

Mechanical strain promotes osteoblastic differentiation through integrin- β 1-mediated β -catenin signaling

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Abstract. As integrins are mechanoresponsive, there exists an intimate relationship between integrins and mechanical strain. Integrin- β 1 mediates the impact of mechanical strain on bone. Mechanical strain induces bone formation through the activation of β -catenin pathways, which suggests that integrin- β 1 mediates β -catenin signaling in osteoblasts in response to mechanical strain. In the present study, we examined the role of integrin- β 1 in Wnt/ β -catenin signal transduction in mechanically strained osteoblasts. MC3T3-E1 osteoblastic cells were transfected with integrin- β 1 small interfering RNA (si-Itg β 1), and exposed to mechanical tensile strain of 2,500 microstrain ($\mu\epsilon$) using a four-point bending device. The mechanical strain enhanced the mRNA expression of integrin- β 1, the protein levels of phosphorylated (p-) glycogen synthase kinase-3 β (GSK-3 β) and β -catenin, simultaneously increased the mRNA levels of runt-related transcriptional factor 2 (Runx2) and osteocalcin (OCN), the protein levels of bone morphogenetic protein (BMP)-2 and -4 and enhanced the alkaline phosphatase (ALP) activity of the MC3T3-E1 cells. The elevations were inhibited by si-Itg β 1. Additionally, the mechanical strain induced the nuclear translocation of β -catenin into the nucleus, which was also inhibited by si-Itg β 1. These findings indicated that mechanical strain promoted osteoblastic differentiation through integrin- β 1-mediated β -catenin signaling.

Introduction

Integrins are the main receptors that connect the cytoskeleton to the extracellular matrix (ECM), and an intimate relationship exists between integrins and mechanical strain. Integrins act as mechanoreceptors in bone and transduce mechanical signals into biochemical responses within bone cells (1,2).

Integrin- β 1 has been reported to be expressed on the surface of osteoblasts (3). Mechanical strain may cause the redistribution of integrin- β 1 on the osteosarcoma cell surface, and integrin- β 1 antibodies inhibit the activity of mechanosensitive ion channels (4). The expression of an osteoblast-specific dominant negative form of integrin- β 1 resulted in reduced bone mass with increased cortical porosity in the long bones of mice (5). Fluid flow shear stress or mechanical tensile strain were demonstrated to upregulate integrin- β 1 expression in osteoblasts, and induce cell proliferation or differentiation (6-8). These studies demonstrated that integrin- β 1 mediates the impact of mechanical strain on the proliferation and the differentiation of osteoblasts. A recent study of ours revealed that integrin- β 1 mediates osteoblastic differentiation and ECM formation which was enhanced by mechanical tensile strain (9).

It has been previously demonstrated by researchers that Wnt/ β -catenin signaling is required for mechanotransduction in bone (10). It has been found that mechanical strain and fluid shear stress induce the nuclear translocation of β -catenin in osteoblasts and periodontal ligament cells, and activate the β -catenin signal pathway (11-13). In the developing chick embryo, integrin- β 1 was demonstrated to regulate cell shape and tissue morphogenesis indirectly by regulating Wnt and Notch signaling (14). Integrin- α 3 β 1, acting in coordination with c-Met, regulated the expression of Wnt 7b transcripts expressed in developing papilla (15). Thus, we hypothesized that integrin- β 1 regulated Wnt/ β -catenin signaling in response to the mechanical stimulation of osteoblasts.

In the present study, we aimed to examine this hypothesis by stimulating MC3T3-E1 cells with mechanical strain in order to explore the involvement of integrin- β 1 in β -catenin signaling in response to the mechanical stimulation of osteoblasts.

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Table I. siRNA sequences of integrin- β 1.

Description	Type	Sequence	Quantity
Itg β 1-MSS205553	RNA	UAGAAAUGUUGGAACACUUUCGUCC	10 nmol
	RNA	GGACGAAAGUGUCCAACAUUUCUA	10 nmol

siRNA, small interfering RNA; Itg β 1, integrin- β 1.

Table II. Primer sequences used in RT-qPCR.

Gene	Primer sequences (5'-3')
Itg β 1	Forward: GCAACGCATATCTGGAAACT
	Reverse: CAAAGTGAAACCCAGCATCC
Runx2	Forward: AGTAGCCAGGTTCAACGAT
	Reverse: GGAGGATTTGTGAAGACTGTT
OCN	Forward: AGTCTGACAAAGCCTTCA
	Reverse: AAGCAGGGTTAAGCTCACA
GAPDH	Forward: TGCACCACCAACTGCTTAGC
	Reverse: GGCATGGACTGTGGTCATGAG

Itg β 1, integrin- β 1; Runx2, runt-related transcriptional factor 2; OCN, osteocalcin.

Materials and methods

Cell culture. MC3T3-E1 cells were provided by the School of Basic Medicine of Peking Union Medical College (Beijing, China). The MC3T3-E1 mouse pre-osteoblastic cell line has been shown to differentiate into osteoblasts and osteocytes (16,17). The cells were maintained in α -minimal essential medium (α -MEM; Invitrogen, San Diego, CA, USA) containing 10% fetal calf serum and 1% penicillin-streptomycin.

Application of mechanical strain to cultured cells. Mechanical tensile strain was generated by a specially designed four-point bending device (provided by the Institute of Medical Equipment, Academy of Military Medical Sciences, Tianjin, China) as previously described (18,19). The four-point bending device has been shown to produce homogenous, predominantly uniaxial strains of the cell culture substrate so that every cell is subjected to the same deformation (20,21). The cells were seeded at a density of $2 \times 10^4/\text{cm}^2$ in the cell culture dishes and cultivated until they reached 80% confluence. For 1 h/day, the cell cultures were subjected to mechanical strain of 2,500 microstrain ($\mu\epsilon$) at 0.5 Hz for 3 days. Unstrained (control) cultures were incubated under the same conditions for the maximum period of mechanical strain application.

RNA interference (RNAi) targeted against integrin- β 1. The specific small interfering RNA (siRNA) targeting mouse integrin- β 1 was purchased from Invitrogen (Table I). At 60-70% confluence, the MC3T3-E1 cells were transfected with integrin- β 1 Stealth siRNA (si-Itg β 1) or negative control

siRNA using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Mechanical strain was applied 48 h after transfection.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the cells using TRIzol LS reagent (Invitrogen) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed to complementary DNA (cDNA) using the SuperScript III reverse transcriptase kit (Invitrogen). qPCR was performed with an ABI StepOne Real-Time PCR machine in a 48-well format using the Fast SYBR-Green Master Mix kit (both from Applied Biosystems, Foster City, CA, USA). The primer sequences for the PCR reactions are listed in Table II. Triplicate samples were used for these experiments. The amplification reaction included a denaturation step at 94°C for 3 min followed by 40 cycles of 94°C for 15 sec, and annealing and extension at each annealing temperature for 30 sec. The PCR products were normalized for the amount of GAPDH in the same sample, which was also standardized on a dilution curve from the sample.

Western blot analysis of integrin- β 1, phosphorylated (p-) glycogen synthase kinase-3 β (GSK-3 β) and β -catenin. Following trypsinization and centrifugation, the cell lysates were harvested with RIPA lysis medium containing protease inhibitors (Tianjin Weike Biotechnology Co., Ltd., Tianjin China). The protein content of the cell lysates was quantified using the Bradford method. Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After blocking with 5% skim milk (Solarbio, Beijing, China), the membranes were incubated overnight with the primary antibodies [integrin- β 1 (sc-374430; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), p-GSK-3 β (sc-11757; Santa Cruz Biotechnology, Inc.), β -catenin (BM1575; Wuhan Boster Bio-engineering Co., Ltd., Wuhan, China), and then incubated with secondary antibody conjugated with horseradish peroxidase. The immunoreactive bands were visualized using an Enhanced Chemiluminescence Detection kit (Santa Cruz Biotechnology, Inc.). The optical density of the protein bands was determined using a Gel Doc 2000 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The expression of GAPDH was used as a loading control and the data were normalized against those of the corresponding GAPDH. The results are expressed relative to the control.

Determination of alkaline phosphatase (ALP) activity. The cells were harvested and lysed by brief sonication in lysis buffer (10 mmol/l HEPES, 250 mmol/l sucrose, 5 mmol/l Tris-HCl,

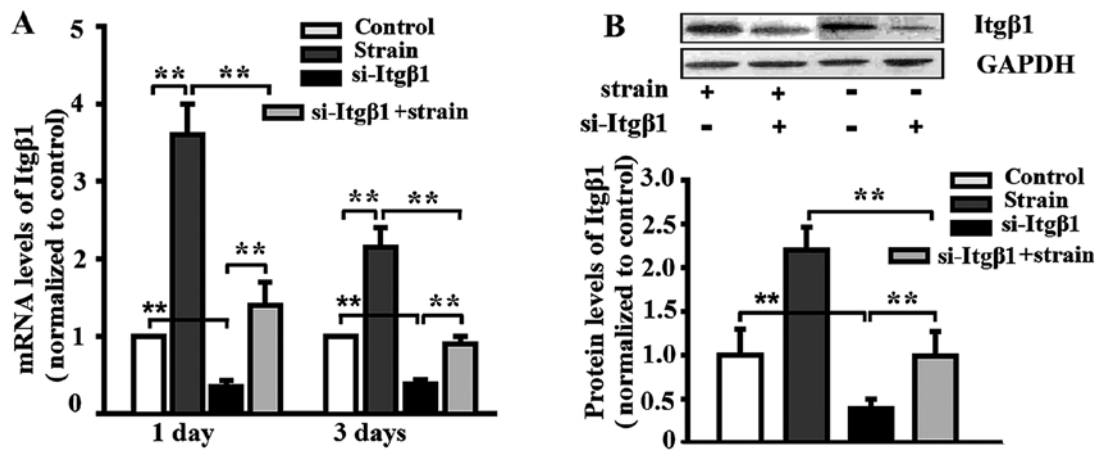


Figure 1. Mechanical strain enhances integrin- β 1 expression. The MC3T3-E1 cells were pre-treated with integrin- β 1 siRNA (si-Itg β 1), and subjected to mechanical strain for 3 days. The mRNA and protein expression of integrin- β 1 were assayed using RT-qPCR and western blot analysis, respectively. Mechanical strain increased (A) the mRNA expression and (B) protein expression of integrin- β 1 in the cells, and si-Itg β 1 inhibited the increases (A and B). In addition, the si-Itg β 1 reduced the mRNA and the protein expression of integrin- β 1 in the unstrained cells (A and B). ** $P < 0.01$, between the indicated groups, $n = 7$.

0.1% Triton X-100, pH 7.5). ALP activity in the cellular fraction was measured using a fluorometric detection kit (Nanjing Jiancheng Biotechnology Co., Ltd., Nanjing, China) according to the manufacturer's instructions. The ALP activity of each sample was normalized to the protein concentration.

Fluorescent immunolocalisation of β -catenin. For this experiment, the cells were fixed in 4% paraformaldehyde for 10 min, and incubated in phosphate-buffered saline (PBS) containing 10% fetal calf serum/0.1% Triton X-100 (v/v) for 20 min to block non-specific binding and to facilitate access to intracellular epitopes. Following incubation overnight at 4°C with β -catenin antibody (1:500 dilution; Santa Cruz Biotechnology, Inc.), the cells were incubated with FITC-conjugated goat anti-rabbit secondary antibody (1:100; Sigma-Aldrich, St. Louis, MO, USA) for 60 min in the dark at room temperature. The cells were mounted in PBS containing 100 μ g propidium iodide (PI) to stain the nuclei. The cells were washed three times in PBS following each incubation. Images were captured with a laser scanning confocal microscope (LSCM; FV500; Olympus, Tokyo, Japan), and analyzed using Image-Pro Plus 6.2 software (Media Cybernetics Inc., Bethesda, MD, USA).

Enzyme-linked immunosorbent assay (ELISA) for bone morphogenetic proteins (BMPs). BMP ELISA kits (Wuhan Boster Bio-engineering Co., Ltd.) were used in order to detect BMP-2 and BMP-4 levels in the culture medium. The culture medium was collected following exposure to mechanical strain, and the samples were placed into microtiter plates coated with BMP-2 or BMP-4 antibody and incubated for 1.5 h at room temperature. After washing, horseradish peroxidase-conjugated streptavidin was added to the plates to catalyze the conversion of a chromogenic substrate (tetramethylbenzidine) to a colored solution. The absorbance was measured at 450 nm on a Microplate Reader (Model 680; Bio-Rad Laboratories, Inc.). The results are presented as the percentage of activity change, compared with the control.

Statistical analysis. The data are presented as the means \pm standard deviation, and analyzed by SPSS 10.0 soft-

ware (SPSS, Inc., Chicago, IL, USA) using one-way analysis of variance (ANOVA). A P -value < 0.05 was considered to indicate a statistically significant difference.

Results

Mechanical strain enhances integrin- β 1 expression. The MC3T3-E1 cells were pre-treated with si-Itg β 1 and then subjected to mechanical strain for 3 days. Mechanical strain enhanced the mRNA and the protein expression of integrin- β 1, and the knockdown of integrin- β 1 attenuated the enhancement (Fig. 1). The mRNA and the protein expression of integrin- β 1 in the MC3T3-E1 cells which were not stimulated by the application of mechanical strain was also reduced by si-Itg β 1 (Fig. 1).

Mechanical strain activates the β -catenin signal pathway, and this effect is inhibited by integrin- β 1 knockdown. Mechanical strain enhanced the protein expression of β -catenin, and pre-treatment with si-Itg β 1 decreased the protein levels of β -catenin (Fig. 2). Similar to β -catenin, mechanical strain increased the protein level of p-GSK-3 β , and the increase was inhibited by integrin- β 1 knockdown (Fig. 2). At the same time, si-Itg β 1 also reduced the protein levels in the unstrained MC3T3-E1 cells on day 1 (Fig. 2A). Under normal conditions, activated GSK-3 β phosphorylates β -catenin, targeting it for degradation. The inactivation of GSK-3 β by phosphorylation leads to β -catenin accumulation and subsequent translocation into the nucleus, where it modulates gene transcription, and subsequently activates the β -catenin signal pathway (22-24).

Additionally, in this study, mechanical strain induced β -catenin translocation into the nuclei of the MC3T3-E1 cells, and si-Itg β 1 hampered the translocation (Fig. 3). The results confirmed that the application of mechanical strain activated the β -catenin signal pathway, and that the activation was inhibited by integrin- β 1 knockdown.

Mechanical strain promotes osteoblastic differentiation, which is attenuated by integrin- β 1 RNAi. Mechanical strain enhanced ALP activity (Fig. 4), the mRNA expression of runt-related transcriptional factor 2 (Runx2) and osteocalcin (OCN) in the

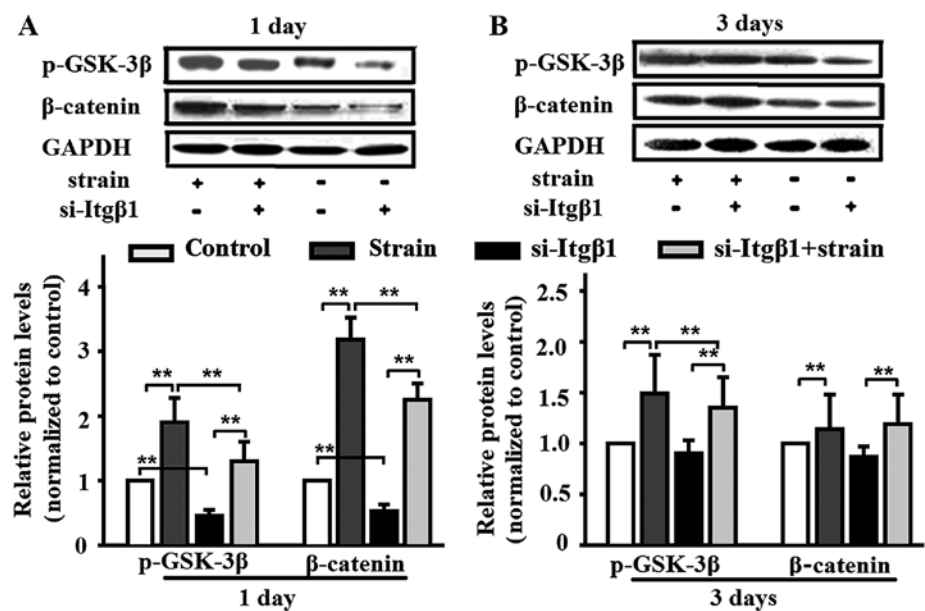


Figure 2. Mechanical strain increases the protein levels of phosphorylated (p-)glycogen synthase kinase- β (GSK-3 β) and β -catenin, and this effect is attenuated by integrin- β 1 siRNA (si-Itg β 1). Mechanical strain increased the protein levels of p-GSK-3 β and β -catenin at (A) day 1 and (B) day 3. Knockdown of integrin- β 1 attenuated the increased protein levels (A and B). In addition, the knockdown reduced the protein levels in the unstrained cells after 1 day (A). ** $P < 0.01$, between the indicated groups, $n = 6$.

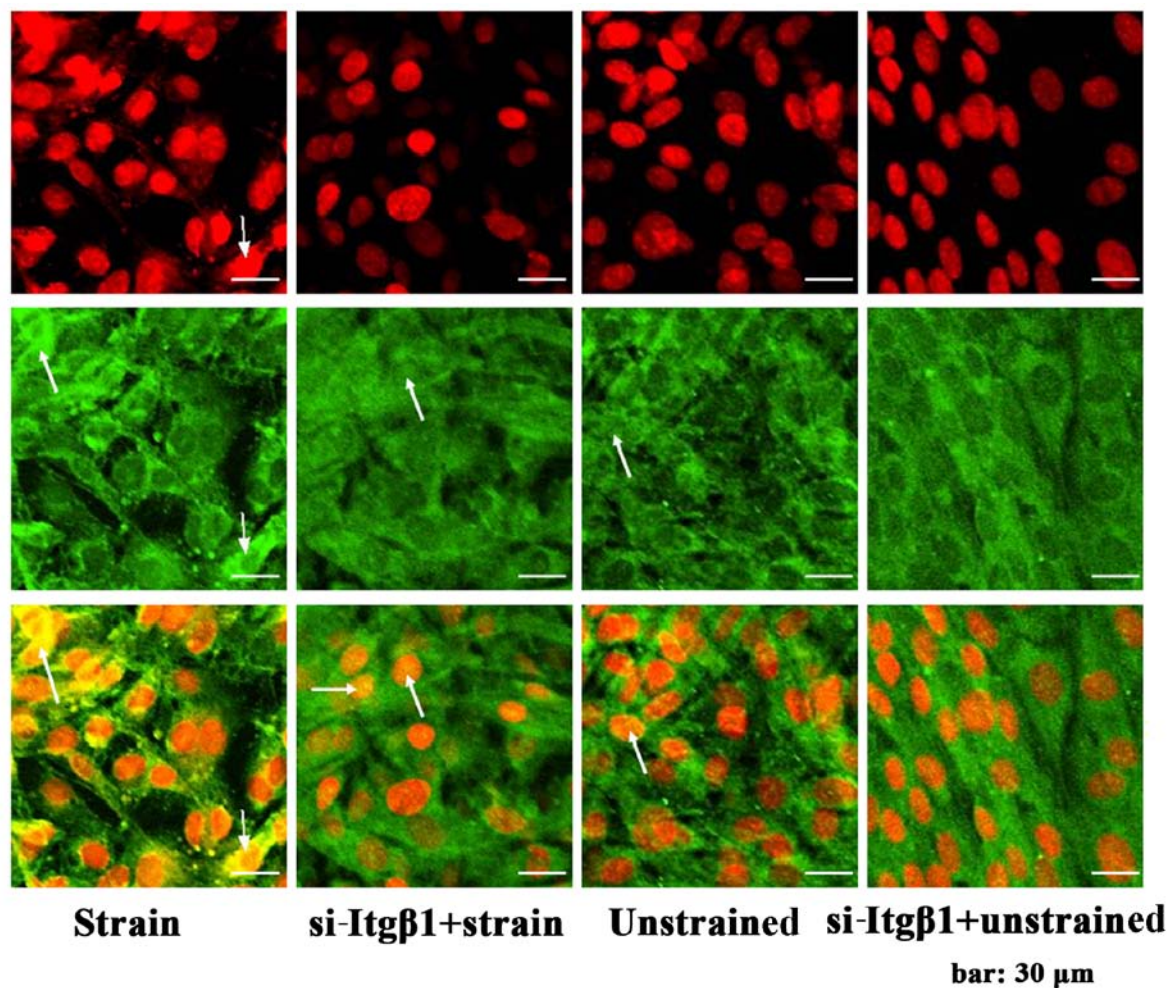


Figure 3. Mechanical strain stimulates β -catenin nuclear translocation and the nuclear translocation is inhibited by integrin- β 1 siRNA (si-Itg β 1) [shown by laser scanning confocal microscopy (LSCM)]. Top panels, propidium iodide (PI) staining; middle panels, immunofluorescent staining of β -catenin; bottom panels, merge. The mechanical strain imposed on MC3T3-E1 cells for 3 days, stimulated the nuclear translocation of β -catenin in the cells (white arrows) compared with the unstrained cells. Pre-treatment with si-Itg β 1 inhibited the nuclear translocation induced by mechanical strain.

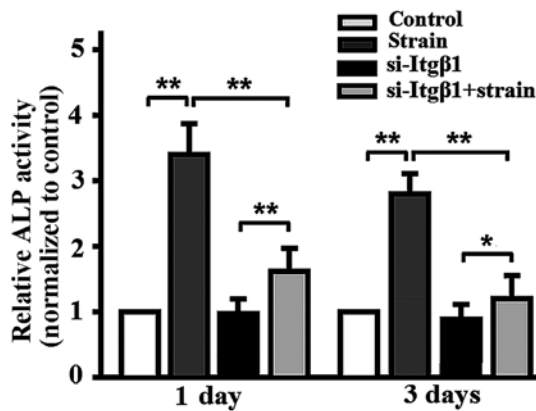


Figure 4. Mechanical strain enhances ALP activity and this effect is weakened by integrin- β 1 siRNA (si-Itg β 1). Mechanical strain enhanced ALP activity at the indicated times, and knockdown of integrin- β 1 resulted in reduced ALP activity. * $P < 0.05$ and ** $P < 0.01$, between the indicated groups, $n = 6$.

MC3T3-E1 cells (Fig. 5A), the protein levels of BMP-2/4 (Fig. 5B), and si-Itg β 1 lowered ALP activity, the mRNA expression of Runx3 and OCN and the protein levels of BMP-2/4 in the mechanically strained MC3T3-E1 cells (Figs. 4 and 5). When the cells were not mechanically strained, si-Itg β 1 had no effect on ALP activity, the mRNA expression of Runx2 or OCN, and the protein levels of BMP-2/4 (Figs. 4 and 5).

ALP is a marker of early osteogenic differentiation (25), Runx2 is the critical transcription factor that regulates osteoblast differentiation (26), and OCN is a late marker of

differentiation corresponding with matrix deposition and mineralization (27). BMP-2/4 are also markers of osteoblastic differentiation (28,29). Thus, the results indicated that the application of mechanical strain promoted osteoblastic differentiation which was attenuated by integrin- β 1 RNAi.

Discussion

Mechanical loading is a potent regulator of bone remodeling and maintenance of bone mass, and many signaling pathways are involved in the response of bone cells to mechanical strain (30,31). However, the characterization of the cellular and molecular events linking loading and bone response remains incomplete.

As an important member of the integrin family, integrin- β 1 is also mechanoresponsive, as it mediates the response of bone to mechanical strain. Mechanical stretching upregulated the expression of integrin- β 1 in osteosarcoma cells (4). Fluid shear stress increased the expression of integrin- β 1 in C57BL/6J mouse osteoblasts (32). In addition, integrin- β 1 was required for focal adhesion kinase-independent activation of MAP kinase induced by mechanical stress (33).

Wnt/ β -catenin signaling has been shown to be important in the osteogenic differentiation of mesenchymal stem cells and bone formation/development, as well as in the mechanical response of osteoblasts (22,23). Mechanical strain induces bone formation through the activation of Wnt/ β -catenin pathways. Consistent with β -catenin activation, osteoblasts respond to mechanical loading with the increased expression of Wnt/ β -catenin target

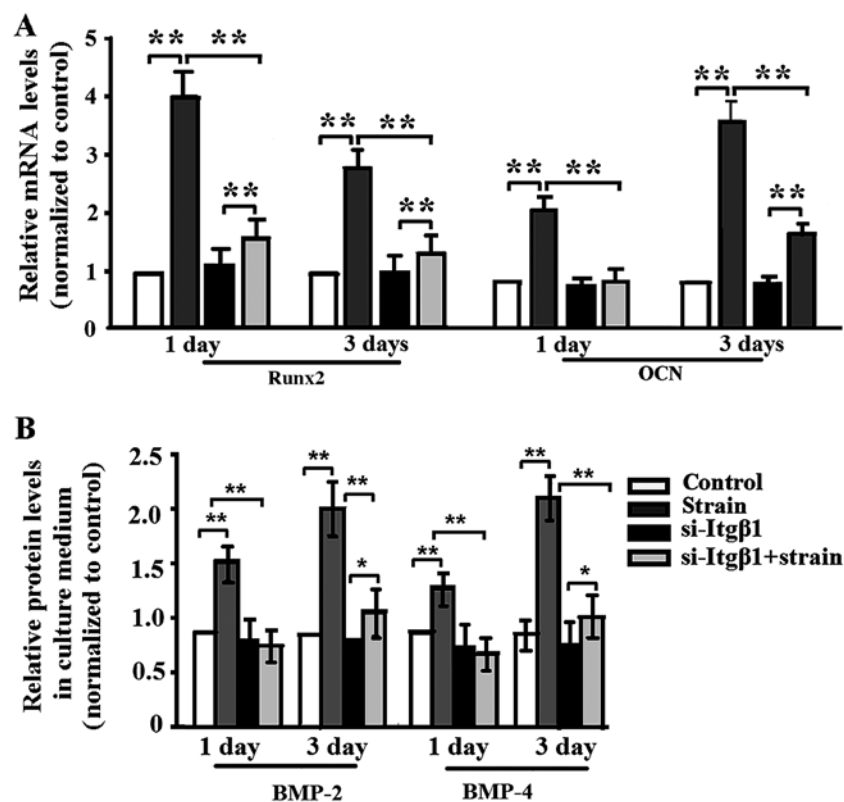


Figure 5. Mechanical strain increases the mRNA levels of (A) runt-related transcriptional factor 2 (Runx2) and osteocalcin (OCN), as well as (B) the protein levels of bone morphogenetic protein (BMP)-2 and -4 at the indicated times, which were diminished by integrin- β 1 siRNA (si-Itg β 1). * $P < 0.05$ and ** $P < 0.01$, between the indicated groups, $n = 6$.

genes (11,34). However, to date, the role of integrin- β 1 in the regulation of Wnt/ β -catenin signaling in osteoblasts subjected to mechanical strain remains poorly understood.

Our previous studies demonstrated that mechanical tensile strain at a frequency of 0.5 Hz and intensities of 2,000–3,000 $\mu\epsilon$ for 1 h/day promoted the osteoblastic differentiation of MC3T3-E1 cells (increased bone ECM proteins/genes such as collagen I, OCN and BMPs) (35–37), suggesting that mechanical tensile strain promoted osteoblast ECM formation. Thus, in this study, we selected 0.5 Hz at 2,500 $\mu\epsilon$ mechanical strain for 1 h/day.

In the present study, integrin- β 1 knockdown reduced the relative protein levels of p-GSK-3 β and β -catenin, which were enhanced following exposure to mechanical strain. The application of mechanical strain to the MC3T3-E1 cells also caused β -catenin accumulation in the cytoplasm and subsequent translocation into the nucleus, and si-Itg β 1 inhibited the accumulation and translocation of β -catenin.

GSK-3 β and β -catenin are important components of the Wnt/ β -catenin signaling pathway. GSK-3 β normally phosphorylates β -catenin, targeting it for degradation. After phosphorylation, p-GSK-3 β is inactivated, which leads to β -catenin accumulation in the cytoplasm and subsequent translocation into the nucleus, where it modulates gene transcription (22–24). The present study demonstrated that mechanical strain activated the β -catenin signal pathway which was inhibited by integrin- β 1 knockdown.

ALP is widely used as a marker of osteogenic differentiation, with increasing enzymatic activity associated with an osteoblastic phenotype (38). Runx2, OCN and BMP-2/4 are also markers of osteogenic differentiation (26–29). In this study, the application of mechanical strain promoted osteoblastic differentiation which was attenuated by integrin- β 1 knockdown. Thus, integrin- β 1 knockdown inhibited Wnt/ β -catenin signal transduction in osteoblasts, in response to mechanical strain.

si-Itg β 1 reduced the protein levels of β -catenin and p-GSK-3 β in the unstrained MC3T3-E1 cells after 1 day although it had no effect on osteoblastic differentiation. Future studies are warranted into the associations among integrin- β 1, β -catenin signaling and osteoblastic differentiation.

In conclusion, the knockdown of integrin- β 1 inhibited the activation of the β -catenin signal pathway and osteoblastic differentiation induced by the application of mechanical strain at 2,500 $\mu\epsilon$, which indicated that mechanical strain promoted osteoblastic differentiation through integrin- β 1-mediated β -catenin signaling.

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