ORIGINAL ARTICLE

Expression of Grhl2 and Its Target Gene Products in Developing Mouse Submandibular Gland

Takumi MATSUSHITA[1], Takayoshi SAKAI[2], Hiroaki YOSHIDA[3], and Shosuke MORITA[3]

[1] Graduate School of Dentistry
(Department of Oral and Maxillofacial Surgery 1)
Osaka Dental University, Osaka, Japan
[2] Department of Oral-facial Disorders,
Osaka University Graduate School of Dentistry, Osaka, Japan
[3] Department of Oral and Maxillofacial Surgery 1,
Osaka Dental University, Osaka, Japan

SYNOPSIS
Development of the salivary gland is characterized by extensive branching morphogenesis and lumen formation as well as differentiation into acinar and ductal cells. Although various molecules have been implicated in salivary gland development, transcription factors regulating the expression of those molecules and salivary gland development are largely unknown. The GRHL2 transcription factor and its target gene product SPINT1 as well as the SPINT1-regualted protease matriptase play crucial roles in epithelial development in several organs. Here we examined the expression of these epithelial regulators in developing mouse SMG. Of the three *Grhl* gene members, *Grhl2* was the most abundant in embryonic SMG. The expression levels of GRHL2, SPINT1 and matriptase were increased during development of the gland. All of these molecules were expressed exclusively in the epithelial tissue. The present data may suggest the involvement of these molecules in SMG development and give a clue to developing a regenerative therapy of damaged salivary glands.

***Key words**: Grhl2, SPINT1, Matriptase, Salivary gland, Development*

INTRODUCTION

The salivary glands consist of several types of epithelial cells, including acinar cells, which secret saliva, and ductal cells, which modify saliva during the passage to the oral cavity. Salivary gland dysfunction under certain conditions, including Sjögren syndrome and radiotherapy for head and neck cancer, causes oral disorders such as dry mouth[1, 2], however, no satisfactory treatment for dry mouth is currently available. One possible way of restoring the damaged glands is the regenerative therapy based on the understanding of the cellular and molecular mechanisms of salivary gland development.

Development of the salivary gland is characterized by extensive branching morphogenesis and lumen formation of the epithelial tissue. In the mouse submandibular salivary gland (SMG), the

initial epithelial bud arises at embryonic day (E)11 to make solid cell masses with no lumens. These epithelial cells lack intercellular junctions such as tight junctions and do not have defined apical and basolateral membrane domains. As the epithelium grows and branches into lobules and stalks, lumens appear first in the stalk at E14, at which time the luminal cell have intercellular junctions and defined membrane domains. Subsequently, the lobules develop lumens covered with polarized cells to form terminal tubules or acini, leading to the establishment of the complete tubular structure in the entire epithelium by E17[3-5]. Although various molecules including signaling molecules and cell adhesion molecules have been implicated in salivary gland development[5], transcription factors regulating the expression of those molecules and SMG development are largely unknown.

The Grainyhead-like (GRHL) family of transcription factors is expressed in many embryonic and adult epithelial tissues and plays a crucial role in the development of epithelial cell types in several organs[6-8]. These factors regulate the expression of genes associated with epithelial cell activities such as cell adhesion, apical–basal polarization, barrier function, and differentiation[6, 9-15]. GRHL2 directly binds genes encoding cell adhesion molecules such as E-cadherin, claudin 3 and claudin 4[9, 11, 16-18]. Another GRHL2 target is the *Spint1* gene encoding a Kunitz type 1 transmembrane serine protease inhibitor (also known as hepatocyte growth factor activator inhibitor (HAI)-1)[15] , which inhibits the proteolytic activity of proteases such as matriptase and plays a role in epithelial development[19-22].

It was previously reported that *Grhl1* and *Grhl2* were detectable in developing mouse SMG by *in situ* hybridization[7]. In the present study we show that, of the three *Grhl* members, *Grhl2* is the most abundant in embryonic mouse SMG and that GRHL2 together with SPINT1 and matriptase is expressed exclusively in the epithelial tissue.

MATERIALS AND METHODS

Animals and tissues

Pregnant and 8-week-old virgin mice (ICR strain) were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). The day of discovery of the vaginal plug was designated as embryonic day 0 (E0). The SMGs were dissected from E13, E15, and E17 embryos and adults in Hanks' balanced salt solution (HBSS) under microscope. For preparation of tissue sections, SMGs were embedded in O.C.T. compounds (Sakura Finetek Japan, Tokyo), frozen in liquid nitrogen, and kept at -80˚C until use. Frozen sections (6μm thick) were cut with a Leica cryostat. All animal experiments were performed according to the Ethical Committee on Animal Testing of Osaka Dental University (14-06003).

Immunofluorescence microscopy

Tissue sections were fixed in 4% paraformaldehyde at room temperature for 20 min. In some cases, tissue sections were incubated in 0.5% Triton X-100 in phosphate buffered saline (PBS) for 10min at room temperature. After wash in PBS and incubation with M.O.M. Mouse Ig Blocking Reagent (Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature, specimens were incubated with primary antibodies for 3 h at room temperature or overnight at 4ºC and then with fluorescein-conjugated secondary antibodies for 2 h at room temperature. After wash in PBS, specimens were mounted in ProLong Gold antifade reagent with DAPI and observed with confocal microscopy (Laser Scanning Microscope SP8; Leica microsystems, Wetzlar, Germany). Images were processed with ImageJ (NIH) and Photoshop (Adobe, San Jose, CA, USA).

Antibodies

Primary antibodies used were mouse anti-E-cadherin (clone 36/E-Cadherin) (BD Biosciences, Franklin Lakes, NJ, USA), rabbit anti-Grhl2 (HPA004820, Sigma-Aldrich, Munich, Germany), sheep anti-matriptase (AF3946, R&D Systems, Minneapolis, MN), and goat anti-HAI-1 (AF1141, R&D Systems). Secondary antibodies used were all donkey antibodies labeled with Alexa Fluor488 and Alexa Fluor555 (Life Technologies, Carlsbad, CA, USA) and with Alexa Flour647 and Cy2 (Jackson ImmunoResearch, West Grove, PA, USA).

Western blot analysis

SMGs were lysed with RIPA buffer (Nacalai Tesque, Kyoto, Japan) supplemented with protease inhibitors (Nacalai Tesque). Cell lysates were subjected to centrifugation (15,000 rpm) for 15 min, and supernatants were heated at 95°C for 5min in denaturing Laemmli buffer (Bio-Rad Laboratories). Proteins were separated by SDS-PAGE and transferred to Polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories). The membranes were blocked with Blocking One (Nacalai Tesque) for 30 min and then incubated with primary antibodies. The bound antibodies were detected with horseradish peroxidase-conjugated secondary antibodies and the ECL detection reagent (Bio-Rad Laboratories). The band intensity was quantified using ImageJ software.

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from freshly prepared SMGs using TRIzol Reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions, followed by treatment with DNase I (Roche Applied Science, Penzberg, Upper Bavaria, Germany). RNA was reverse transcribed to cDNA by using the PrimeScript RT reagent Kit (Takara Bio, Otsu, Shiga, Japan). Quantification of PCR products was performed using the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad Labo- ratories, Hercules, CA, USA) with iQ SYBR Green Supermix (Bio-Rad Laboratories). The amplification program comprised 40 cycles of denaturation at 95°C for 5 s, annealing at 55°C for 20 s, and extension at 72°C for 20 s. Relative levels of mRNA expression were normalized for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression and calculated according to the $\Delta\Delta$CT method. Primer sequences are listed in Table 1. Experiments were repeated three times.

Table 1 Primers used for Real-time RT-PCR

Target Gene	Forward primer sequence 5' to 3'	Reverse primer sequence 5' to 3'	Reference
Grhl1	AAATTCGGGATGAAGAACGA	TCAGCTTAGCAGGAGGAGGA	Werth M *et al*., 2010.
Grhl2	TTTGGTCCAACACCGTCTA	CACTGGCAGCCCATACTT	Xiang X *et al*., 2012.
Grhl3	CCAGACTCCAGTAACAATG	AAGGGTGAGCAGGTTCGCTT	Gustavsson P *et al*., 2007.
SPINT1	CTCAGACCAACCAGAGGAAA	GAGATTCCTTGCACATCCTT	Yamauchi M *et al*., 2004.
Matriptase	CACGAATGATGTGTGTGGGTTTC	CCTGGAACATTCGCCCATCT	Kawaguchi M *et al*., 2011.
GAPDH	CCATCACCATCTTCCAGGAG	GCATGGACTGTGGTCATGAG	Obana-Koshino, A *et al*., 2015.

RESULTS

Our present qRT-PCR analysis showed that *Grhl1* and *Grhl2* mRNAs were expressed in E13 SMG, with being *Grhl2* mRNA much more abundant than *Grhl1* mRNA, and significantly increased in the expression level in the adult (Fig. 1). Although *Grhl3* mRNA was detectable in E13 SMG with less abundance than *Grhl2* mRNA, the expression level in the adult remained almost the same as that at E13.

Given the most abundance of the three Grhl members expressed in embryonic SMG and the involvement in epithelial development of several organs[9, 12, 14, 15], we focused on GRHL2 protein to further analyze its expression in developing SMG. Western blot analysis was performed using a GRHL2-specifc antibody, which had been validated by the Human Protein

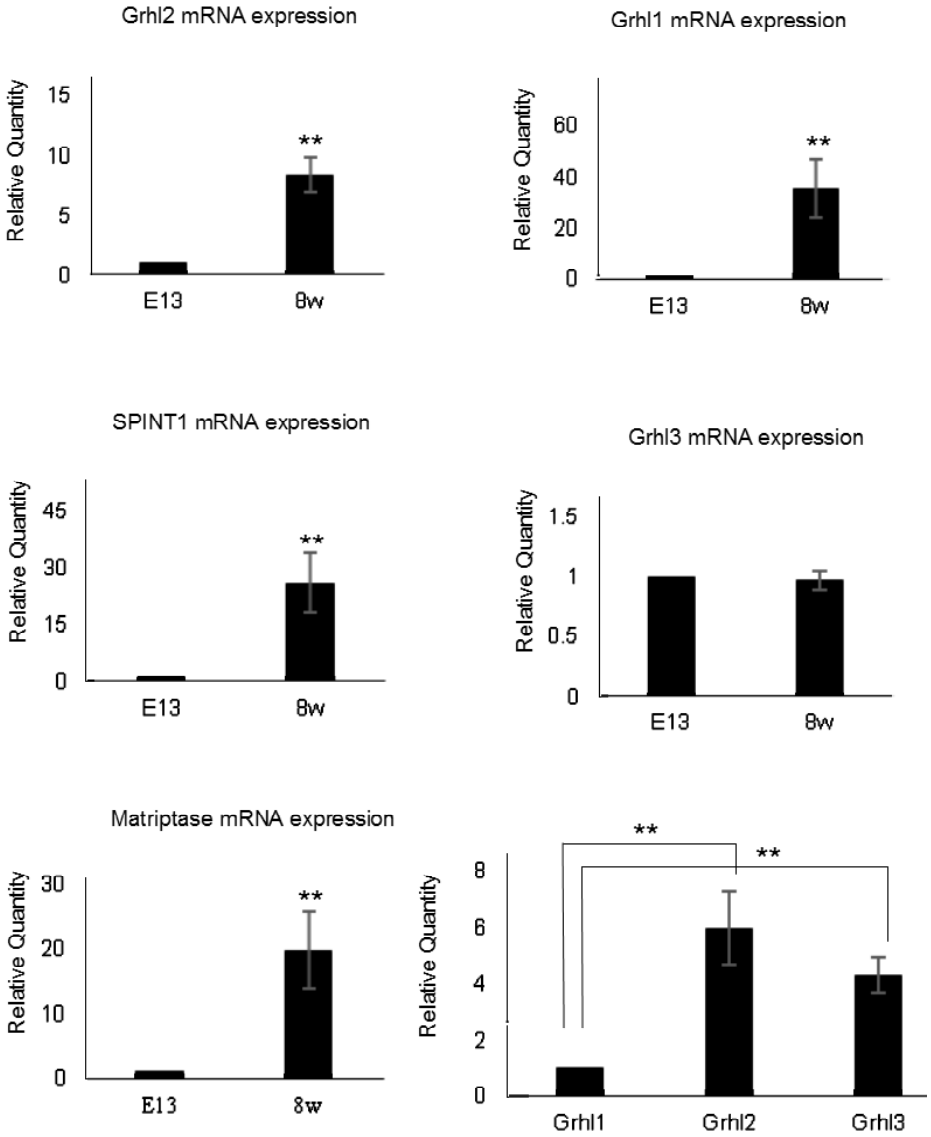

Figure 1 qRT-PCR analysis of the expression of GRHL1, GRHL2, GRHL3, SPINT1, and matriptase mRNAs in the mosue SMG. GRHL2 mRNA is more abundant than GRHL1 and GRHL3 mRNAs in E13 SMG. GRHL1, GRHL2, SPINT1 and matriptase mRNAs but not GRHL3 mRNA are increased during SMG development. Error bars represent the mean ± SEM. **p<0.01, *p<0.05.

Atlas Project (http://www.proteinatlas.org/) and other researchers[15]. As shown in Fig. 2, the expression level of GRHL2 protein was up-regulated during embryonic development of the gland. Furthermore, immunofluorescence microscopy demonstrated, as reported in other organs, that GRHL2 staining was detectable exclusively in the E-cadherin-positive epithelial tissue of SMG at all of the developmental stages examined, being localized at the cellular nuclei (Fig. 3).

As revealed by immunofluorescence microscopy, SPINT1 was expressed only in the epithelial tissue of embryonic and adult SMGs (Fig. 4). Noticeably, SPINT1 staining was strong along the luminal surfaces of cells at E15 and E17. The expression site of SPINT1 in SMG epithelial cells overlapped with the cell adhesion molecule E-cadherin, being consistent with the plasma membrane-bound nature of SPINT1.

Immunofluorescence microscopy showed that although faint in E13 SMG, matriptase staining became evident in the epithelial tissue by E15 (Fig. 5). Notably, strong staining for matriptase was recognized along the luminal surfaces of epithelial cells in E15 and E17 SMGs. In the adult SMG, matriptase exhibited the uniform distribution along the entire surfaces mainly of ductal cells, which was consistent with the expression pattern in the human salivary gland[25].

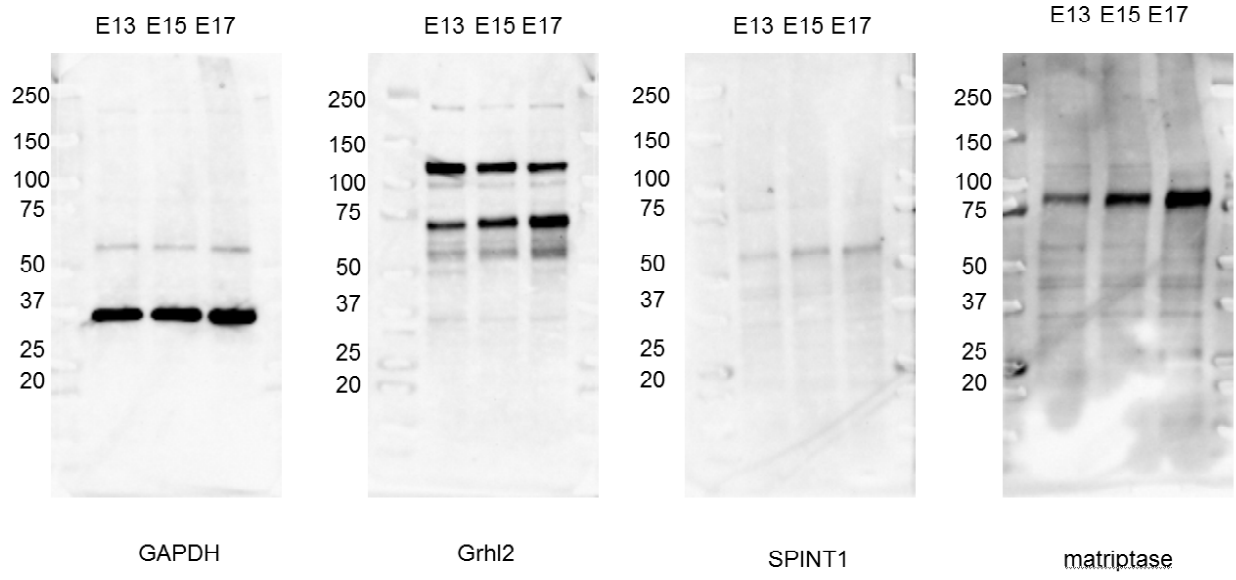

Figure 2 Western blot analysis of the expression of GRHL2, SPINT1 and matriptase in E13, E15, and E17 SMGs. All of these proteins are increased during embryonic development of the gland.

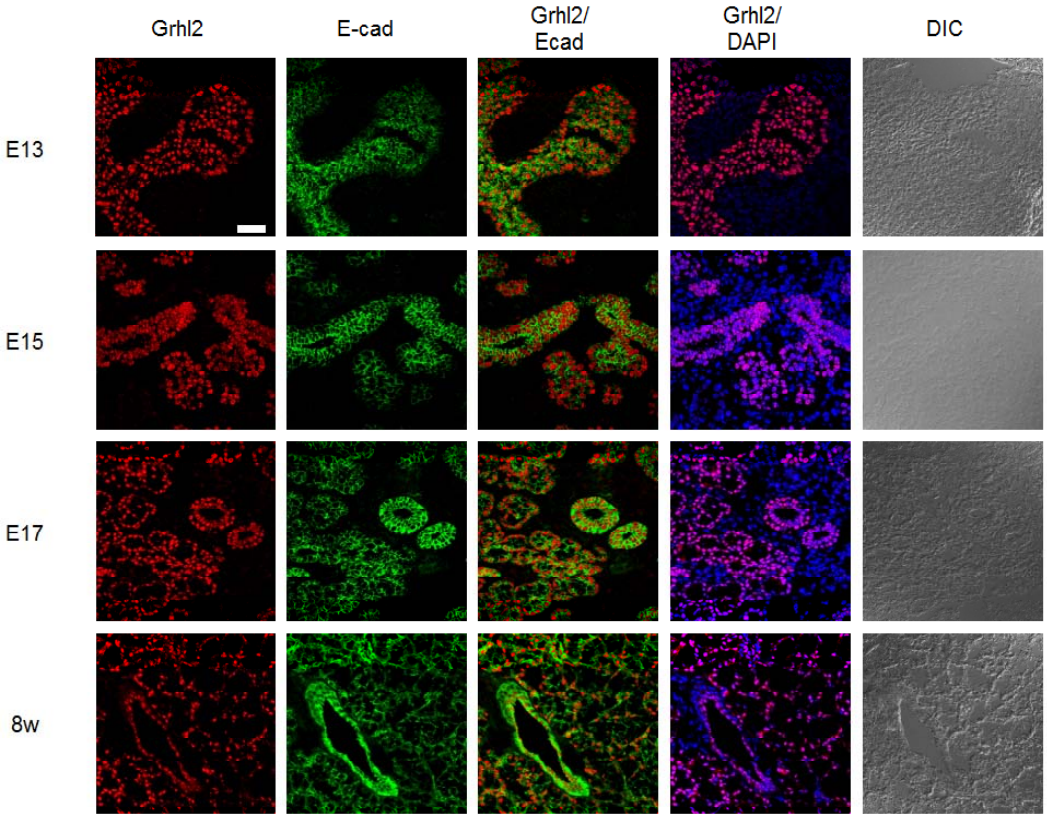

Figure 3 Tissue sections of the mouse SMGs at E13, E15, E17 and 8w were stained for Grhl2 and E-cadherin. Grhl2 expression is exclusively seen in the nuclei of epithelial cells. Scale bar = 25μm.

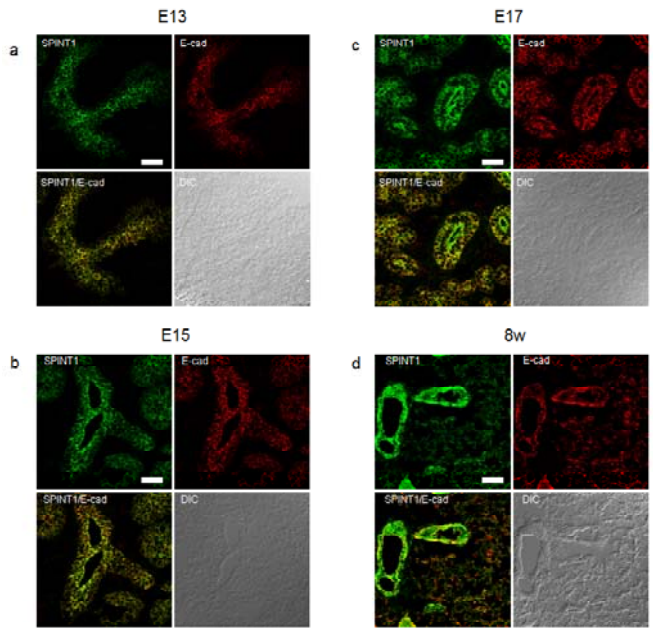

Figure 4 Tissue sections of the mouse SMGs at E13, E15, E17 and 8w were stained for SPINT1 and E-cadherin. SPINT1 expression is seen in the epithelial tissue. SPINT1 is co-localized with E-cadherin. Note the intense SPINT1 staining along the luminal surfaces of the epithelial tissue at E15 and E17. Scale bar = 25μm.

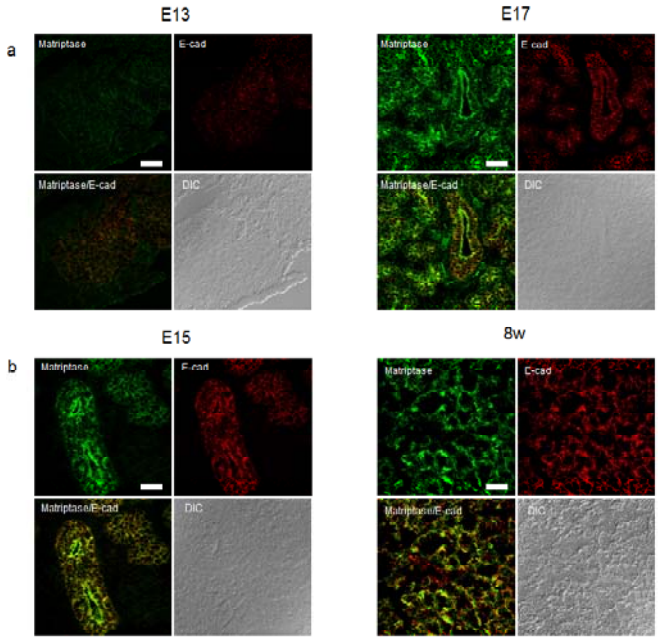

Figure 5 Tissue sections of the mouse SMGs at E13, E15, 17 and 8w were stained for matriptase and E-cadherin. Matriptase expression is seen mainly in the epithelial tissue, being co-localized with E-cadherin. Note the intense matriptase staining along the luminal surfaces of the epithelial tissue at E15 and E17. Scale bar = 25μm.

DISCUSSION

The present study demonstrates the expression of the GRHL2 transcription factor, its target gene product of the SPINT1 protease inhibitor, and the SPINT1 regulated protease matriptase in the SMG of embryonic and adult mice. Their expression begins at the initial developmental stages of gland, increasing later in development. Being expressed exclusively by epithelial cells, GRHL2 is localized at the nuclei, and both SPINT1 and matriptase are present at the cell surfaces, consistent with their membrane-bound nature.

It has recently been reported that the GRHL2 transcription factor directly binds the *Spint1* gene encoding a Kunitz type 1 transmembrane serine protease inhibitor (also known as hepatocyte growth factor activator inhibitor (HAI)-1)[15, 20, 22, 23]. Although there has been no report, as far as we know, on the expression of the *Spint1* gene in salivary glands. In the previous study, we found by comparing gene expression profiles of E13 and E17 mouse SMGs[24], that the expression level of *Spint1* mRNA in embryonic mouse SMG increased approximately 5 folds between E13 and E17 (data not shown). This was supported by qRT-PCR and western blot analyses showing that the Spint1 mRNA and protein were detectable in embryonic and adult mouse SMGs with the expression level increasing during development (Figs. 1, 2). Our data showed that both SPINT1 was localized at the apical cell surfaces facing lumens in developing SMGs at E15 and E17 but not adult SMG. In the mouse SMG, lumen formation occurs first in rod-like stalks at E14 and later in terminal buds by E17, leading to the establishment of the complete tubular structure lined by an epithelial wall. It is of note that lumen formation is closely associated with the *de novo* establishment of cell adhesion structures including claudin-based tight junctions[4, 24, 26]. It has been demonstrated that Spint1 is involved in regulation of epithelial integrity, specifically the structure and barrier function of tight junctions[22]. In addition, Grhl2 promotes lumen expansion and enhances epithelial barrier by up-regulating the expression of Cldn3 and Cldn4 in cultured cells and developing kidenys[11, 12, 14, 27]. It is thus likely that Grhl2 and Spint1 play roles in the formation of tight junctions and lumens in developing SMG.

The expression of both Grhl2 and SPINT1 in the epithelial tissue of the mouse SMG was clearly recognized at E13, at around which branching morphogenesis of the tissue begins. Roles of the two molecules in epithelial branching morphogenesis are less understood; however, it has been demonstrated that they are both involved in trophoblast branching morphogenesis in developing placenta[15, 28]. We are currently investigating possible roles of Grhl2 and SPINT1 in branching morphogenesis of the SMG epithelium.

Matriptase has also been shown to play crucial roles in regulation of epithelial integrity and development of several organs. Matriptase deletion in the salivary glands of mice leads to the impairment of epithelial barrier function along with the altered distribution of the tight junction protein claudin 3 and displays Sjögren's Syndrome-like characteristics including the loss of secretory cell function; however, the glands appear to develop normally with no apparent histological defects[29, 30]. In this study, since SPINT1 forms stable complexes to inhibit the proteolytic activity of proteases such as the transmembrane serine protease matriptase encoded by the *St14* gene[19, 21], we then tried to see the expression of matriptase in developing mouse SMG. qRT-PCR and western blot analyses showed that the matriptase mRNA and protein were de-

tectable in embryonic and adult mouse SMGs with the expression level increasing during development (Figs. 1, 2). Our present data showed the faint expression of matriptase in E13 SMG, and the proteolytic activity of not only matriptase but other proteases, including hepsin and TMPRSS13, is regulated by SPINT1[31, 32]. Proteases other than matriptase might be involved development of the salivary gland.

In conclusion, our findings may suggest that GRHL2 regulation of SPINT1 expression is involved in SMG epithelial development by affecting the proteolytic activity of proteases on epithelial cell surfaces.

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Corresponding author:
Takumi Matsushita, D.D.S.
Department of Oral and Maxillofacial Surgery 1, Osaka Dental University
Kuzuhahanazono 8-1, Hirakata,
Osaka 573-1121, Japan
Phone: +81-6-6910-1509
E-mail: takumi10301986@yahoo.co.jp