

Proteome analysis of developing mice diastema region

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Different from humans, who have a continuous dentition of teeth, mice have only three molars and one incisor separated by a toothless region called the diastema in the hemi mandibular arch. Although tooth buds form in the embryonic diastema, they regress and do not develop into teeth. In this study, we evaluated the proteins that modulate the diastema formation through comparative analysis with molar-forming tissue by liquid chromatography-tandem mass spectroscopy (LC-MS/MS) proteome analysis. From the comparative and semi-quantitative proteome analysis, we identified 147 up- and 173 down-regulated proteins in the diastema compared to the molar forming proteins. Based on this proteome analysis, we selected and evaluated two candidate proteins, EMERIN and RAB7A, as diastema tissue specific markers. This study provides the first list of proteins that were detected in the mouse embryonic diastema region, which will be useful to understand the mechanisms of tooth development. [BMB Reports 2012; 45(6): 337-341]

INTRODUCTION

Teeth develop through sequential and reciprocal interactions between oral epithelium and neural crest-derived mesenchyme (1). The first morphological sign of tooth development is a narrow band of thickened epithelium on the developing jaw primordium. The thickened epithelium progressively takes the form of the bud, cap, and bell configurations as differentiation proceeds. Subsequently, epithelial cells and mesenchymal cells differentiate into enamel-producing ameloblasts and dentin-producing odontoblasts, respectively. It is known that many signaling pathways such as Bmp, Fgf, Wnt, and Shh play critical roles in regulating tooth position, number, and shape (2-4).

Mice have only one incisor and three molars in each jaw quadrant that are divided by a toothless region, the diastema (5). Although mice lost the teeth in the diastema during evolution, the remnants of the evolutionary lost teeth are observed as transient epithelial buds in the wild-type diastema during the early stages of development. In the diastema of mice, rudimentary tooth primordia develop through the initial stages of teeth development as the remnants of evolutionary lost teeth but cease before the cap stage and regress by apoptosis (6). Previous reports have shown that there are rudimentary tooth germs, which develop into the bud stage before their removal by apoptosis, in the lower diastema regions of mice at E13 (7). Peterkova et al. evaluated the apoptotic elimination of the vestigial teeth through agenesis in the diastema region and Tureckova et al. showed sporadic cell death in the diastema region (6, 8). Recently, information on apoptosis during tooth development has been reported. The question is whether proliferation and cell death are involved in the rudimentary development of teeth (9).

In previous studies, many important signaling pathways have been reported that modulate the formation of teeth in the diastema region through the repression of Shh, BMP, and Wnts signaling (10). Shh has important roles in the development of various ectodermal appendages including hair mammary glands and teeth in the diastema-forming region, it is repressed by Gas1, a known potent inhibitor. These results were confirmed by examining Gas1 mutant mice that have extra teeth in the maxilla diastema region where there would be expected agenesis of the tooth structures (11). Gas1 inhibited ectopic Ptc1, a receptor of Shh signaling, expression in the diastema suggesting that Gas1 has a role in inhibiting the activity of Shh signaling to form teeth in the diastema forming region (12).

In addition, previous reports have shown that signaling networks including Shh, Wnt, Fgf, and BMPs would produce supernumerary or ectopic teeth in the diastema region (13). Ectodin integrates BMP signaling with the Shh pathways in teeth formation. Inhibition of BMP signaling by Sostdc1 and negative feedback from Shh controls the number and patterning of the teeth (14). Modulation of these signals can rescue these vestigial tooth rudiments to develop into supernumerary diastema teeth using Fgf8 treatment. A number of mutant mouse strains have been reported exhibiting supernumerary

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diastema teeth, including mice with overexpression of *Eda* (15). *Sostdc1* null mutant mice and *Lrp4* hypomorphic mice also present supernumerary teeth (5).

These region specific expression patterns of genes would determine the fate of the tissue whether to form teeth. However, there are no detailed explanations about the functional mechanisms involved in the agenesis of teeth. To understand the detailed molecular mechanisms in the formation of the diastema region, we elucidated the functional molecules that have crucial roles in tooth agenesis by proteome analysis. We selected the E13 tooth and diastema forming tissues based on the previous reports, which showed the detailed developmental changes in these tissues (7, 8). Since, at E13, this developmental stage showed the obvious differences between molar and diastema forming regions with the distinct molecular expression patterns, such as *Msx1*, *Msx2* and *Wnt* signalings (7, 10).

Proteomics is a large-scale study of proteins, particularly their structures and functions. Proteins are vital parts of living organisms since they are the main components of the physiological metabolic pathways of the cells. Thus, in this study, we used the proteome approach to examine the functional mechanisms involved in the formation of the diastema region during mice embryogenesis.

RESULTS AND DISCUSSION

Identification of proteins in diastema-forming regions by LC-MS/MS

In this study, we evaluated the proteins that modulate the agenesis of the diastema region through comparison with molar-forming tissue by LC-MS/MS proteome analysis. At E13, the

diastema- and molar-forming regions were microdissected from a developing mouse mandible, removed, and then pulverized in a homogenizer. For dissecting the mandible, we first removed the aboral and tongue parts from the mandible, and then we dissected the diastema- and molar-forming tissues under the dissecting microscope (Fig. 1A and B). Extracted proteins from the samples were resolved on a SDS-PAGE (Fig. 1C), and each sample was divided into 15 gel pieces and then subjected to in-gel digestion by trypsin. Each peptide mixture was eluted and analyzed by liquid chromatography with tandem mass spectroscopy (LC-MS/MS). The SEQUEST algorithm in the Sorcerer program was used to search the IPI mice protein database using our MS/MS data. From data combined from two individual samples, 539 proteins were identified.

Next a comparative analysis of the proteins identified from the diastema- and molar-forming regions was performed. One hundred and forty-seven proteins had a 1.5-fold higher peptide hit number in the diastema-forming region compared to the molar-forming region (Supplement Table 1). Conversely, 173 proteins were detected with a 1.5-fold higher peptide hit number in the molar-forming region compared to the diastema-forming region (Supplement Table 2). A sub network was reconstructed using twelve proteins, which showed significant expression patterns, as the seed molecules. The sub network was statistically obvious for indirect connectivity. Ingenuity pathway analysis showed the relationships among selected differentially expressed proteins in the diastema-forming region (Fig. 2).

Up- or down-regulated proteins were classified as intracellular, membrane, nuclear, or cytoplasm proteins, based on their predicted cellular location (Fig. 3A). These proteins were also classified according to biological processes (Fig. 3B) and

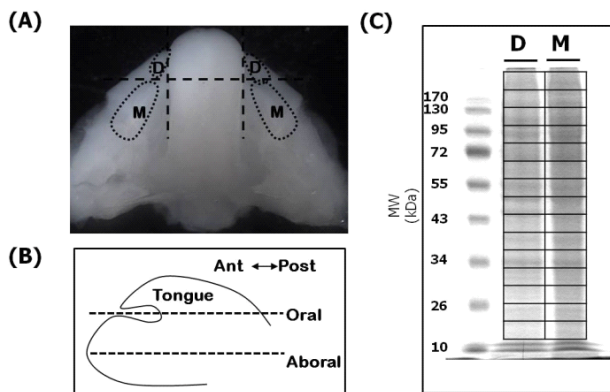


Fig. 1. SDS-PAGE analysis of mice diastema and molar forming regions extracts at E13. (A) Diastema and molar forming regions were microdissected and extracted the region specific proteins. (B) Schematic diagram for dissecting of the mandible (D: diastema forming region, M: molar forming region, Ant: anterior, Post: Posterior). (C) Two different samples and sample buffer were separated by 1-D electrophoresis and stained with the Coomassie blue dye. Whole gel lanes were cut into 15 equally sized pieces along the black lines on the gel images.

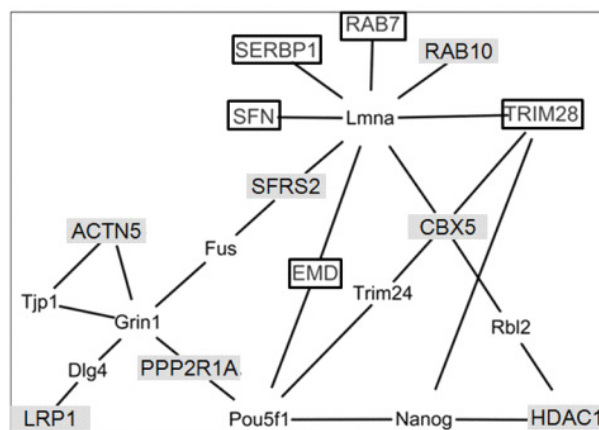


Fig. 2. Expectation of signaling network based on proteomics results. Expectation of signaling interactions among the most regulated protein are presented by using BisoGenet. Signaling network among proteins were reconstructed by using seed candidate genes (square box: up-regulated proteins, shadow background: Down-regulated proteins).

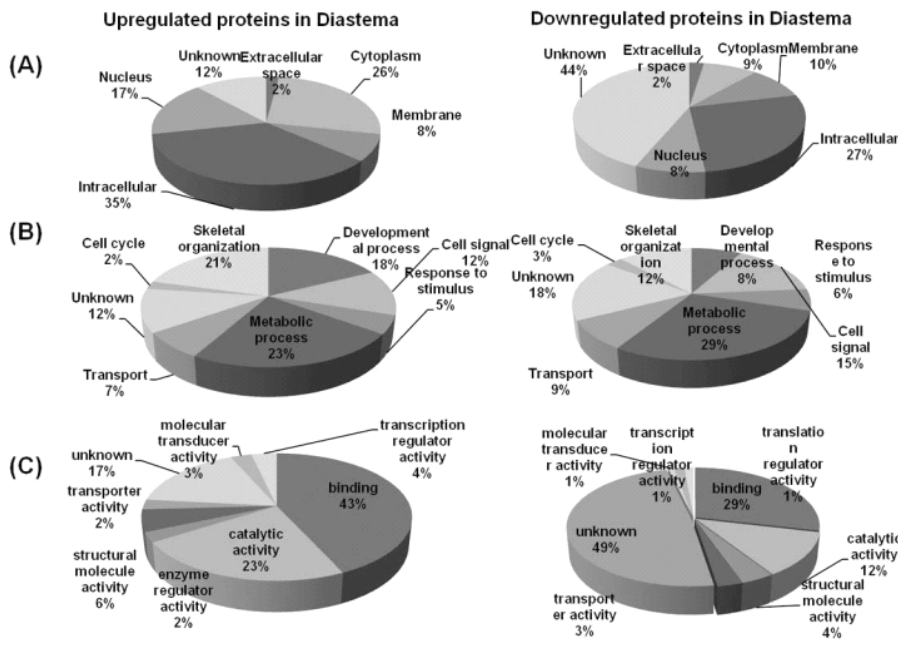


Fig. 3. Distribution and classification of up- or down-regulated proteins that were enriched in the diastema. (A) Cellular distribution profile, (B) Biological process profile and (C) Molecular function profile, for the identified proteins.

molecular functions (Fig. 3C). The cellular localizations of the proteins identified from two individuals are shown in Figure 3. The majority of the proteins identified (35%) originated from the intracellular space followed by the cytoplasm (26%), nucleus (17%), membrane (8%), unknown (12%), and extracellular space (2%). As shown in Figure 2B, the proteins had a variety of functions, including metabolic enzymes (23%), skeletal organization (21%), developmental processes (18%), cell signal transduction (12%), transport (7%), and response to stimulus (5%). Proteins with unknown functions corresponded to 12%. Most of the known metabolic process proteins were identified in our proteome analysis of the diastema (Supplement Table 1). In addition to binding matrix proteins, significant numbers of cellular metabolic proteins were also detected in the diastema of this study.

Selection of candidate proteins

To select candidate proteins that modulate the fate determination of the diastema region, we examined various databases including BisoGenet (16), which show the interactions of signaling networks. First, we examined higher altered signaling in the diastema samples with their known functions. Based on this criterion, we selected two potential proteins, EMERIN and RAB7A, which were highly expressed in the diastema-forming region involved in intracellular signaling pathways.

EMERIN is a type II integral membrane protein of the inner nuclear membrane. EMERIN is known to interact with lamin A/C directly and with chromatin through binding to the barrier-to-autointegration factor (BAF) (17). In addition, EMERIN was reported to possibly influence transforming growth factor

beta (TGF- β) in tissues affected by Emery-Dreifuss muscular dystrophy (18). Mutations of the STA (emerin gene symbol) gene coding for EMERIN cause X-linked Emery-Dreifuss muscular dystrophy (EMD1), a neuromuscular disease characterized by progressive skeletal muscle weakness and wasting and early contractures of the elbows, post-cervical muscles, and Achilles tendons, and cardiomyopathy (19). The localization patterns of EMERIN at the inner nuclear membrane is through its association with the type V intermediate filament proteins and lamins A/C, which form part of the nuclear lamina (20). EMERIN expression is developmentally regulated and it normally appears at the time of organogenesis. EMERIN on its own is dispensable for cell survival and normal development, but has overlapping functions in cell division and chromosome segregation with the inner nuclear membrane protein.

The Rab family of the Ras-related GTPases appears to be essential for the regulation of intracellular membrane traffic in mammalian cells. Ras-related protein Rab-7a, also known as RAB7A, is a member of the Ras superfamily of small Rab GTPases. RAB7A associates with the Rab-interacting lysosomal protein (RIPL) effector protein to control membrane trafficking from early to late endosome and to lysosomes (21). Interestingly, late endosomal Rab7 was found to localize at the ruffled border membrane indicating the late endosomal nature of this specialized plasma membrane domain in resorbing osteoclasts. This also suggests that late endocytotic pathways may play an important role in the secretion of lysosomal enzymes, such as cathepsin K, during bone resorption. Previous studies have identified the sequential recruitment and activation of the small GTPases Rab7 to apoptotic cell-containing phagosomes (22).

Validation of the candidate proteins by western blotting

To validate the candidate proteins identified by MS/MS, we did western blots using EMERIN and RAB7A antibodies. The expression levels of EMERIN and RAB7A were significantly higher in the diastema-forming region than in the molar-forming region (Fig. 4A). The predicted molecular size of EMERIN was approximately 33 kDa and 23 kDa for RAB7A (Fig. 4). The signal intensity was quantified by densitometric analysis, which revealed that EMERIN expression was significantly higher by 3.4 fold in the diastema-forming region compared to the molar-forming region. Densitometry analysis of the RAB7A western blots showed the 1.3 fold increase in the diastema-forming region compared to the molar-forming region. These results suggest that EMERIN and RAB7A are potential factors that are involved in the agenesis of teeth in the diastema region throughout the developmental process. As was amply reported, the regression of tooth germs in the diastema region was shown to occur through apoptosis (7). EMERIN and RAB7A have important roles in the apoptotic pathway. Hiraoka et al. suggested that EMERIN is involved in apoptosis through Bcl-2-associated transcription factor (Btf) signaling in Emery-Dreifuss muscular dystrophy (19). In addition, RAB7A has also been shown to regulate growth receptor endocytic trafficking, degradation, maturation of phagosome autophagic vacuoles, and apoptosis (23). Therefore, EMERIN and RAB7A seem to have crucial roles in the apoptotic pathway and modulate the fate determination of the diastema region.

Based on these previous reports about the apoptotic activities of these two candidate proteins, EMERIN and RAB7A, we concluded that EMERIN and RAB7A would involve in the apoptotic pathway, known as a major event in the developing diastema, to form the diastema region. This study provides the first list of proteins that were detected in the mouse embryonic diastema region, which will be useful to understand the mechanisms of tooth development.

MATERIALS AND METHODS

Extraction of proteins

At embryonic day 13 (E13), molar- and diastema-forming regions were microdissected and the region-specific proteins were extracted. The samples were pulverized using a homogenizer and RIPA buffer was added to the pulverized sample in an Eppendorf tube, and the samples were incubated at 37°C for 30 min with gentle tilting and rotation. After incubation, the samples were centrifuged for 20 min at 12,000 rpm at 4°C. The concentration of the total proteins in the supernatants was assayed with a protein assay kit using BSA as a standard.

SDS-PAGE and in-gel digestion

SDS-PAGE and in-gel digestion were performed as previously reported (24). Briefly, protein bands were excised from Coomassie-stained gels (Fig. 1A) and destained by incubation in 75 mM ammonium bicarbonate/40% ethanol (v/v, 1 : 1).

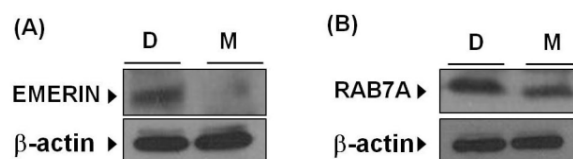


Fig. 4. Validation of EMERIN and RAB7A expression levels by Western blot analysis. (A) Individual samples from diastema and molar forming regions were used for Western blot analysis using anti-EMERIN (1 : 1,000 dilution). (B) Individual samples from diastema and molar forming regions were used for Western blot analysis using anti-RAB7A (1 : 1,000) antibodies.

After destaining, for protein alkylation, the gel was incubated in a solution of 55 mM iodoacetamide at room temperature for 30 min, and the gel pieces were dehydrated in 100% acetonitrile (ACN) and dried. Gel pieces were swollen in 10 μ L of 25 mM ammonium bicarbonate buffer containing 20 μ g/mL modified sequencing grade trypsin (Roche Applied Science), and were incubated overnight at 37°C. The trypsinized peptide mixture was eluted from the gel with 0.1% formic acid.

LC-ESI-MS/MS analysis

LC-MS/MS analysis was also carried out as previously reported (25). Briefly, LC-MS/MS analysis was conducted using Thermo Finnigan's ProteomeX workstation LTQ linear ion trap MS (Thermo Electron, San Jose, CA) equipped with NSI sources (San Jose, CA). Twelve microliters of the peptide mixture was injected and loaded onto a peptide trap cartridge (Agilent, Palo Alto, CA). Trapped peptides were eluted onto a 10-cm reverse-phase PicoFrit column packed in-house with 5 μ m, 300 Å pore size C18, and then separated on an RP column by gradient elution. The solutions used as the mobile phases were H₂O (A) and ACN (B), and both contained 0.1% (v/v) formic acid. The flow rate was maintained at 200/min. The gradient was started at 2% B, reached 60% B in 50 min., 80% B in the next 5 min, and 100% A in the final 15 min. Data-dependent acquisition mode (m/z 300-1,800) was enabled, and each survey MS scan was followed by five MS/MS scans with the 30 s dynamic exclusion option on. The spray voltage was 1.9 kV and the temperature of the ion transfer tube was set at 195°C. For database searching, tandem mass spectra were extracted by Sorcerer version 3.4 beta 2.

Network signaling (BisoGenet)

BisoGenet is a multi-tier application for visualization and analysis of biomolecular relationships. The system consists of three tiers. In the data tier, an in-house database stores genomics information, protein-protein interactions, gene ontology, and metabolic pathways. In the middle tier, a global network is created at the server startup, representing the whole data on bio-entities and their relationships retrieved from the database. BisoGenet is available at <http://bio.cigb.edu.cn/bisogenet-cytoscape> (16).

Western blot analysis

Western blot analysis was performed as previously reported. Briefly, after SDS-PAGE, the transferred nitrocellulose membranes (Whatman, Germany) were incubated with antibodies against EMERIN (1 : 1,000 dilution, AbFRONTIER, Cat.PA40380, Korea), RAB7A (1 : 1,000, EPIPOMICS, Cat.5575-1, USA) followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (1 : 2,000). Signals were developed with the ECL-PLUS detection reagent (Amersham Biosciences, U.K.) and the membranes were exposed to X-ray film for an appropriate time and then developed. The densitometric analysis of the thickness of bands was performed with ImageJ.

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