Research article

RHO KINASE INHIBITORS STIMULATE THE MIGRATION OF HUMAN CULTURED OSTEOBLASTIC CELLS BY REGULATING ACTOMYOSIN ACTIVITY

XUEJIAO ZHANG[1,3], CHENG LI[1], HUILING GAO[1], HIROAKI NABEKA[1], TETSUYA SHIMOKAWA[1], HIROYUKI WAKISAKA[1], SEIJI MATSUDA[1] and NAOTO KOBAYASHI[2]*

[1]Department of Anatomy and Embryology, Ehime University Graduate School of Medicine, [2]Medical Education Center, Ehime University School of Medicine, Shitsukawa, To-on City, Ehime 791-0295, Japan, [3]Current address: Shenyang Women and Children Health Center, Shenyang city, Liaoning, 110032, China

Abstract: We investigated the effects of Rho-associated kinase (ROCK) on migration and cytoskeletal organization in primary human osteoblasts and Saos-2 human osteosarcoma cells. Both cell types were exposed to two different ROCK inhibitors, Y-27632 and HA-1077. In the improved motility assay used in the present study, Y-27632 and HA-1077 significantly increased the migration of both osteoblasts and osteosarcoma cells on plastic in a dose-dependent and reversible manner. Fluorescent images showed that cells of both types cultured with Y-27632 or HA-1077 exhibited a stellate appearance, with poor assembly of stress fibers and focal contacts. Western blotting showed that ROCK inhibitors reduced myosin light chain (MLC) phosphorylation within 5 min without affecting overall myosin light-chain protein levels. Inhibition of ROCK activity is thought to enhance the migration of human osteoblasts through reorganization of the actin cytoskeleton and regulation of myosin activity.

* Author for correspondence. e-mail: naoto@m.ehime-u.ac.jp, phone: 81-89-960-5928, fax: 81-89-960-5931

ROCK inhibitors may be potentially useful as anabolic agents to enhance the biocompatibility of bone and joint prostheses.

Key words: Osteoblast, Migration, ROCK inhibitor, Cytoskeleton, Stress fiber, Focal contact, MLC phosphorylation

INTRODUCTION

Biocompatibility refers to the ability of a biomaterial to perform its desired function with respect to medical therapy, without eliciting any undesirable local or systemic effects in the recipient of that therapy [1]. To assess whether various implant surface modifications are effective in optimizing biocompatibility, the behavior of osteoblasts on the surfaces of implants such as dental implants, bone and joint prostheses is often examined, with considerable attention being paid to their adhesion, spreading, migration, proliferation, and differentiation [2-4]. Positive cell behaviors, such as stronger adhesion, fast migration, and good proliferation often indicate higher biocompatibility of the biomaterial [5, 6].

Cell migration is a dynamic and carefully regulated process. Directed cell migration involves coordination between membrane protrusive activity at a leading edge, movement of the cell body, and retraction of the rear of the cell. Moreover, dynamic changes in stress fiber organization and cell adhesion are necessary for cell migration [7, 8]. Focal contacts play an important role in both formation of new membrane extensions at the leading edge and retraction of the membrane at the rear of the cell [9].

The Rho-associated kinase (ROCK) pathway plays an important role in cell migration. A small GTPase, Rho, promotes the formation of stress fibers and focal contacts, thereby increasing actin–myosin contractility [10]. It acts through its effector, ROCK, to enhance the contraction of both smooth muscle cells and non-muscle cells through either inactivation of myosin phosphatase or direct phosphorylation of myosin light chain (MLC) [10, 11]. Contractile forces can also be modulated by MLC kinase (MLCK), which promotes actin–myosin interactions and the phosphorylation of MLC [12].

Several different ROCK inhibitors have been used to probe the function of ROCK in various cells. ROCK inhibitors have been reported to induce osteoblast differentiation and bone formation [13, 14]. However, although ROCK is reported to be involved in cell differentiation, proliferation, and migration [15-17], the roles of the Rho–ROCK pathway in osteoblast migration remain largely unknown.

In the present study, we examined the effects of the ROCK inhibitors Y-27632 and HA-1077 (also known as fasudil) [18, 19] on migration and cytoskeletal organization in cultured osteoblasts. Using an improved migration assay, we found that ROCK inhibitors enhance the migration of, and induce morphological changes in, human osteoblasts through reorganization of the actin cytoskeleton and regulation of myosin activity.

MATERIALS AND METHODS

Reagents and antibodies

Three ROCK inhibitors (Y-27632, HA-1077, and H1152) were purchased from Wako chemicals (Osaka, Japan), epidermal growth factor (EGF) and fibroblast growth factor (FGF) from Sigma (St. Louis, MO, USA), and platelet-derived growth factor (PDGF) from R&D Systems (Minneapolis, MN, USA). Alexa Fluor 488-conjugated phalloidin was obtained from Molecular Probes (Eugene, OR, USA), Cy3- and Cy5-conjugated secondary antibodies from Rockland (Gilbertsville, PA, USA), and DAPI (4',6-diamidino-2-phenylindole) from Wako. Anti-GAPDH antibody was purchased from Imegenex (San Diego, CA, USA), anti-α-tubulin antibody from CalBiochem (Darmstadt, Germany), and anti-beta-actin, anti-vinculin, anti-vimentin, anti-MLC, and anti-MLCK antibodies from Sigma (St. Louis, MO, USA). Anti-phosphorylated-myosin light-chain 2 (P-MLC2) antibody, which recognizes myosin light-chain 2 (MLC2) phosphorylated at both Thr18 and Ser19 (according to the manufacturer's data), was purchased from Cell Signaling (Danvers, MA, USA). (MLC2, also known as myosin regulatory light chain, is known to be phosphorylated at Thr18 and Ser19 by MLCK in a Ca^{2+}-/calmodulin-dependent manner [20], and at Ser19 by ROCK [21]).

Cell culture

Primary human osteoblasts (HOb cells) purchased from Cell Applications Inc. (San Diego, CA, USA) were maintained in growth medium (Cell Applications Inc.) at 37°C in a humid atmosphere comprising 95% air and 5% CO_2. Saos-2 cells (provided by Dr. H. Kawahara, Institute of Clinical Materials, Osaka, Japan) were cultured in Dulbecco's modified Eagle's medium (Wako) containing 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Monolayers of both cell types were harvested using trypsin/EDTA at over 80% confluence, and were then seeded to plastic dishes at low density for cell immunofluorescence analysis and at a higher density for cell motility assays (for which confluent monolayers were required). Toxicity of the ROCK inhibitors was tested using the cell viability assay by measuring the number of living cells with Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) following the manufacturers' instructions.

Cell motility assays

A sterile glass coverslip was placed at the center of the tissue culture dish and overlaid with two sterile iron blocks (20 mm diameter; 80 g), which tightly pressed it onto the bottom of the dish. HOb cells or Saos-2 cells were then seeded to the culture dish. 24 h later, when the cells had reached full confluence, the glass coverslip and iron blocks were removed to allow cells to migrate into the cell-free rectangular area where the coverslip had prevented cell adhesion and growth (Fig. 1A). Photographs were taken at different time points using a BS-41L cooled charge-coupled device (CCD) camera (Bitran, Tokyo, Japan)

coupled with an inverted microscope (Olympus, Tokyo, Japan). Data were then analyzed using Image software version 1.63 (NIH, Rockville, MD, USA). Cells whose nuclei were located in the rectangular area that had previously been cell-free were judged to be "migrating" (Fig. 1A, arrows). Numbers of migrating cells were counted and ratios of the numbers of migrating cells in the ROCK inhibition groups to those in the control groups then calculated. The migration distance was defined as the distance between the nucleus of an individual cell at different time points (Fig. 1A). "Migrating" cells were identified and tracked for the remainder of the experiment. For each identified cell, the migration distance was defined as "the distance of movement of the farthest forward edge of its nucleus." In each experiment, more than 20 cells were identified and an average migration distance calculated. The mean ± SEM of three independent experiments were calculated.
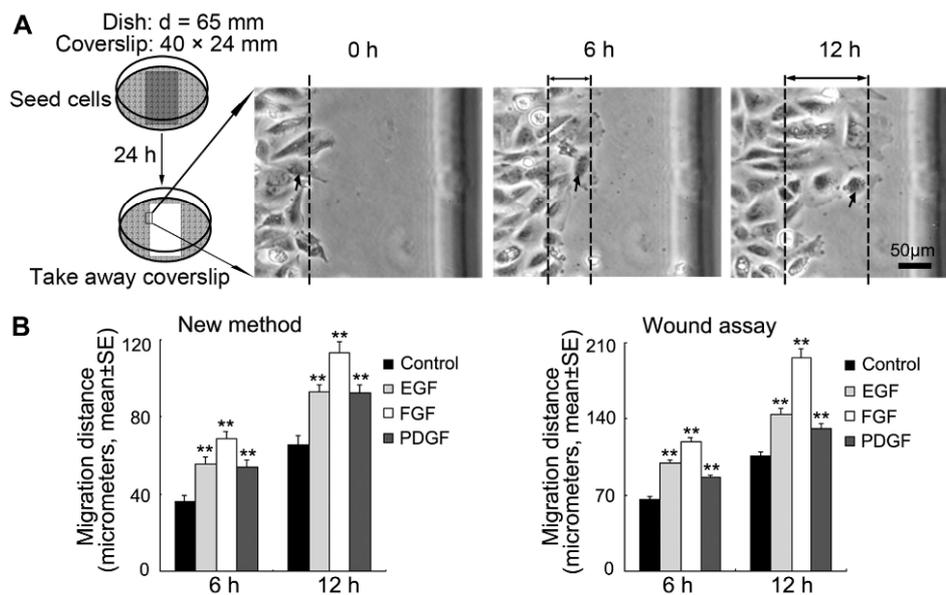

Fig. 1. Cell migration assay designed for the analysis of primary culture of human osteoblasts. A – Schematic illustration of the assay optimized for primary osteoblasts. Cells judged to be "migrating" are identified by arrows. The distance between two dashed lines defines the migration distance. B – The migration of Saos-2 cells in the presence of various growth factors, measured by the improved migration assay (left) and a conventional wound assay (right). The two assays produced comparable results. EGF, FGF, and PDGF were applied at a concentration of 50 ng/ml for 6 and 12 h.**, $P < 0.01$ compared to the control.

Immunocytochemistry

HOb and Saos-2 cells were seeded onto glass coverslips at a low cell density to permit individual cells to grow separately. After incubation for 24 h, cells were treated with ROCK inhibitors for different periods of time. Thereafter, they were

fixed, permeabilized, and blocked as described previously [22]. They were then incubated with primary antibodies for 1 h, and then with the mixture of Cy3- or Cy5-conjugated secondary antibody, Alexa Fluor 488-conjugated phalloidin (to visualize F-actin), and DAPI (to stain nuclei) for 1 h. All procedures were performed at room temperature. Stained cells were then viewed under an A1 confocal laser microscope (Nikon, Tokyo, Japan). Digital images were captured in bitmap format using Nikon A1 software. Data were analyzed using BZ Analyzer Software (Keyence, Tokyo, Japan).

Western blotting
HOb and Saos-2 cells were treated with Y-27632 or HA-1077 (25 μM) for different periods of time. They were then harvested on ice using cold lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100] containing protease inhibitor and phosphatase inhibitor cocktails (Nacalai Tesque, Kyoto, Japan). The resulting homogenates were solubilized in Laemmli sample buffer. 15 μg of each protein sample were loaded onto 10-15% SDS-PAGE gels. Following electrophoretic separation, proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) using a semidry blotting system. Membranes were blocked in tris-buffered saline Tween 20 [20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.1% Triton X-100] containing 5% nonfat dry milk for 2 h. After washing with TBST, they were next incubated with primary antibody (dilution 1:500-1:5,000) at 4°C overnight. After washing with TBST, the membranes were further treated with the anti-mouse or anti-rabbit horseradish perdoxidase-conjugated secondary antibody for 1 h at room temperature. After extensive washing with TBST, antibody binding was visualized through the treatment of membranes with enhanced chemical luminescence (ECL) Detection Reagent (GE Healthcare, Little Chalfont, Buckinghamshire, UK), followed by exposure to instant positive films (FujiFilm, Tokyo, Japan). GAPDH was selected as a housekeeping protein to confirm that equal amounts of protein were loaded to each lane. Band intensities were analyzed using a ChemiDoc XRS imaging system with Quantity One software (Bio-Rad, Hercules, CA, USA).

Statistical analyses
Data are presented as the mean ± SEM. Differences between groups were identified through analysis of variation (ANOVA), performed using StatView software (Abacus Concepts, Berkeley, CA, USA). *P*-values less than 0.05 were considered to be statistically significant.

RESULTS

New cell motility assay designed for the primary culture of human osteoblasts
In the present study, we tested not only Saos-2 osteosarcoma cells, but also primary HOb cells, in a motility assay. Primary cells represent the best *in vitro* models, since they retain their original *in vivo* characteristics, which are

sometimes lost in transformed cell lines. To measure osteoblast motility on plastic, the well-known "*in vitro* wound" assay [23] was found to be successful for Saos-2 cells. However, it could not be applied to HOb cells, which detached from the substratum at the wound margins after wounds had been made using a plastic pipet tip. Moreover, we were unable to create wounds with straight edges. We speculate that HOb cells secrete greater amounts of extracellular matrix and are more adhesive than Saos-2 cells. Therefore, we designed a new method, as described in the Materials and Methods.

During fracture healing, PDGF has been shown to stimulate Saos-2 cell migration [24]. Many other growth factors are also involved in the process [25]. The growth factors PDGF, EGF, and FGF (50 ng/ml each) were tested both by our new technique and a conventional one. Both methods yielded similar results, although the rate of migration of both control and reagent-treated cells in our new assay was only approximately one-half of that in the wound assay (Fig. 1B). As previously reported [26], extracellular matrix left behind when wounds are made using a pipet tip may promote cell motility. We concluded therefore, that our newly developed method compares favourably with the "wound" assay in terms of validity, reliability, and reproducibility.

Effects of ROCK inhibitors on the motility of HOb and Saos-2 cells

To assess the effects of ROCK inhibition, we used two ROCK inhibitors, Y-27632 and HA-1077 [18, 19]. Both compounds are competitive inhibitors of ROCK that bind at the ATP-binding site, although they have different chemical structures [27]. We evaluated their effects on the migration of HOb and Saos-2 cells on plastic (Fig. 2). Both Y-27632 and HA-1077 induced drastic changes in cell shape, inducing the formation of large numbers of protrusions, most of which pointed in the direction of migration (Fig. 2A). Inhibition of ROCK with Y-27632 increased the migration rate of HOb cells nearly two-fold at 6 h and 12 h, and increased the numbers of migrating HOb cells about three-fold compared to the control. HA-1077 produced similar effects on HOb cell migration (Fig. 2B and 2C). Similar responses were also observed in Saos-2 cells, although they were more modest than those in HOb cells (Fig. 2E and 2F). We also tested the effects of a third ROCK inhibitor, H1152 [28], with the conventional assay, and H1152 at 1 μM enhanced Saos-2 cell migration with the extent similar to that with Y-27632 and HA-1077 (data not shown).

Next, we tested whether the effects of Y-27632 and HA-1077 were dose-dependent. Migration was measured in cells treated with Y-27632 and HA-1077 at concentrations ranging from 0 to 50 μM for 12 h. The two ROCK inhibitors significantly increased HOb cell migration in a dose-dependent manner (Fig. 2D). Remarkably, the highest rates of Saos-2 cell migration were detected in cells treated with Y-27632 and HA-1077 at a concentration of 25 μM, not 50 μM (Fig. 2G), which suggests that Saos-2 cells are more susceptible to ROCK inhibition than HOb cells. To confirm the reversibility of the response, we treated cells with 50 μM Y-27632 or HA-1077 for 12 h, and then replaced the

growth medium with medium containing no ROCK inhibitors, before incubating the cells for a further 12 h. Immunofluorescence staining showed that the cell types tested completely regained their original morphology (Fig. 3G). In addition, the cell viability assay showed no significant cell damage with either of the ROCK inhibitors at 50 μM for 12 hours (data not shown). Since all of the ROCK inhibitors tested had similar effects on Saos-2 cells migration, selectivity of the inhibitors is thought to be proven. Taken together, these observations show that ROCK inhibition enhanced the migration of HOb and Saos-2 cells in a dose-dependent and reversible manner.
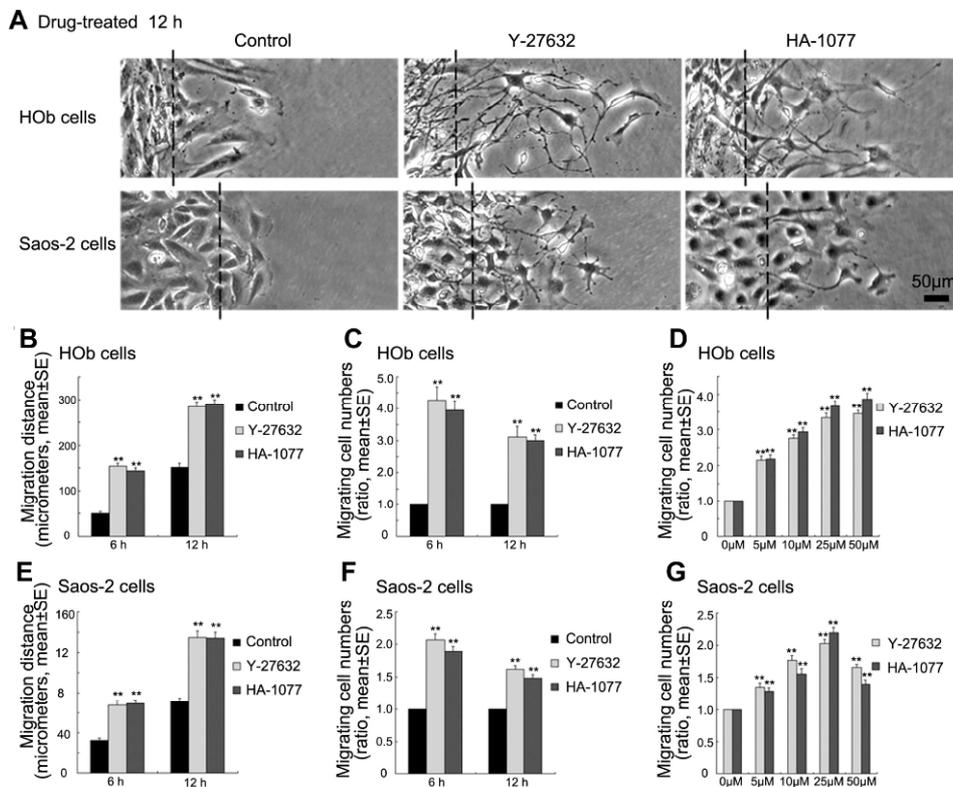

Fig. 2. Dose-dependent and reversible enhancement of migration in HOb and Saos-2 cells by the ROCK inhibitors Y-27632 and HA-1077. A – Treatment with Y-27632 and HA-1077 at a concentration of 50 μM resulted in increased migration into the rectangular cell-free area and an altered cell morphology, characterized by multiple protrusions, the majority extending in the direction of migration. Dashed lines indicate the position of cells at the beginning of the migration assay. B, C, E, F – For both cell types, migration distance and number of "migrating" cells are shown. Y-27632 and HA-1077 were applied at 50 μM. D, G – ROCK inhibitors enhanced the migration of both cell types in a dose-dependent manner. Y-27632 and HA-1077 were applied at a concentration ranging from 0 to 50 μM. **$P < 0.01$ vs. control.
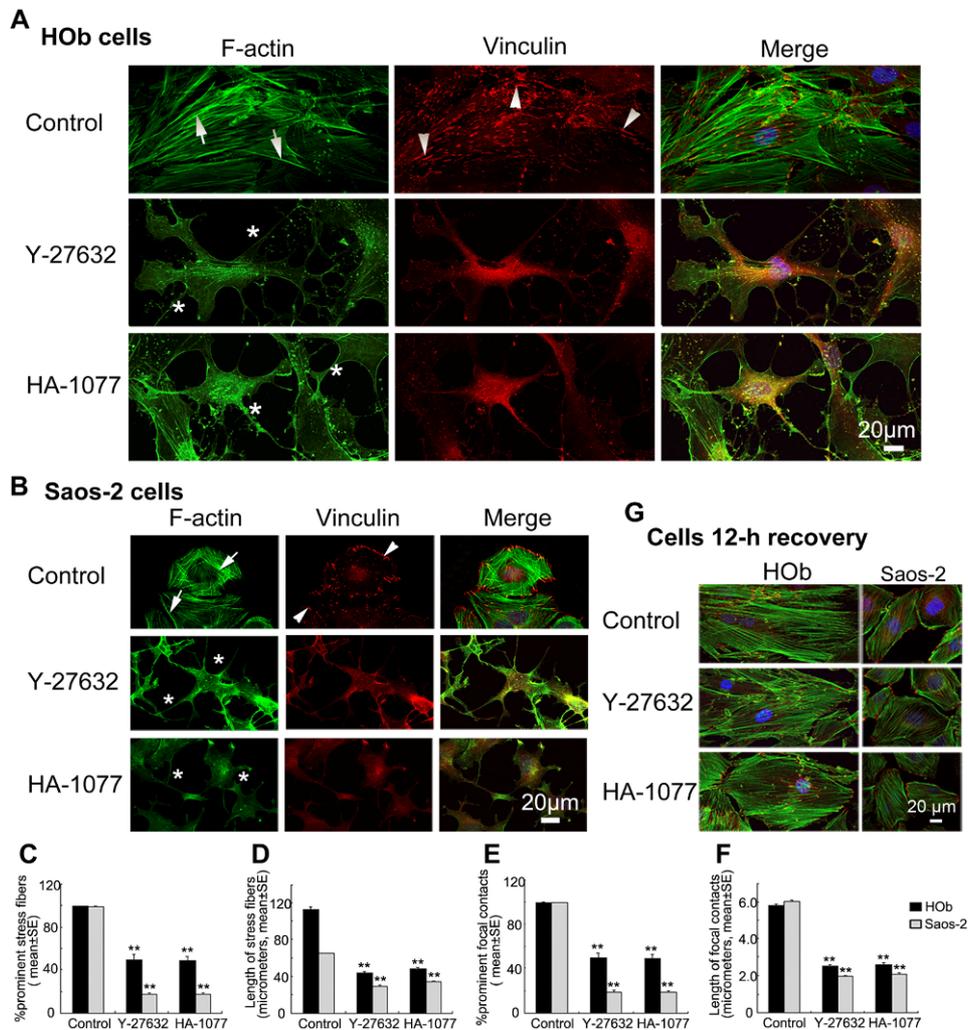
Fig. 3. Effects of ROCK inhibitors on the morphology and cytoskeleton of cultured osteoblasts. Y-27632 and HA-1077 were applied at a concentration of 25 μM for 9 h. A, B – Cells cultured with either Y-27632 or HA-1077 exhibited a stellate appearance, with a limited volume of perinuclear cytoplasm and long, thin, actin-rich protrusions *. Both HOb cells and Saos-2 cells treated with the ROCK inhibitors displayed poor assembly of stress fibers and focal contacts, whereas prominent F-actin (arrows) and vinculin staining (arrow heads) occurred in control cells. (Actin stress fibers were labeled with phalloidin and focal contacts with an anti-vinculin antibody.) C, E – Fewer inhibitor-treated cells displayed prominent F-actin and vinculin labeling than control cells (both kinds of cell). D, F – Stress fibers and the long axes of focal contacts were much shorter in inhibitor-treated cells than in control cells of both types. G – Cells were incubated with Y-27632 and HA-1077 (50 μM) for 12 h and then cultured in drug-free medium for a further 12 h. Overall morphology and cytoskeletal organization recovered completely after removal of the ROCK inhibitors. **$P < 0.01$.

Effects of ROCK inhibitors on the morphology and cytoskeleton of osteoblast cells

As described previously, ROCK inhibitors altered the shape of cultured osteoblasts during their migration (Fig. 2A). Rho GTPases are known to control the organization of the actin cytoskeleton and assembly of focal adhesions [10]. We therefore next examined the effects of ROCK inhibitors on the morphology of HOb and Saos-2 cells (Fig. 3). When treated with either Y-27632 or HA-1077 at a concentration of 25 μM for 9 h, cells of both types displayed a stellate appearance, with a small perinuclear region and long, actin-rich protrusions, as well as poorer assembly of stress fibers and focal contacts than control cells (Fig. 3A and 3B).

We next determined the percentage of cells with prominent stress fibers/focal contacts and measured the lengths of the stress fibers and the long axes of the focal contacts. While nearly all non-treated cells displayed prominent stress fibers and focal contacts, Y-27632 or HA-1077 reduced these proportions to around 50% in HOb cells and about 20% in Saos-2 cells (Fig. 3C and 3E). Moreover, the lengths of the stress fibers and the long axes of focal contacts decreased in both cell types following ROCK inhibition (Fig. 3D and 3F). Therefore, ROCK inhibitors clearly suppressed the assembly of actin stress fibers and vinculin-containing focal contacts in human cultured osteoblasts.

We then examined whether ROCK inhibition impacts upon other cytoskeletal elements in osteoblasts, namely microtubules and intermediate filaments. Western blot analysis showed that treatment with Y-27632 or HA-1077 at a concentration of 25 μM for 12 h did not affect the expression of α-tubulin (one of the subunit proteins of microtubules, Fig. 4A) and vimentin (a major intermediate filament protein in osteoblasts, data not shown) in human osteoblasts. Immunofluorescence staining following treatment with Y-27632 or HA-1077 (25 μM, 9 h) showed that ROCK inhibition did not affect the assembly of microtubules (Fig. 4B) and intermediate filaments (data not shown), although they were concentrated in the long, thin protrusions formed in inhibitor-treated cells.

Effects of ROCK inhibition on MLC and MLCK in HOb and Saos-2 cells

In non-muscle cells, contractile activity, a key contributor to cell shape and migration, is triggered by the phosphorylation of MLC [29]. As revealed by quantitative Western blot analysis, treatment with ROCK inhibitors at a concentration of 25 μM for 5 min significantly reduced the phosphorylation of MLC in both osteoblast cultures, without affecting overall MLC protein levels (Fig. 5A and 5B). Rapid dephosphorylation of MLC was confirmed by immunostaining in both kinds of osteoblastic cells (Fig. 5C and 5D). In control cells, MLC and P-MLC co-localized with stress fibers, while in ROCK inhibitor-treated cells, the fluorescent label was concentrated in membrane ruffles (arrows), especially in Saos-2 cells. In addition, Inhibition of ROCK significantly increased MLCK levels in both kinds of cells (Fig. 5A and 5B).
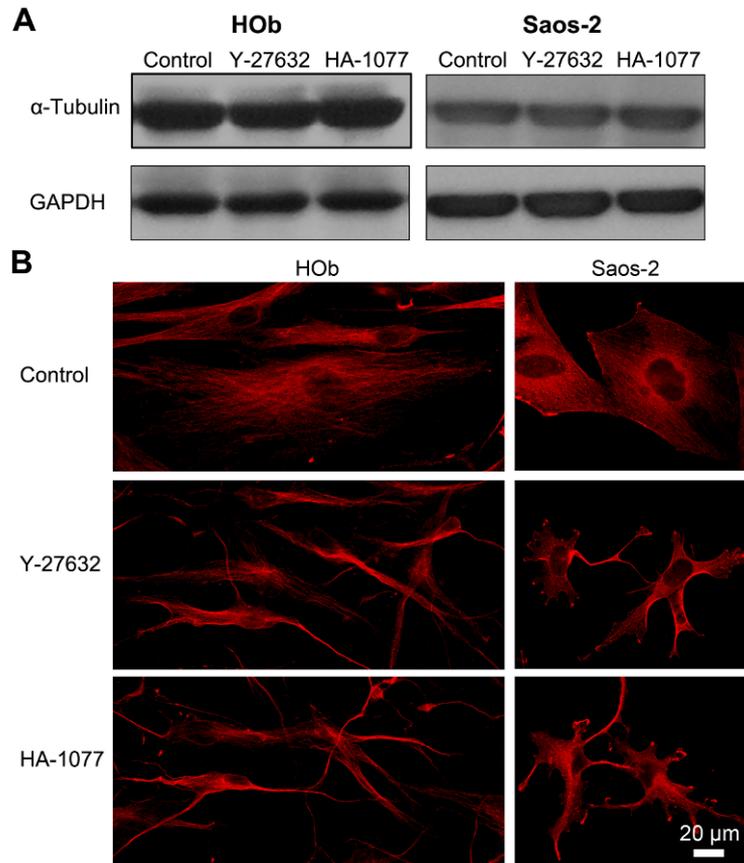
Fig. 4. Effects of inhibition of ROCK on microtubules in both HOb and Saos-2 cells. Cells were incubated with one of the two ROCK inhibitors (concentration 25 μM) for 9 h or 12 h and then subjected to western blot or immunofluorescence analysis. A – Representative western blot images are shown. ROCK inhibitors did not affect the expression of α-tubulin (one of the subunit proteins of microtubules). B – Immunofluorescence staining shows that ROCK inhibition did not affect the assembly of microtubules, although they were concentrated in the long, thin protrusions formed in inhibitor-treated cells.

Fig. 5. Inhibition of ROCK activity reduced the phosphorylation of myosin light chain and increased levels of myosin light-chain kinase (MLCK) in both HOb and Saos-2 cells. Cells were incubated with one of the two ROCK inhibitors (concentration 25 μM) for 5 min and then subjected to Western blot or immunofluorescence analysis. A, B – Representative western blot images (upper panels) and quantitative evaluation of the band intensities (lower panels) are shown. Data represent the mean ± SEM of three independent assays. ROCK inhibitors reduced phosphorylation of MLC, without influencing overall MLC protein levels and increased levels of MLCK. *$P < 0.05$. C, D – Immunofluorescence staining showing the co-localization of MLC and P-MLC on stress fibers in control cells. ROCK inhibition significantly reduced the P-MLC signal. The fluorescent label was concentrated in membrane ruffles (arrows) in ROCK inhibitor-treated cells, especially in Saos-2 cells.
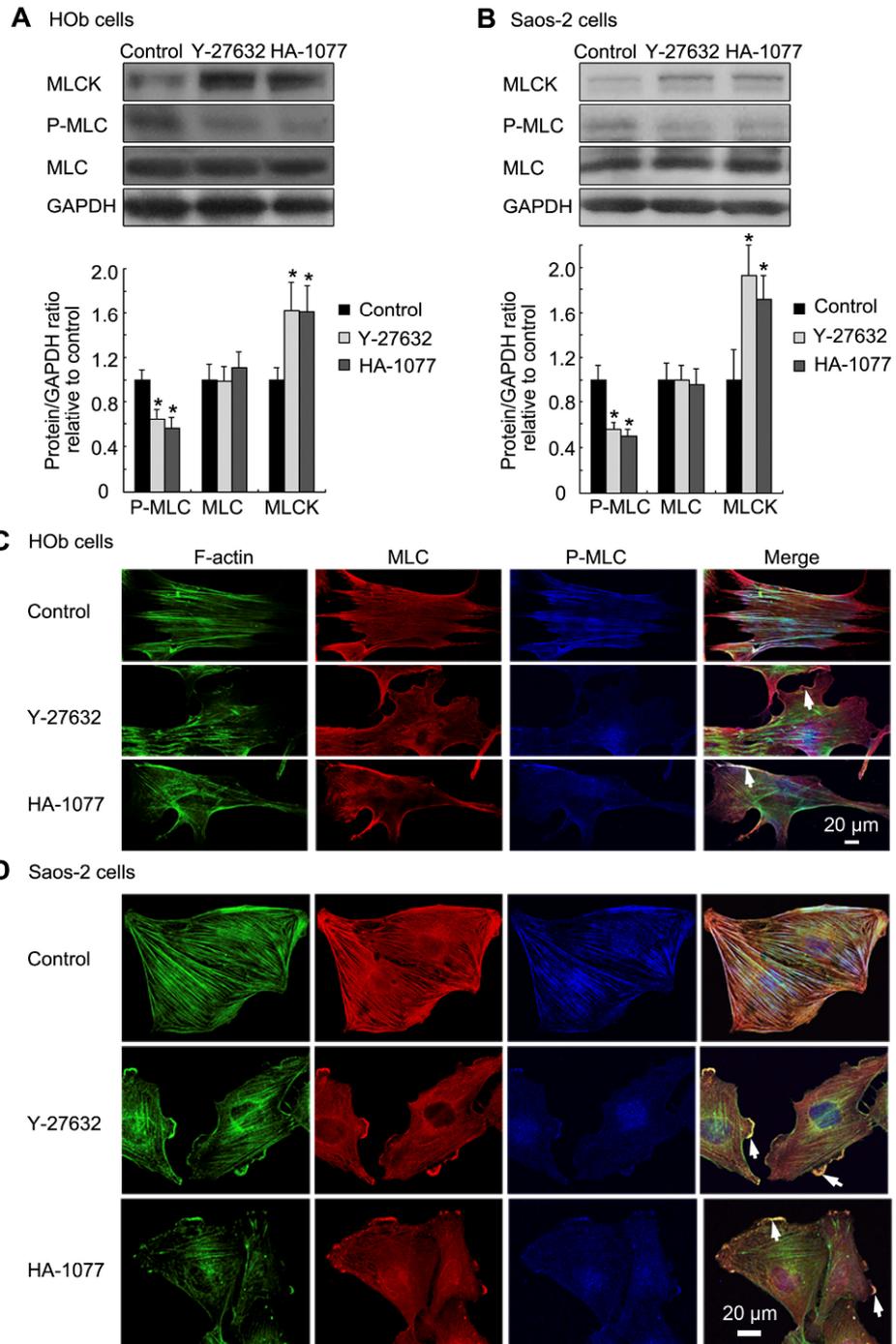
DISCUSSION

Two methods – the Boyden chamber assay and the "*in vitro* wound" assay –have long been used to investigate different aspects of cell movement. The Boyden chamber assay tests directional chemotactic migration, whereas the *in vitro* wound assay measures general cell motility. To analyze the motility of HOb cells, which are far more adhesive than Saos-2 cells, we developed a new method, which is described in the Materials and Methods and Results sections (Fig. 1). With this newly designed assay, we provided, for the first time, data describing the roles of ROCK in the regulation of migration, cell morphology, and the cytoskeleton in primary HOb and Saos-2 human osteosarcoma cells.

Previous studies have shown both increases and decreases in migration rate after ROCK inhibition depending on cell type [30-33]. In the present study, the inhibition of ROCK stimulated the migration of both HOb and Saos-2 cells (Fig. 2). Our fluorescent images showed that the two tested ROCK inhibitors caused cells to adopt a stellate appearance, characterized by a limited volume of perinuclear cytoplasm and long, actin-rich protrusions. They further induced reorganization of the cytoskeleton and induced the disassembly of stress fibers and focal contacts (Fig. 3). As shown by others and according to the findings of the present study, Y-27632 and HA-1077 enhance cell migration through reorganization of the actin cytoskeleton [30, 34, 35]. This is accordance with the idea that stabilization of actin stress fibers limits cell movement [33].

In order to confirm the specificity of ROCK inhibitors, we used two different reagents, Y-27632 and HA-1077, for the migration assay, both of which gave similar results with both types of osteoblastic cells (Fig. 2). Moreover, we tested a third ROCK inhibitor, H1152 [28], after the conventional assay, and again demonstrated similar results compared to those with other two reagents (data not shown). In addition to pharmacological inhibitors, siRNA has been used to suppress ROCK expression. Kroening et al. [36] reported that siRNA against ROCK increased migration of renal epithelial cells and also induced long filopodia at the migration front, showing a result similar to our pharmacological study. Taken together, the specificity of our inhibitor study is thought to be convincing.

Rho GTPases and ROCK have been shown to control the formation of stress fibers and focal adhesion assembly by modulating MLC phosphorylation [21, 37]. In agreement with previous data, ROCK inhibition rapidly decreased MLC phosphorylation in osteoblasts in the present study. Decreased phosphorylation of MLC limits the activity of myosin [38]. Inactive myosin causes cells to lose tension and induces the disappearance of actin–myosin bundles and mature focal cell–matrix adhesions [39]. Destruction of stress fibers after contraction can lead to a shift in mDia1 activity in a way that promotes microtubule stabilization [40], which maintains polarization during cell migration (Fig. 4) [39]. In addition, Rac activity increases when ROCK is inhibited [31]. Active Rac stimulates the protrusion activity and thereby increases the cell migration rate. In addition, we

showed that ROCK inhibition induced the formation of membrane ruffles where phosphorylated MLC molecules were concentrated with actin filaments (Fig. 5C and 5D). Since membrane ruffles are important for cell motility, the induction of their formation may enhance the migration of osteoblasts.

MLC can be phosphorylated by MLCK [41]. The levels of MLCK were increased following ROCK inhibitor treatment (Fig. 5A and 5B). This upregulation may be a feedback effect of decreased MLC phosphorylation. Since high levels of MLCK, and consequently elevated actomyosin contractility, strengthen cell attachment at leading edges and increase the turnover of focal contacts [42, 43], MLCK may indirectly stimulate osteoblast migration.

Based on our findings, we propose a model to describe the effects of ROCK inhibition on osteoblasts (Fig. 6). ROCK inhibitors decrease MLC phosphorylation, and then induce actin reorganization and focal contact disassembly, as well as the upregulation of MLCK levels. While Rac activation stimulates protrusion activity, MLCK is important for the stimulation of cell migration. As a consequence, osteoblasts change their shape and migrate more rapidly after ROCK inhibitor treatment. Other proteins and kinases such as focal adhesion kinase (FAK), Src, and/or paxillin may also be involved in the ROCK signaling pathway.
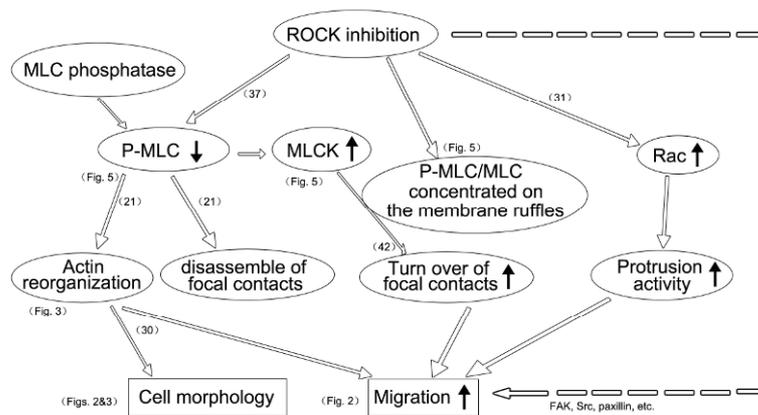

Fig. 6. Proposed mechanism of action of ROCK inhibitors on osteoblasts. ROCK inhibitors reduce MLC phosphorylation, and then induce actin reorganization and focal contact disassembly, as well as the upregulation of MLCK levels. ROCK inhibitors also increase Rac activity. While Rac activation stimulates protrusion activity, high expression of MLCK is important for attachment at the leading edge and increases focal contact turnover. As a consequence, osteoblasts change their shape and migrate more quickly after ROCK inhibitor treatment. The numbers in brackets identify the references used.

In summary, we developed a new method to measure the motility of osteoblastic cells, including primary HOb cells. ROCK inhibitors enhance the migration of human osteoblasts by inducing reorganization of the actin cytoskeleton and by regulating myosin activity. Since the Rho–ROCK pathway is an important

negative regulator of osteoblast behaviors [13, 14, 44], ROCK inhibitors may potentially be useful as anabolic agents to enhance the biocompatibility of bone and joint prostheses.

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