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研究題目：Ameloblastin attenuates RANKL-mediated osteoclastogenesis by suppressing nuclear factor of activated T cells cytoplasmic1 (NFATc1) activation

目　的：

The purpose of the present set of studies is to clarify the effect of Ameloblastin（Ambn）on osteoclast differentiation and attempt to purpose the underlying mechanisms that involve in the process

対象および方法：

Bone marrow cells from 6 week old ddY male mice were induced with 20 ng/ml M-CSF for 3 days. Adherent cells were defined as bone marrow derived macrophage（BMMs）. All procedures were approved by the Animal Care and Use Committee of Kyushu Dental University. RAW264.7 cells were induced with 0-100 ng/ml Ambn for 6 h and then stimulated 50 ng/ml RANKL for 5 days. The number of multinucleated giant cell was determined by TRAP analysis. BMMs were pretreated with 20 ng/ml M-CSF and 100 ng/ml Ambn for 6 h and then induced with 40 ng/ml RANKL at an indicated time. Cells were subjected to TRAP analysis and actin ring formation assay. To determine the effect of Ambn on bone resorption activity, BMMs were plated on Osteo Assay Stripwell Plate® and then pretreated with 20 ng/ml M-CSF and 100 ng/ml Ambn for 6 h before stimulation with 40 ng/ml RANKL for different lengths of time over a period of 7 days. The resorption area was measured by reverse-phase microscope and analyzed using Image J software. The osteoclast specific genes were detected by RT-qPCR.

To identify the underlying mechanisms that Ambn regulates RANKL-induced osteoclastogenesis in RAW264.7 cells or BMMS, the protein or gene expression of NFATc1, RANK, c-Fos, IκB-α, ERK1/2, p-ERK1/2, p38 MAPK, p-p38 MAPK, JNK, p-JNK, CREB, p-CREB, Blimp1, Irf8, MafB and Bcl6 were measured.

To verify the effect of Ambn on RANKL-regulated Ca^{2+}oscillation, RAW 264.7 cells were pretreated with 100 ng/ml Ambn, followed by stimulation with 50 ng/ml RANKL. Cells were then incubated with 5 μM Fluo 4-Am. Fluo 4-AM fluorescence images were recorded at 20-s intervals over 200 s using a fluorescence microscope.

結果および考察：

Firstly we found that Ambn suppressed the formation of TRAP-positive multinucleated

osteoclast-like cells with the strongest suppression achieved with 100 ng/ml Ambn (Fig. 1A, B) in both RAW264.7 cells and bone marrow derived macrophages (BMMs), suggesting that Ambn is able to inhibit RANKL-induced osteoclastogenesis. The effect of Ambn was not due to cytotoxicity, as in a viability assay there was no obvious difference between BMMs and RAW264.7 cells stimulated with RANKL in the presence or absence of Ambn (data not shown). We then examined the effect of Ambn on osteoclast function and cytoskeletal organization. We analyzed the effect of Ambn on actin ring formation in a time course assay evaluating the impact of Ambn on pit formation in BMMs induced with M-CSF and RANKL. As shown in Fig. 2, stimulation of BMMs with M-CSF and RANKL markedly induced actin ring reorganization and pit formation, indicating that the cells had differentiated into functionally active, mature osteoclasts. However, in the presence of Ambn, the number of well-defined actin rings was reduced (Fig. 2A). Between days 0 and 4, Ambn treatment resulted in a notable decrease in the pit resorption area, whereas between 4 and 7 days the inhibitory effect of Ambn was weaker (Fig. 2B, C). These results suggested that Ambn inhibits osteoclast differentiation and function. Normally, RANKL induces the expression of osteoclast specific-genes. In the presence of Ambn, the expression of these genes was remarkably reduced, both in BMMs (Fig. 3A) and in RAW264.7 cells (Fig. 3B) cultured with RANKL for 72 and 48 h, respectively. These data further confirmed the suppressive effect of Ambn on RANKL-mediated osteoclastogenesis.

NFATc1 activation is a hallmark event in the cell fate determination of osteoclasts activated via the RANK-RANKL signaling pathway. In RANKL-stimulated RAW264.7 cells, c-Fos and NFATc1 gene and protein expression was diminished by the presence of Ambn (Fig. 4A, B). The inhibitory effect of Ambn on RANK protein and mRNA expression in RANKL-induced RAW264.7 cells was weaker (Fig. 4A, B). In addition, as shown in Fig. 4C, RANKL activated IκB-α degradation in RAW264.7 cells at 30 min and was not altered by Ambn (Fig. 4D). These findings indicated that Ambn negatively regulates osteoclastogenesis by down-regulating c-Fos /NFATc1 but not NF-κB activation.

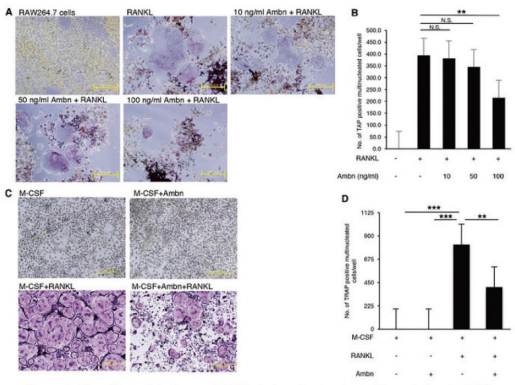

Figure 1. The effect of Ambn on RANKL-induced osteoclast differentiation in BMMs and RAW264.7 cells: A, B, RAW264.7 cells were pretreated with 0-100 ng/ml Ambn for 6 h, then stimulated with 50 ng/ml RANKL for 7 days and stained for TRAP activity. Scale bars = 500 μM. C, D, BMMs were incubated with 20 ng/ml M-CSF and 100 ng/ml Ambn for 6 h prior to their stimulation with 40 ng/ml RANKL for 3 days and stained for TRAP activity. Scale bars = 500 μm. The data were analyzed using Dunnett's post-hoc test after one-way ANOVA and expressed as the mean ± S.D. of independent triplicate samples. N.S., not significant, ***p <0.001 and **p<0.01 compared with RANKL-induced cells.

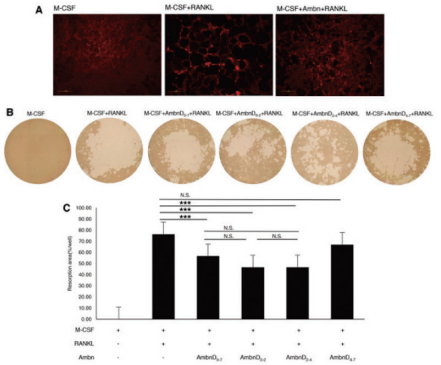

Figure 2. The effect of Ambn on osteoclast activity in BMMs: A, BMMs were pretreated with 20 ng/ml M-CSF and 100 ng/ml Ambn for 6 h, followed by stimulation with 40 ng/ml RANKL for 5 days. They were then fixed and stained for F-actin. Scale bars = 500 μm. B, BMMs on an Osteo Assay Stripwell Plate® were induced with 20 ng/ml M-CSF and 100 ng/ml Ambn prior to their time-dependent stimulation with 40 ng/ml RANKL for 7 days. After removal of the cells, the resorption area was visualized by light microscopy. Scale bars = 1000 μm. C, Resorption areas were analyzed using Image J software. The data show the resorption areas from three independent samples. Error bars represent the mean ±S.D. The data were analyzed using Tukey's post-hoc test after a one-way ANOVA. ***p<0.001 and N.S., not

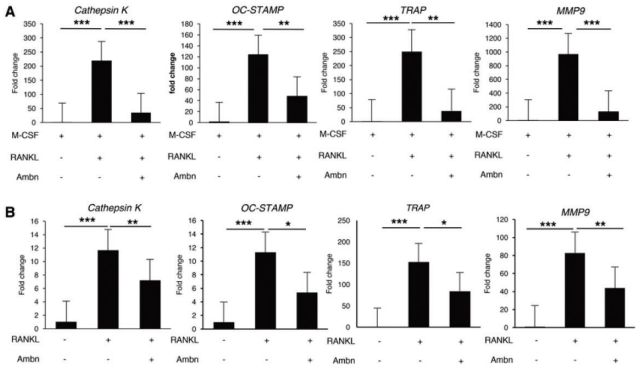

Figure 3. The effect of Ambn on RANKL-induced osteoclast-specific gene expression in BMMs and RAW264.7 cells. A, BMMs were induced with 20 ng/ml M-CSF and 100 ng/ml Ambn for 6 h, followed by stimulation with 40 ng/ml RANKL for 72 h. B, RAW264.7 cells were pretreated with 100 ng/ml Ambn and then induced with 50 ng/ml RANKL for 48 h. The mRNA levels of osteoclast-specific genes (cathepsin K, OC-STAMP, TRAP and MMP9) was measured by real-time RT-qPCR. The data show the fold changes in the mRNA copy number of osteoclast-specific genes from three independent samples. Error bars represent the mean ±S.D. The data were analyzed using Dunnett's post-hoc test after a one-way ANOVA. ***p<0.001, **p<0.01, and *p<0.05 compared with RANKL-induced cells.

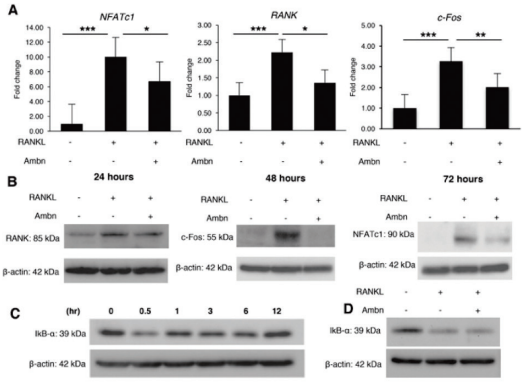

Figure 4. The effect of Ambn on RANKL–induced osteoclast differentiation-related molecules in RAW264.7 cells: RAW264.7 cells were incubated with 100 ng/ml Ambn for 6 h and then induced with 50 ng/ml RANKL for the indicated time. A, NFATc1, RANK, and c-Fos mRNA levels at 48 h were analyzed using real-time RT-qPCR. The data were analyzed using Dunnett's post-hoc test after a one-way ANOVA and expressed as the mean ± S.D. of triplicate samples. ***p<0.001, **p<0.01, and *p<0.05 compared with RANKL-induced cells. B, Whole-cell lysates were subjected to western blot analysis for the detection of RANK, c-Fos, IκB-α, and NFATc1 expression. C, RAW264.7 cells were incubated with 50 ng/ml RANKL for 0–12 h. IκB-α protein expression was detected by western blot analysis. D, RAW264.7 cells were pretreated with 100 ng/ml Ambn for 6 h, followed by stimulation with 50 ng/ml RANKL for 30 min. Whole-cell lysates were subjected to western blot analysis for the detection of IκB-α expression. Equivalent protein aliquots of cell lysates were analyzed based on β-actin.

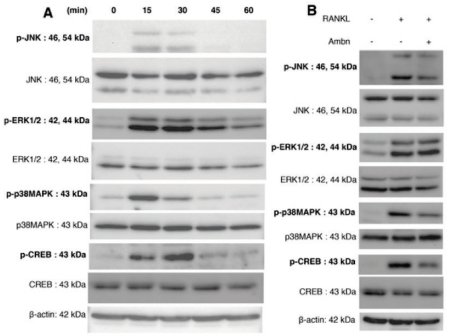

Figure 5. The effect of Ambn on RANKL-induced MAPK and CREB phosphorylation in RAW264.7 cells: A, RAW264.7 cells were induced with 50 ng/ml RANKL for 0–60 min. B, RAW264.7 cells were incubated with 100 ng/ml Ambn for 6 h and then induced with 50 ng/ml RANKL for 15 min. Whole-cell lysates were subjected to western blot analysis for the detection of CREB and MAPK phosphorylation. Equivalent protein aliquots of cell lysates were analyzed based on β-actin.

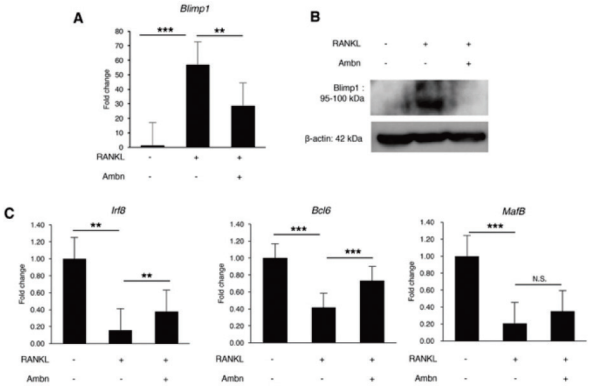

Figure 6. The effect of Ambn on Blimp1–mediated negative regulators of osteoclastogenesis in RAW264.7 cells: RAW264.7 cells were incubated with 100 ng/ml Ambn for 6 h and then induced with 50 ng/ml RANKL for the indicated time. A, Blimp1 mRNA level at 48 h and C, MafB, Irf8, and Bcl6 mRNA levels at 72 h were measured by real-time RT-qPCR. The data were analyzed using Dunnett's post-hoc test after a one-way ANOVA and expressed as the mean ± S.D. of triplicate samples. N.S: not significant, ***p<0.001 and **p<0.01 compared with RANKL-induced cells. B, Whole-cell lysates were subjected to SDS-PAGE and then to western blot analysis for Blimp1. Equivalent protein aliquots of cell lysates were analyzed based on β-actin.

We then examined its effect on the phosphorylation of the early cellular transducers of osteoclastogenesis, including MAPK (ERK1/2, JNK, and p38MAPK) and CREB (Fig. 5A). As shown in Fig. 5B, Ambn abrogated the phosphorylation of CREB, JNK, and p38 MAPK in osteoclasts cultured with RANKL at 15 min, suggesting a role for Ambn in early signal transduction, via the phosphorylation of JNK, p38MAPK, and CREB.

Subsequent to RANKL stimulation, the induction of Blimp1 expression promotes osteoclast differentiation by suppressing the expression of several transcriptional repressors, including MafB, Bcl6, and Irf8. We therefore investigated the effect of Ambn on the negative regulators of osteoclastogenesis. Ambn significantly down-regulated Blimp1 expression (Fig. 6A, B), with a slight, concomitant elevation of MafB, IRF-8, and Bcl6 expression (Fig. 6C). According to these results, inhibition of the transcriptional repressor Blimp1 by Ambn down-regulates osteoclast differentiation by interacting with the negative regulators of osteoclastogenesis.

Ca^{2+} transport plays a critical role in regulating NFATc1 during RANKL-mediated

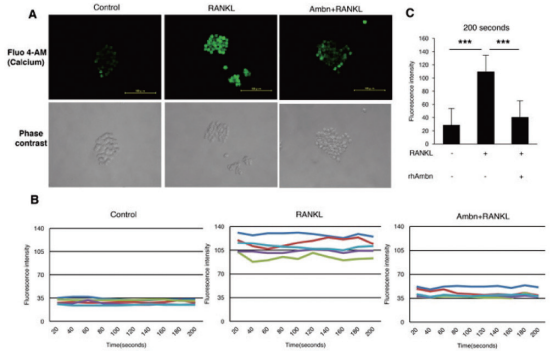

Figure 7. The effect of Ambn on RANKL-induced Ca^{2+}oscillation in RAW264.7 cells: RAW264.7 cells were incubated with 100 ng/ml Ambn for 6 h and then induced with 50 ng/ml RANKL for 48 h. Ca^{2+} oscillation was measured in cells incubated with 5 μM Fluo 4-Am at 37°C for 60 min. The cells were then excited at 480 nm, and fluorescence images for Fluo 4-AM recorded at 20-s intervals over a period of 200 s. A, Intracellular Ca^{2+} was visualized by green fluorescence (Fluo 4-AM) (scale bars = 500 μm). B, Fluorescence intensity over a period of 200 s. Each different-colored line represents the fluorescence intensity of an independent cell in the same field. C, Fluorescence intensity over a period of 200 s. The data were analyzed using Dunnett's post-hoc test after a one-way ANOVA and expressed as the mean ± S.D. of triplicate cultures. ***p<0.001 compared with RANKL-induced cells.

osteoclastogenesis. Treatment of RAW264.7 cells with Ambn attenuated RANKL-induced Ca^{2+} entry（Fig. 7A–C）. These results indicated that the Ambn-mediated reduction in NFATc1 expression and thus the inhibition of RANKL-induced osteoclastogenesis occurred via the reduction of Ca^{2+} oscillation by Ambn

These results suggested that Ambn negative regulated RANKL-induced osteoclast differentiation *in vitro* in three specific pathways：(1) the inhibition of JNK and p38 MAPK-mediated c-Fos activation, (2) Ca^{2+} oscillation and CREB-mediated NFATc1 up-regulation, and (3) Blimp1-mediated negative regulators of osteoclastogenesis.

成果発表：(予定を含めて口頭発表、学術雑誌など)

1. Chaweewannakorn W, Ariyoshi W, Okinaga T, Maki K, Nishihara T. 先端歯学国際教育研究ネットワーク（先端歯学スクール 2017）（August 24-25, 2017, Tokyo）; oral presentation
2. Chaweewannakorn W, Ariyoshi W, Okinaga T, Maki K, Nishihara T. The 55th Annual Conference of The Japanese Society of Pediatric Dentistry,（May 25-26, 2017, Fukuoka）; poster presentation