Fig. 5

A

(a)

Panx3
GAPDH

(b)

Panx3
β-actin

B

ATDC5

C

ATDC5

Mock   Panx3 shRNA

D

ATDC5

Control siRNA   Panx3 siRNA-1   Panx3 siRNA-2

E

Primary chondrocytes

Control siRNA   Panx3 siRNA-1   Panx3 siRNA-2

Fig. 6

A

B

Fig. 7

A

B

Fig. 7

C

Fig. 8

A

B

Fig. 9
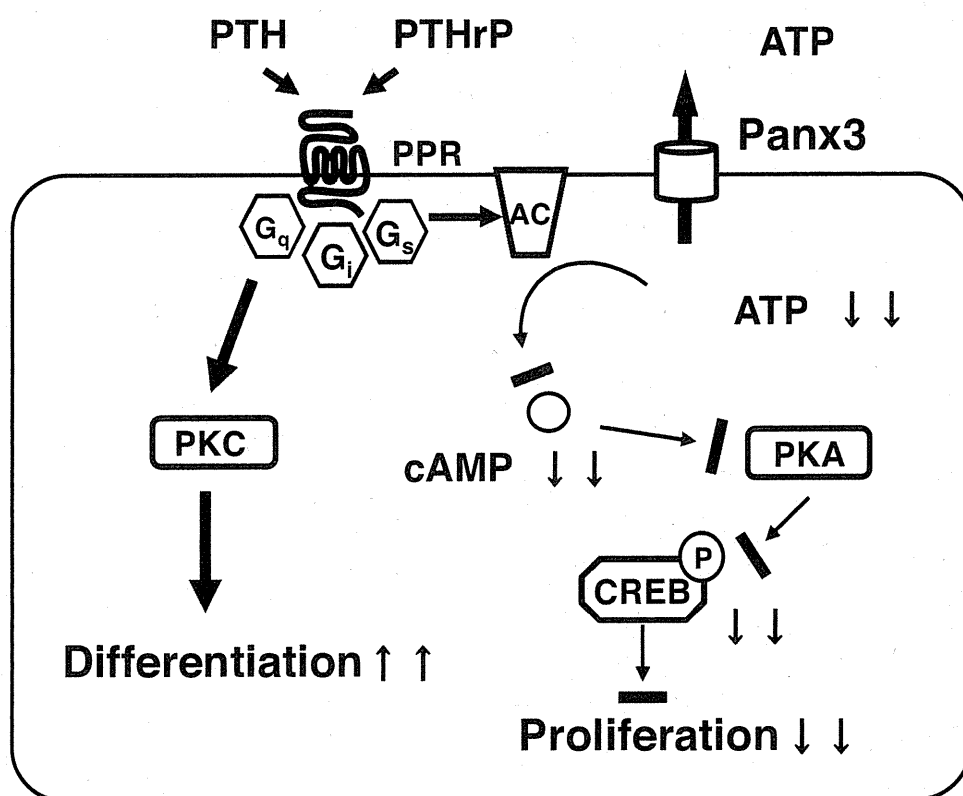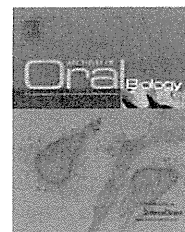
PDGFs regulate tooth germ proliferation and ameloblast differentiation

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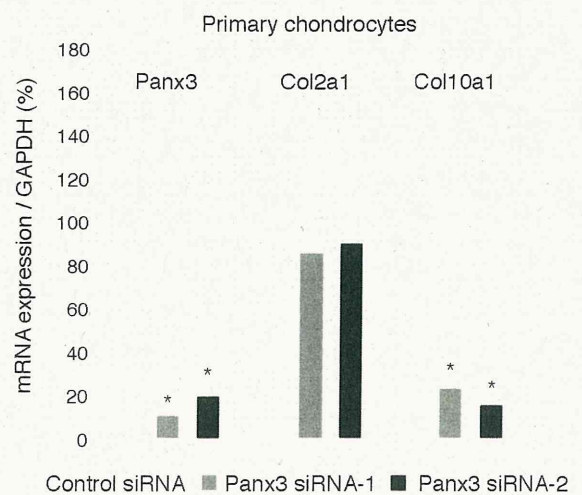Keigo Yoshizaki [a], Shinya Yamamoto [a], Aya Yamada [b], Takashi Nakamura [b],
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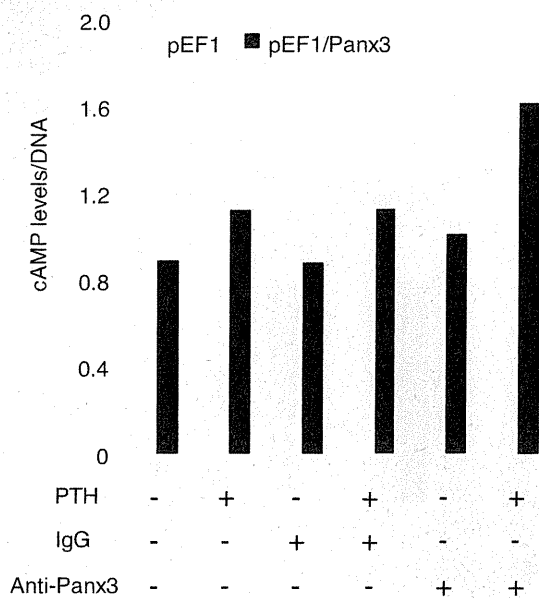
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ABSTRACT

Objective: The purpose of this study was to elucidate the effects of platelet-derived growth factors (PDGFs) during tooth development, as well as the mechanisms underlying the interactions of growth factors with PDGF signalling during odontogenesis.

Design: We used an ex vivo tooth germ organ culture system and two dental cell lines, SF2 cells and mDP cells, as models of odontogenesis. AG17, a tyrosine kinase inhibitor, was utilised for blocking PDGF receptor signalling. To analyse the expressions of PDGFs, reverse transcriptase (RT)-PCR and immunohistochemistry were performed. Proliferation was examined using a BrdU in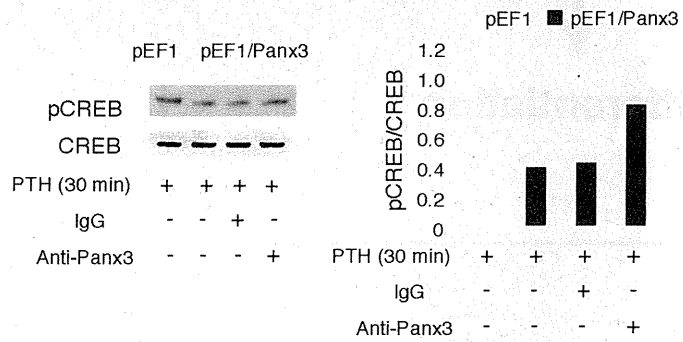corporation assay for the organ cultures and a cell counting kit for the cell lines. The expressions of Fgf2 and ameloblastin were analysed by real-time RT-PCR.

Results: The PDGF ligands PDGF-A and PDGF-B, and their receptors, PDGFRα and PDGFRβ, were expressed throughout the initial stages of tooth development. In the tooth germ organ cultures, PDGF-AA, but not PDGF-BB, accelerated cusp formation. Conversely, AG17 suppressed both growth and cusp formation of tooth germs. Exogenous PDGF-BB promoted mDP cell proliferation. Furthermore, PDGF-AA decreased Fgf2 expression and increased that of ameloblastin, a marker of differentiated ameloblasts.

Conclusion: Our results indicate that PDGFs are involved in initial tooth development and regulate tooth size and shape, as well as ameloblast differentiation.

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1. Introduction

The process of tooth development depends on well-regulated reciprocal epithelial–mesenchymal interactions that are mediated by multiple signalling pathways.[1] Mouse molar tooth development is initiated as a thickening of oral epithelium to form a dental placode, which subsequently continues to grow and invaginate into the

underlying dental mesenchyme derived from cranial neural crest cells. Consequently, differential cell proliferation in the dental epithelium results in the formation of tooth cusps, which pre-pattern the final shape of each individual tooth. Finally, dental epithelial cells differentiate into ameloblasts, which secrete enamel specific matrix proteins, including amelogenin, ameloblastin and enamelin.

Platelet-derived growth factors (PDGFs) bind strongly to their receptors and are thought to play important roles in epithelial–mesenchymal interactions to regulate cell proliferation, migration, extracellular matrix synthesis and deposition in organogenesis.[2] PDGFs consist of four member ligands, PDGF-A, -B, -C and -D, which compose disulphide-linked homodimers, except for PDGF-AB, and act via two different receptor tyrosine kinases, PDGFRα and β. Homodimers of PDGFRα bind PDGF-A, -B and -C, while those of PDGFRβ bind PDGF-B and -D.[3]

PDGF ligands and their receptors are expressed during critical phases of mouse embryogenesis, and play important roles in multiple organ development including tooth.[4-11] PDGF-A null-mice die either as embryos or shortly after birth due to lung emphysema.[4,5] PDGFRα deficient mice also die during embryonic development, and exhibit incomplete cephalic closure.[6,7] In addition, PDGF-B and PDGFRβ knockout mice die during late gestation, due to cardiovascular complications caused by the absence of vascular smooth muscle cells and/or pericytes, and the lack of mesangial cells of the kidney glomerulus suggest the failure of mesenchymal progenitors to locate to their appropriate final destination.[8-11] Our previous work demonstrated that knockdown of PDGF-AA and -BB gene by siRNA dramatically inhibited salivary gland branching morphogenesis, indicated that PDGF signalling is involved in salivary gland morphogenesis, and PDGFs modulate Fgfs expressions through the interaction between epithelial and neural crest-derived mesenchyme.[12] In tooth development, PDGFRα null mutant mice showed inhibition of cusp formation by tooth germs.[13] Transplantation of tooth germ of PDGFRα null mutant mice into a kidney capsule revealed that it does not affect proper odontoblasts proliferation and differentiation in the cranial neural crest-derived odontogenic mesenchyme but perturbs the formation of extracellular matrix and the organisation of odontoblast cells at the forming cusp area, resulting in dental cusp growth defect.[13] However, during tooth morphogenesis, the effects of PDGFs on tooth development and mechanisms of the underlying growth factors that interact with PDGF signalling have not been clearly elucidated.

In the present study, we examined the role of PDGF signalling in tooth development using tooth germ organ culture systems. We found that PDGF-AA accelerated cusp formation, whereas PDGF-BB did not. Furthermore, treatment with AG17, a tyrosine kinase inhibitor, suppressed growth and cusp formation of tooth germs. We also noted that PDGF-AA decreases Fgf2 expression and increases that of ameloblastin. Together, our results indicate that PDGFs regulate tooth size and shape, as well as the differentiation of ameloblasts.

2. Materials and methods

2.1. Animal and tissue preparations

Pregnant ICR mice were provided by Japan SLC, Inc. The presence of a vaginal plug was used as an indication of pregnancy day 0 (E0). The first mandibular molar tooth germs were dissected under stereomicroscope and used for organ cultures. The dental epithelia and mesenchymal tissue samples were separated by treatment with dispase, as reported previously.[12]

2.2. Immunostaining

ICR mouse embryo heads were dissected and embedded in OCT compound (Sakura Finetechnical Co.) for frozen sectioning. OCT blocks were cut into 8-μm sections with a cryostat (2800 FRIGOCUT, Leica), held for 30 min at room temperature and then fixed in 4% PFA for 5 min. After washing 3 times with PBS for 5 min each, liberate antibody binding solution (LAB, Polysciences, Inc.) was applied for 5 min for activation of the binding sites, then washing with PBS was performed. Blocking with Power Block (BioGenex) was performed for 5 min, then the specimens were incubated with the primary antibody for 1 h. After washing 3 times with TPBS for 10 min each, the specimens were incubated with the secondary antibody for 30 min, followed by another washing with TPBS. Finally, the sections were mounted with Vectashield (Vector Labs). A fluorescent microscope (Biozero-8000, Keyence, Japan) was utilised for immunostaining image analysis. The antibodies used were previously described.[12]

2.3. Semiquantitative and real-time RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. First strand cDNA was synthesised at 42 °C for 90 min using oligo(dT)$_{14}$ primer with SuperScript III (Invitrogen). PCR amplification was performed using Taq DNA polymerase (TAKARA, Japan), with the PCR products separated on a 1.5% agarose gel. Real-time RT-PCR was carried out with SYBR Green PCR Master Mix. A TaqMan 7700 Sequencer (Applied Biosystems) was used for the amplification process. PCR was performed for 40 cycles at 95 °C for 1 min and 60 °C for 1 min, and 72 °C for 1 min. The primer sequences used for PDGF-A were 5′-caagaccaggacggtcattt-3′ and 5′-cctcacctggacctct-ttca-3′ (223), while those for PDGF-B were 5′-gcagggtgag-caaggttgtaatg-3′ and 5′-tgaaggaagcagaaggaacgg-3′ (514), for PDGFRα were 5′-acagagactgagcgctgaca-3′ and 5′-caccag-gtccgaggaatcta-3′(226), for PDGFRβ were 5′-acgtaccctacgac-caccag-3′ and 5′-tccattggaagttcaccaca-3′ (234), for Bmp4 were 5′-actgccgcagcttctctgag-3′ and 5′-ttctccagatgttcttcgtg-3′ (485), for CK14 were 5′-tgggtggagatgtcaatgtg-3′ and 5′-ctgccaatcatctcctggat-3′ (446), for HPRT were 5′-gcgtcgt-gattagcgatgatga-3′ and 5′-gtcaagggcatatccaacaaca-3′ (563), for Fgf2 5′-gcgagaagagcgacccacac-3′ and 5′-gaagccag-cagccgtccatc-3′ (124), for ameloblastin 5′-ttgaatgctcctgcca-gaatcg-3′ and 5′-taagggtttgcctgaagcctcc-3′ (118) and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were

5′-ggagcgagacccctctaacatc-3′ and 5′-ctcctggttcacacccatcac-3′ (181).

2.4.   Ex vivo tooth germ organ culture

Mandibular first molar germs from E13.5/E14.5 ICR mouse embryos were dissected using chemically defined medium in a modification of Trowell's system[14] and cultured on cell culture inserts (353090 BD Falcon) at the air/medium interface. Two tooth germs from each mandible were divided into the control and experimental groups. The inserts were floated on 2 ml of BGjb media in 6-well compartments of TC Plates (353502 BD Falcon). To the BGjb we added 0.5%/10% FBS, 100 U/ml of penicillin–streptomycin and 100 µg/ml of ascorbic acid. Ten of the tooth germs were cultured in humidified 5% CO_2/95% air. PDGF-AA (lot 24087, UPSTATE) or PDGF-BB (lot ZT02, SYSTEMS) was added to the media as exogenous PDGF. AG17, a selective inhibitor of the PDGF receptor tyrosine kinase, was used to arrest the function of PDGFRα and PDGFRβ. The tooth germs were photographed every day and tooth size was measured using a computer to evaluate their growth. Each experiment was repeated 3 times.

2.5.   BrdUrd incorporation assay

Tooth germs were cultured with or without AG17 for 5 days, then stained with a 5-Bromo-2′-deoxy-uridine Labeling and Detection Kit I (Roche) according to the manufacturer's protocol. Briefly, BrdUrd was added to the plates (10 mm) for 1 h, and washed with PBS. Then the tooth germs were embedded into paraffin. Paraffin sections from the embedded organ were generated and placed on glass slides. The specimens were dewaxed. After washing 3 times in PBS, the organs were incubated for 30 min with a 1:50 dilution of fluorescein isothiocyanate-conjugated anti-BrdUrd antibody at room temperature. Finally, after washing the organs in PBS 3 times, the nuclei were stained with propidium iodide (Vector Laboratories) and BrdUrd-positive cells were examined under a microscope (Biozero-8000; Keyence, Japan).

2.6.   Dental cell culture and cell proliferation assay

Two different dental cell lines were used to examine the functions of PDGFs; SF2 cells, from an epithelial cell line, and mDP cells, from a dental mesenchymal cell line. They were maintained in Dulbecco's modified Eagle's medium/F-12 medium supplemented with 10% foetal bovine serum, and 1% penicillin and streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2.[15,16] Cell proliferation was assessed using a Cell counting kit-8 containing WST-8 (2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulpho-phenyl]-2H-tetrazolium, monosodium salt) (Dojindo Laboratories). SF2 and mDP cells were separately inoculated at 4000 cell per/well (96-well plates), then cultured in the presence or absence of 5 ng/ml of PDGF-AA or PDGF-BB recombinant proteins for 1, 3 and 5 days. After a 1-h incubation with WST-8 at 0.5 mM, cell proliferation activity was determined calorimetrically at 450 nm, as described in the manual provided by the manufacturer.

3.   Results

3.1.   Expressions of PDGF-A, PDGF-B, PDGFRα and PDGFRβ in developing tooth germs

First, we examined the mRNA expressions of PDGF-A, PDGF-B, PDGFRα and PDGFRβ during tooth germ development by RT-PCR. All PDGF ligands and their receptors were expressed during the initial stages of tooth germ development (Fig. 1A). Tooth development had reached the cap stage by E14.5, at the time when PDGF-A mRNA was strongly expressed in dental epithelium and weakly expressed in dental mesenchyme, while PDGF-B mRNA was expressed in both dental epithelium and mesenchyme and PDGFRα and PDGFRβ were preferentially expressed in dental mesenchyme, and faintly expressed in dental epithelium (Fig. 1B). Immunostaining with specific antibodies for PDGF-A, PDGF-B, PDGFRα and PDGFRβ revealed their protein expressions during initial tooth development. PDGF-A and -B proteins were strongly associated with dental and oral epithelia, and diffusely distributed throughout the dental mesenchyme (Fig. 1C). PDGFRα and PDGFRβ proteins were mainly expressed in the dental mesenchyme during initial tooth development. In the dental epithelium, PDGFRα protein was observed at the inner enamel epithelium by E15.5 (Fig. 1C). PDGFRβ protein was observed in the enamel knot on E14.5, and was also likely in the stratum intermedium on E16.5 (Fig. 1C). Overall, PDGF ligands and their receptors were expressed throughout the initial stages of tooth development, indicating their involvement in that process.

3.2.   AG17 inhibits tooth germ growth and cusp formation

To analyse the functional importance of PDGFs in tooth development, we used a tooth molar organ culture system with or without AG17, a tyrosine kinase inhibitor, to determine its inhibition of PDGFRα and PDGFRβ signalling. Under a normal condition, tooth germs from E13.5 mice grew and formed cusp-like structures after 5 days of culture (Fig. 2A). In contrast, in the presence of AG17, tooth germ growth and cusp formation were blocked (Fig. 2A), with tooth germ width, height and cusp height inhibited by 17%, 22% and 40%, respectively (Fig. 2B). These results suggest that PDGF signalling is essential for tooth germ growth and cusp formation.

Next, we investigated the involvement of PDGFRα and PDGFRβ in cell proliferation and cusp formation. Since AG17 blocked tooth germ growth, we examined its effect on cell proliferation. Tooth germs from E13.5 mice were treated with or without AG17 for 5 days, and cell proliferation was analysed by BrdU incorporation after 1 h (Fig. 3A). Inner enamel epithelium (Fig. 3A) and dental papilla facing dental epithelium (Fig. 3A) had a large number of BrdU-positive cells. On the other hand, the stratum intermedium, stellate reticulum and dental papilla, except for the dental papilla 1 area, showed fewer BrdU-positive cells as compared to the inner enamel epithelium and dental papilla 1 area. Furthermore, the numbers of BrdU-positive cells were decreased in all cells in both dental epithelium and mesenchyme exposed to AG17 (Fig. 3B, C). These results indicate that suppression of PDGFR signalling inhibits cell proliferation in developing tooth germs.
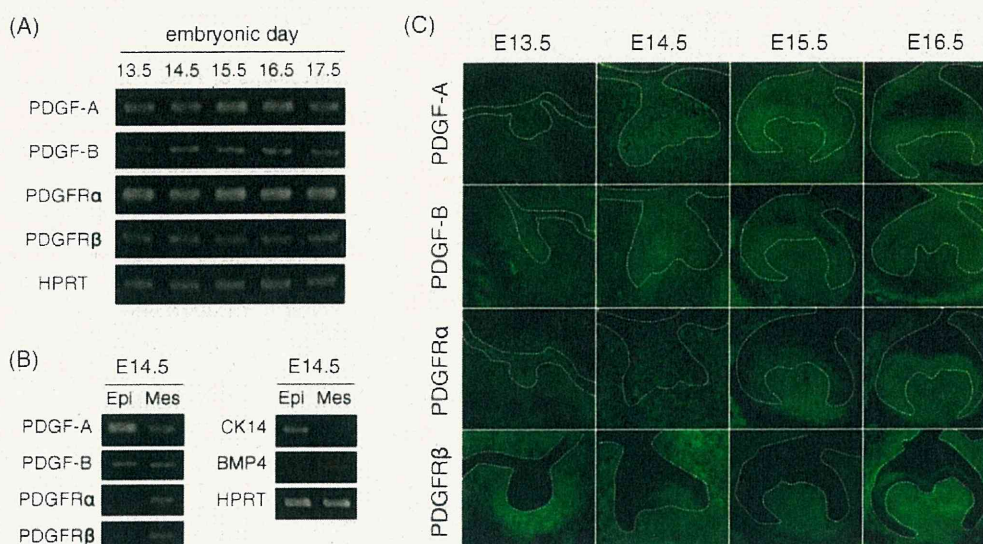
Fig. 1 – Expressions of PDGF-A, PDGF-B, PDGFRα and PDGFRβ in developing tooth germs. (A) The expressions of mRNAs for PDGFs and PDGFRs were analysed in tooth germs from embryonic day (E)13.5 to E17.5 by RT-PCR. PDGFs were expressed in all initial stages of tooth germ development. Hypoxanthine phosphoribosyltransferase (HPRT) gene was used as an internal control. (B) Tooth germs were dissected on E14.5, the cap stage, and dental epithelium (Epi) and mesenchyme (Mes) were separated under a microscope. RT-PCR revealed that PDGF-A mRNA was strongly expressed in the dental epithelium, PDGF-B mRNA was expressed in both dental epithelium and mesenchyme, and PDGFRα and PDGFRβ were preferentially expressed in dental mesenchyme, and faintly expressed in dental epithelium. Cytokeratin-14 (CK14) and Bmp4 were used as markers for dental epithelium and mesenchyme, respectively. (C) Immunostaining for PDGFs in tooth germ development. PDGF-A and -B were strongly expressed in dental epithelium, while PDGFRα and PDGFRβ proteins were mainly expressed in dental mesenchyme. In dental epithelium, PDGFRα protein was observed from E13.5, though its expression eventually shifted to the inner enamel epithelium by E15.5. PDGFRβ protein was located in the enamel knot on E14.5, after which PDGFRβ protein was present in the stratum intermedium on E16.5. Broken white lines, determined by immunostaining with laminin antibody (data not shown), indicate the basement membrane.

3.3. PDGF-BB promotes cell proliferation of dental mesenchymal cells

We also examined the effects of PDGF-AA and -BB on proliferation of the dental epithelial cell line SF2 and dental mesenchyme cell line mDP. Both types of cells were cultured in the presence of PDGF-AA or -BB for 5 days, and cell proliferation was analysed using a cell counting kit. We found that neither had an effect on SF2 proliferation after 5 days (Fig. 4A). In contrast, PDGF-BB promoted mDP cell proliferation, whereas
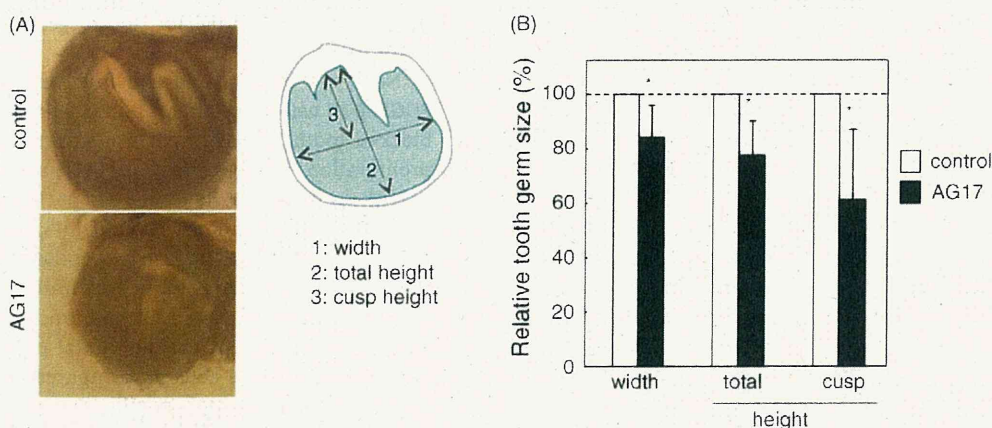

Fig. 2 – AG17 blocks tooth germ growth and cusp formation. (A) Tooth germs obtained on E13.5 were cultured with or without 500 ng/ml of AG17 for 5 days. (B) Tooth germ width, height and cusp height were measured. AG17 was found to inhibit tooth germ growth and cusp formation.
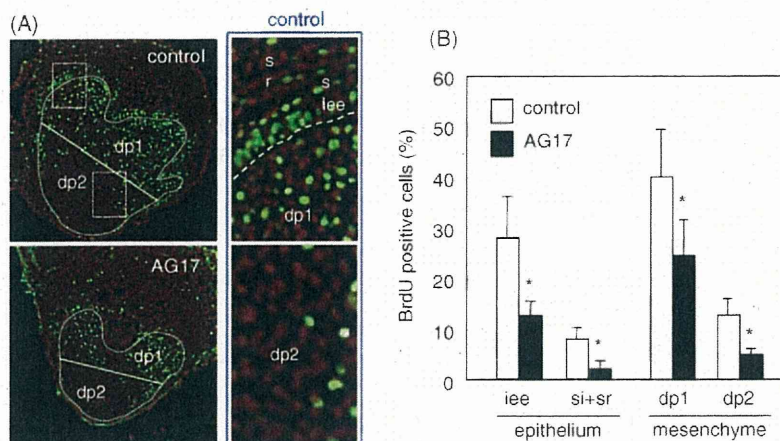
Fig. 3 – AG17 inhibits tooth germ cell proliferation. (A, B) Tooth germs were cultured with or without 500 ng/ml of AG17 for 5 days, then BrdU incorporation after 1 h was analysed using a fluorescence microscope. AG17 inhibited both dental epithelium and mesenchyme cells in the tooth germs. For this experiment, dental papilla sections were divided equally into two parts (DP1 and DP2). Statistical analysis was performed using analysis of variance ($^*p < 0.05$). iee; inner enamel epithelium, si; stratum intermedium, sr; stellate reticulum.

PDGF-AA did not (Fig. 4B). On the other hand, AG17 inhibited cell proliferation of both types of cells regardless of PDGF treatment (Fig. 4A, B). These results indicate that PDGF-AA signalling may not take part in cell proliferation in dental epithelial cells, and PDGF-BB signalling is important for dental mesenchymal cell proliferation. Furthermore, results showing that AG17 has an effect on cell proliferation indicate that autocrine effects of PDGF-AA and -BB may be important for proliferation of tooth cells.

3.4. PDGF-AA accelerates cusp formation

Analysis of cell proliferation using dental cells showed that PDGF-BB accelerates dental mesenchymal proliferation. However, since the role of PDGF-AA in tooth development remains

unclear, we cultured tooth germs taken on E13.5 with PDGF-AA or -BB for 7 days, and analysed dental cusp formation. To evaluate the effects of those PDGFs on cusp formation, we divided tooth development into 4 stages, as follows: the initial stage (S-I), the stage in which the tooth germ grows and becomes round (S-II), the stage in which the initial sign of a tooth cusp can be identified (S-III) and the stage in which the tooth cusp can be clearly identified and becomes sharp (S-IV) (Fig. 5A). When the total percentage of tooth germs classified as S-III and S-IV following treatment with PDGF-AA was compared to non-treated tooth germs, those with PDGF-AA treatment had cusp formation that was greater by 1.5- and 1.2-fold on days 5 and 6, respectively (Fig. 5B). However, there was no difference in cusp formation between tooth germs treated with and without PDGF-BB (data not shown). These findings
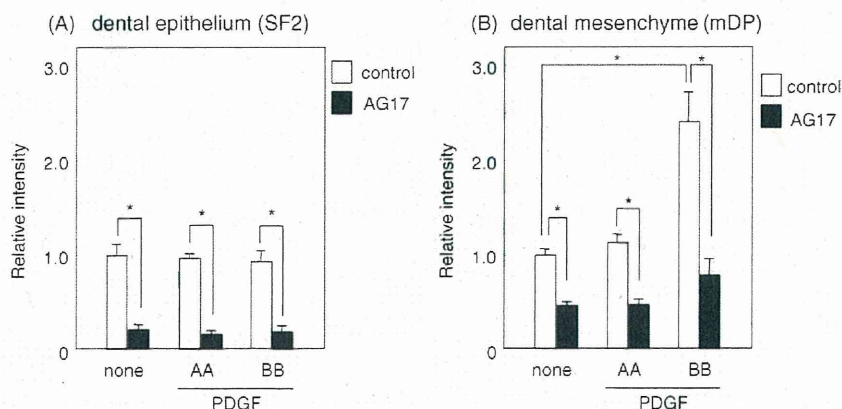
Fig. 4 – AG17 inhibits cell proliferation. The numbers of (A) SF2 cells and (B) mDP cells cultured with or without AG17 were determined using a cell counting kit after 5 days of culture. AG17 inhibited cell proliferation of both SF2 and mDP cells. Neither PDGF-AA nor -BB had effects on SF2 proliferation. However, PDGF-BB promoted mDP cell proliferation, whereas PDGF-AA did not. Statistical analysis was performed using analysis of variance (*, $p < 0.01$).
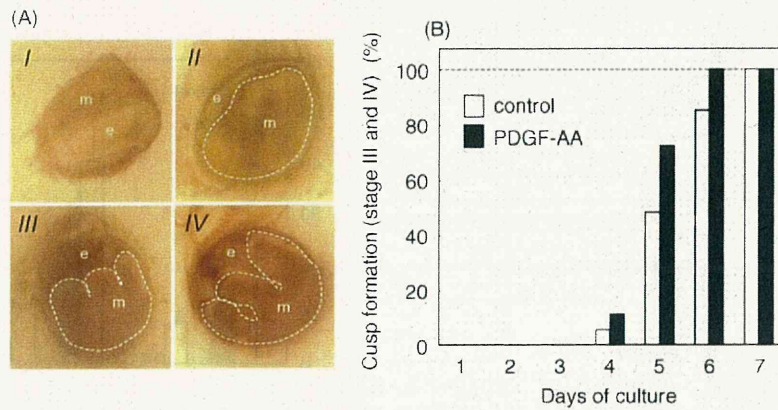
Fig. 5 – PDGF-AA accelerates cusp formation. Tooth germs were cultured with or without 10 ng/ml of PDGF-AA for 7 days. (A) To evaluate the effects of PDGFs, cusp formation was classified into 4 stages: SI, the initial stage; S-II, during which the tooth germ grew and become round; S-III, during which the initial signs of tooth cusp development could be identified; and S-IV, during which the tooth cusp could be clearly identified and became sharp. (B) The total percentage of S-III and S-IV tooth germs following treatment with PDGF-AA was greater than that of untreated tooth germs on days 5 and 6, indicating that cusp formation was accelerated by PDGF-AA.

indicate that cusp formation is accelerated by PDGF-AA. In addition, our results suggest that, in contrast to PDGF-BB, exogenous PDGF-AA via PDGFRα signalling is important for cusp formation.

3.5. PDGF-AA regulates Fgf2 and ameloblastin expression in dental epithelium

In the present experiments, AG17 inhibited the proliferation of tooth germ epithelium and SF2 cells, whereas PDGF-AA and -BB did not have an effect on proliferation of those cells. Furthermore, PDGF-AA via PDGFRα signalling was found to have an effect on cusp formation. The presence of these complimentary functions suggests that other key factors also take part in PDGF signalling. In our previous study, we clearly demonstrated that PDGF signalling regulates the expression of Fgfs in submandibular glands,[12] thus it is possible that a similar system functions in teeth and we investigated the effects of AG17 on the expressions of Fgfs, Bmps and Shh in tooth germ cultures. AG17 partially decreased the expressions
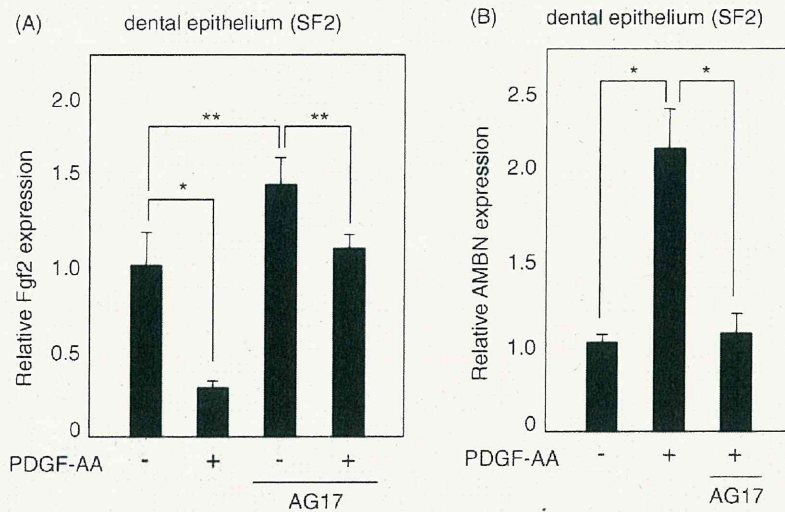
Fig. 6 – PDGF-AA regulates Fgf2 and ameloblastin expressions in SF2 cells. SF2 cells were cultured with or without 10 ng/ml of PDGF-AA and/or AG17 for 48 h, and the mRNA expressions of Fgf2 and ameloblastin (AMBN) were analysed by real-time RT-PCR. (A) Fgf2 expression was inhibited by PDGF-AA and induced by AG17. (B) Ameloblastin expression was induced by PDGF-AA and inhibited by AG17. There were no differences for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression, used as a control, among the various culture conditions. Similar results were obtained in at least three separate experiments. Statistical analysis was performed using analysis of variance (*, $p < 0.01$, **, $p < 0.05$).

of Bmp2, Bmp7, Fgf4, Fgf7 and Shh, while it unexpectedly increased that of Fgf2 (data not shown). To confirm that Fgf2 expression is regulated by AG17 and PDGF, we examined its expression in SF2 cells. Interestingly, AG17 induced Fgf2 expression, whereas PDGF-AA inhibited that expression in SF2 cells (Fig. 6A). Furthermore, PDGF-AA induced ameloblastin expression, whereas AG17 inhibited it (Fig. 6B). These results show that PDGF signalling functions as a modulator of other potent factors, including Fgf2 and ameloblastin, to control dental epithelium differentiation.

4.   Discussion

In the present study, we found that PDGF-BB induces dental mesenchyme proliferation and PDGF-AA accelerates cusp formation. In addition, endogenous PDGFs were shown to be important for tooth germ growth and cusp formation, based on our experiments with AG17 treatment. Furthermore, our results showed that PDGF-AA regulates the expressions of Fgf2 and ameloblastin in dental epithelial cells, indicating its critical role in dental epithelium differentiation.

PDGFα, a gene coding PDGF-A, is expressed in embryos by several different types of progenitor cells that proliferate and migrate in response to PDGF. For example, PDGFα is expressed in precursor cells that become cranial neural crest cells and is thought to be required for migration of those cells into the brachial arches. PDGFα is also expressed by smooth muscle progenitors in developing lungs and widely throughout the embryonic mesenchyme, while PDGFα-deficient mice have a variety of defects in crest-derived tissues, including gross craniofacial, skeletal and cardiac abnormalities. During tooth development, PDGF-A is highly expressed in dental epithelium and weakly in dental mesenchyme (Fig. 1). PDGF-A and PDGF-B form homodimers, PDGF-AA and PDGF–BB, and a heterodimer, PDGF-AB. The only known receptor for PDGF-AA is PDGFRα, which is expressed in dental mesenchyme and inner enamel epithelium, indicating that PDGF-AA may have effects on inner enamel epithelium and neural crest-derived dental mesenchyme.

Dental cusp malformation in PDGFRα null mutants leads to a critical growth defect and shows the requirement for PDGF signalling in the determination of tooth morphology. Loss of the Pdgfrα gene does not have effects on proper odontoblast proliferation and differentiation in the cranial neural crest-derived odontogenic mesenchyme. However, such a lack perturbs formation of the extracellular matrix and organisation of odontoblast cells in the cusp forming area, resulting in a dental cusp growth defect.[13] PDGF-AA and PDGF-BB are able to bind to PDGFRα. In the present experiments, PDGF-BB, but not PDGF-AA, was shown to accelerate dental mesenchymal proliferation. Together, our results suggest that PDGF-BB and PDGFRβ signalling, but not PDGFRα, may be important for dental mesenchymal proliferation.

A previous study used embryonic day 10 mandibular explants cultured in serum-free media and reported that exogenous PDGF-AA enhances tooth development to reach the cap stage with increased tooth size.[17] However, in our experiments with embryonic day 13 tooth germ cultures with a low concentration of serum, we found that treatment with

exogenous PDGFs did not have an effect on tooth germ growth (data not shown), in contrast to treatment with AG17. Based on these results together with those showing a high expression of PDGF-A in tooth germs, we propose that the PDGF-AA isoform and its tyrosine kinase receptor, PDGFRα, regulate tooth size and tooth development during odontogenesis via an autocrine mechanism.

We found that PDGF-A was expressed in submandibular gland epithelium, whereas PDGF-B, PDGFRα and PDGFRβ were expressed in mesenchyme. Exogenous PDGF-AA and -BB in submandibular gland organ cultures demonstrated increased levels of branching and epithelial proliferation, though their receptors were found to be expressed in mesenchyme. PDGF-AA and PDGF-BB induced the expression of Fgf7 and Fgf10, indicating that PDGFs regulate Fgf gene expression in submandibular gland mesenchyme. Also, the PDGF receptor inhibitor AG17 inhibited PDGF-induced branching morphogenesis, whereas exogenous Fgf7 and Fgf10 expressions were fully recovered. Together, these results indicate that fibroblast growth factors function downstream of PDGF signalling, and regulate Fgf expression in neural crest-derived mesenchymal cells and submandibular gland branching morphogenesis.[12] Thus, PDGF signalling is a possible mechanism involved in the interaction between epithelial and neural crest-derived mesenchyme. In tooth germ development, we speculate that an important mechanism of tooth morphogenesis via epithelial and mesenchymal interactions functions in submandibular gland morphogenesis, because of similar patterns of expression of PDGFs and their receptors in both submandibular gland and tooth germ cultures. In fact, AG17 partially decreased the expressions of Bmp2, Bmp7, Fgf4, Fgf7 and Shh, and increased that of Fgf2 in tooth germ organ cultures (data not shown). Previous studies have provided critical information regarding the functional significance of the epithelially derived enamel knot in dental cusp formation. The primary enamel knot in the cap stage tooth germ is a transient structure and serves as a signal centre for regulating cusp formation. Multiple growth factors (such as Shh, Bmps, Fgfs and Wnts), transcription factors (such as Msx2 and Lef1) and cell cycle regulators (such as p21) have important functions in regulating dental cusp formation and the fate of epithelial cells in the enamel knot.[1] A decrease in the expressions of Bmps and Shh may be associated with inhibition of cusp formation in the presence of AG17.

Organisation and remodelling of the basement membrane are also important for determination of tooth size and shape. Laminin α5, a basement membrane component, is highly expressed during tooth germ development. Laminin α5 null-mice have small teeth and inhibited cusp formation, because of decreased expressions of Fgf4 and Shh in the enamel knot.[14] Previous studies have shown a dramatic reduction in MMP-2 in neural crest-derived dental mesenchyme and inhibition of cusp formation in PDGFRα null mutants, which suggest a critical role for that extracellular proteinase in normal tooth development.[13,18] The biological function of MMP-2 is critical for breakdown of the basement membrane prior to the formation of dentin and enamel matrix, as well as for extracellular matrix remodelling, as odontoblast cells retreat towards the central part of dental mesenchyme during dental cusp development.[13] These results indicate that a proper

basement membrane is necessary for cusp formation and PDGF signalling may regulate these related processes.

In our experiments, exogenous PDGF-AA reduced Fgf2 expression in the dental epithelial cell line SF2. Previously, Fgf2 was found to potently induce both proliferation and expression of DSPP in immature pulp cells.[19] Furthermore, exogenous Fgf2 decreased the gene expressions of differentiation markers, such as amelogenin, DSPP and alkaline phosphatase, in molars at the bell stage, while abrogation of endogenous Fgf2 by antisense oligonucleotide increased the gene expressions of those differentiation makers, and also significantly enhanced enamel and dentin formation.[20] These findings suggest that Fgf2 at the bell stage regulates cell differentiation and matrix secretion. In addition, the effects of Fgf2 on tooth cells may be regulated by PDGF-AA, while exogenous PDGF-AA induces ameloblastin expression in dental epithelium. Ameloblastin plays an important role in maintaining the differentiation state of ameloblasts, and also serves as a cell adhesion molecule and regulates ameloblast differentiation.[21] That study also showed that a deficiency of ameloblastin causes severe enamel hypoplasia, accelerates the proliferation of dental epithelium and decreases the expression of amelogenin. Administration of exogenous PDGF-AA to dental epithelial cells may be useful for induction of ameloblastin and differentiation of dental epithelium to ameloblasts.

In conclusion, PDGF-A, PDGF-B, PDGFRα and PDGFRβ were found to be expressed during tooth development. AG17 inhibited tooth germ growth and cusp formation, while exogenous PDGF-BB accelerated the proliferation of dental mesenchymal cells and PDGF-AA induced cusp formation. Furthermore, PDGF-AA reduced the expression of Fgf2 and induced ameloblastin expression. Together, our results indicate that PDGFs and their receptors are necessary for tooth development.

Funding

Conflicts of interest

None declared.

Ethical approval

Not required.

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Critical Role of Heparin Binding Domains of Ameloblastin for Dental Epithelium Cell Adhesion and Ameloblastoma Proliferation[*][s]

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AMBN (ameloblastin) is an enamel matrix protein that regulates cell adhesion, proliferation, and differentiation of ameloblasts. In AMBN-deficient mice, ameloblasts are detached from the enamel matrix, continue to proliferate, and form a multiple cell layer; often, odontogenic tumors develop in the maxilla with age. However, the mechanism of AMBN functions in these biological processes remains unclear. By using recombinant AMBN proteins, we found that AMBN had heparin binding domains at the C-terminal half and that these domains were critical for AMBN binding to dental epithelial cells. Overexpression of full-length AMBN protein inhibited proliferation of human ameloblastoma AM-1 cells, but overexpression of heparin binding domain-deficient AMBN protein had no inhibitory effect. In full-length AMBN-overexpressing AM-1 cells, the expression of Msx2, which is involved in the dental epithelial progenitor phenotype, was decreased, whereas the expression of cell proliferation inhibitors p21 and p27 was increased. We also found that the expression of enamelin, a marker of differentiated ameloblasts, was induced, suggesting that AMBN promotes odontogenic tumor differentiation. Thus, our results suggest that AMBN promotes cell binding through the heparin binding sites and plays an important role in preventing odontogenic tumor development by suppressing cell proliferation and maintaining differentiation phenotype through Msx2, p21, and p27.

The extracellular matrix provides structural support for cells and regulates cell proliferation, migration, differentiation, and apoptosis for tissue development and homeostasis (1). The extracellular matrix also plays a crucial role in pathological processes and diseases, such as wound healing, tumorigenesis, and cancer development (2, 3). AMBN (ameloblastin), also known as amelin and sheathlin, is a tooth-specific extracellular matrix and the most abundant non-amelogenin enamel matrix protein (4–6). AMBN is expressed primarily by ameloblasts, which are differentiated from the oral ectoderm and form a polarized single cell layer underlying the enamel matrix. In a previous study, we created Ambn-null mice and demonstrated that AMBN is required for cell attachment and polarization and for maintaining the differentiation state of ameloblasts and is essential for enamel formation (3). Overexpression of Ambn in transgenic mice causes abnormal enamel crystallite formation and enamel rod morphology (7). These results suggest that enamel formation and rod morphology are influenced by temporal and spatial expressions of AMBN and imply that the AMBN gene locus may be involved in the etiology of a number of cases of undiagnosed hereditary amelogenesis imperfecta (8). Further, it was reported that recombinant AMBN enhances pulpal wound healing and reparative dentine formation following pulpotomy procedures, suggesting that it functions as a signal molecule in epithelial-mesenchymal interactions (9).

We previously reported that about 20% of Ambn-null mice developed an odontogenic tumor of dental epithelium origin in the buccal vestibule of the maxilla (3). The epithelial cells of odontogenic tumors express enamel matrix proteins, including AMEL (amelogenin), ENAM (enamelin), and TUFT (tuftelin), but not AMBN, indicating that AMBN deficiency is probably the primary cause of tumorigenesis seen in those mice. An ameloblastoma appearing in the jaw is the most frequently encountered odontogenic tumor and is characterized by benign but locally invasive behavior with a high rate of recurrence. Since abnormal proliferation and growth of ameloblastoma cells easily destroys surrounding bony tissues, wide excision is required to treat this disorder. It is also reported that ameloblastomas rarely metastasize to other parts of the body, such as the lungs and regional lymph nodes (10, 11). Associations of AMBN mutations were reported in ameloblastomas, adenomatoid odontogenic tumors, and squamous odontogenic tumors (12). These results suggest that AMBN regulates odontogenic tumor formation.

In the present study, we investigated the mechanism of AMBN in dental epithelial cell adhesion and ameloblastoma proliferation. We found that AMBN has heparin binding domains, which are essential for AMBN binding to dental epithelial cells. We demonstrate that overexpression of recombinant AMBN inhibits proliferation of human ameloblastoma cells. This inhibition requires the heparin binding sites of AMBN and is accompanied by dysregulation of Msx2, p21, and p27. These results suggest that AMBN suppresses ameloblastoma cell proliferation by regulating cellular signaling through the heparin binding domains.

EXPERIMENTAL PROCEDURES

*Expression and Purification of Recombinant Ameloblastin—*The expression vector pEF6/V5-His-Topo (Invitrogen) was used to express His-tagged rat AMBN proteins, as described previously (3). The expression plasmids were transfected into COS-7 cells using Lipofectamine 2000 (Invitrogen). After 2 days, transfected cells were lysed using lysis buffer (1% Triton X-100, 10 mM Tris-HCl, pH 7.4, 150 mM BaCl, 10 mM $MgCl_2$), and His-tagged recombinant proteins were purified with a TARON purification system (Clontech), according to the manufacturer's instructions. Purified proteins were separated by SDS-PAGE and analyzed by Western blotting.

*Cell Culture and Transfection—*For dental epithelial cell cultures, molars from P3 mice were dissected. The molars were treated with 0.1% collagenase, 0.05% trypsin, 0.5 mM EDTA for 10 min, and the dental epithelium was separated from the dental mesenchyme. The separated dental epithelium was treated with 0.1% collagenase, 0.05% trypsin, 0.5 mM EDTA for 15 min and then transferred with a pipette up and down into culture wells. Dental epithelial cells were then selected by culturing in keratinocyte-SFM medium (Invitrogen), supplemented with epidermal growth factor and bovine pituitary extract, for 7 days to remove contaminated mesenchymal cells. Cells were then detached with 0.05% EDTA, washed with DME containing 0.1% bovine serum albumin, resuspended to a concentration of 1.0 × 10^5/ml, and used for cell adhesion assays. HAT-7 and SF2 cells from dental epithelium were maintained in Dulbecco's modified Eagle's medium/F-12 medium supplemented with 10% fetal bovine serum (13, 14). AM-1 cells, which were established from human ameloblastoma tissue by human papilloma virus type 16, were maintained in defined keratinocyte-SFM medium supplemented with adjunctive growth supplement (Invitrogen). COS-7 and SQUU cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum. All cells were cultured with 1% penicillin and streptomycin (Invitrogen) at 37 °C in a humidified atmosphere containing 5% CO_2. AM-1, COS-7, and SQUU cells were transiently transfected with an AMBN expression plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

*Cell Adhesion Assays—*Cell adhesion assays were performed in 96-well round bottom microtiter plates (Immulon-2HB; Dynex Technologies, Inc.). The wells were coated overnight at 4 °C with 10 μg/ml recombinant rat AMBN, recombinant mouse AMEL (15), or laminin 10/11 (R&D systems), each

diluted with PBS,[3] and blocked with 3% bovine serum albumin for 1 h at 37 °C. After washing, 10^4 cells were treated with or without heparin (Sigma), heparan sulfate (Sigma), laminin 10/11, or 5 milliunits of heparitinase (Seikagaku Co.) and then added to plates and incubated for 60 min at 37 °C. The plates were washed with PBS three times to remove unattached cells, and then attached cells were treated with 0.05% trypsin, 0.5 mM EDTA and counted under a microscope.

*RNA Isolation and Reverse Transcription-PCR—*Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's protocol. First strand cDNA was synthesized at 42 °C for 90 min using oligo(dT)$_{14}$ primer with SuperScript III (Invitrogen). PCR amplification was performed using the primers listed in supplemental Table S1. The PCR products were separated on a 1.5% agarose gel. The relative expression level was deduced from a standard curve constructed using the positive control sample and normalized against the expression level of glyceraldehyde-3-phosphate dehydrogenase in each sample.

*Western Blotting—*Forty-eight hours after transfection with various AMBN expression vectors, cells were washed twice with 1 mM ice-cold sodium orthovanadate (Sigma) in PBS, lysed with Nonidet P-40 buffer supplemented with a proteinase inhibitor mixture (Sigma) and phenylmethanesulfonyl fluoride at 4 °C for 10 min, and centrifuged, and then the supernatants were transferred to fresh tubes. For a heparin binding assay, protein lysates were incubated with Ni^{2+}-nitrilotriacetic acid beads (Sigma) or heparin-acrylic beads (Sigma) for 12 h at 4 °C and then washed with lysis buffer three times. The cell lysates or purified proteins obtained using nickel or heparin beads were separated by 4–12% SDS-PAGE and analyzed by Western blotting. The blotted membranes were incubated with antibodies for V5-tag (Invitrogen) and AMBN (3), and signals were detected with an ECL kit (Amersham Biosciences).

*Bromodeoxyuridine (BrdUrd) Incorporation and Cell Counting—*For the BrdUrd incorporation assay, cells were incubated at the same cell density for 48 h after transfection with the various vectors. BrdUrd (Sigma) (10 μM) was added to the plates for 60 min, and then the cells were fixed with cold methanol for 20 min, rehydrated in PBS, and incubated for 30 min in 1.5 M HCl. After washing three times in PBS, the plates were incubated with a 1:50 dilution of fluorescein isothiocyanate-conjugated anti-BrdUrd antibody (Roche Applied Science) for 30 min at room temperature. Finally, the cells were washed in PBS three times and incubated with 10 μg/ml propidium iodide (Sigma) in PBS for 30 min at room temperature. BrdUrd-positive cells were examined under a microscope (Biozero-8000; Keyence, Japan). AM-1 cells with or without AMBN expression vector transfection were cultured with serum for 48 h and then plated into 6-well plates at a density of 1 × 10^4 cells/well. Cell numbers were determined using a trypan blue dye exclusion method. Cells were incubated with 10% fetal bovine serum for 0–120 h and then counted in a counting chamber. Cell count analysis was performed in tripli-

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[3] The abbreviations used are: PBS, phosphate-buffered saline; BrdUrd, bromo-2'-deoxyuridine; ERM, epithelial cell rests of Malassez.
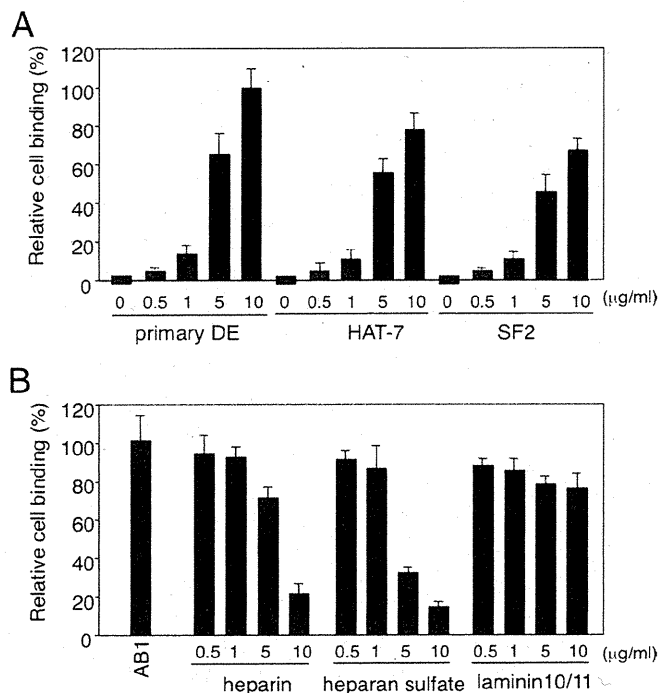
A

B

FIGURE 1. **Dental epithelial cell adhesion to recombinant AMBN and inhibition by heparin and heparan sulfate.** *A*, adhesion of primary dental epithelial cells and rat dental epithelial HAT-7 and SF2 cells to dishes coated with various amounts of full-length recombinant AMBN (AB1). *DE*, primary dental epithelial cells. *B*, inhibition of HAT-7 cell adhesion to AMBN and laminin 10/11 by heparin and heparan sulfate. Heparin and heparan sulfate inhibited cell adhesion to AMBN but not to laminin 10/11.

FIGURE 2. **Inhibition of cell adhesion to AMBN and laminin 10/11 by EDTA.** Adhesion of HAT-7 cells to dishes coated with full-length recombinant AMBN (AB1) and laminin 10/11 in the presence of various concentrations of EDTA. The inhibitory effect of EDTA on dental epithelial cell adhesion to AMBN was less effective than that of laminin 10/11.

cate for each time point, and the presented results are the average of 10 independent experiments.

RESULTS

AMBN Binding to Dental Epithelial Cells Is Inhibited by Heparin and Heparan Sulfate—To analyze cell adhesion to recombinant AMBN, we created V5-His-tagged recombinant AMBN (see Fig. 3A). The anti-V5 antibody was able to detect the recombinant protein as a specific band of about 58 kDa, which is larger than the predicted molecular size of the AMBN-V5-His fusion protein. This higher molecular weight on SDS-PAGE is due to the unconventional protein property of AMBN (16). Primary dental epithelium bound to full-length AMBN (AB1) in a dose-dependent manner, as previously reported. Further, rat dental epithelial lines HAT-7 and SF2 cells also bound to AMBN in a dose-dependent manner (Fig. 1A).

AMBN has a VTKG motif, which is a potential thrombospondin-like cell adhesion domain (6), also known as a heparin binding domain (17). In addition, AMBN has positively charged lysine, arginine, and histidine (KRH) amino acid-rich sequences in the middle and C-terminal regions (Fig. 3A), and a KRH-rich motif has been proposed as a heparin binding domain (17). We found that heparin and heparan sulfate inhibited dental epithelial cell adhesion to AB1 but not laminin 10/11 (Lam-511 and Lam-521, according to a recently proposed nomenclature) (Fig. 1B) (46). These findings suggest that the heparin binding domains are involved in dental epithelium cell adhesion to AMBN. Many extracellular matrix proteins bind to
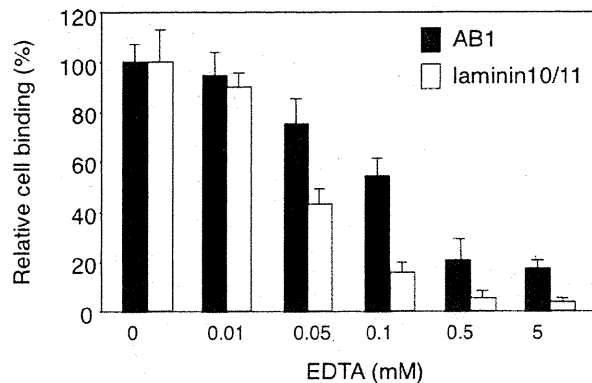
cells through integrins or calcium-dependent cell adhesion molecules, and this binding is inhibited in the presence of EDTA. EDTA inhibited cell adhesion to laminin 10/11, which has integrin binding regions (Fig. 2) (18). However, the inhibitory effect of EDTA on dental epithelial cell adhesion to AMBN was less effective than that of laminin 10/11 (Fig. 2). These results suggest that non-integrin-dependent cell adhesion is important for cell binding of AMBN.

Heparin Binding Domains in AMBN Critical for Heparin Binding and Cell Adhesion—To examine the significance of the potential heparin binding domains of AMBN, we prepared truncated V5-His-tagged AMBN proteins from COS-7 cells transfected with various AMBN cDNA expression vectors (Fig. 3A) and determined their heparin binding properties. All recombinant proteins purified by nickel bead affinity chromatography were detected by anti-V5 antibodies (Fig. 3B). Some proteolytic bands were observed for the AB1, AB2, AB3, and AB4 proteins, whereas the AB6 protein band was weak as compared with the others. AB1 and AB2, which have all three heparin binding domains, and AB3, which lacks the C-terminal heparin binding domain, bound equally well to heparin beads at high levels (Fig. 3B), whereas AB4, which has only the N-terminal heparan binding domain, bound to the beads weaker than those three proteins. AB5, which has a half-portion of the N-terminal heparan binding domain, showed substantially reduced heparin binding activity, and AB6, which lacks all three heparan binding domains, did not bind the heparan beads at all (Fig. 3C). These results indicate that the two N-terminal domains are required for heparin binding of AMBN.

To examine the role of the heparin binding domains in AMBN cell binding, dental epithelial cells were plated on dishes coated with purified AMBN recombinant proteins, and cell binding was measured. The AB2, AB3, and AB4 proteins had about 60% cell binding activity compared with that of AB1, and the levels for AB5 cell binding were further lower by 30% than AB1. AB6 showed little cell binding (Fig. 4A). Heparin inhibited cell binding of AB2 but not AB5 (Fig. 4B). These results suggest that AMBN has a heparin-insensitive cell binding region at the N-terminal half and a heparin-sensitive cell binding region at the C-terminal half.
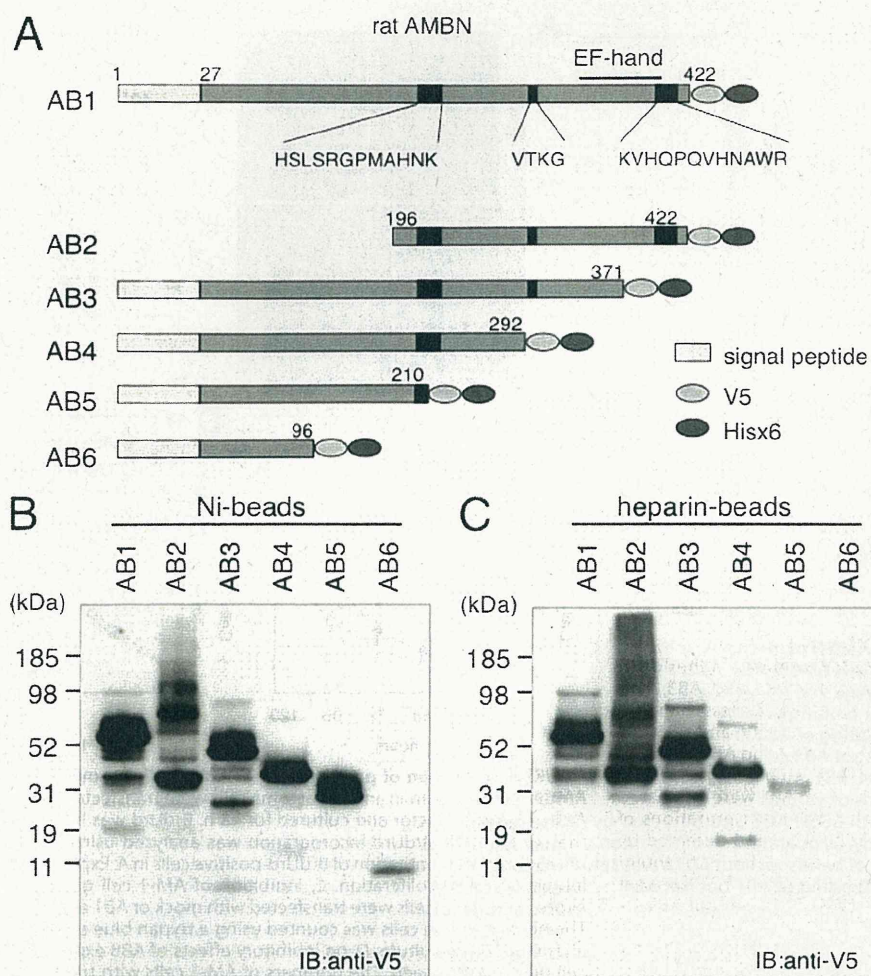
FIGURE 3. **Identification of heparin binding regions of AMBN.** A, creation of deletions in AMBN. All recombinant AMBN proteins have V5 and His tags at the C terminus; the amino acid sequences for potential heparin binding are shown (*black boxes*). B, expression of mutant AMBN proteins. An expression vector for each recombinant AMBN was transfected into COS-7 cells and was purified using Ni^{2+}-nitrilotriacetic acid beads. Purified proteins were separated by SDS-PAGE and visualized with the anti-V5 antibody. C, heparin binding of AMBN proteins. Each cell lysate was mixed with heparin-acrylic beads, and bound proteins were separated by SDS-PAGE and visualized with the anti-V5 antibody. AB1, AB2, and AB4 bound strongly to heparin. AB4 bound less to heparin, and AB5 had substantially reduced heparin binding. AB5 lost heparin binding.

Overexpression of Full-length AMBN (AB1) but Not Heparin Binding Domain-deficient AMBN (AB5) Inhibits Proliferation of Ameloblastoma AM-1 Cell—*Ambn*-null mice develop odontogenic tumors, suggesting that AMBN may function as a tumor suppressor. AM-1 cells are an ameloblastoma cell line, which does not express AMBN (supplemental Fig. 1A). To identify the role of AMBN in odontogenic tumor proliferation, the AB1 expression vector was transfected into AM-1 cells. Transfected AM-1 cells expressed and secreted recombinant AMBN protein (supplemental Fig. 1C). AB1-overexpressing AM-1 cells showed a decrease in the number of BrdUrd-positive cells as compared with mock-transfected cells (Fig. 6, A and B). After transfection, the number of cells was counted every 24 h for 5 days and was found to be substantially decreased (Fig. 6C). However, overexpression of AB8 lacking the heparin binding domains did not affect cell proliferation as compared with mock-transfected cells (Fig. 6D). These results indicate that AMBN expression inhibits proliferation of AM-1 cells via the heparin binding domains.

To examine whether the inhibition of cell proliferation by AMBN is dependent on a cell type, the AB1 expression vector was transfected into COS-7 cells from a kidney fibroblast cell line and SQUU-B cells from a tongue squamous cell carcinoma cell line. BrdUrd incorporation was inhibited in AB1-overexpressing AM-1 cells but not in AB1- overexpressing COS-7 or SQUU-A cells (Fig. 7). Further, proliferation of AM-1 cells was inhibited when the cells were cultured on recombinant AMBN (AB1)-coated dishes but not recombinant AMEL- and laminin 10/11-coated dishes (Fig. 7B). These findings suggest that AMBN suppresses cell proliferation in a cell type-specific manner.

AMBN Expression Induces the Expression of p21 and p27 but Inhibits Msx2 Expression—To identify the inhibitory mechanism of proliferation of AM-1 cells by AMBN, we examined the expressions of *p21* and *p27*, *CDK* inhibitors and negative regulators of cell proliferation, and Msx2, a homeobox-containing transcription factor, which is expressed in undifferentiated ameloblasts (20) and in *Ambn*-null ameloblasts (3). Msx2 was strongly expressed in mock-transfected AM-1 cells; however, its expression was strongly inhibited by overexpression of AMBN (Fig. 8). We found that overexpression of AMBN

AMBN has an EF-hand calcium binding region in the C terminus. Bioinformatic analysis suggests a conformational change in the AMBN protein in the presence of the Ca^{2+} ion (19). We found that heparin binding of AB1 containing an EF-hand motif was increased by EDTA in a dose-dependent manner, whereas heparin binding of AB4 lacking the EF-hand motif did not change by EDTA (Fig. 4, C and D). These results suggest that the EF-hand motif modulates heparin binding activity of AMBN. An internal deletion of the N-terminal heparin binding domain of AB1 (AB7) caused a small reduction in heparin and cell binding (Fig. 5, A–C). AB8, in which three heparin binding domains, but not the N-terminal sequence, were deleted from AB1, showed a nearly complete loss of heparin binding and weak cell binding activity (Fig. 5). Further, cells treated with heparitinase lost the ability to bind to AB1 (Fig. 5C), indicating that heparan sulfate on the surface of ameloblasts is important for cell binding to AMBN.
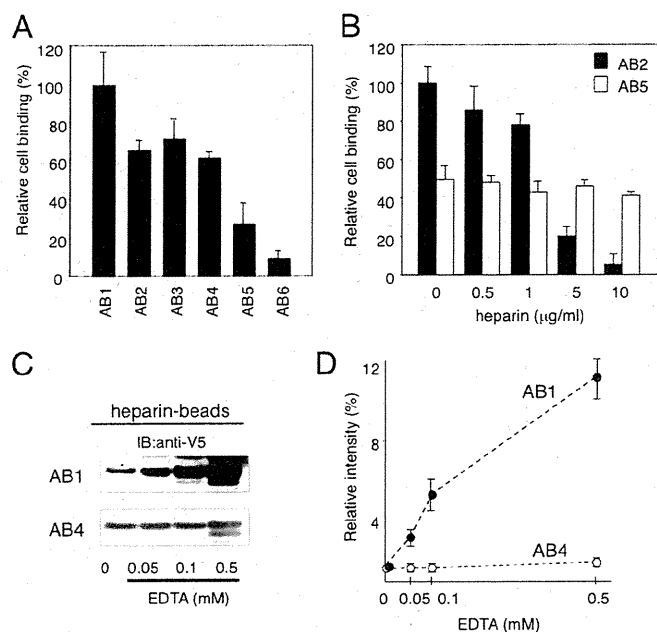
Heparin Binding Domains of Ameloblastin

FIGURE 4. **Cell binding activity of mutant AMBN proteins.** A, adhesion of HAT-7 cells to dishes coated with recombinant AMBN, AB1, AB2, AB3, AB4, AB5, and AB6. Cell binding activity of AB2, AB3, and AB4 was less than that of AB1. AB5 had substantially reduced cell binding activity, and AB6 lost the binding activity. B, inhibition of cell binding of AB2 and AB5 by heparin. Heparin inhibited AB2 cell binding but not AB5 cell binding. C, effects on EDTA on heparin binding of AB1 and AB4. AB1 and AB4 were incubated with heparin-acrylic beads in the presence of various concentrations of EDTA. Bound proteins were separated with SDS-PAGE and detected by Western blotting using the anti-V5 antibody. D, quantitation of the intensity of the protein bands in C. The intensity of bands without EDTA was set at 1 for comparison. EDTA affects heparin binding of AB1 but not AB4.
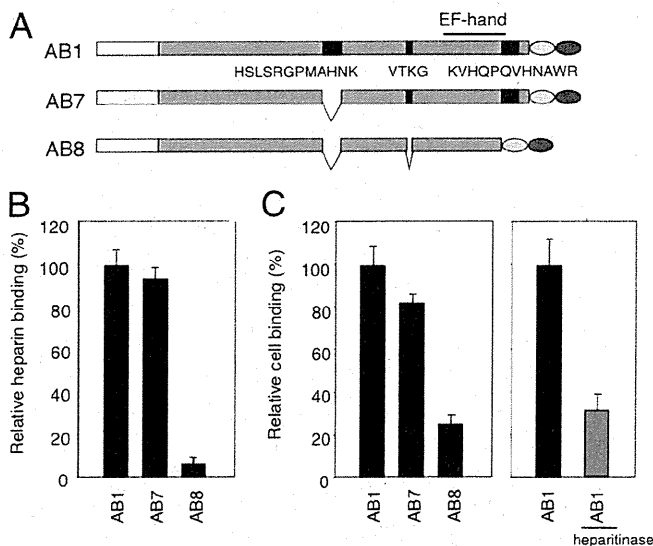

FIGURE 5. **Deletion analysis of heparin binding regions of AMBN for heparin binding and cell adhesion.** A, deletions of recombinant AMBN proteins. AB7 results from deletion of the first heparin binding region, and AB8 is the result of deletion of all three heparin binding regions. B, heparin binding of AB1, AB7, and AB8. AB7 slightly reduced heparin binding activity, but AB8 lost all activity. Heparin binding of AB1 was set at 100%. C, adhesion of HAT-7 cells to dishes coated with AB1, AB7, and AB8. AB7 had reduced cell binding activity. AB8 further reduced cell binding. Cells pretreated with heparitinase showed decreased binding to AB1. Cell adhesion of AB1 was set at 100% for comparison.
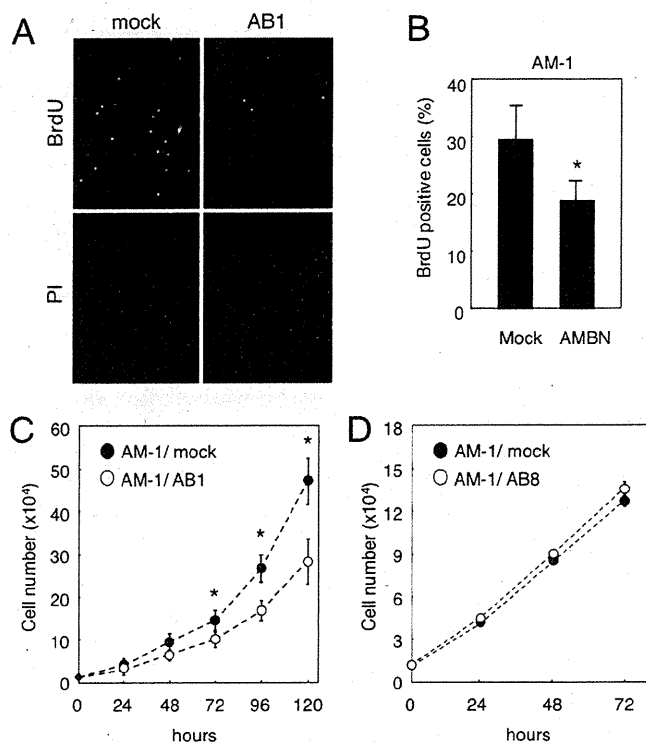

FIGURE 6. **Inhibition of proliferation of AM-1 cells by overexpression of AMBN.** A, AM-1 human ameloblastoma cells were transfected with a mock or AB1 expression vector and cultured for 48 h. BrdUrd was incorporated into the cells for 1 h. BrdUrd incorporation was analyzed using a fluorescence microscope. B, quantitation of BrdUrd-positive cells in A. Expression of AMBN-inhibited AM-1 proliferation. C, inhibition of AM-1 cell growth of by AB1 expression. AM-1 cells were transfected with mock or AB1 expression vector. The number of the cells was counted using a trypan blue exclusion method after 0–120 h of culture. D, no inhibitory effects of AB8 expression on AM-1 cell growth were seen. The numbers of AM-1 cells with transfection of the mock or AB8 expression vector were counted from 0 to 72 h. Statistical analysis was performed using analysis of variance ($*$, $p < 0.01$).

induced the expression of both *p21* and *p27*, whereas the expression of positive cell cycle regulators *CDK1*, *-4*, and *-6* was not changed (Fig. 8). These results suggest that AMBN promotes odontogenic cell differentiation and inhibits proliferation via p21 and p27 expression in a CDK-independent manner.

Overexpression of AMBN in AM-1 Cells Induces Expression of ENAM but Not AMEL—Our finding that AMBN expression inhibited *Msx2* expression suggests that AMBN promotes odontogenic cell differentiation. To better elucidate the function of AMBN in AM-1 cell differentiation, we examined whether AMBN has effects on the expression of other enamel matrix proteins. AMBN overexpression in AM-1 cells strongly induced the expression of *ENAM* mRNA (Fig. 9), whereas the expression of *AMEL* and *TUFT* was not affected. These results indicate that AMBN does not induce all enamel matrix genes but induces selectively in AM-1 cells and suggests that AMBN may serve as a suppressor of odontogenic tumors by regulating cellular signaling for differentiation and proliferation.

DISCUSSION

We previously found using *Ambn*-null mice and cell culture that AMBN is an adhesion molecule for ameloblasts and required for maintaining a single ameloblast cell layer