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Rho-associated coiled-coil-containing protein kinase inhibitor Y-27632 promotes distraction osteogenesis healing by activating both osteoblasts and osteoclasts

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ABSTRACT

Distraction osteogenesis can induce substantial skeletal tissue regeneration; however, the treatment duration is long, making the procedure suboptimal for clinical care. Rho-associated coiled-coil-containing protein kinase inhibitors might promote bone generation in distraction osteogenesis and shorten treatment durations. However, the relationship between Rho-associated coiled-coil-containing protein kinase inhibitors and distraction osteogenesis levels has not been described. In this study, we focused on osteoblasts and osteoclasts, which are essential for bone remodeling and regeneration. Proliferation assay, boyden chamber, and wound healing assay were performed on MC3T3-E1 and RAW264 cells. Osteogenic differentiation assay was performed on MC3T3-E1 cells, and osteoclast differentiation assay was performed on RAW264 cells. Samples collected from distraction osteogenesis model mice were subjected to micro-computed tomography analysis and tissue staining. We found that Y-27632, a Rho-associated coiled-coil-containing protein kinase inhibitor, promoted cell motility and affected cell differentiation and bone differentiation in MC3T3-E1 preosteoblast cells. We also found that Y-27632 promoted cell motility and osteoclast differentiation in the osteoclast precursor RAW264 cells. In vivo experiments showed that the local administration of Y-27632 in a mouse distraction osteogenesis model promoted bone formation and increased the number of osteoblasts and osteoclasts in the distraction osteogenesis gap. These findings demonstrate that Y-27632 promotes bone formation in a mouse distraction osteogenesis model. Collectively, the study findings suggest that Y-27632 can be used as a therapeutic agent to promote distraction osteogenesis healing.

Keywords: bone regeneration, distraction osteogenesis, Rho-associated coiled-coil-containing protein kinase, Y-27632

Abbreviations:
DO: distraction osteogenesis
ROCK: Rho-associated coiled-coil-containing protein kinase
TRAP: tartrate-resistant acid phosphatase

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INTRODUCTION

Distraction osteogenesis (DO) is applied in orthopedic, oral, and maxillofacial surgeries to correct congenital or acquired skeletal deformities.[1] The DO procedure generates new bone in a gap created by osteotomy by gradually lengthening two bone segments. Large-scale tissue regeneration via DO, induced by intermittent mechanical stimulation due to tissue injury, requires angiogenesis and the recruitment of endogenous connective tissue stem/progenitor cells.[2] Although DO offers great benefits to clinicians, it requires patients to use large external fixation devices for long periods, which can be very painful. These devices can encourage pathogen growth and pose a risk of serious infection. Therefore, reducing the duration of DO treatment is desirable.

The Rho/Rho-associated coiled-coil-containing protein kinase (Rho/ROCK) signaling pathway plays an important role in maintaining basic cellular functions. One of the most important actions of the Rho/ROCK signaling pathway is the phosphorylation of myosin-light-chain phosphatase and several other phosphokinases and cytoskeleton-binding proteins.[3] The Rho/ROCK pathway regulates cytoskeletal contraction through these actions, which is indispensable for the maintenance of apoptosis and cell migration, proliferation, and differentiation. Therefore, Rho/ROCK signaling is often pharmacologically targeted to control cell differentiation and proliferation. Several small-molecule inhibitors of ROCK are known and affect cells differently depending on the cell type and the combination of cytokines. Y-27632 is a competitive ROCK inhibitor that can inhibit cell differentiation induced by extracellular matrix or mechanical stimulation, as shown in previous studies.[4,5] Further, it may promote differentiation induced by paracrine factors.[6,7] Administration of ROCK inhibitors in a rat model of skull defects was reported to promote bone formation.[8] These findings suggest that ROCK inhibitors, as bone-active molecules, might promote bone generation in DO and be used to shorten treatment durations.

This study aimed to investigate the effects of Y-27632 on DO treatment. To the best of our knowledge, this is the first study to describe the relationship between ROCK inhibitors and DO levels. Histological and radiographic evaluations were performed in a mouse model of DO to assess the potential of ROCK inhibitors to promote bone generation and shorten the duration of DO treatment.

MATERIALS AND METHODS

Cell culture

MC3T3-E1 and RAW264 cells were purchased from RIKEN BRC (Ibaraki, Japan). MC3T3-E1 were cultured in MEM ALPHA (Gibco) containing 100 U/mL penicillin, 100 mg/mL streptomycin, and 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2 and 95% air. RAW264 cells were cultured in MEM (Sigma-Aldrich) containing 100 U/mL penicillin, 100 mg/mL streptomycin, 10% FBS, and 0.1 mM non-essential amino acids under the same conditions. Cells from the third to sixth passages were used for experiments.

Proliferation assay

MC3T3-E1 and RAW264 cells were seeded in 96-well plates at 5×10^3 cells/well. The cells were either left unstimulated or stimulated with 5–20 μM Y-27632 (Selleckchem, S1049). After 24 or 48 h of incubation, cell proliferation was examined using the Cell Counting Kit-8 (Dojindo, Mashiki, Japan). Cell Counting Kit-8 solution was added to each well, and cells were incubated for 2 h. The absorbance of each well at 450 nm was then measured using a microplate reader (Infinite 200 PRO Microplate Reader; Tecan Japan, Kanagawa, Japan).

Boyden chamber assay

MC3T3-E1 and RAW264 cells were suspended in a culture medium containing 1% FBS and adjusted to a density of 1×10^5 cells/mL. Two hundred microliters of cell suspension were added to the upper chamber of a Transwell plate (8-μm pore size, 24 wells; Greiner, Kremsmünster, Austria). The lower chamber was treated with culture medium containing 1% FBS with or without Y-27632 for 12 h to induce cell migration. The cells were stained with 0.05% crystal violet for 15 min, and non-migrated cells were removed from the inner side of the membrane using a cotton swab. Images were captured in 5 random fields with an optical microscope. The migrated cells were counted using the ImageJ software.[9]

Wound healing assay

MC3T3-E1 and RAW264 cells were seeded in Culture-Insert 24-well plates (Ibidi, Gräfelfing, Germany) at a concentration of 7×10^3 cells/well. After 24 h, the culture insert was removed, and the cells were washed with phosphate-buffered saline to remove cell debris. The cells were cultured in medium with or without Y-27632 for 24 h. Photographs were taken of each well, and wound closure rates were quantified using the ImageJ software.

Alizarin Red S staining

MC3T3-E1 cells were used as preosteoblasts. When the cells reached 90% confluency, they were switched to mineralization medium containing 10 mM β-glycerol phosphate, 50 μg/mL ascorbic acid, and 10 nM dexamethasone. The medium was replaced every three days. After 14 days of incubation, the cells were stained with a saturated solution of Alizarin Red S (pH 6.4). Calcified nodules were extracted using 5% formic acid, and the absorbance of the extracts was measured at 415 nm using a microplate reader.

Tartrate-resistant acid phosphatase staining

RAW264 cells were used as osteoclast precursor cells. For the induction of osteoclastic differentiation, 100 ng/mL sRANKL (Oriental Yeast, Tokyo, Japan) was added to the medium, and the cells were incubated for 5 days. The culture medium in each well was replaced with fresh medium containing sRANKL every two days. Cell staining was performed using a tartrate-resistant acid phosphatase (TRAP)/ALP Stain Kit (Wako, Japan). Images were captured in 10 random fields with an optical microscope. TRAP-positive red cells with three or more nuclei were manually counted as osteoclasts.

Mouse distraction osteogenesis model

All animal experiments were carried out in accordance with the Guidelines for Animal Experimentation of the Nagoya University School of Medicine and were performed with the approval of the Animal Care and Use Committee of Nagoya University School of Medicine (no. M230040-006). We used 8-week-old male ICR mice (Chubu-Kagaku Shizai Corporation, Tokyo, Japan). The surgical DO method was performed as previously described.[10] Mice were anesthetized via intraperitoneal injection of medetomidine hydrochloride (0.3 mg/g), midazolam (4 mg/kg body weight), and butorphanol tartrate (5 mg/kg body weight). The right limb was shaved and prepared using an iodine solution. An anterior longitudinal incision was made on the right leg, and the underlying muscles were bluntly separated, taking care not to remove the entire periosteum. The tibia was fixed to the device with one pair of 25-gauge needles proximally and one pair of 27-gauge needles distally before being glued with acrylic resin. After the resin was completely polymerized, osteotomy was performed in the middle of the diaphysis using a very thin cutting disc, and the wound was closed with a 5–0 nylon suture. After incubation for five

days, distraction was initiated at a rate of 0.2 mm/12 h during the experiment. Twenty microliters of type 1 collagen, mixed with 20 μL phosphate-buffered saline or 20 μL 0.5 μM Y-27632, were administered topically to the distraction zone every two days during bone lengthening. Mice were screened for signs of surgical site infection and/or severe pain or discomfort, which are predefined early termination criteria. None of the mice fulfilled the early termination criteria. On day 17, each mouse was euthanized by perfusion fixation under deep anesthesia and then underwent radiographic and histological examination.

Radiographic and histological analyses

DO tibial segments were scanned using micro-computed tomography (1-mm Al filter, voxel size 0.9 μm; SkyScan1176, Bruker, Billerica, MA, USA). After reconstruction using the Skyscan NRecon software, the images were analyzed using three-dimensional algorithms in the Skyscan CTAn software according to the manufacturer's instructions. The region of interest was determined based on previous studies,[11] as the shape of the cylinder between the proximal and distal tibia. Finally, the bone volume and bone volume to tissue volume ratio were measured.

Tibiae were embedded in SCEM gel (SECTION-LAB). Non-decalcified sections of the tibia were prepared according to Kawamoto's film method.[12] Sections (CM3050S, Leica Biosystems) of 5 μm thickness were stained with hematoxylin-eosin to examine new bone formation. The region of interest was a quadrilateral outlined from the outer corners of the cortical bones proximal and distal to the distraction gap. The percentage of callus (new bone zone) in the region of interest was calculated using ImageJ software. TRAP staining was then performed. For immunohistochemistry, the sections were stained with an antibody specific for osteocalcin (1:100 dilution, Proteintech). The most central section of the medullary cavity was selected, and the number of cells in each section was measured. Histological analyses were performed using a microscope (BZ-X800; KEYENCE) and its analysis software.

Statistical analysis

All data were analyzed using GraphPad Prism 9.0 (GraphPad Software Inc, La Jolla, CA, USA) and are presented as mean ± standard deviation. Comparisons between the two groups were performed using a two-tailed unpaired Student's *t*-test. Comparisons among three or more groups were performed using one-way analysis of variance followed by Dunnett's post-hoc multiple comparison test. Statistical significance was set at $P < 0.05$.

RESULTS

Effects of Y-27632 on preosteoblasts

In the proliferation assay, no effect of 0–20 μM Y-27632 treatment on MC3T3-E1 cell proliferation was seen (Fig. 1A, B) at either 24 or 48 h. In the Boyden chamber assay, no significant difference was observed at 5 and 10 μM Y-27632 compared with 0 μM Y-27632, and the number of migrated cells increased significantly at 20 μM Y-27632 ($P < 0.05$) (Fig. 1C-G). In the wound healing assay, wounds were significantly closed at 5, 10, and 20 μM Y-27632 compared with that at 0 μM ($P < 0.01$, 0.01, 0.001) (Fig. 1H-L). These results indicated that Y-27632 treatment did not promote cell proliferation but promoted cell motility in MC3T3-E1 cells. Alizarin Red S staining showed significantly more calcified nodule formation at 5, 10, and 20 μM compared to that of 0 μM Y-27632 ($P < 0.001$, 0.001, 0.001) on MC3T3-E1 osteoblast differentiation (Fig. 1M, N).

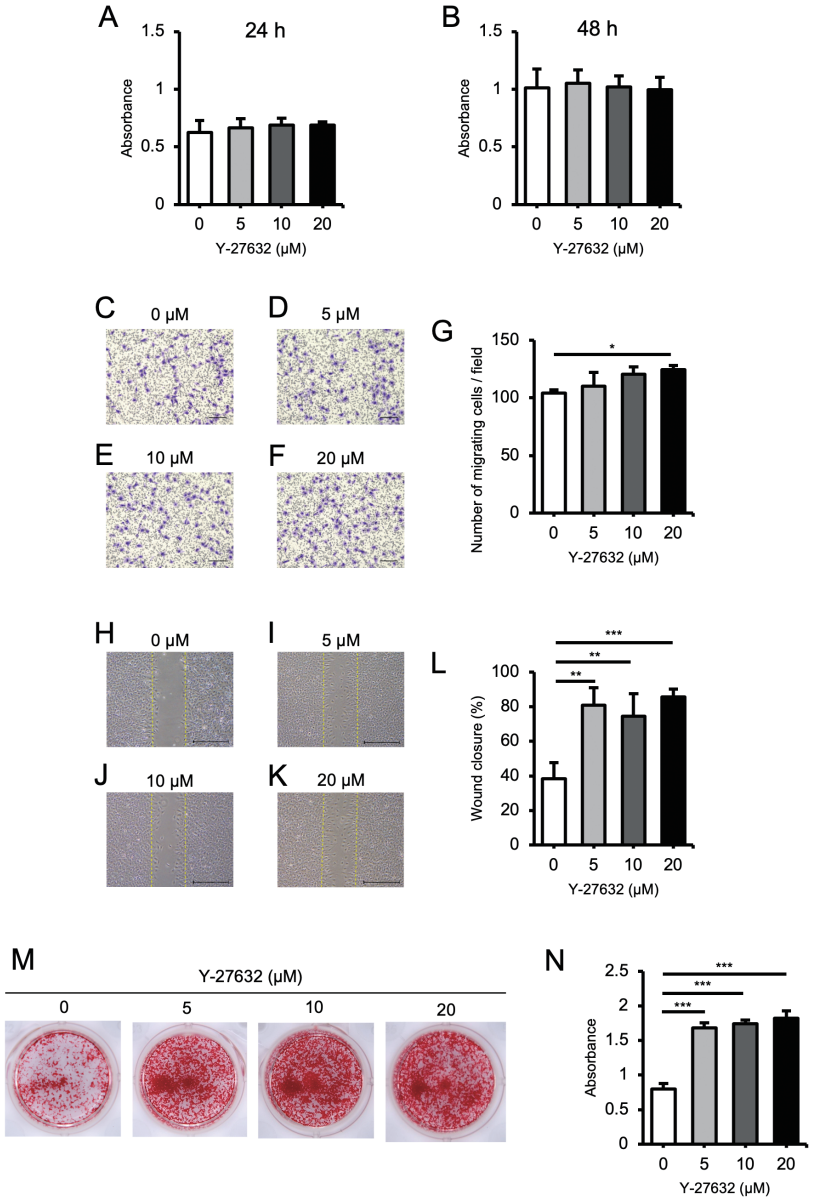

Fig. 1 The ROCK inhibitor Y-27632 promoted cell motility and osteogenesis in preosteoblasts

Fig. 1A, B: Effects of Y-27632 on the proliferative capacity of MC3T3-E1 at (A) 24 and (B) 48 h (n = 3).

Fig. 1C–F: Photographs of membranes from Boyden chamber assays in which (C) 0 μM, (D) 5 μM, (E) 10 μM, and (F) 20 μM of Y-27632 were administered (n = 3). Scale bar = 200 μm.

Fig. 1G: Number of migrated cells in wound healing assays using MC3T3-E1 cells.

Fig. 1H–K: Photographs from wound healing assays in which (H) 0 μM, (I) 5 μM, (J) 10 μM, and (K) 20 μM of Y-27632 were administered (n = 3). Scale bar = 500 μm.

Fig. 1L: Wound closure rate of wound healing assays using MC3T3-E1 cells.

Fig. 1M: Alizarin Red S staining of Mc3T3-E1 cells at 14 days after osteogenic induction (n = 3).

Fig. 1N: Absorbance at 415 nm was measured by dissolving calcium deposits in formic acid.

Data are presented as mean ± standard deviation. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

ROCK: Rho-associated coiled-coil-containing protein kinase

Effects of Y-27632 on osteoclast precursor cells

In the proliferation assay, no effect of 0–20 μM Y-27632 treatment on RAW264 cell proliferation was seen (Fig. 2A, B) at either 24 or 48 h. In the Boyden chamber assay, no significant difference at 20 μM Y-27632 was observed compared with that of 0 μM Y-27632, and the number of migrated cells increased significantly after treatment with 5 and 10 μM Y-27632 ($P < 0.05$, 0.05) (Fig. 2C-G). In the wound healing assay, no significant difference existed at 5 μM Y-27632 compared to that of 0 μM Y-27632, and wounds were significantly closed following treatment of 10 and 20 μM Y-27632 ($P < 0.05$, 0.05) (Fig. 2H-L). These results indicated that Y-27632 treatment did not promote cell proliferation but promoted cell motility in RAW264 cells. In TRAP staining, a significant increase in TRAP-positive cells occurred with three or more nuclei at 5, 10, and 20 μM Y-27632 compared to that of 0 μM Y-27632 ($P < 0.001$, 0.001, 0.001) (Fig. 2M, N). These results suggest that Y-27632 promotes osteoclast differentiation.

Y-27632 accelerates DO healing

To investigate the effects of Y-27632 on DO healing in vivo, we used a mouse model of tibial DO. After incubation for five days following osteotomy, bone extension was performed at a rate of 0.2 mm/12 h for eight days, increasing the total length by 3.2 mm. Y-27632 or phosphate-buffered saline was administered to the extension every two days during bone lengthening, and the mice were euthanized 17 days after surgery. Callus formation was quantified using hematoxylin-eosin staining. Callus formation was observed in both groups on day 17 (Fig. 3A, B). Significantly more calluses were observed in the Y-27632 group compared with those in the control group ($P < 0.05$) (Fig. 3C). Micro-computed tomography was used to validate the hematoxylin-eosin results (Fig. 3D, E). The bone volume and bone volume to tissue volume ratio were significantly higher in the Y-27632 group than in the control group ($P < 0.05$, 0.05) (Fig. 3F, G).

Effects of Y-27632 on osteoblasts and osteoclasts during DO healing

Because osteoblasts and osteoclasts play important roles in bone regeneration, the effect of Y-27632 on these cells was investigated. Immunofluorescence staining showed that more osteocalcin-positive osteoblasts were mobilized at the defect site in the Y-27632 group ($P < 0.05$) (Fig. 4A–C). More TRAP-positive osteoclasts were also observed to move toward the defect site in the Y-27632 group ($P < 0.05$) (Fig. 4D–F).

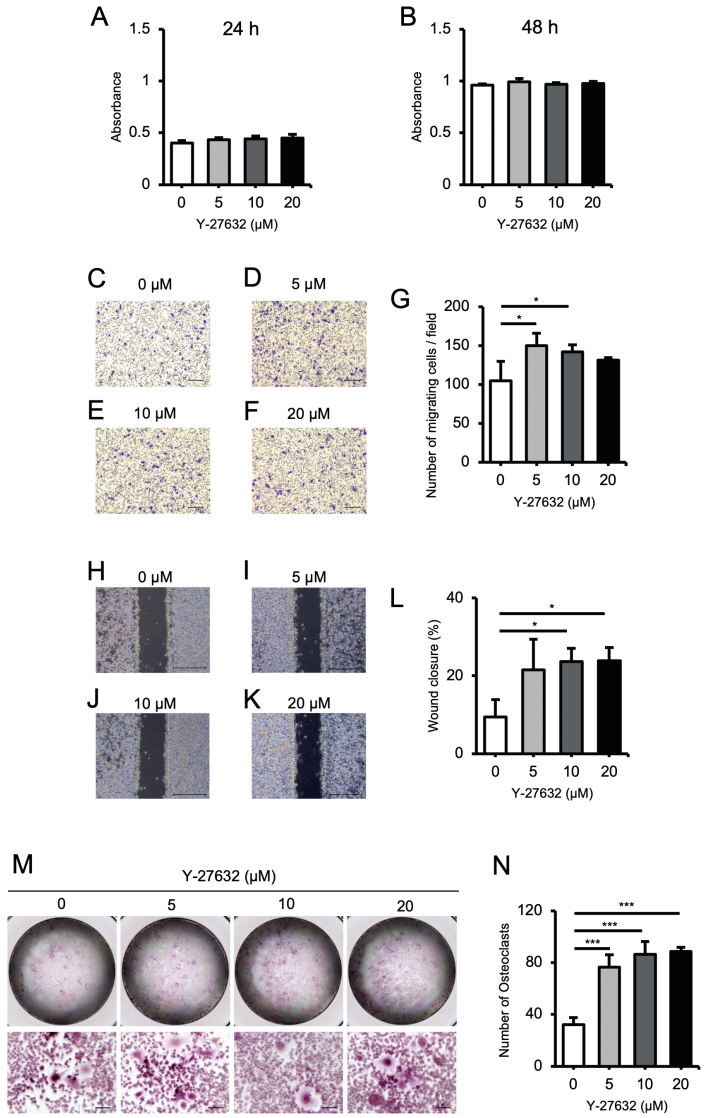

Fig. 2 The ROCK inhibitor Y-27632 promoted cell motility and osteoclast differentiation in osteoclast precursor cells

Fig. 2A, B: Effects of Y-27632 on the proliferative capacity of RAW264 cells at (A) 24 and (B) 48 h (n = 3).

Fig. 2C–F: Photographs of membranes from Boyden chamber assays in which (C) 0 μM, (D) 5 μM, (E) 10 μM, and (F) 20 μM of Y-27632 were administered (n = 3). Scale bar = 200 μm.

Fig. 2G: Number of migrated RAW264 cells.

Fig. 2H–K: Photographs from wound healing assays in which (H) 0 μM, (I) 5 μM, (J) 10 μM, and (K) 20 μM of Y-27632 were administered (n = 3). Scale bar = 500 μm.

Fig. 2L: Wound closure rate of wound healing assays using RAW264 cells.

Fig. 2M: TRAP staining of RAW264 cells seven days after sRANKL (100 ng/mL) was added (n = 3). Bar = 50 μm.

Fig. 2N: The number of osteoclasts differentiated from RAW264 cells.

Data are presented as mean ± standard deviation. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

ROCK: Rho-associated coiled-coil-containing protein kinase

TRAP: tartrate-resistant acid phosphatase

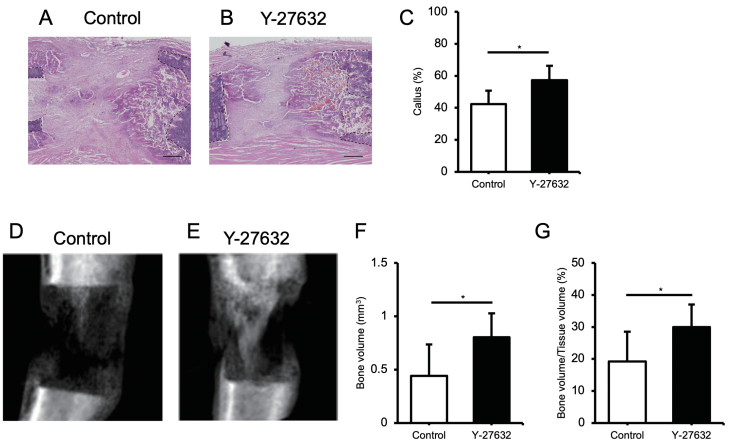

Fig. 3 The ROCK inhibitor Y-27632 accelerated distraction osteogenesis healing

Fig. 3A, B: H & E staining on day 17 of (A) control and (B) Y-27632 groups (n = 6). The black dotted line in each image indicates the proximal bone fragment (right) and the end of the distal portion (left).

Fig. 3C: Histomorphometric analysis of callus formation in the distraction osteogenesis gap.

Fig. 3D, E: Micro-CT images on day 17 of (D) control and (E) Y-27632 groups (n = 6).

Fig. 3F, G: Histomorphometric analysis of callus formation in distraction osteogenesis gap by micro-CT.

Data are presented as mean ± standard deviation. *$P < 0.05$; Scale bar = 500 μm.

ROCK: Rho-associated coiled-coil-containing protein kinase

H & E: hematoxylin-eosin

CT: computed tomography

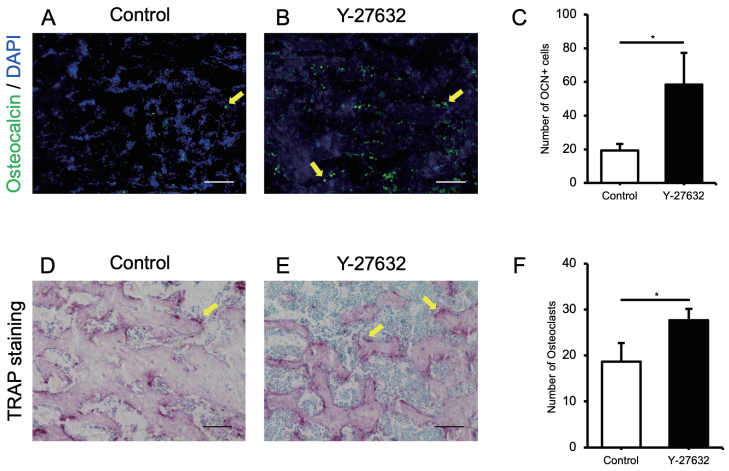

Fig. 4 Y-27632 increased the number of osteoblasts and osteoclasts in the distraction osteogenesis gap

Fig. 4A, B: Immunofluorescence staining of osteocalcin on day 17 of (A) control and (B) Y-27632 groups (n = 3).

Fig. 4C: Number of osteocalcin-positive osteoblasts.

Fig. 4D, E: TRAP staining on day 17 of (D) control and (E) Y-27632 groups (n = 3).

Fig. 4F: Number of TRAP-positive osteoclasts.

Data are presented as mean ± standard deviation. *$P < 0.05$; Scale bar = 100 μm.

TRAP: tartrate-resistant acid phosphatase

DAPI: 4',6-diamidino-2-phenylindole

OCN: osteocalcin

DISCUSSION

Previously, another publication showed that Y-27632 treatment enhanced ectopic bone formation induced by recombinant human BMP-2 in mice and enhanced the osteoblastic differentiation of murine neonatal calvarial and ST2 cells.[13] The present results using different cell lines support this previous reports. Further, the present study also found that a ROCK inhibitor promoted osteoclast differentiation, suggesting that Y-27632 promotes bone regeneration by enhancing both osteoblast and osteoclast differentiation. This is consistent with the results of a previous study.[8] However, as opposed to the results described in the present study, it has been reported that Y-27632 treatment decreased the osteogenic differentiation ability in mesenchymal stem cells.[14] A complex regulatory scenario has been postulated in which the differentiation of mesenchymal stem cells into osteoblasts requires RhoA/ROCK signaling, whereas terminal differentiation requires its repression, which may explain these differing results.[15]

The role of ROCK in osteoclastogenesis is not known in detail. Y-27632 has been reported to inhibit osteoclast differentiation in peripheral blood mononuclear cells from patients with inflammatory arthritis and in osteoclast precursor cells from arthritic mice.[16] That report contradicts the results of the present study. Nevertheless, other studies[8] have observed longer filopodia formation and increased numbers of differentiated osteoclasts in osteoclast progenitor cells in the presence of Y-27632. Filopodia formation of osteoclast precursor cells is required for the initiation of cell-cell contacts in osteoclastogenesis. This supports our finding that ROCK inhibitors increase the number of osteoclasts. However, further knowledge is needed regarding the effects of ROCK inhibitors on osteoclast differentiation.

The present study demonstrated for the first time that ROCK inhibitors promote bone formation in DO. Furthermore, Y-27632 administration was found to enhance the migratory ability of osteoclast precursor cells and preosteoblasts. Considering these results, the enhanced migration and differentiation of osteoclasts and osteoblasts may be involved in the action of Y-27632 to promote bone healing during DO.

Several pharmacological approaches to promote bone healing have been identified. Many reports have demonstrated methods to promote osteoblast activity and inhibit osteoclast activity. Some reagents, such as DZNep, an Enhancer of Zeste Homolog 2 inhibitor, have been reported to enhance both osteoblast and osteoclast activity and promote bone defect healing in mice.[17] Taken together with the results after using ROCK inhibitors, simultaneous activation of bone resorption and bone formation could be a useful pharmacological approach to bone healing.

Safety is an issue associated with the use of new drugs. One advantage of using ROCK inhibitors for bone regeneration therapy is that they are already in clinical use for treating glaucoma and cerebral vasospasms. In the treatment of these diseases, ROCK inhibitors are administered locally and systemically and are considered safe drugs.[18] Therefore, it would not be difficult to repurpose ROCK inhibitors as agents promoting bone healing. Overall, the present study may serve as a basis for clinical treatments aimed at not only promoting bone healing during DO but also improving bone healing in general.

This study has several limitations. First, the effects of local administration of Y-27632 on other tissues and organs must be fully evaluated to identify potential side effects. However, no abnormal behavior or health conditions were observed in any of the mice in this study. Second, the in vivo study was performed using only a single dose of Y-27632. Although the effect of Y-27632 on DO healing was observed at the current dose, healing could be further enhanced by modifying the pharmacokinetics and treatment duration. Furthermore, because DO healing is a process involving multiple cell types,[19] it would be interesting to examine the complex crosstalk between osteoblasts and osteoclasts and the effects of Y-27632 on cells involved in angiogenesis

and neurogenesis.

To the best of our knowledge, this is the first study to demonstrate that Y-27632 promotes DO healing by activating osteoclasts and osteoblasts. Furthermore, these findings suggest that Y-27632 is a candidate therapeutic agent for DO healing via a novel mechanism.

AUTHOR CONTRIBUTIONS

Study conception and design: MT, MF, and HH. Data acquisition: MT, QC, and MW. Data analysis and interpretation: MT, MF, QC, HB, WM, and YL. Drafting and revision of the manuscript: MT and MF. All the authors approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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