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Hydrostatic pressure promotes Wnt10b and Wnt4 expression dependent and independent on ERK signaling in early-osteoinduced MSCs

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ABSTRACT

Recent publications have shown that mechanical stress can regulate the direction of stem cell differentiation. The exact mechanobiological effects of pressure on initial osteodifferentiation have not been determined. Here, we show that ERK signaling participates in early osteodifferentiation and plays a positive but non-critical role in mechanotransduction, whereas p38 MAPK is not involved in this process. Moreover, our findings provide evidence that in response to both types of pressure with high sensitivity, Wnt10b mRNA is ERK-dependent whereas Wnt4 mRNA is upregulated by treatment of the inhibitor of ERK signaling. The findings suggest novel mechanisms of the initial biological responses of bone remodeling and regeneration upon mechanical stimuli.

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Introduction

It has been well accepted that the culture micro-environment of stem cells has a significant influence on differentiation and phenotypic expression. Mechanical signals can regulate the direction of stem cell differentiation [1–3]. Strain alone can induce a significant increase in bone morphogenetic protein-2 (BMP-2) mRNA levels in human MSCs without the addition of osteogenic supplements [4]. Undifferentiated human MSCs are highly sensitive to cyclic tensile strain which transcriptionally controls early osteo-chondrogenic response *in vitro* [5]. However, little is known about the exact mechanobiological effects of dynamic and static pressure on early osteodifferentiation of MSCs.

Recently, the canonical Wnt/β-catenin signaling pathway has been found to play a critical role in skeletal development and osteogenesis [6–10]. The canonical Wnt10b may shift cell fate toward the osteoblast lineage by induction of Runx2, Dlx5, and Osx and suppression of the adipogenic transcription factors [11,12]. Non-canonical Wnt signaling may also play a role in osteodifferentiation. The non-canonical Wnt4 signaling enhances *in vitro* osteodifferentiation of MSCs isolated from human adult craniofacial tissues and promotes bone formation *in vivo* [13]. Since Wnts are secreted growth factors, they may potentially be utilized as recombinant factors to improve bone regeneration. Furthermore, Wnt/β-catenin signaling is shown to be a normal physiological response to load and activation of the Wnt/β-catenin pathway may enhance the sensitivity of osteoblasts/osteocytes to mechanical loading [14,15]. However, little information was known about the relationship between Wnts and the mechanobiological response of MSCs exposed to pressure.

The studies were designed to correlate changes in molecular pathways associated with osteodifferentiation of MSCs with different types of pressure. We investigated the effects of static and dynamic pressure on MSCs during the initial process of osteodifferentiation resulting from treatment with dexamethasone, β-glycerophosphate, and ascorbic acid (for 0, 3, and 7 days, respectively).

Materials and methods

Cell culture. MSCs were isolated from bone marrow of 2-week-old male Sprague–Dawley rats, as reported previously [16,17]. Briefly, both femora and tibias were removed and soft tissues were detached. Metaphysis from both ends were resected and bone marrow cells were collected by flushing the diaphysis with 2 ml/bone of Eagle's alpha minimum essential medium (α-MEM; Gibco) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS; Hy-

Abbreviations: BMP-2, bone morphogenetic protein-2; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde phosphate dehydrogenase; MAPK, mitogen-activated protein kinase; MSCs, mesenchymal stem cells; OS, osteogenic supplements

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Clone), 100 U/ml penicillin, and 100 mg/ml streptomycin. A suspension of bone marrow cells was obtained by repeated aspiration of the cell preparation through a syringe needle (18-gauge). Cells were resuspended in 5 ml of complete medium, plated in a 25-cm^2 glass tissue-culture flask and cultured in a humidified atmosphere of 95% air with 5% CO_2 at 37 °C. After 2 days, the culture medium and non-adherent cells were removed. The medium was changed two or three times a week. As the culture reached almost complete confluence, cells were subcultured or plated for subsequent experiments. MSCs were identified as CD44(+), CD54(+), and CD34(−); furthermore, MSCs cultured in adipogenic, osteogenic, or chondrogenic media differentiated into adipocytes, osteoblasts, and chondrocytes, respectively (data not shown).

MSCs (passage 2–4) were seeded at approximately 1×10^4 cells/cm^2 on culture dishes (diameter 60 mm, Corning) in a culture medium composed of α-MEM medium plus 10% defined FBS and cultured until subconfluence occurred. After this period, cells were grown in the culture medium alone or in osteogenic medium consisting of the same culture medium with the addition of osteogenic supplements (OS)—10 nM dexamethasone, 10 mM β-glycerolphosphate, and 0.05 mM 2-phosphate-ascorbic acid (Sigma). To investigate the effects of mechanical stress on MSCs during their initial osteodifferentiation, we used MSCs that had not yet expressed obvious osteoblastic phenotype such as alkaline phosphatase (ALPase) activity at the stages of 0-, 3-, and 7-day-culture (OS-0d, OS-3d, and OS-7d) [18] (Fig. 1).

Dynamic and static pressure experiments. A custom-made, computer-operated dynamic and static pressure system was designed, fabricated and used in the present study (Supplement 1). The pressure system exposed cells to mechanical stimulation by increasing the pressure of the gaseous phase above the supernatant media, as well as used by other scholars [19]. Briefly, a computer, with software specially written for this system, controlled and maintained a dynamic or static pressure environment inside a sealed chamber, which housed standard tissue-culture plastic ware with cells. During experiments with cells, the pressure system (except for the computer and electronic components) was maintained under standard cell culture conditions, i.e., a 37 °C, humidified, 5% CO_2/95% air environment. MSCs (passage 2–4), seeded at 1×10^4 cells/cm^2 and cultured until subconfluence, were grown in the culture medium alone or in OS for 3 or 7 days. Then OS medium was changed into ordinary culture medium. After that, cells were exposed to dynamic (10–36 kPa, at 0.25 Hz frequency and with a sinusoidal wave) or static (23 kPa) pressure, respectively, for 1 h daily for the next one, three, and five consecutive days. Control cells were cultured on the same dishes and kept in the similar culture conditions and time periods without loading pressure. During these experiments culture medium was initially changed before loading pressure and subsequently half changed every 2 days in the following time periods. In some experiments, cell cultures were treated with 10 μM PD98059 (Sigma) to inhibit ERK activity 4 h before pressure treatment. Cell morphology was observed by phase-contrast microscopy. Fig. 2 schematically outlines the experimental protocol.

Real-time RT-PCR analysis. After loading pressure, cells were washed twice with PBS. The total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's protocol. Total RNA was quantified, in a spectrophotometer, at an absorbance (*A*) of 260 nm. The RNA samples had an *A*260:*A*280 ratio of 2.0 to guarantee high purity. Two micrograms of total RNA from each sample were subjected to reverse transcription using the SYBR® PrimeScript™ RT-PCR Kit (TaKaRa Biotechnology, Dalian, Liaoning, China) according to the manufacturer's protocol. Each real-time PCR was carried out in triplicate in a total of 20 μl reaction mixture (2 μl of cDNA, 10 μl of SYBR® Premix Ex Taq™, 0.4 μl of ROX Reference Dye II, 0.4 μl of each 10 μM forward and reverse primers, and 6.8 μl of H_2O) in an ABI PRISM 7300 Real-time PCR System. Primers used for real-time PCR analysis are presented in Table 1. The PCR program was initiated by 10 s at 95 °C before 40 thermal cycles, each of 5 s at 95 °C and 31 s at 60 °C. The starting copy numbers of unknown samples were calculated by the 7300 System SDS Software from the standard curve. The housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was concurrently amplified in each sample as control and was used for normalization. The cDNA of control MSCs untreated of OS induction and pressure normalized to the level of GAPDH mRNA have been ascribed a fold induction of 1. Melting curves for each PCR were generated to ensure the purity of the amplification product (data not shown).

Western blotting. To obtain whole-cell extracts, cells that were treated with or without pressure were washed twice with ice-cold

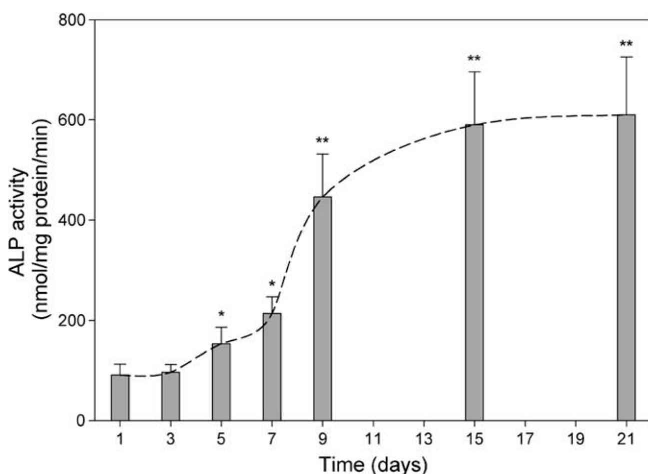

Fig. 1. Time course of alkaline phosphatase (ALPase) activity in osteoinduced rat MSCs. The changing tendency showed as an S-shaped line. During the first 7 days, the ALPase activity had a small rise but remained in low levels. After that, a two- to threefold increase followed. To examine MSCs during their initial osteodifferentiation, we used MSCs that had not yet expressed obvious ALPase activity at the stages of 0- , 3-, and 7-day-culture (OS-0d, OS-3d, and OS-7d). The values are mean ± SD. There were significant differences of $^{*}p < 0.05$ and $^{**}p < 0.01$.

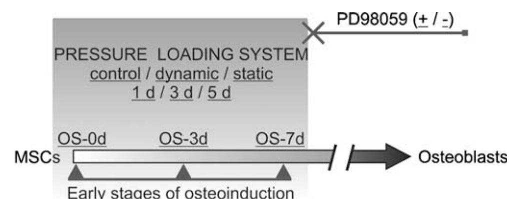

Fig. 2. Experimental design. OS represents osteogenic supplements.

Table 1
Real-time RT-PCR primers used in the experiments.

Target	Primer sequence	PCR product (bp)	GenBank Accession No.
Wnt10b	Forward: 5′-GTAATCACGACATGGACTTTGGAG-3′ Reverse: 5′-GCACTTCCGCTTCAGGTTTT-3′	146	NM_001108111
Wnt4	Forward: 5′-CGTAGCCTTCTCACAGTCCTT-3′ Reverse: 5′-GCTCGCCAGCATGTCTTTA-3′	183	NM_053402
GAPDH	Forward: 5′-GCAAGTTCAACGGCACAG-3′ Reverse: 5′-GCCAGTAGACTCCACGACA-3′	143	NM_017008

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

PBS and then lysed and sonicated in a lysis buffer (Keygen total protein extraction kit, Keygen Biotech., Nanjing, Jiangsu, China). The cytosolic fraction was collected as the supernatant after centrifugation at 14,000*g* at 4 °C for 15 min and assayed it quantitatively with the BCA method. After boiling for 5 min, 20–25 μl of the lysate (50 μg of protein) was applied to SDS–12% PAGE at 120 V for 5 h, and the proteins in the gel were transferred to a PVDF membrane (Millipore). After blocking, the membranes were probed with 1:1000 dilutions of the anti-phospho-ERK1/2 Thr202/Tyr204 (D13.14.4E), anti-ERK1/2 (137F5), anti-phospho-p38 MAPK Thr180/Tyr182 (3D7), and anti-p38 MAPK (Cell Signaling Technology), followed by the addition of horseradish peroxidase (HRP)-conjugated secondary antibody (diluted 1:6000) at 37 °C for 1 h. Immunoreactive proteins were visualized using a chemiluminescence kit (Immobilon Western Chemiluminescent HRP Substrate; Millipore). Band intensities were determined using the ChemiDoc XRS Gel documentation system and Quantity One software (Bio-Rad). Detection of p-ERK1/2 or p-p38 MAPK was performed first. After the targeted bands of p-ERK1/2 or p-p38 MAPK were exposed, PVDF membranes were stripped with eluent (Beyotime Biotech., Haimen, Jiangsu, China). Then the second hybridism was carried out to get the data of ERK1/2 or p38 MAPK, respectively. The band intensity ratio (p-ERK/ERK or p-p38 MAPK/p38 MAPK) was analyzed, respectively.

Statistical analysis. All experiments were performed at a minimum of three times. Measurements are expressed as mean ± SD. Statistical comparisons were made using factorial analysis of variance (ANOVA), followed by multiple comparisons using the SNK test. A value of $p < 0.05$ was statistically considered significant.

Results

Expression of Wnt10b and Wnt4 genes

Both Wnt10b and Wnt4 mRNA levels increased greatly when MSCs (OS-0d, OS-3d, and OS-7d) were exposed to either type of pressure (Fig. 3A and B). Generally, pressure-stimulated OS-0d and OS-3d MSCs expressed higher Wnt10b and Wnt4 mRNA levels than OS-7d MSCs. Both Wnt10b and Wnt4 mRNA levels significantly increased in the control cells after osteogenic induction ($p < 0.05$). It was noted that Wnt10b mRNA level of control OS-3d MSCs decreased on the 5th day after the OS medium was changed and so did Wnt4 mRNA level of control OS-7d MSCs ($p < 0.05$).

PD98059 pretreatment decreased pressure-stimulated Wnt10b mRNA levels of OS-0d and OS-7d MSCs ($p < 0.01$), which were still higher than controls (Fig. 3A). For OS-3d MSCs exposed to pressure for 5 days, slightly stimulated Wnt10b mRNA leve2ls changed insignificantly after usage of PD98059. PD98059 also decreased Wnt10b mRNA levels of control OS-3d and OS-7d MSCs ($p < 0.05$). As showed in Fig. 3B, it was interesting that pressure-stimulated Wnt4 mRNA levels upregulated significantly after PD98059 pretreatment ($p < 0.01$ or $p < 0.05$), though for control OS-3d and OS-7d MSCs levels of Wnt4 mRNA decreased as well as Wnt10b ($p < 0.01$). It suggested that though both Wnts were highly sensitive to pressure signals Wnt10b mRNA was ERK-dependent whereas Wnt4 mRNA was ERK-independent during the mechanobiological process.

Phosphorylation of ERK1/2 and p38 MAPK

When MSCs (OS-0d, OS-3d, and OS-7d) were exposed to both types of pressure for 1 h for one to five consecutive days, ERK1/2 was significantly activated ($p < 0.01$ or $p < 0.05$) at different levels. After dynamic pressure treatment, ERK1/2 phosphorylation reached peaks on the first or third day, whereas static pressure

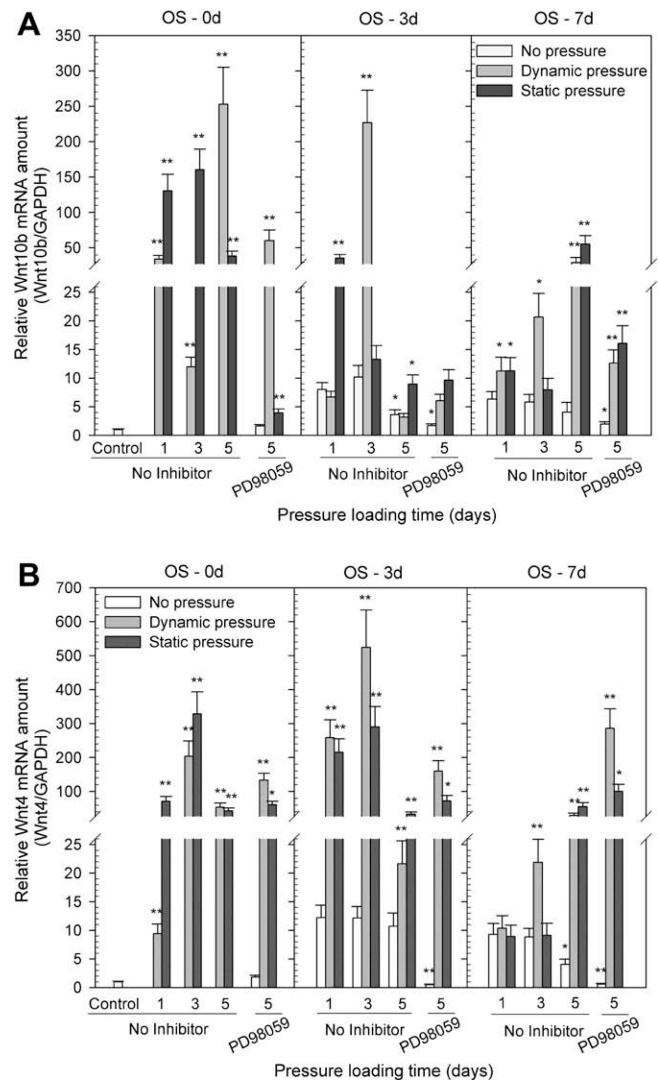

Fig. 3. Expression of Wnt10b and Wnt4 genes affected by dynamic and static pressure for 1–5 days or plus PD98059 pretreatment for 5 days. Transcription of (A) Wnt10b gene and (B) Wnt4 gene. The values are mean ± SD. There were significant differences at $^{*}p < 0.05$ and $^{**}p < 0.01$.

had slower effects (Supplement 2A–C). Supplement 2D demonstrates the peak levels of activated ERK1/2. When exposed to dynamic pressure, OS-0d MSCs (exposed for 1 day) showed significantly lower level of ERK1/2 activation than OS-3d MSCs (exposed for 1 day) and OS-7d MSCs (exposed for 3 days) ($p < 0.05$); but there was no significant difference between the latter two. For cells exposed to static pressure for 5 days, peak levels of ERK1/2 activation increased over the time of osteoinduction ($p < 0.05$). PD98059 pretreatment (10 μM) effectively blocked pressure-induced ERK activity. Supplement 2E illustrates that ERK activities were effectively inhibited by PD98059 when OS-0d and OS-7d MSCs were exposed to both types of pressure for 5 days.

Phosphorylation of p38 MAPK was not found in MSCs (OS-0d, OS-3d, and OS-7d) exposed to either dynamic or static pressure (data not shown).

Discussion

ERK1/2 pathway had been shown to participate in cellular mechanotransduction, turning mechanical signals into intracellular biological signals to regulate cell proliferation and differentia-

tion [20–23]. We demonstrated that both types of pressure activated ERK1/2 signaling at different levels during initial osteodifferentiation of MSCs. Jansen et al. [24] had investigated the effect of mechanical loading on SV-HFO (a human osteoblast cell line) and ERK1/2 signaling in relation to osteodifferentiation. Their study demonstrated that the extent of ERK activation depended on the differentiation stage of the osteoblast. However, their study did not include the mechanoresponse of MSCs during initial osteodifferentiation. The present study showed that after exposure to static pressure for 5 days the peak levels of ERK activation increased over the time of osteoinduction. When treated with dynamic pressure, OS-3d and OS-7d MSCs also expressed higher peak levels of ERK1/2 activation than OS-0d MSCs but at different loading times. Generally, dynamic pressure groups reached the peak levels of ERK activation sooner than static pressure groups.

A major contribution of the present study was the experimental evidence that in response to pressure signals with high sensitivity, Wnt10b mRNA was ERK-dependent whereas Wnt4 mRNA was ERK-independent. Based on the present study, we could not make a simply conclusion of the comparison between the effect of dynamic pressure and the one of static pressure on Wnt10b and Wnt4 mRNA levels. Recently, the existence of crosstalk between canonical Wnt3a and ERK pathways was reported [25–27]. Though both are major signaling pathways for cellular differentiation and function, no significant interactions between Wnts (canonical and non-canonical) and ERK pathway in response to mechanical stimuli had been identified. Our study suggested that ERK signaling played a positive but non-critical role in the mechano-signal transduction. Meanwhile, it was interesting to note that PD98059 pretreatment upregulated pressure-induced Wnt4 mRNA levels significantly, contrary to the downregulation of Wnt10b mRNA. We hypothesize that ERK-independent Wnt4 signaling might be enhanced and act as a substitute for Wnt10b signaling in cellular response to hydrostatic pressure when ERK pathway is blocked by PD98059.

The present study provided evidence that p38 MAPK signaling was not involved in the mechanobiological response of MSCs during their early osteodifferentiation, which contradicted the finding of Simmon et al. [28]. They investigated the effect of cyclic substrate deformation on the proliferation and osteodifferentiation of human MSCs and their results suggested an inhibitory role for p38 signaling in the modulation of strain-induced osteodifferentiation [28]. The relation between the p38 MAPK pathway and mechano-induced osteogenic differentiation requires further investigation.

Our findings showed that different points of initial osteodifferentiation of MSCs had varying responses to either type of pressure. The expression of osteogenesis-related factors in OS-0d MSCs was highly sensitive to pressure exposure; whereas OS-3d or OS-7d MSCs responded slowly to pressure in contrast. We hypothesize that with increased expression of Wnt10b and Wnt4 after OS induction, MSCs could go into a state of low response to pressure. It suggests the complexity of the mechanism of the initial bone remodeling and regeneration upon pressure. Further studies about initial osteodifferentiation are needed to unveil the complex mechanisms.

To examine the exact mechanobiological responses of MSCs and exclude the effects of osteogenic agents, we used the ordinary culture medium instead of the OS medium right before OS-3d and OS-7d MSCs were exposed to pressure. Because the differentiating status of OS-3d and OS-7d MSCs might change over time after that, our study established controls for the different pressure exposure times to exclude the possible bias.

In our study, the magnitudes (dynamic pressure 10–36 kPa and static pressure 23 kPa) and duration (1 h per day) of either type of pressure were chosen according to the macroscopic level physiological values reported in the literature for daily activities of humans and the stress analysis of the periodontal ligament under various orthodontic loadings, considering the side effects of pressure on culture conditions as well [19,29].

In summary, we show that ERK pathway participates in early osteodifferentiation and plays a positive but non-critical role in mechanotransduction, whereas p38 MAPK is not involved in this process. Moreover, we provide evidence that in response to pressure signals with high sensitivity, Wnt10b mRNA is ERK-dependent whereas Wnt4 mRNA is upregulated by treatment of the inhibitor of ERK signaling. The findings should lead to further studies to unveil the complex initial biological mechanisms of bone remodeling and regeneration upon mechanical stimuli.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.12.087.

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