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The role of Dclk1-positive tuft cells in the stomach of germ free mouse

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The role of Dclk1-positive tuft cells in the stomach of germ free mouse

Directed by Professor Ki Taek Nam

The Master's Thesis
submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Master of Medical Science

Nakyum Lee

June 2021

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This certifies that the Master's Thesis
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ABSTRACT

The role of Dclk1-positive tuft cells in the stomach of germ free mouse

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The Graduate School, Yonsei University
(Directed by Professor Ki Taek Nam)

Doublecortin-like kinase1(Dclk1) is a microtubule-associated protein kinase and has been identified as a marker of tuft cells in GI tract. The Dclk1 gene has multiple splicing isoforms; Dclk1-L(full length), CPG16 (Kinase domain only), DCL domain(DCX domain only), PEST(a linker proline/serine rich domain). Tuft cells are minor cell type found throughout gastrointestinal epithelia. The morphology of tuft cells is characterized by apical and blunt microvilli, detected by acetylated-α-tubulin. These cells act as luminal detectors, connecting the intestinal microbiome to the host immune system. Previous studies have reported the Dclk1 as a tumor stem cell marker in various tissues and as luminal sentinels to promote epithelial repair after acute injury. However, among GI tract, there are relatively many studies about these microorganisms related to the tuft cells in the small intestine and large intestine but not the stomach. Here, I studied the Dclk1 positive tuft cells expression utilized by germ free mouse and antibiotics-treated SPF mouse. In pseudo-sterile environment, the number of Dclk1 positive tuft cells increased, and the level of expression varies depending on the type of isoform.

Key words: tuft cell, microbiome, Dclk1, stomach, germ free mouse

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The role of Dclk1-positive tuft cells in the stomach of germ free mouse

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I. INTRODUCTION

The gastrointestinal tract, (GI tract, digestive tract) is the solitary chemosensory system that comprised of single epithelial cells which encounter commensal and pathogenic microbes. Bacteria in GI tract, also called gut flora or microbiome. The intestinal microbiota are necessary for maintenance of physiological homeostasis and also as a modulator of disease processes[1]. The sensory cells in the GI tract react against microbiome then releasing a variety of hormones to mediate physiological response. Microbiome exists in stomach as well as intestine but there are no previous reports about functional differences of sensory cells in stomach depending on the presence or absence of microbiome. Several types of sensory cell present in the GI tract including stomach, and I focus on tuft cells among them. Tuft(also called brush or caveolated)cells are unusual epithelial cells that have been identified in numerous epithelial tissue, in rodents, including salivary glands[2], stomach[3], small intestine[4], colon[5], cecum[6], gall bladder[7] and bile duct[8], pancreatic duct[9], et al. These cells exhibit characteristic long and apical microfilaments and by a well-developed tubulovesicular system in the supranuclear cytoplasm[10]. Decades of research have revealed little about the function of this mysterious cell type, until recently. Broadly, tuft cells are

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characterized by chemosensory cells that monitor their surroundings and transmit the environmental signals. Also, they express several chemoreceptor molecues, such as TRPM5[11] and α-gustducin[12], which suggests that tuft cells are innervated by adjacent neurons. A number of intestinal tuft cell markers have previously been reported including structural(Dclk1, acetylated tubulin), chemosensing (TRPM5, α-gustducin), unknown(Hopx, p-EGFR(Y1068)) markers[13-15].

Previous studies have demonstrated that Dclk1-expressing tuft cells have a morphologically characteristic structure in apical microvilli and acetylated microtubules called microvillar sensory cells (MVSCs), which was not seen in normal gastric mucosa but increased in induced oxyntic atrophy[16]. In the small intestine, tuft cells have emerged as critical sentinels that initiate type2 immunity responses to intestinal parasites and protozoa[17]. In several damage models, tuft cells increase and are involved in immunity activity. It can hypothesize that tuft cells involved in epithelial effector function and implicated in epithelial regeneration, DNA damage repair[9,14,18,19], and tumorigenesis[20-22].

The mouse Dclk1 gene consists of 20 exons that result in several different isoforms through alternative splicing[23]. Dclk1 contains two doublecortin(DCX) domains in the N-terminus, $Ca2^{+}$/calmodulin dependent kinase domain in the C-terminus, and PEST(serine/ proline-rich domain) between DCL and CPG16 domain. Through alternative splicing, four main types of transcript splice forms are generated including Dclk1-L that contains all the domains, CPG16 contains only kinase domain that is a protein serine/threonine kinase (also called Dclk1-S), DCL with only the DCX domain, and PEST[24,25], but I focus three major isoform groups except PEST. Also, acetylated tubulin, required to form microtubule bundles which bound to DCX, enriched at the apical tuft region[26,27]. In the developing mammalian brain, DCLK1 is also highly expressed during neurogenesis, particularly in the neocortex(SVZ/VZ) and cerebellum. DCLK1-L and CPG16 have been reported with important differences between the isoforms in both human and mouse tissues. In the human neoplasia, hypermethylation of

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DCLK1-L causes a predominant switch to the short isoform, conferring a more invasive tumor phenotype[28]. Thus the DCLK1 isoforms likely have distinct functions, which are orchestrated in part by β-catenin and NF-κB signaling pathways[29].

In this study, I aimed to characterize the expression patterns of the three major isoform types of Dclk1-L, CPG16, DCL in SPF and Germ free mouse stomach by regions using multiple platforms.

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II. MATERIALS AND METHODS

1. Mice

1) Wild type mouse

Wild type mice are bred in cages under specific pathogen-free conditions. Mice were maintained on the C57BL/6 background.

2) Germ free mouse

Germ free mice are bred in isolators that completely block exposure to microorganisms, viruses, eukaryotic microbes, and the viruses. Germ free mice allow for study of the complete absence of microbes or for the generation of gnotobiotic animals exclusively colonized by known microbes. Germ free mice must be monitored frequently for contamination using the microscopy, culture for detection germ.

2. Immunohistochemistry(IHC)

For immunostaining, samples were deparaffinized and rehydrated using the ethanol. Antigen retrieval (DAKO, S1699) was performed using a pressure cooker. After cooling on ice for at least 1hr, sections were incubated in 3% H2O2 for 30 min for blocking endogenous peroxidase. Sections were washed in PBS and incubated with protein block serum-free (DAKO, X0909) for 1–2hr. at room temperature to reduce non-specific signals. Treatment with M.O.M (Vector Laboratories, BMK-2202) reagent for 1hr was performed with mouse primary antibodies. Primary antibodies were incubated overnight at 4 ℃. After washing in PBS, sections were incubated in HRP-conjugated secondary antibody for 15 min at room temperature. For immunohistochemistry, DAB (DAKO, K3468) was used for the development of antibodies, and Mayer's hematoxylin (DAKO, S3309) was used for counterstaining. After the dehydration using the ethanol,

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sections were mounted. For immunofluorescence, Alexa-488 conjugated anti-rabbit and Cy3 conjugated anti-mouse was used to secondary antibodies. These secondary antibodies incubated for 1hour at room temperature on sections. Nuclei were stained with DAPI (Vector laboratories, Inc., Burlingame, CA, USA) for 10 min.

3. Flow cytometry

The isolated stomach cell pellet from wild type and germ free mouse using solution A and B filtered through 40μm cell strainer to remove debris and the filtered suspension was centrifuged 10 min at 1000 rpm. The isolated cells were suspended in Fcs (blocking step) for 15 min. To isolate the tuft cell markers population(+) live epithelial cells, isolated cells were incubated with anti-EPCAM antibodies for 15 min and using the antibody conjugation kit for possible to stain several antibodies.

4. Antibodies

The sources of antibodies were commercially purchased; anti-DCAMKL1 (ab109029, Abcam, Cambridge, UK), anti-Doublecortin (DCX) (sc-271390, Santa cruz Biotechnology, Dallas, TX, USA), anti-acetylated tubulin (T7451, Sigma-Aldrich, St. Louis, MO, USA), anti-EPCAM (118215, Biolegend, San Diego, CA, USA), and anti-GAPDH(ab181602, Abcam, Cambridge, UK)

5. Statistical analysis

Statistical analysis data are presented as mean ± SEM and performed using GraphPad Prism software v 9.0. Statistical significance was determined using unpaired Student's t test or One-way ANOVA. $P < 0.05$ was considered

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significant. *P < 0.05, **P < 0.01, ***P < 0.001.

6. Western blotting

Mice were euthanized in a CO_2 chamber. Stomach was cut out and washed with PBS. Fundus and antrum, which are distinct regions within the stomach, were punctured and chopped into smaller pieces with sterile scissor. Tissues were lysed in T-PER tissue protein extraction reagent with protease and phosphatase inhibitor cocktails.(Thermo, Waltham, MA, USA) Lysates were mixed by vortexing 3 times every 5 min and collected by centrifugation. After measuring the concentration of proteins with BCA protein assay(Pierce Biotechnology, Rockford, IL, USA), lysates were boiled in 2x sample buffer at 95℃ 5 min.Then, 30 μg of protein sample was separated by SDS-PAGE and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). After blocking with 2% skim milk for 30min, the membranes were incubated overnight at 4℃ with primary antibodies in shaker. Then, HRP-conjugated secondary antibody was incubated at room temperature for 1hr in shaker. The proteins were detected using ECL reagents(Thermo, Waltham, MA, USA).

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III. RESULT

1. Dclk1 has multiple transcript isoforms

Dclk1 gene contains two doublecortin(DCX) domains, serine/proline-rich domain, and kinase domain. It has four mulitiple transcript variants by alternative splicing; Dclk1-L (full length), CPG16 (kinase domain only), DCL domain (DCX domain only), PEST (a linker pro/ser rich domain). .(Fig1.)

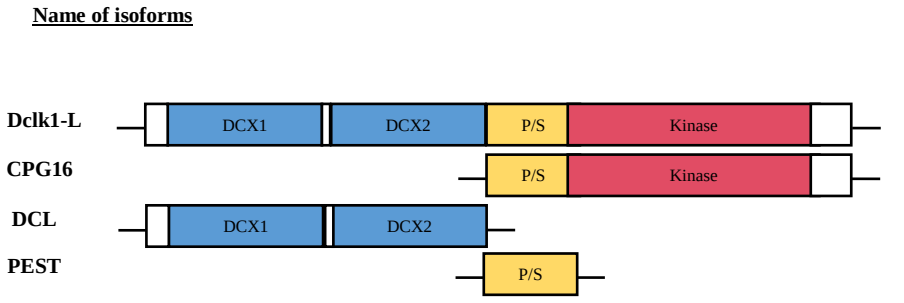

Figure 1. Alternative transcript isoform types of the Dclk1. Dclk1-L contain two doublecortin (DCX) domains, serine/proline domain, and kinase domain. CPG16 has only kinase domain and DCL has only doublecortin domain. PEST sequence has only serine/proline domain.

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2. The expression of Dclk1 isoforms increased in Germ free condition

In order to characterize the expression of Dclk1 and its isoforms in association with germ, immunohistochemical analysis was conducted using SPF and Germ free mouse stomach and compared the expression of Dclk1 in two parts of the stomach; fundus and antrum(Fig2.A.). Interestingly, the number of Dclk1-expressing cells was higher in the condition without germ, especially fundus region.(Fig2.B.). Used Dclk1 antibody detect kinase domain and it means Dclk1-expressing cells containing Dclk1-L and CPG16.

The number of Dcx-expressing cells was also higher in Germ free and, unlike Dclk1-expressing cells, increased in antrum region.(Fig3.A,B) Used Dcx antibody detect the DCL domain and it indicates Dcx-expressing cells containing Dclk1-L and DCL.

To investigate expressional variations of Dclk1 in translational level, immunofluorescence was performed using Dclk1 and Dcx antibody. If two markers double positive means Dclk1-L, Dclk1 only positive represent CPG16, and Dcx only positive corresponds to DCL.(Fig4.A.) Comparing the each population, except for the DCL domain, there was no significant difference in the expression of remaining domains and Dclk1-L is increased than other isoforms. These data confirm Dclk1-L is mostly expressed isotypes and DCL rarely observe in fundus region of Germ free mouse stomach.

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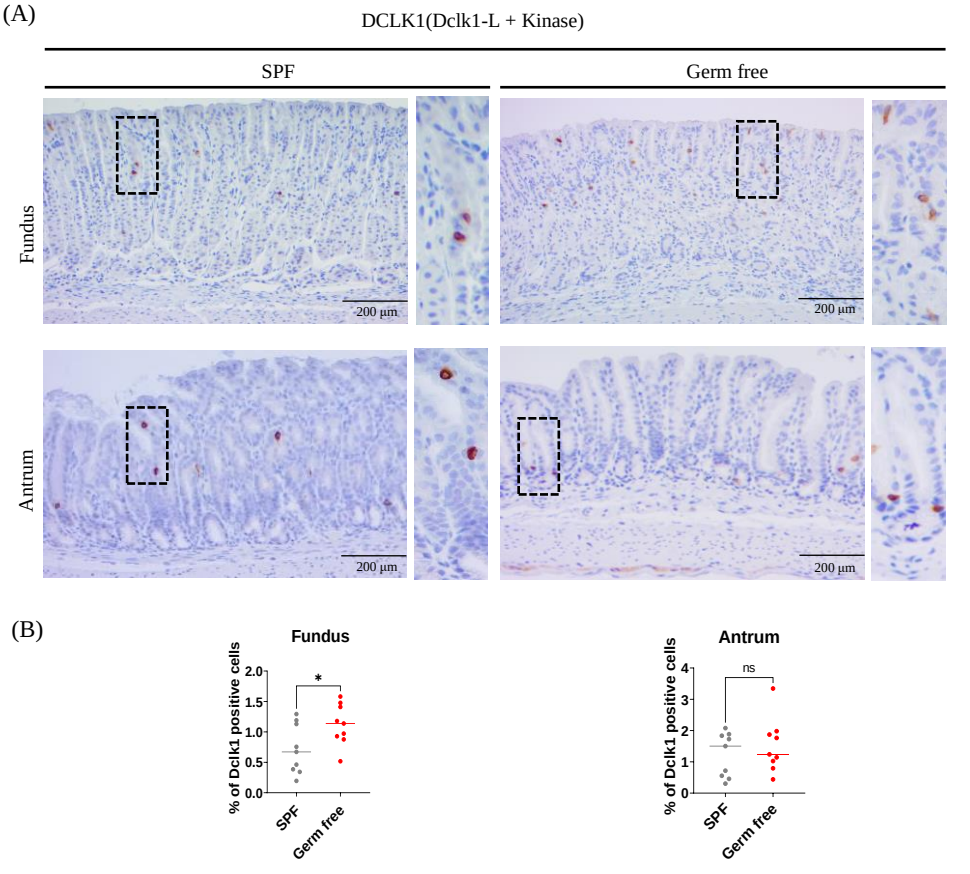

Figure 2. Differences in expression of Dclk1-positive cells in the stomach of SPF and Germ free mouse conditions (A) Dclk1 in SPF and Germ free mouse stomach was analyzed by immunohistochemistry and compared by the region (fundus and antrum). (B) Quantification of Dclk1+ cells population (*P<0.05). Data are expressed as the mean ± SEM of two different groups.

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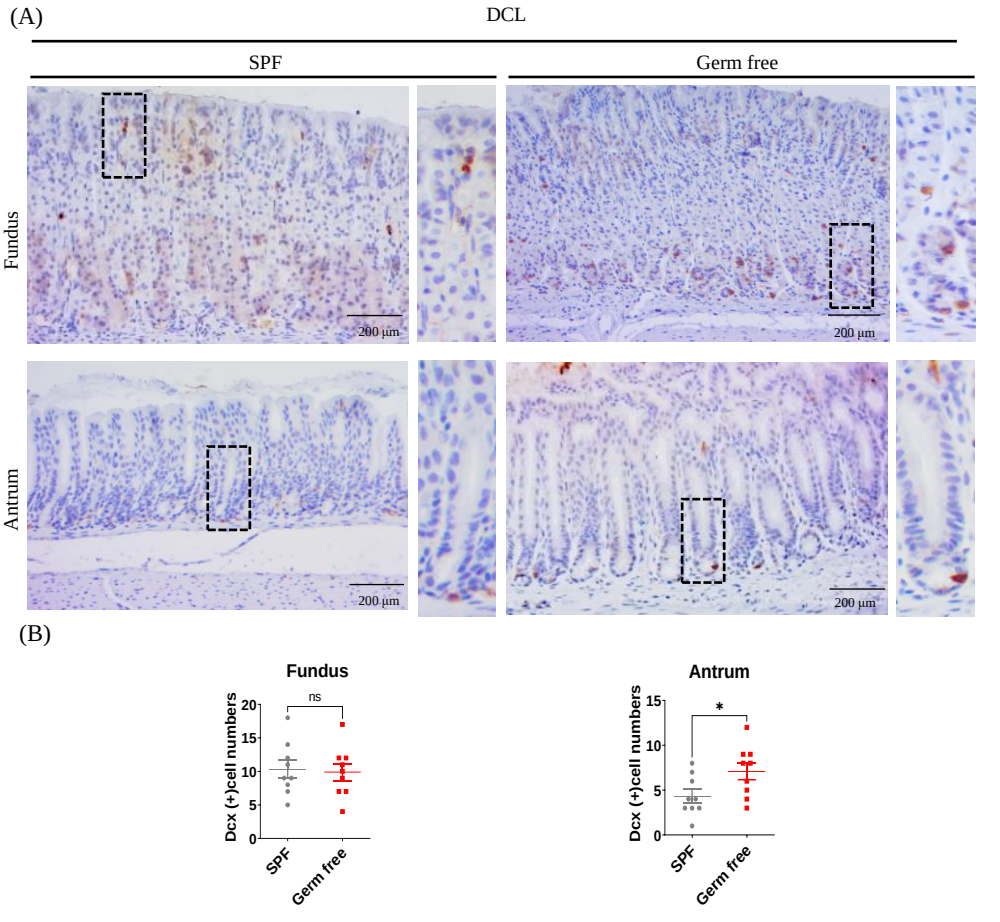

Figure 3. Differences in expression of Dclk1-positive cells in the stomach of SPF and germ free mouse conditions. (A) DCX in wild type and germ free mouse stomach was analyzed by immunohistochemistry and compared by the region (fundus and antrum). (B) Quantification of DCX+ cells population (*P<0.05). Data are expressed as the mean ± SEM of two different groups.

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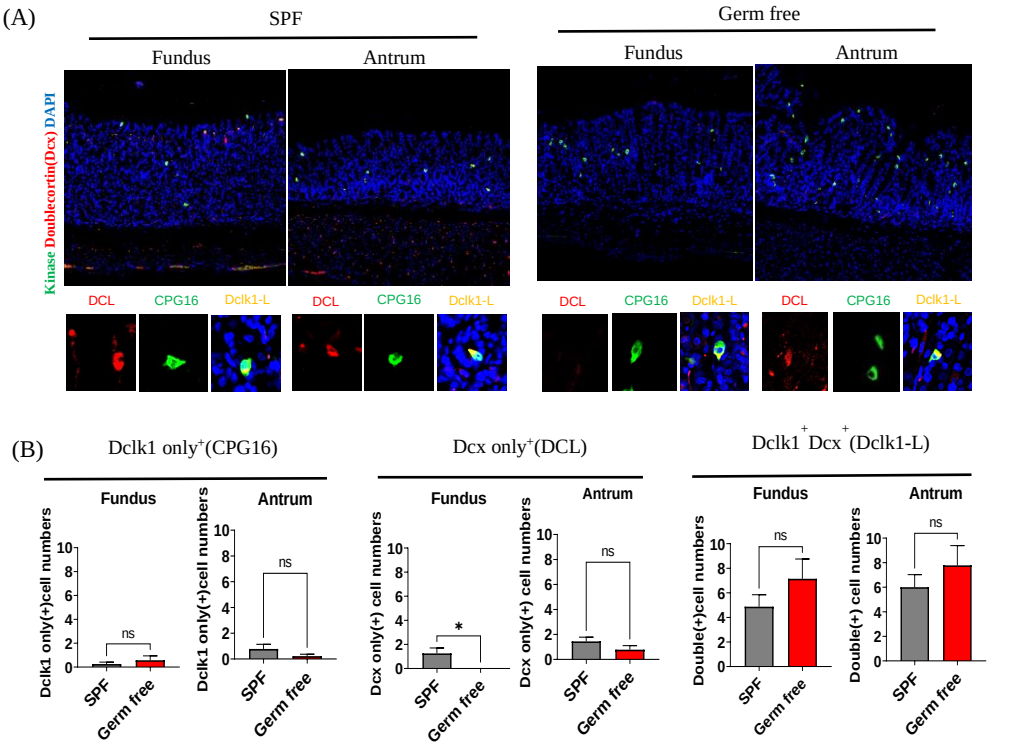

Figure 4. Immunofluorescence analysis of Dclk1 and Dcx in the stomach of SPF and germ free conditions. (A) Double staining using Dclk1 and Dcx to distinguish according to isoform type. (B) Counted each positive cell numbers of Dclk1 only, Dclk1 and Dcx, Dcx only were compared by conditions and regions. (*p<0.05, **p <0.01)

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3. Expression of Dclk1^{+}AcTub^{+} increase in Germ free mouse

Tuft cells have the distinct microvilli and it can detect using structural marker such as AcTub. In this study, Dclk1 and AcTub double positive cells indicate “tuft cell”. Likewise, Dclk1 only (+) cells and double (+) cells were increased in fundus region of Germ free mouse stomach.(Fig.5.A,B). There was no significant difference in antrum region but it was more increase than SPF condition. These data suggested expression of tuft cell increase in Germ free mouse stomach.

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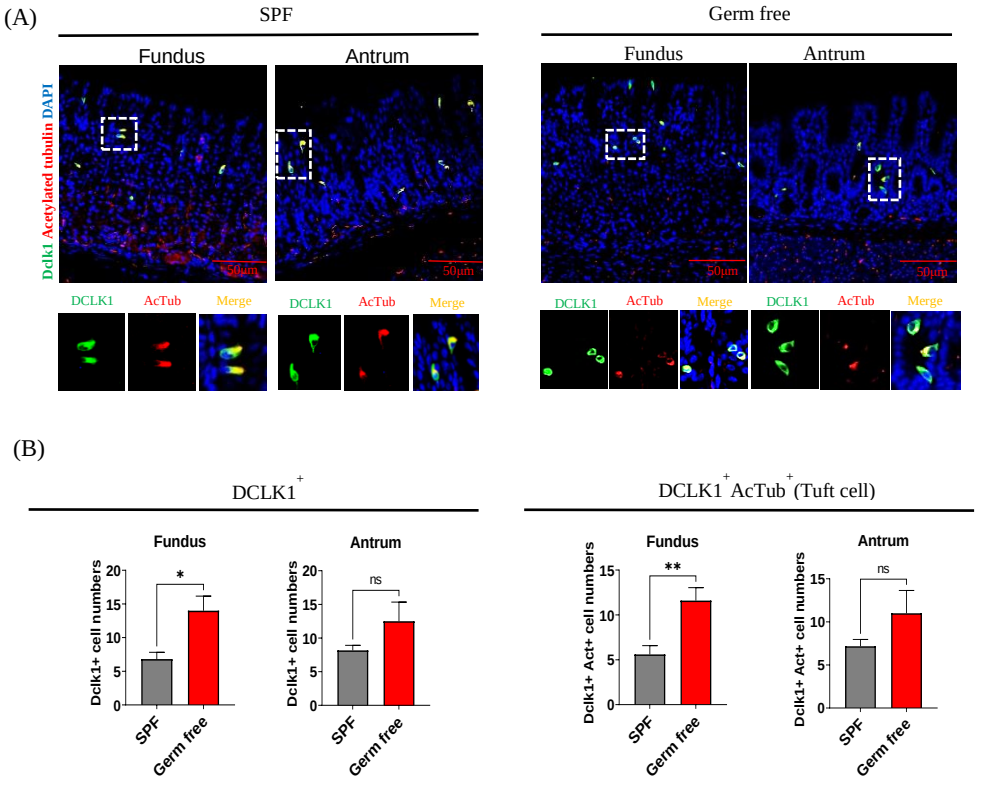

Figure 5. Immunofluorescence staining of Dclk1 and acetylated-α-tubulin in the stomach of SPF and germ free conditions. (A) Double staining using Dclk1 and acetylated-α-tubulin known as morphology markers of tuft cells. (B) Counted each positive cell numbers of Dclk1 only, Dclk1 and acetylated-α-tubulin, were compared by conditions and regions. (*P<0.05, **P <0.01)

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4. Each Dclk1 isoform identified different expression pattern in protein level

To examine whether Dclk1 isoform expression pattern changes in whole stomach, flow cytometry was performed using the antibody conjugation kit for conjugating several antibodies, it can help to detect to isoform types. (Fig.6) Here, after gating with single and epcam, then filter the DCLK1 and acetylated tubulin(AcTub) positive population and gate the DCX positive or negative population. It means Dclk1-L for DCX positive population and CPG16 for DCX negative population. To isolate the DCL domain, first filtering with DCX and acetylated tubulin positive population and then gate the negative population of Dclk1.(Fig6.A,B.) Analyzing the flow cytometry data, we found that differences in expression depending on the isoforms. In contrast to SPF, Dclk1-L and CPG16, included kinase domain, are increased in Germ free mouse, especially fundus region.(Fig6.C.) However, DCL, included only DCX domain, is rarely expressed in Germ free mouse stomach. This analysis provides that Dclk1-L and CPG16, included kinase domain, were increased in Germ free condition.

Next, to examine the changes of Dclk1 isoforms in SPF and Germ free, western blotting was performed.(Fig7.) Dclk1-L were markedly expressed both of regions in Germ free mouse and CPG16 were significantly expressed in fundus of Germ free mouse. These findings suggest kinase domain associates with microbiome.

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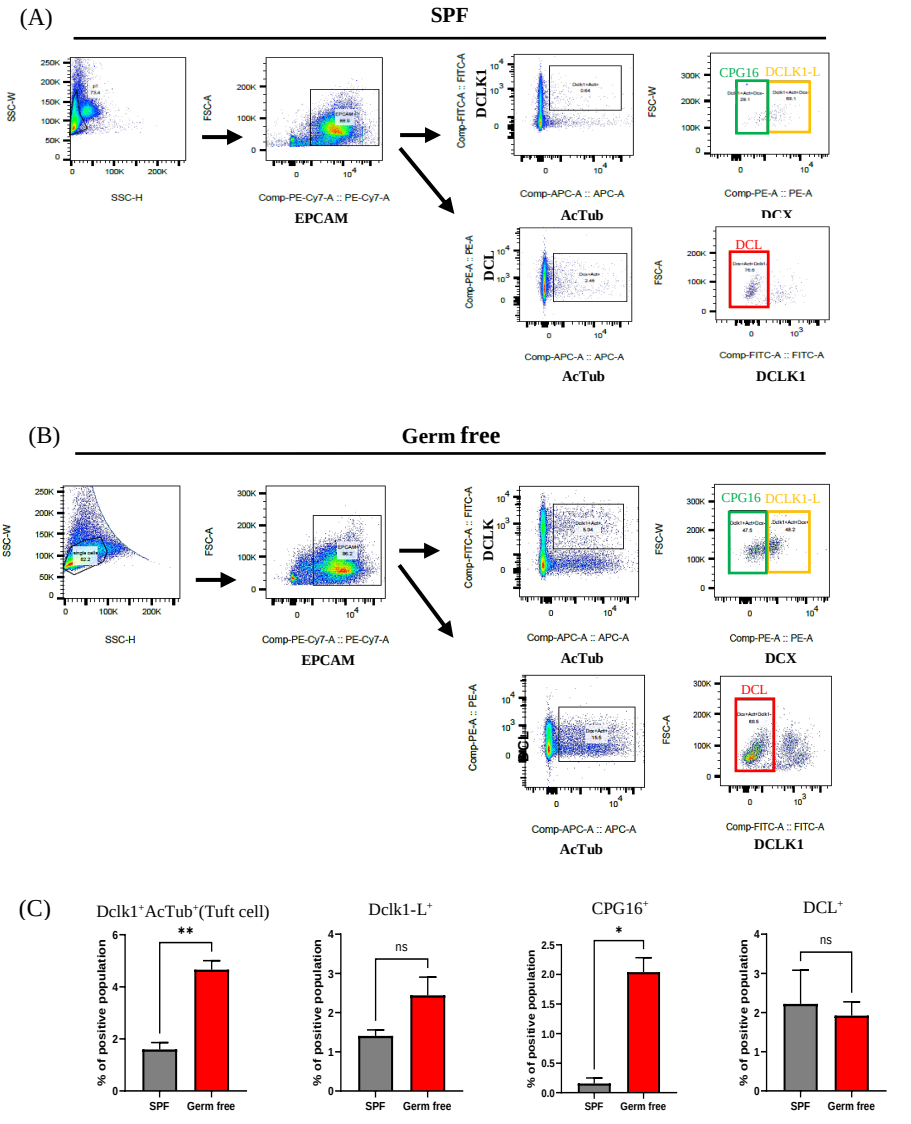

Figure 6. Flow cytometry analysis of the type of Dclk1 isoforms in the stomach of SPF and germ free mouse (A) FACs strategy for isolation of isoform types of Dclk1 Singlet;EpCAM^{+}; tuft cells in SPF mouse(n=3). (B) FACs strategy for isolation of isoform types of Dclk1 Singlet;EpCAM^{+}; tuft cells in SPF mouse(n=3). (C) Quantification of the expression of dclk1 isoform types through flow cytometry data. (*p<0.05, **p <0.01)

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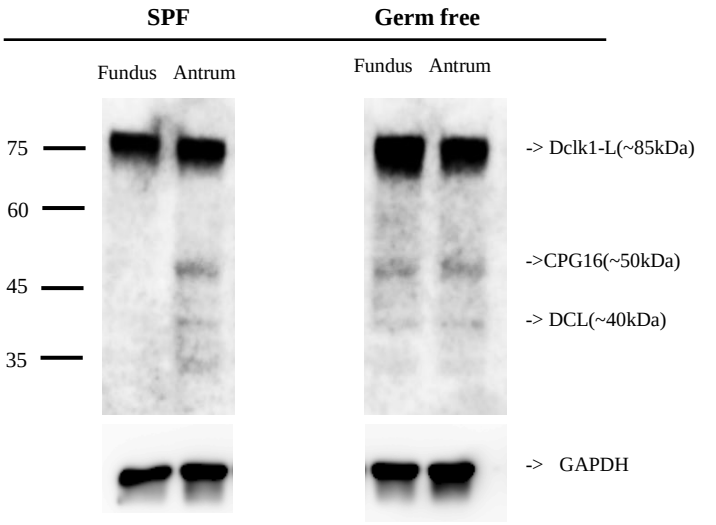

Figure 7. Protein expression of Dclk1 isoforms in SPF and Germ free mouse stomach. Tissue lysates of fundus and antrum in SPF and Germ free mouse stomach were analyzed by western blot. Immunoblot analysis of Dclk1-L and CPG16 expression was remarkably increased in fundus of Germ free mouse stomach.

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5. Expansion of tuft cell number in antibiotic-treated SPF mouse

The removal of microbiota from SPF mice on 8-week-old using antibiotics for 1 week and sacrifice for tissue preparation.(Fig8.A.) To compare the expression of tuft cells, immunostaining was conducted using Dclk1 and AcTub antibody.(Fig8.B.) The expression of tuft cells were significantly increased in fundus region. When microbiome exist and disappear, tuft cells are more reactive and that number is increased.

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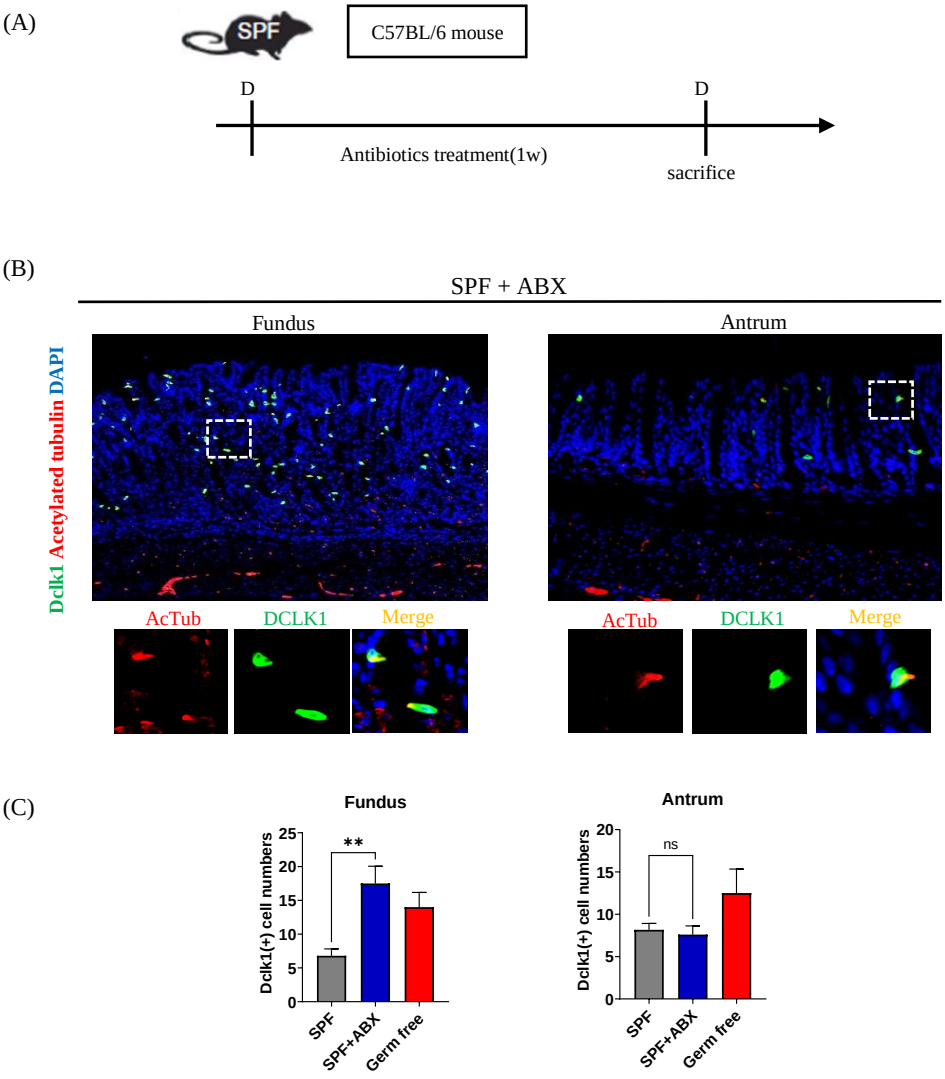

Figure 8. Comparison of the Dclk1-positive tuft cells in antibiotics treated SPF mouse stomach with SPF and Germ free mouse stomach. (A) Scheme of antibiotics treatment in C57BL/6 SPF mouse. Sacrifice the treated mouse after 1week. (B) SPF and Germ free mouse stomach sections were stained with two kinds of antibodies against, Dclk1(Green) indicates Dclk1-L and acetylated tubulin(Red) indicates apical tuft.(C) Quantification of the expression of dclk1 isoform types through flow cytometry data. (**p <0.01)

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IV. DISCUSSION

Doublecortin-like kinase 1 (Dclk1) is accepted as a cancer stem cell (CSC) marker in several tissues and marker of tuft cells. This gene has four major isoform types, Dclk1-L, CPG16, DCL, PEST domain. Dclk1-L and DCL domains are recognized by the antibody detecting the microtubule-binding site. Dclk1-L and CPG16 are recognized by the antibody detecting the kinase site. Dclk1-L and DCL are mainly expressed in the mouse brain, especially cortex region. DCL includes two doublecortin domain (DCX1 and DCX2) which are essential factor in neuronal migration and neurogenesis with microtubules[11]. Both Dclk1 and DCX were highly expressed in the mouse brain, but there is a difference in the degree of expression.

In this study, I used the antibody that can detect the tuft cells; dclk1 as a representative tuft cell marker, and acetylated-α-tubulin as a structural marker. Also, Dclk1 and Dcx antibody that can detect the dclk1 isoforms; dclk1 only positive cells mean CPG16, doublecortin only positive cells represent DCL and double positive cells are Dclk1-L.

Previous studies have noted that tuft cells have interaction with microbiome[30,31] and difference of expression on presence or absence of germ in the intestine[14,32]. Nevertheless, none of these studies have addressed the question on the isoforms of Dclk1 in relation with microbiome. Interestingly, when Germ free mouse stomach were examined for expression of Dclk1 isforms using Dclk1 and Dcx, Dcx only positive cells were rarely present in the fundus region. Conversely, double staining with Dclk1 and AcTub, these cells were increased in Germ free mouse fundus. Confirmed by immunohistochemistry and flow cytometry, tuft cell and kinase domain were more increased in Germ free than SPF. It can be assumed that Dclk1-L is basically expressed in normal stomach and the expression of each isoform is relatively low. As expression of Dclk1 in fundus

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and Dcx in antrum was increased, the number of double-positive cells increased more in Germ free, but it was not significant.

According to previous reports, kinase domain was identified as the major isoform in human colon adenocarcionoma with a few exceptions[29] and DCLK1-isoform2, Dclk1-L, expression in tumor cells to the regulation of a major component of the innate immune system, the M2-macrophage, which is a predominant immune cell type in PDAC[33]. Also, the catalytic activity of the kinase domain impairs phosphorylation-mediated regulation of tubulin polymerization. Dclk1 kinase activity negatively affects the binding affinity of the Dcx domains to tubulin and to lead to its dissociation.[34] It is likely that expression of kinase domains and Dcx domains relatively in Germ free condition. This negative regulation might be a possible explanation for relative decrease in Dcx expression level in germ free mice in spite of significant increase of kinase domain containing isoforms.

When I treated antibiotics to SPF mouse and created an environment similar to Germ free, expression of Dclk1 increases like in Germ free mouse stomach. It can be assumed that Dclk1 is continuously expressed to maintain homeostasis and activated in Germ free conditions for sensing the germ.

In summary, expression of tuft cell and Dclk1 isoforms, especially included kinase domain, was increased in Germ free mouse but it isn't clear that their target cells or where are they from. It needs to further study to clarify their origin and functional study about tuft cell using Dclk1-cre mouse or Dclk1-KO mouse.

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V. CONCLUSION

The study showed that the number of Dclk1 depending on isoform types increased in Germ free mouse stomach. It was found that tuft cells are react to microbiome and these phenomenon can explain that Dclk1 and its isoforms are increased in Germ free condition. Also, creating an environment similar to Germ free can confirm the change in expression. Pseudo-germ free condition made by antibiotic-treated and the expression of Dclk1 isoforms were examined in protein level. One interesting finding was that Dcx only positive cells rarely present in Germ free mouse fundus region. This finding suggest that the expression of CPG16 increased in Germ free mouse, especially fundus region. Addtionally, in whole stomach, flow cytometry analysis confirmed the increased expression of Dclk1 isoforms except DCL. These findings imply possibilities that kinase domain associates with germ. Altogether, the study showed that population of Dclk1-positive tuft cells change by presence or absence of microbiome.

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ABSTRACT (IN KOREAN)

무균 마우스의 위 내에서 dclk1 이 표지된 tuft cell 의 역할

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이 나 겸

Dclk1은 미세 소관 관련 단백질 키나아제이며 위장관 내에서 솔세포(tuft cells)의 마커로서 밝혀진 바 있다. Dclk1 유전자는 다양한 splicing isoforms를 갖고 있다; Dclk1-L(long form), Kinase (Kinase only domain), DCL domain(Dcx domain only), PEST(Proline/Serine rich domain). 솔세포(tuft cells)는 위장 상피 전체에 걸쳐 발견되는 적은 세포 유형이다. 솔세포(tuft cells)의 형태는 아세틸화된-α-튜블린(tubulin)에 의해 검출되는 꼭대기(apical)이고 무딘 미세융모가 특징이다. 이러한 세포는 장내 미생물과 숙주 면역계를 연결하는 내강 탐지기 역할을 한다. 이전 연구에서 Dclk1은 다양한 조직에서 종양 줄기 세포 마커로, 그리고 급성 손상 후 상피 복구를 촉진하기 위한 내강 보초(luminal sentinels)로 보고했다. 그러나, 위장관 중 소장 및 대장에서는 이러한 미생물에 대한 연구가 상대적으로 많이 되어있지만 위는 그렇지 않다. 따라서, 나는 무균마우스와 항생제가 처리된 SPF 마우스를 이용하여 Dclk1 양성의 솔세포 발현을

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연구했다. 유사 무균 환경에서, Dclk1 양성의 솔세포 수는 증가했으며, 동형(isoform)의 유형에 따라 발현량은 달라진다.

핵심 되는 말: 솔 세포, 미생물, Dclk1, 위, 무균 마우스