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The roles of angiotensin II in stretched periodontal ligament cells

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< Information >

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ABSTRACT

The loading caused by occlusion and mastication plays an important role in maintaining periodontal ligament (PDL) tissues. We hypothesized that a loading magnitude would be involved in the production of biological factors that function in the maintenance of PDL tissues. Here, we identified up-regulated gene expressions of transforming growth factor- β 1 (TGF- β 1), alkaline phosphatase (ALP) and angiotensinogen (AGT) in human PDL fibroblastic cells (HPLF) that were exposed to 8% stretch loading. Immuno-localization of angiotensin I/II (Ang I/II), which were converted from AGT, were detected in rat PDL tissues. HPLFs that were stimulated by Ang II also increased their gene expressions of TGF- β 1 and ALP. Furthermore, the antagonist for Ang II type 2 receptor, rather than for type 1, significantly inhibited gene expressions induced by the stretch loading. These data suggest that Ang II mediates the loading signal in stretched HPLFs to induce expressions of TGF- β 1 and ALP.

INTRODUCTION

Periodontal ligament (PDL) is a dense specialized connective tissue that lies between the cementum and the alveolar bone. The homeostasis of PDL tissues is maintained while continuously being subjected to the mechanical tensile loading caused by occlusion and mastication (Yamaguchi *et al.*, 2002). Human PDL cells are known to include osteoblastic properties and to express alkaline phosphatase (ALP) (Somerman *et al.*, 1988). It has been reported that, while the basal ALP activity in human PDL cells tends to increase, the cells also can differentiate into osteoblastic cells and form mineral-like nodules, depending on various extracellular stimuli (Basdra and Komposch, 1997). On the other hand, human PDL cells are also known to express receptor activator of nuclear factor kappa B ligand (RANKL) (Wada *et al.*, 2004), a known regulator of osteoclastogenesis (Udagawa, 2002). RANKL signaling is inhibited by osteoprotegerin (OPG), and one of the biological roles of OPG in the PDL may be the protection of the tooth from attack by osteoclasts activated by various stimuli (Wada *et al.*, 2001).

Mechanical stress is an essential stimulus for the development, function and repair of the major elements of the musculoskeletal system, such as bones,

tendons, ligaments and cartilage. Among these stress, stretching loading is known to be one of the important regulators of ligament and tendon remodeling (Kim *et al.*, 2002). It has been reported that proper mechanical stimulation is required for maintaining PDL tissues (Shi *et al.*, 2005).

The renin-angiotensin system (RAS) has been described as a major regulator of cardiovascular physiology and has been strongly implicated in the development of several cardiovascular diseases including hypertension and cardiac hypertrophy (Senbonmatsu *et al.*, 2003). Angiotensin II (Ang II), a vasoactive octapeptide, is converted from angiotensinogen (AGT) via angiotensin I (Ang I) (Jeunemaitre *et al.*, 1992) and plays an important role as the principal mediator of RAS (Tamura *et al.*, 1998). It has also been reported that Ang II contributes to stretch-induced hypertrophic responses (Yamazaki *et al.*, 1995). In mammals, Ang II acts via the Ang II type 1 receptor (AT1) and type 2 receptor (AT2). AT1 and AT2 exhibit limited sequence homology (~34% amino acid sequence identity) (Inagami *et al.*, 1992). In bone tissue, AT2 is expressed in both osteoblasts and osteoclasts *in vivo*, and the treatment with AT2 blocker has increased bone mass through both enhancement of osteoblastic activity and suppression of osteoclastic activity *in vivo* (Izu *et al.*, 2008).

In the present study, we report the response of human PDL fibroblastic cells (HPLF) exposed to stretch loading and the correlating role of Ang II in the signaling of these cells.

MATERIALS & METHODS

Reagents

Recombinant human Ang II was purchased from Calbiochem (Darmstadt, Germany). Signaling pathways were investigated using specific antagonists: candesartan (10 ng/mL, TRC Inc., NY), an antagonist of AT1, and PD123319 (100 nM, Sigma, St Louis, MO), an antagonist of AT2.

Cell Culture

HPLFs were obtained from healthy third molars that were extracted for orthodontic reasons and prepared as previously described (Fujii *et al.*, 2006). Cells isolated from a 30-year-old female and a 39-year-old female were denoted as HPLF-2D and HPLF-2E, respectively. All cells were cultured in alpha-minimum essential medium (α -MEM, Gibco-BRL, Grand Island, NY), supplemented with 50 μ g/mL streptomycin and 50 U/mL penicillin (Gibco-BRL) and containing 10% fetal bovine serum (FBS, Gibco-BRL; 10% FBS/ α -MEM), at 37°C in a 5% CO₂ incubator. HPLFs underwent 5 to 6 passages prior to use in the experiments. All the procedures in this study were performed in compliance with the regulations of Kyushu University.

Application of Mechanical Stress

Stretch loading was applied to HPLF cultures using STB-140 (STREX, Osaka, Japan) in a CO₂ incubator. HPLFs were pre-cultured in flexible-bottomed culture chambers coated with type I collagen (Cell matrix I-P, Nitta Gelatin Inc., Osaka, Japan) until reaching sub-confluence. HPLFs were subjected to stretch loading (0, 8 and 12% elongation, 0.5 sec stretch and 0.5 sec relaxation per cycle) for 1 h. After loading, HPLFs were subjected to RNA extraction.

Semi-quantitative RT-PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. First-strand cDNA synthesis and PCR were performed using a Thermal Cycler Dice (Takara Bio Inc., Shiga, Japan) as described previously (Maeda *et al.*, 2005; Tomokiyo *et al.*, 2008). Each cycle consisted of a heat denaturation at 94°C for 30 sec, annealing for 30 sec and extension at 72°C for 30 sec. Annealing temperatures were optimized for each primer-pair as follows: GAPDH [452bp]: sense, 5'-ACCACAGTCCATGCCATCCAC-3', antisense,

5'-TCCACCACCCTGTTGCTGTA-3' , 60°C; RANKL [196bp]: sense,

5'-ACCAGCATCAAATCCCAAG-3' , antisense,

5'-CCCCAAAGTATGTTGCATCC-3' , 59°C; OPG [472bp]: sense,

5'-GTACGTCAAGCAGGAGTGCAATC-3' , antisense,

5'-TTCTTGTGAGCTGTGTTGCCG-3' , 55°C. GAPDH primers were used as

internal controls. The PCR products were analyzed using picture-imaging

software (NIH Image; National Institutes of Health, Bethesda, MD).

Quantitative RT-PCR

First-strand cDNA was synthesized from 1 µg of total RNA using ExScript RT Reagent kit (Takara Bio Inc.). Briefly, total RNA was reverse-transcribed with random 6mers and ExScript RTase for 15 min at 42°C, and the reaction was stopped by incubation for 2 min at 99°C, followed by 5 min at 5°C. PCR was performed using SYBR Green I (Takara Bio Inc.) in a Thermal Cycler Dice Real Time System (Takara Bio Inc.) under the following conditions: 95°C for 10 sec (initial denaturation), followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec, followed by a dissociation program at 95°C for 15 sec, 60°C for 30 sec and 95°C for 15 sec. A human β-actin primer was used as an internal control. Expressions

of the target genes were calculated from the delta-delta Ct values. Specific primer sequences for each gene purchased from Takara were as follows: β -actin [89bp] : sense, 5'-ATTGCCGACAGGATGCAGA-3', antisense 5'-GAGTACTTGCCTCAGGAGGA-3'; transforming growth factor- β 1 (TGF- β 1) [125bp]: sense, 5'-AGCGACTCGCCAGAGTGGTTA -3', antisense, 5'-AGTACATGGCGTAACCTCTAGTCA-3'; ALP [118bp]: sense, 5'-GGACCATTCCCACGTCTTCAC-3', antisense, 5'-CCTTGTAGCCAGGCCATTG-3'; AGT [182bp]: sense, 5'-AGCTGCCGTTGTTCTGGGTAATA-3', antisense, 5'-GTGGAGCAGTAGGTGTTACTCTCA-3'; RANKL [174bp]: sense, 5'-TGGATGCCTTGAATAATAAGCAGGA-3', antisense, 5'-AAGGTGTTACGGCGTTTAA-3'; OPG [196bp]: sense, 5'-TGGCACCAAAGTAAACGCAGAG -3', antisense, 5'-CTGTACGATTGGAGTGGAAGCTC-3'.

Detection of Ang II *in vivo* and *in vitro*

All procedures were approved by the Animal Research Committee of Kyushu University.

Five-week-old Sprague-Dawley rats were purchased from Kyudo Co. Ltd. (Saga, Japan) and were perfused through the left ventricle with 4% paraformaldehyde (PFA). The mandibles were dissected, decalcified in 10% EDTA solution and embedded in paraffin. Five μm horizontal sections of the first molars were prepared. Immunohistochemical analysis was performed using an anti-Ang I/II antibody (Santa Cruz, CA, USA) as a primary antibody and a biotinylated anti-goat IgG (Nichirei Biosciences Inc., Tokyo, Japan) as a secondary antibody. Positive reactions were visualized with Simple Stain DAB Solution (Nichirei). HPLFs were fixed with 4%PFA and 0.5% dimethyl sulfoxide (Wako, Osaka, Japan), and the expressions of Ang I/II were also examined immunocytochemically with the same procedure.

Statistical Analysis

All values are expressed as mean \pm SD. Statistical analysis of the results was performed using the Student's paired t-test.

RESULTS

Subjection of HPLFs to stretch loading

Gene expressions of RANKL and OPG were examined from HPLF-2E cultures exposed to stretch loading at a frequency of 60 cycles/min with 0, 8 or 12% elongation. After 1 h, the expression level of RANKL mRNA was significantly down-regulated by exposure to 8% stretch loading as compared with non-loading, whereas the expression was up-regulated by exposure to 12% stretch loading ($P < 0.02$) (Fig. 1A). The expression level of OPG mRNA was up-regulated by exposure to both 8% and 12% stretch loading as compared with non-loading ($P < 0.02$) (Fig. 1B). Because these data suggested that 8% elongation may exert inhibitory effects on osteoclastogenesis in HPLFs, we used this magnitude in subsequent experiments. Next, we examined the effects of 8% stretch loading on gene expression in HPLF-2D and -2E cultures by quantitative RT-PCR. The expression levels of TGF- β 1 and ALP mRNA were up-regulated by exposure to stretch loading as compared with non-loading (Figs. 1C, 1D). Interestingly, stretch loading also up-regulated AGT mRNA expression in both HPLF cultures (Figs. 1C, 1D).

Localization of Ang I/II in rat PDL tissues

The expression of Ang I/II was examined using rat PDL tissues and HPLF-2E cultures. In rat periodontal tissues, the intense immunoreactivity for an anti-Ang I/II antibody could be seen in the entire PDL tissue while bone and dentin matrices showed no positive reactions (Figs. 2A-2C). Ang I/II protein also strongly expressed in the cytoplasm of HPLF-2Es as compared with the negative control (Figs. 2D, 2E), and HPLF-2Ds revealed the same results (data not shown).

The effects of Ang II on gene expression of HPLFs

We next wanted to determine whether Ang II could mimic the effects of stretch loading on gene expressions in HPLFs. Both TGF- β 1 and ALP mRNA expressions were up-regulated dose-dependently by the 1h-stimulus of recombinant Ang II in HPLF-2E cultures (Fig. 2F). Additionally, HPLF-2Es treated with recombinant Ang II for 1 h up-regulated OPG and AGT mRNA expressions and down-regulated RANKL mRNA expression, similar to that stimulated by exposure to stretch loading (Fig. 2G). In HPLF-2Ds, Ang II significantly increased the gene expressions of both TGF- β 1 and ALP (Fig. 2H).

Roles of two Ang II receptors in HPLFs exposed to stretch loading

We examined whether stretch-induced TGF- β 1 and ALP mRNA expressions were modulated through the Ang II receptor in HPLFs. Both HPLF-2D and -2E cultures exhibited gene expressions of AT1 and AT2 (Fig. 3A). Before exposure to the stretch loading, HPLF-2Es were pre-incubated with candesartan or PD123319. After loading, only PD123319 suppressed up-regulation of gene expressions of both TGF- β 1 and ALP by the stretch loading (Figs. 3B, 3C). In contrast, candesartan had no effects on the stretch-mediated gene expressions of TGF- β 1 and ALP.

DISCUSSION

Stretch loading caused by occlusion and mastication functionally contributes to the homeostasis of oxytalan fibers in PDL tissue (Tsuruga *et al.*, 2008). However, excess mechanical loading of PDL tissues has been reported to induce osteoclastogenesis / cementoclastogenesis that resorbs bone or root via up-regulated RANKL expression in human PDL cells (Yamaguchi *et al.*, 2006). Human PDL cells regulate osteoclastogenesis by opposing mechanisms, including stimulation by RANKL combined with inhibition by OPG (Kanzaki *et al.*, 2001), and tensile loading that inhibits osteoclastogenesis through OPG induction (Kanzaki *et al.*, 2006). Therefore, in the present study, we recognized the possibility to reduce RANKL expression and to induce OPG expression during stretch loading, and that this mechanism may be necessary for bone metabolism and maintenance of the PDL tissues. Indeed, our present data demonstrated that 8% stretch loading down-regulated mRNA expression of RANKL and up-regulated that of OPG. Thus, we fixed the elongation rate at 8% for the necessary magnitude of stretch loading to investigate the associated biological effects.

A recent study showed that mechanical stretch activated AGT mRNA

expression and caused secretion of Ang II in cardiomyocytes (Yamazaki *et al.*, 1995), and other reports have also demonstrated that Ang II acts as a mediator of the mechanical stretch signaling in cardiomyocytes (Sadoshima *et al.*, 1993; Tamura *et al.*, 1998). These reports support our present results, which showed that 8% stretch loading up-regulated mRNA expression of AGT in HPLFs. Thus, we hypothesized that RAS, including AGT, may be involved in the signal transduction in HPLFs that have been exposed to stretch loading. In this study, we demonstrated that Ang I/II was localized in PDL tissues, and furthermore, that HPLFs expressed Ang II receptors, AT1 and AT2. Though there are few reports about the role of Ang II in the PDL tissues, these results suggest that Ang II plays a role in maintaining PDL tissues in an autocrine or paracrine manner. Surprisingly, our present data showed that mimicking the stimulus by exposure to 8% stretch loading, exogenous Ang II significantly up-regulated the expression of AGT, TGF- β 1, ALP and OPG, and down-regulated that of RANKL. Therefore, we speculate that Ang II is involved in the cellular signaling of stretch loaded-HPLFs.

Finally, we investigated the signaling pathways in stretched HPLFs using specific antagonists of Ang II receptors. Pre-incubation of HPLF cultures with an

AT2 antagonist, but not an AT1 antagonist, suppressed up-regulation of gene expressions of both TGF- β 1 and ALP by the stretch loading. However, because there is little information about the AT2 pathway with regard to stretch loading, further studies are necessary to elucidate this mechanism.

TGF- β 1 is one of the most multifunctional peptides, which is involved in a wide variety of biological processes (Mehta and Attramadal, 2003). Recent studies have revealed that in human PDL cells exposed to orthodontic force, mRNA expression of TGF- β 1 was augmented in both the compression side and tension side (Garlet *et al.*, 2007). Additionally, in cardiomyocytes, Ang II has been reported to act as a paracrine mediator of stretch-induced TGF- β 1 mRNA expression (van Wamel JET *et al.*, 2002). These reports support our current data. Other researchers have discussed that TGF- β 1, up-regulated by mechanical stretch, plays a critical role in the healing and remodeling process of the human anterior cruciate ligament (Kim *et al.*, 2002). Therefore, the percentage of stretch loading utilized in this study may be suitable to further investigate the metabolism of the PDL tissues that are exposed to mechanical stress.

A couple of studies have shown that human PDL cells express some osteoblastic characteristics *in vitro* (Somerman *et al.*, 1988) and that mechanical

stimulation, including tensile force, can induce the differentiation of human PDL cells into osteoblast-like cells (Matsuda *et al.*, 1998; Yang *et al.*, 2006).

Short-term stretch loading significantly induced ALP mRNA expression in human PDL cells cultured in three dimensions (Ku *et al.*, 2009). The experimental loading system used in our present report resulted in the up-regulation of ALP expression in HPLFs induced by exposure to stretch loading.

We summarize the current results in Fig. 4. PDL cells expressed Ang I/II, and stretch loading up-regulated gene expression of AGT, which is potentially converted into Ang I/II by RAS, in HPLFs. Subsequently, extracellularly-secreted Ang II may up-regulate mRNA expression of TGF- β 1 and ALP in an autocrine/paracrine manner via the stimulation of the AT2 signaling pathway in HPLFs. In conclusion, we have obtained evidence that Ang II is involved as a transducer of the stretch loading signals in HPLFs, which consequently induces expression of TGF- β 1 and ALP.

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Figure Legends

Figure 1.

The effects of stretch loading on gene expression of RANKL (A) and OPG (B) in HPLFs. HPLF-2E cultures were exposed to stretch loading of 0, 8 and 12% elongation for 1 h, and the gene expressions of RANKL and OPG in the cell cultures were assessed by semi-quantitative RT-PCR. The stretch loading of 8% down-regulated mRNA expression of RANKL, as compared with 0%, while the stretch loading of 12% up-regulated the expression. The stretch loading of both 8% and 12% up-regulated mRNA expression of OPG. The gene expressions of TGF- β 1, ALP and AGT in HPLF-2Ds (C) and HPLF-2Es (D) exposed to the stretch loading of 8% were examined by quantitative RT-PCR. The results are shown as mean \pm SD from three different experiments. **p < 0.02.

Figure 2.

Localization of Ang II in rat PDL tissue and HPLFs (A-E). Ang II immunoreactivity was detected in PDL tissues. Dentin, bone matrices and pulp tissues showed negative reactions (A). A magnified view of the rectangle area in (A) is shown in (B). Both PDL cells and extra-cellular-matrix in PDL tissues showed strongly

positive reactions (D, dentin; PDL, periodontal ligament; B, bone) (B). Control staining with serial sections showed negative reactions (C). Ang II immunoreactivity could be seen in the cytoplasm of HPLF-2Es (D). Control staining of HPLF-2Es showed no positive reactions (E). Bars, 100 μ m. Gene expression in HPLF-2Es stimulated with Ang II analyzed by quantitative RT-PCR are shown (F-H). The expressions of TGF- β 1 and ALP mRNA were up-regulated dose-dependently by a 1h-stimulus of recombinant Ang II in HPLF-2Es (F). OPG and AGT mRNA expressions were up-regulated by the 1h-stimulus of recombinant Ang II in HPLF-2Es, while RANKL mRNA expression was down-regulated (G). TGF- β 1 and ALP mRNA expressions were also up-regulated by the 1h-stimulus of recombinant Ang II in HPLF-2Ds (H). The values were compiled as mean \pm SD from three different experiments. ** $p < 0.02$, * $p < 0.05$.

Figure 3.

Gene expression of Ang II receptors in HPLFs and the effects of Ang II receptor antagonists on the mRNA expressions of stretch-induced TGF- β 1 and ALP. The expressions of AT1 and AT2 mRNA both in HPLF-2Ds and in HPLF-2Es were

examined by semi-quantitative RT-PCR (A). mRNA expressions of stretch-induced TGF- β 1 (B) and ALP (C) as they were affected by Ang II receptor antagonists and stretch loading. HPLF-2Es were incubated with or without an AT1 antagonist, candesartan (10 ng/ml), or an AT2 antagonist, PD123319 (100nM), for 30 min before exposure to 1h-stretch loading. The values of quantitative RT-PCR data are reported as mean \pm SD from three different experiments. **p < 0.02, *p < 0.05.

Figure 4.

Proposed Ang II-mediated signaling in HPLFs exposed to stretch loading.

HPLFs exposed to a stretch loading of 8% up-regulated AGT expression and secreted Ang II. Ang II up-regulated TGF- β 1 and ALP expressions in HPLFs via AT2 only, but not AT1, in an autocrine or paracrine manner.

Figure.1

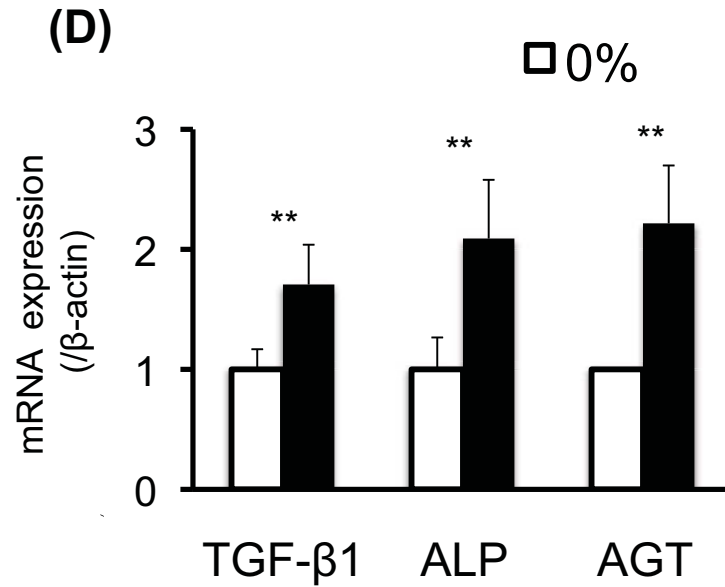
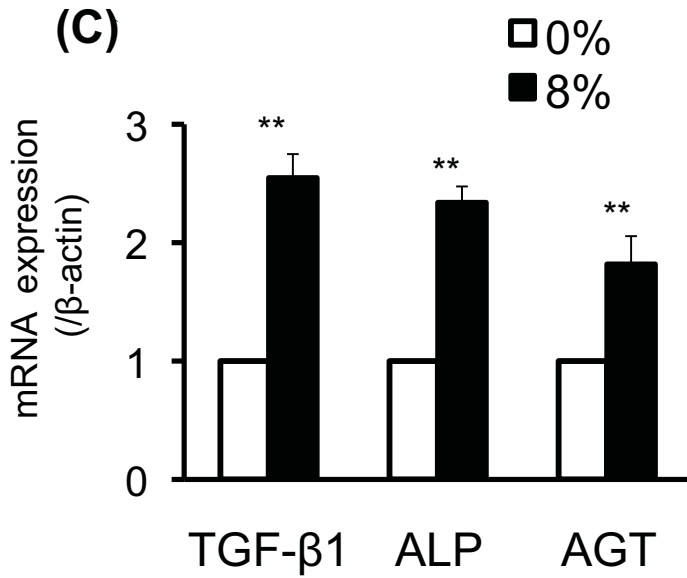
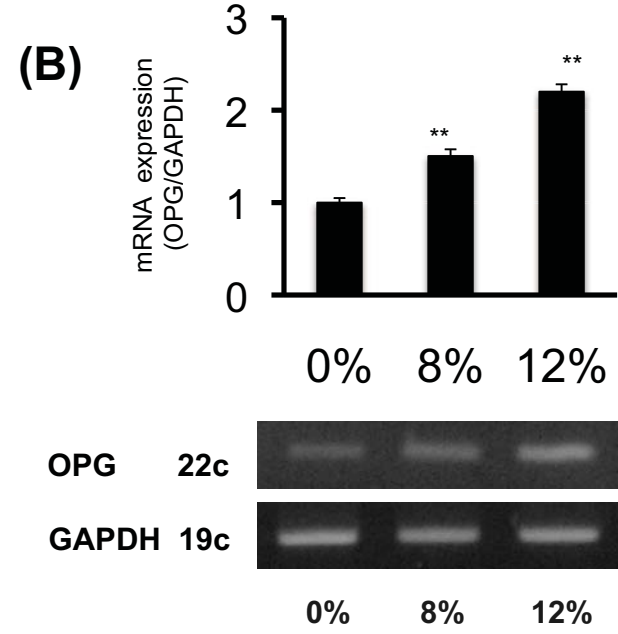
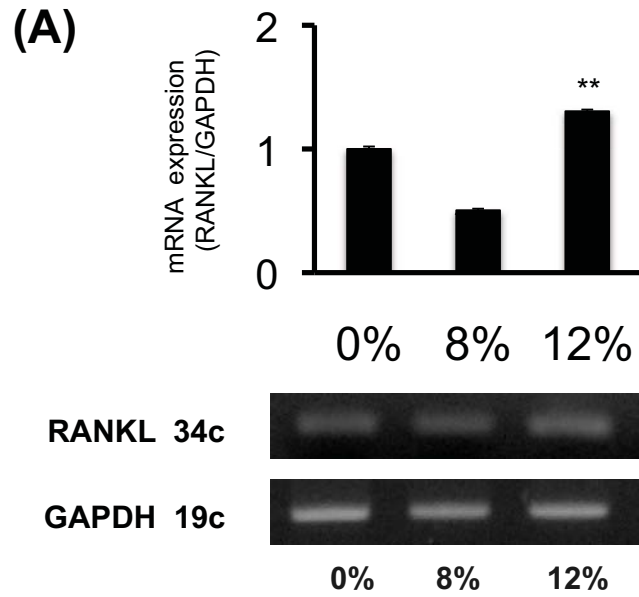


Figure. 2

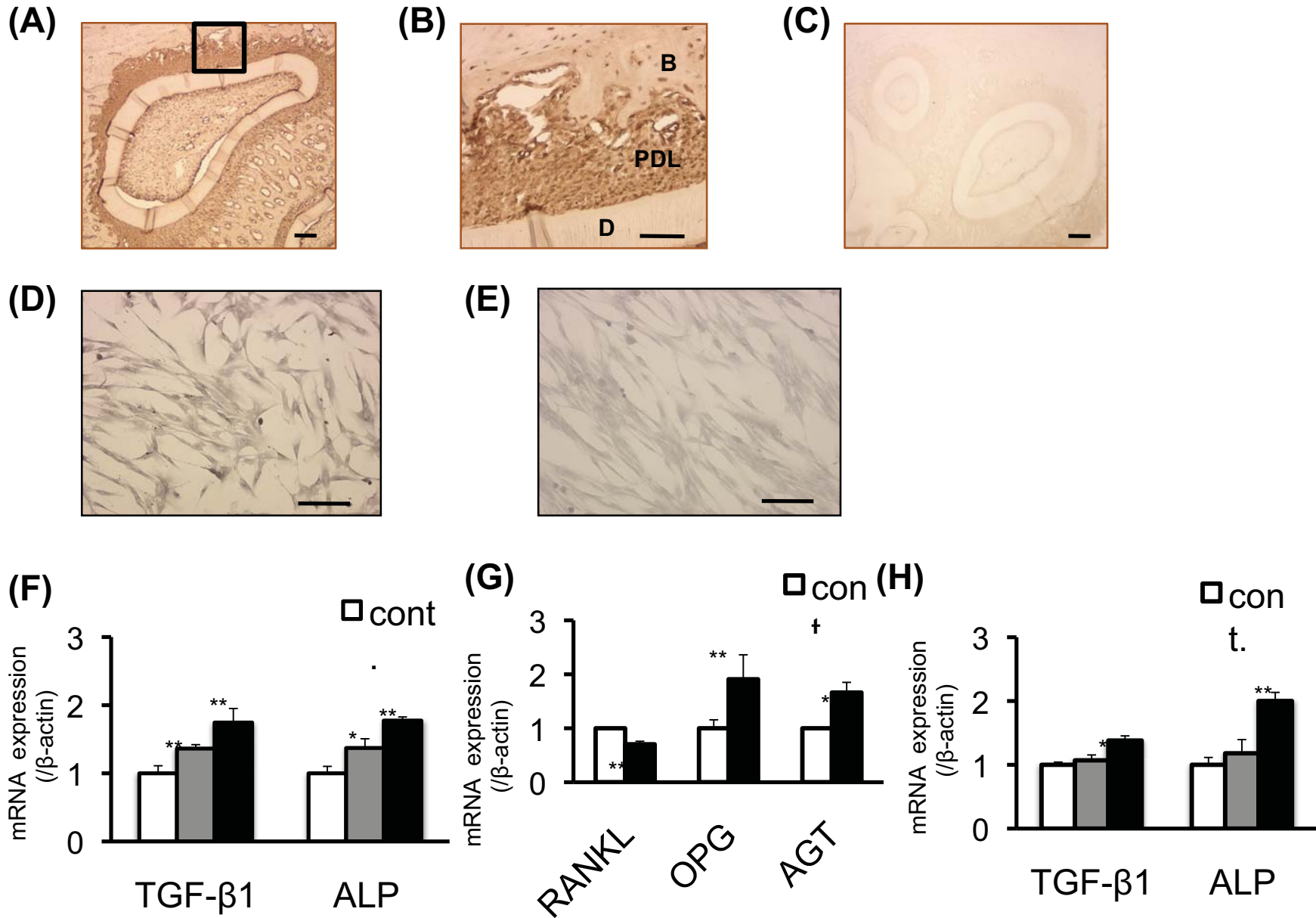


Figure.3

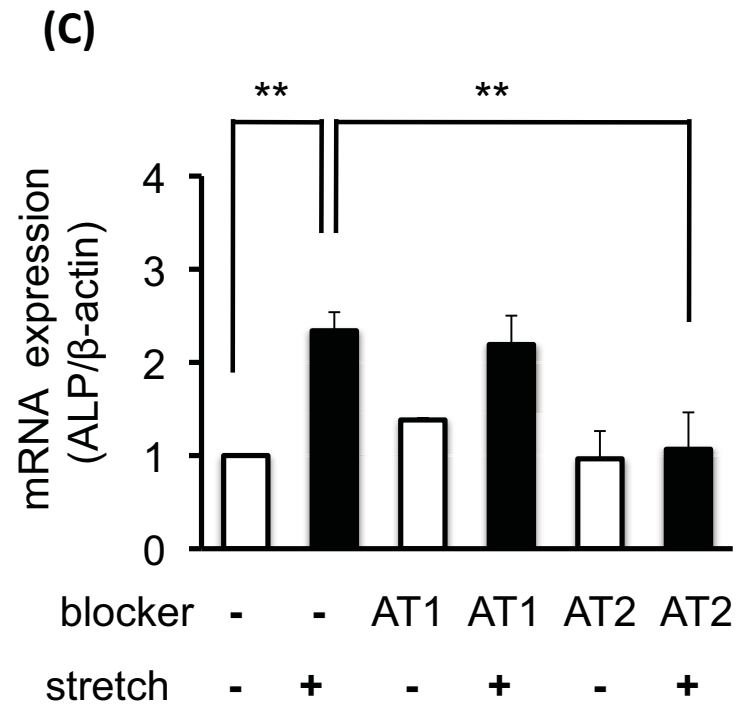
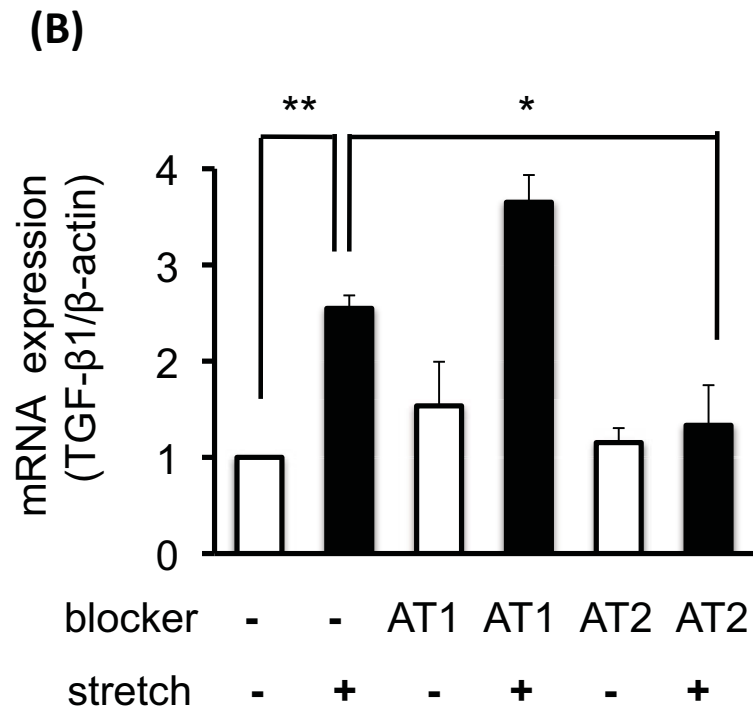
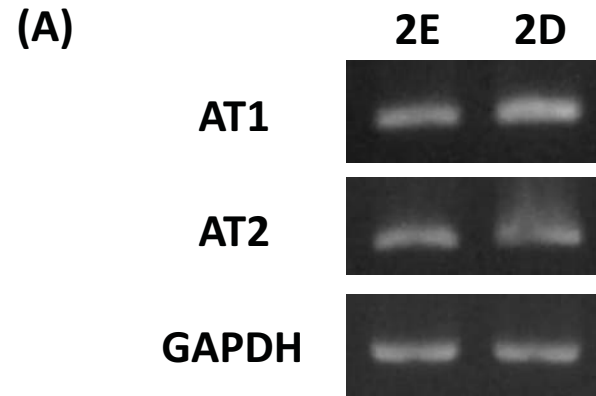


Figure.4

