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# Constant hypoxia inhibits osteoclast differentiation and bone resorption by regulating phosphorylation of JNK and IκBα

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## Abstract

**Background** Osteoclasts are responsible for the bone loss in rheumatoid arthritis (RA). Hypoxia has been suggested to play key roles in pathological bone loss. However, the current understanding of the effects of hypoxia on osteoclastogenesis is controversial. Effects of hypoxia on both the formation and function of osteoclasts requires examination. In the current study, we aimed to explore the effect of hypoxia on osteoclast differentiation and the underlying mechanisms.

**Methods** RAW264.7 cells and murine bone-marrow-derived monocytes were used to induce osteoclastogenesis in the presence of macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B ligand (RANKL). Hypoxic conditions were maintained in a hypoxic chamber at 5% $CO_2$ and 1% $O_2$, balanced with $N_2$. Osteoclasts were detected by tartrate-resistant acid phosphatase (TRAP) staining. A bone resorption assay was carried out in vitro using bone slices. RT-PCR was conducted to detect osteoclast markers and transcription factors. The phosphorylation of nuclear factor-κBα (IκBα), c-Jun N-terminal kinase (JNK), extracellular regulated protein kinase (ERK), and p38 was detected by western blotting. Mann–Whitney *U* test or Student's *t* test was used to compare differences between the two groups.

**Results** TRAP staining and the bone resorption assay revealed that hypoxia-restrained osteoclast differentiation and bone resorption. Expression of osteoclast markers including cathepsin K, RANK, and TRAP decreased during osteoclast differentiation under hypoxic conditions (all $P<0.05$). Hypoxia at 1% $O_2$ did not affect cell viability, whereas it dramatically abated RANKL-dependent phosphorylation of the JNK-mitogen-activated protein kinases (MAPK) and IκBα pathways. Moreover, the expression of nuclear factor of activated T-cell cytoplasmic 1 (*NFATc1*) was inhibited under hypoxic conditions (all $P<0.05$).

**Conclusions** These results suggest that constant hypoxia at 1% $O_2$ significantly restrains osteoclast formation and resorbing function without affecting cell viability. Constant hypoxia might inhibit RANKL-induced osteoclastogenesis by regulating NFATc1 expression via interfering the phosphorylation of JNK and IκBα.

**Keywords** Hypoxia · Osteoclast · Osteoclastogenesis · JNK-mitogen-activated protein kinases · NFATC transcription factors

## Introduction

Rheumatoid arthritis (RA) is a systemic chronic autoimmune disease characterized by synovitis, cartilage, and bone erosion, eventually leading to joint deformities [1]. It has been reported that RA is one of the main causes of disability in China [2], and approximately 15.8% of RA patients in China suffer from functional disability [3]. Bone erosion is a prominent factor contributing to poor functional outcome [4]. The integrity of bone depends on the balance between bone cells, including bone-resorbing osteoclasts and bone-forming osteoblasts. When the homeostasis is disrupted, like abnormal activation of

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osteoclasts, pathological bone loss or bone remodeling can occur. Osteoclasts, derived from the monocyte lineage, are primary cells that mediate bone resorption in RA [5–7]. Imbalanced activation of osteoclasts results in pathological bone loss.

Osteoclasts are large, multinucleated bone-resorbing cells differentiated from the hematopoietic monocyte–macrophage linage [7, 8]. Osteoclast differentiation is mediated by two essential factors: receptor activator of NF-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) [9, 10]. Released RANKL binds to its receptor RANK on the surface of osteoclast precursors, thereby initiating downstream signaling cascades. First, RANKL binding to RANK induces the recruitment and activation of a major adaptor protein, tumor necrosis factor receptor-associated factor 6 (TRAF6) [11]. Next, TRAF6 activates the nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPK) signaling pathways, such as p38, JNK, and ERK [12–15], eventually leading to induction of *c-Fos* [16]. Finally, in the late stage of signaling, transcription factor nuclear factor of activated T-cell cytoplasmic 1 (*NFATc1*), a master transcription factor in bone resorption, is activated. Activated *NFATc1* translocates to the nucleus, where it upregulates *NFATc1* target genes to regulate osteoclast differentiation, cell fusion, and function [17–22].

Hypoxia is a characteristic microenvironment feature in the joints of RA [23–25]. It has been reported that hypoxia exerts various effects on osteoclast differentiation and bone resorption activity [26]. However, there are also contradictory data regarding how hypoxia affects osteoclast formation. Arnett et al. first reported that hypoxia strongly stimulated osteoclast formation and bone resorption derived from murine bone-marrow cells or human peripheral blood mononuclear cells at either 2% $O_2$ or 1% $O_2$ [27, 28]. Another study showed similar results in which hypoxia-induced giant osteoclast formation and extensive bone resorption in cats [29]. In contrast, other studies found that hypoxia did not induce osteoclastogenesis [30–32]. The use of hypoxia mimics cobaltous chloride ($CoCl_2$), dimethyloxallyl glycine (DMOG), and deferoxamine (DFO) exerted inhibitory effects on osteoclast differentiation [30]. Moreover, hypoxia-inducible factor (HIF)-1α activation by the von Hippel–Lindau tumor suppressor protein (VHL) knockdown or prolyl hydroxylas (PHD) enzyme inhibitors strongly reduced osteoclastogenesis and osteoclastic bone resorption [31, 32].

Because the joints of patients with RA are in a chronic hypoxia condition [24, 25], it is important to understand how constant hypoxia affects the differentiation and activity of osteoclasts. We, therefore, examined the effects of constant hypoxia on osteoclast differentiation and bone resorption and further explore the underlying mechanisms.

## Methods

### Ethical approval

All procedures involving mice were performed in accordance with the Peking University Animal Protection and Ethics Committee (No. LA201425). In this study, the national law on the care and use of laboratory animals was followed.

### Regents

Culture medium and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). Murine sRANKL and murine recombinant M-CSF were purchased from PeproTech (Rocky Hill, NJ, USA). Trizol was from Invitrogen (Carlsbad, CA, USA). The RNA reverse transcription kit and Talent qPCR PreMix (SYBR Green) were from Tiangen Biotech (Beijing, China). The tartrate-resistant acid phosphatase (TRAP) Kit (387-A) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The FITC Annexin V Apoptosis Detection Kit I was from BD Biosciences (Franklin Lakes, NJ, USA). The antibody information used in this study was shown in the supplementary material, Table 1.

### Cell culture

RAW264.7 cells (mouse macrophages) were kindly provided by the Medical Research Center of Peking University Third Hospital. RAW264.7 cells were maintained in DMEM containing 10% heat-inactivated fetal bovine serum(FBS).Murine bone-marrow-derived monocytes (BMMs) flushed from the long bones of *C57BL/6* mice aged 6–8 weeks were maintained in α-minimal essential medium (MEM) containing 10% heat-inactivated FBS, penicillin(50 U/ml), and streptomycin sulfate (50 μg/ml). BMMs were cultured as follows: first, marrow cells flushed from the long bones of mice were cultured in α-MEM containing 10% FBS in the presence of M-CSF (10 ng/ml) at 37 °C in 5% $CO_2$ for 3 days. Non-adherent cells were removed by washing the culture dishes with phosphate-buffered saline followed by incubation under different conditions in the presence of M-CSF (10 ng/ml) and sRANKL (50 ng/ml) for 7 days for osteoclast detection. Cells were incubated in a hypoxic chamber (HERACELL 150i, Thermo Fisher Scientific, Waltham, MA, USA) at 5% $CO_2$ and 1% $O_2$, balanced with $N_2$ as indicated or in a normal incubator containing 5% $CO_2$ and approximately 20% $O_2$ (Forma Series II, Thermo Fisher Scientific).

### TRAP staining

TRAP staining was carried out using the TRAP kit (387A-1KT, Sigma) according to the manufacturer's instructions. Briefly, prior to staining, RAW264.7 cells were incubated for 5 days, while BMMs were incubated for 7 days in the presence of M-CSF (10 ng/ml) and sRANKL (50 ng/ml). The cells were fixed in 4% paraformaldehyde for 30 min at room temperature, followed by incubation with a mix prepared according to the manufacturer's instruction for 30 min at 37 °C. Finally, the cells were stained with hematoxylin for 2 min, and then washed two times with water. Images were captured using a Nikon ECLIPSE Ti–S microscope (Nikon, Tokyo, Japan). Multinucleated cells containing three or more nuclei were considered osteoclasts.

### In vitro bone resorption assay

BMMs were seeded on bone slices (DT-1BON1000-96, Biorj, London, UK) in a 96-well plate at a density of $2\times10^4$ cells/well. After 2 day pre-incubation with M-CSF (10 ng/ml) to induce osteoclasts precursors, the cells were stimulated with M-CSF (10 ng/ml) and sRANKL (50 ng/ml) for 6 days until mature osteoclasts formation, and then incubated in a normoxic or hypoxic chamber, respectively. Fresh medium with M-CSF (10 ng/ml) and sRANKL (50 ng/ml) was replaced every 3 days. After 15 day induction, the bone-resorption assay was carried out. Resorption of bone slices was visualized by staining with 1% toluidine blue after removing adherent cells by sonication. Bone slices were photographed using a microcirculation microtester (TongRen Medical, Jiangsu, China).

### Cell-viability assay

RAW264.7 cells were seeded into 96-well plates at a concentration of 3000 cells/well. Cells were incubated under normoxic or hypoxic conditions as described above. After 1–3 days, cell viability was measured using the CellTiter 96 ®AQueousOne Solution Cell Proliferation Assay (MTS assay; Promega, Madison, WI, USA) according to the manufacturer's protocol.

### Flow-cytometry analysis for apoptosis

Cell apoptosis experiments were conducted by flow-cytometry analysis. After incubation for 3 days in the presence of M-CSF (10 ng/ml) and RANKL(50 ng/ml) under normoxic or hypoxic conditions, RAW264.7 cells were harvested and suspended, followed by incubation with FITC Annexin V and propidium iodide (BD Biosciences). The positive control group was induced by incubating the cells in a 55 °C water bath for 5 min. Flow cytometric analysis was performed using a FACSCalibur (BD Biosciences).

### Real-time quantitative PCR

Total RNA was extracted using TRIzol reagent (Invitrogen), and 1 μg of total RNA was reverse-transcribed into cDNA using a FastQuant RT Kit (with gDNase) (Tiangen Biotech). RT-PCR was carried out using the QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific) with Talent qPCR PreMix (SYBR Green) (Tiangen Biotech). Data were normalized to the expression of *GAPDH*. Primers were synthesized by Sangon Biotech (Shanghai, China). The primer nucleotide sequences for PCR were obtained from GenBank database and synthesized by Sangon Biotech (China). The primer sequence information used in the study was shown in the supplementary material, Table 2.

### Western blotting analysis

Total protein was extracted using RIPA lysis buffer (Applygen, Beijing, China). To detect the protein levels of *NFATc1* and *c-Fos*, nucleoprotein was extracted using a Nuc-Cyto-Mem Preparation Kit (Applygen, Beijing, China). A total of 40 μg of proteins was loaded into a 10% sodium dodecyl sulfate–polyacrylamide gel and electrophoretically transferred to polyvinylidene fluoride membranes (Merck Millipore, Billerica, MA, USA). After blocking with 5% milk for 2 h, the membranes were incubated with specific antibodies at the indicated dilutions for 12–16 h at 4 °C. The membranes were scanned using an Odyssey Sa Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

### Statistical analysis

Statistical analysis was performed using SPSS V.17.0 (SPSS, Inc., Chicago, IL, USA). Data were presented as the mean ± standard deviation (SD). Mann–Whitney *U* test or Student's *t* test was used to compare differences between two groups. $P$ value $< 0.05$ was considered to indicate a statistically significant difference.

## Results

### Hypoxia-suppressed osteoclasts differentiation

The effects of hypoxia on the differentiation and function of osteoclasts are controversial and thus additional studies are necessary. The hypoxia model used in this study has been validated in our previous work [33]. The results revealed much larger multinucleated cells in the normoxia–M-CSF–sRANKL group than those in groups under hypoxic

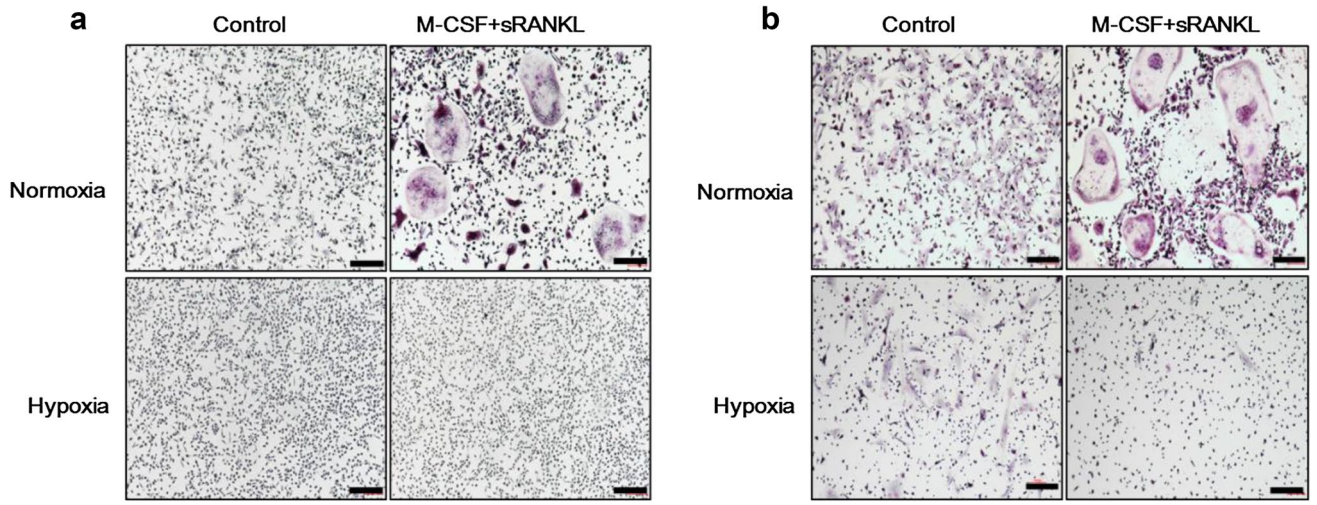


**Fig. 1** Hypoxia-suppressed osteoclast differentiation. RAW264.7 cells (**a**) and BMMs (**b**) were used to induce osteoclast formation. Large multinucleated cells stained by TRAP staining were observed in the normoxia–M-CSF + sRANKL group, but not in other groups. M-CSF + sRANKL: with the addition of M-CSF (10 ng/ml) and sRANKL (50 ng/ml). Data represented three independent experiments, for BMMs $n = 4$ per group. Scale bar 100 μm

conditions (Fig. 1a). The expression of osteoclast markers RANK, cathepsin K, and TRAP was significantly decreased in the hypoxic group after 5 days of stimulation with M-CSF (10 ng/ml) and sRANKL (50 ng/ml) (all $P < 0.05$, Fig. 2a). The protein levels of RANK, cathepsin K and TRAP were consistent with mRNA expression (Fig. 2c). These results suggested that hypoxia inhibited osteoclast formation.

Consistent with the results obtained in RAW264.7 cells, the inhibitory effect of hypoxia on osteoclast formation was validated in murine bone-marrow-derived monocytes. Comparing with hypoxic groups, much larger multinucleated cells in the normoxia–M-CSF–sRANKL group were found (Fig. 1b). The expression of osteoclast markers RANK, cathepsin K, and TRAP was significantly decreased in the hypoxic-M-CSF-sRANKL group after 3 or 5 days stimulating with M-CSF and sRANKL (all $P < 0.05$, Fig. 2b).

## Hypoxia-suppressed osteoclastic bone resorption

Mature osteoclasts were incubated with bone slices in normoxic or hypoxic incubator for 15 days in the presence of M-CSF (10 ng/ml) and sRANKL (50 ng/ml). Toluidine blue staining showed a much larger number of lacunae in the normoxic group compared to in the hypoxic group (Fig. 3). The results demonstrated that hypoxia significantly inhibited osteoclastic bone resorption, which was consistent with the results of the osteoclastogenesis assay.

## Hypoxia had no long-term effect on cell viability

Our results confirmed that hypoxia inhibited RANKL-induced osteoclast differentiation and bone resorption. However, the exact mechanism is unclear. Therefore, we investigated the effect of hypoxia on cell viability. The MTS assay showed that hypoxia had no cytotoxic effect on cell viability (Fig. 4a). To investigate if the inhibition of osteoclastogenesis under hypoxia was due to apoptosis, flow cytometry was conducted to detect cell apoptosis. Cells were cultured at the indicated conditions for 3 days before the detection of apoptosis. No significant differences in the ratio of early or late apoptotic cells were observed among the different groups (Fig. 4b).

## Hypoxia attenuated RANKL-mediated activation of the JNK MAPK and IκBα pathways

To explore the molecular mechanisms by which hypoxia inhibits osteoclastogenesis, the effects of hypoxia on the activation of several signaling pathways were investigated, including the NF-κB-signaling pathway and ERK MAPK, JNK MAPK, and p38 MAPK signaling pathways. RAW264.7 cells were pre-incubated under hypoxic conditions for 24 h to ensure that the cells were in a stable hypoxic state. After stimulation with sRANKL (50 ng/ml), western blotting at different timepoints was conducted to detect the phosphorylation of IκBα, ERK1/2, p38, and JNK. The addition of RANKL under hypoxia conditions dramatically ablated phosphorylation of JNK and IκBα

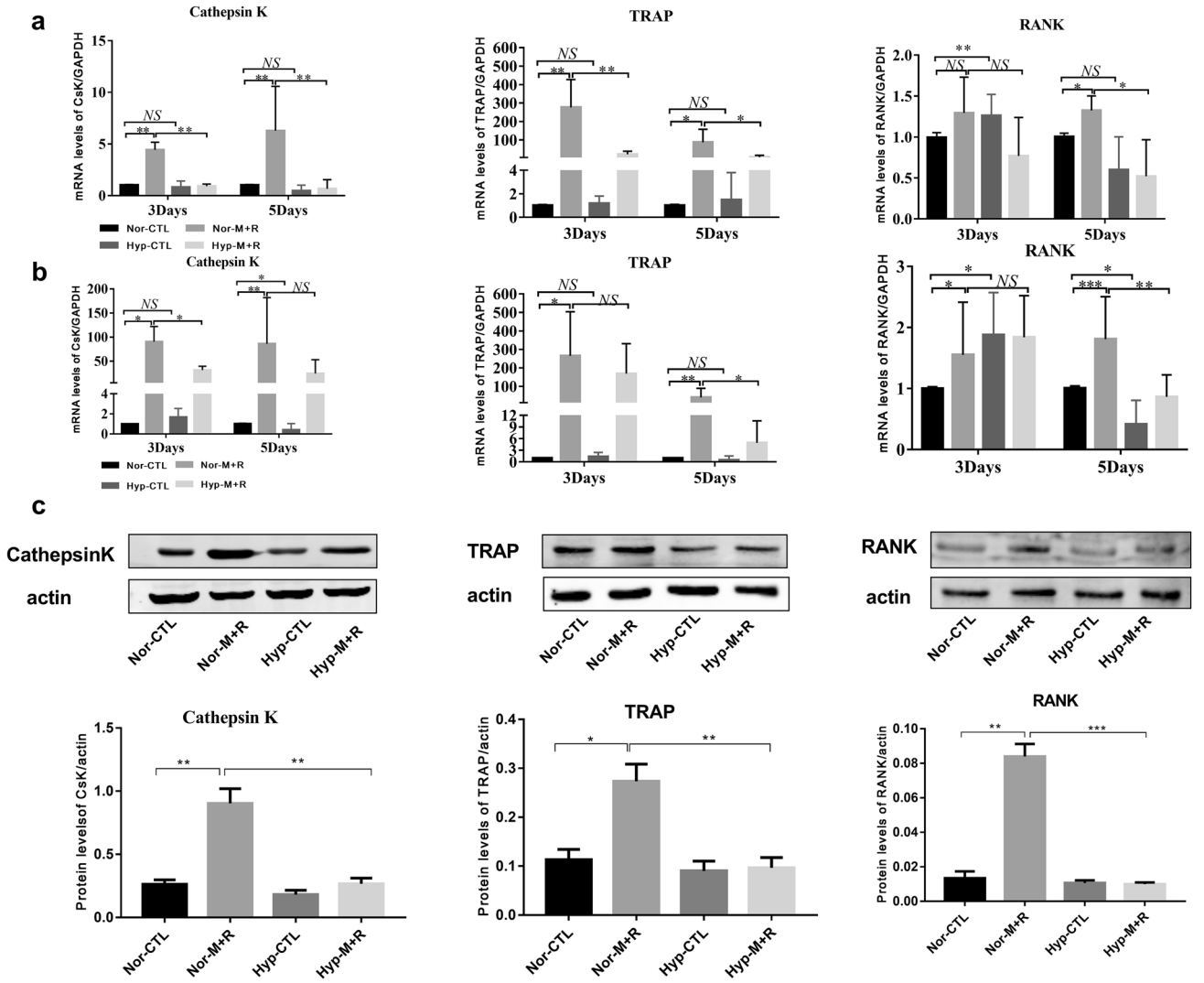


**Fig. 2** Hypoxia decreased the expression of osteoclast markers. Hypoxia decreased the mRNA expression of cathepsin K, TRAP, and RANK in osteoclast induction process at 3 or 5 days in RAW264.7 cell (**a**) and BMMs (**b**). The protein levels of cathepsin K, TRAP, and RANK were detected after 3 days of stimulation with M-CSF and sRANKL. Hypoxia decreased the protein expression of cathepsin K, TRAP, and RANK in RAW264.7 cell (**c**). *Nor-CTL* normoxia control group, *Nor-M-CSF + sRANKL* normoxia group with the addition of M-CSF (10 ng/ml) and sRANKL (50 ng/ml), *Hyp-CTL* hypoxia control group, *Hyp-M-CSF + sRANKL* Hypoxia group with the addition of M-CSF (10 ng/ml) and sRANKL (50 ng/ml). Data represented three independent experiments (mean ± SD), for BMMs $n = 3$ per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; *NS* not significant

(Fig. 5a, b). However, hypoxia conditions did not affect the phosphorylation of ERK1/2 and p38 (Fig. 5c, d).

## Hypoxia-decreased *NFATc1* expression

Activation of transcription factors such as *NFATc1* and *c-Fos* plays a crucial role in the process of RANKL-induced osteoclast differentiation. To further explore the molecular mechanisms involved in inhibition of osteoclast differentiation under hypoxia conditions, the expression of *NFATc1* and *c-Fos* was investigated. The results showed that hypoxia significantly inhibited the expression of *NFATc1* after 3 or 5 days of stimulation with M-CSF (10 ng/ml) and sRANKL (50 ng/ml) ($P < 0.05$), but had no effect on *c-Fos* ($P > 0.05$) (Fig. 6a). The same results were obtained in murine BMMs after 5 day stimulation with M-CSF (10 ng/ml) and sRANKL (50 ng/ml) ($P < 0.05$), while there was no statistical difference in the 3 days of stimulation (Fig. 6b). In addition, the nucleoprotein analysis further confirmed the downregulation of NFATc1 (Fig. 6c). These results suggested that hypoxia inhibits RANKL-induced osteoclast differentiation by down-regulating *NFATc1* expression.

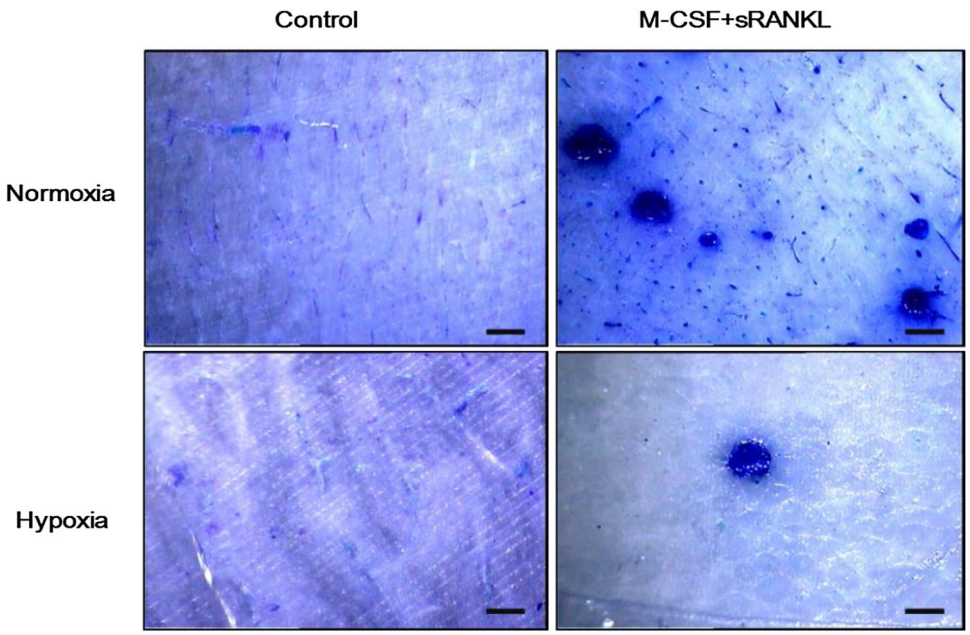


**Fig. 3** Hypoxia-suppressed osteoclast bone resorption. BMMs were used to evaluate the effect of hypoxia on bone resorption. Resorption of bone slices was visualized by staining with 1% toluidine blue. Numerous lacunae were found in the normoxia–M-CSF + sRANKL group, while in other groups, less or no lacunae was observed. Data represented three independent experiments and $n = 3$ per group. Scale bar 100 μm

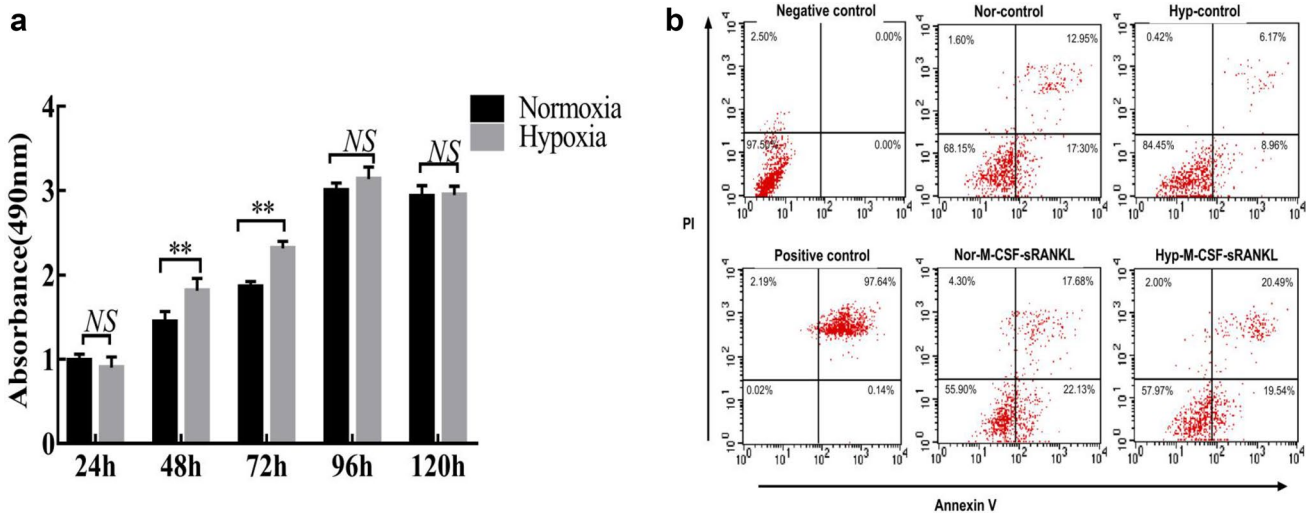


**Fig. 4** Hypoxia had no long-term effect on cell viability. **a** Proliferation of RAW264.7 cells was detected using MTS assay. Results showed that hypoxia had no cytotoxic effect on cell viability. **b** Effect of hypoxia on cell apoptosis was determined by flow cytometry. RAW264.7 cells were incubated under the indicated conditions with or without M-CSF (10 ng/ml) and sRANKL (50 ng/ml) for 72 h. Positive control group was induced by incubating the cells in a 55 °C water bath for 5 min. No significant differences in the ratio of apoptotic cells between different groups were observed. Data represented three independent experiments (mean ± SD). $^{**}P < 0.01$; *NS* not significant

## Discussion

Given the complexity and dynamics of the microenvironment in the body, the specific role of hypoxia in the process of osteoclast differentiation remains unclear. Our study revealed an inhibitory effect of hypoxia on osteoclast formation derived from RAW264.7 cells and murine BMMs, which contrasts the results of some previous studies [27–29, 34]. Arnett et al. examined the effects of hypoxia on osteoclast formation and function using bone-marrow cells from mouse as a source of monocytic precursors [27]. They used 'plug seal' tissue culture flasks to maintain hypoxic conditions by flushing with gas mixtures containing 5% $CO_2$ and indicated $O_2$ (balanced by $N_2$) every day. Their results showed that culturing the cells in 2% $O_2$ strikingly increased the formation and bone resorption of osteoclasts. When the cells were

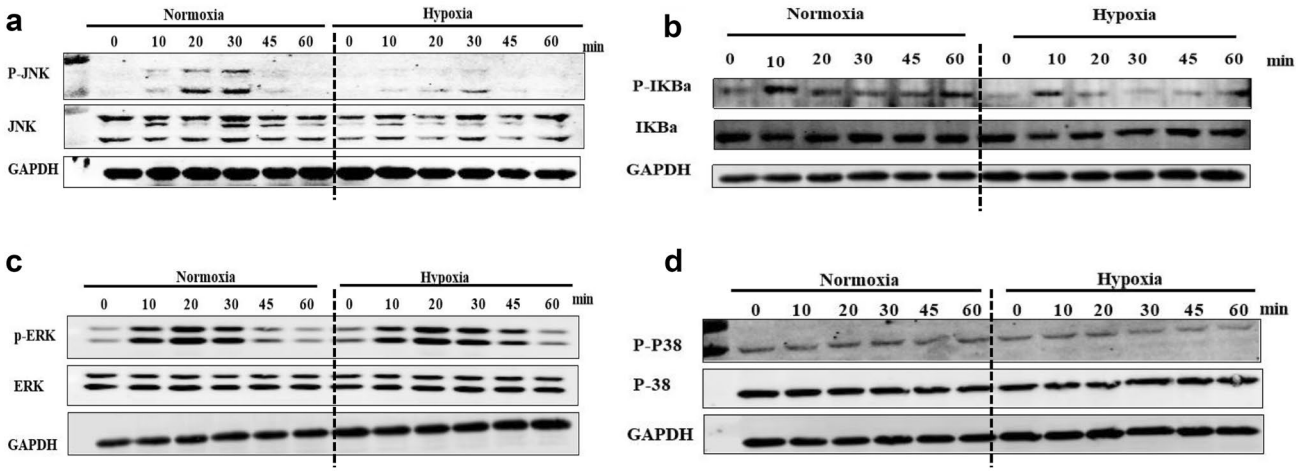


**Fig. 5** Hypoxia-suppressed RANKL-mediated activation of the JNK MAPK and IkBα pathways. Hypoxia inhibited the RANKL-mediated activation of the JNK pathway at 10, 20, and 30 min (**a**). Hypoxia-downregulated RANKL-mediated phosphorylation of IκBα at 10, 20, 30, and 45 min (**b**), but had no effect on phosphorylation of ERK(**c**) and p38 (**d**). Data represented three independent experiments

exposed to severe hypoxia as low as 0.2% $O_2$, a 2.3-fold increase in osteoclast formation and 4.7-fold increase in resorption were observed [27]. They then confirmed that hypoxia strongly stimulated osteoclast formation and bone resorption from human peripheral blood mononuclear at either 2% $O_2$ or 1% $O_2$ [28]. Another study drew a similar conclusion that hypoxia-induced giant osteoclast formation and extensive bone resorption in cats [29]. However, the hypoxia protocol in these studies was a hypoxia/re-oxygenation schedule, rather than constant hypoxia. We used a constant hypoxia model in this study for it is more in line with the local intra-articular environment of RA patients. In the present study, the culture medium was equilibrated in a hypoxic chamber for 24 h before use. When we replaced the culture medium, the cell dishes were brought to normoxic conditions. However, this process was completed within 1 min and had little effect on the oxygen concentration in the hypoxic group. Thus, the hypoxia protocol used in our study is relatively a constant hypoxia model. Considering that the hypoxic condition is difficult to maintain and evaluate, different oxygen conditions used among investigators may explain the heterogeneous results. Another study confirmed that the procedure of hypoxia/re-oxygenation during osteoclast differentiation enhanced osteoclastogenesis. Constant exposure to 2% $O_2$ dramatically inhibited osteoclast formation and resorption due to extensive cell death, suggesting that a delicate balance between hypoxia-induced osteoclast activation and hypoxia-induced osteoclast apoptosis mediates pathological bone resorption [34]. However, in our study, we also evaluated the effect of hypoxia on cell viability using the MTS assay and apoptosis assay, and did not find detect a relationship between hypoxia and cell viability.

Consistent with our present results, some other studies revealed that hypoxia does not induce osteoclastogenesis. Using the hypoxia mimics $CoCl_2$, DMOG, and DFO to simulate hypoxic conditions, osteoclast differentiation was significantly inhibited [30]. Hulley et al. confirmed that induction of HIF using PHD enzyme inhibitors reduced osteoclastogenesis. HIF-1α siRNA only moderately affected osteoclast differentiation, indicating that HIF-1α had little effect on osteoclast differentiation [32]. Moreover, HIF-1α activation by VHL knockdown strongly reduced osteoclastogenesis and osteoclastic bone resorption, with the interleukin-33-miR-34a-5p-Notch1 pathway playing a central role in the inhibitory effect of HIF-1α on osteoclastogenesis [31]. The above data indicate that hypoxia exerts complex effects on osteoclast formation and bone resorption, which requires further investigation in vivo.

After confirming that hypoxia inhibited osteoclast formation and bone resorption in RAW264.7 cells and murine BMMs, we examined how hypoxia inhibited osteoclastogenesis. Effective osteoclastogenesis requires high energy and ATP. As reported previously, hypoxia strongly diminished mitochondrial respiration and OX-PHOS and suppressed mTORC1 activity [35, 36]. This may explain the inhibitory effect of hypoxia on osteoclastogenesis. Additionally, hypoxia promoted interleukin-33 and osteoprotegerin expression [31, 37], which was found to inhibit osteoclastogenesis [37–40]. It is unclear whether other mediators are involved in hypoxia-restrained osteoclast formation. The effects of hypoxia on RANKL-induced activation in the signaling pathway were examined to explore the molecular mechanisms of the inhibitory effect of hypoxia on osteoclastogenesis. The addition of RANKL under hypoxic conditions dramatically ablated phosphorylation of JNK and IκBα, but did not affect phosphorylation of ERK 1/2

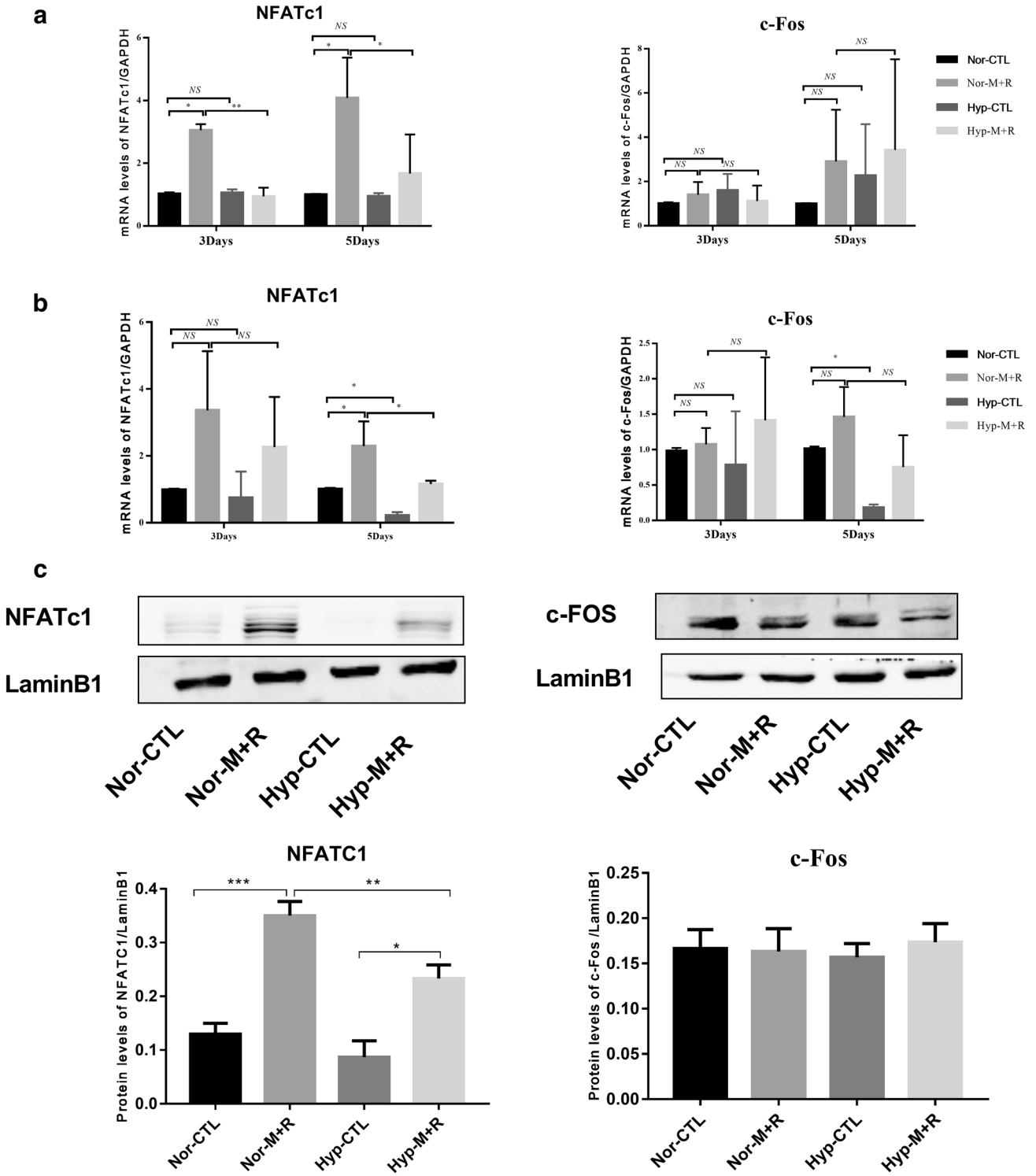


**Fig. 6** Hypoxia inhibited expression of *NFATc1*. Hypoxia decreased the mRNA expression of *NFATc1* during osteoclasts induction at 3 or 5 days, respectively (**a** RAW264.7 cells, **b** BMMs). To detect the protein levels of *NFATc1* and *c-Fos*, nucleoprotein was extracted. Hypoxia decreased the protein expression of *NFATc1* in RAW264.7 cell (**c**). $*P<0.05$, $**P<0.01$, $***P<0.001$. Data represented three independent experiments (mean ± SD), for BMMs $n=3$ per group

and p38. *NFATc1* and *c-Fos* are critical transcription factors in the regulation of osteoclast differentiation [16]. In the current study, we observed that *NFATc1* was decreased significantly in hypoxic group with M-CSF and RANKL. *NFATc1* is an important regulatory factor in the process of osteoclast differentiation mediated by RANKL-activated MAPK and NF-κB-signaling pathways [41]. Therefore, based on our results, we speculated that hypoxia-restrained osteoclast differentiation and bone resorption by regulating NFATc1 expression via interfering the phosphorylation of JNK and IκBα.

Our results indicate a different role for hypoxia in osteoclastogenesis that hypoxia inhibits osteoclast formation and bone resorption. However, these are in vitro studies at the cell and animal levels, and could not completely reflect the pathological condition in RA patients. Considering the complex pH regulation in the body and interaction of cytokines and other cells in vivo, the in vitro conditions do not completely mimic the complex oxygen environment within the joints of RA patients. Hypoxia conditions are not an independent variable in RA joints and may participate in RA bone destruction by interacting with other cells, such as by promoting the invasion of fibroblast-like synoviocytes, secreting inflammatory mediators, and promoting angiogenesis [42]. Therefore, it is necessary to further study the disease model in vivo.

In conclusion, the current study demonstrated that hypoxia at 1% $O_2$ inhibited osteoclast differentiation and bone resorption in vitro. In addition, the phosphorylation of JNK and IκBα might be responsible for the inhibitory effects of hypoxia in osteoclast formation, while still needs further studies. This study expands our understanding of the role of hypoxia on osteoclast formation and activity.

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## Compliance with ethical standards

**Conflict of interest** There are no conflicts of interest.

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