0.5-Gy X-ray irradiation induces reorganization of cytoskeleton and differentiation of osteoblasts

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Received September 17, 2020; Accepted January 29, 2021

DOI: 10.3892/mmr.2021.12018

Abstract. Osteoblasts are sensitive to ionizing radiation. The small GTPase RhoA and its effector Rho-associated protein kinase (ROCK) are critical to several cellular functions, including cytoskeleton reorganization, cell survival, and cell differentiation. However, whether the RhoA/ROCK signaling pathway is involved in the regulation of osteoblast cytoskeleton reorganization and differentiation induced by low-dose X-ray irradiation remains to be determined. The aim of the present study was to investigate the role of the RhoA/ROCK signaling pathway in mediating differentiation of osteoblasts and reorganization of the cytoskeleton under low-dose X-ray irradiation. Osteoblasts were pretreated with the ROCK kinase-specific inhibitor (Y-27632) before exposure to low-dose X-ray irradiation. The changes of F-actin in MC3T3 cells were observed at different time points following X-ray irradiation. Cell Counting Kit-8 assay, alkaline phosphatase activity, Alizarin red staining and western blotting were used to detect the proliferation and differentiation of osteoblasts after 0.5-Gy X-ray irradiation. In the present study, low-dose X-ray irradiation promoted the expression of genes associated with the cytoskeleton reorganization. Indeed, the results showed that, 0.5-Gy X-ray irradiation can induce reorganization of cytoskeleton and promote differentiation of osteoblasts through the RhoA/ROCK signaling pathway. Additionally, inhibiting ROCK activity blocked low-dose X-ray irradiation-induced LIMK2 phosphorylation, stress fiber formation and cell differentiation. Thus, these results demonstrated the excitatory effects of low-dose X-ray irradiation on MC3T3-E1 cells, including reorganization of the cytoskeleton and differentiation of osteoblasts.

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Key words: low-dose X-ray radiation, osteoblasts, cytoskeleton, differentiation

Introduction

X-ray is a type of ionizing radiation that is widely used in medicine, including for radiotherapy of malignant tumors and imaging examinations. It is considered that any dose of ionizing radiation, even a very low dose, is harmful, and the detrimental effects increase linearly with increasing doses. The effects on bone tissues mainly comprise osteonecrosis, delayed union of fracture, non-union and osteoporosis (1,2). Previous studies have found that radiation can destroy the dynamic balance between osteoblasts and osteoclasts, and inhibit proliferation and differentiation of osteoblasts. In addition, it can also reduce bone matrix deposition (3,4), impair the circulation around the bone tissues and callus, and directly destroy osteoblasts and osteoblast progenitor cells (5). This conclusion is based on research using medium and high doses of ionizing radiation. In 1982, Luckey (6) suggested for the first time that a low level of radiation may have beneficial effects on humans, known as an excitatory effect. The excitatory effect has been shown to enhance immune function and reduce the incidence of cancer (7). Previous findings demonstrated that the excitatory effect induced by low-dose ionizing radiation may be associated with the antioxidant defense mechanism of free radicals and DNA double-strand breaks (DSBs) induced by low-dose ionizing radiation (8). Low-dose ionizing radiation can also inhibit inflammation and production of oxygen-free radicals in macrophages (9,10).

Low-dose radiation has markedly attracted scholars' attention in nerve stimulation and anti-tumor therapy (11-13); however, few studies have evaluated the effects of low-dose X-ray irradiation on orthopedic diseases. It has been reported that low-dose irradiation can increase the serum level of alkaline phosphatase (ALP) and promote the secretion of VEGF, leading to an increase in the number of mineralized nodules in osteoblasts (14,15). In a previous study, it was demonstrated that low-dose ionizing radiation can significantly increase the number of trabeculae in the distal femur of mice and improve the microstructure of trabecular bone (16). A relatively low-dose X-ray irradiation can activate osteoclast production, which was closely associated with bone loss (17). Our previous study revealed that low-dose X-ray irradiation could promote callus

formation and mineralization in a rat fracture model (18). The results of an *in vitro* study also showed that X-ray irradiation promoted osteoblast differentiation (19). Using microarray analysis, previous findings have indicated that low-dose X-ray irradiation increases the expression of cytoskeleton-associated genes, which may promote the proliferation and differentiation of osteoblasts through changes in the extracellular matrix (ECM), local adhesion, and actin cytoskeleton (18).

The cytoskeleton is a complex, dynamic network of interlinking protein filaments present in the cytoplasm of all cells, including bacteria and archaea; in eukaryotes, it is composed of three main components: Microfilaments, intermediate filaments, and microtubules. Moreover, the cytoskeleton plays a significant role in maintaining cell morphology, as well as participating in cell migration and cell differentiation (20,21). The cytoskeleton is sensitive to ionizing radiation. Indeed, ionizing radiation was closely associated with the changes of cytoskeleton (12,13,22).

RhoA is a member of the Rho family of GTPases, which is closely associated with the organization of the actin cytoskeleton (23). RhoA is activated via phosphorylation, leading to subsequent loading of Rho GTPases with GTP. Activated Rho GTPases transmit signals to Rho-associated protein kinase (ROCK). ROCK can phosphorylate and activate LIMK1 and LIMK2 (24). Eventually, LIMK2 phosphorylates Cofilin, causing Cofilin to lose its ability to depolymerize actin (24). Previous studies have shown that X-ray irradiation could induce a rearrangement of the actin cytoskeleton and cause an increase in stress fibers (25,26). As a result, the RhoA/ROCK signaling pathway is activated (25,26). RhoA/ROCK signaling is involved in a variety of cellular processes, such as cell differentiation and migration (20,27). RhoA can also promote differentiation of osteoblasts by activating the Wnt signaling pathway (28). As for osteoblasts, whether RhoA/ROCK can mediate the reorganization of actin cytoskeleton and changes in cell function caused by X-ray irradiation remains unknown. Therefore, in the present study, the effects of low-dose X-ray irradiation on differentiation of osteoblasts and reorganization of the cytoskeleton were investigated, with the aim of providing a theoretical basis for improved understanding of the biological effects of low-dose X-ray irradiation.

Materials and methods

Cell culture. MC3T3-E1 cells were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in an α-minimum essential medium (MEM) (HyClone; Cytiva) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin, and 100 mg/ml streptomycin at 37˚C, 5% CO_2 and changed every three days. During the induction of osteogenic differentiation, the osteogenic conditioning medium (containing MEM, 10% FBS, 5 mM β-glycerophosphate, 50 μg/ml ascorbic acid and 100 nM dexamethasone) was replaced. The medium was replaced after low-dose X-ray irradiation, and changed every 3 days.

Treatments. The ROCK inhibitor Y27632 was purchased from EMD Millipore. Cells were divided into four groups: i) Group 1, blank control group, no Y27632 pretreatment and no irradiation; ii) group 2, pretreatment with Y27632 without irradiation; iii) group 3, 0.5 Gy X-ray irradiation without Y27632 pretreatment; and iv) group 4; pretreatment with Y27632 followed by 0.5-Gy X-ray irradiation.

Cells were pretreated with 10 μM Y27632 for 1 h before X-ray irradiation in groups 2 and 4. Third-generation MC3T3-E1 cells-were used, and the cell density was observed daily. When the cells reached 70% confluency, they were irradiated with 0 and 0.5 Gy X-ray at a rate of 200 cGy/min using a medical linear accelerator with a 6 MV radiation source (Siemens Primus).

F-actin staining. MC3T3-E1 cells ($5x10^4$) in each group were washed twice with PBS 2, 24, 36, or 120 h after irradiation. After washing, the cells were fixed with 4% paraformaldehyde for 25 min at room temperature, and then treated with 0.1% Triton X-100 for 10 min at room temperature. After washing with PBS three times, the cells were blocked with 5% bovine serum albumin (Beyotime Institute of Biotechnology) for 30 min at room temperature, then incubated with fluorescein isothiocyanate (FITC)-conjugated phalloidin (Sigma-Aldrich; Merck KGaA) for 1 h at 37˚C. The cell nuclei were additionally counterstained with 100 nM 4',6-diamidino-2-phenylindole (DAPI) for 10 min. After washing, cells on coverslips were visualized using a fluorescence microscope (Carl Zeiss AG). The aforementioned processes were performed in the dark.

ImageJ 1.8.0 software (National Institutes of Health) was used to analyze the images of six cells per field of view on the slide, and the average fluorescence intensity of each cell (average fluorescence intensity=fluorescence intensity/cell area) was measured accordingly (Table I). Area represents the total area of the cells counted. 'Mean ± SD' expresses the average fluorescence intensity of the cells measured. 'Min' denotes the lowest fluorescence intensity of the cells. 'IntDen' indicates the total fluorescence intensity of the cells measured.

Western blot assay. MC3T3-E1 cells ($1x10^6$) were lysed on ice with RIPA buffer containing protease and phosphatase inhibitors (Beyotime Institute of Biotechnology) after being cultured for 1, 3 or 5 days. The supernatant was collected by centrifugation at 12,000 x g for 15 min at 4˚C, and the protein concentration was quantified using a bicinchoninic acid (Beyotime Institute of Biotechnology) assay kit. Protein samples (30 μg/lane) were resolved using 10% SDS-PAGE, then transferred onto PVDF membranes (EMD Millipore). Membranes were blocked with 5% non-fat dried milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 2 h at room temperature. Membranes were incubated overnight at 4˚C with the following antibodies: Rabbit anti-Cofilin (cat. no. 3312; Cell Signaling Technology, Inc.; dilution 1:1,000), rabbit anti-ROCK (cat. no. 4035; Cell Signaling Technology, Inc.; dilution 1:2,000), rabbit anti-phosphorylated (phospho)-LIM domain kinase 2 (LIMK2; cat. no. 3845, Cell Signaling Technology, Inc.; dilution 1:2,000), rabbit anti-phospho-Cofilin (cat. no. 3311; Cell Signaling Technology, Inc.; dilution 1:2,000), rabbit anti-Runx2 (cat. no. ab76956; Abcam; dilution 1:2,000), rabbit anti-Osterix (cat. no. ab209484; Abcam; dilution 1:2,000), rabbit anti-Collagen Type 1 (COL1; cat. no. ab96723, Abcam; dilution 1:2,000), rabbit anti-osteocalcin (OCN; cat. no. ab93876, Abcam; dilution 1:1,000), rabbit anti-alkaline phosphatase (ALP;

Table I. Fluorescence intensity of F-actin staining of cells in each group.

Group	Area (pixel)	Mean ± SD	Min	IntDen
2 h 0 Gy	272,308	37.36±4.91	15	7,479,102
0.5 Gy	272,308	33.60±1.93	12	6,342,378
0 Gy+Y27632	272,308	37.12±3.31	12	6,710,960
0.5 Gy+Y2762	272,308	27.77±2.64	22	8,075,451
1 d 0 Gy	272,308	27.57±2.47	16	10,052,197
0.5 Gy	272,308	35.26±3.66	12	7,362,259
0 Gy+Y27632	272,308	28.18±4.45	10	9,419,783
0.5 Gy+Y2762	272,308	30.57±5.14	15	9,811,449
3 d 0 Gy	190,825	27.97±6.07	12	10,295,261
0.5 Gy	190,825	38.48±4.78	13	4,343,367
0 Gy+Y27632	252,417	25.00±4.50	7	6,640,082
0.5 Gy+Y2762	232,329	35.08±4.73	15	7,266,554
5 d 0 Gy	260,815	20.64±2.62	13	6,912,119
0.5 Gy	238070	32.76±2.63	10	7,227,973
0 Gy+Y27632	284,840	21.44±1.95	13	9,183,526
0.5 Gy+Y2762	284,334	27.90±2.89	8	8,617,311

Min, minimum fluorescence intensity; IntDen, total fluorescence intensity.

cat. no. ab95462, Abcam; dilution 1:2,000), and β-actin (cat. no. ab8227, Abcam; dilution 1:2,000). Following washing with TBST three times, the membranes were incubated with a goat anti-rabbit IgG HRP-conjugated secondary antibody (cat. no. ab97200, Abcam; dilution 1:2,000) for 1 h at room temperature. Immunoreactive bands were visualized by using enhanced chemiluminescence detection reagent (EMD Millipore) and images were captured using a chemiluminescence imaging system (Kodak). The data were quantified using Image J software (version 1.8.0; National Institutes of Health).

RhoA activation assay. MC3T3-E1 cells ($1x10^6$) were harvested on days 1, 3, and 5 following X-ray irradiation and lysed with RIPA buffer. A total of 30 μl of the supernatant was used to determine the expression of total RhoA. The remaining supernatant was used to isolate GTP-bound RhoA using an Active GTPase Pull-down kit (cat. no. 16116; Thermo Fisher Scientific, Inc.), in which the glutathione S-transferase-Rhotekin Rho binding domain was used, according to the manufacturer's protocol. The eluted proteins were then separated using 15% SDS-PAGE, transferred onto PVDF membranes (EMD Millipore), and detected using specific anti-RhoA antibodies (Santa Cruz Biotechnology, Inc.). Total RhoA protein was detected via western blot analysis as described in the previous section. Immunoreactive bands were visualized using ECL, and band intensity was quantified using ImageJ software.

Cell proliferation assay. MC3T3-E1 cells were inoculated into 96-well plates at a density of $3x10^3$ cells/well. After the aforementioned treatment (X-ray irradiation and/or Y27632 pretreatment), the cells were cultured for 1-7 days. Cell viability was measured using Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology). The specific procedure was carried out according to the manufacturer's instructions, in which 10 μl CCK-8 solution was added for a 100-μl volume of medium, and incubated at 37˚C for 2 h. Subsequently, the optical density value of each well was determined using an enzyme-labeling instrument (BioTek Instruments, Inc.) at 450-nm wavelength.

Table II. Effects of 0.5 Gy X-ray irradiation on extracellular ALP activity.

A, Day 7

Group	ALP activity (U/l)
- Y27632 0 Gy	11.69±0.4518
+ Y27632 0 Gy	11.37±0.6026
- Y27632 0.5 Gy	16.89±0.3145
+ Y27632 0.5 Gy	11.49±1.3162

B, Day 10

Group	ALP activity (U/l)
- Y27632 0 Gy	18.75±0.4217
+ Y27632 0 Gy	18.9±0.5201
- Y27632 0.5 Gy	22.37±1.6963
+ Y27632 0.5 Gy	19.6±0.7915

ALP, alkaline phosphatase.

ALP staining and ALP activity. MC3T3-E1 cells were cultured in 24-well plates at a density of $1x10^4$ cells/well. Cells were then treated with 0.5 Gy X-ray irradiation and/or Y27632. The

medium was discarded on day 7. ALP staining was carried out using the ALP assay kit (cat. no. P0321; Beyotime Institute of Biotechnology) at room temperature for 30 min according to the manufacturer's protocol, and visualization was undertaken using an inverted light microscope (magnification, x10; Olympus Corporation).

The activity of ALP, a marker of early differentiation of osteoblasts, was detected using an ALP assay kit. Osteoblasts were inoculated into 96-well plates at a density of $3x10^3$ cells/ml in triplicate wells. The 96-well plates were placed in an incubator at a constant temperature (37˚C) and continued to be cultured until day 7 or day 10. A total of 30 μl culture medium was collected from each well. The absorbance was detected at 520 nm in each group according to the manufacturer's protocol, and the relative ALP activity in each group was calculated according to the measured data.

Alizarin red staining. The mineralization degree of MC3T3-E1 cells ($1.0x10^5$/ml) in each group was determined by Alizarin red staining. A total of 21 days after irradiation, osteoblasts were fixed in 4% paraformaldehyde for 1 h at room temperature. The cells were then washed with deionized water and stained with 40 mM Alizarin red (pH 4.2) for 30 min at 37˚C. After staining, the cells were washed with deionized water to remove the non-specific Alizarin red dye. After drying, the formation of calcium nodules in each group was observed under an inverted phase-contrast microscope. Orange to red staining indicated mineralized calcium nodules.

Statistical analysis. All experiments were repeated three times independently. Data are presented as the mean ± SD. Differences between the groups were analyzed using one-way ANOVA followed by Tukey's post hoc test using SPSS 18.0 software (SPPS, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Low-dose X-ray irradiation induced cytoskeleton reorganization in MC3T3 cells. The cells in 0 Gy-Y27632 group were stretched, and the actin cytoskeleton were clear, complete and neatly arranged, forming a dense network. After 2 h of X-ray irradiation, the cells shrank and the formation of actin tension fibers decreased. In addition, the arrangement was discontinuous, and the green fluorescence intensity was weakened. After 24 h, the F-actin in the 0.5 Gy group began to increase, and the green fluorescence of the actin cytoskeleton was significantly improved. The fluorescence intensity of F-actin in the 0.5 Gy group was markedly higher than that in other groups at day 3 after irradiation. However, the fluorescence intensity of F-actin in the 0.5 Gy X-ray irradiation group tended to be normal on the day after irradiation (Fig. 1A). Cells treated with Y-27632 were unable to induce the formation of new actin filaments, indicating that ROCK is highly essential for actin reorganization by 0.5 Gy X-ray irradiation (Fig. 1B).

Table I and Fig. 2 are the summary data of Fig. 1. At 2 h following X-ray irradiation, the fluorescence intensity of cells in the 0.5 Gy-Y27632 group and 0.5 Gy + Y27632 group was lower than that in the non-irradiated group. The fluorescence intensity of cells in 0.5 Gy +Y27632 group was significantly lower than

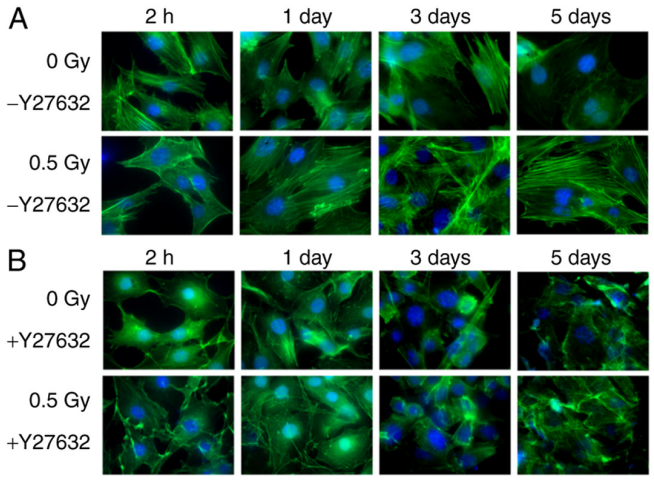

Figure 1. Effects of low-dose X-ray radiation and ROCK inhibitor (Y-27632) on actin cytoskeleton of MC3T3-E1 cells. (A) Low-dose X-ray radiation-induced reorganization of the actin cytoskeleton was visualized by using F-actin staining of MC3T3-E1 cells were irradiated for 24 h with 0 and 0.5 Gy X-ray. Cells were then fixed and fluorescence-labeled with fluorescein isothiocyanate-phalloidin and stained with DAPI. (B) ROCK inhibitor (Y-27632) pretreatment and F-actin staining of MC3T3-E1 cells. F-actin staining of cells on post-irradiation at 2 h, 1, 3, and 5 days. Y-27632 inhibited low-dose irradiation-mediated actin reorganization. Magnification, x200. ROCK, Rho-associated protein kinase.

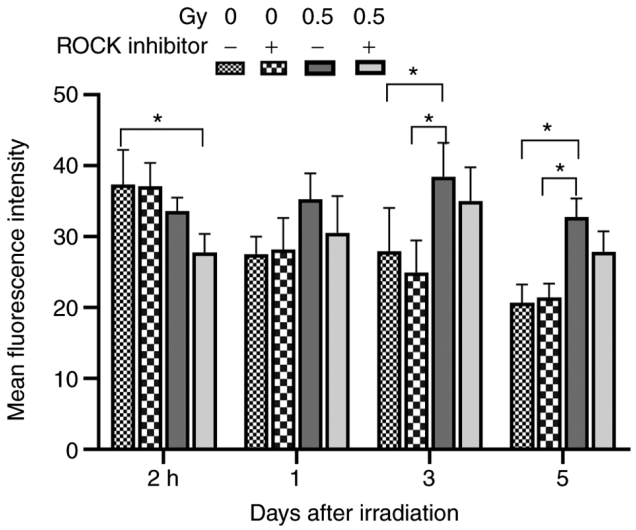

Figure 2. Comparison of mean fluorescence intensity among the groups. The values were presented in bar graphs as mean ± SD of three independent experiments. *P<0.05. ROCK, Rho-associated protein kinase.

that in the 0 GY-Y27632 group (P<0.05). At 1 day after X-ray irradiation of Y27632-pretreated cells, no significant difference was detected in the fluorescence intensity between each irradiated group and the 0 Gy-Y27632 group. Three days after X-ray irradiation, the fluorescence intensity of the cells in the 0.5 Gy group was elevated compared with non-irradiated group. The fluorescence intensity of cells in the 0.5 Gy-Y27632 group was higher than that in other groups (P<0.05). On day 5, the intracellular F-actin gradually returned to normal, and the fluorescence intensity of the cells in the 0.5 Gy group was stronger compared with non-irradiated group (P<0.05; Table I; Fig. 2). These results demonstrated that after 2 h X-ray irradiation, the F-actin

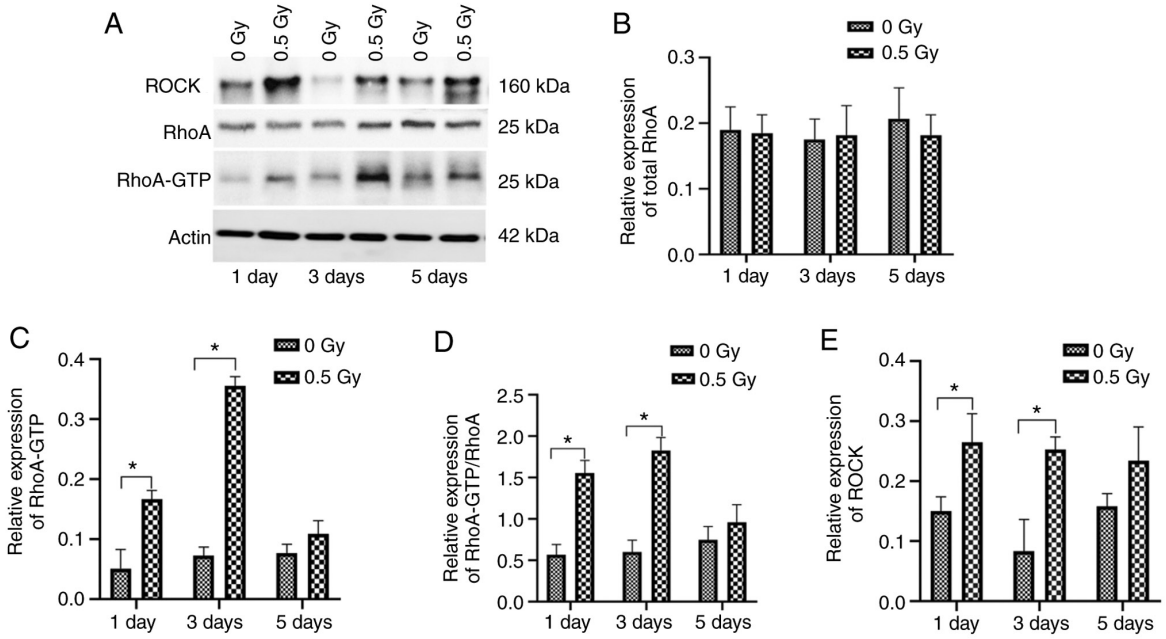

Figure 3. 0.5-Gy X-ray irradiation induced activation of RhoA and ROCK. (A) Representative experiment showing the amount of active GTP-RhoA following 0 or 0.5-Gy X-ray irradiation at different time points. The levels of RhoA and ROCK were detected by immunoblotting with antibodies specific for the respective GTPases following the pulldown or in the total cell extracts. (B-E) Immunoblots were analyzed by densitometry, and the intensity of the GTP-RhoA bands was normalized to the intensity of the corresponding total Rho band. The ratios are presented in bar graphs as mean ± SD of RhoA and ROCK. *P<0.05. ROCK, Rho-associated protein kinase.

depolymerized and the fluorescence intensity decreased. At 24 h after X-ray irradiation, the cytoskeleton was reorganized and the fluorescence intensity was enhanced. The fluorescence intensity of F-actin reached a peak value after 3 days X-ray irradiation. On the 5th day after X-ray irradiation, the fluorescence intensity of F-actin decreased and returned to normal, indicating that cytoskeleton reorganization caused by X-ray irradiation was reversible.

RhoA and ROCK are activated by 0.5 Gy X-ray irradiation of MC3T3-E1 cells. Accumulating evidence indicates that the RhoA/ROCK signaling pathway plays a significant role in regulating actin reorganization through various effectors (24,25). As the RhoA/ROCK signaling pathway triggers the formation of stress fibers, it was hypothesized that these proteins could be activated by 0.5 Gy X-ray irradiation of MC3T3 cells. The activation of RhoA was analysed in X-ray-irradiated osteoblasts. Cells were treated with 0 and 0.5 Gy X-ray irradiation, then analysed at different time points. The protein levels of GTP-RhoA and ROCK were detected in each group (Fig. 3A). Both RhoA and ROCK were activated by 0.5-Gy X-ray irradiation in MC3T3-E1 cells. The levels of GTP-RhoA were elevated at day 1 after 0.5-Gy X-ray irradiation, reached maximum level at day 3 after irradiation, and decreased to baseline at day 5 (Fig. 3B-D). ROCK exhibited similar results after 0.5-Gy irradiation (Fig. 3E). The aforementioned results indicated that 0.5-Gy X-ray irradiation could induce rapid activation of RhoA/ROCK signaling in MC3T3-E1 cells, which was in agreement with the actin stress fiber formation in these cells (Fig. 1).

ROCK mediates LIMK2 activation and actin cytoskeleton reorganization following 0.5-Gy X-ray irradiation of MC3T3-E1 cells. As LIMK2 is a downstream molecule of the RhoA/ROCK signaling pathway (24), it was hypothesized that ROCK could be involved in X-ray irradiation-induced phosphorylation of LIMK2. As before, cells were pretreated with Y27632, followed by 0.5-Gy X-ray irradiation, and western blotting was undertaken to detect the levels of phospho-LIMK2 and phospho-Cofilin in each group at various time points (Fig. 4A). The levels of phospho-LIMK2 and phospho-Cofilin in the 0.5 Gy-Y27632 group were significantly elevated compared with those in the other groups. However, there was no change in the levels of phospho-LIMK2 and phospho-Cofilin between 0.5 Gy + Y27632 and 0 Gy + Y27632 groups, indicating that ROCK-mediated 0.5 Gy X-ray irradiation could induce phosphorylation of LIMK2 (Fig. 4B-G).

Effects of RhoA on cell proliferation. The effects of Y27632 and low-dose irradiation on cell proliferation were then assessed. The results showed that on the first day after irradiation, there was no significant difference in CCK-8 activity in MC3T3-E1 cells between the irradiated group and the non-irradiated group (Fig. 5). However, the cells irradiated with low-dose (0.5 Gy) showed an increase in cell proliferation from days 2-5 ($P<0.05$). When Y27632 was used before irradiation, the cell proliferation was decreased compared with the 0.5-Gy irradiation group. There was no significant difference on days 6 and 7 between the groups, which may be attributed to the stable growth state of MC3T3-E1 cells in each group. These results demonstrated that low-dose X-ray irradiation promoted the proliferation of osteoblasts.

Effects of low-dose X-ray irradiation on cell differentiation: ALP staining and activity. ALP is one of the most important markers of osteoblasts, which increases inorganic phosphate concentration and promotes mineralization of bone

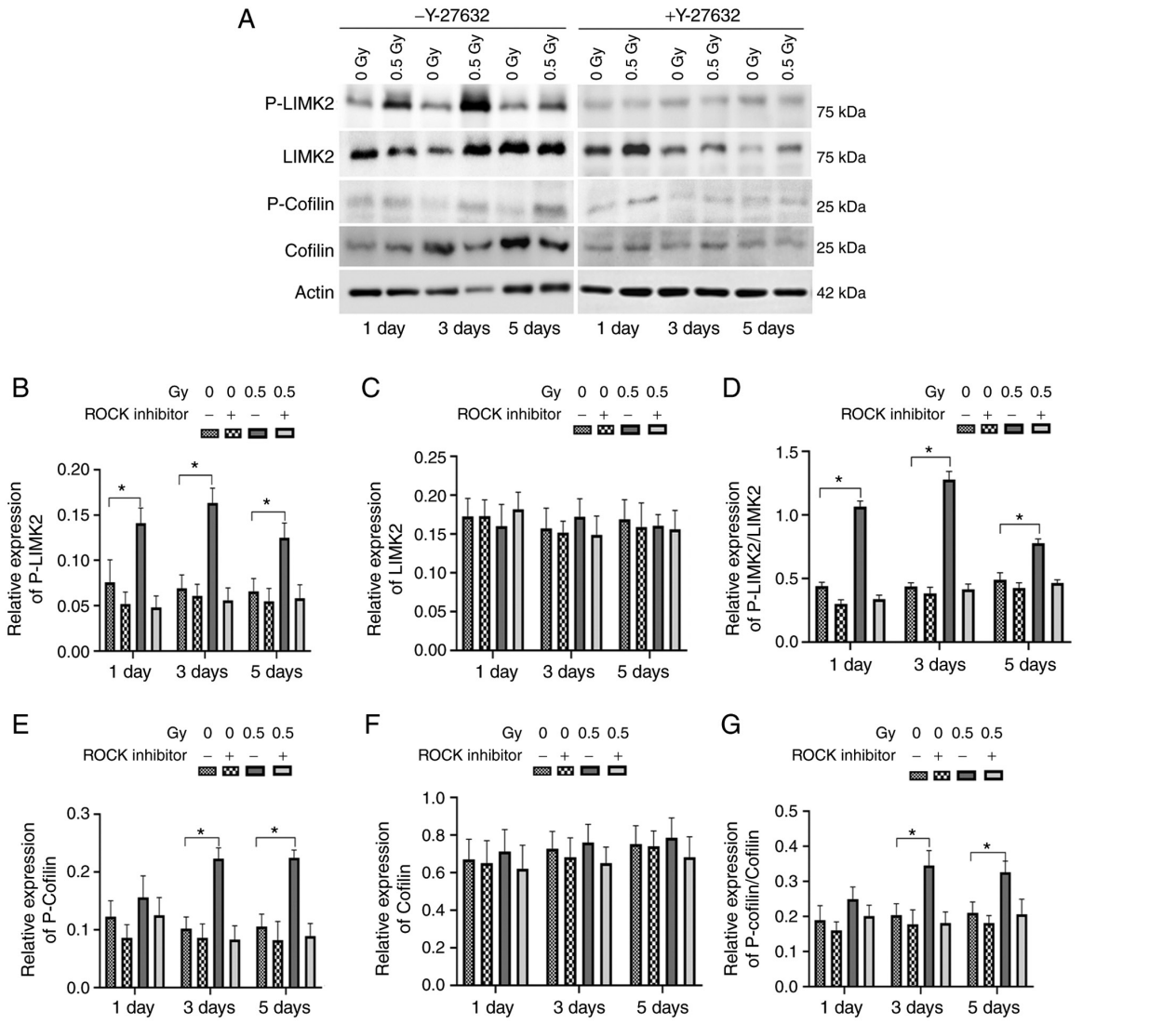

Figure 4. Low dose X-ray irradiation-induces LIMK2 and Cofilin phosphorylation. The ROCK inhibitor blocked 0.5-Gy X-ray irradiation-induced actin reorganization and LIMK2 phosphorylation. Cells were pretreated (+Y-27632) or not (-Y-27632) with 10 μmol/l Y-27632, and subsequently with 0 or 0.5-Gy X-ray irradiation for the indicated time points. Western blotting was performed to detect the levels of P-LIMK2 and P-Cofilin in each group (A). The immunoblots were analyzed by densitometry, and the intensity of the GTP-RhoA bands was normalized to the intensity of the corresponding total Rho band. The ratios presented in bar graphs are shown as mean ± SD of P-LIMK2 and P-Cofilin (B-G) (*P<0.05). ROCK, Rho-associated protein kinase; LIMK2, LIM domain kinase 2; P, phosphorylated.

formation (18). In the present study, following ALP staining, the cytoplasm of osteoblasts was purple granular, and the staining was positive (Fig. 6). The intensity of staining in 0.5-Gy group was higher than that in other groups (Fig. 7).

On the day 7 and 10 after irradiation, ALP activity was detected in MC3T3-E1 cells. The results showed that 0.5-Gy irradiation without Y27632 pretreatment significantly enhanced the ALP activity of osteoblasts, which was notably significantly than that in the other three groups (P<0.05). However, there was no significant difference in ALP activity between the remaining three groups (Table II; Fig. 8).

Alizarin red staining. The number of mineralized nodules indicates the osteogenic capacity of osteoblasts. The results of Alizarin red staining revealed that at 21 days after X-ray irradiation, the degree of red staining in the 0.5-Gy group without pretreatment was higher than that of the other three groups (Fig. 9). Moreover, the Alizarin red staining positive

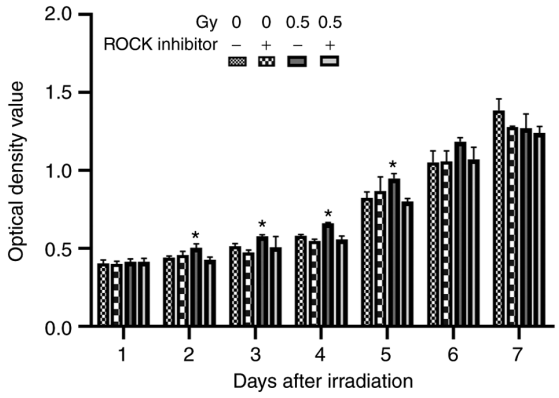

Figure 5. Low-dose X-ray irradiation promotes osteoblasts proliferation via the RhoA/ROCK signaling pathway. A Cell Counting Kit-8 assay was employed to detect the changes in the proliferation of osteoblasts pretreated with Y27632 after 0.5-Gy X-ray irradiation. Compared with the control group (0 Gy), low-dose (0.5 Gy) irradiation significantly improved cell viability from days 2-5. *P<0.05 compared with the control group.

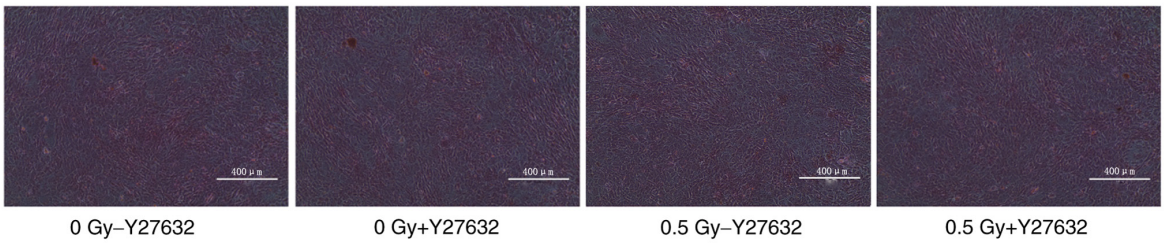

Figure 6. Low-dose X-ray irradiation enhances ALP staining of osteoblasts. ALP was expressed on the surface of the cell membrane of the osteoblasts. It also served as a marker for differentiation and maturation of osteoblasts, indicating various stages of osteoblast differentiation. ALP staining was carried out after cells were cultured until the 7th day. Magnification, x10. ALP, Alkaline phosphatase.

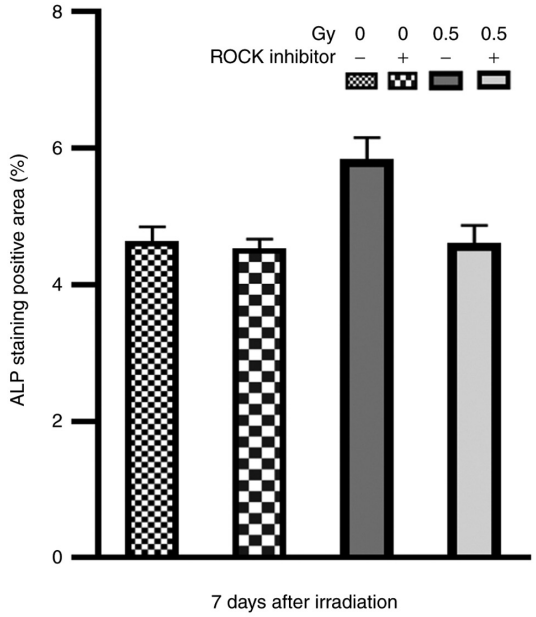

Figure 7. ALP staining positive area in each group. The intensity of staining in the 0.5 Gy group was higher than that in the other groups. ALP, alkaline phosphatase.

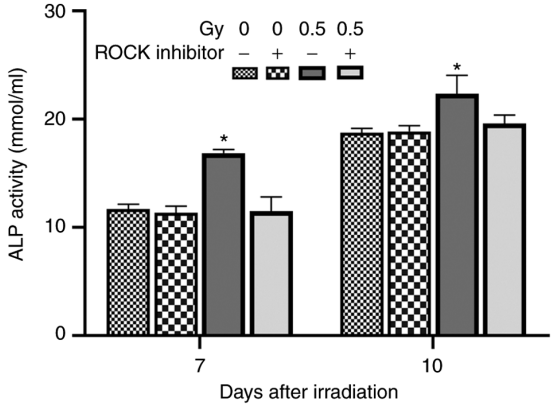

Figure 8. Low-dose X-ray irradiation promotes ALP activity of osteoblasts. The ALP activity of 0.5 Gy-Y27632 group was significantly higher compared with that of the other three groups. *P<0.05. ALP, alkaline phosphatase.

area was markedly greater than that of the +Y27632 0.5 Gy and non-irradiated groups (Fig. 10). The aforementioned findings showed that 0.5-Gy X-ray irradiation promoted the mineralization and maturation of osteoblasts.

Expression levels of osteogenic markers induced by 0.5 Gy X-ray irradiation. The expression levels of OCN, Runx2, ALP, COL1, and Osterix in MC3T3 cells were detected by western blotting (Fig. 11). The results showed that the expression level of ALP was significantly elevated 10 days after 0.5-Gy irradiation, and the difference was statistically significant compared with the other three groups (P<0.05; Fig. 11A). There was no significant difference in the expression of any of the analyzed markers at day 7. However, at day 10 after irradiation, Runx2 (Fig. 11B), Osterix (Fig. 11C), COL1 (Fig. 11D) and OCN levels (Fig. 11E) in the 0.5-Gy without Y27632 pretreatment was significantly elevated, compared with the untreated group (all P<0.05). However, when MC3T3-E1 cells pretreated with Y27632 were exposed to 0.5-Gy X-ray irradiation, the expression levels of osteogenic markers were markedly reduced, compared with irradiated cells that did not receive Y27632 pretreatment (P<0.05). The aforementioned findings suggested that the RhoA/ROCK signaling pathway was involved in differentiation of osteoblasts induced by low-dose X-ray irradiation.

Discussion

In the current study, fluorescence microscopy was used to observe the changes of actin cytoskeleton in MC3T3-E1 cells after low-dose X-ray irradiation and to examine the mechanism of cytoskeleton remodeling induced by low-dose X-ray irradiation. At 2 h after X-ray radiation, the cells became wrinkled. The actin arrangement became discontinuous, and the green fluorescence intensity decreased. These results indicated that the cellular microfilament network was destroyed soon after X-ray irradiation.

The cause of the changes in the cytoskeleton may be associated with direct damage from ionizing radiation. After 24 h of X-ray irradiation, the actin cytoskeleton was neatly arranged, the fluorescence intensity was enhanced in the 0.5 Gy group, indicating that the cytoskeleton was reorganized. It was hypothesized that the remodeling of actin fibers after X-ray irradiation may be related to the activation of injury repair after X-ray irradiation, which may activate DNA-damage repair response. It also increased the expression of related growth factors, and activated downstream signaling pathways by binding to the corresponding receptors, thereby causing cytoskeleton rearrangement.

It was hypothesized that low-dose X-ray exposure caused activation of the Rho/ROCK pathway. The results showed that the levels of phospho-LIMK2 and phospho-Cofilin were

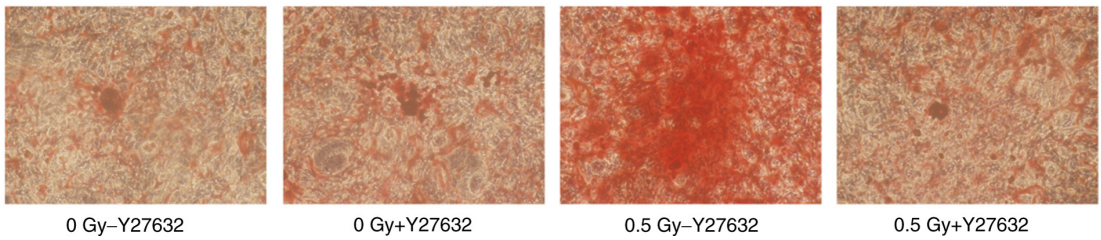

Figure 9. Alizarin red staining of mineralized nodules at different sizes on day 21. In the current study, the number of calcium nodules in the-Y27632 0.5 Gy group was markedly higher than that in the other three groups. Magnification, x20.

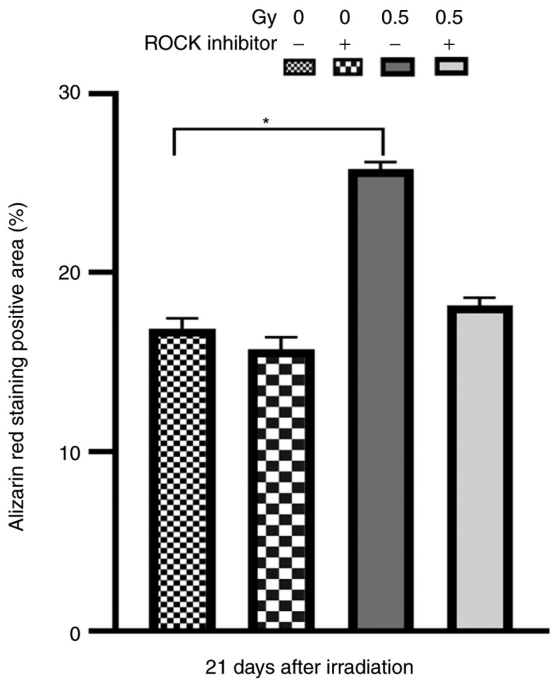

Figure 10. Alizarin red staining positive area in each group. The Alizarin red staining positive area in the-Y27632 0.5 Gy group was higher than that in the other three groups. *P<0.05.

significantly increased on day 3 and 5 after X-ray irradiation. However, the levels of phospho-LIMK2 and phospho-cofilin were decreased after pretreatment with ROCK inhibitors, suggesting that the RhoA/ROCK/LIMK2/Cofilin pathway was involved in cytoskeleton remodeling induced by low-dose X-ray irradiation, but the specific mechanism of cytoskeleton changes caused by ionizing radiation remains to be elucidated (29,30). These changes were apparent on day 3 after irradiation, indicating that RhoA/ROCK signaling pathway was activated, which is consistent with Murata *et al* findings (31). In the present experiment, the expression level of P-Cofilin in the 0.5 Gy group increased compared with that in the non-irradiated group. It was hypothesized that 0.5-Gy X-ray irradiation caused activation of the Rho/ROCK pathway, and the intracellular-synthesized Cofilin was more converted into P-Cofilin. Other studies have reported similar results. For instance, Gabryś *et al* (26) found that radiation could cause rapid rearrangement of actin in capillary endothelial cells, which activated the RhoA/ROCK signaling pathway. The initial factors of RhoA activation caused by ionizing radiation have not been fully clarified. Cells produce a large amount of reactive oxygen species (ROS) after radiation (32), which may activate the RhoA/ROCK signaling pathway. It has been found that RhoA is the target protein of ROS (33).

The RhoA/ROCK signaling pathway plays a pivotal role, not only in regulating the cytoskeleton, but also in cell proliferation and differentiation under various stimuli (20,34-38). RhoA and ROCK are key signaling molecules in respond to various stimuli (such as vasoactive substances, shear force, angiogenic factors, and oxidative stress). The RhoA/ROCK signaling pathway regulates a variety of cellular functions, such as permeability, migration, adhesion (39), cell survival, and apoptosis (40-42).

In the current study, MC3T3-E1 cell proliferation increased after exposure to 0.5-Gy X-ray irradiation. In addition, low-dose X-ray irradiation elevated the expression levels of Runx2, Osterix, ALP, OCN and COLI in MC3T3 cells. These results suggested that low-dose X-ray irradiation may promote the proliferation and differentiation of MC3T3 cells through the RhoA/ROCK signaling pathway. Additionally, 0.5-Gy X-ray irradiation may activate the RhoA/ROCK signaling pathway and promote osteogenic differentiation.

The differentiation process of osteoblasts can be divided into two stages, ECM maturation and ECM mineralization. ALP is one of the appropriate markers for early-stage of osteoblast differentiation (43). RUNX2 is an important transcription factor for osteoblast differentiation and bone formation, which is also very significant for regulating the rate of bone matrix deposition. OCN is secreted by mature osteoblasts during matrix calcification (44). In the current study, the activity of ALP was analyzed, which showed early osteogenic differentiation potential of MC3T3 cells. ALP activity in 0.5 Gy X-ray irradiation group reached the maximum on the 10th day after irradiation, and it was stronger than that of the other three groups. These results indicated that MC3T3 cells showed stress response to 0.5 Gy X-ray irradiation and their early differentiation ability was improved. Osteogenic differentiation requires a complete actin network. The activation of ROCK can maintain a complete and robust actin cytoskeleton, which is indispensable for gene expression caused by low-dose X-ray irradiation.

Thus, identifying the effector molecules of the RhoA/ROCK signaling pathway may be of great significance. Previous studies have shown that Akt, PI3K, P38 phosphorylation and ERK1/2 in MAPK pathway were all associated with RhoA (45-47). Moreover, the ECM regulates bone formation by affecting downstream MAPK signaling pathway and RhoA/ROCK signaling pathway (46). Bone sialic acid glycoprotein is expressed in several types of cells (such as

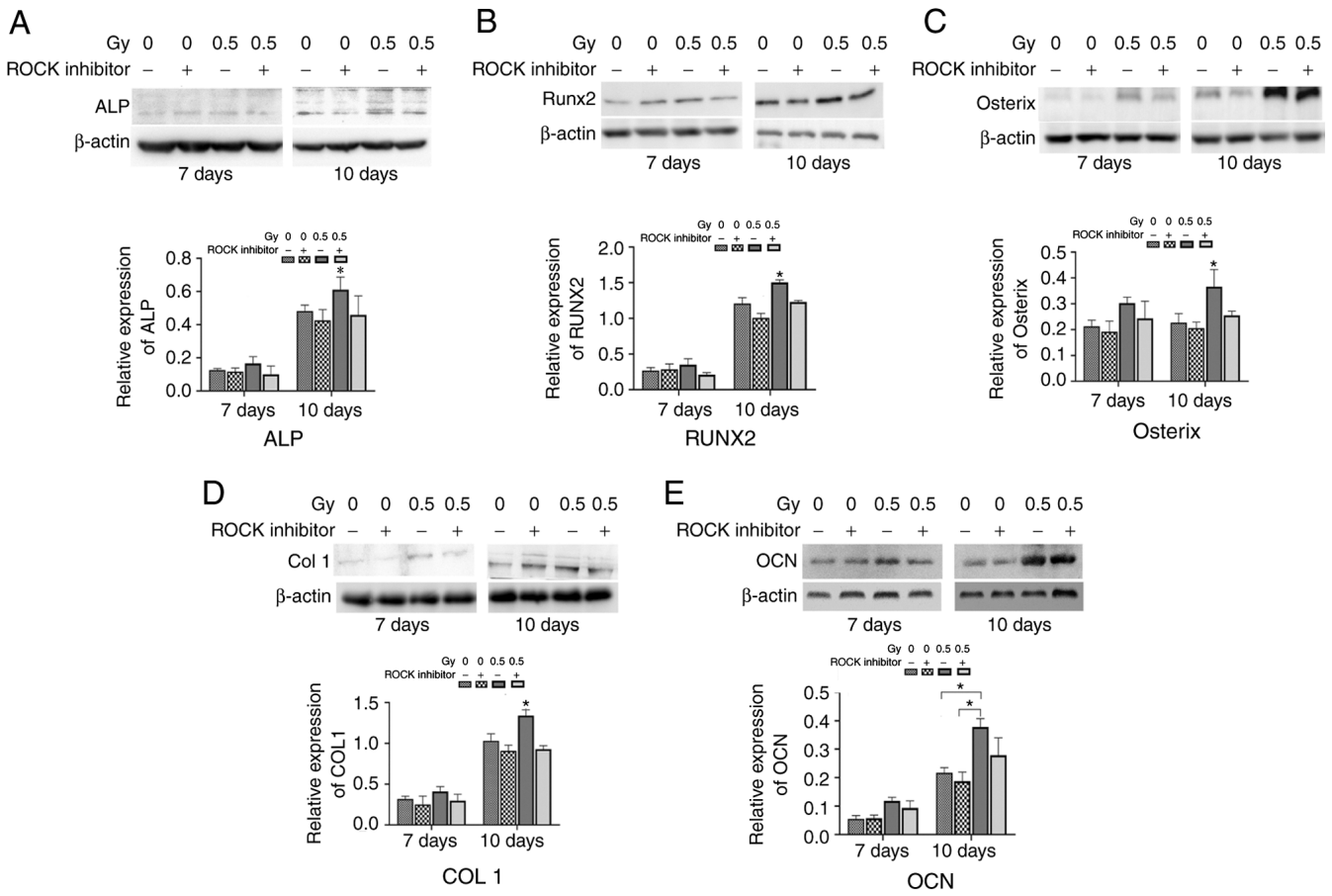

Figure 11. Effects of X-ray irradiation on the expression levels of osteogenic markers. The expression levels of ALP, Runx2, Osterix, COL1 and OCN were detected by western blotting at 7 and 10 days after irradiation. Protein expression levels of (A) ALP, (B) Runx2, (C) Osterix, (D) COL1- and (E) OCN proteins, respectively. *P<0.05. ALP, alkaline phosphatase; OCN, osteocalcin; COL1, Collagen Type 1.

osteoblasts, osteocytes, chondrocytes, fibroblasts, and endothelial cells) (48). Its synthesis can be regulated by the PI3K and MAPK pathways. To some extent, PI3K/MAPK signaling pathway could be mediated by RhoA (49).

The RhoA/ROCK signaling pathway induces PI3K activation in a variety of cells, mediating myocardial protection (50), and proliferating mouse prostate cancer cells (51). In the present study, Y27632 blocked osteogenic differentiation induced by 0.5-Gy X-ray irradiation. The possible mechanism was that Y27632 inhibited the activation of RhoA/ROCK signaling pathway induced by low-dose X-ray irradiation, thereby inhibiting the phosphorylation of ERK1/2, p38, and Akt, and could ultimately reduce the synthesis of osteogenic differentiation proteins. Therefore, it can be concluded that RhoA/ROCK signaling pathway can be involved in regulating osteoblast differentiation induced by 0.5 Gy X-ray irradiation.

In addition, Lumetti *et al* (28) found that RhoA could activate the Wnt signaling pathway and promote the differentiation of osteoblasts. Rossol-Allison *et al* (52) suggested that, RhoA activation is indispensable in the process of osteogenic differentiation of mesenchymal stem cells stimulated by Wnt signaling pathway. RhoA inhibition can significantly inhibit the transcription of target genes depending on Wnt3A-β-catenin pathway. Activation or inhibition of RhoA can affect the nuclear transport of β-catenin and subsequent osteogenic differentiation (53). These results suggest that RhoA activation is highly essential for osteogenic differentiation by Wnt3A/β-catenin signaling pathway.

In conclusion, the results of the present study suggested that low-dose X-ray irradiation could regulate cytoskeleton reorganization and promote the proliferation and differentiation of osteoblasts. This effect may be mediated by activation of the RhoA/ROCK signaling pathway. However, multiple signaling pathways may be involved in the promotion of proliferation and differentiation of osteoblasts by low-dose X-ray irradiation, while the specific mechanism remains to be further clarified.

Acknowledgements

Not applicable.

Funding

This study was financially supported by the National Natural Science Foundation of China (grant no. 81874008).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QH and HC carried out the experiments, participated in collecting data, and drafted the manuscript. SW performed the statistical analysis and participated in its design. YS and WX participated in acquisition, analysis, or interpretation of data and drafting the manuscript. QH and WX confirmed the authenticity of the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that there is no conflict of interest.

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