

DR MITSUKO KONDO (Orcid ID : 0000-0003-3673-5431)

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Chloride ion transport and overexpression of TMEM16A in a guinea pig asthma model

Mitsuko Kondo M.D., Mayoko Tsuji M.D., Kaori Hara M.D., Ken Arimura M.D., Osamitsu Yagi
M.D., Etsuko Tagaya M.D., Kiyoshi Takeyama M.D., Jun Tamaoki M.D.

First Department of Medicine, Tokyo Women's Medical University School of Medicine, Tokyo
162-8666, Japan.

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Key words; asthma, Cl channel, mucus, CaCC, TMEM16A

Address correspondence

Mitsuko Kondo, M.D; First Department of Medicine, Tokyo Women's Medical University,
School of Medicine, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan.

Phone; 81-3-3353-8111

Fax; 81-3-5379-5457

E-mail; mikondo@chi.twmu.ac.jp

Abstract

Background: TMEM16A, a Ca-activated Cl channel, regulates various physiological functions such as mucin secretion. However, the role of TMEM16A in hypersecretion in asthma is not fully understood.

Objective: The aim of this study is to evaluate Cl ion transport via TMEM16A and determine the localization of TMEM16A in a guinea pig asthma model.

Methods: Guinea pigs were sensitized with OVA *ip.* on Day 1 and Day 8. On Day 22, we assessed OVA challenge-induced Cl ion transport in the sensitized tracheas *ex vivo* in an Ussing chamber, compared with the non-sensitized tracheas. We then examined the effect of T16Ainh-A01, a TMEM16A inhibitor, on the increase in Cl ion transport. The tracheal epithelium was immunostained with an anti-TMEM16A antibody. Epithelial cells from guinea pig tracheas were cultured at the air-liquid interface in the presence of IL-13 for *in vitro* study. We studied the effect of TMEM16A inhibitors on Ca-dependent agonist, UTP-induced increases in Cl ion transport in the cultured cells. The cells were immunostained with an anti-TMEM16A antibody, an anti-MUC5AC antibody, and an anti- α -tubulin antibody.

Results: OVA challenge induced an increase in Isc within 1 min in the OVA-sensitized tracheas but not in the non-sensitized tracheas, which was inhibited by pretreatment of T16Ainh-A01.

Sensitized tracheas showed goblet cell metaplasia with more positive TMEM16A immunostaining, particularly in the apical portion compared with the non-sensitized tracheas.

The *in vitro* UTP-induced increase in Cl ion transport was strongly inhibited by pretreatment with

T16Ainh-A01, benzbromarone, and niflumic acid. TMEM16A was positively immunostained at the apical portion and in the MUC5AC-positive area in IL-13-induced goblet cell metaplasia.

Conclusions: Antigen challenge and Ca-dependent agonist treatment increased Cl ion transport via the overexpression of TMEM16A in goblet cell metaplasia in a guinea pig asthma model.

TMEM16A inhibitors may be useful for the treatment of hypersecretion in asthma.

Key words; asthma, epithelium, animal models, CaCC, IL-13, goblet cell

Introduction

Ca-activated Cl channels (CaCCs) play key roles in various physiological functions, ranging from electrolyte transport in the airway epithelium to cellular excitation in sensory neurons and muscle fibers. However, the target genes of CaCCs remained unknown for many years. In various airway diseases, including asthma, the CLCA family that is involved in goblet cell metaplasia [1, 2] had been proposed to encode putative CaCCs. However, CLCA is now thought to not be a Cl channel *per se* and seems instead to be a CaCC modulator due to its protease and cell adhesion properties[3]. In 2008, the TMEM family, particularly TMEM16A,

was discovered to be a critical component of CaCCs[4, 5]. It was then reported that TMEM16A modulates mucus secretion and airway smooth muscle contraction in asthma[6, 7] and that TMEM16A inhibitors may impact multiple causes of asthma symptoms. Recently, Sala-Rabnal reported that secreted and cleaved CLCA1 can stabilize TMEM16A in the apical membrane[8]. We previously reported that niflumic acid inhibited mucus secretion induced by OVA-challenge, histamine and UTP in a guinea pig asthma model[9]. Because niflumic acid is reported to inhibit TMEM16A[10], these events may have been caused via TMEM16A inhibition. Therefore, we studied the role of CaCC, particularly TMEM16A, in antigen challenge-induced increases in Cl⁻ ion transport in the airway epithelium of a guinea pig asthma model. Furthermore, to elucidate the mechanism, we studied the role of TMEM16A in Ca-dependent agonist, UTP-induced Cl⁻ ion transport in IL-13-induced goblet cell metaplasia cultured at the air-liquid interface.

Methods

Animal models

All protocols were approved by Ethical Review Committee of Animal Experiments

Tokyo Women's Medical University The ethics approval number: 15-46.

Pathogen-free male Dunkin-Hartley guinea pigs, weighing between 250 and 300 g, were sensitized with an intraperitoneal injection of OVA (70 mg/kg, Sigma Chemical Co., St. Louis, MO, USA) on Day 1 and Day 8. Control (non-sensitized) guinea pigs were injected with saline.

Ussing chamber study

On Day 22, after anesthetizing the animals with an intraperitoneal injection pentobarbital (50 mg/kg), the animals were sacrificed by exsanguination, and the tracheas were isolated from the surrounding tissues, removed, immersed in 37°C oxygenated Krebs-Henseleit solution (pH 7.4), and opened at the anterior portion. The tissues were immediately mounted in an Ussing chamber (surface area 0.2 cm²) and bathed in Krebs-Henseleit solution (pH 7.4) at 37°C, which was bubbled with 95% O₂ and 5% CO₂. The transepithelial potential difference (PD) was clamped to

zero, and the short circuit current (Isc) was continuously recorded under this condition.

Resistance (R) was calculated by Ohm's law from the change of Isc by a constant voltage pulse (0.5~1 mV). A standard protocol was followed. To control for the effect of Na absorption, amiloride (10^{-4} M, Sigma-Aldrich, St. Louis, MO, USA) was added to the mucosal side of the tracheas. The tissues were challenged by 1% OVA at both the mucosal and serosal sides *ex vivo*.

To examine the mechanism of the OVA-challenged Isc response, the effects of Cl channel inhibitors were examined. A newly synthesized TMEM16A inhibitor, T16Ainh-A01 (10^{-5} M, Calbiochem, San Diego, CA, USA)[11], and a non-specific CFTR inhibitor, diphenylamine-2-carboxylate (DPC) (10^{-4} M, Sigma-Aldrich)[12, 13], were added to the mucosal side before the OVA challenge.

Histology

Tracheal mucosa was fixed for 24 h with 10% formal saline for morphological assessment. The tracheal sections were stained with periodic acid-Schiff (PAS) and alcian blue (AB) to demonstrate acidic and neutral intracellular glycoproteins in goblet cells. A semiautomatic image analysis system (WinROOF2015, Mitani Co. Fukui, Japan) was used to measure the

PAS/AB-positive area and the total tracheal epithelial area. The mucus area was calculated as a percentage of the total area of the tracheal epithelium[9].

Immunostaining of TMEM16A

Paraffin sections were dewaxed and hydrated in distilled water. They were heated in 0.01 M citrate buffer (pH 6.0) with an autoclave at 105°C for 5 minutes and then incubated in 4% Block ace (Dainippon Seiyaku, Osaka, Japan) for 20 minutes to block nonspecific immunoreactions.

Next, immunohistochemistry was performed using the following primary antibody in PBS containing 1% bovine serum albumin (Sigma-Aldrich) overnight: anti-TMEM16A antibody (ab53212, polyclonal, rabbit IgG, 1:200, Abcam, Cambridge, UK). The immunogen for ab53212

is a synthetic peptide of human TMEM16A protein. This antibody has cross-reactivity to mouse, rat and human, and the prediluted form (ab53213) is reported to have cross-reactivity to

guinea-pig TMEM16A[14]. For immunofluorescent staining, Cy3-conjugated goat antibody (Jackson Immuno Research, PN, USA) was used as a secondary antibody. VECTASHIELD mounting medium with DAPI (VECTOR, CA, USA) was used for nuclear counterstaining.

Immunostained sections were examined using a Zeiss LSM-710 confocal laser-scanning

microscope (ZEISS LSM710; Carl Zeiss, Jena, Germany). The TMEM16A-positive area was calculated as a percentage of the total area of tracheal epithelium by the semiautomatic image analysis system (WinROOF 2015)[9].

Cell culture

Epithelial cells from guinea pig tracheas were isolated by digestion with 0.05% protease (type XIV, Sigma-Aldrich) at 4° C overnight. Cells were pelleted (200 g, 10 min) and suspended in a 50:50 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (GIBCO, Grand Island, NY, USA) containing 5% fetal calf serum. Cells were plated at 2.5×10^5 cells per cm² onto polycarbonate inserts that were 12 mm in diameter, had a 0.4-μm pore size, and were 10-μm thick (Costar Transwell, Cambridge, MA, USA), which had been coated with human placental collagen (type XVI, Sigma-Aldrich)[15]. Cells were cultured in serum-free, hormonally defined medium as previously reported[16] from the first day after plating. After the cells reached confluence, the apical medium was removed, and the cells were fed from only the basolateral side with 1 ml of medium containing human recombinant IL-13 (10 ng/ml, Biosource International,

Camarillo, CA, USA). Cells were cultured at the air-liquid interface for 14 days with medium containing IL-13. Cells were maintained in an incubator with 21% O₂ and 5% CO₂ at 37°C, and the culture medium was changed once every two days.

Ussing chamber study for cultured cells

Cultured epithelial cells on a porous membrane were mounted on an Ussing chamber, and PD, I_{sc} and R were measured as described above. In the presence of amiloride (10⁻⁴ M), UTP (10⁻⁵ M, Sigma-Aldrich) was added to the mucosal side; then, isoproterenol (10⁻⁶ M, Sigma-Aldrich) was added to the serosal side. To elucidate the effects of TMEM16A inhibitors[10], T16Ainh-A01 (10⁻⁵ M), benzbromarone (10⁻⁵ M, Sigma-Aldrich), and niflumic acid (10⁻⁵ M, Sigma-Aldrich) were added to the mucosal side.

Immunohistochemistry of cultured cells

Cultured epithelial cells were fixed on ~ Day 14 by adding 200 µl of Bouin's solution (Wako, Osaka, Japan) to the apical side for 15 min at room temperature. Samples were blocked with 4%

Block ace (Bio-Rad Laboratories, OX, UK) before antibody treatment to prevent nonspecific staining for 20 minutes at room temperature. Then, the samples were incubated with 200 µl of each primary antibody diluted in 1% bovine serum albumin (BSA) (Jackson Immuno Research, PN, USA) in phosphate buffered saline (PBS), pH 7.4, at 4°C overnight. The primary antibodies used were as follows: anti- α -tubulin antibody (ab6160, monoclonal [YL1/2], rat IgG2a, 1:1000, Abcam, Cambridge, UK), anti-TMEM16A antibody (ab53212, polyclonal, rabbit IgG, 1:100, Abcam, Cambridge, UK) and biotin-conjugated anti-MUC5AC antibody (ab79082, monoclonal [45M1], mouse IgG, 1:100, Abcam, Cambridge, UK). For immunofluorescent staining, alexa 647 conjugated donkey anti-rat antibody, alexa 594 conjugated donkey anti-rabbit antibody and dichlorotriazylamino fluorescein isothiocyanate (FITC) conjugated streptavidin (1:100, Jackson Immuno Research, PN, USA) were used as secondary antibodies and were diluted in PBS with 1% BSA for 1 h. After three washes with PBS, the porous membranes carrying the cells were cut from the plastic support, placed on microscope slides and mounted with VECTASHIELD mounting medium. Signals were observed with laser scanning spectral confocal microscope.

Statistics

The data were analyzed using the Prism 6 software package (GraphPad Software, San Diego, CA, USA). All data are presented as the mean $\pm$ SEM and were analyzed by two-tailed Student's t-test or one-way analysis of variance (ANOVA) with Tukey's post-test analysis for significant differences. A probability of <0.05 for the null hypothesis was accepted as indicating a statistically significant difference.

Results

1. OVA-sensitized animal model

Ussing chamber study

The baseline Isc was significantly higher in the OVA-sensitized tracheas than in the control tracheas (30.6 ± 3.9 vs. 17.1 ± 4.1 $\mu\text{A}/\text{cm}^2$, $n=7-9$, $p<0.05$). However, R was not significantly different between both groups (47.7 ± 6.9 vs. 49.2 ± 8.6 $\Omega.\text{cm}^2$). The decrease in Isc after addition of amiloride did not differ between the two conditions (4.5 ± 2.0 vs. 3.8 ± 1.1 $\mu\text{A}/\text{cm}^2$). The OVA-challenge induced a monophasic increase in Isc within 1 min, which peaked at ~ 3 min and

then gradually decreased (Fig. 1A). The maximum increase in I_{sc} after OVA challenge was significantly higher in the sensitized tracheas than in the control tracheas (6.7 ± 1.5 vs. 1.1 ± 0.5 $\mu A/cm^2$, $p < 0.01$, Fig. 1B). The increases in I_{sc} induced by OVA challenge in the sensitized epithelium were significantly inhibited by T16Ainh-A01 but not by DPC (no inhibitors, 4.2 ± 0.7 $\mu A/cm^2$; T16Ainh-A01 10^{-5} M, 0.5 ± 0.9 $\mu A/cm^2$; DPC 10^{-4} M, 4.2 ± 1.6 $\mu A/cm^2$; $n = 4-5$, $p < 0.05$, no inhibitor vs. T16Ainh-A01, Fig. 2B).

Histology

The sensitized tracheal epithelium showed more PAS/AB-positive area compared with the control epithelium, suggesting that sensitization induced goblet cell metaplasia (Fig. 3Aa, c). The PAS/AB-positive mucus area at baseline was significantly higher in the sensitized epithelium than in the control epithelium ($37.1 \pm 2.5\%$ vs. $18.0 \pm 1.9\%$, $p < 0.01$, $n = 4-5$, Fig. 3B). After OVA challenge *ex vivo* in an Ussing chamber, the mucus area decreased in the sensitized tracheal epithelium, but not in the control epithelium (Fig. 3Ab, d). The mucus area after OVA challenge was significantly lower than the mucus area at baseline in the sensitized epithelium ($17.2 \pm 2.7\%$ vs. $37.1 \pm 2.5\%$, $p < 0.01$, $n = 5$, Fig. 3B). The decrease in mucus area induced by OVA challenge

was inhibited by pretreatment with T16Ainh-A01 (10^{-5} M) in the sensitized epithelium (Fig. 3Ae). The mucus area induced by OVA challenge in the presence of T16Ainh-A01 was significantly higher than the mucus area induced by OVA challenge ($31.5 \pm 3.4\%$ vs. $17.2 \pm 2.7\%$, $p < 0.01$, $n = 4$, Fig. 3B). Thus, T16Ainh-A01 inhibited mucus secretion induced by OVA challenge.

Immunofluorostaining of TMEM16A

To examine the baseline level of TMEM16A expression, control and sensitized tracheal epithelium was immunofluorostained with an anti-TMEM16A antibody. As a result, TMEM16A was immunofluorostained in both control and sensitized tracheal epithelium (Fig. 4Aa, b). However, TMEM16A immunofluorostaining was more strongly positive in the apical membrane of the sensitized epithelium (Fig. 4Ab). Mucus granules in goblet cells and basal cells were positively immunofluorostained in both the control and sensitized epithelium. The percent of TMEM16A-positive area was significantly higher in the sensitized epithelium than in the control epithelium ($26.4 \pm 3.7\%$ vs. $12.0 \pm 1.0\%$, $n = 4$, $p < 0.05$, Fig. 4B).

2. Cultured airway epithelial experiment

Ussing chamber experiment

To elucidate the role of TMEM16A in goblet cell metaplasia, we studied the effect of various TMEM16A inhibitors on Cl ion transport induced by the Ca-dependent agonist, UTP, and the cAMP-dependent agonist, isoproterenol in the IL-13-treated cultured epithelial cells. As shown in Fig. 5, UTP (10^{-5} M)-induced increase in Isc was greater in the IL-13-treated cells than in the control cells without IL-13 treatment (Δ Isc: $25.3 \pm 5.6 \mu\text{A}/\text{cm}^2$ vs. $4.8 \pm 0.3 \mu\text{A}/\text{cm}^2$, $n=4$, $p < 0.01$). Although T16Ainh-A01(10^{-5} M) had a little inhibitory effect in the control cells (Δ Isc: T16Ainh-A01, $3.3 \pm 0.2 \mu\text{A}/\text{cm}^2$, $n=4$, $p < 0.05$ vs. no inhibitor), the augmenting effect of UTP-induced Cl ion transport in the IL-13-treated cells was strongly inhibited by pretreatment with various TMEM16A inhibitors (all 10^{-5} M): T16Ainh-A01, benzbromarone, and niflumic acid, (Δ Isc: no inhibitor, $25.3 \pm 5.6 \mu\text{A}/\text{cm}^2$; T16Ainh-A01, $1.4 \pm 0.8 \mu\text{A}/\text{cm}^2$; benzbromarone, $3.0 \pm 1.3 \mu\text{A}/\text{cm}^2$; niflumic acid, $4.4 \pm 1.5 \mu\text{A}/\text{cm}^2$, $n=4$, $p < 0.01$ vs. no inhibitor) (Fig. 6A). However, isoproterenol (10^{-6} M)-induced increases in Isc in the IL-13-treated cells were not significantly inhibited by these inhibitors (Δ Isc: no inhibitor, $14.2 \pm 4.7 \mu\text{A}/\text{cm}^2$; T16Ainh-A01,

4.8 ± 3.2 $\mu\text{A}/\text{cm}^2$; benzbromarone, 5.0 ± 4.0 $\mu\text{A}/\text{cm}^2$; niflumic acid, 12.3 ± 7.5 $\mu\text{A}/\text{cm}^2$, Fig. 6B).

Immunofluorostaining of TMEM16A in cultured cells

TMEM16A immunofluorostaining was more strongly positive in the IL-13-treated airway epithelial cells, but not in the control cells without IL-13 treatment (Fig. 7a, d, e). The

IL-13-treated cells showed MUC5AC-positive goblet cell metaplasia that was strongly related to

TMEM16A (Fig. 7a, b, d, Fig. 8). TMEM16A localized at the apical portion and in the

MUC5AC-positive mucus area in the goblet cells (Fig. 7f, Fig. 8). In contrast, α -tubulin-positive

ciliated cells in the IL-13-treated cells were negative for TMEM16A immunofluorostaining (Fig.

7c, d). Similarly, the control cells without IL-13 treatment had only α -tubulin-positive ciliated

cells that were negative for TMEM16A immunofluorostaining (Fig. 7e).

Discussion

In this study, we demonstrated that OVA-challenge induced Ca-dependent Cl secretion along with overexpression of TMEM16A in the apical portion of airway epithelium in an OVA-sensitized asthma model. In addition, we demonstrated that the Ca-dependent agonist, UTP also increased Cl ion transport via TMEM16A in IL-13-induced goblet cell metaplasia cultured at the air-liquid interface.

Various inflammatory mediators associated with asthma, including histamine [17] and leukotrienes [18], released from mast cells in sensitized animals have been shown to stimulate Cl secretion from the submucosa towards the airway lumen[19]. Most of these mediators are known to elevate intracellular Ca^{2+} [20]. We demonstrated that a newly synthesized TMEM16A inhibitor, T16Ainh-A01[11], strongly inhibited OVA-induced Cl ion transport, whereas a non-specific CFTR inhibitor, DPC[12] [13], had little effect. This suggests that OVA challenge induced mainly Ca-dependent Cl ion transport via TMEM16A, but not cAMP-dependent Cl ion transport through CFTR. Furthermore, TMEM16A was overexpressed especially in the apical portion of the sensitized epithelium with goblet cell hyperplasia. This suggests that the airway epithelial Cl secretion induced by antigen challenge was caused not only by mediator release but also by

TMEM16A overexpression at the apical membrane in sensitized animals. Sensitization and IL-13 induce CLCA1 expression in the airway epithelium [21, 22]. CLCA1 was originally annotated as a CaCC because cells display increased Ca-dependent Cl current when the protein is expressed. However, bioinformatics analysis of CLCA1 indicates that CLCA1 is not a Cl channel, but a secreted protein[23]. Therefore, the relationship between CLCA1 and CaCC remains unclear. Recently, it has been reported that secreted and cleaved N-terminal CLCA1 can stabilize TMEM16A at the apical membrane[8]. If so, TMEM16A at the apical membrane may be ready to stimulate Cl ion transport through cooperation with CLCA1 in sensitized animals. Our data supports this hypothesis because TMEM16A was overexpressed in the apical portion especially in sensitized animals.

In accordance with activation of TMEM16A-dependent Cl ion transport, mucus secretion by goblet cell degranulation was also observed. We previously reported that OVA-challenge induced mucus secretion was inhibited by another CaCC inhibitor, niflumic acid[9]. Here, we demonstrated that T16Ainh-A01 also inhibited antigen-induced mucus secretion and confirmed the involvement of TMEM16A in OVA-challenge-induced mucus secretion.

TMEM16A was also strongly immunostained in the area of goblet cell metaplasia in the sensitized epithelium. This suggests that the overexpression of TMEM16A is associated with goblet cell metaplasia. Because IL-13 is a potent inducer of goblet cell metaplasia[15], we then examined the expression of TMEM16A and Ca-dependent agonist, UTP-induced Cl ion transport in IL-13-induced goblet cell metaplasia cultured at an air-liquid interface. As a result, we demonstrated that IL-13 augmented UTP-induced Cl ion transport that was strongly inhibited by three different TMEM16A inhibitors, T16Ainh-A01, benzbromarone, and niflumic acid (Fig.5, Fig.6)[10]. In contrast, T16Ainh-A01 had a little effect of UTP-induced increase in Isc in the non-treated, control cells (Fig.5). In accordance with the ion transport study, TMEM immunofluorostaining was strongly positive in the IL-13-treated cells but not in the control cells as shown in Fig.7. Our previous study showed that IL-13 augments UTP-induced Cl ion transport that is strongly inhibited by TMEM16A inhibitors, niflumic acid and DIDS in a human bronchial epithelial cell line, 16HBE [24]. Recently it has been reported that IL-13-treated 16HBE shows TMEM16A overexpression and Ca-induced current that is inhibited by T16Ainh-A01[25]. Furthermore, it has been reported that IL-4 augments UTP-induced Cl ion transport that is inhibited by T16Ainh-A01 in primary human bronchial epithelial cells [26]. Taken together,

although TMEM16A is a minor contributor to total CaCC in the resting state[11], TMEM16A plays an important role in Ca^{2+} -dependent Cl ion transport in the airway epithelial cells stimulated with Th2 cytokines such as IL-13 and IL-4.

TMEM16A immunofluorostaining was strongly positive at the apical portion and in the mucous area in MUC5AC-positive goblet cells but not α -tubulin-positive ciliated cells in culture. This suggests that TMEM16A is associated with IL-13-induced goblet cells but not with ciliated cells, and supports a recent report that showed that the expression of TMEM16A and MUC5AC is increased in the IL-13-induced nasal epithelial cells from chronic rhinosinusitis patients[27]. However, whether TMEM16A is necessary to cause IL-13-induced goblet cell metaplasia has not been fully elucidated. IL-4 is reported to upregulate TMEM16A expression as an early event of goblet cell differentiation in human cultured bronchial epithelial cells[24]. Moreover, a previous study demonstrates that one TMEM16A inhibitor, niflumic acid, inhibited IL-13-induced goblet cell metaplasia in mice[28]. These reports suggest that TMEM16A is associated with the induction of IL-13-induced goblet cell metaplasia. However, further studies including signaling pathway are needed.

TMEM16A immunostaining was also found in basal cells in the present study. As basal cells have the potency of proliferation, TMEM16A may be involved in cell proliferation. TMEM16A is also referred to anoctamin-1 (ANO-1), which is sometimes overexpressed in cancer cells, including lung cancer[29]. Therefore, goblet cell hyperplasia following overexpression of TMEM16A suggests the role of TMEM16A in epithelial proliferation in asthma.

T16Ainh-A01 inhibited antigen-induced mucus secretion as shown in Fig. 3. These data suggest that TMEM16A expression around mucous granules as well as the apical membrane may be associated with the mucus secretion. Upregulation of Cl ion transport via TMEM16A may be adequate to dilute overloaded glycoconjugate in the airway surface fluid, thereby possibly preventing airway plugging with viscid mucus. However, excessive water increases the depth of periciliary layer, which may result in the ineffectiveness of ciliary motion to transport airway mucus toward the pharynx. Based on the present study, it is suggested that TMEM16A may be a key regulator for hypersecretory diseases such as asthma because TMEM16A is involved in goblet cell metaplasia, mucin secretion and Cl ion transport. Therefore, TMEM16A inhibition may be an attractive candidate for asthma therapeutics.

In conclusion, TMEM16A is associated with Ca-activated Cl ion transport via overexpression of TMEM16A in goblet cell metaplasia in a guinea pig asthma model.

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All authors have no conflict of interest.

Reference

1. Nakanishi A, Morita S, Iwashita H, Sagiya Y, Ashida Y, Shirafuji H, Fujisawa Y, Nishimura O, Fujino M. Role of gob-5 in mucus overproduction and airway hyperresponsiveness in asthma. *Proc Natl Acad Sci U S A* 2001;98: 5175-80.

- Accepted Article
2. Hoshino M, Morita S, Iwashita H, Sagiya Y, Nagi T, Nakanishi A, Ashida Y, Nishimura O, Fujisawa Y, Fujino M. Increased expression of the human Ca^{2+} -activated Cl^- channel 1 (CaCC1) gene in the asthmatic airway. *Am J Respir Crit Care Med* 2002;165: 1132-6.
 3. Yurtsever Z, Sala-Rabanal M, Randolph DT, Scheaffer SM, Roswit WT, Alevy YG, Patel AC, Heier RF, Romero AG, Nichols CG, Holtzman MJ, Brett TJ. Self-cleavage of human CLCA1 protein by a novel internal metalloprotease domain controls calcium-activated chloride channel activation. *J Biol Chem* 2012;287: 42138-49.
 4. Caputo A, Caci E, Ferrera L, Pedemonte N, Barsanti C, Sondo E, Pfeiffer U, Ravazzolo R, Zegarra-Moran O, Galiotta LJ. TMEM16A, a membrane protein associated with calcium-dependent chloride channel activity. *Science* 2008;322: 590-4.
 5. Galiotta LJ. The TMEM16 protein family: a new class of chloride channels? *Biophys J* 2009;97: 3047-53.
 6. Huang F, Zhang H, Wu M, Yang H, Kudo M, Peters CJ, Woodruff PG, Solberg OD, Donne ML, Huang X, Sheppard D, Fahy JV, Wolters PJ, Hogan BL, Finkbeiner WE, Li M, Jan YN, Jan LY, Rock JR. Calcium-activated chloride channel TMEM16A modulates

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mucin secretion and airway smooth muscle contraction. *Proc Natl Acad Sci U S A* 2012;109: 16354-9.

7. Zhang CH, Li Y, Zhao W, Lifshitz LM, Li H, Harfe BD, Zhu MS, ZhuGe R. The transmembrane protein 16A Ca^{2+} -activated Cl^- channel in airway smooth muscle contributes to airway hyperresponsiveness. *Am J Respir Crit Care Med* 2013;187: 374-81.
8. Sala-Rabanal M, Yurtsever Z, Nichols CG, Brett TJ. Secreted CLCA1 modulates TMEM16A to activate Ca^{2+} -dependent chloride currents in human cells. *Elife* 2015;4.
9. Kondo M, Nakata J, Arai N, Izumo T, Tagaya E, Takeyama K, Tamaoki J, Nagai A. Niflumic acid inhibits goblet cell degranulation in a guinea pig asthma model. *Allergol Int* 2012;61: 133-42.
10. Pedemonte N, Galletta LJ. Structure and function of TMEM16 proteins (anoctamins). *Physiol Rev* 2014;94: 419-59.

- Accepted Article
11. Namkung W, Phuan PW, Verkman AS. TMEM16A inhibitors reveal TMEM16A as a minor component of calcium-activated chloride channel conductance in airway and intestinal epithelial cells. *J Biol Chem* 2011;286: 2365-74.
 12. Shen BQ, Mistry RJ, Finkbeiner WE, Widdicombe JH. Role of CFTR in chloride secretion across human tracheal epithelium. *Am J Physiol* 1995;269: L561-6.
 13. Verkman AS, Galiotta LJ. Chloride channels as drug targets. *Nat Rev Drug Discov* 2009;8: 153-71.
 14. Gallos G, Remy KE, Danielsson J, Funayama H, Fu XW, Chang HY, Yim P, Xu D, Emala CW, Sr. Functional expression of the TMEM16 family of calcium-activated chloride channels in airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2013;305: L625-34.
 15. Kondo M, Tamaoki J, Takeyama K, Nakata J, Nagai A. Interleukin-13 induces goblet cell differentiation in primary cell culture from guinea pig tracheal epithelium. *Am J Respir Cell Mol Biol* 2002;27: 536-41.

- Accepted Article
16. Kanoh S, Tanabe T, Rubin BK. IL-13-induced MUC5AC production and goblet cell differentiation is steroid resistant in human airway cells. *Clin Exp Allergy* 2011;41: 1747-56.
 17. Marin MG, Davis B, Nadel JA, Effect of histamine on electrical and ion transport properties of tracheal epithelium. *J Appl Physiol Respir Environ Exerc Physiol* 1977;42: 735-8.
 18. Widdicombe JH, Coleman DL, Finkbeiner WE, Friend DS. Primary cultures of the dog's tracheal epithelium: fine structure, fluid, and electrolyte transport. *Cell Tissue Res* 1987;247: 95-103.
 19. Sestini P, Bienenstock J, Crowe SE, Marshall JS, Stead RH, Kakuta Y, Perdue MH. Ion transport in rat tracheal epithelium in vitro. Role of capsaicin-sensitive nerves in allergic reactions. *Am Rev Respir Dis* 1990;141: 393-7.
 20. Noah TL, Paradiso AM, Madden MC, McKinnon KP, Devlin RB. The response of a human bronchial epithelial cell line to histamine: intracellular calcium changes and

extracellular release of inflammatory mediators. *Am J Respir Cell Mol Biol* 1991;5: 484-92.

21. Yasuo M, Fujimoto K, Tanabe T, Yaegashi H, Tsushima K, Takasuna K, Koike T, Yamaya M, Nikaido T. Relationship between calcium-activated chloride channel 1 and MUC5AC in goblet cell hyperplasia induced by interleukin-13 in human bronchial epithelial cells. *Respiration* 2006;73: 347-59.
22. Kuperman DA, Lewis CC, Woodruff PG, Rodriguez MW, Yang YH, Dolganov GM, Fahy JV, Erle DJ. Dissecting asthma using focused transgenic modeling and functional genomics. *J Allergy Clin Immunol* 2005;116: 305-11.
23. Gibson A, Lewis AP, Affleck K, Aitken AJ, Meldrum E, Thompson N. hCLCA1 and mCLCA3 are secreted non-integral membrane proteins and therefore are not ion channels. *J Biol Chem* 2005;280: 27205-12.
24. Endo Y, Isono K, Kondo M, Tamaoki J, Nagai A. Interleukin-9 and Interleukin-13 augment UTP-induced Cl ion transport via hCLCA1 expression in a human bronchial epithelial cell line. *Clin Exp Allergy* 2007;37: 219-24.

- Accepted Article
25. Qin Y, Jiang Y, Sheikh AS, Shen S, Liu J, Jiang D. Interleukin-13 stimulates MUC5AC expression via a STAT6-TMEM16A-ERK1/2 pathway in human airway epithelial cells. *Int Immunopharmacol.* 2016;40: 106-114.
26. Scudieri P, Caci E, Bruno S, Ferrera L, Schiavon M, Sondo E, Tomati V, Gianotti A, Zegarra-Moran O, Pedemonte N, Rea F, Ravazzolo R, Galletta LJ. Association of TMEM16A chloride channel overexpression with airway goblet cell metaplasia. *J Physiol* 2012;590: 6141-55.
27. Zhang Y, Wang X, Wang H, Jiao J, Li Y, Fan E, Zhang L, Bachert C. TMEM16A-mediated mucin secretion in IL-13-induced nasal epithelial cells from chronic rhinosinusitis patients. *Allergy Asthma Immunol Res* 2015;7: 367-75.
28. Nakano T, Inoue H, Fukuyama S, Matsumoto K, Matsumura M, Tsuda M, Matsumoto T, Aizawa H, Nakanishi Y. Niflumic acid suppresses interleukin-13-induced asthma phenotypes. *Am J Respir Crit Care Med* 2006;173: 1216-21.

29. Jia L, Liu W, Guan L, Lu M, Wang K. Inhibition of calcium-activated chloride channel ANO1/TMEM16A suppresses tumor growth and invasion in human lung cancer. PLoS One 2015;10: e0136584.

Figure legends

Fig. 1 The effect of OVA challenge on short circuit current (Isc).

A) Recordings of Isc induced by OVA challenge in control (non-sensitized) and sensitized tracheas. All groups were pretreated with amiloride (10^{-4} M).

B) Comparison of Δ Isc induced by OVA challenge between control and sensitized tracheas. Data are presented as the mean $\pm$ SEM and were analyzed by unpaired t-test. n=7-9 for different individuals, ** p<0.01.

Fig. 2 The effects of Cl channel inhibitors on the increase in Isc induced by OVA challenge in the sensitized tracheas.

A) Recordings of Isc induced by OVA challenge in the presence or absence of T16Ainh-A01 in the sensitized tracheas.

B) The effects of T16Ainh-A01 and DPC on the increase in Isc after OVA challenge in the sensitized tracheas. The concentrations of inhibitors were 10^{-5} M for T16Ainh-A01, and 10^{-4} M for DPC. T16Ainh-A01 but not DPC inhibited OVA-challenge induced increase in Isc. n=4-5 for different individuals. Data are presented as the mean $\pm$ SEM and were analyzed by ANOVA with Tukey's post-test as appropriate. ** p<0.01 vs. no inhibitor.

Fig. 3 The effects of OVA challenge on mucus secretion in control and sensitized epithelium.

A) Light microscopic photographs of tracheal epithelium stained with PAS/AB.

a) Control (non-sensitized) tracheal epithelium at baseline (before OVA challenge), showing that there are several goblet cells.

b) Control tracheal epithelium after OVA challenge, showing that the mucus remains in goblet cells.

c) Sensitized tracheal epithelium at baseline, showing marked goblet cell hyperplasia compared with control tracheal epithelium at baseline.

d) Sensitized tracheal epithelium after OVA challenge, showing mucus secretion from goblet cells.

e) Sensitized tracheal epithelium after OVA challenge in the presence of T16Ainh-A01 (10^{-5} M), showing that T16Ainh-A01 inhibits OVA-challenge induced mucus secretion.

Scale bar: 20 μ m.

B) Percent of mucus areas at baseline and after OVA challenge in control and sensitized tracheal epithelia. OVA challenge induced mucus secretion in sensitized epithelium but not in control epithelium. T16Ainh-A01 inhibited the OVA-challenge induced mucus secretion in the sensitized epithelium. The mucus area stained with PAS/AB was calculated as a percentage of the total area of tracheal epithelium using a semiautomatic image

analysis system. n=4-5 for different individuals. Data are shown as the mean $\pm$ SEM and were analyzed by ANOVA with Tukey's post-test as appropriate. **p<0.01, control epithelium at baseline vs. sensitized epithelium at baseline. ††p<0.01, sensitized epithelium at baseline vs. sensitized epithelium after OVA challenge. #p<0.05, sensitized epithelium after OVA challenge vs. sensitized epithelium after OVA challenge in the presence of T16Ainh-A01.

Fig. 4 The expression of TMEM16A protein in control and sensitized epithelium.

A) Immunofluorescence photographs stained with anti-TMEM16A antibody.

a) Control tracheal epithelium at baseline, b) Sensitized tracheal epithelium at baseline. Red:

TMEM16A, Blue: DAPI, Scale bar: 50 μ m.

TMEM16A was more positive in OVA-sensitized goblet cell hyperplasia, especially at the apical membrane compared to control epithelium. Basal cells were also stained with anti-TMEM16A antibody.

B) Percent area of TMEM16A immunofluorostaining in the tracheal epithelium at baseline.

TMEM16A-immunofluorostained area was calculated as a percentage of the total area of tracheal epithelium using a semiautomatic image analysis system. n=4 for different individuals. Data are shown as the mean $\pm$ SEM and were analyzed with an unpaired t-test. *p<0.05, control epithelium vs. sensitized epithelium.

Fig. 5 Inhibition of UTP-induced increase in short-circuit current (Isc) by T16Ainh-A01.

Representative recording of traces showing the effect of T16Ainh-A01 on UTP-induced increases in Isc. T16Ainh-A01 (10^{-5} M) strongly inhibited the effect of UTP-induced increase in Isc in the IL-13-treated cells, whereas it had a little effect in the control cells without IL-13 treatment. All experiments were performed in the presence of amiloride (10^{-4} M).

Fig.6 The effects of TMEM16A inhibitors on agonist-induced increases in Isc in IL-13-treated tracheal epithelial cells cultured at the air-liquid interface.

A) UTP (10^{-5} M)-induced increase in Isc. B) Isoproterenol (ISO, 10^{-6} M)-induced increase in Isc.

The concentration of all inhibitors was 10^{-5} M. None: no inhibitor, T16Ainh: T16Ainh-A01, Benz: benzbromarone, NFA: niflumic acid. n=4. Data are shown as the mean $\pm$ SEM and were analyzed by ANOVA with Tukey's post-test as appropriate. ** $p < 0.01$, * $p < 0.05$ vs. None (no inhibitor).

Fig. 7 Immunofluorescence micrographs of tracheal epithelial cells cultured at the air-liquid interface by confocal microscopy. a-d) IL-13 treated cells, xy image, e) control cells without IL-13 treatment, xy image, f) IL-13 treated cells, z image. TMEM16A staining is strongly observed at the apical portion of epithelial cell sheet and in the mucus area in the MUC5AC-positive goblet cells. In contrast, α -tubulin-positive ciliated cells were negative for TMEM16A immunofluorostaining.

Red: TMEM16A. Green: MUC5AC. Blue: α -tubulin. Yellow shows TMEM16A plus MUC5AC.

Scale bar: 20 μ m.

Fig. 8 Immunofluorescence micrographs of goblet cells cultured at the air-liquid interface at high magnification. TMEM16A localizes at the cell membrane (arrow) as well as MUC5AC-positive area. Green: MUC5AC. Red: TMEM16A. Blue: DAPI. Scale bar: 10 μm .

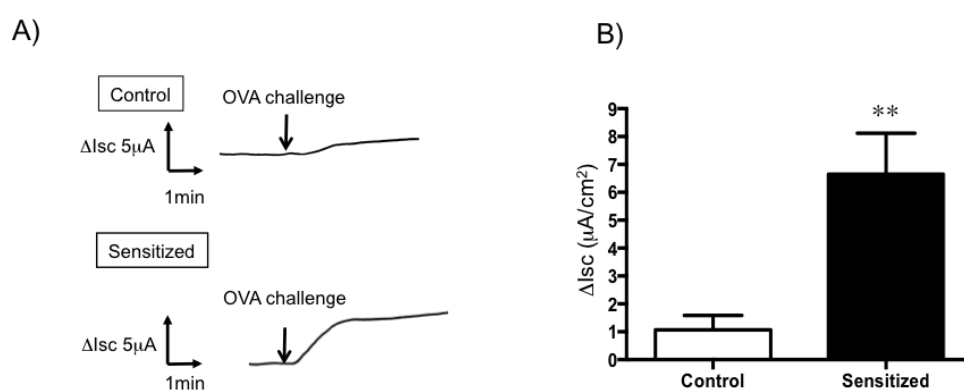


Fig. 1

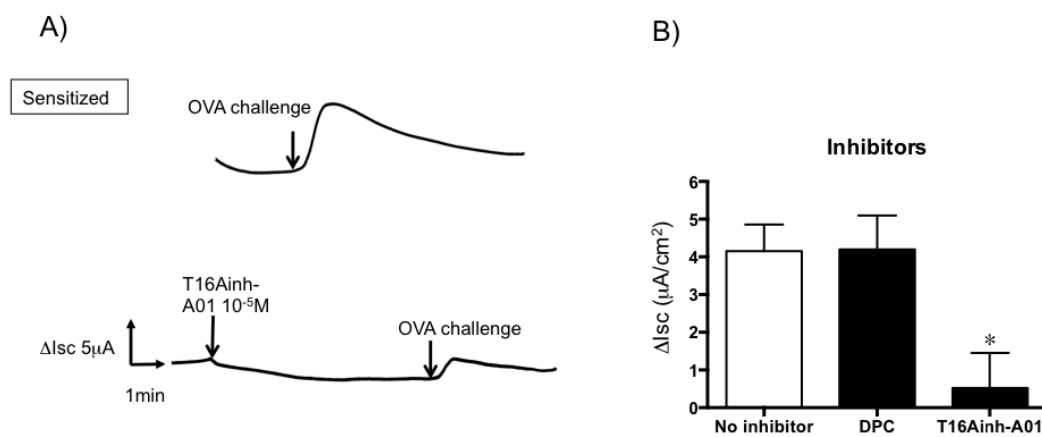


Fig. 2

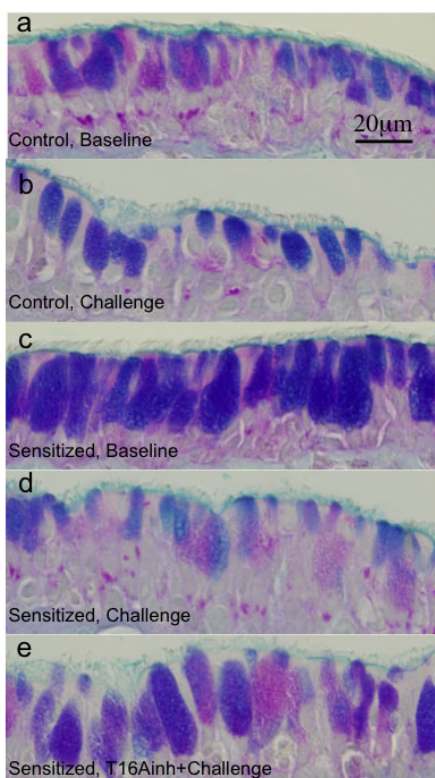


Fig. 3A

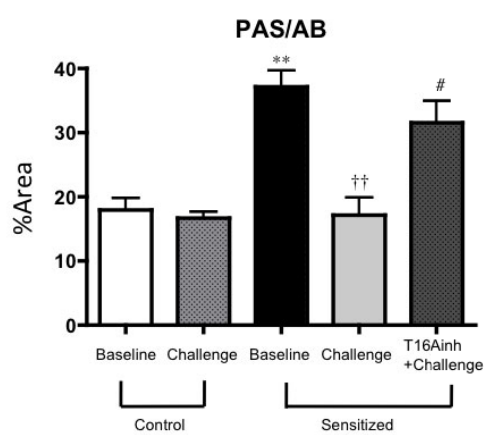


Fig. 3B

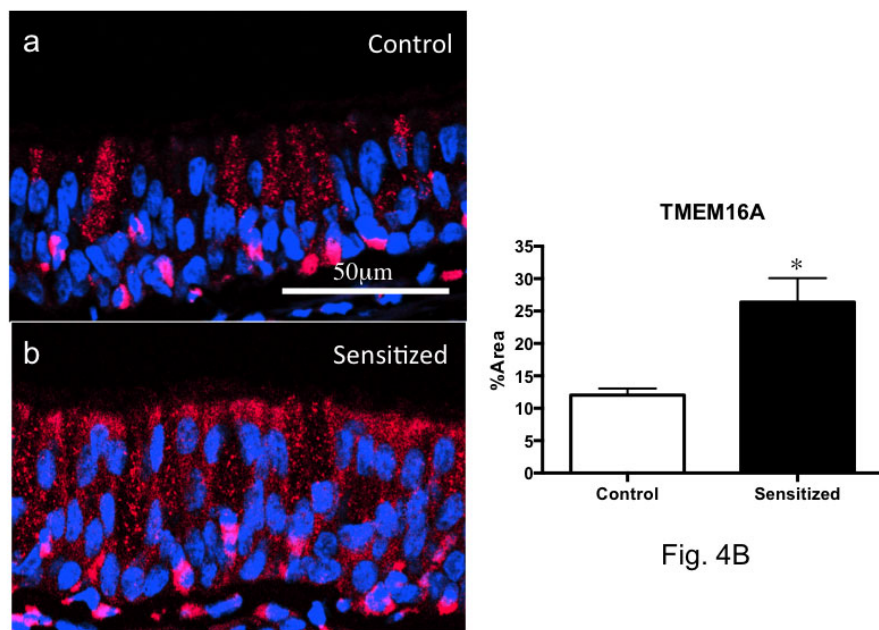


Fig. 4A

Fig. 4B

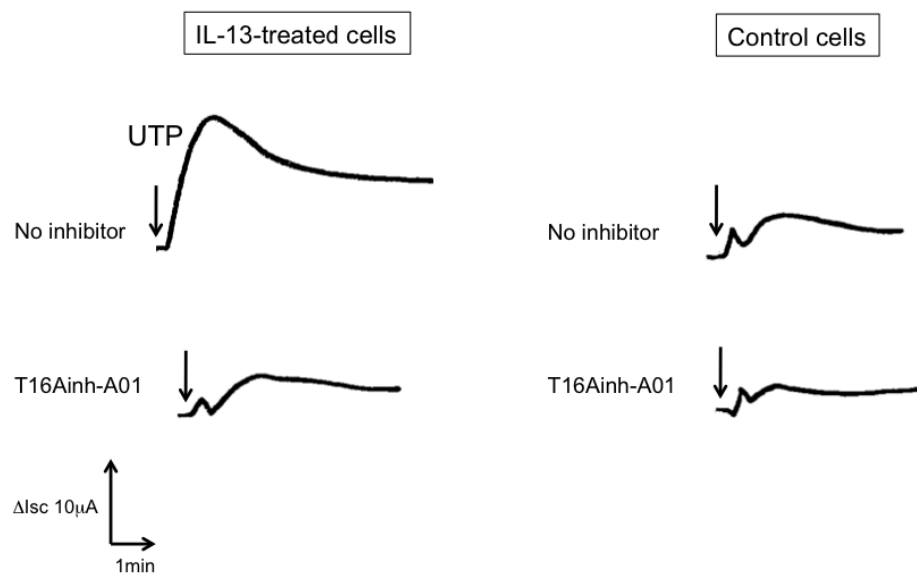


Fig. 5

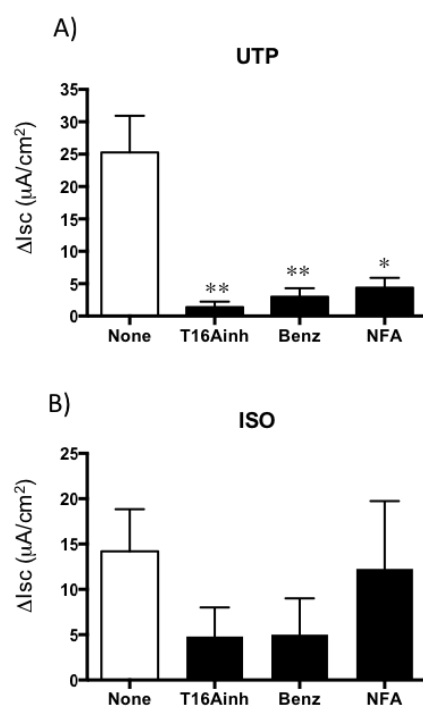


Fig. 6

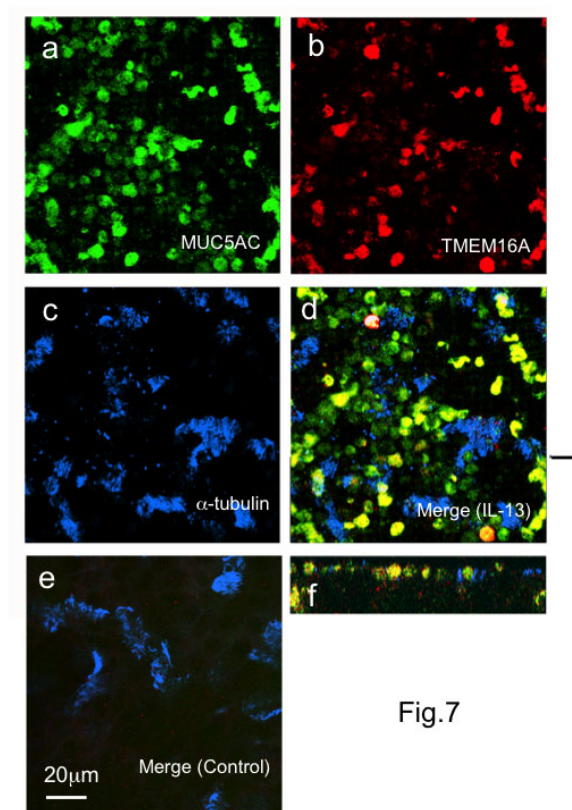


Fig.7

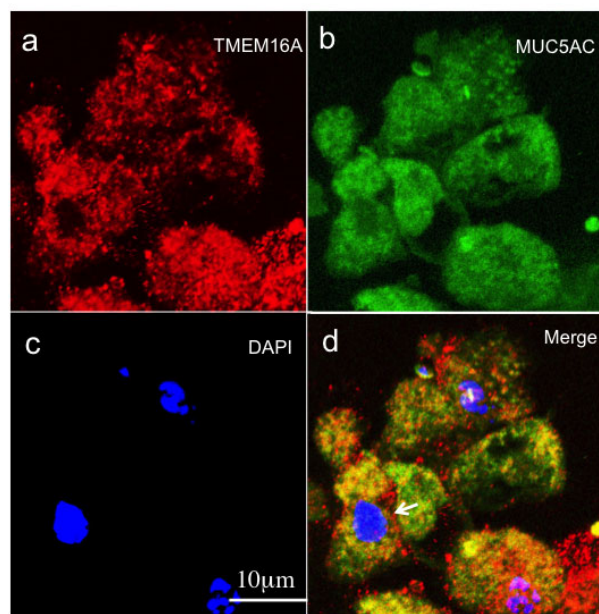


Fig. 8