

Fluid flow modulates the expression of genes involved in the Wnt signaling pathway in osteoblasts in 3D culture conditions

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Received October 29, 2013; Accepted February 26, 2014

DOI: 10.3892/ijmm.2014.1694

Abstract. The balance between osteoclastic bone resorption and osteoblastic bone formation maintains bone mass, while mechanical loads stimulate bone formation and suppress resorption. The molecular mechanisms responsible for this process have not yet been fully elucidated. In the present study, we assessed whether mechanical stimulation by pulsating fluid flow (PFF) leads to functional Wnt production and affects the function of osteoblasts. ROS17/2.8 osteoblasts were submitted to 1-4 h PFF (0.8 Pa) by three-dimensional (3D) cell culture system with fluid flow. PFF upregulated the gene expression levels of adenomatous polyposis coli, alkaline phosphatase, low density lipoprotein receptor-related protein 5 (LRP5), Wnt3a and β -catenin [catenin beta 1 (CTNNB1)] in all the groups of osteoblasts. Our results suggest that mechanical stimulation by PFF induces the differentiation of osteoblasts and the activation of the Wnt/ β -catenin signaling pathway in a 3D cell culture system. Furthermore, mechanical stress plays an important role in the Wnt/ β -catenin signaling pathway and is involved in bone formation.

Introduction

Bone mass in adults is maintained locally by the balance between osteoclastic bone resorption and osteoblastic bone formation, each of which is subject to various factors aimed at fulfilling bone function. This function, termed remodeling, is influenced by a number of factors, including growth factors, systemic hormones and the mechanical environment (1).

Mechanical loads affect the balance of bone formation and resorption. Mechanical stimulation is recognized as a major mediator of both osteogenic and angiogenic activities in bone. An appropriate function of stress is beneficial to the normal metabolism of bone tissue and the formation of new bone. Normal bone tissue activity would normally result in osteoporosis and bone resorption, if not for proper stimulation. Thus, stress stimulation plays an important role in remodeling (2).

When mechanical loads stimulate bone tissue, the interstitial fluid flow has an impact on the osteoblast, which is in the porous bone tissue. By fluid shear stress stimulation, the osteoblast transforms a mechanical signal to a biochemical signal, and then regulates the formation and resorption of bone (3). Increased mechanical loads stimulate bone formation and suppress resorption, whereas unloading has the opposite effect (4). Wnts are involved in this process.

Wnts are a large family of 19 secreted carbohydrate- and lipid-modified polypeptides that mediate important biological processes (5). The Wnt/ β -catenin pathway is one of the Wnt signaling pathways, and it is also a main factor in the mediation of bone remodeling. The Wnt/ β -catenin signaling pathway facilitates the formation of new bone by inducing the differentiation of pluripotent mesenchymal cells into osteoblast progenitors, becoming osteoblasts, thus maintaining the precursor status of these osteoprogenitors, inhibiting osteoblast apoptosis, and promoting osteoblast proliferation and differentiation (6,7).

As both mechanical stimulation and the Wnt pathway are critical anabolic signaling factors affecting bone formation, the demonstration of stress regulation of Wnt expression in osteoblasts may provide new insight into the role of the functional communication between stress and Wnt signaling pathways that affect bone formation. In the present study, we assessed whether mechanical stimulation by pulsating fluid flow (PFF) leads to functional Wnt production.

Historically, two-dimensional (2D) cultures *in vitro* have played a key role in the study of bone mechanobiology; however, 2D experiments are performed on flat surfaces, which do not reflect the environment of three-dimensional (3D) architecture *in vivo*. Studies have proven that 2D models do not always accurately represent what will occur under 3D culture conditions (8-11).

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Key words: osteoblasts, fluid shear stress, Wnt/ β -catenin, scaffolds, gene expression

Barron *et al* (12) demonstrated that a 3D grafting structure and *in vitro* cultures under dynamic conditions provide critical information to defining the fundamental biochemical and biomechanical responses of bone cells. Therefore, in a previous study, we designed and fabricated a 3D fluid flow cell culture system, which can load shear stress on cells that are cultured on scaffolds (13).

Therefore, the aim of this study was to evaluate whether mechanical stimulation by means of PFF affects the function of osteoblasts and Wnt production in osteoblasts.

Materials and methods

Cell culture. The osteoblast-like rat osteosarcoma cells, ROS17/2.8, between passages 20 and 26 were cultured in Dulbecco's modified Eagle's medium (DMEM) - low-glucose medium with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all from Gibco-Invitrogen, Carlsbad, CA, USA), and were set in a 37°C incubator with 5% carbon dioxide and moisture.

Scaffolds. Partially deproteinized bone (PDPB; Yantai Zhenghai Biology Technology Co., Ltd., China) was used as a scaffold for osteoblasts. The scaffolds were round, 10 mm in diameter and 2 mm in thickness with an average porosity of 79±1.2% and a pore diameter of 300-500 µm (Fig. 1A).

Cell seeding. The cells were harvested (trypsinized and resuspended in culture medium) after reaching 80% confluency. Subsequently, 2,000,000 cells in 100 µl medium were seeded onto each 3D calcium phosphate scaffold in 24-well plates (2,000,000 cells/scaffold) (Fig. 1B-a). After 2 h, 2 ml medium were added to each well and the cells were allowed to attach for 24 h in a 37°C incubator (Fig. 1B-b).

Mechanical loading in 3D. The 3D fluid flow cell culture system consists of 8 flow chambers, platinum-cured silicon tubing, reservoirs, a gas filter and a pump (Tianjin Xieda Weiye Electron Co., Ltd., Tianjin, China) (Fig. 2A). Scaffold/cell constructs were then placed into each chamber, which was made of 2 ml glass syringes. The scaffolds were fixed by a cut radial plunger (Fig. 2B).

Experimental design. The PFFs were generated by a roller pump, and the value of 0.8 Pa used for shear stress was based on the outcome of the theoretical model proposed in the study by Weinbaum *et al* (20). There were 4 loading treatments. The scaffolds were thus divided into 4 groups: group A remained incubated at 37°C with 5% CO₂ for the duration of the experiment. Groups B-D were placed in their chambers and connected to the flow circuit. Loading occurred immediately after the 3D fluid flow cell culture system was connected and continued for 1 h and statically incubated for 23 h for group B, 2 h and statically incubated for 22 h for group C, and 4 h and statically incubated for 20 h for group D. All samples were removed for analysis at 24 h.

Scanning electron microscopy (SEM). For SEM, the samples were rinsed twice with calcium-free phosphate-buffered saline (PBS) and fixed in 2.5% glutaraldehyde at 4°C overnight. The

samples were then dehydrated in a graded series of ethanol (30, 50, 70, 85, 90 and 100%), dried with tetramethylsilane, sputter-coated with gold and examined using an Inspect F scanning electron microscope (FEI Inspect F, The Netherlands) at an acceleration voltage of 15 kV.

RNA isolation and qRT-PCR. The scaffolds were transferred into a cryotube and immediately shock-frozen and pulverized in liquid nitrogen. Total RNA was extracted using TRIzol reagent (Invitrogen) and frozen at -70°C for subsequent RNA isolation.

cDNA synthesis was performed using 0.5-1 mg of total RNA in a 20-µl reaction mixture consisting of 5 units of PrimeScript™ RT reagent kit (Takara Bio, Inc., Shiga, Japan) at 37°C for 15 min, and terminated by heating at 85°C for 5 sec followed by cooling at 4°C in a thermal cycler (i-Cycler; Bio-Rad, Hercules, CA, USA). The cDNA was then used for real-time PCR for the genes of interest and glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a house-keeping gene.

Real-time PCR was performed in 20 µl total volume for each sample, containing 2 µl cDNA template, 10 µl SYBR® Premix Ex Taq™ (Takara Bio, Inc.), 0.8 µl each of forward and reverse primers, and 0.4 µl ROX Reference Dye with the 7300 real-time PCR system. The primers of alkaline phosphatase (ALP), low density lipoprotein receptor-related protein 5 (LRP5), catenin beta 1 [(CTNNB1), also known as β-catenin], Wnt3A, adenomatous polyposis coli (APC) and GAPDH (synthesized by Takara Biotechnology Co. Ltd., Dalian, China) are listed in Table I. Cycling conditions were as follows: 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 31 sec, then 95°C for 15 sec and 60°C for 31 sec, and 95°C for 15 sec.

Statistical analysis. All experiments were run 3 times with triplicate samples. Significant differences were determined by one-way analysis of variance (ANOVA) followed by a Newman-Keuls post hoc test, and all data are expressed as the means ± standard deviation (SD). In all the analyses, values of P<0.05 were considered to indicate statistically significant differences. All the data were analyzed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA).

Results

SEM: PFF stimulates osteoblasts that are larger, more spread out and thicker extracellularly, in a 3D fluid flow cell culture system. SEM was used to visualize the scaffold/cell constructs in order to assess cell morphology (x300, x600 and x1,200 magnification). Fig. 3A shows microscopic images of the scaffold/cell constructs after 24 h static culture, while Fig. 3B-D shows microscopic images of group B (PFF for 1 h and statically incubated for 23 h), group C (PFF for 2 h and statically incubated for 22 h) and group D (PFF for 4 h and statically incubated for 20 h). In the PFF culture, the cells were larger, more spread out, and appeared to be surrounded by thicker extracellular matrix constructs than the cells cultured under static conditions.

Fig. 3A shows the SEM view of the control group A, in which the osteoblasts covered most of the scaffold surface, had a long fusiform or polygon shape and were arranged in a disor-

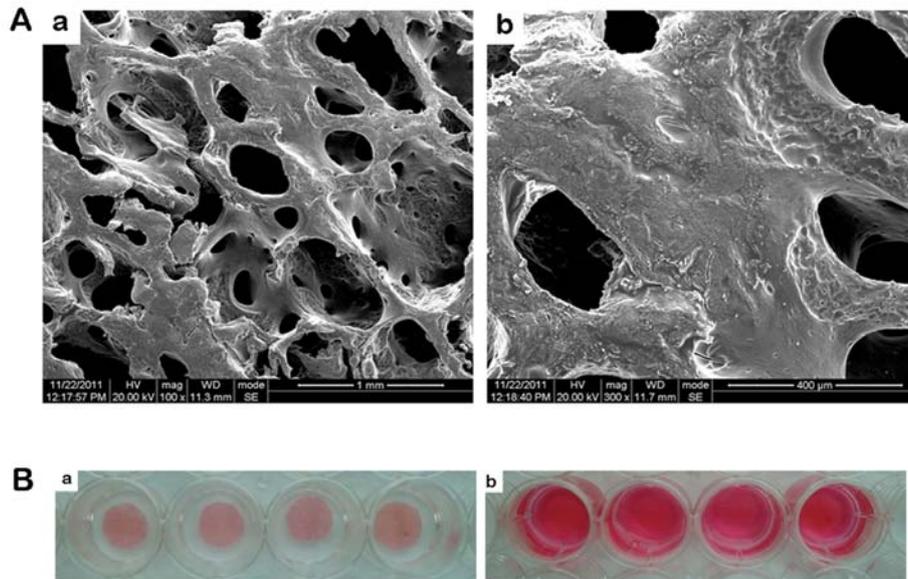


Figure 1. Scanning electron microscopy (SEM) images of the surface of the partially deproteinized bone (PDPB). (A) PDPB with an average porosity of $79 \pm 1.2\%$ and a pore diameter of $300\text{-}500 \mu\text{m}$ (a, $\times 100$; b $\times 300$ magnification). (B) Scaffolds in 24-well plates were seeded with 2,000,000 cells in (a) $100 \mu\text{l}$ medium; (b) 2 ml medium were added to each well 2 h later.

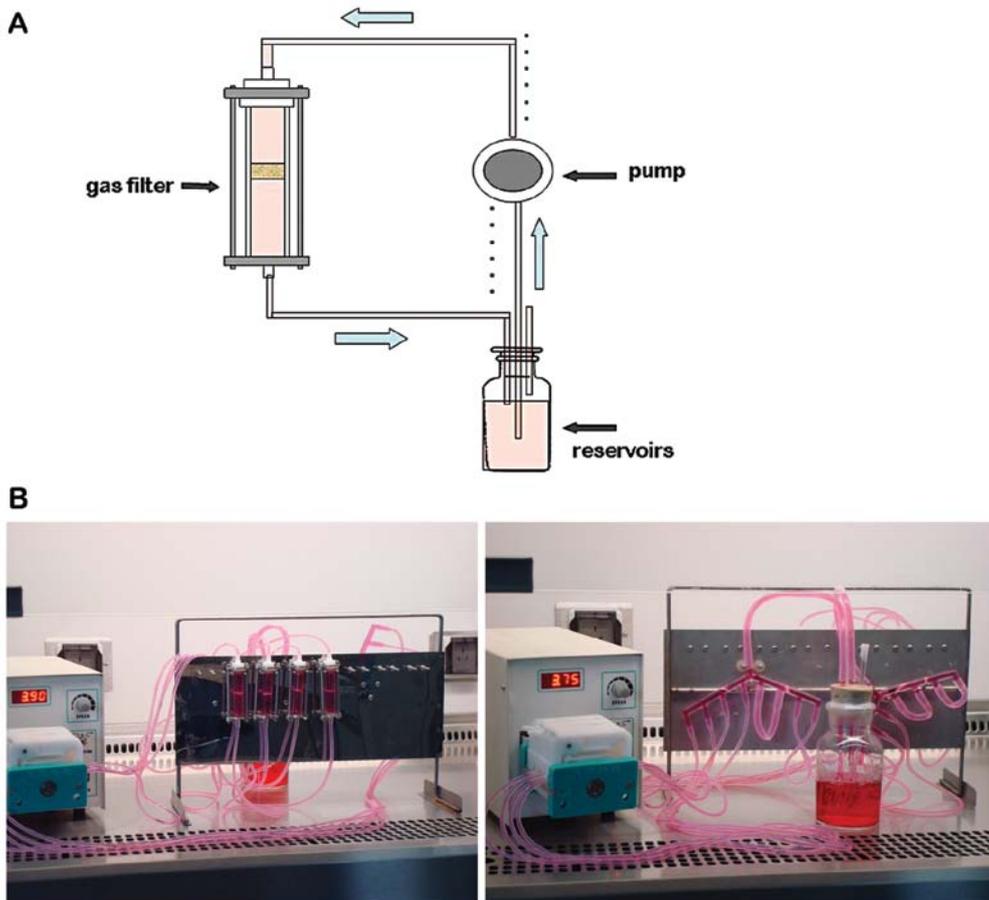


Figure 2. (A) Schematic representation of the chamber and fluid flow cell culture system. (B) Image of the flow perfusion bioreactor.

derly manner. Group B-D scaffolds were completely covered with osteoblasts and the cells were polygonal in shape. In some osteoblasts, many tiny synaptic adhesions had extended from the cells in the scaffolds, and the cells were gathered together

more closely and were integrated. It could be seen the bigger and rougher cells that contained a large number of processes (needle-like structures), indicating osteoblasts with increased secretion. The cells were arrayed with obvious directionality,

Table I. The base of primers for quantitative real-time RT-PCR.

Genes	Left primer	Right primer	Product size (bp)
GAPDH	TATGACTCTACCCACGGCAAGT	ATACTCAGCACCAGCATCACC	138
ALP	GACCCTGCCTTACCAACTCATT	GTGGAGACGCCATAACCATCT	166
APC	GAGTCAGCATCCAAAGGACTGA	GACTGGCGTACTAATGCAGGTCT	140
LRP5	GAGTCAGCATCCAAAGGACTGA	CCAGGCTCACAGAACTCATCA	116
Wnt3a	ACGAGAGGATTGAGAGCGTCA	GATAAGGGTCTTTGAGCGAGTCC	118
CTNNB1	GGGTCCTCTGTGAAGTTGCTC	CTTGTAGTCCTGTGGCTTGTC	167

GAPDH, glyceraldehyde phosphate dehydrogenase; ALP, alkaline phosphatase; APC, adenomatous polyposis coli; LRP5, low density lipoprotein receptor-related protein 5; CTNNB1, catenin beta 1 (also known as β -catenin).

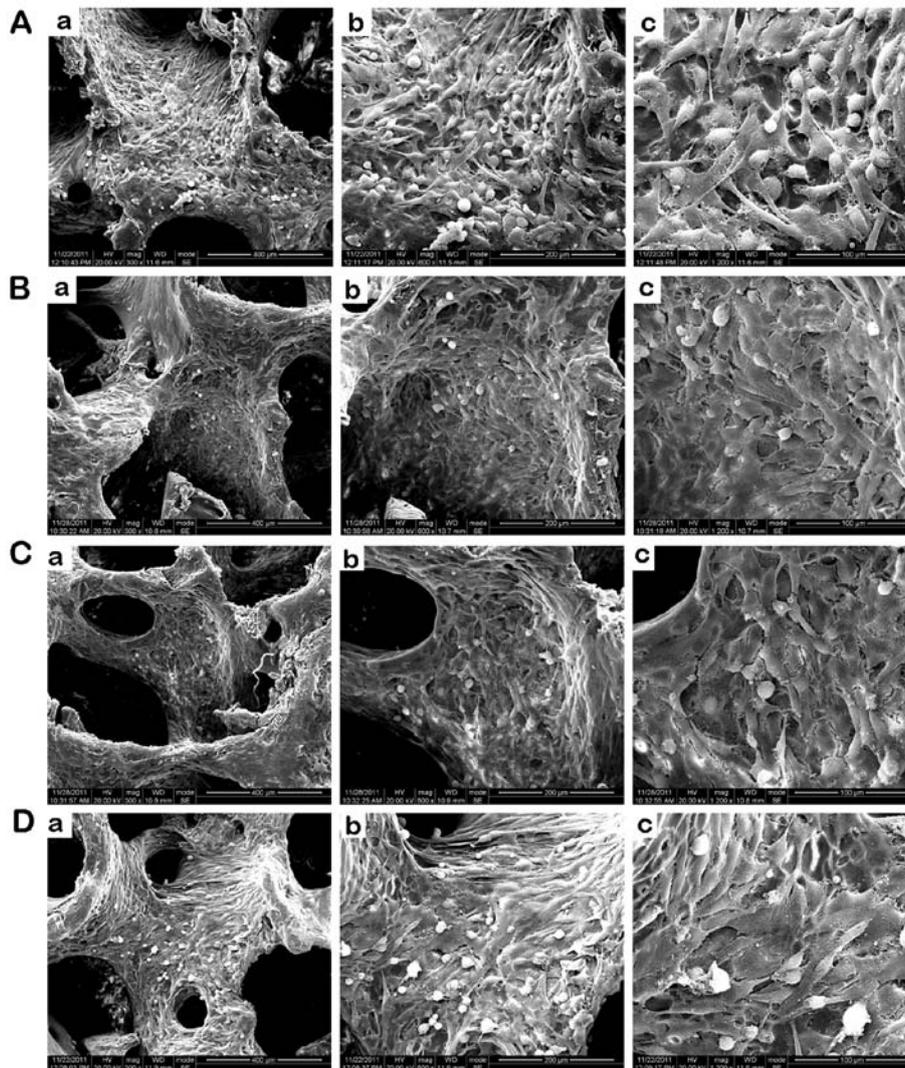


Figure 3. Scanning electron microscopy (SEM) images of the surface of the seeded scaffold after 24 h. (A) Group A: kept under static culture conditions (a, x300; b, x600; c, x1200 magnification). (B) Group B: cells were subjected to pulsating fluid flow (PFF) for 1 h and statically incubated for 23 h (a, x300; b, x600; c, x1200 magnification). (C) Group C: cells subjected to PFF for 2 h and statically incubated for 22 h (a, x300; b, x600; c, x1200 magnification). (D) Group D: cells were subjected to PFF for 4 h and statically incubated for 20 h (a, x300; b, x600; c, x1200 magnification).

according to the direction of PFF. In group C, osteoblasts were covered with layers of some parts of the scaffolds, and the number of cells had increased. In group D, the elongated osteoblasts in the shear direction were more apparent, the surface of

cells became rougher, and some exfoliated cells were observed occasionally. In addition, a higher cell density and more extracellular matrix was observed as compared with the perfusion groups and the static culture group.

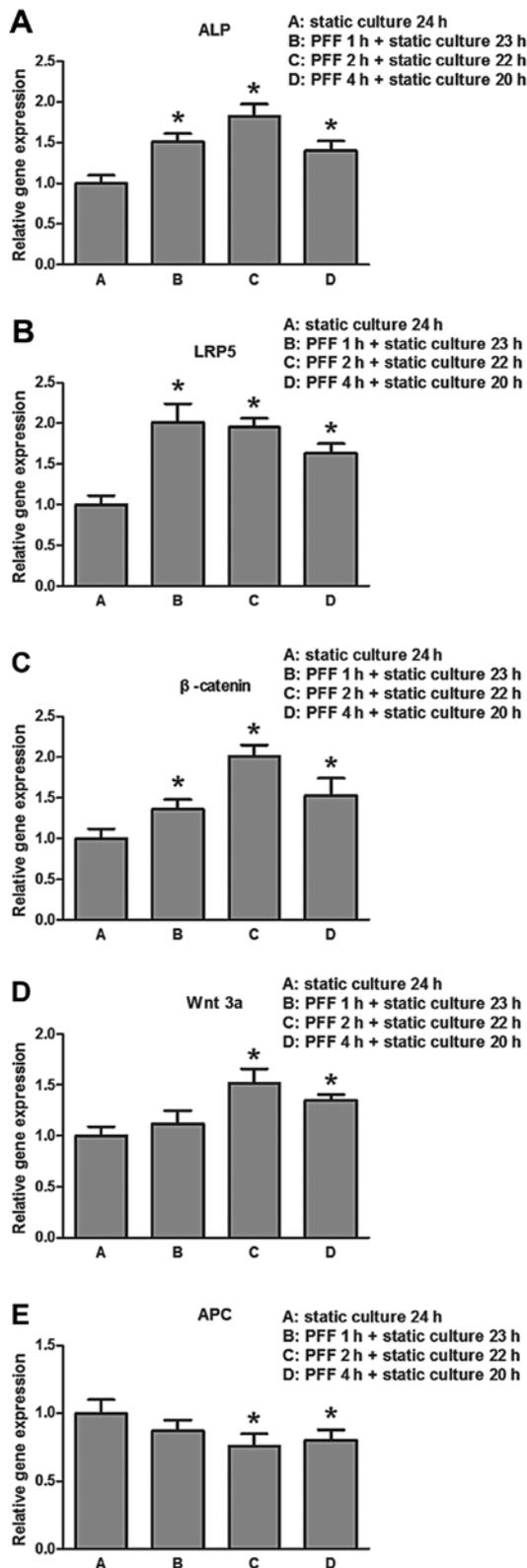


Figure 4. Effects of fluid shear stress on osteogenic differentiation marker genes in osteoblasts. (A) The mRNA expression level of alkaline phosphatase (ALP) was increased in the groups in which cells were induced with pulsating fluid flow (PFF). (B) The mRNA expression level of LRP5 was increased in groups in which cells were stimulated by PFF. (C) The mRNA expression level of β -catenin was increased in groups in which cells were stimulated by PFF. (D) The mRNA expression level of Wnt3a was increased in groups in which cells were stimulated by PFF. (E) The mRNA expression level of adenomatous polyposis coli (APC) was decreased in groups in which cells were stimulated by PFF. Error bars represent the means \pm standard deviation (SD); n=3 experiments. Significant differences were set at *P<0.05.

PCR: PFF upregulates the expression of osteogenic genes of osteoblasts and activates the Wnt/ β -catenin signaling pathway in the 3D fluid flow cell culture system. We assessed whether PFF affects the expression of proteins related to osteogenesis, as well as genes involved in the canonical Wnt signaling pathway. The results revealed that PFF upregulated the expression of osteogenic genes in osteoblasts and activated the Wnt/ β -catenin signaling pathway. The mRNA expression of ALP significantly increased in the groups in which the cells were stimulated by PFF (P<0.05) (Fig. 4A). LRP5, β -catenin and Wnt3a, the makers of the Wnt/ β -catenin signaling pathway, demonstrated an increased mRNA expression in the cells that were stimulated by PFF (P<0.05) (Fig. 4B and C). However, as the negative regulator of the key protein in the Wnt/ β -catenin signaling pathway, the mRNA expression of APC was significantly decreased in all the PFF groups (P<0.05) (Fig. 4E).

Discussion

Bone is a dynamic tissue and is constantly under conditions of growth and remodelling. It adapts to both physiological and physical environments and undergoes continuous remodeling throughout life. Osteoblasts secrete new bone, by sensing and responding to mechanical stimulation via stress-generated fluid flow inside the canalicular-lacunar networks and trabecular spaces within bone tissue. Traditionally, 2D *in vitro* culture [such as the parallel plate flow chamber (PPFC)] (14) is a method normally used in the study of fluid shear stress. A number of studies have shown that the majority of adhered cells respond differently if cultured in 2D and 3D substrates (15-19). In comparison to 2D monolayer cultures, 3D culture system devices represent valuable tools for the establishment of physiologically oriented *in vitro* tissue models.

Previous studies have proven that the mechanical loading of bone engenders the movement of extracellular fluid through the bone lacunar-canalicular system. Such fluid flow may stimulate bone cells via streaming potentials, wall shear stress, or chemo-transport related effects. Biot's porous media theory has been used to relate whole bone stress to canalicular interstitial fluid flow past osteocytic processes (20). It was predicted that loading regimens engendering peak physiological strains will induce fluid-induced shear stresses of 0.8-3 Pa. Subsequently, Bakker *et al* (21) used PFF to generate fluid shear stress of 0.2-1 Pa.

In this study, a higher cell density and greater extracellular matrix were observed in the perfusion groups as compared with the static culture group. In the fluid shear stress group, the cells were larger in size and rougher, and contained a large number of processes (needle-like structures). In some osteoblasts, many tiny synaptic adhesions had extended from the cells in the scaffolds, and the cells were gathered more closely together and were integrated. The cells are arrayed with obvious directionality, according to the direction of PFF. It is possible that following stimulation with PFF, the cells deformed to adapt to the direction of the PFF. These change were used to reduce stress stimulation, maintain physiological function, promote the stress on osteogenesis, and reduce osteoclast activity. With the extended perfusion time, the cells proliferated more actively, cell secretion increased, and the extracellular matrix presented an increasing trend. All of these effects indicated

that the osteogenesis of osteoblasts was enhanced. This study reveals that a fluid shear stress intensity of 0.8 Pa has an effect on osteoblasts, conducive to the proliferation and differentiation of osteoblasts within 4 h.

Recently, an important role for Wnt/ β -catenin signaling has been recognized in promoting bone anabolism. The Wnt family includes more than 15 proteins that have been shown to play an important role in organ development and the homeostasis of several adult tissues, including bone (22). Considerable progress has been made in terms of understanding the role of Wnt/ β -catenin signaling in osteogenesis and particularly in osteoblast biology; however, little is known about the functions of this pathway in osteocytes (23).

The β -catenin degradation complex consists of the scaffold proteins, axin and APC, and the protein kinases, casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3), thereby relieving β -catenin (CTNNB1) from its constant phosphorylation by CK1 and GSK3 and its subsequent degradation by the ubiquitin-proteasome pathway (24,25). Hill *et al* (26) used β -cat ^{Δ Prx^{-/-} mice to study the effects of β -catenin in the limb and head mesenchyme. Their findings revealed that β -catenin activity is required for the early steps of osteoblast differentiation. On the other hand, stabilization of the β -catenin function in the mesenchyme of β -cat Δ ex3^{Prx/+} animals suppressed chondrogenesis, rather than stimulating osteoblastogenesis. Day *et al* (27) demonstrated the similar finding that β -catenin signaling is required for osteoblasts to complete differentiation and to synthesize properly formed bone.}

LRP5 is the most well known member of the LRP family, which constitutes a membrane receptor complex as Wnt ligands. It is a Wnt co-receptor, and Wnt signaling through LRP5 is required for mechanically induced bone formation. Sclerostin is speculated to function as a secreted inhibitor of canonical Wnt/ β -catenin signaling by binding to LRP5 and LRP6 Wnt co-receptors, preventing their association with the Wnt-Frizzled (Fzd) receptor complex (28-30). Kato *et al* (31) reported that the germline deletion of murine LRP5 reduced vertebral total bone volume by 40% at 8 weeks of age when peak bone mass occurred in LRP5^{+/+} mice, as determined by histomorphometry. They then disrupted exon 6 of the gene, and also noted that LRP5^{-/-} mice had tibial fractures by 2 months of age, resulting from low bone mass. Furthermore, when the LRP5^{-/-} mice were only 2 weeks old, the total bone volume was found to be significantly reduced. They also found that the loss of LRP5 did not alter osteoblast apoptosis and differentiation, osteoclastogenesis, or bone resorption. Thus, the deletion of LRP5, by reducing osteoblast proliferation and activity, leads to decreased bone accrual in early post-natal mice.

As shown in a previous study, osteoblast differentiation was arrested at the early progenitor stages, and only type I collagen and ALP were expressed (32). This suggests that β -catenin signaling is required for osteoblasts to complete differentiation and to synthesize properly formed bone (11). Thus, ALP is known to be an early marker for the osteoblastic phenotype, being upregulated at the onset of differentiation and subsequently decreasing as differentiation progresses (33). In this study, we observed the upregulation of the gene expression of ALP in all PFF groups and the difference was not statistically significant.

Wnt3a initiates canonical Wnt signaling by binding to receptor complexes consisting of LRP5/6 and Frizzled on

the cell surface, which results in the nuclear translocation of β -catenin and the activation of Lef1/Tcf transcription factors (34-36). Thus, we examined whether a canonical Wnt pathway ligand (Wnt3A) that acts through the LRP5 receptor would have similar effects on PFF-induced gene expression. We also observed that 1 h of PFF followed by 23 h of incubation without PFF significantly upregulated gene expression.

A great deal of research has indicated that mechanical stress and Wnt/ β -catenin play important roles in bone formation. The involvement of the Wnt/ β -catenin pathway in the mechanotransduction of signals from osteocytes has been revealed (37,38). However, studies on mechanical stress and Wnt signaling are limited. In this study, in a 3D cell culture system, loading resulted in the promotion of osteoblast proliferation and differentiation, as well as an increase in the expression of Wnt pathway and Wnt/ β -catenin target genes. However, there are differences between *in vivo* and *in vitro* environments; shear stress needs to be transformed inside the body in future studies.

In conclusion, the results from our study suggest that mechanical stimulation by PFF induces the differentiation, proliferation and apoptosis of osteoblasts and the activation of the Wnt/ β -catenin signaling pathway in a 3D cell culture system. The Wnt/Wnt/ β -catenin signaling pathway is involved in the progression of bone formation. These data provide a framework for understanding the role of the Wnt/ β -catenin signaling pathway in the mechanical adaptation of bone.

Acknowledgements

We thank Xiaoyu Li, Yurong Liu, Xiangli Kong and Chaoliang Zhang for their technical assistance. This study was supported by the National Natural Science Foundation for the Youth of China (NSFC) (grant 30801310).

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