Ameloblastin as Biomarker of Bone

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Abstract

Regional bone diversity has major public health implications. This is exemplified by the tissue incompatibility problems associated with bone ectopic autografts or the puzzling jaw osteonecrosis induced by antiresorptive agents that are otherwise effective in treating long-bone osteoporosis or metastatic resorptive lesions. Identifying bone site-specific biomarkers is therefore essential, firstly to determine why or how bone cell phenotypes vary depending on the anatomical site and secondly to implement new bone site-specific therapeutics. The present chapter summarizes findings on site-specific bone cell profiles and highlights ameloblastin (AMBN) as an exemplary peptide for jaw bone site-specificity. AMBN was originally discovered in tooth enamel matrix, extracts of which have been successfully applied clinically for regeneration of mineralized tissue. AMBN has also been detected outside the enamel in both mineralized and nonmineralized tissues. In bone, functional studies have demonstrated crucial functions of AMBN in the control of bone balance, notably processes associated with a high bone remodeling rate. In contrast to appendicular and axial bones, jaw bones are highly affected by AMBN. For example, AMBN participates in the physiological control of alveolar bone integrity in response to tooth-associated biomechanical stimulation. Based on these observations, AMBN-based

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treatments have promising clinical potential for craniofacial tissue repair and more specifically for alveolar bone regeneration.

Keywords

Ameloblastin • Enamel • Osteoblast • Bone remodeling • Alveolar bone • Site-specificity • Bone regeneration

List of Abbreviations

ADAS	Adipose-derived adult stem cell
ALP	Alkaline phosphatase
AMBN	Ameloblastin
AMEL	Amelogenin
BMMC	Bone marrow-derived monocyte/macrophage cell
BMP	Bone morphogenetic protein
BMSC	Bone marrow stromal cell
BP	Bisphosphonate
BRONJ	BP-related osteonecrosis of the jaw
DF	Dental follicle
DFDBA	Demineralized freeze dried bone allograft
ECM	Extracellular matrix
EMD	Enamel matrix derivative
EP	Enamel protein
ERM	Epithelial rest of Malassez
ES	Embryonic stem
FDBA	Freeze dried bone allograft
HERS	Hertwig's epithelial root sheath
hOMSC	Human oral mucosa stem cell
HOX	Homeobox
IHC	Immunohistochemistry
ISH	In situ hybridization
IUP	Intrinsically unstructured protein
KLK4	Kallikrein 4
LCM	Laser capture microdissection
LNA	Lock nucleic acid
LRAP	Leucine rich amelogenin peptide
MMP	Metalloprotease
MSC	Mesenchymal stem cell
MSX	Muscle segment homeobox
NB	Northern blot
NBM	Natural bovine mineral
NHO	Normal human osteoblast
Ocn	Osteocalcin
Opg	Osteoprotegerin
Opn	Osteopontin
PBMC	Peripheral blood mononuclear cell

PDL	Periodontal ligament
PTH	Parathyroid hormone
RANK	Receptor activator of nuclear factor kappa b
RANKL	Receptor activator of nuclear factor kappa b ligand
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SCC	Squamous cell carcinoma
SCPP	Secretory calcium-binding phosphoprotein
Seq	Sequencing
SIBLING	Small integrin binding ligand N-linked glycoprotein
SPARC	Secreted protein, acidic, cysteine-rich
STAT	Signal transducer and activator of transcription
WB	Western blot

Key Facts of Ameloblastin

The following are the key facts of ameloblastin (AMBN) and provides a brief description of this protein, its expression and functions in enamel and dental tissues, its role in bone cells, its differential expression in bone compartments, its expression and functions in craniofacial bones, and its putative clinical application to alveolar bone regeneration.

- Ameloblastin (AMBN) is the second most abundant protein in the enamel matrix after amelogenin (AMEL) (~5–10% and 90% of organic matrix proteins for AMBN and AMEL, respectively).
- The gene encoding AMBN as well as many other genes involved in vertebrate skeletal mineralization form the secretory calcium-binding phosphoprotein (SCPP) gene cluster located on human chromosome 4q21 and murine chromosome 5q (for rat and mouse).
- In enamel, AMBN promotes the growth of a crystalline layer and regulates the matrix binding, proliferation, and differentiation of ameloblasts.
- Besides its expression in enamel, AMBN is expressed during early stages of embryogenesis and has been shown to affect a wide variety of cells in mineralized and soft tissues. This suggests that AMBN may act as a signaling molecule in various bodily processes, including epithelial–mesenchymal interactions.
- In bone, AMBN stimulates both osteogenesis and osteoclastogenesis.
- AMBN is considered as a biomarker of the craniofacial bones due to its relatively higher expression in jaw and skull bones compared to long bones.
- AMBN is almost exclusively expressed in bone compartments with neural crest-derived osteoblasts. This suggests that AMBN could be a specific marker of the embryonic origin of bone organs (neuroectoderm versus mesoderm-derived bones).

- AMBN is strongly expressed in jaw bone processes associated with high remodeling rates, such as bone formation, repair, and regeneration.
- AMBN is involved in biomechanical responsiveness of the alveolar bone, and AMBN and Msx2 interact in this process.
- The application of AMBN to mandibular bone defects has been shown to stimulate bone repair in vivo; AMBN-based treatments might therefore have promising clinical potentials for alveolar bone regeneration.

Definition of Words and Terms

Adenomatoid odontogenic tumor	Tumor originating from the enamel organ or dental lamina.
Ameloblastoma	Odontogenic tumor of the buccal cavity, derived from ameloblasts that have lost their ability to build enamel.
Amelogenesis	The process of enamel formation.
Blastema	A group/mass of cells able to grow and differentiate into organs or body parts. Blastemas are composed of undifferentiated pluripotent cells.
Calvaria	The part of the skull consisting of the frontal bone, occipital bone, temporal bone, parietal bones, and sphenoid.
Chondrogenesis	The process of cartilage formation.
LNA	Abbreviation for lock nucleic acid. LNA is a modified nucleic acid with a methyl bridge between 2'-O and 4'-C.
Odontogenic tumor	Refers to a neoplasm and tumor-like malformation originating from cells of the odontogenic apparatus.
Perichondrium	Layer of fibrous connective tissue that surrounds the developing bone cartilage.
Squamous odontogenic tumor	Any group of rare odontogenic tumors originating in alveolar bone.

Introduction

During the processes of bone growth, homeostasis, and healing, terminally differentiated mesenchymal stem cells known as osteoblasts regulate bone apposition and participate in bone remodeling by controlling osteoclast differentiation and activity. Driven by the master genes Runx2 and Osterix (Sp7), osteoblasts synthesize a panel of extracellular matrix (ECM) proteins controlling biomineralization as well as

various other proteins governing cell fate (e.g., WNTs/BMPs for osteoblast differentiation or RANK/RANKL for osteoclastogenesis). Although osteoblasts are traditionally considered to be a unique, differentiated mesenchymal entity, irrespective of their location in the skeleton, several studies have highlighted that major differences in bone physiopathology – specifically between long and craniofacial bones – are associated with bone site-specific osteoblast phenotypes.

For example, disparate hormonal sensitivities have been demonstrated in jaw versus long bones. Using transgenic mouse models, Liu et al. (2009) showed that the mandible is less sensitive to the anabolic action of increased endogenous parathyroid hormone (PTH) than are long bones, and that this differential sensitivity is associated with differential expression of PTH receptor and subsequent regulation of insulin growth factor-1 (IGF1) by PTH in osteoblasts. In addition, estrogen deficiency (modeled using ovariectomy) induced an extensive reduction in bone mineral density and deterioration of the trabecular structure in appendicular and axial bones while jaw and cranial bones showed no or only minor changes (Rawlinson et al. 2009a; Liu et al. 2015). Furthermore, when compared with the tibia, alveolar bone developed ovariectomy-induced bone loss at a later stage (Du et al. 2015).

The site-specificity of bone physiopathology is also exemplified by the localized side effects of certain drugs used to treat bone-destructive disorders, such as the deleterious effect of bisphosphonates (BPs) on jaw bones. Indeed, BPs have been shown to induce BP-related osteonecrosis of the jaw (BRONJ), while no adverse effects in long bones have been observed (Marx 2003). Interestingly, a recent study highlighted that the jaw-specific impairment of bone remodeling by BPs is associated with differential expression of markers of osteoblast activity, including Msx1, Dlx5, Bmp2, Rankl, and Osteopontin (Wehrhan et al. 2015).

Finally, this bone site-specificity is exemplified in the limitations of regional autograft procedures: grafts obtained from craniofacial donor sites (mandible, maxilla, calvaria) show superior therapeutic efficacy in bone regeneration, independently of the bone acceptor-site, compared to grafts obtained from iliac crest, femur, or rib donor sites (Zins and Whitaker 1983; Casey et al. 1995; Leucht et al. 2008). Furthermore, even when successfully incorporated within the jaw, the noncraniofacial grafts are resorbed with time, probably due to inappropriate communication between donor-site osteoblast/matrix and acceptor-site bone cells such as osteoclasts or endothelial cells. In line with these observations, both osteoblasts and bone marrow stromal cells isolated from alveolar bone (mandible or maxilla) and cranial bones show increased osteogenic capacities in vitro when compared to osteoblastic cells isolated from axial or appendicular bone (Kasperk et al. 1995; Leucht et al. 2008; Reichert et al. 2013).

Taken together, these observations demonstrate that osteoblast responsiveness under physiopathological circumstances is bone site-dependent and that osteoblast behavior should no longer be considered independently of the bone site physiologic environment. The mechanisms underlying the site-specific responsiveness of osteoblasts remain poorly understood. However, recent studies have identified factors that are involved in osteoblast site-specificity and highlight ameloblastin (AMBN) as a putative molecular marker of the jaw bones.

This chapter aims to provide an interdisciplinary state-of-the-art review on enamel peptides, with a focus on AMBN and its expression and functions in bone tissue. Because for years amelogenins and AMBN were considered to be exclusive to the tooth compartment, section "Part I" gives an overview of these proteins and their structural and regulatory roles in dental tissues. In the last decade, the detection of the enamel proteins has been expanded to other mineralized and nonmineralized tissues. In this context, section "Part II" provides a detailed description of AMBN expression, functions, and pathways beyond enamel. Section "Part III" focuses on AMBN expression in various bone compartments and bone cells. This part provides supportive data that AMBN is a bone site-specific marker, strongly involved in cranial and jaw bone formation and healing, and their biomechanical responsiveness. Finally, based on the data covered in this chapter, section "Part IV" introduces perspectives on the potential of AMBN-based therapies for orofacial bone regeneration.

Part I: Enamel Matrix and Enamel Proteins

Enamel Matrix, Amelogenesis, and Enamel Proteins

Dental enamel acts as a solid barrier that protects the inner layers of the tooth from masticatory impacts, bacterial invasion, and acid exposure. Enamel is the most mineralized tissue in the human body. Mature enamel is mostly composed of bioapatite crystals (97%) [$Ca_5(PO_4)_3(OH)$, with a high number of ion substitutions (e.g., Ca^{2+} by Sr^{2+} and PO_4^{3-} by CO_3^{2-} or F^-)]. The remainder consists of organic material. Because of its unique composition (hypermineralized, acellular, avascular, no innervation, and very low content of protein), and also its mechanical properties and ectodermal embryonic origin, enamel differs from other mineralized tissues like dentin, cementum, or bone. Finally, enamel is a distinctive tissue because its formation begins with a labile protein scaffold devoid of collagen, which is gradually replaced by highly organized crystalline architecture.

Amelogenesis

Enamel is one of the rare mineralized tissues in the human body that is deposited by epithelial cells. Amelogenesis – the process of enamel formation – is orchestrated by the ameloblasts, which are derived from the basal layer of the oral ectoderm.

Enamel formation per se consists of two steps: (1) secretion of a partially mineralized organic matrix by ameloblasts, and (2) degradation of this matrix temporally linked to termination of the mineralization process [reviewed in Nanci (2013), Lignon et al. (2015)]. Briefly, enamel formation begins at the bell stage of odontogenesis and involves the differentiation of cells of the inner enamel epithelium into polarized ameloblasts. The deposition of enamel matrix requires the functional differentiation of the ameloblasts into effector/secreting cells.

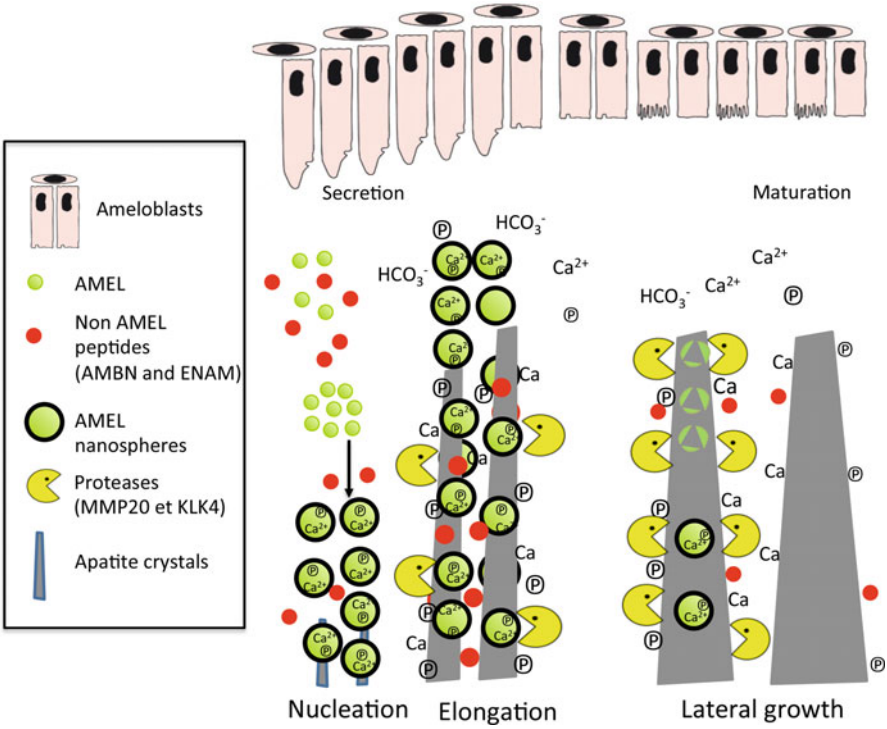

Fig. 1 Schematic representation of crystal growth during amelogenesis. Crystalline nucleation begins with the secretion of enamel peptides – mainly AMEL, AMBN, and ENAM – by ameloblasts at the secretion stage. AMEL self-assembles into nanospheres while the other enamel peptides remain as monomers. The AMEL nanosphere supramolecular structure promotes calcium phosphate formation by concentrating the charge at the nanosphere surface and acting as a nucleation template. At the crystal level, the nucleation step is followed by an elongation step. During this step, crystal elongation and inhibition of its lateral growth are controlled by the adsorption of enamel proteins onto the growing crystal. From the secretion stage to the end of the maturation stage, ameloblasts secrete proteases such as MMPs and KLK4 to degrade the enamel peptides; this results in crystal lateral growth. To counteract acidification of the medium secondary to the crystal growth during amelogenesis, bicarbonate ions are secreted to maintain pH

Amelogenesis is divided into three main sequential cell stages: presecretion/secretion, transition, and maturation (Fig. 1). During the secretion stage, the production and secretion of enamel proteins by the ameloblasts is maximal, while their protease production is limited. The resulting secreted enamel is composed of an organic matrix that interacts with calcium and phosphate to initiate bioapatite crystal formation. The longitudinal growth of crystals is controlled by a transient protein scaffold (Fig. 1). Differential orientation of these elongated crystals generates distinct areas: (1) the innermost and outermost layers called the “aprismatic” enamel and (2) the “prismatic” area of the bulk of enamel composed of intra-prismatic and inter-prismatic zones. Once the final thickness of the enamel is established, ameloblasts

undergo a morphological transition; they become shorter and reduce the volume of their organelles for preparation of the next stage, enamel maturation. This stage is characterized by decreased matrix protein secretion and increased protease synthesis and activity. These proteases [essentially kallikrein 4 (KLK4) and metalloprotease 20 (MMP)] are necessary to clear proteins from the enamel matrix. The matrix proteins are digested and removed, thereby allowing growth and densification of the bioapatite crystals (Hu et al. 2005). The mineral quantity increases from 35% in the secretion stage to 97% in the final mineralized matrix of mature enamel.

Of the matrix peptides (including matrix proteins and their enzymatic degradation products) produced by the ameloblasts during amelogenesis, this chapter focuses on the two main species, amelogenins and AMBN. The synchronized secretion of these enamel proteins is crucial for the formation of organized enamel crystals and the regulation of their growth.

Enamel Proteins and Their Structural Roles in Mineralizing Enamel

Amelogenins in enamel are secreted by ameloblasts and constitute ~90% of the organic matrix during enamel secretion [reviewed in Nanci (2013)]. They are members of a highly conserved protein family (produced by translation of at least 16 mRNAs). Two human amelogenin genes have been mapped to Xp22.1-p22.3 and Yp11.2 (AMELX and AMELY), with ~90% of the transcripts being expressed from the X chromosome. In experimental animal species, such as mouse and rat, only the X-chromosomal amelogenin gene is found, which will be referred to as "AMEL" in this chapter. In addition to the number of isoforms produced by alternative splicing of RNA, AMEL proteins are cleaved into different peptides that differentially regulate crystal growth. Amelogenins are the key proteins of enamel mineralization control: they aggregate into nanospheres that are reservoir of Ca^{2+} and PO_4^{3-} ions and participate in the regulation of crystal development. These nanospheres inhibit lateral crystal growth, guiding their tri-dimensional structure (Fig. 1). The quality of enamel (thickness, molecular organization) is conditioned by the amount and quality of amelogenins (Gibson et al. 2001; Molla et al. 2010).

AMBN is the second most abundant protein in enamel and represents ~5–10% of the organic matrix during enamel secretion. In the enamel matrix, AMBN is located at the boundary between inter- and intra-prismatic enamel crystals, mostly in the newly externally formed enamel. This protein initiates and regulates enamel mineralization and maintains the supracrystalline structure of enamel by inhibiting lateral crystal growth (Fig. 1). Using transgenic mouse models, Lu et al. showed that AMBN promotes the growth of a crystalline enamel layer with short and randomly oriented crystals but lacks the ability to facilitate the formation of mature oriented apatite crystals. This suggests that AMBN is not involved in enamel apatite crystal elongation (Lu et al. 2011). At the cellular level, AMBN was shown to regulate the matrix binding, proliferation, and differentiation of ameloblasts (Fukumoto

et al. 2004; Sonoda et al. 2009). The molecular structure of AMBN and its functions will be detailed in section "Part II."

The major roles of these two proteins in enamel structure are supported by clinical enamel phenotypes. Mutations in the human AMEL and AMBN genes lead to various forms of amelogenesis imperfecta (Lagerstrom et al. 1991; Poulter et al. 2014).

A First Overview of Enamel Protein Expression Outside the Enamel Tissue

For years, enamel proteins were considered to be exclusively produced by the ameloblasts and to specifically control enamel formation and biomineralization. However, more recently, their expression and functions once thought to be restricted to the ectodermal tooth compartment have now been extended to a second odontogenic compartment, the mesenchyme. This localization was definitively established for AMEL when two specific cDNAs comprising AMEL gene exons were cloned and sequenced from a rat tooth odontoblast pulp cDNA library (Veis et al. 2000). Enamel protein expression outside the enamel tissue is now widely documented in the literature. However, detection and quantification of enamel proteins in mineralized tissues is difficult with conventional techniques, and some authors still consider enamel proteins to be exclusive to enamel, with no extra-enamel distribution.

Many studies have demonstrated the presence of AMEL in dentin and dental ligament. For example, using immunohistochemistry (IHC), AMEL peptides were detected in the outermost layer of the dentin during the enamel presecretion stage (Inai et al. 1991). RNA studies have further demonstrated that AMEL is expressed by odontoblasts (Oida et al. 2002). In 2006, Ye et al. suggested that this latter expression is transitory during the first steps of dentinogenesis, and they showed that recombinant AMEL protein enhances pulp cell proliferation (Ye et al. 2006). Other studies have reported sequential AMEL expression during tooth root formation (Diekwisch 2001), both in cementoblasts (Nunez et al. 2010) and in Hertwig's epithelial root sheath (HERS) cells in response to inflammation (Fong and Hammarstrom 2000). In addition to these dental tissues, AMEL has been detected in many nondental tissues, including bone and soft tissues such as the eye, tongue, testis, heart, colon, ovary, and kidney (Deutsch et al. 2006; Li et al. 2006; Haze et al. 2007; Gruenbaum-Cohen et al. 2009; Jacques et al. 2014b) (Table 1).

Shortly after its discovery in secretory ameloblasts (Cerny et al. 1996; Krebsbach et al. 1996), AMBN was detected in dentin matrix, preodontoblasts, polarized odontoblasts, and pulp cells (Begue-Kirn et al. 1998; Fong et al. 1998). AMBN was also shown to be transitorily expressed during mineralization of the dentin outermost layer (Fong et al. 1998) and in trauma-induced reparative dentin (Spahr et al. 2002). AMBN was also detected in HERS cells (Fong and Hammarstrom 2000; Lu et al. 2013). Similar to AMEL, AMBN was identified in epithelial cell rests of

Table 1 The expression of ameloblastin and amelogenin in extra-dental tissues. This table reviews the literature reporting on the expression of ameloblastin and amelogenin in extra-dental tissues. *AMBN* ameloblastin, *AMEL* amelogenin, *ENAM* enamelin, *LRAP* leucine rich amelogenin peptide, *D* day, *E* Theiler sage, *M* month, *RT-qPCR* reverse transcriptase quantitative polymerase chain reaction, *IHC* immunohistochemistry, *ISH* in situ hybridization, *WB* Western blot, *NB* Northern blot, *Seq* sequencing, *MSC* mesenchymal stem cell, *BMSC* bone marrow stromal cell, *ADAS* adipose derived adult stem cell, *PBMC* peripheral blood mononuclear cell, *NHO* normal human osteoblasts, *Saos-2* sarcoma osteogenic cells, *ES* embryonic stem, *hOMSC* human oral mucosa stem cell, *LCM* laser capture microdissection

Reference	Model	Species/cells	Stage	Tissue	Evaluation method	Main conclusion
Deutsch D., 2006	In vitro	RAW 264.7 cells (mouse macrophages)			RT-PCR/seq	Identification of AMEL M180 transcript
	In vivo	Rat (Sabra) and dog	W5-6	Brain and long bones	RT-PCR/seq/ IHC	Identification and localization of AMEL transcripts (M180 and M194) in brain (glial cells) and in bone marrow cells
Spahr A., 2006	In vivo	Rat (Sprague Dawley)	E18, D2-28	Skull, vertebrae, mandible	RT-PCR/HIS/ IHC	Higher expression of AMBN in newly formed bone matrix during endochondral and intramembranous ossification. Decreased expression with aging
Li Y., 2006	In vivo	Mouse (C57Bl/6/ 129)	D2 and D4	Variety of hard and soft tissues	RT-PCR/seq	Identification of AMEL isoforms (LRAP and E4) and detection of AMEL in bones (cavarial and long bones) and in soft tissues (e.g., skin, eye, brain)
Haze A., 2007	In vitro	Human bone cells and bone marrow stromal cells			RT-PCR/seq	Identification of AMEL M180 transcript
	In vivo	Rat (Sabra), Dog	W5-10 (rat), M2 (dog)	Long bones	RT-PCR/seq/ WB/ISH/IHC	Identification of AMEL isoforms (LRAP) and detection in bone cells (osteoblasts, chondrocytes, osteoclasts) and bone marrow (mesenchymal stem cells)
				Bone marrow		Identification of AMEL isoform (10–15 kDa)
Müller W., 2007	In vitro	Saos-2 cells (human osteosarcoma cells)			NB	Detection of ARN coding for AMEL, AMBN, and ENAM

(*continued*)

Table 1 (continued)

Reference	Model	Species/cells	Stage	Tissue	Evaluation method	Main conclusion
Gruenbaum-Cohen Y., 2009	In vivo	Mouse	E10.5-17.5	Whole craniofacial complex	RT-PCR/seq/ IHC	Identification of two AMEL isoforms and detection in all the analyzed tissues. Expression of AMEL in brain and eye before tooth formation
Haze A., 2009	In vivo	Human	Fetus	Mandible	IHC	Higher expression of AMEL in preameloblasts compared to alveolar bone cells
		Dog (Beagle)	M13-16		IHC	High expression of AMEL in newly formed bone with high bone turnover and activity
		Rat (Sabra)	W5-10		ISH	High expression of LRAP in alveolar bone at the boundary of periodontal ligament (PDL)
Rawlinson SC., 2009	In vivo	Rat (CD) (100 g)		Parietal skull bone and ulnar limb	microarray	Higher mRNA levels of AMEL and AMBN in skull bone when compared to limb bone
Iizuka S., 2011	In vitro	Calvaria bone cells (Wistar rat fetuses)			RT-PCR	Higher levels of AMBN during proliferation stages compared to differentiation and maturation stages
		NOS-1, Saos-2, MG63, HOS cells			RT-PCR/IHC	Detection of AMBN in NOS-1 cells
	In vivo	Human		Samples of osteosarcoma	IHC	Expression of AMBN in osteosarcoma tissues
Tamburstuen M., 2011	In vitro	Human and mouse cells (MSC, BMSC, ADAS, PBMC, NHO, pulp and PDL cells, LS8, MC3T3-E1)			RT-PCR/seq	AMBN expression in human hematopoietic and mesenchymal cells, from calcified and noncalcified tissues
					Dot blot/WB	Expression in cell lysate and secretion in cell culture medium of AMBN protein in the tested cells
	In vivo	Rat (Sprague Dawley)	Adult	Ramus	IHC	AMBN expression in newly formed bone after mandibular ramus defect

Landin M., 2012	In vivo	Mouse (CD1)	E12.5-P2	Whole craniofacial complex	ISH	AMBN and ENAM mRNA expression in forming facial bone structures, adjacent to molars
Alikhani M., 2012	In vivo	Rat (Sprague Dawley)	D120	Maxillar bone	RT-qPCR/ Biomechanical assay	Increased mRNA expression of AMBN and ENAM in alveolar bone after high-frequency acceleration
Treves-Manusevitz S., 2013	In vivo	Implantation of human cells from oral gingiva and mucosa (hOMSC) in SCID mice			IHC	Detection of AMEL and AMBN proteins in hOMSC 8 weeks after implantation in mice and in the newly acellular mineralized matrix formed by hOMSC
		Sections obtained from human periodontal tissues			IHC	Detection of AMEL and AMBN proteins in human cementum and PDL tissues
Atsawasuwan P., 2013	In vivo	Mouse (CD1)	D1-20	Calvarial vault and sutures	RT-qPCR/IHC/ WB	AMBN mRNA detection in sutures. Detection of AMBN protein in bone extracts and localization in calvarial bone matrix, dura mater, and adjacent mesenchyme
	In vitro	Skull suture mesenchymal cells (mouse)			RT-qPCR/WB	AMBN expression in suture mesenchymal cells in vitro
Atsawasuwan P., 2013	In vivo	Mouse (CD1)	D3-35	Calvarial vault	RT-qPCR/IHC/ WB	Detection of AMEL, AMBN, and ENAM in calvarial tissues at lower levels compared to teeth. Variation of expression in calvaria with developmental stages
	In vitro	MC3T3-E1 cells/primary calvarial osteoblasts (mouse)			RT-qPCR	Variation of expression of AMEL, AMBN, and ENAM in cultured bone cells during osteoblastic differentiation and matrix mineralization stages

(*continued*)

Table 1 (continued)

Reference	Model	Species/cells	Stage	Tissue	Evaluation method	Main conclusion
Jacques J., 2014	In vivo	Mouse (CD1)	E10.5	Whole body and head	RT-qPCR	Detection of AMBN mRNA before tooth formation in embryos, with higher expression in body compared to head
			D3 and D105	Mandible, tibia, occipital, and frontal bone	RT-qPCR/seq/ LCM/IHC	AMBN expression in both craniofacial and long bone. Variation of expression with anatomical site and ontogenic stage
Jacques J., 2014	In vivo	Mouse (CD1)	W1, W8, and W15	Dental, mandibular, and a variety of soft tissues	RT-qPCR/seq/ ISH	Detection of AMEL and AMBN in both epithelia and mesenchymal-derived tissues. Variation of expression with tissue type, anatomical region, and age
					IHC/WB	Expression of AMEL and AMBN proteins in mineralized matrix and in solubilized form in mandible. Variation of expression with anatomical region

Malassez (ERM), and this expression was increased in response to physiopathological events such as pulp inflammation (Hasegawa et al. 2003) or developmental disorders (Molla et al. 2010). AMBN expression was also detected in cementoblasts at the level of the root (Nunez et al. 2010). Together, these observations suggest that AMBN may have a signaling function in epithelial–mesenchymal interactions occurring during the formation of the tooth/parodontium complex and under physiopathological circumstances.

As seen in this first part, the history of these two enamel proteins is quite different. In contrast to AMEL, as soon as AMBN was discovered, it was detected outside the enamel, notably in mesenchymal cells. In addition, again in contrast to AMEL, AMBN is not involved in the elongation of the apatite crystals of enamel; rather, it acts mostly during the initial steps of crystal growth and regulates cell adhesion and differentiation. These observations therefore suggest that AMBN may act as both a cell adhesion molecule and a signaling factor and be involved in various bodily processes, including epithelial–mesenchymal interactions.

Part II: Ameloblastin

Ameloblastin Gene and Proteins

AMBN, the second most abundant protein in the enamel matrix, was discovered in 1996 (Cerny et al. 1996; Krebsbach et al. 1996). This occurred long after an AMEL cDNA had been cloned (Snead et al. 1983), consistent with a low amount of AMBN in enamel matrix (comprising ~5–10% of the organic matrix proteins, versus 90% for AMEL). AMBN, AMEL, and many genes involved in vertebrate skeletal mineralization (as the noncollagenous SIBLINGs family members [DSPP, DMP1, IBSP, MEPE, SPP1, etc.]) form the secretory calcium-binding phosphoprotein (SCPP) gene cluster. The SCPP genes are evolutionarily related as they all originate from duplications of a common ancestral SPARC gene. With the single exception of the genes encoding the amelogenins, which are located on the X and Y chromosomes (see section "Part I"), AMBN and the other SCPP genes form a cluster located on human chromosome 4q21 and murine chromosome 5q (for rat and mouse).

The AMBN gene contains 11 exons in rat, mouse, and pig, whereas the human AMBN gene consists of 13 exons. Two isoforms have been described for AMBN protein (Krebsbach et al. 1996). AMBN protein is first synthesized as a polypeptide of 44–55 kDa, and after glycosylation it has a molecular weight of 65 kDa; this form is considered as the native protein. In enamel, AMBN is first secreted by ameloblasts, then cleaved by enamel proteases (e.g., MMP20 and KLK4), resulting in two types of peptides: (1) basic peptides with an N-terminus of 13–17 kDa and (2) acidic peptides with a C-terminus of 27–29 kDa. The AMBN protein sequence features binding sites for calcium, heparin, CD63, fibronectin, and integrins (Beyeler et al. 2010; Zhang et al. 2011a). Based on bioinformatics analysis, AMBN is an

intrinsically unstructured protein (IUP); it lacks a fixed 3D structure and contains long disordered regions (Vymetal et al. 2008).

AMBN Expression

Besides its expression in dental tissues (detailed in section "Part I"), some studies have demonstrated that AMBN is also expressed in various body compartments (mineralized and nonmineralized) during embryonic development – AMBN is detected as early as E10.5 in both mouse body and head segments (Jacques et al. 2014a) – and after birth (Table 1).

In bone tissues, different patterns of AMBN expression were found in the processes of intramembranous and endochondral ossification during craniofacial bone formation at embryonic and early postnatal stages (Spahr et al. 2006; Landin et al. 2012). At E18, in intramembranous ossification (e.g., in alveolar bone of developing mandible, frontal, and parietal bones), AMBN RNA and protein were detected in the superficial layer of the condensed vascularized primitive connective tissue and in the cellular layer covering the surface of the newly formed woven bone (Spahr et al. 2006). A similar expression pattern was reported in calvaria by Atsawasuwan et al. (2013a). In endochondral ossification (e.g., in ethmoid, parts of the sphenoid, vertebra), AMBN was expressed within the ECM of the cartilage templates and in the perichondrium. Between postnatal days 2 and 28 the expression decreased markedly, concordant with the maturation of the bone, and was not detected after completion of bone remodeling (Spahr et al. 2006). AMBN RNA expression was also detected in postnatal cranial sutures (Atsawasuwan et al. 2013b). In line with Jacques et al. (2014a), unambiguous AMBN RNA expression was shown in alveolar bone using laser capture microdissection of mandible from 3-day-old mice (Fig. 2). At day 3, AMBN protein was detected in bone-lining cells and recently embedded osteoblasts/osteocytes of the mandibular alveolar bone (Fig. 3). AMBN RNA in adult mice showed a similar localization pattern (Fig. 4). In recent studies, AMBN RNA expression has been measured and compared in different bone compartments, including in craniofacial and long bones isolated from young and old mice (Jacques et al. 2014a, b). In line with studies on AMEL expression in mandible (Haze et al. 2009) and on AMBN expression in appendicular, axial, and cranial bones (Spahr et al. 2006; Atsawasuwan et al. 2013a), Jacques et al. showed that AMBN RNA quantity in jaw bones decreased with age, suggesting that AMBN expression tends to phase out during the differentiation of bone cells, a process which is reduced after completion of bone remodeling (Jacques et al. 2014b). In addition, using PCR analysis, the same group demonstrated that AMBN expression is not restricted to mineralized tissues; it is also expressed in several soft tissues, at a high level in dental follicle of the mouse incisor and at low levels in eye, tongue, and testis (Jacques et al. 2014b). Interestingly, using Western blot on proteins extracted by nondissociative means, Jacques et al. showed that AMBN in mandibular bone is present both in an aggregate form incorporated into

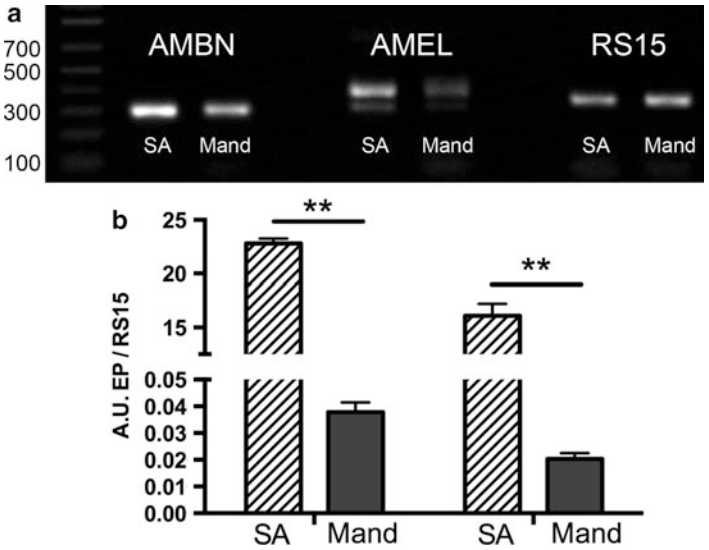

Fig. 2 AMBN and AMEL RNA expression in mandibular bone in 3-day-old mice. Mandibular bone (*Mand*) was isolated from 3-day-old mice by using laser capture microdissection and subjected to RT-qPCR to quantify AMBN and AMEL. Secreting ameloblasts (*SA*) were used as positive control. The expression levels of AMBN and AMEL were significantly higher in SA compared to mandible. The migration of the amplicon products (2% agarose gel) and their sequencing confirmed AMBN and AMEL RNA expression in SA and mandibular bone. RNA levels in these tissues were compared using RT-qPCR, which showed that expression of both AMBN and AMEL was significantly higher in SA compared to mandibular bone (≈600:1 and 700:1, respectively). The DNA molecular size marker is indicated on the side of the gels. RNA levels were normalized to the expression of the housekeeping gene RS15. Significance was evaluated using the Mann–Whitney test (*$p < 0.05$, **$p < 0.01$). *EP* enamel protein, *AU* arbitrary units

matrix and also in a solubilized state (Jacques et al. 2014b). These data support the notion that AMBN has some capacity to diffuse and may function as a growth factor-like molecule solubilized in the aqueous bone microenvironment.

In vitro, Iizuka et al. investigated the expression of AMBN during osteogenesis by RT-PCR using rat embryonic calvaria cells (Iizuka et al. 2011). AMBN RNA was detected during cell growth, and its expression was downregulated when the cells reached confluence. By screening various cell culture models, Tamburstuen et al. showed that AMBN was expressed in many primary cells and in cell lines that had originated from mesenchymal tissues such as bone and adipose tissues (e.g., MSC, BMSC, ADAS, PBMC, and MC3T3) (Tamburstuen et al. 2010, 2011). Of note, AMBN gene expression was also detected in human osteosarcoma tissue (Iizuka et al. 2011) and tumor cell lines such as Saos-2 (Muller et al. 2007) and NOS-1 (Iizuka et al. 2011). Osteoclasts were also shown to contain AMBN protein (Tamburstuen et al. 2010).

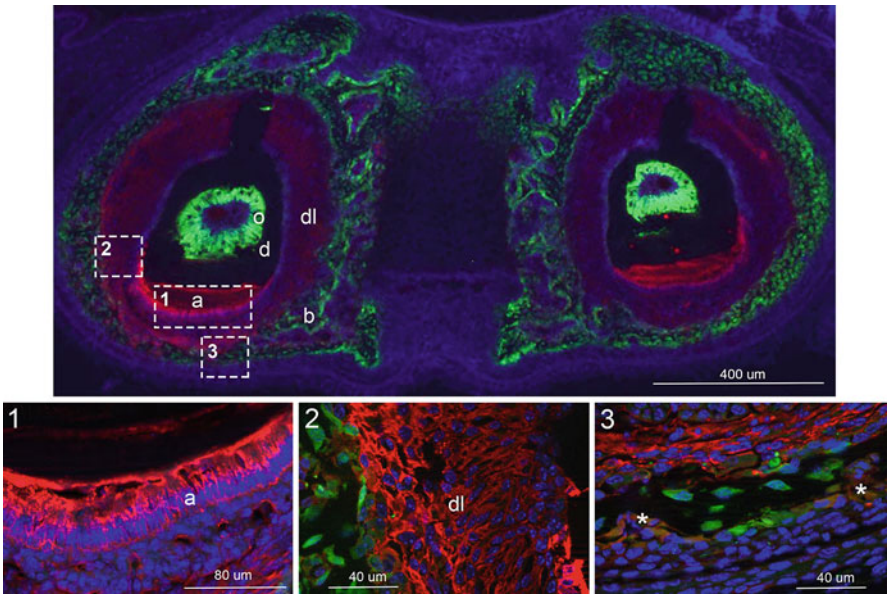

Fig. 3 AMBN protein expression in incisor/parodontium complex in 3-day-old mice. Frontal sections of 3-day-old mouse head were labeled with an antibody to AMBN (*red signal*). Collagen I-expressing cells (e.g., odontoblasts and osteoblasts) appear *green* and nuclei appear *blue*. AMBN protein is strongly expressed in ameloblasts (*a*) and in dental ligament (*dl*). No protein expression was detected in odontoblasts (*o*) or in dentin (*d*). Higher magnification shows strong AMBN signal in ameloblasts (*1*) and in cells of the dental ligament (*2*). Higher magnification of AMBN protein expression in bone (*3*) shows AMBN-positive osteoblastic cells lining bone trabeculae (*white asterisk*) (*3*). *a* ameloblast, *b* bone, *o* odontoblast, *dl* dental ligament, *d* dentin

AMBN Functions and Pathways

The signaling function of enamel peptides was demonstrated for the first time in 1999 when Nebgen et al. showed that implantation into muscle of a peptide fraction derived from bovine dentin matrix induced ectopic chondrogenesis and osteogenesis (Nebgen et al. 1999). These experiments aimed to duplicate in teeth the success story of the bone morphogenetic proteins (BMPs), which are osteoinductive growth factors isolated from decalcified bone matrix. In vitro, a peptide fraction distinct from the BMP family was shown to induce the synthesis of type II collagen and proteoglycans and to stimulate the expression of Sox9 and Runx2 in embryonic muscle-derived fibroblasts (Nebgen et al. 1999). Since then, many studies have reported that recombinant or purified enamel proteins and peptides affect a wide variety of cell types, suggesting direct roles for these molecules in both epithelial and mesenchymal tissues [reviewed in Grandin et al. (2012)]. For some authors, the "unstructured" characteristics of AMEL and AMBN proteins and their cleavage products may explain their signaling potential and their ability to promote cell interactions (Gibson 2008; Vymetal et al. 2008). However, although the biological

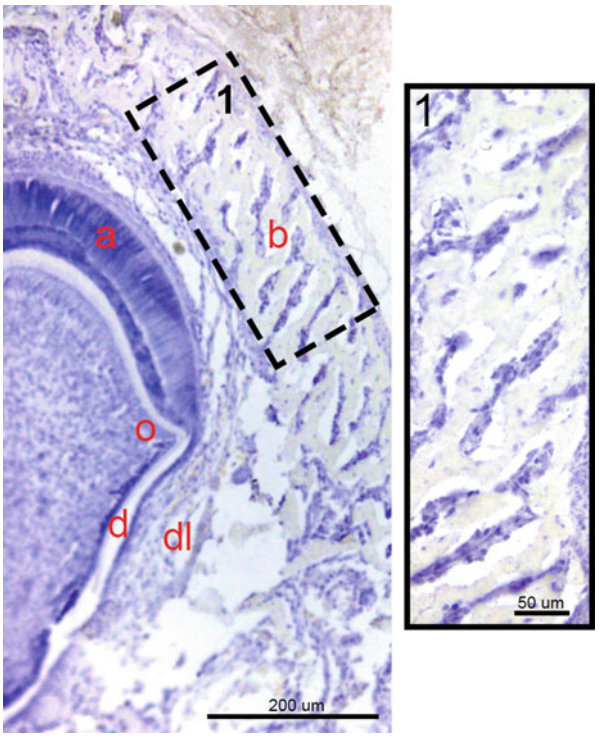

Fig. 4 AMBN RNA expression in incisor/parodontium complex in 3-day-old mice. In situ hybridization was performed using AMBN LNA probes. A strong AMBN RNA signal is observed in ameloblasts (*a*) and odontoblasts (*o*) and in cells from the dental ligament (*dl*). Higher magnification of the bone tissue shows AMBN RNA in osteoblasts and in some recently embedded osteocytes. *a* ameloblast, *b* bone, *o* odontoblast, *dl* dental ligament, *d* dentin

functions of enamel proteins have been widely studied, their mechanisms of action in a physiological context are still under investigation.

The use of transgenic mouse models has provided new insight into the in vivo functions and mechanisms of AMBN.

As mentioned above, results showing AMBN expression inside and outside the enamel have suggested that this protein acts as a signaling molecule in epithelial–mesenchymal interactions and may play a role during the cellular processes of commitment and differentiation, including early tooth development (Begue-Kirn et al. 1998; Fong et al. 1998). In that context, AMBN expression was found to be associated with epithelial odontogenic tumors. In 2004, Fukumoto et al. developed a transgenic mouse model expressing a truncated form of AMBN lacking the portion encoded by exons 5 and 6 (AMBNΔ5–6 mouse) (Fukumoto et al. 2004). Using this mouse model, Fukumoto et al. showed that about 20% of the mutant mice developed an odontogenic tumor of dental epithelium originating in the buccal vestibule of the maxilla (Fukumoto et al. 2004). AMBN mutations were also reported in ameloblastomas, adenomatoid odontogenic tumors, and squamous odontogenic tumors (Perdigao et al. 2004), suggesting that AMBN regulates odontogenic tumor formation. This assertion was confirmed by Sonoda et al., who showed that overexpression of recombinant AMBN protein (rAMBN) inhibited proliferation of human ameloblastoma cells (Sonoda et al. 2009). This inhibition required the AMBN

heparin-binding sites, indicating that the presence of heparin sulfate on the cell surface is important for AMBN to adhere to and act on target cells. Moreover, the AMBN-induced inhibition of proliferation was associated with dysregulation of Msx2, p21, and p27. These data suggest that AMBN acts as an inhibitor of ameloblastoma cell proliferation by regulating cellular signaling through its heparin-binding domains and Msx2/p21/p27 pathways. Recently, the role of AMBN as an inhibitor of proliferation in epithelial carcinoma cells was confirmed by Saito et al., who showed that rAMBN treatment suppressed the proliferation of squamous cell carcinoma of tongue cells (SCC-25) via cell-cycle arrest in G1 phase (Saito et al. 2014).

In 2011, Lu et al. developed a transgenic mouse line (AMBN-K14-Tg) in which AMBN overexpression was under the control of the human keratin 14 promoter (Lu et al. 2011). In 2013, the same group showed that AMBN-K14-Tg mice displayed fivefold elevated AMBN levels in mandibles and suffered from root cementum resorption, delamination, and reduced alveolar bone thickness (Lu et al. 2013). AMBN gain of function resulted in a significant reduction in trabecular bone volume and bone mass in the postnatal mouse jaw. In addition, AMBN-overexpressing mice displayed increased thickness of the periodontal ligament (PDL) as well as significant bone loss at the alveolar bone crest. To investigate the specific role of AMBN in osteoclastogenesis, the same authors used an in vitro model of osteoclastogenesis in which bone marrow derived-monocyte/macrophage cells (BMMCs) were cultured in the presence of rAMBN. AMBN dramatically increased the osteoclast number and modulated osteoclast differentiation, as shown by the upregulation of the osteoclastogenic genes NFATc1 and cFos. AMBN also increased adhesion and accelerated cell spreading of BMMCs; this effect was associated with enhanced RhoA protein expression and elevated ERK1/2 and AKT phosphorylation. Furthermore, blocking the integrin α2β1 and ERK1/2 pathways inhibited the effects of AMBN on osteoclast adhesion and differentiation. The authors therefore concluded that AMBN acts as a signaling molecule that enhances osteoclastogenesis and resorption of mineralized tissue by regulating adhesion of osteoclastic cells via integrin-dependent ECM signaling cascades involving integrins, ERK 1/2, RhoA, MAPK, and phosphorylation of AKT (Lu et al. 2013).

Using the same AMBN-K14-Tg mouse model, Atsawasuwan et al. showed that overexpression of AMBN also resulted in delayed posterior frontal suture fusion and incomplete suture closure. In addition, mutant mice displayed lighter skulls, thinner interfrontal bones, and morphological alterations of the craniofacial bones and their sutures (Atsawasuwan et al. 2013b). These mice also featured reduced cell proliferation in suture blastemas and in mesenchymal cells from frontal sutures. In addition, calvaria and suture mesenchymal cells isolated from these mice displayed significantly reduced expression of Msx2 and its downstream target genes, including the osteogenic transcription factors Runx2 and Sp7, the bone matrix proteins Ibsp, Col1, Ocn, and Opn, and the cell-cycle-related gene CcnD1. Furthermore, using the AMBNΔ5–6 mouse model developed by Fukumoto et al. (2004), Atsawasuwan et al. demonstrated that AMBN expression varied with osteoblast differentiation and affected bone development and mineralization (Atsawasuwan et al. 2013a). Indeed, skulls from AMBNΔ5–6 mice were approximately 15% shorter. In addition,

analysis of calvaria from AMBNΔ5–6 mice and calvaria osteoblast cultures revealed a dramatic reduction in mineralized nodules associated with reduced expression of Runx2, Sp7, Ibsp, and Msx2 and a delay in suture closing. Taken together, these studies suggest that AMBN plays a crucial role in the regulation of cranial bone growth and suture closure and affects osteoblast differentiation and mineralization. AMBN acts on craniofacial bone and suture formation and homeostasis by regulating Msx2 and controlling the proliferation of progenitor cells (Atsawasuwan et al. 2013a, b). Involvement of AMBN in cell proliferation associated with Msx2 alteration, which was identified in an Msx2-knockout transgenic mouse model (Aioub et al. 2007), is supported by the studies on odontogenic tumor mentioned above (Fukumoto et al. 2004; Perdigao et al. 2004; Sonoda et al. 2009).

To further determine the mechanisms involved in AMBN regulation of mesenchymal cells, several studies using rAMBN have been performed in vitro.

In 2010, Tamburstuen et al. demonstrated that rAMBN enhanced the proliferation and migration of osteoprogenitors and osteoblasts (Tamburstuen et al. 2010). Using gene-screening techniques, they showed that rAMBN treatment was associated with increased expression of markers of bone cell differentiation (e.g., Ocn, CD44) as well as immune responses (e.g., IL6, IL8, IL1). Importantly, the genes encoding the transcription factors STAT1 and STAT2 were among the stimulated genes involved in signaling pathways. The authors therefore suggested that AMBN would have the potential to induce osteoprogenitor cell recruitment and growth via the interferon pathway and its downstream factors STAT1, STAT2, and CD44. Using Western and dot blot, the same group demonstrated that mesenchymal stem cells and primary human osteoblasts not only expressed AMBN RNA but also secreted the protein into the cell culture medium (Tamburstuen et al. 2011). Moreover, human rAMBN was shown to stimulate high levels of AMBN RNA expression in primary human mesenchymal stem cells, suggesting there is positive feedback regulation of AMBN expression in these cells. Feedback regulation is a well-recognized mechanism for controlling cell signaling and cell homing; these observations therefore suggested a role for AMBN protein, or its derivatives, in signaling pathways. Interestingly, and in line with an in vivo study described earlier (Lu et al. 2013), Tamburstuen et al. also showed that rAMBN treatment was associated with increased differentiation of osteoclast precursors cells in vitro (Tamburstuen et al. 2010).

To further investigate the role of AMBN in osteoblastic differentiation and mineralization, human oral primary squamous cell osteocarcinoma (NOS-1) cells were treated with AMBN siRNA (Iizuka et al. 2011). Downregulation of Alp, Col1, and Ibsp was observed, suggesting that AMBN is involved in the mineralization process. Furthermore, ectopic overexpression of AMBN in Saos-2 cells resulted in enhanced matrix mineralization and increased Runx2 and Ibsp gene expression. In addition, AMBN was shown to bind to CD63 and promote CD63 binding to integrin β1 in osteosarcoma cells. The interaction between CD63 and integrin β1 led to inactivation of Src kinase. These results suggested that AMBN acts as a promoting factor for osteogenic differentiation of osteosarcoma cells via a pathway involving CD63, integrin β1, and Src.

Furthermore, AMBN was shown to affect cell attachment and mineralization of mouse PDL cells (Zeichner-David et al. 2006). In vitro, rAMBN treatment resulted

in an increased level of cell attachment of PDL cells, modulation of BMP expression, downregulation of Col1 expression, and induction of de novo Ocn expression. These data demonstrated that AMBN has growth factor activity during periodontium development and regeneration. More recently, Zhang et al. provided new insight into the growth factor activity of AMBN in mouse PDL cells and dental follicle (DF) cells (Zhang et al. 2011b). Using rAMBN and AMBN siRNA treatments, the authors showed that AMBN reduced both PDL and DF cell proliferation. This was associated with upregulation of the cell-cycle inhibitor p27. Furthermore, adhesion and spreading of both cell types were significantly enhanced in AMBN-coated dishes, and AMBN treatment resulted in increased expression of RhoA, one of the classic intracellular mediators of ECM-induced cell adhesion. Finally, blocking of CD63, integrin β1 and ERK pathways reduced the effects of AMBN on RhoA expression, cell adhesion, and proliferation. This study indicates that AMBN affects cell-cycle progression through p27 modulation and stimulates cell adhesion by increasing RhoA activity via the already mentioned cross-talk between CD63, integrin β1, and ERK pathways (Iizuka et al. 2011; Lu et al. 2013).

To summarize, the results detailed in section "Part II" demonstrate that AMBN has roles beyond the control of enamel mineralization. Most of the newer articles dedicated to the study of AMBN indicate its presence in bone. During bone growth, AMBN is highly expressed at the boundaries of the bone matrix, including of DF, PDL, and endosteal and periosteal surfaces. Subsequently, AMBN expression fades in mature bone, except for bone with a high remodeling rate, such as the alveolar bone, where AMBN presence is maintained at all stages. Interestingly, even if some of AMBN protein appears to be incorporated into bone matrix, the protein is also detected in a solubilized state in the microenvironment of AMBN-producing superficial mesenchymal dental and bone cells. The role of AMBN in bone formation and remodeling has been demonstrated using various in vivo and in vitro models. AMBN stimulates the recruitment, proliferation, osteoblastic differentiation, and mineralization of many mesenchymal cells, including osteoprogenitors, osteoblasts, and also PDL and DF cells. Besides its osteogenic role, AMBN has been shown to directly stimulate osteoclastogenesis and to be a key factor in the regulation of cell adhesion to the ECM. Taken together, these data demonstrate the crucial function of AMBN in the control of bone balance. However, although AMBN activities have been shown to involve many molecular mechanisms (e.g., interferon pathway, cross-talk between CD63 and integrins, Msx2/p21/p27 pathway, etc.), full knockout and bone-specific conditional transgenic mouse models are now required to address the controversy over AMBN function in bone tissues (Kuroda et al. 2011) and to fully elucidate its mechanisms of action.

Part III: AMBN as a Bone Site-Specific Marker

Enamel is distinct from bone and dentin in its tissue origin, mineralization matrix composition, and mineralization process. As shown in section "Part I," enamel forms by bioapatite crystallization on a noncollagenous protein matrix secreted from

ameloblasts of epithelial origin, whereas bone and dentin form on a collagenous matrix deposited by cells of mesenchymal origin. The enamel matrix mineralizes immediately after secretion, whereas bone and dentin both mineralize on preformed unmineralized collagenous matrix called osteoid and predentin, respectively. Afterwards, the enamel matrix, mainly composed of specialized proteins such as amelogenins and AMBN, matures into a hypermineralized inorganic tissue (~97% inorganic content). However, this traditional distinction between enamel (noncollagenous protein matrix) versus bone and dentin (collagenous protein matrix) as well as the enamel-specificity of AMEL and AMBN has recently been challenged. Indeed, recent studies (detailed in section "Part II") have shown that expression of AMEL and AMBN is not simply restricted to enamel and ameloblasts but also occurs in many nonenamel tissues and cells, notably bone and osteoblasts. Interestingly, recent quantitative studies focusing on bone tissue have confirmed the expression of AMBN in various bone compartments and further demonstrated that AMBN shows significantly higher expression in jaws and skull bones compared to long bones (Rawlinson et al. 2009b; Jacques et al. 2014a, b).

As emphasized in the introduction to this chapter, the osteodiversity evident between different anatomical regions has major public health implications. This is exemplified by the tissue incompatibility problems associated with bone ectopic autografts or the puzzling jaw osteonecrosis induced by antiresorptive agents that are otherwise effective in treating long-bone osteoporosis or metastatic resorptive lesions. Identifying bone site-specific biomarkers is therefore essential to determine why bone cell behaviors vary depending on their anatomical site and to propose new bone site-specific therapeutics.

Bone Site-Specific Molecular Fingerprint and AMBN

In vivo and ex vivo transcriptomic and proteomic studies have shown molecular fingerprint variations between bone sites. These include differential gene and protein expression profiles of bone morphogens (e.g., BMPs, TGFs, IGFs, FGFs), ECM proteins (e.g., collagens, enamel matrix proteins), and remodeling factors (i.e., OPG, RANKL, CSF1) (Kasperk et al. 1995; van den Bos et al. 2008; Rawlinson et al. 2009b; Kingsmill et al. 2013; Reichert et al. 2013; Isaac et al. 2014).

Transcription factors encoded by homeogenes such as Hox genes are also differentially expressed among bone sites (Leucht et al. 2008; Rawlinson et al. 2009b). Differentiation of the vertebral and appendicular segments during patterning is driven by a Hox code that is maintained in adult bones, whereas the craniofacial skeleton is free of Hox-gene expression and mainly depends on divergent homeoproteins (e.g., MSXs and DLXs families). Among these divergent homeoproteins, Msx2, a major actor of osteoblast differentiation, is one of the key markers of the bone site-specific molecular fingerprint. Msx2 is more highly expressed in jaws when compared to long bones (Kingsmill et al. 2013), and human Msx2 mutations specifically impact craniofacial bone morphogenesis (craniosynostosis OMIM #604757, parietal foramina OMIM #168500). In addition,

Msx2-knockout transgenic mice show alterations exclusively in jaw bone remodeling (Aioub et al. 2007). The role of divergent homeoproteins in bone site-specificity was also recently illustrated with the Dlx3 gene. Indeed, in vivo and in vitro models of Dlx3 deletion revealed that Dlx3 has differential bone site-specific gene targets (Isaac et al. 2014).

Based on these observations, the spatio-temporal expression of AMBN was further explored and compared at the RNA and protein levels in various bone compartments of wild-type and knockout mice. Among the investigated enamel proteins, AMBN was identified as a bone site-specific marker, with both AMBN RNA and protein showing higher expression in jaws and skull bones when compared to long bones (Jacques et al. 2014a, b).

AMBN and Embryonic Origin

The regionalized bone physiopathology and associated osteoblast site-specific molecular fingerprint may result from the dual embryonic origin of the different bone compartments. Developmentally, osteoblasts derive from two distinct tissues. The osteoblasts of most of the craniofacial bones, including the mandible, maxillar, and interparietal bones, as well as the dental mesenchyme are derived from neural crest (Chai et al. 2000). In contrast, the osteoblasts of the parietal and occipital bones and of the axial and appendicular skeleton are mesoderm derived. Of note, several studies in both mouse and chicken suggest that frontal bone could have a mixed embryonic origin, with osteoblasts from both neural crest and paraxial mesoderm contributing to this formation (Deckelbaum et al. 2012).

By exploring enamel proteins in bones, Jacques et al. recently demonstrated that AMBN RNA levels in adult mice were graded, from mandibular (1:1), to frontal (≈1:10), occipital (≈1:100), and tibial (≈1:2,000) bones (Fig. 5). Bones with neural crest-derived cell contributions (e.g., mandibular and frontal bones) therefore displayed higher AMBN RNA expression levels compared to mesoderm-derived bones (occipital and tibial bones). These observations, in line with those of Rawlinson et al. (2009b), suggest that AMBN expression in osteoblasts varies depending on their embryonic origin. AMBN may therefore be a useful marker of neural crest-derived bone compartments.

AMBN and Craniofacial Bone Healing

In craniofacial bones, AMBN is involved in processes associated with high remodeling rates, such as bone formation or healing. In calvaria, AMBN expression varied with age: it is increased during bone formation and reduced in mature bone (Atsawasuwan et al. 2013a). In line with these observations, Jacques et al. showed that AMBN RNA expression was 50-fold higher in alveolar bone in young mice (1 week) compared to adults (15 weeks). Notably, no significant variation with age was detected in basal bone, a bone with a lower remodeling rate (Jacques

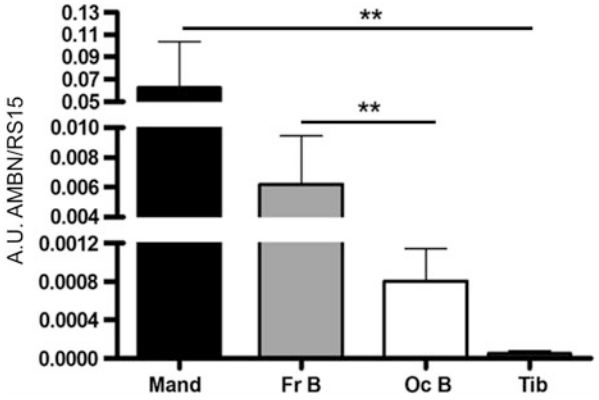

Fig. 5 AMBN expression in different bone compartments in 15-week-old mice. Whole mandible (*Mand*), tibia (*Ti*), frontal bone (*Fr B*), and occipital bone (*Oc B*) from 15-week-old mice were dissected with the aid of a stereomicroscope and transcript expression was analyzed using RT-qPCR. RT-qPCR analyses show that AMBN expression is significantly higher in mandibular bone compared to the other bone compartments: frontal bone (≈10:1), occipital bone (≈100:1), and tibia (≈2,000:1). Moreover, frontal bone shows significantly higher expression when compared to occipital bone (≈10:1) and tibia (≈200:1). Finally, occipital bone displays an increased RNA expression level when compared to tibia (≈20:1). AMBN mRNA levels were normalized to the expression of the housekeeping gene RS15. Significance was evaluated using the Mann–Whitney test (*$p < 0.05$, **$p < 0.01$). *AU* arbitrary unit

et al. 2014b). Using experimentally induced bilateral defects in the mandibular ramus of adult rats as a model, Tamburstuen et al. reported AMBN protein expression in newly formed bone (Tamburstuen et al. 2010). During the bone healing process, AMBN was located in the immature bone ECM adjacent to lining cells, osteoblasts, and perivascular cells. Interestingly, osteocytes were AMBN negative as was the matrix located in the more mature parts of the formed bone and the surrounding original bone. In 2013, Trevez-Manusevitz et al. showed de novo expression of AMBN protein in bone cells, and newly mineralized matrix formed 8 weeks after the implantation of human oral mucosa and gingiva stem cells (hOMSC) in mouse calvaria (Treves-Manusevitz et al. 2013). The role of AMBN in craniofacial bone healing was further consolidated by Tamburstuen et al., who showed that rAMBN treatment of rat mandibular bone defects enhanced bone regeneration (Tamburstuen et al. 2010). Together these findings demonstrate a crucial role for AMBN in early jaw bone formation, repair, and regeneration.

AMBN and Jaw Bone Biomechanical Responsiveness and Plasticity

Bone tissue formation and homeostasis are conditioned by site-specific biomechanical loading to which they are constantly subjected. Weight-bearing bones (e.g., appendicular and axial bones) and non-weight-bearing bones (e.g., jaws and cranial bones) have different ossification processes. Weight-bearing bones form from

endochondral ossification, which enables bone growth to occur under heavy mechanical loads, whereas most of the craniofacial bones, which are not exposed to heavy loads, form directly from mesenchymal cell condensation (intramembranous ossification). During growth and homeostasis, while the weight-bearing bones are exposed to direct loading, the alveolar bone is exposed to indirect loading via the teeth.

Alveolar bone morphology (shape and volume) is conditioned by the existence of the tooth, which is considered to be the organizing "signaling center" of the alveolar bone anlage during jaw development (Jernvall and Thesleff 2000). Thus, tooth extraction results in alveolar bone loss (height and thickness) (Atwood and Coy 1971). In addition, the morphology and properties of the adjacent alveolar bone, and also of the antagonist jaw, are affected by the variations in occlusal stress levels (e.g., mastication, chewing, orthodontic treatments, etc.). The rapid remodeling of the alveolar bone and its exceptional lability may result, at least in part, from the intrinsic specificity of the alveolar bone cells compared to the cranial, axial, and appendicular bone cells. Particularly, the role of the osteoblasts in this site-specific mechanical sensitivity has been confirmed in vitro by Rawlinson et al., who showed that the molecular responses of osteoblasts to mechanical stress vary according to bone localization (Rawlinson et al. 1995).

Interestingly, Alikhani et al. demonstrated that local application of high-frequency acceleration on the occlusal surface of the rat maxillary first molar jointly increased alveolar bone formation and AMBN RNA expression levels (Alikhani et al. 2012). Using a biomechanical assay in which the crowns of the right maxillary molars of mice were reduced occlusally by grinding to simulate the unopposed teeth, Jacques et al. showed a threefold increase in AMBN RNA expression 72 h after altering the tooth occlusion (Jacques et al. 2014b). Based on these observations, the authors hypothesized that the AMBN expression gradient observed in bone compartments (mandible > frontal bone > occipital bone > tibia) (Fig. 5) may result from mechanical stress diffusion in the craniofacial apparatus during mastication and swallowing. This hypothesis is in line with the low AMBN RNA level in tibia and the loss of AMBN protein in edentulous animals such as avian species (Meredith et al. 2014).

The high level of AMBN, described as an osteogenic factor (see section "Part II"), during biomechanical stimulations may contribute to the increase in alveolar bone mineralization consequent to unilateral mandibular hypofunction (removal of maxillary molars) (Rawlinson et al. 2009a). The AMBN increase observed in the mandibular alveolar bone after occlusal alteration (Jacques et al. 2014b) would therefore trigger new alveolar bone formation, allowing mandibular molar "overeruption" in response to the loss of contact with the opposite, grinded molar.

Interestingly, Jacques et al. demonstrated that the increase in AMBN level in response to alteration of tooth occlusion was associated with a threefold decrease in Msx2, one of the candidates for the molecular signature of craniofacial osteoblasts (Jacques et al. 2014b). This observation is in line with those of previous studies showing that Msx2 was affected when mechanical loading was applied in vitro on craniofacial osteoblastic cells (Gonzalez et al. 2008; Fushiki et al. 2015). As detailed

in section "Part II," previous studies have reported that AMBN and Msx2 reciprocally control their expression and are involved in common signaling pathways in the regulation of both mineralized tissues and tumors (Aioub et al. 2007; Sonoda et al. 2009; Molla et al. 2010; Atsawasuwan et al. 2013a, b; Jacques et al. 2014b). Taken together, these results demonstrate that AMBN is involved in the biomechanical responsiveness of the craniofacial osteoblasts and that there could be interplay between AMBN and Msx2 in this process.

Part IV: Potential Applications of AMBN in Jaw Bone Reconstruction

Many studies have demonstrated the high potential of enamel peptides for periodontal tissue healing, including the regeneration of alveolar bone [reviewed in Grandin et al. (2012)]. Considered as "bioactive," the application of these peptides during periodontal healing would recapitulate the epithelial–mesenchymal signaling interactions that orchestrate the normal development of the dento-alveolar complex. This growth factor activity has motivated the development of protein products derived from enamel matrix (Enamel Matrix Derivatives = EMD) in an attempt to regenerate functional periodontal tissues.

The first clinical EMD-based treatment, a commercial formulation of purified extract of porcine fetal tooth enamel matrix called Emdogain® (Straumann, Basel, Switzerland), stimulated the regeneration of tooth-supportive tissues, including alveolar bone, ligament, and cementum, when applied to periodontal defects (Heijl 1997). Since that time, EMD has been shown to stimulate in vitro the proliferation and differentiation of osteoprogenitor cells [reviewed in Grandin et al. (2012)] and to promote the clinical regeneration of alveolar bone and periodontal tissues in patients with intrabony defects (Dori et al. 2013). In order to improve the physicochemical properties for the adsorption of EMD, new formulations of EMD such as Osteogain® are under development (Zhang et al. 2015). Of note, since 2006, Xelma® (Mölnlycke, Göteborg, Sweden), another commercial EMD, has been used to promote fibroblast and keratinocyte cell adhesion in the treatment of soft-tissue wounds, including venous leg and foot ulcers (Vowden et al. 2006).

Today, although EMD is widely used in clinics, knowledge of its exact composition and its cellular and molecular mechanisms of action is far from complete. Because AMEL is the major component (>95%) of EMD, most studies of EMD have focused on AMEL [reviewed in Lyngstadaas et al. (2009)]. However, EMD is a complex mix of proteins, including growth factors and proteases that could also contribute to the clinical bioactivity (Maycock et al. 2002). This composition could explain the contrasting effects of EMD extracts and AMEL observed on osteoclast activity. Indeed, whereas AMEL negatively regulated osteoclastogenesis in vivo (Yagi et al. 2009) and in vitro (Hatakeyama et al. 2006; Nishiguchi et al. 2007), EMD has been shown to promote osteoclastogenesis [reviewed in Grandin et al. (2012)]. Both osteoblastogenesis and osteoclastogenesis are essential to bone regeneration because these two differentiation processes jointly interact in the remodeling of

newly formed bone to functional mature bone. Thus, the beneficial effects of EMD-based treatment on alveolar bone regeneration are likely a consequence of its dual actions; EMD not only enhances bone formation via stimulation of osteoblastic bone-forming activity but it also stimulates osteoclastic bone-resorptive activity.

Similar to EMD, AMBN has been shown to stimulate both osteogenesis and osteoclastogenesis (see section "Part II"). And, as described in section "Part III," craniofacial bone regeneration activity of AMBN (initially hypothesized to be due to enhanced AMBN expression during bone formation, remodeling, and repair) was demonstrated by Tamburstuen et al., who showed that application of rAMBN to rat mandibular bone defects stimulated bone regeneration (Tamburstuen et al. 2010). Together, these data strongly suggest that (1) the clinical effect of EMD in alveolar bone reconstruction might also be associated with its AMBN content and (2) the use of "pure" AMBN protein could optimize the beneficial activity of EMD on bone regeneration. AMBN-based treatment might therefore enhance alveolar bone reconstruction (healing time, bone volume, mineral density) and provide the new bone with high adaptability properties.

The clinical potential of AMBN for alveolar bone regeneration is further supported by in vivo studies showing that AMBN-based treatments stimulated the regeneration of two other mineralized tissues: dentin and cementum. Thus, local application of rat rAMBN (native protein) in a pig pulpotomy model enhanced pulpal wound healing and reparative dentin formation compared with calcium hydroxide treatment (Nakamura et al. 2006). Interestingly, Fukae et al. demonstrated that the 17-kDa N-terminal cleavage product of AMBN (either isolated from developing porcine incisors or commercially synthesized based on human and porcine peptide sequences) showed ALP-inducing activity in vitro on human PDL cells (Fukae et al. 2006). Using a dog mandible bone-defect model, the same group further showed that this peptide also exhibits cementum regeneration activity in vivo (Kanazashi et al. 2006).

In addition to its high potential for alveolar bone reconstruction, the use of human rAMBN (native form or cleavage peptides) could circumvent clinical problems potentially associated with treatment based on animal products (e.g., immune response, disease transmission, ethical issues). From a clinical perspective and similar to EMD (Dori et al. 2013), the bone-regeneration activity of rAMBN-based treatments might later be optimized by combining rAMBN with osteoconductive/osteoinductive scaffolds such as synthetic bone substitutes (e.g., calcium phosphates, bioactive glasses), allografts [e.g., freeze dried bone allograft (FDBA) or demineralized freeze dried bone allograft (DFDBA)], or xenografts [e.g., natural bovine bone mineral (NBM)].

Beyond its stimulatory activity in osteogenesis and osteoclastogenesis, AMBN is also a biomarker of the craniofacial bones and specifically of alveolar bone (see sections "Part II" and "Part III"). Thus, it can be speculated that AMBN-based treatment may be used to direct/redirect the molecular and phenotypic profile of osteoblastic progenitor cells into osteoblasts with either a "craniofacial" or an "alveolar" site-specific fingerprint. Modifying the osteoblast profile would have

many clinical applications. In bone grafting, for example, AMBN treatment might induce the differentiation of progenitor cells isolated from the appendicular and axial skeleton into craniofacial osteoblasts. Theoretically, AMBN-based treatment could thereby improve the clinical efficacy of heterotopic autograft performed in maxillo-facial reconstructive surgeries (see section "Part III") by increasing the adaptability/plasticity of the extra-craniofacial donor site. As previously mentioned in section "Part III," alveolar bone has been shown to be less sensitive to hormonal alterations (such as PTH increase and estrogen deficiency) compared to the appendicular and axial skeleton. Preventive AMBN-based treatment might therefore be used to limit the bone loss associated with postmenopausal osteoporosis.

However, as detailed in section "Part II," mouse AMBN is not only expressed in mineralized tissues it is also detected during early stages of embryogenesis and in many soft tissues in the adult, including reproductive organs such as the testis (Jacques et al. 2014a, b). This expression pattern suggests that AMBN may play additional roles in the organism which are still unknown. These observations, in line with the association between AMBN alterations and ameloblastoma or odontogenic tumor (see section "Part II"), highlight that the delivery of rAMBN (and EMD) to damaged tissues has to be perfectly controlled in terms of localization and quantity. Gene therapy might be used to help circumvent rAMBN delivery problems. Indeed, AMBN DNA could be locally delivered to the damaged bone tissues to transduce the resident bone cells and increase (or induce) regional secretion of AMBN protein over extended periods. Based on tissue engineering and hybrid biomaterials strategies, AMBN DNA could also be used to transduce in vitro autologous stem cells isolated from the patient in order to later graft AMBN-overexpressing osteoprogenitors into the damaged bone site. The feasibility of AMBN gene therapy is supported by Wazen et al., who showed that bone cells could be efficiently transduced by lentiviral vectors encoding AMBN both in vitro and in vivo (Wazen et al. 2006).

As illustrated by the alveolar bone loss subsequent to tooth extraction, mechanical stimulations are essential to maintain a functional alveolar bone (see section "Part III"). Interestingly, recent studies detailed above have shown that local variations of tooth occlusal forces induce increased AMBN RNA expression in the adjacent alveolar bone (Alikhani et al. 2012; Jacques et al. 2014b). These recent results suggest that mechanical therapy using the osteogenic effect of these stimuli could stimulate alveolar bone formation/regeneration in a physiopathological context. Thus, local application of biomechanical stimulation could be a promising alternative method to induce regionally controlled cell secretion of osteogenic factors such as AMBN without using biologically active factors or DNA transduction. Together, these data encourage the use of mechanical therapies for alveolar bone regeneration such as low intensity pulsed ultrasound stimulation (LIPUS) and orthodontic extrusion.

In conclusion, AMBN expression in bone tissues depends on the anatomical site and the ontogenic stage. AMBN is almost exclusively expressed in bone compartments with a neural crest-derived osteoblast contribution, suggesting that this protein could serve as a specific marker of the embryonic origin of bone organs (neuroectoderm- versus mesorderm-derived bones). In addition, in vitro and

in vivo studies support that AMBN (1) is strongly involved in bone processes associated with high remodeling rates, such as bone formation, repair, and regeneration and (2) controls the physiological response of peri-dento-alveolar bone to tooth-associated biomechanical stimulation, thereby maintaining alveolar bone integrity. Owing to its physiological properties, AMBN-based treatments may have promising clinical potential for craniofacial tissue repair and most specifically for alveolar bone regeneration.

Summary Points

- This chapter focuses on ameloblastin (AMBN), which is an extracellular matrix protein initially discovered in enamel matrix and ameloblasts.
- In enamel, AMBN promotes the growth of a crystalline enamel layer and regulates the matrix binding, proliferation, and differentiation of ameloblasts.
- Mutation of human AMBN leads to various forms of amelogenesis imperfecta.
- AMBN is also detected outside the enamel in both mineralized and nonmineralized tissues, including bone.
- In bone, AMBN stimulates osteoblastogenesis and osteoclastogenesis and participates in the regulation of cell adhesion to the extracellular matrix.
- Although many molecular mechanisms have been shown to be involved in AMBN function (e.g., interferon pathway, cross-talk between CD63 and integrins, Msx2/p21/p27 pathway, etc.), full knockout and bone-specific conditional transgenic mouse models are now required to fully elucidate its mechanisms of action.
- AMBN expression in bone tissues depends on the anatomical site and the ontogenic stage; it shows higher expression in jaw and skull bones compared to long bones, and it is highly expressed during processes associated with high levels of bone remodeling, such as bone formation, repair, and regeneration.
- AMBN is a bone site-specific marker that participates in the physiological control of alveolar bone integrity in response to tooth-associated biomechanical stimulation.
- Owing to its physiological properties, AMBN-based treatments may have promising clinical potential for craniofacial tissue repair, and most specifically for alveolar bone regeneration.

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