

Effect and mechanism of propranolol on promoting osteogenic differentiation and early implant osseointegration

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Abstract. The present study aimed to investigate the effect of β -receptor blocker propranolol on early osseointegration of pure titanium implants and the underlying molecular regulatory mechanisms. An implant osseointegration model using the tibial metaphysis of New Zealand rabbits was established. The rabbits were divided into control and low-, medium- and high-dose propranolol groups. The formation of implant osseointegration was detected by X-ray scanning. Mesenchymal stem cells (MSCs) and osteoblasts (OBs) were isolated and cultured *in vitro*, isoproterenol was supplemented to simulate sympathetic action and propranolol was subsequently administered. The effect of propranolol on cell proliferation and osteogenic differentiation were assessed by EdU, flow cytometry, alizarin red staining and alkaline phosphatase (ALP) detection. The expression levels of bone morphogenetic protein (BMP)2, RUNX family transcription factor (RunX)2, collagen (COL)-1, osteocalcin (OCN) and β 2-adrenergic receptor (AR) were detected by immunofluorescence, reverse transcription-quantitative PCR and western blot assay. Propranolol effectively promoted implant osseointegration *in vivo*, facilitated proliferation of OBs, inhibited proliferation of MSCs and enhanced osteogenic differentiation of OBs and MSCs. The calcium content and ALP activity of cells treated with propranolol were markedly higher than in the control group. Propranolol also elevated mRNA and protein expression levels of BMP2, RunX2, COL-1 and OCN in tissue and cells, and decreased the expression of β 2-AR. The present study demonstrated that the β -receptor blocker propranolol promoted osteogenic differentiation of OBs and MSCs and enhanced implant osseointegration. The present

study provided a novel insight into the application and regulatory mechanisms of propranolol.

Introduction

The occurrence of defects and deformity of bone tissue affects normal function and patient mobility (1). Severe cases may also result in psychological problems for patients (1). Multiple treatment options have been available in the past, including bone transplantation (either autologous or allogeneic) and the application of bone substitute materials (2). However, these methods have disadvantages, including rejection, high surgery costs, limited availability of bone, surgical trauma and complicated surgery (3), which result in limited clinical application. Two fundamental processes, bone formation and resorption, are involved in bone metabolic formation, which leads to decreased bone formation and mass; the application of β -receptor blocker propranolol significantly increases bone formation and bone mass (4,5).

As the effects of the sympathetic nervous system on bone remodeling and healing are complex and extensive, the underlying mechanism has not yet been fully recognized (6). Numerous experiments have investigated the effect of propranolol, a sympathetic blocker drug, on bone metabolism (7-9). To the best of our knowledge, however, few reports have illustrated the effect and mechanism of propranolol on proliferation of osteoblasts (OBs) and bone resorption of osteoclasts (OCs). OBs are derived from bone marrow-derived stem cells (BMSCs); OBs generate bone matrix during bone formation and release diverse bioactive substances to regulate and control the function of OCs (10,11). The stimulation of the sympathetic nervous system increases bone resorption and inhibits bone formation (12). This mechanism is associated with the activity of β 2-adrenergic receptors (ARs) on OBs. Takeda *et al* (13) confirmed the presence of β 2-ARs (and absence of other adrenoceptor subtypes) on OBs using reverse transcription-quantitative (RT-q)PCR and northern blotting in OBs. The most frequently administered drugs for hypertension and cardiovascular disease are β 2-AR blockers. A previous study demonstrated the effect of β 2-AR blockers in skeleton tissue (7). AR agonists promote bone resorption in mice (7). Systemic application of β -AR agonists decreases bone formation and mass, while the opposite result has been obtained

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using the β -AR blocker propranolol (14-16). To the best of our knowledge, however, the effects and regulatory mechanisms of propranolol on OBs and MSCs have not been fully explored. Therefore, the present study aimed to investigate whether the β -AR blocker propranolol promotes osteogenic differentiation of OBs and MSCs and enhances osseointegration of implants. OBs and MSCs were treated with propranolol and the effects on cell proliferation and osteogenic differentiation and expression levels of osteoblast-associated proteins were detected. The present study also aimed to serve as a basis for further investigation of the mechanisms and effects of propranolol on implant osseointegration.

Materials and methods

Model establishment of implant osseointegration. The present animal experiment was performed in strict accordance with the regulations of the Ethics Committee of The Affiliated Hospital of Qingdao University (approval no. 201905036). A total of 32 healthy male New Zealand white rabbits (age, 5-6 months; weight \sim 3.5 kg), purchased from Qingdao Kangda Biological Technology Co., Ltd., were randomly divided into four groups ($n=8$ /group): Control and low-(0.1 mg/kg), medium-(1 mg/kg) and high-dose propranolol (10 mg/kg). Rabbits were housed in sterile cages at 22°C and relative humidity of 50-60%, with 12 h light/dark cycle and free access to fodder and sterile water. All rabbits were acclimatized for 7 days before the operation. The anesthetic protocol was based on the Guide for Care and Use of Laboratory Animals (17). For anesthesia, xylazine hydrochloride (Jilin Province Huamu Animal Health Products Co., Ltd.) at 5 mg/kg was intramuscularly injected, followed by slow intravenous injection of 3% pentobarbital sodium (Sigma-Aldrich; Merck KGaA) at 24 mg/kg. Subcutaneous propranolol injections were administered to the propranolol groups close to the surgical site preoperatively and an equal amount of normal saline was given to the control group for 28 successive days. Animals from each group subsequently received conventional surgical cavity preparation at the metaphysis of the tibia and two pure titanium implants (diameter, 3.4 mm; length; 8.0 mm) were inserted using an implant machine, one in each hindleg. On days 1-3 postoperatively, rabbits in each group were subcutaneously injected with buprenorphine hydrochloride (0.03 mg/kg) and doxycycline (3.2 mg/kg) and all healed well following surgery. Bone tissue binding to implants and absorption was observed through X-ray examinations. Then, rabbits in each group were sacrificed with a lethal dose of pentobarbital sodium (100 mg/kg) by intravenous injection and the intact tibia tissue was removed for subsequent experiments.

Extraction of MSCs. Isolation, culture and characterization of MSCs by cell surface markers detection and multi-lineage differentiation were performed as described previously by Farahzadi *et al.* (8,9) and Fathi *et al.* (18,19). Two laboratory rabbits were sacrificed and placed in 75% alcohol at room temperature for 15 min. Following separation on a clean bench under aseptic conditions, the tibia and femur of rabbits were placed into a beaker supplemented with PBS solution. The connective tissue and periosteum were removed

immediately and the medullary cavity was washed with serum-free Dulbecco's modified eagle medium (DMEM) in a syringe and transferred into a centrifuge tube containing DMEM with 15% fetal bovine serum (FBS; both HyClone; Cytiva). When the medullary cavity turned white, ultrasound shaking at room temperature was performed at 240 x g for 5 min. The supernatant was discarded and high-glucose DMEM containing 15% FBS was added for cell suspension preparation. The samples were planted in a culture flask while being blown evenly. The culture medium was refreshed after 24 h and replaced every 2-3 days. Experiments were performed using third passage cells.

OBs isolation and culture. Isolation and culture of OBs were performed as described by Zhao *et al.* (20). New Zealand rabbits were anesthetized by intravenous injection of pentobarbital sodium. The femur was subsequently removed and treated as aforementioned. The marrow was extracted from cleaned bones using DMEM supplemented with 15% FBS (HyClone; Cytiva) and incubated at 37°C with the medium replaced every 3 days. Once the cells were 50% confluent, the medium was replaced with DMEM containing 10% FBS and 1% penicillin + streptomycin (all HyClone; Cytiva). The cells were harvested after reaching 80-90% confluence using 0.25% trypsin. Experiments were performed using third passage cells.

Alizarin red staining. MSCs or OBs were cultured in DMEM containing 50 nM isoproterenol to simulate the effects of the sympathetic nerve (21). Following 24 h culture in DMEM, MSCs or OBs were cultured in osteogenic differentiation medium [5 mM β -glycerophosphate, 50 μ g/ml ascorbic acid phosphate and 10 nM dexamethasone (all Sigma-Aldrich; Merck KGaA) in DMEM supplemented with 15% FBS] containing 50 nM isoproterenol and propranolol at different concentrations for 7-day induction at 37°C. Mineralized nodule alizarin red staining was subsequently performed. The original culture medium was discarded and PBS solution was used for three cycles of washing. Subsequently, 4% paraformaldehyde was used for fixation at room temperature for 20 min. Finally, the PBS solution was used to rinse cells. Cells were stained with 0.1% alizarin red dye for 30 min at 37°C, washed with running water and examined under a light microscope at x200 magnification.

Alkaline phosphatase (ALP) staining. ALP staining was performed using the Gomori modified calcium-cobalt method, as previously described Huang *et al.* (22). Following 3 weeks of induction, MSCs or OBs were inoculated into a petri dish (density, 2×10^4). Following removal of the original culture medium, cells were fixed with 95% ethanol at room temperature for 10 min, and then incubated with incubation buffer (5 3% sodium β -glycerophosphate, 5 2% sodium barbiturate, 10 distilled water, 10 2% CaCl_2 and 1 ml 2% MgSO_4) at 37°C for 4 h. Substrate solution was subsequently added to the petri dish, covered with hydrophobic membrane, incubated in a 37°C oven in the dark for 15 min and washed. Finally, dye solution was added for staining at room temperature for 3 min and samples were washed with running water and examined under a light microscope at x200 magnification.

Cell proliferation assessment via EdU assay. EdU assay was performed as described by Shen *et al* (23). Briefly, MSCs or OBs (1×10^7) were cultured at 37°C in 6-well plates overnight. Cells were supplemented with EdU (10 μ M) and incubated at 37°C for 2 h. After DMEM was removed, the cells were fixed with 1 ml 4% paraformaldehyde for 15 min at room temperature. After fixing medium was removed, cells were washed 3 times by adding 1 ml/well washing solution (3-5 min each). The washing solution was discarded. Then, 1 ml PBS containing 0.3% Triton X-100 was added to each well and incubated at room temperature for 10-15 min. The cells in each well were washed with 1 ml washing solution 1-2 times (3-5 min each). Then, endogenous peroxidase blocking solution was added for incubation at room temperature for 20 min to inactivate endogenous peroxidase activity. The cells were then rinsed with washing solution 3 times (2 min each) and visualized via a fluorescent microscope (Olympus Corporation) at x200 magnification.

Cell proliferation assessment via flow cytometry. To determine cell proliferation, BrdU assay was performed as described by Heo *et al* (24). The MSCs or OBs (density, 1.5×10^5 /ml) were inoculated in a 35-mm-diameter culture dish for 1 day at 37°C and synchronized in DMEM containing 0.4% FBS for 3 days until most cells entered the G₀ phase. Cell proliferation was measured by a BrdU Staining kit for Flow Cytometry (cat. no. 8811-6600-42; Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Anti-BrdU FITC was added and incubated at 37°C for 40 min. The culture medium was discarded. The cells were collected into a flow tube and centrifugated at room temperature and 350 x g for 5 min. The supernatant was discarded. Each tube was fixed at room temperature with 1 ml 4% paraformaldehyde for 15 min and centrifugated at room temperature and 600 x g for 10 min. The supernatant was discarded. After washing, 2 glycine and 1 ml 0.5% triton was added to each tube and incubated at 37°C for 10 min. Following washing with PBS, the cells were resuspended and detected by BD LSRFortessa Flow Cytometer (BD Biosciences). Data was analyzed using CytExpert 2.3 software (Beckman Coulter, Inc.).

Wound healing assay. MSCs or OBs were seeded into 6-well plates at a cell density of 5×10^5 /ml (500 μ l/well) and cultured at 37°C for 24 h in RPMI-1640 complete growth medium (Invitrogen; Thermo Fisher Scientific, Inc.) to form an 80% confluent monolayer, which was scratched using the tip of a 10- μ l pipetting gun. Cells were washed three times with PBS and incubated for 48 h (37°C; 5% CO₂) in serum-free DMEM (HyClone; Cytiva). The cells were observed and photographed at 24 and 48 h under an inverted fluorescence microscope (Olympus Corporation) at x200 magnification and quantification of scratch closure was evaluated using the wound healing measurement tool of ImageJ V1.8.0.112 (National Institutes of Health).

RT-qPCR. Total RNA was isolated from MSCs or OBs using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RT-qPCR was performed as described by Yin *et al* (25). RT was performed using a PrimeScript™ RT reagent kit (cat. no. RR047A; Takara Biotechnology Co., Ltd.) and a

reaction system was established containing 2.2 μ g RNA, 2.0 μ l OligodT, 4.0 μ l dNTP, 4.0 μ l 5X buffer, 1.0 μ l reverse transcriptase, 0.5 μ l RNAase inhibitor and ≤ 20.0 μ l RNAase-free ddH₂O. The reaction conditions were as follows: 25°C for 5 min, 50°C for 15 min, 85°C for 5 min and 4°C for 10 min. qPCR was performed using a 2xT5 SYBR Green Fast qPCR Mix kit (cat. no. TSE202; TsingKe Biological Technology) according to the manufacturer's instructions. The reaction system contained 0.4 forward primer, 0.4 reverse primer, 10.0 SYBR Green and 5.2 μ l H₂O. The thermocycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, 95°C for 30 sec and 60°C for 30 sec for 40 cycles. The 2^{- $\Delta\Delta$ C_q} method was used to calculate the relative expression levels (26). The primers were as follows (5'-3'): BMP2 forward, TGGCCC ATTTAGAGGAGAACC and reverse, AGGCATGATAGC CCGGAGG; RUNX family transcription factor (RunX)2 forward, GAGACTACTGCCGACCAC and reverse, TACCTC TCCGAGGGCTACC; collagen (COL)-1 forward, AGGGCC AAGACGAAGACATC and reverse, AGATCACGTCATCGC ACAACA; osteocalcin (OCN) forward, CTCACACTCCTC GCCCTATT and reverse, CGCCTGGGTCTCTTCACTAC; β 2-AR forward, GGACAACCTCATCCCTAA and reverse, GGACAACCTCATCCCTAA and GAPDH forward, CAC ATGGCCTCCAAGGAGTAA and reverse, GTACATGAC AAGGTGCGGCTC. GAPDH was used as the control for normalization.

Protein expression level detection by western blot analysis. Western blotting was performed as described by Yin *et al* (25). Total protein was extracted from MSCs or OBs and bone tissue using RIPA reagent (Beyotime Institute of Biotechnology), and the concentration of extracted proteins was measured by BCA. Proteins (20 μ g/lane) were isolated using SDS-PAGE (12% separating gel and 5% stacking gel) and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% BSA (Beyotime Institute of Biotechnology) at 37°C for 1 h. The membranes were incubated with primary antibodies against BMP2, RunX2, COL-1, OCN and β 2-AR overnight at 4°C, and using corresponding secondary antibodies incubated for 1.5 h at room temperature. Protein bands were detected by an enhanced chemiluminescent kit (Thermo Fisher Scientific, Inc.). The antibodies (all Chongqing Boai Madison Biotechnology Co., Ltd.) were as follows: BMP2 (1:500; cat. no. BM19970), RunX2 (1:500; cat. no. BM16360), COL-1 (1:500; cat. no. BM2319), OCN (1:500; cat. no. BM18692), β 2-AR (1:500; cat. no. BMP0265), β -actin (1:2,000; cat. no. BMC026) and horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2,000; cat. no. BMS014). Results were normalized to β -actin. Western blots were quantified using Image J V1.8.0.112 (National Institutes of Health).

Immunofluorescence detection. Immunofluorescence detection was performed as described by Fathi *et al* (18). The MSCs or OBs were fixed with 4% paraformaldehyde at 4°C overnight. Paraffin-embedded sections (thickness, 4 μ m) were baked at 60°C for 2 h, dewaxed and hydrated with xylene and alcohol. The sections were placed in citrate buffer solution (pH 6.0) for antigen repair, heated at 98°C in a microwave oven for 20 min and left to cool to room temperature. Three cycles of washing were performed with PBS (5 min each) followed

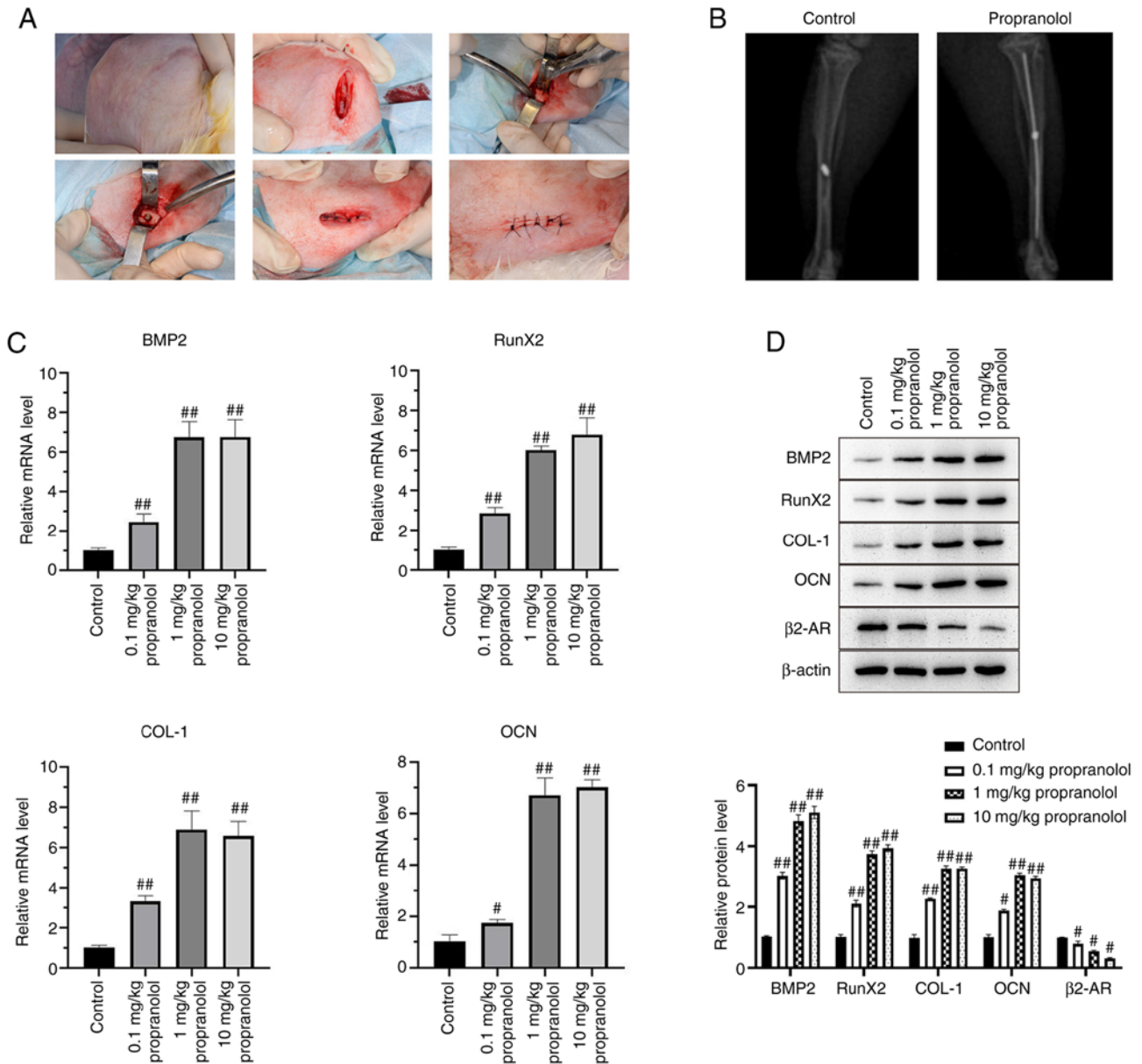


Figure 1. Propranolol promotes osseointegration of implants. (A) Preparation of New Zealand rabbit implant femoral shaft fracture model. (B) X-ray examination of bone binding. Expression of BMP2, RunX2, COL-1 and OCN detected by (C) reverse transcription-quantitative PCR and (D) western blotting. Statistical significance was assessed by ANOVA with Tukey's post hoc test. # $P < 0.05$, ## $P < 0.01$ vs. control. BMP2, bone morphogenetic protein 2; RunX2, RUNX family transcription factor 2; COL-1, collagen 1; OCN, osteocalcin; AR, adrenergic receptor.

by blocking with 5% BSA for 30 min at room temperature. The aforementioned primary antibodies (1:100) were added and incubated overnight at 4°C. Antibodies are described in western blot. Following reheating at 37°C for 30 min, PBS was used for washing (3 times; 5 min each). Corresponding secondary antibodies conjugated to Alexa594 (cat. no. R37117, 1:100; Thermo Fisher Scientific, Inc.) were added and incubated at room temperature for 1 h. PBS washing was repeated 3 times (5 min each). Nuclei were visualized by staining with 0.3 μM DAPI in the dark for 5-10 min at 37°C and then PBS was used to wash 3 times (1 min each). The sections were observed and photographed under a fluorescence microscope at x200 magnification.

Statistical analysis. The experimental data were statistically analyzed using SPSS 23.0 software (IBM Corp.) and are

expressed as the mean \pm SD of three independent experiments. A paired t-test was used to compare groups before and after treatment. One-way ANOVA with Tukey's post hoc test was used for comparison between multiple groups. $P < 0.05$ and $P < 0.01$ were considered to indicate a statistically significant difference.

Results

Propranolol promotes osseointegration of implants. A New Zealand white rabbit model of implant osseointegration was established and injected with different doses of propranolol (0.1, 1.0 and 10.0 mg/kg). At 14 days post-surgery, bone tissue binding to implants and absorption were examined by X-ray scanning (Fig. 1A and B). Bone tissue samples of rabbits near the implants were collected and mRNA expression of BMP2,

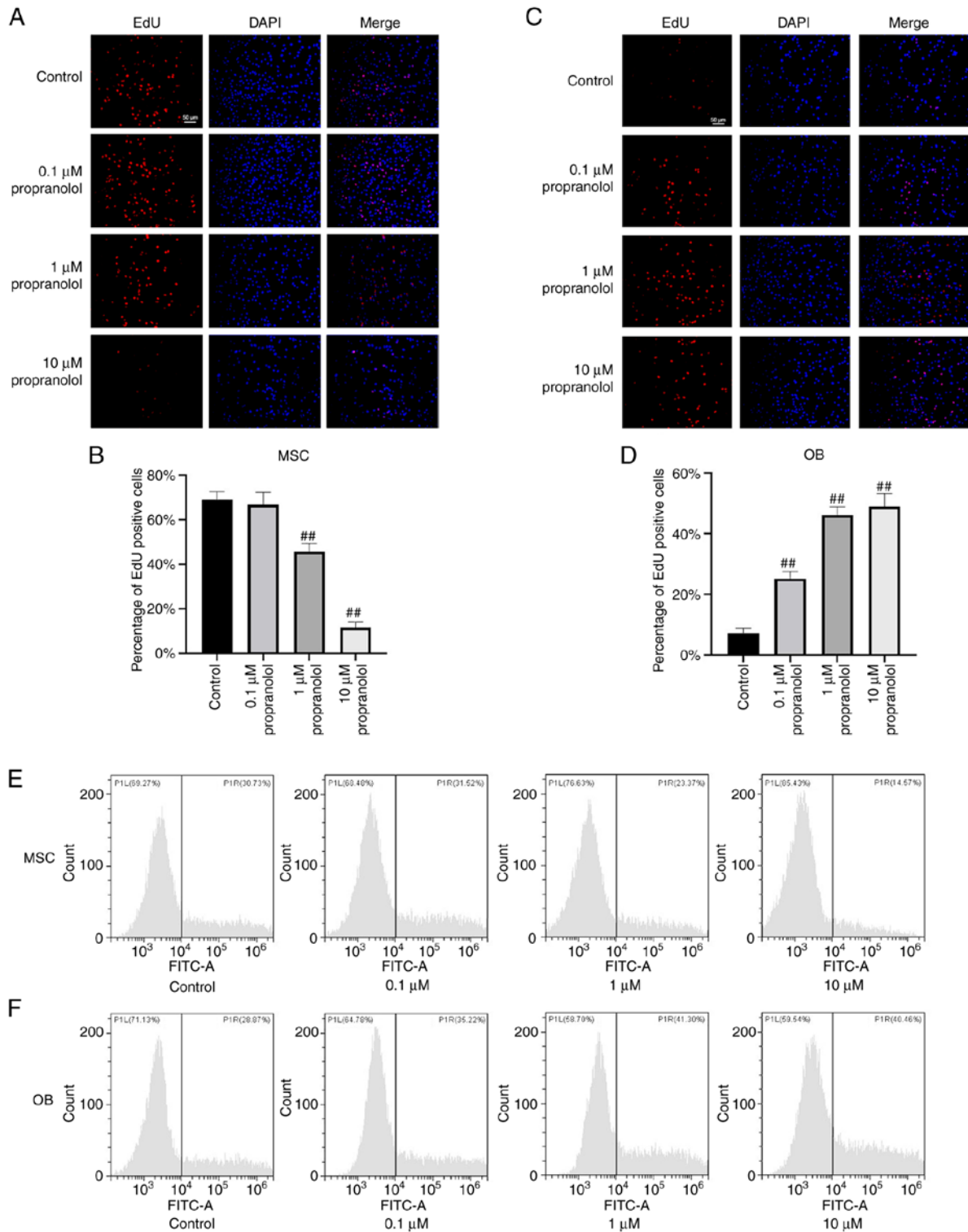


Figure 2. Propranolol inhibits proliferation of MSCs and promotes proliferation of OBs. (A) Effect of propranolol on MSCs proliferation was determined by EdU staining. Magnification, $\times 200$. Scale bar, $50 \mu\text{m}$. (B) Statistical analysis of EDU positive cells in MSCs. (C) Effect of propranolol on OBs proliferation was determined by EdU staining. (D) Statistical analysis of EDU positive cells in OBs. (E) Proliferation rate of MSCs was determined by flow cytometry. (F) Proliferation rate of OBs was determined by flow cytometry. Statistical significance was assessed by ANOVA with Tukey's post hoc test. ^{##} $P < 0.01$ vs. control. MSC, mesenchymal stem cell; OB, osteoblast.

RunX2, COL-1, OCN, and $\beta 2$ -AR was detected by qPCR. Propranolol increased mRNA expressions of BMP2, RunX2, COL-1 and OCN and decreased that of $\beta 2$ -AR. The protein expression of BMP2, RunX2, COL-1, OCN and $\beta 2$ -AR was detected by western blotting; propranolol increased protein

levels of BMP2, RunX2, COL-1 and OCN and decreased $\beta 2$ -AR levels. (Fig. 1C and D).

Propranolol inhibits proliferation of MSCs and promotes proliferation of OBs. To determine the effect of propranolol on

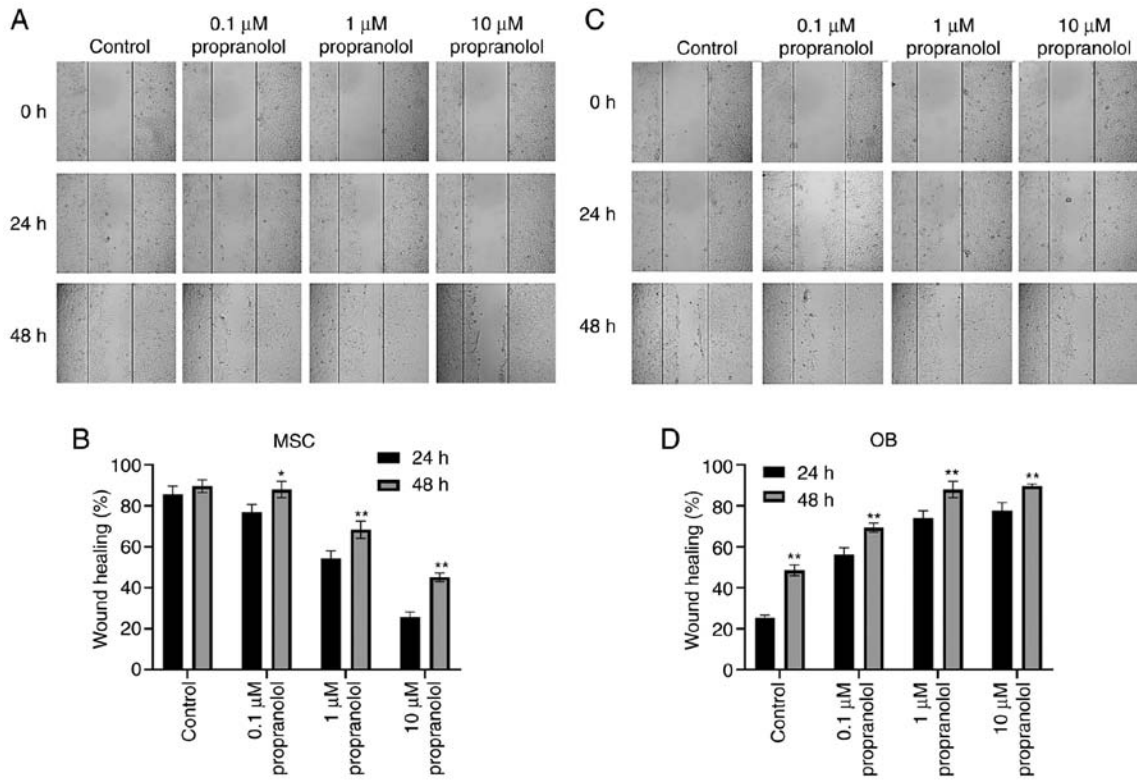


Figure 3. Propranolol inhibits migration of MSCs and promotes migration of OBs. Wound healing assay assessed MSC migration efficiency of (A and B) MSCs and (C and D) OBs following treatment with propranolol. Images were captured at 0, 24, and 48 h (x200 magnification). Statistical significance was assessed by Student's *t*-test. **P*<0.05, ***P*<0.01 vs. 24 h. MSC, mesenchymal stem cell; OB, osteoblast.

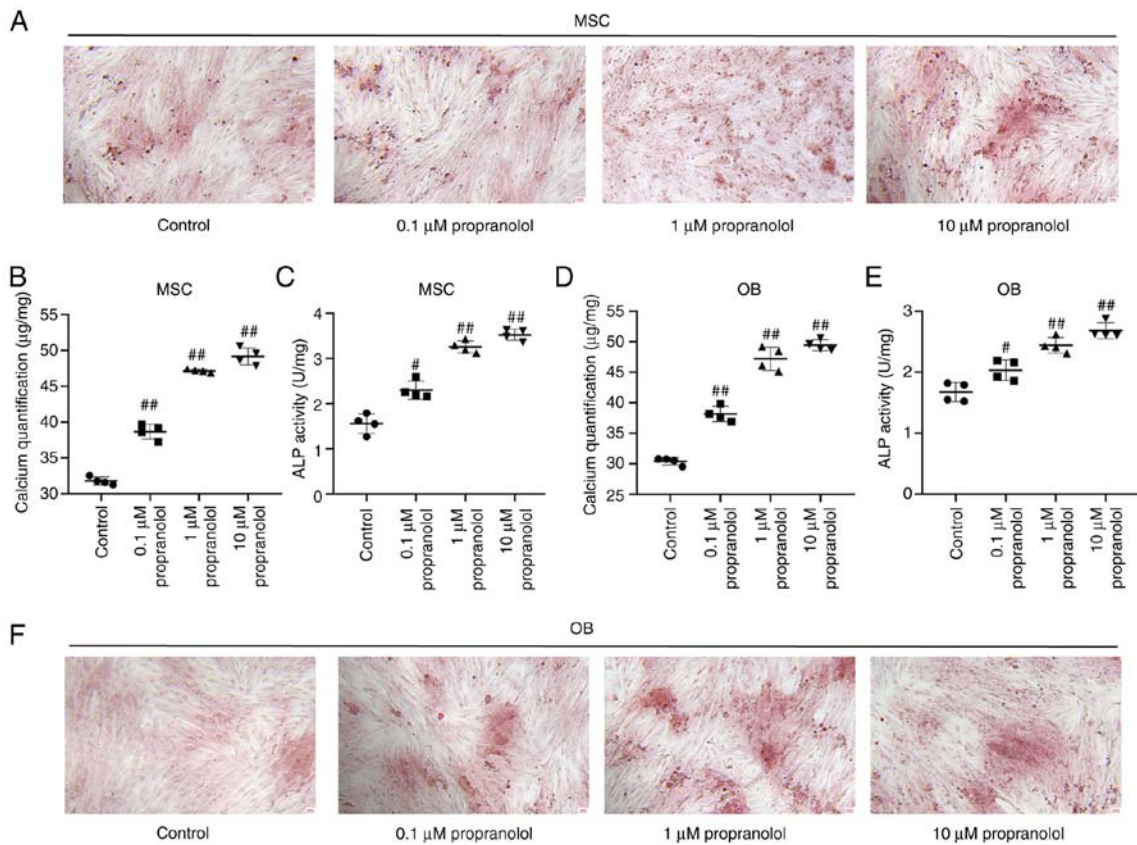


Figure 4. Propranolol promotes osteogenic differentiation of MSCs and OBs. (A) Alizarin red staining detected the osteogenic differentiation of MSCs. Determination of (B) calcium content and (C) ALP activity in MSCs. Determination of (D) calcium and (E) ALP activity in OBs. (F) Determination of osteogenic differentiation of OB by alizarin red staining. Statistical significance was assessed by ANOVA with Tukey's post hoc test. **P*<0.05, ***P*<0.01 vs. control. MSC, mesenchymal stem cell; OB, osteoblast; ALP, alkaline phosphatase.

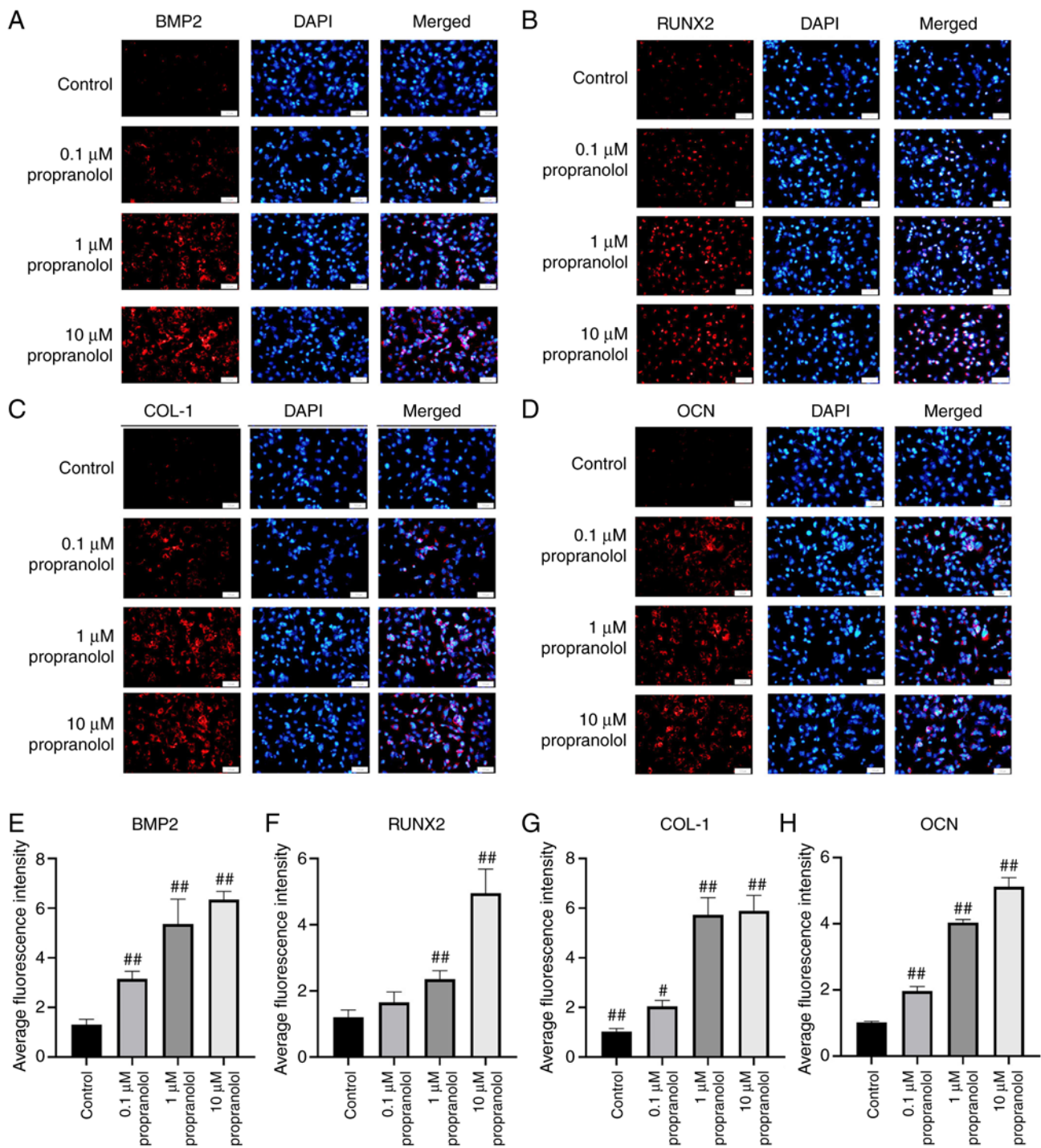


Figure 5. Regulatory effect of propranolol on osteogenesis-associated genes in MSCs. Immunofluorescence was used to detect (A) BMP2, (B) RunX2, (C) COL-1 and (D) OCN expression levels in MSCs. Relative immunofluorescence intensity of (E) BMP2, (F) RunX2, (G) COL-1 and (H) OCN was measured by Image J. Magnification, $\times 200$. Scale bar, $50 \mu\text{m}$. Statistical significance was assessed by ANOVA with Tukey's post hoc test. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. BMP2, bone morphogenetic protein 2; RunX2, RUNX family transcription factor 2; COL-1, collagen 1; OCN, osteocalcin; MSC, mesenchymal stem cell; OB, osteoblast.

proliferation of MSCs and OBs, MSCs and OBs were cultured *in vitro* using complete medium containing isoproterenol and treated with propranolol for 24 h. Cell proliferation was detected by EdU staining. The number of EdU-positive MSCs decreased markedly following the addition of propranolol and was lowest in the $10 \mu\text{M}$ propranolol group, indicating that propranolol inhibited proliferation of MSCs (Fig. 2A and B). In OBs, all concentrations of propranolol significantly promoted cell proliferation and the number of Edu-positive cells in the

1 and $10 \mu\text{M}$ propranolol groups were similar (Fig. 2C and D). These results suggested that propranolol inhibited proliferation of MSCs and promoted proliferation of OBs.

Propranolol inhibits migration of MSCs and promotes migration of OBs. The effect of propranolol on cell migration was detected by wound healing at 24 and 48 h. MSC migration was inhibited following the addition of propranolol to complete medium containing isoproterenol. Moreover, the healing rate

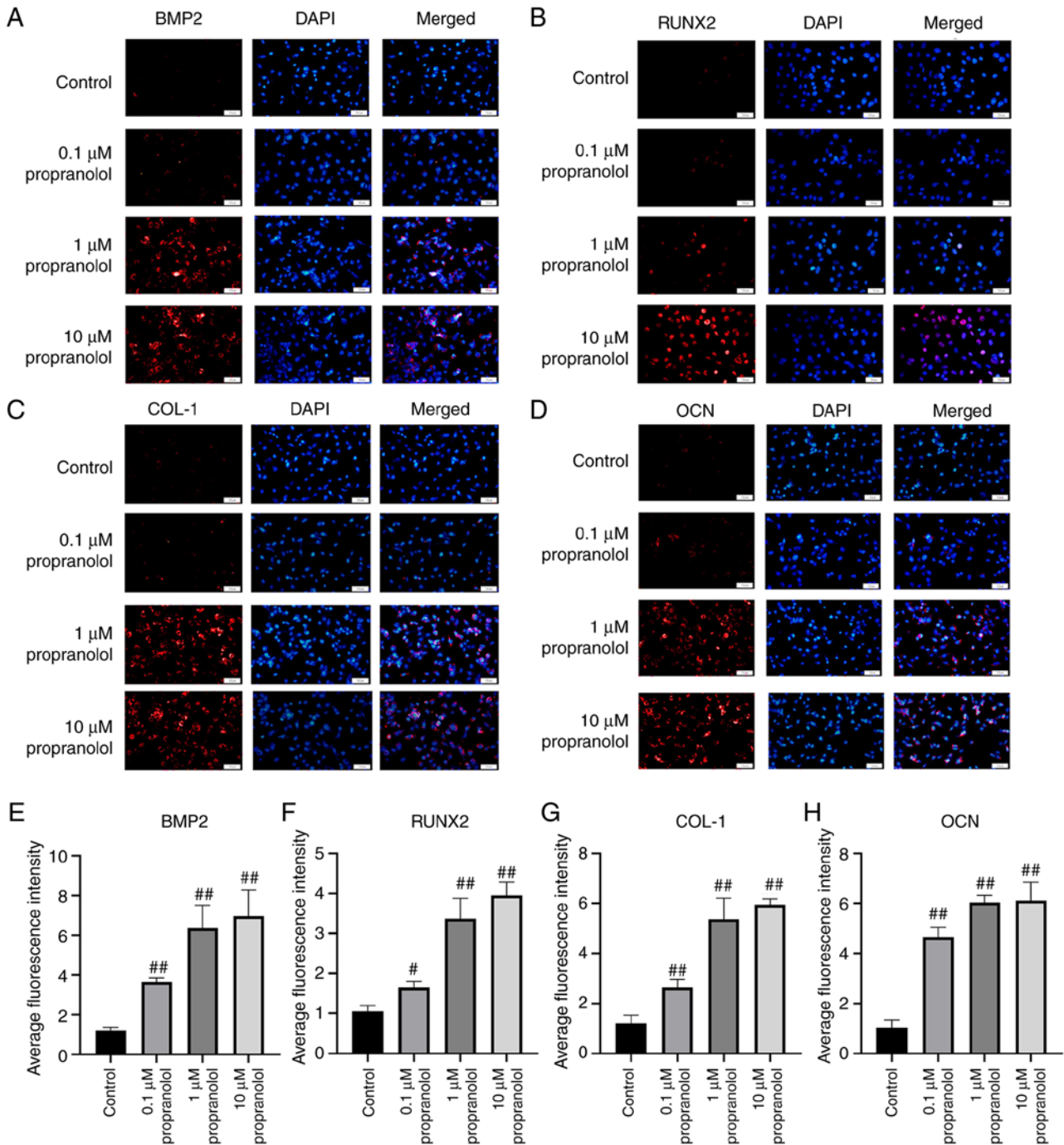


Figure 6. Regulatory effect of propranolol on osteogenesis-associated genes in OBs. Immunofluorescence was used to detect (A) BMP2, (B) RunX2, (C) COL-1 and (D) OCN expression in OBs. Relative immunofluorescence intensity of (E) BMP2, (F) RunX2, (G) COL-1 and (H) OCN was measured by Image J. Magnification, $\times 200$. Scale bar, $50 \mu\text{m}$. Statistical significance was assessed by ANOVA with Tukey's post hoc test. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs. control. BMP2, bone morphogenetic protein 2; RunX2, RUNX family transcription factor 2; COL-1, collagen 1; OCN, osteocalcin; MSC, mesenchymal stem cell; OB, osteoblast.

of scratches was markedly lower in propranolol-treated MSCs compared with the control group; the higher the concentration, the lower the healing rate (Fig. 3A and B). Conversely, propranolol improved the healing rate of OBs and cell migration was higher than that of the control group (Fig. 3C and D). These results suggested that propranolol inhibited MSC migration and promoted OB migration.

Propranolol promotes osteogenic differentiation of MSCs and OBs. To determine the effect of propranolol on osteogenic differentiation of MSCs and OBs, MSCs and OBs cultured

in vitro with osteogenic differentiation medium containing isoproterenol and propranolol. Following induction for 7 days, osteogenic differentiation of cells was detected by alizarin red staining. Propranolol promoted osteogenic differentiation of both MSCs and OBs and increased the number of intracellular calcium nodules following induction. The calcium content and ALP activity of cells treated with propranolol were significantly higher compared with the control group (Fig. 4A-F).

Regulatory effect of propranolol on osteogenesis-associated genes. To elucidate the osteogenic mechanism of propranolol in

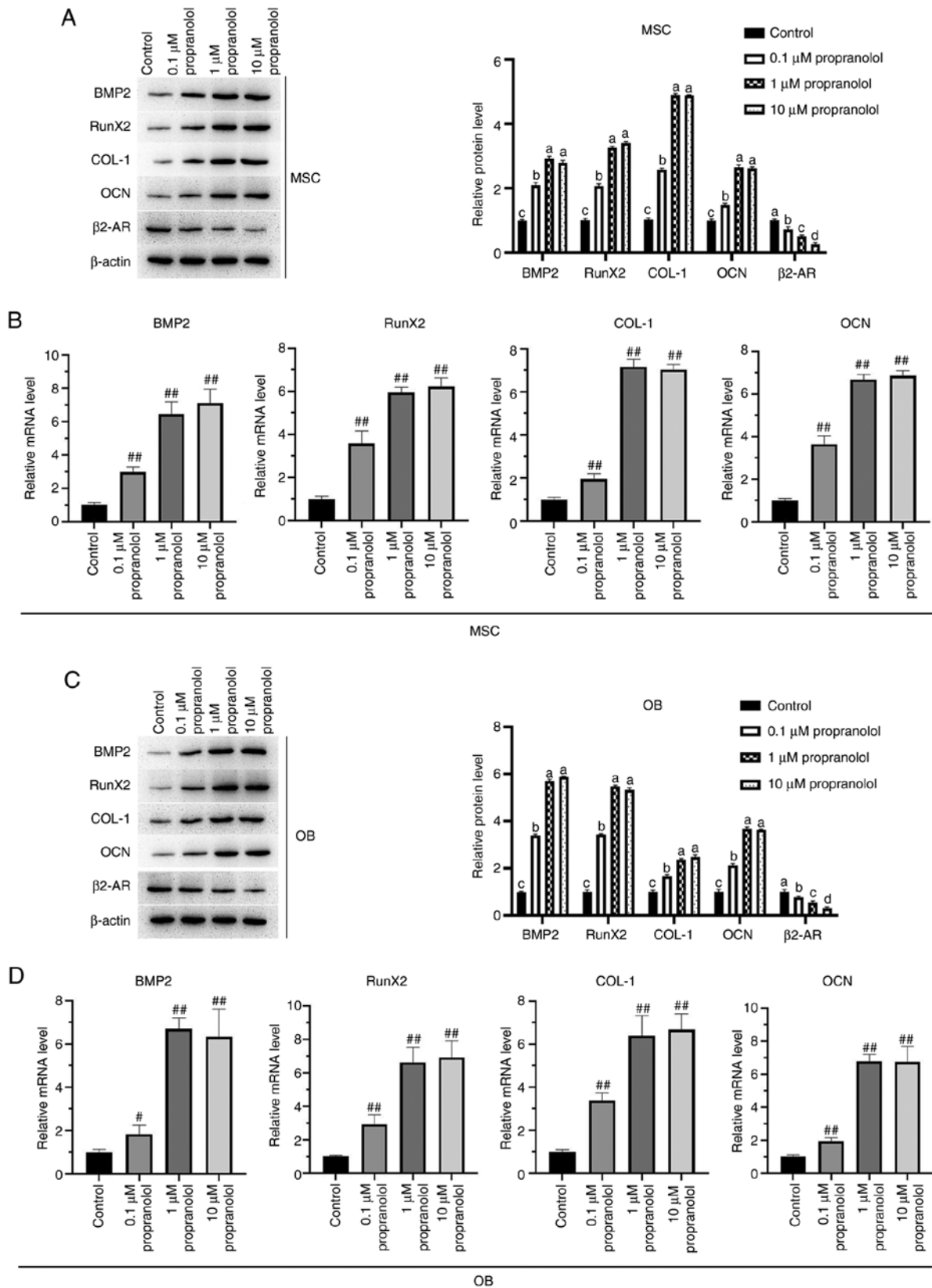


Figure 7. Expression levels of BMP2, RunX2, COL-1, OCN and β 2-AR in MSCs and OBs detected by RT-qPCR and western blotting. Expression levels of BMP2, RunX2, COL-1, OCN and β 2-AR in MSC were detected by (A) western blotting and (B) RT-qPCR. Expression levels of BMP2, RunX2, COL-1, OCN and β 2-AR in OB were detected by (C) western blotting and (D) RT-qPCR. Statistical significance was assessed by ANOVA with Tukey's test. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. control. BMP2, bone morphogenetic protein 2; RunX2, RUNX family transcription factor 2; COL-1, collagen 1; OCN, osteocalcin; MSC, mesenchymal stem cell; OB, osteoblast; AR, adrenergic receptor; RT-q, reverse transcription-quantitative.

regulating MSCs (Fig. 5A-H) and OBs (Fig. 6A-H), expression levels of osteogenesis-associated genes BMP2, RunX2, COL-1,

OCN and β 2-AR were detected by immunofluorescence, RT-qPCR and western blotting. The findings demonstrated

that propranolol significantly increased protein expression levels of BMP2, RunX2, COL-1 and OCN and decreased expression of β 2-AR in a dose-dependent manner. The results of RT-qPCR and western blotting were consistent with those of immunofluorescence analysis, indicating that propranolol upregulated expression levels of osteogenesis-associated genes (BMP2, RunX2, COL-1 and OCN), thereby promoting osteogenic differentiation of MSCs and OBs (Fig. 7A-D).

Discussion

Early rapid osseointegration is a key factor for successful implantation but is limited by biological inertia, high elastic modulus and limited biological effects of the material surface (27). An effective technique to improve the performance of titanium implants and to promote and accelerate osseointegration has been the focus of research in recent years (28-30). Despite titanium and titanium alloy implants are widely used in the fields of dentistry and oral and maxillofacial surgery, postoperative infection and poor osseointegration remain obstacles to implant surgery (31). Drugs, such as zoledronic acid, osteoprotegerin, and kaempferol, also promote early osseointegration of implants (32-34).

As an integrated part of the autonomic nervous system of the human body, the sympathetic nervous system serves a key role in regulating homeostasis. Growing evidence has shown that the sympathetic nervous system is involved in bone remodeling (6,35). In 1977, Duncan and Shim (36) demonstrated that the surface of intraosseous vessels is covered with rich adrenergic nerve fibers by observing the bone tissue of rabbits using histochemistry and fluorescence electron microscopy. Mach *et al* (37) demonstrated that sympathetic nerve fibers are primarily located in the bone marrow cavity, favors areas of abundant blood flow and are rare in the periosteum. When the sympathetic nerve is excited, bone resorption is promoted and bone formation is decreased via β 2-ARs on the surface of OBs (38). β 2-AR has been visualized on the surface of human OBs by immunofluorescence and studies using β 2-AR agonists indicated that they serve a role in inhibiting proliferation of OBs (38,39). Although regulation of sympathetic nerve on bone turnover via β 2-ARs expressed on OBs has been demonstrated, little is known about the effect of β -AR blocker propranolol on osteogenic differentiation of OBs. In the study, propranolol promoted the proliferation and differentiation of OBs, which is consistent with previous studies (40,41).

Propranolol is a recognized drug for treatment of hypertension and cardiovascular disease (42-44). Propranolol is a non-selective β 1 and β 2-AR blocker that has been used since 1964 to treat coronary artery insufficiency (45). Propranolol competitively inhibits the action of epinephrine and norepinephrine on β 1- and β 2-ARs (46). Multiple studies have shown that propranolol inhibits expression of β 2-AR (47,48). Previously, the effect of propranolol on bone metabolism has received attention (41,49,50). Minkowitz *et al* (49) reported that mineral deposition and bone formation increase in a rat model of surgical fracture treated with propranolol for 9 weeks. Bonnet *et al* (41) found that low-dose propranolol improved bone formation and prevented osteoclasts proliferation in ovariectomized rats. Epidemiological studies have also demonstrated that β 2-AR blockers serve as potential candidate drugs

for treatment of osteoporosis and fractures (51,52). β -blockers enhanced bone healing and improved bone metabolism (53). The present study investigated the effects of β -AR blocker propranolol on osteogenesis in an animal model. The results demonstrated that propranolol promoted osseointegration of implants in rabbits, which is consistent with conclusions of previous studies (7,50), suggesting that propranolol enhances bone regeneration and implant osseointegration.

No consensus on the mechanism of β 2-AR in regulating osteogenesis and osteoclast has been reached. By investigating the osteogenic mechanism of MSCs, certain scholars have reported that β -AR activators inhibit osteogenesis of MSCs, while blockers promote osteogenesis of MSCs (54,55). The β -AR activator also inhibits the signaling pathway associated with osteogenesis by regulating expression of MEK and ERK1/2 phosphorylation, thereby inhibiting differentiation of BMSCs into osteoblast-like cells *in vitro* (56,57). The osteogenic capacity of MSCs has been established (58). MSCs serve as a source of osteochondral progenitors that invade bone sites, proliferate and differentiate into cartilage and bones (59). β -AR antagonists promote MSC osteogenesis (50,55). In the present study, propranolol promoted osteogenic differentiation of MSCs while inhibiting their proliferation. This may be due to inhibition of MSC proliferation during differentiation; this has been reported in previous studies, which illustrated that stem cell differentiation is inhibited but proliferation is promoted (60,61). Osteoblastic differentiation of cells is accompanied by upregulation of osteoblast marker genes, including BMP2, RunX2, COL-1 and OCN (62,63). The present study demonstrated that propranolol increased the mRNA and protein expression levels of BMP2, RunX2, COL-1 and OCN in tissue and cells and decreased expression of β 2-AR in OBs and MSCs. The increased calcium content and ALP activity in propranolol-treated OBs and MSCs also indicated osteoblastic differentiation of cells. The findings provide a basis for further studies on the mechanisms underlying the regulatory effects of propranolol.

There effects and mechanisms of propranolol on osteogenesis at different concentrations are disputed (64). Smitham *et al* (2014) (65) demonstrated that low-dose propranolol (0.1 mg/kg) has little effect on bone defect healing, while Bonnet *et al* (2008) (41) suggested that high-dose propranolol (10-100 mg/kg) produces no additional positive effect on osteogenesis compared with low-dose propranolol. The present study demonstrated that the osteogenic effect of propranolol at medium (1 mg/kg) and high (10 mg/kg) doses was markedly enhanced compared with the low dose (0.1 mg/kg), whereas no superior effect was revealed in the high- compared with the medium-dose group. However, further investigation of the clinical use of propranolol in this context is required.

The present study demonstrated that the β -AR blocker propranolol promoted osteogenic differentiation of OBs and MSCs and enhanced osseointegration of implants by regulating expression levels of osteogenic-associated proteins, including BMP2, RunX2, COL-1, OCN and β 2-AR. The present study therefore provided a novel insight into the application and regulatory mechanisms of propranolol.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

All authors contributed to study conception and design. YW wrote the manuscript. YW, QZ, BZ and XW performed experiments and collected and analyzed data. YW and XW reviewed and edited the manuscript. YW and XW confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the institutional ethical review committee of the Ethics Committee of The Affiliated Hospital of Qingdao University (approval no. 201905036).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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