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# Mechanical stretch inhibits mesenchymal stem cell adipogenic differentiation through TGFβ1/Smad2 signaling

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ABSTRACT

Mesenchymal stem cells (MSCs) are the common precursors of several functionally disparate cell lineages. A plethora of chemical and physical stimuli contribute to lineage decisions and guidance, including mechanical stretch concomitant with physical movement. Here, we examined how stretch regulates MSC differentiation into adipocytes and the intracellular signaling pathways involved. MSCs were cultured under adipogenic conditions and divided into a control and an experimental group. Cultures in the experimental group were subjected to a sinusoidal stretch regimen delivered via flexible culture bottoms (5% magnitude, 10 times per min, 6 h/day, 3 or 5 days). Expression levels of the adipocyte markers PPARγ-2, adiponectin, and C/EBPα were measured as indices of differentiation. Compared to controls, MSCs exposed to mechanical stretch exhibited downregulated PPARγ-2, adiponectin, and C/EBPα mRNA expression. Alternatively, stretch upregulated phosphorylation of Smad2. This stretch-induced increase in Smad2 phosphorylation was suppressed by pretreatment with the TGFβ1/Smad2 pathway antagonist SB-431542. Pretreatment with the TGFβ1/Smad2 signaling agonist TGFβ1 facilitated the inhibitory effect of stretch on the expression levels of PPARγ-2, adiponectin, and C/EBPα proteins, while pretreatment with SB-431542 reversed the inhibitory effects of subsequent stretch on the expression levels of these markers. These results strongly suggest that the anti-adipogenic effects of mechanical stretch on MSCs are mediated, at least in part, by activation of the TGFβ1/Smad2 signaling pathway.



## 1. Introduction

Mesenchymal stem cells (MSCs) are the precursors of several cell lineages, including osteoblasts, chondrocytes, myoblasts, adipocytes, and fibroblasts (Liu et al., 2009b; Pittenger et al., 1999). It is thought that complex combinations of growth factors, cytokines, hormones, and physical stimuli act to guide MSC differentiation toward specific lineages. To induce differentiation toward one lineage it is also necessary to prevent MSCs from differentiating into alternative cell types (Heino and Hentunen, 2008; Knothe Tate et al., 2008; Zhao and Hantash, 2011). Hong et al. (2005) demonstrated that the TAZ promoter regulates MSC osteogenic differentiation by activating RunX-dependent transcription cascades while repressing adipocyte-linked PPARγ-dependent transcription cascades. Zhou et al. (2004) demonstrated a synergistic interaction between TGFβ and Wnt signaling pathways in the stimulation of chondrogenesis and inhibition of adipogenesis.

Cells in vivo are exposed to a variety of mechanical stimuli, many of which couple kinetic processes to tissue development (Adams, 2006; Genchev et al., 2009; Park et al., 2007). For instance, physiological loading reportedly helps maintain skeletal integrity (Frost, 2004). Recent studies have also indicated that mechanical stretch regulates MSC lineage decisions, facilitating MSC differentiation into osteoblasts while impeding differentiation into adipocytes. Hossain et al. (2010) found that compressive force could inhibit human adipogenesis by suppressing PPARγ-2 and C/EBPα expression. Khayat et al. (2012) reported that relatively low-frequency mechanical stimulation (0.01 Hz) inhibited C3H10T1/2 mouse MSC adipogenic differentiation, even in the presence of a potent adipogenic differentiation medium. Conversely, physical inactivity leads to progressive obesity, which is characterized by an increase in adipocyte number and individual cell size (Coppack, 2005). Limiting food intake and increasing physical exercise are commonly utilized to prevent and cure obesity. Physical

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activity, massage, and whole body vibration impose mechanical stress on tissues and can effectively alleviate or prevent obesity and mitigate the symptoms of osteoporosis (Leslie and Morin, 2014; Rubin et al., 2007). Thus, physical exercise may control obesity and strengthen the skeletal system by downregulating adipocyte differentiation while promoting the osteocyte lineage (Shoham and Gefen, 2012).

The signaling pathways underlying the inhibitory effects of mechanical stretch on adipogenesis from MSCs remain elusive. Adipocyte differentiation requires a variety of signaling pathways (TGF, BMPs, PTHrP, Wnt, Notch, and MAPK) and transcription factors (PPAR and C/EBP) (Hossain et al., 2010; Potier et al., 2010; Rosen and MacDougald, 2006). TGFβ, an important factor involved in the regulation of MSC adipogenic differentiation (Kaminska et al., 2005), inhibited adipogenesis in preadipocyte cell lines while TGF overexpression reduced adipocyte differentiation in vivo (Choy and Derynck, 2003; Kaminska et al., 2005). Ahdjoudj et al. (2002, 2005) reported that increased adipogenic differentiation from MSCs in the bone marrow of rats subjected to reduced skeletal loading was inhibited by TGFβ treatment. TGFβ signaling is activated by binding to membrane receptors and subsequent phosphorylation and nuclear translocation of the downstream effector molecule Smad (Kamato et al., 2013). However, it is unknown whether mechanical stretch regulates MSC adipogenic differentiation through the TGFβ/Smad signaling pathway.

This study was designed to investigate the role of mechanical stretch in regulating MSC adipogenic differentiation. During chemical induction of adipogenesis, periodic stretch was imposed with or without a TGFβ/Smad signaling agonist or inhibitor, and Smad2 phospho-activation and expression levels of adipogenesis markers PPARγ-2, adiponectin and C/EBPα were measured.

## 2. Materials and methods

### 2.1. Ethics statement

All animal work was conducted according to relevant national and international guidelines. MSCs were isolated from the bone marrow of Sprague–Dawley rats (male or female, 80–100 g) as described below using procedures approved by the Animal Care and Use Committee of Nanfang Hospital at Southern Medical University. Rats were euthanized via cervical dislocation under the guidelines of the Animal Care and Use Committee of Nanfang Hospital. Appropriate steps were taken to ameliorate suffering in accordance with our institutional Animal Care and Use Committee.

### 2.2. Cell culture and identification

MSCs were isolated according to a previously described method (Zeng et al., 2012). Briefly, bone marrow was obtained by flushing the femurs and tibias of rats with general medium (GM) containing DMEM-LG (Gibco, Langley, OK, USA) supplemented with 10% defined fetal calf serum (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin (North China Pharmaceutical Factory, China). Isolated cells were plated in 25 $cm^2$ flasks and incubated at 37 °C in a humidified atmosphere containing 5% $CO_2$. After a 24 h incubation, non-adherent cells were removed by washing with PBS and fresh GM added to allow for further growth. The culture medium was changed every 2–3 days. When the cells reached 80–90% confluence, they were washed with PBS, detached by 0.25% trypsin, and subcultured in two new 25 $cm^2$ flasks at $1 \times 10^4$ cells/$cm^2$. MSCs were collected at the second or third generation and examined by flow cytometry to measure the expression levels of CD29, CD34, CD44, and CD45 (BD, Franklin Lakes, NJ, USA). All further experiments were performed with cells at 3–6 passages.

### 2.3. Experimental grouping and mechanical stretch parameters

Grouping was based on stretch intervention (with/without), culture in general or adipogenic differentiation medium, and treatment with TGFβ1/Smad2 signaling agonists or antagonists (with/without). DMEM-LG was used as the GM, while the adipogenic differentiation medium included DMEM-DG containing 1 μmol/L dexamethasone, 0.5 mmol/L IBMX, 10 mg/L insulin, and 100 mmol/L indomethacin. Mechanical stretch was delivered by seeding MSCs on flexible membranes, with membrane contortion controlled by a Flexcell-5000 device (Hillsborough, NC, USA) using the methods described by Turner et al. (2008) and Lohberger et al. (2014). Strain parameters were as follows: 5% magnitude sine wave at 10 per min, 6 h/day, for 3 or 5 days.

### 2.4. Measuring effect of mechanical stretch on adipogenic differentiation

MSCs were plated in GM onto type IV collagen-coated Bioflex 6-well plates (Flexcell International, Hillsborough, NC, USA) at $\sim 1.5 \times 10^5$/2 ml. After ~24 h, MSCs started to adhere and reached 80% confluence approximately 3 days later. After replacing the culture medium (GM or adipogenic), MSCs in the experimental groups were subjected to mechanical stretch using the Flexcell-5000 device. All conditions except delivery of mechanical stretch were identical between the control and experimental groups. All treatments were repeated three times with independent cultures. MSCs were collected, and lipid droplets, a sign of the adipocyte phenotype, were visualized using oil red O staining. Expression levels of adipogenic marker mRNAs and proteins were estimated using real-time fluorescence quantitative PCR and Western blot, respectively, as detailed below.

### 2.5. TGFβ1/Smad2 signaling modulation

Cells were plated on Bioflex 6-well plates as described, and the medium changed to GM or adipogenic differentiation medium with or without a TGFβ1/Smad2 signaling agonist (TGFβ1, 4 ng/ml) or antagonist (SB-431542, 10 μM). After another 24 h in culture, cells in the experimental group were subjected to stretch stimulation as described while the control group was incubated without stretch stimulation. After stretch intervention or the control period, cells were collected and expression levels of signaling pathway proteins (Smad2 and p-Smad2) and downstream adipogenic differentiation markers (PPARγ-2, adiponectin, and C/EBPα protein) were measured by Western blot (details provided below).

### 2.6. Histochemical staining

Cells were fixed in 4% paraformaldehyde, rinsed three times in deionized water (5 min per rinse), and then stained with oil red O (Khayat et al., 2012; Sen et al., 2008).

### 2.7. Reverse transcription and real-time fluorescence quantitative PCR

Total RNA was extracted after treatment using Trizol reagent (Invitrogen, CA, USA) according to the manufacturers' instructions and concentration/quality assessed by spectrophotometry (ShiMadzu, Japan). First strand cDNAs were synthesized from the total RNA using the Prime Script™ RT Reagent Kit (Takara, Kyoto, Japan) according to the manufacturers' instructions. Real-time fluorescence quantitative PCR analyzes were performed at least in triplicate using the CFX96 Real-time PCR System (Bio-Rad, USA). The primer nucleotide sequences are listed in Table 1. Primers were designed using Primer 3.0 based on sequences in the National Center for Biotechnology Information (NCBI) website database (http://www.ncbi.nlm.nih.gov/gene/). Reactions were performed in a final volume of 20 μl containing 25 ng cDNA, 10 μl SYBR® Premix Ex Taq™ (2 ×), and 1 μl each of the PCR forward and reverse primer (10 μM). The cDNAs were amplified at 95 °C for 10 min, followed by 44 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 20 s, and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was then quantified as an endogenous control, and target gene expression levels in each sample were normalized to that of GAPDH. PCR specificity was determined using dissociation curve analysis. Data were analyzed using Bio-Rad CFX Manager Software (version 1.6, Applied Biosystems) to determine relative expression of genes.

### 2.8. Western blot

Proteins were extracted from the collected cells (Turner et al., 2008) and total concentration measured using a BCA protein assay kit (Boster, China) according to the manufacturers' instructions. Equal quantities of proteins per gel lane were separated on 10% polyacrylamide gels by SDS-PAGE and transferred to nitrocellulose membranes using an electroblotting apparatus (Bio-Rad, Hercules, CA, USA). Membranes were blocked in 5% non-fat milk/PBS-Tween 20 solution, followed by separate incubation with monoclonal antibodies specific for phospho-Smad2 (1:1500, Sigma, St. Louis, MO),

**Table 1**
Design of primer sequences.

| Gene | Reference gene ID | Primer | Primer sequence |
|---|---|---|---|
| **PPARγ-2** | NM_013124.3 | Forward | TCTGGGAGATCCTCCTGTTG |
| | | Reverse | CGAAGTTGGTGGGCCAGAAT |
| **Adiponectin** | NM_144744.3 | Forward | CTGGCTCCAAGTGTATGGGG |
| | | Reverse | AAGCCTGTCGCCTGTTCTTT |
| **C/EBPα** | NM_012524.2 | Forward | GCCGGGAGAACTCTAACTCC |
| | | Reverse | TCGATGTAGGCGCTGATGTC |
| **GAPDH** | NM_017008.3 | Forward | TGCCACTCAGAAGACTGTGG |
| | | Reverse | TTCAGCTCTGGGATGACCTT |