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Ling Jiang

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ABSTRACT

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The common presenting problems of the long duration of treatment and posttreatment relapse still lose simple and effective solutions during orthodontic procedure. Periodontal soft-tissue is one of the key points to both problems. It is known that as a tooth moves through the gingiva, the response of the gingiva is to increase collagen formation to resist this movement. The primary factor causing relapse in orthodontically rotated teeth is the displaced supra-alveolar connective tissue fibers. Surgically cutting the circumferential supracrestal fibers called circumferential supracrestal fiberotomy (CSF) is known as a means of accelerating the kinetics of tooth movement and reducing the tendency to relapse. So far CSF is the unique technique demonstrating advantages both in tooth movement and relapse, however, as an invasive procedure, low patient acceptability suggests that an alternative approach is required. The matrix metalloproteinases (MMPs), a group of zinc-dependent endopeptidases, contribute to the cleavage of various components of the extracellular matrix (ECM), and activate osteoclasts to trigger bone resorption. The expression levels of MMPs and tissue inhibitors of metalloproteinase (Timps) are increased at both the resorption and apposition sides during tooth movement. It is postulated that the alteration of MMPs/Timps balance regulates ECM

degradation. As far as is known, delivering Timp1 siRNA to rat with hepatic fibrogenesis resulted in indirect types I and III collagen fibrolysis by elevating the expression and activity of MMP-13, which is the main substrate of Timp1. Timp1 could inhibit almost all MMPs, except MT1-MMP, MT3-MMP, MT5-MMP and MMP-19. Based on the above, Timp1 is proposed as a potential therapeutic intervention target for tooth movement and relapse. RNAi offers researchers an effortless tool for the interrogation of the biological mechanism, and will likely work as a standard therapeutic agent in illness treatment by selectively silencing genes. In this study, the possibility and potential mechanism of Timp1 siRNA in attenuating the collagen expression was assessed *in vitro* first, using a NIH3T3 cell culture. The Timp1 and collagen1 productions were tested by RT-PCR and western blot. Then the effects on tooth movement and relapse were judged after local subperiosteum Timp1 siRNA injection in a mouse tooth movement model induced by a Ni-Ti closed coil spring. Timp1 siRNA efficiently inhibited Timp1 expression and decreased collagen1 protein level by ~70% after transfection for 48-72 h in vitro. The maximum mean amount of tooth movement in the transfection group and control group on 17 days was 0.94 mm and 0.73 mm, respectively. 80% of the accelerated tooth movement was apparent during the early activation period, between days 0 and 7. During two weeks relapse phase, the relapse percentage of Timp1 siRNA group was 15.96%, significantly less than 64.38% in the control group. This study indicated that Timp1 siRNA and any other medicine which could modify the homeostasis between Timps and MMPs may be a potential pharmacological agent for enhancing tooth movement and inhibiting relapse simultaneously.

Key words: Timp1 siRNA, Timps, MMPs, collagen1, tooth movement, relapse

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I. INTRODUCTION

1. Biological Mechanisms of Orthodontic Tooth Movement

"Pressure Tension" Theory

Three stages of teeth movement were categorized as following: Initial phase (Displacement), an instant very rapid movement observed over a short distance reflects the fact that periodontal ligament holds the teeth loosely in the dental groove. The indirect resorption in this phase which is also described as undermining resorption is characterized by the adjacent marrow space resorption but not the periodontal ligament (PDL) resorption (Krishnan and Davidovitch, 2006). Lag phase (Delay), tooth movement is limited by the formation of hyalinised tissue (necrotic tissue) at the pressure site. The compression to the local vasculature leads to localized

ischemia, resulting in a subsequent degenerated cell-free zone formation in the PDL. Finally, lag phase is ended by the removal of the necrotic tissue by macrophage-like cells from periphery. Post lag phase (Movement), actual tooth movement progresses rapidly as the hyalinized zone is removed and bone undergoes frontal resorption which is taken place directly on the wall of the PDL but not marrow cavities.

"Bone-Bending" Theory ("Fluid Flow" Theory)

The alternative bone-bending theory was popular during the 1960s and 1970s. The possible mechanism explaining bone-bending theory was explored: Deformation of alveolar bone, convex configuration under tension and concave surface under compression (Krishnan and Davidovitch, 2006), changes the fluid flow through the canaliculi and causes a shear stress on the osteocyte surface. Then the integrin at the osteocyte surface which is known as a kind of transmembrane protein with intra- and extracellular domains, is activated to transmit extracellular matrix signals to the cytoskeletal microfilament bundles, so the mechanical forces are tansformed into biochemical signals (Guicheux et al., 1998; Cool et al., 2005).

"Bioelectric Signals" Theory

In 1968, Gillooly proposed that the generation of endogenous electric signals, electronegativity in the bone concave surface and electropositivity in the convex surface, is responsible for regulating bone remolding during tooth movement (Gillooly et al., 1968). It is believed that the mechanical distortion of collagen

matrices in the periodontium contributes to the occurring of this bioelectrical phenomenon (Davidovitch et al., 1980). When hydroxyapatite crystalline materials like bone are deformed, electrons inside of the lattice are displaced to generate an electric potential (Nishiyama et al., 1996). It is likely that the external electrical signals influence some macromolecules which interact with specific sites in cell membranes, or the permeability of ions across cell membranes, thereby increase osteoclastic activity (Sodhi and Tripathi, 2008). However, Meikle speculated that this bioelectrical phenomenon is just a kind of by-product of bone deformation and does not regulate bone remodeling (Meikle, 2006).

"Micro-cracks" Theory

Microcrack is the first damage caused by orthodontic forces which is observed one day after application of orthodontic force (Frost, 1960). There are various types of microdamages in respond to different types of mechanical loads. Tensile strains are related to "difuse microdamage" which is commonly found in trabacular bone, while compression strains are related to "linear microcracks" which usually exists in cortical bone, and frequently limited by osteonal cement line (Boyce et al., 1998). The strain stimulus on the bone matrix causes these microcracks or micro damages which lead to local apoptosis of osteocytes or local necrosis, and thereby recruit osteoclasts to induce bone remodeling (Burr et al., 1985; Verborgt et al., 2000). Bone remodeling by micro-damage is described as an inflammatory repair response as oppose to the mechanism of calcium transport.

2. Therapies of Accelerating Tooth Movement

Both mechanoresponse and inflammation are essential and important for achieving tooth movement which is emphasized by most of the researchers (Krishnan and Davidovitch, 2009). Wilcko mentioned that bone density, bone turnover, and hyalinization of the PDL are the main influences on the velocity of orthodontic tooth movement (Wilcko, 2008). The pharmacological acceleration of tooth movement, such as, injection of PGE1, PGE2, RANKL, and 1a, 25(OH) 2 vitamin D3, has been achieved by enhancing the activation of osteoclasts, however, none of these factors is applied in current orthodontic treatment in clinic (Kamatchi, 2012).

Physical Adjuvant Methods

1) Electrical Current Therapy

Only one preliminary study assessed this therapy. Kim et al. designed a miniature electric device which was fixed on the moving tooth to deliver a 20 μA electrical current to the surrounding mucosa 5 hours per day. The result showed that the exogenous electric current has a potential to reduce one third of the orthodontic treatment duration (Kim et al., 2008).

2) Low-level Laser Therapy

Laser therapy is consistently proved to have no negative effects in terms of the

root and periodontal health. There was only one article suggested that the Low-level laser treatment could significantly accelerate orthodontic movement in humans (Genc et al., 2013). In contrast, the pooled mean differences between the experiment and control groups indicated that low-level laser therapy was unable to accelerate orthodontic tooth movement, as Long described previously (Long et al., 2013).

3) Pulsed Electromagnetic Therapy (vibration)

Noninvasive pulsed electromagnetic field (PEMF) is possible to increase not only the velocity of tooth movement but also the amount of bone deposition through local synthesis of prostaglandins, intracellular calcium, and cAMP (Showkatbakhsh et al., 2010). It is reported that direct currents could not penetrate the cell but cause electrochemical changes in the cell membrane surface. On the other hand, pulsating currents are able to penetrate the cell membrane, and these stimuli could either act at the level of the cell membrane directly or affect intracellular organelles indirectly (Stark and Sinclair, 1987; Darendeliler et al., 2007).

Chemical Adjuvant Methods

1) Osteocalcin (OC)

Osteocalcin (OC) is a small highly conserved bone specific protein, containing 46-50 amino acids and 2–3 *γ*-carboxyglutamic residues, which has strong capacity to combine with Ca^{2+} and hydroxyapatite. OC is also considered as a chemoattractant for

progenitor or mature osteoclasts, therefore it could interrupt mineral apposition and bone formation. The number of TRAP-positive cells on the pressure side was significantly increased (Hashimoto et al., 2001). In addition, osteocalcin did not seem to affect immunological reaction (Kobayashi et al., 1998). Another important local feature of osteocalcin is that it is considered as a differentiation marker for the osteoblastic phenotype. The majority of osteocalcin on the tension side is located in the cytoplasm of osteoblasts, cementoblasts, and periodontal ligament cells, and plays a role in bone deposition. Meanwhile, it mainly deposites in the periodontal extracellular matrix on the compression side, and plays a role in bone resorption (Han et al., 2008).

2) Macrophage colony-stimulating Factor (M-CSF)

M-CSF accelerates tooth movement by targeting preosteoclasts. M-CSF is known to induce osteoclasts (OCs) differentiation, recruit OCs precursors, inhibit apoptosis, and promote the expression of the receptor activator of nuclear factor kappa B (RANK) and tumor necrosis factor family receptors for cell fusion. Additionally, vascular endothelial growth factor (VEGF) proteins and macrophage marker F4/80 are activated after M-CSF binding to receptor c-Fms on monocytic lineage cells. RANK in turn binds its ligand (RANKL), inducing preosteoclastic cell fusion to produce polykaryon cells which could become OCs (Brooks et al., 2011).

3) RANKL

RANKL gene transfer to the local periodontium accelerated tooth movement through activating osteoclastogenesis by RANKL pathway in a rat model (Kanzaki et al., 2006). M-CSF and RANKL are the two major cytokines for osteoclastogenesis regulation. In the skeletal system, RANKL is expressed by the osteoblastic lineage cells, and exerts its osteoclastogenesis regulation effect via binding to its cell surface receptor on osteoclast lineage precursor. This binding induces a rapid precursor differentiation into fully mature osteoclast. Many other cytokines and hormones exert their osteoresorptive effect through RANKL which works as a downstream regulator of osteoclast differentiation and activation (Yamaguchi, 2009).

4) Parathyroid Hormone (PTH)

Orthodontic tooth movement speed in patients undergoing PTH medication treatment was increased (Shirazi et al., 1999). The possible reasons could be explained as following: PTH leads to an increased bone remodeling by increasing bone resorptive activity and reducing bone density. Parathyroidectomy completely inhibited osteoclast formation on the compression side of periodontium during tooth movement, and injection of parathyroid recovered the expression level of osteoclast back to normal. PTH is considered as a potent stimulator of osteoclast formation (Soma et al., 1999). Effects of PTH on bone tissue are also related to the augmentation of bone resorptive factors, such as interleukin-1, interleukin-6, and tumor necrosis factor-a production which are induced by PTH at low concentrations

(Diravidamani et al., 2012). In addition, PTH stimulates bone cells to release proteolytic enzymes, including collagenase, cathepsin B, and cathepsin L which will benefit for necrotic tissue rapid removing, thus unfavorable root resorption could also be avoided by PTH continuous administration (Soma et al., 1999).

5) Prostaglandins (PGEs)

A lower concentration (0.1μg) of PGEs seems to be sufficient for enhancing tooth movement, whereas, higher concentration leads to a side effect of root resorption (Leiker et al., 1995). Both submucosal and intraligamentous PGEs administrations have been assayed to enhance orthodontic tooth movement by increasing bone remodeling process (Caglaroglu and Erdem, 2012). The main side effect is hyperalgesia due to the noxious agents release associated with PGEs local administration. PGEs maybe work as mediators of mechanical stress during tooth movement (Diravidamani et al., 2012). PGEs injection increased the intracellular expression of cAMP and calcium, inducing morphologic changes in osteoclasts and osteoblasts (Yang et al., 1998). The mRNA synthesis and protein expression of RANKL are modified by exogenous PGEs (Kanzaki et al., 2002).

6) Growth Hormone (GH)

GH synthesized by somatotrophic cells in anterior pituitary gland, has growth-stimulating effects on many target tissues, especially liver, muscle, kidney, and bone. GH triggers the liver to product insulin-like growth factor (IGF), which has

stimulatory effects on the activation of chondrocytes, osteoblasts, and osteoclasts (Ueland, 2005). GH/IGF receptors presenting on osteoblasts and osteoclasts could activate these cells directly, and stimulate their proliferation, differentiation, and extracellular matrix production in osteoblast-like cells (Andreassen and Oxlund, 2001). Meanwhile, GH/IGF exerts the effect on bone cells in a more indirect way by stimulating other different factors, such as, IL-1, IL-6, IFN-gamma, TNF-alpha, OPG/RANK, Prostaglandins, Matrix Metalloproteinases (MMPs), Growth factors ,Vitamin D and so on (Swolin and Ohlsson, 1996; Zhang et al., 2004; Pagani et al., 2005).

7) Vitamin D

Collins and Kale demonstrated that local periodontium injection of vitamin D, or vitamin D metabolite, 1, 25-dihydroxy cholecalciferol sped up the rate of tooth movement (Collins and Sinclair, 1988; Kale et al., 2004). The well-balanced bone turnover promoted by vitamin D is essential in enhancing the rate of tooth movement, since vitamin D or vitamin D metabolite could increase the number of osteoclasts, facilitate the mineral apposition, and intensify the re-establishment of supporting alveolar bone after orthodontic treatment, as observed by Kawakami (Kawakami et al., 2004).

8) L-arginine

The nitric oxide (NO) synthesized from the amino acid L-arginine by the enzymatic action of nitric oxide synthase (NOS), has recently been implied in tooth

movement (Nilforoushan and Manolson, 2009). Nitric oxide synthase (NOS) seems to be an important enzyme responsible for initiating bone remodeling during orthodontic force application (Tan et al., 2009). Experimental rats were fed with drinking water consisting of 2% (w/w) dietary L-arginine, the result indicated that tooth movement was accelerated by increasing the number of osteoclasts through Larginine/NO pathway, and no significant increased root resorption was found during tooth movement (Mohammadi and Azar, 2012).

9) Nicotine

In vitro, treating human gingival fibroblasts with nicotine upregulated the expression of cyclooxygenase-2 (COX-2) which is known as one of the most important enzymes for converting arachidonic acid into prostaglandins and thromboxanes (Nakao et al., 2009). It explained the phenomenon that nicotine exerted a positive dose-dependent effect on orthodontic tooth movement in a rat model in vivo. However, considering the adverse effects of nicotine on periodontal tissues, it is not a suitable method for accelerating tooth movement in clinic situation (Sodagar et al., 2011).

Surgical Adjuvant Methods

1) Corticotomy

The experimental evidence demonstrated that the acceleration effect is limited to

approximate the first 1-2 months in a canine model, during which 4-6 mm tooth movement is expected to be obtained (Ortega et al., 2012). The corticotomy-assisted tooth movement is considered as a classical tooth movement combined with bone block movement (Iino et al., 2007). The main reasons explaining for the positive effects of corticotomy on tooth movement were proposed by Iino et al.: 1) Hyalinization within the periodontal ligament is eliminated which causes the disappearance of the lag phase. 2) Bone turnover is increased. 3) Bone density is decreased. Furthermore, root resorption in the corticotomy group was less than the sham group which may be due to the less hyalinization of the PDL in the early stage. The negative effects to the periodontium, ranging from the loss of attached gingiva to periodontal defects, were observed in some short interdental cases (Kwon et al., 1985).

2) Osteoperforation

Data Analysis demonstrated that shallow cortical bone perforations induced higher inflammatory markers levels, such as, CCL20, CCR1 (lymphocytes); LTa,IL-3, CCL5, CCR5, CX3CR1, IL-18rb, IL-1r1(T-cells); IL-1, IL-6, IL-11, IL-18, IL-6ra (monocytes); IL-1, TNF, IL-6, IL-11, IL-18, IL13ra1, CCL2, CCL9, CCL12, CCR5, IL-6ra (macrophages), without changing their expression patterns in response to tooth movement. Increased expressions of inflammatory cytokines facilitate the recruitment of osteoclasts so as to assist tooth movement which depends on the bone remodeling (Teixeira et al., 2010). Osteoperforation surgery is more simple and less invasive than corticotomy, and it could be used instead of corticotomy.

3) Dentoalveolar Distraction vs Periodontal Distraction Osteogenesis

From the clinical point of view, distraction osteogenesis is an established and efficient method for rapid orthodontic canine retraction in some clinical cases (Kisnisci et al., 2002). The dentoalveolar distraction technique reduced 50% overall treatment duration without negtive effects on surrounding tissues (Iseri et al., 2005). Distraction osteogenesis avoids root resorption that is attributed to the decreased force required for periodontal distraction. Moreover, anchorage loss in both techniques is negligible (Long et al., 2013).

4) Circumferential Supracrestal Fiberotomy (CSF)

CSF Used for Accelerating Tooth Movement

In an animal study, at the end of active phase, the fist molar moved twice the distances in fiberotomy rats when compared with apical surgery or no surgery rats. It is interesting that apical flap surgery without detaching the dentogingival fibers had no evident effects on the rate of tooth movement than no surgery group. It seemed that detaching dentogingival fibers from root surfaces immediately relaxed the strain of fibroblasts, and then triggered cellular release of ATP which further activated a pathway, leading to the burst of osteoclastic alveolar bone resorption (Young et al., 2013). Roberts stated that the resorptive activity mediated by osteoclasts is the limiting factor in tooth movement in which the periodontal ligament (PDL) plays a

crucial role (Roberts et al., 2004).

Another mechanism explaining for the accelerated tooth movement by CSF was proposed in the last decades. Reduction or elimination of the forces of supracrestal fibers shift rotation center toward the root apex, thereby affect the crown movement (Tuncay and Killiany, 1986).

CSF Used for Alleviating Relapse

Immediate contraction of transseptal fibers which are mainly composed of collagen and oxytalan has been shown to play a major role in relapse (Redlich et al., 1996; Hirate et al., 2012). The aforementioned authors Young et al. suggest that the instantaneous loss of positional physical memory of the dentition (PPMD) is the key in alleviating relapse by fiberotomy (Young et al., 2013). From clinical point of view, the CSF technique does contribute to inhibiting rotational tooth relapse.

Possible Relationships between Timps, MMPs and Osteoclasts

Since several studies have found that MMPs associated with the degradation of extracellular matrix are also involved in the differentiation, recruitment, and invasive activity of osteoclast in several situations (Delaisse et al., 2000; Engsig et al., 2000; Hirate et al., 2012). MMP-9 is involved in osteoclast migration, and the release of VEGF enticed by MMP-9 may be the reason for osteoclast recruitment (Delaisse et

al., 2003) (Fig. 1). Both osteoclast number and bone resorption were decreased in a MMP-13 knockout mice model, and osteopetrosis was increased. MMP-13 is believed to be involved in the initiation of bone resorption and osteoclast differentiation. It has been proposed that MMP-13 generated collagen fragments are key factors for the activation of osteoclast and the initiation of absorption pit formation (Holliday et al., 1997). MMP-14 induces the release of RANKL which is known as a regulator for osteoclast differentiation and activation. MMPs are also proposed to interfer the access of some messages into cells. MMPs degrade IL-1, PTHrP 1-34, calcitonin (MMP-9), and remove membrance HGF-receptor, TNF-receptor, and interleukin-6-receptor (Delaisse et al., 2003).

Low concentrations of Timp1 promote bone-resorbing activity by directly stimulating osteoclasts, in contrast, higher concentrations of Timp1 reduce bone resorption by alleviating some MMPs activity (Geoffroy et al., 2004) (Fig. 2).

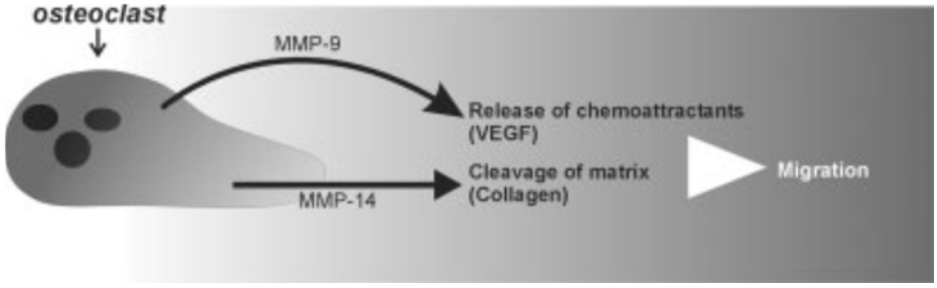

Figure 1. Model for the involvement of MMPs in osteoclast migration.

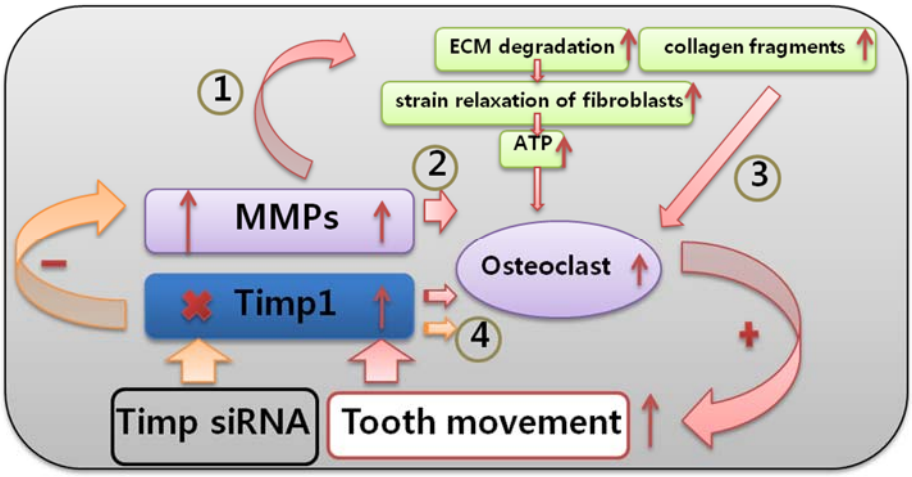

Figure 2. The possible relationships between Timp1 siRNA, MMPs, Timp1, osteoclast, and tooth movement. The expression levels of MMPs and Timps are increased at both the resorption and apposition sides during tooth movement. Timp1 siRNA could attenuate Timp1 inhibitory effect on MMPs. 1) Degragation of periodontic ligaments releases the strain of fibroblasts, which will trigger cellular releasing ATP that activates a pathway to lead to the burst of osteoclastic alveolar bone resorption. 2) MMP-13 may be involved in the initial bone resorption and osteoclast differentiation. MMP-14 induces the release of RANKL which could entice osteoclast differentiation and activation. 3) MMP-13-generated collagen fragments are key factors for the activation of osteoclast and the initiation of the resorption pit formation. 4) Low concentrations of Timp1 promote bone-resorbing activity by directly stimulating osteoclast, in contrast, higher concentrations of Timp1 reduce bone resorption by alleviating some MMPs activity.

Possible Relationships between Timp1, MMPs and Extracellular Matrix

Four kinds of vertebrates Timps (Timp1, Timp2, Timp3, and Timp4) that specific bind MMPs in a 1:1 stoichiometry have been discovered (Brew et al., 2000). As far as is known, Timp1 could inhibit all MMPs, except MT1-MMP, MT3-MMP, MT5-MMP and MMP-19 (Will et al., 1996). The changing of Timps expression level is considered to be important for MMPs activity. The expression levels of MMP-2, MMP-9, and Timps 1-3 at both the compression and tension sides increase instantly during tooth movement. Alteration of MMPs/ Timps expression pattern may determine the rate and extent of ECM remodeling (Takahashi et al., 2006).

As far as is known, delivering Timp1 siRNA to rat with hepatic fibrogenesis resulted in indirect types I and III collagen fibrolysis by elevating the expression and activity of MMP-13, which is the main substrate of Timp1 (Cong et al., 2013).

Hypothsis of This Study

The past decades, some researchers have reported the use of CSF as a means of accelerating the kinetics of tooth movement through enhanced bone remodeling and reduced gingival tissue resistance to orthodontic forces in a variety of animal models (Tuncay and Killiany, 1986; Young et al., 2013). Immediate contraction of transseptal fibers composed of collagen and oxytalan has been considered to play a major role in relapse (Redlich et al., 1996; Hirate et al., 2012). CSF is the unique method that could

affect the tooth movement and relapse simultaneously.

MMPs contribute to the cleavage of various components of the ECM, and activate osteoclasts to trigger bone resorption (Holliday et al., 1997; Holliday et al., 2003). As far as is known, Timp1 could inhibit all MMPs, except MT1-MMP, MT3-MMP, MT5-MMP and MMP-19 (Nagase et al., 2006). Holliday et al. demonstrated the inhibitory effect of Timps on tooth movement in a rodent model (Holliday et al., 2003). An imbalance between MMPs (MMP-1, MMP-8, MMP-9) and Timps, the expression of which increased at both the resorption and apposition sides during tooth movement (Takahashi et al., 2003; Takahashi et al., 2006; Bildt et al., 2009), was quite notably followed by the periodontal connective tissue breakdown (Reynolds and Meikle, 1997). Delivering Timp1 siRNA to a rat with hepatic fibrogenesis resulted in indirect types I and III collagen fibrolysis by elevating the expression and activity of MMP-13, which is the main substrate of Timp1 (Cong et al., 2013).

MMPs or Timps may represent potential targets for the pharmacological reagents for tooth movement and relapse. The Timp1 siRNA was used for local subperiosteum injection in a mouse tooth movement model. As a preliminary experiment in these *in vivo* studies, the NIH3T3 cells were cultured to assess the possibility and potential mechanisms of attenuation of collagen expression by Timp1 siRNA *in vitro*.

II. MATERIALS & METHODS

1. siRNA Design and Synthesis

Formulations of synthetic Small-interfering RNAs (siRNAs) directed against Timp1 were aligned based on the reference genome (UCSC ID: NM_053819) and purchased from Shanghai GenePharma Co., Ltd. The following siRNAs synthesized and purified with HPLC were used:

siRNA283, sense sequence: 5′-CUGGUCAUAAGGGCUAAAUTT -3′ and antisense sequence: 5′- AUUUAGCCCUUAUGACCAGTT-3.

SiRNA340, sense sequence: 5′-CAGCGUUAUAAGAUCAAGATT -3′ and antisense sequence: 5′- UCUUGAUCUUAUAACGCUGTT-3.

SiRNA577, sense sequence: 5′-GCUUUCUCAAAGACCUAUATT -3′and antisense sequence: 5′- UAUAGGUCUUUGAGAAAGCTT-3.

Scramble siRNA was used as negative control under similar conditions. For determining siRNA transfection efficiency, FAM labeled NC-siRNA obtained from GenePharma was used. All of the siRNAs were modified by methoxy for in vivo periosteum injection.

2. In vitro siRNA Transfection

NIH3T3 cells (Mouse embryonic fibroblast cell line) were obtained from Korean Cell Line Bank (KCLB, Seoul, Korea) and were maintained in D-MEM supplemented with 10% FBS, 4 mM gutamine, 1.5 g/L sodium bicarbonate, and 1% penicillin-streptomycin. One day before transfection, cells were diluted in complete growth medium without antibiotics, and plated into 6 well plates. The cell density was 30-50% confluence at the time of transfection. siRNA duplex-Lipofectamine RNAiMAX (Invitrogen Corporation, Carlsbad, CA, USA) complexes were prepared according to the manufacturer's forward transfaction instructions. siRNA (480 pmol) was diluted in Opti-MEM reduced serum medium (Invitrogen Corporation, Carlsbad, CA, USA) without antibiotics to a final volume of 200 μl. Lipid (4 μl) was diluted in 200 μl Opti-MEM and was then combined with the diluted siRNA duplex. After 10-20 min of incubation at room temperature in order to allow complex formation, the siRNA duplex-Lipofectamine RNAiMAX complexes were added to each well containing cells in 2 ml Opti-MEM (200 nM final concentration). Subcellular distribution of FAM-labeled siRNA was observed by fluorescence microscopy. Total RNA and total protein were harvested for RT-PCR, and western blot analysis, respectively, after incubate for 48-72 h at 37°C in a CO_2incubater.

3. Reverse Transcription Polymerase Chain Reaction

Total cellular RNA was extracted by means of a PureLink™ RNA Mini Kit (Ambion, Carlsbad, CA, USA). The following Timp1 gene specific primer set was used for amplifying fragment of the polymerase gene:

FW (5'-CTGGTCQTAAGGGCTAAATTCATG -3')

RV (5'-GCCTTGAATCCTTTTAGCATCTTAG-3')

Specific primers for GAPDH were used for control:

FW (5'-AGGTCGGTGTGAACGGATTTG -3')

RV (5'-TGTAGACCATGTAGTTGAGGTCA-3')

RT-PCR was performed using a one-step RT-PCR kit (Qiagen, Valencia, CA, USA) in a 20 μl reaction volume containing 4.6 μl (1 μg) template RNA, 4 μl 5x QIAGEN OneStep RT-PCR Buffer, 0.8 μl dNTP mix, 1.5 μl (0.6 μm) of each primer, 2.8 μl Rase-free water, 0.8 μl QIAGEN OneStep RT-PCR Enzyme Mix, 4 μl 5x Q-Solution. The optimized thermal cycling conditions were used: reverse transcription at 50°C for 30 min; PCR activation at 95°C for 15 min; 30 cycles of 94°C for 30 s, 58°C for 90 s, and 72°C for 1min; final extension at 72°C for 10 min. Amplified cDNA bands were analyzed by electrophoresis in 1% agarose gels, and visualized by ethidium bromide staining under UV-light.

4. Western Blotting

Cells were lysed in lysis buffer (20 mM Tris PH7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 1% Na_3VO_4, 0.5 μg/ml leupeptin, 1 mM PMSF). Protein lysate (30 μg) were loaded on 12% (6%) SDS-PAGE gels followed by blot onto PVDF membranes (Bio-Rad, Hercules, CA, USA). After blocking, proteins were identified using primary antibodies (Timp1: 1:500; collagen1: 1:500; b-tubulin: 1:10,000) and HRP- conjugated goat anti-rabbit secondary antibody (diluted 1:1000). Bound antibodies were visualized using enhanced chemiluminescence ECL kit (Amersham Biosciences, GE Healthcare, UK).

5. Animals and Orthodontic Appliance

Total twenty eight-week-old male C57BL6/J mice (body weight 23 ± 1.6 g) purchased from Korea Research Institute of Bioscience and Biotechnology (KRIBB, Daejeon, Korea) were used in this study. Twenty mice were randomly divided into 2 groups: 10 mice were local subperiosteum injected with Timp1 siRNA twice per week during the whole experiment period; the others worked as control and were injected with PBS. During the experiment, the mice were fed granular chow and distill water ad libitum in a 12-hour light/dark environment. All animal procedures in this study were performed in accordance with the guidelines of Ethics Committee at Yonsei University College of Dentistry.

As described in the previous lecture (Braga et al., 2011), a Ni-Ti closed-coil spring was inserted between maxillary left first molar and incisors, with the contralateral side used as control (Fig. 3). The springs initially delivered a mesial direction force of 30 g. To prevent the detachment of the appliance and to avoid rehooking the wire repeatedly, the mandibular incisors were polished, and the mandibular first molar was extracted. Ligatures were fixed with light-curing adhesive to the maxillary incisors followed by the removal of suspended known weight, ensuring a precise initial orthodontic force.

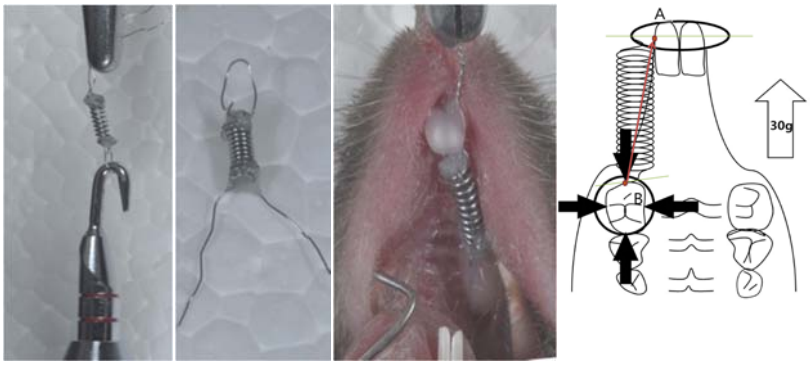

Figure 3. The application of orthodontic device in a mouse tooth movement model. 1) A Ni-Ti closed-coil spring combined with a ligature wire by light-curing resin was prepared first. 2) Ligatures were fixed with light-curing adhesive to the maxillary incisors followed by the removal of the suspended known weight, ensuring a precise initial orthodontic force. 3) The upper left second molar and the lower first molar were extracted to escape the detachment of appliance, and the mandibular incisors were polished. 4) The most right figure showed that the distance between A

(the cemento-enamel junction of the maxillary incisor) and B (the most mesial point of the homolateral maxillary left molar) was measured using a digital caliper, described as the I–M distance. The injection locations surrounding the molar gingiva were marked as the picture shown (black arrow). 5) A 30 g mesial direction force was used for the first molar migration.

6. In vivo siRNA Transfection

The transfection complex of Timp1 siRNA was prepared for local injection according to the manufacturer's instructions (Engreen, Beijing, China). Briefly, 5 μg of Timp1 siRNA was mixed with 5 μl Entranster™-*in vivo* and diluted with 10% glucose to obtain a 20 μl transfection complex at a final concentration of 0.25 μg/μl. For in vivo transfection, the 20 μl siRNA mixtures was delivered into the subperiosteum adjacent to the maxillary right first molar twice per week, beginning from the initial day of force application to the end of the experiment (Fig. 4). The control group was given injection of PBS.

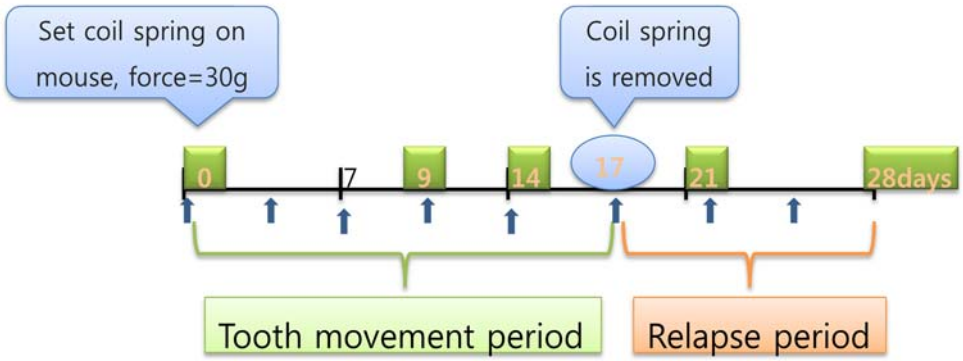

Figure 4. The time schedule for experiment. 1) The spring was inserted between the maxillary left first molar and the incisors on the initial fist day (Day 0). 2) The magnitude of tooth movement was quantified twice per week during the tooth movement period from 0 to 17 days. On the day 17, orthodontic appliance was removed, and then the relapse distances were recorded up to day 28. 3) Total twenty eight-week-old male C57BL6/J mice were used in this study. 10 was local subperiosteum injected with Timp1 siRNA twice per week during the whole experiment period; the other ten was used as control injected with PBS. 4) One mouse from each group was sacrificed for HE and IHC assays on day 1, 9, 14, 21 and 28, separately.

7. Measurement of Tooth Movement and Relapse

Distances of tooth movement and relapse were quantified with a precision digital

caliper (Mitutoyo Co., Kawasaki, Japan) at 0, 3, 7, 10, 14, 17, 21, 24 and 28 days after application of the Ni-Ti coil spring. The magnitude of tooth movement was quantified up to 17 days. On the day 17, orthodontic appliance was removed, and the relapse distances were recorded up to 28 days. The method used for quantifying molar mesial drift involved measuring the relative separation at the gingival level between the cemento-enamel junction of the maxillary incisor and the most mesial point of the homolateral maxillary left molar (I–M distance) (Ren et al., 2003). Every sample was measured 3 times by a single investigator in blind tests, and the final measurement was averaged.

8. Hematoxylin-Eosin Staining

Five animals from each group were sacrificed by intracardiac fixation perfusion with 4% paraformaldehyde on days 1, 9, 14, 21 and 28 (Fig. 4). After perfusion, the maxillary jaw on the tooth movement side, including the first molar, was dissected, immersed in the same fixative overnight at 4°C, decalcified with Rapid Cal Immuno (BBC Biochemical, WA, USA) for 4 days, and finally embedded in paraffin wax. Specimens were sliced into serial 4 μm thick sections at the middle third of the mesial roots in the horizontal direction and were stained with hematoxylin and eosin. The periodontal tissues in compression and tension areas of the mesial roots, the positions of which were determined as previously described (Hirate et al., 2012), were evaluated using light microscope, respectively.

9. Immunohistochemistry

Immunostainings for Col-I/Timp1 were used to demonstrate the Timp1 reducing and collagen1 degradation in the mice injected with Timp1 siRNA. In brief, the slides were deparaffinized and incubated with polyclonal rabbit anti-Timp1 antibody at 1:200 (Santa Cruz Biotechnology Inc., California, USA), or polyclonal rabbit anti-Col-I antibody at 1:100 (Bioss Biotechnology, Beijing, China) overnight at 4°C. Col-I and Timp1 were stained using HRP-conjugated goat anti-rabbit IgG detection system (Beyotime, Shanghai, China), followed by the final colour reactions with diaminobenzidine.

10. Statistics

Repeated-measures ANOVA was used to evaluate intergroup comparison of the mean values, and two group statistical differences at each time point were analyzed by paired t test ($P < 0.05$). Data in each figure represented mean ± SD for each group.

III. RESULTS

1. Timp1 siRNA Complexes Silence the Expression of Timp1 and Facilitate the Degradation of Collagen1 in vitro

Efficient cellular internalization of FAM-siRNA complexes in NIH3T3 cells was indicated by discrete green dots after 12 h incubation (Fig. 5A). RT-PCR analysis of total RNA after 48h showed that siRNA340 and siRNA577 were more potent silencing reagents, inhibiting Timp1 mRNA expression by 80-90% at the 200 nM siRNA level (Fig. 5B). Proliferation was not or only minimally affected by Timp1 siRNA transfer, as assessed by the levels of the internal housekeeping gene GAPDH. Furthermore, siRNA340 and siRNA577 exhibited a near-equivalent knockdown effect of Timp1 at the protein level (Fig. 5C, D). In order to determine whether the down-regulation of Timp1 expression would increase collagen1 degradation activity, collagen1 protein levels were analyzed, and were found to have decreased by approximately 70% (Fig. 5E, F).

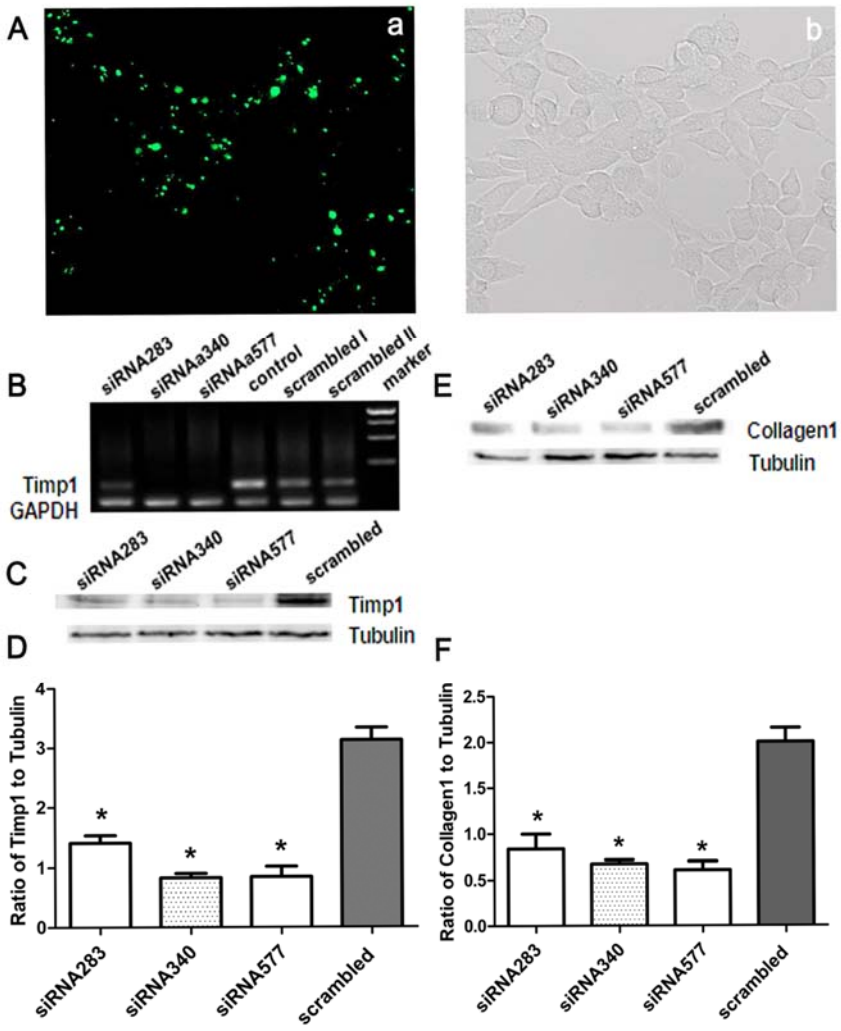

Figure 5. In vitro assay of Timp1 siRNA transfection. (A) Fluorescence microscopic image showing the uptake of FAM-siRNA complexes in green. NIH3T3 cells were treated with 200 nM FAM-siRNA for 12 h. (B) RT-PCR analysis of Timp1 mRNA levels at 48 h post-transfection. (C, D, E, F) Representative western blot analysis of Timp1 and collagen1 expressions at 72 h post-transfection. Analysis of band intensity was represented as the ratio of Timp1 (D) or collagen1 (F) to tubulin. Data indicated mean ± SD of four independent experiments. $^{*}p < 0.05$ as compared with scramble siRNA transfected cells determined by Student's *t*-test.

2. Local Timp1 siRNA Transfer Accelerates Orthodontic Tooth Movement and Alleviates Relapse

The weight of the mice decreased transiently during the first 3 days and then recovered (Fig. 6). During the 17 days activation phase, the Timp1 siRNA transfection group demonstrated a significantly greater tooth movement at every time point compared with the control group ($p < 0.05$) (Fig. 7). On day 17, the maximum mean amount of tooth movement in the Timp1 siRNA transfection group and the control group was 0.94 mm and 0.73 mm, respectively (Table. 1). The most obvious effect of the Timp1 siRNA administration, viz. 80% of the maximum accelerated tooth movement, was apparent in the early activation period, between days 0 and 7 (Table. 2). During the relapse phase, from days 17 to 28, the relapse percentages of Timp1 siRNA group – as 5.32% (Day 21), 9.58% (Day 24) and 15.96% (Day 28), which were significantly less than the 27.34% (Day 21), 49.32% (Day 24), and 64.38% (Day 28) of the control group ($p < 0.05$).

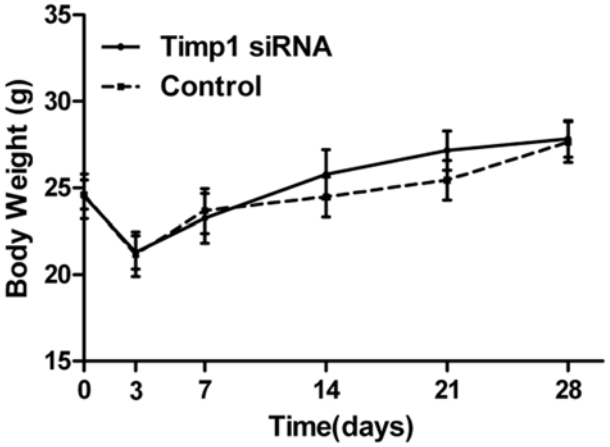

Figure 6. Body weight changes throughout the experiment expressed as the mean ± SD. The weight of the mice decreased transiently during the first 3 days and then recovered.

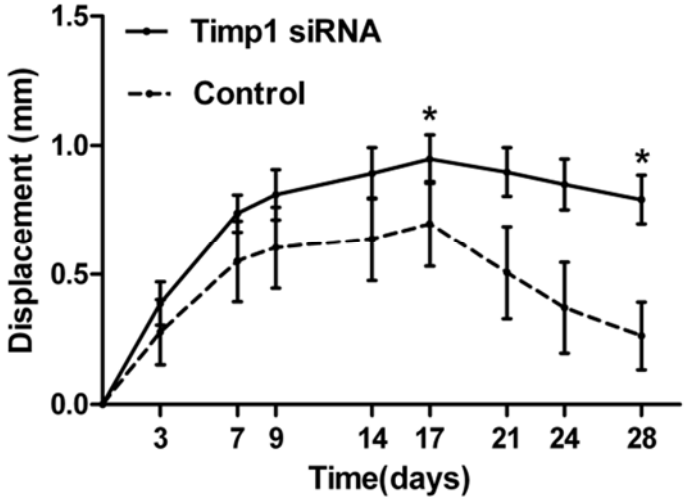

Figure 7. Effect of Timp1 siRNA injections on tooth movement displacement. Each point showed the mean ± SD of 5-10 mice. Two group statistical differences at two important time points were analyzed by paired t test ($^{*}p < 0.05$).

Table 1. Descriptive stasistical results of tooth movement distance (mm) at different time points (mean ± SD)

Day / Group	3	7	9	14	17	21	24	28
Timp1 siRNA	0.39 ± 0.09	0.75 ± 0.06	0.83 ± 0.10	0.88 ± 0.11	0.94 ± 0.10	0.89 ± 0.10	0.85 ± 0.10	0.79 ± 0.09
PBS	0.29 ± 0.14	0.55 ± 0.17	0.60 ± 0.17	0.65 ± 0.17	0.73 ± 0.16	0.53 ± 0.18	0.37 ± 0.18	0.26 ± 0.16

Table 2. Comparisons of the teeth movement distances (mm) between two groups in different time periods

Period (days)	Normality test	Paired t test	
	K-S	t	P
0-3	0.198	3.003*	0.017
3-7	0.169	2.430*	0.041
7-9	0.136	1.525	0.166
9-14	0.208	0.766	0.469

*$p < 0.05$

3. Hematoxylin-Eosin Staining Results

No obvious inflammatory cell infiltration was observed in the periodontium where Timp1 siRNA or PBS was injected repeatedly. Bone resorption on the compression side injected with Timp1 siRNA increased gradually during tooth movement, and the increase was greater than that seen in the control (Fig. 8B', C'). From days 9 to 17, the phase of stasis in tooth movement was generally attributed to the formation of hyalinization which was found on the compression side in some samples, especially in the control (Fig. 8B', D'). Photomicrographs indicated a more coarse and amorphous fiber structure in the Timp1 siRNA group compared to the control group on both the compressed side and tension side (Fig. 8D', E', D", E"). The thickness of the periodontium on the compression side was significantly decreased during tooth movement. The figure also showed serious root resorptions after orthodontic appliance insertion in PBS control group. Multinucleate osteoclasts and lacunae (black arrowhead in Fig. 8C'', D'', E'') were found on the mesial or even the distal bone surfaces.

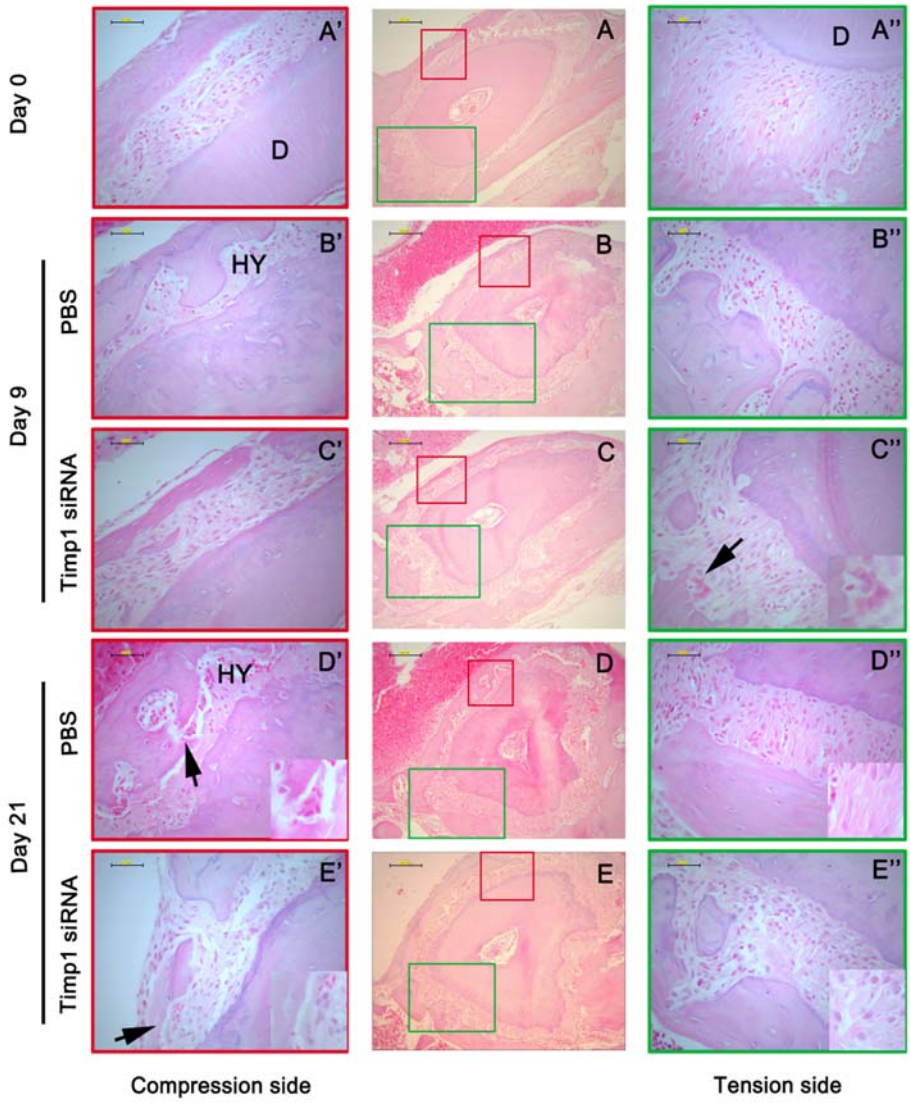

Figure 8. Representative cross section images showing the variations of the periodontal tissue at the level of the middle third of the mesial root. Hyalinization (HY) was formed (B', D') during tooth movement, especially in the PBS group.

Photomicrographs indicated that fiber ligaments in the Timp1 siRNA group in both the compression (E') and tension sides (C", E") became more and more coarse, amorphous and irregular on day 9 and day 21 when compared with day 0 (A', A") and the PBS control group (B", D"). This figure also showed serious root resorptions after orthodontic appliance insertion (B, D, B', D') in the PBS control group. Multinucleate osteoclasts and lacunae were found on the mesial (arrowhead in D', E') and distal (arrowhead in C'') bone surface. Red and green boxes are higher magnification images of A, B, C, D, E. (A', B', C', D', E') Compression sides of the periodontium. (A'', B'', C'', D'', E'') Tension sides of the periodontium. D: dentin; HY: hyalinization. Scale bar- A, B, C, D, E: 400 μm; A', B', C', D', E', A'', B'', C'', D'', E'': 200 μm.

4. Timp1 siRNA effects collagen1/Timp1 distributions in the periodontium

Consistent with previous findings, a marked gradual increase of collagen1 was observed in the periodontium until day 9 after insertion of orthodontic appliances, and then again decreased to normal level (Fig. 9a, c, e). Significantly reduced immunoreactivity to collagen1 in mice treated with Timp1 siRNA transfer was observed (Fig. 9b, d, f), when compared to that in mice treated with PBS (Fig. 9a, c, e), which corresponded to a marked decrease in Timp1 in the Timp1 siRNA group (Fig. 10b, d, f). The Timp1 siRNA transfection met with the requirement for inhibition of the increased Timp1. The knockdown of Timp1 by Timp1 siRNA was shown on both the compression and tension sides and remained at low levels during the whole observation period (Fig. 10).

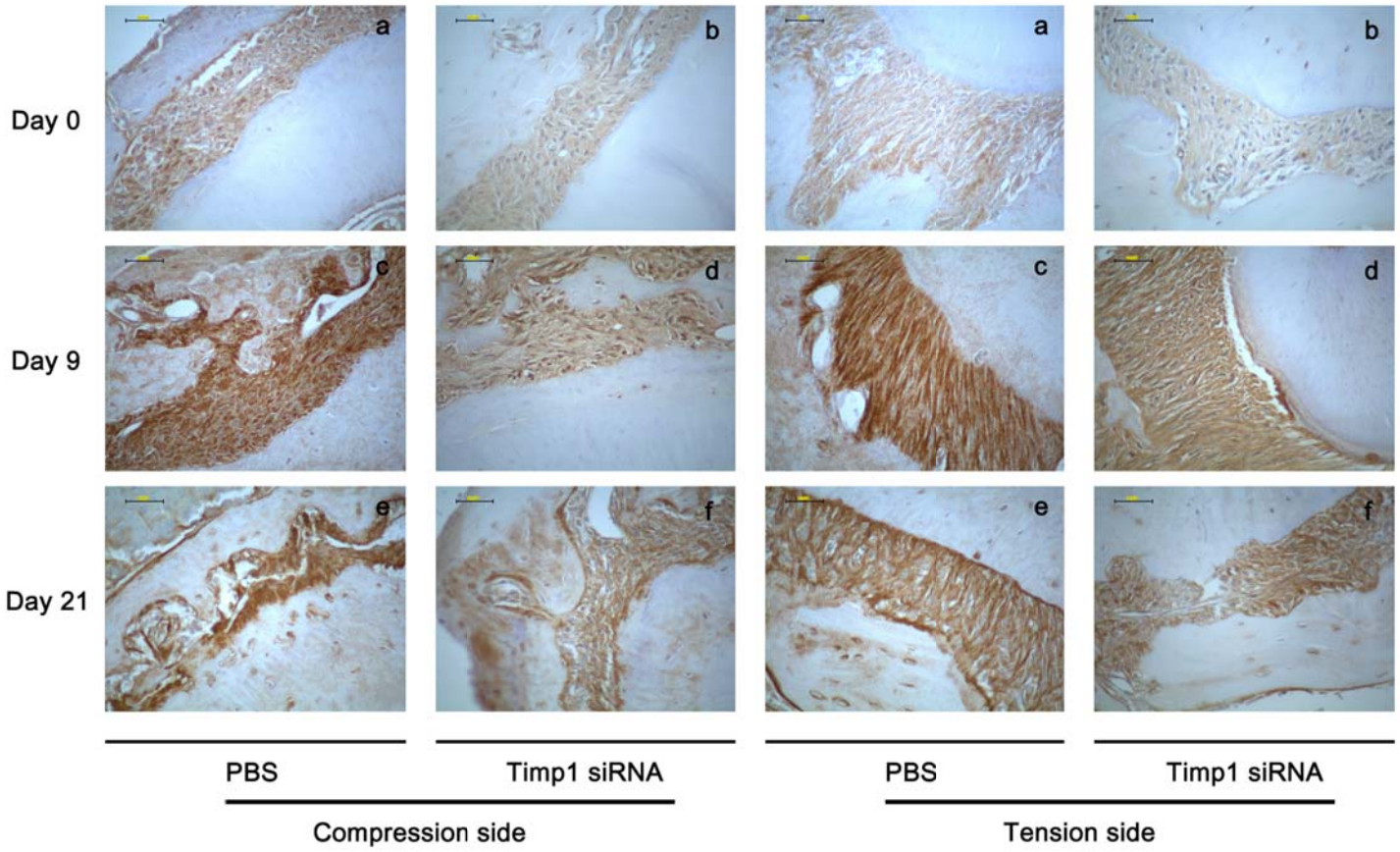
Day 0
Day 9
Day 21
a
b
a
b
c
d
c
d
e
f
e
f
PBS
Timp1 siRNA
PBS
Timp1 siRNA
Compression side
Tension side

Figure 9. Immunohistochemical staining of collagen1 in the periodontal tissue. (A) The two left columns images showing the compression tension sides of the PBS control group (a, c, e) and the Timp1 siRNA group (b, d, f), during tooth movement (Day 0, Day 9) and relapse (Day 21). (B) The two right columns images showing the tension sides of the PBS control group (a, c, e) and the Timp1 siRNA group (b, d, f), during tooth movement (Day 0, Day 9) and relapse (Day 21). Specimens prepared at 0, 9 day after insertion of the orthodontic appliance, and 21day as the appliance has been removed. Original magnification: ×400. Scale bars = 200 μm.

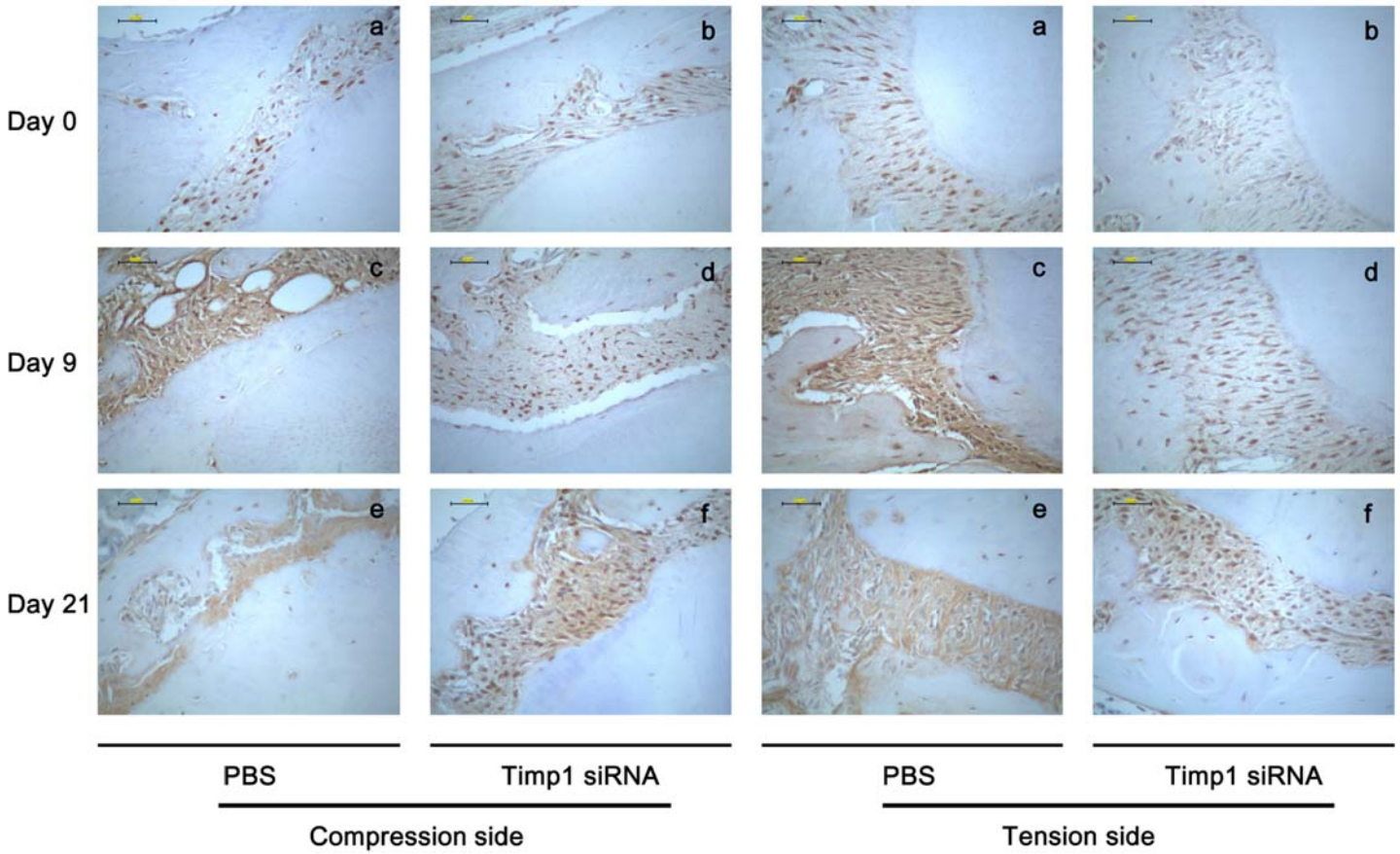
Day 0
Day 9
Day 21
a
b
a
b
c
d
c
d
e
f
e
f
PBS
Timp1 siRNA
PBS
Timp1 siRNA
Compression side
Tension side

Figure 10. Immunohistochemical staining of Timp1 in the periodontal tissue. (A) The two left columns images showing the compression tension sides of the PBS control group (a, c, e) and the Timp1 siRNA group (b, d, f), during tooth movement (Day 0, Day 9) and relapse (Day 21). (B) The two right columns images showing the tension sides of the PBS control group (a, c, e) and the Timp1 siRNA group (b, d, f), during tooth movement (Day 0, Day 9) and relapse (Day 21). Specimens prepared at 0, 9 day after insertion of the orthodontic appliance, and 21day as the appliance has been removed. Original magnification: ×400. Scale bars = 200 μm.

IV. DISCUSSION

Many researchers have attempted to accelerate orthodontic tooth movement through approaches such as surgical procedures, pharmacological agents and so on (Lee, 1990; Soma et al., 2000; Yamaguchi et al., 2010; Sodagar et al., 2011). Most of them sped up the rate of tooth movement through increasing the activation of osteoclasts. However, very few studies have considered both the tooth movement and the relapse that occurred after tooth movement. Fiberotomy of the marginal gingiva connection accelerates orthodontic tooth movement and diminishes relapse simultaneously. During fiberotomy, ATP release caused by transient fibers strain relaxation leads to accelerated bone remodeling; the transient loss of PPMD, thus allows teeth to remain stable at the new position, without retracting back to their origin (Edwards, 1988; Young et al., 2013).

In this study, the findings *in vitro* indicated that the Timp1 siRNA complex transfer provides a possible avenue for mimicking the effects of a gingival fiberotomy in mice, as the inhibition of Timp1 can elevate collagen degradation, possibly by increasing MMPs activation. Several studies have found that MMPs associated with the degradation of extracellular matrix are involved in the differentiation, recruitment, and invasive activity of osteoclasts in several contexts. (Delaisse et al., 2000; Engsig et al., 2000; Delaisse et al., 2003). Inhibitor of MMPs suppresses bone resorption and orthodontic tooth movement by preventing the activation of mature osteoclasts (Holliday et al., 2003).

For maintaining continuous inhibition, the most efficient homology-dependent chemically synthesized Timp1 siRNA was chosen and injected in subperiosteum every 3 days for 28 days. In vivo study showed that the Timp1 siRNA transfer treatment resulted in a decrease in the expressions of Timp1 and collagen1, which were significantly elevated at both the tension and compression sites after orthodontic application inserting, which were detected by immunohistochemical staining.

Both groups maintained a high speed of tooth movement up to days 7, and then slowed down into a lag period. This finding indicated that Timp1 siRNA did not affect the predictable overall pattern of tooth movement curve. It is likely that the tensile strain generated in normal function collagen fiber was not the unique stimulus for osteogenesis, as described previously by Meikle (Meikle, 2006). Tension from collagen fibers of the PDL may not be an absolute necessary for bone formation which was elucidated by an animal study that disrupting rat PDL collagen fibers by the lathyrogen beta-aminopropionitrile (BAPN) did not cause an abnormal bone remodeling in response to orthodontic force (Noda et al., 2007). It was speculated that Timp1 siRNA transfer may confer more appreciable benefits as compared with direct MMPs injection. Because the expression of Timp1 is usually remained at a relative low level, Timp1 siRNA could not perform the severe inhibitory effect until Timp1 expression was raised following the tooth movement. As a consequence, the remodeling of periodontium by Timp1 siRNA would be more close to a kind of physiologic adaptation. In contrast, sudden collagen degradation induced by direct MMPs injection may interfere with the initial signals transduction, and postpone the

tooth movement. The lag phase could not be avoided by Timp1 siRNA injection. Hyalinization is known as one of the most important factors that counteract tooth movement, and probably mainly depend on the irregularities in periodontal and bone morphology rather than on the magnitude of applied force, as a previous study found (Von Bohl et al., 2004).

Assuming that the main cause of relapse is not transseptal fibres, but the rebuilding of the alveolar bone (Yoshida et al., 1999; Franzen et al., 2013), it could be speculated that a burst of osteoclastic alveolar bone resorption in response to fiberotomy would lead to an even more serious relapse. In contrast, fiberotomy is successful in obtaining a favorable result over a short period with reduced replase (Tuncay and Killiany, 1986; Hirate et al., 2012). The findings in present study also suggested that stretching of fibrous bands in the periodontium still plays an important role in relapse, whereas remodeling of the alveolar bone may be a procedure caused by such stretching. Long term studies should investigate this phenomenon further. Relapse is a complex problem with many other potential confounders such as occlusal interference, dynamics of facial growth, dentoalveolar development, and maxillofacial function, and needs to be comprehensively understood before treatment (Birgit *et al*., 2000). Considering these factors, a more reasonable orthodontic treatment plan with concomitant pharmacological agent treatment may benefit some patients eliminating the need for retainers, and avoiding unfavorable dental alignment several years after retention. In this study, Timp1 siRNA was injected from the beginning of orthodontic appliance activity. Unfortunately, due to the lack of the

control group (Timp1 siRNA was only injected after removing the orthodontic appliance), role of the prolonged treatment did not be confirmed in this research.

Increased root resorption observed during tooth movement may be due, at least in part, to the 30 g force used in the mouse model, since 5 cN is considered to be heavy enough for evaluating experimental rat tooth movement, as analyzed by Ren (Ren et al., 2003). Whereas, there was no relative tooth resorption description in the Braga study in which 35 g was applied to a mouse model (Braga et al., 2011). Thus, another possible explanation is that odontoclasts may have been affected simultaneously by Timp1 siRNA injection. Further research is required to identify the main reason for the root resorption.

In this study, local injection of Timp1 siRNA complexes in the periodontal tissue has been used to enhance the absorption effect and decrease systemic diffusion, without impairing the biological activity of Timp1 siRNA. Andersen et al. previously exploited a mucoadhesive and mucopermeable chitosan/siRNA nanoparticle system which may provide a possible platform for the future clinical application of Timp1 siRNA (Andersen et al., 2009), nevertheless, before that, long term researches are required to describe the mechanism action, long term efficacy, safety and toxicity of Timp1 siRNA, and further modifications of the gene transfer are needed.

It is a pity that MMPs expression level after the Timp1 siRNA transfection has not been assayed in this study. However, it was proved that down regulation of Timp1 expression has successfully diminished collagen1 protein levels in vitro and vivo. Thus, it is speculated that MMPs may be one of the essential factors for accelerating

tooth movement and alleviating relapse in this experiment model. The possible mechanisms are explained as following: Timp1 siRNA disturbs the homeostasis between Timp1 and MMPs, and promotes MMPs expression and activity. 1) Degragation of periodontic ligaments releases the strain of fibroblasts, which will trigger cellular releasing ATP that activates a pathway to lead to the burst of osteoclastic alveolar bone resorption. 2) MMP-13 may be involved in the initial bone resorption and osteoclast differentiation directly. MMP-14 induces the release of RANKL which could entice osteoclast differentiation and activation. 3) MMP-13-generated collagen fragments are key factors for the activation of osteoclast and the initiation of resorption pit formation. 4) Low concentrations of Timp1 promote bone-resorbing activity by stimulating osteoclasts, in contrast, higher concentrations of Timp1 reduce bone resorption by alleviating MMPs activity.

V. CONCLUSION

In conclusion, this study demonstrated that downregulation of Timp1 by Timp1 siRNA facilitated degradation of collagen1 in vitro and in vivo. As it has been anticipated, posttranscriptional gene silencing of Timp1 enhanced the initial velocity of tooth movement and diminish instant relapse simultaneously.

(A) RNA interference is an effortless technology to help orthodontists understanding the molecular mechanisms involved in orthodontic procedure, and discovering new targets for therapeutic.

(B) Downregulation of Timp1 by Timp1 siRNA could facilitate the degradation of collagen1 in vitro and in vivo.

(C) 80% of the accelerated tooth movement by Timp1 siRNA was apparent in the early period, between 0 and 7 days.

(D) During two weeks relapse phase, 15.96% relapse in Timp1 siRNA group is significantly less than 64.38% in control.

(E) Timp1 is proposed as a potential therapeutic intervention target for tooth movement and relapse.

(F) Stretching of the fibrous bands in the periodontium plays an important role in relapse.

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