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Original Article

RhoA inhibits the hypoxia-induced apoptosis in osteoblasts

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Abstract: Bone regeneration and fracture healing is dependent on local and systemic factors, including oxygen tension. Disrupted blood supply by fracture always results in reduced oxygen tension (pO2) and hypoxia in bone tissues and inhibits fracture healing. RhoA small GTPase triggers signaling cascades in a variety of cellular responses, including apoptosis suppression via regulating anti-apoptotic Bcl-2 expression. In the present study, we examined the apoptosis promotion by hypoxia in mouse osteoblastic MC3T3-E1 cells by flow-cytometry, caspase 3 activity assay and western blot analysis of apoptosis-associated markers. And then we investigated the regulatory role of RhoA in the hypoxia-promoted apoptosis in MC3T3-E1 cells with gain-of-function and loss-of-function strategies to manipulate the RhoA expression in MC3T3-E1 cells. Results demonstrated that apoptosis was significantly promoted by hypoxia in the osteoblast MC3T3-E1 cells. There were higher levels of apoptotic cells, caspase 3 activity and apoptosis-associated markers such as released cytochrome c, cleaved caspase 3 and lyzed PARP in the hypoxia-treated MC3T3-E1 cells. And such apoptosis was markedly inhibited by the RhoA overexpression with lentivirus vector, whereas was aggravated by the RhoA knockout via the transfection with RhoA-specific siRNA. In conclusion, the present study confirmed the protective role of RhoA in the hypoxia-induced apoptosis in osteoblast cells, implying the RhoA promotion might be a valuable strategy for the therapeutic intervention to the hypoxic-ischemic damage to osteoblasts.

Keywords: RhoA, hypoxia, apoptosis, osteoblast

Introduction

Bone formation during the fracture healing is highly regulated [1], with a complex and sequential set of events. And the bone regeneration and fracture healing is dependent on local and systemic factors, such as growth factors, hormones, pH, oxygen tensions and immune response. Disrupted blood supply by fracture always results in reduced oxygen tension (pO2) and hypoxia [2] in bone tissues and inhibits fracture healing. Low oxygen tension or hypoxia has recently been confirmed to play an important role in skeletal development and cell differentiation [3]. The severely-disrupted and inadequate blood supply accounts for the delayed fracture healing or nonunion [4, 5], via inducing osteoblast cell death, promoting osteoclastogenesis [6], inhibiting the differentiation of chondrocytes and osteoblasts [7]. Severe hypoxia also impaired multiple cellular processes such as aerobic metabolism [8], collagen synthesis process [9] and the expression of several angiogenic genes [10] in osteoblasts and chondrocytes, and inhibited the proliferation, mineralization and differentiation [11] of alveolar osteoblasts. Therefore, the hypoxia might be a key suppressor to fracture healing.

Rho small GTPase family belongs to the Ras-like protein super-family, includes the Ras, Rab, Arf and Ran families [12]. The highly-conserved Rho GTPases trigger signaling cascades in a variety of cellular responses [13, 14], posing an essential role in cancer cell migration and invasion [15], promote the activation of extracellular signal-regulated kinase (ERK) and facilitates glomerular epithelial cells survival [16]. Particularly, RhoA is confirmed to suppress apoptosis via various anti-apoptotic pathways [17]. RhoA up-regulates anti-apoptotic Bcl-2 expression in T cells [18], vascular smooth muscle cells [19] and osteosarcoma cells [20]. On the other side, RhoA inhibits the induction of p53 in

human endothelial cells [21]. Rho associated coil-coil protein kinase (ROCK) has been recently recognized to bind RhoA and to activate the kinase [22], and has been confirmed to be associated with apoptosis [23]. It has been recently demonstrated that the RhoA/ROCK pathway regulates hypoxia-induced apoptosis in myocardial cells [24].

In the present study, we examined the apoptosis promotion by hypoxia in mouse osteoblastic MC3T3-E1 cells, and then investigated the regulatory role of RhoA in the hypoxia-promoted apoptosis in MC3T3-E1 cells with gain-of-function and loss-of-function strategies to manipulate the RhoA expression in MC3T3-E1 cells. Our results confirmed the protective role of RhoA in the hypoxia-induced apoptosis in osteoblastic MC3T3-E1 cells. It is implied that the RhoA promotion might be a valuable strategy for therapeutic intervention to hypoxic-ischemic damage to osteoblasts.

Materials and methods

Reagents, cell culture and treatment

Mouse osteoblastic MC3T3-E1 cell line was purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA), and was cultured in α-Minimal essential medium (α-MEM) (Gibco, Rockville, MD, USA), which was supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) in a incubator with humidified atmosphere (5% CO_2) at 37°C. For the hypoxia treatment, MC3T3-E1 cells were cultured in a hypoxia incubator (Forma 3130; Thermo Scientific, Rockford, IL, USA) infused with a gas mixture of 5% CO_2 and nitrogen to obtain 1% O_2 concentration for 0, 6, 12, 24 or 48 hours. To abrogate the RhoA expression, 30 or 60 nM RhoA-specific siRNA (siRNA-RhoA) or control siRNA (siRNA-Con) (Ambion, Austin, TX, USA) was transfected with Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA, USA) to abrogate the RhoA expression in MC3T3-E1 cells. To overexpress RhoA in MC3T3-E1 cells, the RhoA coding sequence was amplified and was cloned into the pLenti 6/TR vector (Invitrogen, Carlsbad, CA, USA). And the recombinant virus of pLenti-RhoA or control pLenti-GFP (pLenti-Con) was produced by cotransfecting 293T cells with pLenti-RhoA or pLenti-GFP and ViraSafe™ Lentiviral Packaging System (Cell Biolabs, San Diego, CA, USA). And the pLenti-RhoA or pLenti-Con virus with 1 Multiplicity of infection (MOI) was utilized to infect MC3T3-E1 cells for 6, 12, or 24 hours.

Apoptosis assay and caspase 3 assay

Apoptotic MC3T3-E1 cells were examined with an annexin V/FITC apoptosis detection kit (Sigma-Aldrich, St. Louis, Missouri, USA). In brief, approximate 5×10^5 cells post treatment were stained with annexin V-FITC and propidium iodide and detected by a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA). The apoptosis was evaluated by a percentage of apoptotic cells to total cells. The caspase 3 activity in MC3T3-E1 cells was examined with an AMC Caspase Profiling Kit (for caspase 3) (AnaSpec, Fremont, CA, USA) according to the product's guidance. MC3T3-E1 cells (5×10^5 cells) post treatment were collected and were washed with ice-cold phosphate-buffered saline (PBS), then caspase-3-like activity was determined by assessment of Asp-Glu-Val-Asp (DEVD)-AMC cleavage. In brief, cell pellets were resuspended in 100 μL (final volume) of a caspase buffer solution supplemented with the fluorogenic peptide substrate Ac-DEVD-AMC for incubation for 30 min at 37°C. Then the cleavage was monitored in a Fluoroscan II plate reader using an excitation wavelength of 390 nm and an emission wavelength of 460 nm. And the activity was expressed as a relative value of fluorescence intensity of AMC to control.

RNA isolation, reverse transcription, quantitative real-time PCR

Total cellular mRNA from MC3T3-E1 cells was prepared with TRIzol reagent (Life Technologies, Grand Island, NY, USA) according to the product's manual. The expression of RhoA mRNA was quantified by the real-time quantitative PCR method with Takara One Step RT-PCT kit (Takara, Tokyo, Japan). Each mRNA sample was amplified using primer sets specific for RhoA on a Lightcycler 480 II (Roche, Mannheim, Germany). Relative quantification was determined using the ΔΔCt method using β-actin as a reference gene [25]. The primers for RhoA are as following: Forward primer: 5'-ctgccatcaggaagaaactg-3', Reverse primer: 5'-agcatgtcttaccacaagct-3'; and the primers for β-actin are as following: Forward primer: 5'-tgtccaccttccagcagatgt-3', Reverse primer: 5'-agctcagtaacagtccgcctaga-3'.

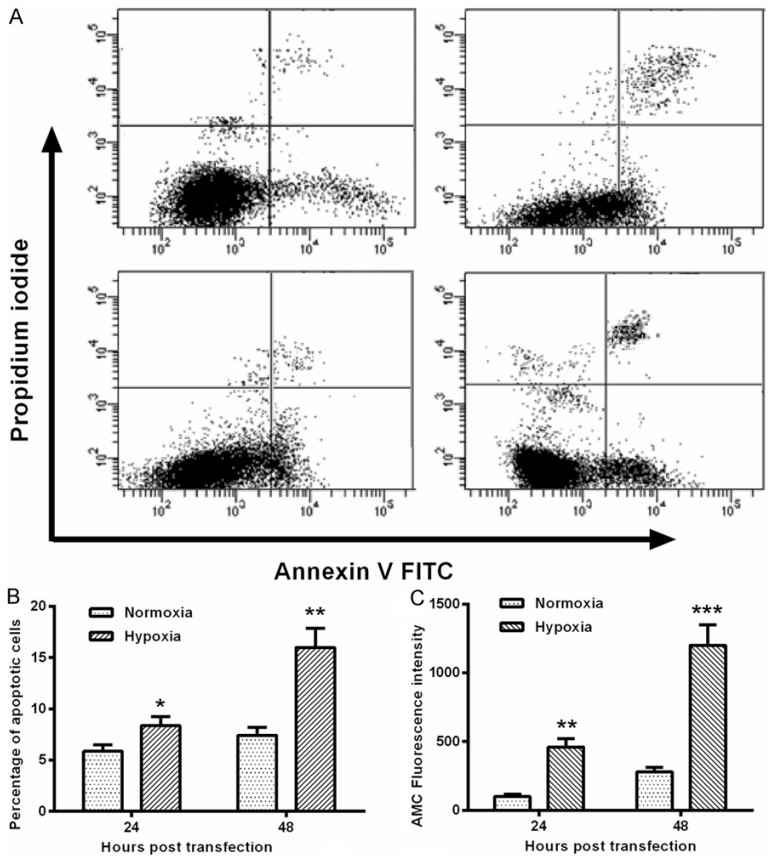

Figure 1. Flow cytometric analysis of hypoxia-induced apoptotic MC3T3-T1 cells. MC3T3-T1 cells were inoculated under hypoxia or normoxia condition for 24 or 48 hours, and then were analyzed for apoptosis with annexin V/FITC apoptosis detection kit. A: Flow cytometric results of the hypoxia- or normoxia-treated MC3T3-T1 cells; B: Percentage of apoptotic cells to total MC3T3-T1 cells; C: Caspase 3 activity in MC3T3-E1 cells under normoxia or hypoxia for 24 or 48 hours; the caspase 3 activity was presented as relative AMC fluorescent units. Each results were averaged for triple independent results, Statistical significance was shown as *P < 0.05, **P < 0.01, or ***P < 0.001.

Western blot analysis

Approximately 1 × 10^6 MC3T3-E1 cells were collected with a cell scratcher, and the cytoplasmic or the mitochondrial proteins were isolated with the Mitochondria/Cytosol Fractionation Kit (Abcam, Cambridge, UK), and were supplemented with the protease inhibitor cocktail (Roche, Basel, Switzerland) according to the manual. Each protein sample was separated by 8 or 10% gradient SDS-PAGE gel, was transferred to nitrocellulose membrane (Millipore, Bedford, MA, USA) and was blocked with 3% skimmed milk. Rabbit polyclonal antibody to cytochrome c (Cyt c) (Abcam, Cambridge, UK), to caspase 3 (either procaspase 3 or cleaved caspase 3) (Sino Biological, Beijing, China), to poly ADP-ribose polymerase (PARP) (Abcam, Cambridge, UK), to RhoA (Abcam, Cambridge, UK), Rock 1 (Abcam, Cambridge, UK), to RhoB (Abcam, Cambridge, UK) or β-actin (Sino Biological, Beijing, China) were utilized to quantify the protein level of each molecule. Goat anti-rabbit IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA, USA) and ECL detection systems (Super Signal West Femto; Pierce, Rockford, IL, USA) were used for detection.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). The RhoA expression in mRNA and in protein levels, the level of released cyto c, cleaved caspase 3 or lyzed PARP, the protein level of Rock 1 or RhoB the percentage of apoptotic cells, and the caspase 3 activity between two groups were analyzed by Student's t test. A p value < 0.05 or less was considered statistically significant.

Results

Hypoxia induces apoptosis in mouse osteoblastic MC3T3-E1 cells

In order to recognize the apoptosis induction by hypoxia in osteoblast cells, we examined the apoptosis level with flow cytometric analysis in mouse osteoblastic MC3T3-E1 cells, under hypoxia. Flow cytometry results (**Figure 1A**) demonstrated that the hypoxia treatment for 24 or 48 hours significantly promoted the apoptosis of MC3T3-E1 cells, compared to the MC3T3-E1 cells under normoxia (P < 0.05 or P < 0.01; **Figure 1B**). Hypoxia-induced apoptosis has been confirmed to be caspase-dependent [26], and procaspase 3 is cleaved into its active form in apoptosis. The activity of active caspase 3 in the hypoxia-treated MC3T3-E1 cells

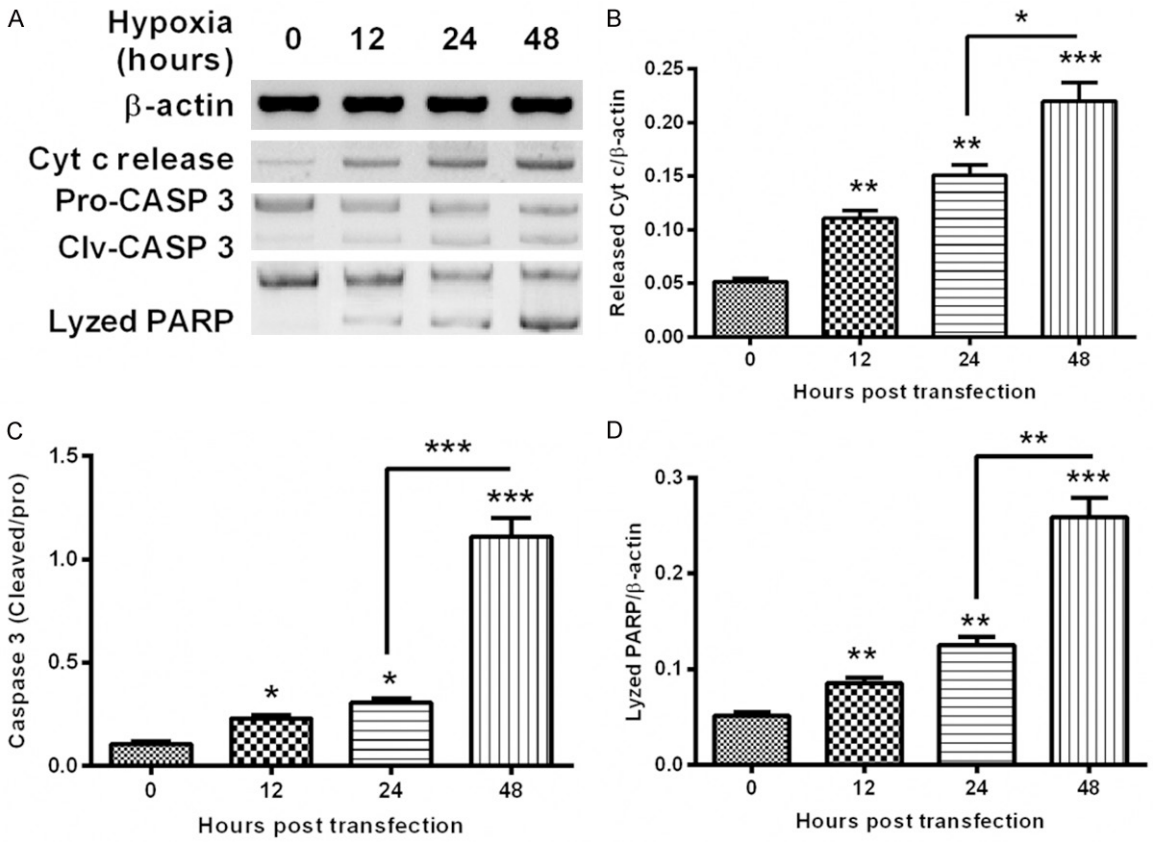

Figure 2. Promotion of apoptosis-associated markers and AMC Caspase 3 activity in hypoxia-treated MC3T3-T1 cells. (A) Western blot analysis of apoptosis-associated markers in MC3T3-T1 cells, post the treatment for 0, 12, 24 or 48 hours; (B) Relative level of released cytochrome c (Cyt c) in the hypoxia-treated MC3T3-T1 cells, with β-actin as internal control; (C and D) Relative level of Cleaved caspase 3 (Clv-CASP 3) level (C) or lyzed poly ADP-ribose polymerase (PARP) by the cleaved caspase 3 in the hypoxia-treated MC3T3-T1 cells, with β-actin as control. All experiments were performed in triplicate. Statistical significance was shown as *P < 0.05, **P < 0.01, or ***P < 0.001.

was also examined. It was indicated in **Figure 1C** that the hypoxia treatment induced higher levels of caspase 3 activity than normoxia, which was evaluated by fluorescence intensity AMC (P < 0.01 for 24 hours or P < 0.001 for 48 hours).

We also analyzed with western blotting the cytochrome c release, procaspase 3 cleavage and the lyzed PARP, which was catalyzed by the active caspase 3 in the hypoxia- or normoxia-treated MC3T3-E1 cells post hypoxia treatment. **Figure 2A** indicated that there were significantly high levels of release cytochrome c (Cyt c release) in the hypoxia-treated MC3T3-E1 cells from 12 to 48 hours post treatment (P < 0.01 for 12 or 24 hours, or P < 0.001 for 48 hours **Figure 2B**), with a marked time-dependence (P < 0.05, Column 4 vs column 3, **Figure 2B**). And the cleaved caspase 3, the subunit with 17 KD was also significantly promoted by the hypoxia treatment (P < 0.05 for 12 or 24 hours, or P < 0.001 for 48 hours, **Figure 2C**), time-dependently (P < 0.001, Column 4 vs column 3, **Figure 2B**). In addition, a promoted PARP cleavage by the activated caspase 3 was confirmed in the hypoxia group, from 12 to 48 hours post treatment (P < 0.01 for 12 or 24 hours, or P < 0.001 for 48 hours, **Figure 2D**), also with a time-dependence (P < 0.01, Column 4 vs column 3, **Figure 2D**). Taken together, we confirmed the apoptosis induction by hypoxia in mouse osteoblastic MC3T3-E1 cells.

RhoA overexpression inhibits the hypoxia-induced apoptosis in MC3T3-E1 cells

RhoA is confirmed to suppress apoptosis via various anti-apoptotic pathways [17, 27]. To determine whether RhoA regulated the hypoxia-induced apoptosis in MC3T3-T1 cells, we over-expressed RhoA with a Lentivirus vector in

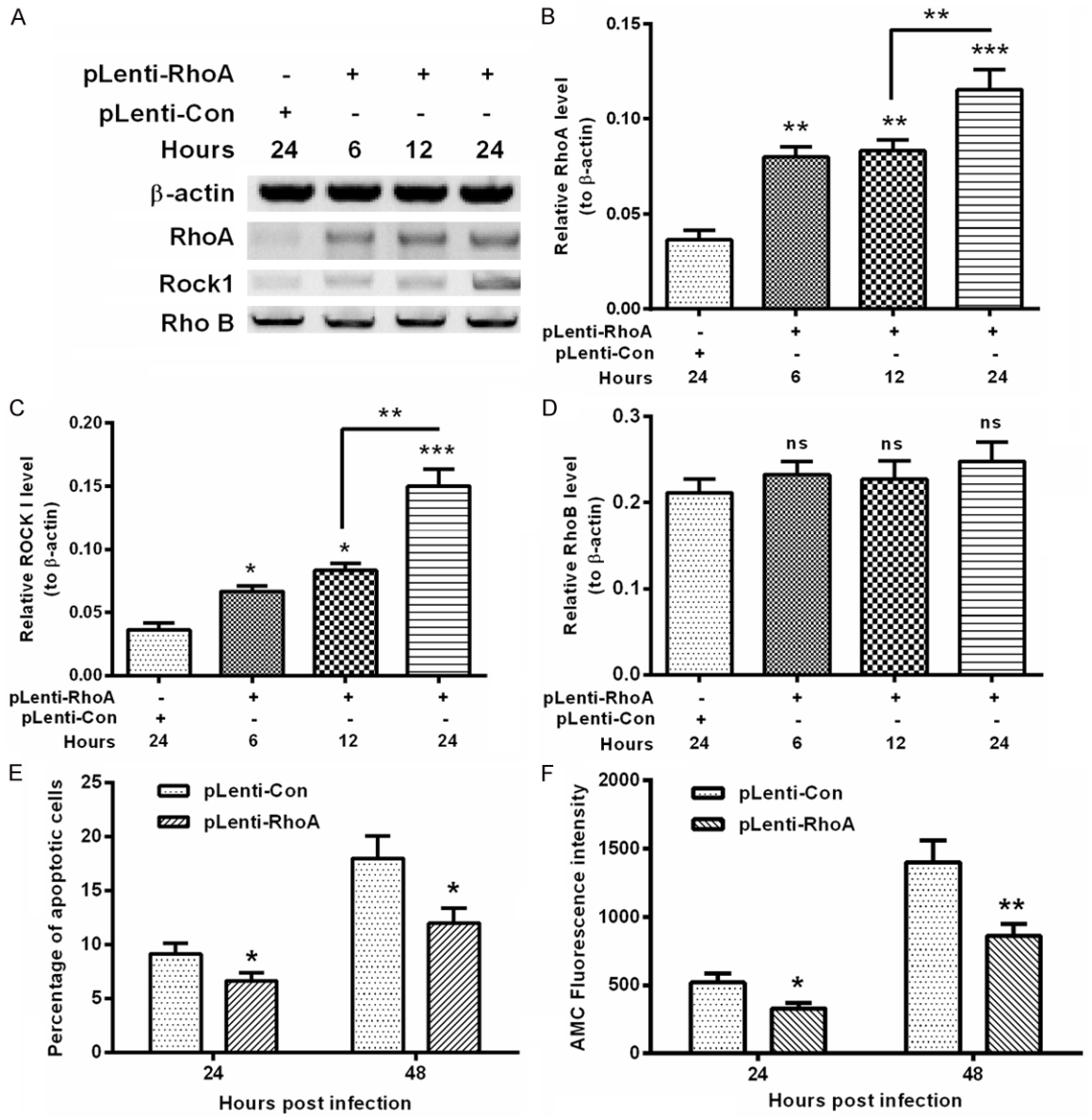

Figure 3. RhoA overexpression antagonizes the hypoxia-induced apoptosis in MC3T3-T1 cells. The MC3T3-T1 cells were infected with pLenti-RhoA or pLenti-Con virus and were incubated under hypoxia for 6, 12 or 24 hours, and then were examined for the expression of RhoA, Rock 1 or RhoB, or for the cell apoptosis; (A) Western blot analysis of RhoA, Rock 1 or RhoB in the RhoA-overexpressed MC3T3-T1 cells, under hypoxia; (B-D) Relative level of RhoA (B), Rock 1 (C) or RhoB (D) to β-actin in the RhoA-overexpressed MC3T3-T1 cells under hypoxia; (E) Flow cytometric analysis of the hypoxia-induced apoptotic MC3T3-T1 cells, with or without RhoA overexpression; (F) Caspase 3 activity in the RhoA-overexpressed or control MC3T3-E1 cells under hypoxia for 24 or 48 hours; the caspase 3 activity was presented as relative AMC fluorescent units. All experiments were performed in triplicate. Statistical significance was shown as *P < 0.05, **P < 0.01 or ***P < 0.001, ns: no significance.

MC3T3-T1 cells. Western blotting in **Figure 3A** demonstrated that and the protein levels of RhoA and ROCK 1 were dramatically upregulated by the pLenti-RhoA virus infection, at 6, 12 or 24 hours post infection, compared to the pLenti-Con (control) virus infection ($P < 0.01$ for 6 or 12 hours, or $P < 0.001$ for 24 hours), time-dependently ($P < 0.01$). And the upregulated RhoA then promoted the ROCK 1 markedly ($P < 0.05$ for 6 or 12 hours, or $P < 0.001$ for 24 hours), with a time-dependence ($P < 0.01$). However, the RhoB was not significantly regulated by the RhoA overexpression (**Figure 3D**). Then we investigated the influence of the over-

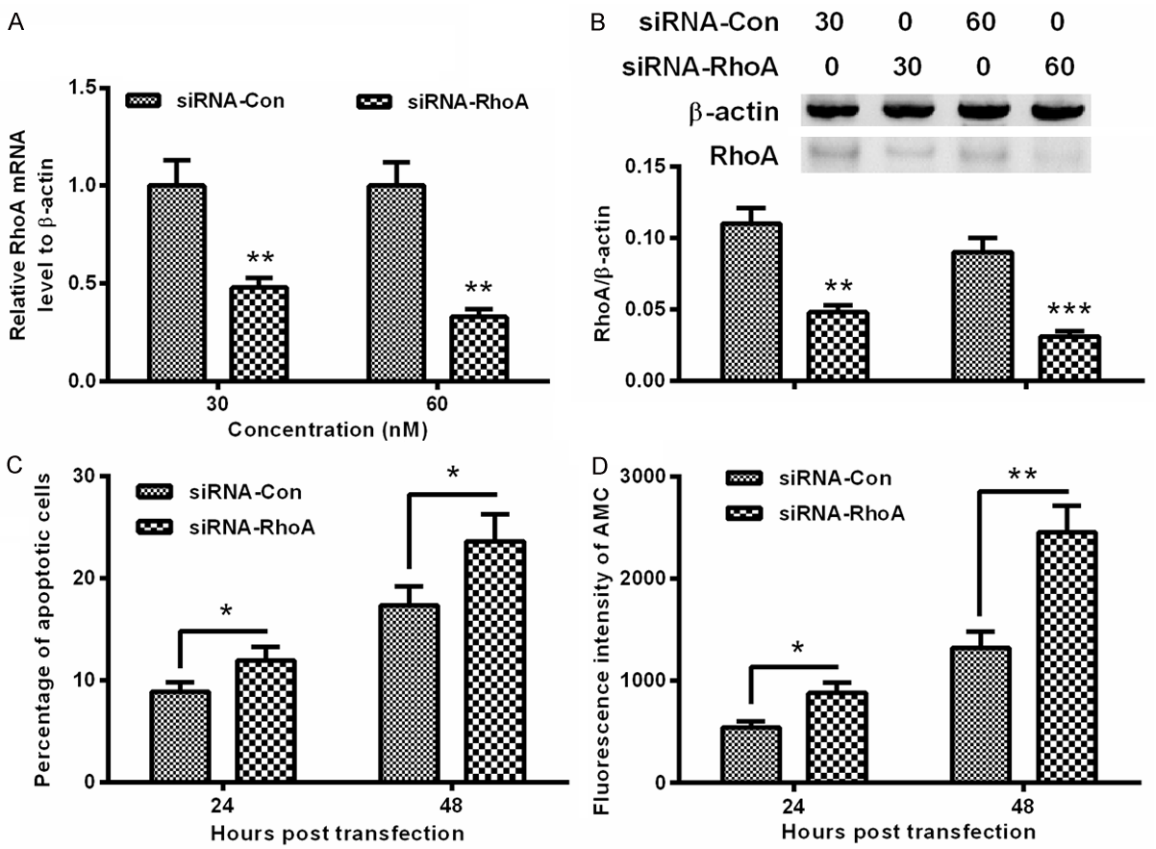

Figure 4. RhoA knockout aggravates the hypoxia-induced apoptosis in MC3T3-T1 cells. A: Relative RhoA mRNA level to β-actin in MC3T3-E1 cells post the transfection with 30 or 60 nM RhoA-targeted siRNA (siRNA-RhoA) transfection, with siRNA-Con as control; B: Western blot analysis of RhoA in MC3T3-E1 cells post the transfection with 30 or 60 nM siRNA-RhoA transfection, with siRNA-Con as control; C: Apoptotic cells induced by hypoxia post the transfection with 60 nM siRNA-RhoA or siRNA-Con for 24 or 48 hours; D: Caspase 3 activity by the Ac-DEVD-AMC substrate lyzation analysis in MC3T3-E1 cells under hypoxia, post the transfection with 60 nM siRNA-RhoA or siRNA-Con for 24 or 48 hours. All experiments were performed in triplicate. Statistical significance was shown as *$P < 0.05$ or **$P < 0.01$.

expressed RhoA on the hypoxia-induced apoptosis in MC3T3-T1 cells. As shown in **Figure 3E**, the flow cytometric analysis indicated that the RhoA overexpression reduced the hypoxia-induced MC3T3-T1 cell apoptosis (either $P < 0.05$ for 24 or 48 hours). And the caspase 3 activity assay demonstrated that the AMC fluorescence intensity in the hypoxia-treated MC3T3-T1 cells was significantly inhibited in the pLenti-RhoA group, than in the pLenti-Con group ($P < 0.05$ for 24 or $P < 0.01$ for 48 hours post infection). Therefore, RhoA overexpression attenuated the hypoxia-induced MC3T3-T1 cell apoptosis.

RhoA knockout aggravates the hypoxia-induced apoptosis in MC3T3-E1 cells

To reconfirm the regulatory role of RhoA in the hypoxia-induced apoptosis in MC3T3-T1 cells, we then used RhoA-specific siRNA (siRNA-RhoA) to knockout RhoA, and then re-examined the hypoxia-induced apoptosis in MC3T3-T1 cells. Firstly, the mRNA level of RhoA was assayed with real-time quantitative PCR in the MC3T3-T1 cells which were transfected with siRNA-RhoA or with siRNA-Con. As shown in **Figure 4A**, 30 or 60 nM siRNA-RhoA significantly reduced the mRNA level of RhoA ($P < 0.01$ for 30 or 60 nM). And the protein level of RhoA was also markedly reduced by the siRNA-RhoA transfection ($P < 0.01$ for 30 nM or $P < 0.001$ for 60 nM). Then we evaluated the regulation of RhoA knockout on the hypoxia-induced apoptosis in MC3T3-T1 cells, the flow cytometric analysis also indicated a significantly higher level of hypoxia-induced apoptotic MC3T3-T1 cells in the siRNA-RhoA group, then in the siRNA-Con group ($P < 0.05$ for 30 or 60 nM, **Figure 4C**). And the AMC fluorescence intensity assay also

indicated a markedly higher caspase 3 activity in the siRNA-RhoA group, then in the siRNA-Con group ($P < 0.05$ for 30 nM or $P < 0.01$ for 60 nM, **Figure 4D**). Therefore, the RhoA knockout aggravated the hypoxia-induced apoptosis.

Discussion

Oxygen affects the activity of multiple skeletogenic cells and is involved in many processes that are important for fracture healing. Hypoxic animal model demonstrated that severe hypoxia decreased tissue vascularity, bone formation and callus remodeling [28]. The trauma-localized and systemic inflammation post trauma was influence by hypoxia and further resulted in poorly healing fractures [29]. The current studies confirmed that hypoxia directly promoted apoptosis in the osteoblast MC3T3-E1 cells, via promoting a higher level of apoptotic cells, via upregulating caspase 3 activity and upregulating apoptosis-associated markers such as released cytochrome c, cleaved caspase 3 and lyzed PARP by the activated caspase 3 in the hypoxia-treated MC3T3-E1 cells. Apoptosis can be initiated through membrane receptor-associated and mitochondrial-initiated pathways that converge and mediate their downstream apoptosis effects [30]. And in this study, the mitochondria dependence was also confirmed in the hypoxia-induced apoptosis in MC3T3-E1 cells. In addition, previous studies recognized the apoptosis induction by hypoxia via disturbing the mitochondrial membrane potential, causing the release of cytochrome c, and further promoting cell apoptosis in cardiomyocytes [31].

Apoptosis appears to function as a limiting factor in bone formation and fracture healing. And it is important to recognize the mechanisms responsible for osteoblast apoptosis. Multiple bone anabolic agents have been confirmed to prevent osteoblast apoptosis, such as parathyroid hormone (PTH) [32], insulin-like growth factor-I [33] and mechanical loading [34]. RhoA promote cell survival and survival-related signaling [35, 36], such as increased Bcl-2 expression [37] via binding and hydrolyzing GTP. RhoA knockout or the chemical Rho kinase activator regulates apoptosis of motor neurons in the spinal cord [36]. The signaling pathway through which RhoA prevents apoptosis varies in different tissues. And our results confirmed that the hypoxia-induced apoptosis was inhibited by the RhoA overexpression, whereas was aggravated by the RhoA knockout. And the Rock 1 was upregulated by the RhoA overexpression in MC3T3-E1 cells, implying the RhoA/Rock pathway is implicated in the anti-apoptosis response in osteoblasts.

In conclusion, the present study confirmed the protective role of RhoA in the hypoxia-induced apoptosis in osteoblast cells, implying the RhoA promotion might be a valuable strategy for the therapeutic intervention to the hypoxic-ischemic damage to osteoblasts.

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Disclosure of conflict of interest

None.

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