MODELING DISEASE AND DEVELOPMENT WITH PLURIPOTENT STEM CELLS

MODELING DISEASE AND DEVELOPMENT WITH PLURIPOTENT STEM CELLS

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Descriptive Note

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Lay Abstract

The development of human *in vitro* models has allowed for the application of basic knowledge of disease mechanisms previously established in animal models. Recent advances in stem cell technology have enhanced researchers' ability to retrieve stem cells from patients in a non-invasive manner. Specific genetic changes found in these stem cells are often linked to specific diseases and provide unique opportunities to study disease progression outside of the human body (*in vitro* modelling). In our lab, we studied genes that may be linked to pulmonary fibrosis in order to determine the impact of these genes on the development and progression of this disease.

Previously, our lab has also been able to develop intestinal organoids, which are groups of cells that contain many of the cell types found in the human intestine. We sought to use these organoids to model human intestinal maturation by culturing them with native gut bacteria and examining any changes that occur. Development of these *in vitro* models will provide researchers with human models without the constant need for patient samples.

Abstract

With recent advances in stem cell research, the use of human pluripotent stem cells (hPSC) provides access to disease-relevant cells that are not easily accessible in patients. The ability of hPSCs to self-renew indefinitely and to differentiate into cell types of all three embryonic germ layers provides a model system to investigate the effects of genetic mutations that are relevant in the context of specific diseases. hPSC carrying disease associated genetic mutations provide access to an unlimited supply of disease-relevant tissue while healthy hPSC can be used to model normal human development.

Our lab has access to samples from a family with multiple cases of pulmonary fibrosis (PF). The high prevalence of PF in this family led us to believe that there is a strong genetic component to the pathogenesis of PF in these cases. We previously were able to derive iPSC lines from each of the patients and have identified genetic variants via exome sequencing, including PACS1 and ITGB6, that may predispose carriers to PF. In the present study, we examine the phenotypic changes caused by the PACS1 and ITGB6 gene variants. We hypothesized that the N217S mutation in PACS1 conveys a resistance to apoptosis in fibroblasts which could lead to the accumulation of fibrotic tissue. This hypothesis was tested through the use of apoptosis assays including annexin flow cytometry and immunostaining for caspase 3 cleavage after treatment with TNF-related apoptosis-inducing ligands. We found that in transfected cell lines, there were no significant changes to apoptotic sensitivity. The results are inconclusive in the patient-derived fibroblasts because of high inter-test variability. The second mutation we examined was the R15K mutation in ITGB6, which occurs within the signal peptide. Thus, we hypothesized that this mutation affects the efficiency of localization and subsequently, function. This hypothesis was tested through confocal microscopy of endogenous

ITGB6 in small airway epithelial cells (SAEC), as well as HEK 293 FT cells transfected with plasmid constructs containing GFP tagged wt or R15K ITGB6. We then tested for changes in the ability of ITGB6 to affect downstream signaling through plating on fibronectin, pulldown of focal adhesion kinase, and analysis for phosphorylated FAK through western blot. We found that endogenous expression of ITGB6 in SAECs, appears mainly in the membrane with a small percentage within the nuclear membrane. In transfected cell lines, there was a shift in localization from plasma membrane to nuclear membrane. The functionality of ITGB6 did not appear to be affected based on our preliminary data.

This thesis also explores the utility of intestinal epithelial organoids derived from hPSCs, termed "human enterospheres" (hEnS), in investigating the process that drives intestinal maturation. The hEnS provide a unique novel model for studying maturation, due to the fetal phenotype of the hEnS. We hypothesized that through changes in culturing medium and addition of bacterial products, it is possible to induce maturation of the hEnS. To test this hypothesis, we cultured the hEnS with heat-killed bacteria that play a role in the natural maturation process and examined changes to markers of maturation. We also performed an in-depth study of key maturation markers at specific gestational stages. By immunostaining the gestational tissue for various markers associated with maturation, we sought to create a scale with which to evaluate the maturation status of hPSC-derived intestinal tissue.

Acknowledgements

First and foremost, I would like to thank my supervisor Dr. Jonathan Draper for the opportunity to pursue my masters in his lab. Words cannot express my appreciation for your support and kindness over the past 2 years. I appreciate how you always made me feel as if I was never alone on this journey. Every time I would feel that the task was impossible, you would encourage me to leave my comfort zone and to try my hardest. Thank you for helping me grow not just as a scientist but also as a person. Thank you for never giving up on me.

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I would like to thank my parents for the love and support that they have given me through these years. I am always grateful for their interest in my work no matter how bad I am at explaining it in another language.

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List of Important Abbreviations

AEC2	Airway epithelial cell type 2
CHGA	Chromogranin A
CK2	Casein kinase 2
CLDN3	Claudin 3
DEFA	α-defensin
EEC	Enteroendocrine cells
FAK	Focal adhesion kinase
HDF	Human derived fibroblast
hEnS	Human enterosphere
hESC	Human embryonic stem cell
hPSC	Human pluripotent stem cell
ITGB6	Integrin β6
IPF	Idiopathic Pulmonary Fibrosis
iPSC	Induced pluripotent stem cell
ISC	Intestinal stem cells
LRGG	*Lactobacillus rhamnosus GG*
OLFM4	Olfactomedin 4
PDGF	Platelet-derived growth factor
PF	Pulmonary Fibrosis
SAEC	Small airway epithelial cell
SI	Sucrase isomaltase
TLR4	Toll-like receptor 4
TNFα	Tumor necrosis factor α
TRAIL	Tumor necrosis factor related apoptosis-inducing ligand
UEA1	Ulex Europaeus Agglutinin I

Declaration of Academic Achievement

Amos Lim performed all experiments including generating plasmid constructs, immunostaining for ITGB6, westerns for phospho-FAK, immunoprecipitation of FAK, Annexin flow for TRAIL induced apoptosis, immunostaining of gestational tissue, and intestinal organoid coculture experiments.

Dr. Jonathan Draper provided funding, helped with experimental design, analyzed data from whole exome sequencing and preliminary investigation into the mutations found.

Dr. Rohan Nadkarni provided support for experimental design and aided with experiments.

Dr. Soumeya Abed generated iPSC and HDF from patient blood samples, aided with analysis of whole exome sequencing data and preliminary investigation of the mutations.

Evaluation of Genetic Variants that may predispose for Familial Pulmonary Fibrosis

Introduction

Pulmonary Fibrosis

Pulmonary fibrosis (PF) is a late-onset interstitial lung disease characterized by scarring of the alveolar membranes where gas exchange occurs (Raghu et al., 2011). This condition is thought to be a result of the cycle of recurrent lung injury, inflammation and aberrant wound repair (Dave et al., 2009). Biochemical findings include the accumulation of fibroblast and myofibroblast cells (fibroblast foci), macrophage infiltration, deposition of excess matrix, malformation of the lung architecture, and the appearance of honeycomb cysts (Raghu et al., 2011). Although PF may occur secondarily to other disease processes, it can also arise spontaneously (Raghu et al., 2011). In these cases, it is known as idiopathic pulmonary fibrosis (IPF). The causes of IPF are unknown but a combination of genetic and environmental factors are thought to contribute to the progression of IPF (Grutters et al., 2005). There are several proposed risk factors which affect the appearance and progression of PF including smoking and inhalation of particulates such as silicates or dusts from metal and wood manufacturing. However, the main contributor is thought to be related to micro-injuries to the ageing alveolar epithelium which subsequently results in aberrant epithelial-fibroblast communication, accumulation of extracellular matrix, activation of myofibroblasts, and remodeling of the lung interstitium. Other causes discovered from genetic variants include telomere dysfunction and cellular stresses. IPF is one of the most frequent manifestations of telomerase-associated disease. Although there is no causal link established yet, shortened telomeres are found in IPF patients regardless of the presence of telomere-related genetic variants. Previously, the immune system was implemented in the initiation of injury and inflammation that drives fibrogenesis but treatment with anti-inflammatory drugs has no effect on disease progression.

Similar to the etiology of IPF, the pathogenesis of this disease is not yet fully elucidated. However, several papers have proposed mechanisms for the pathogenesis of this disease and the development of its characteristic fibroblast foci. These include: 1) epithelial to mesenchymal transition causing lung epithelial cells to adopt the phenotype of fibroblasts, 2) recruitment of bone marrow-derived fibrocytes, 3) activation of resident lung fibroblasts, and 4) stress induced differentiation of pericytes. There are also several genetic variants that contribute to IPF including genes that affect telomere function leading to premature cellular senescence, apoptosis and impairment of wound repair leading to fibrotic scars.

Genetic factors of pulmonary fibrosis

The most common genetic variant associated with IPF is in the MUC5B promoter. The variant rs35705950, was shown to increase MUC5B expression in the lungs. MUC5B encodes mucin-5B precursor protein which is essential for host defense and plays a role in mucocilliary clearance. This genetic variant does not have an effect in isolation and it does not seem to be causative of IPF but it may affect normal mucocilliary clearance or lung repair. Interestingly, patients with IPF and this genetic variant of MUC5B often have a higher rate of survival compared to those without.

Other known but rare genetic variants that are associated with IPF include genes associated with either surfactant dysfunction (SFTPC, SFTPA2) or telomere biology (TERT, TERC, TINF2, PARN, RTEL). With regards to the prior, mutations in genes responsible for surfactant production and processing cause endoplasmic reticulum stress and may promote epithelial to mesenchymal transition. As for the latter, mutations affecting telomere biology result in abnormally shortened telomere length in alveolar epithelial type 2 cells (AEC2), which is often found in patients with sporadic IPF.

Epigenetic reprogramming

As part of the aging process, there are several stochastic changes made to the epigenome. These changes are thought to influence the pathogenesis of IPF. Loss of histones, deregulation of miRNA and DNA methylation drift are some such changes that occur and cause unpredictable differences between individuals. Although no causal relationships have been established, epigenetic modifications have been implicated as a mechanism through which environmental exposures associated with disease risk can translate to pathogenesis (Yang and Shwartz, 2015). The most major example is the effects of cigarette smoke on the methylome in the small airway epithelium. Several studies have shown that with exposure of A549 and BEAS-2B to cigarette smoke condensate results in hypermethylation of specific tumor suppressor genes and repression of E-cadherin which promotes epithelial to mesenchymal transition (Nagathihalli et al., 2012). In patients with PF, miRNAs were found to be modified in a way which may contribute to the pathogenesis of IPF. For example, miRNA Let-7 downregulation in patients may contribute to epithelial-mesenchymal transition (Pandit et al., 2010). Changes in miRNA affects AEC2 and fibroblasts, resulting in the activation of pro-fibrotic signaling pathways. There have also been several studies that have found an upregulation in class I and class II histone deacetylases in myofibroblasts and bronchiolar basal cells from patients with IPF (Korfei et al., 2015). The exact effect of this upregulation is yet unknown. However, other studies have previously linked histone modification to silencing of anti-fibrotic genes (CAV1, PTGS2, FAS, THY1) (Martinez et al. 2017). These genes are linked with various pathways including transforming growth factor beta (TGFβ) signaling, production of prostaglandin E2, and apoptotic resistance in fibroblasts.

Project rationale and hypothesis

Currently, the most common model for PF is bleomycin-treated mice. Bleomycin is shown to induce the production of reactive oxygen species which leads to inflammation and subsequently, pulmonary toxicity (Lederer et al., 2018). This model is readily available and the results are highly reproducible. The use of this model has led to many key discoveries including the role of TGFβ in PF (Reinert et al., 2013). However, although the model recapitulates many of the features of IPF, it is more similar to an acute lung injury and the damage is partially reversible. The model does not recapitulate the chronic disease progression nor does it require a long incubation time to develop like IPF (Tomás et al., 2013). In fact, many of the prospective treatments developed in this mouse model did not show any effect in humans. Therefore, a need for a more representative human model is necessary to better understand this disease. The use of known mutations that lead to PF may provide an *in vitro* model. Using patient-derived iPSCs, we would be able to generate cell types that are relevant to the disease and examine the link between the mutation and the pathogenesis of PF. Understanding the mechanism of action may provide therapeutic targets and aid in research of prophylactic treatment.

Through collaboration with Dr. Martin Kolb (FIHR, Hamilton) samples were collected from 3 patients with PF who are blood relatives from a family of 12 (F12-Family; a total of 5 individuals in this family have an FPF diagnosis). Induced pluripotent stem cell (iPSC) lines have been made from each patient sample and differentiated into fibroblasts for use in future experiments (iPSC lines generated by Soumeya). Whole exome sequencing was then used to identify missense mutations that were common among the FPF patients and subsequently the list was narrowed using online databases MutationTaster and Polyphen. Both databases are used to predict if a mutation will have disease-causing potential but through different methods.

MutationTaster compares the input sequence with other known disease causing and harmless polymorphisms from integrated databases at the DNA and protein level (Shwarz et al., 2010). Polyphen is specific for examining the effect of amino acid substitutions on structure and function of the protein. Using integrated databases, Polyphen performs multiple sequence alignments and machine learning classification to predict if a mutation will cause disease (Adzhubei, Jordan, and Sunyaev, 2013). Figure 1A shows the list of candidate gene variants and the score that was received from MutationTaster and Polyphen. We further narrowed it down by removing the genes that were determined by both databases to be benign. This left us with CTNNA3, DDHD2, DIP2B, ESR1, ITGB6, MKL1, PACS1, and TRPC3. Next, we examined the candidate genes for loss of function intolerance (Figure 1B), frequency of the specific mutations (Figure 1C), and expression in lung tissue. Lastly, we looked at the function of the encoded protein to determine if it might play a role in PF. CTNNA3, DDHD2, ESR1, and TRPC3 are not expressed in the lungs and DIP2B and MKL1 have a higher loss of function intolerance score. The 2 priority candidates that represent putative disease-causing genetic variants we were left with were: Phosphofurin acidic cluster sorting 1 (PACS1) and integrin β6 (ITGB6).

A

	Gene	Chr.	Ref/Alt	Consequence	Annotation	MutTaster	Polyphen
	BRAT1	7	C/T	A540T	mis	Polymorph	Benign
	CTNNA3	10	A/T	S160T	mis	Polymorph	Prob. Dam.
	DDHD2	8	A/C	Q236P	mis	Disease	Prob. Dam.
	DIP2B	12	G/A	D265N	mis	Disease	Benign
	ESR1	6	C/A	P146Q	mis	Disease	Prob. Dam.
*	ITGB6	2	C/T	R15K	mis	Disease	Benign
	KRT85	12	C/T	D189N	mis	Polymorph	Poss. Dam.
	LRBA	4	T/C	M1190V	mis	Polymorph	Benign
	MKL1	22	G/A	R504W	mis	Polymorph	Prob. Dam.
*	PACS1	11	A/G	N217S	mis	Disease	Prob. Dam.
	TRPC3	4	T/C	S896G	mis	Disease	Benign

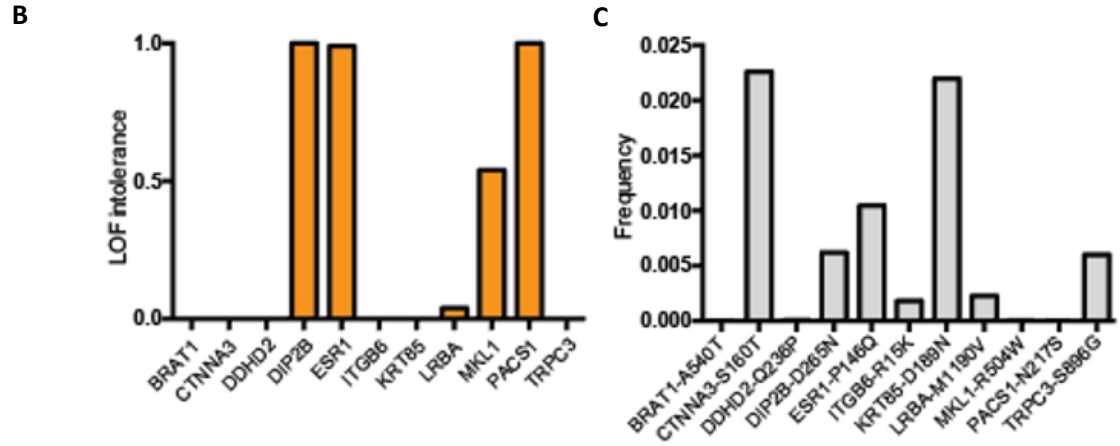
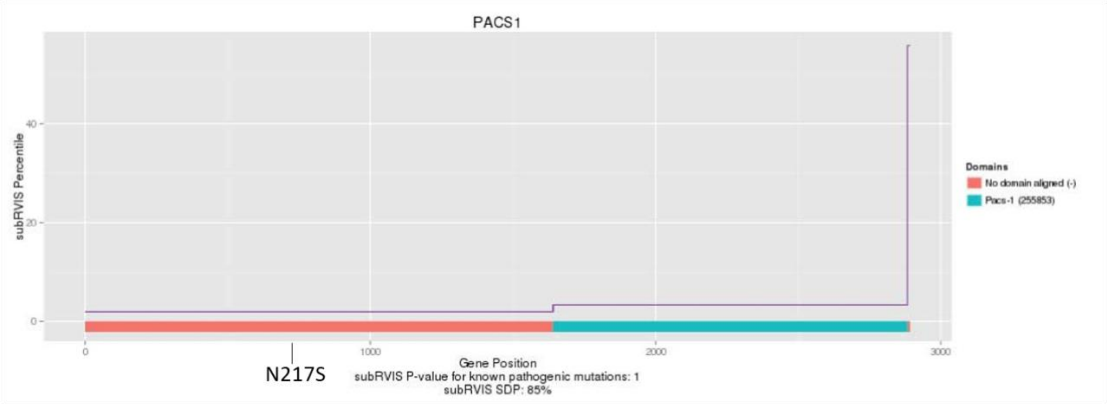


Figure 1. Candidate genes with mutations common to a family of patients with FPF. Whole exome sequencing (WES) was done on 3 patients with FPF from one family. 1A) Mutations common to all 3 patients were tested with Mutation Taster and Polyphen to determine if the mutations are potentially deleterious. The genes with mutations are listed along with the chromosome they appear on, the change in nucleotide, the resulting amino acid change, the type of mutation, and the result from Mutation Taster and Polyphen. 1B) Tolerance of loss of function (LOF) in candidate genes. Candidate genes were analyzed for the loss of function intolerance using ExAC Browser. 1C) Prevalence of candidate mutations in the human population. MutationTaster was used to determine the frequency of mutations.

Phosphofurin Acidic Cluster Sorting Protein 1

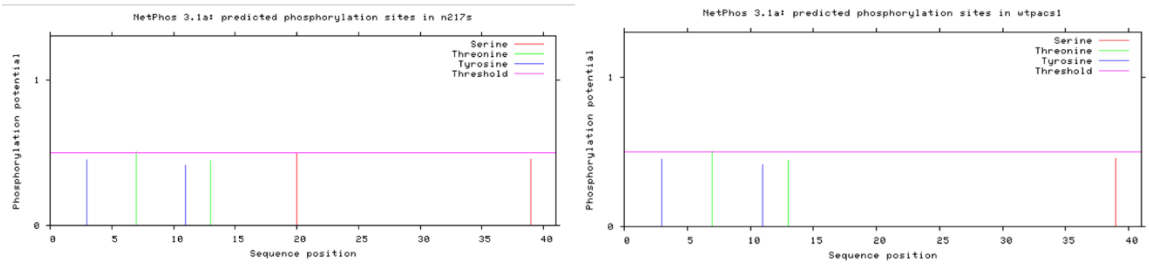
PACS1 is a constitutively expressed protein in most human tissues and loss of function mutations have been found to cause intellectual disability, developmental delay, and cranio-facial defects (Youker 2019). The pervasive presence of this protein and significant role that PACS1 plays in development, provided evidence to warrant further investigation. PACS1 is involved in the localization of trans-golgi network membrane proteins and has been implicated in the regulation of intrinsic apoptosis (Youker 2019). More specifically, PACS1 was found to assist in the association of BAK and BAX to permeabilize the mitochondrial outer membrane (MOM). Permeabilization of the MOM results in the release of cytochrome c, SMAC/diablo, and Omi/HtrA2 which activate the caspase cascade resulting in apoptosis. Knockdown of PACS1 was found to have a protective effect when an apoptotic signal was present because without it, BAK and BAX were not able to permeabilize the MOM. Instead BAK and BAX formed irregular structures in the MOM (Youker 2019). We hypothesized that the F12 mutation affects the activity of PACS1 to modify apoptotic sensitivity. It is important to note that fibroblasts from patients with IPF were found to have decreased sensitivity to apoptosis and alterations to signaling pathways. Thus, aberrant control over the intrinsic apoptosis pathway mediated by PACS1 may lead to excessive proliferation of fibroblasts resulting in a fibrotic phenotype. Through comparison of sequences across species and use of subRvis online data base, it appears that the mutation occurs in a position that is highly conserved across species and in a region where it is likely to result in deleterious effects (Figure 2). The nucleotide change in PACS1 results in a substitution from Asparagine (N) to Serine (S) at position 217. Although the substitution is not a dramatic change in terms of charge, serine acts as a potential

phosphorylation site. We used an online phosphorylation predictor, NetPhos 3.1 Server, which predicted that the new serine acts as a target for casein kinase 2 (CK2) (Figure 3). Phosphorylation via CK2 was found to affect the stability of proteins via targeted degradation or the prevention of caspase-mediated degradation of the target protein. We hypothesize that CK2 phosphorylation mediated degradation results in a decrease in the half-life of PACS1 and decreased sensitivity of affected fibroblasts to apoptosis.



Otolemur_garnettii_PACS1	QRRKRYKNRTILGYKTLAVGLINMAEVMQHPSEGALVLGLHSNVKD	127
Macaca_mulatta_PACS1	QRRKRYKNRTILGYKTLAVGLINMAEVMQHPNEGALVLGLHSNVKD	239
Pongo_abelii_PACS1	QRRKRYKNRTILGYKTLAVGLINMAEVMQHPNEGALVLGLHSNVKD	239
Gorilla_gorilla_PACS1	QRRKRYKNRTILGYKTLAVGLINMAEVMQHPNEGALVLGLHSNVKD	178
Pan_troglodytes_PACS1	QRRKRYKNRTILGYKTLAVGLINMAEVMQHPNEGALVLGLHSNVKD	240
Homo_sapiens_PACS1	**QRRKRYKNRTILGYKTLAVGLINMAEVMQHPNEGALVLGLHSNVKD**	240
Cavia_porcellus_PACS1	QRRKRYKNRTILGYKTLAVGLINMAEVMQHPNEGALVLGLHSNVKD	127
Mus_musculus_PACS1	QRRKRYKNRTILGYKTLAVGLINMAEVMQHPNEGALVLGLHSNVKD	238
Rattus_norvegicus_PACS1	QRRKRYKNRTILGYKTLAVGLINMAEVMQHPNEGALVLGLHSNVKD	238

Figure 2. Missense mutation occurs at a conserved region in PACS1. A) subRVIS percentile indicating missense mutation occurs in a conserved region of the protein (below the 35 percentile) B) Sequence alignment across species of PACS1 highlighting the mutation of a conserved asparagine (yellow) performed using Clustal Omega.




```
# n217s          20 S   VGLISMAEV   0.496   CKII        .
# n217s          20 S   VGLISMAEV   0.447   GSK3        .
# n217s          20 S   VGLISMAEV   0.432   CaM-II      .
# n217s          20 S   VGLISMAEV   0.393   cdc2        .
# n217s          20 S   VGLISMAEV   0.369   CKI         .
# n217s          20 S   VGLISMAEV   0.367   PKG         .
```

Figure 3. Missense mutation creates a new phosphorylation site in PACS1. NetPhos 3.1 prediction of phosphorylation site created n217s missense mutation in PACS1. The input sequence is a 42 amino acid sequence taken from PACS1 (amino acids 198-239). The n217s mutation is at position 20.

Integrin β 6

ITGB6 encodes integrin β6 which forms a heterodimer (αvβ6) with integrin αv. αvβ6 is an epithelial membrane protein known to activate TGF-β, which is well documented in the pathogenesis of fibrosis (Sporn, 1992; Worthington et al., 2011; Simon, 2007). As mentioned earlier, the current mouse model for PF uses bleomycin to induce the fibrotic phenotype (Reinert et al., 2013). Bleomycin is a chemotherapeutic agent used to treat cancer by inducing DNA damage. The implicated mechanism for induction of the fibrotic phenotype involves bleomycin forming oxidants which cause damage to cells but also participate in inflammatory reactions. Inflammation causes recruitment of alveolar macrophages to the site of pulmonary damage to release cytokines such as platelet derived growth factor, interleukin 1, and TGF-β (Reinert et al., 2013). Oxidative damage to pulmonary epithelial cells also results in release of pro-fibrotic growth factors including TGF- β and TNF-α. The release of growth factors and chemokines

promote extracellular matrix production, which triggers integrin signaling and propagates the cycle of recurrent injury (Simon, 2007). Previous studies have found that monoclonal antibodies to αvβ6 prevent PF in bleomycin-treated mice, thus implicating ITGB6 in PF (Worthington et al.,2011). The F12 gene variant of ITGB6 substitutes lysine in place of arginine at position 15. Although lysine and arginine have a similar charge, the position of the lysine is highly conserved across organisms (Figure 4). Using SignalP 4.1, the signal sequence cleavage site was predicted to be at the 22nd amino acid in the sequence (figure 5). This places the mutation within the signal sequence which could alter the localization of the protein. Moreover, subRVIS data also supports that the mutation occurs in a highly conserved region and thus warrants further investigation.

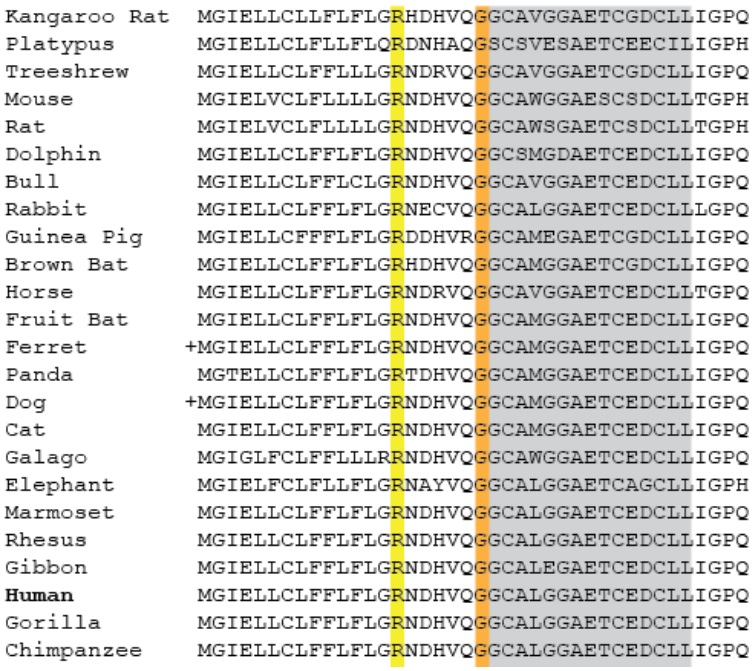
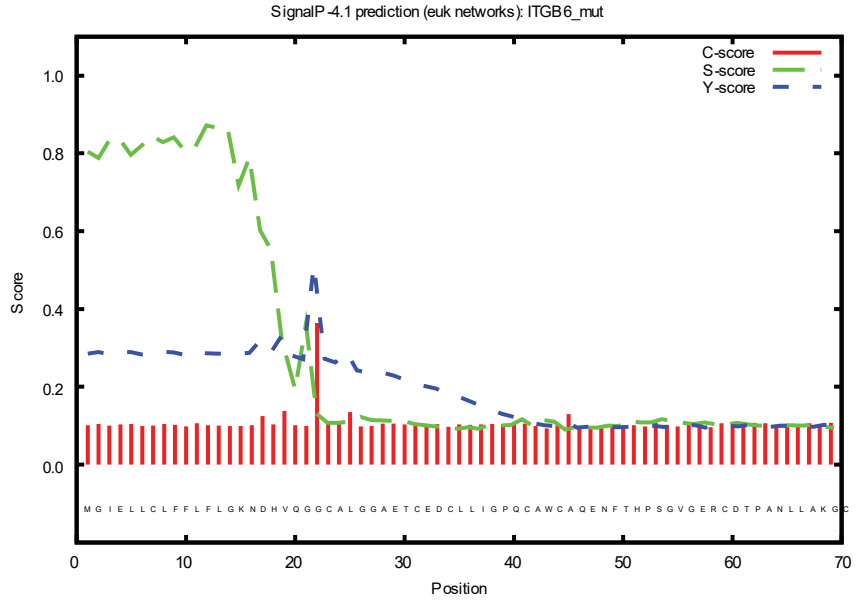


Figure 4. Missense mutation occurs at a conserved amino acid in ITGB6 signal sequence. Sequence alignment across 24 mammalian species of amino acids 1-40 in ITGB6, highlighting the mutation of a conserved arginine (yellow) and sequence cleavage site (orange) performed using Clustal Omega.

A



B

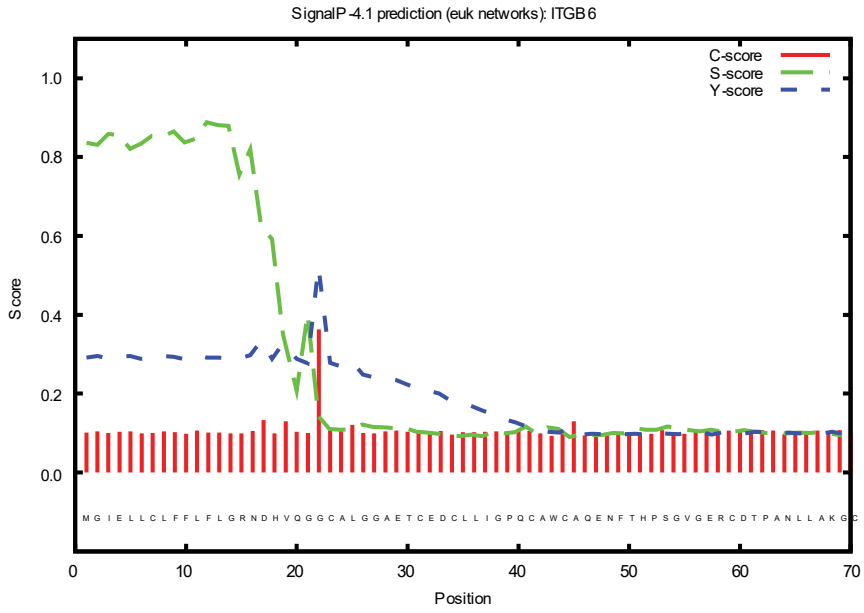


Figure 5. SignalP-4.1 prediction of ITGB6 signal sequence cleavage site. The amino acid sequence of both A) ITGB6 R15K and B) ITGB6 wt were submitted as input. Cleavage is predicted to occur before the amino acid with the highest C-score. S-score marks predicts which

amino acids are part of the signal peptide. Y-score is the geometric average of the C-score and the S-score which is used to distinguish between multiple peaks if present.

We hypothesize the PACS1 or ITGB6 genetic variants present in the PF patients may cause a predisposition for PF. To investigate this, we aimed to elucidate any changes in function caused by the mutation. More specifically, in PACS1 we aimed to determine if the N217S missense mutation altered cellular sensitivity to apoptosis. For ITGB6, we aimed to determine if R15K alters the localization of ITGB6 or changes ITGB6 mediated signal transduction.

Materials and Methods

Cell culture

HEK 293 FT, HeLa, DLD1, and human-derived fibroblasts (HDF) were grown in Dulbecco's Modified Eagle Medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Sigma), 1X Non-Essential Amino Acids (NEAA, Gibco), and 1X Glutamax (Gibco). Media was filter sterilized with 0.22 µm filter (sigma). Cells were passaged when confluent with TrypLE (Gibco) and grown at 37ºC and 5% carbon dioxide in a humidified incubator.

Small airway epithelial cells (SAEC) were grown in small airway epithelial cell growth medium (SAGM bulletkit, Lonza) that was filter sterilized with a 0.22 µm filter (Sigma). Cells were passaged and grown in the same conditions as written above.

Confirmation of the mutations in genomic DNA

Patient genomic DNA (generated previously) was used as a template for PCR amplification of each gene upstream from the location of each mutation (PRIMESTAR). The resulting DNA is then run on a gel and the target band is excised. Gel extraction kit is used to isolate the DNA which is then sent for sanger sequencing at the MOBIX facility at McMaster.

Table 1: Primers used to confirm heterozygous point mutation in genomic DNA

Primer	**Sequence (5'→3')**
PACS1	Forward: CTCCACCAGCAGCAACTTCATG
	Reverse: GCAGGCTGGGAAAATCATCTGG
ITGB6	Forward: GGGATTGAACTGCTTTGCCTG
	Reverse: CTGCCCAATGGAACTTAGTGC

Primer design for site-directed mutagenesis of point mutations in PACS1 and ITGB6.

PACS1-mCherry plasmid was obtained from another lab and ITGB6-GFP plasmid was obtained from Addgene. Primers were designed to use site-directed mutagenesis to introduce a point mutation (CloneAmp). Plasmid constructs were generated using PCR with the respective primer pairs. The parental plasmids were digested using dpn1 (NEB) and allowed to self-circularize before transformation into Mach1 competent cells.

Table 2: Primers used for the generation of mutant plasmid constructs.

Primer	**Sequence**
PACS1	Forward: GCCGTGGGACTCATCAGCATGGCAGAGGTGATGC
	Reverse: GCATCACCTCTGCCATGCTGATGAGTCCCACGGC
ITGB6	Forward: CTTTCTATTTCTAGGAAAGAATGATCACGTACAA
	Reverse: TTGTACGTGATCATTCTTTCCTAGAAATAGAAAG

Annexin V flow cytometry

HEK 293 FT, HeLa, or HDF cells from iPSC derived tissue of each of the patients were used in this experiment. HEK 293 FT and HeLa cells were transiently transfected for 48 hours using lipofectamine LTX with Plus Reagent (Invitrogen) with either wildtype or mutant PACS1. Cells were then treated with fasL for 24 hours to induce apoptosis. Cells were singularized using TrypLE (Gibco), collected in PBS and spun down at 1300 RPM for 3 min before being

resuspended in Annexin binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$). Staining was done at room temperature for 15 minutes. Antibody used was Annexin V, Alexa Fluor® 647 conjugate (ThermoFisher). Additional Annexin binding buffer is added to dilute the samples and 7AAD (BD Biosciences) is added before being assayed using the BD LSR2. FlowJo (Treestar) was used for quantification and analysis of the results.

Assessment of mutant ITGB6 function in transfected DLD1 colon carcinoma cells.

DLD1 cells were transfected with wt or mutant ITGB6 construct for 48 hours. Fibronectin plates were precoated for 30 min with 10 µg/mL fibronectin. After aspirating the liquid, the plates were dried for 45 minutes. Transfected DLD1 cells were singularized with TrypLE (Gibco) and collected with PBS. The cells were then pelleted and plated on the fibronectin plates with or without a blocking antibody P5D2 (integrin beta-1 (CD29), human) (P5D2-s developmental studies hybridoma bank) for 2 hours at 37ºC before being collected for analysis.

Antibodies

Primary antibodies used for Western Blot analysis: mouse anti β-Actin (T7816, Sigma), mouse anti-GFP (B-2) (9996, Santa Cruz), Rabbit Anti-FAK (phospho Y397) antibody [EP2160Y] (ab81298), and Rabbit Anti-FAK antibody [EP695Y] (ab40794). For immunofluorescence, the following antibodies were used: Sheep anti-Human Integrin beta 6 Antibody (AF4155, Novus) and E-cadherin antibody (67A4) PE (21791-PE, Santacruz).

Horseradish peroxidase-conjugated secondary antibodies used for Western Blot analysis: goat anti-mouse (1721011, BioRad) and goat anti-rabbit (1706515, BioRad). Alex Fluor conjugated secondary antibodies used for immunofluorescence studies: donkey anti-sheep 546. DNA was stained using Hoechst 33342 (H1399; Life Technologies).

Cell Lysate Preparation

Whole cell lysate was prepared by cells with PBS before lysing on ice with either 1X RIPA buffer [50 mM Tris-HCl pH 8, 150 mM NaCl, 0.1% Sodium dodecyl Sulfate, 1% NP-40, 0.5% sodium deoxycholate and 1X Complete™ Mini Protease Inhibitor Cocktail (Roche)] for 20 minutes or non-denaturing lysis buffer [2 mM EDTA, 20 mM Tris Cl, 137 mM NaCl, 1% NP-40, 1X Protease and phosphatase inhibitor (Halt protease and phosphatase inhibitor 100X)] for 20 minutes on ice. The lysate is then spun at 12000 g for 10 minutes at 4ºC. Supernatant is collected and quantified using the DC protein assay II kit (BioRad). Samples are then normalized using 1X NuPage® LDS sample buffer (Invitrogen) with 15% TCEP Bond-Breaker Solution (thermo Scientific) and heated at 95ºC.

Immunoprecipitation

After quantification of the lysate and before the addition of LDS, 2 alliquots of 500 µg of protein per reaction was separated and 2 µg of anti-FAK or anti-IgG antibody was added. In a separate tube 10ug of protein was normalized with LDS and bondbreaker as described above and frozen at -80ºC to use as a 2% input control. 20 µL of Protein G dynabeads (10003D; ThermoFisher) was used for each immunoprecipitation.

Western Blot Analysis

Lysates (10-20 ug) from cell lysis or immunoprecipitation were separated using 10% Bis-Tris gels and proteins were transferred to PVDF membranes (Millipore) by wet transfer in 1X Towbins. Blocking buffer made with 1X Tris buffered saline and 5% BSA (Sigma) was incubated with the membrane for 30 minutes before incubation with primary antibody in 3% BSA in tris buffered saline with 0.1% Tween for 2 hours at room temperature or overnight at

4ºC. Primary antibody dilutions used: β-actin (1:50000), GFP (1:1000), FAK (1:1000), and FAK phosphor Y397 (1:1000). After washes in 1X tris buffered saline with 0.1% tween (TBST), the membrane was incubated with secondary antibody in TBST with 3% BSA for 1 hour at room temperature. Blots were developed using Luminata Forte Western HRP substrate (Millipore) and then imaged with ChemiDoc™ MP imaging System (Bio-Rad).

Immunofluorescent Staining

HEK 293FT were transfected with wt or mutant ITGB6 for 48 hours. Transfected HEKS and SAEC were cultured in chambered cover slips (µ-Slide 4 well Ph+; Ibidi) fixed with 4% PFA and permeabilized with ice cold methanol. Cells were blocked with 1% BSA in PBS for 10 minutes before being incubated with primary antibody in 1% BSA in PBS for 2 hours at room temperature or overnight at 4ºC. After washing with PBS, cells were stained with Hoechst 33342 (H1399; Invitrogen) and the appropriate secondary antibody for 1 hour at room temperature. Slides were imaged using confocal microscopy. The following dilution of primary antibodies were used: Sheep anti-Human Integrin beta 6 Antibody (10 µg/mL) and E-cadherin antibody (67A4) PE (1:100). Secondary antibody dilution for donkey anti-sheep 546 (1:250)

Results

Confirmation of the heterozygous mutations found in whole exome sequencing

To confirm that the heterozygous mutations detected using whole exome sequence were present, genomic DNA from 3 PF patients and 1 control were used as a template for PCR of the genes of interest. The resulting DNA was then sent for sequencing using primers specific to the region where each of the mutations are located. The sequencing data shows that each of the patients carry heterozygous mutations for N217S in PACS1 and R15K in ITGB6 (Figure 6).

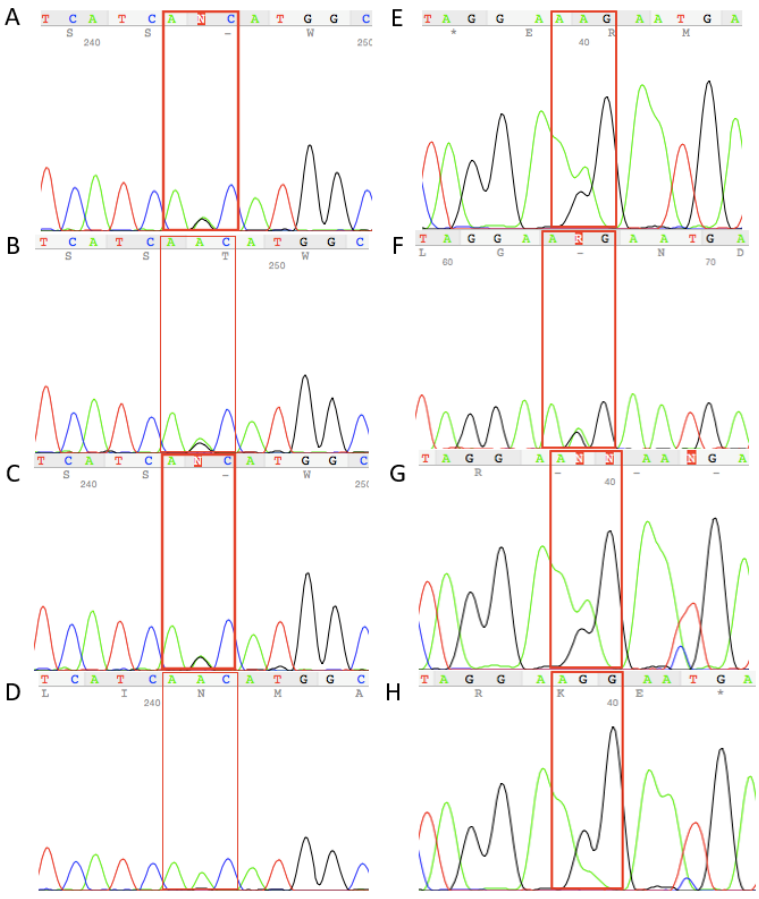


Figure 6. Sequencing of ITGB6 and PACS1 from Genomic DNA. A-D) Sequence of PACS1 at the site of mutation. PACS1 was amplified from genomic DNA of Patients RG, GM, SP and

Control (A-D respectively). E-H) Sequence of ITGB6 at the site of mutation. ITGB6 was amplified from genomic DNA of Patients RG, GM, SP and Control (E-H respectively).

R15K ITGB6 mutation may affect the ability of ITGB6 to localize to the membrane via the change in the signal peptide

The R15K mutation occurs in the signal sequence which is necessary for proper localization of the protein. As shown in multiple sequence alignments, the arginine at position 15 is highly conserved across species which may be an indication that it is crucial for proper function. To investigate if there were any changes in localization, we transfected our GFP tagged construct into HEK 293 FT cells and examined the localization of ITGB6 through immunofluorescence microscopy (Figure 7). We also examined the localization of ITGB6 in small airway epithelial cells (SAEC), a non-transformed cell line that expresses ITGB6 endogenously (for context). In SAEC, ITGB6 mainly localized to the membrane as shown when co-stained with E-cadherin however there appears to be a low percentage of expression seen in the nuclear membrane (Figure 8A). Interestingly, when HEK 293 FT were transfected with our wildtype and R15K ITGB6-GFP construct, localization of ITGB6 shifted to the nuclear membrane (Figure 8B).

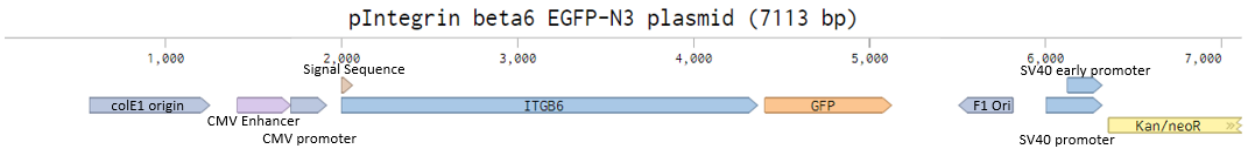
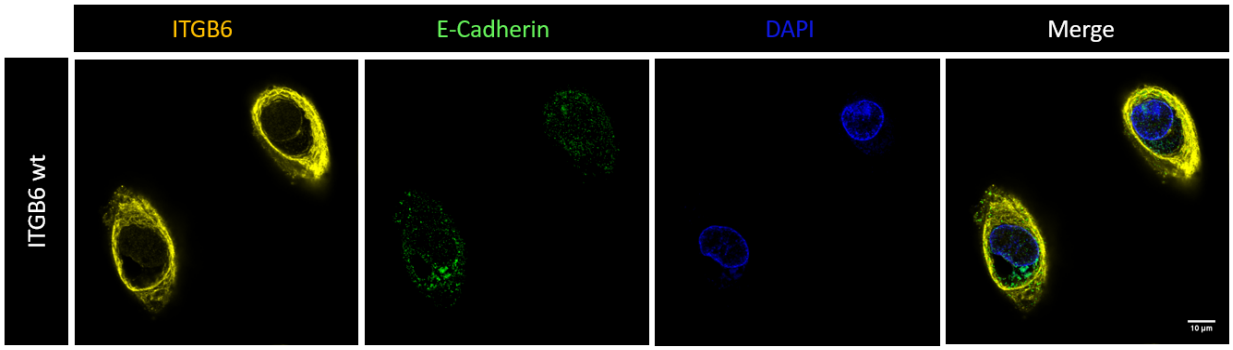


Figure 7. Schematic of pITGB6-GFP translational fusion construct.

A



B

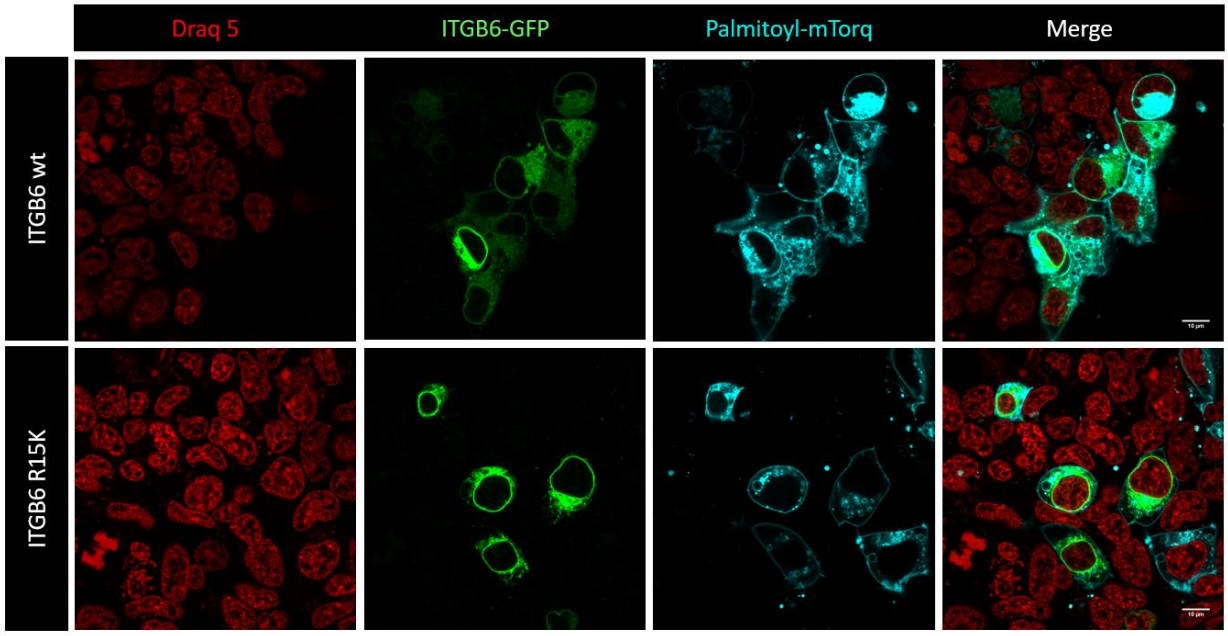


Figure 8. Identifying localization of ITGB6 using confocal images of SAEC and Transfected HEK293 FT. A) SAEC were fixed and stained for ITGB6, ECAD, and DAPI. B) HEK293 FT were co-transfected with either ITGB6 wt or ITGB6 R15K and Palmitoyl-mTorq which marks the plasma membrane. 48 hours after transfection, DRAQ5 was added and cells were imaged live.

R15K mutation may affect the ability of ITGB6 to phosphorylate FAK a downstream mediator

ITGB6 is part of a heterodimer which forms with ITGAV and plays a role in the interaction with the extracellular matrix. Stimulation of the integrin receptor leads to phosphorylation of FAK and downstream mediators including activation of the MAPK pathway. Evidence for the implication of integrin avB6 in PF is shown in the bleomycin-treated mouse model of PF. As previously mentioned, bleomycin is thought to induce the fibrotic phenotype through the formation of oxidants which causes damage and subsequent inflammation (Reinert et al., 2011). The recruitment of macrophages leads to the release of other proinflammatory cytokines such as PDGF, IL-1, and TGF-β. Interestingly, partially blocking the activation of integrin avB6 with monoclonal antibodies successfully blocks formation of PF in bleomycin-treated mouse model of PF. We decided to investigate this using DLD1 which is a colon cancer cell line which does not express ITGB6 but expresses ITGAV. We transfected the dld1 cells with our wt and R15K ITGB6-GFP construct and blocked integrin β1using P5D2 for 30 minutes before re-plating the cells on fibronectin to stimulate integrin avb6. We then collected the cells and, using immunoprecipitation to pulldown FAK, we assayed for levels of phosphorylated FAK using western blot analysis. Despite detecting phosphorylated FAK in control samples, there was no significant differences in the levels of phosphorylation between wt and R15K transfected dld1 (data not shown).

PACS1 may not affect apoptosis in transfected HEKs, HeLas, and human derived fibroblasts.

PACS1 has been shown to play a role in TNF-related apoptosis-inducing ligand (TRAIL) induced apoptosis (Youker et al. 2009). Minor changes to the rate of apoptosis may affect the

inflammatory process and affect wound healing over time which could result in pulmonary fibrosis. To investigate the effects of this mutation on apoptosis, we performed annexinV flow cytometry on fasL treated HEK-293FT and HeLa cells transfected with wild type or N217S PACS1. Upon inducing apoptosis with fasL (a TRAIL), untransfected HEKs showed increased expression of AnnexinV, and this increase was consistent when comparing cells also transfected with wildtype or N217S PACS1 (Figure 10 A). In transfected HeLa cells, apoptosis levels were significantly different with N217S PACS1 showing protective effects against fasL-induced apoptosis (Figure 10B). Since both of these cell lines have endogenous PACS1 and demonstrated disparate results, we decided to use patient derived fibroblasts to understand if N217S PACS1 expression was linked to altered apoptosis. However, as shown in figure 11, the results were inconclusive. Although the treatment remained the same, the results demonstrated significant inter-test inconsistency suggesting that there may be some confounding variable.

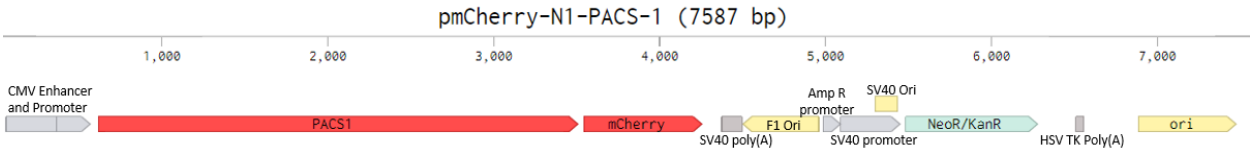


Figure 9. Schematic of pPACS1-mCherry translational fusion construct.

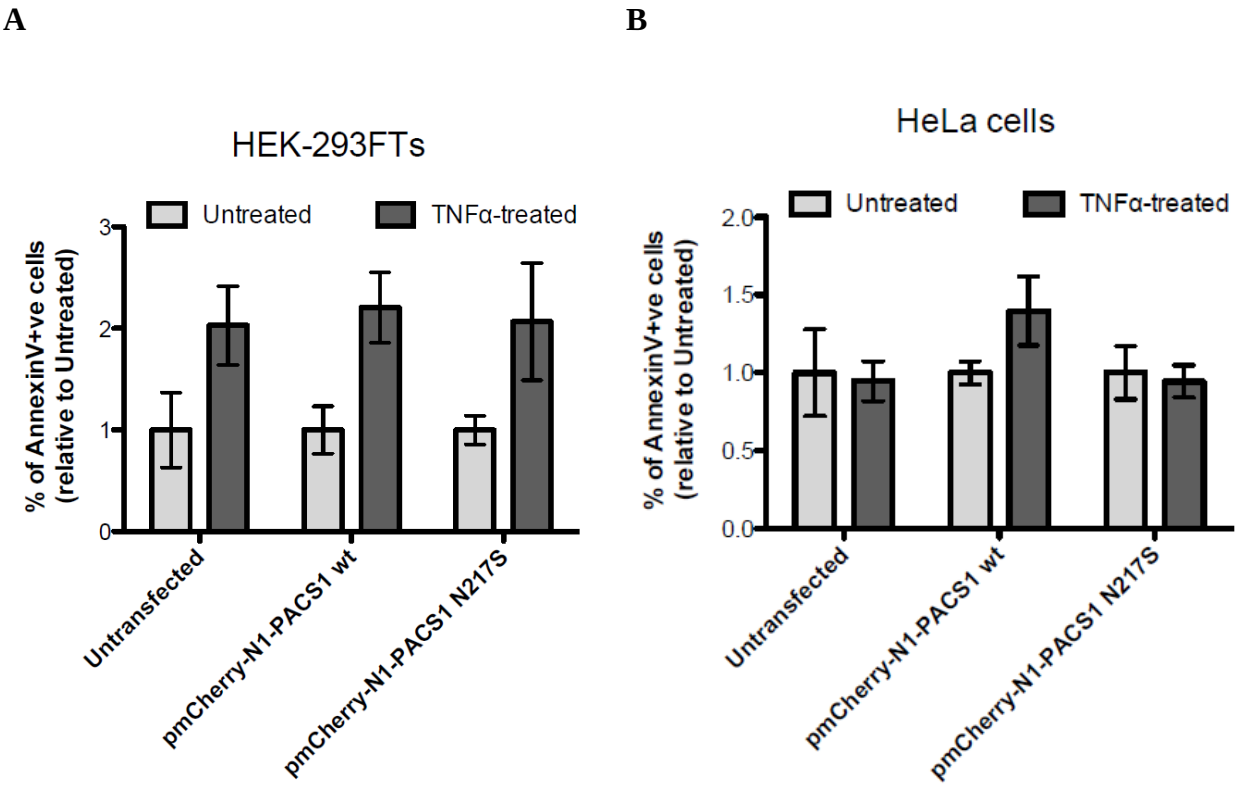


Figure 10. Annexin V flow cytometry of HEK 293 FT and HeLa cells. HEK293 FT and HeLa were transfected with PACS1 wt or PACS1 N217S. 48 hours after transfection, the cells were treated with TNF-α for 24 hours before being harvested for flow cytometry.

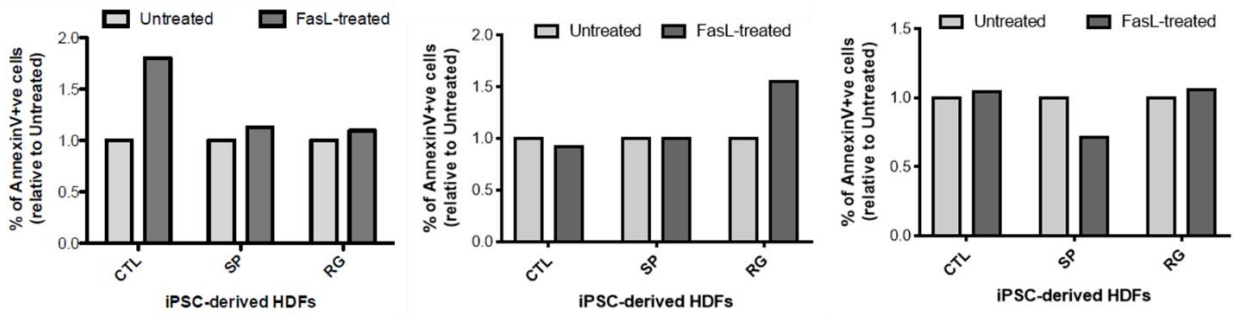


Figure 11. Annexin V flow cytometry of HDF. HDF from patients SP or RG and control HDF were treated with fasL for 12 hours before being harvested for flow cytometry. Each graph shows a separate n.

Discussion

Previously, we were able to conduct whole exome sequencing on 3 members of the F-12 family and generate iPSC and fibroblasts from each of the patients. Here, we were able to confirm the heterozygous missense mutation found in whole exome sequencing and create expression constructs containing the mutation in both PACS1 and ITGB6 to further explore the effects of the mutations. Unfortunately, there was an insufficient amount of time to fully explore the genetic variants. However, this study provides the tools and preliminary data necessary for performing future studies on the roles of these genes.

Previous studies have identified that ITGB6 plays a role in IPF and that treatment of ITGB6 blocking antibodies could prevent PF in bleomycin-treated mice (Youker et al. 2009). After confirmation of the heterozygous R15K mutation, we looked at potential changes in localization because of the location of the mutation. We found that ITGB6 localized mainly to the membrane in small airway epithelial cells (SAEC) but a small portion localized to the nuclear membrane. In comparison the transfected HEK293 FT cells had ITGB6 wt and R15K localized to the nuclear membrane with a minor population found in the membrane. R15K ITGB6 seemed to slightly preferentially localize to the nuclear membrane however more experiments are required for definitive results. The localization is different between HEKS and SAEC which suggests that adding GFP onto ITGB6 may affect its localization or that localization of ITGB6 requires contextual cues. To confirm the reason behind the abnormal localization of ITGB6, SAECs can be transfected with the same constructs. If localization of the wild type remains similar to what is seen in HEKS, the problem would be the GFP and if it becomes localized like endogenous ITGB6, it would show that contextual cues are necessary for proper localization. We

have tried using multiple protocols for the transfection of SAEC including the use of different transfection reagents and electroporation but had no success in the given amount of time.

We also looked into the functional change of the R15K mutation on ITGB6 by using phosphorylation of FAK, a downstream kinase, as an indirect measure of ITGB6 activity. Changes in the phosphorylation of FAK could affect downstream effectors such as TGFβ which plays a major role in the pathogenesis of IPF. Although preliminary, we were able to show that fibronectin activates ITGB6 and causes phosphorylation of FAK. We also found that in order to show a distinct difference in activity, immunoprecipitation was required because of the low amount of protein. We were able to optimize the experiment and show that the system worked but were never able to successfully conduct the full experiment. Given more time, rerunning the experiment would determine if the R15K mutation impacts FAK phosphorylation compared to wild type ITGB6.

Increased epithelial apoptosis and decreased myofibroblast apoptosis are common findings in the lungs of patients with IPF (Uhal, 2008). Because of the major role that PACS1 plays in TRAIL-induced apoptosis, we investigated whether or not N217S mutation would affect this. We found that there were no significant differences in the apoptotic population of HEK293 and HeLa cells when transfected with our construct and treated with TNFα. This may be an indication that this mutation alone may not be sufficient to cause a change in phenotype. It may also indicate that overexpression of transfected PACS1 may not be sufficient to compensate for the effects of endogenous PACS1. Another potential reason for the lack of change may be the use of cell lines that have intrinsic resistance to TRAIL induced apoptosis which would diminish any changes that we would otherwise observe. To investigate if the combination of mutations is required for an effect on apoptosis to be seen, we investigated the effects of TRAIL-induced

apoptosis in patient-derived fibroblasts. We hypothesized that patient derived fibroblasts would be more resilient to apoptosis, but we found no consistency in the result after trying multiple times with the same conditions. In fact, even the control fibroblasts did not show consistent changes upon treatment. These results indicate that there may be another factor which we have yet to consider. Given more time and resources, we would have investigated where this issue originated but some possibilities include: antibodies, the TRAIL used to induce apoptosis, and passage number as these cells have limited proliferative capacities. We would also work on other forms of analysis including staining for caspase 3 cleavage to confirm any results found from the annexin flow cytometry. We also examined the change in structure of PACS1 and hypothesized that the change may allow for phosphorylation via CK2. This was supported by the analysis of a short fragment of PACS1 by NetPhos3. However, the interaction has not yet been thoroughly investigated and there may be other mechanisms through which the mutation could impact the protein.

The genes that have been listed here may not be an exhaustive list of potential genetic links to PF within this family. Whole exome sequencing only examines protein coding genes which leaves out many other heritable regions such as promoters, intronic sequences, and RNA coding regions. Thus, further investigation into these other elements may be necessary to understand the familial connection to PF.

Conclusion

Discovering mutations that are the underlying cause of a disease allows researchers to understand mechanisms of pathogenesis. If cells containing these mutations can recapitulate the disease findings *in vitro*, it would also provide us with a human model of the disease. To date, the bleomycin-treated mouse model has played a pivotal role in many major PF discoveries unfortunately many of the treatments developed using this model have not lent themselves to clinically significant results. It is for this reason that the development of a humanized model is paramount. Here, we have identified potential mutations that could contribute to the pathogenesis of PF. Both of the genes identified as mutated in our patient cohort may be connected to previous proposed mechanisms for the pathogenesis of PF including apoptosis control and the TGFβ pathway. However, further experimentation is required to establish a concrete causal relationship between these mutations and PF. Environmental factors also play a critical role in the pathogenesis of PF. The natural accumulation of damage may normally be tolerable but the heterogenous mutations may lead to a susceptibility to PF. If the mutations are definitively linked to the disease, future work would include the differentiation of iPSCs from each of the patients into lung epithelium and potentially provide a humanized model of IPF. Further characterization of these mutations may lead a deeper understanding of the pathogenesis of IPF.

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Investigating Intestinal Maturation using hPSC-derived Enterospheres and Primary Human Gestational tissues

Introduction

Intestinal Maturation

Maturation of the human intestine involves the transition of fetal intestine to a fully functional adult phenotype. However, due to the difficulty associated with obtaining fetal tissue samples, this process has not been well characterized. To date, the vast majority of the data currently known about maturation has been extrapolated from mouse models. Post-natal maturation of the intestine in the mouse model is defined by several changes, namely: completed morphogenesis, the appearance of Paneth cells and stem cells in the crypts, expression of differentiated cell markers, expression of proteins required for regular intestinal function, and rapid turnover (Finkbeiner et al., 2015; Mallow et al., 1996). Mice require colonization by commensal bacteria for proper maturation as germ-free mice have many defects including altered glycosylation of the luminal surface, impaired immune system development, decreased epithelial barrier integrity, improper villus formation, and decreased turnover rate of epithelial cells (Günzel and Yu, 2013; Lu et al., 2013; Milatz et al., 2010; Patel et al., 2012).

Similar to mouse models, during normal human gut maturation, bacteria colonize the gut and interact with the intestinal epithelium (Chu et al., 2017). This interaction is thought to be essential for proper development, as a growing number of studies report a connection between microbiome dysbiosis and a wide range of diseases that appear later in life (Sartour, 2008). This association has been noted in the case of several inflammatory bowel diseases such as Crohn's disease and ulcerative colitis (Alfaleh et al., 2011). However, the causal mechanisms through which microbiome dysbiosis is associated to these diseases is poorly understood. Due in part to ethical considerations and scientific complexity, there are currently no human models for the interactions of bacteria on the maturation of the human gut. As previously mentioned, mouse

models are the prevalent system used for studying the microbiome. However, a significant pitfall of this model is that the timing at which colonization occurs during maturation differs between mice and humans (Sartour, 2008). Furthermore, the microbiome of mice is optimized to digest standard laboratory food which results in disparities between wild mice and lab mice. To circumvent these issues, we investigated the effects of bacteria on maturation by using pluripotent stem cell derived human enterospheres (hEnS) as an *in vitro* model for the fetal intestine.

Organoid models

Intestinal organoids are 3D structures that contain a monolayer of epithelial cells with the lumen contained within the structure. They initially form cystic spheres but can form budding structures that contain crypt domains. Intestinal organoids can be derived from adult intestinal stem cells (ISC) or from pluripotent stem cells (PSC) (Nadkarni et al., 2017; Spence et al., 2011; Rahmani et al., 2019). Models created from ISC are more mature, as all cell types found in the small intestine can be found in the organoids, and changes in media can induce differentiation into specific lineages. However, PSC-derived models tend to be more fetal, but a period of *in vivo* engraftment in the mouse kidney capsule has been demonstrated to provide an environment that permits the organoid tissue to mature (Spence et al., 2011). PSC-differentiated intestinal organoids may serve as the best model for maturation because of their fetal-like state.

Human enterospheres

Previously, our lab has been able to derive human enterospheres (hEnS) from human pluripotent stem cells (hPSC) (Nadkarni et al., 2017). hEnS are cystic spheroids with simple uniform structure and are composed entirely of epithelial cells. Similar to most PSC-derived

models, hEnS stain for markers similar to fetal intestinal tissue. There are several benefits for using hEnS as a model for maturation. Firstly, using hEnS provides a source of human intestinal tissue containing multiple cell types that are formed from a single cell. Another benefit is that all the cells are epithelial, which isolates influences from the mesenchymal tissue. Furthermore, we can use the sphere-forming capacity as a surrogate measurement of intestinal stem cell proliferation in vitro. Lastly, the formation of a sphere creates a defined basal and luminal surface similar to that in the intestine. We have shown that hEnS are a tractable system for studying intestinal function. Through the use of different media conditions, we can induce changes in the cellular composition of the hEnS. We have also shown that hEnS mount an innate immune response to bacteria and our preliminary data shows that bacteria are able to induce changes in transcription. Therefore, hEnS represent a model system for understanding elements of intestinal development and maturation.

Microbiome Influence on Intestinal Development

The intestinal microbiome has become a highly pervasive topic because of the major role it plays in human health (Sartor, 2008). More specifically, the intestinal microbiome has been implicated in many disease processes including metabolic diseases and autoimmune diseases (Thomas et al., 2017). There is evidence that changes in the microbiome early on in life may lead to susceptibility later in life. Although there is evidence for intrauterine exposure to bacteria, it is generally believed that the first major exposure to bacteria occurs during childbirth (Milani et al., 2017). The initial colonization of neonatal intestine can be affected by several factors including: 1) the mode of childbirth, 2) maternal or pediatric antibiotic treatment, and 3) whether or not the child is breastfed or formula fed. In normal fetal development, facultative anaerobes such as L*actobacillus* and *Enterobactericaea*, typically act as pioneers to establish the intestinal

environment for strict anaerobes such as *Clostridia*, *Bifidobacterium*, and *Bacteroides* (Milani et al., 2017). The composition of bacteria diversifies further and eventually most adult microbiota consists mainly of: *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*. The initial colonization and evolution of bacteria in the intestine is thought to provide cues for maturation.

Project Rationale and hypothesis

There are 3 major changes during maturation that we chose to use as markers of maturation: increased structural integrity, shift in glycosylation pattern, and tolerance of the innate immune response. The first of these changes, increased structural integrity, is crucial to prevent the entry of bacteria and toxins into the host system, which would cause inflammation or injury. In murine gut maturation, bacteria typically colonize in the first weeks of life and modulate expression of genes involved in barrier function. Claudins are a family of proteins found in tight junctions and are shown to be correlated with intestinal barrier function (Gunzel and Yu, 2013). In typical mouse development, Claudin 3 (Cldn3) expression in the small intestine is coincident with maturation in mice. Previously, it has been shown that mice treated with antibiotics did not show the same increase in Claudin 3 expression throughout the small intestine during development (Patel et al. 2012). However, the expression could be rescued through introduction of live or heat killed *Lactobacillus rhamnosus GG* (LRGG) in their feed. To determine if these findings are translatable to the human system, we cultured the same bacteria with our hEnS to determine if there were any changes in expression and production of Claudin 3 between the treatment and control groups.

The second hallmark of maturation we studied is change in glycosylation patterns on intestinal epithelial cells. As the intestine matures, epithelial glycan terminations change from being predominantly sialic acid to fucose (Pickard and Chervonsky, 2015). This shift is not

observed in germ-free mice. However, treatment of germ-free mice with *Bacteroides thetaiotaomicron* is sufficient to recapitulate the fucosylation pattern of mature epithelium (Biol-N'Garagba et al., 2002; Bry et al., 1996). *B. thetaiotaomicron* is a prominent member of the microflora during maturation for both mice and humans. Through culturing hEnS with heat killed *B. thetaiotaomicron* or bacterial supernatant, we can determine if there is an effect on maturation through quantifying changes in expression of genes involving fucosylation.

The last marker of intestinal epithelium maturation studied was decreased innate immune response. In normal physiology, intestinal epithelial cells have ongoing exposure to commensal bacteria and their products which would typically result in an inflammatory response for other cell types. To prevent unnecessary inflammation, these cells become tolerant during maturation towards antigens that are typically present in the gut (Savidge et al., 2006). LPS is a common bacterial product which induces a pro-inflammatory response in most other cell types through toll-like receptor 4 (TLR4). As the intestinal epithelium matures, chronic exposure to LPS leads to the reduced expression of TLR4, resulting in decreased sensitivity of the epithelium to LPS.

Another method to investigate maturation involves looking at the emergence or loss of characteristic proteins that act as markers of functional change. For example, when enterocytes first emerge they express villin, but they are considered functional when they are able to produce glucosidase enzymes such as sucrase isomaltase, trehalase, and lactase (Finkbeiner et al., 2015). Similarly, Paneth cells are able to produce lysozymes but are not considered mature until they are able to produce α-defensins (Cunliffe, 2003). Failure or untimely emergence of these changes may be indicators of intestinal pathology. As mentioned earlier, currently most studies on maturation are done in the murine model. However, the timing of maturation is significantly different. For example, intestinal morphogenesis is complete several weeks before birth in

humans but several weeks after birth in mice. Furthermore, the appearance of Paneth cells occurs at postnatal day 14 in mice but appear at week 20 of gestation in humans (Chin et al., 2017; Kim et al., 2012). These differences suggest that intestinal maturation is temporally different between mice and humans suggesting a need for a human model to better extrapolate the data obtained from mice. To date, the expression of protein marker emergence during intestinal development has not been fully explored because of the difficulty in accessing primary gestational tissue. Our lab has access to gestational intestinal tissue which gives us the opportunity to investigate emergence of protein markers of maturation. We sought to establish a set of observations that would address the gap in our knowledge of maturation. This would also provide a reference for us and others to understand maturation in model systems such as organoids. Through examining the timing of these phenotypic changes, we may be better able to define a timeline for maturation and to gain a better understanding of development.

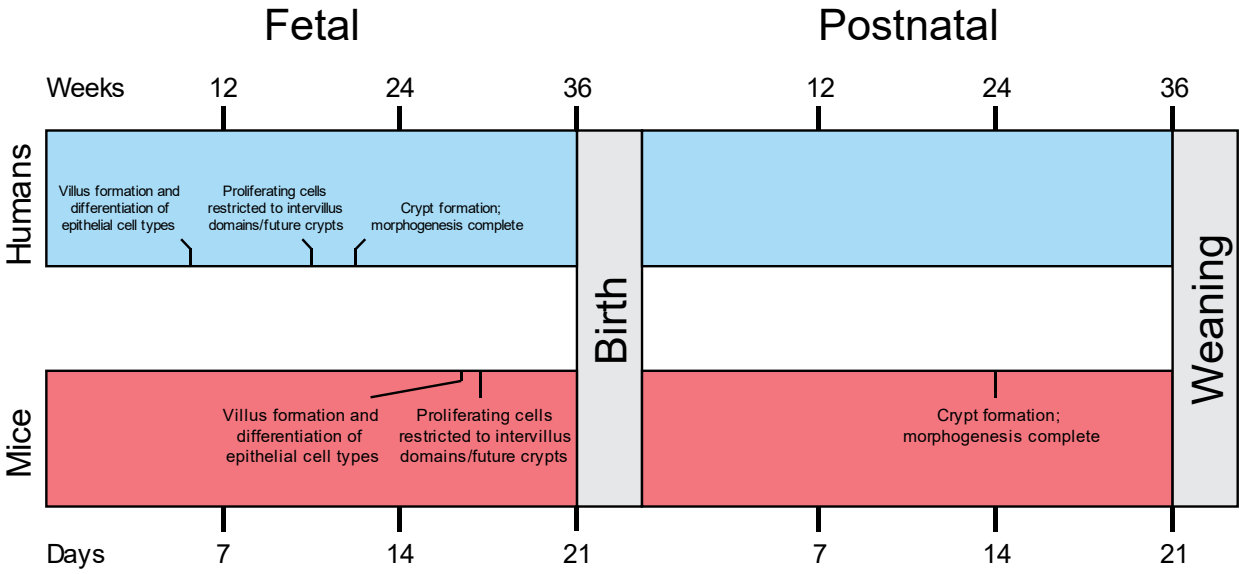

Figure 1. Schematic of the comparison between human and mouse intestinal epithelial development from conception to weaning. Morphogenesis of the intestinal epithelium in

humans is complete several months before birth around the end of the second trimester, whereas in mice it is complete around two weeks after birth. Adapted from McCracken et al., 2001.

Materials and Methods

Maintenance and Differentiation of hESC

H1 and H9 wt hESC (Wicell Research Institute) were cultured on matrigel (Corning; #354234) coated tissue culture plates in mouse embryonic fibroblast-conditioned medium (MEF-CM). Cells were passaged when approaching confluence at about 6-7 days. Cells were detached using collagenase IV (Invitrogen) at 37ºC until the edges started to lift off then mechanically scraped, spun and plated at 1:5. For differentiation, cells were plated in the 48-well format.

Differentiation of hESC into Intestinal Organoids

After allowing the hESC to grow for 2-3 days in the 48-well format we used the 4-stage differentiation protocol developed in our lab previously (Nadkarni et al. 2017). Stage 1 is 3 days long and uses a basal media consisting of RPMI 1640 (ThermoFisher; 11875093), 1X non-essential amino acids, 1X GlutaMAX, and 0.05% BSA. The basal media is supplemented with 100ng/mL Activin A (R&D systems; 338-AC-010) and 25 ng/mL Wnt3A (R&D Systems; 5036-WN-010) for all 3 days and with 0.2% FBS on day 2 and 2% FBS on day 3.

The basal media for stage 2 and stage 3 is DMEM/F12 (ThermoFisher; 11320033), 1X non-essential amino acid, 1X GlutaMAX, 0.05% BSA, 0.4 µM monothioglycerol (Sigma; M6145), 1X B27 (ThermoFisher; 17504044), 1X N2 supplement (ThermoFisher; 17502048) and 50 µg/mL L-ascorbic acid. Stage 2 lasts for 2 days and the basal media is supplemented with 200 ng/mL Noggin (Peprotech; 120-10C) and 10 µM SB431542 (Tocris; 1614). For stage 3, basal media is supplemented with 10 ng/mL BMP4 (Peprotech; 120-05), 20 ng/mL EGF, 10 ng/mL (Peprotech; AF-100-15), FGF10 (Peprotech; 100-26), 10 ng/mL KGF (Peprotech; 100-19B), 0.05 µM all trans retinoic acid (Sigma; R2625) and 100 ng/mL Wnt3A for 6 days.

At the end of stage 3, cells were detached as aggregates by mechanical scraping and seeded in a 3D matrix of 1:1 dilution of growth factor reduced Matrigel (GFRM, Corning; #356231) and 100 µL mouse tracheal epithelial cell (MTEC) media in a 24-well format. MTEC media consists of DMEM/F12 with HEPES (ThermoFisher; 11330032), 1X non-essential amino acids, 1X GlutaMAX, 1X Insulin-Transferin-Selenium (ThermoFisher, 51500056), 30 µg/mL Bovine Pituitary Extract (ThermoFisher; 13028014), 5% FBS, 25 ng/mL EGF, and 10 nM all-trans retinoic acid. After seeding in the gel, the gel was allowed to solidify for 30 minutes at 37ºC before overlaying 500 µL of MTEC media on top. Cells were grown in a humidified incubator at 37ºC and 5% carbon dioxide. The media was replaced every 2 days and the cells were cultured for 21 days before selection.

After 21 days of culture in stage 4, the cells are detached and singularized with TrypLE and prepared for fluorescence activated cell sorting. $ECAD^+$ cells were selected for and replated in hEnS media.

Maintenance of hEnS

Primary human lung fibroblasts (HLF) (Martin Kolb) were grown in preparation to coculture with hEnS. HLF were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% FBS, 1X non-essential amino acids, and 1X Glutamax and kept at 37ºC and 5% carbon dioxide. TrypLE was used to detach the cells when the hEnS were ready to be seeded.

hEnS basal media consists of adv DMEM F12, 1X glutamax, 1X N2, 1X B27, and 10 mM HEPES which is supplemented with 100 ng/mL Wnt3A (R&D Systems; 5036-WN-010), 50 ng/mL EGF (Peprotech; AF-100-15), 100 ng/mL Noggin (Peprotech; 120-10C), 500 ng/mL Rspondin (Peprotech; 120-38), 10 ng/mL Gastrin (Sigma; G9145), and 10 mM Nicotinamide

(Sigma; N0636). In brief, hEnS were washed with PBS and collected with cell recovery solution (Corning). The gel was dissolved for 2 hours on ice. hEnS were washed with PBS and singularized with TrypLE before being seeded with HLF into a 48-well format with 50000 hEnS and 50000 HLF per well. After allowing the gel to solidify for 30 minutes, 200 µL of hEnS media was overlayed on top. Media was changed every 3 days adding extra 50 µL per well with every change.

RNA extraction and qRT-PCR

Total RNA was isolated using Trizol LS reagent or RNeasy Mini Kit (Quiagen) and contaminating DNA removed using DNase treatment (Qiagen). cDNA was made from total RNA using SensiFAST™ cDNA synthesis kit (Bioline). FAM-based detection using PerfeCTa Multiplex qPCR SuperMix (Quanta Biosciences; 97065-230) was used with probes from the Universal Probe Library (Roche) and optimized primer pairs for qRT-PCR analysis of Cldn3 on Bio-Rad CFX96. Values were normalized to GAPDH.

Statistics

Graphs from quantitative data were created on GraphPad Prism 7 and significant differences compared using two-tailed unpaired t-test.

Treatment of hEnS with Enteric Bacteria

Bacterial supernatants were added to the overlaying media during each media change. Heat killed bacteria was added at given MOI during seeding of the hEnS within the gel. Bacteria were cultured by Dr. Michael Surette's lab in cooked meat broth (Sigma) and both supernatant and heat killed bacteria were made using meat broth. Both heat-killed bacteria and supernatant were tested to ensure no living bacterial cells were present.

Obtaining tissue sections of human and mouse intestine

Fixed tissue sections of human intestine from gestation were obtained from Hamilton Health Sciences and St. Joseph's Healthcare Hamilton in Ontario, Canada. Full approval was obtained from the Hamilton Integrated Research Ethics Board (HiREB) for the use of human tissue for research purposes. Tissue blocks of fetal autopsies and products of conception from completed cases were chosen by a staff pathologist. The gestational age of each sample was determined at the time of diagnosis, based on the patient history and gestational age reference values. Only the tissue blocks containing small intestine were considered and identified by the description on the software Meditech.

Murine intestinal samples were obtained from Dr. Tae-Hee Kim's lab in the department of Molecular Genetics at the University of Toronto in Toronto, Ontario. The mouse strain used was C57BL/6 (Black 6). Mice were housed in specific-pathogen-free (SPF) barrier facilities. All experimental work and mouse handling was performed according to protocols approved by The Centre for Phenogenomics (TCP) Animal Care Committee in Toronto, Ontario (AUP #23-0276H). Mice were sacrificed and small intestine was harvested from the chosen embryonic and postnatal ages.

Immunostaining of tissue sections

Formalin-fixed paraffin-embedded (FFPE) tissue sections were stained according to protocols from R&D systems for fluorescent immunohistochemical staining and chromogenic immunohistochemical staining. In brief, sections are rehydrated by immersion in xylene 2 times for 10 minutes, then 100% ethanol for 2 times for 10 minutes, then 95%, 70%, and 50% ethanol for 5 minutes each. Antigen retrieval can then optionally be performed by immersing the sections

in 0.01 M citrate buffer (pH 6) and steaming for 25 minutes. After rehydration in PBS, the sections can be blocked and stained with primary antibody overnight at 4ºC. Sections can then be washed and incubated with appropriate secondary antibodies before mounting and fluorescence imaging. For chromogenic IHC staining, reagents from Anti-mouse (CTS002), Anti-rabbit (CTS005), and Anti-rat HRP-DAB Cell & Tissue Staining Kit (CTS017) from R&D Systems were used. After rehydration in PBS, a series of blocking steps are required including peroxide blocking, avidin blocking, and biotin blocking before incubation with primary antibody. After washing, Biotinylated secondary antibodies and high sensitivity-HRP conjugate can be added. Careful monitoring is required after addition of DAB chromagen solution and during counterstaining with hematoxylin to ensure the slides are not oversaturated.

Table 1. Primary antibody information

Antibody/protein	**Vendor**	**Catalog no.**	**Usage**
CDX2 (m)	Abcam	ab76541	1:100
CLDN3 (h/m)	Abcam	ab15102	1:100
DEFA5 (h)	Novus Biologicals	NB110-60002SS	10 μg/ml (1:235)
DEFA1 (m)	cloud-clone corp	PAB705Mu01	1:100
OLFM4 (h)	Abcam	ab85046	5 μg/ml (1:120)
OLFM4 (m)	Cell Signaling	39141T	1:150
SI (h/m)	Santa Cruz	sc-27603	1:100
UEA1 (h/m)	Vector Laboratories	DL-1067	1:100

H&E, AB-PAS and chromogenic IHC staining for CDX2 and CHGA were performed by technical staff in the HRLMP at St. Joseph's Hospital in Hamilton, Ontario, Canada. Antibody information:

CDX2: Clone - DAK-CDX2; Isotype - IgG1, kappa

CHGA: Clone - DAK-A3; Isotype - IgG2b, kappa

Imaging and processing

IF-stained sections were viewed using an Olympus IX81 inverted fluorescence microscope, and photographs were captured with Hamamatsu C11440 digital camera. IHC-stained sections were scanned on an Aperio ScanScope slide scanner. Image processing and channel merging was done on ImageJ.

Results

H&E, AB/PAS and CDX2 show structural changes, the presence of goblet cells and confirms intestinal identity,

To examine a variety of time points during gestation, we obtained intestinal tissue at 13, 18, 24, and 32 weeks of gestation which represent the end of the first trimester, middle of the second trimester, end of the second trimester, and middle of the third trimester, respectively. Although gestational tissue is logistically difficult to obtain, we were able to obtain 2 sets of tissue around each of these time points. For comparison, we obtained murine intestinal tissue from E17.5, P7, P14, P21 and P60. These ages span from the age of villus formation to the age which the murine intestine is mature. P60 is an adult mouse for comparison.

To identify good quality tissue that stains properly, we selected for tissue which showed proper CDX2 and haematoxylin and eosin (H&E) staining (figure 2). CDX2 is the master regulator of intestinal identity and is present in all intestinal epithelial cells. CDX2 was present in all samples obtained and thus confirmed that the samples were in fact intestinal tissue (Silberg et al., 2000; Zorn and Wells, 2009). H&E staining reveals changes in epithelial structure during development. Most notably, the appearance of crypt structures after 20 weeks of gestation in humans while it appears around P14 (after birth) in the murine sample (Chin et al., 2017). Tissues were also examined for the density of goblet cells to differentiate between the small intestine and colon samples as colonic samples have a significantly higher density of goblet cells.

Using Alcian Blue and Periodic Acid Schiff (ABPAS), we demonstrated that goblet cells are present in the epithelium as early as 13 weeks of gestation, similar to its appearance in mice

after villus formation (figure 3). We then stained for protein markers of maturation including: cell type emergence, functionality, structural integrity and protective role.

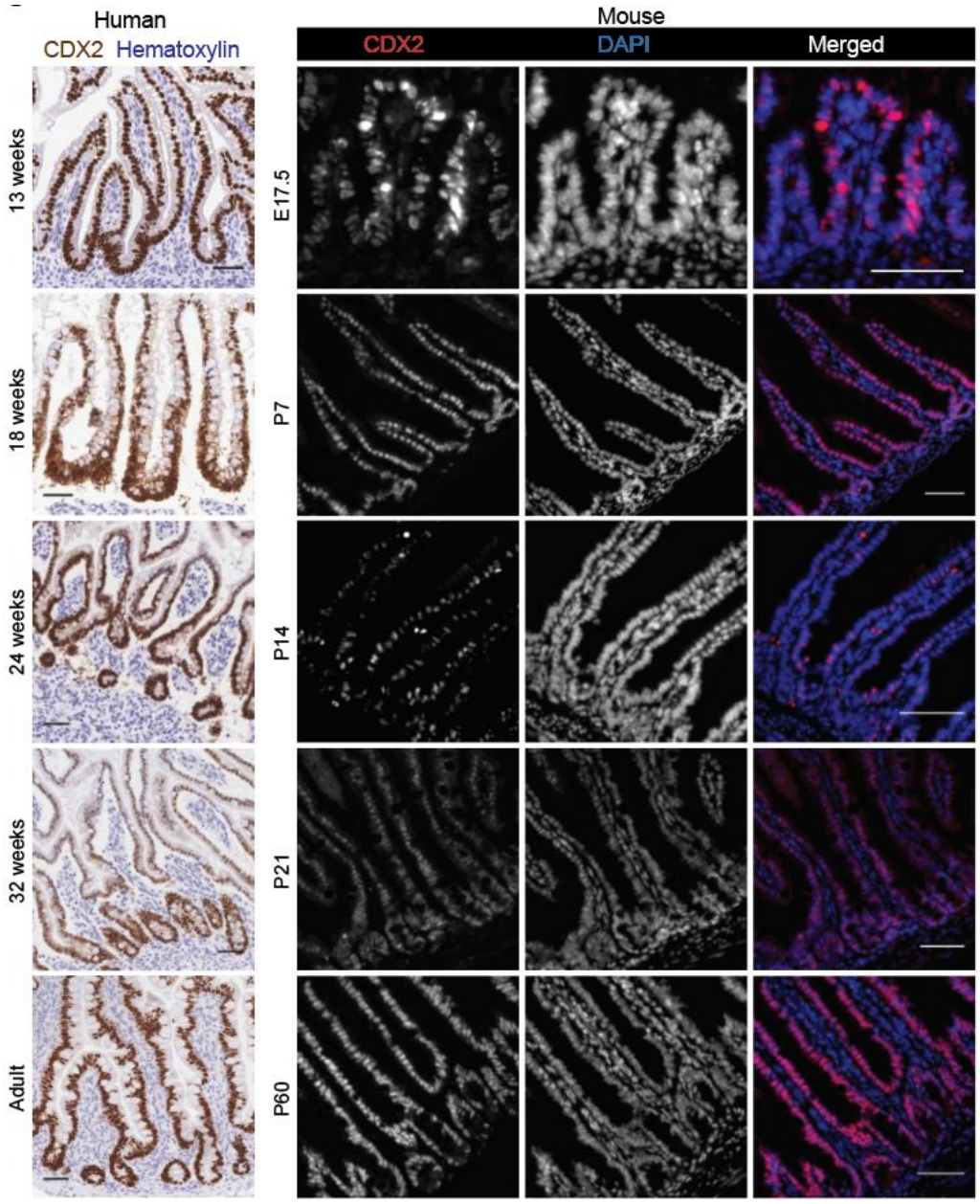

Figure 2. Chromogenic and Fluorescent IHC staining of gestational and postnatal small intestine in humans versus mice for CDX2. Scale bars, 50 μm.

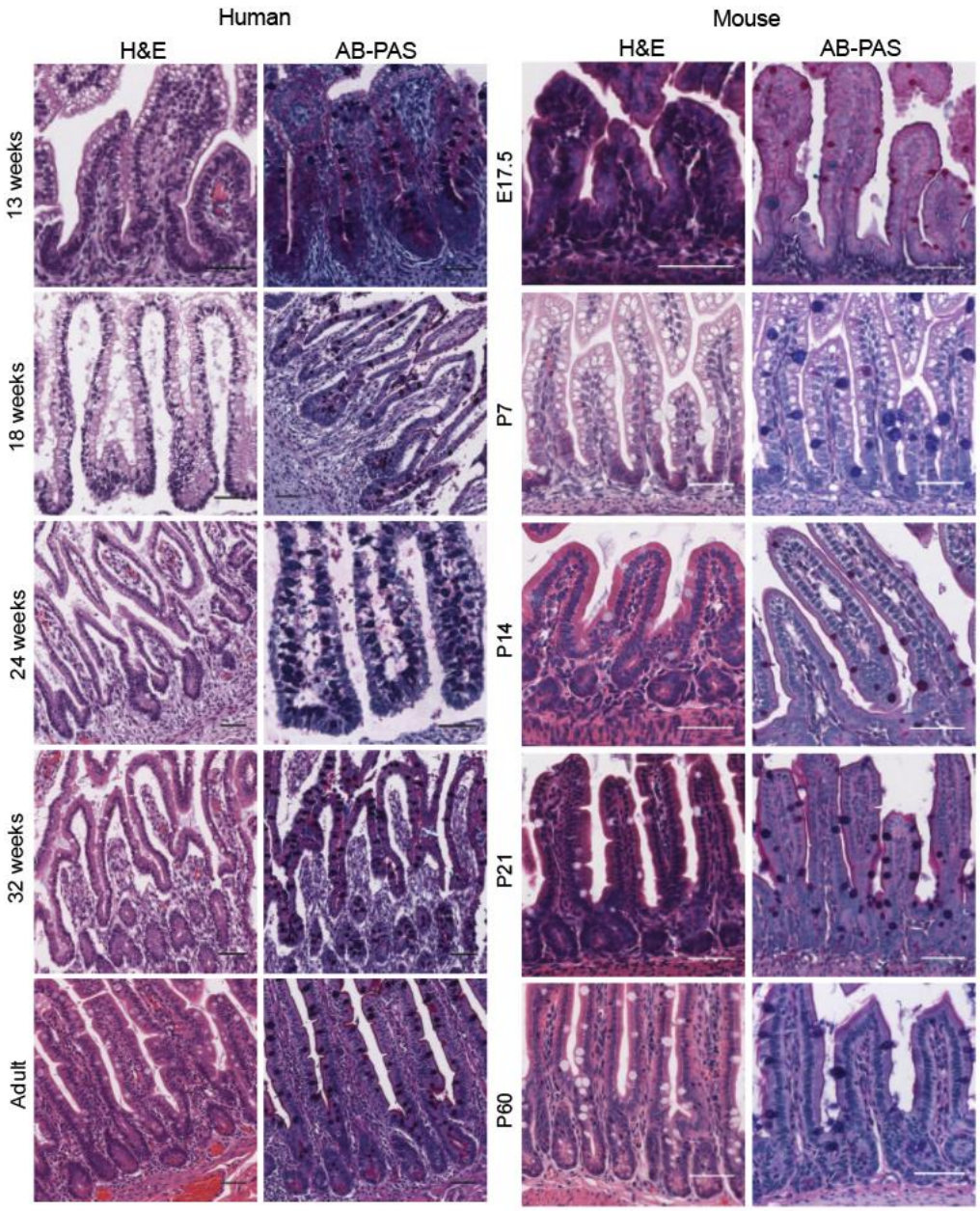

Figure 3. Haematoxylin and eosin (H&E) staining and Alcian Blue and Periodic Acid Schiff (ABPAS) staining of gestational and postnatal small intestine in humans versus mice. Scale bars, 50 μm.

Sucrase Isomaltase and Chromogranin A, markers of brush border maturation and enteroendocrine cells, appear during early gestation

Villus formation occurs early in intestinal development at 9 weeks of gestation in humans and P15 in mice (Chin et al., 2017; Noah et al., 2011). The major cell type present in the developing epithelium is absorptive enterocytes which constitute over 80% of the total surface area and are responsible for absorption of luminal nutrients. The defining presence of microvilli on the surface of the enterocytes make up the characteristic brush border of the intestine. Villin (VIL1) is an actin-binding protein that can be used as a marker of the brush border. However, a major characteristic of a mature intestine is the presence of digestive enzymes which demonstrate the functionality of the brush border. Sucrase isomaltase (SI) is one such enzyme which is found in mice after birth. Expression of SI increases towards the end of the weaning process in mice. In humans, RNA expression of SI was found to be low in the fetal intestine and increases into adulthood (Finkbeiner et al., 2015). These findings suggest that SI can be used as an indicator of brush border maturation. However, there have not been any studies on protein expression to date. As with previous findings, we demonstrate that SI can be found in mice at P21 but is not seen in any earlier ages (figure 4). Contrastingly, protein expression of SI in humans is found in all ages including tissue from as early as 13 weeks of gestation. This shows that SI protein expression begins early in gestation and continues throughout pregnancy which not only challenges the previous notion that SI appears later but also demonstrates that it can be used as a marker of enterocyte maturation.

Intestinal enteroendocrine cells (EEC) are specialized cells which secrete a variety of hormones in response to different stimuli. For example, EECs play an essential role in enzyme secretion, motility, and appetite regulation but also act as modulators of immune activity in

response to microbial products. EECs make up 1% of intestinal epithelium and are located as single cells spread throughout the intestine (Noah et al., 2011). Difficulty differentiating these cells *in vitro* from pluripotent stem cells has led researchers to believe that this cell type may appear later in development and thus has utility as a marker of maturation (Gunawardene et al., 2011). In mice, EEC appear around E16.5 shortly after villus morphogenesis begins but it is not currently known when EEC appear in humans (figure 4). Chromogranin A (CHGA) is a neuroendocrine secretory protein which is found in secretory vesicles. CHGA has multiple functions including regulation of neuroendocrine function. We found that CHGA was present as early as 13 weeks of gestation and persists throughout gestation. CHGA-expressing cells were also present in higher frequency in crypt regions as compared to villi at 32 weeks of gestation and in the adult tissue. This indicates that EECs emerge as early as the end of the first semester after villus morphogenesis begins similar to the timing of the appearance of EECs in the murine model.

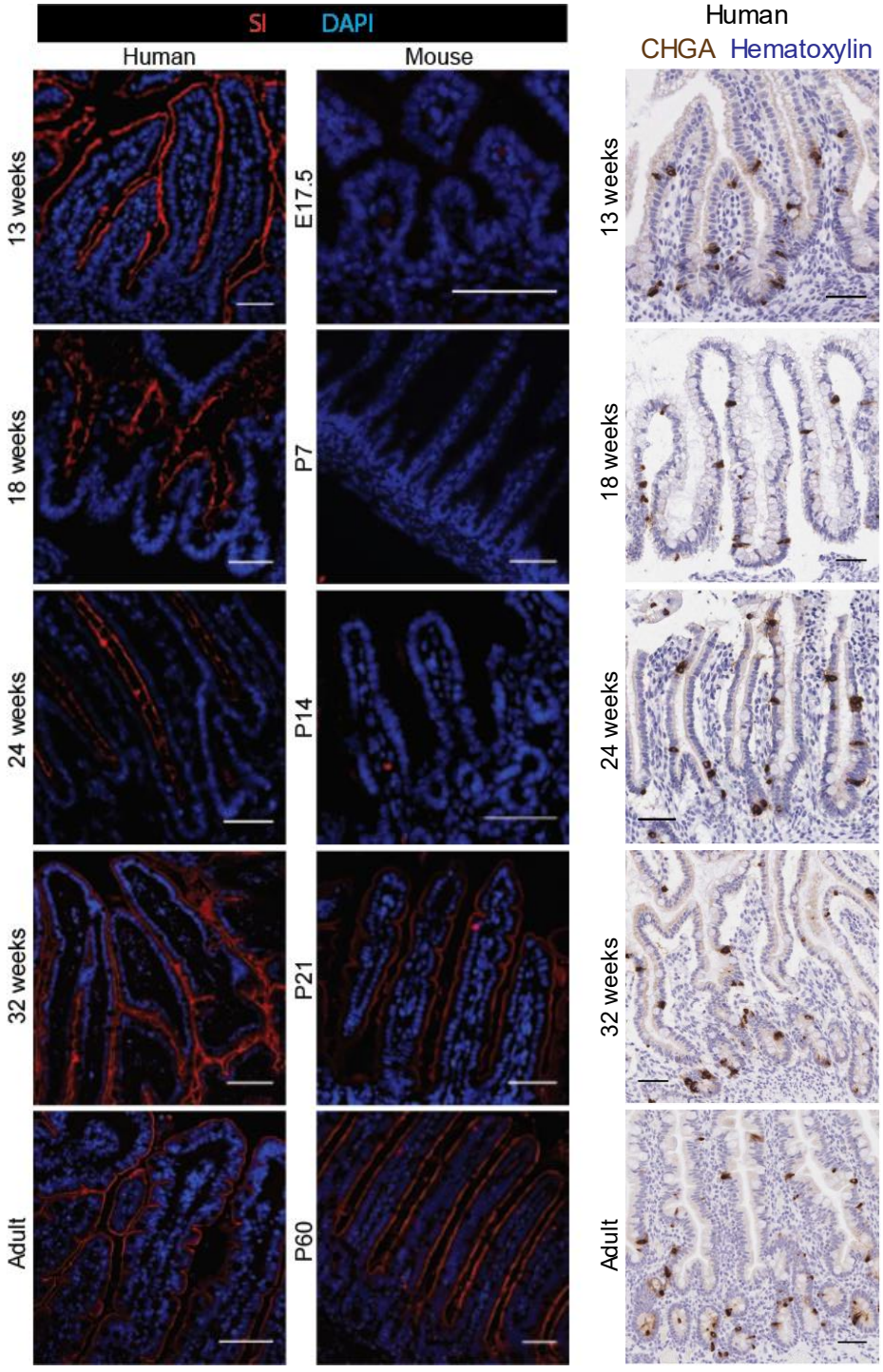

Figure 4. Fluorescent and chromogenic IHC staining of gestational and postnatal small intestine in humans versus mice for SI and Chromogranin A (CHGA). Scale bars, 50 μm.

OLFM4 a marker of intestinal stem cells appears in crypt base columnar cells before birth in the human intestine

The turnover rate of cells is an important characteristic of a mature and fully functional intestine which is why the presence of intestinal stem cells is thought to be a marker of maturation (van der Flier et al., 2009). The most common marker for definitive, long-term, propagating stem cells in the crypts is LGR5 but it is not strongly expressed. Olfactomedin 4 (OLFM4) is expressed by multipotent stem cell population found in crypt base columnar cells which is the same population as $LGR5^{+}$ cells. OLFM4 is a secreted molecule that was found to play a role in apoptosis in multiple types of cancer. We hypothesized that OLFM4 protein expression would be present when crypt morphogenesis occurs at approximately 20 weeks of gestation (Finkbeiner et al., 2015). We found that at 24 weeks, OLFM4 could be seen in the developing crypt regions in the crypt base columnar cells (figure 5). However, OLFM4 was not present at 13 weeks or 18 weeks when crypts were not yet present. In mice, OLFM4 was found sporadically at P14 and more consistently in the crypts at P21, concurrent with crypt development in the mouse. Our results indicate that OLFM4 is present in the human fetal intestine at the end of the second trimester before 24 weeks of gestation and is comparable to expression of OLFM4 in the adult intestine. The expression of OLFM4 in both mice and humans occur after crypt morphogenesis begins although the exact timing of these processes differs between humans and mice, with occurrences before birth in humans and after birth in mice.

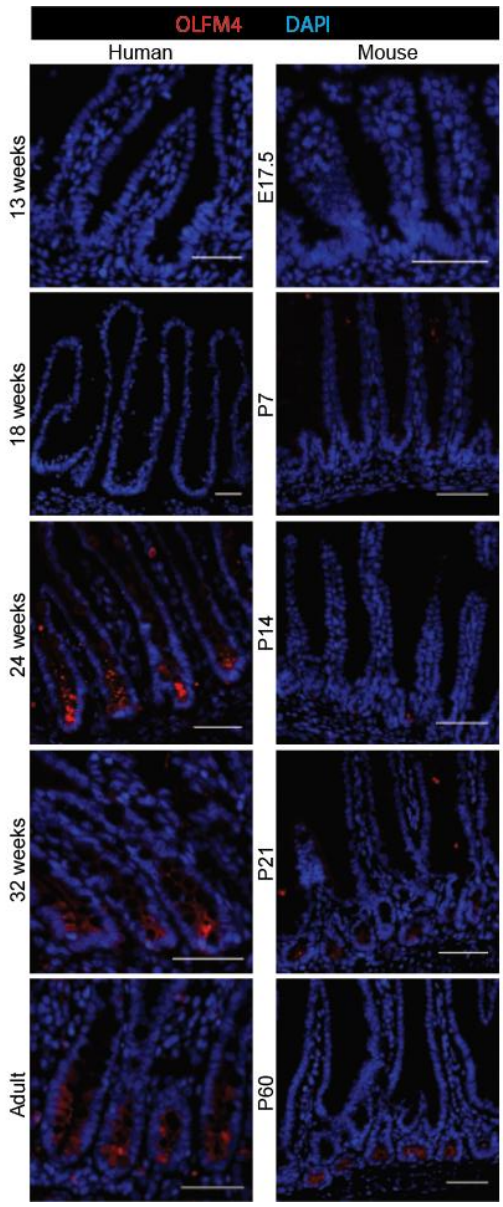

Figure 5. Fluorescent IHC staining of gestational and postnatal small intestine in humans versus mice for OLFM4. Scale bars, 50 μm.

DefA5, a marker of Paneth cell maturation appears before birth in the human intestine

Paneth cells are important for maintaining the stem cell niche and secreting antibacterial compounds to maintain homeostatic balance of microbes. Their differentiation coincides with

crypt emergence which occurs at P14 in mice and 20 weeks of gestation in humans (Chin et al., 2017; Kim et al., 2012). Initially, Paneth cells can be distinguished by production of lysozyme but the production of α-defensins is an indicator of Paneth cell maturation (Ayabe et al., 2000). α-defensins are small antimicrobial cationic peptides that create pores in the cell membrane of bacteria, fungi and even some viruses. We hypothesize that maturation of Paneth cells occurs around the same time that the cells start to appear at 20 weeks of gestation. Here, we use α-defensin 5 (DEFA5) to mark human α-defensins and cryptidin 1 (DEFA1) for murine α-defensin. We found that DEFA5 was present at 24 and 32 weeks of gestation in the crypts concurrent with human crypt morphogenesis (figure 6). DEFA5 was not visible in 13 and 18 weeks of gestation as Paneth cell differentiation would not have occurred before 20 weeks. In mice, DEFA1 is sparingly visible at P7 in the intervillus domains and appears in the crypts at P14 and P21. This suggests that Paneth cell maturation occurs before birth in humans promptly after they emerge.

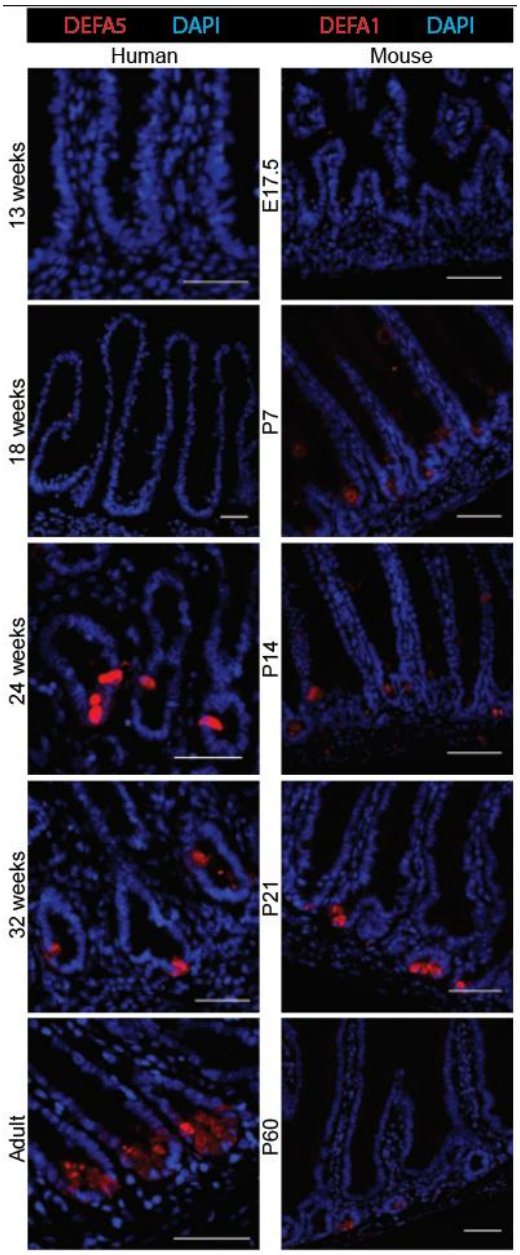

Figure 6. Fluorescent IHC staining of gestational and postnatal small intestine in humans versus mice for DEFA5(h)/DEFA1(m). Scale bars, 50 μm.

Fucosylation in the human intestine occurs during early gestation but changes localization with morphogenesis.

Fucosylation is a type of glucosylation in which fucose units are added to glycoproteins and glycolipids. Although this is thought to play a protective role by suppressing virulence of harmful pathogens, the exact timing at which fucosylation occurs during human development is still unknown (Pickard and Chervonsky, 2015). However, in mice, fucosylation is increased towards the end of the weaning process and is influenced by the colonization of different microbes. For example, colonization by *Bacteroides thetaiotaomicron* or treatment with polyamines was found to induce fucosylation of the brush border and mucinous glycoproteins in germ-free mouse and rat models (Biol-N'Garagba et al., 2002; Bry et al., 1996). Fucosylation can be identified by the expression of fucosyltransferase and by reactivity to the lectin, Ulex europaeus agglutinin 1 (UEA1). Fucosylation is often found on glycoproteins produced by Paneth cells in the crypts and mucins from goblet cells in the villi. We found that in human adults UEA1 could be seen clearly defining the brush border and also in goblet cells and Paneth cells (figure 7). At 24 and 32 weeks of gestation, UEA1 clearly marked goblet cells and Paneth cells but did not line the brush border. At 18 weeks of gestation, UEA1 was seen in the lumen in patches resembling secreted mucins and at 13 week of gestation there was no obvious localization. In mice, at E17.5 and P7, UEA1 can be seen in the goblet cells and patches resembling secreted mucins. At P14 and P21, UEA1 is seen in both Paneth cells and Goblet cells but it is also found sporadically along the brush border. In comparison, P60 has a more definitive lining of the brush border marked by UEA1 along with Paneth cells and Goblet cells. This indicates that in humans fucosylation is localized to goblet cells and Paneth cells at 24 and 32

weeks of gestation but appears in the brush border glycoproteins some time after 32 weeks of gestation.

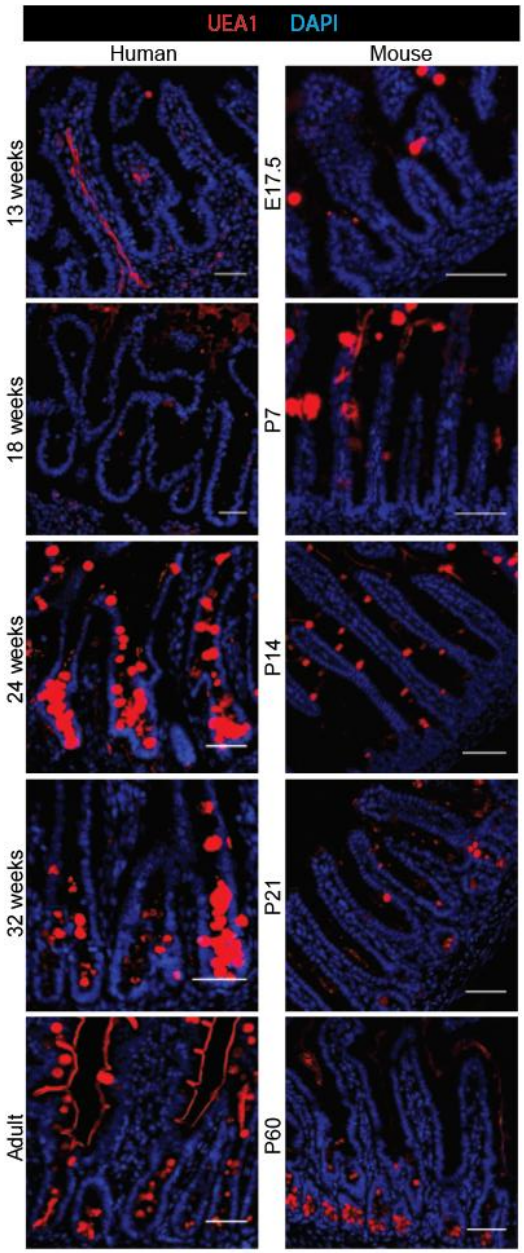

Figure 7. Fluorescent IHC staining of gestational and postnatal small intestine in humans versus mice for lectin UEA1. Scale bars, 50 μm.

Claudin 3 expression emerges before birth in the human intestine

Epithelial barrier function is essential for preventing gut luminal pathogens and toxins from entering (Grave et al., 2007). In the case of premature infants suffering from NEC or patients with inflammatory bowel disease, the intestine is vulnerable to invading bacteria due to compromised barrier integrity (Alfaleh et al., 2011). However, the use of breast milk and probiotics can contribute to proper barrier maturation in premature infants and thus prevent disease. Claudin 3 (CLDN3) is a tight junction marker that acts as a measure of improved intestinal barrier integrity (Patel et al., 2012). In the murine intestine, Cldn3 is thought to be absent at a protein level during gestation but increases as intestinal morphogenesis approaches completion at 2-3 weeks after birth. Germ-free mice maintain a low level of CLDN3 expression but expression can be induced by colonization with probiotic bacteria *Lactobacillus rhamnosus* (Günzel and Yu, 2013; Lu et al., 2013; *Patel et al., 2012)*. We hypothesized that similar to the murine intestine, CLDN3 expression would start low and increase exponentially after birth with the colonization of commensal bacteria. In mice, we found that protein expression was sporadic at E17.5 and became more robust at P7 and P14 with higher expression found towards the base of the villi (figure 8). At P21, CLDN3 can be seen throughout the villi but not as strongly expressed throughout as it is at P60. In humans, weak CLDN3 expression can be seen throughout the villi at 13 weeks of gestation, with expression appearing similar to adults by 18 weeks of gestation. This indicates that CLDN3 protein expression emerges before birth as early as the beginning of the second trimester.

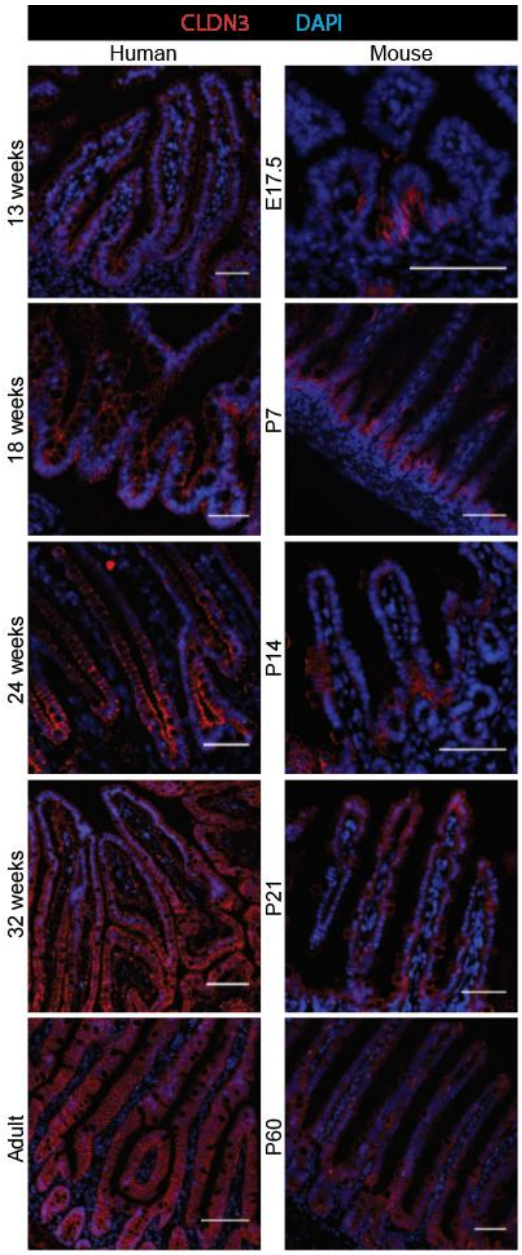

Figure 8. Fluorescent IHC staining of gestational and postnatal small intestine in humans versus mice for CLDN3. Scale bars, 50 μm.

hPSC-derived enterospheres resemble the fetal intestine in maturation status

Similar to other PSC-derived models, our hEnS display a fetal phenotype in comparison to adult stem cell derived organoids and the native intestine. Previously our lab has shown that the hEnS are functional and express a variety of intestinal genes. Here, we stained for all the markers described above to evaluate the maturation status of our hEnS (figure 9). We found that SI and fucosylated surfaces marked by UEA1 were present but CHGA, OLFM4, DEFA5, and CLDN3 were not. When compared to the gestational tissue, the phenotype would be most similar to the fetal intestinal epithelium from before 18 weeks of gestation.

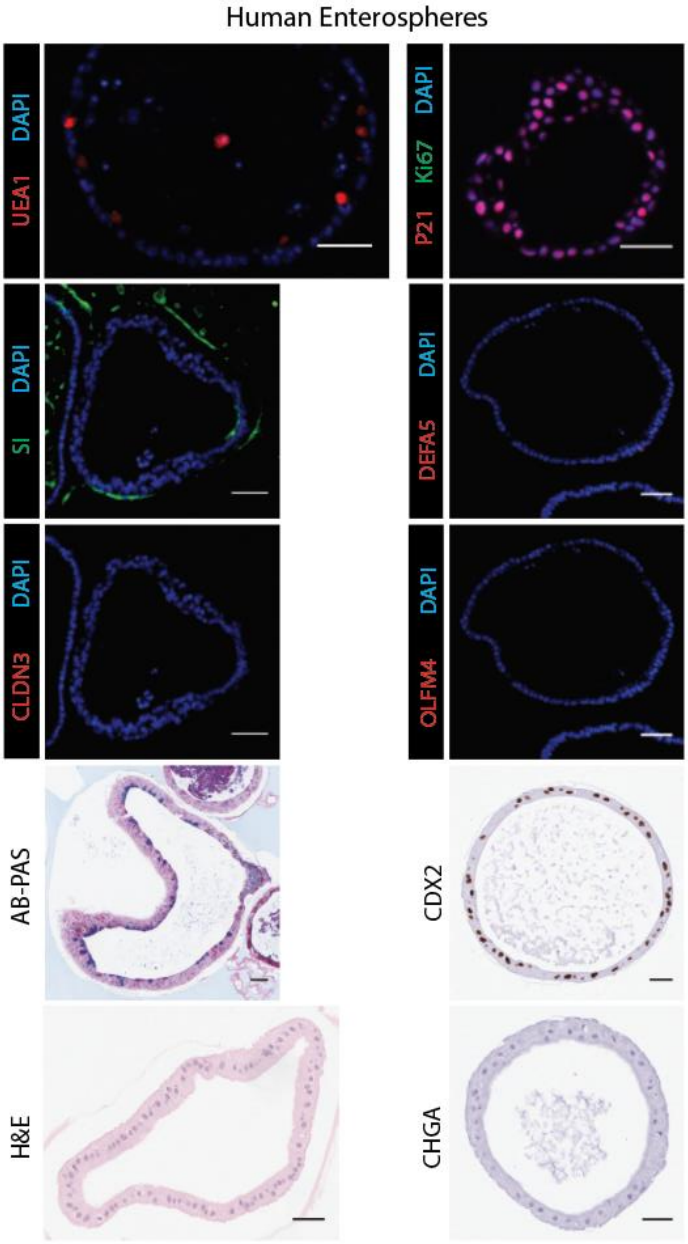

Figure 9. Fluorescent and chromogenic IHC staining for maturation markers of hPSC-derived enterospheres. Scale bars, 100 μm.

Discussion

Previously, studies on development of the fetal intestine during gestation have been investigated in non-human models because of the lack of access to human tissue. Here, we have an opportunity to investigate the changes in phenotypic expression of various protein markers throughout human gestation. We found that there are significant differences between the timing of the appearance of many markers in humans when compared to mice. There have been previous studies on the transcript level of certain markers used in this study, but the results have not been confirmed at the protein level nor at multiple gestational ages (Finkbeiner et al., 2015). The use of tissue sections provides us with information about localization and structure of the intestine allowing us to visualize changes over time in comparison to whole tissue protein analysis such as western blots. However, access to tissue was still a major issue and it was difficult to obtain many samples of higher quality. The study was also limited by the current availability of antibodies and their compatibility with the samples. Despite these limitations, the information provided allows us to take a step towards understanding intestinal development and allows us to better translate findings from other models.

The emergence of EECs, is thought to be a putative indicator of intestinal maturation. Using CHGA, a marker of EECs, we show that CHGA is present in the fetal intestinal epithelium as early as 13 weeks of gestation, indicating EECs are one of the earlier emerging cell types in humans, similar to mice (Chin et al., 2017). Difficulty generating EECs from hPSC *in vitro* is attributed to the lack of exogenous signaling to activate genes specific for promoting EEC differentiation such as *Neurogenin-3* (Sinagoga et al., 2018).

Previous studies on SI, OLFM4, and DEFA5 show that there is a low transcript level in the fetal intestine which is highest in the adult intestine (Finkbeiner et al., 2015). No protein data

has been shown from gestational tissue in humans but in mice all 3 of these markers appear after birth and is thought to indicate maturation. We confirmed these findings in the murine intestine. SI, a digestive enzyme marking the brush border, appears before P21 in mice which is when weening of the pups occurs (Finkbeiner et al., 2015). OLFM4 and DEFA1, intestinal stem cell and mature Paneth cell markers, appear strongly by P21, which is when crypts have formed. In humans, SI appeared as early as 13 weeks of gestation which suggests that brush border maturation occurs before birth. Similarly, OLFM4 and DEFA5 are present before 24 weeks of gestation which indicates that maturation of the crypt also occurs before birth in humans.

In the murine intestine, CLDN3 is absent at the protein level during gestation and does not emerge until exposure to probiotic bacteria (Patel et al., 2012). Similarly, fucosylation of the brushborder by fucosyltransferases occurs after birth and exposure to *Bacteriodes thetaiotaomicron* or polyamines in mice (Bry et al., 1996). However, we found that CLDN3 expression was present robustly as early as 18 weeks of gestation. This suggests that the emergence of CLDN3 may not be linked to probiotic exposure in humans. UEA1 reactivity to fucosylation was localized to goblet cells and Paneth cells at 24 and 32 weeks of gestation but in the adult tissue it was shown to line the brush border. In mice, we found fucosylation in the goblet cells at E17.5 which is early in morphogenesis and eventually in the brush border by P60. The data in the murine intestine supports previous findings of the timing of brush border fucosylation. However, in humans, fucosylation of the brush border does not occur within 32 weeks of gestation. These results suggest that this event occurs after birth and potentially after exposure to probiotic bacteria.

The timing of many intestinal maturation events is significantly different between humans and mice. For example, the completion of morphogenesis occurs before birth in humans

and after birth in mice (McCracken and Lorenz, 2001). The fetal intestine may have systemic access to bacterial products from the maternal microbiome. The intestine was thought to be sterile before birth, but recent studies have showed that there may be some levels of bacterial products present in the placenta and amniotic fluid which may enter the fetal intestine (Perez-Muñoz et al., 2017). Although the levels of bacteria would be nowhere close to the level that is present in the intestine after birth, there is a possibility that bacteria-mediated maturation may be *in utero*. Many of our findings suggest that the maturation happens *in utero* in comparison to murine intestine maturation, but it is likely that additional maturation occurs after birth and during the weaning process that will affect intestinal structure and function.

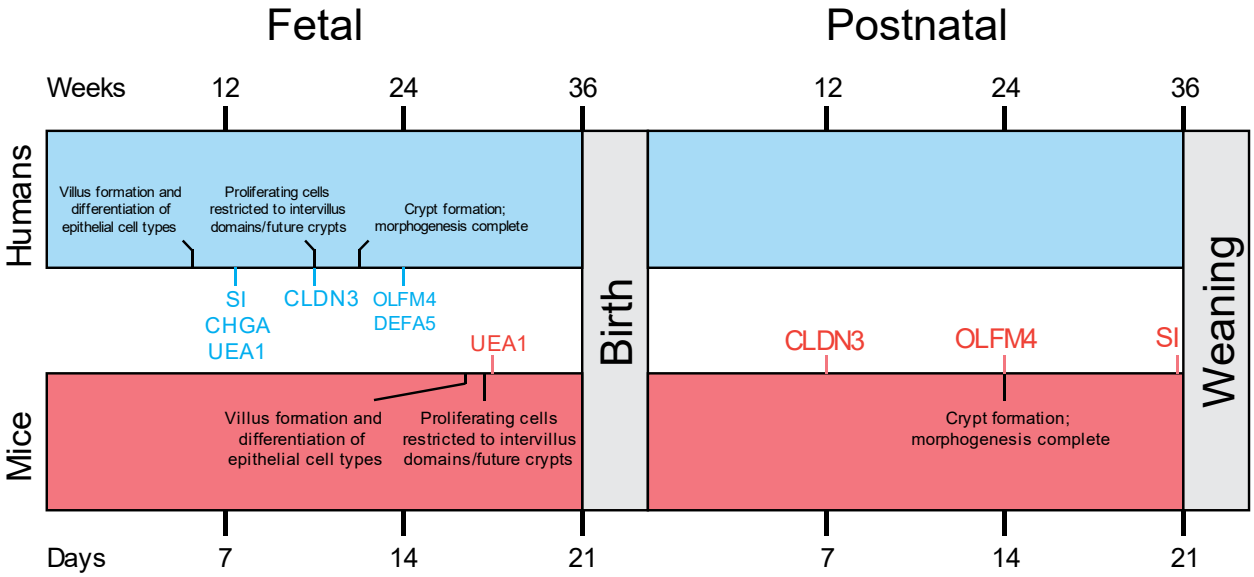

Figure 10. An updated schematic of the comparison between human and mouse intestinal epithelial development from conception to weaning including the appearance of novel staining markers. Adapted from McCracken et al., 2001.

The hEnS model displays a fetal phenotype which resembles the intestinal epithelium before 18 weeks of gestation. Positive staining of SI and UEA1 indicates that enterocytes and goblet cells are present in the spheres, while the absence of OLFM4, DEFA5, and CHGA indicate that adult intestinal stem cells, mature Paneth cells and enteroendocrine cells are not present in the spheres. Previously, we have shown that at a transcript level, stem cell related genes such as LGR5 and OLFM4 are expressed. This finding is consistent with the ability of hEnS to self-propogate indefinitely. We have also shown that lysozyme is present within the spheres when cultured in a secretory lineage-promoting media. Taken together, hEnS contain stem/progenitor cells which are capable of differentiation into enterocytes, goblet cells, and Paneth cells. However, the exact identity of the stem/progenitor cells has yet to be elucidated. Thus further studies are required to characterize this system.

Despite its many benefits, this model does have several limitations. Firstly, mesenchymal tissue has recently been reported as an important supporter of the intestinal stem cell niche through secretion of Wnt ligands (Gregorieff et al. 2005). Due to the fact that this model only contains epithelial cells, the effects of this interaction may not be observed (Nadkarni et al., 2017). Secondly, continuous culturing of the hEnS does not result in maturation as the system is missing environmental cues. While this allows us to isolate maturation based on our experimental procedures, there is also no definitive way to induce a mature phenotype. Thus, there would no positive control to confirm a mature phenotype. This is a common obstacle for all organoid models derived from hPSC. To induce maturation, certain cues from the body must be present. As mentioned before, intestinal organoids can be matured by engraftment in the kidney capsule of mice but that is not possible for humans. Lastly, since the cells are a mixed composition of

different cell types with unknown homogeneity, slight changes in transcription or translation may not be reflected if changes are induced in a cell type that is present in lower quantities.

In the future, we hope to obtain more samples from various other periods during gestation and from the postnatal period to further support our findings and to understand the critical changes that occur with exposure to bacteria and throughout weaning. Further studies on intestinal development may lead to the discovery of more putative markers of maturation which would also be beneficial to examine. We also hope to use our hEnS as a model of maturation and to examine what bacteria or compounds are able to induce maturation of intestinal organoids. Being able to mature intestinal organoids without *in vivo* engraftment would provide a fully defined model of maturation.

Conclusion

Overall, our work here provides a novel set of observational data that documents changes in maturation markers over gestation. Our data shows that intestinal stem cells and mature Paneth cells appear before birth in humans and after birth in mice. We also found that Cldn3 is robustly expressed by 18 weeks which contrasts previous studies that suggests the need for bacteria to induce robust expression of cldn3. We also show that SI appears before 13 weeks of gestation compared to mice in which SI appears at P21. We also examined our hEnS model for the same markers and found that it represents a fetal intestinal epithelium before week 18 of gestation. Our findings further support that there are major differences in the timing of the emergence of key markers of maturation between mice and humans. Our findings also provide a foundational data set that will allow researchers to compare the maturation of *in vitro* models such as intestinal organoids to gestational development.

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