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Changes in gene-expression during development of the murine molar tooth germ

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ABSTRACT

In a matter of a few days the murine tooth germ develops into a complex, mineralized, structure. Murine 30 K microarrays were used to examine gene expression in the mandibular first molar tooth germs isolated at 15.5 dpc and at 2 DPN. Microarray results were validated using real-time RT-PCR.

The results suggested that only 25 genes (3 without known functions) exhibited significantly higher expression at 15.5 dpc compared to 2 DPN. In contrast, almost 1400 genes exhibited significantly ($P < 0.015$) higher expression at 2 DPN compared to 15.5 dpc, about half of which were genes with unknown functions. More than 50 of the 783 known genes exhibited higher than 10-fold increase in expression at 2 DPN, amongst these were genes coding for enamel matrix proteins which were expressed several 100-fold higher at 2 DPN. GO and KEGG analysis showed highly significant associations between families of the 783 known genes and cellular functions relating to energy metabolism, protein metabolism, regulation of cell division, cell growth and apoptosis. The use of bioinformatics analysis therefore yielded a functional profile in agreement with known differences in tissue morphology and cellular composition between these two stages. Such data is therefore useful in directing attention towards genes, or cellular activities, which likely are worthy of further studies as regards their involvement in odontogenesis.

1. Introduction

Tooth development results from interactions between oral epithelium and cranial neural crest derived mesenchyme[1,2] involving a large number of genes, the expression of which are regulated in time and space. So far expression of more than 250 genes and/or their translated proteins have been detected during tooth germ development by in situ hybridization and immunocytochemistry.[3] Main events in odontogenesis are dentition patterning, establishment of tooth morphology and differentiation of ectomesenchymal cells into dentin-producing odontoblasts and epithelial cells into enamel-producing ameloblasts. Synthesis of dentin and enamel, the two main hard tissues of the tooth, involves secretion of two different types of extra-cellular matrices, both of which are subjected to controlled mineralization.

An overview of total gene expression in the tooth organ at different developmental stages should help our understanding of the cellular phenomena operating during odontogenesis. Expression profiling may be useful to highlight differences in gene expression between various stages of odontogenesis. To highlight developmental differences we

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have compared two distinctly different stages in the development of the mouse mandibular first molar; 15.5 dpc (days post coitum) (late cap stage) and 2 DPN (2 days postnatal) (late bell/early secretory stage).[4] To this end 30 k murine microarrays were used.

2. Methods

2.1. Experimental animals

Tooth germs were isolated from mouse embryos and pups (CD-1 strain) at 15.5 days post coitum (dpc) and at 2 DPN (i.e. 2 days postnatal), the day of vaginal plug being set to 0.5 dpc. Embryos were staged according to the Theiler criteria.[5] First mandibular molar tooth germs were micro-dissected from 10 phenotypical embryos/pups at the various developmental stages. Most of the tooth follicle remained with the tooth germ.

The pregnant mice were sacrificed by cervical dislocation, the pups by decapitation. Embryos and pup heads were immediately immersed in RNAlater (Ambion Inc., TX, USA). The dissection of tooth germs was subsequently carried out in diluted RNAlater (diluted 1:1 with PBS). While still immersed, the first right mandibular molar germs were dissected out and transferred to undiluted RNAlater™.

The animal-house had a 12-h light:12-h dark cycle, and was thermostatted at 21 °C with a relative humidity of 60%. Fodder and water were supplied ad lib. The animals were kept according to the regulations of the Norwegian Gene Technology Act of 1994.

2.2. Isolation of RNA from tooth germs

Total RNA was extracted from single tooth germs by using the Qiagen RNA Mini-kit™. This yielded RNA fractions exhibiting a ratio of OD_{260}/OD_{280} of at least 1.7. Concentrations of RNA (dissolved in RNA storage solution™ (Ambion Inc.)) were assayed using ribo-green™ (Molecular Probes, OR, USA). All reagents used were of molecular biology grade.

The quality of isolated RNA was assessed by using the RNA for RT-PCR. RNA solutions giving a signal of correct length from various primer-pairs (i.e. ribosomal protein L27, ß-actin or glutamate dehydrogenase) were considered suitable for use in microarray analysis.

2.3. Microarray analysis of mRNAs from tooth germs

Murine oligo(30 k)-microarrays were purchased from the NTNU Microarray Core Facility, Trondheim, Norway. These slides had been printed using the Operon murine v. 3 oligo set (Qiagen GmbH, Hilden Germany).

cDNA synthesis, labelling and hybridization were carried out as described for the use of the Cy3 and Cy5 labelling using the Genisphere 3DNA Array 900™ detection kit (Genisphere, PA, USA). Each slide was hybridized with cDNA obtained starting with 1 μg of total RNA. The microarrays were scanned in a Packard Bioscience Scanarray Lite microarray scanner (Perkin-Elmer Life and Analytical Sciences, Inc., MA, USA). The Cy3 and Cy5 fluorescence signals were quantified by using the ScanArray Express v. 2.2™ program (Perkin-Elmer Life and Analytical Sciences, Inc.). The resulting fluorescence data (contained in a .gpr-file) was analysed using the Spotfire™ Functional Genomics v. 8 (Spotfire, MA, USA). The .gpr-files have been deposited in the ArrayExpress database with reference no. E-MEXP-947.

In this investigation total RNA was extracted from each of three tooth germs removed at 15.5 dpc and at 2 DPN. RNA from the three separate tooth germs, isolated at the same stage, was mixed in a 1:1:1 ratio (w/w basis). Each of three microarrays was hybridized with cDNA prepared from 1 μg of RNA derived from each of the resulting two RNA solutions. Although this approach is statistically less desirable, it consumed fewer slides compared to an approach involving triplicates of three individual RNA-samples. In these experiments cDNA from 15.5 dpc was Cy3 labelled, and cDNA from 2 DPN was Cy5 labelled.

The printed arrays included probes for 10 different mRNAs from *Arabidopsis thaliana*, enabling checks of hybridization to be carried out using a spike mixture of 10 different mRNAs from *A. thaliana* (purchased from Stratagene, CA, USA). Pairs of the *A. thaliana* mRNAs were mixed in 10 different ratios ranging from 0.1 to 5. The ratios of the resulting Cy5/Cy3 signal ratios were always about 15–20% less than the expected values.

2.4. Validation of microarray results using real-time RT-PCR

Levels of selected mRNAs (Acox1, Arpc3, Actb, Ambn, Amlx, Aplp1, Brd7, Catnb, Clu, Fzd6, Gna11, Ibsp, Itpr3, Pfn2, Ppif, Rbp2, Rpl27, Shh, Slc25a5, Wif1) were also assayed by real-time quantitative RT-PCR using primers primers designed using Primer3.[6] cDNA was synthesized by oligo-dT priming using First Strand Synthesis Kit (Fermentas GmbH, St. Leon-Rot, Germany). Real-time PCR assays were carried out using Stratagene Mx 4000 PCR system (CA, USA) using a qPCR core kit (Eurogentec, Seraing, Belgium). RNA isolated from three to five separate tooth germs from each stage (13.5, 15.5, and 19.5 dpc; 2 and 4 DPN) was used in this analysis, and every analysis was carried out in triplicates. Statistical evaluation of the significance of differences between measured Ct-values was carried out using the REST 2005 program.[7]

Assay of expression of Ambn, Amelx, Brd7, Clu, Ibsp, Ppif and Rbp2 were also carried out at several stages of foetal development (Fig. 4). The primers used here were purchased from Applied Biosystems Inc. (CA, USA). Here the Ct-values were normalized to that of Ppif which was found to exhibit relatively stable expression.

2.5. Statistical analysis

Statistical evaluation (two-tailed *t*-test) of non-microarray data was carried out using GraphPad InStat (Graphpad Software Inc., CA, USA). Statistical analysis of microarray data was carried out on data derived from sets of triplicate slides which were combined into one data-set. Genes exhibiting net fluorescence values of less than 300 in both channels were not included in further analysis. The ANOVA facility of the Spotfire program was used to select genes which exhibited statistically significant differences in levels of expression ($P < 0.015$) with respect to developmental stage. For this purpose the measured fluorescence intensities