The Role of Ion Transport in Enamel Formation

Endocytosis in Maturation-stage Amelogenesis
Dental caries, although largely preventable, is the most common chronic disease for humans from ages 6 – 19 years, and if untreated will result in pulpal pathologies involving severe dental pain, and eventually tooth loss. In a 2005 World Health Organization report on Policy and Practice it was noted that up to 90% of school-aged children, and the vast majority of adults, are affected by dental caries worldwide.


Caries start in enamel
Caries prevention and dental restorations always impacts enamel
General dentists spend the majority of their time doing preventative t’ms, and restorations

Yet,
- the dental practitioner and dental research communities know little about the physiology and microscopic structure of enamel
- in dental schools dental histology (enamel/dentin/periodontal etc) is briefly covered, and the benefits and chemistry of fluoride explained
- but genetic diseases impacting enamel and dentine are poorly covered in the dental curriculum
- the non-mineral building blocks of enamel (organic enamel matrix, ion exchange ….) remain poorly understood

- if we have a better understanding of the genes (and their function) critical for enamel formation, and can correlate genotypes to phenotypes, better preventative and restorative t’m options should result
Stratum intermedium

Ameloblasts

IR

R

Enamel Matrix
TEM images of early enamel crystallites proximal to Tomes’ processes
Enamel and the Dentin Enamel Junction
SEM images of molar cusp sectioned and lightly etched.
SEM images of surface enamel (incisor) sectioned and lightly etched. Right: same section under backscattered SEM indicating relative mineral density.

Images from Rodrigo Lacruz and Tim Bromage - NYU
Amelogenesis Imperfecta

The enamel defects associated with AI are highly variable and are described as hypoplastic, hypocalcified or hypomature.

Hypoplastic defects represent deficiencies in the amount of enamel, characterized by thin enamel or enamel of normal thickness with pits or grooves. These teeth can have small crowns and have normal to opaque white or yellow-brown color.

In contrast, hypocalcified and hypomature AI have a normal enamel thickness but poorly mineralized enamel.

Hypocalcified AI is thought to result from a defect in initial crystallite formation followed by defective growth.

AI involving hypomaturation is caused by a defect in final growth and maturation of enamel crystallites. In both situations, the hypomineralized enamel often abrades and chips easily, leaving exposed dentin. The enamel color ranges from opaque white to yellow-brown, and its surface is soft and rough. Dental sensitivity is a frequent complaint for people with these types of AI.

Coffield et al., JADA 2005 V136, p620-630.
Amelogenin-null

Gibson et al., J. Biol. Chem., V276, p31871-5, 2001
Ameloblastin-null

Photographic examination of the wild type (Enam+/+; top row), heterozygous (Enam+/-; middle row), and null (Enam-/-; bottom row) mouse dentitions at 7 weeks.
SEM analysis of erupted mouse incisors and molars at 7 weeks.

$10 \text{Ca}_2^+ + 6 \text{HPO}_4^{2-} + 2 \text{H}_2\text{O} \leftrightarrow \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 + 8 \text{H}^+$

Hap

$8 \text{H}^+ + 8 \text{HCO}_3^- \rightarrow 8 \text{H}_2\text{O} + 8 \text{CO}_2$

Carbonic Anhydrases

$\text{H}_2\text{O} + \text{CO}_2 \leftrightarrow \text{HCO}_3^- + \text{H}^+$

or ion channels move ions in a unidirectional path
Ion Transport: Summary

Ruffle-ended ameloblasts: possess proximal junctions that are leaky and distal junctions that are tight.

Smooth-ended ameloblasts: possess proximal junctions that are tight and distal junctions that are leaky.

Ion transport from the enamel organ papillary layer to the enamel matrix has more typically been described as “intercellular”, however our, and others’, recent data suggests a “transcellular” passage accounts for some, (if not all) of the ion movement from the papillary layer to the enamel extracellular matrix.
CA2

Early – Late Maturation

Toyosawa et al., Cell Tissue Res, 285 1996

<table>
<thead>
<tr>
<th>Secretory-stage</th>
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<tr>
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DEVELOPMENTAL CHANGES IN THE pH OF ENAMEL FLUID AND ITS EFFECTS ON MATRIX-RESIDENT PROTEINASES

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Enamel develops over time through a series of interactions among compaction, a mineral phase, and other proteinases (reviewed in Nanci and Smi Fincham, 1995). The organic phase is heterogeneous and is comprised of structural matrix proteins and proteoglycans. In the fluid phase, depend on amelogenesis (Aoba and Moreno, 1991; Aoba et al., 1992; Nanci and St. al., 1995; Deutsch et al., 1995a; Fir Oláh, 1995; Simmer and Fincham, 1997). Some proteinases are presumably secretory products of ameloblasts, and function within, the fluid. Some proteinases are secreted into the fluid phase after amelogenin degradation (Fincham et al., 1991; Aoba et al.,

Fig. 3—Categorization plot of mean pH (solid squares) ± SD (lines) and SEM (open boxes; n = 10 teeth) for enamel strips cut at 0.5-mm length across the secretory (S) and maturation (M) stages of amelogenesis (R = location of the molar reference). The average measured pH of enamel strips remains fairly constant across the secretory stage (S) and into the early maturation stage (M) (by 8 mm on the abscissa). The pH then drops to mildly acidic conditions, spikes sharply to near-neutral pH, drops a second time to a more mildly acidic pH, and finally rises in choppy fashion to near-neutral pH as the enamel hardens.
pH regulation by ameloblasts.

Figure 1. The CF mouse incisors (A) always appeared chalky white, unlike the normal mouse incisors, that were a characteristic yellow-brown (B).

Wright et al., J DENT RES 1996;75:966-973
Sui W et al. J DENT RES 2003;82:388-392

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Bronckers et al., Bone 2010
Cftr immunolocalization
Porcine deciduous I2 (WT left, CF pig right)

Animals from Dr. Michael Welsh, Univ. Iowa
Cystic Fibrosis (Domestic Pig mandibular 1st molar)

Chang et. al., Cells Tissues Organs. 2011;194(2-4):249-54.
Better dental health in children with CF

May 20, 2014

Children with CF may have better dental health compared to non-CF children, although it is unclear if a benefit is maintained into adolescence.


Overall, the DMFT score was significantly lower in CF children compared to controls (4.6 vs. 7.7). The incidence of dental caries was not found to be associated with the use of antibiotics among children with CF. In addition, saliva pH and salivary flow rate were not significantly different between the two groups. So in this analysis, it is unclear why the CF group had a lower incidence of dental problems.

In the systematic review (Chi, 2013), 10 of 15 studies included in the analysis found that children with CF had a lower prevalence of dental caries than healthy controls, three studies found a higher prevalence in CF, and two studies reported no difference. When patient age was included, only 1 of 7 studies found a lower prevalence of dental caries in CF patients, suggesting that patients may not enjoy better dental health during adolescence and adulthood.

The author concluded that additional research is needed to determine if CF patients really have a lower risk of dental problems during childhood and adolescence.

Comment

Dr. Mark Montgomery: Based on these articles, we can anticipate a new symposium at the CF meetings—CFDD —CF related dental disease! Attention to dental health is an important part of overall health surveillance. The existing studies provide inconclusive effects of CF on dental health. There is some solace that the use of antibiotics is not associated with incidence of dental caries. However, the studies do highlight that CF has the potential to impair dental health. The CF clinic visit should review the impact of CF on an individual’s health, relationships and quality of life. Moreover, CF clinic visits should be a time to help families keep CF care and disease in perspective. Providing general health advice to our CF families may assist in this goal. Lifelong general health advice should include an age-appropriate review of accident prevention, immunizations, evaluation of high-risk behaviour, school and work life, and dental health.
Are children and adolescents with cystic fibrosis at lower risk of caries?
O'Keefe E.

Related factors of dental caries and molar incisor hypomineralisation in a group of children with cystic fibrosis.
Peker S, Mete S, Gokdemir Y, Karadag B, Kargul B.

Dental caries prevalence in children and adolescents with cystic fibrosis: a qualitative systematic review and recommendations for future research.
Chi DL.

Dental enamel defects in Italian children with cystic fibrosis: an observational study.
Ferrazzano GF, Sangianantoni G, Cantile T, Amato I, Orlando S, Ingenito A.

Dental and periodontal health status in children affected by cystic fibrosis in a southern Italian region.
Ferrazzano GF, Orlando S, Sangianantoni G, Cantile T, Ingenito A.

Assessment of dental status and oral hygiene in the study population of cystic fibrosis patients in the Podlasie province.
Dabrowska E, Blahuszewska K, Minarowska A, Kaczmarski M, Niedźwiecka-Andrzejewicz I, Stokowska W.
S427L-NBCe1 mutation. a, patient's facial manifestations (blind eye with cataract and corneal opacity (top) and abnormal dentition (bottom)). b, sequence analysis of C→T transition (see “Experimental Procedures”): b1, normal; b2, mother; b3, patient. c, et...

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Appearance of the patient's anterior eye segments at age 27. Eyes demonstrate peripheral corneal vascularization and opacification, interpalpebral band keratopathy, and dense, white mature cataract. The right eye also shows pupil deformation (superior retraction) that seems to be due to previous glaucoma surgery.

Winsnes et al. (1979) reported the cases of 2 brothers with severe hyperchloremic acidosis, a maximum tubular capacity for bicarbonate reabsorption about half normal, growth retardation, mental retardation, nystagmus, cataract, corneal opacities, glaucoma, and defects in the enamel of the permanent teeth. Red cells showed increased osmotic resistance. The possibility of a generalized membrane defect was raised.

Dental features in congenital persistent renal tubular acidosis of proximal type.

10yo male, agenesis 2nd bicuspid, delayed development and erruption of permanent dentition, severe enamel hypoplasia

CASE REPORT

Amelogenesis imperfecta with renal disease - a report of two cases

Gross phenotype of NBC1 null mutant mice.

Gawenis L R et al. J. Biol. Chem. 2007;282:9042-9052
Gross phenotype of NBCe1−/− mice at 10 days of age.

Gross anatomy of upper and lower incisor teeth of NBCe1−/− mice at 14 days of age.


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Immunofluorescence (IF) and immunoperoxidase of NBCe1 in polarized ameloblast cells.
SEM images of mature enamel cross-sections in NBCe1−/− mouse incisors.

Backscattered scanning electron microscope (SEM) imaging of NBCe1 mutant animals.


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The anion exchanger Ae2 is required for enamel maturation in mouse teeth.

Role of NbcE1 and AE2 in secretory ameloblasts.

What about AE2 mutations in human?
Targeted overexpression of amelotin disrupts the microstructure of dental enamel.

Regulation of pH During Amelogenesis.
Lacruz RS, Nanci A, Kurtz I, Wright JT, Paine ML.

A survey of carbonic anhydrase mRNA expression in enamel cells.
Lacruz RS, Hilvo M, Kurtz I, Paine ML.

Identification of novel candidate genes involved in mineralization of dental enamel by genome-wide transcript profiling.

Gene-expression analysis of early- and late-maturation-stage rat enamel organ.
Lacruz RS, Smith CE, Chen YB, Hubbard MJ, Hacia JG, Paine ML.
Why Slc24a4/NCKX4 and Stim1?

**Slc24a4 – Ca++ export**

Of the Ca++ exporters, Slc24a4 was the most highly upregulated from the array analysis.

**Stim1 – Ca++ import**

STIM1 mutation associated with a syndrome of immunodeficiency and autoimmunity.


“We report on three siblings from one kindred with a clinical syndrome of immunodeficiency, hepatosplenomegaly, autoimmune hemolytic anemia, thrombocytopenia, muscular hypotonia, and defective enamel dentition. Two of these patients have a homozygous nonsense mutation in STIM1 that abrogates expression of STIM1 and Ca(2+) influx.”
Slc24a4 = NCKX4

(A) DNA gel electrophoresis showing bands for different alleles.

(B) mRNA expression levels relative to β-actin, log scale, across different days.

(C) Fold change normalized to levels at day 6 across different days.

(D) Western blot images showing NCKX4 and β-actin expression levels across different days.

(E) Graph showing intensity relative to β-actin across different days.

Hu 2012
The Coupling of NCX1 and Na, K-ATPase, and Calcium Extrusion, During Enamel Maturation

NCX1 – Ameloblasts (Okumura 2012)

Na, K-ATPase – alpha and beta subunit

α1 ↑ 3 fold in maturation vs secretory

4 alpha subunit genes
4 beta subunit genes

α/β combination not critical to function

in vitro but tissue-specific

α1/β1 combination most common

NCX1 & Na, K-ATPase coupling in heart and brain previously described
The Coupling of NCX1 and Na, K-ATPase, and Calcium Extrusion, During Enamel Maturation

A

B

Expression relative to β-actin

Expression relative to secretory

Atp1a1  Atp1b1  Atp1b3

α1  β1  β3

Actb

S  EM  LM  S  EM  LM  S  EM  LM

Na, K-ATPase

α1  β1  β3
The Coupling of NCX1 and Na, K-ATPase, and Calcium Extrusion, During Enamel Maturation
SLC4A & SLC26A genes
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Table 2. Expression profiles (fold change) of the SLC26 gene family in maturation-stage vs. secretory stage enamel organ cells. Note: N/A indicated SLC26A10 not represented on the Illumina Rat Array.

Figure 1. Real-time PCR results (fold-increase) for SLC26 gene transcripts in maturation-stage compared to secretory-stage enamel organ cells. Data is collected from 4 male rats. Error bars represent plus/minus one SD.
B). Column whole cell lysate; Column 2 negative control where agarose beads used to precipitate all lysate, product centrifuged, and supernatant added to gel; Column 3 CFTR precipitated products SAT1 and SUT2 identified by Western analysis. Antibodies used were SAT1 (Santa Cruz sc-132090); SUT2 (AbCam ab65367); and CFTR (Santa Cruz sc-8909).
From MS/MS studies functional interactions have been described for CFTR and many members of the SLC26 gene family. Examples are SLC26A3, SLC26A4, SLC26A5, SLC26A6, SLC26A8 and SLC26A9. The binding domains described typically involve; 1) the R domain of CFTR, an approximately 150 amino acid region that when phosphorylated is responsible the opening of the Cl⁻ ion channel and; 2) the C-terminal STAS (sulfate transporter and anti-sigma factor antagonist) domain of the SLC26 transporters responsible for protein-protein interactions and biosynthesis.

Another important protein-protein interacting region of CFTR is the terminal 3 amino acids (threonine, arginine and leucine or TRL) recognized as a PDZ-protein binding motif. It is believed that PDZ-containing proteins help couple CFTR to other cytoskeletal elements, and regulatory proteins in the cytoplasm such as phosphatases and kinases.
Figure 5. Landmarks to identify maturation-stage enamel organ cells in 100 gram rats (published) and in 4-week old mice. In mice we are interested in the maturation enamel cells only, so we move slightly anterior to landmarks used in rat.
Figure 4. Selected gene expression profiles in the maturation-stage enamel organ of 4-week old male SAT1 and SUT2 mutant mice. Panel A shows Odam and Enam mRNA levels (controls) in maturation-stage enamel organ cells relative to Actb transcript levels. SD included. Note the vertical axis uses a Log scale. Panel B shows selected gene transcripts in SAT1 and SUT2 mutant mice using the same total RNA samples as used in Panel A. All data in Panel B normalized against sex- and aged-matched wild-type controls. Two-tail t test used to determine significance (transcript numbers in each mutant mice line when compared to controls). Significance is indicated by (*) p<0.05 and (**) p<0.01.
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Summary

CA2  Toyosawa 1996
CFTR  Sui 2003, Bronckers 2010
CA6  Smith 2006
NBCe1  Paine 2008
NHE1  Josephsen 2010
CAR3, CAR12  Lacruz 2012
NCKX4  Hu 2012
NCX1, NCX3  Okumura 2012
Na, K-ATPase  Wen 2014

Na, K-ATPase x NCX1
SLC26A1, SLC26A6, SLC26A7
Conclusion:

- The past 2 decades has resulted in a much clearer picture of enamel biomineralization, first with the discoveries of the enamel matrix-specific proteins and proteinases (1983 [Amelx] – 2006 [Odam] - ongoing) BUT I think the current work looking at ameloblast physiology, ion transport etc (stating perhaps with the papers on CA2 and CFTR – 1996) will result in a much clearer picture of enamel pathologies and genotype-phenotype relationships.
Transport Functions Of Maturation Stage Ameloblasts

Adaptor Protein Complex 2–Mediated, Clathrin-Dependent Endocytosis, and Related Gene Activities, Are a Prominent Feature During Maturation Stage Amelogenesis

Rodrigo S Lacruz,1 Steven J Brookes,2 Xin Wen,1 Jaime M Jimenez,1 Susanna Vikman,3 Ping Hu,14 Shane N White,1 S Petter Lyngstad,2 Curtis T Okamoto,1 Charles E Smith,1 and Michael J Poine1
Matrix Turnover

Endocytosis

The removal of the degraded extracellular enamel organic matrix (protein peptides) has more typically been described as “intercellular”. That is, during enamel maturation small peptides (degraded enamel matrix proteins such as amelogenin, enamelin and ameloblastin) move in an intercellular manner from the enamel extracellular space to the papillary layer as the distal and proximal tight junctions relax during the ruffle-ended and smooth-ended stages.

Our hypothesis: “Receptor-mediated, clathrin-dependent endocytosis is a significant feature of maturation-stage ameloblasts.”
Multiple genes code many of the subunits: i.e. 3 genes code the AP-1 sigma subunit and 2 genes code both the AP-1 and AP-3 mu subunits.

Entire AP complexes can compensate if another non-functional: i.e. if AP3D1 mutated, AP-1 activities are up-regulated.

- **AP-1**: primarily associated with Golgi-endosome trafficking

- **AP-2**: clathrin dependent, found at the plasma membrane

- **AP-3**: role similar to AP-2, with disease states common if subunits mutated.

- **AP-4**: very little information available
Endocytosis vs. pinocytosis in enamel maturation

- 1976 – Clathrin Identified (Pearse; PNAS)

- 1984 – 91 – coated vesicles noted on the cytoplasmic surface of the apical pole of secretory and maturation ameloblasts (Sasaki ‘83, ‘84 & Franklin et al., ’91). “Tomes’ processes of secretory ameloblasts are highly active in endocytosis” and some of this endocytosis is receptor mediated” (Franklin et al., ’91)

- 1969 – 96 – ameloblasts use macropinocytosis involving passive (fluid-phase) cellular uptake via either ameloblasts, or by intercellular movement into cells of the papillary layer (Smith, Nanci, Katchburian, Warshawsky, Josephsen)
Using multiple bioinformatics analyses, we identified groups of maturation-associated genes whose functions are linked to key mineralization processes including pH regulation, calcium handling and matrix turnover.
Array Data (fold increase)

Lamp1 (6.5 fold), Lamp2 (-), Cd63 (-), Cd68 (6 fold)

Real Time PCR
Figure 4. Clathrin light (CLta) and clathrin heavy chain (CLtc), and Ap2a2 immunolocalization in maturation stage.
Cell Culture

Green – Lamp1
Red – Enamel Matrix Proteins

Shapiro et al., 2007
When Emdogain® is added to plated LS8 cells at a concentration of 250 µg/ml, after 6 hours exposure, significant change in mRNA levels, as determined by qPCR, are noted for Ap2a2, Ap2b1, Ap2m1, Ap2s1, Cltc, Lamp1, Lamp2 and Tpp1 ranging from ∼1.4 fold (for Ap2s1) to ∼3.1 fold (for Cltc). All data normalized to Gapdh.

In Experiment 1 the cells were plated at a lower density (0.9 x 10^5) vs. Experiment 2 (1.2 x 10^6).