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Periosteum-derived cells respond to mechanical stretch and activate Wnt and BMP signaling pathways

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ABSTRACT

The periosteum supplies osteoblasts and nutrients for bone metabolism and is important for osteoblast differentiation and osteogenesis. Recently, periosteum-derived cells have been used for orofacial bone regeneration therapy. However, little is known about the function of the periosteum in physiological bone remodeling. On our hypothesis that the periosteum senses a mechanical stress to induce bone remodeling, we subjected human jaw bone periosteum cells (HJBPCs) to uniaxial stretching for 24 h and characterized their gene expression profiles by microarray analysis. Of 62,976 genes detected, 550 genes related to bone metabolism were extracted, and 76 of these genes with large changes in gene expression were short-listed. The results indicated that mechanical stretch in HJBPCs regulated the expression levels of genes involved in the Wingless-type MMTV integration (Wnt) site, bone morphogenetic protein (BMP) signaling pathways, and inflammatory cytokines. We propose that periosteum-derived cells sense mechanical stress and then activate and regulate signals for osteoblast differentiation and osteogenesis.

The periosteum is a fibrous membrane tissue that covers the outer surface of bones. Histologically, it consists of two layers, the cambium layer on the bone side and the outer fibrous layer. The former is characterized by a high-density alignment of osteoblast-like cells and pre-osteoblasts. The latter, on the other hand, is a fibrous tissue containing fibroblasts, with cells arranged at a low density. During bone growth, cells in the cambium layer differentiate into osteoblasts, followed by membranous ossification by the periosteum (20). During the healing process after bone fracture, a complex sequence of events occur: thickening of the periosteum, nutrient supply from the periosteum, and differentiation of the cambium layer cells into osteoblasts, leading to formation of callus, which advances the healing process (33). The periosteum serves as a supplier of osteoblasts and nutrients in bone metabolism, and is capable of inducing osteoblast differentiation and osteogenesis.

In recent years, bone regeneration therapy utilizing the ability of the periosteum to differentiate osteoblasts and promote osteogenesis has been clinically applied for jaw bone regeneration with good outcomes. Bone regeneration has been confirmed in transplantation of sheet-shaped cultures of small pieces of bone tissue to patients with bone resorption due to periodontal diseases and those with a small defective bone cavity after tumorectomy or cystectomy (14). However, while the abilities of the periosteum to induce osteoblast differentiation and promote osteogenesis have drawn attention and are being extensively studied, little is known about the functions of the periosteum in bone remodeling un-

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der mechanical stress.

The relationship between mechanical stress and osteogenesis has been widely studied. Bones are always subject to mechanical stress from gravity and motion. These mechanical stresses are essential to the maintenance of bone structures and functions (1). Low mechanical stresses states, such as gravity-free and bedridden states, are associated with reduced osteogenesis, resulting in reduced bone mass (1, 18). On the one hand, moderate mechanical stresses promote osteogenesis and increase bone mass (7). In the oral area, resorption of alveolar and jaw bones is associated with occlusal traumatic periodontal disease and tooth movement due to orthodontics (21), because mechanical stresses are stronger than that under physiological conditions and because of additional inflammatory factors.

Although it has been established that mechanical stresses induce bone remodeling, as summarized above, mechanisms for sensing mechanical stresses and triggering bone remodeling process have not been fully elucidated. Many studies have suggested that osteocytes, osteoblasts and bone lining cells are responsible for mechanical stress sensing (2, 35). Despite the continued research, this issue remains unclear. Furthermore, roles of the periosteum in the mechanical stress-induced bone remodeling have not been studied extensively and are largely unknown. Given that mechanical stresses are essential for bone remodeling, which maintains bone structures and functions, we suggest that the periosteum, which is capable of inducing osteoblast differentiation and osteogenesis, senses mechanical stresses and participates in bone remodeling.

In this study, we established primary cultured human jaw bone periosteum cells (HJBPCs) from periosteum tissues isolated from human jaw bones and conducted a comprehensive gene expression profiling by microarray analysis to understand the response and functions of HJBPCs under mechanical stress.

MATERIALS AND METHODS

Cell cultures. HJBPCs were isolated from a healthy, non-smoking female volunteer (age, 21 years). The periosteum tissue was aseptically dissected from the periodontal tissues of the buccal side of the retromolar region in the lower jaw bone. Tissue was washed thrice in Dulbecco's phosphate-buffered saline (PBS) lacking Ca^{2+} and Mg^{2+}, cut into small samples (approximately 2×3 mm), and plated on 100-mm dishes. After 15–20 min CO_2 incubation under dry conditions, the samples of periosteum tissue were cultured in a minimal essential medium containing 10% fetal calf serum, penicillin (100 U/mL), streptomycin (100 μg/mL), and gentamicin (80 μg/mL). After HJBPCs emerged, the small sample of the periosteum tissue was removed. HJBPCs were cultured for 20 days unless otherwise specified (13, 14).

The study design and consent form were approved by the ethical committee for human subject use at Hirosaki University Medical Hospital. Procedures were in accordance with the Helsinki Declaration of 1975 (revised in 2000). Human gingival fibroblasts (HGFs) were isolated, and then established, from healthy gingival tissues of the buccal side of the retromolar region of a volunteer in the Department of Dentistry and Oral Surgery, Hirosaki University Graduate School of Medicine as described prviously (17, 27).

Alkaline phosphatase staining of human periosteal cells. HJBPCs were plated in culture and treated as described above. Alkaline phosphatase staining assay was performed using the Sigma Diagnostics Alkaline Phosphatase kit (Sigma Diagnostics, USA), as directed by the manufacturer. In brief, cell layers were rinsed with phosphate-buffered saline (PBS), fixed with citrate-acetone-formaldehyde fixative solution at room temperature for 30 s, and then stained with alkaline-dye mixture at room temperature for 15 min. Naphthol AS-BI alkaline solution and fast red violet B alkaline solution were utilized to detect enzyme activity. Slides were then counterstained with hematoxylin solution (Wako Chemicals, Japan), and evaluated using light microscopy.

Application of mechanical stretch to cultured HJBPCs. Polydimethylsiloxane stretch chambers (STREX, Japan) were coated with 1 mg/mL of fibronectin from bovine plasma (Wako Chemicals). HJBPCs were seeded onto stretch chambers, having a culture surface of 4 or 10 cm^2, at 15,000 cells/cm^2. Cells were incubated in chambers for 24 h before stretching. Uni-axial tensile strain (10% stretch) was applied using an STB-140 system (STREX) for 24 h. Non-stretched HJBPCs cultured on stretch chambers were used as controls. Cells, RNAs, and conditioned media were immediately collected after stretching experiments. Total RNA was isolated from the cells using Trizol reagent.

Microarray analysis. For gene expression profiling, we used the Affymetrix GeneChip Human Genome

U133 Plus 2.0 Array (Affymetrix, USA) and the kit for One-Cycle Target Labeling and Control Reagents, according to Affymetrix protocols. In brief, double-stranded cDNA was generated from 5 μL of total RNA using a T7-oligo (dT) primer. The cDNA was purified and biotin-labeled cRNA was then synthesized by *in vitro* transcription. Purified biotin-labeled cRNA was added to the fragmentation buffer and heated for 35 min at 95°C. Fragmented cRNA (15 μg) was hybridized with the Human Genome U133 Plus 2.0 Array (62,976 genes) for 16 h at 45°C. Arrays were subjected to washing and staining with R-phycoerythrin streptavidin (SAPE; Molecular Probes, USA) using the GeneChip Fluidics Station 400 (Affymetrix). In order to amplify the signals, arrays were further stained with goat biotinylated anti-streptavidin antibodies (Vector Laboratories, USA), followed by SAPE. After scanning (with an Affymetrix scanner), the oligonucleotide hybridization data were exported for gene expression value analysis using the Affymetrix Microarray Suite (version 5.0). The hybridization intensity for each gene was calculated with a probe set containing 24 specific probes for perfect matches and mismatches. The expression of each gene was also categorized as "present," "absent," or "marginal". For quality control, cRNA samples were hybridized to the test microarray chip (Test 3; Affymetrix) in order to ensure equal hybridization to 5'- and 3'-oligonucleotides of the genes chosen for standardization [D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin] before hybridization to the Human Genome U133 Plus 2.0 Array.

Data analysis and pathway analysis. Analysis of oligonucleotide hybridization data was performed using GeneSpring 7.3 (Agilent Technologies, USA). Gene expression intensities were normalized by data transformation (set measurements less than 0.01 to 0.01) and per chip (normalized to the 50th percentile), according to the GeneSpring's protocol for standard normalization of a one-color microarray.

Networks of upregulated and downregulated genes in cells subjected to mechanical stretch were generated through KEGG pathway analysis (KEGG; Kyoto Encyclopedia of Genes and Genomes, Japan). A data set of the regulated genes containing gene identifiers and their corresponding expression values was uploaded into the application. Each identified gene was mapped to its corresponding gene object in the KEGG Pathway Knowledge Base. The uploaded genes, called focus genes, were overlaid onto a global molecular network developed from information contained in the KEGG Pathway Knowledge Base. Networks of these genes were then algorithmically generated based on their connectivity.

RNA extraction and reverse transcription-quantitative polymerase chain reaction. Because of small absolute differences in gene expression of periosteal cells induced by mechanical stretch, reverse real-time PCR was used to confirm a selection of differentially expressed genes identified by the microarray analysis. We chose genes involved in osteogenesis, Wnt signaling pathway, BMP signaling pathway, and inflammatory cytokines. In total, the differential expression levels of 20 genes were examined using real-time PCR. For comparison, HGFs subjected to the same mechanical stretch were examined using real-time PCR.

Single-stranded cDNA for a PCR template was synthesized from 1 μg of total RNA using a primer oligo (dT) $_{12–18}$ and the Superscript II reverse transcriptase under the conditions indicated by the manufacturer. A CFX96 real-time PCR detection system (Bio-Rad, USA) was used for quantitative analyses of the genes. GeneBank accession numbers for gene sequences and primer sequences are provided in Table 1.

The amplification reactions were performed with SsoFast EvaGreen Supermix (BioRad) according to the manufacturer's specifications. The amplification conditions were as follows: heat for 30 s at 98°C, followed by heating consecutively at 98°C and 58°C for 5 s each for 40 cycles. After amplification was completed, a melting curve was generated by slowly heating from 65°C to 95°C at 0.5°C increments with 5 s per step, with continuous monitoring of the fluorescence. The melting curves and quantitative analysis of the data were performed using a CFX manager (BioRad).

Data are expressed as means ± SD of three replicates. Statistical significance of differences between stretched cells and non-stretched cells was assessed on each HJBPCs and HGFs using Student's *t*-test, and a P value of < 0.05 was considered to be statistically significant.

RESULTS

Characterization of human periosteal cells

Cultured HJBPCs stained positive for ALP (Fig. 1), suggesting the presence of osteoblast-like cells. The proportion of ALP-positive cells to total cells in 5 random fields was approximately 55.5%. We suggest that fibroblasts and osteoblast-like cells exist in

Table 1 *Primers of RT-qPCR target genes and internal control 18S rRNA (18S ribosomal RNA)*

Gene/primer	(GeneBank ID)	Forward	Reverse
18S rRNA	(NM_014473)	ACTCAACACGGGAAACCTCA	AACCAGACAAATCGCTCCAC
SHH	(NM_000193)	AGGGCACCATTCTCATCAAC	GGAGCGGTTAGGGCTACTCT
Wnt-5a	(NM_003392)	TGGCTTTGGCCATATTTTTC	CTCCGATGTACTGCATGTGG
DKK-1	(NM_012242)	CCTTGGATGGGTATTCCAGA	GGCAAGACAGACCTTCTCCA
SFRP-1	(NM_003012)	ATCTCTGTGCCAGCGAGTTT	AAGTGGTGGCTGAGGTTGTC
SFRP-2	(NM_003013)	AGGACAACGACCTTTGCATC	TTGCTCTTGGTCTCCAGGAT
LRP-5	(NM_002335)	TGCTGGGGGACTTCATCTAC	GGGTGTGAAGAAGCACAGGT
ROR-2	(NM_004560)	CCGGTTTGGGAAAGTCTACA	CGTGCGAACAGTAGCTGAAG
BMP-2	(NM_01200)	GGCATCCTCTCCACAAAAGA	ACGTCTGAACAATGGCATGA
BMP-4	(NM_001202)	AAGCGTAGCCCTAAGCATCA	GGCTTTGGGGATACTGGAAT
BMP-6	(NM_001204)	GCGACACCACAAAGAGTTCA	CCCATACTACACGGGTGTCC
DLX-5	(NM_005221)	CTCTCCTACCTCGGCTTCCT	TTTGCCATTCACCATTCTCA
CHRDL-1	(NM_001143981)	CTCCTTACCCCCAGTGAACA	TCCATCTCCTCTGCATACCC
IGF-1	(NM_000618)	TCAACAAGCCCACAGGGTAT	CCTGCACTCCCTCTACTTGC
IGF-2	(NM_000612)	ACACCCTCCAGTTCGTCTGT	GGGGTATCTGGGGAAGTTGT
LIF	(NM_002309)	GTCTTGGCGGCAGTACACAG	CTGGGGTTGAGGATCTTCTG
IL-1β	(NM_000576)	GGGCCTCAAGGAAAAGAATC	TTCTGCTTGAGAGGTGCTGA
IL-6	(NM_000600)	CCTTCCAAAGATGGCTGAAA	CAGGGGTGGTTATTGCATCT
IL-11	(NM_000641)	CTGAGCCTGTGGCCAGATA	AGCTGTAGAGCTCCCAGTGC
IL-16	(NM_001172128)	CCGATAAAACACCCACTGCT	AAACTTTGGGAGTGCCATTG
IL-34	(NM_152456)	TTGACGCAGAATGAGGAGTG	GCTCACCAAGACCCACAGAT

HJBPCs at the approximately same rate.

Microarray analysis of mechanical stretch-induced changes in gene expression in HJBPCs

Gene expression changes in HJBPCs subjected to uniaxial stretching were characterized by microarray analysis. Of 62,976 genes detected, 550 genes related to bone metabolism were extracted and 76 of these genes with log2 fold changes of ≥1.00 were short-listed. Functions and pathways associated with these genes were retrieved from various databases provided by the National Center for Biotechnology Information, Gene Ontology, and Kyoto Encyclopedia of Genes and Genomes. Table 2 shows the short-listed genes divided into two groups: increased and decreased gene expression.

Microarray data indicated that cell stretch-induced changes resulted in expression levels of bone metabolism-related genes. Genes upregulated by the stretch-stimulation of HJBPCs included Wnt-5a, LRP-5, ROR-2, Axin-2 (AXIN-2), and lymphoid enhancer-binding factor-1 (LEF-1). These genes are all involved in the Wnt signaling pathway (15, 16) and in the regulation of osteoblast differentiation. Downregulated genes found were DKK-1, SFRP-1, SFRP-2, SFRP-4, and transcription factor 7-like 2 (TCF7L2). These genes are also involved in the Wnt signaling pathway (Fig. 3).

The expression of certain genes in the BMP signaling pathway (9, 30), involved in osteoblast differentiation, was also changed in stretch-treated HJBPCs. Stretch-stimulation of HJBPCs increased the expression of BMP-2, BMP-4, and distal-less homeobox-5 (DLX-5) and decreased the expression of BMP-6, chordin-like 1 (CHRDL-1), and SMAD family-3 and -6 (SMAD-3 and SMAD-6, respectively). Among the inflammatory cytokine genes, expression levels of IL-18, IL-34 and LIF increased and those of IL-1β, IL-6, IL-11, and IL-16 decreased in stretch-treated HJBPCs. Furthermore, changes in the expression of genes involved in osteoblast and osteoclast differentiation were also found. These included expression of increased insulin-like growth factor (IGF)-1 and IGF-2 and decreased expression of IGF-binding protein (IGFBP)-3, IGFBP-5, and IGFBP-6. Among the genes induced by HJBPCs stretching, sonic hedgehog (SHH) showed the highest degree of change.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was conducted for 20 molecules, found by microarray analysis including those constituting Wnt and BMP signaling pathways, inflammatory cytokines, their family molecules, and those closely associated with bone metabolism. Human gingival fibroblasts (HGFs) subjected to stretch

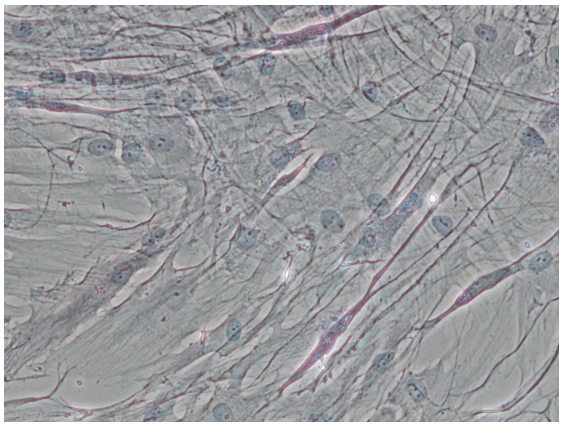

Fig. 1 After isolation and culturing, HJBPCs were stained with ALP. HJBPCs were ALP-positive, suggesting elevated ALP activity and presence of osteoblast-like cells.

in the same manner as HJBPCs were used as control cells (Fig. 2).

Genes of the Wnt signaling pathway were analyzed by RT-qPCR. Wnt-5a expression was significantly increased in stretch-stimulated HJBPCs. Expression levels of SFRP-1, SFRP-2, and DKK-1, extracellular negative regulators of Wnt signaling pathway, were significantly reduced in stretch-stimulated HJBPCs, while no significant changes were noted in identically treated HGFs. In HJBPCs, expression levels of LRP-5 and ROR-2, co-receptors of Wnt, increased significantly upon cell stretching. In HGFs, the stretch treatment induced no significant change in LRP-5 expression, but it induced a significant increase in ROR-2 expression.

Next, we analyzed genes related to the BMP signaling pathway by RT-qPCR. In HJBPCs, the stretch treatment increased the expression levels of BMP-2 and BMP-4, and reduced the expression levels of BMP-6. No significant changes in BMP-2, BMP-4 or BMP-6 occurred in identically treated HGFs. In HJBPCs, the stretch treatment induced a significant increase in the expression of DLX-5, a gene upregulated by BMP-2 and BMP-4. In addition, stretch-stimulation of HJBPCs induced a significant increase in the expression of SHH present in upstream of Wnt and BMP genes.

We also conducted RT-qPCR analysis targeting inflammatory cytokine genes and those in related families. In stretch-stimulated HJBPCs, expression levels of IL-1β and IL-6 were significantly reduced, and expression levels of IL-34 and LIF were significantly increased. No significant change was found in the expression of IL-11 or IL-16.

IGF-1 and IGF-2, genes involved in osteoblast differentiation, were expressed at elevated levels in stretch-stimulated HJBPCs as well as in identically treated HGFs.

DISCUSSION

This study revealed that stretch-stimulation of HJBPCs induces changes in the expression levels of genes encoding bone metabolism-related factors. Wnt signaling pathway is regulated by the secretory glycoprotein SHH that modulates various vital functions (6, 31). Nineteen Wnt members have been identified in humans, and the 7-transmembrane protein Frizzled is known as a receptor for Wnt proteins. Two Wnt signaling pathways are known: the Wnt/β-catenin pathway (canonical pathway) (Fig. 3), which regulates gene expression through β-catenin, and the β-catenin-independent pathway (non-canonical pathway). These pathways are involved in the regulation of various biological functions, including bone metabolism. In the Wnt/β-catenin pathway, ternary binding of the Wnt molecule, Frizzled receptor and co-receptor LRP-5 induces suppression of β-catenin phosphorylation catalyzed by glycogen synthase kinase-3 (GSK-3) and intracytoplasmic accumulation of β-catenin escaped from proteasome-catalyzed degradation. Following translocation to the nucleus, β-catenin activates the transcription factor TCF/LEF, thereby inducing expression of various genes. Subsequently, osteogenesis is induced through differentiation of mesenchymal cells into osteoblasts and suppression of osteoblasts apoptosis, and concomitantly, bone resorption is suppressed through inhibition of osteoclast differentiation (15, 16). Recently, mutated LRP-5 has been identified as the causative gene for osteoporosis-pseudoglioma syndrome (OPPG), and has been found to be involved in bone metabolism-related disease (3, 36). Moreover, inhibition of DKK-1, an LRP-5 inhibitory factor, or SFRP-1 and SFRP-2, the Wnt inhibitory factors, results in enhanced osteogenesis (26, 34). Therefore, Wnt/β-catenin pathway is important to facilitate osteogenesis. In the β-catenin-independent pathway (non-canonical pathway), the binding of the Wnt molecule to Frizzled receptor and co-receptor ROR-2 induces osteoclast differentiation through c-jun N-terminal kinase (Jnk) and mediates the regulation of cell skeleton, movement and planar polarity through Ras homolog family member A (RhoA) and Filamin-α (12, 15, 22). Therefore, the Wnt signaling pathway appears to mediate regulatory signals in bone metabolism as a network formed by multiple pathways.

Table 2-A, B *Genes differently expressed in stretch-stimulated HJBPCs*

Table 2-A: Up regulated genes

Symbol	GeneBank ID	Gene title	Fold change
SHH	NM_000193	Sonic hedgehog	8.63
AMH	NM_000479	Anti-Mullerian hormone	4.21
CYTL-1	NM_018659	Cytokine-like 1	3.88
MMP-1	NM_002421	Matrix metallopeptidase 1	3.44
HAS-2	NM_005328	Hyaluronan synthase 2	2.91
IGF-1	NM_000618	Insulin-like growth factor 1 (somatomedin C)	2.81
TNFAIP-6	NM_007115	Tumor necrosis factor, alpha-induced protein 6	2.60
LIF	NM_002309	Leukemia inhibitory factor	2.49
PDPN	NM_198389	Podoplanin	2.49
HAS-1	NM_001523	Hyaluronan synthase 1	2.29
LRP-5	NM_002335	Low density lipoprotein receptor-related protein 5	2.10
LEF-1	NM_016269	Lymphoid enhancer-binding factor 1	2.06
ALPL	NM_000478	Alkaline phosphatase, liver/bone/kidney	2.05
HMOX-1	NM_002133	Heme oxygenase	2.03
ROR-2	NM_004560	Receptor tyrosine kinase-like orphan receptor 2	1.96
COL11A2	NM_080680	Collagen, type XI, alpha 2	1.93
KIT	NM_000222	V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	1.88
FASN	NM_004104	Fatty acid synthase	1.83
TGFB-3	NM_003239	Transforming growth factor, beta 3	1.83
INHBA	NM_002192	Inhibin, beta A	1.77
PGF	NM_002632	Placental growth factor	1.75
CCL-2	NM_002982	Chemokine (C-C motif) ligand 2	1.74
ICAM1	NM_000201	Intercellular adhesion molecule 1	1.73
RHOA	NM_001664	Ras homolog family member A	1.73
LAMA-4	NM_001105209	Laminin, alpha 4	1.67
Wnt-5a	NM_003392	Wingless-type MMTV integration site family, member 5A	1.65
FLNA	NM_001457	Filamin alpha	1.56
DLX-5	NM_005221	Distal-less homeobox 5	1.51
IL-18	NM_001243211	Interleukin 18	1.46
IGF-2	NM_000612	Insulin-like growth factor 2 (somatomedin A)	1.43
HIF-1A	NM_181054	Hypoxia inducible factor 1, alpha subunit	1.39
BIRC-5	NM_001012271	Baculoviral IAP repeat containing 5	1.30
CDH-2	NM_001792	Cadherin 2, type 1, N-cadherin (neuronal)	1.25
ACTA-2	NM_001613	Actin, alpha 2, smooth muscle, aorta	1.15
BMP-2	NM_01200	Bone morphogenetic protein 2	1.14
COL7A1	NM_000094	Collagen, type VII, alpha 1	1.13
ITGB-3	NM_000212	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	1.13
HGF	NM_001010931	Hepatocyte growth factor (hepapoietin A; scatter factor)	1.12
BMP-4	NM_001202	Bone morphogenetic protein 4	1.11
MEF2C	NM_002397	Myocyte enhancer factor 2C	1.02

In our experiments, stretch-stimulated HJBPCs enhanced the expression of Wnt-5a and the co-receptors, LRP-5 and ROR-2, and suppressed the expression of the inhibitory factors, DKK-1 and SFRP-1 and SFRP-2, suggesting that both the Wnt/β-catenin (canonical pathway) and the β-catenin-independent pathways (non-canonical pathway) were activated by stretch treatment. In contrast, identically treated HGFs showed no significant changes in the expression of the same genes. These results suggest that the periosteum senses the mechanical stress and plays a regulatory role via Wnt signaling pathway in mechanical stress-induced bone remodeling.

HJBPCs stretch-stimulation induced changes in the expression of another group of genes, the genes related to the BMP signaling pathway. BMP-2 and BMP-4 promote osteoblast differentiation and osteogenesis through upregulation of DLX-5 and expres-

Table 2-B: Down regulated genes

Symbol	GeneBank ID	Gene title affy	Fold change
SFRP-2	NM_003013	Secreted frizzled-related protein 2	−4.72
SFRP-4	NM_003014	Secreted frizzled-related protein 4	−3.47
ERBB-3	NM_001982	V-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	−3.46
ASPN	NM_017680	Asporin	−3.11
IL-1β	NM_000576	Interleukin 1, beta	−2.92
IGFBP-3	NM_001013398	Insulin-like growth factor binding protein 3	−2.82
IL-11	NM_000641	Interleukin 11	−2.71
TIMP-3	NM_000362	TIMP metallopeptidase inhibitor 3	−2.62
SFRP-1	NM_003012	Secreted frizzled-related protein 1	−2.47
IL-6	NM_000600	Interleukin 6 (interferon, beta 2)	−2.45
IL-34	NM_152456	Interleukin 34	−2.26
IGFBP-5	NM_000599	Insulin-like growth factor binding protein 5	−2.19
MAP-2	NM_002374	Microtubule-associated protein 2	−2.12
CHRDL-1	NM_001143981	Chordin-like 1	−2.12
MGP	NM_001190839	Matrix Gla protein	−2.1
TCF7L2	NM_001198531	Transcription factor 7-like 2	−2.01
OGN	NM_033014	Osteoglycin	−1.96
IGFBP-6	NM_002178	Insulin-like growth factor binding protein 6	−1.96
ITGA-6	NM_000210	Integrin, alpha 6	−1.9
FOS	NM_005252	FBJ murine osteosarcoma viral oncogene homolog	−1.86
A2M	NM_000014	Alpha-2-macroglobulin	−1.85
PPARG	NM_138711	Peroxisome proliferator-activated receptor gamma	−1.79
DKK-1	NM_012242	Dickkopf homolog 1 (Xenopus laevis)	−1.79
INHBA	NM_002192	Inhibin, beta A	−1.68
BMP-6	NM_001204	Bone morphogenetic protein 6	−1.65
MYC	NM_002467	V-myc myelocytomatosis viral oncogene homolog	−1.61
FHL-2	NM_001039492	Four and a half LIM domains 2	−1.52
FST	NM_013409	Follistatin	−1.49
BCL-2	NM_000633	B-cell CLL/lymphoma 2	−1.44
IL-16	NM_001172128	Interleukin 16	−1.31
MMP-3	NM_002422	Matrix metallopeptidase 3	−1.26
ID-1	NM_002165	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	−1.09
AXIN-2	NM_004655	Axin 2	−1.08
FAS	NM_000043	Fas (TNF receptor superfamily, member 6)	−1.07
S100P	NM_005980	S100 calcium binding protein P	−1.04
APLN	NM_017413	Apelin	−1.03

HJBPCs with and without stretch stimulation were subjected to microarray analysis, and genes with changes in expression were extracted. We selected the genes related to bone metabolism, and further short-listed them based on their fold change (log2 fold change of ≥1.00). These genes were divided into two groups: the increased and decreased expression groups.

sion of Runt-related transcription factor-2 (RUNX-2) and Osterix (OSX), controlled by DLX-5. Expression levels of BMP-2 and BMP-4 and DLX-5 were significantly increased upon stretch-stimulation of HJBPCs. This result suggests that HJBPCs sense the mechanical stress and promotes osteogenesis through the expression of BMP-2 and BMP-4. On the other hand, the stretch-stimulation of HJBPCs suppressed BMP-6 expression. BMP-6, similar to BMP-2 and BMP-4, promotes osteogenesis, but may also play a role in bone resorption, as suggested by studies showing that BMP-6 induces IL-1β and IL-6 expression and osteoclast differentiation (4, 19, 28). The observed increase in BMP-2 and BMP-4 expression and reduction in BMP-6 expression may represent a unique profile associated with the mechanical stress-driven bone remodeling.

SHH expression level was enhanced in stretch-stimulated HJBPCs. Given that SHH lies upstream of Wnt and BMP and induces their expression (6, 31), the increase of Wnt-5a and BMP-2 and BMP-4 expression found in stretch-stimulated HJBPCs may be mediated by SHH.

Stretch-stimulated HJBPCs showed changes in the

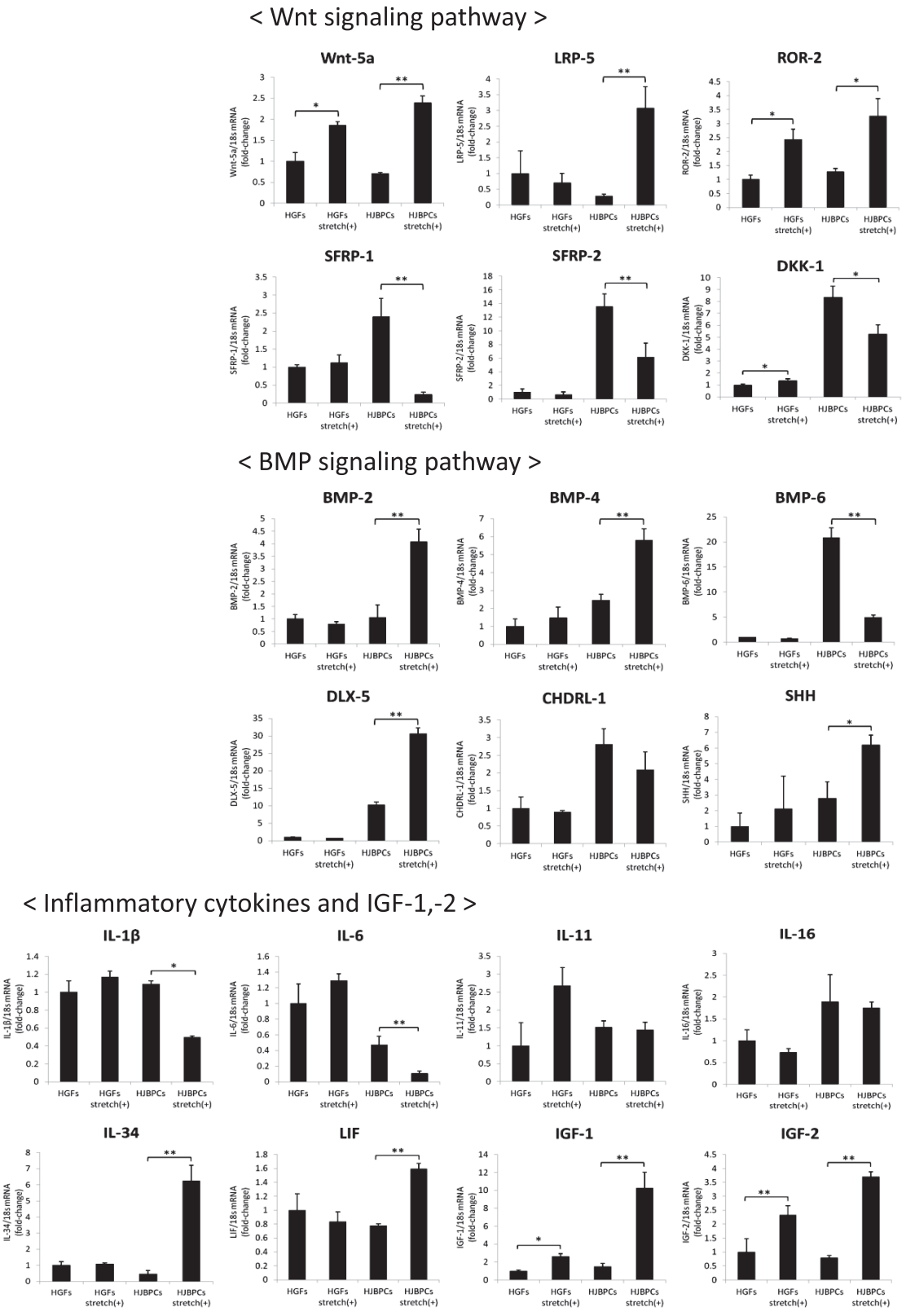

Fig. 2 Expression of genes encoding molecules in the Wnt, BMP, and inflammatory cytokines pathways and other cytokines using real-time PCR. Changes in mRNA levels in stretch-treated HGFs and HJBPCs were measured by real-time PCR. $^{*}P < 0.05$, $^{**}P < 0.005$

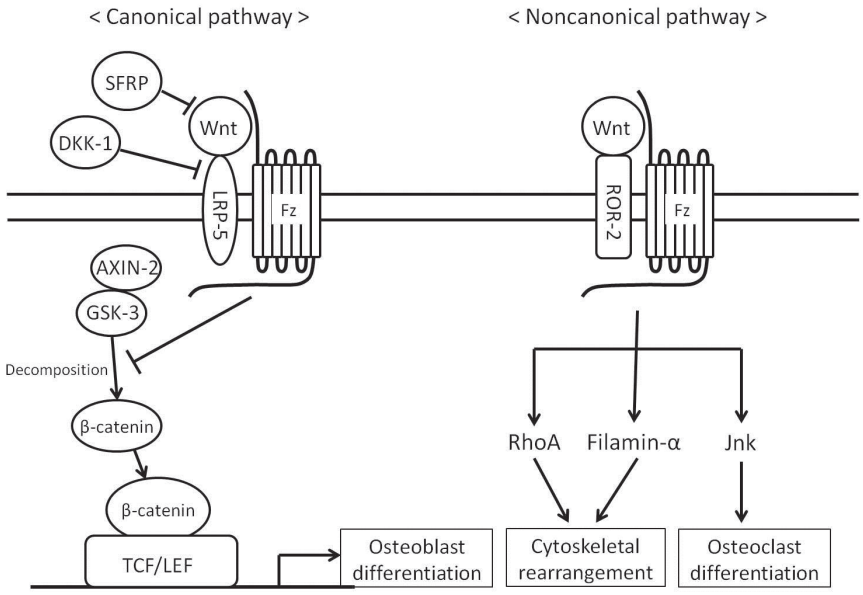

Fig. 3 Wnt signaling pathway. Wnt binds to Frizzled receptor (Fz) and to one of the co-receptors, LRP-5 and ROR-2, to form the ternary complexes, Wnt/Frizzled/LRP-5 and Wnt/Frizzled/ROR-2, which activate the Wnt/β-catenin pathway (canonical pathway) and the β-catenin-independent pathway (non-canonical pathway), respectively. These pathways are mainly involved in the regulation of osteoblast and osteoclast differentiation and cytoskeletal rearrangement. SFRP is an extracellular inhibitory factor of Wnt, and DKK-1 is an inhibitory factor of LRP-5.

expression of certain inflammatory cytokines and their family genes. Expression levels of IL-1β and IL-6 were significantly reduced, whereas expression levels of LIF and IL-34 were significantly increased. Cytokines of the IL-family are known to work in coordination with the BMP signaling pathway in each tissue. IL-1β and IL-6 cause inflammatory responses and promote osteoclast differentiation and bone resorption (23, 25). BMP-6, which promotes osteogenesis mentioned above, induces IL-1β and IL-6 expression, and osteoclast differentiation (4, 19, 28). In this study, we found that expression levels of BMP-2 and BMP-4 were increased, and that of BMP-6 was decreased as well as IL-1β and IL-6 in stretch-stimulated HJBPCs. This suggests that stretch treatment of HJBPCs promotes osteoblast differentiation through induction of BMP-2 and BMP-4, whereas it suppresses osteoclast differentiation through downregulation of BMP-6 expression as well as IL-1β and IL-6. These results suggest that HJBPCs sense the mechanical stretch and transmit signals for inducing osteogenesis.

LIF, a member of the IL-6 family, is known to be involved in bone resorption during inflammation, and it is involved in osteoblast differentiation and osteogenesis through signal transducer and activator of transcription (STAT) in coordination with BMP-2 and BMP-4, during normal bone metabolism (11, 29). Expression levels of LIF, BMP-2 and BMP-4 were all significantly increased in stretch-stimulated HJBPCs. These results suggest that LIF, BMP-2 and BMP-4 expressed upon stretch-stimulation of HJBPCs promote osteogenesis in a coordinated manner.

Expression of IL-34 was induced upon stretch-stimulation of HJBPCs. IL-34 is a cytokine involved in osteoclast differentiation and promotes it independently from macrophage colony-stimulating factor (M-CSF) (8, 32). IL-34 shares a receptor with M-CSF. These findings suggest that a regulatory mechanism mediated by IL-34 is required for osteoclast differentiation as a result of mechanical stress-induced bone remodeling.

Expression levels of IGF-1 and IGF-2 were also increased upon stretch-stimulation of HJBPCs. IGF-1 and IGF-2, which are secreted mainly by osteoblasts, induce osteoblast differentiation and potentiate their functions. At the same time, expression of receptor activator of nuclear factor-κB ligand (RANKL) is also induced by IGF-1 and IGF-2 in osteoblasts, resulting in osteoclast differentiation in the reversal period in bone remodeling (5, 24). The observed increase in IGF-1 and IGF-2 expression levels in stretch-stimulated HJBPCs suggests that mechanically stressed periosteal tissues control bone remodeling through IGF-1 and IGF-2.

In this study, we characterized gene expression

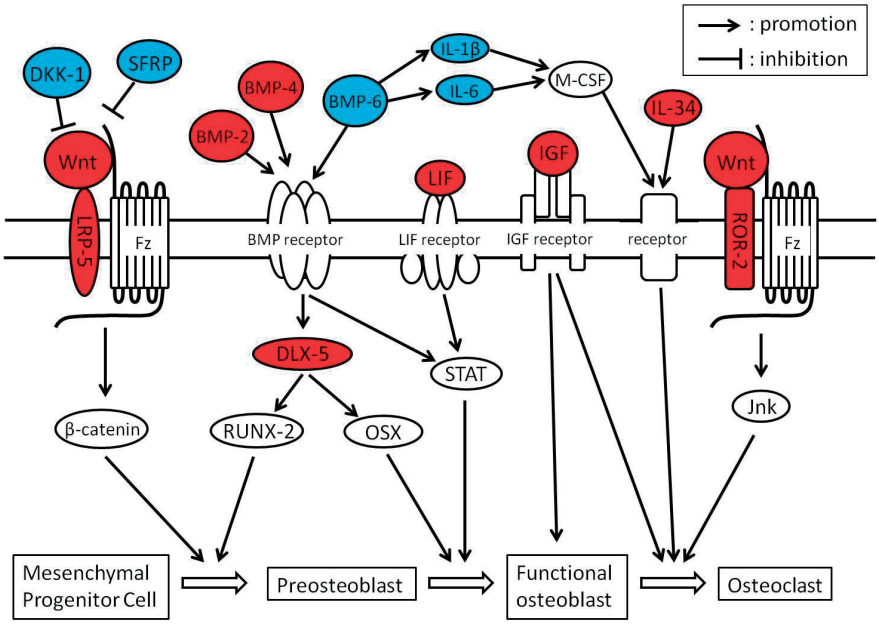

Fig. 4 Wnt signaling pathway, BMP signaling pathway, and bone metabolism-related genes expressed at increased or decreased levels in stretch-stimulated periosteum-derived cells. Genes increased in stretch-induced cells are indicated in red, and those decreased are indicated in blue. Signals from the Wnt signaling pathway, BMP signaling pathway and other bone metabolism-related molecules cooperate. These genes are considered to regulate differentiation of mesenchymal progenitor cells into osteoblasts and their eventual differentiation to osteoclasts that occurs during bone remodeling under mechanical stress.

changes in HJBPCs under stretch stimulus and found significant changes in the expression of genes involved in Wnt and BMP signaling pathways as illustrated in Fig. 4. Stretch-induced changes were also associated with certain genes of the IL family, working in coordination with BMP and IGF, known to be closely related to bone metabolism. In contrast, these changes were not found in HGFs subjected to the identical mechanical stretch. There are numerous reports describing an important signaling cross-talk between Wnt and BMP signaling pathways and its fundamental role in osteoblast differentiation and osteogenesis (10, 37). Therefore, HJBPCs appear to induce, in response to the stretch stimulus, gene expression changes in Wnt signaling pathway, BMP signaling pathway, and various cytokines, which cooperate to promote osteoblast differentiation and osteogenesis. Our results also suggest that during mechanical stress-induced bone remodeling in the body, the periosterum senses the mechanical stress to control signals for promoting osteoblast differentiation and osteogenesis.

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