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Apoptotic gene expression by human periodontal ligament cells following cyclic stretch

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Background and Objective: Periodontal ligament cells play an important role in maintaining homeostasis of periodontal tissue upon mechanical force loading caused by mastication or orthodontic force. Previous studies revealed force-driven periodontal ligament cell death via apoptosis, but the force-sensing genes assigned to the apoptotic pathway have not been fully characterized. The present study aimed to identify force-sensing genes implicated in the apoptotic pathway in periodontal ligament cells.

Material and Methods: Human periodontal ligament cells were exposed to 20% stretch strain for 6 or 24 h, and the differential expression of 84 genes implicated in the apoptotic pathway were quantified by real-time PCR array technology.

Results: Ten and 11 genes showed upregulated expression after 6 and 24 h stretches, respectively, and there were two downregulated genes in response to both 6 and 24 h stretches. These genes included those encoding the tumor necrosis factor ligand family (*TNFSF8*), tumor necrosis factor receptor family (*FAS*, *TNFRSF10B*, *TNFRSF11B*, *TNFRSF25* and *CD27*), the Bcl-2 family (*BAG3*, *BAK1*, *BCL2L11* and *BCLAF1*), the caspase family (*CASP5* and *CASP7*), the inhibitor of apoptosis proteins family (*BIRC3*, *BIRC6* and *NAIP*), the caspase recruitment domain family (*RIPK2* and *PYCARD*) and the death domain family (*DAPK1*), as well as an oncogene (*BRAF*).

Conclusion: This study identified several force-sensing genes implicated in the apoptotic pathway in periodontal ligament cells and should facilitate future studies on force-driven apoptosis by providing putative target genes.

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The periodontal ligament is a specialized connective tissue that connects cementum and alveolar bone, and acts as the major element in tooth mobility and stress distribution to the supporting tissues (1). The periodontal ligament cell is the predominant cell type in the periodontal ligament (2). During occlusal function or orthodontic tooth movement, periodontal ligament cells are directly subject to mechanical stresses, and their response to mechanical stimulation is important in maintaining periodontal homeostasis and in periodontal remodeling (3). Physiological mechanical stimulations are essential for periodontal homeostasis (4), while the application of excessive mechanical stress results in local ischemia, tissue hyalinization and cell death in the periodontal ligament (3,5).

Cells undergo death through one of two major mechanisms: necrosis or apoptosis. Apoptosis is a programmed cell death by which damaged or unneeded cells are individually eliminated to maintain healthy homeostasis in multicellular organisms (6). Recently, several *in vivo* studies revealed that the pressure applied during orthodontic tooth movement caused apoptosis in the periodontal ligament (7,8). In

addition, *in vivo* cell death by apoptosis was found not only at the pressure side of the periodontal ligament, but also at the tension side after orthodontic force acted on the rat tooth (9). *In vitro* application of compressive force was reported to induce apoptosis in MG-63 osteoblast-like cells through the activation of caspase-3 via the caspase-8 signaling cascade (10). Our recent studies also demonstrated that cyclic stretch induced apoptosis in cultured human periodontal ligament cells through the activation of caspase-3 via the caspase-9 signaling cascade (11,12).

Recently, Ritter *et al*. (13) reported 20 force-sensing genes assigned to the apoptotic pathway in cultured human periodontal ligament fibroblasts following 6 h continuous stretch at 2.5% strain, by employing cDNA array chip hybridization. However, in this previous research, cDNA array analysis was carried out only in duplicate; hence, no statistical analysis was carried out for the cDNA array data, and only three genes (*BAD*, *FAS* and *CRADD*) among the 20 force-sensing genes were selected to be confirmed statistically by real-time PCR. Most recently, real-time PCR array analysis has been introduced to cytomechanical research on periodontal ligament cells to profile osteogenic gene expression during cyclic tension (14,15). One advantage of real-time PCR array technology is that the quantitative expression of a large number of genes can be screened at the same time in identical experimental conditions, and by measuring control and experimental samples in triplicate, one can obtain statistically robust data. This technology also provides us with a powerful tool to profile multiple genes involved in force-driven apoptosis in periodontal ligament cells.

As noted above, force-sensing genes assigned to the apoptotic pathway have not been fully characterized. For this purpose, the present study quantified the differential expression of 84 genes implicated in the apoptotic pathway by cultured human periodontal ligament cells in response to cyclic stretch, with real-time PCR array technology. This should contribute to the identification of force-sensing genes implicated in the apoptotic pathway in periodontal ligament cells and help us to understand force-driven apoptosis better by providing putative target genes for further studies.

Material and methods

Preparation of human periodontal ligament cells

Human periodontal ligament cells were obtained from two healthy premolars (extracted for orthodontic reasons) of a 12-year-old female donor, after obtaining informed consent from her parents. The protocol for harvesting human tissue from extracted teeth was approved by the Ethics Committee of Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine (2008; 16). Human periodontal ligament cells were cultured as previously described (17). Pieces of periodontal ligament were obtained from the middle of the roots with a sterile scalpel and were rinsed five times with Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA). Pieces of periodontal ligament were attached to a cell culture flask and cultured in DMEM supplemented with 20% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and fivefold reinforced antibiotics (500 U/mL penicillin and 500 μg/mL streptomycin; Sigma, St Louis, MO, USA) at 37°C in a humidified atmosphere of air containing 5% CO_2. Cells that grew out from the extracts were passaged in DMEM supplemented with 10% (v/v) FBS and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). Cells at passage four were used in the present study.

Stretch loading

Human periodontal ligament cells were stretched by using a cell strain unit, which has been described previously (11,12). The cell strain unit included a strainer, a controller and a personal computer (PC). Cells were seeded in a flexible-bottomed culture dish (diameter 60 mm) whose bottom was made of elastic silicon rubber (Q7-4750; Dow Corning Co., Midland, MI, USA). A spherical cap moved up and down repeatedly and stretched cells attached on the bottom of culture dish by deforming the elastic silicon bottom. All changes in stretching strain and movement of the spherical cap were controlled by the controller and PC. Cells were seeded in the flexible-bottomed culture dishes at a concentration of 1.5×10^6 cells per dish and reached confluence following 3 d of culture, and were then exposed to 20% stretch strain for 6 or 24 h at a frequency of 6 cycles/min, each cycle consisting of a 5 s stretch period followed by a 5 s relaxation period. The treatments were repeated three times for each of the two time intervals (6 and 24 h). Cells cultured in flexible-bottomed culture dishes placed in similar conditions but without stretch served as controls. It is believed that a stretch strain no higher than 24% is reasonable for cultured periodontal ligament cells to mimic the strain which may be confronted by periodontal ligament cells *in vivo* (16). Our recent studies demonstrated that 6 and 24 h cyclic stretches with 20% strain induced notable early and late apoptosis, respectively, in cultured human periodontal ligament cells (11,12). Therefore, 20% stretch strain was chosen to load cells for 6 and 24 h for profiling the apoptotic gene expression in the present study. The loading frequency of 6 cycles/min (5 s stretch and 5 s relaxation) was the same to that in the report by Matsuda *et al*. (18) and also our previous studies on periodontal ligament cells (11,12).

Isolation of RNA and synthesis of cDNA

Total RNA from cells in each stretched group and in the control group was isolated by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After stretch loading, the medium was removed. Cells were washed with phosphate buffered saline (PBS) once. After the PBS was sucked out, an appropriate amount of TRIzol reagent (1 mL/10 cm^2 area of culture dish) was added. The TRIzol solution containing cell lysate was incubated at room temperature for 5 min and then

transferred to a 1.5 mL Eppendorf tube. Then 0.2 mL chloroform per 1 mL TRIzol reagent was added, and the tube was shaken vigorously by hand for 15 s. A further incubation of 2–3 min at room temperature was required prior to centrifugation of the samples at 12,000*g* for 15 min at 4°C. Following centrifugation, the clear aqueous phase was transferred to a fresh tube and mixed with an equal volume of isopropyl alcohol and incubated at room temperature for 10 min to precipitate the RNA. After centrifugation at 12,000*g* for 10 min at 4°C, the RNA pellet was washed once with 75% ethanol and centrifuged at 7500*g* for 5 min at 4°C. The RNA pellet was air-dried for 5–10 min and then dissolved in RNase-free water. Contaminating genomic DNA was removed from total RNA samples by DNase I digestion. The RNA samples were then purified by means of the RNeasy® MinElute™ Cleanup kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's instructions. The RNA concentration was determined by an ND-1000 Spectrophotometer (Nanodrop, Rockland, DE, USA), and the RNA purity was confirmed by 260/280 optical density value of 1.8–2.0. The RNA samples were assessed for degradation status by denaturing agarose gel electrophoresis. First-strand cDNA synthesis was performed with the RT^2 First Strand kit (Superarray, Frederick, MD, USA) in accordance with the manufacturer's instructions.

Real-time PCR array analysis

The cDNA samples were then screened for the expression of 84 key genes involved in apoptosis by means of the Human Apoptosis RT^2 Profiler PCR Array (PAHS-012; Superarray, Frederick, MD, USA) on an ABI 7900 real-time PCR system (Applied Biosystems, Carlsbad, CA, USA), according to the manufacturer's protocols. To obtain statistically sound data, we analyzed control and experimental samples at each of the two time intervals in triplicate. The expressions of target genes were measured relative to the mean threshold cycle (CT) values of three different calibrator genes (β-2-microglobulin, *GAPDH* and β-actin), by the ΔΔCT method described previously (19). The results were expressed as the relative fold change of gene expression in the stretched group compared with the control group, or in the 24 h stretched group with the 6 h stretched group. Genes with relative fold changes greater than ± 2 were considered as up- or downregulated in expression.

Statistical analysis

Student's unpaired *t*-tests were used for statistical comparison between the stretched groups and control groups, or between the 24 h stretched group and the 6 h stretched group, with mean CT values derived from the triplicate samples. Values of $p < 0.05$ were considered statistically significant.

Results

The Human Apoptosis RT^2 Profiler PCR Array profiles the expression of 84 key genes involved in apoptosis. These 84 genes and the effects of cyclic stretch on their differential expression are shown in Appendix S1 in the Supporting Information. A gene was regarded as being constitutively expressed if it was detected at a CT value of < 35, and the data sets of both the 6 and the 24 h stretched groups reflect a pattern of constitutive expression for the majority of genes screened (Fig. 1). The CT values > 35 lie outside the detection threshold of the system; these genes were therefore considered not to have been expressed.

Among the 84 genes that were screened, the expression levels of 10 genes (11.9%) were upregulated, and the expression levels of two genes (2.4%) were downregulated in human periodontal ligament cells after 6 h stretch (Table 1). There were 11 upregulated genes (13.1%) and two downregulated genes (2.4%) after 24 h stretch (Table 2). In addition, two upregulated genes and one downregulated gene were identified in the 24 h stretched group compared with the 6 h stretched group (Table 3). These differentially expressed genes can be allocated to genes encoding the tumor necrosis factor (TNF) ligand family

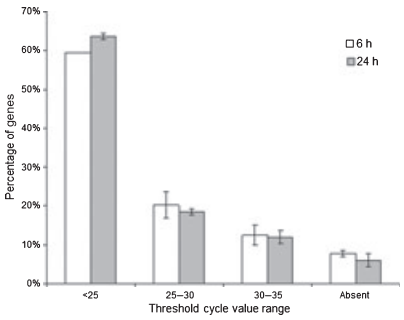

Fig. 1. Histogram showing the mean cycle threshold (CT) distribution for 6 and 24 h stretched groups. The mean values were determined from three replicate microarray plates. Low CT values (< 25) represent genes present at high transcript copy number. Genes with CT values of > 35 (absent calls) were considered to lie outside the detection threshold of the system. Error bars represent standard deviations ($n = 3$).

(*TNFSF8*), the TNF receptor family (*FAS*, *TNFRSF10B*, *TNFRSF11B*, *TNFRSF25* and *CD27*), the Bcl-2 family (*BAG3*, *BAK1*, *BCL2L11* and *BCLAF1*), the caspase family (*CASP5*, *CASP7*), the inhibitor of apoptosis proteins (IAP) family (*BIRC3*, *BIRC6* and *NAIP*), the caspase recruitment domain (CARD) family (*RIPK2* and *PYCARD*) and the death domain family (*DAPK1*), as well as an oncogene (*BRAF*).

Discussion

The initiation and execution phases of apoptosis involved activation of a family of cysteine-dependent aspartate-specific proteases known as caspases (20), which cause cell death by cleavage of critical cellular substrates and activation of proteolytic enzymes in the apoptotic pathway. Initiation of apoptosis involves the activation of initiation caspases, such as caspase-8 and -9, which in turn trigger an amplifying cascade of execution caspases, including caspase-3, -6 and -7, which are responsible for the alteration of cell morphology and the final cell death (21). Currently, there are two identified apoptotic signaling pathways: the extrinsic pathway and the intrinsic pathway. The extrinsic pathway is triggered by the interaction between specific ligands and death receptors

Table 1. Differentially expressed genes in human periodontal ligament cells following 6 h cyclic stretch

Name of gene	Description	Relative fold change (stretched/control)	Accession no. (GeneBank ID)
BAG3	Bcl2-associated athanogene 3	2.99	NM_004281
BAK1	Bcl2-antagonist/killer 1	2.01	NM_001188
BIRC3	Baculoviral IAP repeat-containing protein 3	8.37	NM_001165
BIRC6	Baculoviral IAP repeat-containing protein 6 (appollon)	2.75	NM_016252
CASP5	Caspase-5, apoptosis-related cysteine peptidase	13.50	NM_004347
CASP7	Caspase-7, apoptosis-related cysteine peptidase	2.12	NM_001227
FAS	Fas (tumor necrosis factor receptor superfamily, member 6)	3.07	NM_000043
TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b	2.14	NM_003842
TNFRSF25	Tumor necrosis factor receptor superfamily, member 25	2.68	NM_003790
RIPK2	Receptor-interacting serine-threonine kinase 2	2.52	NM_003821
PYCARD	PYD and CARD containing protein	−3.11	NM_013258
DAPK1	Death-associated protein kinase 1	−7.27	NM_004938

CARD, caspase recruitment domain; IAP, inhibitor of apoptosis proteins; PYD, pyrin domain.

Table 2. Differentially expressed genes in human periodontal ligament cells following 24 h cyclic stretch

Name of gene	Description	Relative fold change (stretched/control)	Accession no. (GeneBank ID)
BCL2L11	Bcl2-like 11 (apoptosis facilitator)	7.80	NM_006538
BCLAF1	Bcl2-associated transcription factor 1	3.68	NM_014739
NAIP	NLR family, apoptosis inhibitory protein	2.73	NM_004536
BIRC6	Baculoviral IAP repeat-containing protein 6 (appollon)	2.01	NM_016252
BRAF	V-raf murine sarcoma viral oncogene homolog B1	2.09	NM_004333
CASP5	Caspase-5, apoptosis-related cysteine peptidase	13.51	NM_004347
CASP7	Caspase-7, apoptosis-related cysteine peptidase	2.27	NM_001227
FAS	Fas (tumor necrosis factor receptor superfamily, member 6)	3.41	NM_000043
TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b	2.84	NM_003842
TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	−2.85	NM_002546
TNFRSF25	Tumor necrosis factor receptor superfamily, member 25	15.14	NM_003790
CD27	CD27 molecule (tumor necrosis factor receptor superfamily, member 7)	2.00	NM_001242
TNFSF8	Tumor necrosis factor (ligand) superfamily, member 8	−5.36	NM_001244

IAP, inhibitor of apoptosis proteins; NLR, nod-like receptor.

(which are mainly members of the TNF receptor superfamily, such as Fas) on the cell surface, and consequently activates caspase-8. The intrinsic pathway is activated by a set of molecules such as cytochrome *c* secreted from the mitochondria and activates caspase-9. Both of these pathways activate executor caspases (22).

Caspase-3 is one of the key executioners of apoptosis, being responsible for the cleavage of crucial substrates in the final degradation phase. Goga *et al*. (10) reported that 24 h *in vitro* application of compressive force induced the activation of caspase-3 in MG-63 cells via caspase-8. Our recent research showed that the caspase-3 activity increased and reached a peak after 24 h cyclic stretch in human periodontal ligament cells via caspase-9 (12). Liu *et al*. (23) also reported that cyclic stretch induced cleavage and activation of caspase-3 in rat skeletal muscle myoblasts and the induction reached a peak at 24 h, while the expression of procaspase-3 remained unchanged. In the present study, the expression of *CASP3* did not change after 6 and 24 h stretch, suggesting that the regulation of stretch-induced apoptosis in human periodontal ligament cells by caspase-3 is mediated primarily at the post-transcriptional level. It should be noted that the expression of *CASP8* after 6 h stretch was 1.96-fold of control values ($p = 0.0274$) and the expression of *CASP9* after 24 h stretch was 1.53-fold of control values ($p = 0.0179$; Appendix S1 in the Supporting Information), although they did not exceed twofold of the control level. This suggests that both caspase-8 and caspase-9 signaling pathways may have contributed to the stretch-induced apoptosis in human periodontal ligament cells, but in different stages of apoptosis. Ritter *et al*. (13) also reported an elevated expression of *CASP8* (2.81-fold relative to control values) in human periodontal ligament fibroblasts following 6 h continuous stretch. Our previous studies demonstrated that 6 and 24 h cyclic stretch induced notable early and late apoptosis, respectively, in human periodontal ligament cells (11,12); therefore, caspase-8, which is involved in the extrinsic pathway, may be mainly

Table 3. Genes differentially expressed in human periodontal ligament cells stretched for 24 compared with 6 h

Name of gene	Description	Relative fold change 24/6 h	Accession no. (GeneBank ID)
BCL2L11	Bcl2-like 11 (apoptosis facilitator)	12.09	NM_006538
TNFRSF25	Tumor necrosis factor receptor superfamily, member 25	5.64	NM_003790
BIRC3	Baculoviral IAP repeat-containing protein 3	−6.12	NM_001165

IAP, inhibitor of apoptosis proteins.

responsible for the early apoptosis, while caspase-9, which is involved in the intrinsic pathway, may be mainly responsible for the late apoptosis. It is not surprising that the expressions of *CASP7* in both 6 and 24 h stretched groups were higher than the control level, because caspase-7 is an execution caspase at downstream of caspase-3 (13). What is more interesting is the much higher expression of *CASP5* in both 6 and 24 h stretched groups in our study, which is consistent with the report of Ritter *et al*. (13) that expression of *CASP5* was elevated (7.05-fold relative to control values) in human periodontal ligament fibroblasts following 6 h stretch. Caspase-5 has been reported to be an inflammatory caspase and has a role in the immune system (21). Little is known about its role in cytomechanics. Further studies are required to elucidate the role and regulatory mechanism of caspase-5 in stretch-induced apoptosis.

The Fas/Fas ligand (FasL) death signaling system is an important regulator of apoptosis. Stimulation of Fas by its ligand, FasL, results in its trimerization and the recruitment of the adapter protein, Fas-associated death domain (FADD), and caspase-8, which form the death-inducing signaling complex (DISC) and lead to subsequent activation of the effector caspases (24). In the present study, the expression of *FAS* was significantly increased by stretch, clearly indicating that it can be allocated to the force-sensing genes. This result is consistent with the report by Ritter *et al*. (13), where an elevated expression of *FAS* (3.31-fold of the control level) in human periodontal ligament fibroblasts following 6 h stretch was revealed. Like *FAS*, genes encoding other members of the TNF receptor superfamily which can mediate apoptosis also exhibited a stretch-dependent increase in expression (*TNFRSF10B*, 2.14- and 2.84-fold relative to the control value, for the 6 and 24 h stretched groups, respectively; *TNFRSF25*, 2.68- and 15.14-fold relative to the control vlaue, for the 6 and 24 h stretched groups, respectively; and *CD27*, 2.0-fold relative to the control value for the 24 h stretched group). Moreover, 24 h stretch induced a higher expression of *TNFRSF25* compared with 6 h stretch. This leads to the assumption that, in periodontal ligament cells, death receptors other than Fas may also be involved in the force-driven apoptosis. Different from the pro-apoptotic role of the above-mentioned TNF receptor family members, the role of TNF receptor superfamily member 11b (TNFRSF11B) in apoptosis has been reported to be anti-apoptotic (25). In this context, the downregulated expression of *TNFR SF11B* after 24 h stretch accords with the upregulated expression of the above genes encoding pro-apoptotic TNF receptor family members.

The Bcl-2 family proteins govern mitochondrial outer membrane permeabilization and can be either pro-apoptotic (Bax, Bak, Bid, Bim and Bad, among others) or anti-apoptotic (Bcl-2, Bcl-x_L and Mcl-1, among others; 26). In the present study, the expression of *BAK1* was significantly increased by 6 h stretch, which is consistent with the report of Ritter *et al*. (13). Besides, in our study, 24 h stretch increased the expression of *BCL2L11* and *BCLAF1*. Bcl2-antagonist/killer 1 (Bak1) is a Bcl-2 family member that functions to induce apoptosis by forming a mitochondrial apoptosis-induced channel and mediating the release of cytochrome *c* (27). Bcl2-like 11 (BCL2L11/Bim) is a Bcl-2 family member that acts as an apoptotic agonist (26). Bcl2-associated transcription factor 1 (BCLAF1) is a transcriptional repressor that interacts with several members of the Bcl-2 family and induces apoptosis (28). All these three proteins are pro-apoptotic factors in the intrinsic apoptotic signaling pathway; therefore, the increased expression of their genes after stretch loading strongly suggests the participation of the intrinsic pathway in the regulation of stretch-induced apoptosis in periodontal ligament cells. In the present study, elevated expression of *BAG3* was observed in 6 h stretched cells, but not in 24 h stretched cells. Bcl2-associated athanogene 3 (Bag3) has been reported to be able to bind to Bcl-2 and protect cells from apoptosis (29). The possible explanation for the elevated expression of *BAG3* after 6 h stretch may be that at the early stage of apoptosis, its high expression will protect cells from death.

Receptor-interacting serine/threonine-protein kinase 2 (RIPK2) contains a caspase recruitment domain (CARD) and has been reported to be an activator of nuclear factor-κB and an inducer of apoptosis in response to various stimuli (30). The elevated expression of *RIPK2* after 6 h stretch in the present study suggests the involvement of PIPK2 and perhaps the nuclear factor-κB pathway in the regulation of stretch-induced early apoptosis in periodontal ligament cells. Further studies are needed to clarify the regulatory mechanism by this protein in stretch-induced apoptosis.

Besides the above mentioned *BAG3*, other anti-apoptotic genes, such as genes encoding members of the IAP family, also showed elevated expression in human periodontal ligament cells after stretch loading (*BIRC3*, 8.37-fold relative to the control value, for 6 h stretched group; *BIRC6*,

2.75- and 2.01-fold relative to the control value, for 6 and 24 h stretched groups, respectively; and *NAIP*, 2.73-fold relative to the control value for 24 h stretched group). The IAP family is a protein family characterized by containing baculovirus IAP repeat (BIR) domains, causing specific inhibition of caspase-3, -7 and -9, and plays an important role in regulating apoptosis (21). It is likely that the elevated expression of these anti-apoptotic genes, together with the downregulation of some pro-apoptotic genes, such as *PYCARD* (31), *DAPK1* (32) and *TNFSF8* (33), found in the present study are parts of a delicate feedback control on the stretch-induced apoptosis.

In the present study, an oncogene (*BRAF*) encoding v-Raf murine sarcoma viral oncogene homolog B1 (BRAF) also showed elevated expression in human periodontal ligament cells after 24 h stretch. BRAF is a serine/threonine-specific protein kinase and plays a role in regulating the MAPK signaling pathway (34). Its role in cytomechanics has not be previously reported and needs to be studied further.

In summary, this study identified several force-sensing genes implicated in the apoptotic pathway in stretched human periodontal ligament cells, by employing quantitative real-time PCR array analysis. These genes include key players in both extrinsic and intrinsic apoptotic signaling pathways, and include not only pro-apoptotic genes but also anti-apoptotic genes, showing the highly complicated network in the stretch-related apoptotic signaling and the delicate balance between survival and death in stretched cells through regulation by many factors. Although message is not always translated into protein, analysis of these data should facilitate future studies on force-driven apoptosis by providing putative target genes.

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Supporting Information

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

Appendix S1. Differentially expressed genes in human periodontal ligament cells following cyclic stretch.

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