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Bioinformatic analysis of responsive genes in two-dimension and three-dimension cultured human periodontal ligament cells subjected to compressive stress

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Background and Objective: Analyzing responses of human periodontal ligament cells to mechanical stress and mechanotransduction is important for understanding periodontal tissue physiology and remodeling. It has been shown that the cellular response to mechanical stress can vary according to the type and duration of force and to extracellular attachment conditions. This study investigated the gene-expression profile of human periodontal ligament cells cultured in two-dimension (2D) and three-dimension (3D) conditions after application of compressive stress for 2 and 48 h.

Material and Methods: Human primary periodontal ligament cells were obtained from premolars extracted for orthodontic purposes. Cells were cultured in a conventional 2D culture dish or in 3D collagen gel and compressive stress was applied for 2 and 48 h. Control cells were cultured under identical conditions but without the application of compressive stress. After the application of compressive stress, total RNA was extracted and a cDNA microarray was performed. Microarray data were analyzed using statistical methods, including DAVID and gene set enrichment analysis to identify significant signaling pathways. Real-time PCR was performed for five mRNAs in order to confirm the cDNA microarray results.

Results: The cDNA microarray analysis revealed that after application of compressive stress for 2 h, 191 and 553 genes showed changes in their expression levels in 2D and 3D cultured cells, respectively. After application of compressive stress for 48 h, 280 and 519 genes showed changes in their expression levels in 2D and 3D cultured cells, respectively. Euclidean clustering method was used to demonstrate the gene-expression kinetics.

Conclusion: Analysis of the results showed that several signaling pathways, including the MAPK pathway and the focal adhesion kinase pathway are relevant

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to the compressive force-induced cellular response. 2D and 3D cultured cells showed significantly different gene-expression profiles, suggesting that cellular attachment to extracellular matrix influences cellular responses to mechanical stresses.

Periodontal ligament (PDL) tissue is interposed between dental root cementum and alveolar bone. It is a unique functional tissue that supports the teeth and maintains the integrity of surrounding periodontal tissue. Also, PDL modulates the external forces, such as occlusal forces and orthodontic forces, applied to the teeth into biochemical signals. Owing to modulation of the forces, tissues surrounding the teeth do not undergo dystrophic changes upon application of extrinsic forces, but rather maintain their integrity and it also helps to strengthen the structure of tissues. Many studies have been performed to characterize the response of PDL cells to mechanical forces and it has been shown that PDL cells are capable of producing and secreting bone-forming factors and also bone-resorbing factors (1–7).

It has been shown that PDL cells respond differentially to various types of force, including tension, compression, fluid shear and hydrostatic force. Different responses to different types of forces indicate that the mechanosensing mechanism of PDL cells is complicated, rather than simple. Various molecules, including ion channels, caveolae, adhesion proteins, cytoskeletons and G protein-coupled receptors, have been proposed as the cellular mechanosensors (8–11). Among them, cellular adhesion to the extracellular matrix has attracted attention as an important mechanosensor. A recent study showed that focal adhesion kinase (FAK), a kinase related to the cell-adhesion structure of integrin, is responsible for prostaglandin E2 production in compression-stressed PDL cells (12). Also, studies have been performed on the signal-transduction phenomena in cells after application of mechanical stress. Various signal-transduction pathways, including MAPK, ERK, JNK, Activator Protein-1, nuclear factor-kappaB (NF-κB) and Rho GTPases (5,13–16), have been shown to be involved in cellular mechanotransduction. An investigation of the mechanosensing and mechanotransducing mechanisms of PDL cells is necessary to understand the physiology of PDL cells and the utilization of PDL cells in clinical treatment.

Genome-wide expression analysis using DNA microarrays is a powerful method for investigating cellular behavior. This method is useful, not only for obtaining gene-expression profiles, but also in interpreting the results to gain insights into biological mechanisms (17–20). Combining DNA microarray results and databases focused on signaling pathways by using computer programs provides a systematic overview of the cellular response.

Several studies have investigated gene-expression profile changes after mechanical stress application to PDL cells (21–23). Yamashiro *et al*. conducted a DNA microarray study of PDL cells after application of cyclic tension. Their study included bioinformatic analysis of gene-expression profiles, using databases, computer programs and statistical analysis. As mentioned above, cellular responses vary according to the type of mechanical stimulation applied. Not many DNA microarray studies have been performed on compressive stress, which is known to elicit the osteoclastogenic potential of PDL cells, and no studies conducted bioinformatic analysis that involves mining for biological signaling pathways. This study was performed to investigate the gene-expression profiles of PDL cells after compressive stimulation and also to investigate the relevant signaling pathways by analyzing the gene-expression profile. Because it has been shown that cellular attachment to the extracellular matrix acts as a mechanoreceptor, conventional two-dimension (2D) cultured and three-dimension (3D) cultured cells, which mimick the *in vivo* conditions and are also expected to increase in the number of ECM attachments, were compared.

Material and methods

Primary human PDL cell culture

Human PDL (hPDL) cells were acquired from freshly extracted first bicuspids obtained from patients (three women, 21, 24 and 27 years of age) undergoing bicuspid extraction for orthodontic purposes. Immediately following extraction, teeth were placed in α-minimum essential medium (α-MEM) containing 15% fetal bovine serum (FBS) and 1% threefold-reinforced antibiotic/antimycotic (penicillin G and streptomycin). All reagents used for cell culture, including α-MEM, FBS and antibiotic/antimycotic, were purchased from Invitrogen (Gibco, Carlsbad, CA, USA).

Pieces of PDL were obtained exclusively from the middle of the tooth roots using a #15 surgical blade in order to exclude the intermixture of the gingivae and dental pulp (3). The PDL tissue thus obtained was treated with 1.10 units/mL of dispase and with 264 units/mL of collagenase for 1 h at 37°C. After washing with α-MEM, PDL samples were cultured in α-MEM containing 10% FBS and 1% antibiotic/antimycotic on 100-mm primary culture dishes. Cells proliferating in the extracts were passaged. For all experiments, PDL cells between the fourth and eighth passages were employed. All procedures were conducted with appropriate informed consent. The Institutional Review Board of Kyung Hee University Hospital at Gangdong approved the protocol for this study (approval number: KHNMC GRRB 2011-006).

2D and 3D culture of PDL cells

To mimic the *in vivo* conditions and to evaluate cellular attachment to the extracellular matrix in response to mechanical stress, a 3D culture system as well as conventional 2D culture were included in the study. The 2D culture was conducted by transferring 4×10^5 PDL cells onto a 35-mm culture dish and the 3D culture was conducted by using 3D collagen gel. The 3D collagen gel was fabricated using a commercially available kit (3D collagen gel ECM675; Millipore, Billerica, MA, USA) specially designed for cell culture. Type I collagen was the main collagen in the collagen gel and is also the main collagen in the PDL tissue. In accordance with the manufacturer's instructions, collagen gel solution was prepared by mixing collagen solution, 5× α-MEM and neutralizing solution. Approximately 4×10^5 PDL cells were incorporated into the mixture and placed in a 35-mm-diameter culture dish (Fig. 1). After placing the PDL cells into the culture dish, the dish was transferred to a 36.5°C incubator for 60 min in order to polymerize the collagen gel. Nutrition media were added to the culture dish after polymerization.

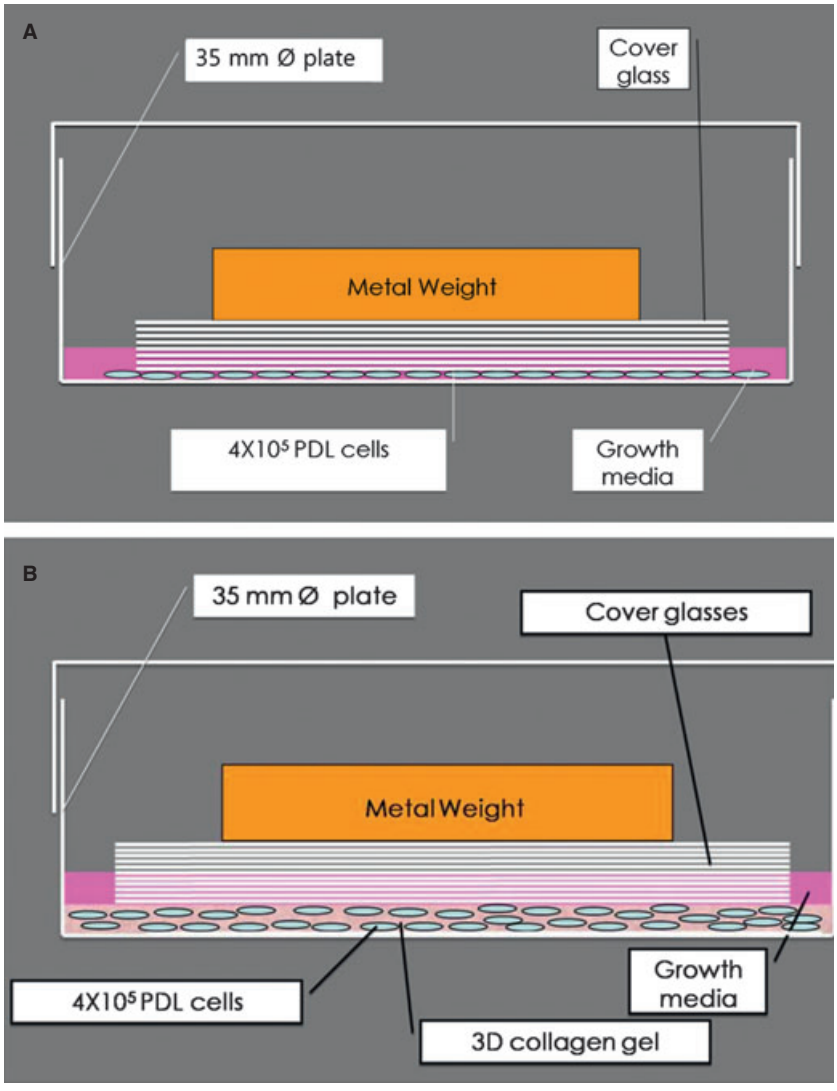

Fig. 1. Schematic drawing of compression stress application on cultured human periodontal ligament (PDL) cells. (A) Compressive stress on two-dimension (2D) cultured PDL cells; (B) Compressive stress on three-dimension (3D) collagen gel-cultured PDL cells.

Application of compressive stimulation

Before the application of mechanical stress, the PDL cells were allowed to stabilize completely for 48 h. The PDL cells were compressed continuously using the uniform compression method shown in Fig. 1. Briefly, 30 layers of 24 mm × 24 mm-sized cover glasses (Muto, Tokyo, Japan), and additive metal weights positioned on the top of the cover glasses, were placed over the cell layer of 2D cultured cells and over the collagen gel of 3D cultured cells. The PDL cells were subjected to a total compressive force of 2.0 g/cm^2 for 2 and 48 h. This compressive force (2.0 g/cm^2) was selected because it has been reported that the production of cytokines and the expression of mRNA coding for osteoclastogenic molecules reaches a peak at this level (2,3,24). Time periods of 2 and 48 h were used in order to identify the early- and late-response genes, respectively. Control cells (without application of compressive stimulation) were also cultured in the same 2D and 3D conditions.

cDNA microarray

After compressive stimulation, RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized from 1 μg of total RNA using T7oligo(dT) reverse transcriptase at 42°C for 2 h. Then, the second strand of cDNA was synthesized from the first strand of cDNA using DNA polymerase at 16°C for 2 h. The synthesized second strand of cDNA was purified using columns. Then, amplified RNA was synthesized from the purified cDNA by *in vitro* transcription using T7 enzyme at 37°C overnight. Amplified RNA was purified using columns. Purified amplified RNA was labeled with Cy3-ULS dye. Labeled amplified RNA was mixed with blocking solution and 2× hybridization buffer, placed on a Roche NimbleGen Human whole-genome 12-plex array (Roche NimbleGen, Madison, WI, USA) and covered with a NimbleGen H12 mixer (Roche NimbleGen). The microarray chip used in this study was the NimbleGen

Human Whole Oligo 12-plex chip, which was designed based upon the Human Genome Build 36 database and covers 44,049 human genes (35,778 known-function genes and 8271 unknown-function genes, including the expressed sequence tag sequence). The slides were hybridized for 16 h at 42°C using the MAUI system (Biomicro Systems, Salt Lake City, UT, USA) and then were washed in 2 × saline sodium citrate (SSC), 0.1% sodium dodecyl sulfate for 2 min, in 1 × SSC for 3 min and then in 0.2 × SSC for 2 min at room temperature. The slides were centrifuged at 1977 ***g*** for 20 s to dry. The arrays were scanned using an Axon GenePix 4000B scanner with the associated software (Molecular Devices Corp., Sunnyvale, CA, USA). The whole procedure was repeated in triplicate.

Analysis of differentially expressed genes

The 2D and 3D compressed cells were analyzed using corresponding control cells: 2D compressed cells were compared with 2D control cells without compressive stress; and 3D compressed cells were compared with 3D control cells without compressive stress. The gene expression levels were calculated using NimbleScan Version 2.4 (Roche NimbleGen). The relative signal intensities for each gene were generated using the Robust Multi-Array Average algorithm. The data were processed based on median polish normalization method using the NimbleScan Version 2.4 (Roche NimbleGen). This normalization method aims to make the distribution of intensities for each array in a set of arrays the same. The normalized, and log-transformed intensity values were then analyzed using GeneSpring GX 11 (Agilent Technologies, Santa Clara, CA, USA). Fold-change filters included the requirement that the genes be present in at least 200% of controls for up-regulated genes and in less than 50% of controls for down-regulated genes. Signal intensities were transformed to log2 intensity, and −log10 of the *t*-test *p*-value was used to select statistically significant genes between replicate samples. Hierarchical clustering data were clustered groups that behaved similarly across experiments using GeneSpring GX 11 (Agilent Technologies). The functional annotations of the differentially expressed genes were examined using the Gene Ontology database. Clustering analysis was performed to visualize kinetic patterns of changes in gene expression. The clustering algorithm included Euclidean distance, average linkage. Gene pathway and network analysis was carried out using GeneSpring GX 11 (Agilent Technologies), which has over 9500 references in Google Scholar, including over 1600 in peer-reviewed publications. Gene set enrichment analysis (GSEA) was also performed (17). GSEA evaluates microarray data at the level of gene sets defined according to the relevant functions. Additionally, Gene Ontology analysis was performed using DAVID (Database for Annotation, Visualization, and Integrated Discovery version 6.7, http://david.abcc.ncifcrf.gov/home.jsp) which was used to cluster the genes based on gene-ontology term enrichment and to identify the relevant pathways in KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway maps.

Real-time PCR

To confirm the microarray results, five genes were selected for quantitative real-time PCR analysis. These five genes were interleukin-1β (*IL1*β), tumor necrosis factor-α (*TNF*α), *RANKL*, *MMP3* and *MMP13*. A total amount of 1 μg of RNA was reverse transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's recommendations. Real-time PCR analysis was conducted using the TaqMan Gene Expression Assay kit (Applied Biosystems Inc., Carlsbad, CA, USA) with a predesigned probe and primer set (gene ID: *IL1*β, HS99999029_M1; *TNF*α, Hs99999043_m1; *RANKL*, HS01092186_M1; *MMP3*, HS00968305_M1; *MMP13*, Hs00233992_m1; and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), SH99999905_M1). Expression was quantified using Chromo4 Reverse Transcription-Polymerase Chain Reaction (real time RT-PCR) analysis (Bio-Rad Laboratories, Hemel Hempstead, UK) with IQ Supermix (Bio-Rad Laboratories, Hercules, CA, USA). To obtain the relative quantification of gene expression, the MJ Opticon Monitor Analysis Software (Bio-Rad Laboratories, Hemel Hempstead, UK) was used. Samples were assayed in triplicate, and the values were normalized to the relative quantities of *GAPDH*.

Results

Changes in gene expression after application of compressive stress

The cDNA microarray detected different numbers of over-expressed genes (> 2-fold) and under-expressed genes (< 0.5-fold) according to the culture conditions (2D or 3D) and the duration of application of compressive stress (2 h or 48 h). The genes showing changes in expression levels are listed in Table 1. The 3D cultured PDL cells showed changes in the expression levels of more genes after application of compressive stress compared with 2D cultured PDL cells. The detailed number of genes showing changes in expression levels according to their gene ontology functions is presented in Table 2. The five genes that showed the greatest increase and decrease in

Table 1. Summary of number of differentially expressed genes ($p < 0.05$)

	2-fold up	0.5-fold down	Total 2-fold
2D 2H	127	64	191
2D 48H	189	91	280
3D 2H	379	154	553
3D 48H	368	151	519

2D, two dimension cultured; 3D, three dimension cultured; 2H, compression stress for 2 h; 48H, compression stress for 48 h.

Table 2. Number of genes with expression changes of over 1.5-fold after compression stress, classified according to gene-ontology terms functional classification

	2D 2H	2D 48H	3D 2H	3D 48H
Cell proliferation	6	4	13	16
Protein biosynthesis	4	6	10	10
Inflammatory response	4	6	7	8
Immune response	10	12	24	25
Cell adhesion	10	13	13	14
Cell migration	2	3	3	3
Cell growth	4	8	1	3
Cell differentiation	9	9	16	14
Apoptosis	8	7	14	20
Transport	15	34	53	68
Cell cycle	5	10	14	19
Transcription	22	38	41	49
Signal transduction	29	28	56	67
Metastasis	1	1	2	1
Total	191	280	533	519

2D, two dimension cultured; 3D, three dimension cultured; 2H, compression stress for 2 h; 48H, compression stress for 48 h.

expression levels are listed in Tables 3 and 4. Clustering analysis classified gene-expression changes into a similar pattern of time-course changes. Six patterns were identified in both the 2D and 3D cultured PDL cells, which are depicted in Fig. 2. To validate the microarray results, real-time PCR was performed for five mRNAs. Comparison of the fold changes measured by microarray and real-time PCR is provided in Table 5.

Gene-expression difference between 2D cultured cells and 3D cultured cells before compressive stress

Because the 2D cultured cells and the 3D cultured cells were compared with each corresponding control cell, the gene-expression change shown after compressive stress might be a mixture of the influence of culture condition and compressive stress. The results from these two variables cannot be distinguished and therefore direct comparison of 2D cultured compressed cells and 3D cultured compressed cells was meaningless. Instead, to give an idea of culture condition-induced changes, 2D cultured cells without compressive stress and 3D cultured cells without compressive stress were compared. The results showed a two-fold up-regulation of two genes and a 0.5-fold down-regulation of six genes in 3D cells compared with 2D cells. The gene lists are given in Table 6. Among them, only one gene [NHL repeat containing 2 (GenBank ID: BX647641)] showed a significantly decreased expression in 2D cultured for 2 h and 2D cultured for 48 h compressed PDL cells. The genes listed in Tables 3, 4 and 5 were checked among 2D and 3D control cells and showed nonsignificant differences within 0.6- to 1.4-fold changes, except for the NHL repeat containing two genes (GenBank ID: BX647641).

Gene set enrichment analysis (GSEA)

GSEA revealed one up-regulated pathway in 2D cultured compressed PDL cells after 2 and 48 h of compression, and three up-regulated pathways in the

Table 3. Top five increased or decreased gene lists in two dimension (2D) cultured cells after compression stress

	2 h compression		48 h compression	
	Name (GenBank ID)	Fold changes	Name (GenBank ID)	Fold changes
Top five increased genes	Solute carrier family 26, member 9 (NM_052934)	3.71318	Chloride channel 4 (BC036068)	4.66247
	Polymerase (DNA directed), theta (NM_199420)	3.2913	Inhibitor of DNA binding 2B, dominant negative helix-loop-helix protein (NM_001039082)	3.1933
	Actin filament associated protein (NM_021638)	3.28145	Centromere protein F, 350/400ka (mitosin) (NM_016343)	3.0949
	Chromosome 1 open reading frame 107 (NM_014388)	3.17537	Cystathionase (cystathionine gamma-lyase) (NM_153742)	3.03378
	KIAA0692 (XM_930821)	3.09091	Hyaluronoglucosaminidase 4 (NM_012269)	3.03318
Top 5 decreased genes	Hypothetical protein LOC643072 (XM_931281)	0.28525	Chromosome 14 open reading frame 147 (BC021701)	0.25344
	Chromosome 14 open reading frame 147 (BC021701)	0.29415	Sterol O-acyltransferase 2 (NM_003578)	0.29186
	NHL repeat containing 2 (BX647641)	0.30221	Membrane-bound transcription factor peptidase, site 1 (NM_003791)	0.30029
	Integrin, alpha D (AK097160)	0.33333	NHL repeat containing 2 (BX647641)	0.34391
	Myosin, heavy polypeptide 11, smooth muscle (BC101677)	0.35149	Hypothetical protein MGC7036 (NM_145058)	0.35845

Table 4. Top five increased or decreased gene lists in three dimension (3D) cultured cells after compression stress

	2 h compression		48 h compression	
	Name (GenBank ID)	Fold changes	Name (GenBank ID)	Fold changes
Top five increased genes	Peptidylprolyl isomerase (cyclophilin)-like 2 (NM_014337)	5.02111	Hypothetical LOC554208 (BC031277)	4.08636
	Hemoglobin, alpha 2 (NM_000517)	4.9046	Mannosyl (beta-1,4-)-glycoprotein beta-1,4-*N*-acetylglucosaminyltransferase (NM_002409)	3.97011
	Hypothetical LOC554208 (BC031277)	3.69039	Similar to CG1550-PA (XM_928636)	3.96554
	Hypothetical protein LOC645022 (XM_928081)	3.68166	Mannosidase, alpha, class 2A, member 1 (BC043416)	3.49062
	Caveolin 2 (BC005256)	3.64877	COBW domain containing 1 (BC005996)	3.40725
Top 5 decreased genes	Scribbled homolog (NM_182706)	0.30564	Hypothetical protein FLJ14327 (AK024389)	0.27007
	Similar to RIKEN cDNA B230118G17 gene (NM_080665)	0.33431	Homo sapiens cDNA FLJ25836 fis, clone TST08317 (AK098702)	0.30289
	InaD-like (NM_176877)	0.33938	Cytochrome P450, family 3, subfamily A, polypeptide 43 (BC100982)	0.30711
	Zinc finger, SWIM-type containing 2 (NM_182521)	0.34859	4-Hydroxyphenylpyruvate dioxygenase (NM_002150)	0.3184
	FLJ41733 protein (NM_207473)	0.34957	Kinesin 2 (CR607419)	0.32892

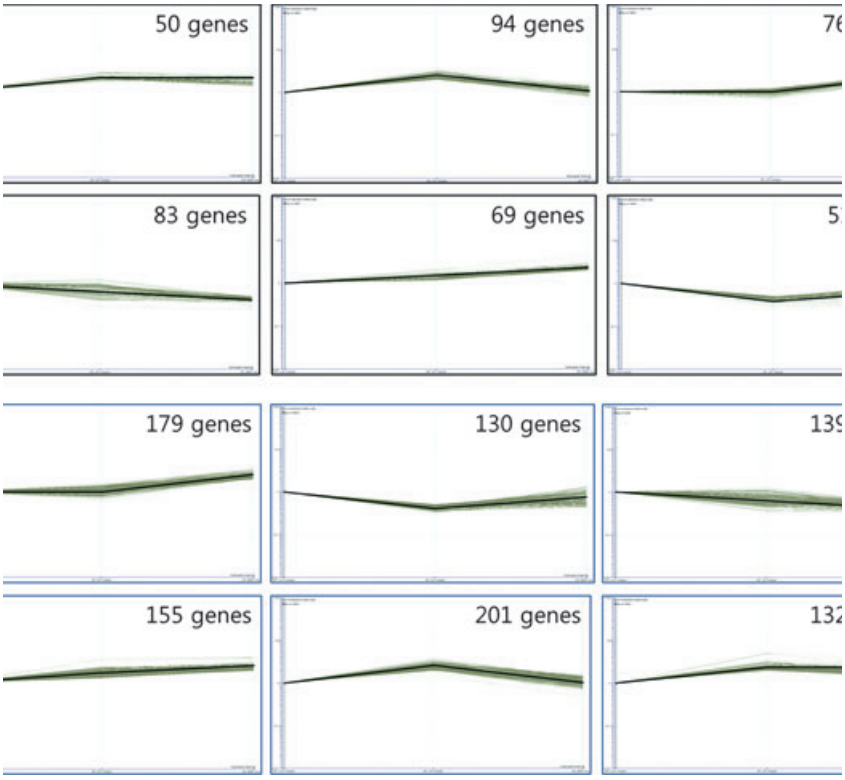

Fig. 2. Kinematic changes in gene expression after 2 and 48 h of compressive stress on two-dimension (2D) cultured (A) and three-dimension (3D) cultured (B) periodontal ligament (PDL) cells. Euclidean clustering analysis was used. Numbers in each panel indicate the number of genes showing similar time-course changes of expression. The *x*-axis is the time course, and the *y*-axis is the gene-expression level. The thick black line in the middle represents the observed trend of gene-expression changes according to the time course. For example, the upper left panel shows 50 genes in 2D cultured compressed PDL cells up-regulated immediately after 2 h of compressive stress application and kept up-regulated until 48 h of compressive stress application.

3D cultured compressed PDL cells with a false discovery rate of < 0.25 after 2 h of compression (Table 7). Regulation of catalytic activity was down-regulated in 2D cultured compressed PDL cells after 2 h of compression. The focal adhesion pathway was up-regulated in 2D cultured PDL cells after 48 h of compression. No significant pathways were found in 3D cultured compressed PDL cells with a false discovery rate of < 0.25 after 48 h of compression, but three pathways, including the MAPK signaling pathway, the focal adhesion pathway and the regulation of actin cytoskeleton pathway were up-regulated in 3D cultured compressed PDL cells after 2 h of compression.

DAVID analysis

Gene Ontology analysis was performed using DAVID, an online source that clusters genes showing changes in their expression levels into gene ontology terms. Table 8 displays the results of DAVID analysis that evaluated the up-regulated and down-regulated functional gene sets.

Gene network map

The gene network map was fabricated based upon the genes showing changes in their expression levels using

Table 5. Confirmation of five selected genes using real-time PCR

Encoded gene	Microarray				Real-time PCR			
	2D 2H	2D 48H	3D 2H	3D 48H	2D 2H	2D 48H	3D 2H	3D 48H
IL1β	1.16	1.69	0.93	1.14	1.10	1.06	1.08	2.02
TNFα	1.23	0.65	1.18	1.01	1.16	1.16	0.97	1.56
RANKL	1.49	1.05	1.2	1.08	1.93	2.07	1.45	1.42
MMP3	0.76	0.78	1.02	0.92	0.94	0.46	0.39	0.30
MMP13	1.19	1.28	1.47	1.48	1.29	4.26	1.01	1.32

Numbers are fold changes relative to vehicle control.
2D, two dimension cultured; 3D, three dimension cultured; 2H, compression stress for 2 h; 48H, compression stress for 48 h.

Table 6. Expression changed genes in three dimension (3D) cultured control cells compared with two dimension (2D) cultured control cells

	Name	GenBank ID	Fold change
Up-regulated genes	Abhydrolase domain containing 5	NM_016006	2.0735
	Hypothetical protein FLJ10159	BC091526	2.0581
Down-regulated genes	Chromosome 5 open reading frame 13	BC011050	0.4146
	Glucuronidase, beta-like 1	NM_001033523	0.4663
	NHL repeat containing 2	BX647641	0.4727
	Chromosome 5 open reading frame 13	NM_004772	0.4747
	nudE nuclear distribution gene E homolog like 1 (*A. nidulans*)	AL832648	0.4816
	Replication factor C (activator 1) 3, 38 kDa	NM_002915	0.4976

Table 7. Results of gene set enrichment analysis

Experimental group	Gene set	Set size	ES	NES	FDR	Rank	
2D 2H	Regulation of catalytic activity	15	−0.64021814	−1.7137179	0.1408435	44	Ontology
2D 48H	Focal adhesion	25	0.51977175	1.6580087	0.034422	100	Pathway
3D 2H	MAPK signaling pathway	26	0.5128271	1.5520194	0.0932914	82	Pathway
	Focal adhesion	25	0.49192715	1.4335557	0.103774	114	Pathway
	Regulation of actin cytoskeleton	17	0.52446616	1.3882401	0.092942	80	Pathway

2D, two dimension cultured; 3D, three dimension cultured; 2H, compression stress for 2 h; 48H, compression stress for 48 h.

GeneSpring GX 11.0 (Agilent Technologies) and is presented in Figs S1–S4. Genes showing changes in their expression levels were connected to each other based on the known relations. These relationships include catalytic relationships, modulatory relationships, regulatory relationships, target relationships, etc. These maps enable visualization of the relationships between genes showing changes in their expression levels and also identification of the most relevant genes or activities at a glance. From Fig. S1, 2D cultured compressed PDL cells after 2 h of compression demonstrated that the genes linked to transcription-related functions were mostly up-regulated. Also, positive regulation of NF-κB transcription factor activity and cell adhesion function were seemingly concentrated. Among the proteins, signal transducer and activator of transcription 5A had the maximum number of connections. Figure S4 shows the map for changes in relationship between genes in 2D cultured compressed PDL cells after 48 h of compression. The RNA interference function had the maximum number of connections, and NFKB1 (the NF-κB DNA-binding subunit) was the protein that had the maximum number of connections. Figure S2 shows the relationship between genes showing changes in their expression levels in 3D cultured compressed PDL cells after 2 h of compression. RNA interference function seemed to be the major function and other functions, such as osteoclast differentiation and tumor necrosis factor biosynthesis, were also noted. EDN1 (endothelin 1) was the protein that had the maximum number of connections. Figure S4 shows the relationship between genes showing changes in their expression levels in 3D cultured compressed PDL cells after 48 h of compression. Many functions were related but it seemed that no major functions existed. MAPK1 was the protein that had the maximum number of connections. Table 9 is a summary of genes and functions with most connecting lines.

Real-time PCR results

Real-time PCR results of mRNA expression of various osteoclastogenesis-related genes are shown in Table 7. Despite the differences in the amount of changes in the gene expression levels, *RANKL*, *RANK*, *TNFα*, *MMP3* and *MMP13* showed similar up- or down-regulated patterns between the 2D and 3D cultured PDL cells. Also, the real time PCR results were generally in accordance with the microarray results.

Discussion

This study investigated genome-wide changes in gene expression using the

Table 8. Summary of gene ontology analysis using DAVID with Kyoto Encyclopedia of Genes and Genomes (KEGG) database

Treatment group	Regulation	Biological pathways	*p*-Value	Benjamini FDR	Matched genes	Gene list
2D 2H	+2 fold	Neurotrophin signaling pathway	3.9E−2	9.3E−1	4	Rho GDP dissociation inhibitor (GDI) alpha, receptor-interacting serine-threonine kinase 2, ribosomal protein S6 kinase 90 kDa polypeptide 2, ribosomal protein S6 kinase 90 kDa polypeptide 3
		mTOR signaling pathway	3.9E−2	7.4E−1	3	Ras homolog enriched in brain, ribosomal protein S6 kinase 90 kDa polypeptide 2, ribosomal protein S6 kinase 90kDa polypeptide 3
		Long-term potentiation	6.4E−2	7.7E−1	3	CREB binding protein, ribosomal protein S6 kinase 90 kDa polypeptide 2, ribosomal protein S6 kinase 90 kDa polypeptide 3
		Purine metabolism	6.5E−2	6.8E−1	4	Deoxyguanosine kinase, ectonucleoside triphosphate diphosphohydrolase 2,phosphodiesterase 1A calmodulin-dependent, phosphodiesterase 6C cGMP-specific cone alpha prime
2D 48H	+2 fold	Antigen processing and presentation	2.5E−2	8.5E−1	4	CD8b molecule, cathepsin B, nuclear transcription factor Y, gamma, protein disulfide isomerase family A, member 3
		Cytosolic DNA-sensing pathway	6.6E−2	9.2E−1	3	Chemokine (C-C motif) ligand 4, chemokine (C-C motif) ligand 4-like 1; chemokine (C-C motif) ligand 4-like 2, nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
		Spliceosome	7.1E−2	8.4E−1	4	Mago-nashi homolog proliferation-associated (Drosophila), poly-U binding splicing factor 60 kDa, small nuclear ribonucleoprotein polypeptide A', splicing factor arginine/serine-rich 1
3D 2H	+2 fold	MAPK signaling pathway	5.6E−2	1.0E0	8	RAP1B, calcium channel voltage-dependent alpha 2/delta subunit 3, epidermal growth factor receptor, fibroblast growth factor 14, fibroblast growth factor receptor 1, serine/threonine kinase 4, stathmin 1, v-fos FBJ murine osteosarcoma viral oncogene homolog
		p53 signaling pathway	5.8E−2	9.5E−1	4	Cyclin B3, protein kinase CHK2-like, ribonucleotide reductase M2 polypeptide, shisa homolog 5 (*Xenopus laevis*)
	−2 fold	Fc gamma R-mediated phagocytosis	3.1E−2	9.0E−1	4	Actin related protein 2/3 complex subunit 5-like, myosin X, spleen tyrosine kinase, vav 3 guanine nucleotide exchange factor
3D48H	−2 fold	N-Glycan biosynthesis	4.8E−2	9.3E−1	4	Asparagine-linked glycosylation 13 homolog (*S. cerevisiae*), mannosidase, alpha, class 2A, member 1, mannosidase alpha class 2A member 2, mannosyl (beta-1,4-)-glycoprotein beta-1,4-*N*-acetylglucosaminyltransferase
		TGF-beta signaling pathway	7.0E−2	9.3E−1	5	SMAD family member 4, SMAD specific E3 ubiquitin protein ligase 2, bone morphogenetic protein 5, mitogen-activated protein kinase 1, zinc finger FYVE domain containing 16

2D, two dimension cultured; 3D, three dimension cultured; 2H, compression stress for 2 h; 48H, compression stress for 48 h.

DNA microarray technique after compressive stimulation of 2D and 3D cultured human PDL cells. With respect to the number of genes showing changes in their expression levels, 3D cultured PDL cells were found to have almost three times as many up-regulated genes after 2 h of compressive stimulation and twice as many up-regulated genes after 48 h of compressive stimulation compared with 2D cultured PDL cells. The 3D cultured PDL cells showed about 2.4 times more down-regulated genes after 2 h of compression and 1.7 times more down-regulated genes after 48 h of compression compared with 2D cultured PDL cells. Simply thinking about the number of genes showing changes in their expression levels, it appears that 3D cultured PDL cells are more sensitive and actively respond to compressive stimulation. In another aspect, after 48 h of compression, 2D cultured PDL cells showed more genes with changes in their expression levels compared with 2D cultured PDL cells after 2 h of compression, while the 3D cultured PDL cells showed a similar number of genes showing changes in

Table 9. Genes and functions with more than 10 connected lines from gene network map

Group	Gene	Function
2D 2H	*STAT5A* (13)	Transcription function (20)
	CREBBP (13)	Mutation function (18)
2D 48H	*NF*-κB1 (26)	RNA interference (14)
	PML (13)	
3D 2H	*EDN1* (20)	RNA interference (22)
3D 48H	*MAPK1* (26)	
	IL13 (13)	

2D, two dimension cultured; 3D, three dimension cultured; 2H, compression stress for 2 h; 48H, compression stress for 48 h. Numbers in () indicates number of connection lines.

their expression levels at both time-points. This can also be simply interpreted in terms of the fact that the 2D cultured PDL cells respond slowly to compressive stimulation, such as application of compression stress. Because the mechanical force (the manner and the amount of force) was delivered to the cells identically, the force itself was not a direct regulator of cellular response. In other words, mechanical force induced changes in cell shape or in intracellular fluid flow, which may be responsible for < 1/3rd of the cellular response to compressive stimulation, supposing that the changes in cell shape after compressive stimulation were similar between the 2D and 3D cultured PDL cells. Instead, the 3D environment seemed to regulate the cellular response to the compression stimulus, and intercellular and/or cell-to-matrix attachment played an important role in cellular mechanoreception. Also, the 3D environment was responsible for the quick or immediate and extensive response of PDL cells to compressive stimulation: 2D cultured PDL cells showed an increase in the number of fold changes in the expression level of genes after 48 h of compressive stress compared with after 2 h of compressive stress; however, in 3D cultured PDL cells, there was no significant change in the number of genes showing changes in their expression levels.

The 3D culture condition itself does not seem to alter cellular response to a great extent. Only eight genes showed a change in gene expression of over twofold in 3D cultured cells compared with 2D cultured cells. It seems that the culture system itself, without external stimuli, does not result in much change to the cellular physiology. It could be generally assumed that genes with altered expression after compressive stress can be directly compared because inherent gene-expression differences between 2D and 3D cultures seem minor.

Numerous studies have shown that the molecules related to cellular attachment, such as cadherins and integrins, are responsible for the mechanoreception in osteoblasts (13,25). Especially, integrin and its associated kinase (FAK) have been focused upon. The GSEA results of this study also indicate the importance of the focal adhesion pathway because the 2D cultured compressed PDL cells after 48 h of compression and the 3D cultured compressed PDL cells after 2 h of compression showed up-regulation of focal adhesion pathway genes. It is believed that the mechanical force causes conformational change in focal adhesion, resulting in tyrosine autophosphorylation of FAK (25). Phosphorylated FAK leads to the activation of JNK, MEK1/2 and ERK1/2 (13,25–27). It is believed that the 3D cultured PDL cells have a greater number of activated integrin–FAK complexes than do 2D cultured PDL cells because the 3D environment permits more focal adhesion complexes to be formed. Similarly, activation of the "Regulation of actin cytoskeleton" signaling pathway in 3D cultured compressed PDL cells after 2 h of compression can be understood. The actin cytoskeleton is closely related to the integrin complex and is thereby also connected to FAK.

The 3D cultured compressed PDL cells showed up-regulation of the *RAB7* (member RAS oncogene family-like 1) gene after both 2 and 48 h of compression, whereas 2D cultured compressed PDL cells showed no changes in *RAB7* expression. Also, the *RAB33A* gene was up-regulated in the 3D cultured compressed PDL cells after 48 h of compression. The *RAB* family is one of the members of the five groups of the RAS superfamily and is known to be important in regulating signal transduction and cellular processes such as differentiation, proliferation, vesicle transport, nuclear assembly and cytoskeleton formation (28). Also these proteins are involved in the recycling of integrins and because integrin is a transmembrane protein that mediates cellular attachment and associated signaling mechanisms (9), the reason for up-regulation of *RAB* genes in the 3D cultured compressed PDL cells can be understood. In contrast, the signal-induced proliferation-associated gene 1 (*SIPA1*) was up-regulated in the 2D cultured compressed PDL cells after both 2 and 48 h of compression, whereas the 3D cultured compressed PDL cells showed no changes in *SIPA1* expression. SIPA1 is a GTPase-activating protein that inhibits the Ras-related regulatory proteins Rap1 and Rap2 (29). It is also known to be involved in cell adhesion; its suppression increases cell adhesion and it has a negative impact on actin cytoskeleton remodeling (30,31). Myosin binding protein H gene was up-regulated in 3D cultured compressed PDL cells after both 2 and 48 h of compression, whereas the 2D cultured compressed PDL cells showed no changes in expression of the myosin binding protein H gene. Myosin binding protein H has been reported to inhibit ROCK1 and to negatively regulate actomyosin organization (32). This may cause alterations in cellular morphology and motility. This also may indicate that cellular morphological adaptation to the compressive force is different according to the attachment conditions.

The results of this study also suggest that several other signaling pathways are responsible for the cellular response to compressive stress. Among them, the MAPK pathway is a well-known mechanical force-related signaling pathway involved in cellular proliferation, differentiation and apoptosis upon external stimulation

(16,33). Ziegler *et al* (16) showed that activation of MAPKs p42/44 is associated with tensile stress-induced expression of the *MMP13* gene in PDL cells. Other pathways, such as the neurotrophin signaling pathway, the mTOR signaling pathway and the TGF-β signaling pathway, were also proposed to be involved. However, most of the proposed pathways were not consistent according to the culture conditions and compressive stress duration. This suggests different cellular responses depending upon the culture conditions and the duration of compressive stress. Such canonical pathways do not always reflect a common cellular response and therefore it seems logical to draw new signaling network maps for different external stimuli. The gene network map provided in Figs S1, S2, and S4 may give an idea of the mechanotransduction signaling pathway. Other pathways that were not up-regulated in this study may not be counted as unrelated pathways. Kang *et al.* (12) showed activation of FAK after compressive stress but without up-regulation of *FAK* mRNA. The possibility of activation of kinases without an increase in its amount is possible and also mRNA expression is not always linked to an increase in its protein products.

From the gene network map (Figs S1,S2, and S4), it can be visualized that several genes with their products have multiple functions. Table 9 summarizes the genes and functions that had more than 10 connection lines from the gene network map. The 2D cultured compressed PDL cells after 2 h of compression showed that *STAT5A* (signal transducer and activator of transcription 5A) and *CREBBP* (cyclic AMP response-element binding protein (CREB) binding protein) had many connections. The STAT5A/B signaling pathway is related to Janus kinase (JAK), and active STAT5A/B is transported from the cytoplasm to the nucleus and binds to DNA to promote transcription (34). CREBBP is a co-activator of CREB, and CREB is a transcription factor that regulates cellular proliferation, survival and differentiation (35). Phosphorylated CREB inhibits NF-κB activation and thereby limits proinflammatory responses (35). Ogasawara *et al.* (14) has shown that CREB has a crucial role in the shear stress-induced expression of cyclooxygenase-2 in MC3T3-E1 cells. It has been shown that NF-κB1 may play an important regulatory role in the 2D cultured compressed PDL cells after 48 h of compression. NF-κB is a well-known transcriptional factor and is a regulator of many cellular processes, including cell survival and inflammation (36). It has been shown that NF-κB signaling is involved in the tension-induced response of PDL cells (15,37). It was shown in the 3D cultured compressed PDL cells that EDN1 had many regulatory connections after 2 h of compression. EDN1 was shown to be up-regulated after tensional stress in gingival fibroblasts and was also found to be involved in the mechanical stress signaling pathways in various cell types (38,39). MAPK1 (mitogen-activated protein kinase 1, extracellular signal-regulated kinase-2) was shown to be a key regulatory molecule in the 3D cultured compressed PDL cells after 48 h of compression. MAPK1 is involved in a wide variety of cellular processes, including proliferation, differentiation, transcription regulation and development. Also, abundant studies have investigated its role in mechanotransduction in various cell types, including PDL cells (13,15,16).

According to the DAVID and GSEA results, and also according to the clustering analysis results, the signaling pathways and cellular gene response might change according to the time course. Also, the DAVID and GSEA results did not match. DAVID is classified as a singular enrichment analysis (SEA) that takes the ''preselected genes'' and iteratively tests the enrichment of each annotation term one-by-one in a linear mode (40). GSEA has a algorithm distinct from SEA and takes experimental values of ''all genes'' into calculation for each annotation term (40). These differences may have resulted in the different outcomes.

Both the quantity and the quality of cellular attachment to the extracellular matrix may influence the cellular response to mechanical stress. In this study, only collagen matrix was used. However, considering that the PDL cells in living tissues are also attached to the bone surface and dental root cementum, these cells might respond differently than only the collagen-attached PDL cells. This assumption is out of the spectrum of the present study and hence should be evaluated further.

Conclusions

The culture conditions and duration of compressive stress might alter the cellular response to mechanical stress. The 2D and 3D cultured PDL cells showed differential gene expression after application of identical compressive stresses. Several possible signaling pathways associated with compressive stress were suggested and these results will facilitate further investigation regarding the mechanical stress-related response of the PDL cells.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Gene network map of expression changed genes in 2D cultured 2 hours compressed PDL cells.

Figure S2. Gene network map of expression changed genes in 2D cultured 48 hours compressed PDL cells.

Figure S3. Gene network map of expression changed genes in 3D cultured 2 hours compressed PDL cells.

Figure S4. Gene network map of expression changed genes in 3D cultured 48 hours compressed PDL cells.

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