Gene expression profiling during murine tooth development

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The aim of this study was to describe the expression of genes, including ameloblastin (Ambn), amelogenin X chromosome (Amelx), and enamelin (Enam) during early (pre-secretory) tooth development. The expression of these genes has predominantly been studied at post-secretory stages. Deoxyoligonucleotide microarrays were used to study gene expression during development of the murine first molar tooth germ at 24 h intervals, starting at the 11th embryonic day (E11.5), and up to the 7th day after birth (P7). The profile search function of Spotfire software was used to select genes with similar expression profile as the enamel genes (Ambn, Amelx, and Enam). Microarray results where validated using real-time reverse transcription-polymerase chain reaction (real-time RT-PCR), and translated proteins identified by Western-blotting. In situ localization of the Ambn, Amelx, and Enam mRNAs were monitored from E12.5 to E17.5 using deoxyoligonucleotide probes. Bioinformatics analysis was used to associate biological functions with differentially expressed (DE; p ≤ 0.05) genes. Microarray results showed a total of 4362 genes including Ambn, Amelx, and Enam to be significant DE throughout the time-course. The expression of the three enamel genes was low at pre-natal stages (E11.5–P0) increasing after birth (P1–P7). Profile search lead to isolation of 87 genes with significantly similar expression to the three enamel proteins. These mRNAs were expressed in dental epithelium and epithelium derived cells. Although expression of Ambn, Amelx, and Enam were lower during early tooth development compared to secretory stages enamel proteins were detectable by Western-blotting. Bioinformatic analysis associated the 87 genes with multiple biological functions. Around 35 genes were associated with 15 transcription factors.

Keywords: tooth development, ameloblastin, amelogenin, enamelin

INTRODUCTION

Interactions between oral epithelium and neural crest derived mesenchyme are considered essential for tooth development. Cells of the epithelium expand and proliferate, invaginating into the condensing mesenchyme, and subsequently forms the tooth germ (Thesleff, 1995; Chai et al., 2000; Sharpe, 2001), this process been modulated by several growth factors (Thesleff and Mikkola, 2002; Zhang et al., 2005). As the invaginating epithelium expands it is surrounded by condensing mesenchyme transforming into a bud and cap, subsequently developing into the bell stage (Tucker and Sharpe, 1999; Fleischmannova et al., 2008). Mesenchymal cells facing the basement membrane differentiate into dentin producing odontoblasts, while the adjacent layer of epithelial cells differentiates into ameloblasts which secrete the organic enamel matrix (Thesleff and Hurmerinta, 1981).

Ameloblastin (encoded by Ambn) is expressed in the mineralizing matrix of bones and teeth. This matrix protein inhibits ameloblast proliferation and is essential for ameloblast adhesion affecting thickness of the enamel layer (Zhang et al., 2011).

Amelogenins (encoded by Amel) are expressed in epithelium derived cells, bone marrow, and mesenchymal stem cells (MSCs). Amelogenins are the main component in the developing enamel matrix and essential for normal enamel thickness and structure (Feng et al., 2012).

Enamelin (encoded by Enam) is a minor constituent of the extracellular matrix but plays a critical role in normal enamel formation. Enamelin is required for the deposition of tooth enamel, but it is also necessary to maintain the ameloblast phenotype, as is the case for ameloblastin (Hu et al., 2011).

The expression of about 300 genes has been mapped1 and has contributed to our understanding of tooth development. The expression of a substantially higher number of genes is likely to be involved. The use of microarrays facilitates the global mapping of genes at various stages of tooth development.

Recent results obtained from gene expression profiling, between two developmental stages (E15.5 and P2) of murine tooth germs using microarrays indicated that Amelx, Ambn, and Enam exhibited low levels of expression at the studied pre-natal stage (E15.5; Osmundsen et al., 2007). We investigated if these three

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1http://bite-it.helsinki.fi
genes were expressed prior to tooth mineralization and if other genes followed the same expression pattern; low levels of expression at pre-natal stages followed by increase at post-natal stages, during murine tooth development. The study of global gene expression during both pre- and post-natal stages (16 time points) of murine molar tooth development using microarrays should provide data capable of capturing dynamic gene expression profiles during tooth development, combined with bioinformatics might help our understanding of cellular processes underlying development of the murine tooth germ.

**MATERIALS AND METHODS**

**EXPERIMENTAL ANIMALS**

The day of the vaginal plug was set to embryonic day E0.5 in pregnant female CD-1 mice (n = 3–5). The embryo/fetus developmental stage was assessed using the Thelier criteria (Kaufman, 1992).

The animal house had 12 h light/dark cycle and was thermostated at 21°C with relative humidity at 55 ± 5%. Water and fodder was provided ad lib. The animals were housed according to the regulations of the Norwegian Gene Technology Act of 1994.

Female mice pregnant at various stages were sacrificed by cervical dislocation and embryos quickly removed from the amnion sac and decapitated.

**TISSUE PREPARATION**

The first mandibular molar tooth germs was dissected for microarray and real-time RT-PCR analysis from CD-1 embryos at various developmental stages starting at the 11th embryonic day (E11.5) and ending at the 7th day after birth (P7).

For in situ hybridization (ISH) embryos heads were dissected and fixed in 4% (v/v) cold, neutral buffered formalin (NBF; AppliChem, Darmstadt, Germany). Heads dissected at P2 up to P7 were decalcified in 12.5% (w/v) ethylenediaminetetraacetic acid (EDTA), 2.5% (v/v) formalin. Paraffin embedding was carried out essentially as described by Hougaard et al. (1997). Serial sections 8 µm thick were used. Batches of three to nine molar tooth germs were dissected for Western blot analysis at each of the various developmental time points used in this investigation. The tooth germs were lysed using CellLytic MT (Sigma, St Louis, MO, USA) and Halt Protease Inhibitor Cocktail (Pierce Biotechnology, Rockford, IL, USA). Protein concentrations were assayed using Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

**MICROARRAY ANALYSIS OF mRNAs ISOLATED FROM TOOTH GERMS**

Total RNA was extracted from tooth germs as described previously by Osmundsen et al. Murine deoxoyribonucleotide (30 k)-microarrays printed with Operon murine v3 oligo-set (Qia-gen GmbH, Hilden, Germany) were purchased from the NTNU Microarray Core Facility (Norwegian University of Science and Technology, Trondheim, Norway). Spikes from A. thaliana (purchased from Stratagene, La Jolla, CA, USA) were used to normalize the fluorescence within each microarray and to monitor the quality of the hybridization. Complementary DNA synthesis, labeling, and hybridization were carried out as described previously (Osmundsen et al., 2007).

After hybridization and scanning the resulting expression data (48 arrays) was assembled into a single data file. Cy3 and Cy5 channels of each slide were treated as single channel data as if derived from single color arrays to facilitate statistical and bioinformatic analysis. The genes exhibiting a net fluorescence fewer than 200 were excluded. LOESS normalized fluorescence intensities (median values, with background subtracted) from each of the two channels were converted to log2-scale, and the log2-values were subjected to z-score normalization (Cheadle et al., 2003).

Statistical analysis of microarray data was carried out using Spotfire v. 9™Decision Site for Microarray Analysis (Spotfire, MA, USA) from sets of three arrays at each time point.

The ANOVA facility of the Spotfire program was used to select genes which exhibited statistically significant differences in levels of expression (p < 0.05) between various developmental stages. False discovery rate (FDR; 0.05; Benjamini and Hochberg, 1995; Reiner et al., 2003) was used to correct selection of genes for false positives.

Experimental design and resulting microarray files have been deposited in the MIAME database with reference E-MEXP-3581.

**ISOLATION OF GENES USING PROFILE SEARCH**

Profile search function of Spotfire software v.9 Decision Site for Microarray analysis software (TIBCO Spotfire, Somerville, MA, USA) was used to select differentially expressed (DE) genes with a similar expression pattern to that of pre-selected genes Ambn, Amelx, and Enam. The mean time-course for Ambn, Amelx, and Enam genes (normalized data) throughout the studied time-course was used as search criteria (master profile). The resulting expression profile was subjected to hierarchical clustering and the result presented as heat map. Unknown genes without an Entrez ID were omitted from this analysis.

**REAL-TIME RT-PCR**

Triplicates of tooth germs for each time point were used in cDNA synthesis (Fermentas, St. Leon Route, Germany). The subsequent real-time PCR was carried out in a Stratagene MX3005P (Stratagene, La Jolla, CA, USA), using SYBR Green-based assay Ampliqon III (Ampliqon, Rødovre, Denmark). Real-time RT-PCR data was analyzed using the ∆∆Ct method 2−∆∆Ct method 2− ∆Ct (Ctarget − CtRpl27) Time point x = (Ctarget − CRpl27)E11.5. Where time x = E11.5 up to P7. All time points were subjected to E11.5 and normalized against ribosomal protein L27 (Rpl27; Pfaffl, 2001). The primer sequences are listed in Table 1.

**BIOINFORMATIC ANALYSIS OF DIFFERENTIALLY EXPRESSED GENES**

Bioinformatic analysis using Ingenuity Pathway Analysis (IPA; Ingenuity Systems Inc., Redwood City, CA, USA) was carried out to identify significant associations (Fisher’s Exact Test, p ≤ 0.05) with canonical pathways, signaling pathways, transcription factors, molecular, and cellular functions for the genes DE, isolated using the profile search function of the Spotfire software. Transcription factors with p-value of overlap <0.01 where considered to be significantly associated with the DE expressed genes.

**IN SITU HYBRIDIZATION**

In situ hybridization was used to visualize microarray and real-Time RT-PCR results for the genes of the expression profile and
Table 1 | Sequences of primers used for real-time RT-PCR assays.

| Gene | Sequence of left primer | Sequence of right primer |
|------|-------------------------|--------------------------|
| Amelx | 5′-CTC TGC CTC CAC TG TCT CC-3′ | 5′-ACT TCT CCC CCG TTG TT-3′ |
| Enam | 5′-GCT TGG GCT CCA ATT CAA AA-3′ | 5′-AGG ACT TCC AGT GGG TGG-3′ |
| Ambn | 5′-CTG TTA CCA AAG GCC CTG AA-3′ | 5′-GCT ATT TGG CAA AGG AGA GC-3′ |
| Rpl27 | 5′-GGG AAA GTG GTG GTG CTG T3′ | 5′-CAC AGT GGC ATG GCT GTA AG-3′ |
| Wif1 | 5′-ACC CTC CGG AAA TGG AGG T3′ | 5′-TTG GTG TTC TGG CAG GTT-3′ |
| Krt17 | 5′-TGT TTG GTG ATG TGA AGA CA-3′ | 5′-ATG CTT AAC GGG TGG TC-3′ |
| Clu | 5′-CAC ATG TCC GGC GAG TA-3′ | 5′-ATG TCT CCG CAA GCA CA-3′ |
| Prnp | 5′-TTC AGG TCC CTT TGA TGG AA-3′ | 5′-CCA AAA CAA CCC CCA ACT A-3′ |
| MMP20 | 5′-AGG GAC GAA GAG AGC TGT GA-3′ | 5′-AAC CTG CAA CCC TCA CG-3′ |
| Col1a2 | 5′-GTC CTA GTC GAT GGC TGC TC-3′ | 5′-CA TGT CCA GAG GTG CAA TG-3′ |
| Wint4 | 5′-ACG CCT GCA GGA GTG ATG CR A-3′ | 5′-GTA TGT CCC TGG GGG TAC-3′ |
| Wint6 | 5′-CTA TCC AGG CCT TGG GAA AT-3′ | 5′-CCT GCA GGA ACTA GCA AAG G-3′ |
| TGFBI | 5′-GTT TGG AGC AAC ATG TGG GA-3′ | 5′-CGT CAA AAG ACA GCCA CCA CTCA-3′ |
| SerpinB5 | 5′-AAC CAG TCC AAC TCG ACC AC-3′ | 5′-TGC TCA TAA AGT CGG TG-3′ |

FIGURE 1 | Expression pattern of Amelx, Ambn, and Enam during development of the molar tooth germ. Expression of Ambn, Amelx, and Enam mRNA monitored using deoxyoligonucleotide microarrays at the various stages of murine tooth development. The plotted normalized net fluorescence intensities (raw fluorescence intensity minus background) are means derived from biological triplicates at each time point with SD indicated. Expression pattern showed low fluorescence intensities at the pre-natal stages (E11.5–P0), with increasing signal throughout the post-natal stages (P1–P7).

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The mean net normalized fluorescence intensities for Ambn, Amelx, and Enam genes (A) at each time point was used as search criteria (master profile) for profile search. The resulting 87 genes were hierarchically clustered using the Euclidean distance and the result is presented as heat map (B). Columns represent different developmental stages and rows represent each gene. Each cell in the matrix represents the expression level of a gene at a certain time point/developmental stage. Green and red in cells reflect low and high expression levels, respectively.
FIGURE 3 | Validation of microarray data. The mean ratios (expression at E11.5 up to P7/expression at E11.5) for some of the differentially expressed (DE) genes (Amelx, Ambn, Enam, Wif1, Clu, Prnp, Mmp20, Col1a1, Tgfb1, Wnt6, and Serpinb5) from the microarray data (black) and real-time RT-PCR (gray). Plotted microarray and real-time RT-PCR data are means derived from at least three biological triplicates at each time point and presented with SD.
separate tissue sections prior to pre-hybridization with either RNase (RNaseA, Qiagen, Hilden, Germany) or DNaseI (DNAfree™ Ambion, CA, USA). RNase was used at a final concentration of 20 µg µL−1, the tissue sections being incubated for 30–120 min at 37°C. The DNase treatment was performed for 15–30 min at 90°C using a final concentration of 5 µg DNase I µL−1. ISH micrographs were obtained using a Nicon Elipse E400 instrument (Nikon Corporation, Tokyo, Japan).

WESTERN-BLOTTING
Aliquots containing 100 µg of total protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using ReadyGel (4–20%) with Tris-HCl buffer (Bio-Rad Laboratories). Proteins were blotted onto 0.45 µm Trans-blot nitrocellulose membranes (Bio-Rad Laboratories) and incubated with antibodies (1:500) against Amelogenin X (SC-32892), or Enamelin (C-18; SC-33107; Santa Cruz Biotechnology, CA, USA), using goat anti-rabbit as secondary antibody (1:10,000, Vector Laboratories, CA, USA). Enzymatic activity was detected by Enhanced Chemifluorescence (ECF) substrate (GE Healthcare, Bucks, UK) using the Storm 860 imaging scanner (GE Healthcare). The specificity of the antibodies was confirmed using full-length Amelogenin X or Enamelin (C-18; SC-33107; Santa Cruz Biotechnology, CA, USA), using goat anti-rabbit as secondary antibody (1:10,000, Vector Laboratories, CA, USA). Antigenic activity was detected by Enhanced Chemifluorescence (ECF) substrate (GE Healthcare, Bucks, UK) using the Storm 860 imaging scanner (GE Healthcare). The specificity of the antibodies was tested incubating with only the secondary antibody.

RESULTS
MICROARRAY ANALYSIS OF mRNAs ISOLATED FROM TOOTH GEM
Ambn, Amelx, and Enam had similar expression pattern throughout the developmental stages (Figure 1), showing low pre-natal levels of expression (net fluorescence intensities of about 800). At P1–P5 about 33-fold increase in fluorescence intensity was observed, further increasing to 100-fold at P6–P7, compared to the pre-natal stages (Figure 1).

The microarray results showed a total of 4362 genes to be differentially (p ≤ 0.05) expressed at every time point studied (E11.5–P7), 1169 of which being without an Entrez ID and were consequently not used during further analysis. The remaining 2441 genes were used in a profile search using the mean time-course expression for Ambn, Amelx, and Enam (Figure 2A). From the 2441 genes screened with the profile search function in Spotfire, 87 genes (Figure 2B) exhibited at time-course of expression similar that of Amelx, Ambn, and Enam.

REAL-TIME RT-PCR
The microarray results of mRNA expression for the enamel genes Ambn, Amelx, Enam, and some random genes from the profile search clusterin (Clu), Wnt inhibitory factor 1 (Wif1), keratin 17 (Krt17), prion protein (Prnp), matrix metalloproteinase 20 (Mmp20), Collagen type I, alpha 2 (Col1a2), transforming growth factor, beta 1 (Tgfb1), wingless-related MMTV integration site 4 (Wnt4), wingless-related MMTV integration site 6 (Wnt6), and serine (or cysteine) peptidase inhibitor, clade B, member 5 (Serpinb5), were verified using real-time RT-PCR. The results show good agreement with the results obtained from microarray (Figure 3 and Table 2), confirming that pre-natal levels of mRNAs of Amelx, Ambn, Enam, Wif1, Krt17, Clu, Prnp, Mmp20, Col1a1, Tgfb1, Wnt4, Wnt6, and Serpinb5 are markedly lower compared to post-natal levels (Table 2). The microarray and real-time RT-PCR results showed good agreement.

| Table 2 | Expression of genes measured with real-time RT-PCR. | Tgfb1 | Serpinb5 |
|---------|------------------------------------------------|-------|----------|
| E11.5   | 1.0 ± 0.01 | 1.0 ± 0.01 |
| E12.5   | 1.2 ± 0.20 | 1.6 ± 0.20 |
| E13.5   | 1.2 ± 0.20 | 1.4 ± 0.20 |
| E14.5   | 1.7 ± 0.40 | 1.3 ± 0.40 |
| E15.5   | 2.3 ± 0.60 | 1.1 ± 0.60 |
| E16.5   | 3.8 ± 0.80 | 1.2 ± 0.80 |
| E17.5   | 6.3 ± 1.00 | 1.6 ± 1.00 |
| E18.5   | 7.1 ± 1.10 | 2.9 ± 1.10 |
| P0      | 175.4 ± 16.3 | 26.3 ± 11.0 |
| P1      | 679.8 ± 120 | 55 ± 0.75 |
| P2      | 1373 ± 150 | 384.9 ± 76.0 |
| P3      | 2258 ± 210 | 636.3 ± 180 |
| P4      | 2855 ± 300 | 954.1 ± 150 |
| P6      | 2541 ± 500 | 599.7 ± 76 |
| P7      | 1004 ± 45 | 346 ± 50 |

The tabled values represent mean fold changes in expression relative to levels of expression at E15. Data from assays on these separate batches of mRNA (RNA) have been scaled from separate experiments of tooth germ (with SD indicated). All analyses were carried using biological triplicates.
Table 3 | The 87 genes isolated using profile search were investigated using IPA to determine significant associations ($p = 0.01$) with “Category” or with “Functions” as judged by Fisher’s exact test.

| Category                      | Functions                              | Molecules                                                                 | $p$-Value  | # Molecules |
|-------------------------------|----------------------------------------|----------------------------------------------------------------------------|------------|-------------|
| Gene expression               | Transcription of DNA                   | Aebp1, Agrn, Bglap2, Blnk, Foxo1, Pdlim1, Pml, Sp6, Tgfb1, Tlx2, Wnt4, Wnt6 | 1.36E−02   | 12          |
| Tissue development            | Development of skeletal system          | Col11a2, Ltbp3, Tgfb1                                                     | 9.88E−03   | 3           |
|                               | Developmental process of enamel         | Klk4, Mmp20                                                                | 9.35E−05   | 2           |
|                               | Aggregation of cells                   | Amelx, Atpl1b1, Ciu, Tgfb1                                                | 9.33E−03   | 4           |
|                               | Adhesion of neuronal cells              | Agrn, Atpl1b1                                                             | 9.04E−03   | 2           |
| Tissue development            |                                                                                       | Aebp1, Agrn, Amelx, Atpl1, Bglap2, Calb1, Chst3, Cldn1, Col11a2, Cst6, Cyp26a1, Foxo1, Kcnj8, Ltbp3, Mmp20, Pml, Prnp, Serpinb5, Sfn, Tgfb1, Tlx2, Wnt4, Wnt6 | 6.55E−07   | 23          |
| Organization of tissue        |                                                                                       | Ambn, Aplp1, Serpinb5, Tgfb1                                              | 3.96E−03   | 4           |
| Adhesion of connective tissue cells |                                                                                   | Ambn, Ciu, Tgfb1                                                          | 3.85E−03   | 3           |
| Development of embryonic tissue |                                                                                   | Cyp26a1, Serpinb5, Tgfb1, Tlx2, Wnt4, Wnt6                                | 3.44E−03   | 6           |
| Formation of connective tissue |                                                                                   | Col11a2, Cyp26a1                                                          | 3.00E−03   | 2           |
| clustering of cells           |                                                                                       | Ciu, Tgfb1                                                                | 2.30E−03   | 2           |
| Deposition of extracellular matrix |                                                                                   | Serpinb5, Tgfb1                                                          | 2.05E−03   | 2           |
| Development of connective tissue |                                                                                   | Calb1, Chst3, Col11a2, Cyp26a1, Foxo1, Ltbp3, Pml, Tgfb1                   | 1.77E−03   | 8           |
| Formation of tissue           |                                                                                       | Chst3, Col11a2, Cyp26a1, Foxo1, Ltbp3, Tgfb1, Tlx2                       | 1.48E−03   | 7           |
| Morphogenesis of tissue       |                                                                                       | Ltbp3, Serpinb5, Tgfb1, Wnt4, Wnt6                                       | 1.36E−03   | 5           |
| Embryonic development         | Organogenesis                           | Aebp1, Amelx, Aplp1, Bglap2, Cldn1, Cst6, Cyp26a1, Foxo1, Kcnj8, Mmp20, Serpinb5, Sfn, Tgfb1, Wnt4, Wnt6 | 1.25E−04   | 15          |
| Organ development             | Aging                                   | Ciu, Pml, Prnp                                                            | 1.84E−04   | 3           |
| Cellular development          | Differentiation of bone marrow cells   | Pml, Tgfb1, Wnt4                                                          | 9.64E−03   | 3           |
|                               | Maturation of cells                     | Blnk, Ciu, Pml, Prnp, Tgfb1                                               | 8.78E−03   | 5           |
|                               | Differentiation of cells                | Agrn, Amelx, Blnk, Ciu, Col11a2, Cyp26a1, Ltbp3, Ndrg1, Plac8, Pml, Prnp, Sfn, Sp6 | 5.40E−04   | 16          |
| Cell-to-cell signaling and interaction |                                                                                   | Foxo1, Tgfb1                                                             | 3.75E−03   | 2           |
|                               | Differentiation of mesenchymal cells    | Tgfb1, Wnt4                                                               | 3.13E−03   | 2           |
|                               | Adhesion of neuronal cells              | Agrn, Atpl1b1                                                             | 9.04E−03   | 2           |
|                               | Adhesion of endodermal cells           | Serpinb5                                                                 | 3.99E−03   | 1           |
|                               | Adhesion of connective tissue cells     | Ambn, Ciu, Tgfb1                                                          | 3.85E−03   | 3           |
| Cell signaling                | Retinoic acid receptor signaling        | Cyp26a1, Pml                                                              | 5.54E−04   | 2           |
| Cellular assembly and         | Opening of pore                         | Ckb, Ckmt1a/Ckmt1b                                                        | 4.69E−05   | 2           |
| organization                  | Deposition of amyloid fibrils           | Ciu, Tgfb1                                                                | 1.59E−03   | 2           |
| Cellular growth and           | Proliferation of smooth muscle cells    | Ciu, Serpinb5, Tgfb1                                                      | 7.42E−03   | 3           |
| proliferation                 | Growth of fibroblast cell lines         | Hspb8, Ldtiap1, Pml, Tgfb1                                                | 7.33E−03   | 4           |
|                               | Proliferation of connective tissue cells | Aebp1, Amelx, Foxo1, Pml, Tgfb1                                            | 6.73E−03   | 5           |
|                               | Proliferation of muscle cells           | Ciu, Prnp, Serpinb5, Tgfb1                                                | 2.66E−03   | 4           |
| Cell death                    | Cell cycle progression                  | Blnk, Ciu, Foxo1, Pml, Serpinb5, Sfn, Tgfb1                               | 8.65E−03   | 7           |

(Continued)
Table 3 | Continued

| Category                                      | Functions                              | Molecules                                                                 | p-Value | # Molecules |
|-----------------------------------------------|----------------------------------------|---------------------------------------------------------------------------|---------|-------------|
| Hair and skin development and function        | Skin development                       | Cldn1, Cst6, Sfn, Tgfb1, Wnt4                                              | 6.07E-04| 5           |
| Tissue morphology                             | Repair of tissue                       | Ambn, Tgfb1                                                               | 3.44E-03| 2           |
| Lipid metabolism                              | Metabolism of lipid                    | Calb1, Clu, Cyp26a1, Foxo1, Gm2a, Lrp10, Prnp, Tgfb1                       | 9.80E-04| 9           |
| Lipid metabolism                              | Synthesis of lipid                     | Aebp1, Clu, Cyp26a1, Foxo1, Moc4r, Tgfb1, Calb1, Foxo1, Ppap2a, Prnp, Tgfb1 | 3.87E-03| 7           |
| Dental disease                                |                                        | Amelx, Enam, Klk4, Mmmp20                                                | 3.65E-03| 9           |
| Developmental disorder                        |                                        | Amelx, Aplp1, Clu, Col1a2, Cyp26a1, Hspb8, Moc4r, Pml, Tgfb1               | 1.91E-03| 9           |

The genes shown in the table exhibited similar levels of expression to the preselected genes of the master profile.

FIGURE 4 | Bioinformatic analysis. Transcription factor analysis performed using Ingenuity IPA for the 87 genes exhibited a p-value of overlap <0.01 associating significantly 35 genes with 15 transcription factors. The genes verified by real-time RT-PCR highlighted with colored outlines and lines together with most of the associated genes (shaded gray).
Amelogenin was identified as a band of approximately 50 kDa. Here we present global gene expression across a range of time points (E11.5–P7) during tooth development. The expression of several genes, including Amelx, Ambn, Enam, Wif1, Clu, Prnp, Mmp20, Col1a2, Tgfb1, Wnt6, and Serpinb5 at pre-secretory stages has not been described earlier. The resulting data indicate that some of the genes mentioned above are expressed in detectable levels at stages prior to the secretory and late maturation stages of murine tooth development.

During early tooth development invagination of the dental epithelium and condensation of the mesenchyme requires fine control and modulation of both short and long-range cellular communication. Members of both fibroblast growth factor (FGF) and wingless-related MMTV integration site (Wnt) families are expressed in the dental epithelium and have been proposed to regulate gene expression in the underlying mesenchyme during early odontogenesis (Kettunen et al., 2005), e.g., Wif1 is known to bind through the WIF domain to several Wnts, e.g., Wnt4 controlling and modulating gradients of cell-to-cell signaling and interaction. Several studies focus on gene expression profiles of tooth development (Osmundsen et al., 2007; Kim et al., 2012).
secreted morphogens which in turn regulate cellular communication during early tooth formation. At post-natal stages these genes play an active role in cell differentiation, e.g., Wnt6 facilitate mineralizing of the murine tooth (Wang et al., 2010). Bioinformatic analysis suggests that some molecules; Amelx, Ambn, Clu, Tgfb1, Serpinb5 have multiple functions, playing different roles during early murine tooth development and mineralization. Most of the genes resulting from Ambn, Amelx, and Enam profile search play an important role in cell proliferation, migration, adhesion during early tooth development (E11.5–E16.5) while during the differentiation and mineralization stages (E17.5 up to P7) these genes contribute to attachment, organization, polarity of either ameloblasts or odontoblasts (Karavanova et al., 1992; Aberg et al., 2004; Nakamura et al., 2008). This multifunctionality may be due to the fact that expression of many of the 87 genes are induced or regulated by different transcription factors and regulators, e.g., Runx2 (Ambn, Bglap, Ibsp, and Col1a2), Fosl2 (Bglap, Col1a2), and VitaminD3/-VDR-RXR (Calb1, CST6, FOXO1), triggering different cellular responses during tooth development (McMahon et al., 1990; Fukumoto et al., 2004; Rufini et al., 2011; Romano et al., 2012). Runx2 suppresses the expression of Ambn and Amel in cultured tooth germs (Kobayashi et al., 2006). Fos is associated with a variety of biological processes, e.g., proliferation and differentiation. Fosl2 (Fra-2) knockout mice show an aberrant tooth formation (Smeyne et al., 1993). Dietary deficiencies in Vitamin D3 affect Calbindin (Calb1) expression in ameloblasts and odontoblasts in rat incisors leading to hypocalcemia and dentin hypomineralization (Berdal et al., 1995).

The in situ results for Amelx, Ambn, and Enam mRNA showed similarities with immunohistochemical localization of Serpinb5, Clusterin, and Prnp (French et al., 1993; Davaadorj et al., 2010; Khan et al., 2010). Expression of enamel proteins in bone cells has previously been reported (Spahr et al., 2006; Tamburstuen et al., 2010; Tamburstuen et al., 2011) and is supported by the presented in situ data.

The combination of several techniques like microarrays, real-time RT-PCR, ISH, and immuno-blotting combined with bioinformatics may serve to provide a more comprehensive view of the cellular consequences of changes in gene expression occurring
during murine tooth development. The major challenge is at the level of analysis of microarray data, i.e., isolation of DE genes which are functionally related. The profile search function, used in this study, has shown itself as a useful tool when searching for related genes in large sets of microarray gene expression data. This may also be useful to identify novel genes during murine tooth development.

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REFERENCES

Aberg, T., Wang, X. P., Kim, J. H., Yamashiro, T., Bei, M., Rice, R., Ryos, H. M., and Thesleff, I. (2004). Runx2 mediates FGF signaling from epithelium to mesenchyme during tooth morphogenesis. Dev. Biol. 270, 76–93.

Benjaminy, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Series B Stat. Methodol. 57, 289–300.

Berdal, A., Papagerakis, P., Hotton, D., Baillieul-Forestier, I., and Davideau, J. L. (1995). Ameloblasts and odontoblasts, target-cells for a 1.25-dihydroxyvitamin D3: a review. Int. J. Dev. Biol. 39, 257–262.

Chai, Y., Jiang, X., Do, Y., Bringas, P. Jr., Han, J., Rowitch, D. H., Soriano, P., Mcmahon, A. P., and Sucov, H. M. (2000). Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. Development 127, 1671–1679.

Cheadle, C., Vawter, M. P., Freed, W. J., and Becker, K. G. (2001). Analysis of microarray data using Z score transformation. J. Mol. Diagn. 5, 73–81.

Davaadorj, P., Tokuyama, R., Ide, S., Tadokoro, S., Kudoh, K., and Satomura, K. (2010). Possible involvement of maspin in tooth development. Histochem. Cell Biol. 134, 603–614.

Farquharson, M., Harvie, R., and McNicol, A. M. (1990). Detection of messenger RNA using a digoxigenin end labelled oligodeoxynucleotide probe. J. Clin. Pathol. 43, 424–428.

French, L. E., Chonn, A., Ducrèt, D., Baumann, B., Belin, D., Wohlhend, A., Kiss, J. Z., Suppio, A. P., Tischhop, J., and Schifferli, J. A. (1993). Murine clustiner: molecular cloning and mRNA localization of a gene associated with epithelial differentiation processes during embryogenesis. J. Cell Biol. 122, 1119–1130.

Fukumoto, S., Kiba, T., Hall, B., Iehara, N., Nakamura, T., Longe-necker, G., Krebsbach, P. H., Nanci, A., Kulkarni, A. B., and Yamada, Y. (2004). Ameoloblastin is a cell adhesion molecule required for maintaining the differentiation state of ameloblasts. J. Cell Biol. 167, 973–983.

Hougaard, D. M., Hansen, H., and Larsen, L. I. (1997). Non-radioactive in situ hybridization for mRNA with emphasis on the use of oligodeoxynucleotide probes. Histochem. Cell Biol. 108, 355–344.

Hu, J. C., Lerlam, R., Richardson, A. S., Smith, C. E., McKeer, M. D., and Simmer, J. P. (2011). Cell proliferation and apoptosis in enamelin null mice. Eur. J. Oral Sci. 119(Suppl 1), 529–537.

Karavanova, I., Vainio, S., and Thesleff, I. (1992). Transient and recurrent expression of the Egr-1 gene in epithelial and mesenchymal cells during tooth morphogenesis suggests involvement in tissue interactions and in determination of cell fate. Mech. Dev. 39, 41–50.

Kaufman, M. (1992). The Atlas of Mouse Development. London: Elsevier Aca- demic Press.

Kettunen, P., Loes, S., Furmanek, T., Fjeld, K., Kiviniemi, I. H., Behar, O., Yagi, T., Fujinawa, H., Yamao, S., Taniguchi, M., and Luukko, K. (2005). Coordination of trigeminal axon navigation and patterning with tooth organ formation: epithelial-mesenchymal interactions, and epithelial Wnt3 and Tgfbeta regulat-

Romano, R. A., Solomon, L. W., and Sinha, S. (2012). Tp63 in oral develop-
morphogenesis, and patterning: the right shape in the right place. J. Dent. Res. 91, 125–132.

Romano, R. A., Solomon, L. W., and Sinha, S. (2012). Tp63 in oral develop-
morphogenesis, and patterning: the right shape in the right place. J. Dent. Res. 91, 125–132.

Romano, R. A., Solomon, L. W., and Sinha, S. (2012). Tp63 in oral develop-
morphogenesis, and patterning: the right shape in the right place. J. Dent. Res. 91, 125–132.

Romano, R. A., Solomon, L. W., and Sinha, S. (2012). Tp63 in oral develop-
morphogenesis, and patterning: the right shape in the right place. J. Dent. Res. 91, 125–132.

Romano, R. A., Solomon, L. W., and Sinha, S. (2012). Tp63 in oral develop-
morphogenesis, and patterning: the right shape in the right place. J. Dent. Res. 91, 125–132.

Romano, R. A., Solomon, L. W., and Sinha, S. (2012). Tp63 in oral develop-
morphogenesis, and patterning: the right shape in the right place. J. Dent. Res. 91, 125–132.
tooth: growth factors, transcription factors, and stem cells. Cell Res. 15, 301–316.

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