Tracking Endogenous Amelogenin and Ameloblastin In Vivo

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Abstract

Research on enamel matrix proteins (EMPs) is centered on understanding their role in enamel bio mineralization and their bioactivity for tissue engineering. While therapeutic application of EMPs has been widely documented, their expression and biological function in non-enamel tissues is unclear. Our first aim was to screen for amelogenin (AMELX) and ameloblastin (AMBN) gene expression in mandibular bones and soft tissues isolated from adult mice (15 weeks old). Using RT-PCR, we showed mRNA expression of AMELX and AMBN in mandibular alveolar and basal bones and, at low levels, in several soft tissues; eyes and ovaries were RNA-positive for AMELX and eyes, tongues and testicles for AMBN. Moreover, in mandibular tissues AMELX and AMBN mRNA levels varied according to two parameters: 1) ontogenic stage (decreasing with age), and 2) tissue-type (e.g. higher level in dental epithelial cells and alveolar bone when compared to basal bone and dental mesenchymal cells in 1 week old mice). In situ hybridization and immunohistodetection were performed in mandibular tissues using AMELX KO mice as controls. We identified AMELX-producing (RNA-positive) cells lining the adjacent alveolar bone and AMBN and AMELX proteins in the microenvironment surrounding EMPs-producing cells. Western blotting of proteins extracted by non-dissociative means revealed that AMELX and AMBN are not exclusive to mineralized matrix; they are present to some degree in a solubilized state in mandibular bone and presumably have some capacity to diffuse. Our data support the notion that AMELX and AMBN may function as growth factor-like molecules solubilized in the aqueous microenvironment. In jaws, they might play some role in bone physiology through autocrine/paracrine pathways, particularly during development and stress-induced remodeling.

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Introduction

The specific properties of mineralized tissues result from their unique extracellular matrix (ECM) composition. ECM has multiple effects on the biological behavior of skeletal cells and extracellular mineralization. As illustrated by the SIBLING family of proteins [1], ECM proteins not only provide template for ordered nucleation and crystal growth [2] but also control fate and activity of cells responsible for odontogenesis and cells regulating bone formation and turn-over. The organic matrix of bone, dentin and cementum is based on type I collagen associated with number of bone/tooth non-collagenous proteins [3]. In contrast, enamel is composed of specific enamel matrix proteins (EMPs) such as amelogenin (AMELX) and ameloblastin (AMBN). Contrary to bone, dentin or cementum ECM proteins, EMPs are ephemeral; after their secretion in enamel ECM and their aggregation into nanospheric structures, AMELX and AMBN are subject to proteolytic processing [4,5].

In recent years, EMPs have been identified in root epithelial cells [6] and non-enamel dental and bone cells [7–12]. Presence of EMPs RNA/proteins were also reported during early tooth development at the pre-mineralization stage [13] and in organs neither related to ectodermal appendages nor mineralized tissues, such as brain [14–16]. Based on these observations, AMELX [14] and AMBN [17] might be functional in non-enamel tissues.

EMPs exhibit cell signaling properties that impact on a wide range of cell activities. A commercially available enamel matrix derivative (EMD) is used for periodontal regeneration as well as epidermal wound healing [for review, [17]]. More specifically, using recombinant AMELX and AMBN and transgenic mice that
overexpressed EMPs and their splicing forms, previous studies have demonstrated that EMPs control cell adhesion, proliferation, polarity, commitment, differentiation and act on key-cellular pathways [18–22]. To date, nearly all the cells of dental-periodontal, epidermal and bone compartments have been found to respond to EMPs (for review, [23]). Transgenic mouse studies indicated that osteoblast and osteoclast cell activities are influenced by AMELX and AMBN [7,24,25]. Thus, an extensive number of investigations have documented in vitro and in vivo cell responses to under- or over-expression of EMPs, knockdown of EMPs, ectopic expression or addition of specific recombinants, synthetic peptides or EMD fractions. Herein we describe the endogenous expression of both AMELX and AMBN in mandibular bone and soft tissues. We also report the potential mobility and diffusibility of AMELX and AMBN in mandibular bone. This last point is an important consideration when ascribing growth factor-like or cell signaling attributes to AMELX and AMBN.

**Materials and Methods**

**Animals and Tissue Sampling**

The experimental animal protocol was reviewed and approved by the French Ministry of Agriculture for care and use of laboratory animals (B2 231010EA). All experiments were performed in accordance with the French National Consultative Bioethics Committee for Health and Life Science, following the ethical guidelines for animal care. All procedures related to AMELX KO and their Wild-Type (WT) littermates were reviewed and approved by The Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania (Protocol # 803067, “Enamel Mineral Formation during Murine Odontogenesis”).

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**Figure 1. AMBN and AMELX mRNA expression in mandible tissues from 1 and 15 week old WT mice.**

A. Illustration of the harvested zones in mandible. Alveolar bone (AB), basal bone (BB), dental epithelial cells (EP) and mesenchymal cells (ME) were microdissected under a stereomicroscope (red dotted lines). Soft tissues, erupted (root and crown) and unerupted (dental germ) molars were carefully removed. AB is composed of bone tissue surrounding extracted molars and, when molars are not erupted, of bone cavity surrounding molar tooth germs (Orange zone). BB is collected from the mandible angular process (Grey zone). Harvested zones of EP (Yellow zone) and ME (Dark blue zone) from the continuously erupting incisor are delimited by red dotted lines. B–C. Quantitative PCR reactions were performed on EP, ME, AB and BB tissues from 1 week and 15 week old WT mice with AMBN or AMELX primers (see primer sets in Material and Methods). mRNA expression levels were normalized against expression of the housekeeping gene GAPDH. Significant differences for each tissue between different stages (MW test) and between tissues at the same stage (KW test) are indicated on the graphs. Note that the apparent reduction in AMBN and AMELX mRNA expression in enamel epithelium in older mice might be due to increased proportion of maturation and post maturation stage ameloblasts in the enamel epithelium harvested from older mice.

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WT Swiss male mice (Janvier, St Berthevin, France) at 1, 8 and 15 weeks of age and 1 and 8 week old AMELX KO mice [26] were obtained.

As detailed in Fig. 1, alveolar and basal mandible bones and dental epithelial and mesenchymal cells from 1 and 15 week old WT mice were microdissected under a stereomicroscope (Leica MZ FLIII, Leica Microscopy Systems, Ltd., Heerbrugg, Switzerland). The molar alveolar bone (AB) was harvested after removal of the mandibular soft tissues and molars. The exfoliation of forming tooth germs and formed teeth was performed by careful observation under stereomicroscope and using miniaturized Gracey curette. Basal bone (BB) was collected from the mandible angular process in order to exclude both dental and cartilage tissues. The harvested bone tissues (AB and BB) were carefully rinsed in 1× Dulbecco’s phosphate-buffered saline (DPBS, Invitrogen, Carlsbad, CA, USA) to avoid soft tissue contamination. Dental epithelial cells (EP) and dental mesenchymal cells (ME) were dissected as previously described [27]. Briefly, continuously erupting incisors were carefully extracted and the dental epithelial cells (EP) were harvested by stripping the entire epithelium tissue off the incisor buccal surface, excluding the cervical loop to prevent contamination by dental stem cells (Fig. 1). Consequently, dental epithelial cells isolated from incisors of 1 week old mice were mainly composed of enamel-forming cells harvested from the secretion and maturation stages. Dental epithelial cells were similarly harvested from 15 week old mice but samples obtained from these older animals contained proportionally less tissue from the secretory stage as the proportion of epithelial cells present in the maturation stage and post maturation stage (i.e. protective reduced enamel epithelium) increases with age. Incisors were then cleaned and fractured to collect dental mesenchymal cells (ME) from dental pulp. Finally, a panel of non-mineralized tissues (eye, tongue, liver, heart, lung, kidney, colon, ovary, testicle and striated muscle) were dissected from 15 week old WT mice and washed in 1× DPBS. All quantitative experiments were performed in triplicate with at least three animals for each time point. After dissection, tissues were immediately frozen and stored at −80°C.

RNA Extraction and RT-qPCR

Tissues were mechanically ground to a fine powder in liquid nitrogen and RNA was extracted using the TriReagent kit (Euromedex, Soufflweyersheim, France), following the manufacturer’s instructions. In brief, total RNA was precipitated with isopropanol and centrifuged at 12,000 g at 4°C. Then, the RNA pellet was washed with 75% ethanol and resuspended in RNA-free water. The concentration and purity of total RNA in each sample were determined by the A260/A280 ratio. The integrity of RNA was confirmed by electrophoresis on an agarose ethidium bromide gel. One microgram of total RNA from each sample was reverse transcribed into cDNA using 200 units of Superscript II (Invitrogen), and 250 ng of random primers according to the manufacturer’s instructions. Real-time PCR reactions were performed using a MiniOpticon Real-Time PCR Detection System (Bio-Rad Laboratory, Hercules, CA, USA). According to manufacturer’s recommendations, a 15 μl volume containing 7.5 μl of IQ SYBR Green Supermix (Bio-Rad Laboratory), 50 ng of cDNA as template and 0.3 μM of the appropriate primer-pairs (Eurogentec, Liège, Belgium) was used. The final mixture was subjected to PCR under the following conditions: denatured at 98°C for 10 s, followed by 40 amplification cycles (98°C for 10 s, 60°C for 30 s, and 72°C for 20 s). AMBN and AMELX PCR amplified products were resolved on a 2% agarose gel and their specificity was confirmed by sequencing (GATCBio-tech, Mulhouse, France). mRNA levels of genes of interest were normalized against gene expression of the housekeeping gene GAPDH. The following specific primers sets were used: AMBN (F5′-agctgtgacaggagctg-3′/R5′-gctgacccagaaagcttg-3′), AMELX F5′-aagatctgagccataggct-3′/R5′-aagccttctgctaaatc-3′ and GAPDH (F5′-gacctcctcattgcctca-3′/R5′-aagttgctaggtgacccagaaagcttg-3′).

In situ hybridization using Amelogenin oligonucleotide probes

In situ hybridization using digoxigenin (DIG)-labeled oligonucleotidic probes (sequence - gagggttaggctagcataaaa - Exiqon, Vedbaek, Denmark) was performed as previously described [28]. Briefly, sections were deparaffinized using Cleararene solvent (Leica Microsystems, Nattemre, France) and rehydrated through ethanol solutions diluted in in situ hybridization buffer (0.1% Diethyl pycrocarbonate (DEPC, Sigma-Aldrich Co., St. Louis, MO, USA) in DPBS). After three washes in in situ hybridization buffer, sections were subjected to proteinase-K treatment in a humid chamber. After washing with saline-sodium citrate (SSC) buffers, sections were blocked using DIG blocking reagent in buffer containing 10% heat-inactivated sheep serum, and hybridization detected using alkaline phosphatase-conjugated anti-DIG, in conjunction with 4-nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) substrate (Roche, Mannheim, Germany) together with 2 mM Levamisole (Dako, Glostrup, Denmark). Sections were mounted with mounting resin (Eukit, Agar Scientific, Freiburg, Germany) and observed using a Leica DMRB microscope (Leica Microscopy Systems).

Immunohistochemistry (IHC)

Tissue Preparation. After dissection, mandibles were fixed for 24 h in 4% paraformaldehyde (PFA, Sigma-Aldrich Co.) in 1× DPBS (Invitrogen) and washed with 1× DPBS for 1 h. Samples isolated from mice older than one week (i.e. 8 and 15 week old mice) were then decalcified in buffered 4% ethylene diamine tetraacetic acid (EDTA) solution at 4°C (under agitation from 1 up to 15 weeks). Finally, the samples were dehydrated, cleared in xylene and embedded in paraffin. Then, 8–10 μm sections were cut.

Immunohistoperoxidase. After deparaffinization and rehydration, endogenous peroxidase was inactivated by treatment with 3% H2O2 (Merck, Darmstadt, Germany) in 1× DPBS for 15 min. Sections from 1 and 8 week old WT and AMELX KO mice were then rinsed in 1× DPBS and blocked overnight at 4°C with ready-to-use (2.5%) normal horse blocking reagent (ImmPRESS reagent kit, Vector Laboratories, Burlingame, CA, USA). Sections were then probed with primary anti-AMBN antibody (1/100) (sc 50534 (M-300), Santa Cruz Biotechnology - rabbit polyclonal IgG to AMBN - Immunogen: amino acids 108–407 mapping at the C-terminus of AMBN mouse origin - Reacts with mouse) and anti-AMELX antibody (1/100) (ab 59705, Abcam, Cambridge, MA, USA - rabbit polyclonal IgG to AMELX - Immunogen: purified full length native protein of AMELX cow origin – Reacts with mouse, rat and cow) for 1 h at room temperature. Tissue sections were washed three times with 1× DPBS for 5 min and incubated for 30 min with ImmPRESS reagent (ImmPRESS reagent anti-rabbit Ig Kit, Vector Laboratories, Burlingame, CA, USA). After three washes in DPBS 1X, immunohistochemistry was visualized using diaminobenzidine peroxidase substrate (Novared, Vector Laboratories). Sections were dehydrated and mounted in resin (Eukit, Agar Scientific, Freiburg, Germany). Sections with no primary antibodies were used as negative controls.
Samples were heated for 15 min at 70°C in Laemmli sample loading buffer containing 10% DTT (Bio-Rad, Philadelphia, PA, USA). Sections were mounted using an aqueous mounting medium followed by DAPI nuclear staining (1/100,000) (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature. Sections were rinsed 3 times for 5 min each under agitation. Nonspecific binding sites were blocked by 30 min incubation in 1% bovine serum albumin (BSA, Euromedex, Mundolsheim, France) diluted in DPBS. Sections were incubated overnight at 4°C with primary anti-AMBN antibody (1/200) (sc 50534 (M-300), Santa Cruz Biotechnology) and anti-AMELX antibody (1/200) (ab 59705, Abcam). After washes with 1× DPBS, Alexa Fluor 594 or 488 Goat Anti-Rabbit IgG antibodies (1/500) (Thermo Fisher Scientific Inc) were applied for 1 h at room temperature followed by DAPI nuclear staining (1/100,000) (Sigma-Aldrich Co.). Sections were mounted using an aqueous mounting medium (FluoPrep, Biomérieux, France).

Western Blot Analysis

To extract total proteins, mashed tissues from 1 and 15 week old WT mice were homogenized in 300 μL of commercial protein extraction reagent T-PER (Thermo Fisher Scientific Inc) which contains the detergent bicine. Samples were sonicated and tissue debris, removed by centrifugation for 5 min at 10,000 rpm. Supernatants were recovered and protein concentration determined using the BCA protein assay (Thermo Fisher Scientific Inc). Protein samples were thawed and heated 5 min at 100°C, before loading at 10 μg protein per well on a 10% polyacrylamide gel.

Immunohistofluorescence. After deparaffinization and rehydration, mandible tissue sections from 1 week old WT and AMELX KO mice were permeabilized for 10 min in 1% Triton X-100 (Thermo Fisher Scientific Inc, Waltham, MA, USA), then rinsed with 1× DPBS 3 times for 5 min each under agitation. Nonspecific binding sites were blocked by 30 min incubation in 1% bovine serum albumin (BSA, Euromedex, Mundolsheim, France) diluted in DPBS. Sections were incubated overnight at 4°C with primary anti-AMBN antibody (1/200) (sc 50534 (M-300), Santa Cruz Biotechnology) and anti-AMELX antibody (1/200) (ab 59705, Abcam). After washes with 1× DPBS, Alexa Fluor 594 or 488 Goat Anti-Rabbit IgG antibodies (1/500) (Thermo Fisher Scientific Inc) were applied for 1 h at room temperature followed by DAPI nuclear staining (1/100,000) (Sigma-Aldrich Co.). Sections were mounted using an aqueous mounting medium (FluoPrep, Biomérieux, France).

Statistical analysis

Data were presented with values expressed as the mean ± standard error of the mean (m ± SEM). Statistical analyses of the Q-PCR data were performed using Prism 5 statistical software (GraphPad Software Inc., San Diego, CA). Non-parametric two-tailed tests were used to compare EMP expression; Mann-Whitney (MW) test to investigate the EMP expression in each tissue between different stages (1 week vs 15 week old mice) and Kruskal-Wallis (KW) test to compare the difference between tissues (EP, ME, AB, BB) at one specific stage. The overall risk was fixed at p<0.05.
Results

**AMBN and AMELX RNA expression in mineralized and non-mineralized tissues**

Screening of AMBN and AMELX gene expression in murine tissues revealed the expression of transcripts coding for matrix enamel proteins in tooth, bone and soft tissues (Fig. 1 and Table 1). Quantitative analysis of AMBN and AMELX mRNA expression in the mandible tissues from 1 week old and 15 week old mice was performed using RT-qPCR (Fig. 1). In mice of both age groups AMBN and AMELX mRNA expression was not only detected in dental epithelial cells (EP) and mesenchymal cells (ME) but also in alveolar bone (AB) and basal bone (BB) (Fig. 1B-C). Overall, AMBN and AMELX genes had similar expression patterns, with RNA levels highly dependent on the investigated tissue and the age of the donor mouse. In 1 week old WT mice, AMBN and AMELX mRNA levels were significantly enhanced in EP when compared to ME and BB (p < 0.05–0.001), whereas mRNA levels in EP and in AB remained comparable (NS). In 15 week old mice, however, AMBN and AMELX mRNA levels were significantly higher in EP when compared to the three other tissues (p < 0.05–0.001). Finally, when compared to 1 week old mice, AMBN and AMELX mRNA expression was significantly decreased in 15 week old mice in all investigated tissues (p < 0.01), except in BB where mRNA levels remained comparable (NS). A series of non-mineralized extra-dental tissues from adult WT mice (15 week old) were also screened for AMBN and AMELX mRNA expression. Soft tissues were dissected and RNA was prepared for RT-PCR. Dental epithelial cells (EP) harvested from incisors were used as positive control tissue. RT-PCR results for these tissues are listed in Table 1. It is important to note that, when detected in soft tissues, AMBN and AMELX showed low mRNA levels when compared to mandibular tissues. Among the 10 soft tissues screened, only liver, lung and striated muscle showed no AMBN and AMELX mRNA expression; identifying these tissues...
as reliable negative controls for expression of these two EMP genes. Based on these results, striated muscle tissue was subsequently used as negative control tissue for both AMELX and AMBN gene expression in in situ hybridization and immunohistochemistry (IHC) performed on jaws.

Spatiotemporal localization of AMBN and AMELX

Based on RT-PCR data (Fig. 1 and Table 1) and a range of primary antibody dilutions tested against sections of mandible containing EMPs-expressing ameloblasts (positive control tissue), jaw bones and striated muscle (negative biological control, as evidenced by RT-PCR described above - Table 1), we determined two dilution thresholds for detection of EMPs; one for enamel and one for bone (Fig. S1). Additionally, to avoid non-specific staining we used a “triple negative-control system” including: 1- sections of dental epithelium where primary antibody was omitted (data not shown); 2- mandibular sections from WT mice including striated muscle, and, for anti-AMELX IHC, 3- mandibular sections from AMELX KO mice. AMELX labelling in AMELX KO tissues and in striated muscle (Orange box) from WT mice was negative (Fig. 2, Fig. 3, Fig. S1 and Fig. S2).

In contrast to the age-dependent differential gene expression revealed by RT-qPCR (Fig. 1), the distribution pattern of AMELX and AMBN was identical at all studied ages in the mandibular tissues surrounding the incisor. In situ hybridization in mandible confirmed AMELX mRNA expression in ameloblasts (positive control tissue) and, with lower intensity, in the alveolar bone area; where bone-lining cells showed AMELX mRNA expression.
AMELX protein was visualized using IHC and showed similar but more diffuse localization pattern when compared to AMELX mRNA distribution (Fig. 2D). No RNA nor protein signal was observed in mandible tissues from 8 week old AMELX KO mice (Fig. 2A–C).

AMBN and AMELX protein distribution in mandibular sections from 1 week old WT and AMELX KO mice was further investigated using immunohistofluorescence staining (Fig. 3-Fig. S2). Besides their presence within enamel matrix, both AMBN and AMELX proteins were detected in mandibular bone-lining cells (Fig. 3 A–B, white arrows, Fig. 3C, inset blue box) and in dental follicle (DF) (Fig. 3A-B-C, inset yellow box) in WT mice. Additionally, a slight diffuse expression of AMELX was detected in the periosteum (Po) and bone (Bo) areas (Fig. 3B and Fig. 3C).

Western blot detection of AMELX and AMBN in mandibular bone

To analyze the protein expression profile of EMPs in alveolar (AB) and basal bones (BB), we first studied total protein extracts from 15 week old mice using a detergent-based dissociative extraction procedure. Dental epithelium cells (EP) were used as positive controls. Extracted proteins were separated by SDS PAGE (10 μg total protein per well) and transferred to membranes for western blotting. Anti-AMBN immunostaining of the western-blot (Fig. 4A) revealed the presence of the nascent AMBN molecule at 67 kDa and a range of lower molecular weight processing products in dissociative extracts of dental epithelial cells. A similar molecular weight profile was reported for ameloblastin extracted from rat incisor enamel organ [30]. The nascent 67 kDa ameloblastin was also detected in dissociative extracts of alveolar bone (AB) with lower molecular weight processing products. AMBN immuno-cross reactivity in basal bone (BB) was much weaker and was near the limit of detection. Anti-AMBN immunostaining of blots of non-dissociative extracts of EP, AB and BB (Fig. 4B) revealed a spectrum of AMBN staining similar to the dissociative extracts. Anti-AMELX immune staining of dissociative extracts showed the presence of multiple AMELX proteins migrating below 26 kDa in the enamel epithelium (Fig. 4C). The additional lower weight AMELX components may be AMELX processing products from enamel matrix present as a contaminant in dental epithelium cell extracts. The identity of the stained bands migrating at 43 kDa and above is unclear though amelogenin is known to form high molecular weight complexes that are stable during SDS PAGE [13,31]. Dissociative extracts of AB and BB exhibited a single band of amelogenin cross-reactivity at 26 kDa though at levels near the limits of detection. Anti-AMELX immune staining of non-dissociative extracts showed the presence of AMELX as a doublet at around 26 kDa in enamel epithelium while alveolar bone exhibited a single band of amelogenin cross-reactivity at 26 kDa. A similar band was present in the basal bone extract but at such low levels that it was only visible after digitally enhancing the image (not shown).

Discussion

To the best of our knowledge, no previous studies have quantified gene expression levels and examined the solubility states of endogenously expressed EMPs in mandible bones using dental epithelial cells as a reference in rodents. Using a similar strategy, we previously demonstrated the regulation of EMPs gene expression by nuclear effectors in vivo in dental [11,27,32] and bone tissues [33,34]. Pioneer reports described AMELX [12,35] and AMBN [36] in non-enamel mineralized tissues. Here we confirm and extend these data; dental epithelial cells and alveolar

Figure 4. Western blot of AMBN and AMELX in 15 week old WT mice. Proteins were extracted from dental epithelial cells (EP) (positive control), alveolar bone (AB), basal bone (BB) under dissociative and non-dissociative conditions. Proteins were loaded at 10 μg per lane and blots probed with anti-AMBN and anti-AMELX antibodies. A. Anti-AMBN probing of dissociative extracts show cross reactive species with molecular weights ranging from 20–67 kDa for AMBN (the 67 kDa band corresponds to nascent ameloblastin). 67 kDa AMBN is present at similar relative amounts in the EP and AB extracts but far less readily detectable in BB. B. A similar situation exists for non-dissociative extracts. C. Anti-AMELX probing of dissociative extracts show cross reactive species at 26 kDa and below in EP samples (higher molecular weight staining may be due to AMELX aggregation). Feint cross reactivity at ~25 kDa is visible in AB samples with even less been detected in BB; the relative amount of AMELX present in bone samples is far less than that seen in EP samples. D. A similar situation exists for non-dissociative extracts; AMELX is detectable in AB but it is present in the extract in much lower amounts compared to EP extracts.

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bone displayed higher levels of AMELX and AMBN mRNA expression when compared to basal bone and dental mesenchymal cells from post-natal mice (one week old).

By screening AMELX and AMBN gene expression in 10 soft tissues, we were able to identify the lung, liver and striated muscle as negative control tissues for in situ studies. In line with previous studies [15,16], repeated or occasional mRNA expression of AMBN and AMELX was detected in several soft tissues but at low level when compared to mandibular tissues. This occasional expression could result from endogenous oscillations of EMPs gene expression orchestrated by clock genes; both AMELX and enamelin being downstream targets of the “clock genes” family of transcription factors [37,38]. Alternatively, these variations may result from the existence of EMPs-positive circulating cells (e.g. macrophages, megakaryocytes and some hematopoietic stem cells [15]).

Our RT-qPCR and western-blot results show that AMELX and AMBN expression is higher in alveolar bone compared to basal bone. This finding raises the possibility that AMELX and AMBN are locally associated with high bone turn-over; this being a classic characteristic of alveolar bone [39]. Indeed, AMELX [25] and AMBN [40] have been shown to impact on osteoblast and osteoclast differentiation and activity. Consistently and in line with previous studies [40], aging was associated with decreased gene expression of EMPs in jaw bones. Recently, increased expression of AMBN was also shown to be associated with mechanical bone stimulation [41] and healing [42]. Interestingly, MSX2, a regulator of bone homeostasis [33] represses AMELX transcription by targeting the AMELX promoter [43] and both AMELX and AMBN inhibit Msx2 expression [44,45]. EMPs-Msx2 reciprocal cross-talk may thus play a part in localized jaw modeling as supported by Msc2 and AMBN null-mutants [34,44] and our preliminary biomechanical assay (Fig. S3). Indeed, 72 h after altering occlusion, we observed a significant increase in AMBN and AMELX mRNA expression associated with decrease in Msc2 gene expression.

Protein occupies 20–30% of the secretory stage enamel matrix by volume and of this protein 90% is derived from the AMELX gene whereas AMBN is present in the matrix at far lower concentrations. AMELX is highly aggregative under physiological conditions and acts as a structural scaffold during enamel development. Nascent AMBN is solubilized in the developing enamel matrix and is a mobile species. In contrast, AMBN processing products are more aggregative [29]. Identifying solubilized and diffusible proteins in tissues using histology-based techniques may be compromised by the fact that otherwise soluble factors become immobilized by chemical fixation. Here AMELX and AMBN proteins were detectable in the microenvironment surrounding EMPs-producing (RNA-positive) cells. In addition, high protein levels detected in the dental follicle suggest that dental and bone cells may secrete these peptides toward this adjacent tissue. If EMPs function in some capacity as signaling molecules [12] then endogenously secreted EMPs would require a certain degree of solubility in the extracellular environment in order for them to diffuse and interact with nearby cells (paracrine signaling) or receptors on the secreting cell itself (autocrine signaling) (Fig. 5).

To test this hypothesis, we extracted dental epithelial cells (EP), alveolar bone and basal bone under dissociative and non-dissociative conditions and subjected equal amounts of total protein extracted to western blot probing for AMBN and

Figure 5. Proposed model for EMPs expression and signaling in extra-dental tissues. This model is based on the presently described RNA and protein patterns and published data. EMP-based autocrine and paracrine cell-cell communications would participate to distinct physiopathological events. They may play a role during tooth growth and alveolar bone modeling processes. In adults, EMPs would be involved in alveolar bone responses to mechanical stimuli. Supramolecular structures generated by self-assembly of EMPs might also intervene in these processes. Bibliographical references. 1. Haze, 2009 [8]; Tamburstuen, 2011 [10], 2. Zeichner-David, 2006 [57]; Fukae, 2006 [58]; Matsuzawa, 2009 [59]; Tamburstuen, 2010 [42]; Kakegawa, 2010 [60]; Zhang, 2011 [54]; Kitagawa, 2011 [53]; Kunimatsu, 2011 [61]; Izumikawa, 2012 [62]; Amin, 2013 [63], 3. Spahr, 2006 [36]; Haze, 2009 [8]; Iizuka, 2011 [18]; Tamburstuen, 2011 [10]; Atsawasuwan, 2013 [40], 4. Matsuzawa, 2009 [59]; Iizuka, 2011 [18]; Tamburstuen, 2010 [42]; Amin, 2013 [64]; Atsawasuwan, 2013 [24], 5. Haze, 2009 [8]; Tamburstuen, 2011 [10], 6. Tamburstuen, 2010 [42]; Lu, 2013 [19], 7. Haze, 2009 [8,20], 8. Saito, 2008; Wang, 2005 [48], 9. Wang, 2005 [48], 10. Beleyer, 2010 [47], 11. Fincham, 1994 [65]; Wald, 2013 [66]. doi:10.1371/journal.pone.0099626.g005
AMELX. We provide evidence that AMBN, particularly the nascent 67 kDa molecule, is readily detectable on blots of dissociative extracts of alveolar bone; and it is present in these extracts at a similar ratio relative to other components as found in EP. The ratio of freely soluble 67 kDa AMBN in the non-dissociative extracts is also comparable to the ratio found in EP; i.e., to some degree, AMBN is present in alveolar bone in a solubilized state; or at least it is not tightly associated with the bone matrix. AMELX was also detectable in blots of non-dissociative extracts of alveolar bone but it comprised a far smaller ratio of the dissociatively extracted proteins when compared to EP. Likewise, AMELX was detectable in non-dissociative extracts of alveolar bone but again at ratios far lower than the ratio of AMELX in EP.

We were unable to detect EMPs in serum (data not shown), which suggests that, contrary to osteocalcin that behaves as hormonal signal in general metabolism [46], EMPs might not play a role in long-range endocrine signaling. Some matrix ligands, omnipresent in mesenchymal extracellular compartments, could perhaps entrap these secreted EMPs and limit their diffusion. Indeed, fibronectin [47], heparan sulfate [20] and biglycan [48] bind AMELX or AMBN in vitro. However, the inability to detect EMPs in serum may simply be due to dilution of the bone-derived EMP peptides once they enter the blood stream.

Autocrine and paracrine activities of growth factors control local bone modeling and remodeling. Solubilized EMPs may behave in the same way (Fig. 5). Indeed, distinct dysmorphologies characterize bones in which AMELX [16,49] or AMBN [24,50] expression are genetically modified, even though calcium and phosphorus content and the degree of mineralization are normal [51,52]. Disturbed EMP expression patterns are associated with alveolar and jaw bone malformations [33,34]. These findings and the present data suggest that solubilized EMPs may play a role in controlling cell fate through short-distance paracrine and autocrine functions and thus control local bone morphology via signals previously evidenced for AMBN [19,53,54] and AMELX [55,56]. We thus propose a model in which EMPs play a role in mandibular physiology (Fig. 5).

Conclusion

Based on our findings on the solubilized state and the localization of AMELX and AMBN in mandibular bones, we hypothesize that these EMPs play a dual role: one as components of a structural extracellular matrix in developing enamel and second as growth factor-like molecules in bones responsible for supporting the teeth in the mandible.

Supporting Information

Figure S1 Optimization of AMBN and AMELX immunodetection in 1 week old WT and AMELX KO mice. A-B. When using a classic dilution of antibody (1/1,000), cross reactivity to AMELX and AMBN proteins is restricted to the dental epithelial cells (EP) (positive control tissue) in WT mouse, a pattern well documented in the literature. Dilutions of 1/500 show the presence of AMBN and AMELX protein expression in EP, but also in some bone stromal cells. Increased concentration of antibody (1/100 for AMBN and 1/50 for AMELX) results in stronger staining in osteoblasts, osteocytes and in mandibular bone matrix. At dilution 1/100 (AMBN) or 1/50 (AMELX), striated muscle (negative control tissue) shows diffuse staining indicating the limit of specificity for these antibodies. At dilution 1/10, the striated muscle was clearly stained for both AMBN and AMELX, determining the non-specificity threshold. This threshold was confirmed using AMELX KO mouse as a bona fide control, non-specific cross reactivity signal being observed when using 1/10 antibody dilution. For detailed immunohistoperoxidase methods see File S1.

Figure S2 AMELX protein expression in 1 week old WT and AMELX KO mice. A. When using 1/500 antibody dilution, AMELX (green staining) is not detected in AMELX KO mice. B. Using the same antibody dilution, WT section show anti-AMELX cross reactivity in enamel (En) and ameloblasts (Ambl).

Figure S3 Impact of mechanical stimuli on AMBN and AMELX mRNA expression in jaw. A. Right maxillary molar cusps in 15 week old WT mice were ground flat to the level indicated by the red dotted line. B. 72 h after grinding, significant increase in mRNA levels of AMELX (x7) and AMBN (x3) is observed in treated mandibular alveolar bone. Concurrently, a significant reduction in MSX2 mRNA level (x3) is observed; MSX2 being a transcriptional repressor of AMELX gene. mRNA expression of those genes are unaffected in basal bone (data not shown). mRNA levels of genes of interest are normalized against nuclear gene expression of GAPDH. This study represents part of the dissertation of Jaime Jacques’s PhD degree at Paris Diderot University – Paris 7, France.

File S1 Detailed procedures.

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Author Contributions

Conceived and designed the experiments: JJ DH MDM SJB AB JI. Performed the experiments: JJ DH MDM SP AA JI. Analyzed the data: JJ DH SP ABK CWG SJB AB JI. Contributed reagents/materials/analysis tools: ABK CWG. Wrote the paper: JJ SJB AB JI.

References

1. Staines KA, Macrae VE, Farquharson C (2012) The importance of the SIBLING family of proteins on skeletal mineralisation and bone remodelling. J Endocrinol 214: 241–255.
2. George A, Veis A (2008) Phosphorylated proteins and control over apatite nucleation, crystal growth, and inhibition. Chem Rev 108: 4670–4693.
3. Kawasaki K, Buchanan AV, Weiss KM (2009) Biominalerization in humans: making the hard choices in life. Annu Rev Genet 43: 119–142.
4. Iwata T, Yamakoshi Y, Hu JC, Ishikawa I, Bartlett JD, et al. (2007) Processing of ameloblastin by MMP-20. J Dent Res 86: 153–157.
5. Bartlett JD (2013) Dental Enamel Development: Proteinases and Their Enamel Matrix Substrates. ISRN Dent 2013: 684607.
6. Boushardt DD (2008) Biological mediators and periodontal regeneration: a review of enamel matrix proteins at the cellular and molecular levels. J Clin Periodontol 35: 87–105.
7. Hatakeyama J, Philp D, Hatakeyama Y, Haruyama N, Shum L, et al. (2006) Amelogenin-mediated regulation of osteoclastogenesis, and periodontal cell proliferation and migration. J Dent Res 85: 144–149.
35. Haze A, Taylor AL, Blumenfeld A, Rosenfeld E, Leiser Y, et al. (2007) Amelogenin expression in long bone and cartilage cells and in bone marrow progenitor cells. Anat Rec (Hoboken) 290: 453–460.
36. Spur A, Lyngstadta SP, Slaby I, Peshkii G (2006) Ameloblastin expression during craniofacial bone formation in rats. Eur J Oral Sci 114: 504–511.
37. Athanasiou-Paphitiemmon M, Kim D, Harbron L, Papagarikas S, Schnell S, et al. Molecular and circadian controls of ameloblasts. Eur J Oral Sci 119 Suppl 1: 33–40.
38. Zheng L, Sun Y, Mourao MA, Schnell S, Kim D et al. Circadian rhythms regulate amelogenesis. Bone 55: 158–165.
39. Schol R, Mckee MD (2000) Molecular and cellular biology of alveolar bone. Periodontol 2000 24: 99–126.
40. Aikah M, Khoe E, Aiyami B, Rapis M, Salgnevo JM, et al. (2012) Osteogenic effect of high-frequency acceleration on alveolar bone. J Dent Res 91: 413–419.
41. Tambrustuen MV, Repp F, Spur A, Salhuenske A, Kvalheim G, et al. (2010) Ameloblastin promotes bone growth by enhancing proliferation of progenitor cells and by stimulating immunoregulators. Eur J Oral Sci 119 Suppl 1: 33–40.
42. Zhou YL, Slaad ML (2000) Identification of CCAAT/enhancer-binding protein alpha as a transactivator of the mouse amelogenin gene. J Biol Chem 275: 12273–12280.
43. Wang H, Tannukit S, Zhu D, Slaad ML, Paine ML (2005) Enamel matrix protein interactions. J Bone Miner Res 20: 1032–1040.
44. Coxon TL, Broch AH, Barron MJ, Smith RN (2012) Phenotype-genotype correlations in mouse models of amelogenesis imperfecta caused by Amel and Enam mutations. Cells Tissues Organs 199: 460–490.
45. Wazen RM, Mofatt P, Zalal SF, Yamada Y, Nanci A (2009) A mouse model expressing a truncated form of ameloblastin exhibits dental and junctional epithelial defects. Matrix Biol 28: 292–303.
46. Kurada S, Wazen R, Selli K, Tanaka E, Mofatt P, et al. (2011) Ameloblastin is not implicated in bone remodelling and repair. Eur Cell Mater 22: 56–66; discussion 66–57.
47. Smith CE, Wazen R, Hu V, Zalal SF, Nanci A, et al. (2009) Consequences for enamel development and mineralization resulting from loss of function of ameloblastin or enamlin. Eur J Oral Sci 117: 485–497.
48. Kitagawa M, Kitagawa S, Nagasaki A, Miyasuchi M, Uchida T, et al. (2011) Synthetic ameloblastin peptide stimulates differentiation of human periodontal ligament cells. Tissue Eng Part A 18: 1145–1152.
49. Zhang Y, Zhang X, Xu X, Atsawaspan P, Luan S (2011) Ameloblastin regulates cell attachment and proliferation through RhoA and p27. J Oral Sci 114 Suppl 1: 280–283.
50. Huang VG, Tanimoto K, Tanne Y, Kamiya T, Kitagawa M, et al. (2010) Effects of human full-length ameloblastin on the proliferation of human mesenchymal stem cells derived from bone marrow. Cell Tissue Res 342: 205–212.
51. Wen X, Cawthorn WP, MacDougald OA, Stupp SI, Snead ML, et al. (2011) The influence of Leucine-rich amelogenin peptide on MSC fate by inducing Wnt10b expression. Biomaterials 32: 6478–6486.
52. Zeidner-David M, Chen LS, Hau Z, Reyna J, Eaton J, et al. (2006) Amelogenin and ameloblastin show growth-factor-like activity in periodontal ligament cells. Eur J Oral Sci 114 Suppl 1: 244–253; discussion 254–261, 301–242.
53. Fukau M, Kanaizashi M, Nagano T, Tanabe T, Oida S, et al. (2006) Porcine sheath proteins show periodontal ligament regeneration activity. Eur J Oral Sci 114 Suppl 1: 212–218; discussion 254–261, 301–242.
54. Tsuchi T, Kitagawa M, Shuto T, Lee Y, Chen M, Li TF, et al. (2009) Putative signaling action of ameloblastin utilizes the Wnt/beta-catenin pathway. J Periodontal Res 44: 289–296.
55. Kakeda A, Oida S, Gomi K, Nagano T, Yamashita Y, et al. (2010) Cytodifferentiation activity of synthetic human enamel sheath protein peptides. J Periodontal Res 45: 643–649.
56. Kumimatsu R, Tanimoto K, Tanne Y, Kamiya T, Okhuma S, et al. (2011) Amelogenin enhances the proliferation of cementoblast lineage cells. J Periodontol 82: 1632–1638.
57. Izumikawa M, Hayashi K, Polan MA, Tanig T, Saito T (2012) Effects of ameloblastin on proliferation, differentiation, and mineralization of rat bone mesenchymal osteogenic stem cells in vitro. ScientificWorldJournal 2012: 879371.
58. Amin JD, Olsen I, Knowles JC, Dard M, Donas N (2013) Effects of enamel matrix proteins on multi-lineage differentiation of periodontal ligament cells in vitro. Acta Biomater 9: 4796–4805.
64. Amin HD, Olsen I, Knowles JC, Donos N (2012) Differential effect of amelogenin peptides on osteogenic differentiation in vitro: identification of possible new drugs for bone repair and regeneration. Tissue Eng Part A 18: 1193–1202.

65. Fincham AG, Moradian-Oldak J, Simmer JP, Sarte P, Lau EC, et al. (1994) Self-assembly of a recombinant amelogenin protein generates supramolecular structures. J Struct Biol 112: 103–109.

66. Wald T, Osickova A, Sulc M, Benada O, Semeradlova A, et al. (2013) Intrinsically disordered enamel matrix protein ameloblastin forms ribbonlike supramolecular structures via an N-terminal segment encoded by exon 5. J Biol Chem 288: 22333–22345.

67. Bartlett Jd, Skobe Z, Lee Dh, Wright Jt, Li Y, et al. (2006) A developmental comparison of matrix metalloproteinase-20 and amelogenin null mouse enamel. Eur J Oral Sci 114 Suppl 1: 18–23; discussion 39–41, 379.

68. Simmer JP, Hu CC, Lau EC, Sarte P, Slavkin HC, et al. (1994) Alternative splicing of the mouse amelogenin primary RNA transcript. Calcif Tissue Int 55: 302–310.

69. Li W, Mathews C, Gao C, DenBesten PK (1998) Identification of two additional exons at the 3’ end of the amelogenin gene. Arch Oral Biol 43: 497–504.

70. Lau EC, Simmer JP, Bringas P, Jr., Hsu DD, Hu CC, et al. (1992) Alternative splicing of the mouse amelogenin primary RNA transcript contributes to amelogenin heterogeneity. Biochem Biophys Res Commun 188: 1253–1260.