Hydrostatic pressure promotes Wnt10b and Wnt4 expression dependent and independent on ERK signaling in early-osteinduced 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-
Cloning, 100 U/ml penicillin, and 100 mg/ml streptomycin. A sus-

pension 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-

cm² glass tissue-culture flask and cultured in a humidified atmos-

phere of 95% air with 5% CO₂ 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 subse-

quent experiments. MSCs were identified as CD44(+), CD54(+),

osteogenic, or chondrogenic media differentiated into adipocytes, osteo-

blasts, and chondrocytes, respectively (data not shown).

MSCs (passage 2–4) were seeded at approximately

1 × 10⁴ cells/cm² 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 (Sig-

ma). To investigate the effects of mechanical stress on MSCs during

their initial osteodifferentiation, we used MSCs that had not yet ex-

pressed obvious osteoblastic phenotype such as alkaline phospha-
tase (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, com-
puter-operated dynamic and static pressure system was designed,
fabricated and used in the present study (Supplement 1). The pres-

sure 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 soft-

ware specially written for this system, controlled and maintained a

well as used by other scholars [19].

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 osteo-

differentiation, 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.

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer sequence</th>
<th>PCR product (bp)</th>
<th>GenBank Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wnt10b</td>
<td>Forward: 5'-GAAATCAGCAGATGGACTTTGCGG-3'</td>
<td>146</td>
<td>NM_001108111</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GGCTGCAGCGAGAGGTTT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wnt4</td>
<td>Forward: 5'-CTGACCGCTTCAAGTGCTT-3'</td>
<td>183</td>
<td>NM_053402</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CGGCCGTTTGCAACCTT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: 5'-GGAGGTCGCTACGGCGGACG-3'</td>
<td>143</td>
<td>NM_017008</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GCAAGTTCAACGGCCACGCA-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 14,000g 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 hybridization 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 levels 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 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 acti-
verted ERK1/2 signaling at different levels during initial osteo-
differentiation 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 osteo-
differentiation. 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 dy-
namic 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 trans-
duction. Meanwhile, it was interesting to note that PD98059 pre-
treatment 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 dur-
ing their early osteodifferentiation, which contradicted the finding
of Simmon et al. [28]. They investigated the effect of cyclic sub-
strate 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 osteodifferenti-
ation [28]. The relation between the p38 MAPK pathway and mech-
nano-induced osteogenic differentiation requires further investiga-
tion.

Our findings showed that different points of initial osteodif-
ferentiation 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
eclude the effects of osteogenic agents, we used the ordinary cul-
ture medium instead of the OS medium right before OS-3d and OS-
7d MSCs were exposed to pressure. Because the differentiating sta-
tus 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 physio-
logical values reported in the literature for daily activities of hu-
mans and the stress analysis of the periodontal ligament under
various orthodontic loadings, considering the side effects of pres-
sure 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 pres-
sure signals with high sensitivity, Wnt10b mRNA is ERK-depen-
dent whereas Wnt4 mRNA is upregulated by treatment of the
inhibitor of ERK signaling. The findings should lead to further stud-
ies 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

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