

## Original Article

# Doxorubicin restrains osteogenesis and promotes osteoclastogenesis in vitro

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**Abstract:** Clinical evidence suggests that doxorubicin (DOX), as a chemotherapeutic drug, can induce severe bone damage in cancer patients. However, the effect of DOX on osteoporosis has not been fully elucidated. Therefore our study aims to investigate the effect and mechanism of DOX in osteoporosis. In our study, we co-cultured rat BMSCs with different concentrations of DOX solution, then the osteogenic differentiation markers and proliferation ability were analyzed. The results indicated that a certain concentration of the DOX solution may restrain the osteogenic differentiation of rat BMSCs by bmp-2/smads signalling pathway. Also, we found DOX promoted the receptor activator of nuclear factor- $\kappa$ B (NF- $\kappa$ B) ligand (RANKL)-induced osteoclast formation. Our research explains excellently the induce-osteoporotic mechanism of DOX in vitro, which maybe contributing to the exploration of a new way to prevent osteoporosis caused by chemotherapy.

**Keywords:** Doxorubicin, mesenchymal stem cells, osteoporosis, smad1/5/9, Bmp-2, RANKL/OPG

## Introduction

Anthracyclines (such as epirubicin and DOX) have been used as first-line treatment for breast cancer patients in the past 40 years and are still used today because of the role in improving survival rates [1]. The current standard regimens for anthracyclines include DOX/cyclophosphamide (AC), cyclophosphamide/DOX/5-fluorouracil (CAF) and cyclophosphamide/epidoxorubicin/5-fluorouracil (CEF) [2]. DOX has been reported to increase the risk of bone metastasis and osteolytic injury in patients [3]. It also can promote the differentiation of BMSCs into adipogenic cells through oxidative stress. However, there are scarcely any studies on the effect of DOX in osteoporosis from the progenitor cell.

BMSCs are primary cells that show self-renewal, high proliferation and multidirectional differentiation. They can differentiate into mesoderm-type cells including osteoblast, adipocyte and chondrocyte [4]. Several pre-clinical and clinical studies have shown the bone regenera-

tive capacity of adult BMSCs-based therapy for the treatment of several bone loss disease including osteoporosis and oral, cranial, maxillo-facial and long bone defects [5]. The depletion of hematopoietic populations and changes in hematopoietic and stromal precursor differentiation are related to intensive and long-term cancer chemotherapy. However, following with repeated assaults, the number of stem cells that enables restoration of the bone marrow are reduced and the recovery efficiency is diminished, following with the destruction of intraosseous balance and eventually leading to osteoporosis [4].

Osteogenesis is controlled by a number of factors, including bone morphogenetic proteins (bmps) and platelet-derived growth factor (PDGFs). As the name suggests, BMPs were initially discovered by inducing new bone formation [6]. Bmp receptor (BMPRs) has serine/threonine kinase activity in the four types I (alk1, 2, 3, 6) and three types II (BMPRII, BMPRIIA, BMPRIIB) and participates in the bmps signalling pathway [7]. Ligand-bound type II

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receptors activate type I receptor kinases by phosphorylation in the glycine serine domain, leading to direct phosphorylation of smads (including smad1, smad5 and smad9) or activation of mitogen-activated protein kinase (MAPK) pathways, including ERK, JNK, and p38. Phosphorylated smad1/5/9 forms a heterozygous compound with smad4, which is transported into the nucleus, regulates the transcription of multiple target genes, including Id-1, and promotes osteogenesis [6, 8-10]. In addition, the activity or expression of Runx2, a key transcription factor for bone differentiation, is positively regulated by bmp-activated signaling pathways such as ERK, JNK, p38 and smads [11, 12]. This upregulated/activated Runx2 in turn promotes the expression of a variety of osteogenic genes, including ALP, col-1, OSX and OCN.

Receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) is a pivotal cytokine that promotes the differentiation of BMMs into osteoclasts [13]. RANKL binds to RANK, a member of the TNF receptor superfamily, and recruits members of the TNF receptor activator family [14, 15]. TRAFs activate the recruitment of many signaling pathways (I $\kappa$ B $\alpha$ /NF- $\kappa$ B, ERK, JNK, and p38) that are important for osteoclastogenesis [16, 17]. Meanwhile, RANKL stimulates the expression of nuclear factor of activated NFATc1 [17], c-Fos [18] and nuclear factors, as well as the expression of matrix metalloproteinase 9 (MMP9), cathepsin K (Ctsk), tartaric phosphate (TRAP), carbonic anhydrase II (Car2) [19] and other osteoclast genes. The biological effect of DOX on osteoclast development remains unclear. Therefore, studying the exact role of DOX in the regulation of rankl-mediated osteoclast formation is necessary. Here, we aimed to investigate the effects of DOX in osteoporosis and elucidate the underlying molecular mechanism in vitro.

### Materials and methods

#### *Media, antibodies, and reagents*

DOX, Alizarin Red kits, InvitrogenTRIZOL reagent and TRAP staining kits were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA), DOX was dissolved in ddH<sub>2</sub>O and stored at -20°C. The final concentrations of DOX were 0 nM (control), 2.5 nM, 5 nM and 10 nM,  $\alpha$ -Minimum Essential Medium ( $\alpha$ -MEM) was

obtained from Corning Company, FBS, trypsin and viability/cytotoxicity Kit were obtained from Thermo Fisher Scientific Company (Cambridge, MA, USA). Cell counting kit-8 (CCK-8) obtained from Dojindo Laboratories (Kumamoto, Japan), ALP Staining kit, Rhodamine-conjugated phalloidin for F-actin staining, 4',6-diamidino-2-phenylindole (DAPI) were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Anti-smad1/5/9 (cat. no. ab66737), anti-bmp-2 (cat. no. ab14933), anti-osterix (cat. no. ab209484), anti-ALP (cat. no. ab194297), anti-col-1 (cat. no. ab34710), anti-osteocalcin (cat. no. ab13420), anti-Runx2 (cat. no. ab76956), anti-RANKL (cat. no. ab239607), anti-OPG (cat. no. ab73400), anti-ALP (cat. no. ab83259), anti- $\beta$ -actin (cat. no. ab8226) were purchased from Abcam (Cambridge, MA, USA). Primer sequences were obtained from the literature and purchased from Golden Wisdom Biotechnology Co. LTD. (Soochow, China).

#### *Cell culture*

BMSCs and BMMs were isolated from the whole bone marrow of SD rat aged 4-6 weeks as described previously [20]. In brief, BMSCs and BMMs were acutely isolated from femoral and tibial bone marrow and cultured in  $\alpha$ -MEM containing 10% FBS, 1% penicillin/streptomycin in 5% CO<sub>2</sub> at 37°C. BMMs were cultured in complete medium with 30 ng/ml M-CSF in an incubator at 37°C with 5% CO<sub>2</sub> until they reached 80% confluence. The BMMs were seeded into 96-well plates at a density of 8 $\times$ 10<sup>3</sup> cells/well. The medium was replaced every 2 days, and the cells were subcultured using 0.25% trypsin.

#### *Osteoblast differentiation*

For the determination of osteoblast differentiation in vitro, all experiments were performed using BMSCs from third passage. Cells were plated at 10 $\times$ 10<sup>3</sup> cells per well. At 80% confluence, osteogenic medium (50 mg/ml of ascorbic acid, 0.04 mg/ml dexamethasone and 10 mM  $\beta$ -glycerol-phosphate) with different concentrations of DOX or without it were replaced. Medium was replaced every 2 days.

#### *Cytotoxicity test*

The cytotoxic effects of DOX on BMSCs and BMMs were determined using the CCK-8 assay

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according to the manufacturer's instructions. Briefly, BMSCs ( $3.0 \times 10^3$  cells per well) were plated in 96-well plates and cultured with different concentrations of DOX (5 nM, 10 nM, 20 nM and 50 nM) for 24 h, 72 h or 120 h, five parallel control (0 nM) wells were set in each group. Similarly, BMMs ( $10 \times 10^3$  cells per well) were incubated with different concentration of DOX (2.5 nM, 5 nM and 10 nM) for 24 h, 48 h or 72 h. Afterward, 10  $\mu$ l of CCK-8 buffer was added to each well for 4 h at 37°C. The absorbance was measured at 450 nm wavelength on an ELX800 absorbance microplate reader.

### *Live death staining*

To measure the viability of DOX-treated BMSCs and BMMs, we used the Viability/Cytotoxicity Kit (Invitrogen) to determine the intracellular esterase activity and plasma membrane integrity. BMSCs were planted in 24-well plates,  $2 \times 10^4$  cells/well and three parallel control wells were set. In brief, the cultured BMSCs were incubated with different concentrations of DOX (0 nM, 5 nM, 10 nM, 20 nM and 50 nM) for 24 h, 72 h or 120 h. Similarly, the cultured BMMs were incubated with different concentrations of DOX (0 nM, 2.5 nM, 5 nM and 10 nM) for 24 h, 48 h or 72 h. Liquid A and liquid B in the Live/Dead kit were prepared with working liquid according to the concentration indicated in the instructions. Afterward, working liquid was added to each well, and plates were incubated for the additional 30 minutes at 37°C. Images were captured using a fluorescence microscope.

### *Alp and alizarin red s staining*

ALP staining was performed at early-stage of osteogenic differentiation by the ALP staining kit after 7 days. Briefly, cells were fixed with 4% paraformaldehyde for 2 min and washed by 0.05% Tween 20 in PBS for 3 times. Then cells were stained with the ALP staining solution for 20 min in the dark. To investigate the effect of DOX in mineralization, Alizarin Red S staining was carried out at late-stage of osteogenic differentiation after 14 days. Briefly, the cells were fixed with 4% paraformaldehyde for 20 min, then stained with 0.4% Alizarin Red S solution for 15 min. Untreated cells were used as a control. Stained cells were washed and then examined under an inverted fluorescence micro-

scope, the absorbance was measured at 562 nm for quantitation.

### *Alp activity*

To assess the ALP activity of BMSCs, the ALP activity detection kit was used. Briefly, the BMSCs cultured with osteogenic medium (OM) with different concentrations of DOX or without it for 7 days then lysed with RIPA lysis buffer on ice. The samples were then centrifuged at 148,000 r/min for 30 min, and ALP activity was measured according to manufacturer's instructions.

### *Trap staining*

BMMs were cultured in 96 well plates at the density of  $2 \times 10^4$  cells/well in complete medium with 30 ng/ml M-CSF. After 24 h, the BMMs were incubated with 50 ng/mL RANKL and different concentrations of DOX (1.25 nM, 2.5 nM and 5 nM) for direct study. On the other hand, BMSCs were incubated with different concentrations of DOX (0 nM, 2.5 nM, 5 nM and 10 nM) for two days then were cultured in complete medium without DOX. Two days later, 20% supernatant of every well was extracted, and BMMs were incubated with 30 ng/mL M-CSF, 50 ng/mL RANKL, and supernatant (20%) for indirect study. TRAP staining was performed after 5 days. Untreated cells were included as controls. According to the manufacturer's instructions, cells were fixed in 37% formaldehyde for 5 min and stained with a tartaric phosphate kit. Trap-positive cells with more than five nuclei were considered osteoclasts. TRAP-positive multinucleated cells were visualized and counted in each well ( $n=3$ ) under light microscopy.

### *Immunofluorescence assay*

After incubated with different doses of DOX for 7 days, BMSCs in every group were fixed with 4% paraformaldehyde for 15 min at room temperature and then permeabilized with 0.5% Triton X-100 for 20 min. Cells were incubated with 5% bovine serum albumin (BSA) for 20 min to block the nonspecific antibody binding sites and then incubated with primary col-1 antibodies (1:500) overnight at 4°C. After washing three times with PBS, cells were incubated with a 1:500 dilution of an APC-labeled rabbit secondary antibody for 1 h. Next, BMSCs were

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**Table 1.** The primers used for quantitative PCR

Gene	Sequence
RANKL	forward 5'-CGATGGTGGATGGCTCATG-3' reverse 5'-TGAGCAAAGGCTGAGCTTCA-3'
OPG	forward 5'-CGGCACATTGGACATGCTA-3' reverse 5'-TCCCGGTAAGCTTTCCATCA-3'
Runx2	forward 5'-GTTCCAGGCATTTTCATCCC-3' reverse 5'-AAGGTGGCTGGATAGTGCAT-3'
ALP	forward 5'-CAAGGATGCTGGGAAGTCCG-3' reverse 5'-CTCTGGGCGCATCTCATTGT-3'
Col-I	forward 5'-TAA AGGGTCATCGTGGCTTC-3' reverse 5'-ACTCTCCGCTCT TCCAGTCA-3'
OCN	forward 5'-CTTGAAGACCGCTACAAAC-3' reverse 5'-GCTGCTGTGACATCCATAC-3'
$\beta$ -catenin	forward 5'-CCTGTACGCCAACACAGTGC-3' reverse 5'-ATACTCTGCTTGCTGATCC-3'
TRAP	forward 5'-TCCTGGCTCAAAAAGCAGTT-3' reverse 5'-ACATAGCCACACCGTTCTC-3'
c-Fos	forward 5'-CCAGTCAAGAGCATCAGCAA-3' reverse 5'-AAGTAGTGCAGCCCGGAGTA-3'
NFATc1	forward 5'-GGGTCAAGTGTGACCGAAGAT-3' reverse 5'-GGAAGTCAGAAGTGGGTGGA-3'
GAPDH	forward 5'-ACCCAGAAGACTGTGGATGG-3' reverse 5'-CACATTGGGGTAGGAACAC-3'

stained with 4'-6-diamidino-2-phenylindole (DAPI) and then examined using a fluorescence microscope, and images were captured by Image Manager software. Similarly, BMMs were treated as previously described. Next, the cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.3% Triton X-100 for 5 min, followed by incubation with 2% BSA for 1 h in order to block nonspecific binding of antibodies. Then the cells were stained with phalloidin for 30 min at 25°C. After washing three times with PBS, the nuclei were counterstained with DAPI. Images were captured using a fluorescence microscope.

### Real-time quantitative pcr (qRT-PCR)

Quantitative real-time PCR (qRT-PCR) was used to determine gene expression levels during osteoblast and osteoclast formation. BMSCs were seeded in 6-well plates at a density of  $2 \times 10^4$  cells/well and cultured in the HSM or OM with different doses of DOX (0 nM, 2.5 nM, 5 nM and 10 nM) for 2 days. Similarly, BMMs were seeded in 6-well plates at a density of  $1 \times 10^5$  cells/well and cultured in the complete  $\alpha$ -MEM supplemented with 30 ng/mL of M-CSF

and 50 ng/mL RANKL. Cells were treated with 5 nM DOX for 24 h or 48 h. Then the cells were washed three times with PBS and RNA was extracted using TRIzol reagent. Next, cDNA was synthesized using the Biometra TGradient First-Strand Synthesis System (Whatman Biometra, Germany). Quantitative real-time PCR was performed using the Real-Time PCR System (Bio-Rad Laboratories, USA) and SYBR Green SuperMix (Bio-Rad, USA) according to the manufacturer's instructions. The primers used for qRT-PCR are listed in **Table 1**. All data are displayed as means  $\pm$  SD of three independent experiments.

### Western blot validation

To determine the activity of osteoblast/osteoclast formation and signalling pathways affected by DOX, treated BMSCs and BMMs were lysed and total proteins were extracted using RIPA lysis buffer (Beyotime, China). The protein samples were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked with Blocking Buffer (Beyotime, China) for 1 h and incubated with the primary antibodies ALP, col-1, OCN, smad1/5/9, bmp-2, Runx 2, OSX, RANKL, OPG, c-Fos, NFATc1 and  $\beta$ -actin overnight at 4°C, according to the manufacturer's instructions. After incubation with the secondary antibodies in blocking buffer at room temperature for 1 h, protein bands were visualized using ChemiDoc™ Touch Imaging System (Bio-Rad Laboratories, USA).

### Statistical analysis

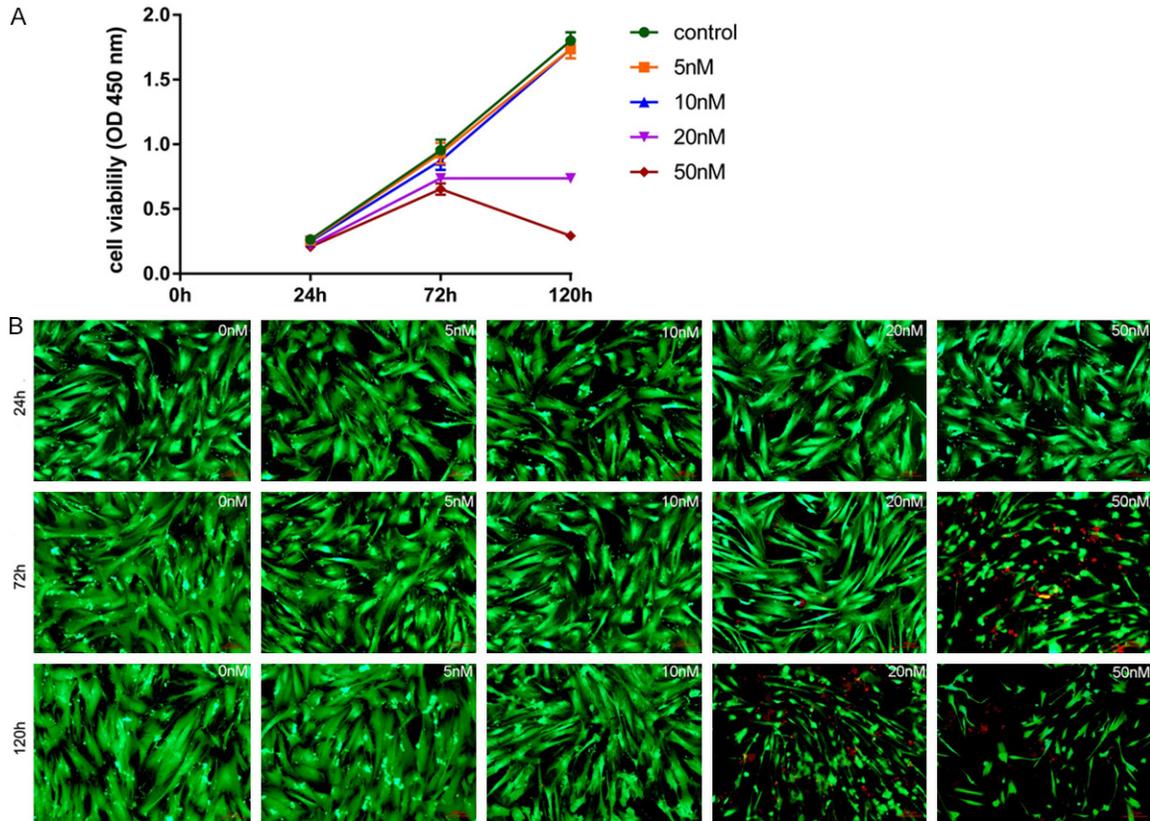
Experiments were performed in triplicate and repeated more than three times independently. The data were presented as mean  $\pm$  standard error of the mean and analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc. La Jolla, CA, USA). Changes were assessed using statistical test or analysis of variance, and *p*-values  $< 0.05$  were considered statistically significant.

## Results

### DOX restrained the proliferation of BMSCs

To investigate the molecular mechanisms underlying the effects of DOX on osteogenesis differentiation, CCK-8 assay was performed to analyze the potential cytotoxicity against BMSCs. As shown in **Figure 1A**, 10 nM of DOX

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**Figure 1.** DOX inhibits the proliferation of BMSCs. Primary BMSCs were treated with DOX before measuring cell viability. The CCK-8 assay (A) and live/dead staining (B) showed that the number of dead BMSCs increased significantly after 72 hours of DOX treatment at a concentration higher than 10 nM. Cytotoxicity (live/dead) assay images show the live (green) and dead cells (red) in a cellulose sample.

did not affect cell viability of BMSCs. Subsequently, Calcein AM (labeled living cells, green) and PI (labeled dead cells, red) staining assays demonstrated that the proliferation of BMSCs were not affected by treatment with DOX at the indicated concentrations (0 nM, 5 nM and 10 nM) (**Figure 1B**). The dose of 10 nM was defined as the sublethal concentration of BMSCs treated with DOX [21].

### *DOX restrained osteogenesis differentiation in BMSCs*

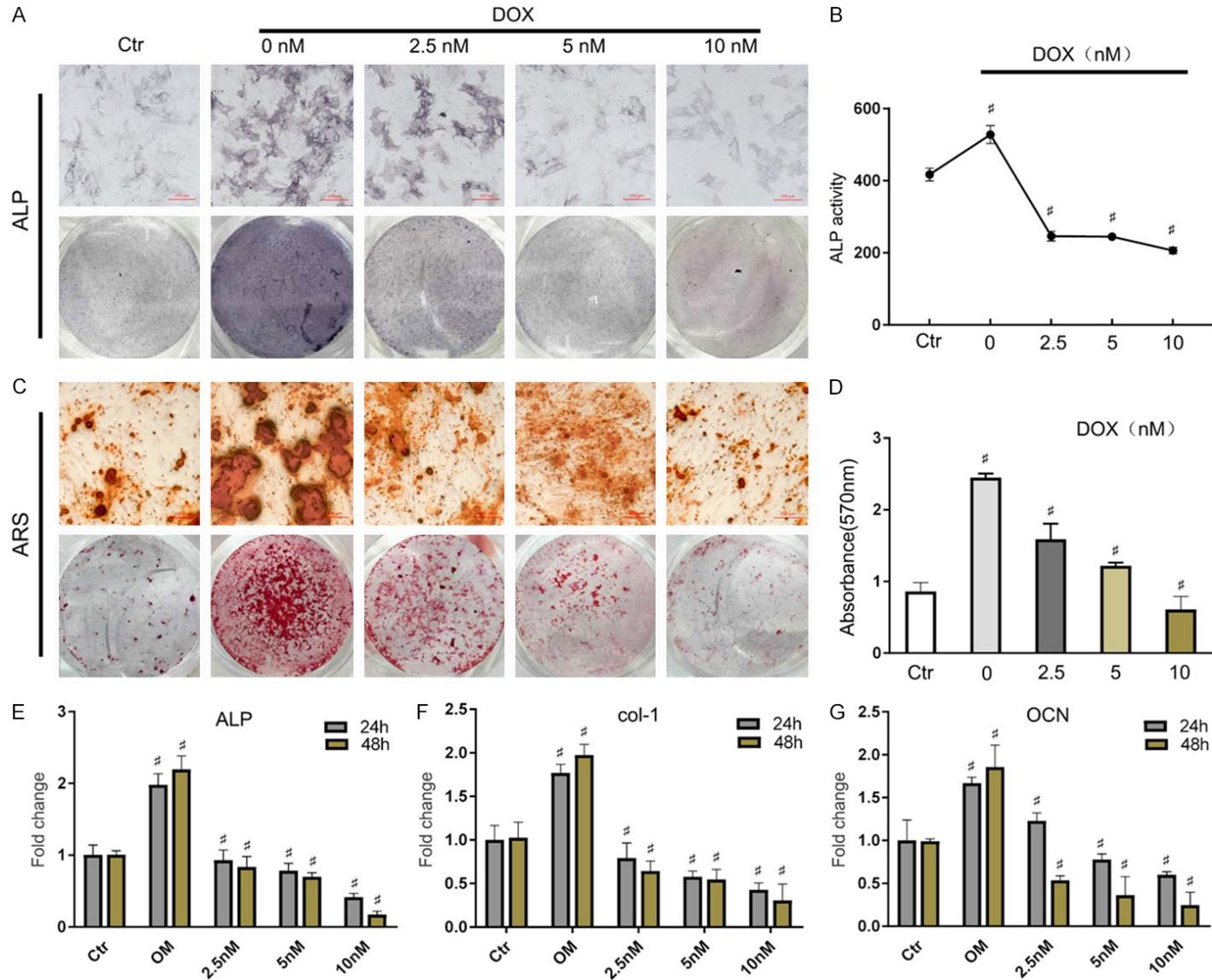
To determine the anabolic activity of osteogenesis differentiation in BMSCs treated with different concentrations of DOX, ALP staining/activity and Alizarin red S staining were evaluated. **Figure 2A** shows that DOX dose-dependently decreased the number of ALP-positive cells on 7 days, as evidenced by ALP staining and quantitative analysis of the ALP activity (**Figure 2B**). In addition, we also used alizarin red S staining to investigate the effects of DOX

on mineral deposition in extracellular matrices. Less plaque calcified extracellular matrices were found following the treatment with 5 and 10 nM DOX, compared with those cells in controls, 0 and 2.5 nM DOX after 14 days (**Figure 2C, 2D**). Similarly, the level of mRNA expression of ALP, col-1 and OCN were decreased with the treatment of DOX in a time and dose-dependent manner, as determined by qRT-PCR (**Figure 2E-G**).

### *DOX restrained osteoblasts differentiation through bmp-2/smads signalling pathway and upregulated the ratio of rankl/opg*

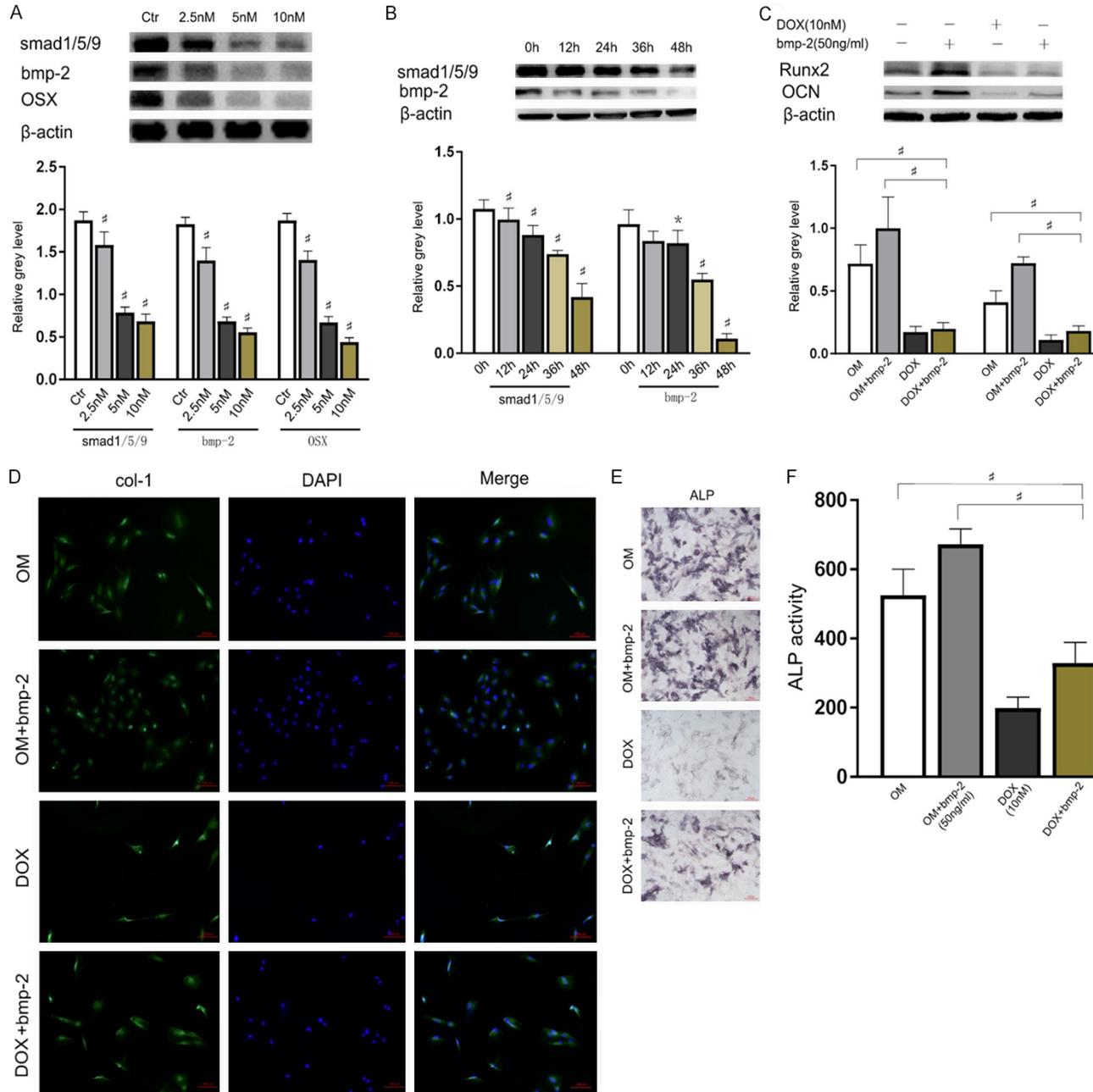
The bmp-2/smads pathway plays an important role in stimulating osteoblastogenesis. To clarify the mechanisms underlying DOX-induced inhibition of osteogenic differentiation, the protein expression levels of smad1/5/9, bmp-2, OSX, Runx2, ALP, col-1 and OCN were detected by WB. Here, results demonstrated that DOX restrained osteoblast formation (**Figure 3C**)

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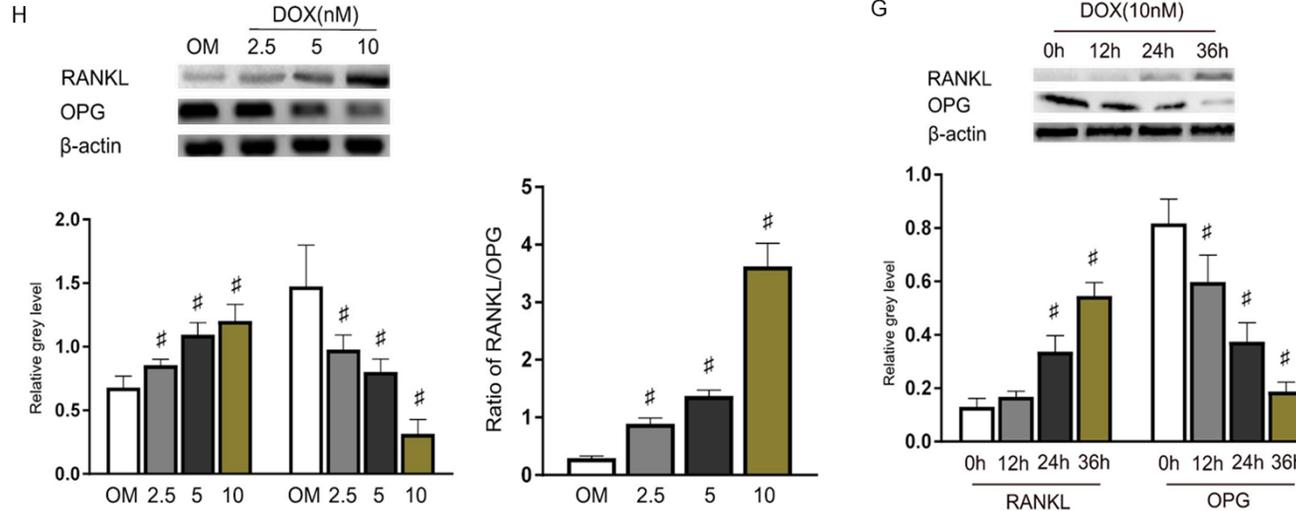


**Figure 2.** Effect of DOX on osteogenic differentiation in BMSCs. A. BMSCs were cultured in HSM (control), OM and DOX. All the assays were performed using ALP staining assay after 7 days. B. ALP activity was tested on BMSCs cultured with HSM, OM, and DOX for 7 days. \* $P < 0.05$  and  $\#P < 0.01$  vs the control group ( $n = 3$ ). C, D. BMSCs were cultured with HSM (control), with or without DOX for 14 days. All the assays were performed and quantified by Alizarin Red S staining. \* $P < 0.05$  and  $\#P < 0.01$  vs the control group ( $n = 3$ ). E-G. BMSCs cultured with OM (control), DOX (2.5, 5, 10 nM) were harvested on days 2. The mRNA expression levels of ALP, col-1 and OCN were assessed by qRT-PCR and quantified \* $P < 0.05$  and  $\#P < 0.01$  vs the control group.

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**Figure 3.** DOX restrained the bmp-2/smad signalling pathway of osteogenesis differentiation in BMSCs. A. BMSCs cultured with OM (control), DOX (2.5, 5, 10 nM) were harvested on days 2. The protein expression levels of smad1/5/9, bmp-2, and OSX were assessed by WB and quantified \* $P < 0.05$  and  $^{\#}P < 0.01$  vs the control group. B. The protein expression levels of smad1/5/9 and bmp-2 were quantified \* $P < 0.05$  and  $^{\#}P < 0.01$  vs the control group. C. BMSCs cultured with OM (control), with or without DOX (10 nM) or bmp-2 (50 ng/ml) were harvested on 48 h, the protein expression of Runx-2 and OCN were quantified. \* $P < 0.05$  and  $^{\#}P < 0.01$  vs the control group. D. Immunofluorescence detection of col-1 translocation in cultured. BMSCs were treated with OM (control), with or without DOX (10 nM) or bmp-2 (50 ng/ml). Col-1 expressed in both the cytoplasm and nucleus, as well as the fluorescent density and intensity was increased dose dependently for 5 days. The nuclei were stained with DAPI and were shown as blue fluorescence. Scale bar = 100  $\mu$ m. E, F. BMSCs cultured with OM (control), with or without DOX (10 nM) or bmp-2 (50 ng/ml) were harvested on 7 days, ALP staining/activity was assessed. G. BMSCs treated with OM (control), DOX (10 nM) were harvested on 0 h, 12 h, 24 h, 36 h. The protein expression levels of RANKL and OPG were assessed by WB and quantified \* $P < 0.05$  and  $^{\#}P < 0.01$  vs the control group. H. BMSCs treated with OM (control), DOX (2.5, 5, 10 nM) were harvested on 48 h. The protein expression levels of RANKL and OPG were assessed by WB and quantified \* $P < 0.05$  and  $^{\#}P < 0.01$  vs the control group.

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through the inhibition of smad1/1/5/9 and bmp-2 activity in a dose and time-dependent manner (**Figure 3A, 3B**). Further immunofluorescence showed the inhibition of col-1 following the treatment with 10 nM DOX, compared with those in the controls and cells treated with DOX+bmp-2 (50 ng/ml) (**Figure 3D**). Our finding indicated that DOX significantly restrained the number of osteoblast and ALP activity through bmp-2/smads pathway (**Figure 3E, 3F**).

The ratio of RANKL/OPG is important for osteoclastogenesis. We found that DOX could upregulate RANKL expression of BMSCs, while it could downregulate OPG expression. The WB results (**Figure 3G, 3H**) showed that the protein expression levels of RANKL increased in both time and dose-dependent manner. By contrast, OPG was expressed in an opposite manner.

*DOX restrained proliferation and osteoclast differentiation of rankl-induced BMMs in a direct manner*

Cell viability assay was performed to analyze the potential cytotoxicity of DOX against BMMs. The result demonstrated that DOX did not show cytotoxicity toward BMMs cells at the investigated concentrations (0-5 nM) (**Figure 4A**). Live/dead staining also demonstrated that BMMs showed obvious apoptosis compared with the control group when the DOX concentration was higher than 5 nM (**Figure 4B**). Since DOX can increase the ratio of RANKL/OPG, BMMs treated without DOX were used as controls. As shown in **Figure 4C-E**, the formation of TRAP-positive cells was enhanced following with the DOX treatment in a dose-dependent manner in comparison with the control group. C-Fos and NFATc1 are crucial transcription factors in osteoclast differentiation which can initiate the expression of TRAP. The acceleration of DOX on osteoclast formation were further supported by the promotion of osteoclast-related gene expression. The expression of genes, including NFATc1 and c-Fos were promoted in a time (**Figure 4F**) and dose-dependent manner (**Figure 4G**), which indicated that DOX promoted osteoclastogenesis in vitro.

*DOX promoted osteoclast differentiation of rankl-induced BMMs in an indirect manner*

The supernatant extracted from the medium of BMSCs which treated with DOX increased the

related gene expression of osteoclast (**Figure 5A**) and the formation of TRAP-positive cells (**Figure 5B**). Thus, we found that DOX dose-dependently promote RANKL-mediated osteoclastogenesis in an indirect manner.

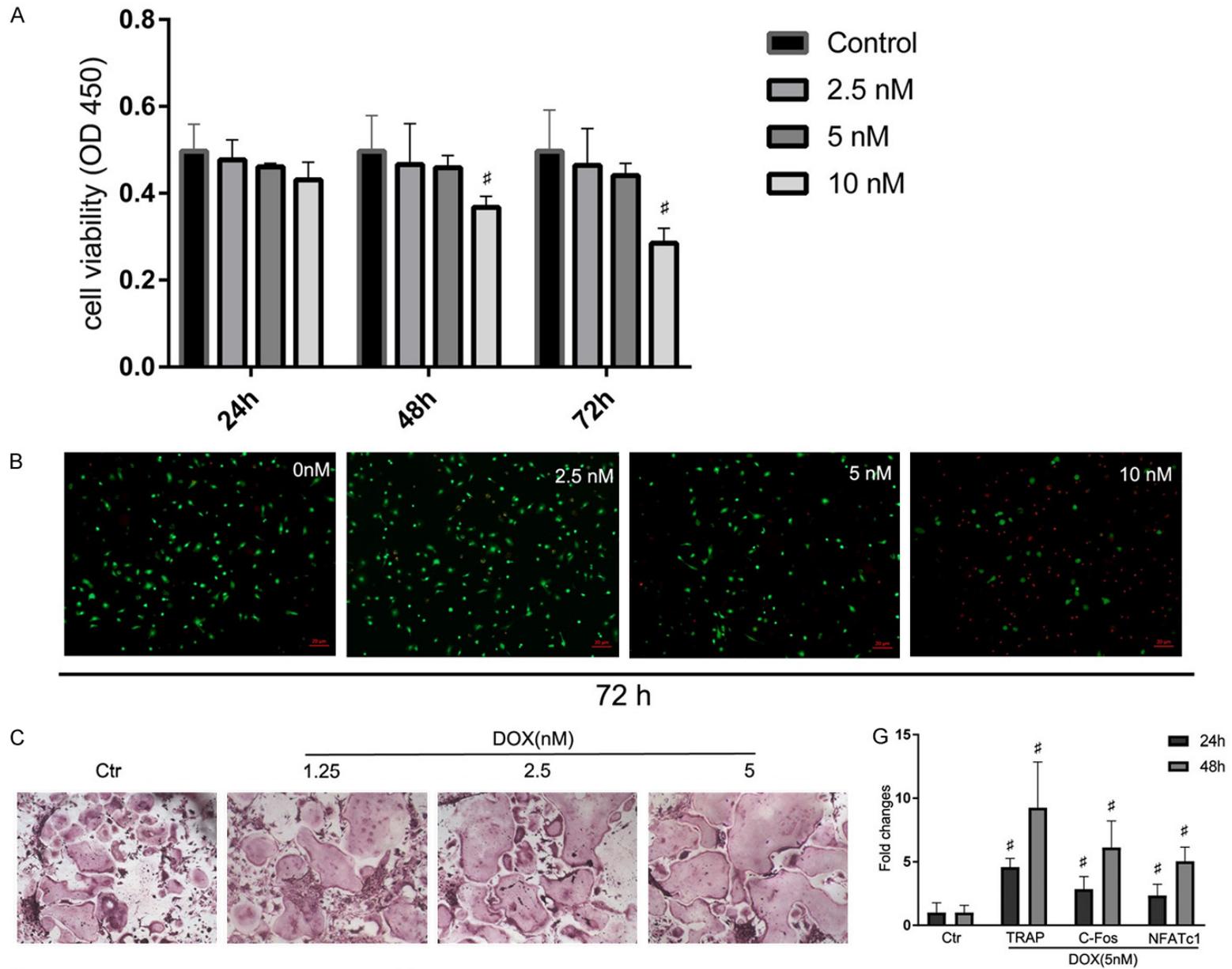
### Discussion

In our study, DOX exerted the inhibitory effects on osteoblastogenesis through suppressing the bmp-2/smads signalling shown by the decreased expression of bmp-2, smad1/5/9 and OSX. Consequently, DOX decreased the expression of osteoblast-specific factors like Runx2, ALP, col-1, OCN and the number of BMSCs. On the other hand, rankl-mediated osteoclastogenesis was also promoted by DOX, which up-regulated the expression of TRAP, NFATc1 and c-Fos. This study has provided new insights into the roles of DOX in bone remodeling, especially for understanding the underlying mechanism for bone loss of DOX-treated patients.

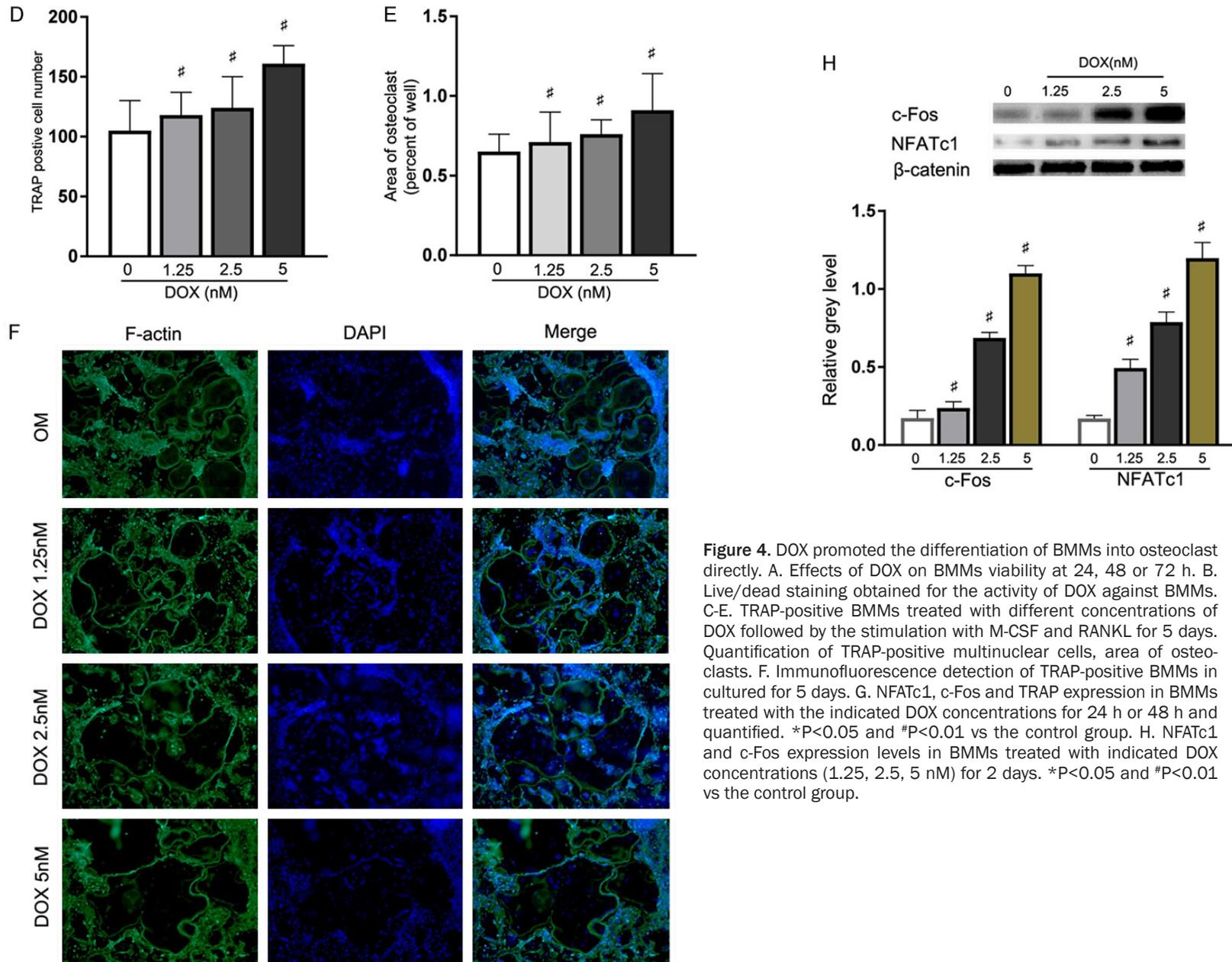
DOX, an anthracycline drug, has been widely used for the clinical treatment of patients suffering from primary and metastatic breast cancer over the last few decades [22]. However, the side effects of DOX, following with the destructive effect on bones, including diminished bone formation, reduced bone density and increased pathological fractures are also deserved to be taken seriously. A reduction in the number of BMSCs is one biological manifestation of DOX-induced microenvironment changes during bone metastasis [23]. It has been reported that DOX causes an increase in ROS and depolarization of the mitochondrial membrane potential by activating p38, JNK and p53 signalling and induces apoptosis and dysfunction of BMSCs [21]. Oxidative stress also inhibits the osteoblastic differentiation of BMSCs by ERK and NF- $\kappa$ B [24].

It is reported that BMSCs have the potential to differentiate into a variety of cell lines. Autologous BMSCs will not undergo immune rejection after re-transfusion and possess high gene transfection rate in vitro [25]. Decreased bone density is associated with decreased numbers of BMSCs and increased bone loss induced by osteoclasts. Since chondroblast and adipoblast differentiation occurred at a cost of reduced numbers of osteoblasts [26-28], differentiation of BMSCs into osteoblasts can

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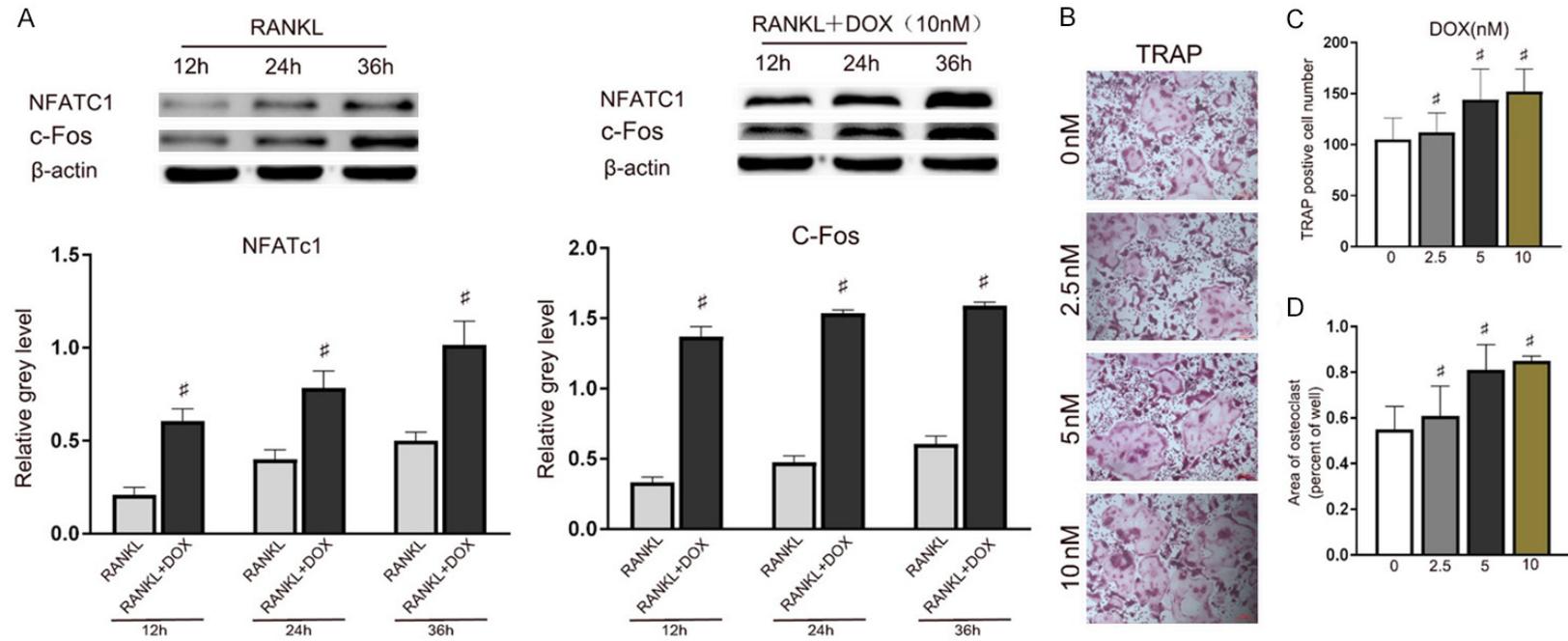


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**Figure 4.** DOX promoted the differentiation of BMMs into osteoclast directly. A. Effects of DOX on BMMs viability at 24, 48 or 72 h. B. Live/dead staining obtained for the activity of DOX against BMMs. C-E. TRAP-positive BMMs treated with different concentrations of DOX followed by the stimulation with M-CSF and RANKL for 5 days. Quantification of TRAP-positive multinuclear cells, area of osteoclasts. F. Immunofluorescence detection of TRAP-positive BMMs in cultured for 5 days. G. NFATc1, c-Fos and TRAP expression in BMMs treated with the indicated DOX concentrations for 24 h or 48 h and quantified. \*P<0.05 and #P<0.01 vs the control group. H. NFATc1 and c-Fos expression levels in BMMs treated with indicated DOX concentrations (1.25, 2.5, 5 nM) for 2 days. \*P<0.05 and #P<0.01 vs the control group.

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**Figure 5.** DOX promoted the differentiation of BMMs into osteoclast indirectly. A. NFATc1 and c-Fos expression in BMMs treated with the indicated DOX concentrations for 12 h, 24 h or 36 h and quantified. \* $P < 0.05$  and  $\#P < 0.01$  vs the control group. B-D. TRAP-positive BMMs treated with different concentrations of DOX followed by the stimulation with M-CSF and RANKL for 5 days. Quantification of TRAP-positive multinuclear cells, area of osteoclasts.

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increase bone mass and prevent osteoporosis. In this study, BMSCs from juvenile animals with multidirectional differentiation potential were selected as the source of osteoblast induction.

BMPs promotes osteoblast maturation and induces the typical expression of mature osteoblasts, including ALP, parathyroid hormone receptor and OCN [29]. Therefore, osteoblastic BMPs have specific intracellular signalling pathways. Smad proteins (smads 1-9) are important transcriptional regulators of the intracellular TGF- $\beta$  family signalling pathway [30, 31]. According to the structure and biochemical function of smads, they are divided into three subgroups: R-smads, Co-smads and I-smads [31]. Osteogenic BMPs induce phosphorylation of smad1, smad5 and smad9 (also known as smad8), while non-osteogenic members of the TGF- $\beta$  family induce phosphorylation of smad2 and smad3 [31, 32]. Runx2 interacts with Smad1/5/9, inducing OCN expression [33-35]. The overexpression of this molecule induces the expression of osteoblast phenotypic markers in different types of cells, including MSCs, primary myoblasts and marrow stromal cells [36].

Additionally, previous studies also have indicated that the bmp-2/smads pathway might participate in apoptosis and the process of osteogenesis [37, 38]. Therefore, the down-regulated expression of bmp-2/smads may account for DOX-induced bone loss because of the direct inhibitory effects on osteoblast-mediated bone formation. The results obtained here demonstrate that DOX induced bone loss in bone remodeling by decreasing the number of BMSCs, restraining osteoblastogenesis, and contributing to the suppressed bone formation and bone loss.

On the other hand, the molecular mechanism of DOX in promoting osteoclast differentiation mediated by RANKL needs to be further studied. The signalling pathway activated by RANKL plays a key role in osteoclast differentiation, survival, activity and bone resorption [39]. This study demonstrates that DOX promotes osteoclast formation and that its underlying mechanism may be increased expression of TRAP, NFATc1 and c-Fos, which are involved in rankl-mediated osteoclast formation [17]. In this

study, we found that DOX could not only promote the differentiation of BMMs into osteoclasts directly but also stimulate the secretion of RANKL from BMSCs/osteoblast promote the formation of osteoclasts indirectly. In conclusion, we demonstrated that DOX modulates bone metabolism through restraining the bmp-2/smads signalling pathway. It can increase the ratio of RANKL/OPG and promote osteoclast formation. The results obtained in this study indicate that DOX inhibited bone formation while accelerated bone destruction. However, due to lack of in vivo and clinical studies, it is still insufficient to explain the mechanism of DOX induced osteoporosis, so further experimental studies are needed.

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### Disclosure of conflict of interest

None.

### Abbreviations

BMSCs, bone marrow mesenchymal stem cells; BMMs, bone marrow macrophages; CCK-8, cell counting kit-8; FBS, Fetal bovine serum; RANKL, receptor activator of nuclear factor- $\kappa$ B ligand; RANK, receptor activator of nuclear factor- $\kappa$ B; OPG, osteoprotegerin; qRT-PCR, real-time fluorescence quantitative-Polymerase Chain Reaction; PBS, phosphate buffered solution; M-CSF, macrophage colony-stimulating factor; ALP, alkaline phosphatase; col-1, collagen I; OCN, osteocalcin; FITC, fluorescein isothiocyanate; DAPI, diamidine-2'-phenylindole dihydrochloride; IF, immunofluorescence; NFATc1, nuclear factor of activated T-cells-cytoplasm 1; TRAP, tartrate-resistant acid phosphatase; Runx2, Runt-related transcription factor; OSX, osteorix; bmp-2, Bone Morphogenetic Protein-2; OM, osteoblast Medium; HSM, high sugar medium.

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