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# Quantitative proteomic analysis of deciduous molars during cap to bell transition in miniature pig

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ABSTRACT

Taking advantage of genetic manipulation tools and accessibility, almost all molecular knowledge on vertebrate tooth development was obtained from rodent models that only have one dentition in their entire lives. Whether the tooth development in other vertebrates such as swine or human follows the same rules remains elusive. Rodent dentitions differ considerably from human dentitions, therefore limiting the application of knowledge from rodent tooth to human tooth. Signal-mediated communication between cells and complex gene and protein regulatory networks are key components of tooth development. By combining isobaric tandem mass tag (TMT) labeling with liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) technology, we constructed the proteomic profile of deciduous molars at embryonic days 40 and 50 in miniature pig (*Sus scrofa*). During the ten days of prenatal development of the miniature pig, the morphology of the lower deciduous molar moves from the early cap to the bell stage. Thus, we identified proteins that are associated with these developing stages and identified differentially regulated proteins (DRPs) that are potential or novel drivers of tooth morphogenesis. Three candidate proteins were validated via qRT-PCR, western blotting analysis, and the location of those proteins in tooth germ were observed by immunohistochemical staining. Multiple signaling pathways and protein interaction network revealed potential mechanisms of early tooth programming in a large mammal. Bioinformatic analysis also showed that cross interaction of Wnt and Sonic hedgehog pathways may play a key role in deciduous development during cap to bell transition in miniature pig.

*Significance:* We performed the most comprehensive study of the whole tooth germ proteome in mammals to date. The high-throughput proteomic analysis identifies differentially regulated proteins and pathways that will help elucidate the mechanisms of tooth development.

## 1. Introduction

Tooth development is a successive process with morphological complexity, and understanding the mechanisms at the level of genes, molecules and cells will lay the basis for tooth regeneration, and prevention of dental defects or diseases [1–3]. Early development of the crown pattern includes the initiation, bud, cap, bell, and maturation stage for all teeth types [4,5]. Deciduous teeth are considered essential for the development of permanent teeth and the oral cavity, and the jaw muscles and bone formation depend on deciduous teeth to maintain proper spacing for permanent teeth [6,7]. When the morphology of the deciduous molars moves from the early cap to the bell stage, the tooth germ grows, the cervical loop develops, the enamel organ morphs, the epithelial-mesenchymal junction fold associated with cusps forms, and cellular heterogeneity changes in the mesenchyme [8,9]. Histological approaches, as well as functional tests in vitro, allow searching for some possible relationships between simultaneous changes occurring in both the epithelial and ecto-mesenchymal compartments. However, the complex mechanisms underlying the histo-morphogenetic events have not been comprehensively documented using histological sections and 3D reconstructions as functional tests in vitro [10]. Patterns of gene expression have been studied in developing teeth around the world over the past few decades. Studies on signal receptors, growth factors, and transcription factors provided a relatively sound understanding of the

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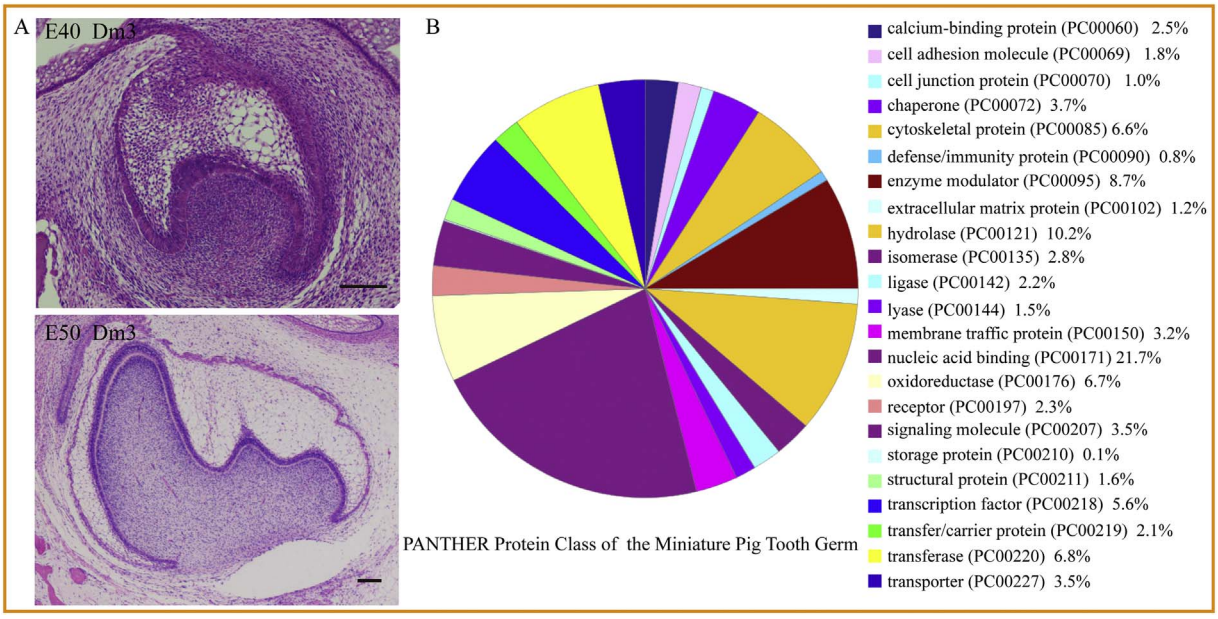


**Fig. 1.** The PANTHER protein classification of the 2872 detected proteins in the third deciduous molar (Dm3) during cap to bell transition. (A) Coronal histological sections (hematoxylin and eosin staining) of Dm3 at the cap (E40) and bell stage (E50). Scale bar, 100 μm. (B) The protein categories in PANTHER protein classification system. Different colors represent different protein classes. The category name, the category accession number, and the percentages of specific protein categories are shown.

genetic mechanisms during early tooth development [11,12]. About 300 genes can be viewed in the 'Bite-it' database (http://bite-it.helsinki.fi) which provides a tool for learning and comparing the expression patterns of different genes in tooth development. Almost all the molecular knowledge on vertebrate tooth development was obtained from the rodent models [1]. Rodents have one dentition set, lack canines, and have highly derived premolars. Because rodent teeth are different from human teeth in number and morphology, application of rodent tooth knowledge to human tooth is limited [13]. The biochemical process and molecular mechanisms underlying tooth germ development remains unaddressed in large mammals. The miniature pig (*Sus scrofa*) resembles humans in anatomy, physiology, pathophysiology, and development, providing a good experimental model for studying tooth development [14,15]. Previously, we have reported developing patterns and spatiotemporal features of gene expression through analysis of transcriptome, microRNA expression profile and DNA methylation profiles of deciduous molars in miniature pigs [16–18].

There are few reports on the proteome of the whole tooth germ in human, rodent, or swine [19]. Proteins are the direct executors of most cellular activities, such as the physiological and biochemical reactions that link phenotypes to genotypes [20,21]. It is important to investigate the molecular mechanisms of tooth origination and development at the protein level. At the present stage, bottom-up proteomics is a viable approach that has generated valuable information on many cellular processes, such as viral infection, tumor biology, and systems biology [22,23]. The changes of specific proteins and protein complexes identified by proteomic analysis can uncover the physiological condition of an organism. The use of the tandem mass tag (TMT) labeling quantitative proteomics approach has been used in many biological applications [24–27]. To date, the *Sus scrofa* reference proteome has > 26,000 proteins in the NCBI and Uniprot platforms. Availability of a swine protein database allowed the direct identification of pig proteins by in-depth, high-accuracy proteomic analysis.

The present proteomic study focused on the early stages of deciduous teeth crown development in miniature pig. Approximately 2800 proteins and 204 differentially regulated proteins (DRPs) with high confidence were identified, and bioinformatic analysis showed the different types of molecules operating in complex protein networks. The cross interaction of Wnt and Sonic hedgehog pathways may play a key role in deciduous molars during cap to bell transition in miniature pig. The present study provided the most comprehensive protein resource for understanding mammalian tooth development.

## 2. Materials and methods

### 2.1. Ethics statement

Surgical procedures and animal care were certified by the Institutional Animal Care and Use Committee of the Capital Medical University, Beijing, China (No. AEEI-2015-095) and were also guided by the Animal Ethics Procedures and Guidelines of the People's Republic of China.

### 2.2. Tissue collection

To obtain miniature pig embryos at embryonic days 40 and 50 (E40 and E50), we performed caesarean operations on pregnant miniature pigs from the Institute of Animal Science, China Agriculture University, as previously reported [16]. After the operation, the pregnant miniature pigs were killed via over-anesthetization. The two mandibular third deciduous molars (Dm3) were removed from the mandible of each pig embryo under a stereomicroscope (LEICA). Three biological replicates were operated at each developmental stage. A total of six pregnant pigs with 51 embryos at E40 as well as six pregnant pigs with 45 embryos at E50 were used. Sample pooling has been widely used for small samples in proteomic studies to eliminate individual variations [28,29]. For each biological replicate, we pooled 34 deciduous molar germs from 17 E40 embryos as the E40 protein sample, and 30 deciduous molars from 15 E50 embryos as the E50 sample. The morphology of Dm3 at the cap stage corresponded to E40 and at the bell stage to E50 (Supplementary Fig. 1). For proteomic analysis, tooth germs were stored in liquid nitrogen. For total RNA extraction, tooth germs were placed into 150 μL RNAlater Stabilization Solution (ThermoFisher Scientific) at − 20 °C.

### 2.3. Protein extraction

Tooth germs were homogenized on ice with an electric hand-held grinder and ultrasonicated on a Branson Sonifier Model 1200 (Hengqi).