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Hydrostatic pressure promotes the proliferation and osteogenic/chondrogenic differentiation of mesenchymal stem cells: The roles of RhoA and Rac1 ☆

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Abstract

Our previous studies have shown that hydrostatic pressure can serve as an active regulator for bone marrow mesenchymal stem cells (BMSCs). The current work further investigates the roles of cytoskeletal regulatory proteins Ras homolog gene family member A (RhoA) and Ras-related C3 botulinum toxin substrate 1 (Rac1) in hydrostatic pressure-related effects on BMSCs. Flow cytometry assays showed that the hydrostatic pressure promoted cell cycle initiation in a RhoA- and Rac1-dependent manner. Furthermore, fluorescence assays confirmed that RhoA played a positive and Rac1 displayed a negative role in the hydrostatic pressure-induced F-actin stress fiber assembly. Western blots suggested that RhoA and Rac1 play central roles in the pressure-inhibited ERK phosphorylation, and Rac1 but not RhoA was involved in the pressure-promoted JNK phosphorylation. Finally, real-time polymerase chain reaction (PCR) experiments showed that pressure promoted the expression of osteogenic marker genes in BMSCs at an early stage of osteogenic differentiation through the up-regulation of RhoA activity. Additionally, the PCR results showed that pressure enhanced the expression of chondrogenic marker genes in BMSCs during chondrogenic differentiation via the up-regulation of Rac1 activity. Collectively, our results suggested that RhoA and Rac1 are critical to the pressure-induced proliferation and differentiation, the stress fiber assembly, and MAPK activation in BMSCs.

Introduction

Inflammation, trauma and tumors may all lead to articular cartilage defects. However, the treatment of these defects remains a clinical challenge. Currently, clinical treatments for articular cartilage defects primarily include autologous cartilage repair (Steadman et al., 2003) and autologous periosteum, cartilage or chondrocyte transplantation (Hangody and Fules, 2003; Outerbridge et al., 1995). The therapeutic efficacies of these approaches are far from ideal. The application of tissue engineering for the repair of articular cartilage defects has become a clinical focus (Johnstone et al., 2013; Wei et al., 2005). Currently, bone marrow mesenchymal stem cells (BMSCs) have become one of the most important seed cell options in cartilage tissue engineering (Dezawa et al., 2004; Luk et al., 2005) due to readily available sources, strong in vitro proliferative abilities, weak immune rejection responses, and strong chondrogenic differentiation potentials (Alford and Cole, 2005; Hangody and Fules, 2003; Hunziker, 2001; Panseri et al., 2012; Shapiro et al., 1993; Smith et al., 2003). Articular cartilage plays an important role in mechanical load support and load transfer in a joint. Mechanical loading stimulates cartilage matrix synthesis and enhances the biomechanical properties of the cartilage (De Croos et al., 2006; Hunter et al., 2002). Therefore, numerous studies have focused on the effects of the mechanoenvironment on seed cells in tissue-engineered cartilage (Carter et al., 1998; Carter and Wong, 1988; Henderson and Carter, 2002; Loboa et al., 2001; Thompson et al., 2012). Hydrostatic pressure applications are methods of applying mechanical loading that mimics the compressive forces borne by cartilage in a joint cavity (Gray et al., 1988). Studies have shown that mechanical stimulation by hydrostatic pressure promotes the expression of the chondrogenic marker genes of BMSCs (Angele et al., 2003; Miyanishi et al., 2006a, 2006b; Wagner et al., 2008; Zeiter et al., 2009). Our preliminary studies have also demonstrated that hydrostatic pressure promotes BMSC proliferation and cytoskeletal assembly (Zhang et al., 2012). However, the mechanisms through which the mechanical signals are transmitted to BMSCs and induce the above-mentioned responses remain unclear.

The perception and transduction of the stimulus by cells are closely related to the cytoskeleton, which plays a number of key roles in cellular mechanotransduction (Ingber, 2002; Wang et al., 2002). A wide variety of cellular activities involving actin dynamics are regulated by the Ras homolog gene family (Rho) subfamily of small guanosine triphosphatases (GTPases) (Etienne-Manneville and Hall, 2002). Rho GTPases not only function as key regulators of stress fiber assembly and focal adhesion formation (Darling and Guilak, 2008) but also play a central role in the regulation of the actin cytoskeleton (Shifrin et al., 2009). Studies have shown that compared with undifferentiated cells, stem cells are softer and more sensitive to local stress stimulation, and the extraordinary sensitivity of embryonic stem cells to stress is closely regulated by the Rho family of GTPases (Chowdhury et al., 2010). Rho GTPases are involved in integrin-mediated mechanotransduction (Burridge and Wennerberg, 2004; Matthews et al., 2006) and regulate stress fiber formation in adult stem cells under mechanical stimulation (Discher et al., 2009). Rac1 plays an important

regulatory role in cytoskeletal assembly during lamellipodia formation in cells under mechanical stimulation (Hu et al., 2002; Masuda and Fujiwara, 1993a, 1993b). However, the exact roles of RhoA and Rac1 in the regulation of hydrostatic pressure-induced BMSC cytoskeleton assembly and its downstream molecular mechanisms are unclear. In addition, studies have found that the inhibition of the RhoA-Rho-associated protein kinase (ROCK) signaling pathway results in an enhanced expression of chondrogenic genes (Woods et al., 2005). The activation of Rac1 results in the increased expression of cadherin protein and chondrogenic genes in BMSCs (Woods et al., 2007). These results indicate that the activities of Rho GTPase signaling molecules are closely related to the differentiation and the fate of BMSCs. Nevertheless, the roles of Rho GTPases in BMSC differentiation induced by the hydrostatic microenvironment have yet to be elucidated.

In this study, agonists and inhibitors of the RhoA or Rac1 pathway were administered to BMSCs to investigate the roles of RhoA and Rac1 in the pressure-related changes of cell proliferation and osteogenic and chondrogenic differentiations and in the possible down-stream signaling molecules, including the F-actin and mitogen-activated protein kinase (MAPK) proteins. We speculated that RhoA and Rac1 were involved in the regulation of hydrostatic pressure-induced proliferation and differentiation, the cytoskeletal assembly and the MAPK activation in BMSCs.

Materials and methods

Isolation and culture of BMSCs

The isolation and primary culture of BMSCs from the femoral and tibial bones of Sprague−Dawley rats (8 weeks old) were performed. BMSCs were cultured using the whole marrow-adherence method. Flow cytometry was used to detect the surface markers of BMSCs. The cells were trypsinized and washed and then incubated for 30 min at 4 °C with FITC-conjugated antibodies against CD29 (β1-integrin) (BioLegend Inc., 9727 Pacific Heights Blvd, San Diego, CA), CD44 (receptor for hyaluronate and osteopontin) (Abcam, Cambridge Science Park, Cambridge,U.K.), CD90 (Thy-1 glycoprotein) (Epitomics, Burlingame, CA, USA), CD34 (stem/progenitor hematopoietic cells; (Abcam)) or conjugated isotype controls. The labeled cells were analyzed using a FACS calibur Cytometer (Beckton Dickinson, Franklin Lakes, NJ) and CellQuest Software (BD).

Application of hydrostatic pressure to the BMSCs

In this study, we focused on articular cartilage regeneration and the repair of the temporomandibular joint. First, we investigated the biomechanical characteristics of articular cartilage in vivo. Then, we explored the mechanobiological effects of chondrogenic differentiation on BMSCs and its underlying mechanotransduction mechanism under the simulated biomechanical environment. We showed by finite element analysis that the stress in the condylar cartilage was a compressive-type stress of approximately 300 kPa under normal occlusion. As such, we applied a self-designed hydraulic pressure-controlling cellular strain unit following the model developed by Yousefian et al. (1995), which

imitates the mechanical circumstances of the chondrocytes or the cartilage-targeted stem cells in vivo. The hydrostatic pressure apparatus allows sterile manipulation and can apply up to 300 kPa of pressure to cultured cells at a constant 37 °C. The device continuously compresses gas (3% CO_2 in air) in a closed culture chamber (98% humidity) and mimics the compressive physiological environment of the cells in vivo. A similar assembly was used in previous studies (Chen et al., 2007; Zhang et al., 2012; M. Zhang et al., 2006; Z. Zhang et al., 2006). In our previous studies on the pressure-induced mechanobiology of primary chondrocytes, we studied a range of pressures on the cells from 30 to 300 kPa (calculated by the finite element method). We found that under high pressures, the morphology and function of the cells were significantly affected. Because of these findings, we limited the range of pressure below 100 kPa. Based on cell proliferation and ALP activity observations, we selected feasible and excessive pressure conditions by combining different pressure values and durations for the in vitro cultured MCCs. The feasible pressure condition was fixed at 90 kPa for 60 min, under which the ALP activity of the cells were promoted without affecting cell proliferation rates. In the studies focused on the mechanotransduction of BMSCs adopted for cartilage regeneration, the same mechanical units as those used in previous works were utilized, and the feasible pressure conditions for mandibular chondrocytes, 90 kPa/h, was adopted. Our previous study demonstrated that a constant pressure of 90 kPa over 1 h promoted the proliferation of rat BMSCs and cytoskeletal assembly (Zhang et al., 2012). Therefore, in the present study, the same experimental condition (1 h of 90 kPa pressure) was utilized to apply mechanical stimulation to BMSCs.

Experimental grouping

Prior to the experiments, the BMSCs were synchronized. Then the cells were divided into 10 groups: control group, cells without treatment; RhoA+ group, cells with 1 nM U46619 (RhoA agonist, Sigma-Aldrich, St. Louis, MO) treatment for 1 h; RhoA− group, cells with 10 nM Y27632 (RhoA inhibitor, Santa Cruz Biotechnology, Santa Cruz, CA) treatment for 1 h; Rac1+ group, cells with 20 nM PDGF (Rac1 agonist, PeproTech, Rocky Hill, NJ) treatment for 1 h; Rac1− group, cells with 100 nM NSC23766 (Rac1 inhibitor, Santa Cruz) treatment for 1 h; P group, cells with 90 kPa hydrostatic pressure treatment for 1 h; P/RhoA+ group, cells with 1 nM U46619 pretreatment for 1 h and then, 90 kPa pressure treatment for 1 h; P/RhoA− group, cells with 10 nM Y27632 pretreatment for 1 h and then, 90 kPa pressure treatment for 1 h; P/Rac1+ group, cells with 20 nM PDGF pretreatment for 1 h and then, 90 kPa pressure treatment for 1 h; and P/Rac1− group, cells with 100 nM NSC23766 pretreatment for 1 h and then 90 kPa pressure treatment for 1 h. For the examination of the cell cycle, proliferation, F-actin production and phosphorylation of ERK and JNK detection, the cells were collected and analyzed immediately after the chemical and mechanical treatments in each group. For the detection of osteogenic and chondrogenic markers, the culture medium was switched to osteogenic or chondrogenic differentiation induction media. The cells were divided into the 10 groups mentioned. The cells were cultured

without any treatment for the control group; with continuous treatments of 1 nM U46619, 10 nM Y27632, 20 nM PDGF and 100 nM NSC23766 in the RhoA+, RhoA−, Rac1+ and Rac1− groups, respectively, without any mechanical stimulation; and with RhoA/Rac1 agonists/inhibitors, and 90 kPa for 1 h per day in the P, P/RhoA+, P/RhoA−, P/Rac1+ and P/Rac1− groups. Then, the cells were collected and analyzed after continuous osteogenic culture for 2 weeks or chondrogenic culture for 4 weeks.

Cell cycle analysis

The BMSCs (10×10^4) were trypsinized, washed once with PBS and fixed in 70% ethanol for 12 h at −20 °C. After washing, the fixed cells were incubated with a propidium iodide (PI, Sigma) staining solution (100 μg/mL PI; 0.7 mg/mL RNase) for 30 min at room temperature in the dark. The stained cells were analyzed by flow cytometry (BD), and the data were analyzed with Cellquest software (BD). The S-phase fraction was calculated as follows: SPF = S / (G0 / 1 + S + G2 / M) × 100%. The index of proliferation was calculated as follows: proliferation index = (S + G2 / M) / (G0 / 1 + S + G2 / M) × 100%.

Fluorescent staining of F-actin

The BMSCs were cultured on glass coverslips in 24-well plates at 10×10^3 cells/cm^2 for 24 h in growth medium. After washing with phosphate buffered saline (PBS), the cells were fixed for 5 min in 3.7% formaldehyde solution in PBS. Cells were dehydrated with acetone and then permeabilized with 0.1% Triton X-100 in PBS. The slips were stained with a 50 μg/mL fluorescent phalloidin conjugate solution in PBS for 40 min at room temperature. After washing three times with PBS to remove the unbound phalloidin conjugates, the slips were sealed. The images were documented with a Leica SP2 laser scanning confocal microscope (Leica Microsystems, Heerbrugg, Switzerland).

Western blotting analysis

Cells (10×10^4) were washed once in PBS and lysed in a lysis buffer. Total cellular protein was quantified by Bradford protein assay kit (Bio-Rad, Hercules, CA) and electrophoresed in a 12% SDS-polyacrylamide gel and transferred to PVDF membrane (EMD Millipore Corporation, Billerica, MA, USA). Membranes were incubated with blocking buffer (TBS, 0.1% Tween-20 and 5% w/v nonfat dry milk) for 1 h at room temperature, and then washed three times with TBS-T and incubated with primary antibodies ERK1/2 (1:2000, (Cell Signaling Technology Danvers, USA), p-ERK1/2, JNK1/2 and p-JNK1/2 (1:1000, Cell Signaling) overnight at 4 °C. After the membranes were washed three times with TBS-T for 5 min, and incubated for 1 h at room temperature with HRP-conjugated secondary antibodies (1:2000, Cell Signaling). The bands were detected by ECL reagent (Thermo Fisher Scientific, Lausanne, Switzerland), and the intensities of the brands were quantified by the Photo-Image System (Gel-Pro Analyzer, Media Cybernetics Inc, Bethesda, MD, USA).

Osteogenic and chondrogenic gene analysis

Osteogenic induction

The BMSCs (10×10^4) were cultured in 6-well plates at 37 °C in a 5% CO_2 humidified incubator. At 70–80% confluence, the culture medium was changed to osteogenic medium consisting of high-glucose DMEM, fetal bovine serum, penicillin–streptomycin, glutamine, ascorbate, β-glycerophosphate and dexamethasone (Cyagen Biosciences, Inc., Santa Clara, CA, USA). The medium was changed every three days. After one and two weeks of differentiation, the cells were fixed and stained with Alizarin Red.

Detection and quantification of mineralization

To quantify the degree of mineralization, 800 μL 10% (v/v) acetic acid were added to each well of the stained monolayers in 6-well plates and incubated at room temperature for 30 min with shaking. The cells were scraped and transferred in acetic acid to 1.5-mL microcentrifuge tubes. After vortexing, the slurry was overlaid with 500 μL mineral oil (Sigma), heated to 85 °C for 10 min, and transferred to ice for 5 min. Then, the slurry was centrifuged at 20,000 g for 15 min and the supernatant was removed to a new tube. Subsequently, 200 μL of 10% (v/v) ammonium hydroxide was added to neutralize the acid. The supernatant samples were read in triplicate at 405 nM in 96-well plates.

Chondrogenic induction

The BMSCs (10×10^4) were resuspended in complete chondrogenic medium consisting of high-glucose DMEM, dexamethasone, ascorbate, ITS + supplement, sodium pyruvate, proline and TGF-β3 (Cyagen). The cells were centrifuged at 150 g for 5 min at room temperature. The tubes were incubated at 37 °C in a humidified atmosphere of 5% CO_2. Freshly prepared complete chondrogenic medium was added to each tube every 2–3 days. Cell pellets were harvested after 2 w or 4 w of culture for subsequent toluidine blue staining, glycosaminoglycan (GAG) quantification and PCR assays.

Detection and quantification of GAG

Cell pellets were harvested and digested in papain (0.56 U/mL in 0.1 M sodium acetate, 10 M cysteine HCl, 0.05 M EDTA, pH 6.0) at 60 °C for 16 h. Following digestion, constructs were evaluated for sulfated GAG content against a standard curve of chondroitin-6-sulfate using the 1,9-dimethylmethylene blue dye-binding assay (Farndale et al., 1986).

Real time-PCR analysis

Total RNA was extracted from the samples using Trizol reagent (Invitrogen, Carlsbad, CA) to detect the expression of osteogenesis-related genes, including collagen I (*COL-I*), osteocalcin (*OCN*), osteopontin (*OPN*), and bone sialoprotein (*BSP*), and chondrogensis-related genes, including *SOX-9*, collagen II (*COL-II*) and *Aggrecan*, using real-time PCR (TaKaRa Bio, Tokyo, Japan). The sequences for the primers used for these experiments are listed in Table 1. The reaction products were quantified using a relative quantification tool (Bio-Rad). All gene expression levels were normalized to the endogenous control β-actin. The expression levels of the target genes in the samples were compared with those in the controls using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

The results were expressed as the means ± standard deviations. Statistical analyses were performed using one-way analysis of variance (ANOVA) combined with the Dunnett t test. Relationships were considered significant for $P < 0.05$.

Results

Down-regulation of RhoA and Rac1 kinase activities inhibited pressure-induced DNA synthesis and cell proliferation

The statistical analysis showed that the S-phase fraction (Fig. 1A) and the cell proliferation index (Fig. 1B) in the RhoA antagonist group (RhoA− group) were significantly lower than those in the control group ($P \leq 0.01$). In contrast, the S-phase fractions in all other experimental groups were significantly higher than that of the control group ($P \leq 0.05$). This result indicated that both the RhoA pathway activation and hydrostatic pressure promoted cell cycle initiation, enhanced DNA synthesis and accelerated cell proliferation, whereas the inhibition of RhoA signaling blocked cell cycle initiation. Compared with the P group, the S-phase fraction (Fig. 1A) and the cell proliferation index (Fig. 1B) in the P/RhoA− group were significantly decreased ($P \leq 0.05$), indicating that the down-regulation of RhoA signaling inhibited the pressure-induced cell cycle initiation and cell proliferation. These results also confirmed that RhoA signaling activation was involved in the pressure-induced cell cycle initiation and cell proliferation.

The S-phase fraction (Fig. 1C) and the cell proliferation index (Fig. 1D) in the Rac1+, P and P/Rac1+ groups were significantly higher than those of the control group ($P \leq 0.01$), whereas those of the Rac1− group were significantly lower than those of the control group ($P \leq 0.05$). These results indicated that both the Rac1 pathway activation and the pressure stimulation promoted cell cycle initiation and cell

Genes	Sequences
	Table 1 Primer sequences for real-time PCR.
ColI	F GGAGAGTACTGGATCGACCCTAAC
	R CTGACCTGTCTCCATGTTGCA
OCN	F TGCAAAGCCCAGCGACTCT
	R AGTCCATTGTTGAGGTAGCG
OPN	F AGACCATGCAGAGAGCGAG
	R ACGTCTGCTTGTGTGCTGG
BSP	F TGTGGAATGGTGCTACGGTCTC
	R GATCAACAGCCCTGATTTACGATG
Sox9	F AGAAAGACCACCCCGATTAC
	R TGCTGATGCCGTAACTGCC
ColII	F GCCTCGCGGTGAGCCATGATC
	R CTCCATCTCTGCCACGGGGT
Aggrecan	F GCGGGTCATCGCTGCAGTG
	R GCTCGGTCAAAGTCCAGTGTG
β-actin	F GAGAGGGAAATCGTGCGTGAC
	R CATCTGCTGGAAGGTGGACA

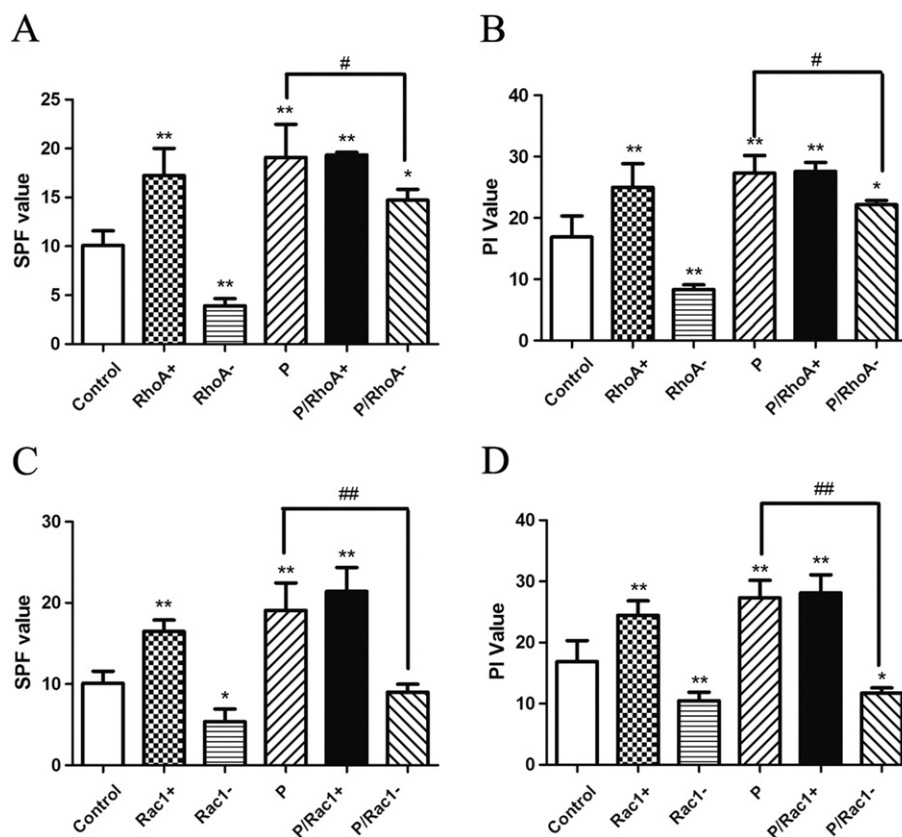**Figure 1** Histograms showing the S-phase fraction (SPF) and proliferation index (PI) of BMSCs. After the cells were synchronized, BMSCs (10×10^4) were treated with RhoA+, RhoA−, Rac1+ or Rac1− and either maintained as untreated controls or subjected to 1 h of 90 kPa hydrostatic pressure. After the treatments, the cells were immediately collected and stained with propidium iodide. The cell cycle phases were analyzed by flow cytometry. The CellQuest program was used for the acquisition and analysis of the FACS scans. Both the SPF (*A*) and PI (*B*) in the P, P/RhoA+, and P/RhoA− groups were compared. Similarly, both the SPF (*C*) and PI (*D*) in the P, P/Rac1+, and P/Rac1− groups were compared. *, $P \leq 0.05$ vs. group control; **, $P \leq 0.01$ vs. group control; ##, $P \leq 0.01$ vs. P group.

proliferation, whereas the blockage of Rac1 signaling inhibited cell cycle initiation. Compared with the P group, the S-phase fraction (Fig. 1C) and the cell proliferation index (Fig. 1D) in the P/Rac1− group were significantly reduced ($P \leq 0.01$), indicating that blocking the Rac1 pathway inhibited the pressure-induced DNA synthesis and cell proliferation.

RhoA positively regulated and Rac1 negatively regulated pressure-induced F-actin cytoskeleton assembly

After the fluorescent staining of F-actin, BMSCs were observed under a laser scanning confocal microscope (Fig. 2A). The mean optical density values in the RhoA+, P, P/RhoA+, Rac1− and P/Rac1− groups were higher than that in the control group ($P \leq 0.05$; Figs. 2B and C). In contrast, the mean optical density values in the RhoA−, P/RhoA−, Rac1+ and P/Rac1+ groups were significantly lower than that in the control group ($P \leq 0.05$; Figs. 2B and C). These results indicated that the activation of the RhoA pathway and the inhibition of the Rac1 pathway under hydrostatic pressure promoted the assembly of the F-actin cytoskeleton, whereas the inhibition of the RhoA pathway and the activation of the Rac1 pathway blocked the F-actin cytoskeleton assembly. Compared with the P group, the optical density values in the

P/RhoA− (Fig. 2B) and P/Rac1+ groups (Fig. 2C) significantly decreased. This demonstrated that RhoA positively regulated and Rac1 negatively regulated the hydrostatic pressure-induced F-actin assembly.

The pressure-induced inhibition of ERK1/2 phosphorylation was associated with the regulation of RhoA signaling

Western blot analysis results showed that the level of ERK1/2 phosphorylation was significantly lower in the RhoA−, P, P/RhoA+ and P/RhoA− groups s compared with that of the control group ($P \leq 0.01$). This indicated that the down-regulation of RhoA activity and/or pressure significantly blocked the phosphorylation of ERK1/2 ($P \leq 0.01$). Compared with the P group, the level of ERK1/2 phosphorylation in the P/RhoA+ group was elevated (Fig. 3A). This indicated that RhoA activation inhibited the pressure-induced down-regulation of P-ERK1/2 expression and that RhoA played an important role in the regulation of ERK1/2 phosphorylation upon pressure stimulation. The Rac1 pathway agonist, PDGF, could significantly inhibit the activation of ERK1/2 in BMSCs. However, the up-regulation or down-regulation of Rac1 activity in BMSCs by the Rac1 agonist or inhibitor showed no significant impact on the inhibitory effect of pressure on the level of ERK1/2
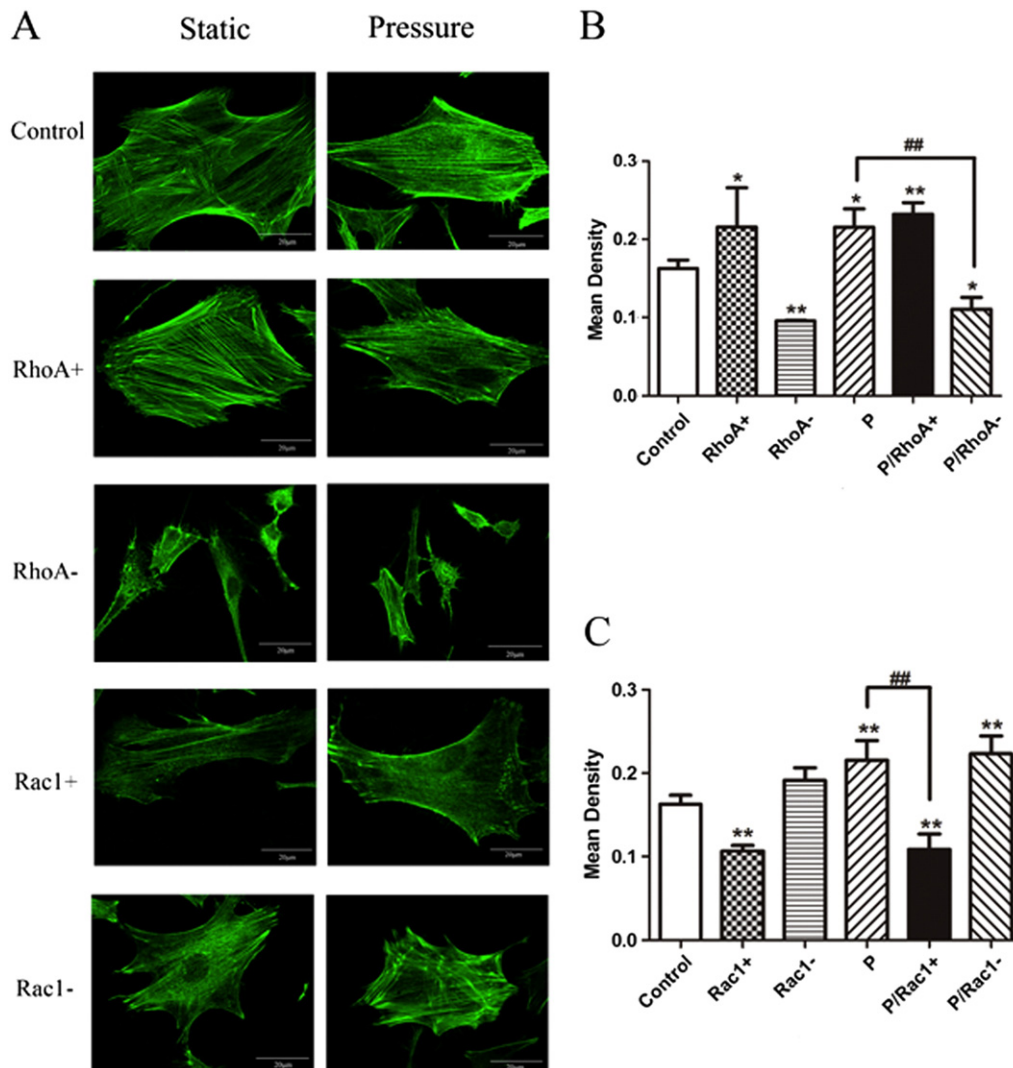
Figure 2 Cooperative and interactive effects between RhoA or Rac1 agitation or inhibition and hydrostatic pressure on stress fibers in BMSCs. (*A*) Representative micrographs are shown of confluent BMSCs treated with RhoA+, RhoA−, Rac1+ or Rac1− that were either kept as untreated controls or subjected to 90 kPa/1 h hydrostatic pressure. After the treatments, the cells were immediately collected and stained with FITC-Phalloidin to identify F-actin bundles (magnification: 600× bar = 20 μm). (B−C) Data analyses of F-actin fluorescence density using the integrated optical density (IOD) of representative images. **, $P \leq 0.01$ vs. control group; #, $P \leq 0.05$ vs. P group; ##, $P \leq 0.01$ vs. P group.

phosphorylation (Fig. 3C). These results suggested that Rac1 may not be involved in the regulation of pressure stimulation-induced ERK phosphorylation.

The promoting effect of pressure on JNK1/2 phosphorylation was associated with RhoA and Rac1 signaling

The phosphorylation of JNK proteins in the experimental groups was examined, and the averaged grayscale values of the images of phosphorylated JNK1/2 (P-JNK1/2) in the P, P/RhoA+ and P/RhoA− groups were significantly higher than that in the control group ($P \leq 0.05$). These results indicated that pressure stimulation resulted in an up-regulation of P-JNK1/2 expression. Compared with the P group, the level of JNK1/2 phosphorylation was significantly reduced in the P/RhoA+ group ($P \leq 0.05$; Fig. 3B). This indicated that the

up-regulation of RhoA activity inhibited the mechanical stimulation-induced up-regulation of P-JNK1/2 expression. The level of P-JNK1/2 expression in P/Rac1+ group was significantly lower than that in the P group ($P \leq 0.05$), which demonstrated that Rac1 also played an active role in the pressure-induced JNK1/2 phosphorylation.

RhoA played a regulatory role in pressure-induced osteogenic marker genes expression in BMSCs under osteogenic induction

BMSCs were cultured in osteogenic induction medium for 2 weeks. Nodular structures stained positive for calcium were observed in the cells in all experimental groups (Fig. 4A). However, the amount of calcium deposits varied among the groups (Fig. 4B). The results showed that the mineralization levels of the RhoA agonist and Rac1 inhibitor
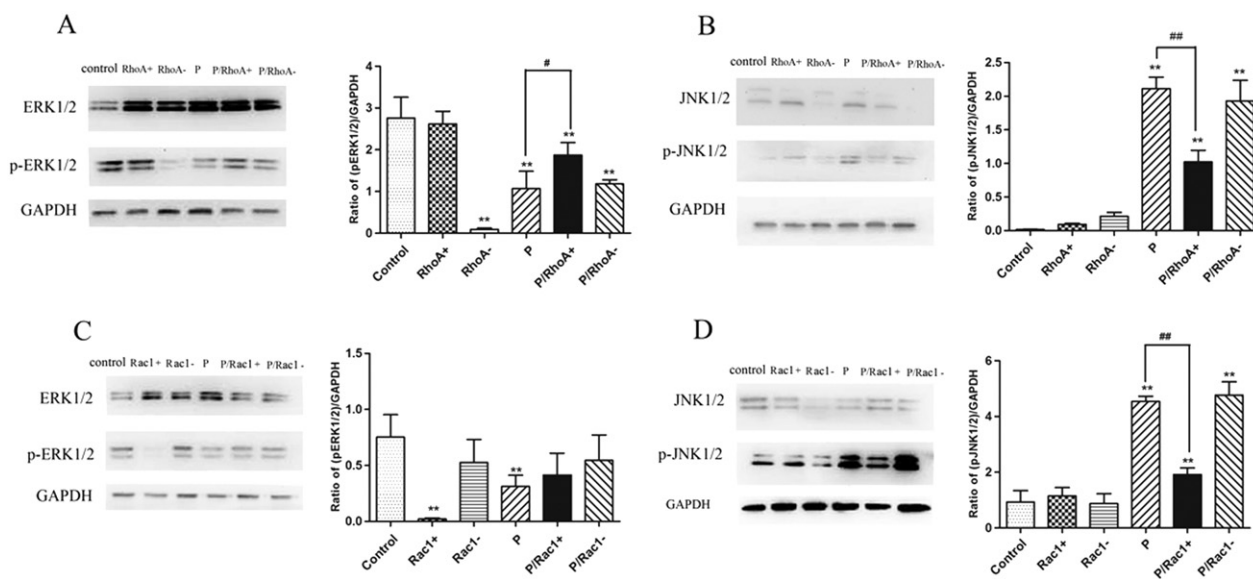
Figure 3 The expression of unphosphorylated and phosphorylated ERK1/2 and JNK1/2 in BMSCs. BMSCs were cultured in the presence or absence hydrostatic pressure with Rho agitation or inhibition (*A*, *B*) or with Rac1 agitation or inhibition (*C*, *D*). The expression levels of pERK1/2 relative to GAPDH (*A*, *C*) and pJNK1/2 relative to GAPDH (*B*, *D*) were derived from Quantity One density analysis. The data are expressed as the means ± SD; **, $P \leq 0.01$ vs. control group; #, $P \leq 0.05$ vs. P group; ##, $P \leq 0.01$ vs. P group.

groups were significantly higher than that of the control group ($P \leq 0.01$). However, the degree of mineralization of the RhoA inhibitor and pressure group and the RhoA inhibitor group were significantly lower than that of the control ($P \leq 0.05$).

The expression of osteogenic marker genes was examined using real-time PCR. Osteogenic induction for one week resulted in the significantly increased expression of *Col I* and *OCN* in the RhoA+, P and P/RhoA+ groups compared with those of the control group ($P \leq 0.05$). In addition, the levels of *Col I* and *OCN* expression in the P/RhoA+ group were significantly higher than those in the P group ($P \leq 0.01$). However, the P/RhoA− group expressed significantly lower levels of *Col I* and *OCN* than those in the P group ($P \leq 0.01$; Fig. 4D). These results indicated that hydrostatic pressure promoted the osteogenic differentiation of BMSCs through the regulation of the *Col I* and *OCN* genes after 1 week of osteogenic induction. The RhoA signaling pathway participated in the pressure-regulated early osteogenic differentiation. After 2 weeks of osteogenic induction, the expression of *OPN* and *BSP* was significantly increased in the RhoA+ group and significantly decreased in RhoA− group. However, the regulatory effect of the pressure stimulation on the osteogenic differentiation of BMSCs vanished. The expression levels of the osteogenic marker genes in the pressure stimulation group showed no significant differences compared with those of the control group ($P > 0.05$; Fig. 4D). These results demonstrated that pressure was incapable of exerting a promoting effect on BMSCs after 2 weeks of osteogenic differentiation.

Among the Rac1 groups, the quantification of mineralization in BMSCs, as measured by Alizarin red-based assays for different groups (Fig. 4C), showed the same variations as observed for the osteogenic genes detected by real-time PCR. That is, the Rac1− group expressed significantly increased levels of the four osteogenic marker genes, *COL-I*,

OCN, *OPN* and *BSP*, after 1 week and 2 weeks of osteogenic induction ($P \leq 0.01$; Fig. 4E). This indicated that the inhibition of Rac1 activity promoted the osteogenic differentiation of BMSCs upon osteogenic induction. However, the up-regulation of Rac1 activity could abolish and even reverse the promoting effect of pressure on *COL-I* and *OCN* expression, with their expression levels in the P/Rac1+ group being significantly lower than those in the pressure-only group ($P \leq 0.01$). At 2 weeks of osteogenic induction, the regulatory effect of pressure stimulation on the osteogenic differentiation of BMSCs vanished completely. Moreover, pressure exerted an inhibitory effect on *OCN* expression. These results suggested that pressure may, to a certain degree, promote osteogenic differentiation by antagonizing the Rac1 activity at 1 week of osteogenesis induction.

RhoA negatively and Rac1 positively regulated the pressure-induced expression of chondrogenic marker genes in BMSCs

After 4 weeks of chondrogenic induction, the BMSCs were stained using toluidine blue. Blue metachromatic granules were visible in the cell pellets in all experimental groups (Fig. 5A). This result confirmed the chondrogenic differentiation of the stem cells and the secretion of a cartilage matrix. Biochemical analyses showed that the GAG content in the RhoA−, Rac1+, P, P/RhoA−, and P/Rac1+ groups were significantly higher than that of the control group ($P \leq 0.01$; Figs. 5B and C). Compared with the P group, the GAG content in the P/RhoA− and P/Rac1+ groups was significantly increased ($P \leq 0.01$). However, the GAG content in the P/RhoA+ and P/Rac1− groups was significantly decreased ($P \leq 0.01$).

The real-time PCR analyses showed that after 2 or 4 weeks of chondrogenic induction, both the inhibition of RhoA activity
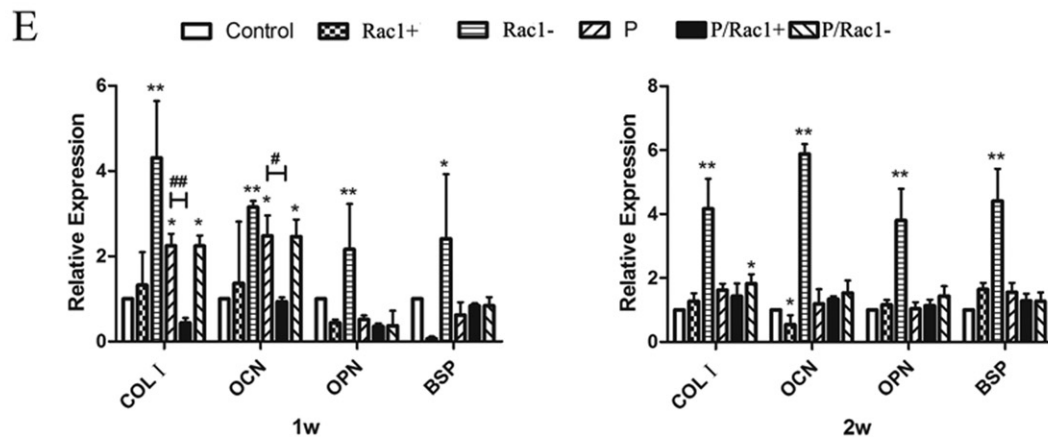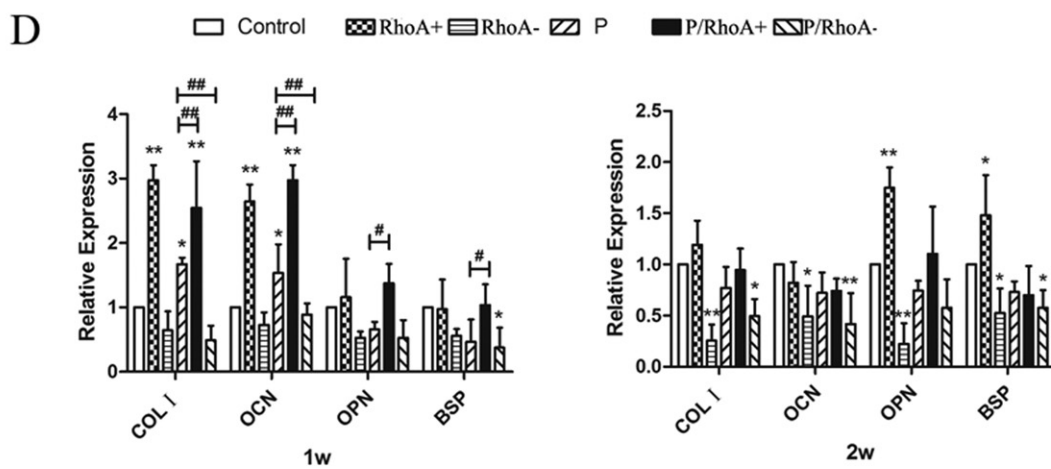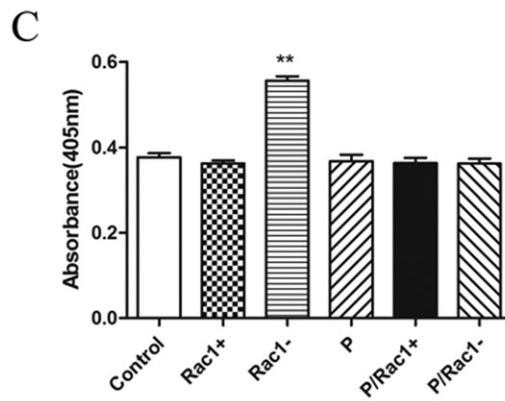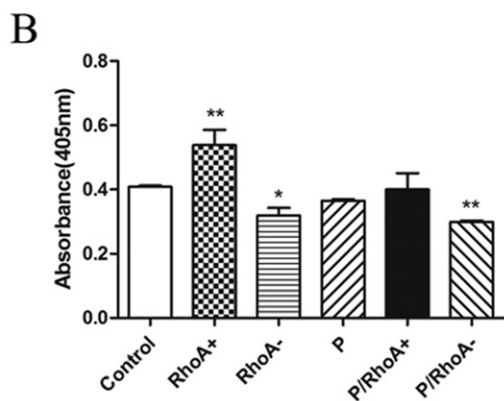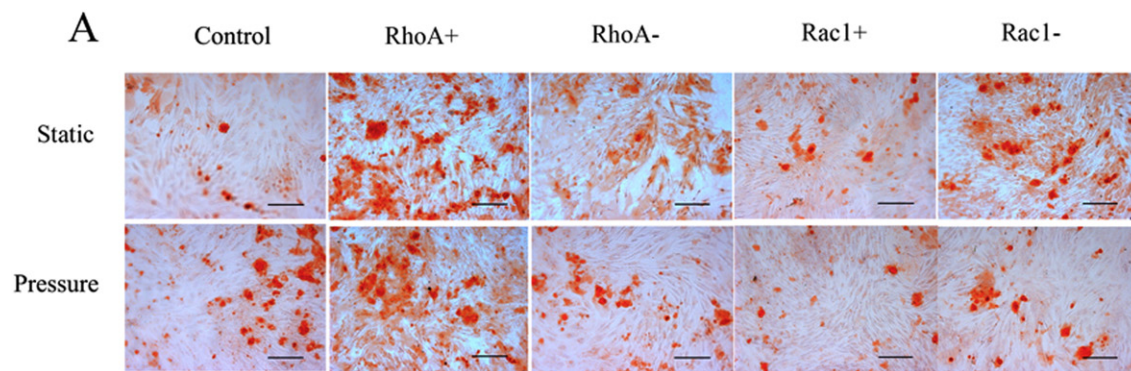
(P/RhoA− group) and the pressure stimulation (P group) significantly promoted the expression of chondrogenic marker genes in the BMSCs, including *Sox-9*, *Aggrecan* and *Col II* ($P \leq 0.01$ vs. control). A combination of decreased RhoA activity and pressure stimulation (P/RhoA− group) achieved the maximum expression of the chondrogenic marker genes in BMSCs. After two weeks of chondrogenic induction, the expression levels of the chondrogenic genes in the P/RhoA+ group were significantly reduced compared with those of the P group ($P \leq 0.01$; Fig. 5D). This suggested that the up-regulation of RhoA antagonized the promoting effect of pressure on the chondrogenic differentiation of the BMSCs. After 4 weeks of chondrogenic induction, the expression levels of *Sox-9*, *Aggrecan* and *Col II* in the RhoA−, P and P/RhoA− groups were significantly higher than those of the control group ($P \leq 0.05$). Compared with the P group, the P/RhoA− group expressed *Sox-9* at a significantly increased level ($P \leq 0.01$; Fig. 5D). This indicated that RhoA kinase was involved in the regulation of the hydrostatic pressure-induced chondrogenic differentiation of the BMSCs and primarily played a negative regulatory role.

An analysis of the regulation of Rac1 activity showed that after 2 and 4 weeks of chondrogenic induction, the up-regulation of Rac1 activity or pressure stimulation significantly promoted the expression of all marker genes for chondrogenic differentiation in the BMSCs, including *Sox-9*, *Aggrecan* and *Col II* ($P \leq 0.01$ vs. control). The combined Rac1 agonist and pressure treatment resulted in the maximum expression of the chondrogenic markers genes in the BMSCs. Compared with the P group, the P/Rac1− group expressed the three chondrogenic genes at significantly reduced levels ($P \leq 0.01$; Fig. 5E), suggesting that the promoting effect of pressure on the chondrogenic differentiation of the BMSCs was Rac1-dependent. Rac1 was critical for the regulation of the hydrostatic pressure-induced chondrogenic differentiation of the BMSCs and primarily played a positive regulatory role.

Discussion

This present study and previous works in the literature have shown that mechanical stimulation promotes the proliferation of BMSCs (Luu et al., 2009). In this study, the presented results revealed that pressure promoted BMSC proliferation by facilitating cell cycle initiation. The G1 phase progression was positively regulated primarily by the cyclin-dependent kinase (CDK)/cyclin D and CDK/cyclin E complexes (Dulic et al., 1992; Koff et al., 1992; Matsushime et al., 1994). The G1 phase progression was negatively regulated by the interactions between CDK and the inhibitor of kinase 4A (INK4A) and the interactions between CDK and the members of the CDK-interacting protein/kinase inhibitory protein (Cip/Kip)

family (McConnell et al., 1999; Parry et al., 1995, 1999)]. Welsh et al. found that the normal expression of the cyclin D protein required the activities of the RhoA/ROCK pathway (Welsh et al., 2001). RhoA/ROCK stabilized ERK activity in cells through the activation of the downstream protein LIM domain kinase (LIMK) and eventually regulated the concentration of the cyclin D protein. In addition, RhoA/ROCK regulated the activities of the CDK inhibitors p21cip1 and p27kip1 (Hu et al., 1999; Olson et al., 1998; Vidal et al., 2002; Weber et al., 1997). The present study showed that pressure promoted cell cycle initiation. However, the simultaneous suppression of RhoA/ROCK activities and application of pressure resulted in cell cycle arrest, indicating that the pressure-induced cell cycle initiation depended on RhoA/ROCK activities. A western blot analysis showed that pressure inhibited ERK phosphorylation, suggesting that the induction of cell cycle initiation by pressure may not require a modulation of the cyclin D concentration, which involved the regulation of ERK activity through the LIMK protein. Therefore, it was speculated that under the present experimental conditions, mechanical pressure promoted cell cycle initiation through the suppression of the activities of the CDK inhibitors p21cip1 and p27kip1. In addition to RhoA/ROCK, which were capable of regulating the cell cycle, the Rac1 pathways have been found to promote cyclin D mRNA translation through integrin activities (Mettouchi et al., 2001). When the Rho pathways were inhibited, Rac1 regulated the expression of cyclin D precursor proteins (Welsh et al., 2001). In agreement with the literature, the present study found that the down-regulation of Rac1 activity effectively antagonized the pressure-induced cell cycle initiation.

It has been reported that mechanical stimulation activated the MAPK signaling cascades in BMSCs and affected their proliferation and differentiation (Riddle et al., 2006; Stanton et al., 2003). Jun Liu et al. applied a combination of hydrostatic pressure and osteogenic agents to BMSCs and found that the exposure to either dynamic or static pressure induced an initial osteodifferentiation of BMSCs. ERK signaling participated in early osteodifferentiation and played a positive but non-critical role in mechanotransduction, whereas p38 MAPK was not involved in this process (Liu et al., 2009). However, the present study showed that pressure promoted the phosphorylation of JNKs but not ERKs in the BMSCs. The discrepancy may be explained by the differences in the types of mechanical forces and biochemical factors applied to the cells. Proteins in the Rho family are closely related to proteins in the MAPK pathways. It was reported that Rac1 functioned as an upstream regulatory factor of JNK (Jin et al., 2006; Kukekov et al., 2006). The present study showed that hydrostatic pressure promoted the phosphorylation of the JNK proteins and up-regulation of both RhoA and Rac1 activities and effectively antagonized the pressure-induced activation of JNK1/2. These results, for the

Figure 4 Effect of RhoA+, RhoA−, Rac1+, Rac1− and pressure on osteogenic genes expression in BMSCs. The BMSCs were cultured in osteogenic medium. After treatment with RhoA+, RhoA−, Rac1+ or Rac1−, the cells were either maintained as no-pressure controls or subjected to 1 h of 90 kPa pressure per day. The cells were then fixed and stained using Alizarin Red (*A*) (scale bar = 200 μm). Quantification of mineralization in BMSCs was performed using an alizarin red-based assay (*B, C*). Analyses of osteogenic markers (Col I, OCN, OPN and BSP) by real-time PCR after one or two week(s) of osteoblast differentiation under the treatment with RhoA+ and RhoA− (*D*), or Rac1+ and Rac1− (*E*) are shown. All gene expression levels were normalized to the endogenous control β-actin. The data are expressed as the means ± SD; **, $P \leq 0.01$ vs. control group; #, $P \leq 0.05$ vs. P group; ##, $P \leq 0.01$ vs. P group.
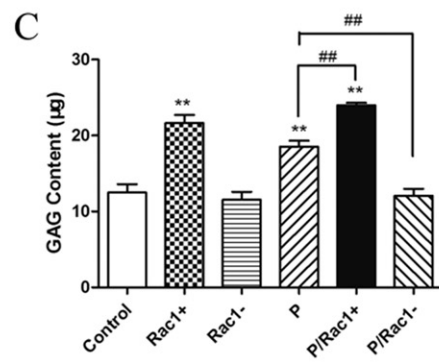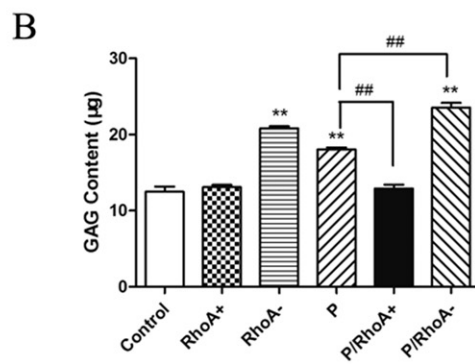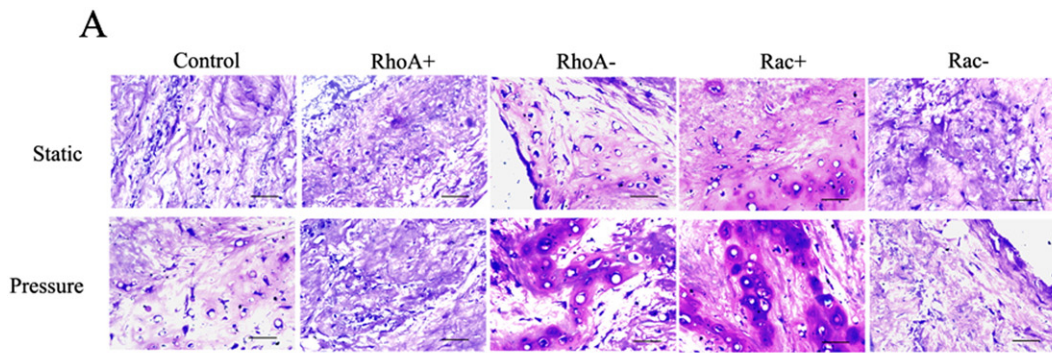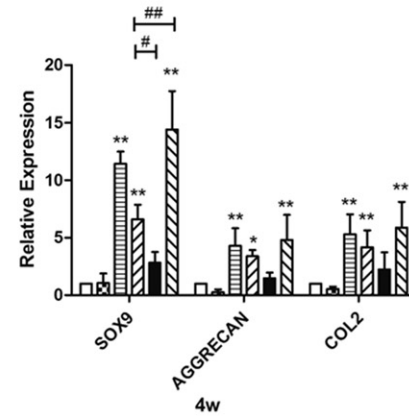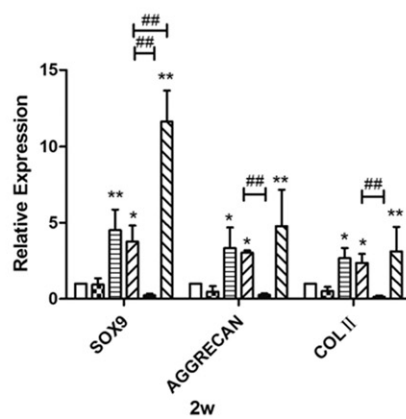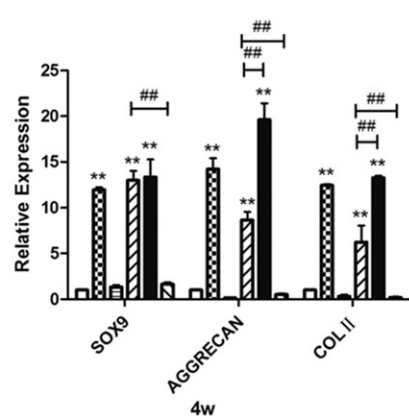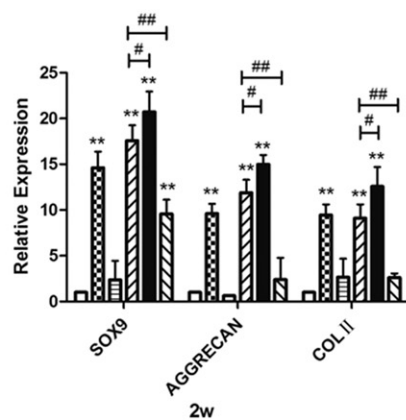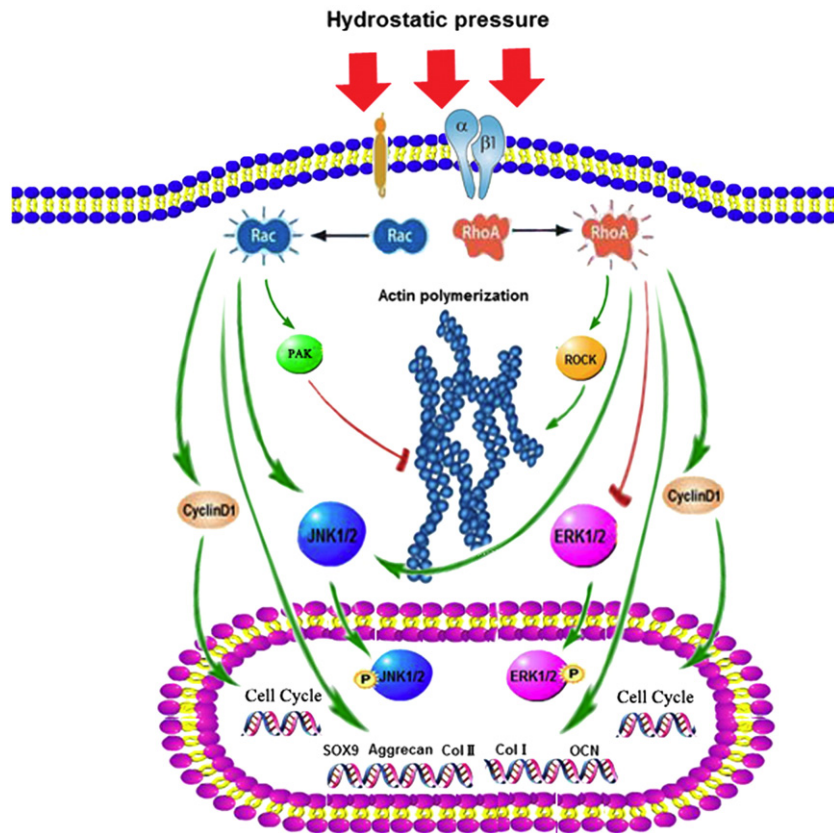
Figure 6 Schematic diagram shows the details of RhoA and Rac1 involved in the mechanotransduction, proliferation and osteogenic/chondrogenic differentiation of BMSCs. Hydrostatic pressure regulates cell cycle initiation through both the RhoA/Rock and the Rac1 signaling pathways. At the same time, the mechanical stimulation promoted cytoskeletal assembly in BMSCs through the up-regulation of RhoA/ROCK activities, and activation of the JNK1/2 pathway by down-regulation of Rac1 activity (A). Hydrostatic pressure could also enhance expression of marker genes for early osteogenic differentiation through the up-regulation of RhoA activation or enhance the expression of chondrogenic marker genes in BMSCs during chondrogenic differentiation via the up-regulation of Rac1 activity (B).

first time, confirmed that the activation of the JNK pathway by hydrostatic pressure may be negatively regulated by RhoA and Rac1. Previous studies have shown that there were associations between active phosphorylated JNK and stress fibers in cells (Hamel et al., 2006; Yang et al., 2007). Furthermore, a proximity ligation assay demonstrated the co-localization of phospho-JNK and F-actin under mechanical stimulation (Mengistu et al., 2011). In the present study, we also observed the increased F-actin cytoskeleton assembly and the up-regulated JNK1/2 phosphorylation in BMSCs upon exposure to hydrostatic pressure. Mengistu et al. reported that the initial co-localization of phospho-JNK with the actin pool outside the nucleus and later with cortical actin at cell peripheries could imply a role of JNK in the transport of actin to form cortical actin (Mengistu et al., 2011). Therefore, we inferred that

inhibition of phospho-JNK under mechanical forces by the agonists of RhoA and Rac1, as observed in our work, was highly dependent on the regulation of the cytoskeleton-modulating proteins to the cytoskeletal assembly.

The present results found that under osteogenic induction, hydrostatic pressure promoted the expression of marker genes for early osteogenic differentiation in BMSCs through the activation of RhoA/ROCK. It was reported that the activated RhoA/ROCK proteins enhanced myosin motor activity through an increase in the level of myosin light chain (MLC) phosphorylation and the inhibition of MLC phosphatase (Amano et al., 1996), which ultimately promoted cytoskeletal assembly. Our results were consistent with the observations of McBeath et al. who had reported that the activation of RhoA/ROCK proteins promoted cytoskeletal assembly,

Figure 5 Effects of RhoA+, RhoA−, Rac1+, Rac1− and pressure on chondrogenic gene expression in BMSCs. BMSCs pellets were cultured in complete chondrogenic medium. After treatment with RhoA+, RhoA−, Rac1+ or Rac1−, the pellets were either maintained as no-pressure controls or subjected to 1 h of 90 kPa pressure per day. Then, the cell pellets were sectioned and stained using toluidine blue (A) (scale bar = 100 μm). GAG content in cell pellets were analyzed by 1,9-dimethylmethylene blue dye-binding assay (B, C). Real-time PCR analysis of the expression levels of chondrogenic markers (Sox9, Col-II and Aggrecan) after two or four weeks of chondroblast differentiation under the treatment with RhoA+ and RhoA− (D), or Rac1+ and Rac1− (E) were analyzed. All gene expression levels were normalized to the endogenous control β-actin. The data are expressed as the means ± SD; **, $P \leq 0.01$ vs. control group; #, $P \leq 0.05$ vs. P group; ##, $P \leq 0.01$ vs. P group.

resulting in a well-spread and flattened cell morphology and a differentiation toward the osteoblast lineage (McBeath et al., 2004). In contrast, as it was suggested in earlier studies that, hydrostatic pressure could also simulate the compressive forces borne by the cartilage in the joint cavity well, which could provide a rather favorable biomechanical environment for tissue-engineered cartilage (Carter and Wong, 1988) and promote the differentiation of BMSCs toward the chondroblast lineage (Angele et al., 2003; Miyanishi et al., 2006a, 2006b) even in the absence of chondrogenic differentiation-inducing factors, such as transforming growth factor beta 3 (TGF-β_3) (Miyanishi et al., 2006a, 2006b). Studies have also shown that the damage to the cytoskeletal structure (M. Zhang et al., 2006; Z. Zhang et al., 2006) and the inhibition of ROCK activity induced rounded cell morphologies and promoted the expression of chondrogenic marker genes in the BMSCs (Woods and Beier, 2006; Woods et al., 2005). These findings were consistent with our results, which showed that biomechanical stimulation promoted the chondrogenic differentiation process of BMSCs when the BMSC pellets cultured in chondrogenic induction media (at high densities and in suspension culture) were subjected to constant hydrostatic pressure for two or four weeks. Therefore, it could be speculated that the mechanical stimulation may be transduced through an inhibited RhoA/ROCK pathway. This would further decrease the level of MLC phosphorylation, inhibit cytoskeletal assembly, induce rounded cell morphologies and promote the chondrogenic differentiation of BMSCs. However, other studies have shown that Rac1 could inhibit the activity of the downstream protein myosin light-chain kinase (MLCK) through the activation of p21-activated kinase (PAK). This reduced the level of MLC phosphorylation and eventually affected cytoskeletal assembly (Sanders et al., 1999). The activation of Rac1 has also been shown to promote the expression of N-cadherin and ultimately enhance the transcription of *Sox-5*, *Sox-6, Sox-9, Col-II* and *Aggrecan* mRNAs (Woods et al., 2007). These findings suggested that in the present study, hydrostatic pressure may have induced N-cadherin activation, promoted the formation of intercellular connections between the BMSCs, and stimulated chondrogenic differentiation through the activation of Rac1. Our results have well established that RhoA and Rac1 have the potential of regulating stem cell fate via intrinsic mechanisms. To our knowledge, this is the first study showing that hydrostatic pressure could induce RhoA activation and further enhance the early osteogenic differentiation of BMSCs. However, hydrostatic pressure could also up-regulate Rac1 and down-regulate RhoA activities to further enhance the chondrogenic differentiation of the BMSCs. These findings suggest that further studies will be required to determine the complex initial biological mechanisms of bone or cartilage formation and regeneration in response to mechanical stimuli.

Conclusions

This study provides new insight to the regulation of the proliferation and the osteogenic/chondrogenic differentiation of BMSCs by hydrostatic pressure. RhoA and Rac1 play important regulatory roles in the pressure-promoted proliferation and differentiation and in F-actin stress fiber assembly and JNK1/2 activation in the BMSCs (Fig. 6). This study showed that the

application of hydrostatic pressure can be adopted as an effective stimulation method for inducing the biological activity of BMSCs and that RhoA and Rac1 can be used as effective modulating molecules for controlling the mechanobiological response of BMSCs.

Acknowledgment

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