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Neuromedin B receptor mediates neuromedin B-induced COX-2 and IL-6 expression in human primary myometrial cells

Jingfei Chen,[1] Yun Shi,[1] Jingrui Huang,[1] Jiefeng Luo,[1] Weishe Zhang [1,2]

[1]Department of Obstetrics and Gynecology, Xiangya Hospital, Central South University, Changsha, Hunan, China
[2]Hunan Engineering Research Center of Early Life Development and Disease Prevention, Changsha, Hunan, China

Correspondence to
Dr Weishe Zhang, Department of Obstetrics and Gynecology, Xiangya Hospital, Central South University, Changsha, Hunan 410008, China; weishezhang@csu.edu.cn

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ABSTRACT

The precise mechanisms that lead to parturition remain unclear. In our initial complementary DNA (cDNA) microarray experiment, we found that the neuromedin B receptor (NMBR) was differentially expressed in the human myometrium during spontaneous or oxytocin-induced labor. We have previously shown that neuromedin B (NMB) could induce interleukin 6 (IL-6) and type 2 cyclo-oxygenase enzyme (COX-2) expression in the primary human myometrial cells via nuclear factor kappa B (NF-κB) transcription factor p65 (p65) and Jun proto-oncogene, activator protein 1 (AP-1) transcription factor subunit (c-Jun). This study is aimed to investigate whether NMBR is required for NMB-induced effect. Primary myometrial cell culture was established to provide a suitable model to investigate the mechanism of NMB in labor initiation. Immunochemical staining was conducted to validate the NMBR expression in primary myometrial cells. The mRNA and protein expression of NMBR, p65, c-Jun, COX-2 and IL-6 were assessed by Quantitative Real Time PCR (RT-qPCR) and western blotting. Lentiviruses with shRNAs targeting NMBR or containing cDNA sequence of NMBR were transfected to primary myometrial cells to knockdown or overexpress NMBR. Cell death was determined by annexin V and propidium iodide staining and analyzed by flow cytometry. The upregulation of COX-2 and IL-6 and phosphorylation of p65 and c-Jun were significantly attenuated by knockdown of NMBR and enhanced by overexpressed NMBR following NMB treatment, with no significant change in total p65 and c-Jun. In summary, this study showed that NMBR-mediated NMB-induced NF-κB and AP-1 activation, which in turn, induce expression of IL-6 and COX-2 in primary myometrial cells.

Significance of this study

What is already known about this subject?
- Myometrial neuromedin B receptor (NMBR) increased with gestation advanced but decreased after delivery.
- Neuromedin B (NMB) could induce interleukin 6 (IL-6) and type 2 cyclo-oxygenase enzyme (COX-2) expression via nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1) in primary cultured myometrial cells.
- Maternal exposure to the NMB shortened the gestational age of pups in mice.

What are the new findings?
- NMBR is critical for NMB-induced COX-2 and IL-6 upregulation.
- NMB-related activation of NF-κB and AP-1 is mediated by NMBR.
- NMBR could be a therapeutic target for preterm birth in the future.

How might these results change the focus of research or clinical practice?
- NMBR might be a novel therapeutic target and develop novel NMB antagonist as a preventive or therapeutic method for preterm birth.

INTRODUCTION

More than 1 in 10 babies are born prematurely worldwide and rates of preterm birth (PTB) were increasing steadily in most countries in the past decades, making it the leading cause of neonatal mortality and morbidity.[1 2] However, the underlying mechanism remains unclear. Current therapy for PTB mainly includes β_2 blockers (ritodrine), calcium channel blockers (magnesium sulfate) or oxytocin receptor antagonists, but their effects are limited.[3] Therefore, the identification of novel effective targets and the development of corresponding small molecules to treat this disease are urgent.

Through our previous complementary DNA (cDNA) microarray screening, we found that the mRNA-encoding neuromedin B receptor (NMBR) was differentially expressed in the human uterine myometrium during spontaneous or oxytocin-induced labor.[4] As a G protein-coupled receptors (GPCR) family member, NMBR is expressed on the cell surface of different tissues such as brain, testis, esophagus, duodenum, rectum and uterus.[5 6] Emerging evidences suggest that NMBR have a wide spectrum of physiological effects, including regulation of exocrine and endocrine secretions, smooth muscle contraction, feeding, blood pressure, blood glucose, body temperature and cell growth.[6] The levels of

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Nmbr mRNA and protein were found to peak at parturition and decrease sharply after delivery in mouse myometrium.[7] All above indicated that NMBR is likely an ideal candidate target in regulating labor initiation.

The bombesin (BN) family of peptides received their unusual name because BN and most of the subsequent other invertebrate members of this family were originally isolated from frog skins and they were named after the genus of the frog. Neuromedin B (NMB) and gastrin-releasing peptide (GRP) are members of BN-like peptide family in mammals.[6] Three classes of closely related receptors comprise the mammalian BN family of receptors: a 384-amino acid GRP-preferring receptor (called GRPR or BB2 receptor), a 390-amino acid NMB-preferring receptor (NMBR or BB1 receptor) and a 399-amino acid orphan receptor termed BN-receptor subtype 3 (called BRS-3 receptor or BB3 receptor).[8] In the rat central nervous system, autoradiographic study demonstrated that NMBR and GRPR having high affinity for NMB or GRP, respectively.[6] Similarly, we found that the administration of NMB could induce an increase of $[Ca^{2+}]_i$ in pregnant smooth muscle cells (SMCs),[9] and maternal exposure to the NMB shortened the gestational age of pups in mice.[7] Studies also showed that maintenance of body temperature after a change in ambient temperature and contraction of the gastric smooth muscle elicited by NMB or GRP were not affected by the elimination of NMBR,[10] suggesting NMB could function through other receptors in the absence of NMBR. Therefore, whether NMB acts mainly through NMBR in primary myometrial cells needs to be validated.

Many researchers demonstrated that nuclear factor kappa B (NF-κB) transcription factor p65 (p65) and activator protein 1 (AP-1) are associated with labor onset. P65 was shown to regulate many cytokines such as interleukin (IL)-6, type 2 cyclo-oxygenase enzyme (COX-2), IL-8, IL-1β, matrix metalloproteinase 9 and tumor necrosis factor-α.[11–18] c-Jun (Jun proto-oncogene, AP-1 transcription factor subunit), the most widely investigated protein of AP-1, is involved in the expression of various inflammatory genes such as IL-6 and COX-2 by binding to their transcription factor-binding sites to increase their expression in several cell types.[19–24] Our previous study reported that NMB-induced COX-2 and IL-6 expression was mediated via p65 and c-Jun in human primary myometrial cells.[25] However, whether NMBR is required in the effect of NMB on p65 and c-Jun activation and IL-6 and COX-2 expression has never been investigated.

Therefore, those findings prompted us to investigate whether NMBR is indispensable in p65 and c-Jun activation and regulation of COX-2 and IL-6 expression by NMB in human primary myometrial cells.

MATERIALS AND METHODS

Sample collection

Human uterine smooth muscle tissues were collected from full-term deliveries at selective cesarean section without labor under a protocol approved by the ethics committee of Central South University (No: 2019030155). Women who had complications such as pre-eclampsia, fetal growth restriction and gestational diabetes were excluded from the study. Human myometrial cells were collected according to our previous publication.[9] Briefly, myometrial tissues (1 cm×1 cm×1 cm) were obtained from the upper edge of the uterine incision after fetus delivery and before oxytocin injection. Myometrial tissues were then digested and dissociated, and myometrial cells were collected and cultured.

Cell culture and treatment

The cells were cultured at in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin (100 units/mL), and 1% streptomycin (100 mg/mL) (all from Hyclone, Utah, USA) in 5% CO_2 at 37°C in a humidified incubator. Cells were treated with 1 μM NMB (H-3280.0001; Bachem, Switzerland) for 24 hours. NMB was dissolved in water, and the same amount of water was used as vehicle control.

Stable knockdown of NMBR

Primary myometrial cells were infected with lentiviruses with small hairpin RNA (shRNAs) targeting NMBR (Oligo-Engine, Seattle, Washington, USA) and then selected with blasticidin (4 μg/mL; Invitrogen) for 2 weeks. The stable clones were pooled and used for further downstream experiments as indicated.

Stable overexpression of NMBR

Primary myometrial cells were infected with lentiviruses containing cDNA sequence of NMBR (Oligo-Engine) and then selected with puromycin (2 μg/mL; Invitrogen) for 3 weeks to generate stable clones, which were pooled and used for further downstream experiments as indicated.

Western blotting

Total cellular protein was extracted from the above treated cells using ice-cold radioimmunoprecipitation assay lysis buffer (Beyotime Biotechnology, China) containing protease inhibitor cocktail (Selleck, Houston, Texas, USA) and phosphatase inhibitor (Selleck). After determination of protein concentration with Bicinchoninic acid (BCA) assay, equivalent protein samples (30 μg protein extract was loaded on each lane) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto Polyvinylidene fluoride (PVDF) membranes (Millipore) and blocked with 5% non-fat milk for 1 hour at room temperature. The membrane was incubated with NMBR (1:1000, Abcam), p65 (1:1000, Cell Signaling), phosphorylated p65 Ser^{536} (1:1000, Cell Signaling), c-Jun (1:1000, Cell Signaling), phosphorylated c-Jun Ser^{63} and Ser^{73} (1:1000, Cell Signaling), COX-2 (1:1000, Cell Signaling), IL-6 (1:1000, Cell Signaling) and GAPDH primary antibody (1:1000, Santa Cruz) overnight at 4°C. Primary antibody was detected by binding horseradish peroxidase-conjugated antirabbit or antimouse secondary antibody with an ECL plus kit. To normalize the western blotting data, relative levels of phospho-p65 and phospho-c-Jun protein were normalized to the total p65 or c-Jun, respectively. Other non-phosphorylated proteins, like COX-2, were normalized to the internal control Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

RT-qPCR analysis

Total RNA was extracted from untreated or NMB-treated cells using a Mini RNA Isolation II kit (Zymo Research,

Table 1 Primer sequences used for real-time qPCR

Gene	Orientation	5′–3′ sequence
IL6	Forward	5′-TACATCCTCGACGGCATCTC-3′
	Reverse	5′-GCCATCTTTGGAAGGTTCAG-3′
PTGS2	Forward	5′-GGTTGCTGGTGGTAGGAATG-3′
	Reverse	5′-TAAAGCGTTTGCGGTACTCA-3′
NMBR	Forward	5′-CCGTCGAGGCCACCATGATCTCGGAATGATGCGAC-3′
	Reverse	5′-CGGGATCCAAATGTTTGCAACTGCTGC-3′
Actb	Forward	5′-CTCCATCCTGGCCTCGCTGT-3′
	Reverse	5′-GCTGTCACCTTCACCGTTCC-3′

qPCR, quantitative PCR.

Irvine, California, USA) according to the manufacturer's instructions. RNA concentration and quality were determined by measuring OD260 (optical density at 260 nm) and the ratio of OD260/OD280 with NanoDrop ND-2000. cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen). Real-time PCR was performed using iTaq SYBR Green Supermix (Bio-Rad, Hercules, California, USA) on an ABI ViiATM real-time PCR detection system (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The value of $2^{-\Delta\Delta Ct}$ was used to determine the fold difference between samples. The housekeeping gene *Actb* was amplified in parallel as an internal loading control. The ratio of the target gene over *Actb* in each sample was obtained as an indication of the target gene expression. The primer sequences used for amplifying *NMBR*, *PTGS2*, *IL-6* and *Actb* were shown in table 1.

Flow cytometry

Cells were suspended at 1×10^6 cells/mL, and 5 µL of annexin V and propidium iodide staining solution was added to 200 µL of the cell suspension. After the cells were incubated at room temperature for 15 min in the dark, cells were centrifuged at 1000 g for 5 min and resuspended in 200 µL phosphate-buffered saline. Stained cells were then assayed and quantified using a FACSort Flow Cytometer (Beckman Coulter, Brea, California, USA).

Statistical analysis

All data are expressed as means±SD of at least three independent experiments. The number for each study indicates repeated experiments using uterine smooth muscle cells from different pregnancies. All statistical analyses were performed using GraphPad Prism V software. Student's t-test or one-way analysis of variance test followed by the Newman-Keuls multiple comparison test were used, where appropriate, to assess significant differences. Significance was set at $p<0.05$ (marked as *). Higher significance levels were set at $p<0.01$ (marked as **).

RESULTS

Primary cell culture and identification

Cultured primary myometrial cells were long and fusiform or polygonal in shape under inverted microscopy (figure 1A). Cell cloning was performed after 48 hours of culture, the medium was first changed on day 5 and fusion was apparent in some clones 1 week later. Cells nested on top of each other on day 14 (figure 1B). Immunocytochemistry results showed NMBR was present in the membrane of the cultured myometrial cells (figure 1C, D). Taken together, these results indicate that the cultured myometrial cells isolated from term myometrium of patients showed significant amounts of NMBR proteins.

NMBR mediates NMB-induced COX-2 and IL-6 upregulation

To determine whether NMBR mediates COX-2 and IL-6 upregulation in myometrial cells, we depleted or overexpressed NMBR. The efficacy of NMBR knockdown and overexpress was verified by RT-qPCR and western blotting. NMBR dramatically reduced in knockdown group at both mRNA and protein level in comparison to empty vector group (figure 2A, C), and significantly increased in overexpression group at both RNA and protein level, compared with empty vector group (figure 2B, D). Neither knockdown or overexpress NMBR induce apoptosis in primary cultured myometrial cells (figure 2E, F). Our previous study established the ideal dose of NMB in our system as 1 µM.[9] NMBR knockdown led to decreased COX-2 and IL-6 mRNA and protein levels in the presence of NMB, compared with the empty vector group (figure 3A, B and E), while overexpression of NMBR increased NMB-induced IL-6 and COX-2 expression at both mRNA and protein level following NMB stimulation (figure 3C, D and F). These data demonstrated that NMBR is required for NMB-induced IL-6 and COX-2 expression.

Activation of NF-κB and AP-1 in primary myometrial cells is mediated by NMBR

Our previous data showed that NMB induced IL-6 and COX-2 via p65 and c-Jun in primary cultured myometrial cells[25]; here, we would like to know whether knockdown and overexpress NMBR could affect p65 and c-Jun phosphorylation following NMB treatment. NMBR knockdown reduced NMB-induced phosphorylation of p65 on s536 and c-Jun on s63 and s73 sites with total p65 and c-Jun proteins slightly reduced (figure 4A, C, D and E). Overexpression of NMBR increased NMB-induced phosphorylation of p65 on s536 and c-Jun on s63 and s73 sites with no significant reduction in total p65 and c-Jun proteins (figure 4B, F, G and H). Therefore, we can conclude that NMB-induced NF-κB and AP-1 activation relies on NMBR.

DISCUSSION

Bombesin (BB) is a 14-amino acid peptide that was originally isolated from the skin of the frog, *Bombina bombina*. Subsequently, two mammalian counterparts of BB were identified, namely NMB and GRP. These peptides may serve as endogenous ligands for the three different receptor subtypes, designated as the NMBR or BB1 receptor, and the GRPR or BB2 receptor, an orphan receptor, named BRS-3 or BB3 receptor.[8] NMBR, a typical G-protein coupled receptor with seven membrane-spanning regions, once bound by its ligands, several intracellular signaling cascades including phospholipase activation, calcium mobilization and protein kinase C activation lead to the expression of several genes, DNA synthesis or cellular effects such as secretion and contraction.[6] Surprisedly, NMBR was found in our initial cDNA microarray screening differentially expressed in the human uterine myometrium during spontaneous

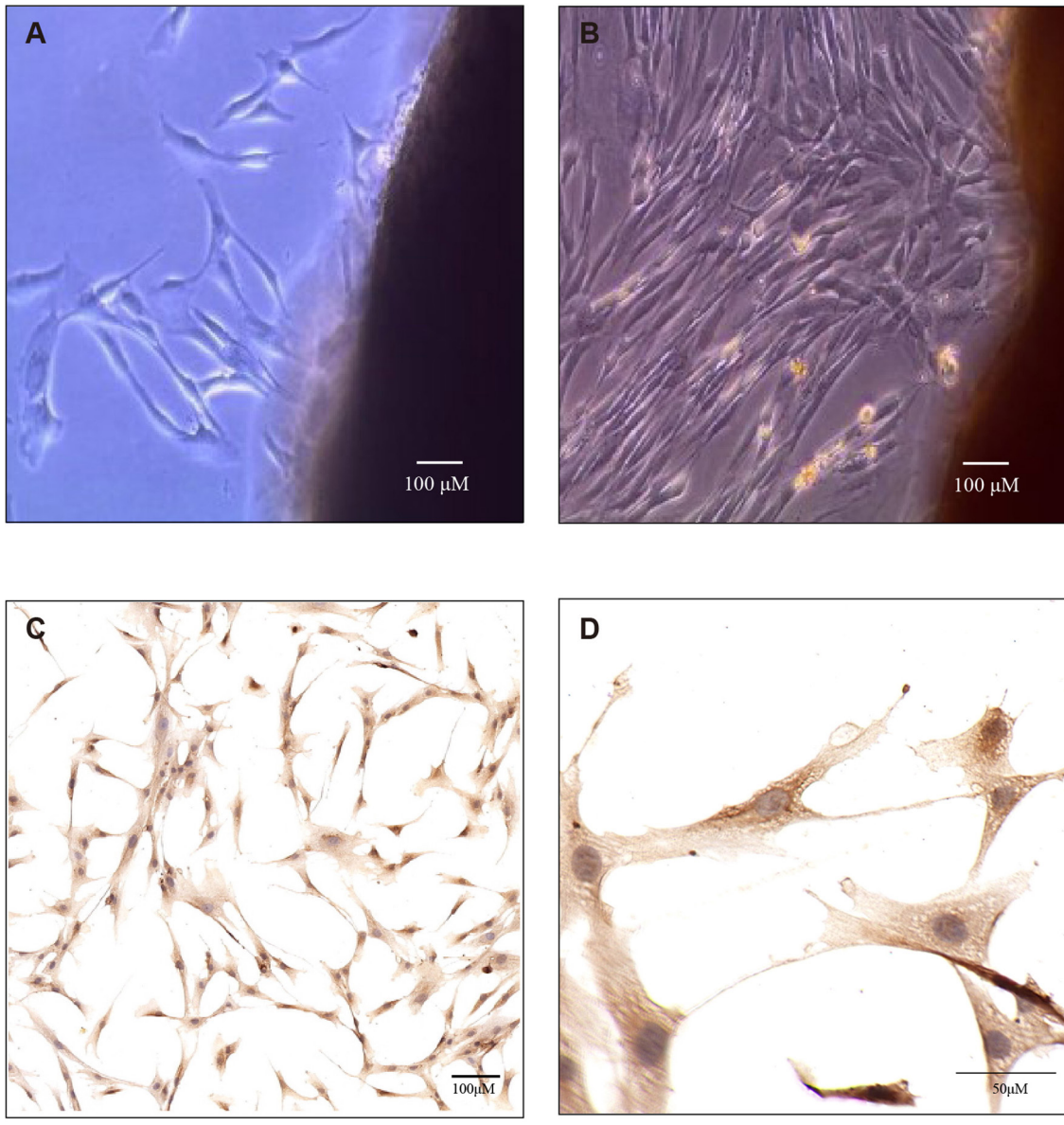

Figure 1 The cultured primary myometrial cells from term myometrium and their verification. (A) Cultured primary myometrial cells at day 5 under inverted microscopy. At least five fields were counted. Original magnification 40×. (B) Cultured primary myometrial cells at day 14 under inverted microscopy. At least five fields were counted. Original magnification 40×. (C) The positive expression of neuromedin B receptor (NMBR), as assessed by immunocytochemistry. At least five fields were counted. Original magnification 40×. (D) The positive expression of NMBR, as assessed by immunocytochemistry. Original magnification 400×.

and oxytocin-induced labor.[26] By establishing mouse pregnant model, we also observed that *Nmbr* is differentially expressed in mouse myometrium during gestation, with maximal mRNA and protein expression during labor onset and a sharp decrease after delivery.[7]

Many studies reported increased concentrations of certain cytokines, most notably IL-6 in the serum and amniotic fluid of patients with preterm labor.[27 28] Our previous study showed that NMB could increase IL-6 expression via the rela/p65 pathway in cultured mouse primary myometrial cells in vitro.[7] Prostaglandins (PGs) are believed to be involved in uterine contractions, cervical ripening and fetal membrane rupture during parturition.[29] COX-2 is the rate-limiting enzyme involved in PG synthesis. It increases in amnion cells and human myometrium in both term and preterm labor.[11 18] We previously observed that the expression of COX-2 and IL-6 were significant increased following NMB stimulation in human primary cultured myometrial cells.[25] This study validated that NMB induced COX-2 and IL-6 expression in a NMBR-dependent manner in human primary myometrial cells.

Studies from others' laboratory showed that NF-κB and AP-1 could induce the expression of IL-6 and COX-2 in macrophages.[25 30 31] Our previous study elegantly showed that both p65 and c-Jun are indispensable for the induction of IL-6 and COX-2 following NMB treatment in human primary cultured myometrial cells. Here we found that p-p65 and p-c-Jun expression were significantly increased

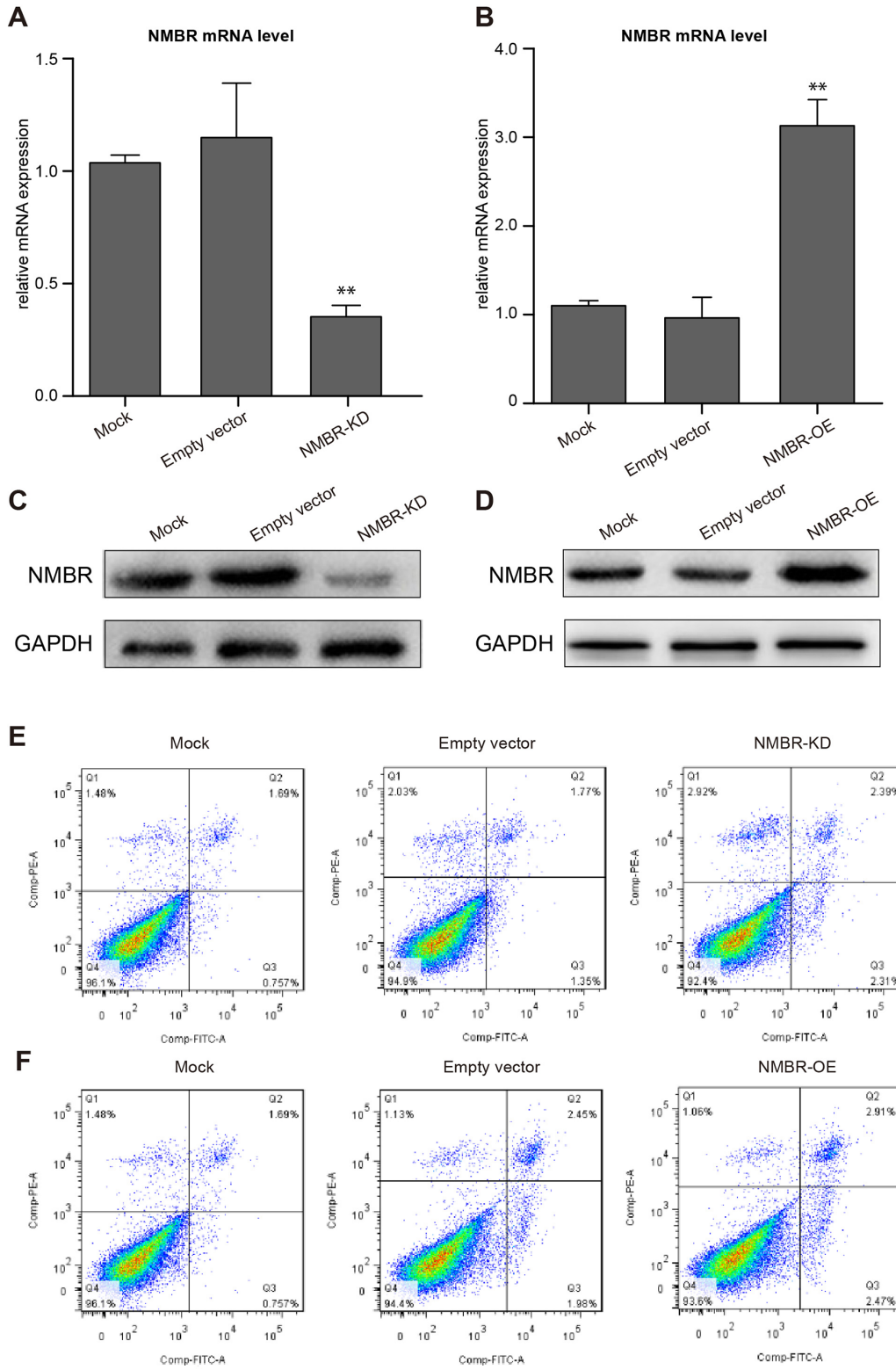

Figure 2 The efficiency of lentiviruse-mediated neuromedin B receptor (NMBR) knockdown or overexpression in primary myometrial cells and the effect of NMBR knockdown or overexpression on apoptosis of primary myometrial cells. (A–D) The protein (A) and mRNA (C) levels of NMBR in primary myometrial cells infected with lentiviruses with NMBR knockdown. The protein (B) and mRNA (D) levels of NMBR in primary myometrial cells infected with lentiviruses with NMBR overexpression. GAPDH was used as an internal control. Representative western blotting results are shown. Data are expressed as mean±SD of six independent experiments (n=6). *p<0.05; **p<0.01, against the empty vector group. (E and F) Apoptosis of primary myometrial cells infected with lentiviruses with NMBR knockdown (E) or NMBR overexpression (F) was determined by flow cytometry. OE: Overexpression, KD: Knockdown, Comp-PE-A: Compensation for PE channel,Comp-FITC-A: Compensation for FITC channel.

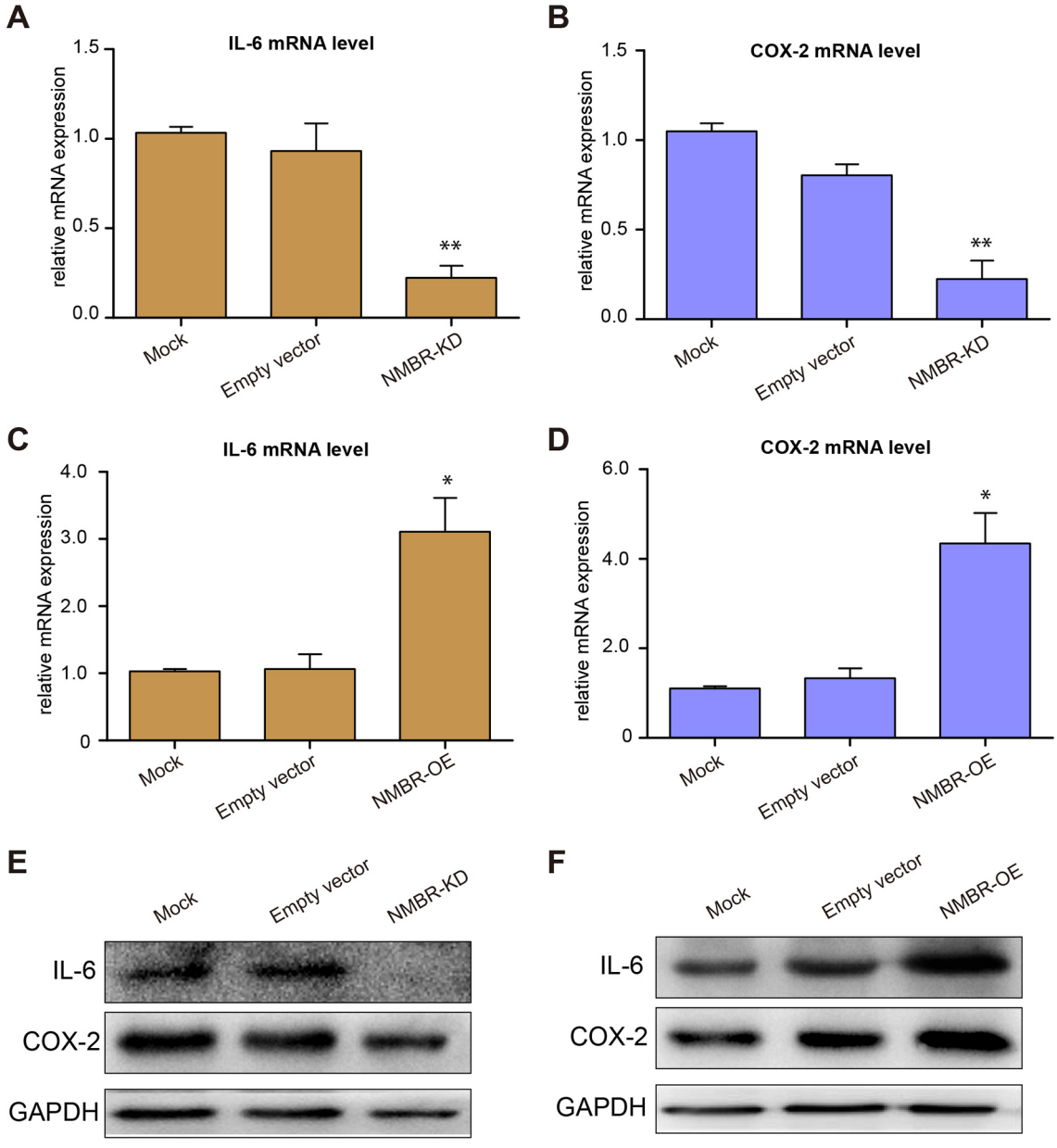

Figure 3 Neuromedin B receptor (NMBR) is indispensable for type-2 cyclo-oxygenase enzyme (COX-2) and interleukin 6 (IL-6) expression after neuromedin B (NMB) treatment in cultured primary myometrial cells. Primary myometrial cells were infected with lentiviruses with NMBR overexpression or knockdown or the empty vector, and then treated with 1 µM NMB for 1 hour. (A and C) *IL6* mRNA levels were analyzed by RT-qPCR. (B and D) *PTGS2* mRNA levels were analyzed by RT-qPCR. (E and F) IL-6 and COX-2 protein levels were probed by western blotting with GAPDH as an internal standard. Blots are representative. Data are expressed as mean±SD of six independent experiments (n=6). *p<0.05; **p<0.01, against the empty vector group. OE: Overexpression, KD: Knockdown, GAPDH was used as an internal control.

in response to overexpression of NMBR, while NMBR knockdown decreased NMB-induced p-p65 and p-c-Jun expression in the presence of NMB, which indicates NMB, via binding to NMBR, activated p65 and c-Jun, which in turn increased IL-6 and COX-2 expression in primary myometrial cells. In addition, NMBR knockdown or overexpression does not lead to cultured primary myometrial cells apoptosis, which pave a good way for our subsequent experiments (figure 2E, F).

There are some limitations of our study. First, although we demonstrated that NMBR is required for NMB-induced increase of COX-2 and IL-6 expression by upregulating p65 and c-Jun expression in vitro, we did not investigate whether such a phenomenon can be replicated in vivo. To establish an NMBR knockout mouse model is a good way to solve the problem. Second, we cannot directly rule out the possibility of GPCR contributing to NMB's effect without investigating the effect of GPCR knockdown or overexpression.

In conclusion, we have demonstrated in this study that NMBR is required for the phosphorylation of p65 and c-Jun and subsequent induction of COX-2 and IL-6

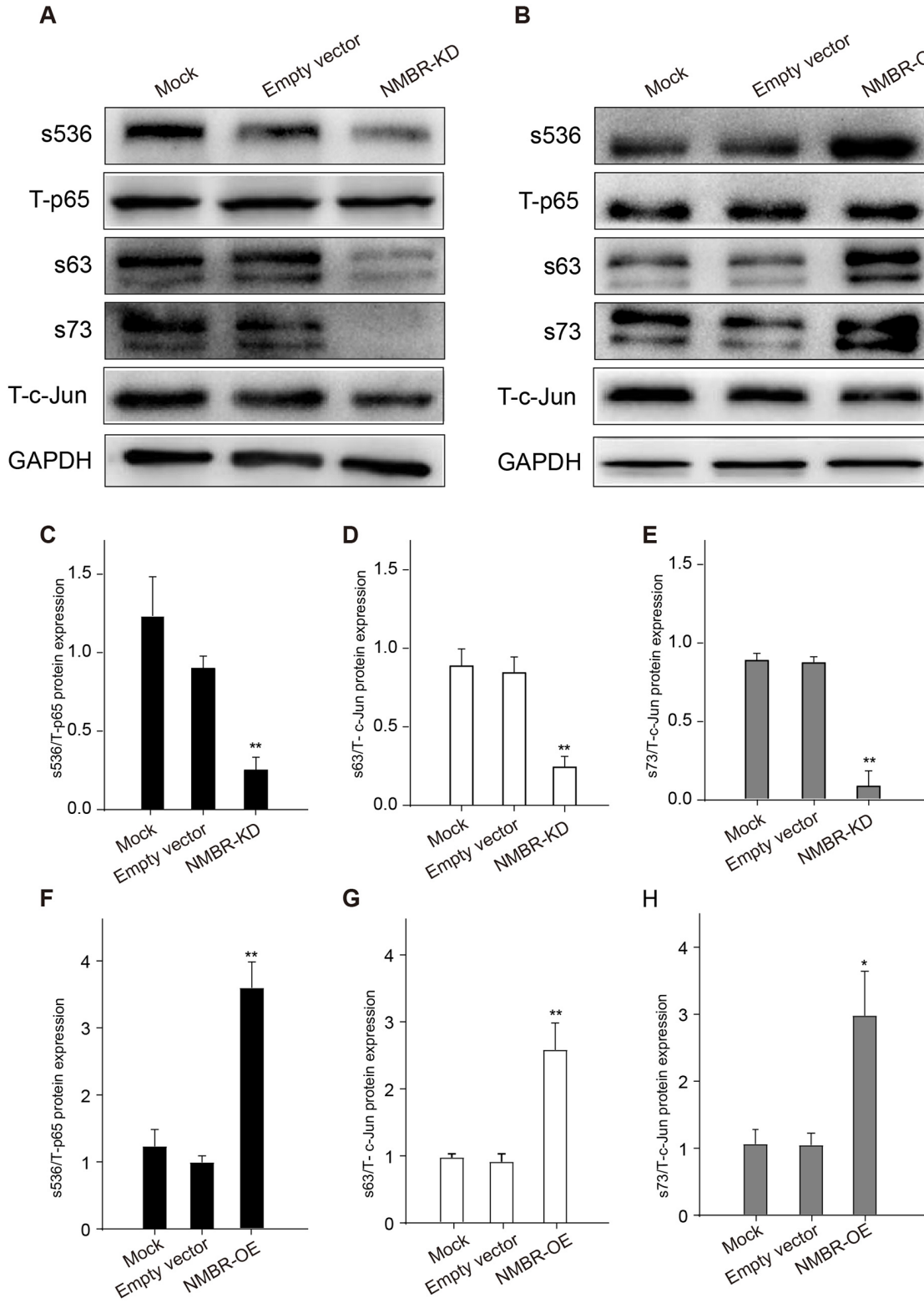

Figure 4 Activation of transcription factor p65 (p65) and AP-1 transcription factor subunit (c-Jun) by neuromedin B (NMB) is mediated via neuromedin B receptor (NMBR). (A) Primary myometrial cells were infected with lentiviruses with NMBR knockdown or the empty vector, and then treated with 1 μM NMB for 1 hour. P-p65 (s536), T-p65, P-c-Jun (s63 and s73) and T-c-Jun levels were probed by western blotting with GAPDH as an internal standard. (B) Primary myometrial cells were infected with lentiviruses with NMBR overexpression or the empty vector, and then treated with 1 μM NMB for 1 hour. P-p65 (s536), T-p65, P-c-Jun (s63 and s73) and T-c-Jun levels were probed by immunoblotting with GAPDH as an internal standard. Blots are representative. (C–H) The relative abundance of P-p65 (s536), P-c-Jun (s63 and s73) was quantified. Blots are representative. Data are expressed as mean±SD of five independent experiments (n=5). *p<0.05; **p<0.01, against the empty vector group. OE: Overexpression, KD: Knockdown, GAPDH was used as an internal control.

following NMB stimulation in human primary myometrial cells. These results help us better understand mechanisms of NMB effect, providing more evidence to explore NMBR as a novel therapeutic target and develop novel NMB antagonist as a preventive or therapeutic method for PTB.

Contributors This study was done by all the authors. WZ guided the project. JC and YS conducted all the experiments. JC wrote the manuscript.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval Samples were collected from full-term deliveries at selective cesarean section without labor under a protocol approved by the ethics committee of Central South University (No: 2019030155).

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available in a public, open access repository.

ORCID iD

Weishe Zhang http://orcid.org/0000-0002-1633-0587

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