R.S. Lacruz^{1*}, C.E. Smith²,
I. Kurtz³, M.J. Hubbard⁴,
and M.L. Paine^{1*}

¹Center for Craniofacial Molecular Biology, Herman Ostrow School of Dentistry, University of Southern California, Los Angeles, USA; ²Facility for Electron Microscopy Research, Department of Anatomy & Cell Biology, and Faculty of Dentistry, McGill University, Montreal, Canada; ³Division of Nephrology, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA; and ⁴Departments of Paediatrics and Pharmacology, The University of Melbourne, Australia; *corresponding authors, paine@usc.edu and rodrigo@usc.edu

J Dent Res 92(2):122-129, 2013

ABSTRACT

Fully matured dental enamel is an architecturally and mechanically complex hydroxyapatite-based bioceramic devoid of most of the organic material that was essential in its making. Enamel formation is a staged process principally involving secretory and maturation stages, each associated with major changes in gene expression and cellular function. Cellular activities that define the maturation stage of amelogenesis include ion (*e.g.*, calcium and phosphate) transport and storage, control of intracellular and extracellular pH (*e.g.*, bicarbonate and hydrogen ion movements), and endocytosis. Recent studies on rodent amelogenesis have identified a multitude of gene products that appear to be linked to these cellular activities. This review describes the main cellular activities of these genes during the maturation stage of amelogenesis.

KEY WORDS: amelogenesis, biomineralization, enamel, ion transport, pH homeostasis, endocytosis.

DOI: 10.1177/0022034512470954

Received October 2, 2012; Last revision November 20, 2012; Accepted November 20, 2012

A supplemental appendix to this article is published electronically only at <http://jdr.sagepub.com/supplemental>.

© International & American Associations for Dental Research

New Paradigms on the Transport Functions of Maturation-stage Ameloblasts

INTRODUCTION

The mineralization of dental enamel is a unique and complex process involving the sequential passage of mineral ions across cell and fluid barriers. The 2 main stages recognized in the formation of enamel are the secretory and maturation stages, both regulated by the formative ameloblast cells. Secretory ameloblasts are tall, polarized cells that often reach up to 60 to 100 μm in length while maintaining a relatively narrow 5- to 8- μm diameter. The secretory stage provides an organic template form in which the enamel crystals grow under spatio-temporal guidelines dictated by the enamel-specific proteins secreted by the ameloblasts (Smith, 1998). The main goal at this stage is to build, in an organized manner, the full volume of enamel tissue, consisting of mineral crystals bundled into structures called prisms (rods) and other mineral crystals filling the spaces between the prisms (interrod). The patterns of enamel scaffolding thereby created are complex and largely species-specific; thus, this review will focus on rodents, one of the best-known enamel models. Enamel maturation is synonymous with calcification and mineralization, widely used terms in the literature (Glick, 1979). Maturation-stage ameloblasts re-organize and change morphology to become shorter and somewhat more ‘squat’ cells. These cells then orchestrate the mineralization of enamel, creating a biomaterial containing 95% mineral by weight (Smith and Nanci, 1996). They accomplish this by removing the organic content and water, originally provided by the cells, and by actively engaging in the organized passage of various ions required for the expansion of enamel mineral crystals (Smith, 1998; Hubbard, 2000). The intricate pathways of active movement of ions across ameloblasts and in between these cells are the primary focus of this review. Moreover, our research, as well as research conducted by other scientists, describes similarities between the physiology of ion transport by ameloblasts and epithelial-derived cells of other organs, allowing us to draw parallels between enamel formation and the formation of non-mineralizing tissues such as the pancreas and kidney, and because of this, the transport properties for these 2 soft-tissue organs will be briefly introduced.

CONTROL OF pH IN PANCREATIC DUCT CELLS AND KIDNEY PROXIMAL TUBULES

Although all cells regulate their intracellular pH *via* a wide variety of hydrogen ion (H^+) and bicarbonate (HCO_3^-) transport proteins, there are specific tissues that mediate vectorial transepithelial bicarbonate transport. For example,

the kidney proximal tubule absorbs ~3,000 mmol of HCO_3^- per day, whereas the pancreatic duct secretes ~300 mmol of HCO_3^- per day. Salivary glands and intestine also mediate transepithelial bicarbonate transport. Vectorial transport in these epithelia is facilitated by the polarized expression of specific acid-base transporters. We will briefly discuss bicarbonate secretion in the pancreas and bicarbonate absorption in the proximal tubule as model systems. As evidenced by the research summarized in this section and in the following sections, many transporters identified in the pancreas and kidney are also associated with similar transport functions in the enamel organ.

In the pancreas, basolateral HCO_3^- uptake into pancreatic duct cells is mediated by the electrogenic $\text{Na}^+/\text{HCO}_3^-$ co-transporter NBCe1-B, which in turn is regulated by IRBIT (adenosylhomocysteinase-like 1) (Yang *et al.*, 2011). The duct also expresses the sodium hydrogen exchanger NHE1 and the anion exchanger AE2 at the basolateral pole, although their contribution to transepithelial HCO_3^- transport is minimal (Yang *et al.*, 2011). Secretion of apical HCO_3^- is mediated by the cystic fibrosis transmembrane conductance regulator (CFTR) and the solute carrier Slc26a6 (Singh *et al.*, 2010). CFTR has limited permeability to HCO_3^- . The major apical HCO_3^- transporter is SLC26a6 (pendrin-like protein 1), functioning as a $2\text{HCO}_3^-/1\text{Cl}^-$ exchanger. SLC26 transporters and CFTR are characterized by mutual regulation. The STAS domain of SLC26 transporters binds the R domain of CFTR and can activate both (Singh *et al.*, 2010). The sodium hydrogen exchangers NHE2 and NHE3 are also expressed in the apical membrane and may function when bicarbonate secretion is decreased to salvage luminal HCO_3^- (Singh *et al.*, 2010). NHE3 interacts with CFTR via PDZ-containing scaffolding proteins and is regulated by CFTR and IRBIT. Together, NBCe1-B, CFTR, and Slc26a6 achieve a luminal HCO_3^- concentration of ~140 mM (Singh *et al.*, 2010; Yang *et al.*, 2011). The carbonic anhydrase (CA) inhibitor acetazolamide significantly blocks ductal HCO_3^- secretion (Sener *et al.*, 2007). CA2, CA4, CA9, and CA12 are expressed in the pancreatic duct (Lee and Muallem, 2008). The role of each CA isoform has not yet been clearly delineated.

In the renal proximal tubule, bicarbonate uptake across the apical membrane is indirect *via* the protonation of luminal HCO_3^- . Proton secretion is mediated by apical NHE3 (Girardi and Di Sole, 2012). Luminal HCO_3^- is converted CO_2 , which is passively absorbed across the luminal membrane. In proximal tubule cells, intracellular CO_2 is re-converted to HCO_3^- and a proton (Girardi and Di Sole, 2012). The proton is recycled across the apical membrane *via* NHE3, and HCO_3^- is absorbed across the basolateral membrane *via* the electrogenic $\text{Na}^+/\text{HCO}_3^-$ co-transporter NBCe1-A. As in the pancreatic duct, various CA isoforms are expressed (CA2, CA4, CA9, CA12) (Purkerson and Schwartz, 2007).

ENAMEL MATURATION

Ameloblasts are highly polarized cells, acquiring this characteristic apical-basal polarity soon after differentiation. This polarity does not change until the enamel organ becomes reduced at the end of amelogenesis. During the post-secretory stage,

enamel crystals experience large volumetric expansion within a mildly acidic environment, concurrent with rapid nucleation events and release of H^+ (Smith and Nanci, 1995; Smith, 1998). A common mechanism to produce this volumetric expansion in mammals is associated with supersaturation of the enamel fluid, primarily with calcium and phosphate ions, thereby optimizing the driving force for mineralization and assisting to degrade and remove organic matrix to create space for crystal expansion, processes mediated by the ameloblasts (Nanci and Smith, 1992; Smith, 1998; Lacruz *et al.*, 2012a).

Secretory-stage ameloblasts present a tight barrier for diffusion of mineral ions, as suggested by differential concentrations of various elements between the tissue and the enamel fluid (Takano, 1995; Hubbard, 2000). This is not the case in maturation-stage ameloblasts and associated cell layers. One of the most intriguing and as-yet-unexplained aspects of ameloblast maturation is the cyclical and rapid changes in morphology (within hours) from what has been described as “ruffle-ended” to “smooth-ended” cell phenotypes (Josephsen and Fejerskov, 1977; Smith, 1998). Smooth-ended ameloblasts (SA) are presumed to be impermeable to ion movement across the basal pole, whereas ruffle-ended ameloblasts (RA) have this barrier at the apical pole (Smith, 1998; Hubbard, 2000). This dynamic permeability pattern allows for bi-directional diffusion of small molecules into, and removal from, the enamel area (Smith, 1998; Josephsen *et al.*, 2010). Prior to enamel maturation, the stratum intermedium (the cell layer in contact with the basal pole of ameloblasts), the stellate reticulum, and the outer dental epithelium undergo morphological transformations, resulting in the formation of the papillary layer (PL) (Garant and Nalbandian, 1968b; Sasaki *et al.*, 1990). The paucity of protein-synthesizing organelles (rough ER and Golgi) suggests little protein-secretory function for the PL. However, the well-developed mitochondrial apparatus has been implicated as an important indicator of substantial energy-generating potential, permitting, for example, active ion transport function (Garant and Nalbandian, 1968a; Hubbard, 2000). The PL is deeply invaginated by numerous blood vessels surrounded by a basal membrane, thereby allowing for more rapid diffusion of ions and nutrients into the ameloblast layer (Decker, 1963). The proximity of capillary systems to ameloblasts ultimately permits rapid incorporation of ions into the enamel crystals (Takano, 1995) and improves the efficiency of local tissue fluid-buffering capacity. Extensive gap junctions connect the basal poles of maturation-stage ameloblasts to the PL, allowing for rapid intercellular communications (Sasaki *et al.*, 1990).

CALCIUM TRANSPORT

Calcium regulation manifests as one of the more difficult challenges facing enamel-forming cells, since they produce the most highly calcified tissue of all. Recent studies support a new paradigm for calcium transport (termed “calcium transcytosis”) that now warrants further investigation in dental tissues. For over 20 years, enamel biologists have contemplated a perplexing question about the need for enamel-forming cells to supply calcium in sufficient bulk for mineralization, yet avoid the potentially

cytotoxic effects of elevated cytosolic calcium (Hubbard, 2000). Cell viability depends on cytosolic-free calcium being maintained at low average concentrations ($\sim 0.2 \mu\text{mol/L}$). Although calcium-signaling transients are well-tolerated, more extreme increases can be toxic and even lead to cell death (Hubbard, 2000). Building from anatomical evidence that the enamel epithelium acts as a semi-permeable barrier to calcium, previous biochemical studies revealed an exceptionally high abundance of calcium-handling proteins in enamel-forming rat and mouse cells. Moreover, various calcium-handling proteins were found to be up-regulated ~ 3 -fold relative to the secretion phase, closely paralleling the increased calcium flux at maturation (Hubbard, 2000). However, contradicting the “calbindin ferry” dogma long held by the calcium-transport field (based largely on studies of gut and kidney), the major calbindin (Calb1/CB28) was found to be down-regulated during maturation, CB28-null mice lacked the dental phenotype, and the other 2 types of calbindin (Calb2 and S100g) were recently ruled out as alternative calcium transporters (Turnbull *et al.*, 2004; Hubbard *et al.*, 2011). Instead of a cytosolic transport mechanism, the biochemical data invoked an organellar route involving endoplasmic-reticulum-related calcium stores (ER/Ca stores). As such, calcium transcytosis is conceptually appealing, because it encompasses safety against calcium cytotoxicity as well as providing a high-flux transcellular pipeline (Hubbard, 2000; Hubbard *et al.*, 2011).

Recent genome-wide transcript-profiling studies have strengthened this emerging paradigm, not only by illustrating that ameloblasts undergo a comprehensive molecular change at maturation, but also by invoking several new “key players” in calcium transcytosis (Lacruz *et al.*, 2011a, 2012a). When one considers how calcium enters the putative transcellular pipeline, it is tantalizing that Stim1, a plasmalemmal calcium channel component associated with ER/Ca-stores, was expressed at exceptionally high levels during maturation (~ 8 -fold increase over secretion at the protein level). At the other end of the putative pipeline, a recently discovered type of sodium-calcium exchanger (NCKX4; coded by *Slc24a4*) has been shown to be expressed at moderately high levels (Hu *et al.*, 2012). Importantly, unlike the 5 other isoforms of NCKX and all 3 isoforms of the “classic” NCX sodium-calcium exchangers (Okumura *et al.*, 2010), NCKX4 protein levels were up-regulated ~ 2 -fold at maturation, producing a particularly strong candidate for mineralization-related calcium extrusion (Hu *et al.*, 2012). In addition to novel machinery for calcium transport, the transcript studies identified S100a4 and Rcan1 as 2 calcium-signaling proteins up-regulated during maturation (Lacruz *et al.*, 2012a). Analysis of these transcriptomics data, collectively, has broadened our theory about how enamel-forming cells might accomplish safe handling of bulk calcium, by balancing sequestration within organelles against the ongoing need for calcium-signaling dynamics in the cytosol. Further investigation of these newly suggested mechanisms appears justified, given the practical significance of calcium regulation with respect to dental bioengineering and prevention of enamel malformations. Furthermore, when one considers the significance for other biological research, it is noteworthy that recent

calbindin-null studies failed to support the calbindin-ferry dogma in gut and kidney (although questions about compensation remain to be addressed) (Hubbard *et al.*, 2011). Murine-enamel models now stand as particularly strong comparators for future investigations into transepithelial calcium transport and the functions of calbindins.

PHOSPHATE AND SODIUM TRANSPORT

Whereas some advances have developed from an understanding of calcium transport in the enamel organ as discussed above, phosphate and sodium transport remains poorly defined. Phosphate homeostasis is essential for cellular function, bone formation, and DNA replication (Murer *et al.*, 2004). In enamel, phosphate is a fundamental element in the formation and growth of hydroxyapatite-based crystals (Smith, 1998). Most studies on phosphate transport in the enamel organ focus on observations of radiolabeled P (^{32}P or ^{33}P) injections in rodents to identify its incorporation into the enamel crystals or to identify phosphate concentrations at various developmental stages (Robinson *et al.*, 1974; Wennberg and Bawden, 1978; McKee *et al.*, 1989). The evidence described in these studies indicated that phosphate uptake increased in the maturation stage and might also be linked with the passive SA phase (Reith and Boyde, 1981). However, the mechanisms of phosphate transport remain obscure, and both transcellular and paracellular transport mechanisms may be involved. We have recently identified a member of the solute carrier 34 (SLC34) gene family as a possible candidate for phosphate transport in the enamel organ (Lacruz *et al.*, 2012a). The SLC34 gene family is composed of 3 sodium-phosphate cotransporters, or Na/Pi's, coded by genes *Slc34a1*, *Slc34a2*, and *Slc34a3*, and coded for the proteins NaPi-IIa, NaPi-IIb, and NaPi-IIc, respectively (Murer *et al.*, 2004). A 64-fold increase in the expression of NaPi-IIb (*Slc34a2*) mRNA was found in maturation-stage enamel organ cells relative to secretory-stage cells (Lacruz *et al.*, 2012a), providing a good candidate gene for major phosphate transport in the enamel organ. Alternative mechanisms for phosphate trafficking, from the cell to the enamel matrix, may play a role in amelogenesis, such as a possible storage and transport mechanism proposed for bone (Omelson and Grynpas, 2008). Here (Omelson and Grynpas, 2008), polyphosphates and/or calcium phosphates would be stored in the mitochondria and released to the extracellular matrix as needed through secretory vesicles. Polyphosphates would then be enzymatically cleaved into orthophosphates, allowing for hydroxyapatite (Hap) formation. For such an activity to occur, the expression of alkaline phosphatase would be required, and it has been shown that alkaline phosphatase activity is expressed at the apical pole of polarized ameloblasts, during both secretion and maturation (Kurahashi and Yoshiki, 1972).

Maintenance of basal cellular levels of sodium and potassium is typically achieved by the membrane-bound Na^+K^+ -ATPase pump (Glynn, 2002; Morth *et al.*, 2011). The Na^+K^+ -ATPase pump is active as a dimer of 2 protein subunits, referred to as the α -subunit and the β -subunit (Glynn, 2002; Morth *et al.*, 2011) and functions to move 3 Na^+ out of cells in exchange for 2 K^+ for every ATP that is hydrolyzed (Morth

et al., 2011). Four unique genes code the α -subunit, and 4 unique genes code the β -subunit, while the α/β dimer combination appears to be somewhat tissue-specific (Morth *et al.*, 2011). The presence of a Na^+, K^+ -ATPase pump in enamel organ cells has previously been investigated by chemical and biochemical approaches suggesting that expression of such a pump localizes in the lateral plasma membranes of secretory ameloblasts and the stratum intermedium, and also in the PL cells of rat incisor enamel organs (Kashgarian *et al.*, 1985; Garant *et al.*, 1987). Analysis of recent immunolocalization data, however, suggests that a Na^+, K^+ -ATPase pump, or at least one of the α subunits, localizes predominantly to the PL (Josephsen *et al.*, 2010).

H^+ /BASE TRANSPORT

Several review papers discussing pH regulation during amelogenesis are available (Smith *et al.*, 1996; Smith, 1998; Lacruz *et al.*, 2010a). During maturation, enamel organ cells express significant levels of carbonic anhydrases: Car2 and Car3 (intracellular CAs), Car6 (secreted), and Car12 (membrane-bound) (Lin *et al.*, 1994; Toyosawa *et al.*, 1996; Smith *et al.*, 2006; Josephsen *et al.*, 2010; Lacruz *et al.*, 2012b). Enamel organ cells, including ameloblasts, also express at least 2 bicarbonate carrier proteins (NBCe1 and AE2) (Lyaru *et al.*, 2008; Paine *et al.*, 2008; Bronckers *et al.*, 2009). Based on the available antibodies and immunolocalizations, various models have been presented in recent years illustrating the spatial location of these bicarbonate transporters (Lyaru *et al.*, 2008; Paine *et al.*, 2008; Bronckers *et al.*, 2009; Josephsen *et al.*, 2010) and an associated chloride channel CFTR (Bronckers *et al.*, 2010) (Fig. 1). Analysis of our data indicated that NBCe1 is located at the basal pole of polarized (secretory)-stage ameloblasts, allowing for bicarbonate movement from the PL cells and/or blood circulation into ameloblasts, while AE2 and CFTR are located at the apical pole of ameloblasts, allowing for movement of bicarbonate from ameloblasts to the enamel matrix (Paine *et al.*, 2008; Lacruz *et al.*, 2010b). For AE2, our immunolocalization data were generated from frozen, unfixed sections of the mandibular incisors of 3-day-old mice (Paine *et al.*,

Figure 1. Schematic representation of transepithelial transporters in pancreatic duct cells and ameloblasts. **(A)** Pancreatic duct cell. The main transepithelial transporters regulating pH via HCO_3^- movement, exhibited above, are modified from Park and Lee (2012). At the basolateral pole, HCO_3^- is regulated by NBCe1. Sodium uptake is mediated by NHE1, whereas extrusion involves Na^+/K^+ ATPases. Non-voltage-gated Na^+ channels may also contribute to Na^+ absorption at the basolateral pole (not shown). The apical pole of these cells expresses the $\text{Na}^+/\text{HCO}_3^-$ co-transporter (NBCn1) and the Na^+/H^+ exchanger (NHE3) that regulate Na^+ intake, whereas Cl^- is extruded by Cfr and exchanged by Slc26a6 for HCO_3^- . **(B)** Maturation-stage ameloblast. This model is not restricted to HCO_3^- transport but depicts our current knowledge of the proteins associated with the main activities of ameloblasts during maturation. Our model has been constructed based on previous reports (Bronckers *et al.*, 2010; Josephsen *et al.*, 2010) together with our own data. In this scheme, Ca^{2+} may be transported out of the cells at the apical pole by at least one of the NCKX members of sodium-calcium-potassium exchangers (NCKX4) (Hu *et al.*, 2012). Cl^- may be transported into the lysosomal system by Clcn7 to acidify luminal pH (Lacruz *et al.*, 2013). We base our diagram on an osteoclast model (Jentsch, 2007) that commonly includes H^+ ATPases in the endosomal-lysosomal system. These pumps, as well as the function of a variety of CLC transporters, are ubiquitous features of endosomes-lysosomes required for luminal acidification (Jentsch, 2007). Chloride is also transported at the plasma membrane by Cfr and may also be linked to the anion exchanger AE2, as has been proposed (Bronckers *et al.*, 2010). Two versions have been reported for the localization of AE2. In the schematic, AE2 localization at the apical pole was reported by Paine *et al.* (2008), whereas AE2 in the basolateral pole was described by Lyaru *et al.* (2008) and Bronckers *et al.* (2009). Carbonic anhydrases 2 and 6 (Car2, Car6) remain important sources of localized bicarbonate production, as described elsewhere (Lacruz *et al.*, 2012b). We hypothesize that, in addition, the isoform Car3 may also contribute to this function, which illustrates increased mRNA expression levels during maturation (Lacruz *et al.*, 2012b). Intracellular passage of bicarbonate appears to be mediated by NBCe1 at the basal pole (Lacruz *et al.*, 2010b). Such buffering may be required for ameliorating H^+ release during Car2 and Car3 activities in the cytosol. Perhaps some of these H^+ are shunted to the enamel area and others are used by the H^+ ATPase pumps of the endosome-lysosome. Extracellular bicarbonate transport to the enamel area may also be mediated by Cfr and AE2. In addition, as discussed in the text, if future research shows the presence of a Na^+, K^+ -ATPase pump in ameloblasts, this would explain Na^+ export to some extent. Endocytotic functions appear to be mediated, at least in part, by the AP-2/clathrin-dependent pathway in maturation-stage ameloblasts (Lacruz *et al.*, 2013). It may also be the case that either the protease Ctrc functions in association with Klk4 in the processing of matrix breakdown which may subsequently undergo endocytosis, or it is perhaps directly associated with the lysosomal/endosomal apparatus. *Note:* The current diagram should be viewed as work in progress.

2008), and, for NBCe1, 3-day-old mouse mandibles were fixed in Carnoy's fixative (Paine *et al.*, 2008; Lacruz *et al.*, 2010b). Other investigators, working with formaldehyde, immersion-fixed, and paraffin-embedded mandibles from older mice

Figure 2. Relative expression of CLCA and CLIC mRNA transcripts in rat mandibular incisor enamel organ by qPCR. Following methods described in Lacruz *et al.* (2012a,b), we investigated the expression levels in the enamel organ of CLCA and CLIC gene families involved in Cl⁻ transport. CLCAs are Cl⁻ channels activated by cytosolic Ca²⁺ concentrations showing a strong outward rectification current (Begenisich and Melvin, 1998). The family is comprised of 5 different members (CLCA1-5), illustrating wide tissue distribution (Kidd and Thorn, 2000; Hartzell *et al.*, 2005). Note that the *Clca3* gene does not appear to be part of the rat genome and was not investigated. The CLIC family is comprised of 6 members (CLIC1-6), also exhibiting wide tissue distribution, but their physiological roles remain poorly described (Cromer *et al.*, 2007). (A) Real-time PCR shows that levels of *Clca2* and *Clca5* significantly increased (one-way ANOVA; $p < 0.05$) in the maturation stage relative to the secretory stage. For the CLIC genes, differences in expression were observed only for *Clc6* (2.3-fold increase in maturation). Levels of *Clc1* and *Clc4* were high in secretory and maturation stages, but no changes in transcript abundance were identified between stages. Values were normalized to β -actin (Y-axis) for each stage and calculated by the $\Delta\Delta$ CT method (Livak and Schmittgen, 2001). Real-time PCR primers used are listed in the Appendix Table.

Figure 3. Immunostaining for Ctrc in mouse molars. Twelve-day-old mouse mandibles were fixed in 10% formalin, demineralized for 7 days in 10% EDTA, embedded in paraffin, and sectioned. Following immunohistochemistry protocols as described (Lacruz *et al.*, 2013), sagittal sections of molars were immunostained with Ctrc antibody (ab35694, Abcam, Cambridge, UK). Ctrc is expressed at the apical pole of maturation-stage ameloblasts (Am) but not in the enamel matrix (E). Secretory ameloblasts did not stain for Ctrc (data not shown).

(Lyaru *et al.*, 2008; Bronckers *et al.*, 2009), or perfusion-fixed rats (Josephsen *et al.*, 2010), have shown somewhat contradictory results related to AE2 and NBCe1 localization in ameloblasts, suggesting that AE2 is located to “cytoplasmic and in basolateral membranes” in maturation-stage ameloblasts (Bronckers *et al.*, 2009), and NBCe1 expression is mostly, or exclusively, seen in the PL cells of the enamel organ (Josephsen *et al.*, 2010). This would suggest that epitope presentation, antibody choice, and immunohistochemical methodologies have to be carefully and critically evaluated as data are gathered and presented. While there is still much to explore, it is clear, even

at this time, that ameloblasts and the PL cells have an extraordinary capacity to buffer the enamel matrix against extreme pH changes arising from enamel Hap crystallite formation and growth (Smith *et al.*, 1996; Smith, 1998; Lacruz *et al.*, 2010a).

FUTURE DIRECTIONS

Enamel maturation and calcium transport were the subjects of 2 seminal papers published in the review series of this journal about 15 years ago (Smith, 1998; Hubbard, 2000). The resurgence of interest on the part of enamel biologists in gaining a broader understanding of the processes involved in the mineralization of enamel is reflected in the recent increase in publications on this subject. Our work has focused and will continue to focus on gaining a better understanding of chloride (Cl⁻) transport, endocytosis, and Ca²⁺ transport and on better defining proteolysis and associated processes. Regarding Cl⁻ transport, we have shown abnormal mineralization in deciduous and permanent porcine teeth with cystic fibrosis

transmembrane conductance regulator (CFTR) mutations (Lacruz *et al.*, 2012b), highlighting the critical functions of this anion in amelogenesis. Chloride is the most abundant biological anion regulating intracellular pH, fluid secretion, cell volume, and changes in membrane excitability, functioning at the plasma membrane or in the membranes of intracellular organelles (al-Awqati *et al.*, 1992; Jentsch, 2007). Our recent report on the expression of one chloride channel family (CLC) in maturation-stage ameloblasts associated with the lysosomal/endosomal pathway (Lacruz *et al.*, 2013) prompted us to further investigate the possible expression of additional Cl⁻ transporters in the enamel organ. Fig. 2 shows the expression levels of mRNA transcripts for the calcium-activated chloride channels (CLCA) and the chloride intracellular channels (CLIC), comparing secretory- and maturation-stage enamel organ cells from rats. These data add to the growing list of Cl⁻ transporters with potential functions in enamel mineralization, either acting as Cl⁻ exchangers, permitting entry into or exit from the cell or to be incorporated intracellularly into the lysosomal/endosomal system of ameloblasts. It remains to be elucidated whether the vast array of chloride channels detected in the enamel organ can function as alternative or complementary paths for the movement of this ion. What has become apparent is that the endocytosis of enamel matrix proteins (EMP) remains a challenging area of study. The resorptive functions of ameloblasts during maturation were recently clarified with the identification of several endocytosis-related gene products, such as clathrin and adaptor protein complex 2 (AP-2) subunits, in the enamel organ (Lacruz *et al.*, 2013). The up-regulation of these mRNA transcripts and their cellular localization in the maturation-stage enamel organ prompted us to suggest that the endocytosis of

Table. Summary of Rodent Genes/Proteins Associated with Transport Functions in Maturation-stage Enamel Organ

Gene Symbol	Gene Name	Protein	Function	Localization in Enamel Organ	References
Ap2a1	adaptor protein complex 2, alpha 1 subunit	Ap2a1	endocytosis	apical pole Am	Lacruz <i>et al.</i> , 2013
Ap2a2	adaptor protein complex 2, alpha 2 subunit	Ap2a2	endocytosis	apical pole Am	Lacruz <i>et al.</i> , 2013
Ap2b1	adaptor protein complex-2, beta 1 subunit	Ap2b1	endocytosis	apical pole Am	Lacruz <i>et al.</i> , 2013
Ap2m1	adaptor protein complex 2, mu 1 subunit	Ap2m1	endocytosis	apical pole Am	Lacruz <i>et al.</i> , 2013
Ap2s1	adaptor protein complex 2, sigma 1 subunit	Ap2s1	endocytosis	apical pole Am	Lacruz <i>et al.</i> , 2013
Atp6v0a1	ATPase, H ⁺ transporting, lysosomal V0 subunit A1	Atp6v0a1	vacuolar proton pump	apical in RA and SA Am	Josephsen <i>et al.</i> , 2010
Atp6v0d2	ATPase, H ⁺ transporting, lysosomal V0 subunit D2	Atp6v0d2	vacuolar proton pump	Am	Lacruz <i>et al.</i> , 2013
Calb1	calbindin 1	Calb1	calcium handling	cytoplasmic	Hubbard, 2000; Turnbull <i>et al.</i> , 2004; Hubbard <i>et al.</i> , 2011
Calb2	calbindin 2	Calb2	calcium handling	cytoplasmic	Hubbard, 2000; Turnbull <i>et al.</i> , 2004; Hubbard <i>et al.</i> , 2011
Car12	carbonic anhydrase 12	Car12	generate bicarbonate, H ⁺ , CO ₂	membrane-bound	Lacruz <i>et al.</i> , 2012b
Car2	carbonic anhydrase 2	Car2	generate bicarbonate, H ⁺ , CO ₂	intracellular	Lin <i>et al.</i> , 1994; Toyosawa <i>et al.</i> , 1996; Josephsen <i>et al.</i> , 2010
Car3	carbonic anhydrase 3	Car2	generate bicarbonate, H ⁺ , CO ₂	cytoplasmic	Lacruz <i>et al.</i> , 2012b
Car6	carbonic anhydrase 6	Car6	generate bicarbonate, H ⁺ , CO ₂	extracellular (enamel matrix)	Smith <i>et al.</i> , 2006
Cfr	cystic fibrosis transmembrane conductance regulator	Cfr	Cl ⁻ transport	apical pole of Am	Bronckers <i>et al.</i> , 2010
Clcn7	chloride channel voltage sensitive 7	Clc7	Cl ⁻ transport (H ⁺ /Cl ⁻ exchanger?)	intracellular (lysosomes?)	Lacruz <i>et al.</i> , 2013
Clta	clathrin light-chain	Clta	endocytosis	apical pole Am	Lacruz <i>et al.</i> , 2013
Cltc	clathrin heavy-chain	Cltc	endocytosis	apical pole Am and PL	Lacruz <i>et al.</i> , 2013
Ctrc	caldecrin (chymotrypsin c)	Ctrc	enzyme	apical pole Am	Lacruz <i>et al.</i> , 2011
Klk4	kallikrein-related peptidase 4	Klk4	enzyme	secreted to enamel matrix	Bartlett and Simmer, 1999; Lu <i>et al.</i> , 2008
Mcoln1	lysosomal membrane-bound transient receptor potential cation channel	Mcoln1	endocytosis	Am and PL	Lacruz <i>et al.</i> , 2012a, 2013
Nhe1	Na ⁺ /H ⁺ exchanger 1	NHE1	sodium/hydrogen exchanger	basolateral pole of Am	Josephsen <i>et al.</i> , 2010
Rab10	member RAS oncogene family 10	Rab10	endocytosis	Am and PL	Lacruz <i>et al.</i> , 2013
Rab24	member RAS oncogene family 24	Rab24	endocytosis	Am and PL	Lacruz <i>et al.</i> , 2013
Rcan1	regulator of calcineurin 1	Rcan1	calcium handling	cytoplasmic	Lacruz <i>et al.</i> , 2012a
S100a4	S100 calcium-binding protein A4	S100a4	calcium handling	cytoplasmic	Hubbard <i>et al.</i> , 2011; Lacruz <i>et al.</i> , 2012a
S100g	S100 calcium-binding protein G	S100g	calcium handling	cytoplasmic	Lacruz <i>et al.</i> , 2012a
Slc4a2	anion-exchanger 2	AE2	possibly exchange Cl ⁻	apical pole of Am	Iyaru <i>et al.</i> , 2008; Paine <i>et al.</i> , 2008
Slc4a4	electrogenic sodium bicarbonate co-transporter	NBCE1	import of bicarbonate	basolateral pole of Am and also in PL	Paine <i>et al.</i> , 2008; Josephsen <i>et al.</i> , 2010; Lacruz <i>et al.</i> , 2010
Slc24a4	solute carrier family 24	NCKX4	sodium/potassium/calcium exchanger	apical pole of Am	Hu <i>et al.</i> , 2012
Slc34a2	solute carrier family 34 (sodium phosphate), member 2	NaPi-2b	membrane-bound	unknown	Lacruz <i>et al.</i> , 2012a
Tfrc	transferrin receptor	Tfrc	ion transport	Am and PL/ membrane-bound	McKee <i>et al.</i> , 1987; Lacruz <i>et al.</i> , 2013
unknown*	ATPase, Na ⁺ /K ⁺ transporting-alpha subunit	?	Na ⁺ /K ⁺ transporting ATPase	PL, Am, Si	Kashgarian <i>et al.</i> , 1985; Garant <i>et al.</i> , 1987; Josephsen <i>et al.</i> , 2010

Am = ameloblasts; PL = papillary layer; Si = stratum intermedium. *Note that the antibody used in the study by Josephsen and co-workers (2010) was an ATPase, Na⁺/K⁺ transporting-alpha subunit, of which there are 4 unique genes. It is unclear which of the 4 gene products this particular antibody recognizes.

enamel matrix protein debris involves ligand-receptor signaling activities (Lacruz *et al.*, 2012a, 2013). Moreover, the identification in the enamel organ of the protease caldecrin (also known as chymotrypsin C or Ctrc) (Lacruz *et al.*, 2011b, 2012a) may also be associated with endocytosis (see below). Originally, the similar expression pattern of Ctrc (Lacruz *et al.*, 2011b) to that of the better known enamel protease kallikrein-4 (Klk4) (Bartlett and Simmer, 1999; Lu *et al.*, 2008) led us to hypothesize that Ctrc may function to degrade enamel products; possibly smaller fragments generated by Klk4, although this was not tested. To help clarify the function of Ctrc in enamel, we have conducted an immunohistologic study of the cellular localization of Ctrc in enamel cells (Lacruz *et al.*, 2011b). Fig. 3 shows the first molar of a 12-day-old mouse stained with Ctrc antibody, suggesting that Ctrc is localized to the apical pole of maturation-stage ameloblasts. Hence, it remains unclear whether Ctrc functions as an extracellular protease or at the plasma membrane/intracellular level, perhaps associated with the early endosomal apparatus.

Many of these ion channels/transporters described in the enamel organ are already well-known in studies of the physiology of other soft-tissue organs, such as the kidney and pancreas, permitting the construction of a model for the mechanistic physiology of transport in enamel. Fig. 1 (see also the Table for gene products associated with the mature enamel organ cells that have been discussed in this review) is a schematic for interpreting intra- and extracellular transport in pancreatic duct cells and maturation-stage ameloblasts. From recent works (Bronckers *et al.*, 2009; Josephsen *et al.*, 2010; Okumura *et al.*, 2010; Lacruz *et al.*, 2012a), we understand that the solute carrier (SLC) gene family is highly represented in amelogenesis, indicating that there is an enormous void of knowledge as to the functions and relevance of other ions (*i.e.*, sodium, potassium, zinc, iron) in enamel maturation (McKee *et al.*, 1987; Lacruz *et al.*, 2013). Similarities of ion transport with kidney and pancreas physiology indicate that enamel development should not be viewed as an isolated occurrence within mammalian development, but rather, ion transport biology must be integrated within overall organismic phenomena and thus is equally affected by physiological anomalies.

ACKNOWLEDGMENTS

This work was supported by grants DE013404 and DE019629 (MLP), DE022799 (RSL), and DK058563 and DK077162 (IK) from the US National Institutes of Health, as well as by the Melbourne Research Unit for Facial Disorders (MJH). The authors also thank the anonymous reviewers for their comments and critique. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

REFERENCES

- al-Awqati Q, Barasch J, Landry D (1992). Chloride channels of intracellular organelles and their potential role in cystic fibrosis. *J Exp Biol* 172:245-266.
- Bartlett JD, Simmer JP (1999). Proteinases in developing dental enamel. *Crit Rev Oral Biol Med* 10:425-441.
- Begenisich T, Melvin JE (1998). Regulation of chloride channels in secretory epithelia. *J Membr Biol* 163:77-85.
- Bronckers AL, Lyaruu DM, Jansen ID, Medina JF, Kellokumpu S, Hoebe KA, *et al.* (2009). Localization and function of the anion exchanger Ae2 in developing teeth and orofacial bone in rodents. *J Exp Zool B Mol Dev Evol* 312(B):375-387.
- Bronckers A, Kalogeraki L, Jorna HJ, Wilke M, Bervoets TJ, Lyaruu DM, *et al.* (2010). The cystic fibrosis transmembrane conductance regulator (CFTR) is expressed in maturation stage ameloblasts, odontoblasts and bone cells. *Bone* 46:1188-1196.
- Cromer BA, Gorman MA, Hansen G, Adams JJ, Coggan M, Littler DR, *et al.* (2007). Structure of the Janus protein human CLIC2. *J Mol Biol* 374:719-731.
- Decker JD (1963). A light and electron microscope study of the rat molar enamel organ. *Arch Oral Biol* 8:301-310.
- Garant PR, Nalbandian J (1968a). Observations on the ultrastructure of ameloblasts with special reference to the golgi complex and related components. *J Ultrastruct Res* 23:427-443.
- Garant PR, Nalbandian J (1968b). The fine structure of the papillary region of the mouse enamel organ. *Arch Oral Biol* 13:1167-1185.
- Garant PR, Sasaki T, Colflesh PE (1987). Na-K-ATPase in the enamel organ: localization and possible roles in enamel formation. *Adv Dent Res* 1:267-275.
- Girardi AC, Di Sole F (2012). Deciphering the mechanisms of the Na⁺/H⁺ exchanger-3 regulation in organ dysfunction. *Am J Physiol Cell Physiol* 302:C1569-C1587.
- Glick PL (1979). Patterns of enamel maturation. *J Dent Res* 58(Spec Iss B):883-895.
- Glynn IM (2002). A hundred years of sodium pumping. *Annu Rev Physiol* 64:1-18.
- Hartzell C, Putzier I, Arreola J (2005). Calcium-activated chloride channels. *Annu Rev Physiol* 67:719-758.
- Hu P, Lacruz RS, Smith CE, Smith SM, Kurtz I, Paine ML (2012). Expression of the sodium/calcium/potassium exchanger, NCKX4, in ameloblasts. *Cells Tissues Organs* 196:501-509.
- Hubbard MJ (2000). Calcium transport across the dental enamel epithelium. *Crit Rev Oral Biol Med* 11:437-466.
- Hubbard MJ, McHugh NJ, Mangum JE (2011). Exclusion of all three calbindins from a calcium-ferry role in rat enamel cells. *Eur J Oral Sci* 119(Suppl 1):112-119.
- Jentsch TJ (2007). Chloride and the endosomal-lysosomal pathway: emerging roles of CLC chloride transporters. *J Physiol* 578(Pt 3):633-640.
- Josephsen K, Fejerskov O (1977). Ameloblast modulation in the maturation zone of the rat incisor enamel organ. A light and electron microscopic study. *J Anat* 124(Pt 1):45-70.
- Josephsen K, Takano Y, Frische S, Praetorius J, Nielsen S, Aoba T, *et al.* (2010). Ion transporters in secretory and cyclically modulating ameloblasts. A new hypothesis for cellular control of preeruptive enamel maturation. *Am J Physiol Cell Physiol* 299:C1299-C1307.
- Kashgarian M, Biemesderfer D, Caplan M, Forbush B 3rd (1985). Monoclonal antibody to Na,K-ATPase: immunocytochemical localization along nephron segments. *Kidney Int* 28:899-913.
- Kidd JF, Thorn P (2000). Intracellular Ca²⁺ and Cl⁻ channel activation in secretory cells. *Annu Rev Physiol* 62:493-513.
- Kurahashi Y, Yoshiki S (1972). Electron microscopic localization of alkaline phosphatase in the enamel organ of the young rat. *Arch Oral Biol* 17:155-163.
- Lacruz RS, Nanci A, Kurtz I, Wright JT, Paine ML (2010a). Regulation of pH during amelogenesis. *Calcif Tissue Int* 86:91-103.
- Lacruz RS, Nanci A, White SN, Wen X, Wang H, Zalzal SF, *et al.* (2010b). The sodium bicarbonate cotransporter (NBCe1) is essential for normal development of mouse dentition. *J Biol Chem* 285:24432-24438.
- Lacruz RS, Smith CE, Chen YB, Hubbard MJ, Hacia JG, Paine ML (2011a). Gene-expression analysis of early- and late-maturation-stage rat enamel organ. *Eur J Oral Sci* 119(Suppl 1):149-157.
- Lacruz RS, Smith CE, Smith SM, Hu P, Bringas P Jr, Sahin-Tóth M, *et al.* (2011b). Chymotrypsin C (caldecrin) is associated with enamel development. *J Dent Res* 90:1228-1233.
- Lacruz RS, Smith CE, Moffatt P, Chang EH, Bromage TG, Bringas P Jr, *et al.* (2012a). Requirements for ion and solute transport, and pH regulation during enamel maturation. *J Cell Physiol* 227:1776-1785.

- Lacruz RS, Smith CE, Bringas P Jr, Chen YB, Smith SM, Snead ML, *et al.* (2012b). Identification of novel candidate genes involved in mineralization of dental enamel by genome-wide transcript profiling. *J Cell Physiol* 227:2264-2275.
- Lacruz RS, Brookes SJ, Wen X, Jimenez JM, Vikman S, Hu P, *et al.* (2013). Adaptor protein complex 2 (AP-2) mediated, clathrin dependent endocytosis, and related gene activities, are a prominent feature during maturation stage amelogenesis. *J Bone Miner Res* (Epub ahead of print 10/8/2012) (in press).
- Lee MG, Muallem S (2008). Physiology of duct cell secretion. In: *Pancreas: an integrated textbook of basic science, medicine, and surgery*. Beger H, Buchler M, Kozarek R, editors. Oxford, UK: Blackwell Publishing, pp. 78-90.
- Lin HM, Nakamura H, Noda T, Ozawa H (1994). Localization of H(+)-ATPase and carbonic anhydrase II in ameloblasts at maturation. *Calcif Tissue Int* 55:38-45.
- Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25:402-408.
- Lu Y, Papagerakis P, Yamakoshi Y, Hu JC, Bartlett JD, Simmer JP (2008). Functions of KLK4 and MMP-20 in dental enamel formation. *Biol Chem* 389:695-700.
- Lyaru DM, Bronckers AL, Mulder L, Mardones P, Medina JF, Kellokumpu S, *et al.* (2008). The anion exchanger Ae2 is required for enamel maturation in mouse teeth. *Matrix Biol* 27:119-127.
- McKee MD, Zerounian C, Martineau-Doize B, Warshawsky H (1987). Specific binding sites for transferrin on ameloblasts of the enamel maturation zone in the rat incisor. *Anat Rec* 218:123-127.
- McKee MD, Warshawsky H, Nanci A (1989). Cyclical incorporation of 33P into rat incisor enamel in vivo as visualized by whole-mount radioautography. *Arch Oral Biol* 34:989-993.
- Morth JP, Pedersen BP, Buch-Pedersen MJ, Andersen JP, Vilsen B, Palmgren MG, *et al.* (2011). A structural overview of the plasma membrane Na⁺,K⁺-ATPase and H⁺-ATPase ion pumps. *Nat Rev Mol Cell Biol* 12:60-70.
- Murer H, Forster I, Biber J (2004). The sodium phosphate cotransporter family SLC34. *Pflugers Arch* 447:763-767.
- Nanci A, Smith CE (1992). Development and calcification of enamel. In: *Calcification in biological systems*. Bonucci E, editor. Boca Raton, FL: CRC Press, pp. 313-343.
- Okumura R, Shibukawa Y, Muramatsu T, Hashimoto S, Nakagawa K, Tazaki M, *et al.* (2010). Sodium-calcium exchangers in rat ameloblasts. *J Pharmacol Sci* 112:223-230.
- Omelon SJ, Grynaps MD (2008). Relationships between polyphosphate chemistry, biochemistry and apatite biomineralization. *Chem Rev* 108:4694-4715.
- Paine ML, Snead ML, Wang HJ, Abuladze N, Pushkin A, Liu W, *et al.* (2008). Role of NBCe1 and AE2 in secretory ameloblasts. *J Dent Res* 87:391-395.
- Park HW, Lee MG (2012). Transepithelial bicarbonate secretion: lessons from the pancreas. *Cold Spring Harb Perspect Med* 2:a009571.
- Purkerson JM, Schwartz GJ (2007). The role of carbonic anhydrases in renal physiology. *Kidney Int* 71:103-115.
- Reith EJ, Boyde A (1981). Autoradiographic evidence of cyclical entry of calcium into maturing enamel of the rat incisor tooth. *Arch Oral Biol* 26:983-987.
- Robinson C, Hiller CR, Weatherell JA (1974). Uptake of 32P-labelled phosphate into developing rat incisor enamel. *Calcif Tissue Res* 15:143-152.
- Sasaki T, Goldberg M, Takuma S, Garant PR (1990). Cell biology of tooth enamel formation. Functional electron microscopic monographs. *Monogr Oral Sci* 14:1-199.
- Sener A, Jijakli H, Zahedi Asl S, Courtois P, Yates AP, Meuris S, *et al.* (2007). Possible role of carbonic anhydrase in rat pancreatic islets: enzymatic, secretory, metabolic, ionic, and electrical aspects. *Am J Physiol Endocrinol Metab* 292:E1624-E1630.
- Singh AK, Riederer B, Chen M, Xiao F, Krabbenhoft A, Engelhardt R, *et al.* (2010). The switch of intestinal Slc26 exchangers from anion absorptive to HCO₃⁻ secretory mode is dependent on CFTR anion channel function. *Am J Physiol Cell Physiol* 298:C1057-C1065.
- Smith CE (1998). Cellular and chemical events during enamel maturation. *Crit Rev Oral Biol Med* 9:128-161.
- Smith CE, Nanci A (1995). Overview of morphological changes in enamel organ cells associated with major events in amelogenesis. *Int J Dev Biol* 39:153-161.
- Smith CE, Nanci A (1996). Protein dynamics of amelogenesis. *Anat Rec* 245:186-207.
- Smith CE, Issid M, Margolis HC, Moreno EC (1996). Developmental changes in the pH of enamel fluid and its effects on matrix-resident proteinases. *Adv Dent Res* 10:159-169.
- Smith CE, Nanci A, Moffatt P (2006). Evidence by signal peptide trap technology for the expression of carbonic anhydrase 6 in rat incisor enamel organs. *Eur J Oral Sci* 114(Suppl 1):147-153.
- Takano Y (1995). Enamel mineralization and the role of ameloblasts in calcium transport. *Connect Tissue Res* 33:127-137.
- Toyosawa S, Ogawa Y, Inagaki T, Ijuhin N (1996). Immunohistochemical localization of carbonic anhydrase isozyme II in rat incisor epithelial cells at various stages of amelogenesis. *Cell Tissue Res* 285:217-225.
- Turnbull CI, Looi K, Mangum JE, Meyer M, Sayer RJ, Hubbard MJ (2004). Calbindin independence of calcium transport in developing teeth contradicts the calcium ferry dogma. *J Biol Chem* 279:55850-55854.
- Wennberg A, Bawden JW (1978). Comparison of 33P with 45Ca distribution in developing rat molar enamel in vivo and in vitro. *J Dent Res* 57:111-117.
- Yang D, Li Q, So I, Huang CL, Ando H, Mizutani A, *et al.* (2011). IRBIT governs epithelial secretion in mice by antagonizing the WNK/SPAK kinase pathway. *J Clin Invest* 121:956-965.