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Original Article

Orthodontic force regulates metalloproteinase-3 promoter in osteoblasts and transgenic mouse models

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Abstract *Background/purpose:* Previously we demonstrated up-regulation of matrix metalloproteinase-3 (*MMP-3*) in human osteoblasts under compression and in bony specimens of experimental orthodontic tooth movement (OTM). Here, we studied the temporal characteristics of compression stimulation in human and mouse osteoblast cell lines, and generated a transgenic mouse model for assessing the *MMP-3* expression during OTM.

Materials and methods: We investigated *MMP-3* expressions in human and murine osteoblasts through RT-PCR and luciferase assay, after compressive force loading. Inhibitors were added to identify the possible mechanisms for signal transduction. A human *MMP-3* promoter was isolated, cloned and transfected to generate a transgenic mouse with a green fluorescent protein reporter. OTM was then initiated to observe the location and time course of transcriptional regulation of *MMP-3* signals.

Results: We found changes in the transcription of *MMP-3* in response to mechanical force applied to both human and mouse osteoblast cell lines, suggesting that the response is positive

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across species. Cloned human *MMP-3* promoter may cause the response of luciferase to 1% compression. Moreover, p38 inhibitor exerted a down-regulatory effect on *MMP-3* promoter expression, although the inhibitory effect didn't reach a significant level. In the transgenic mouse OTM model, we again found increased expression of *MMP-3* in response to mechanical force loading around the periodontal ligament.

Conclusion: Mechanical force can stimulate *MMP-3* expression, possibly through the p38 MAPK pathway, with its strongest signal occurring at 24 h. The mechanical responsiveness in *MMP-3* promoter regions can be observed in both humans and rodents in vitro and in vivo.

Introduction

Bone remodeling under mechanical force has been investigated to determine its ability to increase osteogenesis or slow down osteolysis in osteoporosis patients. Orthodontic loading is an intentional mechanical stimulation using orthodontic devices to increase bone turnover around teeth to achieve the desired positions for improved function and esthetics. An early increase in the signaling of chemokines or cytokines, including Prostaglandin E2 (PGE-2), cyclic adenosine monophosphate (cAMP), tumor necrotic factor-α (TNF-α), interleukin-1 β (IL-1 β) occurs during orthodontic tooth movement (OTM)[1–4] and the downstream effectors, matrix metalloproteinases (MMPs), exhibit increased expression upon mechanical loading on either the tension or pressure side,[5–7] indicating that MMPs play a critical role in increasing extracellular matrix turnover, a fundamental part of OTM.

Previously, we used a microarray approach with confirmation via a polymerase chain reaction (PCR) assay to show that compressive force on human osteosarcoma cells, MG-63, leads to upregulation of *cyclooxygenase-2* (*COX-2*), *ornithine decarboxylase*, and *MMP-3*. Among these, *MMP-3* production was further clinically proved through a human third molar uprighting experiment; increased *MMP-3* staining was found on Days 3 and 7 after compression force was applied on the molars.[8]

However, as a potent enzyme, the temporal characteristics of *MMP-3* response to mechanical force stimulation remain unclear. Thus, in the present study, we investigated the expression of *MMP-3* in a human osteoblast cell line (MG-63) in response to compressive force. To verify the force responsiveness of the *MMP-3* gene in human and mouse cell lines, a human promoter was cloned and transfected to both cell lines, and the promoter activity was analyzed using a luciferase assay. Lastly, after verification of cross-species activation of the *MMP-3* promoter in response to mechanical force, we created a *MMP-3* promoter—*green fluorescent protein* (*GFP*) transgenic (TG) mouse model to investigate the *MMP-3* transcription pattern during OTM in vivo.

Materials and methods

Cyclic compression force experiments

Cells were plated in collagen gels for one day with 10% fetal bovine serum (FBS) before being switched to 2% FBS medium one night before the experiment.[8] The cells cast in collagen gels were then placed in BioPress™ plates (Flexercell International, Burlington, NC, USA) with 1% air pressure and 5.3 kPa intermittent compression loading for 24 h, with 10-s stimulation followed by a 10-min rest using Flexercell strain unit (Flexercell International). Cells without loading were used as controls. Subsequently, 10 μM inhibitors including mitogen-activated protein kinase/extraceullar signal-regulated kinase 1/2 (MEK1/2) inhibitor (U0126), p38 inhibitor (SB203580), c-Jun N-terminal kinase II inhibitor (420128), NF-κB inhibitor inhibitor (BAY 11-7082), or PI3-K inhibitor (LY 29400) were added 1 h to each medium and force being loaded.

Real-time PCR for *MMP-3* expression

Cells were lysed immediately at the allocated time point with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and RNA was isolated with chloroform and precipitated with isopropyl alcohol. After being dissolved in DEPC-H_2O, RNA was reverse transcribed into cDNA and further amplified using real-time PCR with TaqMan gene expression assays (Applied Biosystems, Foster City, CA, USA). For MG-63, *MMP-3* (Hs00233962) and *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) (Hs99999905) were used. For MC3T3-E1 cells, custom made primers for *MMP-3-Mus* (AIGJAPS)($5'$ to $3'$: aaagagatccaaggaaggca, $3'$ to $5'$: gctttgttcagcatgtgctat) (HS 99999905) were used.

Cloning of the *MMP-3* promoter

Using a National Center for Biotechnology Information database, we designed primer sets (a sense primer containing XhoI site $5'$-GCCCT CGAGA CTCAG ATACT TGATA

AATG-3′ and an antisense primer containing HindIII site 5′-CACAA GCTTT ACTTA GCTCT ATGTT GTCT-3′) to generate PCR fragments containing human *MMP-3* promoter 3200 bp in HeLa cells. We cloned them to a *pGEM-T-easy* vector first, and then to p*GL3-basic* vector which containing Luciferase reporter and *pEGFP-1* vector which containing enhanced green fluorescent protein reporter, respectively (Promega, Madison, WI, USA). Gene sequencing verified successful cloning of *MMP-3* promoter regions.

Transfection

Lipofectamine 2000 (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to transfect MG-63 and MC3T3-E1 cells according to the recommended protocols with ratios of reporter genes (*MMP-3 promoter-pGL3*) to internal control genes (*pRL-TK*) of 4:1 for 6 h and culture for one day before the experiment.

Luciferase assay

Transfected cells were collected at different time points after force loading (with collagenase type IA digestion of 30 min for collagen-embedded samples), lysed in a passive lysis buffer, and processed as described in the protocol for the Dual-Luciferase Reporter Assay System (Promega). Luciferase activity was measured using a luminometer (BioTek, Winooski, VT, USA) and expressed as the ratio of firefly luminescence to Renilla luminescence.

Establishing TG mice carrying the *MMP-3* human 3.2 kb promoter—*GFP* reporter

After verification that the human promoter activity was mechanically stimulated in murine cells, this promoter was ligated to plasmid containing *pEGFP*, and linearized *MMP-3* promoter-*pEGFP-1* DNA was injected into blastocysts of B6, and the tails of newborns were screened for the presence of the transgene. Among 72 samples, 4 were found to be positive. Three lines (#52, #70, #72) were found to be germline transmitted.

Identifying TG mice carrying human *MMP-3* 3.2 kb promoter—GFP genes

For genomic DNA extraction, the tails of newborn mice were placed in a buffer containing protease K at 57 °C to mix overnight. After centrifuging at 12,000 rpm for 10 min, supernatants were moved to a new tube containing isopropanol. DNA pellets were collected after a 10-min spin, then washed with 70% ethanol, air dried, and dissolved in ddH$_2$O. Two sets of primers were used to identify the inserted transgene: outer forward primer GCAAGGATGAGTCAAGCTGCGGGTG, outer reverse primer CAGCTTGCCG GTGGTGCAGATGAAC, and inner forward primer GCTGCGC TCCCGAGGTTGGACCT; and inner reverse primer CAGCTTGC CGTAGGTGGCATCGCCC were used for nested PCR.

GFP expression in a calcified tissue section

GFP is sensitive to changes in temperature and decalcification for paraffin embedding. A fluorescent signal was detected in the tissue embedded in a resin block without decalcification within one week after termination of the experiment. For induction of GFP with mechanical force, two types of orthodontic loading were applied (for each group n = 2). For molar movement, a 0.010-inch NiTi alloy was bent into a U shape with short sharp bends at the ends and inserted into the first and second molars and secured with light curing resin to achieve 10 g of expansion force. For incisor movement, elastic rings were inserted between the incisors and secured with light curing resin. At different time points of Days 1—6, the mice were euthanized using deep anesthesia with perfusion of 4% paraformaldehyde in phosphate-buffered saline, and the maxilla was removed for further tissue processing. For calcified tissue sections, the maxilla was dehydrated and embedded in resin using an Osteo-Bed Bone Embedding Kit (PolySciences, Warrington, PA, USA). A Leica SP1600 (Leica Biosystems, Wetzlar, Germany) saw microtome was used for sectioning and resin sections were then polished before observation using fluorescein isothiocyanate, a long pass filter (480-nm excitation, dichroic 505-nm DCLP, 535-nm emission), and a Nikon SMZ 1500 dissecting microscope (Nikon, Tokyo, Japan). Resin sections were scanned using a Zeiss LSM 880 confocal microscope (Zeiss, Jena, Germany) with excitation of 488 nm to acquire 500—550-nm emissions signals (Image Core, The 1st core Laboratory, Medical College, National Taiwan University, Taipei, Taiwan). These animal experiments were conducted under the Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines. The experiments also had the approval of IACUC 20140495 from College of Medicine, National Taiwan University.

Statistical analysis

Student's *t*-test was used to determine if there's statistical significance existed with alpha level = 0.05 and power of 0.8. The error bars presented on each bar of figures was standard deviation of the mean. For multiple group comparisons, ANOVA was used and post-hoc analysis was done with Scheffe test. All statistic tests were run on SPSS (IBM, Armonk, NY, USA), p values < 0.05 were defined as statistically significant.

Results

Effect of compressive force on *MMP-3* mRNA expression in a human osteoblast cell line

MMP-3 mRNA levels increased after 24-h compressive force loading, more so in the 5% compressive force group (8.63-fold) than in the 1% group (6.95-fold). No significant increase was found in the 4-h and 8-h groups, indicating that *MMP-3* gene expression responds in a force- and time-

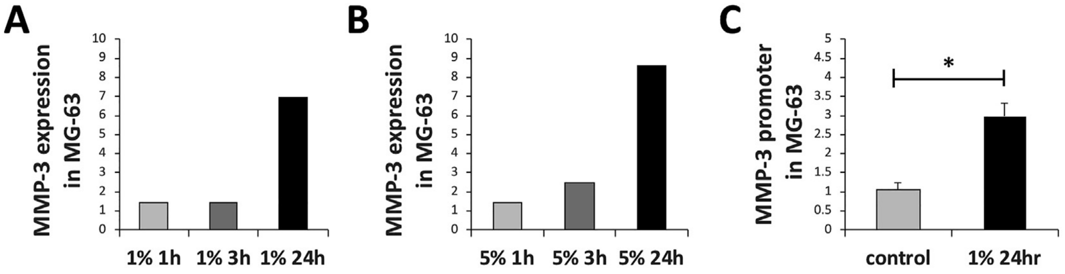

Figure 1 Relative expression of *MMP-3* compared to the control group without pressure loading at different compression force levels and time points in human MG-63 cells: (A) Compression force of 1%. (B) Compression force of 5%. (C) After transfection of *pGL3 Basic-MMP-3* promoter, relative luciferase expression after 1% cyclic compression force for 24 h. * Indicates $p < 0.05$.

dependent manner upon compressive force. The luciferase assay using *MMP-3* promoter in MG-63 cells also confirmed the same trend of *MMP-3* promoter activity that increased significantly after 1% 24-h compressive force loading (Fig. 1).

Effect of compressive force on *MMP-3* mRNA and *MMP-3* promoter expression in a murine osteoblast cell line

MMP-3 mRNA levels also increased significantly after 1% compressive force loading in the 24-h group (4.2-fold). After transfection of human *MMP-3* promoter-*LUC* into the mice osteoblast cell line MC3T3-E1, luciferase activity was also up-regulated after 1%, 24-h cyclic compression force (4.62-fold). This suggests that the mechanism of mechanical stimulation of this promoter gene region is similar across humans and rodents (Fig. 2).

Inhibitors in MG-63 and MC3T3-E1

Cloned human *MMP-3* promoter may cause the response of luciferase to 1% compression, but when five inhibitors were added 1 h prior to compressive force stimulation, including MEK1/2 inhibitor (U0126), p38 inhibitor (SB203580), JNK II inhibitor (420128), NF-κB inhibitor (BAY 11-7082), and PI3-K inhibitor (LY 29400), only p38 inhibitor exerted a down-regulatory effect on *MMP-3* promoter expression. However, the inhibitory effect didn't reach significant level (Fig. 3).

Spatio-temporal regulation of *MMP-3* promoter-GFP in TG mice

We performed OTM on incisors and molars (Fig. 4) and observed green fluorescence in hard tissue sections. Compared to the controls, periodontal tissue connected to alveolar bone at the tension sites exhibited strong fluorescence on Day 1 in all three lines of TG mice (Fig. 5). In detailed observation, the expression was initiated from the anterior-mesial part of incisor periodontal ligament (PDL) as well as the anterior maxillary bony front, then the signal disseminated the whole width to the root surface on Day 2, which subsided after day 4. This pattern of GFP, a reporter gene of *MMP-3* promoter, was similar to endogenous *MMP-3*, which could be secreted outside the cells, presenting more intensive signals.

Discussion

We found consistent up-regulation of *MMP-3* mRNA and protein expression in the 24-h group of human osteoblast cell line MG-63 subjected to both 1% and 5% compressive force, whereas only a slight increase was observed in the 1-h and 3-h compressive force groups, without reaching significance. These results were similar to those of Tasevski

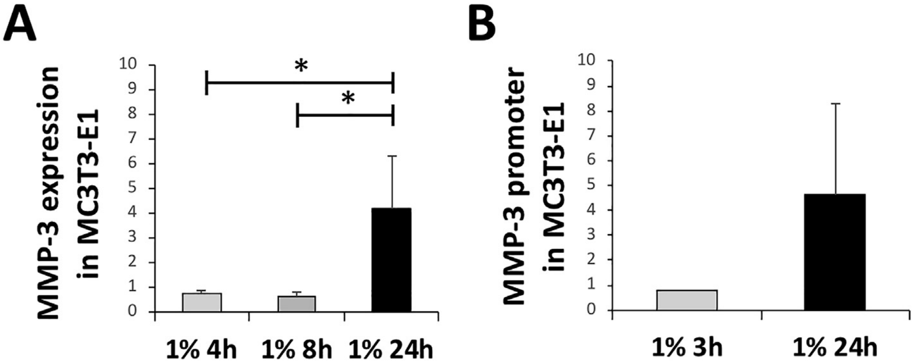

Figure 2 Changes in *MMP-3* expression based on real-time PCR and promoter assay results after compression force loading in MC3T3-E1 cells. (A) Results of real-time PCR for *MMP-3* expression after 1% force loading, reported as the fold change compared to the control group (without force loading). (B) Luciferase assay results for promoter intensity with 1% force loading, reported as the fold change compared to the control group. * Indicates $p < 0.05$.

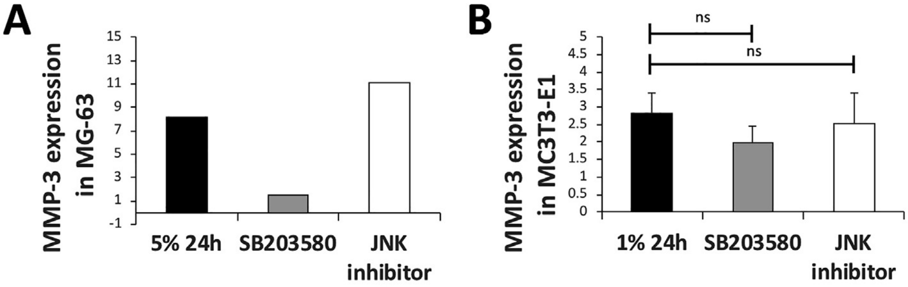

Figure 3 Effect of p38 inhibitor (SB203580) and JNK II inhibitor on *MMP-3* expression prior to compressive force loading in human MG-63 cells and MC3T3-E1 cells. (A) Real-time PCR results for *MMP-3* expression, reported as the fold change compared to the control group (without force loading). (B) Luciferase assay results for promoter intensity reported as the fold change compared to the control group. Abbreviation: ns, not significant difference between groups.

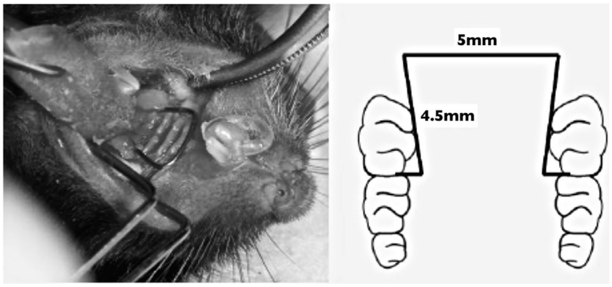

Figure 4 Intraoral setting of mouse OTM: the separator between mouse incisors was secured with resin, pushing two incisors laterally. The molars were expanded using a Ni–Ti preformed spring. Schematic illustration at the right side.

et al.[9] who found that both *MMP-1* and *MMP-3* mRNA are upregulated after 4–12 h of cyclic hydrostatic pressure, although the hydrostatic pressure adopted in their experiment is higher (0.8 MPa) compared to that in our study. According to the literature, to simulate the physiological load range along the pressure side of OTM, the pressure setting should not exceed 0.3 MPa or 5%.[10] In our applied setting, 5% compressive pressure applied to 12-well collagen gel was nearly 0.25 MPa after conversion using Fermor's equation.[11] This might explain the different responses of *MMP-3* mRNA transcription with time.

When this vector was transfected into MC3T3-E1, a mouse osteoblast cell line, compression also affected this human *MMP-3* promoter, showing increased luciferase

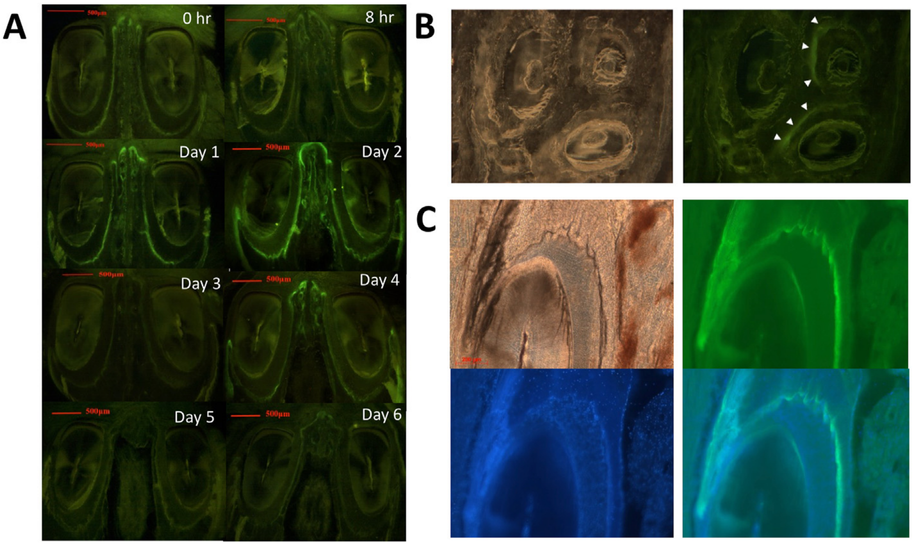

Figure 5 In vivo *MMP-3* promoter-*GFP*-TG mouse model of OTM. (A) GFP expression of the incisor region under fluoroscopy at different time points from Days 1–6. The intensity was greatest on Days 1–2, over the bony margin of the medial (tension) side of the incisor roots. (B) The section of molar expansion showed green fluorescence over the tension side of the first molar multi-roots on Day 3. Left, bright field; Right, green fluorescent excitation through GFP-targeted *MMP-3* promoter. Arrowheads showed marked GFR expression over the tension side bone margin. (C) Overlay fluorescence in a close-up view of the molar roots, showing a clear GFP signal over the PDL-alveolar bone margin, compared to no signal over the PDL space and root surface. Upper left, bright field; upper right, green fluorescent excitation through GFP-targeted *MMP-3* promoter; lower left, nuclear staining using DAPI; lower right, overlaid upper right and lower left images.

activity but with less compressive force of 1% air pressure. The activation of *MMP-3* promoter was not detected after either 4 h or 8 h, but significant activation (4-fold) was observed after 24 h. This finding differs from previous research conducted by Sanchez et al.[12] who reported increased *MMP-3* upon 10% compressive force in 4–8-h groups. However, the force level was much higher in the latter study, and the two distinct force levels might be attributable to the disparity in the timing of *MMP-3* induction causing the earlier and more dramatic response in the study of Sanchez et al.[12]

The mammalian p38 MAPK pathway is activated by a wide range of extracellular stress as well as various inflammatory cytokines.[13] Previous researchers have identified the p38 MAPK pathway in TNF-α-induced up-regulation of MMP-1 and stromelysin-1 (*MMP-3*) in human skin fibroblasts.[14] They recognized that these signaling mechanisms are AP-1-independent, and the primary effect is a marked stabilization of *MMP-1* and *MMP-3* mRNA. This p38 MAPK pathway-induced *MMP-3* up-regulation has also been observed in human chondrosarcoma cells and in murine cementoblast cells.[15,16] The results of the present study were in agreement with the aforementioned findings. After treatment with three different MAPK pathway inhibitors prior to compressive force, we found that increased expression of *MMP-3* under compressive force stimulation could be inhibited by SB203580 (p38 inhibitor) in a human osteoblast cell line, and a slight inhibitory effect was also observed in a mouse osteoblast cell line. On the other hand, although the JNK pathway has demonstrated a suppression effect on *MMP-3* production in human rheumatoid arthritis fibroblast-like synoviocytes,[17] the present study revealed no inhibitory effect on *MMP-3* expression after compressive force loading in MG-63 cells treated with JNK inhibitor and MEK inhibitor. Further study on quantifying pathway-associated downstream transcriptional factor expression will be beneficial to elucidate the overall mechanism.

Our findings in *MMP-3-GFP*-transgenic mice experiments suggest that the OTM model can induce *MMP-3* promoter-GFP expression, especially on the tension side of the bone surface. The lack of expression on the pressure side of the bone might be explained by the hyalinization theory;[18,19] whereas heavy orthodontic force is exerted on a tooth, the vessels over the compression side of the PDL are occluded, causing reduced blood flow and therefore less transcription and protein synthesis. When we closely observed the manifestation time and signal distribution, a signal was noted on the alveolar bone in front of the PDL from Day 1 to Day 2 at its highest intensity, gradually fading after Day 4. Almost no signal was noted on the root surface in front of or within the PDL space (Fig. 5). This *MMP-3* up-regulation was consistent with that in a previous report by Tantilertanant et al.[20] on tension force-induced IL-6-mediated *MMP-3* up-regulation in human PDL cells. However, cells of PDL origin are a mixed population, including fibroblasts, endothelial cells, cementoblasts, osteoblasts, and osteoclasts. Based on our in vitro data, the response may be derived from cells of osteoblast origin. The temporal aspect of the *MMP-3* promoter-GFP signal corresponds to the findings of osteoblasts, with the highest *MMP-3* mRNA expression in the 24-h group. The tight control of its up- and down-regulation was elucidated by our experiment.

MMP-3 is responsive to both tension and compression forces, which are shown in both human and mouse osteoblast cell lines. The results of the promoter assay indicate that the regulation of *MMP-3* promoter is similar across humans and mice, with expression after compressive force loading in both. Regarding the physiological orthodontic pressure level, the expression of *MMP-3* is highest after 24 h and lowest in the earlier stages. The in vivo OTM model of TG mice also correspond to this time frame. Further researches are needed to determine the specific pathway-related gene expression.

Declaration of competing interests

The author and all co-authors declare that there’s no conflict of interests to reveal in regard to this study.

Acknowledgments

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