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# RhoA-Mediated Signaling in Mechanotransduction of Osteoblasts

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**Osteoblasts play a pivotal role in load-driven bone formation by activating Wnt signaling through a signal from osteocytes as a mechanosensor. Osteoblasts are also sensitive to mechanical stimulation, but the role of RhoA, a small GTPase involved in the regulation of cytoskeleton adhesion complexes, in mechanotransduction of osteoblasts is not completely understood. Using MC3T3-E1 osteoblast-like cells under 1 hr flow treatment at 10 dyn/cm$^2$, we examined a hypothesis that RhoA signaling mediates the cellular responses to flow-induced shear stress. To test the hypothesis, we conducted genome-wide pathway analysis and evaluated the role of RhoA in molecular signaling. Activity of RhoA was determined with a RhoA biosensor, which determined the activation state of RhoA based on a fluorescence resonance energy transfer between CFP and YFP fluorophores. A pathway analysis indicated that flow treatment activated phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling as well as a circadian regulatory pathway. Western blot analysis revealed that in response to flow treatment phosphorylation of Akt in PI3K signaling and phosphorylation of p38 and ERK1/2 in MAPK signaling were induced. FRET measurement showed that RhoA was activated by flow treatment, and an inhibitor to a Rho kinase significantly reduced flow-induced phosphorylation of p38, ERK1/2, and Akt as well as flow-driven elevation of the mRNA levels of osteopontin and cyclooxygenase-2. Collectively, the result demonstrates that in response to 1 hr flow treatment to MC3T3-E1 cells at 10 dyn/cm$^2$, RhoA plays a critical role in activating PI3K and MAPK signaling as well as modulating the circadian regulatory pathway.**

**Keywords:** osteoblasts, mechanical stimulation, MAPK, PI3K, Rho, FRET

## Introduction

Bone is a dynamic tissue which undergoes constant remodeling under the stimuli of various hormones, cytokines, and mechanical stimulus. Mechanical loading is one of the critical stimuli that activates bone remodeling and matrix deposition and enhances bone formation [1–3]. Bone tissue consists of an extracellular fluid compartment, which communicates with the vascular compartment, and therefore an external mechanical loading increases the fluid exchange between the two spaces, which results in increased bone formation and decreased bone resorption [4–6]. Osteocytes, the most abundant cells in the bone matrix, are considered to act as a mechanosensor and direct osteoblast to activate osteogenic genes such as collagen type I and osteocalcin [7].

Although osteoblasts and osteocytes have similarities because they share a common lineage, they have distinct differences, like their responses to mechanical loading and utilization of the various biochemical signaling pathways to perform their respective functions. For example, Wnt signaling is important in osteoblasts for differentiation, proliferation, and mineralization, whereas osteocytes use the Wnt signaling to relay signals of mechanical loading to osteoblasts on the bone surface [8,9]. It has been shown that osteoblasts are also sensitive to mechanical stimulation in the absence of osteocytes [10–12]. In response to mechanical stimulus, a group of genes such as c-Fos, Egr-1, and Atf-3 are known to be transcriptionally activated [13]. There are various reports which show that fluid flow on bone cells results in intracellular calcium mobilization, production of nitric oxide (NO) [14–16] and prostaglandin $E_2$ [14,17,18], and increased expression of genes for osteopontin (Opn) and cyclooxygenase-2 (Cox2) [12,19,20]. Earlier in vitro studies have demonstrated mitogen-activated protein kinase (MAPK), NO synthase pathway [21], Cox pathway, and $G_{i/o}$ pathway are involved in fluid shear-induced upregulation of osteoblast proliferation and differentiation [22]. Many of these studies used oscillatory fluid flow in two- or three-dimensional cell culture model.

In this study we proposed to study the effect of continuous unidirectional fluid flow-induced shear on osteoblast cells and the associated pathways using whole genome microarray studies. The role of an MAPK pathway has been previously established in regulating osteoblast function under oscillatory fluid flow shear condition in MC3T3-E1 cells [12]. We first studied the regulation of various MAPK (ERK1/2 and p38) components and expression of Opn and Cox2 under the effect of unidirectional flow in osteoblast. Opn plays an important role in bone remodeling by helping osteoclast to anchor to the bone matrix [23]. Cox2 has been reported to increase osteoblast proliferation via Akt/FOXO3a/p27(Kip1) and PTEN/Akt/p27(Kip1) signaling [24]. Also fluid flow increases Cox2 expression via a protein kinase A signaling [25].

We hypothesized that fluid flow stress stimulates osteoblast proliferation through activation of proliferative signals. To examine our hypothesis we conducted a genome-wide microarray analysis and predicted molecular signaling driven by unidirectional fluid shear stress. Microarray results showed the involvement of Clock genes and phosphoinositide 3-kinase (PI3K)/Akt signaling in reg-

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ulating Opn and Cox2 expression under fluid flow stress. Being a homeostatic function, bone remodeling may also be subject to circadian regulation, which is evident by the diurnal variation in the protein expression levels of type I collagen and osteocalcin in osteoblasts. Mice lacking molecular clock components (Per and Cry genes) in osteoblast showed increase in bone mass [26]. Here we first report that fluid flow shear stress can also regulate the expression of these clock genes in osteoblast and may control the osteoblast function under mechanical stimuli. To validate the PI3K/Akt signaling, we used pharmacological inhibitors of PI3K signaling, LY294002, and performed polymerase chain reactions (PCR) and western blot for Opn and Cox2 mRNA expression and the phosphorylation level of Akt, respectively.

Since the involvement of MAPK is well known in regulating the osteoblast function under fluid shear stress condition, we hypothesized the involvement of a common effector signal upstream to PI3K and MAPK signaling. Research showed that GTPase RhoA/ROCK signaling plays a significant role in regulating cytoskeletal dynamics and has been shown to be crucial for osteoblast proliferation, differentiation, and survival as well as osteogenic differentiation of bone marrow mesenchymal cells [27]. Other reports have shown that one of the possible mechanisms via which extracellular matrix (ECM) regulates osteoblast differentiation involves MAPK activation downstream of the RhoA/ROCK signaling pathway [28]. RhoA/ROCK signaling also directs the PI3K signaling in various cells. RhoA activates Akt and mediates protection of cardiomyocytes [29] and proliferation in murine prostate cancer (TRAMP) cells [30]. Therefore, using fluorescence resonance energy transfer (FRET) we showed the activation of RhoA under unidirectional fluid shear stress. We investigated the role of Rho/ROCK in regulating both PI3K and MAPK signaling under unidirectional fluid shear stress condition using a ROCK-specific inhibitor, Y27632. Our study clearly demonstrates that RhoA/ROCK plays an important role upstream to PI3K and MAPK signaling in regulated flow-induced osteoblast functions.

## Materials and Methods

### Cell Culture and Flow Treatment

Mouse osteoblast-like cells (MC3T3-E1, clone 4; ATCC CRL-2593) were cultured in αMEM medium containing 10% fetal bovine serum and antibiotics (50 units/mL penicillin and 50 μg/mL streptomycin; Invitrogen, Grand Island, NY, USA). Cells were harvested 1, 3, and 5 hr after the application of fluid flow. Prior to flow treatment, cells were grown on a glass slide coated with type I collagen and incubated in the αMEM medium containing 0.5% fetal bovine serum for 24 hr. Unidirectional flow treatment was applied using Streamer Gold flow device (Flexcell International, Hillsborough, NC, USA) for 1 hr at an intensity of 10 dyn/cm$^2$ [31]. To examine the role of PI3K signaling and RhoA, cells were incubated for 1 hr before flow treatment with 10–40 μM LY294002 and 20 μM Y27632, respectively. The supplementary figure lists the genes altered by 1 h flow treatment and is provided online.

### Quantitative Real-Time PCR

Total RNA was isolated using an RNeasy mini kit (Qiagen, Germantown, MD, USA). Reverse transcription was conducted using ~50 ng of total RNA with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was performed using ABI 7500 with a Power SYBR green PCR master mix kit (Applied Biosystems). The mRNA levels of the selected genes (Cox2, Opn, Per1, Per2, Per3, Cry1, Cry2, Clock, and Bmal1) were evaluated. The PCR primers are listed in Table 1. The mRNA level of Gapdh was used as an internal control to calibrate potential variations in RNA isolation. Relative mRNA abundance was determined as a ratio of the mRNA levels in the presence and absence of flow treatment.

### Microarray and Pathway Analysis

Microarray experiments were conducted using Agilent whole mouse genome arrays (G4122F, Agilent, Santa Clara, CA, USA). A total of six RNA samples were isolated from three pairs of cells grown in the presence and absence of flow treatment. They were labeled with Agilent low RNA input fluorescent linear amplification kits. Isolated RNA samples were hybridized to six one-color arrays using in situ hybridization kits (Agilent). Microarray-derived expression data were filtered to remove background noise, and a modified *t*-test was performed to identify a group of genes that were altered >2-fold or <0.5-fold with statistical significance at $p < 0.01$. The list of genes identified above was imported into Pathway-Express (Wayne State University, Detroit, MI, USA), which was used to predict molecular signaling pathways [32].

### Western Blotting

Cells were harvested in a radio-immunoprecipitation assay lysis buffer containing inhibitors for proteases and phosphatases (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Isolated proteins were fractionated using 10% SDS gels (BioRad, Hercules, CA, USA) and electrotransferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). Immunoblots were carried out using antibodies specific to ERK1/2, phosphorylated ERK1/2 (p-ERK1/2), p38 MAPK, phosphorylated p38 MAPK (p-p38 MAPK), JNK, phosphorylated p46 (p-p46), phosphorylated p54 (p-p54), Akt, and p-Akt (Cell Signaling, Danvers, MA, USA). After incubation with antirabbit IgG antibodies conjugated with horseradish peroxidase (Cell Signaling), signals were detected with enhanced chemiluminescence. Images were captured using a Fujifilm Luminescent image analyzer (Tokyo, Japan).

Table 1. Real-time PCR primers used in this study

| Target | Forward primer | Backward primer |
|---|---|---|
| Cox2 | 5′-ATGACATCGATGCCATGGAA-3′ | 5′-TCAGTAGACTCTTACAGCT-3′ |
| Opn | 5′-ACACTTTCACTCCAATCGTCC-3′ | 5′-TGCCCTTTCCGTTGTTGTCC-3′ |
| Per1 | 5′-CTCAGGTATTTGGAGAGCTGCAA-3′ | 5′-TTGCTGACGACGGATCTTTCT-3′ |
| Per2 | 5′-CTGGCTTCACCATGCCTGTT-3′ | 5′-AAGGCCTGAGGCAGGTTTG-3′ |
| Per3 | 5′-GTGTACACAGTGTGCAAGCAAACA-3′ | 5′-ACGGCCGCGAAGGTATCT-3′ |
| Cry1 | 5′-CCTCTGTCTGATGACCATGATGA-3′ | 5′-CCCAGGCCTTTCTTTCCAA-3′ |
| Cry2 | 5′-AGGCTGCCAAGTGCATCAT-3′ | 5′-AGGAAGGGACAGATGCCAATAG-3′ |
| Clock | 5′-CACCGACAAAGATCCCTACTGAT-3′ | 5′-TGAGACATCGCTGGCTGTGT-3′ |
| Bmal1 | 5′-CAACCTTCCCGCAGCTAACA-3′ | 5′-TCCGCGATCATTCGACCTAT-3′ |
| Gapdh | 5′-TGCACCACCAACTGCTTAG-3′ | 5′- GGATGCAGGGATGATGTTC-3′ |

### FRET Imaging

The RhoA biosensor was a gift from Dr. Michiyuki Matsuda at Kyoto University, Japan [33]. The RhoA FRET biosensor consists of a truncated RhoA, a RhoA-binding domain of an effector protein (protein kinase N; PKN), and a pair of green fluorescent protein mutants, cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). When RhoA is activated, the intramolecular binding of activated RhoA (RhoA-GTP) to the RhoA-binding domain of protein kinase N brings CFP in close proximity to YFP, resulting in an increase in FRET from CFP to YFP. Hence, the activation level of RhoA can be determined by monitoring the FRET changes between CFP emission and YFP emission.

The biosensor was transfected into MC3T3-E1 cells using the Neon transfection system (Invitrogen), and cells were analyzed 24–48 hr after transfection. Prior to flow treatment, cells were plated on type I collagen-coated μ-slides (Ibidi, Martinsried, Germany) for 24 hr in the αMEM medium supplemented with 0.5% fetal bovine serum. During imaging, cells were maintained at 37°C and subjected to flow shear at 10 dyn/cm$^2$. Cells were illuminated with a 100 W Hg lamp, and fluorescent images were recorded every 2 min using a Nikon Ti-E inverted microscope equipped with an Evolve CCD camera (Photometrics, Tucson, AZ, USA), a 40X Plan Fluor 0.75 NA objective, and NIS-Elements software (Nikon, Melville, NY, USA). Activity of RhoA was determined by monitoring the ratio of YFP to CFP emission with the following filter sets (Semrock, Lake Forest, IL, USA): CFP excitation at 438 ± 12 nm; dichroic at 458 nm; CFP emission at 483 ± 16 nm; and YFP emission at 542 ± 14 nm. CFP and YFP emission filters were controlled by a Lambda 10-3 filter changer (Sutter, Novato, CA, USA), and three images were recorded with the following exposure times: DIC (0.1 s), CFP (1 s), and YFP (1 s). Cell-permeable C3 transferase (1 μg/mL, 4 hr; Cytoskeleton Inc., Denver, CO, USA) and dominant negative RhoA-N19 were used to inhibit the activity of RhoA.

### Statistical Analysis

Microarray experiments were conducted using three pairs of samples, and other experiments were carried out three times. Microarray and PCR data were expressed as mean ± SD. Statistical significance from a *t*-test was evaluated at $p < 0.05$. The single and double asterisks indicate $p < 0.05$ and $p < 0.01$, respectively.

## Results

### Flow-Driven Elevation of the Phosphorylation of ERK and p38

In response to 1 hr flow treatment, the phosphorylation levels of ERK and p38 were elevated (Figure 1B). The level of JNK phosphorylation (p46 and p54) was not significantly altered. It has been shown that the mRNA levels of Cox2 and Opn are upregulated by mechanical stimulation. Our PCR results showed that the Cox2 mRNA level was increased at 1 and 3 hr, while the Opn mRNA level was elevated at 3 and 5 hr (Figure 1C). The upregulation of these genes is consistent with previous observation of osteoblasts in response to fluid flow [34].

### Evaluation of PI3K Pathway

Signaling pathway analysis with Pathway-Express using mRNA samples at 1 hr revealed that flow treatment activated PI3K signaling (Figure 2A). To validate the involvement of PI3K in mechanotransduction of MC3T3-E1 cells, we examined the level of p-Akt. Compared with control, western blots showed that the p-Akt level was significantly elevated at 1 hr and reduced to a control level at 3 and 5 hr after flow treatment (Figure 2B). Blocking PI3K signaling by LY294002, an inhibitor selective to PI3K [35], suppressed flow-driven upregulation of the mRNA level of Opn (Figure 2C and D).

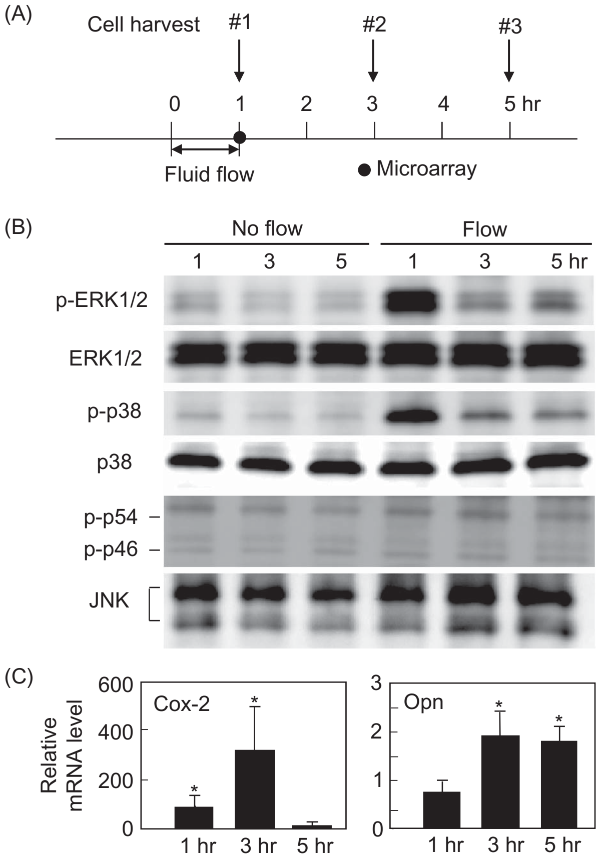

Figure 1. (A) Timeline for the flow experiment. (B) Phosphorylation levels of ERK, p38 MAPK, and JNK (p46 and p54). (C) Relative mRNA abundance of Cox2 and Opn in response to 1 hr flow treatment. The levels are expressed as a fold change to control cells harvested at the same time points. $^*p < 0.05$.

### Involvement of Circadian Regulatory Pathway

Pathway-Express also indicated that the circadian regulatory pathway was involved in the responses to 1 hr fluid flow (Figure 3A). To evaluate the role of mechanical stimulation in the modulation of circadian genes, we determined the mRNA levels of the selected genes (Per1, Per2, Per3, Cry1, Cry2, Clock, and Bmal1) at 1, 3, and 5 hr (Figure 3B). The mRNA levels of four genes (Per1, Per2, Clock, and Bmal1) were significantly elevated at 1 hr, while the level of Per3 mRNA was decreased. At 3 hr, the mRNA levels of Per1, Per3, and Clock were reduced while the levels of Per2 mRNA, Cry1 mRNA, Cry2 mRNA, and Bmal1 mRNA were increased. Thus, although flow treatment changed the mRNA levels of the circadian genes, their temporal profiles did not present the same pattern.

### FRET-Based Activation of RhoA

To evaluate the potential role of RhoA in mechanotransduction of osteoblasts, MC3T3-E1 cells were transfected with the RhoA FRET biosensor that consisted of a pair of fluorophores, CFP and YFP. In response to flow-induced shear stress at 10 dyn/cm$^2$, the RhoA biosensor in the same cell altered its emission ratio of YFP to CFP, which is an index of RhoA. In the color-labeled diagram, the white arrow indicates the direction of flow applied to the

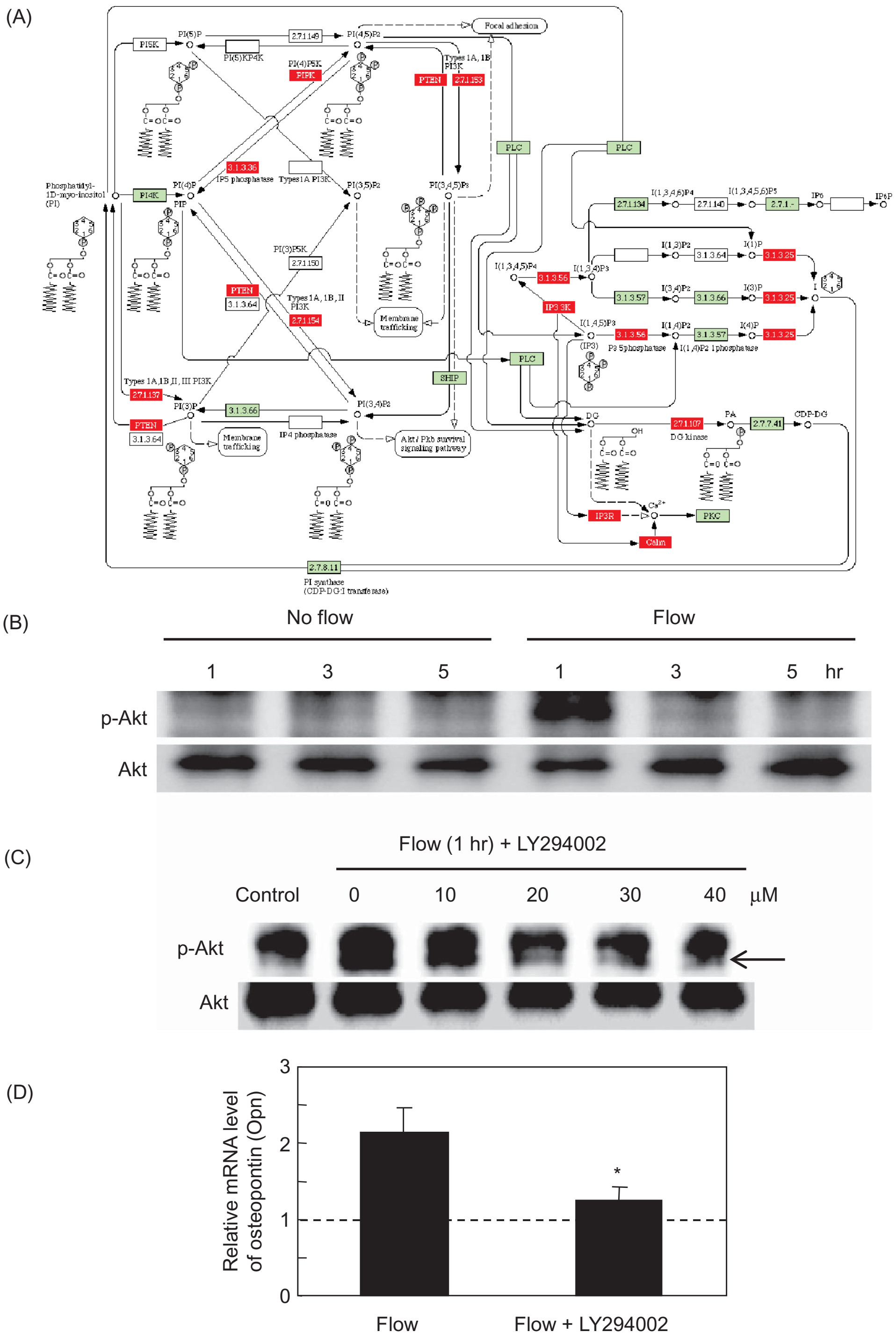

Figure 2. Flow-driven activation of PI3K signaling. (A) PI3K pathway predicted by Pathway-Express. The mRNA levels in the red boxes were significantly altered. (B) Western blots illustrating the elevated phosphorylation level of Akt. (C) Effects of the inhibitor (LY294002), selective to PI3K signaling, on the phosphorylation level of Akt. The band, indicated by the arrow, corresponds to the phosphorylated Akt. (D) Suppression of load-driven upregulation of Opn mRNA by LY294002. $^{*}p < 0.05$.

cells. The hot colors indicate strong RhoA activation, while the cold colors weak activation. The insets, taken from the image at 0 min (no flow) and 56 min (flow), highlight the alterations of FRET-based RhoA activity (Figure 4A). In the presence of a cell-permeable C3 transferase that prevents RhoA activation, however, no significant alteration in the emission ratio of YFP to CFP was observed (Figure 4B). To further examine the specificity of RhoA, we co-transfected the cell with a dominant negative RhoA (RhoA-N19) and RhoA biosensor. RhoA activity of the cell transfected with a dominant mutant RhoA was not altered in response to shear stress (Figure 4C).

## Effects of RhoA on MAPK and PI3K Signaling

Flow treatment to MC3T3-E1 cells induced MAPK and PI3K signaling together with the activation of RhoA. To examine the potential effect of RhoA on MAPK and PI3K signaling, we determined the phosphorylation level of ERK1/2, p38, and Akt in the presence of Y27632, the selective inhibitor of Rho kinase, with and

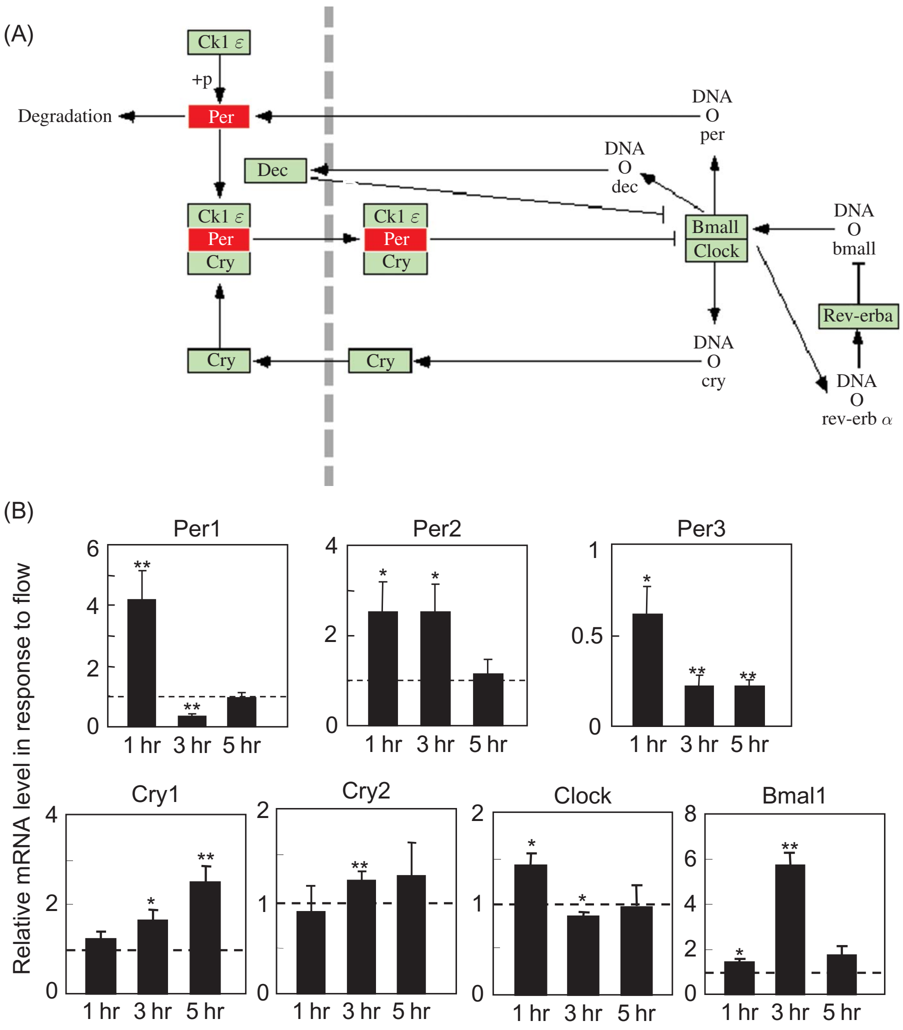

Figure 3. Involvement of a circadian regulator pathway. (A) Circadian regulator pathway predicted by Pathway-Express. (B) Relative mRNA abundance in response to 1 hr flow treatment. The data include the relative mRNA levels of Per1, Per2, Per3, Cry1, Cry2, Clock, and Bmal1 at 1, 3, and 5 hr. $^{*}p < 0.05$; $^{**}p < 0.01$.

without flow treatment (Figure 5A). Y27632 suppressed flow-induced phosphorylation of ERK1/2, p38, and Akt by 41%, 58%, and 16%, respectively, at 1 hr. Furthermore, it significantly reduced the mRNA levels of Cox2 and Opn at 3 hr, although it did not abolish flow-driven upregulation of their mRNA levels. The inhibitor decreased the mRNA level of Per2, but it did not alter Per1 mRNA and Per3 mRNA levels.

## Discussion

Mechanical stimulation plays a critical role in the development and differentiation of osteoblasts [36]. This study demonstrated that the application of 1 hr flow treatment to MC3T3-E1 cells activated a small GTPase, RhoA, and induced the phosphorylation of ERK1/2, p38, and Akt. The activation of RhoA was visualized using the FRET biosensor, whose emission ratio of YFP to CFP was significantly altered in response to flow shear at 10 dyn/cm$^2$. The inhibition of RhoA with cell-permeable C3 transferase reduced a basal level of RhoA activity and abolished a fluorescence energy transfer from CFP to YFP. The inhibitor selective to Rho kinase (Y27632) significantly reduced the phosphorylation levels of ERK1/2, p38, and Akt. The mRNA level of Opn was elevated by flow treatment, and its flow-induced elevation was partially suppressed by the inhibitor of PI3K signaling (LY294002) as well as the inhibitor of Rho kinase. Furthermore, flow treatment modulated the circadian regulatory pathway. The mRNA expression of Per2 was affected by Y27632, while those of Per1 and Per3 were not mediated by RhoA.

Rho GTPase signaling is known to be involved in cell proliferation through integrin and focal adhesion molecules [28]. Single cell analysis with the FRET biosensor in this study suggested that the activation of RhoA altered its molecular conformation and elevated intramolecular FRET from CFP to YFP. An activated domain was observed throughout the cell, and it was particularly noticeable on cellular boundaries in the insets (Figure 4A). Together with a Rho-associated kinase, ROCK, Rho mediates transmission of mechanical forces from ECM to the cytoskeleton and facilitates actin assembly. Our FRET-based observation is consistent with the role of mechanical stimulation to cytoskeletal regulation of osteoblasts, in which external force stimulates development of focal adhesion formation and polymerization of actin through Rho and ROCK [37]. In our FRET experiment, the activation of Rho was not detected at a stress level of 2–5 dyn/cm$^2$ (data not shown). We specifically examined the involvement of RhoA using its wild-type and dominant negative biosensors. However, it is possible that other GTPases in the Rho family such as RhoB and RhoC are also involved in the observed mechanotransduction of osteoblasts. Furthermore, GTPases such as Rac have been reported to be involved in mechanotransduction [38]. Further investigation is needed to understand the potential cross-talks between Rho and Rac in flow-driven alterations in integrin–actin cytoskeleton adhesion complexes in response to varying shear stress.

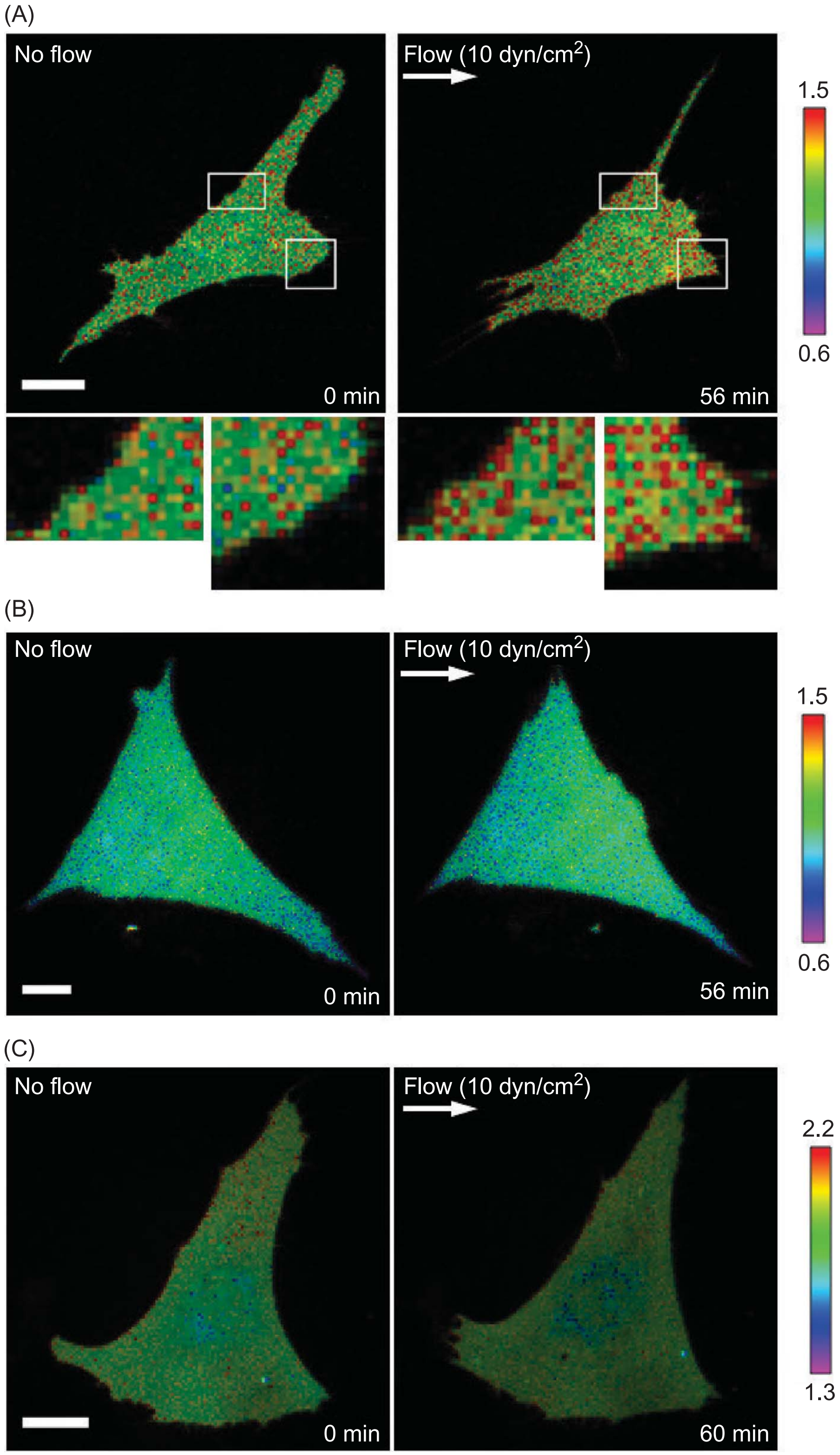

Figure 4. Flow-induced RhoA activation using a CFP–YFP RhoA biosensor. The color bars represent the fluorescence emission ratio of YFP to CFP, an index of RhoA activity. The white arrows indicate the flow direction. Scale bars, 20 μm. (A) Temporal change of a single cell displaying an increase in RhoA activity from 0 min (no flow) to 56 min (flow at 10 dyn/cm$^2$). (B) Single cell imaged at 0 min (no flow) and 56 min (flow at 10 dyn/cm$^2$) with a reduced level of RhoA activity in the presence of a cell-permeable C3 transferase. (C) Single cell co-transfected with a RhoA biosensor and a dominant negative RhoA (RhoA-19) mutant. No RhoA activation was observed in response to shear stress at 10 dyn/cm$^2$.

This study supports the notion that both the phosphorylation of Akt in PI3K signaling and the phosphorylation of p38 and ERK1/2 in MAPK signaling are linked to RhoA activity in MC3T3-E1 cells (Figure 6). Previous studies report linkages of PI3K and Rho as well as MAPK and Rho [27]. For instance, PI3K and Rho were shown to be involved in hypoxia-induced responses in endothelial cells [39], and TGFβ in smooth muscle cells are reported to activate RhoA and PI3K [40]. Furthermore, it is reported that ECM compliance regulates osteogenesis by influencing MAPK signaling downstream of RhoA and ROCK [28]. Our results are consistent with previous findings, and they reveal that flow-driven expression of Opn is regulated through PI3K and MAPK signaling, which are in part mediated by RhoA. Opn is a 33 kD sialoprotein in ECM and expressed in many types of cells including osteoblasts, osteocytes, chondrocytes, myoblasts, and endothelial cells [41–43]. It is known to be responsive to

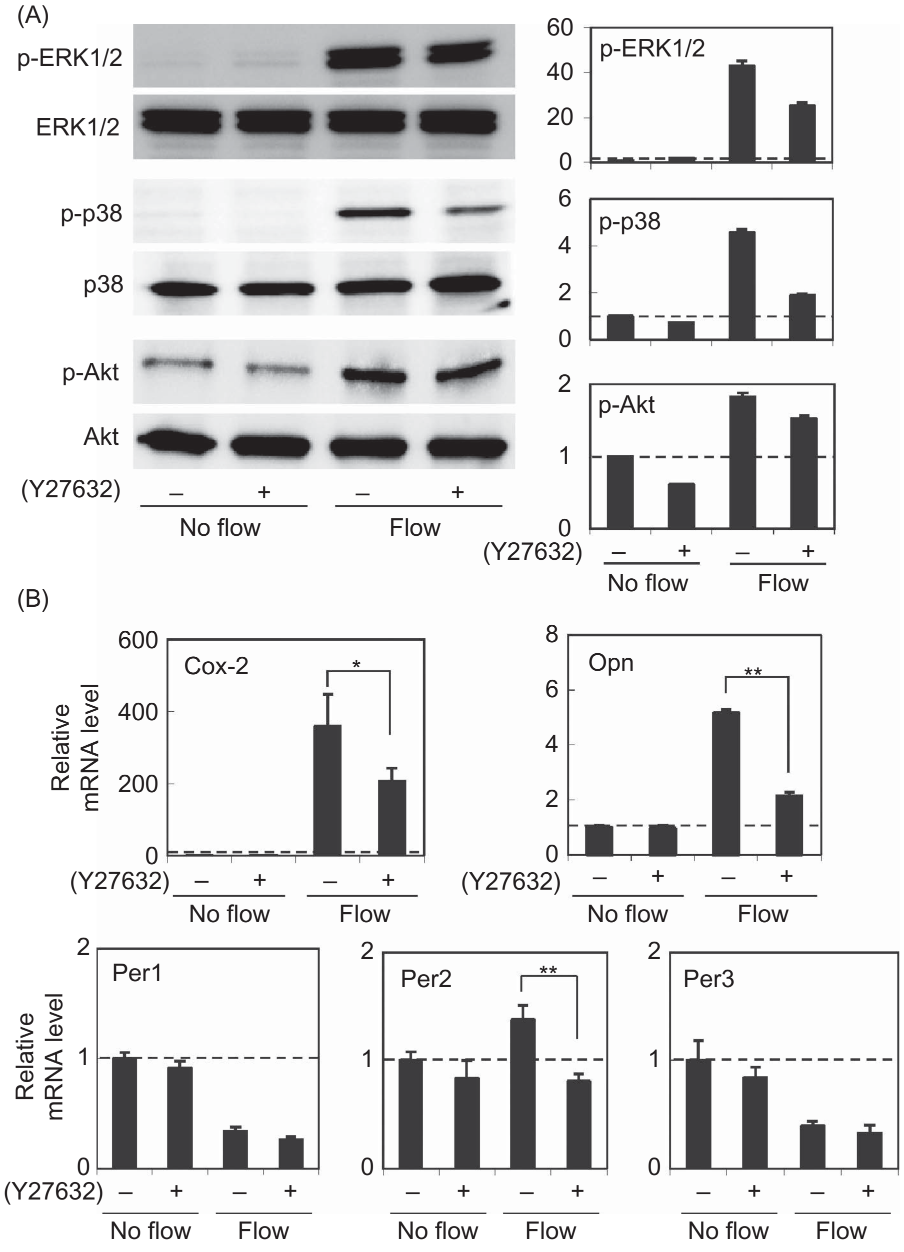

Figure 5. Effects of an inhibitor to Rho kinase, Y27632. Cells were harvested 1 hr after the onset of flow treatment. (A) Reduction of flow-induced elevation of the phosphorylated levels of ERK1/2, p38, and Akt. (B) Effects of Y27632 on the mRNA levels of Cox2, Opn, Per1, Per2, and Per3 in the presence and absence of 3 hr flow treatment. $^{*}p < 0.05$.

proinflammatory cytokines and assists in protection from apoptosis [44,45], although its role in osteoblast development is not completely understood. In our FRET experiment, load-driven activation of RhoA was observed approximately 40 min after the onset of flow at 10 dyn/cm$^2$. Since the inhibition of phosphorylation of the described molecules is partial and their phosphorylation may initiate prior to 40 min, it is conceivable that other signaling factors besides RhoA may also be involved in the activation of PI3K and MAPK signaling.

The circadian regulatory pathway was identified as a flow-stimulated signaling network using microarray-derived data. The real-time PCR results showed various modulation patterns of the mRNA levels of the selected circadian genes. These genes are known to mediate bone formation, and their expression in osteoblasts is reported to be regulated by a sympathetic nervous system and leptin [26]. Furthermore, parathyroid hormone is known to activate expression of Per1 in osteoblasts in association with the anabolic action of parathyroid hormone in bone [46]. Consistent with the role of Rho in mechanotransduction of osteoblasts, flow-induced upregulation of the Per2 mRNA level was suppressed by the inhibitor to the Rho kinase. Modulation of the circadian genes in response to mechanical stimulation is likely to be involved in osteoblast development, and further investigation is necessary to understand the observed variable responses among them.

This study employed an osteoblast-like cell line, MC3T3-E1 cells, which were derived from calvarias that are not typically under mechanical loading [47]. Validation of the results in this study using primary osteoblast cells might be necessary. We also examined FRET-based molecular signaling at 0–1 hr and gene expression at 1–5 hr in response to 1 hr flow treatment, but cellular responses may differ at other time windows. In animal models osteoblasts are reported to activate Wnt signaling in response to mechanically loaded osteocytes [48]. Further studies should be conducted to investigate any linkage of Wnt signaling to Rho-mediated mechanotransduction of osteoblasts. In summary, we demonstrated that 1 hr flow treatment at 10 dyn/cm$^2$ activated

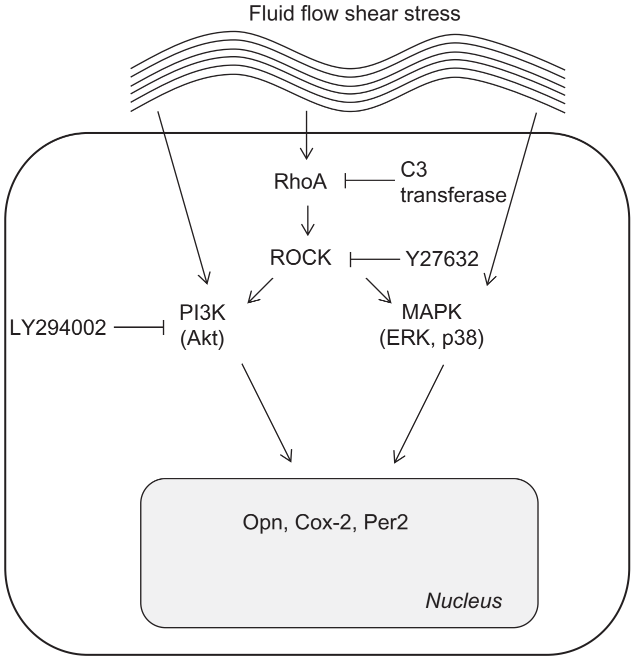

Figure 6. Potential mechanisms of the action by fluid flow shear stress in osteoblasts.

PI3K and MAPK signaling as well as the circadian regulatory pathway, which were at least in part mediated by RhoA.

## Acknowledgment

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## Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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