alizarin red staining. NMB treatment did not significantly affect osteoblast differentiation (Fig. 4) but did cause a dose-dependent increase in osteoblast proliferation (Fig. 5). We found that, compared with control cells, cultured cells treated with 10 nM NMB showed 1.9- and 1.7-fold increases in the cell number and DNA synthesis respectively (Fig. 5A and B). Treatment with the NMBR antagonist BIM23127 (1000 nM) ameliorated these responses (Fig. 5C and D).

NMB-induced osteoblast proliferation via ERK1/2 activation

ERK1/2 MAPK signaling is activated by growth factors and is associated with cell proliferation (Alessi et al. 1994, Cowley et al. 1994). To determine the role of the ERK1/2 signaling pathway in NMB-induced osteoblast proliferation, we assessed ERK1/2 activation after treating the cells with 10 nM NMB; NMB increased pERK1/2 levels over time (Fig. 6A). The cell number and DNA synthesis were significantly attenuated by pretreating the cells with the MEK inhibitor U0126 (100 nM; Fig. 6B and C).

Figure 4

Roles of NMB in osteoblast differentiation. (A and B) Cells were cultured in differentiation medium (β-modified essential medium containing 0.1% BSA, 5 mM β-glycerophosphate, and 50 mg/ml ascorbic acid). (A) Cells were treated with 10 nM NMB for 7 and 14 days. ALP activity was assessed in ALP assay. (B) Cells were treated with 10 nM NMB for 21 days. Bone nodule formation was assessed by alizarin red staining. Data are presented as means ± S.E.M. (n = 4). NS, P value not significant. Full colour version of this figure available via http://dx.doi.org/10.1530/JOE-12-0488.
Effects of BMP2, E2, and TGFβ1 on Nmb expression

We then examined the effects of BMP2, E2, and TGFβ1 on levels of Nmb mRNA (Fig. 7A). BMP2 had no effect on Nmb expression, whereas E2 and TGFβ1 increased and decreased Nmb expression respectively. Of note, none of these potential regulators affected Nmbr mRNA levels (data not shown). To determine the role of NMB in E2-induced cell proliferation, we counted the cells after treating them with 0.01 or 0.1 nM E2; E2 dose dependently increased osteoblast proliferation, and 0.1 nM E2 resulted in 1.7-fold increment in the number of cultured cells (Fig. 7B). Cell proliferation was significantly inhibited by pretreating the cells with the NMBR antagonist BIM23127 (1–1000 nM). BIM23127 (1 μM) suppressed E2-induced proliferation by 61% (Fig. 7C). In addition, a RIA specific for NMB demonstrated that E2 treatment significantly augmented NMB secretion from the cultured osteoblasts compared with control samples (data not shown).

Discussion

Bone is constantly remodeled as old bone is resorbed by osteoclasts and new bone is formed by osteoblasts. Although various skeletogenic factors are known to regulate these processes, the expression profile and functional role of NMB in bone have not been examined. We found that Nmb mRNA was expressed in all the examined rat tissues, including brain, bone, heart, lung, kidney, jejunum, ileum, and colon (Fig. 1). On the other hand, Nmbr mRNA was expressed in rat brain, bone, kidney, jejunum, ileum, and colon. Our results agree with previous reports showing immunoreactive NMB in rat brain and gastrointestinal tissues (Sakamoto et al. 1987) and Nmbr mRNA expression in mouse brain and intestine tissues (Ohki-Hamazaki 2000). Nmb and Nmbr expression in bone was more abundant than those in kidney, jejunum, or ileum, suggesting a prominent functional role for NMB in bone.

Immunohistochemical analysis detected NMB and NMBR in osteoblasts from mouse hind limb tissue (Figs 2 and 3A, B, C, D, E and F). NMB and NMBR were also detected in epiphyseal chondrocytes from mouse limb tissue (Supplementary Figure 1B and D). Chondrocytes regulate endochondral bone formation, which occurs in the growth plate. Our immunohistochemical data showed higher levels of NMB and NMBR in proliferative chondrocytes of the lower zone of mouse limb growth plates compared with cells in the upper proliferative and hypertrophic zones, suggesting that NMB functions during a later stage of chondrocyte proliferation (Supplementary Figure 1C and E). Taken together, these data demonstrate largely overlapping distributions of NMB and NMBR in the epiphysis and the growth plate, indicating...
that NMB is primarily secreted by osteoblasts, epiphyseal chondrocytes, and proliferative chondrocytes in the lower zone of the growth plate to regulate osteoblast and chondrocyte proliferation through NMBR. Thus, both NMB and NMBR were found to colocalize in osteoblasts from mouse hind limb tissue, suggesting that endogenous NMB is likely an autocrine or a paracrine factor that is secreted from osteoblasts. Also in rat, Nmb and Nmbr were coexpressed in the primary cultured osteoblasts, but less abundantly than in the bone tissue (Fig. 3 G). As our preliminary study with RT-PCR demonstrated that the expression of Nmb and Nmbr in the primary osteoblasts did not change under the differentiation condition (data not shown), this difference might be due to the coexistence of other Nmb/Nmbr expressing cells, such as chondrocytes (Supplementary Figure 1) in bone tissue or the phenotype alteration of isolated primary osteoblasts during the prolonged culture.

Figure 6
Role of ERK1/2 MAPK in NMB-induced osteoblast proliferation. (A) Cells were treated with 10 nM NMB for 0, 5, or 30 min. ERK1/2 activation was assessed on western blots. (B and C) Cells were treated with 10 nM NMB for 24 h with or without pretreatment with the MEK inhibitor U0126 (100 nM). Cell number (B) and DNA synthesis (C) was analyzed in proliferation and BrdU incorporation assays respectively. Data are presented as means ± S.E.M. (n = 4). *P < 0.05 vs vehicle control group and #P < 0.05 vs the group not treated with U0126.

Figure 7
Effects of potential regulatory molecules on osteoblastic Nmb expression. (A) Effects of BMP2, E2, and TGFβ1 on Nmb mRNA levels in osteoblasts. Cells were treated with BMP2 (100 ng/ml), E2 (0.1 nM), or TGFβ1 (5 ng/ml) for 24 h. Nmb expression levels were assessed by real-time PCRs and normalized based on Gapdh expression levels. (B) Effects of E2 on osteoblast proliferation. Cells were treated with 0.01–0.1 nM E2 for 24 h. (C) Cells were treated with 0.1 nM E2 for 24 h with or without pretreatment with the NMBR antagonist BIM23127 (0.1–1000 nM). Cell number was analyzed (B and C) in proliferation assays. Data are presented as means ± S.E.M. (n = 4). *P < 0.05 vs the vehicle-treated control group and #P < 0.05 vs the group not treated with BIM23127.
Primary calvarial cultures are often used to study osteoblast differentiation in vitro (Owen et al. 1990). These cells undergo an initial proliferative phase before they reach confluence and lay down a collagenous matrix, which is eventually mineralized. We examined the role of NMB/NMBR signaling in rat primary osteoblasts. NMB did not significantly affect osteoblast differentiation (Fig. 4). We, however, found that NMB induced osteoblast proliferation through NMBR, an effect that was inhibited by the NMBR antagonist BIM23127 (Fig. 5). These results suggest that NMB contributes to osteoblast proliferation rather than differentiation. Moreover, the data agree with a previous study showing that NMB is an autocrine growth factor for non-small-cell lung carcinoma (Siegfried et al. 1999).

Skeletal development requires precise control of osteoblast proliferation and differentiation. Osteoblast proliferation is associated with ERK1/2 MAPK signaling, which has been shown to be activated by growth factors in mouse primary osteoblast, mouse and human osteoblast cell lines (Shimoaka et al. 2002, Engelbrecht et al. 2003). Similar results have been observed in the mouse ATDC5, chondrogenic cell line, in which insulin, insulin-like growth factor 1 (IGF1), and parathyroid hormone induce ERK1/2 activation to regulate cell proliferation (Fujita et al. 2002, Phornphutkul et al. 2006, MacRae et al. 2007). We recently found that NMB stimulates ATDC5 cell proliferation through NMBR (Saito et al. 2012). Here, we showed that NMB activated ERK1/2 to induce osteoblast proliferation, which was inhibited by the MEK inhibitor U0126, indicating that the effect of NMB on osteoblast proliferation is mediated by ERK1/2 (Fig. 6). Therefore, NMB augmented osteoblast proliferation but not osteoblast differentiation. This finding is consistent with a previous report demonstrating that active MAPK/ERK-expressing osteoblasts, which are derived from human bone marrow stromal cells, attenuate osteoblast differentiation (Ozychka & Leboy 2005).

We also examined the effects of skeletogenic regulators on Nmb expression in osteoblasts (Fig. 7A). BMP2 – an inducer of osteoblast differentiation (Hino et al. 1999, Selvamurugan et al. 2007) – did not affect Nmb mRNA levels in osteoblasts. TGFβ1 induces bone formation in vivo (Noda & Camilli 1989) but produces inconsistent effects on osteoblast proliferation and differentiation in vitro depending on the TGFβ1 concentration, cell density, differentiation stage, and presence of other skeletogenic factors (Centrella et al. 1987, Pfeilschifter et al. 1987, Hock et al. 1990, Chen et al. 1991, de Gorter et al. 2011). In general, however, TGFβ1 stimulates osteoblast proliferation and differentiation (Antosz et al. 1989, Bonevald & Dallas 1994, Ghayor et al. 2005). Our data indicated that TGFβ1 suppresses Nmb mRNA expression, which is supported by reports of TGFβ1-mediated inhibition of osteoblast proliferation (Noda & Rodan 1986, Cabiling et al. 2007, van der Zande et al. 2008). On the other hand, low levels of E2 are associated with osteoporosis in postmenopausal women. E2 induces the proliferation and differentiation of primary osteoblasts derived from mice, rats, and humans (Ernest et al. 1988, Scheven et al. 1992, Verhaar et al. 1994, Qu et al. 1998, O'Shaughnessy et al. 2000, Chen et al. 2002). Further, E2 increases IGF1 expression in osteoblasts, and IGF1 secreted from osteoblasts stimulates osteoblast proliferation (Ernst et al. 1989, Centrella et al. 1990, Kassem et al. 1998). Our data demonstrated that E2 increased Nmb mRNA levels, and E2-induced osteoblast proliferation was significantly suppressed by the NMBR antagonist BIM23127, suggesting that NMB is partially but distinctly involved in the effects of E2 on osteoblast proliferation as well as IGF1 (Fig. 7).

In conclusion, this study is the first to show that NMB binds NMBR to augment osteoblast proliferation, an effect that is mediated by activation of the ERK1/2 MAPK pathway and underlies, at least in part, E2-induced proliferation. To understand the functions of NMB in bone physiology, further studies are needed, including examinations of potential interactions between NMB and other growth factors. Taken together, our data suggest important roles for NMB as an autocrine or paracrine agent during bone formation and skeletal development.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1677/JOE-12-0488.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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