International Journal of Oral Biology, Vol. 33, No. 2, June 31 2008, p. 51~58

International Journal of Oral Biology

The effect of *UNCL* inactivation on the expression of mechanical stress related genes in cultured human PDL fibroblasts

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(Received April 14, 2008 ; Revised May 13, 2008 ; Accepted May 14, 2008)

A mutation of UNCL, an inner nuclear membrane RNA-binding protein, has been found to eliminate mechano-transduction in Drosophila. UNCL is expressed in human periodontal tissue including in periodontal ligament (PDL) fibroblasts. However, it is unclear how a mechanical stimulus is translated into cellular responses in PDL fibroblasts. The aim of this study was to evaluate the effect of UNCl on mechanical stress related genes in PDL fibroblasts in response to mechanical stress. The mRNA of TGF-β, COX-2, and MMP-2 was up-regulated after UNCL inactivation in PDL fibroblasts under the compression force. Under the tensile force, inactivation of UNCL decreased the expression of Biglycan, RANKL, MMP-2, and TIMP-2 mRNAs while it increased the expression of TIMP-1. p38-MAPK was expressed in PDL fibroblasts under compression forces whereas phospho-ERK1/2, p65-NFkB, and c-fos were expressed under tension forces. The expression and phosphorylation of the mechanical stress related genes, kinases, and transcription factors were changed according to the types of stress. Furthermore, most of them were regulated by the inactivation of UNCL. This suggests that UNCL is involved in the regulation of mechanical stress related genes through the signaling pathway in PDL fibroblasts.

Key words: UNCL, Mechanical stress, PDL fibroblast, Inactivation

Introduction

The periodontal ligament (PDL) is a highly specialized connective tissue located between the cementum of the tooth root and alveolus of the alveolar bone. PDL is very important for maintaining the health and homeostasis of the tooth, such as nutrition, inhibition of root resorption, and the absorption of shock from stress (Liu *et al*., 2006). PDL fibroblasts, in particular, can differentiate in order to contribute to the regeneration and repair of the PDL and the remodeling of the surrounding hard tissue, cementum and alveolar bone (Lekic and MacCulloch, 1996). Furthermore, PDL fibroblasts can undergo differentiation in response to a variety of extracellular stimuli (Carnes *et al*., 1997). These cells are subject to continuous mechanical strain under physiological conditions, i.e., masticatory forces, and during orthodontic and orthopedic treatment (Wilde *et al*., 2003). However, it is unclear how a mechanical stimulus is translated into cellular responses in PDL fibroblasts.

Mechanical stress to the PDL is divided into tensile, compressive, and shear stress (Kaneko *et al*., 2001). The appropriate force to the PDL is very important for maintaining the health of the PDL. These forces induce a variety of effects through various pathways. In previous reports, mechanical stress plays important roles in the PDL, including cell proliferation, differentiation, survival, and gene expression (Katz *et al*., 2006). Many studies have focused on maintaining the periodontium from various

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mechanical stresses (Yamaguchi *et al*., 2005).

UNCL was identified and isolated from *C. elegance.* The mammalian homologue of *UNCL* (uncoordinated-like) or *UNC* (uncoordinated) was found in the mouse and rat. UNCL was expressed preferentially in human PDL fibroblasts compared with gingival fibroblasts (Park et al., 2001). UNCL protein is an integral membrane protein that localizes to the inner nuclear membrane, endoplasmic reticulum, and Golgi complex. In yeast, UNCL is involved in membrane trafficking (Chantalat *et al*., 2003). However, a mutation of *UNCL* in *Drosophila* larvae caused no responses to mechanical stress (Fitzgerald *et al*., 2000). We recently suggested that *UNCL* might play an important role in the development, differentiation, and maintenance of the periodontal tissue and PDL fibroblasts (Kim *et al*., 2007). It was also observed that the application of mechanical stress on PDL fibroblasts *in vivo* and *in vitro* changed the expression of UNCL mRNA.

To gain more insights into the specific roles of *UNCL* in response of PDL fibroblasts to mechanical stress, we examined the gene expression involved in the responses of PDL fibroblasts to controlled mechanical stimuli after *UNCL* inactivation through RNA interference. In addition, the altered expression of mechanical stress-induced kinases and some transcription factors were investigated by western analysis.

Material and Methods

Cell culture

PDL fibroblasts were obtained from an explant culture of healthy human PDL tissue. This study protocol was approved by the Chosun University Dental Hospital Institutional Review Board (CIRB2007-3). Informed consent was obtained from all subjects. These biopsies were performed under the guidelines of the Chosun University for Animal and Human Subjects Research (Korea). Briefly, the PDL tissue attached to the middle third of the root were minced, plated and cultured in Dulbecco's modified Eagle medium (Gibco BRL, USA) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics at 37°C in humidified air containing 5% CO_2 (Kim *et al*., 2007). Three to five passage cells used in this study.

siRNA synthesis and reverse transfection

The human *UNCL* gene based small interference RNA duplex, siRNA, was designed, synthesized and provided with the cooperation of Sigma-Proligo (Sigma-Proligo, Singapore). Table 1 shows the siRNA duplex sequences. Reverse-transfection to PDL fibroblasts was performed using the Lipofectamin RNAi MAX® reagent (Invitrogen, USA) according to the manufacture's instructions. 10 pmol and 100 pmol of the siRNA duplexes and 5×10^4 cells/dish of PDL fibroblasts were used in this study. After introducing siRNA to the cells, 10 mM glycerol-2-phospahte (Sigma-Aldrich, USA) and 50 μg/ml ascorbic acid (Sigma-Aldrich) were added. Finally, DMEM was added to 6 ml.

Mechanical stress application

Mechanical stresses were applied to the following six groups; control group, siRNA only treated group, compressive force only applied group, compression plus siRNA treated group, tensile force only applied group, and tension plus siRNA treated groups. For the application of compressive force, the PDL fibroblasts were compressed using a pair of weights for 1 hour and 2 hours each. A flame sterilized 24 × 24 mm glass was laid on the cells after they had reached 90% confluence. Two of the 3 g metal weights were placed over the cover glass. This mechanical stress application model was modified from a previous report (Kanzaki *et al*., 2006). Another mechanical stress, tensile force were applied using a flexible bottom cell culture dish, petri-PERM® (Vivascience, Germany), and a pair of 88 g metal weights. petri-PERM® was laid on a 35 mm cell culture dish and a pair of weights were laid on the cover of the petri-PERM®. A tensile force was applied for 1 hour and 2 hours after inactivation of UNCL mRNA. This method was modified from a previous report (Yamaguchi *et al*., 2005).

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

After transfection, the cells were washed with PBS. The total RNA was isolated using the TRIzol® reagent (Invitrogen) according to the manufacture's instruction. The cDNA was synthesized using a SuperScrip II First strand cDNA synthesis kit® (Invitrogen) and oligo-dT primer (Invitrogen).

The inactivation efficiency of UNCL mRNA was evaluated

Table 1. siRNA sequences used for the RNA interference of UNCL.

siRNA-1	Forward	GGAAAUACCUUAUGGUUGAdTdT	POS
	Reverse	UCAACCAUAAGGUAUUUCCdTdT	873
siRNA-2	Forward	CUUCUGAUCUCAACGUUAAdTdT	POS
	Reverse	UUAACGUUGAGAUCAGAAGdTdT	672
siRNA-3	Forward	CUCUUUCGAUUUCGGCAGAdTdT	POS
	Reverse	UCUGCCGAAAUCGAAAGAGdTdT	405

Table 2. Primers used for RT-PCR.

Target gene	Forward (5'-3')	Reverse (5'-3')
UNC-50	ACG TGG GAA TCG CAG GAT	CCT TCC CAA CAC CAG ACA GT
GAPDH	CCA GAT CAT GTT TGA GAC CT	GTT GCC AAT AGT GAT GAC CT
RANKL	AGG GAG CAC GAA AAA CTG GT	CCA GAG ACT GTG ACC CCC TT
OPG	GAC ACC TTG AAG GGC CTG AT	CAT GCT TGG CTT TCT GGG TA
Osteocalcin	GGA CCT GTG CTG CCC TAA AG	CTG CTG TGA CAT CCA TAC TTG
MMP-2	AGA AAA GAT TGA CGC TGT GT	CTT CAC GCT CTT GAG ACT TT
TIMP-1	ACC ACC TTA TAC CAG CGT TA	AGT GTC ACT CTC CAG TTT GC
TIMP-2	TCA GAT CCA TCT CAT TTT CC	GGA GTC CTT AAC CGT TTC TT
Cox-2	AGA AGG GTT CCC AAT TAA AG	ATA ATT TTT CCC TCC AAA GG
TGF-β	TGG TGG AGA GAA GAG GAA AA	TAA TTT GAG GTT GAG GGA GA

at a siRNA concentration of 10 and 100 pmol. In addition, the continuance of siRNA was evaluated for up to 72 hours at 10 and 100 pmol of siRNA. The expression of the mechanical stress related mRNAs were evaluated by RT-PCR using the specific mRNA primers for decorin, biglycan, osteoprotegrin (OPG), cyclooxygenase-2 (COX-2), transforming growth factor-β (TGF-β), receptor activator of NF-kappa B ligand (RANKL), tissue specific inhibitor of metalloprotease-1, -2 (TIMP-1, TIMP-2), osteocalcin, and matrix metalloprotease-2 (MMP-2). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA specific primer was used as the control. Table 2 shows the specific primer sets used in this study. The target bands were imaged using image capture equipment Gel Doc (Bio-Rad, USA) and examined using NIH image software Image J V1.37 (NIH). Volume densities of the samples were normalized against the GAPDH. Triplicated samples were analyzed. Statistical analysis was performed using one-way analysis of variance (ANOVA, $P < 0.05$).

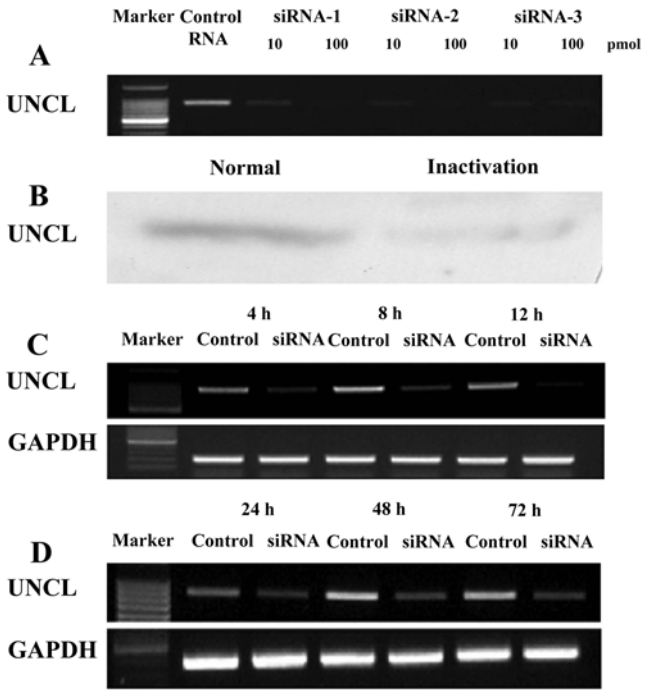

Fig. 1. Inactivation efficiency of the UNCL siRNA in PDL fibroblasts. Among the three duplex strains tested, the siRNA-2 showed the best inactivation efficiency at 100 pmol and 12 hrs after transfection in RT-PCR analysis (A). The inactivation of UNCL mRNA decreased the expression of UNCL protein in western analysis (B). UNCL mRNA was most effectively inactivated after 8 to 12 hours transfection in RT-PCR analysis (C and D).

Protein extraction and Western blot

For nuclear membrane integrated protein extraction, the cells were harvested 12 hours after transfection and the hydrophobic membrane bounded UNCL protein of the PDL fibroblasts were isolated using a Mem-PER® protein extraction kit (Pierce, USA) according to the manufacture's instruction.

Twelve hours after transfection, the cells were washed with PBS and treated with 0.5 ml of the hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF). After homogenization and centrifugation, the supernatant was taken and used for cytoplasmic protein analysis. The pellet was treated with 10 μl of a high salt lysis buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 25% Glycerol, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT) and incubated on ice for 20 min. A 5 fold (v/v) excess of the storage buffer (20 mM HEPES, pH 7.9, 100 mM NaCl, 20 % Glycerol, 0.2 mM EDTA, 0.5 mM PMSF) were added. The mixture was then centrifuged and used as the nuclear extracts.

After protein transfer from 10% SDS PAGE, the nitrocellulose membrane (ECL hybond; Amersham Bioscience, UK) was blocked with 5% non-fat dry milk in 0.05% Tween-20/PBS (PBS-T). The membranes were washed 3 times with PBS-T. The activation of mechanical stress induced kinases was evaluated using anti-ERK1/2 (Cell signaling, UK; 1 : 1000), anti-p38 MAPK (Cell signaling; 1 : 1000), anti-*c-fos* (Oncogene, USA; 1 : 400), anti-p65 NFkB (Santa Cruz, USA; 1 : 1000) as the primary antibodies for 1 h at room temperature. Anti-UNCL (Peptron, Korea; 1 : 500) and anti-GAPDH (Lab frontier, Korea; 1 : 10,000), as the primary antibodies, were added and the mixture was

incubated for 1 hour at room. The membranes were exposed to HRP-conjugated goat anti-rabbit IgG, as the secondary antibody, for 30 min at room temperature. The bands were revealed using ECL hyper film and an ECL chemiluminescence detection system (Amersham Bioscience).

Results

Inactivation of UNCL by siRNA in PDL fibroblasts

Among the three types of duplexes, the siRNA duplex-2 showed the best inactivation efficiency at 100 pmol and 12 hours after transfection. The inactivation efficiencies of UNCL mRNA were approximately 70~80% at 10 and 100 pmol of siRNA (Figure. 1A). The hydrophobic membrane-bounded UNCL protein was weakly detected in western blot after inactivation of UNCL mRNA (Figure 1B). Although the continuous basal expression was detected steadily for up to 72 hours, UNCL mRNA was most effectively inactivated after 8 to 12 hours transfection (Figure 1C, D).

Morphologically, PDL fibroblasts were elongated in the compressive force applied group, and the cellular processes were augmented in the compression plus siRNA treated group compared with the compressive force only applied group. The cells also showed a slightly slender shape in the tension plus siRNA treated group compared with the tensile force only applied group (data not shown). However, there were no morphological changes among the control, siRNA treated group, and tensile force applied group.

Effect of UNCL inactivation on the expression of mechanical stress-related genes in PDL fibroblasts

The level of UNCL mRNA expression had decreased by approximately 60% of the level observed under compression (Figure 2A), whereas it had increased by approximately 20% of the level under tension (Figure 2B).

The expression of decorin, osteocalcin, TGF-β, RANKL, and MMP-2 mRNA was increased under the compressive force (Figure 3A, B, D, E, G). Furthermore, TGF-â, MMP-2, and Cox-2 mRNAs were significantly increased by inactivation of *UNCL* in PDL fibroblasts under the compressive force ($P < 0.05$, Figure 3D, G, H). In contrast, the level of decorin, osteocalcin, biglycan, RANKL, osteoprotegrin, MMP-2, Cox-2, and TIMP-2 mRNA was increased under the tensile force (Figure 3A, B, C, E, F, G, H, I). Under tension, inactivation of *UNCL* by siRNA significantly decreased the expression of biglycan, RANKL, MMP-2, and TIMP-2 mRNAs in PDL fibroblasts ($P < 0.05$, Figure 3C, E, G, I). Decorin, osteocalcin, TGF-β, and osteoprotegerin mRNAs did not show any significant differences in their expression between tensile force only applied and tensile force plus siRNA treated group ($P>0.05$, Figure 3A, B, D, F, G).

Effect of UNCL inactivation on the expression of mechanical stress related kinases and transcription factors in in PDL fibroblasts

UNCL protein expression was not detected in either the compressive force applied group or compression plus siRNA treated group. However, UNCL protein was strongly expressed in PDL fibroblasts under the tensile force. When compared with the control group, the level of UNCL protein expression was slightly lower in the tension plus siRNA treated group than in the tensile forces only applied group (Figure 4A). A type of mechanical stress related transcription factor, NFkB, was also analyzed. The level of p65-NFkB expression was slightly decreased by *UNCL* inactivation during tensile force application (Figure 4B). However, there was no translocation to the nucleus detected in any group (data not shown). p38-MAPK was only expressed under the compression force. The expression was slightly increased by inactivation of *UNCL* during compressive force application in PDL fibroblasts (Fig. 4C). Between ERK-1 and ERK-2, phospho-ERK-2 (p42) was only detected in the tensile force only applied and tension plus siRNA treated group (Figure 4D). The other mecha-

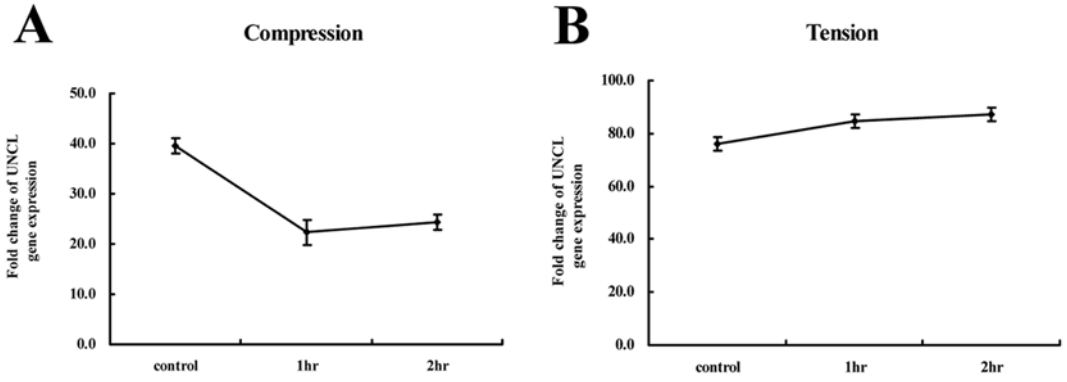

Fig. 2. Expression of UNCL mRNA in PDL fibroblasts during the compressive and tensile force application. Expressions of UNCL mRNA were analyzed by RT-PCR 12 hours after transfection under compression and tension. Under the compression force, UNCL mRNA was decreased in a time dependent manner (A), while it was increased under the tensile force (B).

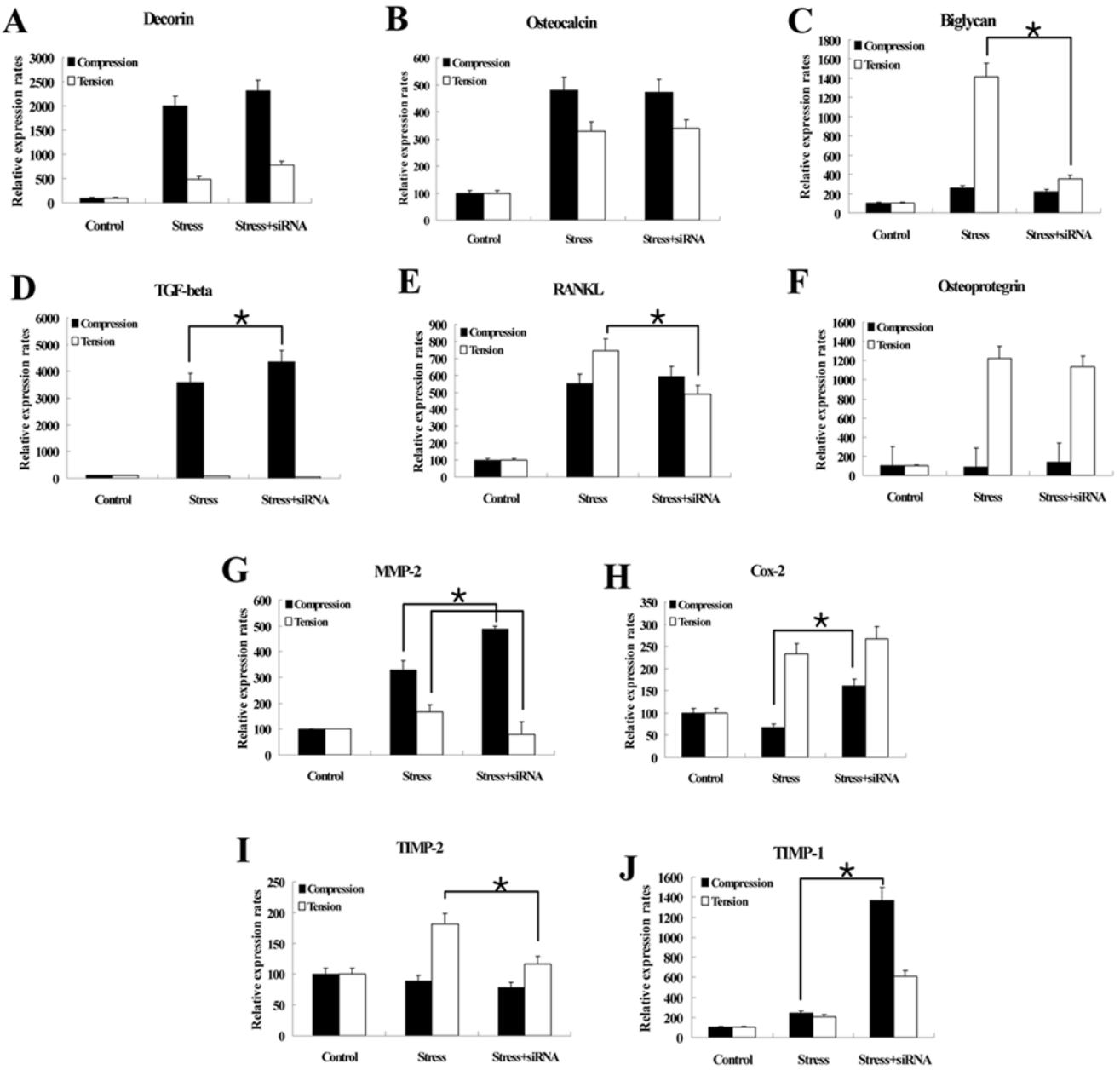

Fig. 3. Expression of mechanical stress related genes in PDL fibroblasts after UNCL inactivation. The gene expression was analyzed by RT-PCR. The genes were decorin (A), osteocalcin (B), biglycan (C), TGF-β (D), RANKL (E), oteoprotegrin (F), MMP-2 (G), COX-2 (H), TIMP-2 (I), TIMP-1 (J). Significant differences after UNCL inactivation were noted by asterisks (*, $P<0.05$).

nical stress related transcription factor, *c-fos*, was expressed in the control and tensile force only applied group. However, there was no remarkable difference between the control and tensile force only applied group (Figure 4E). GAPDH was used to normalize the expression levels (Figure 4F).

Discussion

There are a few reports that *UNCl* is involved in mechano-transduction in various tissues, including PDL (Fitzgerald *et al*., 2000; Park *et al*., 2001; Kim *et al*., 2007). At the base of this supposition, the involvement of *UNCl* in mechano-transduction of PDL fibroblasts was analyzed by evaluating the expression and phosphorylation of the mechanical stress related genes, kinase, and transcription factors after *UNCl* inactivation. In the present study, the highest UNCL mRNA inactivation efficiency was observed 8 to 12 hours after transfection. Considering the inactivation efficiency, the RNA and protein were extracted 12 hours after transfection and used for RT-PCR and western analysis. The siRNA

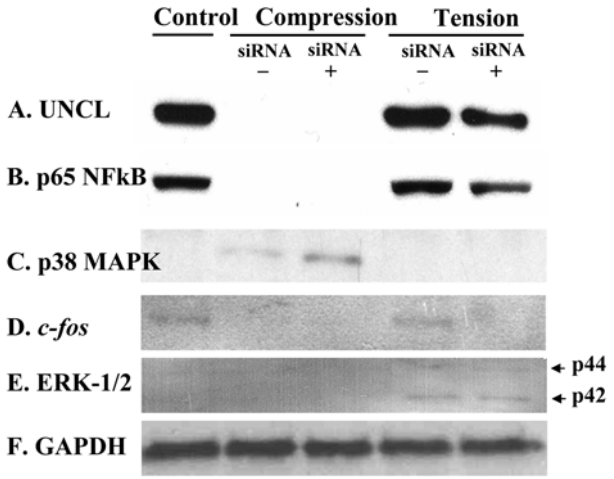

Fig. 4. Expression of mechanical stress related kinases and transcription factors in PDL fibroblasts after UNCL inactivation. The UNCL protein was decreased in the tension plus siRNA treated group compared with the control in western blot analysis (A). The level of p65-NFkB expression was slightly decreased by UNCL inactivation during tensile force application (B). p38-MAPK was expressed in the compressive force applied and compression plus siRNA treated group (C). Phospho-ERK-2 (p42) was detected in the tensile force only applied and tension plus siRNA treated group (D). c-fos was expressed in the control and tensile force only applied group (E). GAPDH was used to normalize the expression levels (F).

concentration was 20 nM at 100 pmol, which was sufficient for inactivating the target gene. Furthermore, the reverse transfection method showed a high transfection and inactivation efficiency than the forward transfection methods (data not shown). The reverse transfection method also has some advantages in the experiments with the cell, which include a low risk of contamination and low stress to the cell (Ovcharenko *et al*., 2005).

The molecular interactions between the receptor activator of NF-kappa B (RANK) and RANK ligand (RANKL) confirm the hypothesis that osteoblasts play a key role in osteoclastic differentiation (Khosla, 2001). The PDL induced osteoclastogenesis through the up-regulation of RANKL under compression. However, PDL does not induce osteoclastogenesis *via* the up-regulation of OPG and TGF-β under the tensile force (Kanzaki et al., 2002). In the UNCL mRNA inactivated state, the level of RANKL mRNA expression showed a significant difference under the tensile force. These differences were strongly induced by the inactivation of UNCL mRNA by siRNA, e.g. RANKL mRNA was suppressed under tension. These regulation patterns of RANKL and OPG mRNA in PDL fibroblasts resemble those of the osteoblasts cell line, MG-63 (Liu *et al*., 2006).

In the present study, MMP-2 and TIMP-2 mRNA expression was decreased after inactivation of *UNCL* under tension. Tissue degradation inducible protease, MMP-2 mRNA, was expressed differently according to the types of mechanical stresses in PDL and osteoblasts (Tsuji *et al*., 2004). An increase in MMP-2 mRNA expression induces an increase in their inhibitors, TIMP-1 and TIMP-2, in human PDL and osteoblasts (Bolcato-Bellemin *et al*., 2000; Morgunova *et al*., 2002). An increase in TIMPs and MMPs facilitates bone and periodontium turn-over (Takahashi *et al*., 2003).

Osteocalcin, decorin, and biglycan are well known mechanical stress inducible periodontium remodeling related genes (Long *et al*., 2002). Decorin mRNA was changed or increased under compression but not changed under tension (Majima *et al*., 2000). The level of biglycan mRNA was increased slightly under compression while unaffected by tension (Evanko *et al*., 1993). Osteoclacin mRNA was decreased under hydrostatic pressure or compression (Tasevski *et al*., 2005). However, osteocalcin production was stimulated at the initial stage of culture by cyclic tension and relaxation force, and its secretion might decrease with time (Miyajima *et al*., 1991). In this study, the level of osteoclacin mRNA was increased in the mechanical stress applied groups but there was no differences compared with siRNA treated groups. This might be caused by the time needed to facilitate and inhibit osteocalcin mRNA expression. The level of decorin mRNA expression was increased after the mechanical stress application. However, inactivation of *UNCL* dramatically decreased the expression of biglycan mRNA under the tension. These results suggest that *UNCL* is involved in the mechano-transduction pathway to control the expression of biglycan. Another tissue degradation and immune response related enzyme, COX-2, is also important in maintaining the periodontium under mechanical stress. The level of COX-2 and TGF-â mRNA was increased under stress in human PDL, bone and cementoblasts (Shimizu *et al*., 1998; Mada *et al*., 2006).

The activation of kinases and transcription factors occur when cells are exposed to stress. Among the mechanical stresses related transcription factors, NFkB and *c-fos* are very important in the cellular responses (Li *et al*., 2006; Mogi *et al*., 2004). In this study, p65 NFkB, a sub-unit of NFkB, was expressed during tensile force application but the expression was slightly down-regulated by *UNCL* inactivation. However, the translocation of the p65-NFkB protein to nucleus could not detect in nuclear extracts (data not shown). The expression of c-fos was also decreased by *UNCL* inactivation. It suggests that *UNCL* might mediate the function of NFkB and *c-fos* during mechanical stress application in PDL fibroblasts.

Mechanical stress also activates ERK1/2, and p38-MAPK in osteoblasts and osteoclasts. However, it was reported that there were no changes in ERK phosphorylation in PDL after stretching (Kapur et al., 2003; Ogata, 2003). A member of the MAP kinase, p38-MAPK, plays an important role in the induction of MMP-2 by tensile force (Grabellus *et al*., 2007). In this study, phosphorylations of ERK-1 and -2 were

detected by western blot under tension. However, there were no remarkable differences between the tension applied and tension plus siRNA treated group. Therefore, mechano-transduction through the ERK pathway was not mediated by *UNCL* in PDL fibroblasts. The activation of p38-MAPK was increased considerably by *UNCL* inactivation under the compression. It also suggests that p38-MAPK might be regulated by *UNCL* under the compression. In the present study, the role of *UNCL* in mechano-tranduction of PDL fibroblasts was investigated. The expression and phosphorylation of the mechanical stress related genes, kinases, and transcription factors were changed according to the types of stress. Furthermore, most of them were regulated by the inactivation of *UNCL*. The results suggest that *UNCL* plays important roles in the mechano-transduction by regulating the mechanical stress related genes, kinases and transcription factors in PDL fibroblasts.

Acknowledgement

This work was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MOST) (No. M10646010002-06N4601-00210).

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