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Effect of UNC-50 inactivation on the expression of genes related to mechanical stress in cementoblast cells (OCCM-30)

조 선 대 학 교 대 학 원

치 의 학 과

최 용 석

백악모세포 *(OCCM-30)*에서 기계적 자극과 관련된 유전자 발현에 미치는 *UNC-50*의 기능억제 효과

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지도교수 박 주 철

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조 선 대 학 교 대 학 원

치 의 학 과

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By Yong-Seok Choi

Advisor : Prof. Joo-Cheol Park, D.D.S., M.S.D., Ph.D.

Department of Dentistry

Chosun University

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Approved by

Professor Joo-Cheol Park

Major Advisor :

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ABSTRACT

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Yong-Seok Choi

Advisor : Prof. Joo-Cheol Park, D.D.S., M.S.D., Ph.D.

Graduate School of Chosun University

The Periodontal ligament (PDL) is a soft tissue that is continuously exposed to mechanical loading by occlusion and mastication. To keep the health of PDL is very important to maintain health of periodontium. Among the periodontium, the cementum is a mineralized tissue around root surface that acts as a shield from mechanical stress-induced root absorption. Mammalian homologue of the *C. elegans* gene UNC-50 (UNCL) was found in human, rat and mouse PDL fibroblasts. In a previous study, it was suggested that the UNC-50 might be related to mechanical stress signal transduction in PDL. Although the function of UNC-50 during periodontium development were suggested, Its effects on signal transduction in PDL and cementoblast is not well known. Small interfering RNA (siRNA) or RNA interference (RNAi) phenomenon was first discovered in *C. elegans* and was characterized by sequence-specific, post-transcriptional gene silencing. RNAi is rapidly adopted for functional genomics and pathway analysis. Recently, siRNA has been widely used in mammalian genomic experiments. In this

study, to reveal the effect of the UNC-50 to expression of mechanical stress related genes, kinases and transcription factors in cementoblast cell (OCCM-30), RT-PCR and western blot were performed under various conditions, including stress application and inactivation of UNC-50 mRNA by siRNA.

In the present study, the cell culture conditions were divided into six groups; control, inactivation of UNC-50 by siRNA, tensile force applied, compressive force applied, tension plus siRNA and compression plus siRNA group. The effects of UNC-50 on mechanical stress in OCCM-30 were analyzed by RT-PCR and western blot analyses.

MMP-2, TIMP-1, byglycan, RANKL, decorin, GP130 and TGF-β mRNAs were up-regulated under compression by RT-PCR. Cox-2, TIMP-1, MMP-2, and decorin mRNAs were also up-regulated at compression plus siRNA. However, the expression of MMP-2, TIMP-1, RANKL, and OPG mRNAs were down-regulated under tension. Expression of p38-MAPK was detected only under compression plus siRNA group by western blot. Expression of phospho-ERK-2, *c-fos*, and p65-NFkB was observed under tension. However, *c-fos* was not expressed and p65-NFkB weakly expressed at tension plus siRNA.

These results support that inactivation of UNC-50 mRNA affected the expression of mechanical stress related gene, kinase and transcription factors in cementoblast cell line, OCCM-30. It was also suggested that mechano-transduction pathway of UNC-50 was thought to be involved in p38-MAP kinase pathway and partially involved with NFkB signaling. Therefore, up-regulation or inactivation of UNC-50 mRNA caused

expression of various mechanical stress related gene, kinase and transcription factors and showed different expression patterns by types of mechanical stress in cementoblasts cell, OCCM-30.

I. INTRODUCTION

Cementoblast synthesizes cementum and are mainly found in lacunae at apical third of tooth root. Cementoblast has similarities with osteoblast in reaction to mechanical stress and their products, cementum and bone. However, there are several differences between cementum and bone. The most distinct differences are reaction pattern between bone and cementum to environmental changes such as remodeling in bone and limited repair capacity in cementum [1]. In addition, cementum is avascular tissue. Therefore, formation and destruction of cementum are very important in reaction pattern to keep health of periodontal ligament (PDL) and maintain periodontium.

To keep the health of PDL, appropriate force to PDL is very important. Mechanical stresses to PDL are divided into some kinds of categories which are tensile, compressive and shear stress [2]. These forces induce various effect and throughout the various signal transduction pathways. In the previous reports, mechanical stress plays important roles in PDL, such as cell proliferation, differentiation, survival and gene expression [3,4]. Many researchers are focused on the maintenance of periodomtium from various mechanical stresses.

UNC-50 was originally identified and isolated from the *C. elegance*. The mammalian homologue of UNC-50 (*unc; uncoordinated*) or (*uncl; uncoordinated-like*) was also found and isolated in mouse and rat. The UNC-50 was differently expressed in human PDL fibroblast compared to gingival fibroblast [5]. It is consisted of 256 amino acids and has five

transmembrane domains. It is generally located inner nuclear membrane, endoplasmic recticulum and Golgi complex. In yeast, UNC-50 involved in membrane trafficking [6]. Mutation of UNC-50 leads no responses to mechanical stress in *Drosophila* larvae [7]. Recently, it was suggested that UNC-50 might play an important role in the development, differentiation, and maintenance of periodontal tissue. It was also suggesting potential role of UNC-50 in the mechanotrasduction of PDL fibroblast [8]. However, the effect of UNC-50 on the expression of mechanical stress induced genes, kinases and transcription factors in cementoblast was not well understood.

Small interfering RNA (siRNAi) is widely used in recently to study gene function, validate candidate drug targets, and perhaps even treat disease [9]. One of the critical function of siRNA is defined in the control of gene expression and chromosome behavior [10]. Long double strand RNA (dsRNA) is cleaved by RNase III endonuclease and produce 21-23 base pair short siRNA. The siRNA is bind to complementary mRNA and cleavage. Therefore, siRNA acts as sequence-specific gene silencer [11].

In this study, the effect of UNC-50 on mechanical stress responsive gene were investigated after application of mechanical stress.

II. MATERIALS AND METHODS

1.Cell culture

Cementoblast cell line, OCCM-30, was kindly provided from professor Sommerman M.J. (School of Dentistry, University of Washington, Seattle, Washington). Collected cells were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L glucose (Gibco, Invitrogen Lifetechnology, CA), non-essential amino acid (NEAA; Invitrogen) of a ratio of 100 : 1 supplemented with 5 % fetal bovine serum (FBS; Invirtrogen), penicillin (100 IU/㎖) (Invitrogen) and streptomycin (100 ㎎/㎖) (Invitrogen) in 5 % of humidified CO_2 incubator.

2. siRNA synthesis and Reverse transfection to OCCM-30

Small interference RNA duplex, siRNA, was synthesized and provided from Sigma-Proligo Cooperation (Sigma-Proligo, Boulder). Three duplexes were designed based on mouse homolog of the *C. elegance* UNC-50 (Gene bank No.***BC019484***). Designed sequences of siRNA are shown in Table 1. Reverse transfection to OCCM-30 was performed using Lipofectamin RNAi MAX® reagent (Invitrogen). Ten or a hundred pmol of siRNA duplexes was mixed with 500 ㎕ of Opti-MEM® (Invitrogen) in a 1.7 ㎖ micro-centrifuge tube. Lipofectamin RNAi MAX® reagent was mixed with 500 ㎕ of opti-MEM in another 1.7 ㎖ micro-centrifuge tube. siRNA mixture blended into lipofectamin mixture in a new 1.7 ㎖ micro-centrifuge tube and incubate 15 min at room temperature. At the same time, cultured OCCM-30

cells were washed with phosphate buffed saline (PBS) and harvested with 0.025 % Trypsin-EDTA (Invitrogen) solution. After 15 min, OCCM-30 cells (5×10^4) were mixed with lipofectamin-siRNA complex solution. After mixing, cell suspensions were poured into 60 mm dish and added 5 ㎖ of medium containing 10 mM glyceol-2-phospahte (Sigma) and 50 ㎍/㎖ ascorbic acid (Sigma). Consequently, final concentration of siRNA is 20 nM at 100 pmol.

Table 1. UNC-50 specific siRNA duplex .

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

3. Immunofluorescence staining of UNC-50

Cells were fixed with ice-cold methylalcohol for 10 min and washed out with PBS containing 0.2 % bovine serum albumin (BSA; Sigma). After washing, permeabilization was performed with 0.1 % triton X-100 (Sigma) in PBS for 10 min. After washing, polyclonal anti-UNC-50 antibody was treated as primary antibody and fluoresceine-conjugated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) was treated as secondary antibody. Cells were observed under fluorescent microscope (Axioscope, Carl Zeiss, Halbergmoos, Germany) and took picture with image capture software axovision V4.2 (Carl Zeiss).

4. Mechanical stress application

According to the mechanical stress methods the experimental groups were divided into six groups; control, transfection of siRNA only, compressive force applied, compression and siRNA, tensile force applied and tension plus siRNA groups.

For the compressive force application, OCCM-30 cells were compressed using a pair of weights for 15 min, 30 min, 1 hr, 2 hr and 10 hrs. A flame sterilized 100 × 100 size cover glass was laid on OCCM-30 mono layer at 90 % of confluent. Two of 3 g metal weights were placed on the cover glass. After appropriate time, the cells were harvested and subjected to next experiments (Figure 1). This method was modified from previous report [12]. Other type of force, tensile force, was applied using flexible bottom cell culture dish, petri-PERM® hydrophilic (Vivascience,. Hannover, Germany), and a pair of cylinder shape metal weights. Each of weight has 88 g. petri-PERM® was laid on 35 mm cell culture dish and a pair of weights were laid on the cover of petri-PERM®. Tensile force were applied for 1 and 2 hrs after inactivation of UNC-50 and normal state (Figure 2).

In the present study, mechanical stress model was established by modification of previously reported methods by Yamaguchi and Kasai [13].

5. Reverse treascriptional polymerase chain reaction (RT-PCR)

After transfection, cells were washed out with PBS. Total RNA was extracted using by TRIzol® reagent (Invitrogen) according to the manufacture's instruction. cDNA was synthesized using by SuperScrip II

First strand cDNA synthesis kit® (Invitrogen) and oligo-dT primer (Invitrogen) according to the manufacture's instruction.

Inactivation efficiency of UNC-50 mRNA was evaluated at siRNA concentration of 10 and 100 pmol. In addition, continuous inactivation efficiency of siRNA was evaluated up to 72 hrs at 100 pmol. Regulation of mechanical stress related mRNAs were estimated by biglycan, osteoprotegrin (OPG), cyclooxygenase-2 (Cox-2), decorin, transforming growth factor-β (TGF-β), soluble receptor activator of NF-kappa B ligand (RANKL), tissue specific inhibitor of metalloprotease-1, -2 (TIMP-1 and TIMP-2) and matrix metalloprotease-2 (MMP-2) mRNA specific primers. As a control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA specific primer was used. Specific primer sets used in this study are shown at Table 2. In addition, Target bands were took picture with image capture equipment Gel Doc (BioRad laboratory, Hercules, CA) and analyzed with NIH image software Image J V1.37 (NIH).

Table 2. Specific primer sets

Target gene	Forward / Reverse	sequences (5`-3`)	Tm(℃) / Product
UNC-50	F	*ACG TGG GAA TCG CAG GAT*	55
	R	*CCT TCC CAA CAC CAG ACA GT*	861
β-actin	F	*CCA GAT CAT GTT TGA GAC CT*	55
	R	*GTT GCC AAT AGT GAT GAC CT*	397
Mechanical stress induced menieralization related gene			
RANKL	F	*AGG GAG CAC GAA AAA CTG GT*	55
	R	*CCA GAG ACT GTG ACC CCC TT*	492
OPG	F	*GAC ACC TTG AAG GGC CTG AT*	55
	R	*CAT GCT TGG CTT TCT GGG TA*	419
Osteocalcin	F	*GGA CCT GTG CTG CCC TAA AG*	49
	R	*CTG CTG TGA CAT CCA TAC TTG C*	122
Mechanical stress induced periodontal remodeling related genes			
MMP-2	F	*AGA AAA GAT TGA CGC TGT GT*	55
	R	*CTT CAC GCT CTT GAG ACT TT*	397
TIMP-1	F	*ACC ACC TTA TAC CAG CGT TA*	49
	R	*AGT GTC ACT CTC CAG TTT GC*	342
TIMP-2	F	*TCA GAT CCA TCT CAT TTT CC*	55
	R	*GGA GTC CTT AAC CGT TTC TT*	417
Mechanical stress induced pro-inflammation related genes			
Cox-2	F	*AGA AGG GTT CCC AAT TAA AG*	55
	R	*ATA ATT TTT CCC TCC AAA GG*	501
TGF-β	F	*TGG TGG AGA GAA GAG GAA AA*	55
	R	*TAA TTT GAG GTT GAG GGA GA*	486
GP-130	F	*CAG GAA GAC GCT ACC GTG AA*	55
	R	*TCC TTG AGC GAA CTT TGG TG*	435

6. Western blot analysis

Protein isolation

In this study, protein isolation was performed by two ways. First, high hydrophobic and membrane-bounded UNC-50 protein was isolated using by Mem-PER® protein extraction kit (Pierce, Rockford, IL). Cells were harvested using trypsin. Cells were washed with PBS and resuspended in

50 ㎕ of room temperature Mem-PER reagent A. For solubilization of the membrane protein, Mem-PER reagent C diluted 2 : 1 with Mem-PER reagent B were added into cell suspension and incubate on ice. The solublized protein mixture was centrifuged and take upper phase. After then, The supernatant was heated at 37 ℃ for 10 min. After incubation, solution was separated lower membrane and upper hydrophilic protein fractions. Lower hydrophobic phase was used in this study as membrane bounded protein phase. This hydrophobic phase was normalized by 4-fold diluted Mem-per reagent B solution. This diluted solution was applied as membrane protein extracts. Second, cytosolic and nuclear protein were isolated by general lysis buffers. For evaluation of expression patterns of mechanical stress related kinase and translocation to nucleus, hypotonic protein isolation buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF) was used. Cells were washed out with PBS and 0.5 ㎖ of hypotonic buffer was applied. After then, cells were collected in 1.7 ㎖ micro-centrifuge tube and incubate for 15 min on ice. Cell suspension was homogenized and centrifuged at 4,000 rpm for 10 min. After centrifugation, carefully took supernatant and transfer to a new 1.7 ㎖ tube and used for cytoplasmic protein analysis. Add 10 ㎕ of high salt lysis solution (20 mM HEPES, pH 7.9, 420 mM NaCl 25 % Glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT) was added to pellet and was incubated for 20 min on ice. After 20 min, 5 fold (v/v) of storage buffer (20 mM HEPES, pH 7.9, 100 mM NaCl, 20 % Glycerol, 0.2 mM EDTA, 0.5 mM PMSF) were added to above tube. Then centrifugation was peformed at 14,000 rpm for 20 min.

Western blot analysis

Collected proteins were subjected to 10 % sodium-dodesyl-sulfate polyacrylamide gel electrophoresis (SDS PAGE). After PAGE, proteins were transferred to nitrocellulose membrane (ECL hybond; Amersham UK, Little Chalfont, UK). After transfer, anti-ERK1/2 (Cell signaling), anti 38-MAPK (Cell signaling), anti-*c-fos* (Oncogene Research, MA), anti-NFkB (Santa Cruz, CA), anti-UNC-50 (Peptron, Daejeon, KOR), and anti-GAPDH (Lab frontier, Seoul, KOR) were used as primary antibodies for 1 h at room temperature and goat anti-rabbit IgG was used as secondary antibody for 30 min at room temperature. Target bands were developed on ECL hyper film and ECL chemoluminescence detection system (Amersham).

III. RESULTS

1. Inactivation of UNC-50 mRNA by siRNA

UNC-50 siRNA duplexes showed about 70～80 % of inactivation efficiencies for UNC-50 mRNA at 10 and 100 pmol after transfection 8 to 12 hrs. Among three kinds of duplexes, siRNA duplex strain-2 showed the best inactivation efficiency (Figure 3A). In the subsequent experiments, the siRNA duplex strain-2 was used for inactivaton of UNC-50 mRNA. In addition, membrane bounded UNC-50 protein was not detected with treatment of UNC-50 siRNA (Figure 3B). UNC-50 mRNA was effectively inactivated after transfection 12 hr (Figure 4A). However, continuous basal expression was detected steadily up to 72 hrs (Figure 4B). UNC-50 protein was localized nuclear membrane in normal OCCM-30. However, after inactivation of UNC-50 by siRNA, UNC-50 expression was moderately decreased the cells (Figure 5).

Morphologically, changes were not observed between control and siRNA treated groups. However, OCCM-30 cell was elongated under compression and cellular processes were augmented under compressive force plus siRNA group than control. OCCM-30 cells did not showed remarkable differences between control and tension group. However, OCCM-30 cells showed slender shape under tensile force plus siRNA group than tension group (Figure 6).

2. Effect of UNC-50 on mechanical stress related genes and kinases under compressive force

Expression of UNC-50 mRNA was down-regulated under compression by RT-PCR analysis (Figure 7A). Among the tested mechanical stress induced mineralization related genes, RANKL, OPG, MMP-2, TIMP-1 and decorin mRNA were up-regulated under compression group. Osteocalcin mRNA did not changed under compression. Biglycan and TIMP-2 mRNAs were down-regulated under compressive force and siRNA group (Figure 8).

In addition, a kind of mechanical stress induced pro-inflammatory response related gene, Cox-2 was up regulated and a kind of mechanical stress induced cytokine Interlukin-6 receptor, GP130 mRNA, was down-regulated. Mechanical stress related growth factor, TGF-β, was up-regulated by siRNA under compressive force (Figure 9).

3. Effect of UNC-50 on mechanical stress related genes and kinases under tension force

UNC-50 mRNA was up-regulated under tension by RT-PCR analysis (Figure 7B). Among the tested mechanical stress induced mineralization related genes, RANKL and decorin mRNA were up-regulated under tension plus siRNA. Especially, TIMP-1 was strongly up-regulated under tensile force plus siRNA group than tension group.

However, OPG, MMP-2, TIMP-2, and biglycan mRNA were down-regulated at tensile force plus siRNA group than tension group (Figure 8). Ostaeocalcin did not changed in each of tension group and tension plus siRNA group. Mechanical stress induced IL-6 receptor, GP130 mRNA, and transforming growth factor, TGF-β mRNA, was down-regulated. However, mechanical stress induced pro-inflammation

related gene Cox-2 was up-regulated under tensile force plus siRNA group (Figure 9).

4. Expressions of mechanical stress induced kinases and transcription factors

After inactivation of UNC-50 mRNA by siRNA, expression p38-MAPK, NFkB, c-fos and ERK1/2 were analyzed by western blot.

Expression and phosphorylation of p-38 MAPK was up-regulated only in compression plus siRNA group. Mechanical stress related transcription factor NFkB was also analyzed. Expression of NFkB was slightly decreased both of tension and tensile force plus siRNA group. However, trans-location to nucleus was not detected in all groups. Up-regulation of *c-fos* was only detected in tension group. Among the tested kinases, phosphorylation of ERK1/2 was detected at compression group. ERK-2 was strongly phosphorylated than phosphorylation of ERK-1 under compression. However, there were no expressional differences between compression and compressive force plus siRNA groups. (Figure 10).

IV. DISCUSSION

1. Inactivation of UNC-50 mRNA by siRNA

Inactivation of UNC-50 mRNA using siRNA could not lead complete inactivation of UNC-50 protein synthesis. 70~80 % of inactivation efficiency of UNC-50 caused steady basal mRNA expression up to 3 days (Figure 1C, D). It was described in previous report, about 20 nM of siRNA concentration was sufficient for inactivating target gene [14]. In the present study, 100 pmol of siRNA-2 strain showed best inactivation efficiency after 8 to 12 hrs. Inactivation efficiencies of siRNA did not showed reliable differences between each concentrations of siRNA.

2. Mechanical stress responsive gene expression in OCCM-30

PDL controls homeostasis of periodontium from various stresses such as mastication or occlusion. PDL induced osteoclastogenesis *via* up-regulation of RANKL under compression. However, PDL does not induce osteoclastogenesis *via* up-regulation of OPG and TGF-β under tensile force [15]. Regulations of RANKL and OPG mRNAs in OCCM-30 are similar to that of osteoblast cell line, MG-63 [16,17].

Although, osteocalcin, decorin and biglycan were known as mechanical stress induced periodontium remodeling related gene [18]. It was reported that osteocalcin mRNA was increased under mechanical stress under long term stress. Increase of biglycan and decorin mRNA were reported. However, signal transduction pathways of decorin and biglycan were not fully understood [2,19].

In the present study, RANKL mRNA showed definite differences in

mRNA expression between stress groups and stress plus siRNA groups. Expression of RANKL was up-regulated by compressive force plus siRNA applied group and decreased under tension plus siRNA (Table 3).

Table 3. Mechanical stress induced mineralization related genes

	Compression	Tension
Decorin	++	++
Biglycan	-	---
RANKL	++	---
OPG	+	-
Osteocalcin	-	-

Decorin mRNA was up-regulated in all groups. Biglycan mRNA was down-regulated at tension plus siRNA group. It mean that UNC-50 weakly influences to mineralization of cementogenesis under tension in OCCM-30. It suggested that UNC-50 might be involved in periodontium remodeling *via* facilitation of osteoblastogenesis or periodontium degradation under compressive force. However, UNC-50 might be not or weakly influence to periodontium remodeling *via* osteoblastogenesis or periodontium degradation under tension. This supposition is supported by the report of Mada *et al.* and these regulations may be essential to protect root surface from absorption [20]. This response was also reported as different magnitudes of mechanical strain induce different effects in osteoblastic cell [17].

In previous report, MMP-2 was differently expressed by types of stress. Up-regulation of MMP-2 mRNA induces up-regulations of TIMP-1 and up-regulation a specific inhibitor of MMP-2 (TIMP-2) in human osteoblast and PDL [21,22]. Regulations of TIMPs and MMPs influence to facilitation of turn-over rate in bone and periodontium [23,24].

In this study, expression patterns of MMP-2, TIMP-1 and TIMP-2 suggested that inactivation of UNC-50 influence to mechanical stress induced signal transduction in OCCM-30. The cementoblast showed

differently reacted to the stresses. Therefore, UNC-50 might influence to be formation and resorption of the cementum *via* regulation of RANKL and OPG mRNA expression under mechanical stress. It was synchronized in the tissue remodeling process by periodontium degradation by MMP-2, TIMP-1 and TIMP-2 under mechanical stress (Table 4).

Table 4. Mechanical stress induced matrix degradation related genes

	Compression	Tension
MMP-2	++	-
TIMP-1	+++	+
TIMP-2	-	+++

Inflammation reactions were commonly observed under mechanical stress. Among the known mechanical stress induced pro-inflammation related factors, IL-6 and its receptor GP130 were well known. It was also known that up-regulation of growth factors under appropriate stress. Increase of GP130 and TGF-β induced tissue degradation by inflammatory responses and osteogenesis in PDL and bone [25-27].

In the present study, GP130 mRNA, was down-regulated and Cox-2 mRNA was up-regulated at all groups (Table 5). Cox-2 is also important in maintaining of periodontium under mechanical stress. In the previous reports, Cox-2 mRNA was up-regulated under tensile force in human PDL.

Table 5. Mechanical stress induced pro-inflammation related genes

	Compression	Tension
TGF-β	--	+++
GP-130	--	-
Cox-2	++	+++

3. UNC-50 influence to expression of p38-MAPK, NFkB, ERK1/2 and c-fos

Mechanical stress induced signal transduction pathway was not fully understood in cementoblast and PDL fibroblast. However, some of the

mechanical stress ineducable gene, kinases and transcription factors were reported [28,29]. It was only suggested that mechanical stress was involved in the Rho kinase pathway in human PDL fibroblast and activate *c-jun*, *c-fos*, ERK1/2 and p38-MAPK. Matsuda et al. also reported no changes in ERK phosphorylation after stretch in PDL [28,30,32-33]. A member of MAP kinase, p38-MAPK, plays an important role in induction of MMP-2 activation by mechanical stress [34]. Mechanical stress induced up-regulation of p38-MAPK. Subsequently, expression of MMP-2 was increased by p38-MAPK-dependent manner in endothelial cells [35]. However, It was also reported that MMP-2 expression from mechanical stress was not involved in ERK1/2 pathway in muscular arteris under tensile force [36].

NFkB plays important roles in various cells, such as, osteoblast and fibroblast. NFkB effects on the expression of mechanical stress induced gene and kinases [37,38]. NFkB is inactivated by IkB. When cells were exposed to stress, IkB is separated from p65-NFkB protein and the p65-NFkB protein is translocate to nucleus. Translocation of p65-NFkB protein stimulates various gene expressions. Another important mechanical stress induced transcription factor, *c-fos*, was reported under uniaxial stretching [38]. A member of mechanical stress related transcription factor *c-jun* and *c-fos* also control gene expression [29]. Mechanical stress induced *c-fos* and *c-jun* had been reported in bone and fibroblast. Especially, expressions of *c-jun* and *c-fos* were mainly reported in osteoblast under compression force [39,40].

In this study, activation of p38-MAPK under compression and strongly up-regulated at compress plus siRNA. It is supposing that UNC-50 might to facilitate mineralization in OCCM-30 under compressive force. Phosphorylation of ERK1/2 was also detected by western blot under tension.

However, there were no expressional differences between tension and tension plus siRNA. It suggested that UNC-50 might not be involved in mechano-transduction *via* ERK pathway in OCCM-30. Although, decrease of p65-NFkB was detected at tension plus siRNA, translocation of p65-NFkB protein was not detected (data not shown). It suggested that UNC-50 might to inhibit phosphorylation of NFkB under tension.

Expression of *c-fos* was also strongly suggested the involvement of UNC-50 in mechano-transduction in OCCM-30.

4. UNC-50 involved in mechano-transduction in cementoblast cell line, OCCM-30

UNC-50 regulates signal transduction on nuclear membrane, endoplasmic recticulum, and Golgi apparatus. However, it is not identified where is the functional location in OCCM-30. The signals from mechanical stress were introduced form external cellular environment to cytoplasm and nucleus. Responses to mechanical stress of OCCM-30 *via* UNC-50 were represented in this study. Although, expression of UNC-50 mRNA was not completely inactivated, expression and phosphorylation of mechanical stress responsive mRNA, kinase, and transcription factor was changed according to the mechanical stresses.

In conclusion, nuclear membrane bounded UNC-50 protein was involved in mechano-transduction. UNC-50 plays a role in mechanical stress induced signal transduction and in regulate expression of gene, kinase and transcription factor in cementoblast cell line, OCCM-30 by types of force. mechano-transduction pathway in OCCM-30 might be involved with NFkB and MAP kinase. Therefore, if expression of UNC-50 was regulated, it might to be a useful way to keep health of PDL and to be a appropriate way for clinical treatments.

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VI. Figure Legends

Figure 1. Artificial mechanical stress model for compressive force.

siRNA transfected and normal cementoblast cell line, OCCM-30 (5×10^4 cell/dish) were seeded on 60 ㎜ culture dish. After 8 hours from transfection, flame sterilized cover glass were laid on the cells. Sterilized a pair of metal weights (2 × 3 g) were also laid on the cover glass.

Figure 2. Artificial mechanical stress model for tensile force.

siRNA transfected and normal OCCM-30 (5×10^4 cell/dish) were seeded on flexible bottom 60 ㎜ culture dish, petri-PERM®. After 8 hours from transfection, petri-PERM is laying in 35 ㎜ culture dish. Sterilized a pair of metal weights (88 X 2 g) were also laid over the cover.

Figure 3. Determination of siRNA concentration and effective duplex strain of siRNA

After transfection 8 hrs, three kinds of siRNA strains showed effectively inactivation of UNC-50 mRNA expression. About 70~80 % of Inactivation rate were detected at 10 and 100 pmol. Furthermore, among the tested siRNA duplexes, siRNA-2 strain showed the best efficiency (***A***). UNC-50 protein synthesis was effectively inactivated by siRNA duplex strain-2 (***B***)

Figure 4. Time course inactivation efficiencies of siRNA-2 starin.

Inactivation effect of siRNA was tested using siRNA-2 strain and by

time course. 100 pmol of siRNA-2 was transfected into OCCM-30 on 60 ㎜ culture dish. Total RNA were extracted from normal and transfected cells by 4, 8, 12, 24, 48, and 72 hrs. Expression of UNC-50 mRNA was analyzed by RT-PCR. Effective inactivation of UNC-50 mRNA expression was detected at 12 hrs after transfection. However, basal expression of UNC-50 mRNA was steadily detected up to 72 hrs.

Figure 5. Immunofluorescence staining of UNC-50 in cementoblast

To evaluate expression pattern of UNC-50 in OCCM-30, Immunofluorescence using polyclonal FITC-conjugated anti-UNC-50 antibody. At normal state, expression of UNC-50 was detected around nuclear membrane (***A***). Meanwhile, expression of UNC-50 was decreased at inactivation of UNC-50 by siRNA. However, basal expression of UNC-50 was steadily detected (***B***).

Figure 6. Morphological changes of cementoblast cell line, OCCM-30, at various conditions.

OCCM-30 showed polygonal shape at normal condition (***A***). At inactivation of UNC-50, OCCM-30 showed similar morphology with normal cell morphology (***B***). Under compressive force, OCCM-30 showed elongated in length and lost polygonal shape (***C***). Under compression and UNC-50 mRNA inactivation state, OCCM-30 showed similar morphology in plate C. However, polygonal shape of OCCM-30 is still remained (***D***). When tensile force was applied to OCCM-30, cell size was decreased. However, remarkable polygonal shape was observed (***E***). Under tensile force was

applied with siRNA, cell size was not changed and polygonal shape slightly lost (***F***). Original magnifications of all above picture are × 100.

Figure 7. Expressions of UNC-50 mRNA under compression and tension.

Expression of UNC-50 under compression (A) and tension (B) were analyzed by RT-PCR. Under compression, expression of UNC-50 showed gradient decrease. Although, slightly increased 2 hrs after force applied. Over all pattern showed decreasing manner. However, expression of UNC-50 showed gradient increasing pattern under tensile force. Expression of UNC-50 was changed about 50 % of decreasing pattern under compression and 20 % of increasing pattern under tension force. Expression rates were normalized with expression of beta-actin.

Figure 8. Expressions of mechanical stress induced mineralization related gene by RT-PCR

Under mechanical stress plus siRNA, mechanical stress induced meralization related gene, RANKL, and its decoy receptor, OPG, were analyzed. OPG and RANKL were decreased after 12 hrs. Although, osteocalcin mRNA was up-regulated under tension plus siRNA and down-regulated compression plus siRNA, negligible differences were observed. Compressive force induced gene, MMP-2 was increased at stress group. Between mechanical stress induced mineralization related gene biglycan and decorin, decorin showed weakly changed in expression under stress and siRNA. However, biglycan showed reliably decreased in

expression under tension plus siRNA. Furthermore, a member of tissue specific inhibitor of MMP family, TIMP-1 and TIMP-2 were slightly decreased under inactivation of UNC-50. Above all expression rates were normalized with expression of beta-actin. Control; Ct, stress applied group; St, stress plus siRNA group; Stsi.

Figure 9. Expressional changes of mechanical stress induced cytokine receptor (GP130) and trnascription factor (TGF-β and Cox-2)

A kind of mechanical stress induced cytokine, IL-6 receptor, GP130 (***A***) and a kine of growth factor, TGF-β (***B***), were analyzed by RT-PCR. Expressions of GP130 was down-regulated under tension force and TGF-β was also slightly expressed. Under stress plus siRNA treated groups, expression of GP130 was more down-regulated. However, expression of TGF-β is greatly expressed under compression force and siRNA applied group. Cox-2 plays in a NFkB activation under mechanical stress. expression of Cox-2 showed increasing pattern at inactivation state of UNC-50 (***C***). Control; Ct, stress applied group; St, stress plus siRNA group; Stsi

Figure 10. Expressions of mechanical stress related kinases and transcription factors by western blot analysis

Among the tested proteins by western blot analysis, p38 MAP kinase were increased unser compression force plus siRNA treated group. p42-ERK is only detected and p-44 was slightly expressed. Although, expressions of

ERK1/2, difference of expression level was not observed. expression of *c-fos* was detected only in tension force applied group. Also, it was not expressed under tension plus siRNA treated group. UNC-50 was detected every lanes, However, it was weakly expressed under compression force applied and more strong expressions were strong under tension applied group.

VII. FIGURES

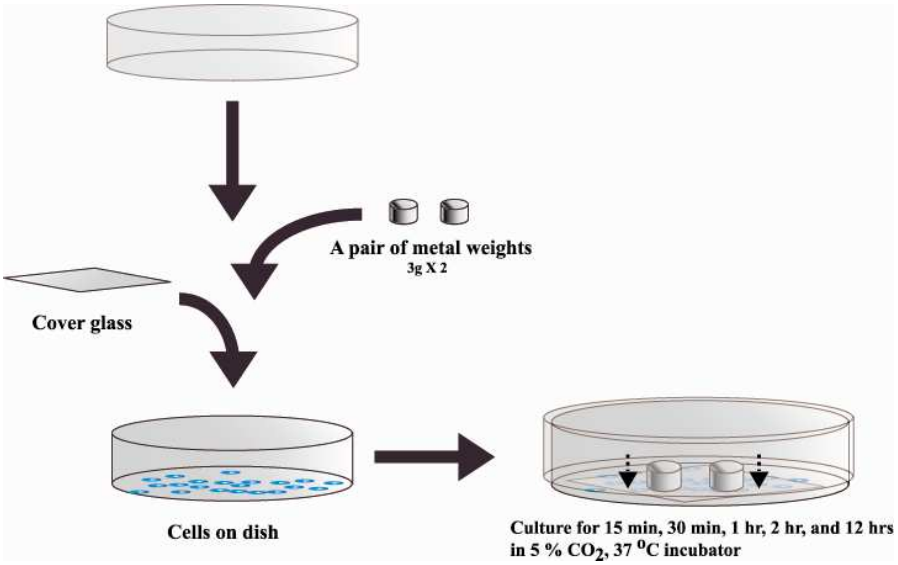

Figure 1 Artificial mechanical stress model for compressive force

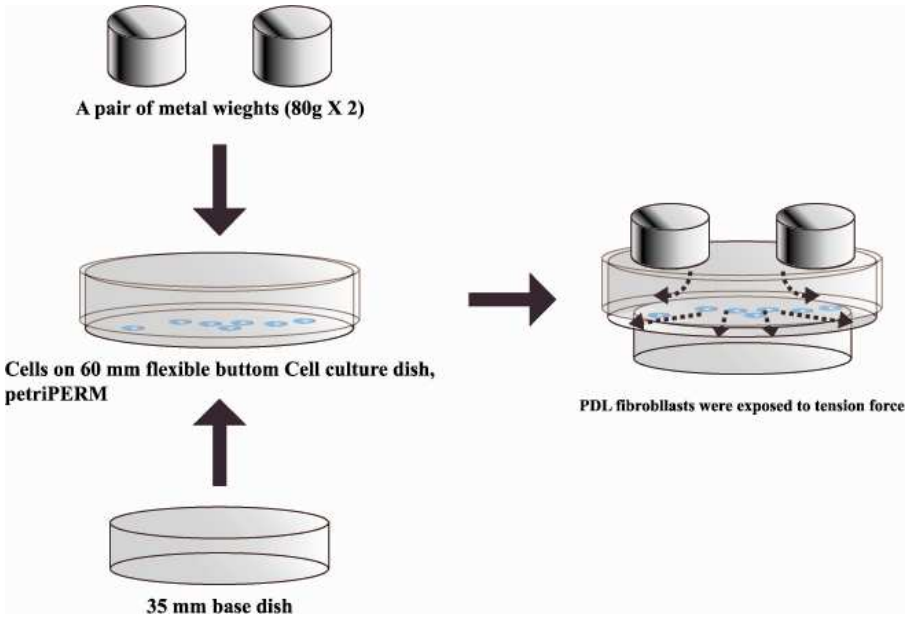

Figure 2. Artificial mechanical stress model for tensile force

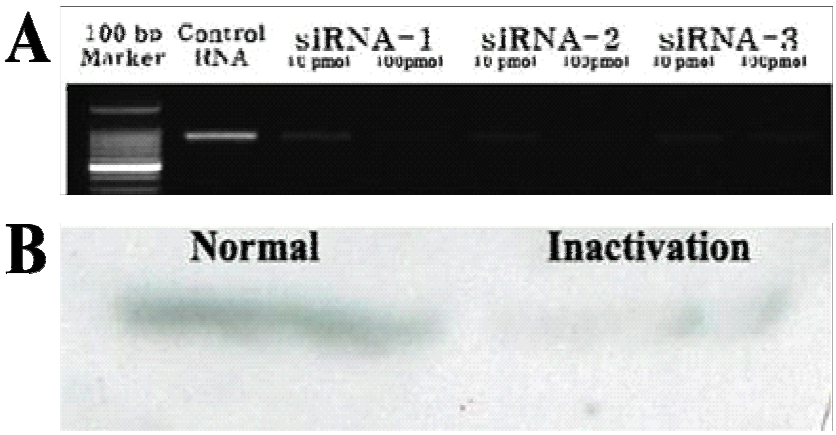

Figure 3. Determination of siRNA concentration and effective duplex strain of siRNA

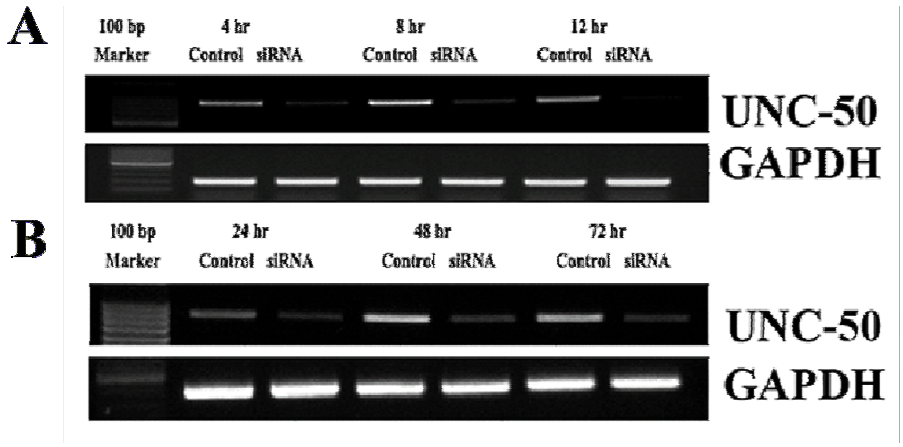

Figure 4. Time course inactivation efficiencies of siRNA-2 strain

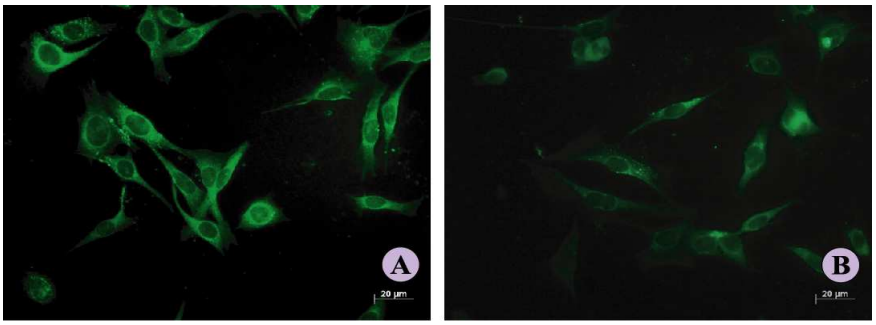

Figure 5. Immunofluorescence staining of UNC-50 in cementoblast

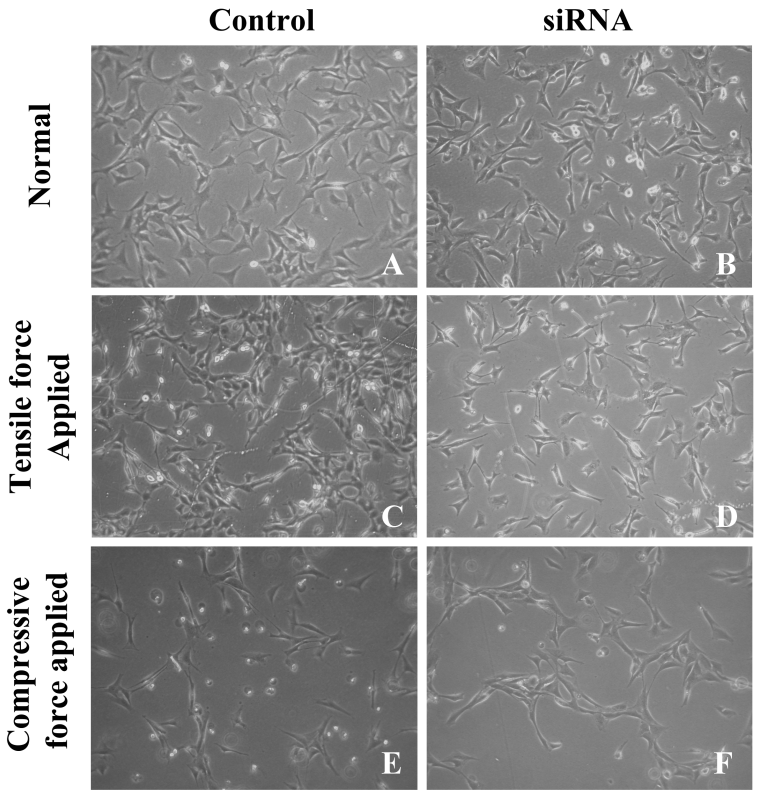

Figure 6. Morphological changes of the cementoblast cell line, OCCM-30, at various stresses.

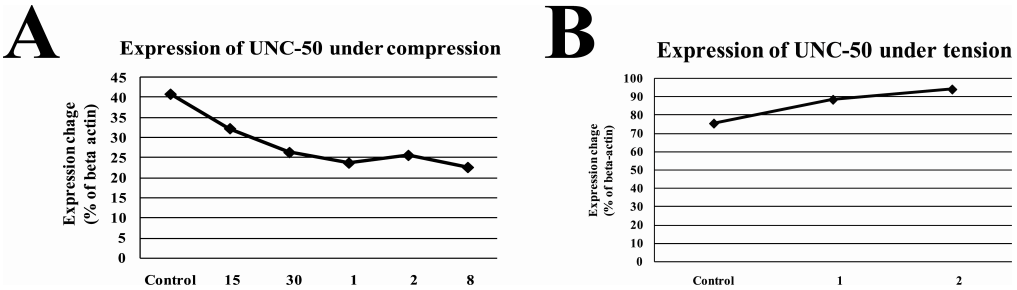

Figure 7. Expression of UNC-50 under compression and tension

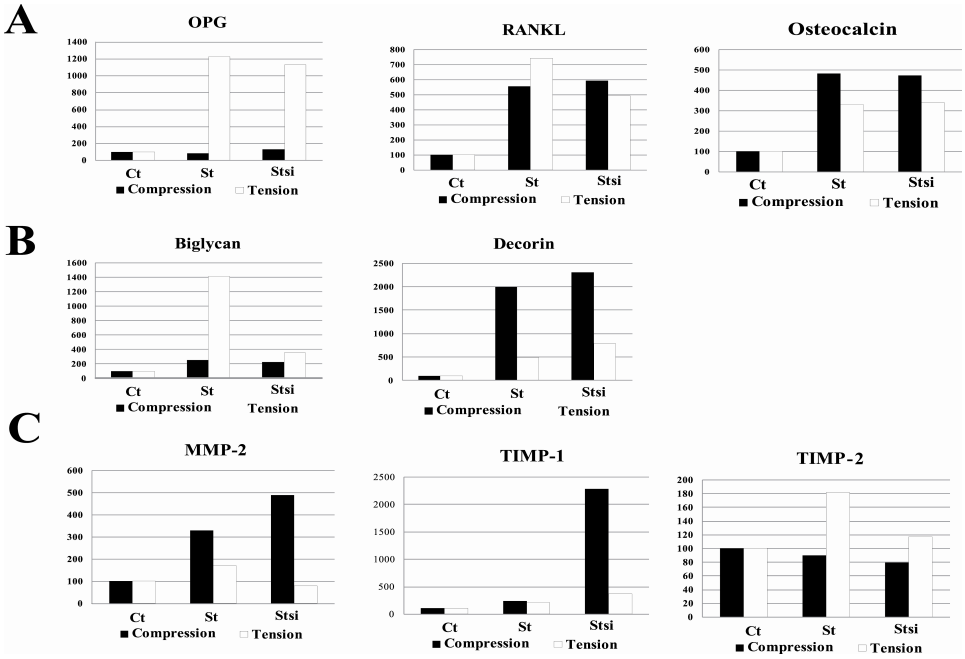

Figure 8. Expression of mechanical stress induced mineralization related gene by RT-PCR

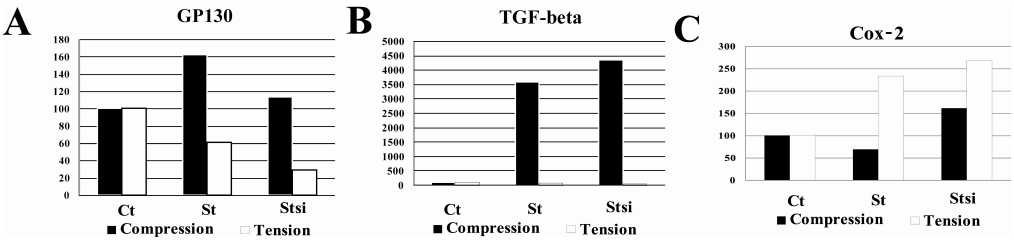

Figure 9. Expressional changes of mechanical stress induced cytocine receptor (GP130) and transcription factor (TGF-beta and Cox-2)

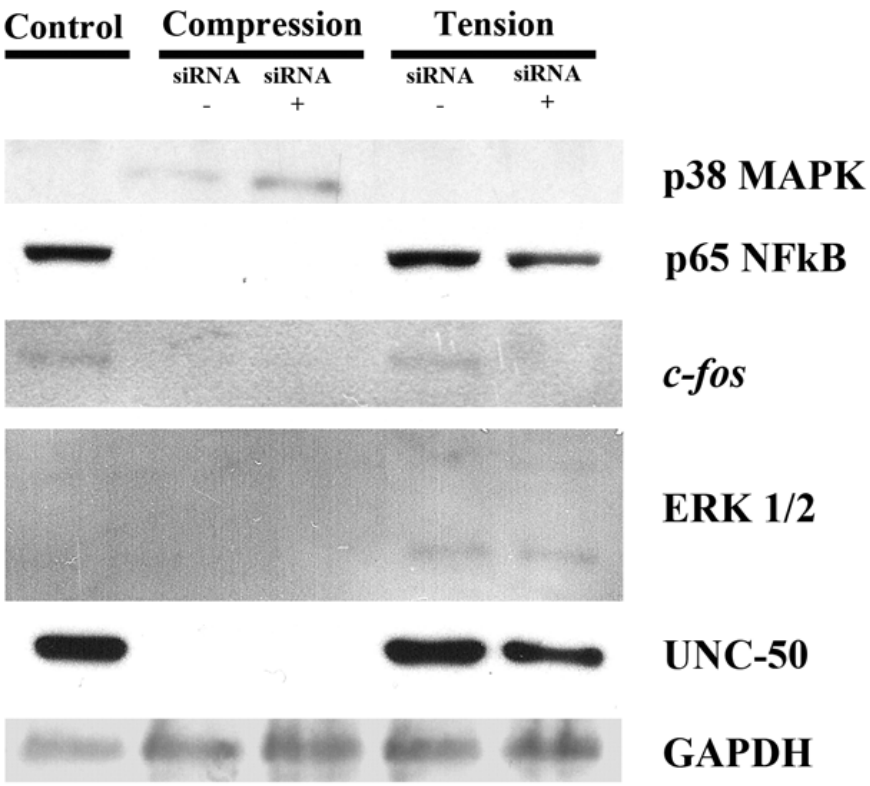

Figure 10. Expressions of mechanical stress related kinases and transcription factors by western blot analysis

ABSTRACT in KOREAN

백악모세포 *(OCCM-30)*에서 기계적 자극과 관련된 유전자 발현에 미치는 *UNC-50*의 기능억제 효과

최 용 석

조선대학교 대학원 치의학과

(지도교수 : 박주철)

치주조직은 일생동안 저작압 및 교합압에 의한 기계적인 자극에 노출되어있는 조직이다. UNC-50 유전자는 *C.elegance*에서 최초로 동정되었고, 최근에는 생쥐와 흰쥐를 포함한 고등 포유동물에서도 보고되었으며, 최근에는 사람의 치주조직에서 발현됨이 보고되었다. 최근의 보고들에 의하면, UNC-50은 치주조직의 발생과 형성과정에서 치주인대섬유모세포에 가해지는 기계적인 자극에 대한 반응에 있어서 중요한 역할을 할 것으로 생각된다. 그러나, 치주조직의 형성과 분화과정에서 UNC-50의 이런 기능들이 제시되었음도 불구하고, 기계적 자극의 전달과 관련된 경로는 아직 밝혀지지 않았다.

siRNA는 *C.elegance*에서 그 존재와 기능이 최초로 확인되었으며, 특정 상보적 서열에 대한 특이적인 결합을 유도하여, 상보적 mRNA를 21-23 뉴클레오타이드로 분해함으로 대상 단백질의 합성을 억제하여 대상 유전자의 기능을 억제하는 기전이다. 이러한 siRNA는 비교적 쉽고 빠르고 정확하게 작용함으로, 포유동물 유전자의 기능과 작용경로를 분석하는데 최근 들어 널리 사용되고 있다. 본 연구는 UNC-50 siRNA를 사용하여 UNC-50의 기능 억제를 유도한 후, 기계적 자극을 가한 군과 가하지 않은 군을 통하여 자극의 수용 및 전달과 관계되어 발현되는 유전자와 전사인자 및 kinase의 발현 변화를 확인하였다. 이들의 발현 변화를 통하여 UNC-50 유전자가 백악모세포(OCCM-30)에서의 자

극 수용 및 전달에 있어서의 영향을 평가하였다.

본 연구는 세포에 가해지는 자극의 종류와 siRNA의 적용유무에 따라 6개의 집단으로 구분하여 수행하였다; 정상대조군, siRNA만을 처리한 군, 압력(compressive force)만을 가한 군과 압력과 siRNA를 동시에 처리한 군, 장력(tensile force)만을 가한 군 및, 장력과 siRNA를 동시에 처리한 군. 본 실험에서 위와 같은 환경과 UNC-50의 기능억제를 통한 백악모세포주인 OCCM-30세포에 가해지는 자극에 대한 유전자, kinases 및 전사인자들의 변화를 immunocytochemical staining, RT-PCR, 및 western blot을 통하여 확인하였다.

RT-PCR 결과, 압력이 가해진 환경에서 MMP-2, TIMP-1, TIMP-2, GP130, TGF-β, biglycan, RANKL 및 decorin mRNA 들의 발현이 압력이 가해진 환경에서 증가 하였으며 Cox-2, TIMP-1, MMP-2 및 decorin mRNA의 발현은 압력과 siRNA가 동시에 주어진 상황에서 더욱 증가하였다. 그러나, MMP-2, TIMP-1, RANKL 및 osteoprotegrin mRNA의 발현은 장력 하에서 감소하였다.

Western blot 분석 결과, p38 MAPK의 발현은 압력과 siRNA가 동시에 주어진 환경 하에서 증가하였다. Phospho-ERK-2의 증가와 p65-NFkB의 감소가 확인되었지만, p65-NFkB의 발현은 세포질에서만 확인 되었고, 핵으로 이동은 관찰되지 않았다.

이러한 결과는 UNC-50이 치주인대에서 기계적 자극전달과 관련이 있다는 것을 의미하며, 자극을 통한 치주인대의 분화에서 중요한 역할을 한다는 것을 시사한다. 또한, UNC-50은 p38 MAPK 신호전달기전에 관여하고, 부분적으로 NFkB의 기전에도 관여한다고 볼 수 있다. 따라서 UNC-50은 백악모세포유사세포주인 OCCM-30세포에서 기계적 자극의 종류에 따라 작용을 달리하고 치주조직 및 백악질의 형성과 파괴를 조절함으로서 치주조직의 유지에 중요한 역할을 할 것으로 사료된다.

저작물 이용 허락서

학 과	치 의 학 과	학 번	20057506	과 정	박사
성 명	한글: 최 용 석	한문 : 崔 容 碩		영문 : Yong-Seok, Choi	
주 소	인천광역시 부평구 삼산동 삼산주공아파트				
연락처	016-284-2848		E-MAIL	Nostalgia19@hanmil.net	
논문제목	한글 : 백악모세포(OCCM-30)에서 기계적 극과 관련된 유전자발현에 미치는 UNC-50의 기능억제 효과				
	영어 : Effect of UNC-50 inactivation on the expression of genes related to mechanical stress in cementoblast cells (OCCM-30)				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(V) 반대( )

2007년 8 월 일

저작자: 최 용 석 (서명 또는 인)

조선대학교 총장 귀하