UNIVERSITY OF CALIFORNIA, SAN DIEGO

A deficiency in the congenital tufting enteropathy gene, *EpCAM*, results in intestinal barrier and ion transport dysfunction

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by

Philip Andrew Kozan

Committee in charge:

Kim E. Barrett, Chair
James Golden, Co-Chair
Mamata Sivagnanam
Ronald Marchelletta
Randy Hampton

2014

The Thesis of Philip Andrew Kozan is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

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All sections of this thesis are currently being prepared for submission for publication of the material. Kozan, Philip; McGeough, Matthew; Marchelletta, Ronald; Barrett, Kim; Sivagnanam, Mamata. The thesis author was the primary investigator and author of this material.

ABSTRACT OF THE THESIS

A deficiency in the Congenital Tufting Enteropathy gene, EpCAM, results in Intestinal Barrier Function and Ion Transport dysfunction

by

Philip Andrew Kozan

Master of Science in Biology

University of California, San Diego, 2014

Professor Kim E. Barrett, Chair

Professor James Golden, Co-Chair

Congenital tufting enteropathy (CTE) is a devastating diarrheal disease seen in infancy associated with villous changes and the appearance of epithelial tufts. Sivagnanam et al., found mutations in epithelial cell adhesion molecule (*EpCAM*) to be the causative factor in CTE. We developed a knock-down cell model of CTE through transfection of an *EpCAM* shRNA construct into T84 cells to elucidate the *in vitro* role of EpCAM in barrier function and ion transport. Transfected cells exhibited decreased electrical resistance, increased

permeability, and decreased ion transport as assessed through the use of western blotting, immunohistochemistry and Ussing chambers. An *in vivo* mouse model, based on a mutation found in CTE patients, was developed allowing for inducible deletion of exon 4 in *Epcam* resulting in a mutant protein with decreased expression. Tamoxifen-induced *Epcam*$^{\Delta 4/\Delta 4}$ mice demonstrated pathological features of villous atrophy, epithelial tufts and intracellular gaps, similar to human CTE patients, within four days post-induction. *Epcam*$^{\Delta 4/\Delta 4}$ mice also showed decreased expression of tight junctional proteins, increased permeability and decreased ion transport in the intestines. These findings, together with the knock-down model, reveal potential underlying disease mechanisms in CTE.

I. Introduction

Gastrointestinal Barrier Function

The lumen of the small and large intestines is comprised of the mucosa which is lined by epithelial cells. The mucosa must establish a robust barrier between the outside intestinal flora and the submucosa and concurrently be responsible for absorption of water and nutrients and ionic homeostasis. A selectively permeable barrier is required for the maintenance of these critical functions (1).

The barrier formed between epithelial cells is partly comprised of the apically located tight junction complex. Tight junctions can be split into two general categories: cytoplasmic and integral membrane proteins. They are composed of tetra-spanning membrane proteins such as the occludins and Claudins, which contain N and C terminuses residing in the cytosol and extracellular loop regions, junctional adhesion molecules, and zonula occludens (ZO). ZO are vital proteins known to link membrane proteins, occludin and claudin, to the actin cytoskeleton together. In the absence of ZO's, cells can fail to form tight junctions (2,3). Occludin is important in regulating paracellular barriers through the C terminus binding to ZO-1 allowing the localization of occludin to tight junctions (4,18). Claudins most likely form the essential backbone of tight junctions because occludin does not absolutely have to be present for the formation of tight junctions (24). Lastly, apart from tight junctions

being vital in barrier function, tight junctions can affect cellular proliferation and metastasis inhibition (3).

Areas that are critical in absorption and secretion are found in specialized structures within the small and large intestines. The intestine has a large capacity for fluid exchange due to the large surface area created by folds in the mucosa, villi and crypts, and the presence of microvilli on epithelial cells (20). The small intestine contains villi pointing into the lumen and crypts, located at the base of villi, while the large intestine is lined with surface epithelial cells and colonic crypts. Absorption in the small intestine is accomplished through various co-transporters including Na^+ coupled nutrient absorption and electroneutral NaCl absorption which in turn creates osmotic pressure drawing water from the lumen through tight junctions. Similarly, in the colon there exist electrogenic Na^+ absorption and electroneutral NaCl absorption on epithelial cells which draws in water through tight junctions (20).

Ion Transport

In addition to a properly formed cellular monolayer, ion transport plays an important role in barrier function. Abnormalities in intestinal transport can lead to problems in water and electrolyte absorption and secretion resulting in diarrhea. The majority of fluid crossing the gastrointestinal barrier is due to the active transport of Na^+, K^+, Cl^-, HCO_3^-. The active transport of Na^+ and Cl^- is dependent on a variety of channels. Na^+ can be taken up by a co transporter of Na^+ and glucose and a Na^+/H^+ anti-porter. Cl^- is primarily absorbed by anion exchanger

DRA. It is necessary to have functional Na^+/K^+ ATPase pumps and the indirect involvement of basolateral K^+ channels and Cl^- for absorption to work (20, 22, 23).

The apical membrane contains essential Cl^- secretion channels, Cystic fibrosis transmembrane conductance regulator (CFTR) being one of them. In the presence of elevated cyclic AMP (cAMP), Protein Kinase A becomes elevated and in turn phosphorylates CFTR, causing Cl^- secretion into the lumen. The basolateral Na^+ K^+ $2Cl^-$ (NKCC1) cotransporter serves as a rate limiting step in Cl^- secreting epithelia, limiting the amount of Cl^- ions entering the cell. Taken together with the Na^+/K^+ ATPase pump, these basolateral proteins work together in controlling the amount of Cl^- transported into and secreted out of epithelial cells (22).

Diarrhea

The net fluid movement across the epithelial cell barrier can be disturbed due to a variety of reasons including genetics, pathogens, and spontaneous dysregulation lead to either problems in absorption, secretion or both (23). Secretory diarrhea is when there is an increase of cell secretion into the lumen which results in water being pulled into the lumen. For example cholera toxin produces a secretory diarrhea by activation of CFTR channels with cAMP resulting in massive chloride out flux and thus drawing water into the lumen (25).

Diarrhea can also be caused by changes in paracellular transport and tight junctions. Bacteria, viruses and parasites may play a role in disrupting cellular tight junctions by affecting specific tight junction proteins, occludin, ZOs, claudins and/or the association between the actin cytoskeleton. For example, a change in the expression/localization of claudins would affect the ability of tight junctions to form a tight barrier (24). A disruption in expression of these could drastically cause changes in paracellular transport resulting in a leaky barrier (22). Intestinal epithelia normally have some amount of paracellular flux but when the size selectivity for ions diminishes, ions such as Na^+ and Cl^- are able to go back out into the lumen (26) which would draw water out as well.

Congenital Tufting Enteropahy

Congenital Tufting Enteropathy (CTE) is a severe intractable diarrheal disease presenting in the neonatal period with chronic watery diarrhea, imbalances in electrolyte levels, and impaired growth. The prevalence of CTE is thought to be at 1/50,000 - 1/100,000 live births in Western Europe (27). CTE is an autosomal recessive disease, often seen in families with a history of consanguinity (32). A diagnosis of CTE is made with histological recognition of changes in the villi within the small intestinal epithelium. CTE is usually accompanied by villous atrophy, crypt hyperplasia, formation of focal epithelial tufts (bunching of enterocytes) in the small intestinal and colonic mucosa, and absent or mild inflammation (5,31). CTE often results in intestinal failure, and patients must therefore rely on total parenteral nutrition to receive necessary

caloric intake for growth and development (6). Periods of prolonged parenteral nutrition are not optimal due to both quality of life and health considerations such as vascular complications and liver disease (7,8).

Genetic bases of CTE

Sivagnanam *et al.* identified mutations in EpCAM as causative in CTE and this has been confirmed by other groups (9,35,36,37). CTE patients express mutant EpCAM at significantly decreased levels in biopsied intestinal tissue as seen by immunohistochemistry and western blot analysis (9). Staining with an antibody to EpCAM , MOC31, has been established as a diagnostic tool to determine if patients have CTE (31).

Epithelial Cell Adhesion Molecule (EpCAM)

EpCAM is part of the family of cell adhesion receptors that control cellular communication (33). EpCAM was first recognized as an antigen overexpressed on human carcinoma cells in the digestive tract, breasts and kidneys (10). In healthy adult tissue, EpCAM is typically expressed on the basolateral surface of simple and pseudostratified epithelial cells in the gastrointestinal and respiratory tracts and the reproductive system. EpCAM has been shown to be involved in cellular adhesion and proliferation but its exact role in intestinal function has not been fully elucidated (11, 12). In further elucidating the role of EpCAM in tight junctions, EpCAM has been shown to co-localize with tight junctions and the

actin cytoskeleton through two binding sites for α-actinin within a short intracellular domain (34).

T84 cell and murine models

The T84 colonic adenocarcinoma cell line has been widely used as a model for studies of epithelial electrolyte transport. When T84 cells are grown as a monolayer they form tight junctions and have unidirectional secretion of chloride through apical channels when stimulated with secretagogues, creating a model for secretory crypt cells within the colon (16). In this study, we generated an shRNA-transfected T84 cell line targeting *EpCAM* to create a stable knock-down (KD) model to elucidate EpCAM's role in tight junction formation between crypt-like cells, as well as its role in secretory function. Because EpCAM is expressed in both the small and large intestines within CTE and normal patients (31), knocking down EpCAM in colonic T84 cells represents an appropriate *in vitro* model to investigate mechanisms of intestinal dysfunction that might account for diarrhea in the absence of EpCAM.

EpCAM is highly conserved in mammals and its distribution in murine models is very similar to that of human EpCAM, with the highest expression being in the intestines (14). The laboratory of Professor Sivagnanam previously created a constitutive mouse model of CTE. This involves a mutation that corresponds to an *EpCAM* mutation found in patients, in which a homozygous G>A substitution at the donor splice site of exon 4 results in a mRNA splice product lacking exon 4 (13, 17). Constitutive mice display intestinal pathology

similar to CTE patients. Their lifespan is less than one week, limiting ability to pursue further studies. Our newly developed murine model allows for inducible deletion of exon 4 in *Epcam* to further elucidate the *in vivo* role of EpCAM in barrier formation and electrolyte transport within the intestines of adult mice. With the inducible mouse model, we were able to validate the T84 cell model in studying mechanisms that bear on the emergence of CTE. We hypothesized that both the cell knockdown and murine models would exhibit attenuated expression of tight junctional proteins, altered ion transport, and increased permeability of the intestinal barrier.

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II. Methods

Animals and development of inducible Epcam$^{\Delta 4/\Delta 4}$ mice

The generation of our *Epcam* targeting construct was developed as previously described (13). Mice homozygous for the mutant Epcam Δ4 construct in which the neomycin-resistant positive selection marker had been removed were bred to B6.Cg-Tg(Cre/Esr1)5Amc/J mice obtained from (Jackson Labs, Bar Harbor, ME) to allow for Cre-LoxP recombination and efficient deletion of exon 4 in *Epcam* following administration of tamoxifen (43). Mice were orally gavaged with 25 mg/kg tamoxifen free base (MP Biomedicals, Solon, OH) in 90% sunflower seed oil from *Helianthus annus* (Sigma, St. Louis, MO) /10% ethanol once daily for two consecutive days generating *Epcam*$^{\Delta 4/\Delta 4}$ mice. The UCSD Institutional Animal Care and Use Committee approved all protocols.

Cell culture

T84 colonic cells were cultured in 1:1 Dulbecco's modified Eagle's Medium/F-12 Ham's medium with 15 mM L-glutamine (Corning Cellgro), 5% Bovine Calf Serum (Invitrogen), 1% Penicillin-Streptomycin (Corning Cellgro) and maintained according to a standard protocol (21).

Transfection of cells was performed according to recommended procedure (Life Technologies). As a control, T84 cells were transfected with scrambled shRNA (Life Technologies) and a KD cell line was developed with transfecting shRNA specifically targeting transcription of *EpCAM*. In order to retain the

phenotype in cells transfected with shRNA, 40 µg/mL G-418 Sulfate (Life Technologies) was used as a selective agent since the shRNA construct used carried G418 resistance.

For resistance and permeability studies, cells were grown on 12 mm Millicell-HA culture plate inserts (semipermeable inserts). 500,000 cells were added per insert. Cells were maintained with media as previously described (21). Cell culture media was changed every 3 days for approximately 2 weeks until cells formed a monolayer and were ready for studies.

Electrical resistance

To assess the formation of a monolayer, a voltohmmeter (Millipore) was used to measure the transepithelial electrical resistance (TER) between the apical and basolateral sides of the monolayer. Once the TER reached a value of 1000 Ohms, typical of wild type (WT) T84 cells, TERs were recorded between the control and KD cell monolayers.

Epcam$^{\Delta 4/\Delta 4}$ and control mice were gavaged with tamoxifen and then were sacrificed on day 5. Subsequently, the resistance of intestinal tissues from control and *Epcam*$^{\Delta 4/\Delta 4}$ mice was assessed by mounting them in Ussing chambers (Physiological Instruments) and imposing a 10 mV pulse under short-circuited conditions, and applying Ohm's law.

Permeability

When a mature monolayer of KD or control T84 cells was formed and confirmed by a TER measurement of 1000 Ohms, a permeability experiment fluorescein isothiocyanate-dextran (FITC-dextran) 4kD (Sigma-Aldrich) was performed. Cells grown in the semipermeable inserts had 200 µL of cell culture media of the apical side replaced with media containing FITC-dextran (1 mg/mL). At each hour thereafter for three hours, 200 µL of cell culture media was removed from the basolateral side and the media was replaced accordingly.

After *Epcam*$^{\Delta 4/\Delta 4}$ and control mice were gavaged with tamoxifen, they were sacrificed on day 5 and distal ileum and proximal colon tissue was mounted in Ussing chambers (Physiological Instruments). FITC dextran-4kD was added to the serosal side (1 mg/mL) and 100 µL of Ringers solution was taken from the mucosal side each hour thereafter.

All samples were analyzed with a Spectramax Plus384 Absorbance Microplate reader (Molecular Devices) calibrated with a standard curve.

Ion transport

To measure active ion transport, either cells grown on semipermeable membranes or distal ileum and proximal colon tissue from *Epcam*$^{\Delta 4/\Delta 4}$ mice and control animals were studied with the use of Ussing chambers (Physiological Instruments) and a Power lab amp (AD Instruments). A standard Ringer's solution composed of 6.5g NaCl, 0.42g KCl, 0.25g $MgCl_2$, and 1 mole $NaHCO_3$ was used, along with bubbled O_2. To ensure a stable barrier, a single 10 mV

pulse was administered upon mounting of cells. Once mounted into the chambers, inserts/tissue were allowed 20 minutes of equilibration. Changes in short circuit current (ΔIsc) were measured after exposure to forskolin (fsk), an activator of CFTR through the cAMP pathway and carbachol (cch) an activator of Cl^- secretion through a Ca^{2+} dependent pathway. The readings from the Ussing Chamber were recorded using LabChart software (AD Instruments).

Western blotting

Cells from inserts containing T84 monolayers were suspended in ice-cold lysis buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 20 μM NaF, 1 mM EDTA, 1 μg/ml antipain, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM $NaVO_3$, and 100 μg/ml phenylmethylsulfonyl fluoride), vortexed thoroughly, and subjected to lysis using a 22-gauge needle. Cells were centrifuged at 10,000 rpm for 10 min to remove insoluble material, and an aliquot was removed from each sample to determine protein content (Bio-Rad protein assay according to the manufacturer's instructions). Samples were resuspended in loading buffer (50 mM Tris (pH 6.8), 2% SDS, 100 mM dithiothreitol, 0.2% bromphenol blue, and 20% glycerol) and boiled for 5 min.

Samples of distal ileum and proximal colon were placed in 10% NP-40 detergent buffer and lysed using a mini bead beater (BioSpec Products). The lysate was centrifuged and the supernatant containing protein was removed. The lysate was then assayed (Bio-rad protein assay according to recommended instructions) for protein using a SpectraMax instrument (Molecular Devices). With

a 5 to 1 ratio of lysate to loading dye, samples were boiled and loaded into a SDS-PAGE gel (BioRad), electrophoresed, and subsequently transferred onto a nitrocellulose membrane. Membranes were blocked with 5% Bovine Serum Albumin/TBST for subsequent detection of occludin, EpCAM, or ZO-1, and with 5% Dry milk/TBST for detection of NKCC-1 or CFTR.

Western blotting was performed using mouse antibodies to occludin and EpCAM (Life Technologies), goat antibodies to NKCC-1 (Santa Cruz), rabbit antibodies to ZO-1 (Life Technologies) and CFTR (GenTex) diluted 1:1000. A mouse monoclonal antibody to β-actin (Sigma Aldrich) was used to identify the loading control, diluted 1:2000. Horseradish peroxidase-conjugated anti-mouse, anti-rabbit and anti-goat IgG (Cell Signaling Technologies) secondary antibodies were used at 1:2000 dilution. A semiquantitative measurement of band density was performed using Scion Image for Windows software.

Q-PCR

Total RNA from T84 cells was isolated using RNeasy Mini kits (Qiagen) and distal ileum and proximal colon were excised and placed in TRIzol (Invitrogen). First strand cDNA was synthesized with SuperScript VILO (Invitrogen) using the recommended protocol. Real time PCR reactions were set up by using FastStart Universal SYBR Green Master Mix (Life Technologies) and thermal cycling performed on a StepOnePlus Real-Time PCR System using Step One software v2.0 (Applied Biosystems). Primers were designed using IDT software and ordered from IDT (Integrated DNA Technologies). All primers were

diluted to a concentration of 100μM. GAPDH and villin were used as endogenous controls.

Immunofluorescence

Control and EpCAM knockdown T84 cells were grown on glass coverslips sterilized with 70% ethanol and a Bunsen burner flame. The cells were washed with PBS and fixed with 3.7% paraformaldehyde for 30 minutes at room temperature. Cells were blocked using 5% Bovine serum albumin/PBS and then incubated at 4^{o}C with antibodies to actin (SigmaAldrich), NKCC-1 (SantaCruz), or EpCAM (Life Technologies) (1:200) overnight. Secondary detection was performed by incubation with Alexa Fluor 568-conjugated donkey anti-mouse antibody, Alexa Fluor 488-conjugated goat anti-rabbit antibody (1:200, Jackson Immunoresearch Laboratories). Nuclei were stained with Hoechst 33258 (Invitrogen).

Distal ileum and proximal colon were excised and fixed with 4% paraformaldehyde for 24 hours at room temperature, then paraffin-embedded and sectioned onto glass slides. De-paraffination was done with xylene, graded concentrations of ethanol, and samples boiled in 10mM sodium citrate buffer for 20 minutes. Blocking was done as previously described (39). Specimens were incubated with antibodies (1:200) to NKCC1 (SantaCruz) and actin (SigmaAldrich) for 1 hour. Secondary detection was done with conjugated secondary antibodies, Alexa Fluor 568 donkey anti-mouse (1:200), Alexa Fluor

488 donkey anti-rabbit (1:200). Nuclei were stained with Hoechst 33258 (Invitrogen).

Statistical Analysis

Linear regression, one-way Anova, and t-test analyses were performed with GraphPad Prism version 2.00 for Windows (GraphPad Software).

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III. Results

EpCAM knocked down in T84 cell model

The T84 cell line used in this study has previously been shown to be an excellent model in which to study ion transport and barrier function within the colon (16). To determine the role of EpCAM in barrier function, a knock-down model utilizing this cell line and EpCAM targetting shRNA was generated. To ensure that the decrease in EpCAM was not due to non-specific activity of the shRNA, cells transfected with scrambled shRNA were used as a control, and compared to shRNA transfected cells specific to EpCAM. Protein expression was quantified through western blotting showing decreased levels of EpCAM in KD cells (Fig. 1A). Densitometry of the results from several samples showed that the decrease in EpCAM was statistically significant.

Expression and localization of EpCAM between control and KD cell cultures was compared using confocal microscopy. We found that there was a decrease in staining intensity for EpCAM in KD cells (Fig. 1B). Normally Epcam is contained at tight junctions as observed in control cells. In KD cells however it became unclear as to where Epcam was localizing (Fig. 1B).

Inducible Epcam$^{\Delta 4/\Delta 4}$ mice exhibit intestinal defects characteristic of CTE

Five days after oral gavage with tamoxifen, small intestinal tissue from Epcam$^{\Delta 4/\Delta 4}$ mice was isolated and processed for western blotting. This revealed a significant decrease in Epcam protein expression compared to wild-type mice

(Fig. 1C). Small intestinal tissue was also evaluated using H&E staining. *Epcam*$^{\Delta 4/\Delta 4}$ mice displayed several characteristics of CTE including villous atrophy, bunching of enterocytes and formation of tufts on villi (Fig. 1D). Quantitatively there was a significant decrease in overall villus length (Fig. 1E).

Knockdown of EpCAM results in barrier dysfunction

T84 KD and WT cells were grown on semipermeable inserts and evaluated for their electrical resistance (TER), permeability, and expression of tight junctional proteins. Measurement of TER revealed a two-fold significant decrease (Fig. 2A) in KD cells compared to the WT cell line.

Permeability measurements with FITC dextran-4kD were taken each hour, up to three hours after the addition of FITC dextran-4kD to the apical side of cells, KD cells showed significant increases in FITC dextran-4kD permeability compared to WT cells (Fig. 2B).

To investigate molecular events that might account for the defects in barrier function, two critical tight junctional proteins were examined, occludin and zonula occludens-1 (ZO-1). Through the use of western blotting, protein levels for both occludin and ZO-1 were decreased in KD compared to WT cells (Fig.3A, B). Occludin and ZO-1 were also studied at the transcriptional level using qPCR, revealing an a significant decrease of mRNA levels for occludin in KD cells (Fig.3C). However, no significant change in mRNA for ZO-1 was appreciable (Fig. 3D).

Tissues from *Epcam*$^{\Delta 4/\Delta 4}$ mice, along with those from wild-type controls, were subjected to permeability measurements with FITC dextran-4kD. At 1, 2, and 3 hours, distal ileum and proximal colon from *Epcam*$^{\Delta 4/\Delta 4}$ mice exhibited significantly increased permeability to FITC dextran-4kD compared to findings in tissues from control animals (Fig. 4A).

To corroborate the findings in the murine and the T84 cell model, tight junctional proteins were investigated in the murine model. Western blotting revealed that occludin expression was decreased in both distal ileum and proximal colon in *Epcam*$^{\Delta 4/\Delta 4}$ mice (Fig 4, B) compared to controls. On the other hand, evaluation of ZO-1 mRNA levels by qPCR showed comparable expression in *Epcam*$^{\Delta 4/\Delta 4}$ and control animals (Fig. 4C).

Knockdown of EpCAM results in ion transport abnormalities

EpCAM KD T84 cells were found to have changes in ion transport. Cells mounted onto Ussing chambers revealed markedly decreased ΔIsc responses following exposure to the chloride secretagogues forskolin and carbachol, activators of cAMP- and Ca^{2+} dependent pathways, respectively (Fig 5A), compared to control cells.

To understand the basis of the decreased ΔIsc responses, protein levels of the two key transporters involved in chloride movement, CFTR and NKCC-1, were studied by western blotting. No appreciable difference was found in the expression of CFTR between WT and the KD cell lines (Fig. 5B). On the other

hand, NKCC-1, a basolateral ion transporter that loads Na^+, 2 Cl^-, and K^+ into the cell was decreased in KD cells compared with controls (Fig. 5C). Further investigation of NKCC-1 was done with confocal microscopy. Decreased fluorescence of NKCC-1 was observed in the KD cell line although its localization remained confined to the basolateral surface as seen in control cells (Fig. 5D).

Investigation of ion transport in tamoxifen-treated *Epcam*$^{\Delta 4/\Delta 4}$ mice revealed similar findings. Sections of distal ileum and proximal colon were mounted in Ussing chambers and stimulated with forskolin. The ability of this agonist to stimulate an increase in Isc was significantly attenuated in *Epcam*$^{\Delta 4/\Delta 4}$ mice compared to control animals (Fig. 6A).

CFTR and NKCC-1 were analyzed in comparable tissues by Western blotting. No significant difference in the expression of CFTR in *Epcam*$^{\Delta 4/\Delta 4}$ tissues compared with controls was appreciated (Fig. 6B), although there was significantly decreased expression of NKCC-1 within the small intestine (Fig. 6C). Similarly, immunohistochemistry revealed decreased fluorescence staining of NKCC-1 throughout the small and large intestines in *Epcam*$^{\Delta 4/\Delta 4}$ mice compared to controls, although NKCC-1 remained localized to the basolateral membrane (Fig. 6D).

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IV. Discussion

Our study highlights and clarifies the role of EpCAM in maintaining proper intestinal epithelial barrier function in terms of both permeability and ion transport. Congenital Tufting Enteropathy presents with a serious clinical phenotype that is severely debilitating to children. Through the use of *in vitro* and *in vivo* models of CTE, we have demonstrated that mutations in *EpCAM* lead to barrier formation abnormalities encompassing compromised resistance, increased permeability, tight junction integrity and ion transport.

Although the T84 cell line cannot model the absorptive aspect of the intestines, these cells are able to form a polarized monolayer with tight junctions (19). Our studies have shown that KD of EpCAM in T84 cells results in cells that were incapable of forming a robust monolayer compared to controls. Markers of this include the decreased TER measurements and increased permeability found in KD cells. In our inducible mouse model, $Epcam^{\Delta 4/\Delta 4}$ mice exhibited a severely leaky epithelial barrier. In light of these results, a mechanistic approach to understand barrier dysfunction was taken by evaluating the expression of key tight junction proteins. ZO-1, a protein essential for the assembly of tight junctions in epithelial cells (2,45) is known to interact with the C-terminus of occludin (40). This led us to investigate how mutations in EpCAM might affect occludin expression and function. In fact, overexpression of occludin has been found to cause increases in TER, highlighting its importance in the maintenance of the tight junction (41). Conversely, we found that both occludin and ZO-1

showed decreased expression in our models of CTE. Further studies aimed at quantifying mRNA expression also found decreases in occludin, suggesting that decreased expression of EpCAM reduces its transcription. However, no significant changes were found for the ZO-1 transcript, suggesting that a relative deficit in EpCAM expression results in changes at the posttranscriptional level. This warrants future studies into the protein-protein interactions of EpCAM. EpCAM has been previously shown to control the composition and function of tight junctions (44). Based on our immunohistochemistry of EpCAM localizing to tight junctions, it may be the case that EpCAM is required to stabilize occludin and ZO-1 at this site via protein-protein interactions. Similarly, the mechanisms underlying the effect on occludin transcription would be valuable to explore.

In furthering the study of EpCAM's role in intestinal function, we analyzed the effect of a deficit in EpCAM expression on ion transport. We found that in EpCAM KD T84 cells and in tissues from *Epcam*$^{\Delta 4/\Delta 4}$ mice, ion transport was attenuated. The method of comparing ion transport was to look at the ΔIsc- reflective of net ion transport. T84 cells model crypt cells of the colon, which are predominantly secretory cells. The main ion transporter on the apical side of these cells is CFTR, secreting Cl^- ions out into the lumen. CFTR also plays a major role in ion secretion within the distal ileum and proximal colon. The change in ion transport was studied by using agonists of chloride secretion: Forskolin, an activator of the cAMP-dependent pathway causing secretion through CFTR (42), and carbachol, an activator of a Ca^{2+} dependent pathway that activates a

basolateral K^+ channel, but also resulting in chloride secretion (46). The ΔIsc response to either of these agonists was attenuated in EpCAM KD cells vs. control cells as well as in *Epcam*$^{\Delta 4/\Delta 4}$ mouse distal ileum and proximal colon. In an attempt to understand why there was a decrease in ion transport when EpCAM was knocked down, ion transporters were studied. NKCC-1, a basolateral loader of Na^+, K^+, and 2 Cl^- ions, showed decreased protein expression in cell and murine models. Because forskolin and carbachol both have the same end result of Cl^- secretion, and both depend on NKCC-1 activity, the decrease in Cl^- loading ability of the cells caused by a decrease in NKCC-1 may explain the decrease in ion transport seen (38). The localization of NKCC-1 was also studied using immunohistochemistry. We found there to be a decrease in NKCC-1 fluorescence, confirming the decrease in protein expression. However NKCC-1 was still localized to the basolateral pole of the epithelium. Thus, the overall decrease in NKCC-1 abundance in the membrane, rather than mislocalization of the transporter, most likely causes the end result of decreased ion transport in the setting of reduced EpCAM expression.

During our studies of NKCC-1 with immunohistochemistry, we found that there was severe actin disorganization with KD-EpCAM cells. ZO-1 has been found to be important in linking membrane proteins to the actin cytoskeleton and is also known to associate with α-catenin (3,28). Recently, it was found that when there is a disruption in the ZO-1 and α-catenin complex, actin disorganization occurs (29). Based on what we see in EpCAM KD cells how the decreased

expression of ZO-1, this suggest that EpCAM can have an effect on the ZO-1 and catenin complex which could result in the disorganization we observe. Additionally, EpCAM is also known to interact with the actin cytoskeleton via α-actinin and associates with actin by crosslinking at each end of the actin filament (30). The cobble stone pattern seen in control T84 cells is most likely a result of actin forming a belt-like structure at the tight junctions. EpCAM is known to directly associate with α-actinin (34) and thus the disorganization of EpCAM KD cells may be due to a disruption in α-actinin-EpCAM complex and the actin cytoskeleton.

Our study on EpCAM in cell and murine models has elucidated the important role that EpCAM plays in maintaining proper intestinal function. In the case of mutated or absent EpCAM, not only can a proper barrier not be maintained based on our permeability and resistance studies, but additionally there is dysregulation of ion transport and secretion. Our study demonstrates the role EpCAM plays in the formation of tight junctions, paracellular transport and regulating transcellular ion transport through epithelial cells. We have corroborated the findings of the cell model with an *in vivo* murine model of disease. The cell model retains its relevance as an important tool to allow the study the mechanisms of CTE quickly and efficiently. Our findings suggest that in CTE, the inability to form a robust barrier outweighs the decrease of chloride secretion. Normally, it would be expected that in diarrhea there would be an increase in ion transport, however the attenuated expression of tight junction

proteins suggests that paracellular flux becomes prominent which may explain the diarrhea phenotype seen. Our results will prompt future studies on the role of EpCAM in tight junction formation and how it may associate with the other major families of cell adhesion molecules. Our findings of actin reorganization also lead to investigation of EpCAM's effects on the actin cytoskeleton. Ultimately, a full understanding of how EpCAM disrupts the intestinal barrier may guide the creation of future therapies for CTE that target EpCAM or its downstream effects.

This section of the thesis is currently being prepared for submission for publication of the material. Kozan, Philip; McGeough, Matthew; Marchelletta, Ronald; Barrett, Kim; Sivagnanam, Mamata. The thesis author was the primary investigator and author of this material.

V. Figures

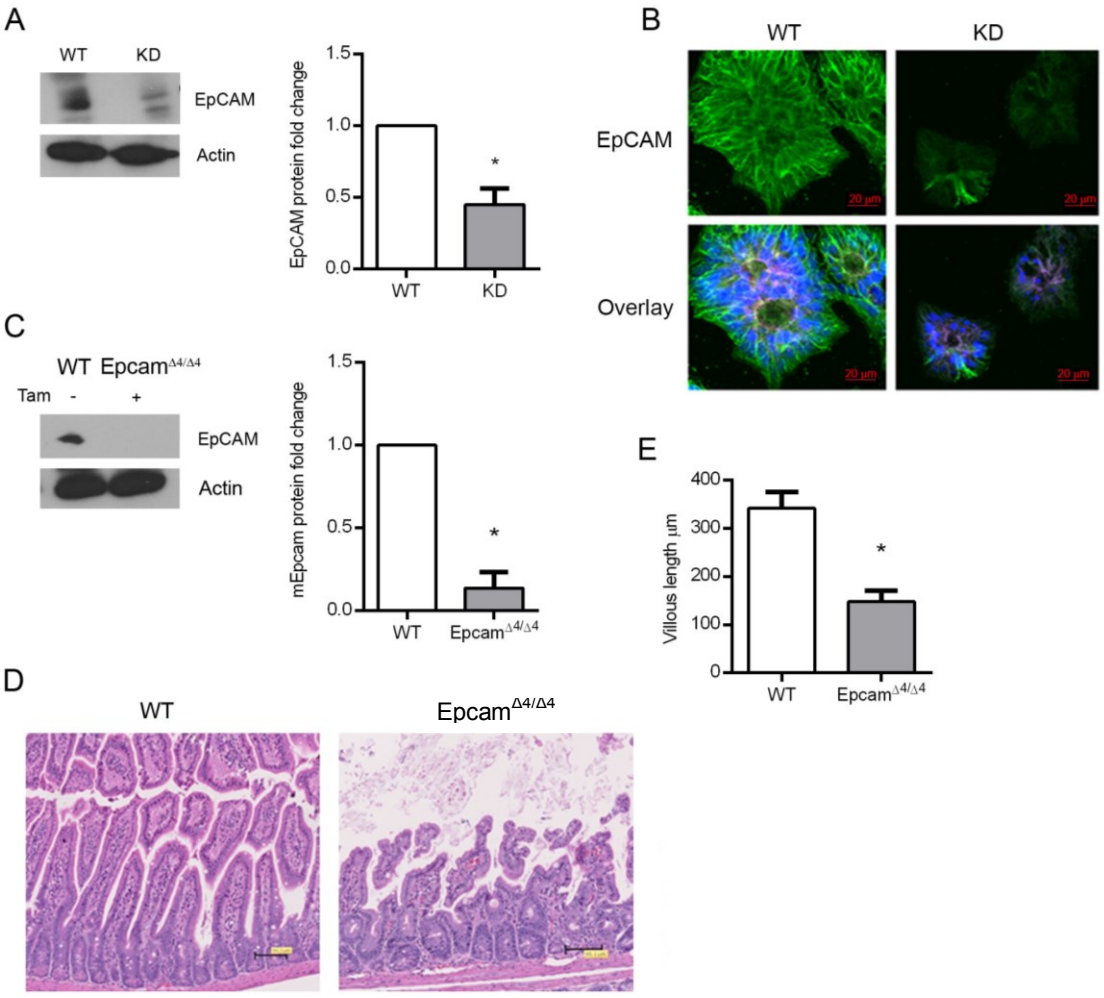

Fig 1. Targeting EpCAM in cell and murine models results in less EpCAM expression and morphological changes in murine intestine. A: Western blot demonstrating a decrease in protein expression of EpCAM in KD-EpCAM T84 cells along with quantification (*p<0.05, mean±SEM, n=3). B: Fluorescent immunohistochemistry demonstrating a decrease in EpCAM fluorescence in KD-EpCAM T84 cells. EpCAM was found to be localized to cellular tight junctions which was unchanged in KD-EpCAM cells. Scale bars represent 20 µm. EpCAM green, Actin purple, DAPI blue. C: Western blot of EpCAM and quantification shows decrease in mutant mice (*p<0.05, mean±SEM, n=3) D: H&E staining of ileum shows morphological changes in villi and crypt cells. E: Mice induced with tamoxifen demonstrated a decrease in villous length (*p<0.05, mean±SEM, n=6)

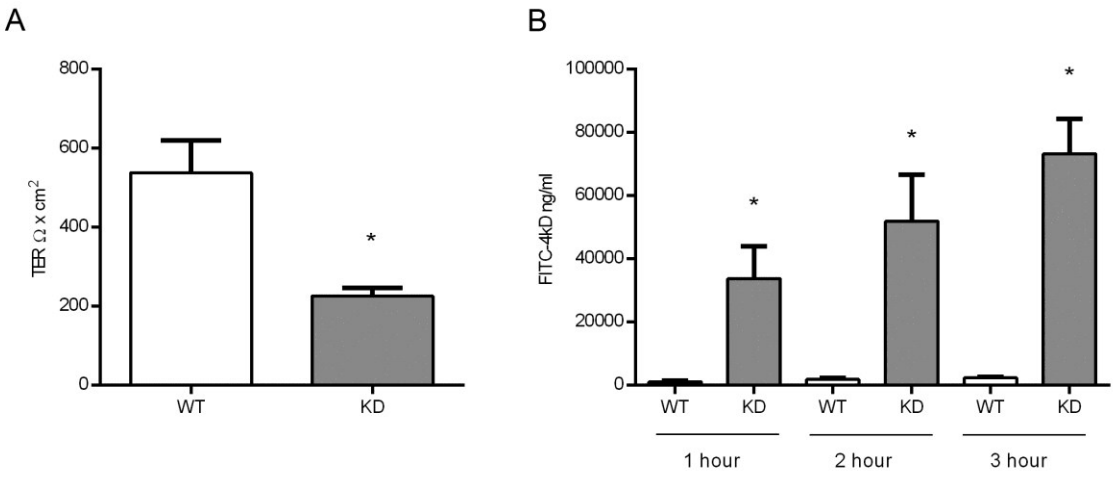

Fig 2. A knockdown of EpCAM attenuates TER and FITC - 4kD permeability. A: TER of T84 KD-EpCAM cells grown in semipermeable inserts was decreased compared with WT. (* $p<0.05$, mean±SEM, n=8) B: FITC dextran-4kD permeability study showed significantly increased FITC traversing the membrane in KD-EpCAM cells for each hour thereafter. (* $p<0.0001$, mean±SEM, n=6).

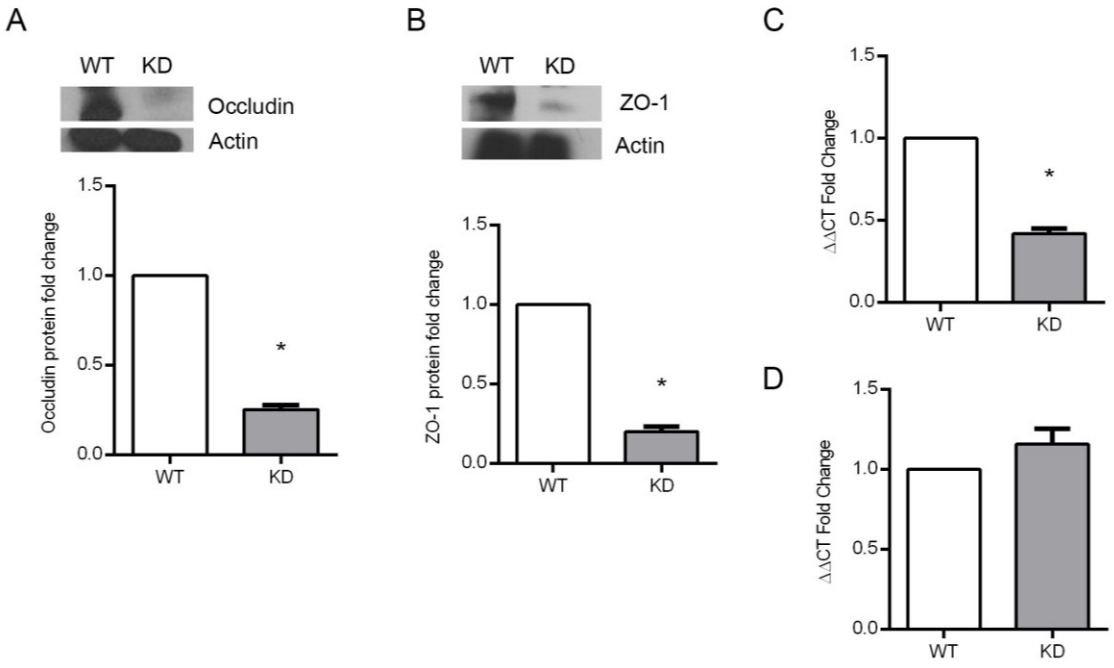

Fig 3. EpCAM knockdown shows changes in tight junction proteins. A and B: Decreased expression of occludin and ZO-1 tight junction proteins in KD EpCAM cell line compared with wildtype (*p<0.05, mean±SEM, n=3). C and D: Occludin (c) and ZO-1 (d) RNA levels by qPCR in KD EpCAM cell line compared with control T84 cell line. Only occludin was found to be decreased (* p<0.05, mean±SEM, n=3)

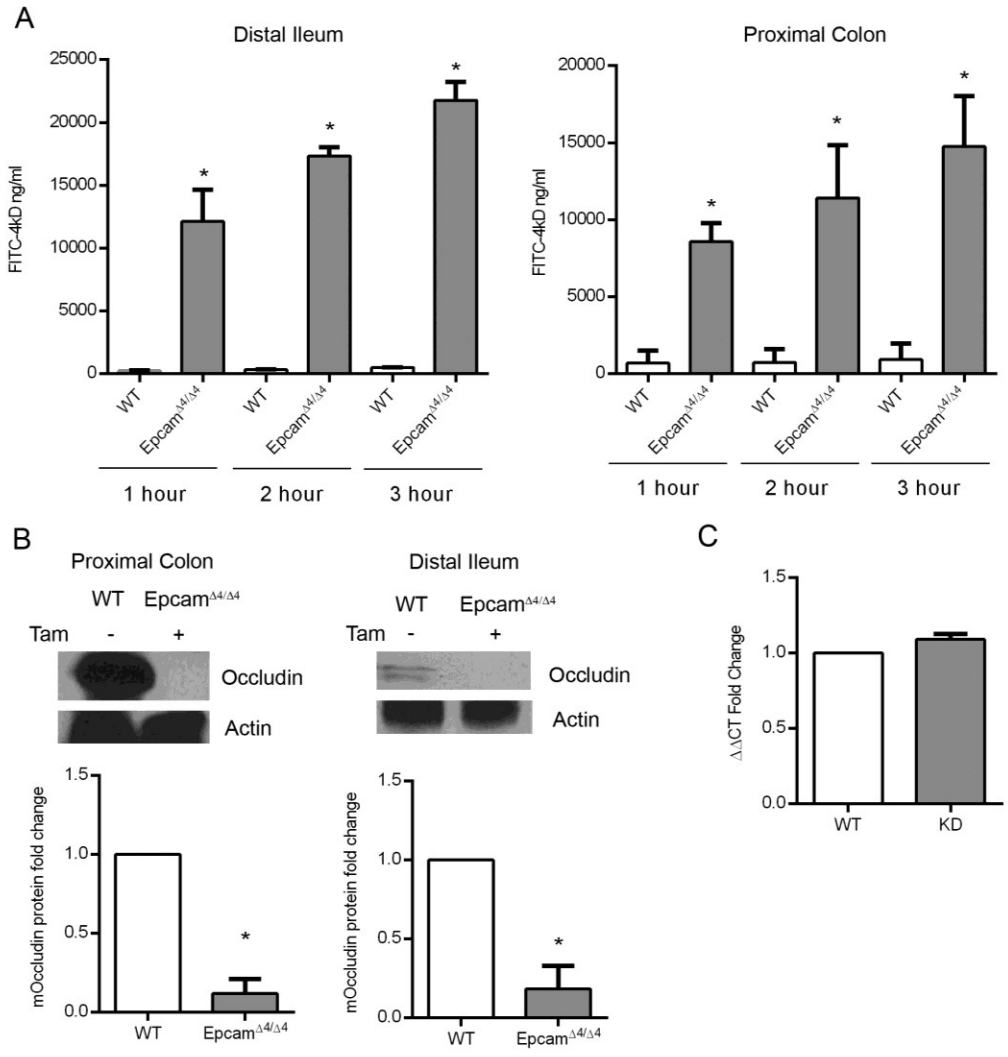

Fig 4. Mutated EpCAM in mice has deleterious effects on permeability and occludin. A.FITC dextran-4kD permeability study showed significant crossing of FITC in both distal ileum and proximal colon over 3 hours (*p<0.001, mean±SEM). B: Occludin protein levels in the proximal colon and distal ileum by western blotting and quantification of protein fold change are decreased in the induced mouse compared to the litter mate control. (*p<0.05, mean±SEM, n=3). C: ZO-1 RNA levels by qPCR in the small intestines of constitutive EpCAM$^{\Delta 4/\Delta 4}$ mice were unchanged compared to EpCAM$^{WT/WT}$.

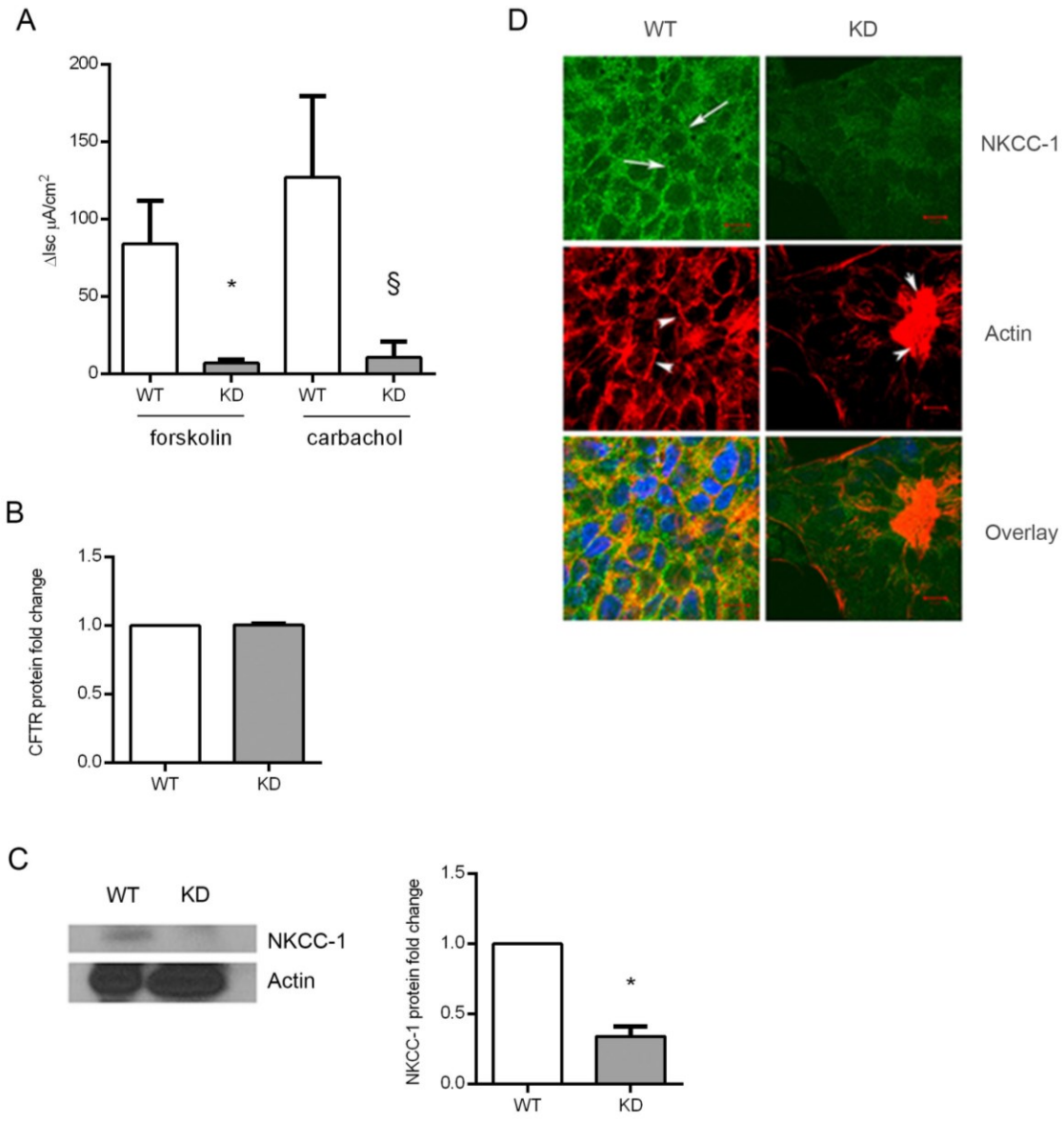

Fig 5. Kockdown of EpCAM results in decreased ion transport. A: ΔIsc measured in response to forskolin and carbachol in Ussing chambers show decreased response in KD EpCAM cells (*p<0.001, mean±SEM, n=6). B and C: Protein expression of CFTR and NKCC-1 measured by Western blotting quantified for protein fold change showed no change in CFTR (B) and a decrease in NKCC-1 (C) in KD EpCAM cells (*p<0.05, mean±SEM, n=3). D: Fluorescent immunohistochemistry of NKCC-1 showed normal localization and decreased fluorescence in KD EpCAM cells. Arrows point out difference in Actin organization. NKCC-1 green, Actin red, DAPI blue. Scale bars are 20 μm.

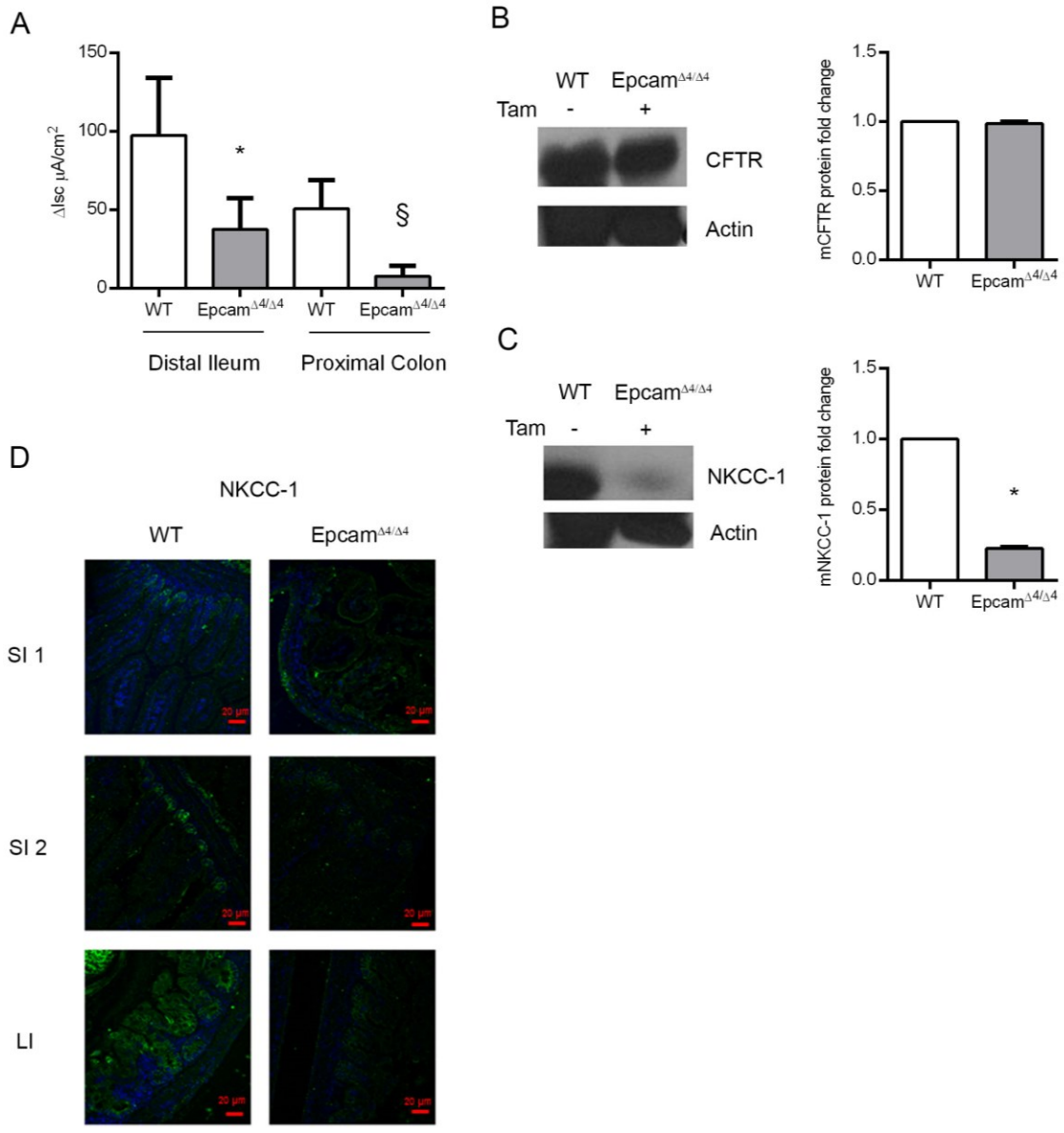

Fig 6. Mutated EpCAM causes ion transport dysfunction in the murine model. A: Δisc measured in response to forskolin in the distal ileum and proximal colon showed a decrease in mice with mutant EpCAM. (* § $p<0.05$, mean±SEM, n=5). B and C: Protein expression of CFTR and NKCC-1 measured in the small intestines by western blot showed and quantified protein expression change showed no change in CFTR (B), mutant mice had decreased NKCC-1 (C) (*$p<0.05$, mean±SEM, n=3). D: Immunofluorescence of NKCC-1 in the small and large intestines shows decreased fluorescence in mice with mutated EpCAM. NKCC-1 Green, DAPI blue, SI1: duodenum/jejunum, SI2: Ileum, LI: Large intestines. Scale bars are 20 μm.

This section of the thesis is currently being prepared for submission for publication of the material. Kozan, Philip; McGeough, Matthew; Marchelletta, Ronald; Barrett, Kim; Sivagnanam, Mamata. The thesis author was the primary investigator and author of this material.

VI. Tables

Type of Reactions	Name of Primer	Primer sequence
Mouse ZO1 RTPCR F	mZO1 forward	CATCTCCAGTCCCTTACCTTTC
Mouse ZO1 RTPCR R	mZO1 reverse	CCTCCAGGCTGACATTAGTTAC
Mouse Villin RTPCR F	mVillin forward	AGCTGCCATCTACACCACACAGAT
Mouse Villin RTPCR R	mVillin reverse	AGTCGCTGGACATCACAGGAGTTT
Cell ZO1 RTPCR F	hZO1 forward	CCTGAGTTTGACAGTGGAGTT
Cell ZO1 RTPCR R	hZO1 reverse	GCTGAAGGACTCACAGGAATAG
Cell Occludin RTPCR F	hOcc forward	GGTTCACTTCTCCCAGTCTTTC
Cell Occludin RTPCR R	hOcc reverse	AGACACAATCAACAGGGTTAGG
Cell GAPDH RTPCR F	hGAPDH forward	CATGTTCGTCATGGGTGTGAACCA
Cell GAPDH RTPCR R	hGAPDH reverse	AGTGATGGCATGGACTGTGGTCAT

Table 1: List of Primers. All primers were ordered from Integrated DNA technologies (IDT) and reconstituted with double DI water. The final concentrations of all primers was 100 μM.

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