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出版情報：Pathology - Research and Practice. 236, pp.153991-, 2022-08. Elsevier
バージョン：

The Semaphorin 3A-AKT axis-mediated cell proliferation in salivary gland morphogenesis and adenoid cystic carcinoma pathogenesis

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Key words: Sema3A, Salivary gland, Morphogenesis, Pathogenesis

Abstract

We recently demonstrated that Semaphorin 3A (Sema3A), the expression of which is negatively regulated by Wnt/β-catenin signaling, promotes odontogenic epithelial cell proliferation, suggesting the involvement of Sema3A in tooth germ development. Salivary glands have a similar developmental process to tooth germ development, in which reciprocal interactions between the oral epithelium and adjacent mesenchyme proceeds via stimulation with several growth factors; however, the role of Sema3A in the development of salivary glands is unknown. There may thus be a common mechanism between epithelial morphogenesis and pathogenesis; however, the role of Sema3A in salivary gland tumors is also unclear. The current study investigated the involvement of Sema3A in submandibular gland (SMG) development and its expression in adenoid cystic carcinoma (ACC) specimens. Quantitative RT-PCR and immunohistochemical analyses revealed that Sema3A was expressed both in epithelium and in mesenchyme in the initial developmental stages of SMG and their expressions were decreased during the developmental processes. Loss-of-function experiments using an inhibitor revealed that Sema3A was required for AKT activation-mediated cellular growth and formation of cleft and bud in SMG rudiment culture. In addition, Wnt/β-catenin signaling decreased the Sema3A expression in the rudiment culture. ACC arising from salivary glands frequently exhibits malignant potential. Immunohistochemical analyses of tissue specimens obtained from 10 ACC patients showed that Sema3A was hardly observed in non-tumor regions but was strongly expressed in tumor lesions, especially in myoepithelial neoplastic cells, at high frequencies where phosphorylated AKT expression was frequently detected. These results suggest that the Sema3A-AKT axis promotes cell

growth, thereby contributing to morphogenesis and pathogenesis, at least in ACC, of salivary glands.

1. Introduction

Murine tooth germ development involves continuous and sequential steps, starting from the invagination of odontogenic epithelium into the underlying mesenchymal tissue at embryonic day (E) 12.5 [1], and is regulated by reciprocal interactions between odontogenic epithelium and the adjacent mesenchyme [2,3]. Several growth factors, including Wnt, bone morphogenetic protein (BMP), fibroblast growth factor (FGF) and sonic hedgehog (SHH), are reported to be essential for tooth germ development [2,3]. Murine submandibular gland (SMG) also exhibits a similar developmental process, where thickening of the oral epithelium protrudes into the underlying mesenchyme at E12.5 and begins branching morphogenesis through epithelial-mesenchymal interactions controlled by growth factors, such as Wnt, BMP, FGF and SHH [4-8]. These reports suggest that the development of tooth germ and SMG is likely controlled by common molecular mechanisms, such as growth factor signaling-mediated cellular behaviors.

The SMG development of rodents and humans exhibits similar developmental patterns involving the initiation by the thickening of the oral epithelium that invaginates into a condensed mesenchyme (6–8 weeks of embryonic life in humans) and the beginning of branching morphogenesis [9].

We recently demonstrated that Wnt/β-catenin signaling negatively regulated cellular growth through reduced expression of Semaphorin 3A (Sema3A), a secreted protein, in odontogenic epithelial cells and described its involvement in tooth germ development [10]. Sema3A was originally identified as an axonal guidance factor for controlling nerve system development in embryogenesis [11]. We hypothesized that Sema3A could regulate SMG development like several growth factors [4-8], while its role remains unclear.

Previous reports have suggested that Sema3A may function as an oncogene-related gene [12-14], but its effects on salivary gland tumors are unclear. Adenoid cystic carcinoma (ACC) is a slow-growing and relentless salivary gland malignancy that forms various patterns, including tubular, cribriform and solid forms [15]. Although the annual incidence of ACC is about 2 cases per 100,000 population [16], ACC exhibits malignant potential, including unpredictable growth, extensive perineural invasion, high rates of metastasis and low survival rates [15,17]. Since ACC is thought to be an important cancer of SMG in the clinical setting, given with its malignant potential, a novel anticancer therapy based on the molecular mechanisms underlying ACC tumorigenesis is awaited. Recently, several gene abnormalities, such as *MYB-NFIB* translocations and overexpression of *KIT* and *EGFR*, and their involvement in the etiology and pathogenesis of ACC have been reported [15,17-19]. But the function of Sema3A in the pathogenesis in ACC is unknown.

Herein, we explored the effects of Sema3A on controlling cellular growth in the development of SMG and investigated its expression in pathological specimens.

2. Material and methods

2.1. SMG rudiment culture

Embryonic submandibular gland (SMG) (SMG rudiments) isolated from ICR mice at E13 were cultured at an air-liquid interface on ThinCert™ tissue culture inserts with 1.0 μm pores (Greiner Bio-One, Berlin, Germany) in DMEM/Ham's F12 supplemented with 150 μg/ml vitamin C, 50 μg/ml transferrin, 100 IU/ml penicillin and 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) [4]. When necessary, the inhibitors, SM-345431, which was kindly provided from Sumitomo Pharma Co., Ltd. [10,20], CHIR99021 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and AKT inhibitor VIII [21] (Merck Millipore, Tokyo, Japan) were added to the same growth media. All protocols used for all animal experiments in this study were approved by the Animal Research Committee of Kyushu University, Japan (No. A30-236-0). After the culture was finished, SMG rudiments were analyzed by immunofluorescence staining and RT-PCR as follows.

2.2. Immunofluorescence staining for SMG rudiments and immunohistochemical analysis for SMG

SMG rudiments were fixed for 30 min at room temperature (RT) in 4% paraformaldehyde (PFA) buffered by phosphate buffered saline (PBS), and then permeabilized in PBS containing 0.5% (w/v) Triton X-100 and 40 mg/ml BSA (Wako) for 30 min. SMG rudiments were incubated with primary antibodies (used at 1:300) for 3 h at RT and then with secondary antibodies for 3 h at RT in accordance with the manufacturer's protocols (Jackson ImmunoResearch Inc., West Grove, PA, USA) [22,23]. The samples were viewed and analyzed with an All-in-one Fluorescence Microscope BZ-X800 (Keyence, Osaka, Japan) and confocal microscope C2si^{+} (NIKON, Tokyo, Japan) [24,25]. Total

epithelium area, in which E-cadherin was positive, and the number of buds, which was defined as "bud" in morphological [4], were measured using BZ-X800.

SMGs isolated from ICR mice at E13, E15 and 8 weeks (W) were fixed for 1 day at RT in 4% PFA buffered by PBS and embedded in paraffin blocks. Subsequently, the paraffin-embedded specimens were sliced into 5-µm-thick sections. Antigen retrieval for staining was done using a decloaking chamber (Biocare Medical, Walnat Creek, CA, USA). Antigen retrieval (Dako, Carpentaria, CA, USA), elimination of the endogenous peroxidase activity (Dako), and blocking (Dako) were carried out as previously mentioned [26,27]. Then, the sections were reacted with primary antibody (used at 1:300 for Sema3A) at 4°C overnight. The sections were incubated with secondary antibody (Histofine Simple Stain MAX PO, Nichirei, Tokyo, Japan) for 1 h at RT. The immunoreactivity was visualized with a solution of 3,3'-diaminobenzidine and <0.1% hydrogen peroxide (DAB substrate solution, Nichirei). Subsequently, the sections were counterstained with hematoxylin.

2.3. Antibodies

Anti-phospho-AKT (4060S), anti-pan-AKT (4691S), anti-phospho-ERK1/2 (4370S) and anti-ERK1/2 (4695S) (for western blotting and immunohistochemistry) antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-Ki-67 (ab15580), anti-Sema3A (ab199475) and anti-p63 (ab53039) (for immunofluorescence and immunohistochemistry) antibodies were from Abcam (Cambridge, UK). Anti-E-cadherin (610181) (for immunofluorescence) antibody was from BD Biosciences (San Jose, CA, USA).

2.4. RT-PCR

Quantitative RT-PCR and semi-quantitative RT-PCR were performed as described previously [24,28,29]. Luna universal qPCR master mix (New England Biolabs, Ipswich, MA, USA) and KOD one PCR master mix (Toyobo, Osaka, Japan) were used in accordance with the manufacturer's protocols. The primers are listed in Supplementary Table 1.

2.5. Patients and immunohistochemistry

Human ACC (n = 10) tissues and human pleomorphic adenoma (PA) (n = 10) tissues were obtained from patients who visited at the Department of Oral and Maxillofacial Surgery, Kyushu University Hospital from May 1998 to April 2021 and October 2013 to February 2022, respectively, who were diagnosed with ACC and PA according to the recent WHO Classification [15], and who underwent surgery. The clinicopathological data of the patients with ACC are presented in Table 1. Patients' informed consent and the approval of the local ethics committee were given (No. 29-392). Following the initial biopsy, all specimens were fixed in 10% (v/v) formalin and embedded in paraffin blocks. Subsequently, the paraffin-embedded specimens were sliced into 4-µm-thick sections, which were stained with hematoxylin-eosin (HE) for independent evaluation by three pathologists.

Immunohistochemical staining was performed on 5-µm-thick paraffin sections. Antigen retrieval for staining was done using a decloaking chamber (Biocare Medical). Antigen retrieval (Dako), elimination of the endogenous peroxidase activity (Dako) and blocking (Dako) were carried out as previously mentioned [26,27]. Then, the sections were reacted with each primary antibodies (used at 1:300 for Sema3A, Ki-67 and p63 or

used at 1:100 for phospho-AKT) at 4°C overnight. The sections were incubated with secondary antibody (Nichirei) for 1 h at RT. The immunoreactivity was visualized with DAB substrate solution (Nichirei). Subsequently, the sections were counterstained with hematoxylin. When the total area of a tumor lesion or a non-tumor region showed >40% staining, the results were defined as Sema3A, Ki-67, p63 or pAKT positive.

2.6. Statistical analysis

Statistical analyses were performed using JMP Pro 15 software. Significant differences were determined using Fisher's exact test for clinicopathological analyses. For other experiments, significant differences were determined using Student's *t*-test and one-way ANOVA with *post hoc* Tukey's test. *P* value of <0.05 was considered to indicate statistical significance.

2.7. Additional Assays

Western blotting data were representative of at least three independent experiments.

3. Results

3.1. Sema3A expression in SMG rudiments

Recently, we demonstrated that Wnt/β-catenin signaling negatively regulated cellular growth through a reduced Sema3A expression in odontogenic epithelial cells and its involvement in tooth germ development [10]. Although the developmental process may involve the same mechanisms for both tooth germ and salivary gland, the effect of Sema3A signaling on salivary gland development, including the proliferation capabilities and cytodifferentiation, remains unclear. To elucidate the role of Sema3A in salivary gland development, we employed an organ culture of SMG rudiments of E13 mouse embryos [4].

In the culture, an increase in the total epithelial area, the number of buds, Ki-67-positive cells and total cells, was observed over time, suggesting that the development of SMG could be proceeded in our hand (Fig. S1A, B). Consistent with a previous report [4], cytodifferentiation processes, such as an increase in the expression of *AQP5*, an early marker of proacinar cells [30,31], *PSP*, which is expressed transiently in developing AQP5-positive cells [32], or *KRT19*, a ductal differentiation marker [31], and a decrease in *Axin2* expression, were also observed (Fig. S1C). *Sema3A* mRNA levels were downregulated towards day 3 (Fig. 1A), but whether or not Sema3A is expressed in epithelium or mesenchyme was unclear. SMG rudiments (E13 and E15) and SMG (8W) were then mechanically separated into epithelium and mesenchyme, and the correct separation of the regions was confirmed by assessing the *Cdh1* (*E-cadherin*) (epithelium marker) and *Vimentin* (mesenchyme marker) mRNA expression (Fig. S2A). The expression of *Sema3A* and its receptors *Nrp1*, *Nrp2*, *PlexinA1*, *PlexinA2*, *PlexinA3*, *PlexinA4*, *PlexinB2*, *PlexinC1* and *PlexinD1* was higher in the mesenchyme than in the

epithelium of E13 SMG rudiments (Fig. 1B and Fig. S2B). Although the expression of *Sema3A* in epithelium and mesenchyme was altered dependent on developmental stages, *Sema3A* expression was downregulated towards the maturation of SMG (Fig. 1B). Similar to these results, immunohistochemical and immunofluorescence analyses demonstrated that Sema3A was expressed both in epithelium and mesenchyme of E13 and E15 SMGs (Fig. 1C and Fig. S2C). In 8W SMG, Sema3A was hardly detected in epithelium, while Sema3A was highly expressed in the vascular tissue (Fig. 1C). These data indicated that Sema3A signaling, the expression of Sema3A might be altered in the developmental processes, would be activated both in epithelium and mesenchyme of SMG in an autocrine manner and/or a paracrine manner in the initial developmental stages of SMG.

As a recent study revealed that ectopic activation of the β-catenin pathway suppresses the Sema3A expression in odontogenic epithelial cells and tooth germ rudiments [10], we examined the effect of the β-catenin pathway on the Sema3A expression in SMG rudiments. Treatment with CHIR99021, an activator of the β-catenin pathway [33], reduced *Sema3A* expression but promoted *Axin2* mRNA expression in SMG rudiments (Fig. 1D), suggesting that the Sema3A expression mechanism in tooth germ and SMG in the developmental processes may share the same machinery.

3.2. Sema3A expression is required for SMG epithelial cell proliferation through AKT activation

As Sema3A was shown to be capable of regulating cell proliferation in tooth germ rudiments of E15 mouse embryos, we examined the effect of Sema3A on the development of SMG rudiments. Treatments with SM-345431, an inhibitor of Sema3A [20], decreased

the total epithelial area, the number of buds and clefts, and the ratio of Ki-67-positive proliferating cells in a dose-dependent manner but increased the average area of a single bud (Fig. 2A, B and Fig. S3A). It is noteworthy that Ki-67-positive cells were predominantly observed in the epithelium (Fig. 2B), suggesting that SM-345431-dependent decreased cellular growth may be the result of loss-of-function phenotypes in the epithelium rather than in the mesenchyme. In contrast, SM-345431 stimulation did not change the mRNA levels of cytodifferentiation markers, such as *AQP5*, *PSP*, *KRT19* and *Axin2*, or cleft formation-related molecules, such as *Fibronectin* (*FN*) [34], *BTB/POZ domain-containing protein 7* (*Btbd7*) [35], *Epiregulin* (*Ereg*) [36] and *Grainyhead-like 2* (*Grhl2*) [37] (Fig. S3B, C). These results suggest that Sema3A signaling-dependent cell proliferation, except for regulating gene expressions, may be involved in cleft formation of SMG rudiments. In addition, SM-345431 stimulation decreased AKT activation but not ERK activation (Fig. 2C). Furthermore, AKT inhibitor VIII decreased the total epithelial area and the number of buds (Fig. 2D). The AKT family consists of three different but highly homologous gene products, such as AKT1, AKT2 and AKT3, and regulates morphogenesis as well as tumorigenesis [38-40]. A recent report demonstrated the potent oncogenic role of AKT3 in ACC pathogenesis using genetically modified mice [40]. The expression of each isoform of AKT was comparable and their mRNA levels were not changed by SM-345431 stimulation in SMG rudiments (Fig. S3D). These results indicated that AKT activation through phosphorylation, not their expression, might be important in SMG development, whereas the limitation of specificity of antibodies and inhibitors might be raised in the evaluation of the involvement of AKT isoforms in SMG development. Taken together, these results indicate that Sema3A signaling can regulate AKT activation to promote epithelial cell proliferation in SMG rudiments.

3.3. Sema3A is expressed in human salivary gland tumor tissues

As previous reports demonstrated that Sema3A could function as an oncogene-related gene [12-14], we examined its role in ACC, which is an important cancer of SMG in the clinical setting with its malignant potential. Immunohistochemical analyses were carried out to examine Sema3A expression in human ACC specimens. The Sema3A expression was detected in 9/10 (90%) tumor lesions, whereas it was hardly detected in adjacent oral non-tumor stratified squamous and/or stromal cell region (Fig. 3A and Table 1). Sema3A was strongly expressed in the cellular cytoplasm of the tumor cells with Ki-67 expression (Fig. 3A). Notably, Sema3A-expressing ACC tumor cells were also stained with p63 (myoepithelial marker [15]) (Fig. 3A and Fig. S4A), indicating that Sema3A could be a specific marker of myoepithelial neoplastic cells in ACC. As reported previously [41,42], the phosphorylated AKT expression was detected in 6/10 (60%) tumor cells, but positive signals were hardly detected in non-tumor region (Fig. 3A, Table 1 and Fig. S4B). Importantly, the phosphorylated AKT expression was more frequently, but not significantly, detected along with Sema3A expression in consecutive tumor lesion sections (6/6; 100%) (Fig. 3A and Table 1). These results suggest that the region-specific expression pattern of phosphorylated AKT in tumor lesions tends to merge with the Sema3A expression in ACC. Furthermore, the perineural invading cells were frequently positive for Sema3A (6/7; 85.7%) (Fig. 3B and Table 1).

To investigate the roles of Sema3A in other salivary gland tumors, Sema3A expression was examined in human PA specimens using immunohistochemical analyses. PA, which consists of epithelial and myoepithelial/stromal components involving ductal epithelial cells, myoepithelial cells and chondromyxoid stroma, is a benign and most

common salivary gland tumor [43]. The Sema3A expression was detected in the cellular cytoplasm of the tumor cells of 6/10 (60%) tumor cases (Figs. S5, S6). In Sema3A-positive cases, Sema3A was detected both in ductal cells and myoepithelial cells without Ki-67 expression and phosphorylated AKT expression (Figs. S5, S6). These results suggested that the underlying pathogenic mechanisms to regulate cell proliferation in salivary gland tumors could be different at least in ACC and in PA.

4. Discussion

It has been suggested that organ formation by stem cells and tumor formation by cancer cells may share a common mechanism, including epithelial-to-mesenchymal or mesenchymal-to-epithelial transition [44], mechanotransduction [24,45] and cell proliferation [22,25,26,46-48]. Consistent with these reports, Sema3A may function to promote morphogenesis and relate to pathogenesis in epithelial cells of salivary grands and tooth germ [10] through regulating cell proliferation.

In the present study, Sema3A and its receptors were predominantly expressed in mesenchyme rather than in epithelium of SMG rudiments of E13 mouse embryos, and the *Sema3A* expression was decreased in a day-dependent manner (see Fig. 1A, B). Although Sema3A signaling, which may depend on its expression, would be diminished in the developmental processes both in epithelium and mesenchyme (see Fig. 1B), Sema3A signaling might be activated both in epithelium and mesenchyme in an autocrine manner and/or a paracrine manner. The treatments with Sema3A inhibitor predominantly suppressed epithelial cellular growth, and budding and cleft formation in SMG rudiments (see Fig. 2A, B and Fig. S3A), while some limitations, especially in the phenotypes of mesenchyme, should be raised in this culture. Although Sema3A inhibitor might affect mesenchymal cells to regulate SMG development, the roles of Sema3A signaling in mesenchyme remain to be clarified. Reportedly, mesenchymal cells secrete growth factors critical for epithelial growth and morphogenesis in the process of branching morphogenesis with cleft formation in lung, kidney and salivary glands [49-51]. However, when suitable growth factors are provided, epithelium of many organs can branch without mesenchyme [6,52-54]. Given the present and previous findings, we speculated that Sema3A, the expression of which is limited in the initial developmental stages, may be

sufficient to exert a positive effect on epithelial cellular growth through AKT activation in SMG rudiments.

ACC exhibits malignant potential, including unpredictable growth, extensive perineural invasion, high rates of metastasis and low survival rates [15,17]. Several gene abnormalities, such as *MYB-NFIB* translocations and the overexpression of *KIT* and *EGFR*, have been reported, along with its involvement in the etiology and the pathogenesis of ACC [15,17-19]. Considering a recent report demonstrating the involvement of AKT3 in ACC pathogenesis using genetically modified mice [40], we propose that the elevated expression of Sema3A might function as a novel oncogene-related molecule to activate AKT signaling, resulting in enhanced cell proliferation in ACC. A recent report demonstrated that Sema3A activates soluble guanylate cyclase (sGC)-cGMP signaling in osteocytes [55]. While the function of Sema3A-AKT signaling may depend on cell context, our immunohistochemical analysis indeed revealed the presence of Sema3A expression with phosphorylated AKT expression at high frequencies in tumor lesions. In addition, Sema3A was detected without phosphorylated AKT expression and Ki-67 expression in PA, suggesting the specificity of Sema3A-phosphorylated AKT-mediated cell proliferation in the pathogenesis of ACC. Sema3A expression was hardly detected in non-tumor regions of ACC (see Fig. 3A), PA (data not shown) and adult murine SMG (see Fig. 1C); therefore, the findings support the idea that a reduction in Sema3A expression may be a novel antitumor therapeutic strategy for human ACC to prevent side effects in non-tumor regions. To reveal the precise function of Sema3A in ACC pathogenesis and tumorigenesis, it is necessary to explore ACC-derived cell lines and mouse ACC models in the future. Furthermore, the expressions of Sema3A receptors, which is involved in ACC tumorigenesis, also need to be clarified.

Although several molecules, such as p63 and smooth muscle actin (SMA) [15], are reported as a marker of myoepithelial neoplastic cells, the expression of Sema3A in the cells were not reported yet. Therefore, Sema3A could be proposed as a specific marker of myoepithelial neoplastic cells at least in ACC, but not in PA. The precise mechanism underlying how Sema3A expression, the expression of which is decreased in the developmental processes of SMG (see Fig. 1B), is upregulated in tumor lesions of ACC, especially in myoepithelial neoplastic cells, is unknown. However, it is noteworthy that Sema3A signaling may exert similar proliferation capabilities in morphogenesis and pathogenesis in salivary glands, despite the expression pattern of Sema3A could be changed. In addition, Sema3A, which is a secreted protein, was originally identified as an axonal guidance factor [11], and whether or not ACC derived-Sema3A would affect nerve systems in perineurally invaded by ACC remains unclear.

In summary, we demonstrated that Sema3A expression regulates cell proliferation positively through AKT activation in SMG rudiments. We also found that Sema3A is highly expressed in proliferating myoepithelial neoplastic cells in ACC specimens. Taken together, these results suggest that the Sema3A-AKT axis contributes to morphogenesis and pathogenesis of salivary glands.

Funding: This work was supported by JSPS KAKENHI Grants to S.F. (2020-2022) (20K09906), K.H. (2021-2023) (21K09843), K.H. (2020-2022) (20K10107) and T.K. (2020-2022) (20K10096), Takeda Science Foundation, The Shin-Nihon Foundation of Advanced Medical Research and SGH Foundation to S.F.

Conflict of Interest Disclosures: None of the authors have any relevant financial relationship(s) with a commercial interest.

Author Contributions

Shinsuke Fujii: Conceptualization, Investigation, Methodology, Writing - Original Draft, Funding acquisition.: **Tatsufumi Fujimoto, Takuma Ishibashi:** Investigation, Writing - Review & Editing. **Kana Hasegawa:** Investigation, Writing - Review & Editing, Funding acquisition.: **Ryoko Nagano, Kari J Kurppa, Yurie Mikami, Megumi Kokura, Yudai Tajiri, Toshiro Kibe, Hiroko Wada, Shosei Kishida, Yoshinori Higuchi:** Supervision, Writing - Review & Editing.: **Naohisa Wada:** Resources.: **Tamotsu Kiyoshima:** Project administration, Writing - Review & Editing, Funding acquisition. All authors were involved in writing the paper and had final approval of the submitted and published versions.

Acknowledgments

The authors thank Drs. K. Nagata, S. Nakamura and Y. Mori for valuable technical support in this research. The authors also thank the Research Support Center, Graduate School of Medical Sciences, Kyushu University. We appreciate the technical assistance from The Joint Use Laboratories, Faculty of Dental Science, Kyushu University.

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Figure Legends

Figure 1. Sema3A expression in SMG rudiments.

(A) E13 SMG rudiments were cultured for 0 (6 h), 1, 2 or 3 days. *Sema3A* mRNA levels were measured in the rudiments using quantitative RT-PCR. Relative levels of *Sema3A* mRNA expression were normalized to *GAPDH* and expressed as fold-changes compared with expression in the rudiments cultured for 0 day. (B) E13 and E15 SMG rudiments and 8W SMGs were separated into epithelium and mesenchyme. *Sema3A* mRNA levels were measured in the separated epithelium and mesenchyme using quantitative RT-PCR. Relative levels of *Sema3A* mRNA expression were normalized to *GAPDH* and expressed as fold-changes compared with expression in the epithelium of each stage and/or in the E13 epithelium or mesenchyme. (C) Tissue sections of mouse SMG at E13, E15 and 8W were stained with anti-Sema3A antibody and hematoxylin. Dotted lines indicate the border between epithelium and mesenchyme. Black arrowheads indicate epithelial cells. White arrowheads indicate vascular tissue. (D) E13 SMG rudiments were cultured without or with 0.1, 1 and 2.5 μM CHIR99021 for 1 day. *Sema3A* or *Axin2* mRNA levels were measured in the rudiments using quantitative RT-PCR. Relative levels of indicated mRNA expression were normalized to *GAPDH* and expressed as fold-changes compared with expression in control rudiments. Scale bars, 20 μm. Results are shown as means ± s.d. of three independent experiments. **P*<0.01.

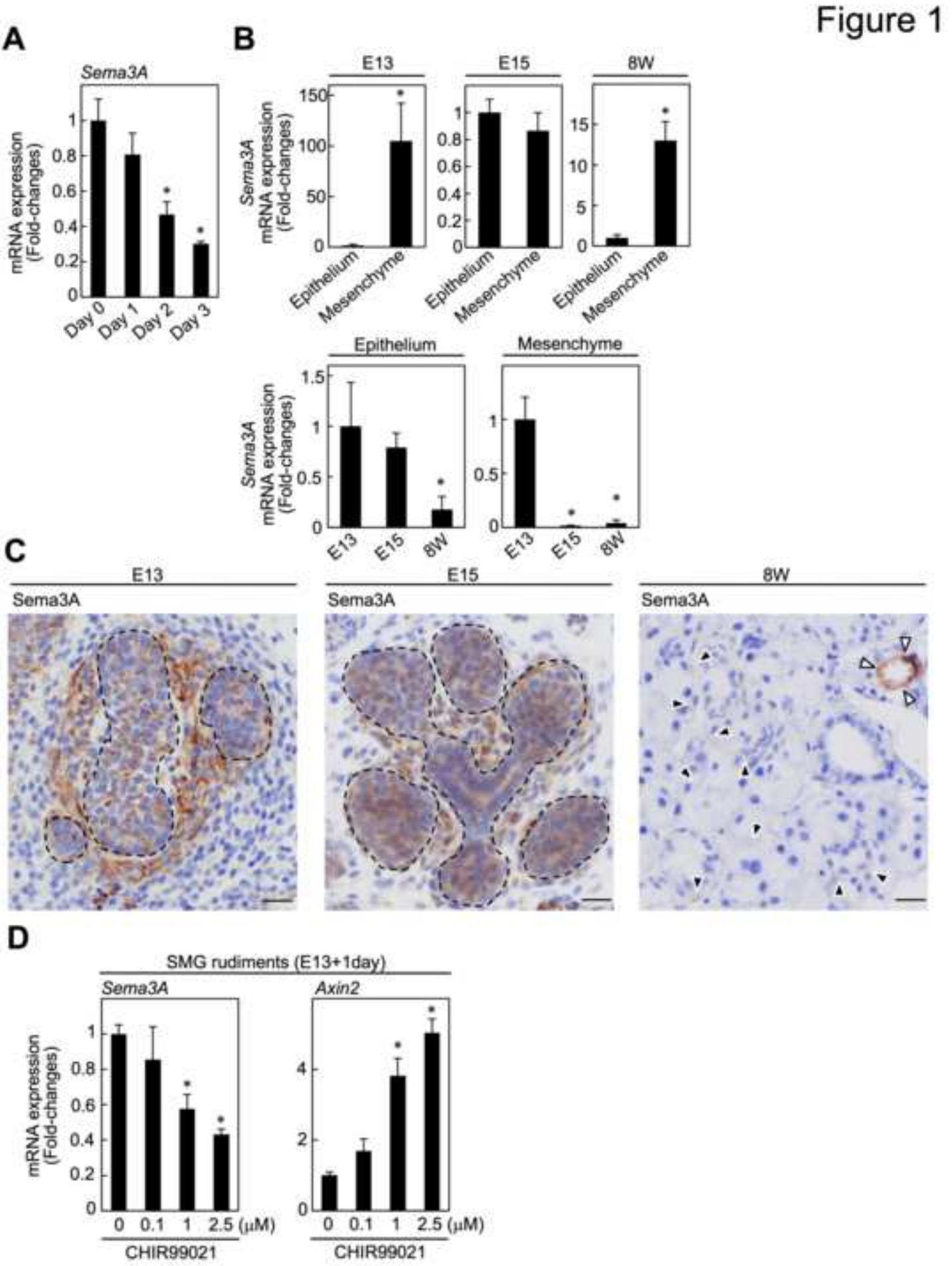
Figure 2. Sema3A expression is required for cell proliferation through AKT activation in SMG rudiments.

(A and B) E13 SMG rudiments were cultured without or with 1, 2.5 and 5 μM SM-345431 for 3 days. (A) The rudiments were stained with anti-E-cadherin antibody (left panels).

The areas of total epithelia and number of buds were counted (right graphs). (B) The rudiments were stained with anti-Ki-67 antibody and Hoechst 33342, and then Ki-67-positive cells and Hoechst 33342-stained cells were counted (left panels). Results are expressed as the percentage of Ki-67-positive cells compared with total Hoechst 33342-stained cells (right graph). White boxes show enlarged images. (C) E13 SMG rudiments were cultured for 6 days without or with 2.5 μM SM-345431 for last 3 days. The lysates were probed with anti-phospho-AKT, anti-pan-AKT, anti-phospho-ERK1/2 and anti-ERK1/2 antibodies. (D) E13 SMG rudiments were cultured without or with 10 μM AKT inhibitor VIII for 3 days. Then, the rudiments were stained with anti-E-cadherin antibody (left panels). The areas of total epithelia and number of buds were counted (right graphs). Scale bars, 400 μm (A), 200 μm (B and D). *P<0.01.

Figure 3. Sema3A is expressed in human adenoid cystic carcinoma tissues.

(A) Adenoid cystic carcinoma tissues (n = 10) were stained with anti-Sema3A, anti-Ki-67, anti-phospho-AKT and anti-p63 antibodies, and hematoxylin. Dashed box and solid box show enlarged images of non-tumor squamous region and tumor lesion, respectively. (B) Representative adenoid cystic carcinoma tissues stained with anti-Sema3A and hematoxylin are shown. N; nerve bundle, Scale bars, 1 mm (A; upper left), 20 μm (A; upper right, lower panels, B).



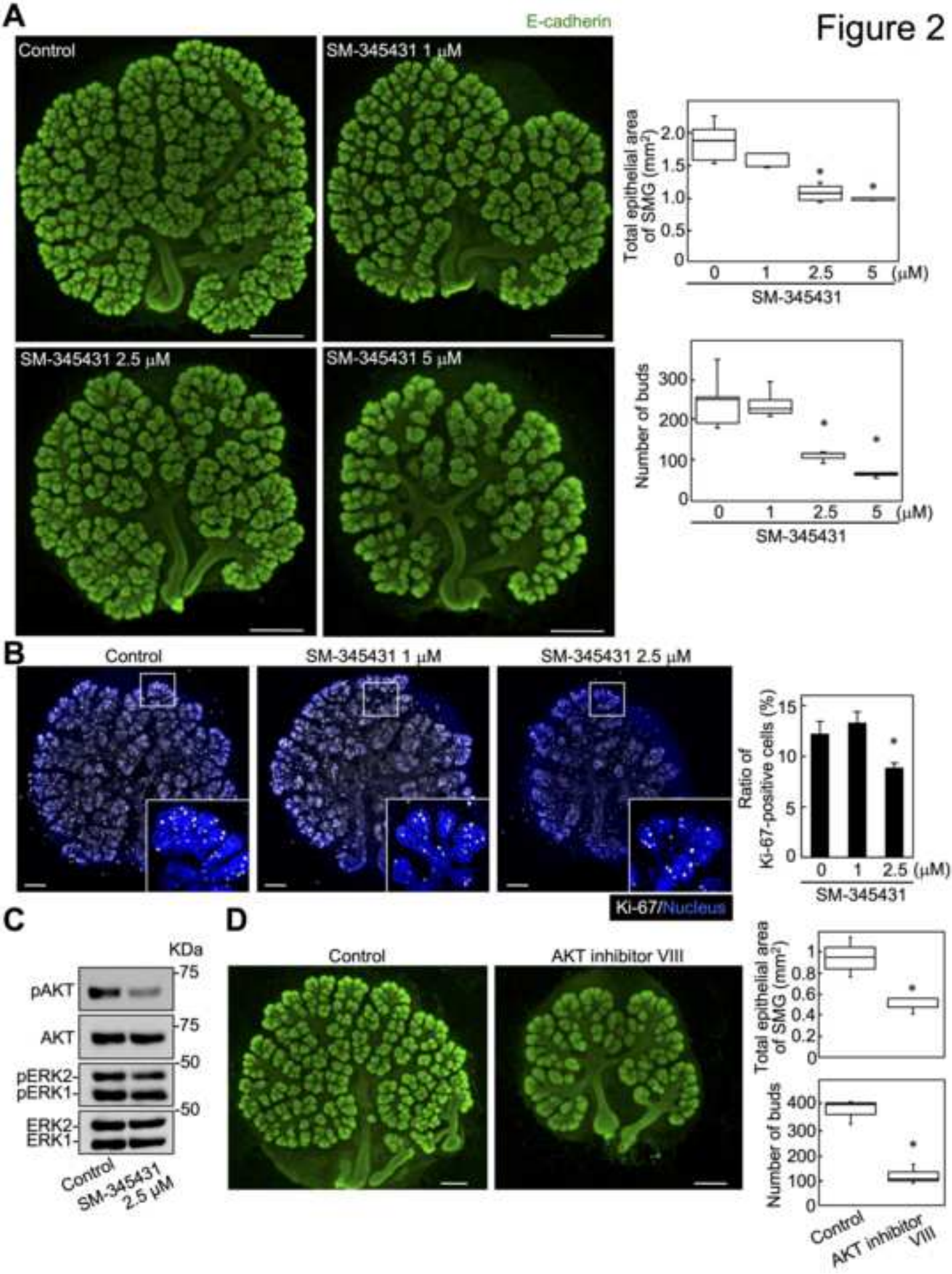


Figure 2

Figure 3

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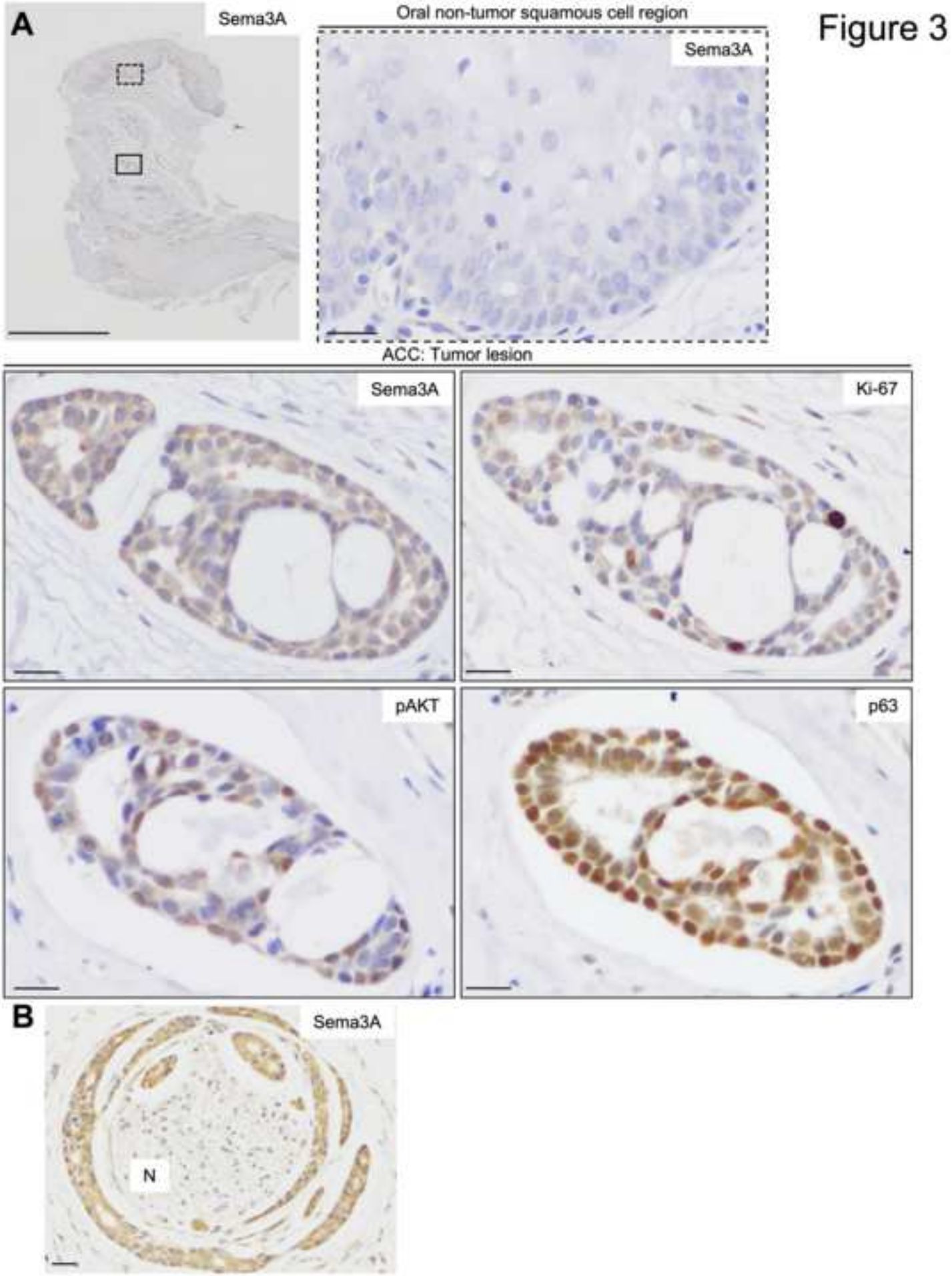

Table 1. Clinical characteristics of ACC patients with Sema3A expression.

Sample no.	Age	Sex	Location	IHC		Perineural invasion
				Sema3A	pAKT	
1	58	Female	Palate	+	+	Negative
2	37	Female	Buccal mucosa	+	+	Negative
3	81	Female	Floor of the mouth	+	+	Positive
4	67	Female	Palate	+	+	Positive
5	72	Male	Palate	+	+	Negative
6	71	Female	Maxilla	+	+	Positive
7	55	Male	Submandibular gland	+	−	Positive
8	84	Female	Palate	+	−	Positive
9	72	Female	Sublingual gland	+	−	Positive
10	71	Male	Tongue	−	−	Positive

Conflict of Interest Disclosures: None of the authors have any relevant financial relationship(s) with a commercial interest.

Author Contributions

Shinsuke Fujii: Conceptualization, Investigation, Methodology, Writing - Original Draft, Funding acquisition.: **Tatsufumi Fujimoto, Takuma Ishibashi:** Investigation, Writing - Review & Editing. **Kana Hasegawa:** Investigation, Writing - Review & Editing, Funding acquisition.: **Ryoko Nagano, Kari J Kurppa, Yurie Mikami, Megumi Kokura, Yudai Tajiri, Toshiro Kibe, Hiroko Wada, Shosei Kishida, Yoshinori Higuchi:** Supervision, Writing - Review & Editing.: **Naohisa Wada:** Resources.: **Tamotsu Kiyoshima:** Project administration, Writing - Review & Editing, Funding acquisition. All authors were involved in writing the paper and had final approval of the submitted and published versions.

Acknowledgments

The authors thank Drs. K. Nagata, S. Nakamura and Y. Mori for valuable technical support in this research. The authors also thank the Research Support Center, Graduate School of Medical Sciences, Kyushu University. We appreciate the technical assistance from The Joint Use Laboratories, Faculty of Dental Science, Kyushu University.