UNIVERSITY OF CALIFORNIA SAN DIEGO

Aberrant Stem Cell Differentiation in Mutant EpCAM Mice

A Thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

In

Biology

by

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2019

This Thesis of John Paul-Nguyen Rabalais is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California San Diego

2019

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Acknowledgements

I would like to acknowledge Dr. Mamata Sivagnanam for her continued guidance and advice about all topics, both within and outside of the realm of the lab. She has tremendously aided me in my successes from my time as an undergraduate as well as a master's student.

I would also like to acknowledge Dr. Kim Barrett, who has also contributed wonderful insights to all my projects. Her wisdom has often helped me during times of confusion within the lab.

All sections of this thesis are currently being prepared for submission for publication of the material. Rabalais, John; Das, Barun; Okamoto, Kevin; McGeough, Matthew; Das, Soumita; Barrett, Kim; Sivagnanam, Mamata. The thesis author was the primary investigator and author of this material.

ABSTRACT OF THE THESIS

Aberrant Stem Cell Differentiation in Mutant EpCAM Mice

by

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Master of Science in Biology

University of California San Diego, 2019

Professor Mamata Sivagnanam, Chair

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Congenital Tufting Enteropathy (CTE) is a rare intestinal disease which presents in newborns, causing intestinal failure and failure to thrive. Mutations in

EpCAM, Epithelial Cell Adhesion Molecule, have been shown to be causative in CTE. In addition to its role in mediating cell adhesion, EpCAM has also been linked to cell proliferation and differentiation. In this project, we investigated the role of EPCAM mutation on intestinal epithelial cell differentiation in the small intestine. To do so, inducible EpCAM$^{\Delta 4/\Delta 4}$ mice were used to investigate these changes. We found there to be significant decreases in cells of the secretory lineage (Paneth, goblet, enteroendocrine cells) in the intestinal epithelium of mutant mice compared to control mice. Characteristic tufting of the villi was also noted in the mutant. We discovered alterations in differentiation factor levels via qPCR and immunoblotting. *Atoh1* was decreased at both the RNA and protein levels, along with a decrease in *Klf4*, which is implicated in terminal goblet cell differentiation, at the RNA level. We also discovered increased NOTCH1 signaling via immunoblot, which activates the enterocyte differentiation pathway. We conclude that in the presence of mutated EpCAM, there is improper differentiation signaling in intestinal epithelial cells leading to a reduction in secretory cell lineages.

I. Introduction

Congenital Tufting Enteropathy (CTE) is a rare, autosomal recessive disease affecting roughly 1 in 50,000 to 100,000 infants in Western Europe (1). It is characterized by intestinal failure, severe intractable diarrhea, and failure to thrive. Pathologically, patients with CTE display focal epithelial tufts, partial to full villous atrophy, and crypt hyperplasia (2). In affected patients, villi lose their finger-like structure and instead form disorganized aggregates of enterocytes, known as tufts. Murine studies have demonstrated that changes to the villi become readily apparent roughly three days after birth, with no detectable differences occurring before then (2). Currently, there is no direct treatment for CTE; treatments are supportive in nature. Patients with CTE are dependent on parenteral nutrition to survive, which itself is intensive and presents a low quality of life. Some patients require intestinal transplant, which carries its own risk factors including a high rate of mortality (3).

Epithelial Cell Adhesion Molecule, or EpCAM, has been identified as the causative gene in most patients with CTE (4). Currently, there are 42 known mutations of EpCAM which lead to CTE, with mutations in SPINT2 also being shown to be syndromic. SPINT2 functions as a proteolytic enzyme for EpCAM, cleaving the intracellular domain which then translocates to the nucleus to participate in cell signaling. Many of the mutations of EpCAM appear to cause loss of function via truncation or loss of intracellular or extracellular domains. One such mutation found in multiple patients leads to an alternative splice site

formation, generating EpCAM transcripts lacking exon 4. The function of exon 4 currently remains unknown, however this mutant EpCAM has been shown to have decreased levels in patients as well as mislocalize to the endoplasmic reticulum of affected animals and patients (5). In this particular mutant, it is unknown whether CTE is caused by a lack of fully functional EpCAM or whether the disease is due to the failure of EpCAM to leave the endoplasmic reticulum, causing ER stress.

Although the pathophysiology by which EpCAM mutation causes CTE has not been fully delineated, the role of EpCAM in healthy cellular function is vast and therefore mutations in EpCAM can affect a variety of cellular processes. One of EpCAM's roles is mediating cell-cell interactions. L929 fibroblasts expressing exogenous EpCAM formed aggregates of cells and EpCAM has been shown to associate with Claudin-7, a key protein in tight junctional function (6). However, conflicting studies have also shown that EpCAM negatively impacts cellular adhesion by impairing E-cadherin association to cellular cytoskeletons (7). *In vivo* studies of EpCAM and its role in cell junctions have shown that both EpCAM and E-cadherin are necessary for proper morphogenesis of tissues in zebrafish (8).

EpCAM has also been implicated in stem cell maintenance and differentiation. EpCAM was found to be highly upregulated in pluripotent stem cells and cancers and downregulated upon terminal differentiation (9). Within the crypt villus axis, EpCAM is found in higher levels in the crypts and decreases

along the villus (10). Accordingly, EpCAM levels are found to be highest within the crypts, which contain the rapidly proliferating LGR5+ intestinal stem cell populations. EpCAM has been shown to play a key role in proliferation of malignant tumors (11). EpCAM signaling occurs via proteolytic cleavage of its intracellular domain, which then relocates to the nucleus and complexes with β catenin and Lef, which are components of Wnt signaling.

Wnt signaling has been shown to participate in crosstalk with the Notch signaling pathway (12). Notch signaling plays key role in determining the fate of LGR5+ stem cells as they migrate from the crypts into the villi (13). These LGR5+ stem cells, located within the basal crypts, are responsible for the generation of all other IECs located within the crypts and villi during standard intestinal renewal. The completed process takes place over the course of 2-6 days, although some IECs have a significantly longer life span(13).

Given the rapid epithelial turnover, differentiation of the stem cells as they migrate from the base of the crypts towards the villi must be highly regulated to ensure normal populations of cells. IECs can generally be split into two categories, absorptive enterocytes and professional secretory cells. Enterocytes, which are the main absorptive cells found in the villus, are responsible for nutrient uptake within the intestines. Cells that are exposed to Notch1 ligands, such as Dll-1 and Dll-4, are pushed towards the absorptive cell fate containing an intermediary progenitor cell before becoming mature enterocytes via Hes1 signaling (14). Failure to receive Notch1 signaling results in cells moving

towards a secretory fate, where they can become Paneth cells, goblet cells, or enteroendocrine cells (13). This pathway is mediated by multiple intermediate progenitor cells, with large branching points controlled by Hnf1β and ATOH1, while terminal differentiation into Paneth, goblet, and enteroendocrine cells are controlled by Sox9, Klf4, and Neurogenin3 respectively (15-17). There are also indications that the two differentiation pathways mutually repress each other in the intestinal system. Knockout studies of Dll-1 showed reduced Notch1 signaling and increased goblet cell counts in murine models while ATOH1 repression increased Hes1 expression (18).

To explore the possible outcome of mutant EpCAM on differentiation, we will be using an inducible $EpCAM^{\Delta 4 \Delta 4}$ system (referred to as mutant from this point on) in adult mice. Mice expressing this inducible mutant EpCAM have been shown to exhibit CTE like phenotype, particularly recapitulating the physiology of the disease [2]. Previous studies have shown that mice expressing mutant EpCAM have the characteristic tufts of CTE patients and show significant weight loss, although they do not experience extreme diarrhea. These mice make for effective models for CTE as the disease is fatal in neonatal mice very early on in life and proper intestinal formation occurs several days post birth.

II. Methods

Generation of mutant EpCAM$^{\Delta 4/\Delta 4}$ mice

Mutant (EpCAM$^{\Delta 4/\Delta 4}$) mice were generated with Cre-Lox system. Upon tamoxifen injection, exon 4 of EpCAM is deleted from the genome. Adult mice aged between 11-13 weeks were given two injections of tamoxifen during days 1 and 2, as well as days 9 and 10. Mice were then sacrificed with CO_2 asphyxiation and cervical dislocation. Duodenal sections were collected for study.

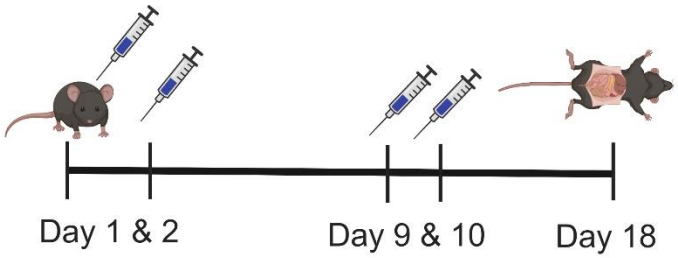

Figure 1. Generation of Mutant EpCAM$^{\Delta 4/\Delta 4}$. Schematic of tamoxifen injections to generate mutant mice.

Immunohistochemistry

Duodenal sections were obtained and fixed in 4% paraformaldehyde for 24 hours and paraffin embedded. Sections were deparaffinized and hydrated prior to low-pH based antigen retrieval (Vector Labs, Burlingame, CA). Sections were blocked and incubated in primary antibody overnight. An anti-Lysozyme antibody (Thermo, Rockford, IL) was diluted 1:1000 for Paneth cell detection and anti-Chromogranin A antibody (Abcam, Cambridge, UK) was diluted 1:800 for enteroendocrine cell detection. 3% H_2O_2 quenching was performed for 15

minutes after primary incubation followed by ABC secondary antibody incubation per manufacturer instructions (Vector Labs, Burlingame, CA). AEC staining was performed to manufacturer instructions (Vector Labs, Burlingame, CA) followed by hematoxylin staining for 3 minutes and Scott's Bluing Reagent for approximately 1 minute. Gold antifade mounting (Vector Labs, Burlingame, CA) was used to mount coverslips.

PAS staining was performed according to manufacturer instructions (Sigma Aldrich, cat#395B) after slides were deparaffinized and rehydrated.

Images was performed using a Leica DMi1 inverted microscope and LAS 4.10 EZ acquisition software at 20x magnification for chromogranin A and PAS images and 40x magnification for lysozyme images.

Sections were analyzed systematically to obtain percentage of cells of each cell type. For each experiment, 2 representative images were taken of each duodenal section at random. The total epithelial cells were counted in frame. The number of positively stained cells (Lysozyme for Paneth cells, Chromogranin A for enteroendocrine cells, and magenta stained cells in PAS staining) were counted in each representative picture. The number of each cell type was divided by total IECs to achieve a percentage. The percentage of positive cells were averaged from the 2 images to give an average percentage of positive cells for each mouse.

Q-PCR

Total RNA from duodenum sections was isolated using Direct-zol RNA MiniPrep kits (Zymo, Irvine, California). RNA was extracted initially using TRIzol (Invitrogen, Carlsbad, California). First strand cDNA was synthesized with iScript cDNA Synthesis kit (Bio Rad, Irvine, California) using the recommended protocol. Real time PCR reactions were set up using FastStart Universal SYBR Green Master Mix (Invitrogen) and thermal cycling performed on a StepOnePlus Real-Time PCR System using Step One software v2.0. (Applied Biosystems, Carlsbad, CA). Primers were obtained from IDT (Integrated DNA Technologies, Coralville, IA). An 18s rRNA gene was used as the housekeeping gene. All primers were diluted to 100µM.

Western blotting

Murine duodenal sections were suspended in 1X RIPA lysis buffer (Cell Signaling Technology, Danvers, MA) with 1.0mm Zirconia beads (Biospec, Irvine, CA) and homogenized for 90 seconds. The lysate was then centrifuged at 4°C for 15 minutes and the supernatant was collected. Protein quantification was performed with Bio-Rad DC protein assay kit according to manufacturer instructions (Bio-Rad, Irvine, California). Lysates were diluted at a 1 to 1 ratio of lysate to loading buffer and boiled for 10 minutes prior to loading. 50 µg of protein was loaded onto Mini-Protean TGX precast gels (Bio-Rad), electrophoresed, then transferred onto PVDF membranes. Membranes were blocked with 5% skim milk/TBST.

Western blotting was performed using antibodies at the following concentrations: activated Notch-1, 1:500 (Abcam, Cambridge, UK), Hes-1, 1:1000 (Thermo Fisher Scientific, Rockford, IL), β-actin 1:5000 (Sigma, St. Louis, MO). Math1 (Atoh1) was deposited to the DSHB by Johnson, J. (DSHB Hybridoma Product Math1 (Atoh1)) and used at 0.5 μg/mL. Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (Cell Signaling Technologies, Beverly, MA) were used at 1:5000 dilutions. A semiquantitative measurement of band density was performed using ImageJ for Windows software.

III. Results

Intestinal epithelial cell populations disturbed in mutant CTE model mice

Tamoxifen-inducible CTE model mice were used to investigate possible differences in cell fate in the intestines during CTE. Under the presence of tamoxifen, CRE recombinase excises exons 4 of EpCAM, generating $EpCAM^{\Delta 4/\Delta 4}$ and a phenotype similar to that of CTE. Depletion of wild type EpCAM was confirmed via PCR (Figure 2A, B).

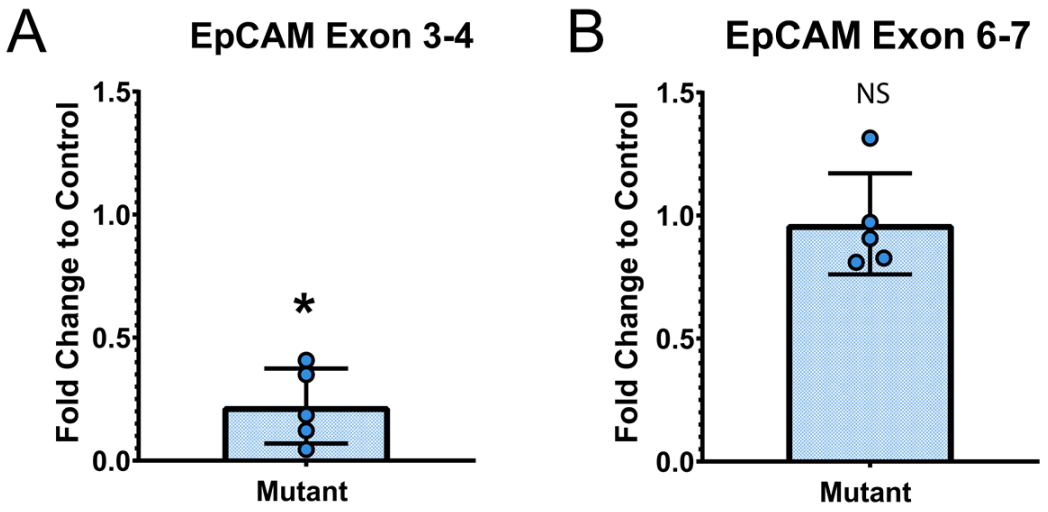

Figure 2. Deletion of Exon 4 in Mutant Mice. (A-B) qRT-PCR of using primers for EpCAM to detect levels of (A) exon 3-4 and (B) exon 6-7. * = P <0.05. N = 5

Three major secretory cell types located in the villi (enteroendocrine and goblet) and crypts (Paneth) were studied and their frequencies were compared to that of non-induced mice. Lysozyme, PAS, and chromogranin A stainings were used to identify Paneth, goblet, and enteroendocrine cells respectively. Paneth

cells were found to be significantly decreased in mutant mice, representing roughly 2.9% of IECs compared to 4.9% in control mice (Fig. 2 A, B, C).

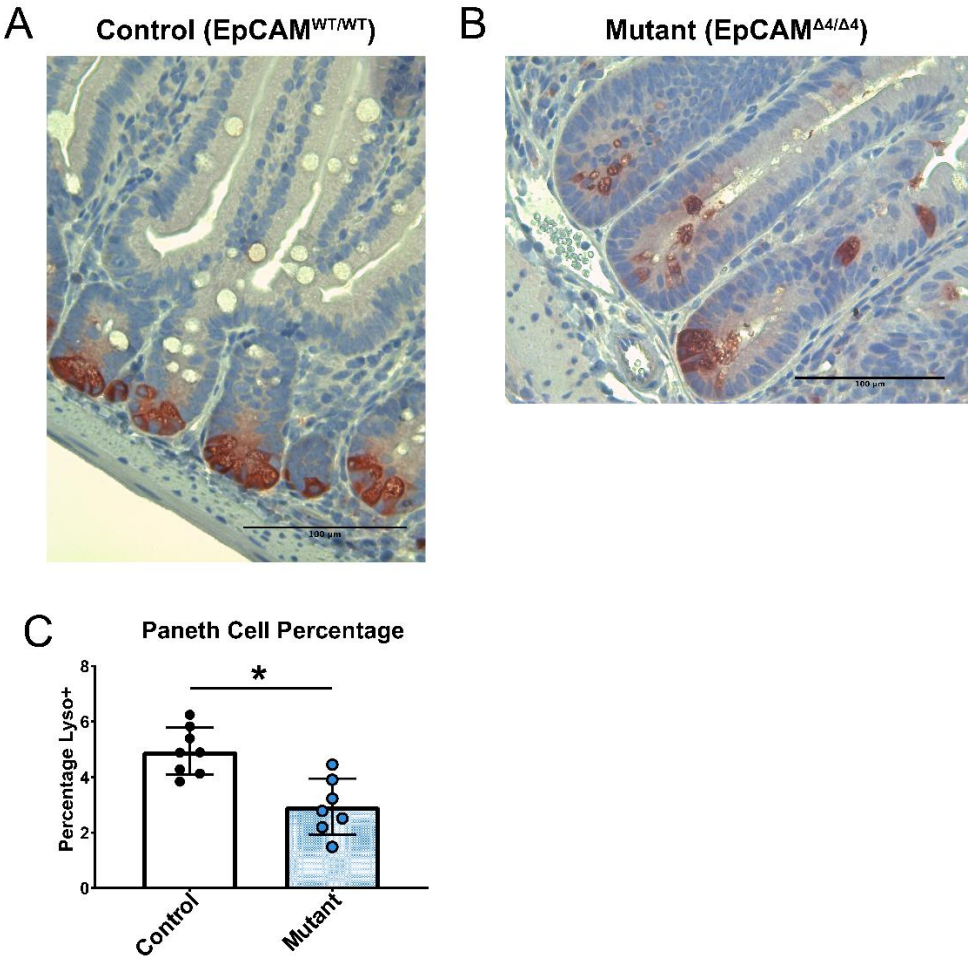

Figure 3. Paneth Cell Population Decreased in mutant EpCAM mice. (A-B) Representative staining of Lysozyme (red) to detect Paneth cells in (A) control and (B) mutant mice. (C) Quantification of Paneth cells as a percentage of IECs. * = P < 0.05. N = 8 control mice, N = 7 mutant mice

Similarly, goblet cells were also found to be significantly decreased in the mutant mice, comprising 4.5% of the intestinal population, compared to 9.7% of the population in non-induced mice (Fig. 3 A, B, C).

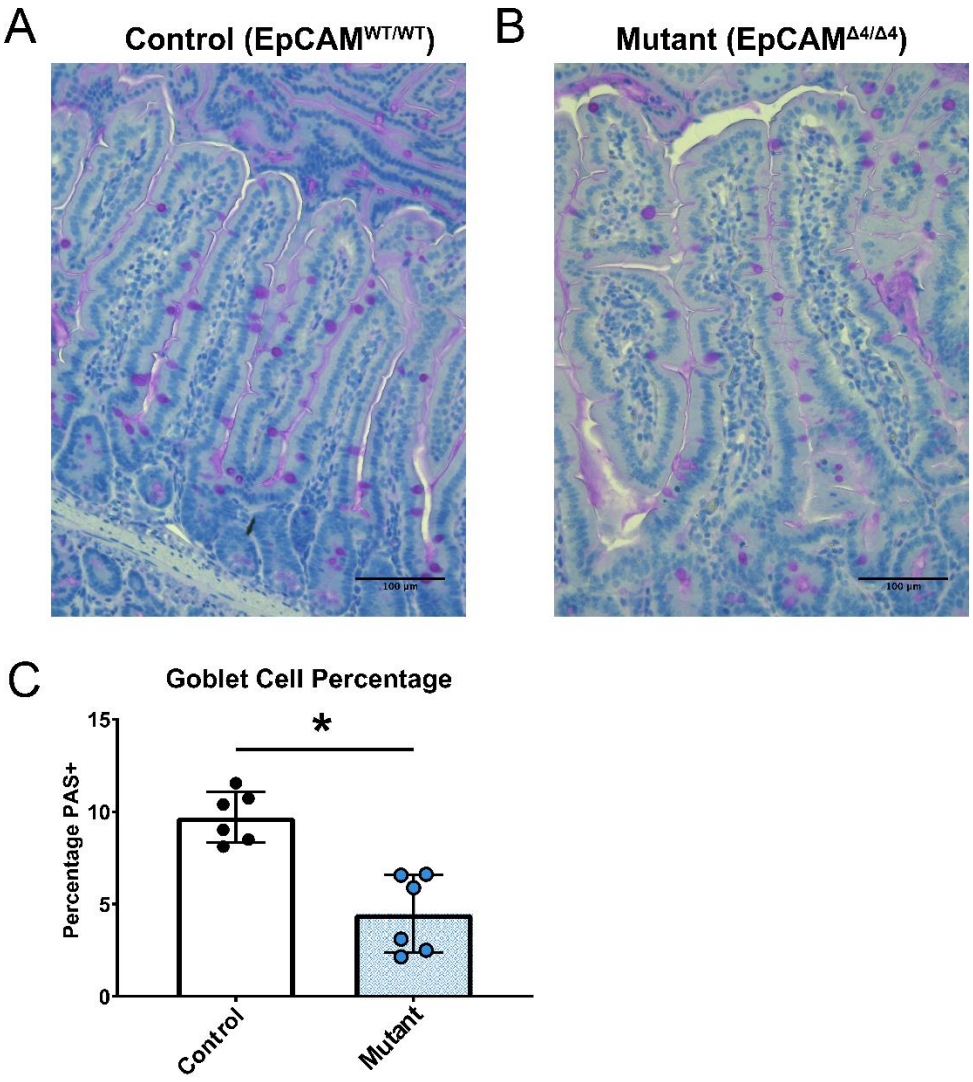

Figure 4. Goblet Cell Population Decreased in mutant EpCAM mice. (A-B) Representative PAS staining (magenta) to detect goblet cells in (A) control and (B) mutant mice. (C) Quantification of goblet cells as a percentage of IECs. * = P < 0.05. N = 6 control mice, N = 6 mutant mice

Enteroendocrine cells were also found to be significantly decreased in mutant mice, constituting 0.35% of the intestinal population, compared to 0.70% in non-induced mice (Fig. 4 A, B, C).

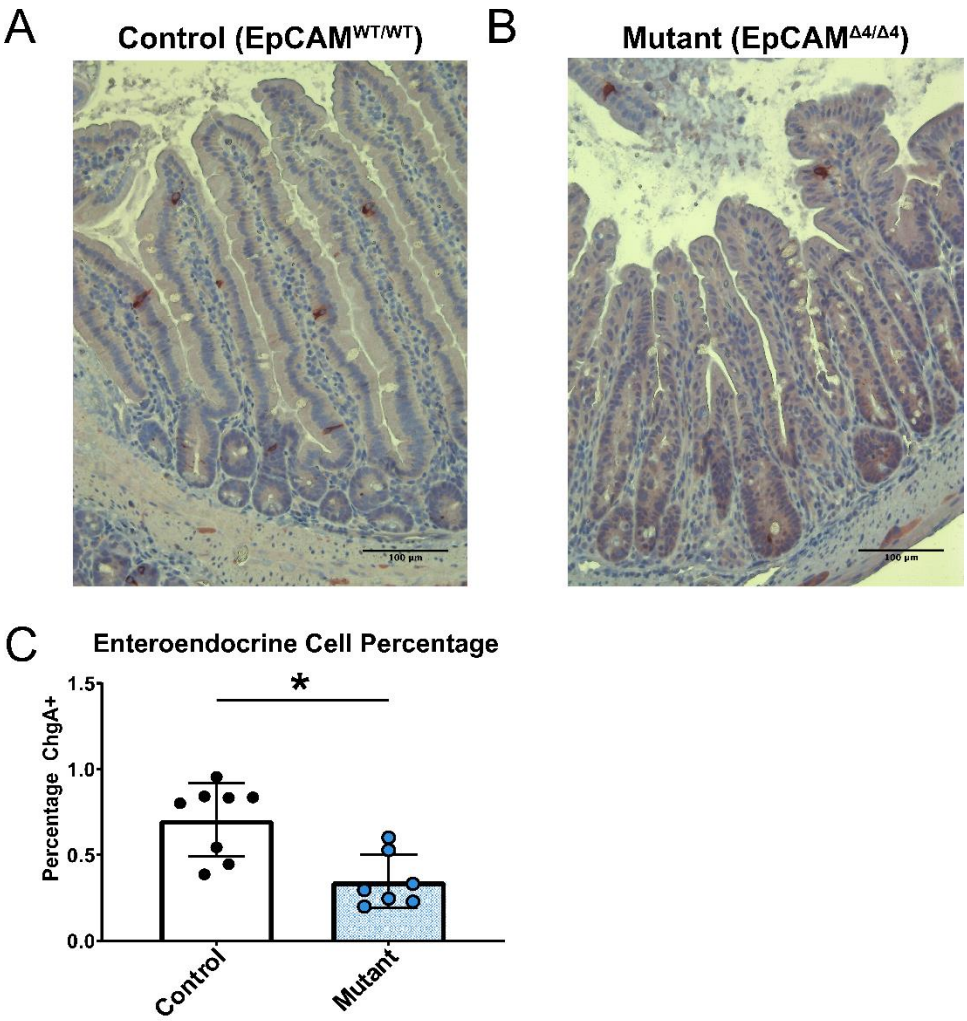

Figure 5. Enteroendocrine Cell Population Decreased in mutant EpCAM mice. (A-B) Representative staining of chromogranin A (red) to detect enteroendocrine cells in (A) control and (B) mutant mice. (C) Quantification of enteroendocrine cells as a percentage of IECs. * = P < 0.05. N = 8 control mice, N = 7 mutant mice

Enterocyte population counts were also determined indirectly, by summing Paneth, goblet, and enteroendocrine cell populations and subtracting their combined percentage from 100% in each mouse. In mutant mice, enterocytes comprised a significantly higher proportion of IECs as compared to control mice (Figure 6).

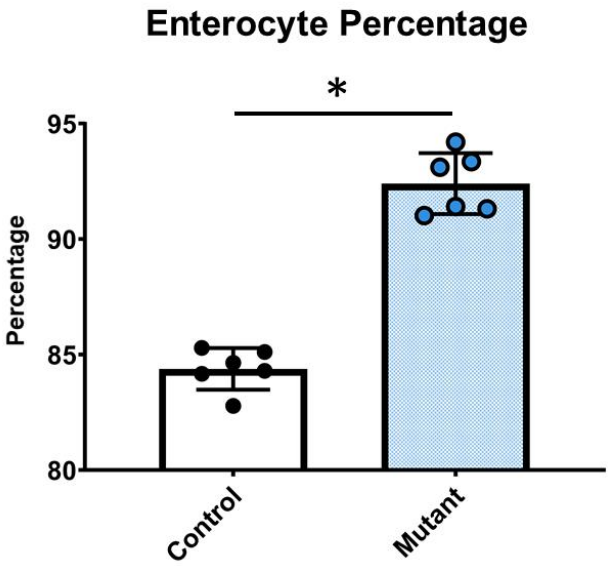

Figure 6. Enterocyte Population is Significantly Increased in Mutant Mice. Enterocytes proportion was calculated by subtracting Paneth, goblet, and enteroendocrine cell populations in mutant mice. N = 6. * = P <0.001

Transcription of differentiation factors reflect decreases in secretory cells in mutant mice

After noting altered cell populations in mutant mice, differentiation factors were studied to determine where any defects in differentiation existed, if any at all. qRT-PCR analysis was performed to ascertain the transcript levels of each differentiation factor during various stages of differentiation (Fig. 5).

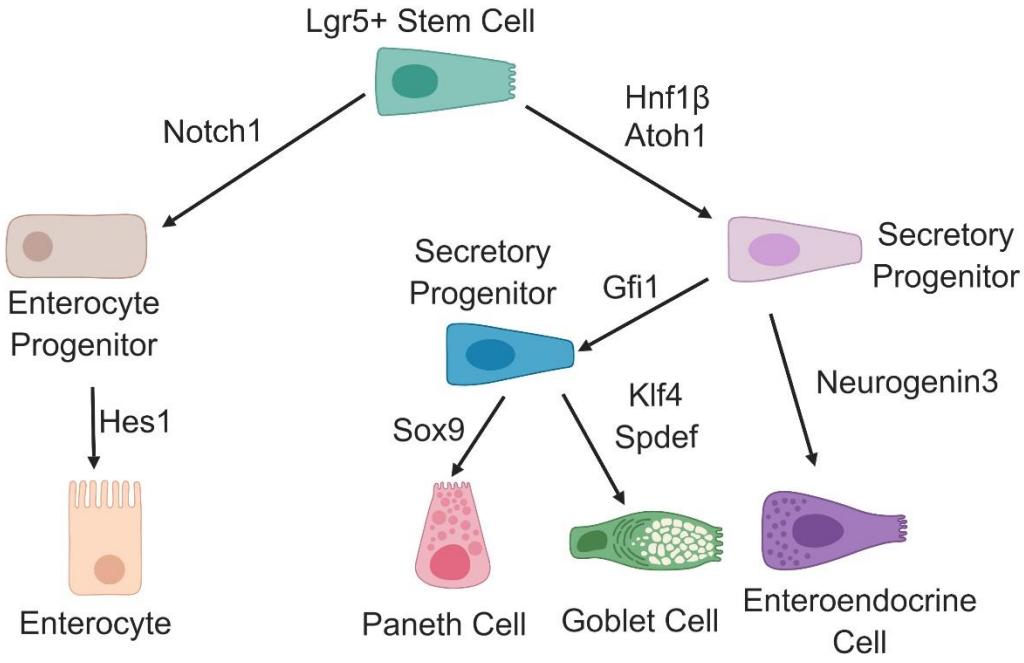

Figure 7. Differentiation of Lgr5+ Stem Cells in Intestines. Differentiation of intestinal stem cells shows two main branches, enterocyte and secretory, with various progenitors along each path.

Two early secretory cell fate transcription factors, *Atoh1* and *Hnf1*β, were significantly down regulated in intestines from mutant mice compared to control mice (Fig. 6 A, B). *Atoh1* was reduced by 65% in the mutant mice compared to controls, while *Hnf1*β was decreased by 34%.

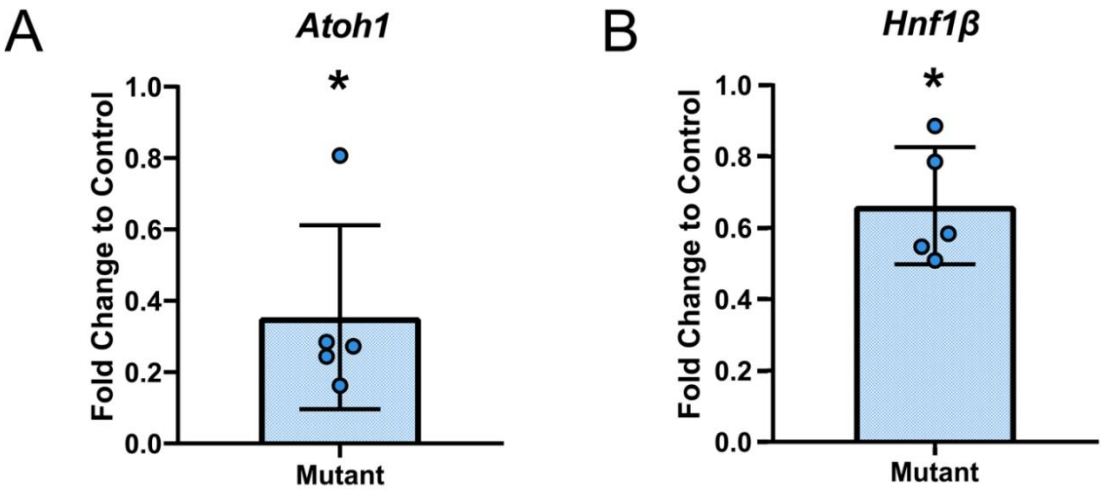

Figure 8. *Atoh1* and *Hnf1β* are decreased in mutant mice. (A-B) qRT-PCR of (A) *Atoh1* and (B) *Hnf1β* show decreases in mRNA transcript compared to control mice. * = P < 0.05. N = 5 mice.

Absorptive cell fate receptor *Notch1* showed no significant changes in mutant mice; accordingly, *Hes1*, the downstream effector of *Notch1*, also did not display any significant changes in mutant mice (Fig 7 A, B).

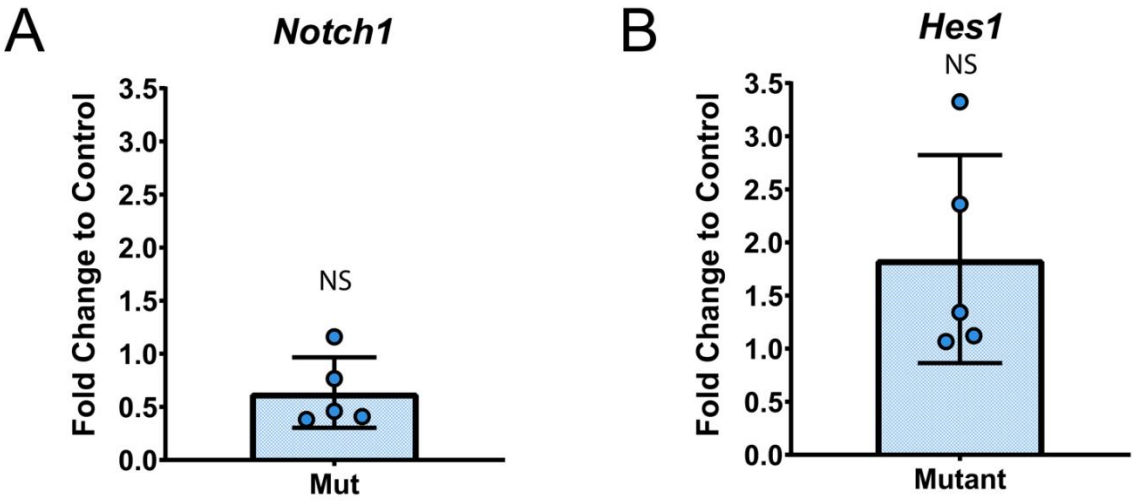

Figure 9. *Notch1* and *Hes1* are unaffected in mutant mice. (A-B) qRT-PCR of (A) *Notch1* and (B) *Hes1* show no changes in mRNA transcript compared to control mice. NS = P > 0.05. N = 5 mice.

Downstream transcription factors leading to Paneth and enteroendocrine cell fates, *Sox9* and *Neurogenin*-3, showed no significant changes in mutant mice (Fig. 8 A, C). *Klf4*, a transcriptional factor implicated in goblet cell differentiation, was shown to be significantly down regulated in mutant mice (Fig. 8 B).

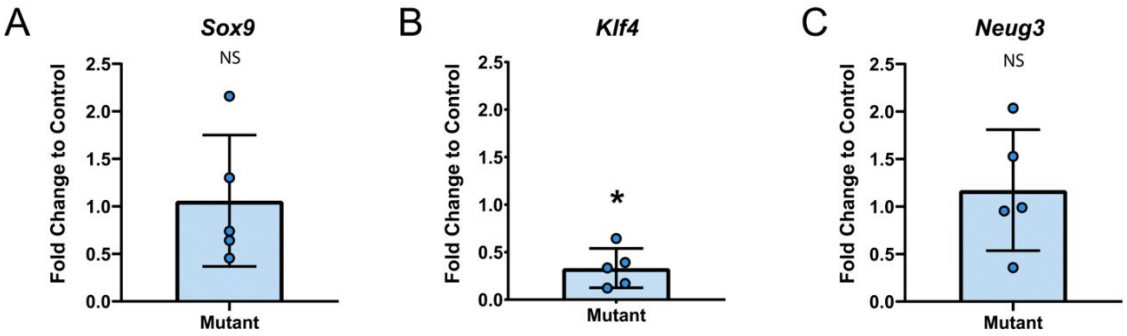

Figure 10. Terminal differentiation factor transcripts in mutant mice. (A-C) qRT-PCR of (A) *Sox9*, (B) *Klf4*, and (C) *Neug3*. *Klf4* is decreased in mutant mice as compared to controls. Both *Sox9* and *Neug3* show no changes in mutant mice. * = P < 0.05. NS = P > 0.05. N = 5 mice.

In summary, transcription of differentiation factors relating to secretory cell fates is significantly decreased, while absorptive cell fate differentiation factors are increased in mutant mice intestines compared with control.

Differentiation factors show altered expression in EpCAM$^{\Delta 4/\Delta 4}$ mice

To further access the possible impairment of differentiation present in mutant mice, western blots were carried out on protein products of differentiation factors that were affected in the previous qRT-PCR studies (*Atoh1, Hnf1β, Hes1, Klf4, Notch1)*. In mutant mice, we found there to be significant decreases in ATOH-1 (Figure 9 a, b) proteins, mirroring previous data demonstrating decreases of mRNA transcripts of these two genes.

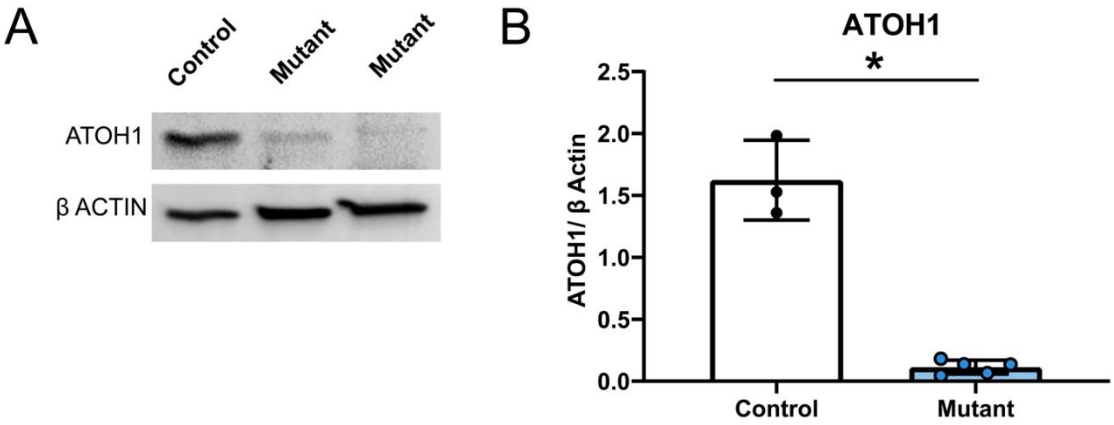

Figure 11. ATOH1 is significantly decreased in mutant mice. (A) Representative western blot of ATOH1 in control and mutant mice. (B) Quantification of ATOH1 protein shows significant decrease in mutant mice. * = P < 0.05. N = 5 mice.

Interestingly, while no decrease in *Notch-1* transcript was found, there was a significant increase in NOTCH-1 signaling, evidenced by increased intracellular NOTCH-1 domain (NICD) (Figure 10 a, b).

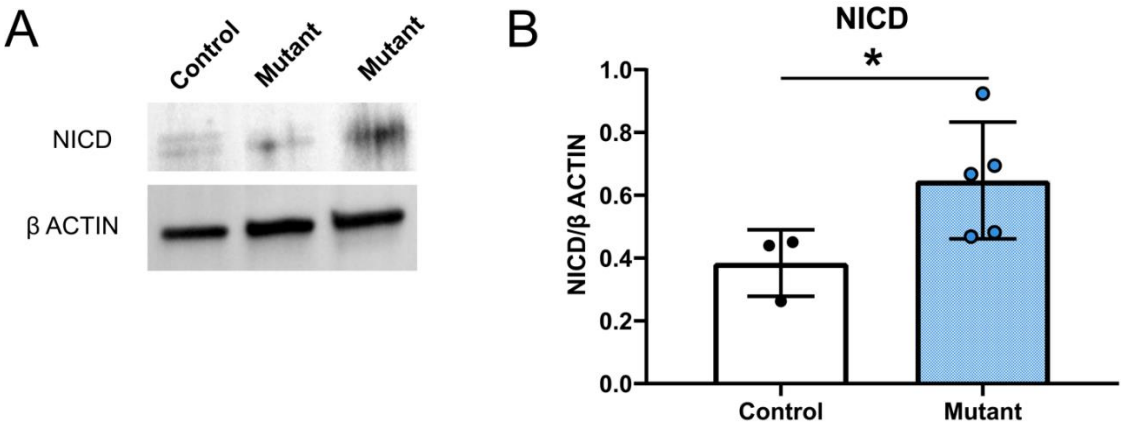

Figure 12. NICD is significantly increased in mutant mice. (A) Representative western blot of NICD in control and mutant mice. (B) Quantification of NICD protein shows significant increase in mutant mice. * = P < 0.05. N = 5 mice.

However, this did not lead to an increase in HES-1 protein (Figure 11 a, b).

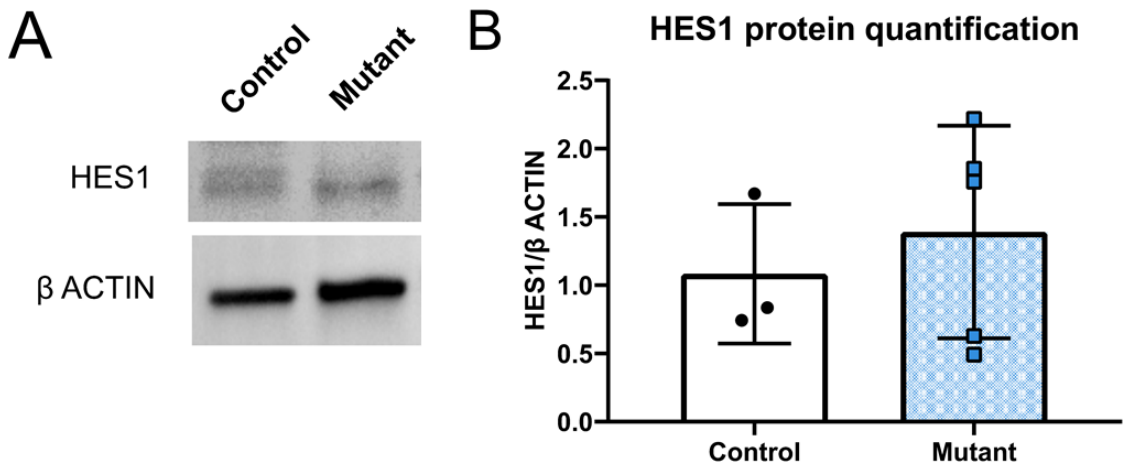

Figure 13. HES1 shows no significant changes in mutant mice. (A) Representative western blot of HES1 in control and mutant mice. (B) Quantification of HES1 protein shows no significant changes in mutant mice. NS = P > 0.05. N = 5 mice.

There was no significant change in HES1 protein in individual mice, as mice samples that showed high levels of NICD did not correlate with increased levels of HES1.

In summary, there are significant decreases in secretory IECs paired with an increase in absorptive IECs in mutant mice compared to control mice. Additionally, there were significant decreases in secretory IEC differentiation factors *Atoh1, Hnf1*β, and *Klf4*. Finally, ATOH1 protein was found to be significantly reduced in mutant mice, which also displayed increased NOTCH1 signaling when compared to control mice (Figure 14).

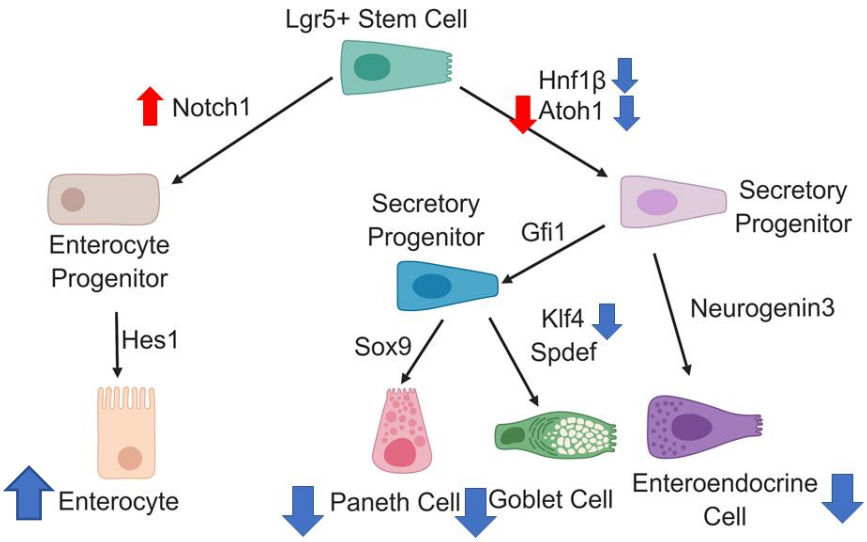

Figure 14. Summary of Impaired IEC Differentiation in Mutant Mice. EpCAM mutant mice were found to host impaired secretory IEC differentiation, leading to decreased secretory IEC populations and an increase in absorptive IECs.

IV. Discussion

In this study, we have demonstrated that secretory cell populations are severely disturbed in CTE model mice. Paneth, goblet, and enteroendocrine cells were all diminished within the intestinal epithelium, with each of the three populations representing only half of their expected values. This reduction in Paneth and goblet cells is in line with initial studies using human biopsies of patients with CTE, indicating that our mouse model does recapitulate other aspects of the disease in addition to the pathology (19).

As the reduction was relatively uniform across all three cell types, it is possible that an early progenitor stage is being disturbed and would then affect all downstream progenitors similarly. This rationale is supported by the significantly decreased ATOH1 levels at both the mRNA and protein levels. ATOH1 is an early differentiation factor for secretory cell types and its downregulation could be a possible cause for a loss of progenitors. Loss of secretory cells have been associated with other diseases or prolonged infections. In Inflammatory Bowel Disease, decreases in both Paneth and goblet cells have been noted (20, 21). Changes in Paneth cell numbers can be particularly stressing on the intestines, as one of their roles is secretion of factors to maintain the stem cell niche. Likewise, goblet cells are responsible for the generation of mucous membranes for the intestines, which is necessary for protection of the epithelial lining, but is also vital in commensal bacteria interactions. Commensal bacteria have been shown to interact with the glycoproteins secreted by goblet

cells as part of a signaling network to prevent pathogen response. Although the mucosal lining has not been studied in CTE patients or model mice, if there is a reduction in the lining preventing commensal bacteria from colonizing the intestines properly, impaired immune response as well as nutrient absorption and generation may be affected.

Notch signaling was also shown to be increased in the mutant mice as compared to the control mice, indicating that the stem cell niche is being driven towards an even more enterocyte dominated population. However, there was no according increase in HES1 protein to accompany the increase in Notch signaling; this may be due to HES1 being a repressor for its own transcription and therefore HES1 protein levels oscillate as a result.

It is noted that there were no decreases in mRNA product for *Sox9* and *Neurogenin3*. This finding was surprising given the decreases in Paneth and enteroendocrine cells found in mutant mice. It is possible that since early secretory progenitor formation could have been disrupted on account of the decrease in *Atoh1*, terminal differentiation factors may not be activated as a result. However, *Klf4* did show a decrease at the mRNA level, which suggests an alternative explanation may be needed. *Klf4* does play a role in many different functions in the intestines, such as mislocalization of Paneth cells to the upper crypt region and has been shown to affect the number and migration rates of epithelial cells, indicating other possible pathways may play a role in *Klf4* regulation outside of the intestinal stem cell differentiation.

However, this model is slightly imperfect, as the disease itself presents neonatally. Using adult mice which have already formed proper intestinal physiology prior to induction of mutant EpCAM may have different effect from mice expressing mutant EpCAM constitutively. While there are some differences in architecture and homeostasis between adult and infant mice, there do not appear to be major differences between the two in intestinal tissue renewal. In the future, studies should be done to confirm the findings from these experiments in infant mice.

The exact role of EpCAM in the changes in differentiation is unclear from our current experiments. Further mechanist studies to understand this are needed. Unlike other cell-cell signaling pathways, Notch signaling is unique in that it requires direct cell contact in order to initiate the signal transduction. As EpCAM generally weakens cell-cell interactions, it is possible that mutant EpCAM does not get trafficked to the cell membrane and cannot play a role in weakening these interactions, there may be increased opportunity for inappropriate NOTCH1 signaling as a result as Notch signaling is depending on cell-cell contact. In order to dampen this response, a γ-secretase inhibitor such as DAPT, which would prevent the cleavage of the intracellular domain of NOTCH1, could be used to attempt to reverse this aspect of the mutant phenotype. Alternatively, ATOH1 could also be explored as a possible therapeutic avenue in an attempt to boost secretory progenitor levels directly. The use of an enteroid model could also prove fruitful in determining the exact

role of the mutant EpCAM. As an *ex vivo* model of the intestines, it is possible to transfect enteroids expressing mutant EpCAM with wild-type EpCAM to determine whether the presence of mutant EpCAM or lack of wild-type EpCAM is causing the defects in cell differentiation.

The delicate balance of intestinal renewal necessitates a tightly controlled proliferation and differentiation process within the intestinal crypts. In this study, mutant EpCAM has been shown to disrupt the differentiation of intestinal stem cells by decreasing secretory IEC differentiation factors, leading to a reduction of secretory populations in mutant mice.

All sections of this thesis are currently being prepared for submission for publication of the material. Rabalais, John; Das, Barun; Okamoto, Kevin; McGeough, Matthew; Das, Soumita; Barrett, Kim; Sivagnanam, Mamata. The thesis author was the primary investigator and author of this material.

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