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Localization and Function of the Anion Exchanger Ae2 in Developing Teeth and Orofacial Bone in Rodents

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Abstract

To explore functions of anion exchanger 2 (Ae2) in the development of bones and teeth we examined the distribution of Ae2 in cells involved in formation of teeth and surrounding bone in young hamsters, mice and rats. In all three species strongest immunostaining for Ae2 was obtained in basolateral membranes of maturation ameloblasts and in osteoclasts resorbing bone. In hamsters a weaker staining was also seen in the Golgi apparatus of secretory ameloblasts, young osteoblasts and osteocytes, odontoblasts and fibroblasts of the forming periodontal ligament. In adult *Ae2_{a,b}^{-/-}* mice, in which Ae2 targeted disruption precluded the expression of Ae2a, Ae2b1 and Ae2b2 isoforms, the immunostaining for Ae2 in ameloblasts and osteoclasts was totally abolished. Enamel formation was abnormal but teeth erupted, osteoclasts in jaw bone were functional and structure of dentin and bone was normal. In another mouse model, *Ae2^{-/-}* mice in which expression of all five Ae2 isoforms was disrupted, teeth failed to erupt and the alveolar bone proved poorly formed with giant but apparently functional osteoclasts. Our data indicate that basolaterally located Ae2a, Ae2b1, or Ae2b2 (or a combination of these) is present in maturation ameloblasts critical for the cells' normal functioning. While isoforms of AE2 were also present in basolateral membranes of osteoclasts they proved not critical to osteoclast resorption of orofacial bone. Poorly formed bone and the failure of teeth to erupt seen in the *Ae2^{-/-}* mice with gene disruption affecting all isoforms may result from secondary (systemic) changes that are different from *Ae2_{a,b}^{-/-}* mice.

Enamel is basically formed in two stages, a secretory stage in which the ameloblasts secrete large quantities of a proteinaceous enamel matrix, and a maturation stage during which the ameloblasts obtain characteristics of resorbing cells and the enamel gradually hardens (Smith, '98). During the maturation phase the matrix proteins are massively degraded and removed from the enamel space along with a progressive increase in mineral content. It has been proposed that during the secretion phase the buffering capacity of amelogenins (the principal enamel matrix proteins) is large enough to neutralise all of the protons formed during initial mineralization (Simmer and Fincham, '95; Smith et al., 2005). However when

these amelogenins are removed in the maturation phase another mechanism is needed to maintain a neutral pH. In kidney, exchange of bicarbonate for chloride by ionic exchangers is used to regulate pH (Alper et al., '97), a mechanism also used to generate acid in stomach cells to digest food and in osteoclasts to resorb bone (Helfrich, 2003). The bicarbonate needed for this process is generated by activity of cytosolic carbonic anhydrase (Helfrich, 2003), an enzyme also present in maturation stage ameloblasts (Lin et al., '94).

Sodium-independent anion exchangers (Ae1, Ae2 and Ae3) are transmembrane proteins that exchange electroneutrally bicarbonate for chloride and regulate intracellular pH, cell volume, transepithelial hydroionic fluxes and acid/base transport (Alper, 2002 review). Among the three main members of the Ae (Slc4) family, Ae2 (Slc4a2) is the most widely distributed. The *Ae2* gene, similarly to the other *Ae* genes, may drive transcription from different alternate promoter sequences. Alternative exons 1b1 and 1b2 are transcribed from overlapping promoter sequences within intron 2, each being spliced to exon 3 in corresponding 5'-variants Ae2b1 (Wang et al., '96) and Ae2b2 (Medina et al., 2000). The alternative transcription from intron 2 is conserved between rodents and humans (Aranda et al., 2004), although expression of the type "b" variants is more tissue specific in humans (Medina et al., 2000). The 5'-diversity of these variants results in small changes in encoded polypeptides, the initial 17 amino acids of the complete Ae2a isoform being replaced by 3 residues (MTQ) in Ae2b1 and by 8 residues (MDFLLRPQ) in Ae2b2 (Medina et al., 2000). Additional alternative exons 1c1 and 1c2 may be transcribed from overlapping sequences within intron 5 in mouse, rabbit, and rat (Lecanda et al., 2000; Rossmann et al., 2000; Wang et al., '96), but not in humans (Medina et al., 2000). These alternative exons are either spliced to or proceed with exon 6 in variants Ae2c1 and Ae2c2, respectively. Ae2c1 expression appears to be rather stomach-specific, whereas Ae2c2 expression is virtually negligible in most tissues (Kurschat et al., 2006; Recalde et al., 2006). Systematic functional characterization of mouse Ae2 variants in *Xenopus* oocytes have indicated an alkaline-shifted $\text{pH}_{\text{O}(50)}$ sensitivity of the Ae2c1-mediated anion exchange compared to the anion exchange activity displayed by Ae2a, Ae2b1, and Ae2b2 polypeptides (Kurschat et al., 2006). On the other hand, Ae2c2 polypeptide lacks any anion exchange activity, while Ae2b2 is the most active polypeptide among the remaining four isoforms (Kurschat et al., 2006).

The importance of Ae2 for bone and tooth formation was suggested in a mouse strain in which a vector insertion replacing exons 14-17 in the *Ae2* gene resulted in protein disruption of all Ae2 isoforms, including the stomach-specific Ae2c1 (Gawenis et al., 2004). In addition to absent gastric acid secretion and hyperkeratosis of the epithelium of non-glandular mucosa in the stomach, these mice with entire disruptions of Ae2 protein isoforms (*Ae2*^{-/-} mice) had alterations in bone development and impaired tooth eruption, although no details were presented (Gawenis et al., 2004). A less severe phenotype was obtained in mice in which the gene had been targeted to disrupt just the Ae2a, Ae2b1 and Ae2b2 isoforms (Medina et al., 2003). In these mice (hereby referred to as *Ae2a,b*^{-/-} mice), long bones became osteopetrotic but structure of the jaw bone was normal and teeth erupted (Jansen et al., 2009; Lyaruu et al., 2008). The data suggested that osteoclasts in the craniofacial area but not in long bones were functional. The enamel of the teeth of these mutants however wore down much faster and contained less mineral than in wild type mice indicating that Ae2 is also required for enamel formation (Lyaruu et al., 2008). Ae2 protein was localized in membranes of mouse incisor ameloblasts and osteoclasts (Lyaruu et al., 2008; Jansen et al., 2009). Altogether these data indicated a critical function of Ae2a, Ae2b1, and/or Ae2b2 isoforms in maturation stage ameloblasts and long bone osteoclasts.

In the present study we describe in much more detail the localization of Ae2 protein during various stages of amelogenesis both in adult and neonatal incisors in three rodent species.

We also extended our study to the distribution of Ae2 during development of molar tooth germs, and the formation of dentin and surrounding bone, since primary human osteoblasts cell cultures as well as rat osteosarcoma cells have been reported to express Ae2 (Kellokumpu et al., '88; Mobasheri et al., '98). Finally we examined these tissues in the *Ae2^{-/-}* mice with protein disruption of all Ae2 isoforms.

MATERIALS AND METHODS

Animals

Newly born golden hamsters (*Mesocricetus auratus*, 4-10 days old), mice (Swiss albino, 9-14 days old) and rats (Wistar, 15 days old) were used for most immunohistochemical studies. Two strains of knockout mice were examined: one with a moderate phenotype (*Ae2_{a,b}^{-/-}* mice) and one with a severe phenotype (*Ae2^{-/-}* mice). The *Ae2_{a,b}^{-/-}* mice with the moderate phenotype (with a mixed 129/Ola en FVB/N background) carried a targeted disruption of *Ae2* that prevents the expression of *Ae2a*, *Ae2b1* and *Ae2b2* isoforms (for details of targeting strategy see Medina et al., 2003). In the *Ae2^{-/-}* mice with a severe phenotype (with a mixed 129S6/SvEv and Black Swiss background) all *Ae2* isoforms had been disrupted because of gene replacement of exons 14-17 in the *Ae2* gene with a neo cassette (for details: see Gawenis et al., 2004). All animal studies were approved and procedures complied with institutional and national guidelines.

Histological procedures

Animals were decapitated, the head skinned and mandibles and maxillae excised and fixed by immersion in 5% formaldehyde in 0.1 M phosphate buffer +2% sucrose for 4-6 h at 4 °C. Some tissues of newly born rodents were not decalcified whereas others were decalcified in 5% EDTA containing 0.8 % formaldehyde at 4 °C for 2-3 weeks. Three heads from the *Ae2_{a,b}^{-/-}* mice and three wild type littermates (12 weeks) were fixed by immersion for 24 h and decalcified in EDTA solution with 0.8 % formaldehyde for 6 weeks at 4 °C, with once-twice a week changes. Tissues were then rinsed in phosphate buffered saline (PBS) and embedded in paraffin under reduced pressure. Sagittal sections 5 µm thick were cut, mounted on polylysine coated glass slides and incubated at 37°C overnight in a dry incubator to adhere the sections.

Three heads of *Ae2^{-/-}* mice with the severe phenotype and three wild type littermates (aged 13 days-22 days) were fixed in a mixture of 1% glutaraldehyde, 4% formaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 5 days, decalcified in 10% EDTA and embedded in epon resin. Sections 1 µm thick were cut, mounted on glass slides and stained with Richardson (a mixture of 0.5% methylene blue and 0.5% azure II in 0.5% sodium borate).

Immunohistochemical procedure

After deparaffination in xylene and rehydration in a descending series of ethanol sections were rinsed in PBS. Some sections were then incubated in 10 mM citrate buffer (pH 6.0) at 95°C for 20 min (antigen retrieval), cooled down and transferred to PBS. Endogenous peroxidase was blocked by incubation in 3% H₂O₂ in water for 3 min at room temperature. Next, non-specific binding sites were blocked by incubating sections with normal goat serum (30%) followed by overnight incubation at 4 °C with affinity purified primary polyclonal rabbit antibodies raised against a synthetic peptide, identical to the 12 C-terminal amino acids of the Ae2a protein as described by Holappa et al. (2001). These antibodies can recognise all five Ae2 isotypes (*Ae2a*, *Ae2b1*, *Ae2b2* and *Ae2c1* and *Ae2c2*) as well as erythrocyte Ae1 (9 of the 12 C- terminal amino acid residues are identical to Ae2). The antisera were affinity purified and verified by Western blotting against the erythrocyte Ae1 and Ae2 protein. Working dilutions for immunohistochemistry were 1:200-1:400 and after

antigen retrieval 1:600 to 1:800. After rinsing in PBS sections were incubated with biotinylated goat anti-rabbit IgG (ABC peroxidase technique, Vectastain, Elite kit, Vector, Burlingame, Ca, USA) for 1 h at room temperature, rinsed and incubated with ABC-peroxidase complex for 1 h at room temperature. After rinsing the bound peroxidase-complex was developed with DAB (kit Vector) and sections counterstained with methyl green, hematoxylin or hematoxylineosin. As positive controls kidney sections were used, a rich source of Ae2 and Ae1 (Alper et al., '97). As negative controls primary antibodies were replaced by normal rabbit IgG (Vector). The specificity of the antibody to Ae2 was tested on tissues of *Ae2_{a,b}^{-/-}* mice. To localize osteoclasts, sections (not subjected to antigen retrieval in hot citrate buffer) were first immunostained for Ae2, rinsed in PBS and then stained for tartrate resistant acid phosphatase (TRACP, a cytoplasmic marker for osteoclasts), using a Leucocyte Acid Phosphatase kit, 386A, Sigma Diagnostics, St Louis, MO, USA).

RESULTS

Immunostaining in developing incisors

Incisors of rodents are continuously erupting and form dental tissues throughout life. Parasagittal sections through the mandible of wild type adult mice revealed all stages of incisor tooth development from the apical end where new cells are generated towards the incisal end where the tooth erupts into the oral cavity and is gradually lost by attrition.

Presecretory and secretory stage ameloblasts of wild type adult mice were negative for Ae2 (Fig. 1a, 1b). Membranes and cytoplasm of other cells of the enamel organ (stratum intermedium and particularly the cells of outer enamel epithelium in close contact with blood vessels of the surrounding connective tissue) reacted weakly with anti-Ae2 (Fig. 1b). Membranes and cytoplasm of red blood cells within blood vessel lumina were intensely stained (Fig. 1a)

The most prominent staining for Ae2 in mouse incisors started in ameloblasts in late-secretory stage/transitional stage and staining became intense during maturation stage (Fig. 1a). Staining intensity increased as development progressed in incisal direction and was highest in cells near incisal end closely before tooth eruption (Fig. 1a, second part, bottom right). Staining in maturation stage ameloblast was cytoplasmic and in basolateral membranes (Fig. 1c, 1d). Remarkably not all maturation stage ameloblasts were immunostained equally. Two types of gaps of negative staining were noted. The first type contained a small group of maturation stage ameloblasts that abruptly turned immunonegative and shortly after became suddenly positive again (Fig. 1a brackets, Fig. 1e). In adult mouse incisors about three of such sharp gaps were noted. In incisors of 1-2 week old mice and hamsters normally only one such gap was seen (Fig. 1f). Rat incisors were not examined for this phenomenon. The second type of gap contained small irregularly stained maturation stage ameloblasts with a fuzzy boundary and these gaps were less conspicuous than the sharp gaps (Fig. 1a, black arrows). The basal part of the cells in these fuzzy gaps was immunopositive but the distal part not. A weak membrane bound staining was furthermore noted in cells of Malassez, the epithelial remnants of the Hertwig epithelial root sheath (Fig. 3a).

The odontoblastic layer of adult mouse incisors was immunonegative but in newly born hamsters a weak reaction was noticed in odontoblasts distally from the nuclei in the area of the Golgi apparatus (not shown). Dental pulp cells were negative but intense staining was always seen in erythrocytes in pulp blood vessels (not shown). No staining was found in negative controls in which non-immune IgG replaced the primary antibody (not shown).

In *Ae2_{a,b}^{-/-}* mice the maturation stage ameloblasts were less well organized, had shortened and polarization of the nuclei was lost. These cells as well as all other enamel organ cells completely failed to stain for Ae2 (Fig. 1g), validating the specificity of the antibodies. Only erythrocytes present in blood vessels stained intensely.

Immunostaining in developing molars

Unlike incisors rodent molar tooth germs develop within a limited time frame, with enamel and crown dentin formed roughly the first 2-3 weeks of age. Basically, in all three rodent species similar findings were obtained for molar tooth germs as for incisors. A weak staining (hamster) or no staining (rat and mice) for Ae2 was noted in secretory phase ameloblasts in the Golgi area (Fig. 2a) but intense cytoplasmic and basolateral staining in maturation stage ameloblasts that had finished deposition of matrix (all three species; Fig. 2b, 2c). In molar tooth germs, no sharply delineated gaps of negative staining maturation ameloblasts were seen as in incisors, only groups with fuzzy boundaries and partially staining ameloblasts (Fig. 2c). Other cells of the enamel organ soft tissue also stained but far less intense than maturation stage ameloblasts (Fig. 2b, 2c).

In hamster but not in mice and rats the Golgi area of young odontoblasts both in crown and forming root was immunopositive as well. This staining was strong when the Golgi apparatus was round (Fig. 2a, 2c-e) and much less intense when Golgi apparatus was elongated at later stages when cell bodies were long and cells more developed (Fig. 2c). Pulpal cells did not stain for Ae2. During root formation discrete staining was also seen in the Golgi area of the fibroblasts of the forming periodontal ligament (Fig. 2d).

Immunostaining in developing bones

Most conspicuous staining for Ae2 in bone tissue of wild type mice, rats and hamsters was seen in large multinucleated cells lining the bone surface of the tooth socket particularly in front of the tip of the incisors with intensely stained ameloblasts (Fig. 3a) and above the developing crowns of the molars (not shown). Staining in these cells was confined to the basolateral membranes and was not present in the distal membranes in contact with the bone surface (Fig. 3b). Double staining for Ae2 and TRACP identified these cells as osteoclasts (Fig. 3c). Small mononuclear TRACP-positive cells, possibly osteoclast precursors near blood vessels or in the soft connective tissue away from bone were negative for Ae2.

Osteoclasts in the periodontium of adult *Ae2_{a,b}^{-/-}* mice failed to stain for Ae2 (Fig. 3d) in contrast to osteoclasts of wild type littermates (Fig. 3e). However, these immunonegative osteoclasts were located in resorption pits indicating they were active similar as wild type osteoclasts. Erythrocytes stained positive in both wild type and mutated tissues.

Periosteal cells at some distance from the osteoblast layer lining the bone surface were negative but weak to moderate staining for Ae2 became apparent in the Golgi area of highly active osteoblasts and young osteocytes, but not in older osteocytes, both in embryonic woven bone (Fig. 3f) and trabecular bone (Fig. 3g)

Histological changes in *Ae2^{-/-}* mice with general Ae2 isoform disruptions

Previously we showed that the absence of Ae2a, Ae2b1 and/or Ae2b2 isoforms resulted in decreased enamel mineralization but had no effect on tooth eruption and on structure of jaw bone and dentin compared to wild type mice (Lyaruu et al., 2008). Visual inspection of the *Ae2^{-/-}* mice with a more severe phenotype in which protein disruption involved all Ae2 isoforms had indicated that teeth failed to erupt into the oral cavity (Gawenis et al., 2004). Histology confirmed this. In wild type and *Ae2_{a,b}^{-/-}* mice, incisors and molars had erupted or were erupting (Fig. 4a) whereas in the severely affected *Ae2^{-/-}* littermates the teeth were

smaller, remained in the jaws and were still surrounded by bone (Fig. 4b). The roots developed very poorly, were distorted and in close contact with bone (Fig. 4c). The shape of the crowns changed although crown development was relatively normal. In wild type mice maturation stage ameloblasts were well organized and located at some distance from overlying bone (Fig. 4d) while in the *Ae2^{-/-}* mice these cells were as irregular as seen in the *Ae2_{a,b}^{-/-}* mice. (Fig. 4e). Abnormalities in crown and root development (small local disruptions, or cyst formation in enamel organ) were seen at locations where bone tissue was very close or in contact with developing dental tissues not seen in wild type mice. Crown dentin was remarkably well developed, of the tubular type and of normal structure except for a widening of predentin layer and formation of some globular dentin (Fig 4b, 4c). Secretory ameloblasts and odontoblasts had a prominent well developed Golgi apparatus and seem to function as in wild type.

Bone formation in the *Ae2^{-/-}* jaws however was severely affected. (Fig. 4f, 4g); bone was poorly formed, trabeculae were very thin, capricious and with large areas of osteoid compared to wild type bone. Osteocytes were round and large, embedded in widely spaced irregular osteocyte lacunae and lacunar walls stained dark blue after staining with Richardson. Wild type osteocytes and their lacunae were much flatter and clearly oriented. Osteoblasts in *Ae2^{-/-}* tissues were less organized along the bone surface as in wild type bone, which was thicker, regularly structured and covered with a clear layer of osteoblasts. Very conspicuous in bone of *Ae2^{-/-}* mice were large numbers of giant multinucleated osteoclasts that seemed to be at least partially functional as indicated by the presence of many resorption lacunae and cement/reversal lines.

DISCUSSION

Our data show that in orofacial skeletal tissues Ae2 is present in two distinctly different locations: (1) in basolateral membranes of maturation stage ameloblasts and osteoclasts and (2) in the Golgi apparatus of many other cell types. It is feasible to assume that these differences reflect different functions of Ae2 in the various cell types.

Ae2 and ameloblasts

We previously showed that Ae2 is present in basolateral membranes of maturation stage ameloblasts in adult mouse incisors and that absence of Ae2a, Ae2b1 and/or Ae2b2 in *Ae2_{a,b}^{-/-}* mice significantly reduced calcium content in maturation stage enamel of incisors (Lyyaruu et al., 2008). We proposed that this plasma membrane-bound Ae2 was involved in intracellular pH regulation by exchanging bicarbonate for chloride and that an absence of Ae2 interferes with terminal mineralization of enamel. The changes in mineral content of enamel in molars were considerably less than in incisors (Lyyaruu et al., 2008). One could argue that pH regulation during enamel development in molars is different from that in incisors to explain that the effect of the absence of Ae2 is less severe in molars. Here we report that also in wild type developing molars, Ae2 staining is present in maturation stage ameloblasts in all three species, indicating that in molar tooth development Ae2 is functional as well. Why the effect of Ae2 disruption in molars is less severe than in incisors is unknown.

Our polyclonal antibody recognizes a portion of the C-terminal end present in all five isoforms of Ae2 as well as in Ae1. Maturation stage ameloblasts of adult *Ae2_{a,b}^{-/-}* mice did not stain with our antibodies, but erythrocytes stained intensely. Our antibody to Ae2 crossreacts with Ae1 that is abundantly present in erythrocytes (Alper et al., '97). This crossreaction explains the strong immunostaining in erythrocytes both in wild type and in *Ae2_{a,b}^{-/-}* tissues. These data validate the specificity of our antibody and identify the Ae2 isoform(s) in maturation ameloblasts to be either Ae2a, Ae2b1 or Ae2b2, or a combination

of these isoforms but not Ae1 or the Ae2c isoforms. Because of the extreme difficulty of breeding these mutants we could not conclusively validate this same feature in ameloblasts of developing molars.

Paine et al. (2008) examined molars and incisors of 3-day old wild type mice for the presence of Ae2 and reported Ae2 staining of the apical membranes of secretory stage ameloblasts. We found that secretory stage ameloblasts either did not stain for Ae2 (mouse, rat) or only weakly stained the Golgi apparatus (hamster) but never the apical or lateral plasma membranes. We also found no structural changes in secretory ameloblasts or secretory stage enamel in either *Ae2_{a,b}^{-/-}* or *Ae2^{-/-}* mice. Thus, our data do not support a function of Ae2 in transcellular bicarbonate secretion by secretory ameloblasts as proposed by Paine et al. (2008). Their PCR data on the presence of Ae2a in a secretory ameloblast-like transformed cell line likely reflects Golgi associated Ae2, rather than plasma membrane bound Ae2.

We also found no basic structural differences between the effect of *Ae2* gene disruption in *Ae2_{a,b}^{-/-}* and *Ae2^{-/-}* mice on enamel formation or ameloblasts structure, except for secondary changes in the latter that seemed caused by the failure of the overlying bone tissue to become timely degraded. Accordingly, the Ae2c1 polypeptide appears to play no direct role in tooth development.

Normally, maturation ameloblasts modulate in cycles from a cell-type with a ruffle-ended membrane facing the enamel surface into one with a smooth-ended membrane and back to the ruffle-ended type again. In the adult rat incisor, 5-7 of such modulation cycles are found along the entire length of developing enamel. Most prevalent are ruffle-ended ameloblasts that make up 80% of the maturation ameloblasts (Smith, '98). The pH of the enamel below the ruffle-ended cells is slightly acidic (pH 6.1-6.8), whereas pH in enamel below smooth-ended cells is physiologic (pH 7.2-7.4) (Smith, '98). We found in adult wild type mouse incisor maturation stage small groups of ameloblasts virtually negative for Ae2. It is tempting to assume that these gaps in the staining for Ae2 are the smooth-ended ameloblasts. Assuming this to be the case, the abrupt change from a positive staining into a sharp unstained gap suggests that Ae2 protein synthesis is shut off suddenly and the Ae2 protein in the plasma membrane broken down very quickly at the onset of smooth ended phase to be synthesized again shortly later at the onset of ruffle ended stage. How this rapid change is regulated is unknown. The changes in the pH in the mineralizing enamel below the ruffle-ended ameloblasts may be the trigger to change the pattern of Ae2 synthesis in order to regulate intracellular pH, similar as pH changes in the lumen of renal tubular epithelium that upregulate Ae2 synthesis during acidosis (Quentin et al., 2004; Frische et al., 2004). Cyclic modulation of maturation stage ameloblasts is essential for completion of enamel mineralization; interruption of modulation as seen at exposure to fluoride reduces enamel hardness and breakdown of matrix proteins (Smith et al., '93).

Ae2 and osteoclasts—Basolateral membranes of osteoclast also stained prominently for Ae2 (Jansen et al., 2009). Within the sensitivity of immunostaining technique absence of immunostaining in osteoclasts in *Ae2_{a,b}^{-/-}* mice demonstrates that these cells normally express Ae2a, Ae2b1 or Ae2b2 or combinations of these isoforms, rather than the Ae2c1 isoform. Our finding in wild type animals that TRACP-positive small mononucleated cells in soft tissue stroma around blood vessels are immunonegative for Ae2 but large multinucleated TRACP-positive osteoclasts are immunopositive indicates that Ae2 is a rather late marker in the osteoclast differentiation pathway, likely produced when also vacuolar type of H⁺-ATPase is synthesized, needed for extrusion of protons into the bone. The observation that in *Ae2_{a,b}^{-/-}* mice teeth erupt and that osteoclasts in periodontium are

resorbing bone supports the concept that osteoclasts in craniofacial area are functional despite the lack of Ae2.

The failure of teeth to erupt in *Ae2*^{-/-} mice with a severe phenotype cannot be attributed to inactivation of the Ae2c1 isoform in osteoclasts, but is likely due to systemic changes that influence bone metabolism and osteoclast activity indirectly. This is also suggested by the widening of predentin and formation of globular dentin, seen after disturbance of calcium metabolism or vit D deficiency (Weinmann and Schour, '45). The general disruption of all Ae2 isoforms in *Ae2*^{-/-} mice is associated with high morbidity and mortality of the animals after 15 days of age. Failure to secrete gastric acid, hyperkeratosis of epithelium of non-glandular mucosa of the stomach and other abnormalities of gastric epithelium indicate that the stomach alterations in these mutants are more severe than those found in the *Ae2*_{a,b}^{-/-} mice (Gawenis et al., 2004; Recalde et al., 2006). We hypothesize that the osteopenic changes seen in alveolar bone in the *Ae2*^{-/-} mice with a general Ae2 disruption are associated with the poorly functioning stomach which causes malnutrition, disturbed calcium and phosphate metabolism and/or disruption of production of bone stimulating factors made by stomach epithelium, such as ghrelin (Fukushima et al., 2005; Ariyasu et al., 2001).

However, we cannot totally exclude that differences in genetic background are responsible for the different bone phenotypes seen in the *Ae2*_{a,b}^{-/-} and the *Ae2*^{-/-} mice. By difference in background some genes may be more expressed in the *Ae2*_{a,b}^{-/-} than in *Ae2*^{-/-} mice. This may partially compensate for the more severe phenotype and give rise to a milder phenotype. Jansen et al., 2009 found that osteoclasts in orofacial bone also express the Na⁺/HCO₃⁻ cotransporter (*Slc4a4*) but osteoclasts in long bones do not. This finding strongly suggested that *Slc4a4* can compensate the lack of functional Ae2 isoforms in osteoclasts in *Ae2*_{a,b}^{-/-} mice and thus prevents the development of an osteopetrotic phenotype in orofacial bones. Since osteoclasts in long bone do not express *Slc4a4* loss of functional Ae2 is not compensated which results in osteopetrosis in long bones (Jansen et al., 2009). Hence, different expression levels of genes as *Slc4a4* between targeted mouse strains with different genetic background may influence the severity of gene targeting.

Ae2 and Golgi—We detected immunostaining in the Golgi apparatus of a variety of hamster cell types that are directly involved in synthesis of extracellular matrix proteins, including secretory ameloblasts, odontoblasts, osteoblasts, young osteocytes and differentiating periodontal ligament cells. This is consistent with reports that the Golgi complex of many cell types such as rat fibroblasts, rat osteosarcoma cell line ROS 17/2.8, mouse NIH 3T3 fibroblasts, articular cartilage cells and human osteoblast cell cultures stain for Ae2 (Kellokumpu et al., '88; Golding et al., '97; Mobasheri et al., '98; Holappa et al., 2001). The most prominent staining in the Golgi area was found in youngest cells in newly born animals that were highly active in depositing and secreting matrix proteins.

Which isoform Ae2 is present in the Golgi apparatus is not clear but likely it is the Ae2a isoform as found in primary human embryonic fibroblasts (Holappa et al., 2001) and in immortalized enamel organ mouse epithelium cells with ameloblast-like characteristics (Paine et al., 2008).

The function of the Golgi associated Ae2 in skeletal tissues is also not clear. Its distinct location in the Golgi apparatus suggests that Ae2 is involved in pH regulation in the Golgi complex. By controlling pH in the Golgi Ae2 could control posttranslational modification of matrix proteins, their packaging, and transfer to the outer membrane and exocytosis. A structural role of Ae2 in stabilizing the structure of the Golgi complex has also been proposed (Holappa et al., 2004). Alternatively, it has been speculated that Ae2 is involved in uptake of sulphate in the Golgi complex to concentrate this anion for posttranslational

modification of matrix proteins (Kellokumpu et al., '88; Mobasheri et al., '98). In our *Ae2_{a,b}^{-/-}* mice we found no major structural changes in skeletal connective tissue cells or in the gross anatomy and histological structure of dentin and bone or their mineral content (Lyyaruu et al., 2008). Likewise, entire *Ae2* protein disruptions in *Ae2^{-/-}* mice was neither associated with marked changes in the structure or function of odontoblasts and secretory ameloblasts nor with other skeletal tissue cells which in wild type littermates show a positive staining for *Ae2* in their Golgi apparatus. As discussed before, changes in bone formation in the *Ae2^{-/-}* mice were attributed to systemic changes rather than to changes in osteoblasts. Our data suggest that *Ae2* in the Golgi apparatus is not essential for the apparently normally functioning of skeletal tissue cells.

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Fig. 1. Ae2 localization in rodent incisors

(a) Major part of an adult mouse mandibular incisor, presented in two parts. Left top represents apical end with secretory stage ameloblasts (SA) that develop downwards into maturation ameloblasts (MA). The part at the bottom (asterisk) continues in the second part at the top (asterisk) towards incisal end (bottom). Note that secretory ameloblasts are immunonegative, that transitional ameloblast (TA) gradually become positive, and that maturation ameloblasts are intensely stained, particularly near the incisal end. The immunopositive maturation zone is abruptly interrupted three times by small groups of ameloblasts that are virtually negative (sharp gaps; brackets), whereas other groups of ameloblasts stain variably (fuzzy gaps, arrows). Intense staining is seen in erythrocytes in the lumen of blood vessels (BV). Some erythrocytes are detached and spread over the enamel. E, enamel matrix (blue). ES, enamel space; D, dentin (pink); B, Bone.

(b) A high power detail of mouse incisor secretory ameloblasts (SA), negative for Ae2, whereas the outer enamel epithelium of the enamel organ (EO) is weakly positive.

(c, d) A longitudinal (c, not decalcified, E enamel) and cross section (d) through mouse maturation stage ameloblasts showing the membrane associated staining for Ae2.

(e) Detail of an abrupt change in Ae2 staining in maturation stage ameloblast (MA) of an adult mouse incisor. PL: papillary layer (epithelial tissue)

(f) Abrupt change in Ae2 staining in maturation stage in neonatal hamster incisor. Weak staining is seen in the papillary layer (PL).

(g) Absence of staining of maturation stage ameloblasts (MA) in an adult *Ae2_{a,b}^{-/-}* mouse incisor. Note disorganization of ameloblasts and papillary layer (PL). Strong staining is seen in erythrocytes in blood vessel (BV) which are stained for Ae1.

Original magnifications: Fig.1a, 50x; Fig 1b-d & 1f, 400x; Figs 1e&g, 250x;. Figs 1c&1d, counterstaining methyl green, Figs 1a,b, e &g, hematoxylin, Fig 1f hematoxylin and eosin. Scale bars represent indicated length in micrometer

Fig. 2. Localization of Ae2 in developing hamster molar tooth germs

(a) A weak immunostaining (brown) is seen in the Golgi area of secretory ameloblasts (SA) depositing the first layer of enamel (E, blue). The odontoblasts (o) contain a well delineated staining in the Golgi area; pulp cells do not stain. D, dentin. Asterisks indicate shrinkage artefacts.

(b) Low power image of a developing molar indicating the different intensities of staining in the various cell types. Secretory ameloblasts (SA) and enamel organ cells (EO) stain weakly, maturation stage ameloblast (MA) near the tip of the cusp and erythrocytes in dental pulp (DP) intensely. Odontoblast staining is weak and pulp cells do not stain.

(c) Detail of molar cusp. Maturation stage ameloblast (MA) are intensely stained and staining is continuous, but at some locations (arrows) staining in distal part of a group of cells is weak or lacking, reminiscent of the fuzzy gap in incisors. Note the elongated Golgi apparatus in the odontoblasts (O). EO, enamel organ; OE, oral epithelium. Asterisk indicates shrinkage artefact

(d) Staining in cytoplasm of fibroblasts (arrows) of developing periodontal ligament (PDL) along the root dentine (RD) formed by the root odontoblasts (O). BV, blood vessel

(e) Staining along the forming root dentin (RD) near the cemento-enamel junction (CEJ). B, bone with positive osteocytes; PDL, periodontal ligament. Strong Golgi staining is seen in both young root and crown odontoblasts (O), not seen in dental pulp cells (DP). BV, blood vessel.

Original magnifications Figs 2a&2c, 250x; 2b, 50x; Figs 2d&2e, 400x; Fig 2d methyl green; Figs 2a-c& Fig 2e haematoxylin. Scale bars represent indicated length in micrometer.

Fig. 3. Ae2 localization in osteoclasts and bone cells

(a) Low power view of tip of incisor of lower hamster jaw containing strongly positive maturation stage ameloblasts (MA) with cytoplasmic and membrane staining. No counterstaining. ES, enamel space; D, dentin. The enamel organ (EO) is weakly stained and separated from the alveolar bone (B) by soft connective tissue (SCT) with blood vessels (BV). The inner side of the bone is being resorbed by osteoclasts whose plasma membranes are stained (arrows) to make way for tooth eruption. M, epithelial cells of Malassez in the developing root analogue (lingual portion) stain weakly.

(b) Large multinucleated cell at the bone (B) surface with basolateral staining for Ae2; the arrow indicates the membrane portion containing the alleged ruffled border that is immunonegative (7 days old hamster; nuclei counterstained blue with hematoxylin).

(c) Double staining for Ae2 (brown) and TRACP (pink), counterstained with methyl green (green blue, nuclei) identifying the large multinucleated cells as osteoclasts (wild type mouse, 9 days old).

(d) Osteoclast (OCL) in a resorption lacunae at the alveolar bone (B) surface of the periodontal gap of an adult *Ae2_{a,b}^{-/-}* mouse immunonegative for Ae2. Note that the red blood cells in the vessels of the periodontal ligament are immunopositive, likely staining for Ae1.

(e) Control section of osteoclast (OCL) against alveolar bone (B) of periodontium of wild type adult littermate mouse positive for Ae2.

(f) Osteoblasts (OB) lining embryonic woven bone, and osteocytes (OC) immunostain in Golgi area (9 days hamster). Note that periosteal cells (upper part) at some distance from the bone surface are immunonegative.

(g) Osteoblasts (OB) forming bone (B) trabeculae in the jaw as well as osteocytes (OC) embedded in bone are immunopositive (9 days hamster).

Original magnifications: Fig. 3a, 250x; Figs 3b, 3c, 3f & 3g, 1000x; Figs 3d&3e, 400x; hematoxylin counterstaining. Scale bars represent indicated length in micrometer.

Fig. 4. Effect of disruption of all Ae2 isoforms in the $Ae2_{a,b}^{-/-}$ mice on tooth eruption and jaw bone structure

(a) Low power of two wild type molars that are erupting into the oral cavity (ORC). Roots are well developing. Root dentin (RD) is surrounded by bone (B) present at some distance from the roots. G, gingiva.

(b) Low power of two knockout molars. The molars are smaller than wild type and irregularly formed. Bone (B) is still covering the tooth crowns. Enamel space (ES) on top of the dark blue crown dentin contains no enamel matrix indicating maturation stage has progressed. Roots are distorted and short. Arrow indicates globular dentin matrix in the predentin (pd). Predentin is wider than usual. RD is root dentin, C is a cyst in the enamel organ

(c) Detail of 5b. The root is poorly developed and bone (B) is very close by. Odontoblasts are well structured whereas predentin (PD) is wider than normal. D, dentin.

(d) Wild type incisor with maturation stage ameloblasts (MA) near enamel space (ES). Double arrow indicates distance between ameloblasts and bone (B) surface.

(e) $Ae2^{-/-}$ incisor with maturation stage ameloblasts (MA), shorter and more irregular than in wild type. Note that bone (B) is closer to the ameloblast layer than in Fig 5d. Arrow in dentin (D) indicates a defect (not stained) in blue stained dentin.

(f, g) Alveolar bone covering incisors in $Ae2^{-/-}$ (f) and wild type (g) mice. In $Ae2^{-/-}$ mice bone trabeculae are very thin and irregular with widely spaced osteocyte lacunae. There is relatively much osteoid. OC, osteocytes; OCL, osteoclasts; OB, osteoblasts.

(h) Alveolar bone of an $Ae2^{-/-}$ mouse with giant multinuclear osteoclasts (OCL). Arrow indicates a dark blue stained reversal line at the surface of a resorption pit indicating bone remodelling.

(i) A giant multinuclear osteoclast at the surface of irregularly formed bone with large osteocyte lacunae in an $Ae2^{-/-}$ mouse.

Plastic sections, Richardson staining. Original magnifications: Fig. 4a-b, 50x; Fig 4c, 250x; Figs 4d-g, 400x. Scale bars represent indicated length in micrometer.