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The Role of the Wnt Signaling Pathway in the Osteogenic Differentiation of Human Adipose-derived Stem Cells under Mechanical Stimulation

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Abstract: This study investigates the effects of the Wnt signaling pathway on the osteogenic differentiation of human adipose-derived stem cells (hASCs) under tensile stress. hASCs cultured in vitro were divided into 4 groups: Group A, hASCs; Group B, Wnt5a RNAi-treated hASCs; Group C, hASCs under tensile stress; and Group D, Wnt5a RNAi-treated hASCs under tensile stress. Five days after treatment, the genes associated with the Wnt/ β -catenin and Wnt/ Ca^{2+} pathways were analyzed in all groups by real-time RT-PCR; the Wnt10b, Wnt5a, RUNX2 and SPP1 proteins were analyzed by western blot analysis. Compared with the expression in Groups A and B, all the genes and proteins in Groups C and D had higher expression, except for Wnt5a in Group D. Compared with Group C, Wnt5a, RhoA, RUNX2 and ALPL had lower expression in Group D, but the markers associated with Wnt/ β -catenin had higher expression. The results suggest that tensile stress can promote maturation and osteogenic differentiation in hASCs and also activate the Wnt/ β -catenin and Wnt/ Ca^{2+} pathways. The Wnt/ Ca^{2+} pathway may have the potential to inhibit the Wnt/ β -catenin pathway. Wnt5a knock down seemed to increase the expression of the Wnt/ β -catenin pathway, which is activated by Wnt10b.

Key words: Human adipose-derived stem cell, Tensile stress, Osteogenic differentiation, Wnt signaling pathway

Introduction

Previous research has shown that cells and tissues in their native environments are subjected to various forms of mechanical loading, such as compression, tension, and shear stress. These mechanical forces play important roles in the regulation of in vivo cellular functions, including gene induction, protein synthesis, cell growth, and differentiation, all of which are essential to maintain or alter tissue homeostasis. Previously, scholars have extensively studied a variety of cells¹⁻³⁾, particularly mesenchymal stem cells (MSCs) and the effect of mechanical stimulation on their differentiation⁴⁻⁶⁾. Some studies have indicated that tensile stress can promote an osteogenic response in human adipose-derived stem cells (hASCs), a type of MSCs⁷⁻⁹⁾.

The advantages of using adipose-derived stem cells (ASCs) are obvious, including their abundance and accessibility as a cell source and their ease of expansion in culture. Furthermore, animal experiments have shown that the osteogenic differentiation ability

of murine ASCs does not significantly decrease with age¹⁰⁾. Recently, many studies have demonstrated that hASCs represent an important new cell source for bone tissue engineering¹¹⁻¹³⁾.

The process of osteogenic differentiation of hASCs under tensile stress is regulated by a series of factors, including cytokines, signaling molecules and surrounding environmental factors^{14,15)}. Wnt signaling plays a key role in osteogenic differentiation¹⁶⁻¹⁹⁾. The Wnt signaling pathway mainly consists of three branches: the Wnt/ β -catenin pathway (the canonical Wnt signaling pathway), the Wnt/PCP pathway (the planar cell polarity pathway) and the Wnt/ Ca^{2+} pathway²⁰⁻²³⁾. Some studies have confirmed that genes of the Wnt/ β -catenin and Wnt/ Ca^{2+} pathways have significantly different expression patterns during the osteogenic differentiation of stem cells induced by mechanical and chemical stimuli, suggesting they play distinct roles in development and osteogenic differentiation^{16,19)}. High expression of Wnt10b, one of the important Wnt/ β -catenin-activating molecules, can promote osteogenesis²⁴⁻²⁷⁾. The non-canonical Wnt5a, an important Wnt/ Ca^{2+} pathway-activating molecule, can cause the Ror2 extracellular domain to fuse with the LPR6 intracellular domain in vitro, activating the Wnt/ Ca^{2+} pathway through a series of complex

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Figure 1. Classification tree showing the group treatment scheme of 108 specimens in experiments 1 and 2 according to the experimental design.

processes²⁸⁻³¹). Recently, a study demonstrated that Wnt5a and its downstream molecule RhoA can act on the RUNX2 gene and increase its expression to promote osteogenic differentiation³². These results indicate that the roles of the Wnt/Ca²⁺ and Wnt/ β -catenin pathways in the osteogenic differentiation of MSCs are closely related. However, the results of similar studies remain controversial³³⁻³⁵, and the effect of Wnt signaling on the osteogenic response of hASCs under mechanical stimulation remains unclear. In this study, we investigated the effect of Wnt signaling on the osteogenic differentiation of hASCs under tensile stress in vitro using a four-point bending apparatus. This work focused on the effects of tensile stress on the gene and protein expression of Wnt signaling components and the relationship between the Wnt/ β -catenin pathway, which is activated by Wnt10b, and the Wnt/Ca²⁺ pathway, which is activated by Wnt5a.

Materials and Methods

Experimental Design

In this study, we obtained human adipose tissue from patients who received skin flap graft transplantations. The enzyme-digestion method was used to isolate hASCs from the human adipose tissue. The grouping is illustrated in Fig 1 in detail. A total of 108 hASCs specimens were divided randomly into 2 groups of 54 specimens each: Group I hASCs were cultured normally and Group II hASCs were subjected to Wnt5a knock down by RNAi. To verify the Wnt5a knock down, 18 specimens from each group were examined by cellular immunofluorescence, real-time RT-PCR and western blots. The remaining hASCs were then assigned to 4 groups (A, B, C, D): Group A, unstimulated hASCs; Group B, Wnt5a RNAi-treated hASCs; Group C, hASCs under tensile stress; and Group D, Wnt5a RNAi-treated hASCs under tensile stress. The cells in groups A and C (18 specimens each) came from the residual specimens from Group I, and the cells in groups B and D (18 specimens each) came from the residual

specimens in Group II. The cells from all groups were cultured in α -modified minimum essential medium (α -MEM) with 10 % fetal bovine serum (FBS). Tensile stress was applied using a four-point bending apparatus. Five days after the tensile stress, Group C and D cells were harvested; Group A and B cells were harvested at the same time point. All of the Wnt component and target genes (Wnt10b, Wnt5a, LRP5, LRP6, β -catenin, RhoA, RUNX2, ALPL, c-Fos, c-Jun, eNOS, COX2 and Cx43) were analyzed by real-time RT-PCR. Wnt10b, Wnt5a, RUNX2 and SPP1 protein levels were analyzed by western blot. All of these independent experiments were repeated 3 times.

Cell isolation and culture

Six patients who had received skin flap grafting transposition operations at the West China Hospital of Stomatology of Sichuan University donated subcutaneous adipose tissue, three males and three females. Their ages ranged from 18 to 25 years. The ethics committee of the West China Hospital of Stomatology of Sichuan University approved this study, and informed consent was obtained from all subjects. The subcutaneous adipose tissues obtained from the patients were immersed in α -MEM (Thermo Scientific HyClone, Logan, UT, USA) with 10 % FBS (Thermo Scientific HyClone, Logan, UT, USA) and stored at -4°C. Subsequently, the subcutaneous adipose tissue was digested for 30 min with 0.075 % collagenase type I (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C. The relatively dense cells released by digestion were separated from the adipocytes by centrifugation (1200 $\times$ g, 10 min) and resuspended in 160 mM ammonium chloride to lyse the red blood cells. These resuspended cells were cultured in α -MEM with 10% FBS and 100 U/ml penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA) under the standard conditions of 37 °C, 5 % CO₂, and 95 % humidity. After 2 days, phosphate-buffered saline (PBS) was used to wash the cell monolayer, and fresh medium was then added to the plastic

Table 1. Primers used in real-time RT-PCR

Symbol	Gene	Accession No	Primer sequence
Wnt10B	wingless-type MMTV integration site family, member 10b	NM_003394	5'-CATCCAGGCACGAATGCGA 5'-CGGTTGTGGGTATCAATGAAGA
Wnt5a	wingless-type MMTV integration site family, member 5a	NM_003392	5'-TGGCAGGACTTTCTCAAGGACA 5'-TTGACGTCGTGACACCTATTGTG
LRP5	low-density lipoprotein receptor-related protein 5	NM_002335	5'-ACTCGCTGTGAGGAGGACAAT 5'-GGCAGGCGCATGTGTAGAA
LRP6	low-density lipoprotein receptor-related protein 6	NM_002336	5'-TTTATGCAAACAGACGGGACTT 5'-GCCTCCAACCTACAATCGTAGC
â-catenin	catenin (cadherin-associated protein), beta 1	NM_001904	5'-TGGACTCTGGAATCCATTCTG 5'-ACTCACCCCTGTCCCTAAAA
RhoA	ras homolog family member A	NM_001664	5'-CTGGTGATTGTTGGTGATGG 5'-CCGTCCTTCTAATACTAGCG
RUNX2	Runt-related transcription factor 2	NM_001024630	5'-ACAGTAGATGGACCTCGGGAAC 5'-AAACAAGAGACTGGCGGAGT
ALPL	Alkaline phosphatase, liver/bone/kidney	NM_000478	5'-CCTGGACCTCGTTGACACCT 5'-AGAGAAGCTCGGTCCCCTG
c-Fos	FBJ murine osteosarcoma viral oncogene homolog	NM_003394	5'-CATCCAGGCACGAATGCGA 5'-CGGTTGTGGGTATCAATGAAGA
c-Jun	proto-oncogene c-Jun	NM_002228	5'-TCCAAGTGCCGAAAAAGGAAG 5'-CGAGTTCTGAGCTTTCAAGGT
eNOS	endothelial nitric-oxide synthase	NM_000603	5'-TGATGGCGAAGCGAGTGAAG 5'-ACTCATCCATACACAGGACCC
COX2	cyclooxygenase-2	NM_000963	5'-CTGGCGCTCAGCCATACAG 5'-CGCACTTATACTGGTCAAATCCC
Cxn43	connexin 43	NM_000165	5'-CAATCTCTCATGTGCGCTTCT 5'-GGCAACCTTGAGTTCTTCTCT
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	AF106860	5'-TGGGTGTGAACCACGAGAA 5'-GGCATCGACTGTGGTCATGA

flasks (Corning, Corning, NY, USA). We then changed the culture medium every 2-4 days. The cells reached 80 % confluence in 5-7 days and were then resuspended for subculturing. The multi-differentiation potential of these cells was demonstrated previously in our laboratory³⁶. We used cells at passage 5 in all of the experiments in this study.

Expression of RNAi-silenced *Wnt5a* in hASCs

Group II cells were subjected to RNAi against *Wnt5a*. The cells were seeded in 6-well plates (Corning, Corning, NY, USA) in α -MEM with FBS and treated with the siRNAs at 30-50 %

confluence. One day prior to the siRNA transfection, the medium was changed to α -MEM without FBS. The *Wnt5a* siRNA sequence was as follows: F: 5'-CCGGAUAACCUUGUAACAUAU-3' R: 5'-AUAUGUUACAAGGUUAUCCGG-3' (Biomics Biotechnology, Nantong, China). First, 20 pmol of siRNA was mixed with 50 μ l of α -MEM medium. One microliter of Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) was added to 50 μ l of α -MEM medium and incubated at room temperature (RT) for 5 minutes; the siRNA- α -MEM mixture was then added to this mixture and incubated at RT for 20 minutes. This Lipofectamine™ 2000-siRNA mixture was added to the Group II

Figure 2. The four-point bending apparatus. (A) Computer controlled unit and the actuator unit. (B) Petri dish with a special cell culture plate. (C) Schematic of cyclic uniaxial tensile strain on the cultured cells. The culture plate is placed between the four supporting rods in the Petri dish and is submerged in 100 mL medium in the bottom of the Petri dish. The plate is bent by the punching head of the actuator unit, and the cells attached on its surface are exposed to uniaxial stretch strain. (F, punching head; P, cell cultured plates; U, Petri dish cover; D, Petri dish bottom.) The strain loaded onto the cells can be calculated using this formula: $\hat{a}=cd/b(a-1.33b)$, where $\hat{a}$ = strain capacity, a = the distance between the two support rods of the Petri dish cover, b = the distance between the same side support rods of the Petri dish cover and bottom, c = the thickness of the cell culture plates, and d = vertical displacement.

cells, which were then incubated at 37 °C, 5 % CO₂, and 95 % humidity for 48 hours. After that the cells in Group I and II were harvested immediately, and 18 specimens of them were selected randomly for examinations to verify the effects of Wnt5a RNAi and the rest were seeded for further works. The effect of the Wnt5a RNAi was verified using cellular immunofluorescence, real-time RT-PCR and western blots.

Determination of Wnt5a Expression in hASCs treated with Wnt5a siRNA using Cellular Immunofluorescence, Real-Time RT-PCR and Western blot examination

Six specimens from Groups I and II (Wnt5a RNAi) were randomly chosen for cellular immunofluorescence to detect the expression of the Wnt5a protein. The specimens from each group were washed three times with PBS for 5 min each. The specimens were fixed with 10 % polyoxymethylene for 15 min and then washed three times with PBS for 5 min each. The specimens were covered with 0.2 % Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) at RT for 30 min and then washed three times for 5 min each. The specimens were then covered with 1 % BSA and incubated at 37°C for 30 min. Wnt5a primary antibody (Abcam, Hong Kong, China) was diluted in PBS according to the manufacturer's instructions and was added to the specimens. The specimens were then incubated overnight at 4°C and subsequently rewarmed at 37 °C for 1 hour the following day. The specimens were washed three times for 5 min with PBS. The fluorescein isothiocyanate-labeled secondary antibody (goat anti-rabbit IgG, Zhongshan Biotechnology, Beijing, China) was diluted in PBS following the manufacturer's instructions and then added to the surface of the specimens. The specimens were then incubated in

the dark at 37°C for 45 min, washed three times for 5 min with PBS, and finally observed and photographed using an optical microscope (Nikon, Tokyo, Japan).

Six specimens from Groups I and II (Wnt5a-RNAi) were randomly chosen for real-time RT-PCR to detect the expression of the Wnt5a gene. The Wnt5a and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers are listed in Table 1; they were synthesized by Invitrogen (Guangzhou, China). Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA concentrations and purity were determined using a spectrophotometer (Nano Vue; GE Healthcare Bio-Science, Piscataway, NJ, USA). Reverse transcription was carried out using a PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). The cDNA preparations were checked using spectrophotometry (i.e., A260/A280 analysis). Real-time PCR was run on an ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA) using the SYBR Premix Ex TaqII Kit (TaKaRa, Dalian, China) with the following program: initial activation of the Fast Start Taq polymerase at 95 °C for 30 sec, followed by approximately 40 cycles of 95 °C for 10 sec and 60 °C for 31 sec. The threshold cycle values were determined automatically by the ABI 7300 real-time PCR system software (Applied Biosystems, Foster City, CA, USA). For each reaction, a melting curve was generated to test for primer dimer formation and false priming.

Six specimens from Groups I and II (Wnt5a-RNAi) were randomly chosen for Western blots to detect the expression of the Wnt5a protein. Briefly, the cells were washed with PBS twice, and total cellular protein was extracted using the Total Protein Extraction Kit (KeyGen, China) according to the manufacturer's

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instructions. Sample protein concentrations were detected using a BCA Kit (Beyotime, Jiangsu, China) according to the manufacturer's instructions. The protein samples were diluted approximately 1 to 4 using 5×SDS loading buffer, heated to 100 °C for 3 min and stored at -20 °C. Equal amounts of protein (20–40 µg) from each sample were loaded onto a 10% SDS-PAGE gel for electrophoresis (Bio-Rad, Hercules, CA, USA). The separated proteins were then transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked in 5% non-fat milk in TBS-Tween 20 for 2 hours at 4°C. The membrane was then incubated with Wnt5a or GAPDH primary antibodies (Abcam, Hong Kong, China) diluted in 5 % non-fat milk/TBS-Tween 20 according to the manufacturer's instructions for 2 hours at 37°C. The secondary antibody (goat anti-rabbit IgG; Zhongshan Biotechnology, China) was diluted in 5% non-fat milk/TBS-Tween 20 and applied to the membrane for 2 hours at 37°C. The membrane was washed 3 times with 0.01 % TBS/Tween 20 for 5 min after the antibody incubation. Immunocomplexes were visualized using an enhanced chemiluminescence reagent (Millipore, Billerica, MA, USA) and photographed using the Quantity One software (Bio-Rad, Hercules, CA, USA).

Cell seeding and application of mechanical stretching

Mechanical stimulation of tensile stress was applied using a four-point bending apparatus (Fig. 2) designed by West China Medical University and the University of Electronic Science and Technology of China. The apparatus included an actuator containing a special resilient cell culture plate (Fig. 2B), which was driven by an electrical stepper motor and a computer-controlled unit (Fig. 2A). The special cell culture plate (length × width, 8.0 × 3.0 cm) was cut from the bottom of a plastic culture flask (Corning, Corning, NY, USA). The mechanism of this apparatus was previously described³⁷. Our previous work confirmed that the tensile stress generated by the four-point bending apparatus was successfully transferred to cells.

According to the experimental design, untreated or Wnt5a siRNA-treated cells at passage 5 were seeded onto 150 cm² plastic culture plates at a density of 1.5×10^5 cells/plate and cultured for 48 hours under standard conditions (α -MEM containing 10 % FBS at 37 °C, 5 % CO₂ and 95 % humidity). The plastic cell culture plates were then placed in stretching dishes filled with α -MEM plus 10 % FBS and subjected to cyclic uniaxial tensile stress (0.5 Hz, 2000 µε, 2 h/d). After daily mechanical loading for 5 days, the cell plates were put in plastic culture dishes for continued culture. Group A and B cells were also seeded at passage 5 on plastic cell culture flasks and cultured for 48 hours under standard conditions, and they remained in standard culture for the duration of the experiment. After 5 days of mechanical loading, the Group C and D cells were collected immediately; at the same time point, Group A and B cells were also collected for examination.

Analysis of Wnt- and Osteogenesis-associated Gene Expression and Cells under Tensile Stress using Real-Time RT-PCR

Cell samples from each group (A–D, 9 specimens each) were randomly chosen for real-time RT-PCR detection. The expression of genes associated with the Wnt signaling pathway and osteogenesis. (Wnt10b, Wnt5a, LRP5, LRP6, β -catenin, RhoA, RUNX2, ALPL, c-Fos, c-Jun, eNOS, COX2 and Cx43; Table 1) was analyzed. Gene-specific primers were synthesized by Invitrogen (Guangzhou, China). The Real-Time RT-PCR procedure was performed as described above in the *Materials and Methods* section 2.4.

Analysis of Wnt- and Osteogenesis-associated Protein Expression in Cells under Tensile Stress using Western blots

Cell samples from each group (A–D, 9 specimens each) were randomly chosen for Western blot analysis. The expression of proteins associated with the Wnt signaling pathway and osteogenesis, such as Wnt10b, Wnt5a, RUNX2, and SPP1 (Abcam, Hong Kong, China), was analyzed. The Western blot procedure was performed as described above in the *Materials and Methods* section 2.4.

Statistical Analysis

The data acquired from this study were analyzed by one way and two way ANOVA using the SPSS 17.0 software package (SPSS, Chicago, IL, USA). $p < 0.05$ was considered statistically significant.

Results

Expression of Wnt5a in siRNA-treated hASCs

The immunofluorescence results are shown in Fig. 3A. Compared with Group I, the fluorescence intensity of Wnt5a in Group II cells was significantly reduced. The results of real-time RT-PCR (Fig. 3B-a) and Western blot analysis (Fig. 3B-b) are shown in Fig. 3B; the relative quantitative analysis showed that Wnt5a expression was significantly lower in Group II than in Group I ($p < 0.05$).

Analysis of Wnt- and Osteogenesis-associated Gene Expression in Cells under Tensile Stress using Real-Time RT-PCR

In this study, the expression of genes associated with the Wnt signaling pathway (Wnt10b, Wnt5a, LRP5, LRP6, β -catenin, and RhoA) and Wnt target genes (RUNX2, ALPL, c-Fos, c-Jun, eNOS, COX2 and Cx43) was analyzed using real-time RT-PCR. The results of the real-time RT-PCR analyses are shown in Figure 4. Compared with Group, the expression of many genes (Wnt10b, LRP5, LRP6, β -catenin, c-Fos, c-Jun, eNOS, COX2 and Cx43) was significantly higher in group B ($p < 0.05$). Conversely, Wnt5a and RhoA expression levels were significantly lower ($p < 0.05$), and there was no significant difference in RUNX2 and

Figure 3. Expression of Wnt5a in hASCs after Wnt5a siRNA knock down. (A) Compared with Group I, the fluorescence intensity of Wnt5a in Group II was significantly reduced (white bar = 100 μm). (B) Wnt5a gene expression (a) and Wnt5a protein expression (b) analyzed by relative quantitative analyses. These results showed that Wnt5a expression was significantly lower in Group II than in Group I ($p < 0.05$). (C) Western blot bands representing the Wnt5a protein in Groups I and II. (*significant differences between this two groups)

ALPL expression ($p < 0.05$). Compared with Groups A and B, the expression of all the genes analyzed was significantly higher in Groups C and D ($p < 0.05$), with the exception of Wnt5a in Group D. Compared with Group B, Wnt5a expression was significantly higher in Group D ($p < 0.05$). Compared with Group C, Wnt5a, RhoA, RUNX2 and ALPL expression levels were significantly lower in Group D ($p < 0.05$); however, the levels of Wnt10b, LRP5, LRP6, β -catenin, c-Fos, c-Jun, eNOS, COX2 and Cx43 were significantly higher ($p < 0.05$). Two-way ANOVA test showed the tensile stress ($p < 0.05$), Wnt5a siRNA ($p < 0.05$), combination of tensile stress and Wnt5a siRNA ($p < 0.05$) affected the genes expression associated with osteogenic differentiation of human adipose-derived stem cells significantly.

Analysis of Wnt- and osteogenesis-associated Protein Expression in Cells under Tensile Stress by Western blots

In this study, the protein expression of Wnt5a, Wnt10b, RUNX2 and SPP1 was analyzed using western blots. The western blot results are shown in Figure 5. Compared with Group A, the protein levels of Wnt10b were significantly higher in Group B cells ($p < 0.05$), and the protein levels of Wnt5a were significantly lower ($p < 0.05$); however, the protein levels of RUNX2 and SPP1 were not significantly different between Groups A and B ($p > 0.05$). Compared with Groups A and B, the levels of all the protein analyzed were significantly higher in Group C ($p < 0.05$). Compared with Group C, the protein levels of Wnt5a and RUNX2 were significantly lower in Group D ($p < 0.05$), while Wnt10b and SPP1 protein expression were significantly higher ($p < 0.05$).

Figure 4. mRNA expression of Wnt- and osteogenesis-associated genes based on real-time RT-PCR. Fold changes are shown for the expression of (A) Wnt10b, (B) Wnt5a, (C) LRP5, (D) LRP6, (E) β -catenin, (F) RhoA, (G) RUNX2, (H) ALPL (I) c-Fos, (J) c-Jun, (K) eNOS, (L) COX2, and (M) Cx43 for every group. Compared with Group A—the expression levels of most of the genes (Wnt10b, LRP5, LRP6, β -catenin, c-Fos, c-Jun, eNOS, COX2 and Cx43) were significantly higher in group B ($p < 0.05$). Conversely, the Wnt5a and RhoA expression levels were significantly lower ($p < 0.05$), and no significant difference was observed for RUNX2 and ALPL ($p < 0.05$). Compared with Groups A and B, the expression levels of all the genes analyzed were significantly higher in Groups C and D ($p < 0.05$), with the exception of Wnt5a in Group D. Compared with Group B, the expression level of Wnt5a was significantly higher in Group D

($p < 0.05$). Compared with Group C, the expression levels of Wnt5a, RhoA, RUNX2 and ALPL were significantly lower in Group D ($p < 0.05$), but the expression levels of Wnt10b, LRP5, LRP6, β -catenin, c-Fos, c-Jun, eNOS, COX2 and Cx43 were significant higher ($p < 0.05$). Two-way ANOVA test showed the tensile stress ($p < 0.05$), Wnt5a siRNA ($p < 0.05$), combination of tensile stress and Wnt5a siRNA ($p < 0.05$) affected the genes expression associated with osteogenic differentiation of human adipose-derived stem cells. (* significant difference between this group and the others; # significant difference between this group and Group B)

Compared with Groups A and B, the expression of all the proteins analyzed was significantly higher in Group D ($p < 0.05$), with the exception of Wnt5a. Two-way ANOVA test showed the tensile stress ($p < 0.05$), Wnt5a siRNA ($p < 0.05$), combination of tensile stress and Wnt5a siRNA ($p < 0.05$) affect the proteins expression associated with osteogenic differentiation of human adipose-derived stem cells significantly.

Discussion

ASCs were selected as the experimental system in this study

because of their many obvious advantages mentioned in *Introduction*. In recent years, cellular biomechanical experiments *in vitro* have been widely used to simulate the biomechanical environment *in vivo*³⁸⁻⁴⁰. In this study, we chose tensile stress applied by a four-point bending system as the mechanical stimulation because tensile stress is considered an important osteogenesis-promoting factor based on previous studies³⁷. The tensile stress value was restricted to 2000 $\mu\epsilon$ to avoid pathological responses in cells caused by supra-physiological loading and because 2000 $\mu\epsilon$ is considered equivalent to *in vivo* physiological

Figure 5. Protein expression of Wnt- and osteogenesis-associated factors in cells under tensile stress detected by Western blot analysis. (A) Compared with Group A, the expression level of Wnt10b was significantly higher ($p < 0.05$), and the expression level of Wnt5a was significantly lower ($p < 0.05$). No significant changes were observed for RUNX2 and SPP1 ($p < 0.05$). Compared with Groups A and B, the expression of all the proteins analyzed was significantly higher in Group C ($p < 0.05$). Compared with Group C, the protein expression levels of Wnt5a and RUNX2 were significantly lower in Group D ($p < 0.05$), but Wnt10b and SPP1 expression were significantly higher ($p < 0.05$). Compared with Groups A and B, the expression of all the proteins analyzed was significantly higher in Group D ($p < 0.05$), with the exception of Wnt5a. (B) Western blot bands representing the Wnt5a, Wnt10b, RUNX2 and SPP1 proteins. Two-way ANOVA test showed the tensile stress ($p < 0.05$), Wnt5a siRNA ($p < 0.05$), combination of tensile stress and Wnt5a siRNA ($p < 0.05$) affected the proteins expression associated with osteogenic differentiation of human adipose-derived stem cells. (*significant differences between this group and the others)

Hong-ming Du *et al.*: Wnt Signaling in the Osteogenic Differentiation of Human Adipose-derived Stem Cells under Mechanical Stimulation loading^{41,42}). Our previous study confirmed that 2000 μe tensile stress can indeed effectively promote the osteogenic differentiation of hASCs³⁷).

RNAi has been widely used in gene silencing studies³⁹). In this study, Wnt5a expression was reduced in hASCs using this technique. The fluorescence intensity of Wnt5a in Group II was significantly lower than that in Group I. The results of real-time RT-PCR and Western blot analysis showed that Wnt5a expression was significantly lower in Group II than in Group I. This result indicates that the RNAi technique successfully reduced Wnt5a expression in hASCs in this experiment.

Cawthorn *et al.* found that Wnt10b is an upstream regulator of PPAR γ and CEBP/ α that can inhibit their expression²⁷) and promote osteogenic differentiation of stem cells^{24,25}). In our experiment, expression at the mRNA and protein levels was detected using real-time RT-PCR and western blot analysis. Compared with Group A, Wnt10b expression was significantly higher in Group C, non-siRNA-treated hASCs under tensile stress, and the expression levels of the Wnt10b downstream targets LRP5/6 and β -catenin were also significantly higher. The important Wnt/ β -catenin target genes c-Fos, c-Jun, eNOS, COX2 and Cx43 all exhibited increased expression in Group C under tensile stress^{32,43,44}). Thus, tensile stress stimulation activated the Wnt/ β -catenin pathway. Based on existing evidence, the Wnt/ β -catenin pathway can directly stimulate RUNX2 expression and thus promote osteogenic differentiation³²). Furthermore, the SPP1 protein is an important promoter of MSCs maturity during the osteogenic differentiation process⁴⁵). Compared with Group A, RUNX2, ALPL and SPP1 exhibited increased expression in hASCs after tensile stress loading in Group C, suggesting that these cells were in the process of osteogenic differentiation. Therefore, tensile stress stimulation activated the Wnt/ β -catenin pathway, promoted osteogenic differentiation, and indirectly promoted maturation in hASCs. After tensile stress loading, Wnt5a expression was also significantly higher in the non-siRNA-treated hASCs of Group C than in Group A, hASCs without mechanical stimulation or Group B or D hASCs treated with Wnt5a siRNA. Furthermore, expression of RhoA, a key downstream target, was significantly higher in Group C than in Groups B and D. RhoA can act on the RUNX2 gene directly and increase its expression. RhoA and its related proteins play an important role in promoting the osteogenic and chondrogenic differentiation of MSCs⁴⁶). Therefore, intermittent tensile stress stimulation not only activates the canonical pathway but also activates the non-canonical Wnt/ Ca^{2+} pathway, which mainly consists of Wnt5a and RhoA, to promote the osteogenic differentiation of hASCs.

Bolzoni *et al.* have confirmed that Wnt5a is involved in the osteogenic differentiation process of hASCs under tensile stress³⁰), and we believe that it is an important molecule for development and osteogenic differentiation. Compared with the non-siRNA-

treated hASCs in Group C, the protein levels of Wnt5a, RhoA, RUNX2 and SPP1 were significantly lower in the Wnt5a-siRNA-treated hASCs in Group D. These results showed that the RNAi successfully inactivated the non-canonical Wnt/ Ca^{2+} pathway, which is mainly composed of Wnt5a and RhoA, and thereby directly inhibited the process of osteogenic differentiation in hASCs. Norvell *et al.* showed that Wnt5a significantly inhibits the expression of β -catenin *in vitro*⁴⁷). In the present study, β -catenin expression was higher in the Wnt5a-siRNA-treated hASCs in Group B than in Group A, indicating that the presence of Wnt5a inhibits the effect of β -catenin *in vitro*. After tensile stress loading, Wnt5a expression in Group D declined compared with Group C, which means that the RNAi decreased the expression of Wnt5a and enhanced the expression of β -catenin *in vitro*. These results indicate that Wnt/ Ca^{2+} and Wnt/ β -catenin promote osteogenesis and inhibit with each other *in vitro*, and the interaction between these two pathways is very complex.

Although Wnt5a expression is lower in the Wnt5a-siRNA-treated hASCs from Group D than in Group A, it is higher in Group D than in Group B. This phenomenon suggests that tensile stress increases the potential for Wnt5a activation and the expression of Wnt5a was not inhibited completely by RNAi during tensile stress.

It is well known that RUNX2 plays an important regulatory role in the osteogenic differentiation of MSCs, ALPL and SPP1 are important maturation-promoting factors during the osteogenic differentiation of MSCs. High expression of these genes can promote the osteogenic differentiation of hASCs and accelerate their maturation. In this study, RUNX2, ALPL and SPP1 expression levels were significantly higher in Group C hASCs under tensile stress than in Group A cells without tensile stress loading; this indicates that tensile stress stimulation can effectively promote osteogenic differentiation in hASCs. The expression levels of RUNX2, ALPL and SPP1 were also significantly higher in Group D than in Group A hASCs without tensile stress loading. This indicates that although the osteogenic stimulatory effect was decreased by Wnt5a siRNA, tensile stress stimulation activated the canonical Wnt signaling pathway and thus significantly promoted osteogenesis.

RUNX2 is considered a target of the Wnt/ β -catenin pathway³²) and RhoA in the Wnt/ Ca^{2+} pathway³⁰), and RUNX2 expression should be activated by elevated expression of the Wnt/ β -catenin and Wnt/ Ca^{2+} pathways. In this study, although Wnt5a was knocked down in group B, RUNX2 expression was the same in Groups A and B, suggesting that the osteogenic differentiation of hASCs was not affected by Wnt5a knock down. However, as we know, if the Wnt/ Ca^{2+} pathway was blocked, the osteogenic response of the cells should also be inhibited. Therefore, the RUNX2 result in this study was interesting. One possible explanation for this result may be that Wnt5a siRNA increased the expression of

Wnt10b and its downstream targets in Group B; thus, Wnt10b promoted the expression of RUNX2 to counteract RUNX2 down-regulation caused by Wnt5a knock down. Finally, the expression level of RUNX2 was not obviously affected by the interaction between the two pathways. Expression of RUNX2 in Groups C and D correlated with the expression levels of Wnt5a and Wnt10b, suggesting that tensile stress activated the Wnt/ β -catenin and Wnt/ Ca^{2+} pathways at the same time.

In summary, our study further confirmed that intermittent tensile stress can promote the maturation and osteogenic differentiation of hASCs and can also activate the Wnt/ β -catenin pathway and a non-canonical Wnt pathway that is made up of Wnt5a and RhoA in hASCs. This non-canonical Wnt pathway may have the potential to inhibit the Wnt/ β -catenin pathway, which is activated by Wnt10b. Wnt5a knock down seemed to increase expression of the Wnt/ β -catenin pathway, which is activated by Wnt10b.

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References

1. Weyts FA, Bosmans B, Niesing R, van Leeuwen JP and Weinans H. Mechanical control of human osteoblast apoptosis and proliferation in relation to differentiation. *Calcif Tissue Int* 72: 505-512, 2003
2. Brighton CT, Strafford B, Gross SB, Leatherwood DF, Williams JL and Pollack SR. The proliferative and synthetic response of isolated calvarial bone cells of rats to cyclic biaxial mechanical strain. *J Bone Joint Surg Am* 73: 320-331, 1991
3. Li YJ, Batra NN, You L, Meier SC, Coe IA, Yellowley CE and Jacobs CR. Oscillatory fluid flow affects human marrow stromal cell proliferation and differentiation. *J Orthop Res* 22: 1283-1289, 2003
4. Sahoo S, Ouyang H, Goh JC, Tay TE and Toh SL. Characterization of a novel polymeric scaffold for potential application in tendon/ligament tissue engineering. *Tissue Eng* 12: 91-99, 2006
5. Kafienah W, Mistry S, Dickinson SC, Sims TJ, Learmonth I and Hollander AP. Three-dimensional cartilage tissue engineering using adult stem cells from osteoarthritis patients. *Arthritis Rheum* 56: 177-187, 2007
6. Derubeis AR and Cancedda R. Bone marrow stromal cells (BMSCs) in bone engineering: limitations and recent advances. *Ann Biomed Eng* 32: 160-165, 2004
7. Koike M, Shimokawa H, Kanno Z, Ohya K and Soma K. Effects of mechanical strain on proliferation and differentiation of bone marrow stromal cell line ST2. *J Bone Miner Metab* 23: 219-225, 2005
8. Park JS, Chu JS, Cheng C, Chen F, Chen D and Li S. Differential effects of equiaxial and uniaxial strain on mesenchymal stem cells. *Biotechnol Bioeng* 88: 359-368, 2004
9. Sumanasinghe RD, Bernacki SH and Lobo EG. Osteogenic differentiation of human mesenchymal stem cells in collagen matrices: effect of uniaxial cyclic tensile strain on bone morphogenetic protein (BMP-2) mRNA expression. *Tissue Eng* 12: 3459-3465, 2006
10. De Ugarte DA, Morizono K, Elbarbary A, Alfonso Z, Zuk PA, Zhu M, Dragoo JL, Ashjian P, Thomas B, Benhaim P, Chen I, Fraser J and Hedrick MH. Comparison of multi-lineage cells from human adipose tissue and bone marrow. *Cell Tissue Organ* 174: 101-109, 2003
11. Kakudo N, Shimotsuma A, Miyake S, Kushida S and Kusumoto K. Bone tissue engineering using human adipose-derived stem cells and honeycomb collagen scaffold. *J Biomed Mater Res A* 84: 191-197, 2008
12. Lin Y, Wang T, Wu L, Jing W, Chen X, Li Z, Liu L, Tang W, Zheng X and Tian W. Ectopic and in situ bone formation of adipose tissue-derived stromal cells in biphasic calcium phosphate nano composite. *J Biomed Mater Res A* 81: 900-910, 2007
13. Wu L, Wu Y, Lin Y, Jing W, Nie X, Qiao J, Liu L, Tang W and Tian W. Osteogenic differentiation of adipose derived stem cells promoted by overexpression of osterix. *Mol Cell Biochem* 301: 83-92, 2007
14. Yu HC, Wu TC, Chen MR, Liu SW, Chen JH and Lin KM. Mechanical stretching induces osteoprotegerin in differentiating C2C12 precursor cells through noncanonical Wnt pathways. *J Bone Miner Res* 25: 1128-1137, 2010
15. Yu B, Yu D, Cao L, Zhao X, Long T, Liu G, Tang T and Zhu Z. Simulated microgravity using a rotary cell culture system promotes chondrogenesis of human adipose-derived mesenchymal stem cells via the p38 MAPK pathway. *Biochem Biophys Res Commun* 414: 412-418, 2011
16. Kahler RA and Westendorf JJ. Lymphoid enhancer factor-1 and beta-catenin inhibit Runx2-dependent transcriptional activation of the osteocalcin promoter. *J Biol Chem* 278: 11937-11944, 2003
17. Kahler RA, Galindo M, Lian J, Stein GS, van Wijnen AJ and Westendorf JJ. Lymphocyte enhancer-binding factor 1 (Lef1) inhibits terminal differentiation of osteoblasts. *J Cell Biochem* 97: 969-983, 2006.
18. Eijken M, Meijer IM, Westbroek I, Koedam M, Chiba H,

- Uitterlinden AG, Pols HA and van Leeuwen JP. Wnt signaling acts and is regulated in a human osteoblast differentiation dependent manner. *J Cell Biochem* 104: 568-579, 2008
19. Kahler RA, Yingst SM, Hoepfner LH, Jensen ED, Krawczak D, Oxford JT and Westendorf JJ. Collagen 11a1 is indirectly activated by lymphocyte enhancer-binding factor 1 (Lef1) and negatively regulates osteoblast maturation. *Matrix Biol* 27: 330-338, 2008
20. Ikeda S, Kishida S, Yamamoto H, Murai H, Koyama S and Kikuchi A. Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3 β and β -catenin and promotes GSK-3 β -dependent phosphorylation of β -catenin. *EMBO J* 17: 1371-1384, 1998
21. Liu C, Li Y, Semenov M, Han C, Baeg GH, Tan Y, Zhang Z, Lin X and He X. Control of β -catenin phosphorylation/degradation by a dual-kinase mechanism. *Cell* 108: 837-847, 2002
22. Ishitani T, Ninomiya-Tsuji J and Matsumoto K. Regulation of lymphoid enhancer factor 1/T-cell factor by mitogen-activated protein kinase-related Nemo-like kinase-dependent phosphorylation in Wnt/ β -catenin signaling. *Mol Cell Biol* 23: 1379-1389, 2003
23. Barker N and Catenins CH. Wnt signaling and cancer. *Bioessays* 22: 961-965, 2000
24. Bennett CN, Longo KA, Wright WS, Suva LJ, Lane TF, Hankenson KD and MacDougald OA. Regulation of osteoblastogenesis and bone mass by Wnt10b. *Proc Natl Acad Sci USA* 102: 324-3329, 2005
25. Bennett CN, Ouyang H, Ma YL, Zeng Q, Gerin I, Sousa KM, Lane TF, Krishnan V, Hankenson KD and MacDougald OA. Wnt10b increases postnatal bone formation by enhancing osteoblast differentiation. *J Bone Miner Res* 22: 1924-1932, 2007
26. Zmuda JM, Yerges LM, Kammerer CM, Cauley JA, Wang X, Nestlerode CS, Wheeler VW, Patrick AL, Bunker CH, Moffett SP and Ferrell RE. Association analysis of WNT10B with bone mass and structure among individuals of African ancestry. *J Bone Miner Res* 24: 437-447, 2009
27. Cawthorn WP, Bree AJ, Yao Y, Du B, Hemati N, Martinez-Santibañez G and MacDougald OA. Wnt6, Wnt10a and Wnt10b inhibit adipogenesis and stimulate osteoblastogenesis through a β -catenin-dependent mechanism. *Bone* 50: 477-489, 2012
28. Takada I, Mihara M, Suzawa M, Ohtake F, Kobayashi S, Igarashi M, Youn MY, Takeyama K, Nakamura T, Mezaki Y, Takezawa S, Yogiashi Y, Kitagawa H, Yamada G, Takada S, Minami Y, Shibuya H, Matsumoto K and Kato S. A histone lysine methyltransferase activated by non-canonical Wnt signalling suppresses PPAR- γ transactivation. *Nat Cell Biol*, 9: 1273-1285, 2007.
29. Fukuda T and Takeda S. Regulation of osteoblastic differentiation by Wnt signals. *Clin Calcium* 23: 825-830, 2013
30. Bolzoni M, Donofrio G, Storti P, Guasco D, Toscani D, Lazzaretti M, Bonomini S, Agnelli L, Capocéfalo A, Dalla Palma B, Neri A, Nicolini F, Lisignoli G, Russo F, Colla S, Aversa F and Giuliani N. Myeloma cells inhibit non-canonical wnt co-receptor ror2 expression in human bone marrow osteoprogenitor cells: effect of wnt5a/ror2 pathway activation on the osteogenic differentiation impairment induced by myeloma cells. *Leukemia* 27: 451-463, 2013
31. Yamashita T, Takahashi N and Udagawa N. New roles of osteoblasts involved in osteoclast differentiation. *World J Orthop* 3: 175-181, 2012
32. Gaur T, Lengner CJ, Hovhannisyan H, Bhat RA, Bodine PV, Komm BS, Javed A, van Wijnen AJ, Stein JL, Stein GS and Lian JB. Canonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression. *Biol Chem* 280: 33132-33140, 2005
33. Kobayashi Y. Roles of Wnt signaling in bone metabolism. *Clin Calcium* 22: 1701-1706, 2012
34. Marcellini S, Henriquez JP and Bertin A. Control of osteogenesis by the canonical Wnt and BMP pathways in vivo: cooperation and antagonism between the canonical Wnt and BMP pathways as cells differentiate from osteochondroprogenitors to osteoblasts and osteocytes. *Bioessays* 34: 953-962, 2012
35. Galli C, Passeri G and Macaluso GM. Osteocytes and WNT: The mechanical control of bone formation. *J Dent Res* 89: 331-343, 2010
36. Lin Y, Liu L, Li Z, Qiao J, Wu L, Tang W, Zheng X, Chen X, Yan Z and Tian W. Pluripotency potential of human adipose-derived stem cells marked with exogenous green fluorescent protein. *Mol Cell Biochem* 291: 1-10, 2006
37. Du HM, Zheng XH, Wang LY, Tang W, Liu L, Jing W, Lin YF, Tian WD and Long J. The osteogenic response of undifferentiated human adipose-derived stem cells under mechanical stimulation. *Cell Tissue Organ* 196: 313-324, 2012
38. Katanosaka Y, Bao JH, Komatsu T, Suemori T, Yamada A, Mohri S and Naruse K. Analysis of cyclic-stretching responses using cell-adhesion-patterned cells. *J Biotechnol* 133: 82-89, 2008
39. Al-Salleeh F, Beatty MW, Reinhardt RA, Petro TM and Crouch L. Human osteogenic protein-1 induces osteogenic differentiation of adipose-derived stem cells harvested from mice. *Arch Oral Biol* 53: 928-936, 2008
40. Wang JH, Yang G and Li Z. Controlling cell responses to cyclic mechanical stretching. *Ann Biomed Eng* 33: 337-342, 2005

41. Martin I, Wendt D and Hebere M. The role of bioreactors in tissue engineering. *Trends Biotechnol* 22: 80-86, 2004
42. Butler DL, Goldstein SA and Guilak F. Functional tissue engineering: the role of biomechanics. *J Biomech Eng* 122: 570-575, 2000
43. Ramachandran PV and Ignacimuthu S. RNA interference—a silent but an efficient therapeutic tool. *Appl Biochem Biotechnol* 169: 1774-1789, 2013
44. Robinson JA, Chatterjee-Kishore M, Yaworsky PJ, Cullen DM, Zhao W, Li C, Kharode Y, Sauter L, Babij P, Brown EL, Hill AA, Akhter MP, Johnson ML, Recker RR, Komm BS and Bex FJ. Wnt/beta-catenin signaling is a normal physiological response to mechanical loading in bone. *J Biol Chem* 281: 31720-31728, 2006
45. Malaval L, Wade-Guye NM, Boudiffa M, Fei J, Zirngibl R, Chen F, Laroche N, Roux JP, Burt-Pichat B, Duboeuf F, Boivin G, Jurdic P, Lafage-Proust MH, Amde J, Vico L, Rossant J and Aubin JE. Bone sialoprotein plays a functional role in bone formation and osteoclastogenesis. *J Exp Med* 205: 1145-1153, 2008
46. Arnsdorf EJ, Kwon RY and Jacobs CR. Mechanically induced osteogenic differentiation - the role of RhoA, ROCKII and cytoskeletal dynamics. *J Cell Sci* 122: 546-553, 2009
47. Norvell SM, Alvarez M, Bidwell JP and Pavalko FM. Fluid shear stress induces beta-catenin signaling in osteoblasts. *Calcif Tissue Int* 75: 396-404, 2004