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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.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Fig. S1.

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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-

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

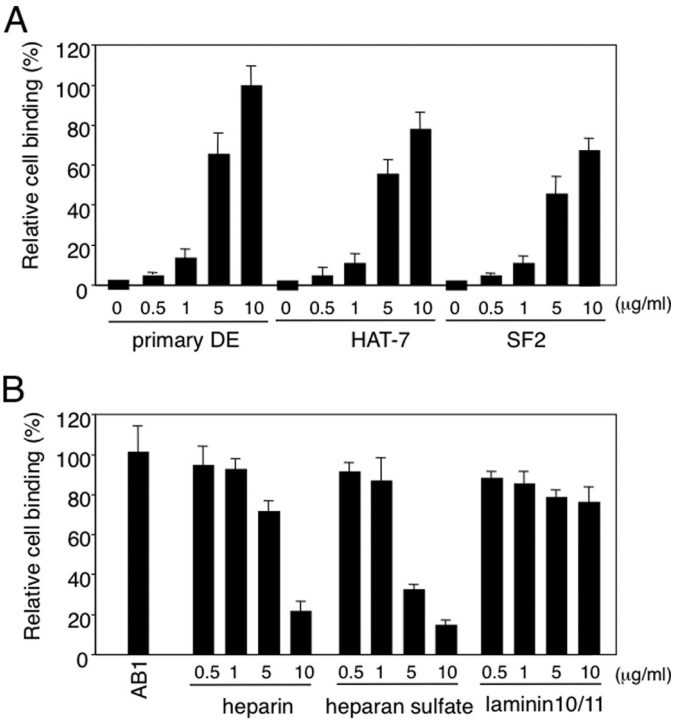

FIGURE 1. **Dental epithelial cell adhesion to recombinant AMBN and inhibition by heparin and heparan sulfate.** *A*, adhesion of primary dental epithelial cells and rat dental epithelial HAT-7 and SF2 cells to dishes coated with various amounts of full-length recombinant AMBN (AB1). *DE*, primary dental 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.

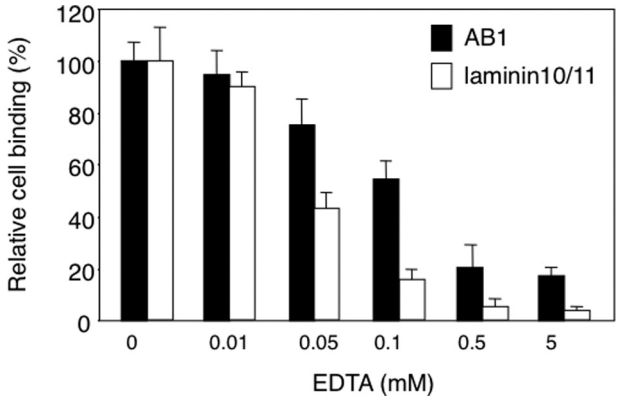

FIGURE 2. **Inhibition of cell adhesion to AMBN and laminin 10/11 by EDTA.** Adhesion of HAT-7 cells to dishes coated with full-length recombinant AMBN (AB1) and laminin 10/11 in the presence of various concentrations of EDTA. The inhibitory effect of EDTA on dental epithelial cell adhesion to AMBN was 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. 3*A*). 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. 1*A*).

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. 3*A*), 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. 1*B*) (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. 3*A*) and determined their heparin binding properties. All recombinant proteins purified by nickel bead affinity chromatography were detected by anti-V5 antibodies (Fig. 3*B*). 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. 3*B*), 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. 3*C*). 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. 4*A*). Heparin inhibited cell binding of AB2 but not AB5 (Fig. 4*B*). 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.

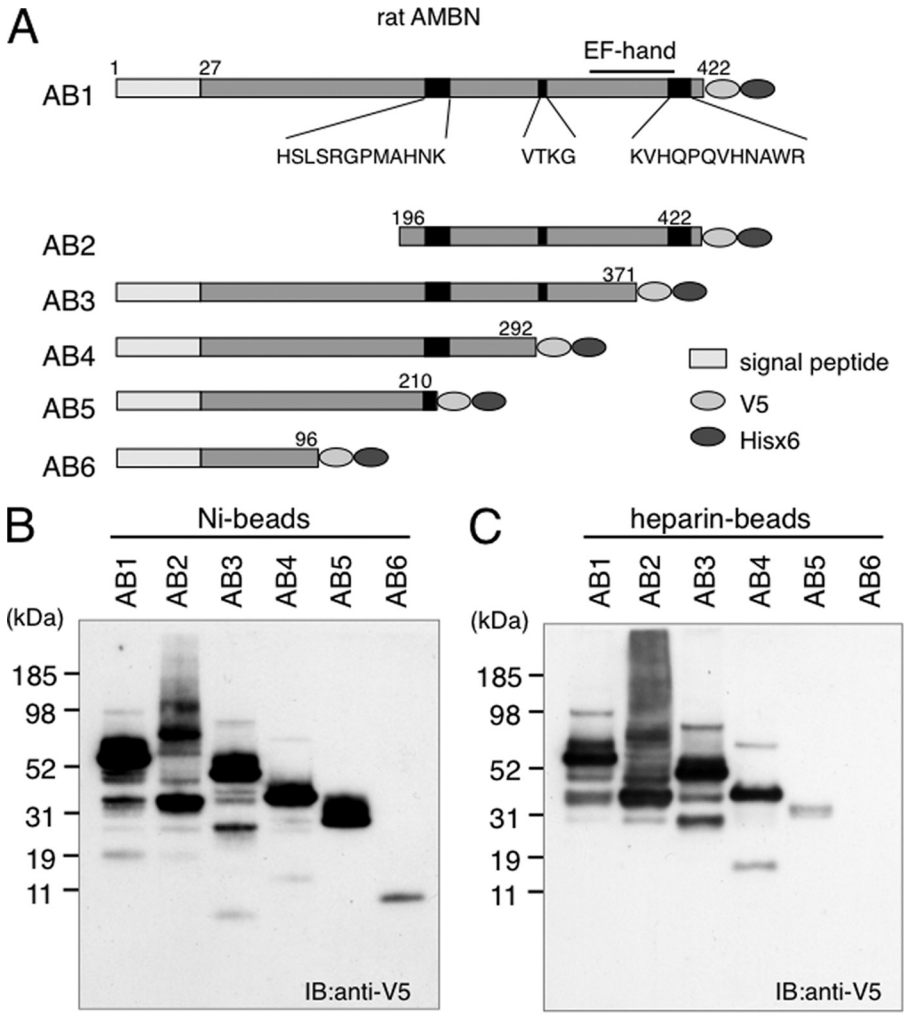

FIGURE 3. **Identification of heparin binding regions of AMBN.** *A*, creation of deletions in AMBN. All recombinant AMBN proteins have V5 and His tags at the C terminus; the amino acid sequences for potential heparin binding are shown (*black boxes*). *B*, expression of mutant AMBN proteins. An expression 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.

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. 5*C*), indicating that heparan sulfate on the surface of ameloblasts is important for cell binding to AMBN.

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. 1*A*). 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. 1*C*). 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. 6*C*). However, overexpression of AB8 lacking the heparin binding domains did not affect cell proliferation as compared with mock-transfected cells (Fig. 6*D*). 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. 7*B*). 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

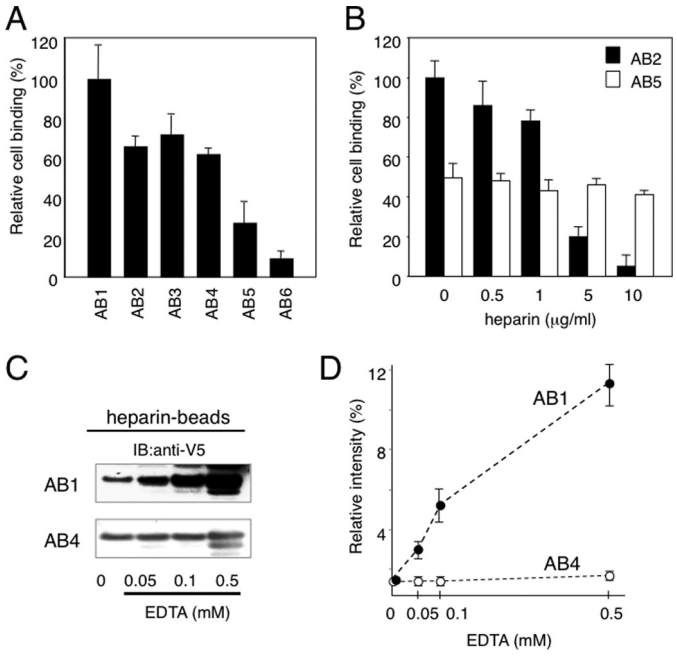

FIGURE 4. **Cell binding activity of mutant AMBN proteins.** *A*, adhesion of HAT-7 cells to dishes coated with recombinant AMBN, AB1, AB2, AB3, AB4, AB5, and AB6. Cell binding activity of AB2, AB3, and AB4 was less than that of AB1. AB5 had substantially reduced cell binding activity, and AB6 lost the 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.

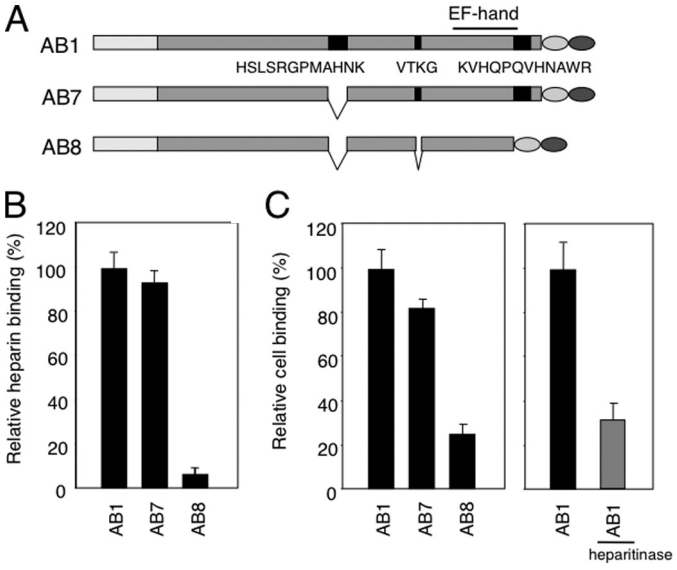

FIGURE 5. **Deletion analysis of heparin binding regions of AMBN for heparin binding and cell adhesion.** *A*, deletions of recombinant AMBN proteins. AB7 results from deletion of the first heparin binding region, and AB8 is the result of deletion of all three heparin binding regions. *B*, heparin binding 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.

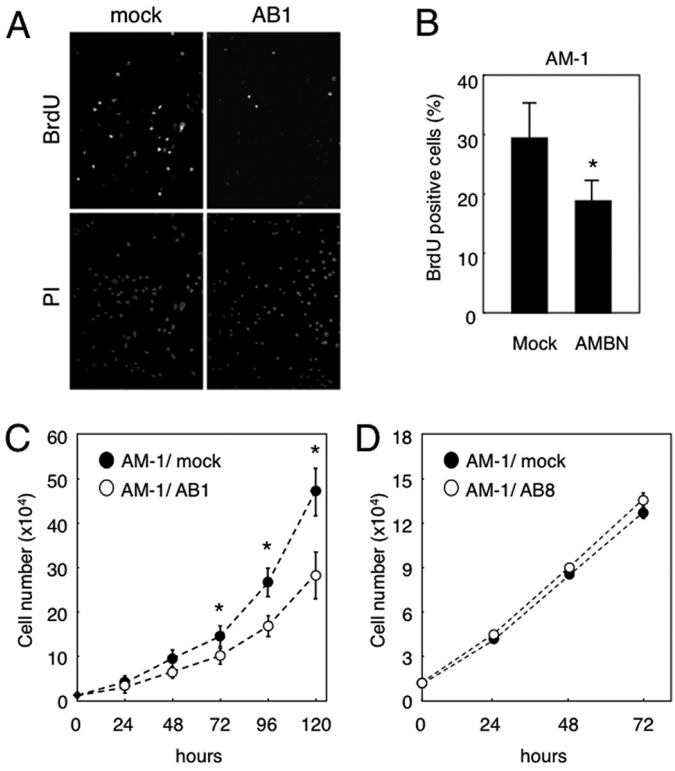

FIGURE 6. **Inhibition of proliferation of AM-1 cells by overexpression of AMBN.** *A*, AM-1 human ameloblastoma cells were transfected with a mock or AB1 expression vector and cultured for 48 h. BrdUrd was incorporated into the cells for 1 h. BrdUrd incorporation was analyzed using a fluorescence microscope. *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

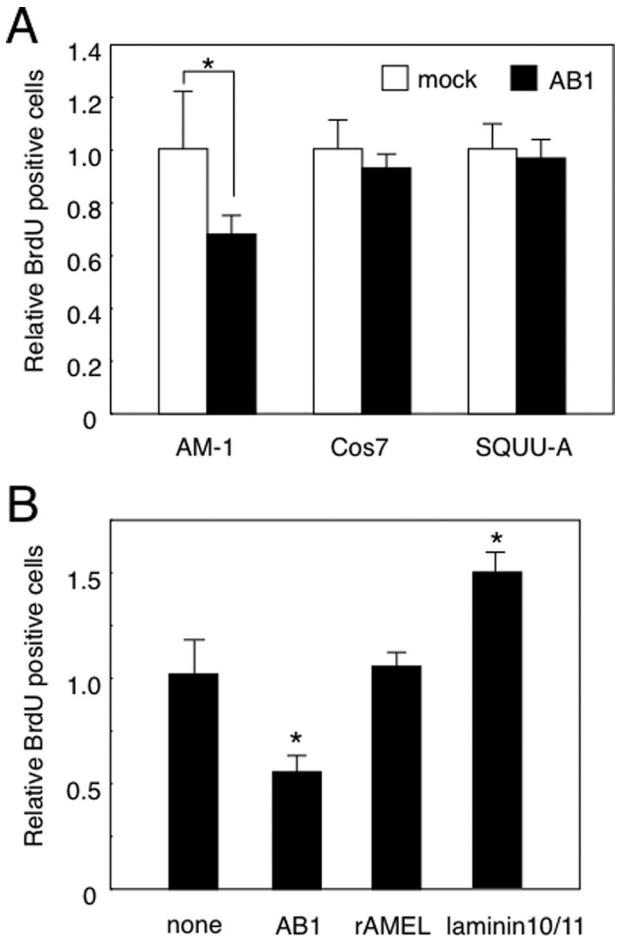

FIGURE 7. **Cell type-specific inhibition of proliferation by AMBN.** *A*, AB1. AM-1, COS-7, and SQUU-A cells were plated on dishes coated with AMBN (10 mg/ml) and cultured for 48 h. BrdUrd was incorporated into the cells for 1 h. BrdUrd-positive cells were counted. Proliferation of AM-1 cells, but not COS-7 and SQUU-A cells, was reduced on AB1 substrate. The number of BrdUrd-positive cells with mock-transfected cells was set at 100%. *B*, adhesion of AM-1 cells to dishes coated with AB1, recombinant AMEL (*rAMEL*), and laminin 10/11. The number of BrdUrd-positive cells with cells cultured on a non-coated dish was set at 100%. Statistical analysis was performed using analysis of variance (*, $p < 0.01$).

attached to the enamel matrix and the differentiation state of ameloblasts. In this paper, we identify the heparin binding domains of AMBN and demonstrate that these domains play a critical role in AMBN binding to dental epithelial cells. We also show that AMBN inhibits proliferation of human ameloblastoma cells. This inhibition is accompanied by the induction of *p21* and *p27* and *ENAM* and the reduction of *Msx2*. It was recently reported that AMBN fusion protein enhances pulpal healing and dentin formation in porcine teeth (21). In addition, AMBN promotes adhesion of periodontal ligament cells and modulates the expression of bone morphogenic protein, collagen type I, and osteocalcin (22). Those results implicate that AMBN regulates cell proliferation and differentiation through cellular signaling induced by the AMBN interaction with cells. Our finding that AMBN cell binding is mediated through the heparin binding domains suggests that AMBN interacts with heparan sulfate (HS) cell surface receptors. However, we could not detect AMBN binding to lymphoid cell lines expressing individual HS cell surface receptors, including syndecan-1, -2, and -4 (data not shown) (23). We also could not find the interaction of AMBN with glypican-1, which is expressed in ameloblasts (data not shown). Laminin is one of the heparin and integrin binding molecules. The laminin α1 chain LG4 module promotes cell attachment through syndecans and cell spreading through integrin $\alpha 2\beta 1$ (23). Active sequences in the LG4 of other laminin α chains have also been identified for cell attachment and heparin and syndecan binding (24–26). Further, Lys and Arg amino acid-rich regions of the LG4 module are important for heparin and HS binding. These regions may be similar to those of AMBN. A recent study (27) showed that laminin-sulfatide interaction modulates basement membrane assembly and regulates cellular signaling. It is possible that AMBN may bind cells through interacting with other co-receptors, including cell surface glycolipids and extracellular matrix.

Rodent AMBN contains a DGEA motif, which is a potential integrin-binding site of collagen I, and a thrombospondin-like cell adhesion motif, VTKG (6, 28). We found that recombinant full-length AMBN produced in mammalian cells binds specifically to primary dental epithelial cells but not to other cell types. Primary dental epithelial cells contain mixed epithelial cell populations, including inner dental epithelial cells, ameloblasts, stratum intermedium cells, and stellate cells. We found that ameloblasts, but not the other cell types, adhere to recombinant AMBN (3). In the present study, we showed that ameloblast cell lines HAT-7 and SF2 (Fig. 1) and ameloblastoma cell line AM-1 (data not shown) also bind to AMBN. The DGEA and VTKG sequences are conserved only in rodents, with little or no conservation of those motifs in other species, including human, pig, bovine, and caiman (28). Further, integrin expression disappears in differentiated ameloblasts, suggesting that other motifs in AMBN may be important for cell adhesion (18, 28, 29). The VTKG region is also known as a heparin binding domain. AMBN has positively charged Lys, Arg, and His amino acid-rich sequences in the middle and C-terminal regions (Fig. 3*A*), and KRH-rich motifs have been proposed as candidate heparin binding domains (17). The middle and C-terminal KRH-rich motifs in AMBN are conserved in human, rodent, and bovine cells, indicating that these domains may be important for cell binding. We found that full-length rat AMBN had a high affinity to heparin, whereas deletions of the heparin binding domains abrogated binding to heparin and resulted in reduced cell binding, indicating that VTKG and KRH-rich motifs serve as heparin and cell binding domains.

The EF-hand structure, which has a helix-loop-helix design, is the most common Ca^{2+}-binding motif (30). AMBN has an EF-hand motif in the C-terminal region, and proteolytic peptides from that region, particularly those migrating at 27 and 29 kDa, can be seen on SDS-PAGE assays of calcium binding. Previously, these peptides were identified in a direct ^{45}C-binding study (31) and by "Stain-all" solution, which is a detector of calcium-binding protein of enamel extract samples (32). In addition, a bioinfomatics model supports earlier experimental observations that AMBN is a bipolar,

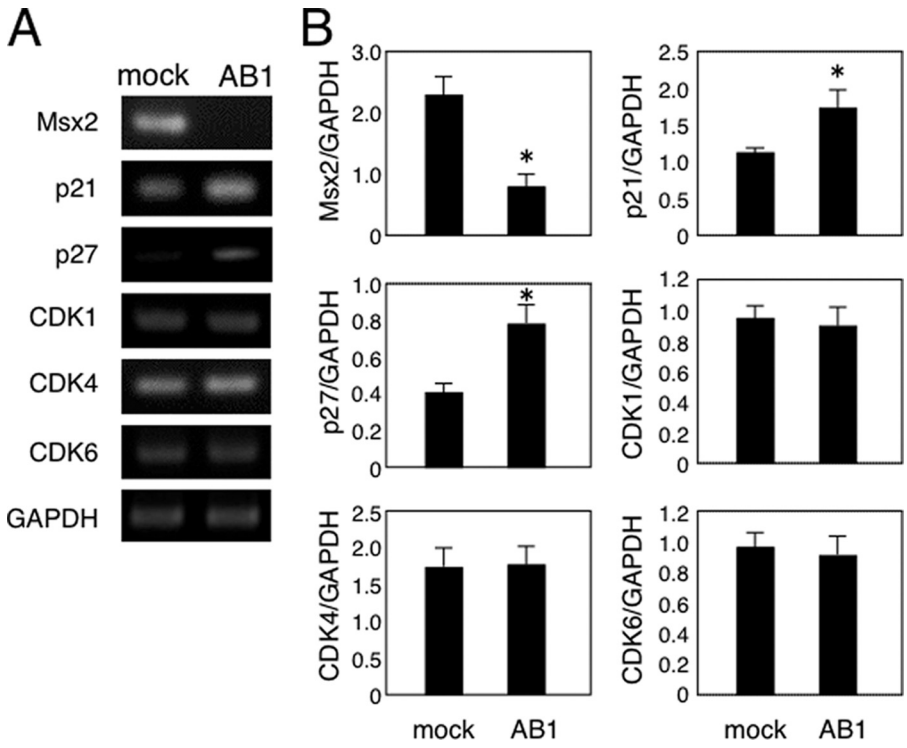

FIGURE 8. **Expression of cell cycle-regulatory genes in AMBN-overexpressing AM-1 cells.** *A*, total RNA from AM-1 cells transfected with a mock or AB1 expression vector was amplified using a semiquantitative reverse transcription-PCR method with specific primer sets. *B*, the intensity of each band was standardized with that of the glyceraldehyde-3-phosphate dehydrogenase band. *Msx2* expression was reduced, whereas expression of *p27* and *p21* was increased in AMBN-overexpressing cells. Statistical analysis was performed using analysis of variance (*, $p < 0.01$).

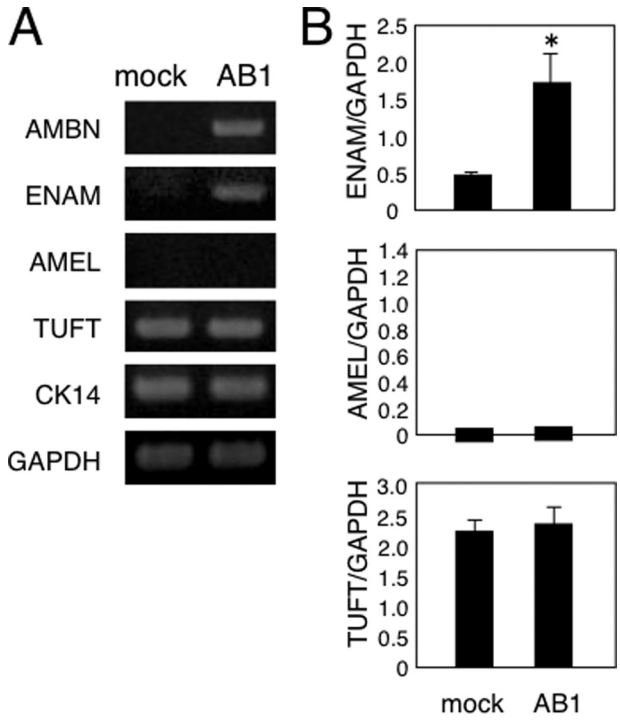

FIGURE 9. **Expression of enamel matrix proteins in AMBN-overexpressing AM-1 cells.** *A*, total RNA from AM-1 cells transfected with a mock or AB1 expression vector was amplified using a semiquantitative reverse transcription-PCR method with specific primer sets for tooth-specific genes. *B*, the intensity of the *AMBN*, *AMEL*, and *TUFT* bands was standardized with that of the glyceraldehyde-3-phosphate dehydrogenase band. *ENAM*, but not *AMEL*, was induced by AMBN overexpression. Statistical analysis was performed using analysis of variance (*, $p < 0.01$).

two-domain protein that interacts with Ca^{2+} ions. The primary structure of AMBN can be divided into two chemically and physically distinct regions: a basic N-terminal region and acidic C-terminal region. It has been speculated that the three-dimensional structure of AMBN is dramatically changed in water and in the presence of Ca^{2+} ions after molecular dynamics stimulation and energy optimization (19). Our findings agree with these reports, since AMBN heparin binding ability changed dramatically in the presence of EDTA, and this change was dependent on the EF-hand motif (Fig. 4, *C* and *D*). However, attachment of dental epithelium to AMBN did not increase in the presence of EDTA (Fig. 2), suggesting that other calcium-dependent molecules may be involved in cell binding. Further, in amelogenesis, AMBN is cleaved after secretion by several protease, including MMP-20 (33). The cleaved C-terminal half of AMBN is located near the cell surface of ameloblasts, and the N-terminal half of AMBN is present in the calcified front of enamel (34), indicating that the C-terminal region of AMBN may be important for cell binding *in vivo*. Recently, *AMBN* has been reported to appear in three different molecular sizes (37, 55, and 66 kDa) in both ameloblasts and enamel matrix during postnatal development (35). There may be various transcripts of *Ambn* that are developmentally expressed and interact with AMEL (16). Interestingly, 37-kDa AMBN containing three heparin binding domains is expressed in the early stage of ameloblast differentiation. These results indicate that the C-terminal region of AMBN is important for cell adhesion, ameloblast differentiation, and enamel nucleation.

Amelogenin and AMBN were shown to be expressed in not only ameloblasts but also odontoblasts (36). The surface of dentin has a layer of keratan sulfate rich in sulfated sialic acids and GlcNAc emanating from the dentinal tubules, which is a potent ligand for amelogenin (16). Ameloblastin may have a similar function with dentin matrix and odontoblast differentiation. Additional experiments are needed to resolve this issue.

Epithelial odontogenic tumors are histologically related to the remnants of odontogenic epithelium, which includes the dental lamina, enamel organ, and Hertwig's epithelial root sheath (37). Actively growing dental lamina is present within the jaws for a considerable time after birth, and because of the widespread presence of odontogenic epithelium, some tumors may arise from residues of those cells. AMBN is expressed by differentiated ameloblasts and also in forming Hertwig's epithelial root sheath cells and can be used as a marker of their migration (38). Previous immunohistochem-

ical studies have attempted to investigate the differentiation of neoplastic cells in odontogenic tumors; however, it was reported that AMBN, AMEL, and ENAM were not expressed in ameloblastoma cells (39, 40). *Ambn*-null mice develop odontogenic tumors of dental epithelium origin in addition to severe enamel hypoplasia. Further, because the ameloblasts disappear after eruption, tooth enamel is never replaced or repaired, and odontogenic epithelium almost completely disappears when tooth formation is completed in those mice. However, it is known that discrete clusters of odontogenic epithelial cells remain in the periodontal ligament as the epithelial cell rests of Malassez (ERM) (41). Although the function of ERM cells is still unclear, it is considered that a number of odontogenic tumors arise from them (41, 42). Recently, it was reported that ERM cells express AMBN but not AMEL or ENAM (43). It was also reported that *AMBN* gene mutations are associated with odontogenic tumors, including ameloblastomas (12, 44). In the study, we showed that AM-1 cells do not express AMBN, but overexpression of AMBN suppresses proliferation of AM-1 cells. Taken together, we speculate that AMBN functions as an odontogenic tumor suppressor.

Msx2, a homeobox-containing transcription factor, was previously shown to be expressed in undifferentiated ameloblasts, whereas it is down-regulated in differentiated ameloblasts (20). In *Ambn*-null ameloblasts, an abnormal up-regulation of Msx2 was observed (3), suggesting that AMBN inhibits the expression of Msx2 in normal tooth development. Our finding that AMBN transfection dramatically reduced Msx2 expression supports the notion that AMBN negatively regulates Msx2 expression. It has been suggested that Msx homeobox genes inhibit differentiation through up-regulation of cyclin D1 (45). In AMBN-transfected AM-1 cells, the cyclin-dependent kinase inhibitors *p21* and *p27* were up-regulated, whereas the expressions of *CDK1*, *-4*, and *-6* were not changed. Thus, down-regulation of *Msx2* and up-regulation of *p21* and *p27* by AMBN expression probably cause reduced proliferation of AM-1 cells. Further, the overexpression of AMBN lacking three heparin binding domains did not inhibit proliferation of AM-1 cells, suggesting the crucial role of the heparin binding domains of AMBN for the inhibition of AM-1 proliferation. It is conceivable that AMBN induces cellular signaling for these cellular changes by its interaction with AM-1 cells.

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