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Down-regulated Genes in Mouse Dental Papillae and Pulp

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ABSTRACT

Important factors involved in odontogenesis in mouse dental papillae disappear between the pre- and post-natal stages of development. Therefore, we hypothesized that certain genes involved in odontogenesis in dental papillae were subject to pre-/post-natal down-regulation. Our goal was to identify, by microarray analysis, which genes were down-regulated. Dental papillae were isolated from embryonic 16-day-, 18-day- (E16, E18), and post-natal 3-day-old (P3) murine first mandibular molar germs and analyzed by microarray. The number of down-regulated genes was 2269 between E16 and E18, and 3130 between E18 and P3. Drastic down-regulation (fold change > 10.0) of Adamts4, Aldha1a2, and Lef1 was observed at both E16 and E18, and quantitative RT-PCR revealed a post-natal reduction in their expression (Adamts4, 1/3; Aldha1a2, 1/13; and Lef1, 1/37). These results suggest that down-regulation of these three genes is an important factor in normal odontogenesis in dental papillae.

KEY WORDS: dental papilla, dental pulp, odontogenesis, gene expression, tooth morphogenesis.

INTRODUCTION

Tooth morphogenesis is a complex process that has been characterized as a series of reciprocal epithelial-mesenchymal interactions, culminating in differentiation of the interacting tissues. At the molecular level, these interactions are mainly regulated by signaling molecules, including bone morphogenetic proteins (Bmp), fibroblast growth factors (Fgf), Sonic hedgehog and Wnt (Hurmerinta et al., 1980; Jernvall and Thesleff, 2000). The functions of these molecules have been investigated in knockout and transgenic mice, and by experiments with antisense oligonucleotides. However, many of the molecules, signal transcriptions, processes, and functions involved in tooth morphogenesis remain to be determined.

Classic studies on tissue recombinations have investigated interactions between dental epithelium and dental papillae. Such studies have demonstrated that dental epithelium can induce tooth formation prior to the bud stage. However, after the bud stage, this initiative shifts to the dental mesenchyme (Kollar and Baird, 1970; Mina and Kollar, 1987). It has been demonstrated that the combination of embryonic 16-day-old (E16) enamel organs and E16 dental papillae formed teeth, while that of E16 enamel organs and post-natal 3-day-old (P3) pulps did not, producing dysplastic dentin only (Palmer and Lumsden, 1987). This led us to hypothesize that certain genes related to odontogenesis were down-regulated in dental papillae after the late bell stage, which corresponds to mouse tooth development between the pre- and post-natal stages. To confirm this hypothesis, we investigated and compared the gene profiles of pre- and post-natal-stage dental papillae.

One current advance in biomolecular technology, GeneChip® microarray technology, allows for precise genetic profiling during tooth morphogenesis. These arrays consist of more than 30,000 oligonucleotide probes positioned in a known location within the target cell. By this method, it is possible for samples to be compared at different stages of tooth development, facilitating the identification of genes and variations in levels of expression during tooth morphogenesis.

The aim of this study was to identify, by microarray analysis, which genes were down-regulated between the pre- and post-natal stages of development in dental papillae.
MATERIALS & METHODS

Animals & Tissue Preparation

ICR mice were obtained from Sankyo Laboratory Service Corporation (Tokyo, Japan). E16, E18, and P3 mice were killed under deep anesthesia with sodium thiopental. The ages of embryos were determined by confirmation of vaginal plugs on the day after mating and confirmed by external developmental features. Stages of tooth germ development were classified as (1) early bell-stage (E16), (2) late bell-stage (E18), and (3) dentin and enamel maturation period (P3). All experiments were carried out according to the Guidelines for the Treatment of Animals at Tokyo Dental College.

First-molar tooth germs were dissected from the mandible under a dissecting microscope. After being washed in phosphate-buffered saline, tooth germs were incubated in 1.2 U/mL dispase I (Roche, Mannheim, Germany) for 5 min at room temperature (RT) to separate dental epithelium and mesenchyme, and de-activated in minimum essential medium (MEM, Invitrogen, Grand Island, NY, USA) with 15% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA). Dental papillae were isolated from the tooth germs under a dissecting microscope with tungsten needles. Dental papillae and pulp tissues obtained at E16, E18, and P3 were used for microarray analysis.

RNA Extraction

Total RNA was extracted from 3 pooled groups of tissue—E16-papillae (n = 20), E18-papillae (n = 20), and P3-pulp tissues (n = 20)—with TRIzol® (Invitrogen) according to the manufacturer’s instructions. RNA extractions were performed in duplicate for each of the 3 pooled groups of tissue from each developmental time-point, to provide 2 biological replicates at each time-point for which the tissue pool n = 20 for each replicate at each developmental time-point. The RNA was concentrated from the 20 homogenates. RNA samples were quantified spectrophotometrically, and the integrity of the RNA was confirmed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Foster City, CA, USA).

Microarray Hybridization

Double-stranded complementary DNA (cDNA) was synthesized by a two-cycle target-labeling method. A 200-ng quantity of total RNA was converted into first-cycle double-stranded cDNA. As the first cycle, in vitro transcription (IVT) amplification of cRNA was carried out with MEGAscript T7 Kit (Ambion, Austin, TX, USA). As a second-cycle, double-stranded cDNA synthesis was performed with the SuperScript Choice System (Invitrogen). The samples were synthesized into Biotin-labeled cRNA by means of a transcription labeling kit (Enzo Diagnostics, Farmingdale, NY, USA) and fragmented randomly. Fragmented cRNA was hybridized with the GeneChip® Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA, USA) at 45°C for 16 hrs. The microarray chip was analyzed with the Affymetrix® Fluidic Station 450, and the images scanned. RNA was applied from each of the 2 biological replicates of each pooled tissue (n = 20 per replicate) at each developmental time-point to the microarray without inclusion of any technical replicates.

Data Analysis

Data were analyzed with the GeneChip® Operating Software version 1.1 (GCOS; Affymetrix). The data were subsequently transferred to GeneSpring® software (Silicon Genetics, Redwood City, CA, USA) for normalization and statistical analysis. Where the software indicated low or unstable gene expression, these data were excluded from further analysis, and only those genes flagged as “present” were selected. From these data, which included unknown genes, we selected only those genes whose difference in expression exhibited a more than two-fold change. Down-regulated genes selected from those data were categorized according to the Gene Ontology database (GO). Those genes among the three stages (E16, E18, and P3) were then categorized with the GeneSpring® software according to their corresponding ontologies as follows: Calcification, Development, or Cell Death.

Quantitative Real-time PCR (qRT-PCR)

Total RNA was reverse-transcribed into complementary DNA (cDNA) with Sensiscript® Reverse Transcript (QIAGEN, Alameda, CA, USA), and qRT-PCR with TaqMan MGB probes (Applied Biosystems, Foster City, CA, USA) was carried out. The TaqMan MGB probes and primer sets for the mouse genes (Adams4, Aldh1a2, Left1) and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as an internal control, were purchased from Applied Biosystems. Quantification and differentiation of mRNA expression was analyzed with ABI 7700 system software (Applied Biosystems) and compared by the ΔΔCT method. Differences among E16, E18, and P3 were analyzed statistically by the non-parametric Mann-Whitney U-test with Bonferroni correction. Values of p < 0.01 or 0.05 were considered significant.

In situ Hybridization

The upper and lower first molars were dissected from E16, E18, and P3 mice and hybridized in situ with Adams4, Aldh1a2, and Left RNA probes. Digoxigenin-labeled antisense RNA probes were generated from cDNA subcloned in pBluescript SK with T7 RNA polymerase, and sense probe was done with SP6 polymerase, as described elsewhere (Cho et al., 2007).

RESULTS

Gene Expression Analysis in Dental Papillae by Microarray

We utilized microarray technology to compare multiple gene expression profiles between pre- and post-natal dental papillae. Information and profile of gene expression were submitted to the Gene Expression Omnibus (GEO) database: E16 (GEO accession number GSM485768), E18 (GEO accession number GSM485769), and P3 (GEO accession number GSM485770).

Downloaded from jdr.sagepub.com at Tokyo Dental College Lib on July 14, 2011 For personal use only. No other uses without permission.
The number of up-regulated and down-regulated genes showing a more than two-fold change between each period is shown in Table 1. More than 2000 genes showed either up- or down-regulation between E16 and E18. The number of up-regulated genes decreased following tooth morphogenesis. In contrast, the largest number of down-regulated genes (3130) was found between E18 and P3.

Down-regulated genes showing a more than four-fold change were categorized according to GO (Appendix Table), and genes showing a more than 10-fold change are listed in Table 2. In the “Calcification” category, expression of \( \text{Adams4} \) and \( \text{Lef1} \) decreased considerably (fold change > 10) between E16 and E18. In the “Development” category, expression of \( \text{Aldh1a2} \) showed a significant reduction (fold change > 10) between E16 and E18. In the “Cell Death” category, no gene showed a more than 10-fold change. Moreover, in all categories, no genes showed a more than 10-fold change between E18 and P3.

Based on the results of the microarray analysis, we focused on 3 genes—\( \text{Adams4} \), \( \text{Aldh1a2} \), and \( \text{Lef1} \)—since all of them displayed conspicuous change (> 10-fold change) following tooth germ maturation.

**Confirmation of Gene Expression by qRT-PCR**

The expression of these 3 genes as measured by qRT-PCR was similar to that obtained with the microarray (Fig. 1). QRT-PCR for \( \text{Adams4} \) confirmed a 3.1-fold reduction in its mRNA expression between E16 and E18 (Fig. 1A), at a statistically significant level (\( p < 0.05 \)). QRT-PCR demonstrated a 12.8-fold reduction in expression of \( \text{Aldh1a2} \) mRNA between E16 and E18 (Fig. 1B), at a statistically significant level (\( p < 0.01 \)). QRT-PCR indicated a 37.4-fold reduction in expression of \( \text{Lef1} \) between E16 and E18, at a statistically significant level (\( p < 0.01 \)) (Fig. 1C).

**Localization of Identified Genes by \textit{in situ} Hybridization**

The three genes, \( \text{Adams4} \) (Figs. 2A-2C), \( \text{Aldh1a2} \) (Figs. 2D-2F), and \( \text{Lef1} \) (Figs. 2G-2I), showed similar patterns of expression during tooth development. At E16, they were localized in inner and outer dental epithelia and dental papilla cells adjacent to the inner dental epithelium (Figs. 2A, 2D, 2G). At E18, localization of \( \text{Aldh1a2} \) and \( \text{Adams4} \) was also observed in dental epithelia consisting of inner dental epithelium and stratum intermediate (Figs. 2B, 2E). However, expression of \( \text{Lef1} \) showed the greatest decrease, and localization was observed in the outer dental epithelium at the cervical area (Fig. 2H). Expression of \( \text{Aldh1a2} \) and \( \text{Adams4} \) became weaker or disappeared in odontoblasts differentiated from dental papilla cells and pulp cells at P3, but persisted in ameloblasts differentiated from inner dental epithelial cells (Figs. 2C, 2F). No expression of \( \text{Lef1} \) was observed in odontoblasts, dental pulp cells, or dental epithelium (Fig. 2I).

**DISCUSSION**

The tooth is an ectodermal organ like hair, feathers, lungs, and mammary glands, and its formation is controlled by reciprocal interactions between dental epithelium and dental mesenchyme (Thesleff, 2003). However, this interaction is dynamic and complex. Many of the molecules, signal transcriptions, processes, and functions that play a part in tooth morphogenesis remain to be characterized, and more than 300 genes have been reported to be involved (http://bite-it.helsinki.fi). High-throughput technologies such as DNA microarrays can comprehensively profile and monitor gene expression in biological processes, including development (Hiratsuka et al., 2002; Brown et al., 2003). In this study, we compared pre- and post-natal gene profiles using microarray technology, and found over 2000 expressed, a higher number than that found in previous reports. Our results also showed that the highest number of down-regulated genes was recognized between E18 and P3, which corresponds to the period between pre- and post-natal mouse dental papillae, when pre-odontoblasts differentiate into mature odontoblasts. Furthermore, down-regulated genes showing a more than four-fold change belonged to the “Development” category (data in Appendix). These results suggest that the expression of development-related signaling molecules and interactions in dental papillae is quite complicated during tooth development, and that as-yet-unreported genes are involved in the process of tooth development.

The Adams family is a zinc-dependent metalloproteinase that plays an important role in normal and pathological events (Kuno et al., 1997). It is composed of at least 19 molecules, and \( \text{Adams4} \) (aggrecanase-1) degrades proteoglycans, including aggrecan, versican, and brevican (Nakamura et al., 2000; Tortorella et al., 2000; Westling et al., 2004). Expression of \( \text{Adams4} \) has been identified, by \textit{in situ} hybridization, in odontoblasts, pulp cells, and periodontal ligament cells during rat molar eruption (Sone et al., 2005). Furthermore, proteoglycans were found in dental papillae after the early bell stage and in the post-natal dental stage, and may play an important role in differentiation of odontoblasts (Hikake et al., 2003). In the present study, we found that expression of \( \text{Adams4} \), which degrades proteoglycans, decreased significantly between E16 and E18, suggesting an increase in proteoglycans in the dental papillae. Our results suggest that reduced expression of \( \text{Adams4} \) induces an increase in proteoglycans in dental papillae, leading to differentiation of odontoblasts.

In the present study, mRNA expression of \( \text{Aldh1a2} \) (Raldh2) showed a marked reduction between E16 and E18. \( \text{Aldh1a2} \) belongs to the aldehyde dehydrogenase family, and has been reported to play a significant role during early embryogenesis of limb, heart, and tooth germ (Zhao et al., 1996; Niederreither et al., 2004). Further study is necessary to understand the role of \( \text{Aldh1a2} \) in the development of dental tissues.

<table>
<thead>
<tr>
<th>Table 1. Number of Up- and Down-Regulated Genes Showing Greater than Two-Fold Change between Each Period</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Up-regulated Genes</strong></td>
</tr>
<tr>
<td>Between E16 and E18</td>
</tr>
<tr>
<td>Between E18 and P3</td>
</tr>
</tbody>
</table>

Number of up-regulated genes decreased following tooth morphogenesis. Largest number of down-regulated genes was found between E18 and P3.
Table 2. Summary of Down-regulated Genes in Dental Papillae during Odontogenesis

<table>
<thead>
<tr>
<th>Probe Set ID</th>
<th>Accession No.</th>
<th>Gene Symbol</th>
<th>Between E16 and E18</th>
<th>Between E18 and P3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1455965_at</td>
<td>BG064671</td>
<td>Adamts4</td>
<td>14.1</td>
<td></td>
</tr>
<tr>
<td>1421299_a_at</td>
<td>NM_010703</td>
<td>Lef1</td>
<td>12.8</td>
<td></td>
</tr>
<tr>
<td>1422789_at</td>
<td>NM_009022</td>
<td>Aldh1a2</td>
<td>70.8</td>
<td></td>
</tr>
</tbody>
</table>

List of genes showing four-fold change in down-regulation and categorized by gene ontology (GO) as “Calcification”, “Development”, or “Cell Death”. In the “Calcification” category, expression of Adamts4 and Lef1 decreased considerably (fold change > 10.0) between E16 and E18. In the “Development” category, a greater than 70-fold change was recognized in the expression of Aldh1a2 between E16 and E18. However, no genes showing a greater than 10-fold reduction were categorized as “Cell Death”.

Figure 1. mRNA expression of Adamts4 (A), Aldh1a2 (B), and Lef1 (C) by qRT-PCR. Vertical bars indicate mean ± SD (significant difference: *p < 0.05, **p < 0.01). (A) qRT-PCR confirmed a 3.1-fold reduction in mRNA expression of Adamts4 between E16 and E18 (p < 0.05). (B) qRT-PCR demonstrated a 12.8-fold reduction in expression of Aldh1a2 mRNA between E16 and E18 (p < 0.01). (C) qRT-PCR showed a 37.4-fold decrease in expression of Lef1 between E16 and E18 (p < 0.001). Mann-Whitney’s U-test with Bonferroni correction. A, n = 4; B, n = 5; C, n = 5.

ACKNOWLEDGMENTS

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REFERENCES


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Figure 2. Expression of Adamts4, Aldh1a2, and Lef1 during tooth development (E16, E18, and P3) in mouse tooth germs. In situ hybridization analysis was carried out with frontal sections of E16 (A, D, G), E18 (B, E, H), and P3 (C, F, I) mouse tooth germs. Adamts4 (A-C), Aldh1a2 (D-F), and Lef1 (G-I) showed similar expression patterns during tooth development. At E16, they were localized in inner and outer dental epithelia, stratum intermedium, stellate reticulum, and dental papilla cells adjacent to inner dental epithelium (A, D, G). At E18, localization of Aldh1a2 and Adamts4 was also observed in dental epithelia consisting of inner dental epithelium and stratum intermedium (B, E). However, expression of Lef1 showed the greatest decrease, and localization was observed in outer dental epithelium of the cervical area (H). Expression of Aldh1a2 and Adamts4 became weaker or disappeared in odontoblasts differentiated from dental papilla cells and pulp cells at P3, but persisted in ameloblasts differentiated from inner dental epithelial cells (C, F). No expression of Lef1 was observed in odontoblasts, dental pulp cells, or dental epithelium (I). Bar = 100 μm.