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Gene expression analysis of early and late maturation stage rat enamel organ

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Abstract

Enamel maturation is a dynamic process that involves high rates of mineral acquisition, associated fluctuations in extracellular pH and resorption of extracellular enamel proteins. During maturation, ameloblasts change from a tall, thin and highly polarized organization characteristic of the secretory stage, to a low columnar and widened morphology in the maturation stage. To identify potential differences in gene expression throughout maturation, we obtained enamel organ epithelial cells derived from the early and late maturation stages from rat incisor and analyzed global gene expression profiles at each stage. Sixty three candidate genes were identified with potential roles in the maturation process. qPCR was used to confirm results from this genome-wide analysis in a subset of genes. Enriched transcripts in late maturation (n= 38) included those associated with lysosomal activity, solute carrier transport and calcium signaling. Cellular responses to oxidative stress, proton transport, cell death and immune system-related transcripts were also up-regulated. Transcripts down-regulated in the late maturation stage (n= 25) included those with functions related to cell adhesion, cell signaling, and T-cell activation. These results indicate that ameloblasts undergo widespread molecular changes during the maturation stage of amelogenesis and so provide the bases for future functional investigations into the mechanistic basis of enamel mineralization.

Keywords

amelogenesis; early maturation; late maturation; enamel organ; gene expression

Enamel development is most commonly demarcated by the time successive pre-secretory, secretory, transitional and maturation stages. The morphologic appearance of ameloblasts

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differs at each stage with corresponding marked changes in expression of several well-characterized genes (1-4). The relatively limited number of available reports on the biology and chemistry of enamel maturation suggest that this is a dynamic process, with cellular, biochemical, genetic, and epigenetic changes occurring during the progression from the earlier to the later stages. However, to date, the molecular differences between early and late maturation stages have been characterized with only a few gene products. For instance, enamelin mRNA is present in early but not in late maturation, and although amelogenin persists in late maturation, its level decreases in relation to early maturation (1). The cellular differences between early and late maturation stages include changes in volume of ameloblasts and in their organelles. By examining serial sections of rat incisor enamel organs by transmission electron microscopy (animals weighting ca. 150g), one study provided quantitative information regarding the volumetric fraction of ameloblasts and other cells comprising the enamel organ (5). Ameloblast organelles were also quantified (5). The most notable differences were a general increase in several major organelles (mitochondria, granules, vacuoles and autophagic vacuoles) with the exception of lysosomal dense bodies which decreased in late maturation (5). The latter observation was subsequently confirmed by histochemical quantification of trimetaphosphatase, a lysosomal-specific marker (6). A greater density of tubular lysosomes was also found within the papillary cell layer in early maturation compared to late maturation (6). Compared to earlier stages of maturation in rat incisors, late maturation ameloblasts contain a large number of ferritin-filled pigment vesicles suggesting that ameloblast function at this stage involves an increase of iron transfer into the final stages of enamel development in rodents (7).

The pH of mouse incisors has been measured by injections of radiolabeled [^{14}C]DMO (5,5-dimethyl-2,4-oxazolidine dione), a compound that distributes throughout body fluids depending on local concentrations of water and pH with higher concentrations found in areas of high pH (8). In these studies involving 11 day old animals, ^{14}C -labelling differences were observed between the most incisal zone of the incisor and areas putatively in the maturation stage, but at a less advanced stage (8). Values of pH 8.0 and pH 8.5 were determined for the most incisal and less matured area respectively. The pH levels of enamel homogenates reconstituted from freeze-dried enamel strip of ca.0.5mm from rat incisors (95g) were measured after solubilizing proteins in the strips (9). While this study did not focus on differences between early and late maturation, clear changes in pH throughout maturation were observed. Early stages showed mildly acidic pH whereas mid-late maturation stages showed more neutral values related to the spatial distributions of smooth-ended ameloblasts (9). Enamel mineralization is influenced by pH and mineralization rates also differ throughout maturation (10-13). The rates of mineral acquisition in the incisors of rats (95g) and adult mice from sections obtained along the enamel crowns shows a steady increase throughout maturation, with late maturation enamel displaying greater rates than early maturation (13). Total dry weight of enamel per unit of size and per unit of volume sampled, also increases throughout maturation (13).

In addition to these cellular phenomena, several other molecular changes have been reported to occur during amelogenesis. In rats, moderate differences in expression of insulin-like growth factor (IGF) type I and II ligand and IGF receptor (IGF-IR and IGF-IIR) transcripts were observed between early maturation and the area described as the most incisal part (14). Biochemical studies of whole enamel homogenates derived from early and late maturation stages of enamel development in rat incisors incubated *in vitro*, revealed that late maturation enamel proteins were rapidly degraded to completion within 48 hours; however, this was not the case for early maturation stage samples where many protein fragments remained undigested (15). Furthermore, activity levels of cytochrome oxidase, a membrane-bound mitochondrial enzyme, vary between early and late maturation (7).

Although not comprehensive, the seminal studies discussed above suggest that substantial changes in functional activity might exist between the early and late maturation stages of amelogenesis. To gain broader insight at the molecular level, we have performed global gene expression profiling analyses of enamel organ cells from early and late maturation. Given that the need for ion transport, pH stress control and protein degradation increases from early to late maturation, we hypothesize that genes involved in these activities would be up-regulated at the later stage, in keeping with the studies described above. The goal of this study was to identify genes that may be regulating these functions in the final stage of amelogenesis. From our results, we highlight a suite of gene expression changes that occur during these two phases of the maturation stage and discuss their possible biological relevance. Our results provide strong molecular support for the notion that enamel maturation is a dynamic and nonuniform process which involves a complex network of changes in cellular morphology and functions.

MATERIALS AND METHODS

Rat tissue dissections

Two different Wistar Hannover rat populations were sampled. Group 1 consisted of 10 rats (170-190 g) and group 2 consisted of 5 rats (100 g). All rat samples were processed using the exact same methods as follows. Animals were euthanized and their mandibles were immediately dissected out. The surrounding soft tissues were removed and mandibles were then frozen in liquid nitrogen. On average, the time taken from tissue dissection to liquid nitrogen freezing was less than 10 min. Samples were kept in liquid nitrogen overnight and subsequently lyophilized for 24 h. The mandibular bone encasing the lower incisors was carefully removed to expose the entire labial surface. A molar reference line was used to isolate enamel organ cells from early-mid maturation and from mid-late maturation stages, following the method previously described for 100 g rats (16). For the 170-190 g rats, the line was positioned at ~3mm apically from the original molar line. A third group of four rats ~110 g were used obtain material for qPCR analysis. Cells dissected from the rat enamel organ include ameloblasts but may also contain other cell types (i.e. papillary layer cells, small blood vessels and some connective tissue). All vertebrate animal manipulation complied with Institutional and Federal guidelines.

mRNA extraction and gene expression profiling

Isolated cells from the early-mid maturation area from all 170-190 g animals were pooled together to extract RNA. The same procedure was done for the mid-late maturation. This process was also repeated for the 100 g rats. Each of the different sets of pooled cells was homogenized and RNA was isolated using Qiagen RNeasy Mini Kit. RNA was obtained from cells at early-mid and mid-late maturation for the 170-190 g rats. The same stages were also sampled in the other group (100 g rats). Gene expression profiling analysis was performed on RNA samples obtained from the 170-190 g rats independently of the 100 g rats. Quadruplicate technical replicate analyses were performed for each RNA batch (4 unique batches, 16 total global gene expression profiling analyses). Gene expression profiling was performed using RatRef-12-v1 Expression BeadChips (Illumina, San Diego, CA, USA) that interrogate approximately 21,910 transcripts selected primarily from the National Center for Biotechnology Information (NCBI) RefSeq database (Release 16). RNA samples collected from Group 1 and Group 2 were analyzed on separate BeadChips. All sample preparation, hybridization, BeadChip processing, and data acquisition were performed at the Southern California Genotyping Consortium (SCGC) according to the manufacturers recommended protocols.

Real time PCR

Only enamel organ cells from the 110 g rats were used for real time PCR analysis to substantiate the gene expression profiling analysis as follows. Pooled enamel organ cells from the early-mid and mid-late maturation were homogenized and total RNA was obtained using Qiagen RNeasy Mini Kit. Reverse-transcribed PCR was obtained using iScript cDNA Synthesis kit (Bio-Rad, City?, State?, USA). Real time PCR reactions were performed with iQ SYBR Green Supermix (Bio-Rad) using rat-specific primers (Table 1). All primer pairs were designed to span intronic regions. Data was normalized to β -actin.

Data processing

The raw data from each of the two BeadArrays (corresponding to data generated from Groups 1 and 2) were processed independently in the Bioconductor R environment according a newly reported method (17) to produce normalized log(2) transformed gene expression scores for the early-mid maturation and mid-late maturation samples. Herein, we defined a gene as being differentially expressed between the early-mid and mid-late maturation stages in an experimental group if there was: (i) a >1.2 -fold change in the geometric means of the gene expression scores and (ii) two-tailed Student's t-test $P < 0.05$ subjected to Benjamini and Hochberg correction for multiple comparisons (18) among the technical replicates in the group. We focused on genes identified as being differentially expressed in the same direction in both Group 1 and Group 2.

Data analysis

GeneOntology, KEGG, transcription factor binding site, and miRNA enrichment analyses were conducted using WebGestalt software (19, 20). Ingenuity Pathways Analysis (IPA) v8 software (Ingenuity Systems, Redwood City, CA, USA) was also used to analyze functional relationships among differentially expressed genes.

RESULTS

Identification of differentially expressed genes (DEGs)

A total of 148 transcripts were differentially expressed in the first experiment and 136 transcripts were differentially expressed in the second experiment (see Methods and Materials for the criteria used to assign DEGs). Thirty eight differentially expressed transcripts were up-regulated in the mid-late maturation stage relative to the early-mid maturation stage in both Groups 1 and 2 (Table 2). Thirty four (89%) of these transcripts showed at least a 2-fold change in both groups (Table 2). Conversely, 25 differentially expressed transcripts were more abundant in the mid-late maturation stage relative to the early-mid maturation stage in both Groups 1 and 2 (Table 3). Twenty-three (90%) of these transcripts showed at least a 2-fold change in both groups (Table 3).

GeneOntology (GO) analysis

To analyze broad patterns in their biological processes, molecular functions, and cellular components, we conducted Gene Ontology (GO) analyses of the DEGs shared between both broad time points in the maturation stage across the two rat samples. We used stringent criteria (Fisher's exact test Benjamini and Hochberg corrected $P < 0.05$, minimum of two genes) for selection of enriched categories. The enriched categories of DEGs that were up-regulated in late maturation included those related to superoxide metabolism (*Mt3*, *Cyba*, and *Sod1*), removal of superoxide radicals (*Mt3* and *Sod1*), mitochondrial membrane potential (*Cck* and *Sod1*), innate immune response (*Cyba*, *Cfd*, and *Lbp*) and proton-transporting two-sector ATPase complex (*Atp6v0d2* and *Tcirg1*) (see Supporting Table S1A). The enriched categories of DEGs that were down-regulated in late maturation

included those related to protein stabilization (*Eraf* and *Rbm38*), systems development (*Alox15*, *Satb1*, *Amelx*, *Sostdc1*, *Krt27*, *Cd24*, *Pbx3*, *Ctnna2*, and *Eraf*) and odontogenesis (*Sostdc1* and *Amelx*) (see also Table S1B). Graphical representation of GO analysis in up- and down-regulated genes is shown in Supporting Figs. S1 and S2.

KEGG analysis

We also conducted KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analyses of the DEGs shared between both broad time points in the maturation stage across the two rat samples. As above, we used stringent criteria (Fisher's exact test Benjamini and Hochberg corrected $P < 0.05$) for selection of enriched categories using a minimum of two genes per category. Only two enriched categories of DEGs were up-regulated in the late maturation. These were the lysosomal pathway-related transcripts (*Cd68*, *Lamp1*, *Atp6v0d2*, and *Tcirg1*) and oxidative phosphorylation (*Atp6v0d2* and *Tcirg1*). A full list of genes for each category is shown in Table S2. KEGG analysis did not reveal any enriched pathways for down-regulated gene transcripts in the late maturation samples.

Gene network analysis

We used Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, Ca, USA) to analyze functional relationships among DEGs. The three most significant gene networks for DEGs up-regulated in late maturation were: 1) Free radical Scavenging, Cell-to-cell signaling interaction, Hematological system development and function, 2) Cell cycle, Cellular development, Cellular growth and proliferation, 3) Inflammatory response, Cellular development. The three most down-regulated networks were: 1) Post-translational modification, Small molecule biochemistry, Carbohydrate metabolism, 2) Lipid metabolism, Small molecule biochemistry, Cell-To-cell signaling and interaction, 3) Cellular function and maintenance, Cellular movement, Hematological system development and function. A graphical representation of these network analyses highlighting the core processes involved is shown in Figs. S3 and S4.

Transcriptional factor (TF) binding site analysis

In order to explore the higher-order regulation of DEGs, we screened for the possible enrichment of transcription factor binding sites in their promoters. No significant enrichments for TF binding sites (defined as Benjamini and Hochberg corrected $P < 0.05$) were identified for the DEGs of up-regulated transcripts in late maturation. The most significant TF binding sites (Benjamini and Hochberg corrected $P < 0.05$, at least two genes) for DEGs down-regulated in late maturation are included in Table S3.

MicroRNA target enrichment analysis

To further explore the regulation of DEGs, we used computational approaches to screen for miRNAs known to regulate their expression; however, we did not measure the abundance of these miRNAs in our study. The only significantly enriched miRNA (Benjamini and Hochberg corrected $P < 0.05$) corresponding to DEGs up-regulated in late maturation was rno_AGGAGTG, represented by the transcripts corresponding to *Slc68a* and *Il1rl1*. For DEGs down-regulated in late maturation, two significantly enriched miRNAs included rno_GTCTTCC, represented by *Pbx3* and *Satb1*, and rno_ACTACCT represented by *Pbx3* and *Col14a1*. Further details of these miRNAs data are shown in Table S4.

Real time PCR

Fig. 1 show results for mRNA expression comparing the early-mid and the mid-late maturation stage for a selected group of genes based on their fold change expression identified in the genome-wide profiling analysis. The well known enamel gene *Amelx* was

used as a control. *Amelx* expression significantly decreased in mid-late maturation relative to early-mid maturation stage as previously reported (1) and as also identified in this study by genome-wide array (Table 3). The most highly up-regulated gene transcript in the genome-wide analysis is *Cck* (Table 2), which also increased expression between early-mid and mid-late maturation by 20 fold as measured by qPCR (Fig. 1B). The expression of the chloride channel *Clca2* increased 12 fold whereas *Mt3* increased by 3 fold and *Slc6a8* 1.3 fold. With the exception of *Slc6a8*, changes between early-mid and mid-late for these genes were statistically significant (one way ANOVA; $P < 0.05$). All values are relative to the expression of β -actin.

DISCUSSION

The main aim of this study was to survey a broad range of gene expression differences that occur in the early-mid and mid-late maturation stages of amelogenesis in rat incisor enamel organ cells. In order to begin the process of cataloging these differences, we used global gene expression profiling methods to characterize the transcriptomes. As expected, the well-known enamel genes *Amelx* and *Enam* showed markedly decreased expression by late maturation (Table 3). The changes observed for *Ambn* were less remarkable and although its expression also decreased, it did not meet our criteria for a DEG. This finding is in agreement with reports that *Ambn* is expressed throughout the maturation stage (2). Collectively; these results reflect the ability of our system to detect major gene expression changes expected to occur in the transition from early-mid to mid-late maturation stages and the robustness of our analytical methods.

Although no gross morphological changes have been described between early and late maturation in the enamel organ, a number of ultrastructural (vacuolation patterns, lysosomal content) as well as inferred physiological activities such as those possibly derived from differences in pH and differences in rates of mineral acquisition described earlier between these two stages (5, 6) are likely related to differences in gene regulation. Consistent with these features of enamel maturation, the most up-regulated genes in late maturation include those potentially involved in matrix endocytosis associated with lysosomal and endosomal membranes (*Lamp1* and *Atp6v0d2*). Other up-regulated lysosomal-associated membrane proteins include *Rab24*, *Tspan4*, *Tcirg1* and *Cd68*. These data contrast with previous descriptions of a reduction of lysosomal bodies within the late stage ameloblasts (5). One possible explanation is that LAMP1 and ATP6V0D2 expression may be confined to the papillary layer, but this requires further morphological confirmation.

Our list of differentially expressed genes (DEGs) includes those highly relevant to intracellular signaling and ion transport. In keeping with previous reports that chloride deficiencies affect enamel growth (21-23), the levels of the *Clca2* transcript, encoding an anion channel protein putatively involved in chloride transport, protein secretion, and cell adhesion (24, 25), were more abundant in the mid-late stage of maturation in both experimental groups (10.2 and 8.5-fold, respectively). CLCA2 proteins are commonly localized in the plasma membranes of secretory epithelial cells (26, 27). The neuropeptide cholecystokinin (*Cck*) was one of the most highly up-regulated transcripts in both experiments (30.5-fold and 25.3-fold, in Groups 1 and 2, respectively) (Table 2), suggesting a role in calcium-signaling during late maturation. The role of *Cck* as a calcium signaling molecule in various cell types is becoming increasingly appreciated (28, 29). Two members of the S100 family of calcium-binding proteins (S100a4 and S100a8) were up-regulated in late maturation but did not meet our criteria for DEG.

The association between members of the solute carrier (SLC) gene family and enamel development is an important area of research, and recent data suggests that many of these

genes are expressed in the enamel organ and some are critically important for enamel development (21, 30, 31). The *Slc6a8* gene identified in our study as being up-regulated in the late maturation stage is associated with creatine transport (32, 33), a molecule not previously identified in the enamel organ. Its localization and putative function in amelogenesis warrants further attention.

As emphasized by the GO analysis (Fig. S1), several key genes with antioxidant response functions (*Mt3*, *Sod1*, *Sesn1*, and *Nqo1*) were up-regulated in late maturation. Metallothionein gene family member 3 (*Mt3*) is a component of a large gene family that plays a major role in cellular homeostasis by sequestering metal cations. Members of this family are up-regulated in response to oxidative stress (34, 35). Interestingly, *Mt3* has been implicated in regulating lysosomal functions and autophagy (36), which is consistent with the differential expression of genes encoding the lysosomal-associated membrane proteins LAMP1, RAB24, TSPAN4, CD68 and the lysosomal proton pump ATP6V0D2. Although metallothioneins are typically associated with cellular survival responses to environmental insults such as reactive oxygen species and metals, there are multiple reports where *Mt3* expression is associated with cell death in brain cells and tissues (37, 38). This has been ascribed to the release of toxic levels of *Mt3*-bound intracellular metals, such as zinc, in response to oxidative stress (37, 38). The superoxide dismutase (*Sod1*) gene has been long appreciated as playing a major role in cellular antioxidant stress responses (39). *Sesn1* is a member of a gene family reported to protect cells from oxidative stress (40). *Nqo1* is a target gene of the Nrf2 transcription factor and a reported part of cellular antioxidant stress responses (41, 42). Overall the greater expression of these four genes (*Mt3*, *Sod1*, *Sesn1*, and *Nqo1*) suggests the possibility of increased oxidative stress and toxic intracellular levels of metals in mid-late maturation ameloblasts relative to early-mid stages of enamel maturation. Calcium is of particular interest in this regard, having a strong link with oxidative stress and cellular toxicity, and being strongly associated with the maturation phase (43, 44). Additionally, the transferring receptor *Tfrc*, commonly involved in iron uptake, was up-regulated in late maturation although it did not meet our criteria for DEG. While the source of the putative oxidative stress is unclear, we note that the *Cyba* gene is up-regulated in late maturation and is well-documented to play a major role in the generation of ROS in vascular and immune cells (45, 46). Collectively, these data could provide a candidate mechanism for the molecular basis of programmed cell death in ameloblasts during the later stages of enamel maturation. Functional biochemical analyses will be required to test this and other related hypotheses.

The inter-relationships of the down-regulated genes in late maturation were less clear than those that were up-regulated. In addition to the above discussed genes (*Amelx*, *Enam*), the expression of *Sostdc1*, a gene known to be involved in the early stages of tooth formation antagonizing several pathways such as FGF (47), was down-regulated. Other down-regulated transcriptional regulators include *Pbx3*, *Satb1*, and *Tcea3* whose identification in amelogenesis is novel and their function in late-stage amelogenesis is unknown. The broad functional GO category of cell adhesion genes was enriched for down-regulated genes (*Cd24*, *Ctnna2*, *Col14a1*, and *Amelx*). Likewise the levels of *Panx3* transcript, which encodes a structural component of gap junctions (48), were reduced 14.7 and 20.5-fold in the two comparisons. Several enzymes were greatly down-regulated in the later maturation. For example, acid phosphatase *Acpt*, which has a structural similarity to lysosomal acid phosphatases (49), was down-regulated >9-fold in both groups. *Gba3*, a predominantly liver cytoplasmic enzyme that hydrolyzes beta-D-glucoside and beta-D-galactoside (50), was down-regulated >75-fold in both groups.

Collectively, our list of DEGs also contained genes associated with immune functions normally not linked with enamel formation, such as *Lbp*, *Il1r1l*, *Lypd3*, *Ccl19*, *Cfd*, *Cyba*,

RGD1564553, *RT1-Da*, and *Cd24*. Gene network analysis also highlighted chemokines as being a major functional category related to the genes up-regulated in later maturation (Fig. S3). While it is possible that these genes are differentially expressed in ameloblasts, we favor the hypothesis that these transcripts collectively reflect the role of the immune system in the later stage of maturation. The apoptosis associated with later stage maturation could promote an immune response to remove dead cells. This emphasizes the fact that enamel formation is a complex system that involves the interplay of multiple cell types and processes. Studies aimed to analyze the localization of these molecules in the enamel organ are warranted.

In our search for biological networks represented by our DEGs, we conducted transcription factor binding site enrichment and miRNA enrichment analyses (Tables S3 and S4). We note that β -catenin/LEF1 (represented by *Cdc42ep3*, *Ctnna2*, *Pbx3*, *Rasl11b*, *Panx3*, and *Satb1*) was an over-represented regulatory motif among genes down-regulated in late maturation. Whereas it is recognized that β -catenin/LEF1 interaction is important in the earlier development of tooth formation (51), the observed decrease in expression may be associated with the function of *Lef1* in the regulation of *Enam* expression (52). These results suggest that other transcription factors listed in Table S3 could also be relevant to enamel formation. For instance, two members of the CEBP family (CEBP-alpha, CEBP-delta) are known for their activity as transcriptional regulators of amelogenin (53). Our analysis revealed that one additional member of this family, CEBP-gamma, is down-regulated in late maturation suggesting a possible role at this stage. Our miRNA analyses could also provide novel leads in the rapidly growing area of identifying miRNAs relevant to tooth or enamel formation (54). These computational approaches promise to provide better candidates as the annotation and discovery of novel miRNA species improve.

Finally, results from our global gene expression profiling analyses confirmed that maturation is not a uniform process. It is well known that ameloblasts engage in a dynamic series of modulations between ruffle-ended (RA) and smooth-ended (SA) morphology throughout maturation (55-57). On average, histological sections of rat incisors display ~70% of cells in the RA stage and ~23% of cells in the SA stage (56-58). Cells in transition from SA to RA may also be identified (55). Therefore, possible differences in gene expression between RA and SA phases (58) are not distinguished in this analysis but rather the differences identified in this study likely reflect global differences in gene expression of the whole enamel organ at each stage. Early and late maturation should then be viewed as two important subdivisions of the maturation stage each characterized by their own distinct transcriptomes, which we have begun to elucidate in this study.

It is becoming increasingly clear that in rodents, there are some differences in gene expression between incisors and molars. An example is of this is the enamel of *Enam*-deficient mice in which this deletion affects incisors but not the molars (59). However, with the exception of pigmentation, a phenomenon of rodent incisor but not of molars, the stages of amelogenesis and the sequences of changes in ameloblasts and papillary layer cells undergo are identical on all mammalian teeth (11). Thus gene expression by ameloblasts in molars might be qualitatively, but likely not quantitatively similar.

In summary, we provide novel insights into the maturation process by analyzing changes between early and late maturation in rat incisor enamel organ based on two independent global gene expression profiling comparisons involving multiple pooled samples. These results were also confirmed by qPCR for a subset of genes (Fig. 1). We have shown that the early and late stages of enamel maturation have distinct transcriptomes, and identified a novel suite of candidate genes associated with the late stages of amelogenesis. Based on these results, we have provided candidate mechanisms for the programmed cell death known

to occur during the later stages of enamel formation. Future studies will help clarify the mechanistic relevance of the genes described. For instance, members of the SLC family and other membrane bound proteins discussed here likely have differential sub-cellular localization, and hence participate in entry or extrusion of ions and molecules. It may be possible that in some instances these products may be associated with ruffle-ended or smooth-ended ameloblasts, or may in fact be localized to the papillary layer. Identification of these tissue and cellular localizations will be important future steps to understand better the intricacies of enamel maturation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Real time PCR analysis of selected gene transcripts based on fold changes identified in the genome-wide study (see Table 2)

A) *Amelx* expression significantly decreases from secretory to early-mid maturation ($P < 0.01$), and from early-mid to mid-late maturation ($P < 0.01$). This pattern of expression is in keeping with previous reports for *Amelx* (ref. 1). We sampled the secretory stage as a reference point for *Amelx* expression. B) The expression of *Cck*, *Mt3*, *Clca2* and *Slc6a8* increased in mid-late maturation relative to early-mid maturation with fold increases of 20, 3, 12 and 1.3 respectively. Stars indicate significance (one way ANOVA; $P < 0.05$). All values are relative to β -actin.

Table 1

Primers used for real time PCR analysis.

Gene	GenBank Ref.	Forward	Reverse	product
<i>Mt3</i>	NM_053968	AGTGTGCCAAGGACTGTGTT	GCTGTGCATGGGATTTATTC	182
<i>Slc6a8</i>	NM_017348	TCCGTGTAGCAGCTTAAACC	GCTATGTTTGCAGTGGCTTT	175
<i>Clea2</i>	NM_001013202	TCTCAATTCCGTGGTTGAAT	ACTTGAGCAAGGAAAACGTG	185
<i>Cck</i>	NM_012829	GTCCCTGTAGAAGCTGTGGA	ATCCTATGGCTAGGGTCCAG	206
<i>Actb</i>	NM_031144	AGTGTGACGTTGACATCCGTA	GCCAGGGCAGTAATCTCCTTCT	112
<i>Amelx</i>	NM_019154	ATAAGGCAGCCGTATCCTTCC	GTTGGGTTGGAGTCATGGAGT	208

Table 2

List of up-regulated genes in late maturation compared to early maturation

Probe ID*	Symbol	Brief Description	Entrez GeneID	Fold change	
				Group 1**	Group 2**
ILMN_1365636	<i>Cck</i>	cholecystokinin	25298	30.5	25.3
ILMN_1359784	<i>Fam129B</i>	family with sequence similarity 129, member B	362115	26.4	27.3
ILMN_1369752	<i>Nr2f6</i>	nuclear receptor subfamily 2, group F, member 6	245980	11.1	13.8
ILMN_1350246	<i>Rab24</i>	RAB24, member RAS oncogene family	361208	9.8	12.6
ILMN_1363177	<i>Clca2</i>	chloride channel calcium activated 2	362052	10.2	8.5
ILMN_1357541	<i>Dnase1l1</i>	deoxyribonuclease 1-like 1	363522	8.3	7.0
ILMN_1359735	<i>Snrp70</i>	U1 small nuclear ribonucleoprotein polypeptide A	361574	8.2	5.7
ILMN_1374039	<i>Mt3</i>	metallothionein 3	117038	9.6	4.2
ILMN_1376661	<i>Lbp</i>	lipopolysaccharide binding protein	29469	6.7	3.3
ILMN_2040501	<i>Dclk2</i>	similar to doublecortin-like kinase 2	310698	4.9	4.6
ILMN_2039341	<i>Tspan4</i>	tetraspanin 4	293627	3.9	5.2
ILMN_1351677	<i>Ccl19</i>	chemokine (C-C motif) ligand 19	362506	5.6	3.2
ILMN_1376603	<i>Atp6v0d2</i>	ATPase, H+ transporting, V0 subunit D, isoform 2	297932	5.6	2.8
ILMN_1363606	<i>Lcn2</i>	lipocalin 2	170496	4.8	3.3
ILMN_1370938	<i>CD68</i>	CD68 molecule	501872	4.8	2.9
ILMN_2039396	<i>Nqo1</i>	NAD(P)H dehydrogenase, quinone 1	24314	4.2	3.3
ILMN_1350232	<i>Phyh2</i>	phytanoyl-CoA 2-hydroxylase 2	85255	3.8	3.6
ILMN_1376744	<i>Slc6a8</i>	solute carrier family 6, member 8	50690	3.8	3.7
ILMN_1352451	<i>LOC500150</i>	similar to tigger transposable element derived 2	500150	3.6	3.5
ILMN_1357538	<i>Lamp1</i>	lysosomal membrane glycoprotein 1	25328	3.5	3.4
ILMN_1361478	<i>Cthrc1</i>	collagen triple helix repeat containing 1	282836	3.0	3.8
ILMN_1357240	<i>Tmem160</i>	transmembrane protein 160	292654	3.2	3.1
ILMN_1349832	<i>Arhgap18</i>	Rho GTPase activating protein 18	293947	3.5	2.7
ILMN_1357380	<i>Gpha2</i>	glycoprotein hormone alpha 2	171158	3.2	3.1
ILMN_1365188	<i>Bglap2</i>	bone gamma-carboxyglutamate protein 2	25295	3.2	2.9
ILMN_1351254	<i>Tcrrg1</i>	T-cell, immune regulator 1	293650	3.4	2.6
ILMN_1351083	<i>Tmem14a</i>	transmembrane protein 14A	363206	2.8	2.9

Probe ID*	Symbol	Brief Description	Entrez GeneID	Fold change	
				Group 1**	Group 2**
ILMN_1349635	<i>Lib4dh</i>	leukotriene B4 12-hydroxydehydrogenase	192227	2.7	2.9
ILMN_1370763	<i>Serpine2</i>	serine proteinase inhibitor, clade E, member 2	29366	2.8	2.3
ILMN_1353544	<i>Sod1</i>	superoxide dismutase 1	24786	2.4	2.7
ILMN_1369374	<i>Il1rl1</i>	interleukin 1 receptor-like 1	25556	2.6	2.5
ILMN_1371456	<i>Cfd</i>	complement factor D	54249	2.4	2.4
ILMN_1366566	<i>Cd68</i>	CD68 antigen	287435	2.7	2.0
ILMN_1370862	<i>Upp1</i>	uridine phosphorylase 1	289801	2.2	2.4
ILMN_1376935	<i>RT1-Da</i>	RT1 class II, locus Da	294269	1.9	2.4
ILMN_1368022	<i>Lypd3</i>	Ly6/Plaur domain containing 3	60378	1.9	2.2
ILMN_1353298	<i>Sesn1</i>	sestrin 1	294518	2.0	1.9
ILMN_1366276	<i>Cyba</i>	cytochrome b-245, alpha polypeptide	79129	1.7	1.8

* Illumina probe identifier.

** Fold-change of expression scores from late maturation relative to early maturation stages based on geometric means. All differentially expressed genes meet the statistical criteria described in the Materials and Methods section.

Table 3

List of down-regulated genes in late maturation compared to early maturation

Probe ID*	Symbol	Brief Description	Entrez GeneID	Fold change Group 1**	Fold change Group 2**
ILMN_1366951	<i>Gba3</i>	glucosidase, beta, acid 3	289687	-122.6	-76.3
ILMN_1370394	<i>Enam</i>	enamelin	289525	-24.2	-36.4
ILMN_1359815	<i>Pannx3</i>	pannexin 3	315567	-14.7	-20.5
ILMN_1354038	<i>Ctna2</i>	catenin, alpha 2	297357	-20.2	-14.8
ILMN_1374245	<i>Kat40</i>	type I keratin KA40	450229	-10.1	-15.8
ILMN_1367363	<i>Ame1x</i>	amelogenin X chromosome	29160	-5.2	-19.1
ILMN_1351599	<i>Gal3s4</i>	galactose-3-O-sulfotransferase 4	498166	-12.5	-9.4
ILMN_1351102	<i>Acpt</i>	acid phosphatase, testicular	308569	-9.4	-11.3
ILMN_1364569	<i>Cd24</i>	CD24 antigen	25145	-10.4	-9.4
ILMN_1376641	<i>Chst1</i>	carbohydrate sulfotransferase 1	295934	-9.3	-7.8
ILMN_1367351	<i>Papln</i>	papilin	314297	-9.1	-6.5
ILMN_1350091	<i>G0s2</i>	G0/G1 switch gene 2	289388	-8.0	-6.7
ILMN_1361779	<i>Tcea3</i>	transcription elongation factor A, 3	298559	-7.6	-6.2
ILMN_1371621	<i>Cst9</i>	cystatin 9	362231	-8.5	-4.1
ILMN_1376648	<i>Satb1</i>	special AT-rich sequence binding protein 1	316164	-4.0	-4.0
ILMN_1352748	<i>Sostdc1</i>	sclerostin domain containing 1	266803	-4.3	-3.5
ILMN_1357730	<i>Npy</i>	neuropeptide Y	24604	-2.5	-3.9
ILMN_1374380	<i>Col14a1</i>	procollagen, type XIV, alpha 1	314981	-3.0	-3.1
ILMN_1371267	<i>Alox15</i>	arachidonate 15-lipoxygenase	81639	-3.1	-2.9
ILMN_1364703	<i>Wdr54</i>	WD repeat domain 54	500226	-2.9	-2.6
ILMN_1352848	<i>Cdc42ep3</i>	CDC42 effector protein 3	313838	-2.4	-2.3
ILMN_1357931	<i>Phx3</i>	pre B-cell leukemia transcription factor 3	311876	-2.4	-2.2
ILMN_1357070	<i>Rnpe1</i>	RNA-binding region containing 1	366262	-2.3	-2.1
ILMN_1363087	<i>Ras111b</i>	RAS-like family 11 member B	305302	-1.4	-2.6
ILMN_1374043	<i>Eraf</i>	erythroid associated factor	293522	-1.7	-1.7

* Illumina probe identifier.

** Fold-change of expression scores from late maturation relative to early maturation stages based on geometric means. All differentially expressed genes meet the statistical criteria described in the Materials and Methods section.