ACTA ODONTOLOGICA SCANDINAVICA, 2017
VOL. 75, NO. 7, 507–516
https://doi.org/10.1080/00016357.2017.1347823

ORIGINAL ARTICLE

Cyclic stretch-induced the cytoskeleton rearrangement and gene expression of cytoskeletal regulators in human periodontal ligament cells

Yaqin Wu[a,b*], Jiabao Zhuang[a,b*], Dan Zhao[a,b], Fuqiang Zhang[a,b], Jiayin Ma[a,b] and Chun Xu[a,b]

[a]Department of Prosthodontics, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China; [b]Shanghai Key Laboratory of Stomatology & Shanghai Research Institute of Stomatology, Shanghai, China

ABSTRACT

Objective: This study aimed to explore the mechanism of the stretch-induced cell realignment and cytoskeletal rearrangement by identifying several mechanoresponsive genes related to cytoskeletal regulators in human PDL cells.

Material and methods: After the cells were stretched by 1, 10 and 20% strains for 0.5, 1, 2, 4, 6, 12 or 24 h, the changes of the morphology and content of microfilaments were recorded and calculated. Meanwhile, the expression of 84 key genes encoding cytoskeletal regulators after 6 and 24 h stretches with 20% strain was detected by using real-time PCR array. Western blot was applied to identify the protein expression level of several cytoskeletal regulators encoded by these differentially expressed genes.

Results: The confocal fluorescent staining results confirmed that stretch-induced realignment of cells and rearrangement of microfilaments. Among the 84 genes screened, one gene was up-regulated while two genes were down-regulated after 6 h stretch. Meanwhile, three genes were up-regulated while two genes were down-regulated after 24 h stretch. These genes displaying differential expression included genes regulating polymerization/depolymerization of microfilaments (CDC42EP2, FNBP1L, NCK2, PIKFYVE, WASL), polymerization/depolymerization of microtubules (STMN1), interacting between microfilaments and microtubules (MACF1), as well as a phosphatase (PPP1R12B). Among the proteins encoded by these genes, the protein expression level of Cdc42 effector protein-2 (encoded by CDC42EP2) and Stathmin-1 (encoded by STMN1) was down-regulated, while the protein expression level of N-WASP (encoded by WASL) was up-regulated.

Conclusion: The present study confirmed the cyclic stretch-induced cellular realignment and rearrangement of microfilaments in the human PDL cells and indicated several force-sensitive genes with regard to cytoskeletal regulators.

ARTICLE HISTORY
Received 9 December 2016
Revised 1 June 2017
Accepted 12 June 2017

KEYWORDS
Cyclic stretch; genes; cytoskeleton; periodontal ligament cells

Introduction

Periodontal ligament (PDL) is believed to be a highly specialized structure that interfaces the tooth with its surrounding alveolar bone and anchors the tooth in the socket [1]. PDL cells perceive mechanical stress generated from normal mastication, occlusal trauma and tooth movement in orthodontic treatment, and are of significant importance in remodeling of periodontal tissue and in maintenance of periodontal health [2,3]. There are growing evidences that mechanical forces could cause biomechanical reactions in PDL cells [3–6]. In particular, quite a few researches published so far have presented the realignment of the PDL cells after being stretched *in vitro* [7,8]. The phenomenon has also been reported in our previous *in vitro* studies that the human PDL cells were prone to be paralleled to each other and reoriented their long axes perpendicular to the direction of the stretching force in response to cyclic stretch [9,10].

Cells are connected to extracellular matrix (ECM) and adjacent cells through cytoskeleton inside the cell and the adhesion molecules on the cell membrane and in the ECM. In the mechanical strain-loading experiments by using the Flexercell system [7] or the cell strain unit (CSU) [10], the attachment of the cultured cells to the matrix on the surface of the flexible membrane was allowed by the connection between cytoskeleton inside the cell and the adhesion molecules of the ECM, via the transmembrane integrins. The cytoskeleton, comprised of three basic components, including microfilaments, microtubules and intermediate filaments, is a complex structural network in eukaryotic cells and is essential in multiple cellular processes, including cell migration, maintenance of cell morphology and signalling transduction [11]. Concomitant with the stretch-induced morphological alteration and realignment, the PDL cells will unavoidably go through breakup and setup of the connection with each other and with the ECM. Consequently, it is quite rational to

CONTACT Jiayin Ma gangnin@sina.com; Chun Xu imxuchun@163.com Department of Prosthodontics, Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, 500 Quxi Road, Shanghai 200011, China
*These authors contributed equally to this work.

Supplemental data for this article can be accessed here.

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consider the cytoskeleton and its regulators as candidate targets in the mechanotransduction pathway in PDL cells, especially in the process of cell realignment.

It has been previously reported that similar to the cell realignment under stretch conditions, rearrangement of microfilaments and microtubules in response to stretch also occurred in the human PDL cells [7,8]. Although a recently published study showed that Rho-signalling pathway might have been involved in the stretch-induced rearrangement of microfilaments in the human PDL cells [12], the exact signalling pathway of stretch-induced cytoskeletal rearrangement remains to be elucidated.

In the recent cytomechanical researches, real-time PCR array has been reported to be widely used to profile gene expression of the cultured PDL cells after stretch loading [13–15]. More recently, researchers including us [9,16] reported that in the cultured human PDL cells, it was detected that cyclic stretch resulted in altered expression of the genes related to the ECM and the adhesion molecules, by using real-time PCR array. Since cytoskeletons are tightly connected to adhesion molecules on the cell membrane and in the ECM structurally and functionally, it is meaningful to investigate the gene expression of cytoskeletal regulators in the human PDL cells after stretch loading.

The aim of this study is to probe into the mechanisms of the stretch-induced cytoskeletal rearrangement in PDL cells. For this purpose, the cultured human PDL cells were loaded with cyclic stretch and the changes of the morphology and content of microfilaments were recorded and calculated. The expression of 84 key genes encoding cytoskeletal regulators was screened and the differentially expressed genes were found by real-time PCR array analysis. Among the proteins encoded by these genes displaying differential expression, the protein expression level of several important proteins was analyzed by using western blot. To our knowledge, the present study is the first report to date with respect to the effect of mechanical stress on the genes assigned to cytoskeletal regulators in human PDL cells. We hope the findings presented in this article shall contribute to exploring the mechanisms of the stretch-induced cellular and cytoskeletal rearrangement and may have meaningful implications for the mechanotransduction pathway in PDL cells by indicating potential target genes.

Material and methods

Cells culture

Healthy premolars were collected from four young girls (one 11-year-old, two 12 year-old and one 13-year-old, totally 16 teeth, four from each donor) undergoing orthodontic treatment, and informed consents were obtained from their patients. The experimental protocol was approved by the Ethics Committee, Ninth People's Hospital, Shanghai JiaoTong University School of Medicine. The relevant Judgement's reference number: [2008]17. The human PDL cells were incubated *in vitro* as formerly reported [9,10,17]. After tooth extraction, the middle-third of root surfaces was scraped gently to obtain PDL tissue. Pieces of PDL tissue were incubated in the high glucose Dulbecco's Modified Eagle's Medium (DMEM, HyClone, Logan, UT) containing with 20% (v/v) fetal bovine serum (FBS, Gibco, Carlsbad, CA) and five-fold reinforced antibiotics (500 U/mL penicillin and 500 μg/mL streptomycin, HyClone) in a 37 °C humidified atmosphere with 5% CO_2 in air. The human PDL cells grew out from the pieces in ∼1 week, and were passaged after they reached confluence. The cells were then cultured in DMEM with 10% FBS and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin, HyClone). Cells were used for subsequent experiments at passage 4.

Stretch loading

A CSU was used to stretch the human PDL cells cultured *in vitro* in the present study, which has been reported in our previous studies [9,15,18]. Cells were passaged onto a cell culture dish (diameter 60 mm) with an elastic silicon rubber membrane in the bottom, at a concentration of 1.5×10^6 cells per dish. The flexible-bottomed cell culture dishes were treated with type I collagen (Sigma, St. Louis, MO) before the human PDL cells were passaged onto it. For the observation of microfilaments, the cultured cells were exposed to stretch strains of 1, 10 and 20% for 0.5, 1, 2, 4, 6, 12 or 24 h at a frequency of six cycles/min (5 s stretch and 5 s relaxation), after they reached confluence. Three independent experiments ($n = 3$) were carried out for each of the combinations of stretch strain (1, 10 or 20%) and loading time (0.5, 1, 2, 4, 6, 12 or 24 h), with the cells obtained from three different donors, respectively. The loading frequency adopted in this study was the same to that in Matsuda et al.'s [8] report and our previous studies [9,10]. Cells cultured in the similar condition but without cyclic stretch were used as non-stretching controls. It has been reported that cyclic stretch <24% strain is appropriate to mimic the strain suffered by the human PDL cells *in vivo* [19]. As notable rearrangement of microfilaments in the cultured human PDL cells was detected after 20% 6 h cyclic stretching and the phenomenon of rearrangement became much more apparent after 24 h cyclic stretching (see the result part), the cultured cells were stretched by 20% cyclic stretch strain for 6 and 24 h for the real-time PCR array analysis and the western blot analysis. For the real-time PCR array analysis, three independent experiments ($n = 3$) were carried out for each of the two time intervals (6 or 24 h), with the cells obtained from three different donors, respectively. While for each experiment, cells in control group, 6 and 24 h stretching groups were from the same donor. For the Western blot analysis, the experiments were conducted only once with the non-stretching control cells, 6 and 24 h stretched cells obtained from one same donor.

Observation of microfilaments and calculation

The human PDL cells were rinsed twice with phosphate-buffered saline (PBS), fixed with 40 g/L paraformaldehyde at room temperature for 10 min, and then rinsed with PBS again. The rinsed PDL cells were incubated with Alexa Fluor®

488 Phalloidin (1:40, Molecular Probes, Eugene, OR) at room temperature for 20 min to label microfilaments and then with DAPI (1:1000, Sigma, St. Louis, MO) at room temperature for 5 min to label nuclei. After being rinsed with PBS, the labeled cells were observed under a confocal laser scanning microscope (Leica, Bensheim, Germany) and fluorescent photos were recorded. For each sample, totally 12 cells with clear outline from four randomly selected confocal fluorescent photos were analyzed with an image analysis software (Image-Pro Plus, Version4.5, Media Cybernetics Inc., Silver Spring, MD) and the cross-section area (Ac) of the cells, cell length (measured along the major axis) and cell width (measured along the minor axis) were measured. The length-to-width ratio (L/W ratio) and the integrated optical density (IOD) of the cells were also calculated by using this software. The content of fluorescently labeled microfilaments was quantified by IOD.

RNA extraction and cDNA synthesis

Total RNA was isolated from the human PDL cells by means of Trizol reagent (Invitrogen, Carlsbad, CA) as described previously [13]. In brief, after cells were rinsed with PBS once, Trizol reagent was added in the cell culture dish (1 ml/10 cm^2 area of cell culture dish). After incubated for 5 min, the cell lysate was added with 0.2 ml chloroform per 1 ml Trizol reagent, and then the tube containing the cell lysate was shaken by hand for 15 s vigorously. Subsequently, the cell lysate was incubated again at room temperature for 2–3 min and was then centrifuged at 12,000*g* at 4 °C for 15 min; afterwards, an equal volume of isopropyl alcohol was mixed with the supernatant. The mixture was then incubated for 10 min at room temperature and was centrifuged for 10 min at 12,000*g* at 4 °C to get the precipitation. The RNA precipitation was rinsed with 75% ethanol once and was centrifuged again for 5 min at 7500*g* at 4 °C. After air-dried for 5–10 min, the RNA precipitation was dissolved in Rnase-free water. Dnase I digestion was used to remove the contaminating genomic DNA from total RNA samples. Then the RNA samples were purified by using the RNeasy® MinElute™ Cleanup Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The RNA concentration was measured by using a ND-1000 Spectrophotometer (Nanodrop, Rockland, DE) and the RNA purity was confirmed by using 260/280 OD value of 1.8–2.0. The RNA samples were evaluated for degradation status by using denaturing agarose gel electrophoresis. First-strand cDNA synthesis was carried out with the RT^2 First Strand Kit (SABiosciences, Frederick, MD) according to the manufacturer's instructions.

Real-time PCR array analysis

The real-time PCR array analysis was performed by using the Human Cytoskeleton Regulators RT^2 Profiler™ PCR Array (PAHS-088, SABiosciences, Frederick, MD) on an ABI 7900 real-time PCR system (Applied Biosystems, Carlsbad, CA), in accordance with the manufacturer's protocols, to detect the expression of 84 key genes related to cytoskeletal regulators. The expressions of target genes were assessed relative to the mean threshold cycle [20] values of two different calibrator genes (β-2-microglobulin, Ribosomal protein L13a), by the ΔΔCT method described previously [17]. The results were presented as the relative fold of gene expression to control. Genes with relative folds $> \pm 1.5$ were considered as up- or down-regulated in expression.

Western blot analysis

Based on the result of real-time PCR array analysis, the protein expression level of Cdc42 effector protein-2, N-WASP and Stathmin-1 was detected by Western blot. The collected cells were lysed on ice by using the RIPA buffer (Sigma) to obtain the total protein. The protein concentration was measured by using the BCA method with the Enhanced BCA Protein Assay Kit (Beyotime, Shanghai, China), according to the manufacturer's instruction, at 590 nm spectrophotometrically. Protein with equal amount was separated by SDS–PAGE (12%) (Beyotime), and then the protein bands were transferred to a 0.22 μm PVDF membrane (KeyGEN, Nanjing, China). After being blocked at room temperature with 5% nonfat milk for 1.5 h, the membrane was cut according to the predicted molecular weight and was incubated with the specific primary antibody, including the monoclonal Stathmin-1 Rabbit mAb (1:10000) (Abcam, Cambridge, MA), the monoclonal N-WASP Rabbit mAb (1:1000) (Abcam), the polyclonal Cdc42 effector protein-2 Rabbit mAb (1:1000) (Abgent, San Diego, CA) and the GAPDH Mouse Monoclonal Antibody (1:1000) (Biotech Well, Shanghai, China), at 4 °C overnight. On the second day, the membrane was incubated at room temperature with the secondary antibody (anti-rabbit, 1:5000; anti-mouse, 1:2000; KangChen, Shanghai, China) for 1 h. Next, the protein bands were detected by using an ECL reagent (SuperSignal West Femto Substrate, Thermo Scientific, Waltham, MA). The protein expression level was analyzed by using the Image-Pro Plus 6.0 (MediaCybernetics, Inc., Bethesda, MD).

Statistical analysis

The results of the calculation of Ac, L/W ratio and IOD of all the samples were expressed as mean ± standard deviation and analyzed statistically with one-way analysis of variance (ANOVA) followed by the least-significant difference (LSD) test. *T*-tests were employed for statistical comparison between the control group and the stretched group, in the real-time PCR array analysis, with mean CT values derived from the triplicate samples in each group. The statistical significance level was set at $p < .05$.

Results

Stretch-induced realignment of cells and rearrangement of microfilaments in the human PDL cells

The confocal fluorescent photos are shown in Figure 1. The cells in control group aligned multidirectionally

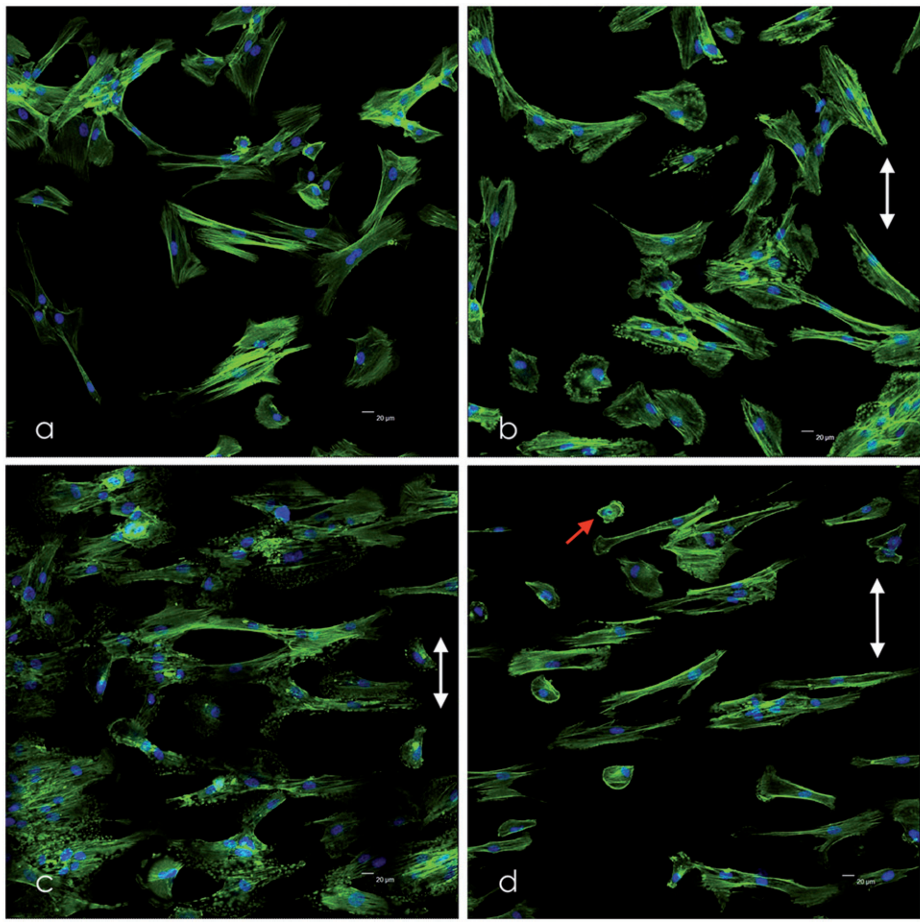

Figure 1. The confocal fluorescent photos showing the stretch-induced realignment of cells and rearrangement of microfilaments in human PDL cells. (a) Control cells. (b) Cells stretched with 1% strain for 24 h. (c) Cells stretched with 10% strain for 24 h. (d) Cells stretched with 20% strain for 24 h. The double headed arrows indicate the direction of stretching force vector. The single headed arrow indicates a shrunken cell.

and the microfilaments were of clearly visible filamentous configuration and were arranged parallelly to the long axes of cells (Figure 1(a)). After being stretched, the human PDL cells were prone to be paralleled to each other and realigned their long axis perpendicular to the direction of the stretching force (Figure 1(b–d), white double headed arrows). This phenomenon was most obvious after 24 h stretch (Figure 1). After 24 h stretch with 1% strain, the microfilaments remained clearly visible and orderly arranged, but were elongated a little bit (Figure 1(b)). After 24 h stretch with 10% strain, the microfilaments were elongated and thickened, with their long axis perpendicular to the direction of the stretching force (Figure 1(c)). Almost all cells were parallel to each other, perpendicular to the stretching force vector and the microfilaments were elongated remarkably after being stretched with 20% strain for 24 h (Figure 1(d)). Some cells shrank to oval shape after 24 h stretch with 20% strain and the microfilaments can hardly be optically discerned (Figure 1(d), red arrow).

Stretch-induced changes of ac, L/W ratio and IOD in the human PDL cells

The results of the calculation of Ac, L/W ratio and IOD are shown in Figure 2. The Ac of the cells in control group remained constant (Figure 2(a)). While the average Ac of the cells increased after 6 h stretch with 1, 10 and 20% strain and was bigger than that of the control group cells (Figure 2(a)). The L/W ratio of the cells in control group remained constant (Figure 2(b)). The L/W ratio of the cells increased after 0.5 h stretch with 10 and 20% strain and remained higher than that of the control group cells until 4 h stretch and then decreased to the control level after 6 h stretch. Two hour stretch with 1% strain also increased the L/W ratio of the cells (Figure 2(b)). The IOD of the cells decreased after 0.5 h stretch with 1, 10 and 20% strain, and then returned to the control lever after 1 h stretch. The IOD of the cells after 12 h stretch with 1 and 10% strain were higher than that of the control group cells (Figure 2(c)).

Stretch-induced gene expression of cytoskeletal regulators in the human PDL cells

In the present study, the 84 key genes related to cytoskeletal regulators were screened, and the Appendix table in the supporting material showed the effects of cyclic stretch on the expressions of these genes. A gene detected at a CT value of <35 was considered as being constitutively expressed.

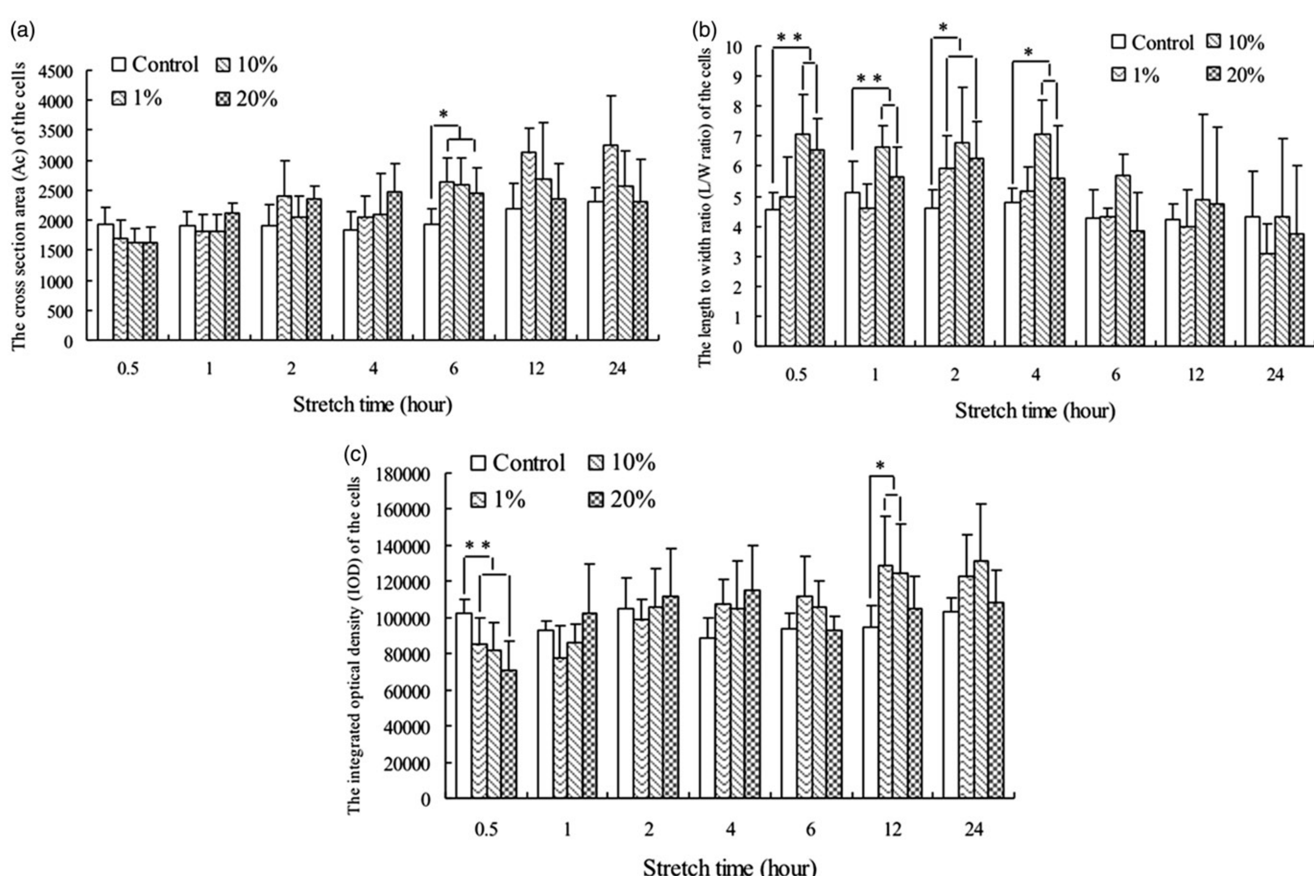

Figure 2. Changes of Ac, L/W ratio and IOD in human PDL cells in response to stretch. (a) Changes of Ac in human PDL cells in response to stretch. (b) Changes of L/W ratio in human PDL cells in response to stretch. (c) Changes of IOD in human PDL cells in response to stretch. Error bars stand for standard deviations ($n = 3$). $*p < .05$ versus control. $**p < .01$ versus control.

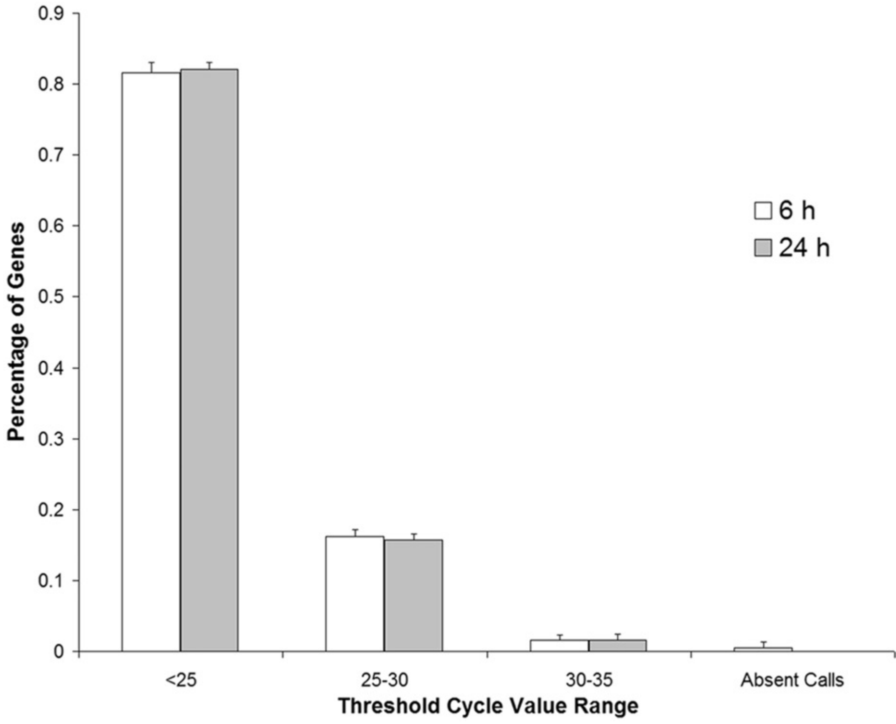

Figure 3. Histogram showing the mean cycle threshold [20] distribution for 6 and 24 h stretched cells. The mean values were determined from three replicate samples. Genes with CT values >35 (absent calls) were considered to lie outside the detection threshold of the system and were classified as not expressed. Error bars stand for standard deviations ($n = 3$).

Table 1. Differentially expressed genes in human PDL cells in response to 6 h cyclic stretch.

Name of gene	Description	Relative fold (stretched/control)	p value	Accession no. (GeneBank ID)
FNBP1L	Formin-binding protein-1-like	−2.29	.0246	NM_017737
MACF1	Microtubule-actin crosslinking factor 1	−1.66	.0243	NM_012090
PPP1R12B	Protein phosphatase-1, regulatory (inhibitor) subunit-12B	1.97	.0450	NM_002481

Table 2. Differentially expressed genes in human PDL cells in response to 24 h cyclic stretch.

Name of gene	Description	Relative fold (stretched/control)	p value	Accession no. (GeneBank ID)
CDC42EP2	CDC42 effector protein (Rho GTPase binding)-2	−4.42	.0339	NM_006779
NCK2	NCK adaptor protein-2	1.99	.0313	NM_003581
PIKFYVE	Phosphoinositide kinase, FYVE finger containing	2.43	.0315	NM_015040
STMN1	Stathmin-1	−4.71	.0342	NM_005563
WASL	Wiskott-Aldrich syndrome-like	2.23	.0247	NM_003941

The majority of these genes showed constitutive expressions after 6 and 24 h stretches (Figure 3).

Among the 84 screened genes, one gene (1.2%) was up-regulated while two genes (2.4%) were down-regulated after 6 h stretch (Table 1). Three genes (3.6%) were up-regulated while two genes (2.4%) were down-regulated after 24 h stretch (Table 2). These genes that were differentially expressed can be assigned to genes regulating polymerization/depolymerization of microfilaments (CDC42EP2, FNBP1L, NCK2, PIKFYVE and WASL), polymerization/depolymerization of microtubules (STMN1), interacting between microfilaments and microtubules (MACF1), as well as a phosphatase (PPP1R12B).

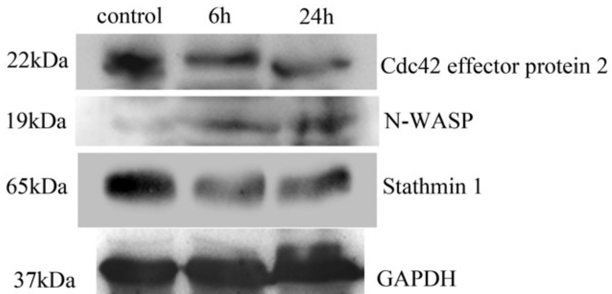

Figure 4. Protein bands of Western Blot experiments for the protein level of Cdc42 effector protein-2, N-WASP and Stathmin-1. The protein expression level of Cdc42 effector protein-2 and Stathmin-1 was down-regulated, while the protein expression level of N-WASP was up-regulated after both 6 and 24 h stretches.

Stretch-induced differential protein expression of cytoskeletal regulators in the human PDL cells

In the present study, the protein expression level of Cdc42 effector protein-2 and Stathmin-1 was down-regulated, while the protein expression level of N-WASP was up-regulated after both 6 and 24 h stretches (Figure 4).

Discussion

The cytoskeleton is a complex organ in eukaryotic cells and has been reported to function in multiple important cellular processes including cell migration, maintenance of cell morphology and signalling transduction [11]. Among the basic cytoskeletal components, microfilaments (also known as actin stress fibers) are known to be the basic tension-bearing structures in nonmuscle cells and are significantly important in lots of cell functions involving alteration in cell morphology such as cell realignment [21].

The confocal fluorescent photos confirmed that the cyclic stretch resulted in the realignment of the cells and rearrangement of microfilaments in the human PDL cells. After being stretched, the human PDL cells were prone to be paralleled to each other and realigned their long axis perpendicular to the direction of the stretching force. The microfilaments were elongated and thickened, with their long axis perpendicular to the stretching force vector, similar to the results of the previous reports [7,8,12]. The stretch-induced rearrangement of microfilaments could be explained in a self-protective mechanism: the microfilaments oriented along the direction of stretching force are predicted to experience excessive tension which leads to the disassembly of the microfilaments and the new microfilaments are reassembled in a new direction away from the stretching force vector. The thickened microfilaments are also a response of the cells to resist the stretch loading. In this way, the balance of cytoskeletal tension is re-established and the cells are protected from the injury of excessive tension. This self-protective mechanism may also be applied to the vital body in maintaining the periodontal homeostasis and remodeling of the periodontal tissue. The occlusal force applied to the teeth resulted in the proliferation of PDL cells and strengthened PDL fibers, and subsequently a widened PDL space, while reduction or lack of occlusal stimuli induced a decrease in the thickness of the PDL. Thus, the alignment of PDL cells perpendicular to the stretching force vector may be a characteristic essential to the maintenance of the PDL architecture [7,10]. Besides the realignment of the cells and rearrangement of microfilaments in response to stretch, several cells shrank to oval shape with their microfilaments hardly being optically discerned were observed after 24 h stretch with 20% strain. This phenomenon was consistent with the report that while tension was needed to assemble the actin stress fibers, excessive tension would destabilize them [21]. Take into account our previous finding that 24 h stretch with 20% strain could induce notable apoptosis in the human PDL cells [10,18], whether there is a link between the stretch-induced rearrangement of

microfilaments and the apoptosis is an interesting question and need to be further investigated.

The calculation of cross section area of the cells showed that the average Ac of the cells increased after 6 h stretch with 1, 10 and 20% strain, suggesting that stretch can directly deform the cells. With the stretch time increased to 12 and 24 h, the average Ac of the cells got back to the control level, showing the capability of the cells to adapt to and resist the stretch loading. Comparing with Ac, L/W ratio is a more sensitive index for the cell deformation. Half hour stretch with 10 and 20% strain increased the L/W ratio of the cells, showing that the cells were rapidly elongated by the stretch. The L/W ratio of the cells stretched with 10 and 20% strain remained higher than that of the control cells until 4 h stretch and then decreased to the control level after 6 h stretch. Accordingly, it is suggested that after the initial stage of passive deformation caused by the stretch loading, the cells re-established the balance of cytoskeletal tension and protected themselves from being elongated too much by the stretch. As for the 1% strain stretching group, 2 h stretch increased the L/W ratio of the cells as well, but the L/W ratio decreased to the control level with the stretch duration further elongated. This result indicated that cells are more capable of resisting and overcoming the deforming caused by minor stretch strain. The IOD represents the content of microfilaments in the cells. There was a rapid decrease of the IOD after 0.5 h stretch with 1, 10 and 20% strain, and then it returned to the control level after 1 h stretch, indicating that a rapid and transient disassembly of the microfilaments occurred at the initial stage of the stretch loading. The finding is consistent with Lee et al.'s [21] report that actin stress fiber disassembly in U2OS osteosarcoma cells was apparent within 5 min after initiation of 10% cyclic stretch at 1 Hz. The increase of the IOD after 12 h stretch with 1 and 10% strain but not the 20% strain indicated that physical mechanical stress enhanced the reassembly and formation of the microfilaments. Cheng et al. [22] also reported that shear stress of 12 dyne/cm^2 increased the stress fibers in endothelial progenitor cells following the rapid initial decrease in stress fibers after 5 min shear stress loading.

Microfilaments, as a key element of cytoskeleton, consist of polymers of actin filaments and can interact with many different proteins. In the process of rearrangement of cytoskeleton in response to many environmental stimuli, polymerization and depolymerization of microfilaments are quite necessary. In the present study, five genes regulating polymerization/depolymerization of microfilaments (CDC42EP2, FNBP1L, NCK2, PIKFYVE and WASL) were found to differentially expressed in response to cyclic stretch in the human PDL cells, and most of these genes have been reported to be related with the Rho family GTPase-signaling pathway. Rho family GTPases have been reported to play an important role in the force-driven reorganization of cytoskeleton. Lee et al. [21] reported that inhibition of Rho-kinase (ROCK) largely suppressed the stretch-induced rearrangement of stress fibers in endothelial cells. A recent report by Pan et al. [12] also concluded that cyclic stretch promoted cytoskeletal rearrangement of the human PDL cells by down-regulating the expression of Rho-GDIa and up-regulating the expression of GTP-Rho, ROCK and p-cofilin. Small Rho family GTPase, including Cdc42, RhoA and Rac1, relays cell surface signals to F-actin cytoskeleton [23]. Cdc42 can facilitate the activation of Neural Wiskott–Aldrich syndrome protein (N-WASP), which in turn contributes to the actin-nucleating activity of actin-related protein-2/3 (Arp2/3) complex at the leading edge of cells [24]. Actin nucleation appears to initiate the polymerization of actin filaments and to improve the cytoskeletal remodeling [25]. In the present study, FNBP1L was significantly down-regulated after 6 h stretch. FNBP1L encodes the formin-binding protein 1-like (FNBP1L). FNBP1L (also known as Toca-1) protein binds to both Cdc42 and N-WASP, and is an essential factor in activating N-WASP [26]. In the Cdc42 pathway mentioned above, the activated Cdc42 has interaction with both FNBP1L and the N-WASP-WIP complex, a predominant form of N-WASP in cells, which in turn leads to the activation of N-WASP and the polymerization of actin filaments [24]. Take into account the confocal fluorescent staining results showing the rapid decrease of the IOD which suggested the disassembly of the microfilaments at the initial stage of stretch loading, the down-regulated expression of FNBP1L (−2.29-fold to control) after 6 h stretch may reflect the disassembly of the microfilaments through the decrease of the polymerization of actin filaments in the human PDL cells at the early stage of stretching. After 24 h stretch, NCK2 (1.99-fold to control) and WASL (2.23-fold to control) were significantly up-regulated, while CDC42EP2 (−4.42-fold to control) was greatly down-regulated. NCK2 gene encodes NCK2 protein [27], while WASL gene encodes the above mentioned N-WASP protein (also known as Wiskott–Aldrich syndrome-like, WASL) [28]. NCK2 protein interacts with the proline-rich region of N-WASP through its SH3 domain, and acts as the most potent activator of N-WASP [29,30]. As mentioned above, N-WASP is a crucial component in the Cdc42-induced actin filaments polymerization and is ubiquitously expressed [31,32]. N-WASP can bind with actin and Arp2/3 simultaneously, which in turn activates the Arp2/3 complex [33]. As shown by the Western blot analysis, the protein expression level of N-WASP was up-regulated in response to 6 and 24 h stretches. This result was consistent with that of the real-time PCR array analysis and the up-regulation of both the gene and protein of N-WASP indicates the polymerization of actin filaments in response to cyclic stretching. Cdc42 effector protein-2, encoded by CDC42EP2 gene, is a downstream effector protein of Cdc42 [34] and can bind to, and negatively regulate Cdc42 [33]. Consistent with the result of the real-time PCR assay analysis, the protein expression level of Cdc42 effector protein-2 was detected down-regulated as well. Taking together the rearrangement of microfilaments confirmed by the confocal fluorescent staining with the stretch loading proceeded, the coordinated up-regulation of NCK2 and WASL and the down-regulation of CDC42EP2 after 24 h stretch may well reflect the enhanced polymerization of actin filaments during the process of cytoskeletal rearrangement in the human PDL cells to resist the stretch loading and avoid the injury of excessive tension. Furthermore, the up-regulation of PIKFYVE (2.43-fold to control) was also detected after 24 h stretch in the present study. The PIKFYVE gene encodes a FYVE finger-containing phosphoinositide kinase (PIKfyve) in human. It has been identified that PIKfyve could

produce PtdIns(3,5)P2 and PtdIns5P [35]. Recent studies have shown that PtdIns5P could facilitate the microfilaments dynamics. Although the detailed linkage between PtdIns5P and microfilaments remains to be elucidated, the clues point to the Rho GTPase pathway components [36]. It has been reported recently that PtdIns(3,5)P2 could function in actin nucleation and filament elongation for polarized growth [37]. The up-regulation of PIKFYVE may also be well-explained by the high demand for the actin nucleation and filament elongation during the process of the rearrangement of F-actin cytoskeleton in the human PDL cells with the stretching time increased. It requires for further studies to elucidate the delicate regulatory network regulating the polymerization/depolymerization of microfilaments in the periodontal remodeling in response to mechanical stress.

Microtubules, like microfilaments, play key roles in regulating the structure of cells as well. There was a remarkable decrease in the expression of the STMN1 gene (−4.71-fold to control) after 24 h stretch in the present study. The protein expression level of STMN1 was also down-regulated after both 6 and 24 h stretches, as detected by the Western blot analysis. STMN1 encodes Stathmin-1 (STMN1) protein, also known as oncoprotein-18. This protein has been characterized as a negatively regulatory protein of the microtubules assembly [38]. It has been reported that STMN1 blocks the polymerization of tubulin dimers, and in this way, inhibits the assembly of microtubules [38]. Meanwhile, STMN1 interacts directly with the ends of microtubules, and facilitates their disassembly as well [39,40]. D'Addario et al. [41] found that tensile force increased the accumulation of α-tubulin in human gingival fibroblasts. Therefore, the reduced expression of STMN1 gene and protein after 24 h stretch may be reasonably attributed to the increasing demand for the assembly of microtubules in the 24 h stretched human PDL cells.

Besides the aforementioned genes with regard to microfilaments and microtubules, the gene MACF1 encoding an interacting protein between actin filaments and microtubules was also detected down-regulated (−1.66-fold to control) after 6 h stretch in the present study. MACF1 is the gene encoding microtubule-actin crosslinking factor-1 (MACF1) [42,43]. MACF1 has a distinctive domain binding both microtubules and microfilaments [44]. This protein supports the actin–microtubule interaction and acts as a bridge between the two key cytoskeleton elements [45]. Like the down-regulation of the gene FNBP1L after 6 h stretch, the possible explanation for the down-regulation of MACF1 is that its reduced expression at the initial stage of stretching may protect the stretched cells from being excessively deformed and injured by the stretch loading, through the disassembly of the existing cytoskeletons which were incompatible with the stretching force. What's more, it is worth mentioning that cytoskeleton functions not only in cell mobility and cell shape, but also in many other cellular processes, including signalling transduction, intracellular transport and so on [46–48]. Thus, it is quite conceivable that the cytoskeleton-mediated signalling transduction and intracellular transport may be involved in the complicated and delicate mechanotransduction pathway in the cultured human PDL cells and requires for further investigations.

In the present study, there was a gene which encoded a phosphatase showing decreased expression in response to 6 h stretch (PPP1R12B, 1.97-fold to control). PPP1R12B encodes protein phosphatase-1 regulatory subunit-12B (PPP1R12B, also known as myosin phosphatase target subunit-2, MYPT2) [49], and a small regulatory subunit of myosin phosphatase (M20). The MYPT2 dephosphorylates myosin, and thus myosin cannot interact with actin filaments [50]. Consequently, the rearrangement of actin filaments is inhibited and the cell mobility is reduced [51]. The up-regulation of PPP1R12B together with the aforementioned down-regulation of FNBP1L and MACF1 after 6 h stretch may be well-explained by the self-protective mechanism that the stretched cells were protected from being excessively deformed and injured at the initial stage of stretch loading through the disassembly of the existing cytoskeletons which were incompatible with the stretching force. Additionally, reports have documented the inhibition effect of ROCK on MYPT2 [51,52], suggesting the pivotal role of ROCK in cytoskeleton rearrangement. Furthermore, PPP1R12B protein was also detected to have interaction with interleukin (IL)-16, indicating a possible connection between cytoskeleton rearrangement and immune responses [53]. Our previous studies have reported the up-regulation of CASP5 gene (encoding caspase-5, an inflammatory caspase) in response to stretch loading in the human PDL cells [15]. It has been reported that mechanical force provoked inflammatory reactions in periodontal tissue, including the release of several cytokines such as IL-1β, IL-6, IL-8, tumor necrosis factor-α (TNF-α) and interferon-γ [54–56]. Whether there is an interaction between force-induced cytoskeleton rearrangement and inflammatory reactions is an interesting question and requires for further investigation.

In summary, the present study confirmed that cyclic stretch-induced cell realignment and rearrangement of microfilaments in the human PDL cells. Several force-sensing genes implicated to cytoskeletal regulating were detected by real-time PCR array analysis. Furthermore, mechanical stress could induce several indirect effects on PDL cells. Many studies revealed programmed cell death in PDL under orthodontic forces in animal experiment *in vivo* [57,58]. Our previous studies have also reported the cyclic stretch-induced programmed cell death in PDL cells, together with the activation of NLRP inflammasomes and the release of pro-inflammation cytokine IL-1β [59,60]. However, the relationship between the cytoskeletal rearrangement and the stretch-induced cell death and inflammation remains unclear and need to be further studied. The results of the present study would provide potential target genes and proteins for further investigations on the cell realignment and the periodontal remodeling in response to mechanical stress. We hope the findings in the present study could help to probe into the mechanism of the force-related periodontal remodeling and destruction, and facilitate finding a new therapeutic target in the prevention and treatment of periodontal diseases in the future.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by National Natural Science Foundation of China (grant numbers 31470903, 31270991, 30900282); Shanghai Pujiang Program (grant number 13PJD021); Science and Technology Commission of Shanghai Municipality (grant numbers 10QA1404200, 08411961500, 07ZR14070); Shanghai Summit & Plateau Disciplines; Shanghai Leading Academic Discipline Project (grant numbers S30206-sms02, T0202).

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