

## Original Article

# Laminar shear stress delivers cell cycle arrest and anti-apoptosis to mesenchymal stem cells

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**Biomechanical forces are emerging as critical regulators of cell function and fluid flow is a potent mechanical stimulus. Although the mechanisms of osteoblasts and osteocytes responding to fluid flow are being elucidated, little is known about how the osteoprogenitors, mesenchymal stem cells (MSCs), respond to fluid flow. Here, we examined the effects of laminar shear stress (LSS) on MSCs *in vitro*. MSCs from bone marrow of Sprague–Dawley rats were isolated, purified, and subjected to physiological levels of LSS. DNA synthesis and cell cycle were measured through [<sup>3</sup>H]thymidine and by flow cytometry, respectively, to detect the cellular proliferation. Annexin V immunostaining and *Bcl-2/Bax* mRNA expression were evaluated to determine the effect of LSS on MSCs apoptosis. Results showed that fluid shear stress caused a dose-related reduction of MSCs' proliferation rate with the majority of cells being arrested in the G<sub>0</sub> or G<sub>1</sub> phase. Moreover, it was found that physiological levels of LSS exerted a potent suppression effect on MSC apoptosis. In summary, these data revealed a critical role of LSS in maintaining the quiescence of MSCs.**

**Keywords** mesenchymal stem cells; laminar shear stress; cell cycle arrest; apoptosis

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## Introduction

Biomechanical forces are important regulators of skeletal homeostasis and fluid flow is a potent mechanical stimulus [1]. The deformation of skeletal tissue in response to load induces the pressurization of interstitial fluid [2]. Interstitial fluid flow produces a number of stimuli that bone cells may perceive, including streaming potentials, fluid shear

stress, and chemotransport [3,4]. Fluid shear stress stimulates the proliferation and differentiation of human osteoblast, while inhibits osteocyte apoptosis through the production and release of a number of paracrine factors including nitric oxide and prostaglandin E, as well as alteration of cyclooxygenase 2, c-fos expression [5–8]. Although it has been elucidated how osteoblasts and osteocytes respond to fluid shear stress, little is known about the manner in which the osteoprogenitors, mesenchymal stem cells (MSCs), respond to these stimuli.

*In vivo* MSCs contain progenitor cells with the potential to differentiate along the osteoblastic, adipocytic, and chondrogenic lineages [9] and could be exposed to fluid shear stress resulting from the generation of intramedullary pressure associated with mechanical loads [10]. As MSCs migrate to the sites of bone formation through Haversian systems, flow is predicted to induce shear stress similar to those hypothesized for the lacunar–right network [11]. The deformation of bone tissue caused by mechanical load at physiological levels induces a cyclic movement of interstitial fluid flow, which was found to predict fluid shear rates of 8–30 dyn/cm<sup>2</sup> [12].

Several studies have reported the mechanosensitivity of human MSCs to oscillatory fluid flow (OFF). Li *et al.* [13] found that MSCs subject to OFF exhibited increased intracellular Ca<sup>2+</sup> mobilization, cell proliferation, and mRNA levels of osteopontin and osteocalcin genes. While Riddle *et al.* [14–16] demonstrated that the exposure of MSCs to OFF triggered the release of ATP, the increase of intracellular calcium, and the activation of intracellular signaling cascades such as MAP kinase, and also showed that chemotransport was a prerequisite for MSCs to respond to OFF. Grellier *et al.* [17] suggested that functional p38 and ERK1/2 signaling were both required for MSCs to respond to mechanical signals and regulate osteoblastic phenotype.

As bone tissue is loaded *in vivo*, fluid in lacunar/canalicular network experiences a heterogeneous pressurization in response to the deformation of the mineralized matrix, which leads to fluid flow along pressure gradients. When loading is removed, pressure gradients and flows are reversed. These fluid motions are dynamic and oscillatory in nature. Furthermore, although load-induced fluid flow rates have not been measured directly, the oscillatory component of the bone cell's fluid flow environment has the potential to greatly exceed the steady component of fluid flow driven by the arterial pressure head, suggesting that oscillating flow may be the most appropriate flow regime for *in vitro* study [11].

As oscillatory flow approximates physiological conditions of MSCs experienced *in vivo*, the effect on MSCs metabolism of steady and pulsatile flow regimes should be studied in detail for at least two reasons: (i) interstitial fluid flow includes the steady and pulsatile flow, and in general terms oscillating flow appears to be significantly less stimulative than steady or pulsatile flow [18]; (ii) the current systemic- or site-directed MSCs therapy, for damaged myocardium [19,20] or immunosuppressive effect [21], needs such characterization of the physiological consequences of MSCs in response to the steady and pulsatile flow. However, to date, *in vitro* fluid flow experiments with MSCs have been conducted using oscillatory flow only. Here, we exposed adult rat bone marrow-derived MSCs to precisely controlled laminar shear stress (LSS) and monitored the proliferation and apoptosis.

## Materials and Methods

### Isolation and cell culture of MSCs

The animal experiments conformed to the Guide for the Care and Use of Laboratory Animals published by Peking University and was approved by the local Ethics Committee. Femur and tibia bones from Sprague–Dawley (SD) rats (80–100 g,  $n = 3$ ) were sawn and gelatinous bone marrow was washed out with 5 ml of Dulbecco's modified Eagle's medium (DMEM) solution (low glucose; Gibco, Carlsbad, USA). The suspension of gelatinous bone marrow was filtered through a 74  $\mu\text{m}$  nylon mesh and then cultured in DMEM supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% fetal bovine serum (FBS; Hyclone, Logan, USA) on 25  $\text{cm}^2$  plastic flasks in 37°C with 5%  $\text{CO}_2$ . After 24 h, the non-adherent cells were removed by refreshing the medium and thereafter the medium was refreshed every 3–4 days. After 14 days, cells reached confluence and were treated with 0.25% trypsin with 1 mM ethylenediaminetetraacetic acid (EDTA; Gibco, Carlsbad, USA) and replated at a split ratio of 1:2.

### Exposure to LSS

Shear stress experiments were carried out in flow chambers using the flow loop apparatus designed by Frangos *et al.* [22] and adapted by Bai *et al.* [23] in our laboratory. Briefly, the medium was driven by a constant hydrostatic pressure through the channel of the flow chamber, and cells were exposed to steady laminar fluid flow and well-defined fluid shear stress. The glass slide formed the top of the flow chamber, which was created by sandwiching a silicone gasket between the glass slide and an acrylic plate. LSS applied to the cells (1, 5, 15  $\text{dyn/cm}^2$ ) was corrected using the Poiseuille Law equation [24]. The passage-three MSCs were plated on glass slides (7.5  $\times$  3.8 cm) at  $5 \times 10^4$  cells/slide and flow experiments were carried out. The pH 7.4 was kept by gassing the flow system with a mixture of 95% air and 5%  $\text{CO}_2$  and the temperature was maintained at 37°C. A confluent monolayer of MSCs in a parallel-plate flow chamber was exposed to steady LSS and monitored during and after the stimulation for 4, 12, and 24 h. Control groups were placed under static conditions but without the shear stress for same periods.

### DNA synthesis

Immediately after confluent MSCs were exposed to 15, 5, or 1  $\text{dyn/cm}^2$  LSS for the indicated times (4, 12, and 24 h), the cells were exposed to 2  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]thymidine for 2 h. After the cells were treated with trichloroacetic acid, they were dissolved with 0.1 M NaOH, and the incorporated radioactivity was counted and normalized by cellular protein, which was measured in parallel samples according to the method of Lowry.

### Analysis of cellular DNA content by flow cytometry

For each assay,  $5 \times 10^5$  cells were collected using 0.25% trypsin, fixed with 70% ice-cold ethanol, and treated with 0.02 mg/ml RNase and EDTA. The DNA was stained with 0.1 mg/ml propidium iodide (PI). Cells were incubated in dark for 30 min and then filtered using 70  $\mu\text{m}$  cell strainers. Samples were analyzed on an FACS (Becton Dickinson, San Jose, USA) and using the standard procedure of the Cell Quest software and the ModFitLT software version 3 (Becton Dickinson).

### Cell apoptosis assay

After being exposed to LSS (15  $\text{dyn/cm}^2$ ), cells were harvested using trypsin/EDTA in phosphate-buffered saline (PBS), counted, and collected by centrifugation in PBS. Phosphatidylserine (PS) exposure on the outer leaflet of the plasma membrane was detected using the fluorescent dye Annexin V-FITC Apoptosis Detection Kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. In brief, cells were rinsed with ice-cold PBS and then resuspended in 200  $\mu\text{l}$  of binding buffer. Ten

microliters of Annexin V stock solution was added to cells and incubated for 30 min at 4°C. The cells were then incubated with 5 µl of PI and immediately analyzed on an FACSC-LSR (Becton Dickinson) equipped with CellQuest (Becton Dickinson) software; approximately  $5 \times 10^5$  cells were collected in each of the samples and  $5 \times 10^4$  cells were analyzed.

### RT-PCR analysis

The total RNA was extracted from samples using Trizol reagent (Trizol; TIAN GEN, Beijing, China) to detect the expression of apoptosis-related genes (*Bcl-2* and *Bax*). A single-stranded cDNA was synthesized from 1 µg of total RNA with the use of Oligo(dT) 20 primer and a ReverTra Ace- $\alpha$ - first-strand cDNA synthesis kit (Toyobo, Osaka, Japan). Porphobilinogen dehydrogenase gene (*PBGD*) was served as endogenous reference and used to normalize *Bcl-2* and *Bax* mRNA expression. Primer sequences of the amplified products were as follows: for *Bax*, 5'-TCC CCC-CGA-GAG-GTC-TTT-T-3' (forward), 5'-CGG-CCC CAG-TTG-AAG-TTG-3' (reverse); for *Bcl-2*, 5'-AGA-ACC TTG-TGT-GAC-AAA-TGA-GAA-3' (forward), 5'-TAC-CCA TTA-GAC-ATA-TCC-AGC-TTG-A-3' (reverse); for *PBGD*, 5'-CTG-GTA-ACG-GCA-ATG-CGG-CT-3' (forward), 5'-GCA GAT-GGC-TCC-GAT-GGT-GA-3' (reverse). PCR procedure was carried out by preheating the samples for 10 min at 95°C, consisting of 15 s at 95°C and 1 min at 60°C for 40 cycles. The RT-PCR products were electrophoresed and quantified by scanning with an optical densitometer. The ratio of target genes/*PBGD* was calculated and used as indicators of relative levels.

### Statistical analysis

Experiment was repeated thrice. Data were presented as the mean  $\pm$  SD and statistical analysis was carried out employing the SPSS software package (Version 12.0). *Bax/Bcl-2* data were not normally distributed and therefore tested using the Wilcoxon signed-rank test.  $P < 0.05$  was considered statistically significant.

## Results

### Effects of LSS on DNA synthesis

There was no obvious detachment of MSCs subjected to LSS. The DNA synthesis of MSCs significantly decreased after exposure to 15 dyn/cm<sup>2</sup> LSS (4, 12, and 24 h), which was evident 2 h after the start of exposure to LSS, reaching 64.3% of static control level 4 h later, and 44.5% at 24 h (Fig. 1). An inhibitory effect on DNA synthesis was also observed with 5 dyn/cm<sup>2</sup> LSS. However, no significant inhibition was observed at 1 dyn/cm<sup>2</sup> LSS, suggesting that there was a threshold of growth inhibition due to steady laminar flow between 1 and 5 dyn/cm<sup>2</sup> shear stress in SD rats' MSCs.

### Cell cycle arrest by LSS

Flow cytometric analysis for the DNA content in MSCs indicated that the cell populations in the S and G<sub>2</sub>/M phases gradually decreased after cells were subjected to 15 dyn/cm<sup>2</sup> LSS. The percentage of cells in the S–G<sub>2</sub>/M phase was 22.31 versus 33.94%, 13.64 versus 38.54%, and 11.50 versus 32.86% in sheared and control group after 4, 12, and 24 h, respectively. This reduction was accompanied by an increase in the percentage of cells in G<sub>0</sub>/G<sub>1</sub> phase (Fig. 2). These results indicated that LSS inhibited DNA synthesis of MSCs by inhibition of cell transition from G<sub>0</sub>/G<sub>1</sub> to S phase of the cell cycle.

### Effects of LSS on MSC apoptosis

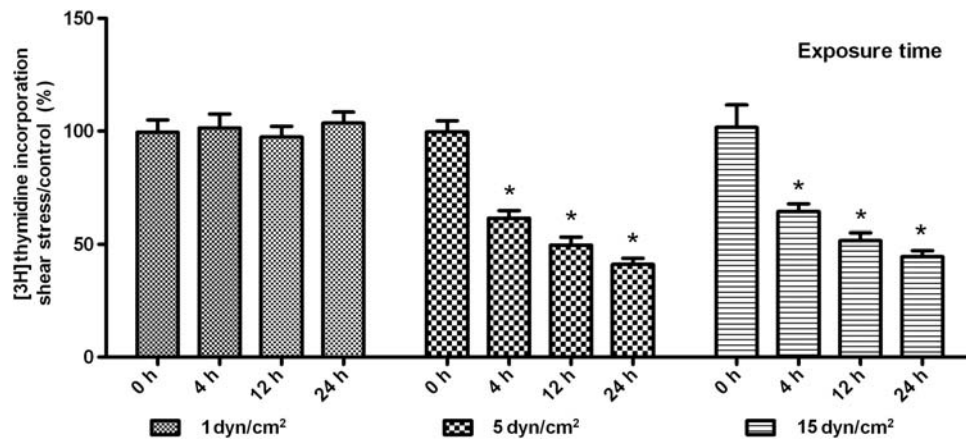
Cell apoptosis was measured using fluorescent dye Annexin V-FITC, which bound to PS residues that were redistributed from the inner to the outer leaflet of the cell membrane as an early event in apoptosis. After loss of membrane integrity, PI could enter the cell and intercalate into DNA. Annexin V<sup>−</sup>/PI<sup>−</sup> cells were considered viable cells, whereas Annexin V<sup>+</sup>/PI<sup>−</sup> cells were counted as apoptotic cells. Figure 3(A,B,C) showed that the percentages of Annexin V- and PI-stained cells in response to 15 dyn/cm<sup>2</sup> LSS treatment after exposure of 12 and 24 h. The percentage of viable cells increased to 82.37% compared with the static control value of 59.39% after 12 h, and 81.55% after 24 h [Fig. 3(C)]. The percentage of apoptotic cells was reduced to 17.49% compared with control of 40.61% after 12 h, and 18.33% after 24 h [Fig. 3(B)]. These data suggested that physiological levels of LSS at 15 dyn/cm<sup>2</sup> exhibited a potential suppression effect on MSCs apoptosis.

### RT-PCR results on LSS

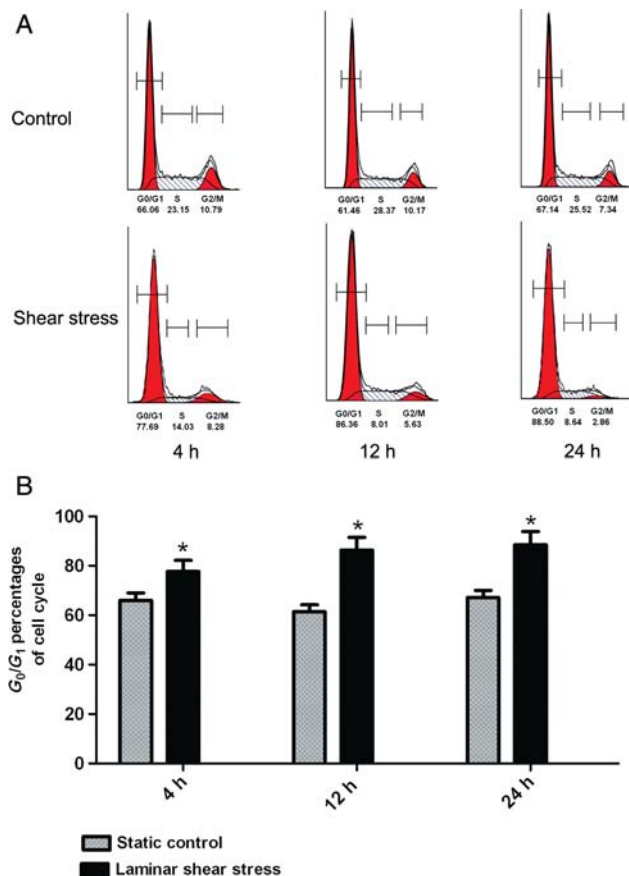
RT-PCR analysis confirmed the apoptosis observations above. As shown in Fig. 4, LSS treatment changed the expression of apoptosis-regulating genes toward greater cell viability, as indicated by a higher *Bcl-2* expression level and a higher *Bcl-2/Bax* ratio. This effect occurred as early as 1 h after the beginning of LSS treatment and in a dose-dependent manner, suggesting that shear stress initiated a rescue program for cell survival (data not shown). The LSS simulation at physiological levels led to increased expression of *Bcl-2*, reaching 5.42 folds (5 dyn/cm<sup>2</sup>) and 7.54 folds (15 dyn/cm<sup>2</sup>), respectively, after 12 h of treatment. The *Bcl-2/Bax* ratio was shown in Fig. 4(B).

## Discussion

Previous reports have indicated that the exposure of MSCs to steady laminar flow decreases the proliferation [25], whereas turbulent flow stimulates the proliferation [13]. In the present study, steady LSS blocked the cell cycle event



**Figure 1 Time course of the LSS-induced inhibition of MSCs DNA synthesis** The confluent MSCs were exposed to 1, 5, and 15 dyn/cm<sup>2</sup> LSS for the indicated times (0, 4, 12, and 24 h). Data represent averages of three experiments. The data are presented as values relative to the static control, which was set at 100%. \* $P < 0.05$  versus static control.



**Figure 2 Effect of LSS on the cell cycle** After MSCs were subjected to 15 dyn/cm<sup>2</sup> shear stress for the indicated times (4, 12, and 24 h), sheared and control MSCs were harvested by trypsin, fixed with ethanol, and treated with RNase and EDTA. After DNA was stained with PI, the fluorescence was evaluated by flow cytometry (A) and the histogram (B) was present. \* $P < 0.05$  versus static control.

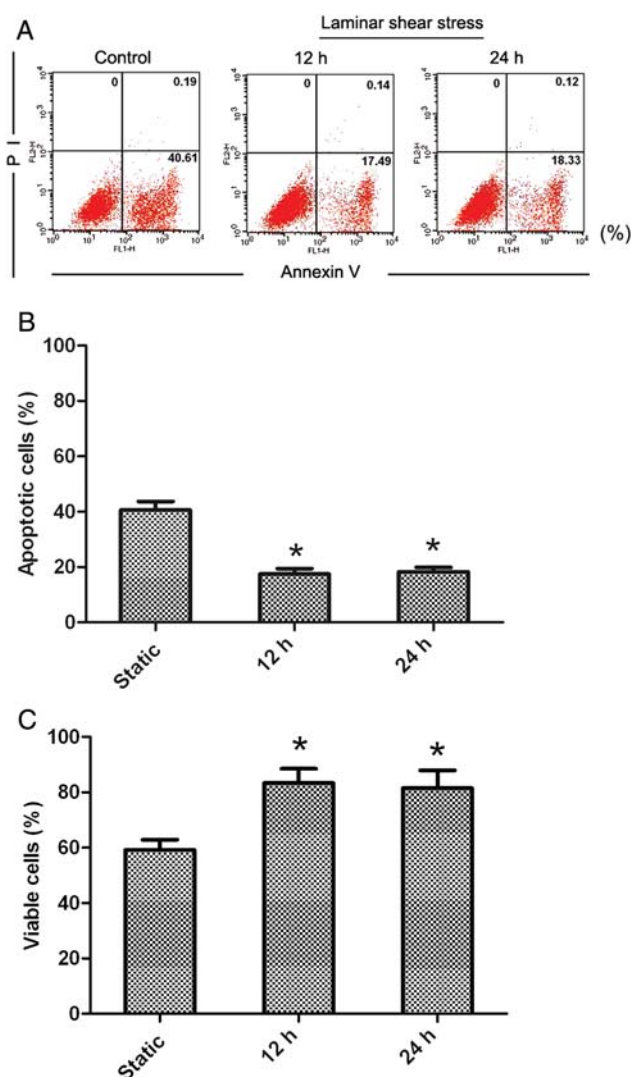
from G<sub>0</sub>/G<sub>1</sub> phase into the S phase. The accumulation of the cell population in the G<sub>0</sub>/G<sub>1</sub> phase after exposure to LSS was accompanied by a decrease in the number of cells in the S phase as well as a decrease in the G<sub>2</sub>/M

population. These results suggest that LSS may play a regulatory role in the inhibition of proliferation, in association with maintaining cells in G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle.

However, the inhibition of proliferation in association with maintaining cells in G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle cannot address the question of cell cycle arrest in quiescence or cell cycle arrest in senescence, since the classic features of both phenotypes include growth arrest in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle [26]. When MSCs approach the end of their lifespan, the senescence concurs. The abnormalities in mitochondrial metabolism include increased activity of cytochrome *c*, oxidase and nicotinamide adenine dinucleotide dehydrogenase, decreased mitochondrial membrane potential, and augmented mitochondria biogenesis [27]. We further detected mitochondrial metabolism of MSCs in physiological levels of LSS.

Twelve-hour treatment with LSS of physiological magnitude reduced the prevalence of apoptotic MSCs by 17.49% and 24 h later by 18.33%. Static control conditions that represent a situation of no mechanical stress and no LSS on the cell membrane caused a high prevalence of apoptosis in MSCs. This reduction of apoptosis was not due to the removal of pre-apoptotic MSCs under the shear stress, since the total number of MSCs per microscopic field was similar before and after treatment (data not shown). We further measured the expression of *Bcl-2/Bax* mRNA. The *Bcl-2/Bax* are apoptosis-regulating genes and most of its function protein anchored in the outer mitochondrial membrane [28,29]. LSS treatment changed the expression of apoptosis-regulating genes toward greater cell viability, as indicated by a higher *Bcl-2* expression and a higher ratio of *Bcl-2/Bax*. This effect occurred as early as 1 h after the beginning of LSS treatment and in a time-dependent manner, suggesting that LSS initiated a rescue program for cell survival. We concluded from the present results that

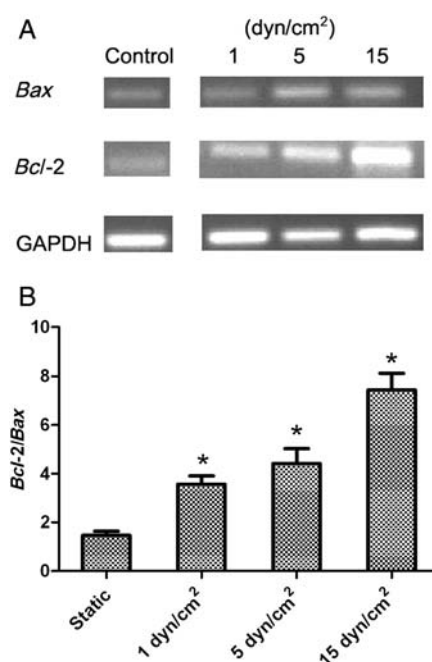




**Figure 3** LSS triggers anti-apoptosis in MSCs (A) MSCs were incubated for 12 and 24 h, then apoptosis was quantified by FACS analysis after staining with Annexin V and PI. The Annexin V<sup>+</sup>/PI<sup>-</sup> cells were early in the apoptotic process (B) and viable cells were Annexin V<sup>-</sup>/PI<sup>-</sup> (C). Data were present as the mean  $\pm$  SD of three independent experiments. \* $P < 0.05$  versus static control.

for MSCs, LSS might be an important survival factor for MSCs.

Greider *et al.* [30] and Valentin *et al.* [31] have reported that the anti-apoptotic regulators BCL-2 and BCL-x(L) promote G<sub>0</sub> by increasing p27 protein level, decreasing cell size and RNA content, reflecting anti-proliferative effects through their ability to enhance quiescence. In cellular quiescence, proliferative genes such as Myc and cyclins are turned off; the RB family member p130 and p27 are up-regulated [29]. These regulatory events are further accentuated in cells overexpressing BCL-2 or BCL-x(L) [32]. Collectively, the results of cell cycle arrest, together with anti-apoptosis effect, lead MSCs to quiescence in physiological levels of LSS.



**Figure 4** Effect of 12 h treatment with or without LSS on mRNA expression of *Bcl-2* and *Bax* (A) and on the *Bcl-2/Bax* ratio (B) in MSCs. LSS was used at 1, 5, or 15 dyn/cm<sup>2</sup>. Data represent averages of three experiments. \* $P < 0.05$  versus static control.

Anchorage of cells to the extracellular matrix (ECM) and integrin-mediated signals play crucial roles in cell growth, differentiation, and survival [33]. Most normal cells need to adhere to serum-derived ECM components in order to survive *in vitro*, a phenomenon called anchorage dependence. The interchange of metabolic waste and nutrients in flow chambers should be superior to that occurring in static condition, acting as a counterbalance to matrix deprivation. This is probably one reason for the high rate of apoptosis in static MSCs. Furthermore, cell adhesion to ECM is primarily mediated by integrins. Integrins, architectural and signaling mediators, are present on the cell surface in an inactive state. Several signal transduction components are activated by integrins, including focal adhesion kinase [33,34], phosphatidylinositol 3-kinase [35], mitogen-activated protein kinase, and extracellular signal-regulated kinase (ERK) [36]. Recent publications have shown that ERK signaling pathway participates in early-osteoinduced MSCs under hydrostatic pressure [37]. Here in our study, the LSS may result in a significant alteration of cytoskeletal structures through the ERK signaling pathway, which leads to a suppression in cell cycle arrest. The mechanical mechanisms underlying the association of integrins with ERK, or other signal transduction pathway for LSS-induced MSCs changes, remain to be investigated.

In summary, our results clearly demonstrated that physiological levels of LSS were contributing to quiescence of MSCs and to maintenance of the stem cell pool. This may

also help explaining why *in vivo* propagation of MSCs may lead to quiescence in niches [38]. Furthermore, such characterization of the physiological consequences of MSCs in response to LSS will provide important information for the systemic or site-directed MSCs, as the blood fluency may change the function of transplanted cells. The therapy provided a potential approach for damaged myocardium or for immunosuppressive effect.

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