NOTCH-REGULATED MECHANISMS OF EPITHELIAL CELL FATE SELECTION IN THE INTESTINE

by

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This thesis is dedicated to 5 great mentors:

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ABSTRACT

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Chair: Linda C. Samuelson

Throughout the lifetime of an organism, progenitor cells in the intestine proliferate and differentiate to form cells of the secretory and absorptive lineages. Many intercellular signaling pathways, including the Notch pathway, coordinate to develop and maintain the intestine. My thesis work has investigated how Notch signaling regulates intestinal cell fate using several novel genetically engineered and pharmacological mouse models. Developmental analysis of a transgenic mouse model with forced expression of the Notch-regulated transcription factor Mouse atonal homolog 1 (Math1) showed increased numbers of all secretory cell types and loss of absorptive cells, demonstrating that Math1 is the key factor regulating intestinal secretory cell differentiation. Furthermore, these data suggest that Math1 can redirect a bipotential progenitor cell to the secretory cell fate. To study the role of Math1 in adult intestine, I describe an inducible Math1 transgenic model; however, Math1 protein was not increased subsequent to

transgene activation, suggesting that there may be active degradation of Math1 protein in the intestine. Previous studies have shown that inhibition of Notch signaling resulted in decreased epithelial cell proliferation and altered cell fate, suggesting that a stem or progenitor cell is targeted by Notch signaling; however, the identity of this target was unknown. Pharmacological inhibition of Notch signaling in both fetal and adult intestine showed that expression of Olfactomedin 4 (Olfm4), a crypt base columnar stem cell gene, was markedly decreased upon Notch inhibition. Transcriptional studies in the human colon cancer cell line LS174T confirmed that Notch signaling activated OLFM4 gene expression and identified a region containing critical *cis*-regulatory DNA elements. Finally, I made the novel observation that a population of intermediate cells that express both goblet and Paneth cell markers emerged upon Notch inhibition in adult mouse ileum and colon. In conclusion, my thesis research has shown that Math1 is the key regulator of secretory cell differentiation in the intestine and that Notch signaling directly targets the crypt base columnar stem cell. Collectively, these studies have provided important information about Notch-regulated mechanisms of intestinal development and cell lineage determination.

CHAPTER 1

INTRODUCTION

Throughout the life of an organism, intestinal stem and progenitor cells are continuously dividing and differentiating to replenish the epithelial layer. This process is integral to preserving sterility of the internal environment and a sufficient epithelial surface for digestion and absorption of nutrients. Due to the close proximity of the epithelium to the external environment, the stem and progenitor cells must be able to respond and adapt to not only intrinsic cues, but also to a variety of environmental cues, in order to establish the correct balance of proliferation versus differentiation at any given time. As such, the processes of intestinal development and maintenance involve integration of many signals and are orchestrated by fundamental signaling pathways including Notch, Wnt, Hedgehog (Hh) and Bone Morphogenetic Protein (BMP)[1]. During adaptive responses or disease states, the equilibrium of signaling is changed, resulting in altered proportions of undifferentiated (proliferative) cells versus differentiated cells or of specific differentiated cell types. Thus, a fine-tuned and complex balance of many signals controls intestinal development, maintenance and response to stressors.

The general structure of the developing and adult intestine is shown in Figure 1-1. The mature intestine is composed of four main differentiated cell types including the absorptive enterocytes which are the most abundant intestinal cell and three secretory (granulocytic) cell types: the mucus-producing goblet cells, the anti-microbial peptide-producing Paneth cells and the hormone-releasing endocrine cells. With the exception of Paneth cells, differentiation of

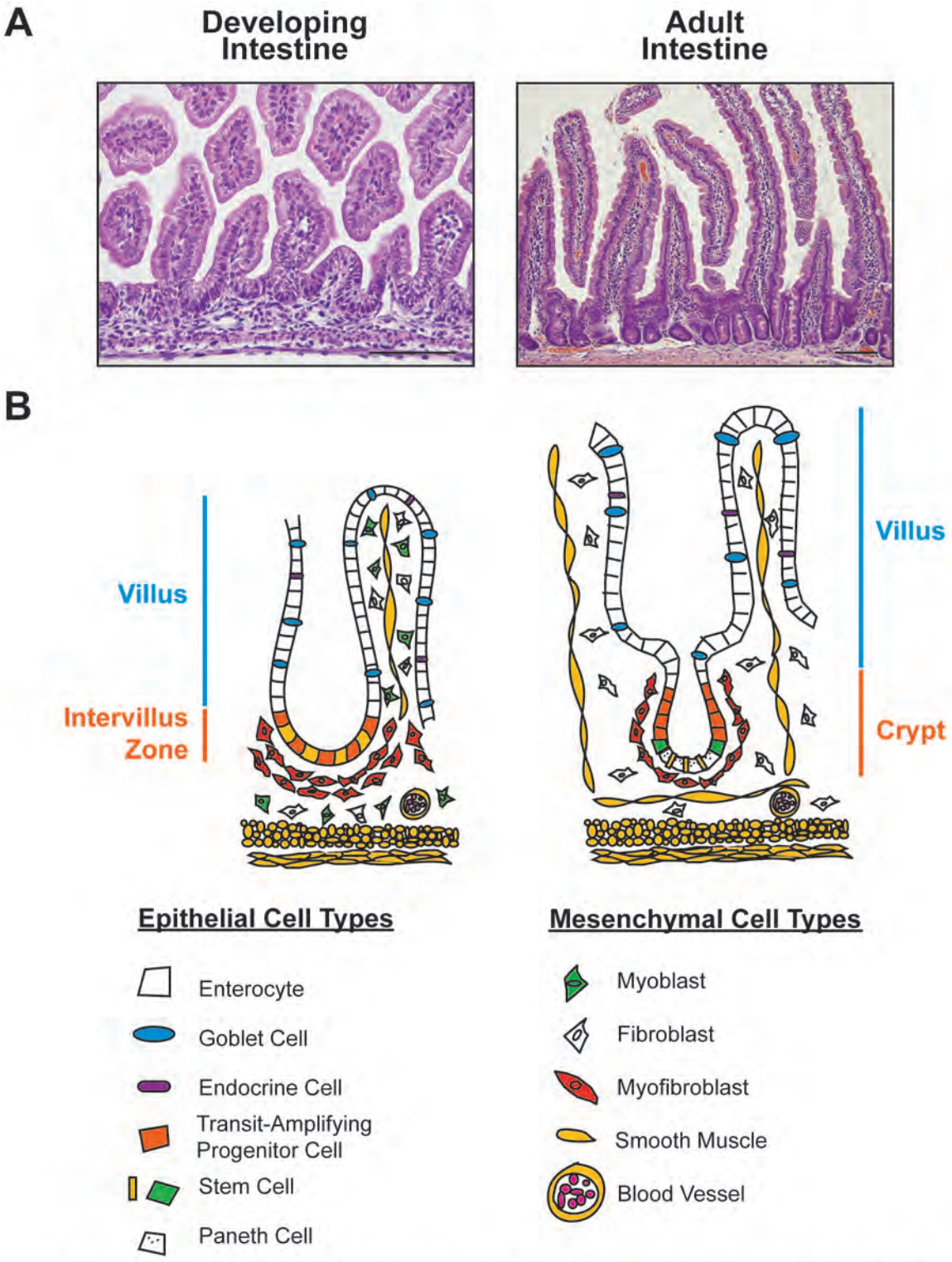

Figure 1-1. General Morphology of Developing and Adult Intestine. (A) Hematoxylin and eosin staining of developing (E18.5) and adult duodenum. (B) The major cell populations of the intestinal epithelium and mesenchyme are shown schematically with the villus and intervillus zone/crypt regions highlighted at each age. Scale bars, 100 μm.

intestinal progenitor cells occurs with a specific spatial distribution with the least differentiated cells at the base of the crypt and the oldest differentiated cells at the tip of the villus. Stem cells, progenitor cells, and Paneth cells are anchored in the crypts while mature enterocytes, goblet cells and endocrine cells are located on the villi. Stem cells give rise to the rapidly proliferating transit amplifying progenitors located in the mid to top of the crypts. Several crypts surround the base of each villus and provide the mature cell types that migrate out of the crypts and onto the villi in organized columns where they eventually reach the villus tip, undergo apoptosis, and are shed into the intestinal lumen[2]. It is unclear whether apoptosis initiates the shedding event or whether it is secondary to detaching from the basement membrane; however, intestinal barrier function is maintained by a yet unidentified substance that seals the gap created by the shed cell[3]. The migratory path from crypt to villus tip takes 3-5 days. In contrast to the other mature cell types, Paneth cells migrate to the base of the crypts where they reside for approximately 20 days before being phagocytosed by neighboring cells[2, 4]. The perinatal intestine does not have crypts or Paneth cells; these structures and cells arise 2-3 weeks after birth. Instead, proliferating cells are located in the so-called “intervillus zone”. Relatively little is known about how the processes of cytodifferentiation, allocation to the absorptive versus secretory lineage, and terminal differentiation of the mature epithelial cell lineages are regulated, although critical transcription factors and signaling pathways are beginning to be elucidated. This thesis addresses the critical role of Notch signaling in epithelial lineage decisions during intestinal development and maintenance of the adult intestine.

Formation and Patterning of the Gut Tube

The gastrointestinal tract is composed of cells originating from each of the three germ layers: the epithelium from endoderm, the mesenchyme (muscle, myofibroblasts, etc.) from mesoderm, and the enteric nervous system from ectoderm. Although differentiation of the endoderm is not fully understood, cell fate mapping studies have identified key morphological movements during

embryogenesis and determined which regions of the gut tube contribute to various digestive organs[5]. In mouse, the primitive, endoderm-derived gut tube closes by embryonic day 9 (E9). During formation of the gut tube, cellular migration and morphological changes are occurring at the same time, as stratified squamous endodermal cells are becoming a pseudostratified columnar epithelium layer which will eventually form a single-cell thick columnar epithelium. The gut tube can be subdivided into ventral foregut, dorsal foregut, midgut, and hindgut regions, which begin to express different gene sets that control formation of the endodermal organs[5]. The ventral foregut gives rise to liver, ventral pancreas, lung, and stomach while the dorsal foregut gives rise to esophagus, dorsal pancreas, stomach and duodenum. The midgut and hindgut give rise to the small and large intestines, respectively[5].

The mouse homeodomain transcription factor caudal type homeobox 2 (Cdx2) is part of the parahox gene cluster[6] and has a conserved function in gut tube formation from flies to humans. Following gastrulation in mice (E7.5+), Cdx2 is expressed highly in posterior gut endoderm[7], prior to expression of most hox genes. Expression of this gene becomes restricted to the intestinal epithelium over time with high nuclear expression throughout the small intestine and proximal colon[8]. Until recently, the function of Cdx2 in the endoderm was unable to be studied *in vivo* due to early lethality of Cdx2 null mice; it was thought that Cdx2 null embryos did not survive the pre-implantation period due to loss of Cdx2 expression in the trophectoderm during this early developmental stage[9]. The early lethality was circumvented by using a Cre-loxP transgenic mouse approach. The Foxa3-Cre transgenic line which expresses Cre in early endoderm was used to cause recombination of floxed Cdx2 alleles, effectively generating a Cdx2 null mouse post-implantation (Foxa3-Cre; $Cdx2^{flox/flox}$)[10]. These Cdx2 mutant mice died shortly after birth; however, the intestinal phenotype could be analyzed during developmental stages. Cdx2-deficient intestine showed loss of all differentiated intestinal cell types and down-regulation of many intestinal genes. Instead, many esophageal-type genes were expressed in the distal intestinal domain suggesting that anterior-posterior patterning had

been disrupted and the intestine had adopted a more anterior identity[10]. Thus, Cdx2 is essential for proper anterior-posterior patterning of the gut tube and functions to promote intestinal fate and inhibit anterior (esophageal) fate in the midgut and hindgut.

Two other mouse Cdx homologs, Cdx1 and Cdx4, did not display gut phenotypes in mouse[11, 12]. Interestingly, Cdx1 was found to be significantly decreased in the Foxa3-Cre;$Cdx2^{flox/flox}$ mutant. Together with data showing that Cdx1 expression is activated in the gut endoderm about 3 days later than Cdx2[8], this suggests that Cdx2 may be upstream of Cdx1. Indeed, Cdx2 appears to be a master regulator of intestinal identity that functions upstream of multiple intestinal transcription factors including Cdx1, Isx, Hnf1α, and HNF4α, which in turn can function with Cdx2 to activate the intestinal gene program[10].

In frog, the Cdx homologue Xcad3 activates posterior hox gene expression to regulate development of the posterior gut[13]. A similar mechanism does not appear to be acting in the development of the mouse gut since the hox gene code was largely unaffected in the Foxa3-Cre;$Cdx2^{flox/flox}$ mouse[10]. In addition, another gene belonging to the parahox cluster, Pdx1, which is expressed in the antrum, duodenum, and pancreas, was found to be unaffected in the Cdx2 mutant mouse intestine suggesting that some endodermal patterning can be maintained in the absence of Cdx2.

In addition to its patterning role during development, Cdx2 appears to be important for maintenance of the mature intestinal epithelium; loss of Cdx2 in the intestine is associated with colorectal cancer. Ninety percent of heterozygous $Cdx2^{+/-}$ mice developed metaplastic colonic polyps by 3 months of age in regions in which Cdx2 expression was lost by an unknown mechanism[9]. Moreover, Cdx2 expression is reduced or lost in many human colon cancer cell lines[14] and mutations in Cdx2 were discovered in a subtype of human colorectal tumors[15]. In contrast to Cdx2 expression being lost in intestinal cancers, ectopic Cdx2 in non-intestinal tissues is associated with cancer progression in Barrett's esophagus[16] and gastric cancer with an intestinal metaplasia phenotype[17]. Transgenic expression of Cdx2 in the stomach resulted in emergence of

intestinal-type goblet cells and expression of intestinal genes including alkaline phosphatase, villin, intestinal fatty acid binding protein, and trefoil factor 3 (Tff3), suggesting that Cdx2 is the driving force behind intestinal metaplasias[18]. Thus, Cdx2 functions as a master regulator of the intestinal differentiation gene program and can ectopically activate this gene program in metaplasias of other endoderm-derived organs.

Role of Epithelial-Mesenchymal Signaling in Intestinal Development and Homeostasis

In addition to anterior-posterior patterning, the developing intestine is patterned along the crypt-villus axis. Interactions between the epithelium and mesenchyme are thought to be key for villus formation, crypt formation and epithelial maintenance. Key pathways participating in intestinal epithelial-mesenchymal "cross-talk" are Hh, BMP, and Wnt signaling. Mutations in these pathways cause general loss of villus and/or crypt morphology or changes to the distribution of cell types along the crypt-villus axis. Current research in the field is attempting to understand the particular effect of each signaling pathway; however, since there is considerable interaction between these pathways, it has been difficult to dissect the direct and indirect phenotypes resulting from perturbations of this complex regulation network. As an example, in Chapter 2, I used an epithelial-specific transgenic mouse to alter cell lineage allocation and, as a consequence, the mesenchyme was remodeled. This study demonstrates the close relationship between the epithelial and mesenchymal compartments.

Hedgehog Signaling Restricts the Proliferative Zone in the Intestine

In the intestine, Hh signaling is strictly paracrine with the pathway ligands Sonic hedgehog (Shh) and Indian hedgehog (Ihh) being secreted from the epithelium to activate their receptors Patched1/2 and effectors Gli1/2/3 in the mesenchyme[19, 20]. Ihh and Shh are expressed in the gut endoderm in overlapping patterns at E8.5[21, 22]. This expression pattern is restricted towards the epithelium of the intervillus zone by late fetal development[23]. Mice deficient

in Shh ($Shh^{-/-}$) or Ihh ($Ihh^{-/-}$) died perinatally and exhibited many gastrointestinal defects[23]. $Shh^{-/-}$ mutants displayed excessive and ectopic neuronal differentiation while $Ihh^{-/-}$ mutants often lacked neurons in portions of the small intestine demonstrating that these ligands regulate migration, proliferation and/or differentiation of the enteric nervous system during development[23]. A redundant role for the Hh ligands was observed with respect to muscle development with both mutants exhibiting loss of smooth muscle[23]. In addition to their mesenchymal abnormalities, Shh and Ihh mutants also displayed epithelial phenotypes suggesting that there is a feedback mechanism that can signal from the mesenchyme back to the epithelium in response to Hh signaling. $Ihh^{-/-}$ mice had reduced proliferation in the intervillus zone with reduced villus size; in contrast, $Shh^{-/-}$ mice had overgrowth of villi suggesting that Shh and Ihh play opposing roles in epithelial stem cell maintenance[23]. A mouse model that drove expression of a secreted form of the pan-hedgehog inhibitor Hhip from the intestinal epithelium via the villin promoter showed increased proliferation and formation of ectopic crypt-like structures, impaired villus formation, and reduced smooth muscle[20]. A similar phenotype was observed when neonatal mice were treated with a Hh neutralizing antibody[24]. Thus, the combined effect of the Shh and Ihh signal is important for smooth muscle proliferation and/or differentiation, villus formation, and restriction of the proliferative compartment to the intervillus zone in perinatal intestine.

BMP Signaling Restricts Crypt Number in the Intestine

Similar to Hh signaling, BMP signals are also paracrine; however, the direction of the BMP signal is from the mesenchyme to the epithelium. The BMP ligands, BMP2 and BMP4, are expressed in the intestinal mesenchyme with their receptor, BMPR1a, expressed in the epithelium[25, 26]. Signaling activity is transduced through the cytoplasm to the nucleus by the SMAD transcription factors. SMAD4 is a common mediator shared by the TGFβ, activin, and BMP pathways and, for BMP signaling, SMAD1, 5, and 8 are the receptor-activated SMADs[27]. Thus, active BMP signaling as determined by the presence of nuclear

phospho-SMAD1, 5, and 8 transcription factors occurs in the villus epithelium[25]. BMP signaling is inhibited in the crypt epithelium due to expression of the BMP antagonist noggin in this region[26]. A transgenic mouse in which the villin promoter was used to drive expression of xenopus noggin (*X-noggin*) presented with ectopic crypt structures in 3-month old adult mice and adenomatous foci development in older adult mice[25] suggesting that BMP signaling normally functions to limit crypt number. These types of epithelial changes are reminiscent of a rare, autosomal-dominant gastrointestinal syndrome called familial juvenile polyposis (FJP), which is characterized by development of many hamartomatous polyps throughout the gut. Mutations in SMAD4 and Bmpr1a have been identified in patients with FJP[28-30] implying alterations in BMP signaling in the pathogenesis of this syndrome. Indeed, a second mouse model in which the Bmp1ra receptor was conditionally inactivated using the interferon-inducible Mx1-Cre also exhibited polyp formation[26]. Loss of Bmpr1a specifically in the epithelium showed increased epithelial proliferation and a defect in secretory cell differentiation, but not formation of ectopic crypts or polyps[31]. Comparison of the epithelial-specific Bmpr1a knock-out mouse model with the *X-noggin* and Mx1-Cre;Bmpr1a models, which had inhibition of BMP signaling in both the epithelium and mesenchyme, suggests that mesenchyme has a larger role in progression to polyps in FJP because epithelial-specific loss of BMP signaling did not result in polyp formation. In addition, these data show that BMP signaling is important for limiting epithelial stem cell proliferation and blocking the formation of ectopic crypts in the intestine. Furthermore, since BMP4 is positively-regulated by Hh signaling and the ectopic crypt phenotype in villin-Hhip and *X-noggin* mice is similar[20, 25], it is likely that BMP signaling is a key factor mediating Hh signaling in the intestine to restrict the proliferative zone.

Wnt Signaling Promotes Intestinal Proliferation

In opposition to the anti-proliferation effect of the Hh and BMP signaling pathways, Wnt signaling is the key pathway promoting proliferation in the intestinal crypts. Overactive Wnt signaling such as that seen in the APC^{min}

mouse model leads to enlarged crypts with enhanced proliferation and progression to adenocarcinoma[32, 33]. Conversely, blocking Wnt signaling by forced expression of the secreted Wnt inhibitor Dickopff (Dkk1) or inactivation of the critical Wnt pathway components T-cell-specific transcription factor 4 (Tcf4) or β-catenin leads to decreased proliferation and crypt loss[34-37]. There is evidence that non-canonical Wnt signaling is important for aspects of gastrointestinal development[38]; however, canonical Wnt signaling through β-catenin and Tcf4 appears to be the most important for stem cell proliferation in the intestine[34, 35]. Downstream targets of Tcf4-mediated Wnt signaling include critical pro-proliferation genes such as c-Myc[39, 40] and Cyclin D1[41, 42]. The canonical Wnt ligands Wnt3, Wnt6, and Wnt9B are expressed in the crypt epithelium as are the Frizzled receptors 5, 6, and 7 and LRP5 and 6 co-receptors[43]. Thus, the pathway components for activation of signaling are present in the epithelium. In addition, subepithelial myofibroblasts have been shown to express Wnt family genes and may also be a source of Wnt signaling[43]. However, to date, the "true source" of Wnt ligand has not been identified and, in fact, could potentially be coming from multiple sources. Whatever the source, active Wnt signaling appears to be restricted to the crypts based on location of nuclear β-catenin and Wnt target gene expression[40, 44]. It is likely that multiple signals act to restrict Wnt to the crypt base with Hh signaling being one of these opposing signals. Accordingly, Villin-Hhip mice had increased Wnt target gene expression and transfection of Ihh into colon cancer cells led to down-regulation of Wnt signaling[20, 45]. Thus, a simplified model of epithelial maintenance in the intestine emerges: active Wnt signaling in the crypts drives stem and progenitor cells to proliferate and as maturing cells leave the base of the crypts and Wnt signaling zone, they exit the cell cycle and terminally differentiate.

In summary, Wnt, Hh, and BMP integrate and relay information between the epithelium and mesenchyme to regulate stem cell renewal and proliferation, crypt-villus patterning and cellular differentiation in the intestine. When these pathways are deregulated, loss of proliferation can lead to villus and/or crypt atrophy, compromising the nutritional status of an organism, or in the opposing

situation, excessive proliferation and lack of cell differentiation can lead to polyp formation and progression to adenoma.

The Notch Signaling Pathway

Notch signaling (Figure 1-2) is a critical regulator of proliferation and differentiation in the intestinal epithelium. The work presented in this thesis has further elucidated the mechanisms by which Notch signaling regulates these processes. Both the Notch ligands and receptors are trans-membrane proteins; thus, signaling occurs between neighboring cells. Upon ligand engagement with the Notch receptor, a series of receptor cleavage events is initiated. The first cleavage (S2 cleavage) is performed by the A Disintegrin and Metalloprotease (ADAM) protease, which removes the extracellular domain of the Notch receptor. It has recently been shown that ADAM10 (and not ADAM17) performs this essential cleavage *in vivo*[46]. ADAM10-mediated cleavage is the key regulatory step in the activation of Notch signaling and produces a substrate for the subsequent γ-secretase complex-mediated S3 cleavage. As part of a negative feedback regulatory mechanism, the interacting ligand (and possibly the ectodomain of the cleaved Notch receptor) are trans-endocytosed into the signal-sending cell to reduce ligand availability; in addition, this may initiate other signaling events in the signal-sending cell[47]. The γ-secretase complex is comprised of the membrane proteins presenillin (Psen1/2), nicastrin, presenillin enhancer 2 homolog (Pen2) and alphaprotein 1 (Aph1) in a 1:1:1:1 stoichiometry[48]. At least six possible γ-secretase complexes can be formed in rodents due to two isoforms of presenillin and three isoforms of Aph1[49]. The biological significance of the different complex configurations is not currently known; however, biochemically, it appears that different complexes may exhibit differential processing efficiency for certain substrates and susceptibility to pharmacological inhibitors[50, 51]. Thus, it will be important to identify the temporal and spatial expression patterns of each of the components of the γ-secretase complex in the intestine to determine which complexes are formed because complex composition likely affects substrate specificity and biological function *in*

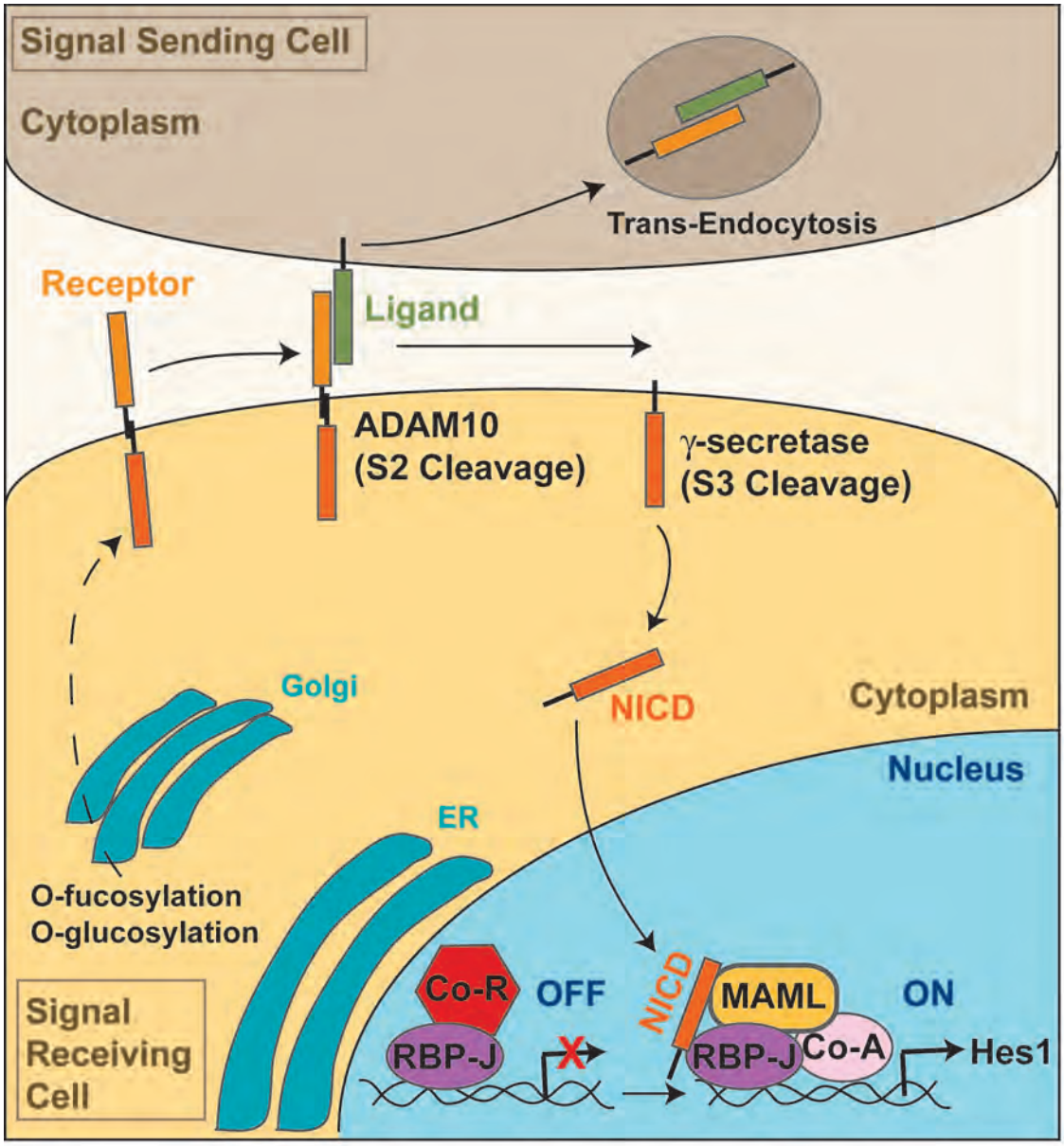

Figure 1-2. The Notch Signaling Pathway. Protein modifications, including fucosylation and glucosylation, are added to the Notch receptor as it traffics through the ER and Golgi, prior to membrane insertion. In the absence of signaling, the DNA binding protein RBP-J interacts with co-repressors (Co-R) to suppress transcription of Notch target genes. Upon ligand engagement, the Notch receptor is cleaved by the ADAM protease which removes the receptor ectodomain, producing a substrate for the γ-secretase complex. The Notch ectodomain and ligand are trans-endocytosed. Subsequent γ-secretase cleavage releases the bioactive form of the Notch receptor, the Notch Intraceullar Domain (NICD), from the membrane. NICD translocates to the nucleus where it interacts with RBP-J, mastermind (MAML) and other co-activators to activate transcription of target genes, including hairy and enhancer of split 1 (Hes1).

vivo. Following cleavage by the γ-secretase complex, the biologically active Notch intracellular domain (NICD) is released from the membrane. The NICD translocates to the nucleus of the signal-receiving cell and binds to its co-activators Recombination signal binding protein for immunoglobulin kappa J region (RBP-J; also known as CSL), a DNA-binding protein, and Mastermind (MAML) to initiate transcription of target genes including hairy and enhancer of split 1 (Hes1). In the absence of NICD, RBP-J appears to occupy its DNA binding sites some of the time and participate in a repressor complex[47].

Study of the Notch signaling pathway is challenging because of the difficulty of detecting nuclear NICD due to rapid degradation of this bioactive receptor fragment. As an alternative to NICD detection, assessment of the transcriptional target of Notch signaling, Hes1, is used as an indicator of Notch signaling activity; however, there are caveats to this method of detection as well because Hes1 can be regulated by other pathways, including the fibroblast growth factor pathway[52], the GATA transcription factors[53], Ids[54], and Pax transcription factors[55].

Regulation of the Notch Signaling Pathway

The key rate-limiting step for the initiation of a Notch signaling event is ADAM cleavage[47]; however, other types of regulation can occur at many steps of the pathway. These regulatory mechanisms may affect either the amplitude or duration of a signaling event. Evidence suggests that pathway activity is regulated by restriction of ligand or receptor availability (both spatially and temporally), modulation of ligand and receptor trafficking, and post-translational modification of receptor and ligand proteins[47]. In addition to protein regulation, miRNA's are also involved in controlling pathway component availability[56, 57]. This field of research is still relatively nascent and it is likely that mammalian Notch activity is differentially regulated based on temporal, tissue-specific, and even cellular-specific contexts.

Notch Signaling is Important for Cell Fate Selection and Progenitor Cell Maintenance in Multiple Tissues

Notch signaling plays key roles in determination of cell fate in many tissues including the nervous system, hematopoietic system, the developing lung and pancreas, and intestine. Notch signaling promotes glial cell fate over neuronal cell fate in the nervous system and exocrine versus endocrine cell fate in the pancreas[58-60]. Notch determines cell fate choice at many points during hematopoiesis including the selection of T-cell fate over B-cell fate and during further differentiation of each of these immune cell lineages (e.g. generation of CD4 versus CD8 T-cells)[61]. A role for Notch signaling has also been demonstrated in the lung during developmental patterning of proximal and distal progenitors and in selection of Clara cell fate over ciliated cell fate[62-66]. In addition to its role in cell fate selection, Notch signaling is important for stem and progenitor cell maintenance in many tissues. Loss-of-function Notch mutants displayed precocious differentiation and progenitor depletion[67-69]. Gain-of-function Notch mutations have been associated with progenitor expansion and cancer, including (but not limited to) T-ALL leukemia, brain tumors, lung cancer, and colorectal cancer[70-73]. This thesis focuses on Notch regulation of cell fate in the intestine.

Notch Signaling in the Intestine

All Notch ligands (Delta-like ligands; Dll1, Dll3, Dll4 and Jagged ligands; Jag1 and Jag2) and Notch receptors (Notch1-4) are expressed in the mouse gut during early development (E13.5) through adulthood except Dll3, whose expression recedes after early development[74, 75]. Based on mRNA expression patterns, Dll1, Dll4 and Jag1 are most likely to mediate the effects of epithelial Notch signaling in the intestine with the critical epithelial receptors being Notch1 and Notch2[75]; however, it is currently not understood which intestinal cell populations are sending or receiving the Notch signal. Immunostaining for NICD or Hes1 labels many cells in the crypts and not cells of the villus epithelium[68, 76] suggesting that active Notch signaling is occurring in stem cells or progenitor

cells, but not in terminally differentiated cell types (with the possible exception of Paneth cells). Novel results that I report in Chapter 4 have identified the crypt base columnar (CBC) stem cell as one direct cellular target of Notch signaling. In addition, many Notch signaling components are also expressed in the intestinal mesenchyme[75]; however, their role is largely unknown.

In the intestine, Notch signaling appears to be the key pathway regulating epithelial cell fate with Notch promoting the absorptive cell fate over the secretory cell fate. Notch signaling is thought to mediate its affect on cell fate selection through the process of lateral inhibition. For example, secretory cell progenitors may begin to express high levels of Notch ligand which, in turn, activates the Notch receptors and Notch signaling pathway in their neighboring cells to inhibit them from also adopting the secretory cell fate[77]. Analysis of intestinal morphology paired with mathematical modeling suggests that lateral inhibition in the intestine is likely to occur in early progenitor cells in the crypts to initially split differentiating cells to the absorptive and secretory lineages. This is followed by disproportionate subsequent cell divisions with absorptive progenitors dividing approximately 4 more times and secretory progenitors 1-2 more times. This model would yield the observed result with ~4% of villus cells belonging to the secretory lineage and these cells are separated by many absorptive cells[77, 78].

The Intestinal Phenotypes of Notch Signaling Mutants

The intestinal phenotypes of mouse models with alterations in key Notch pathway components show altered proportions of proliferating versus non-proliferating cells and of absorptive versus secretory cell types (Table 1-1). Disruption of intestinal Notch signaling via a conditional knock-out mouse model for the essential NICD co-effector RBP-J or administration of the γ-secretase inhibitor dibenzapine (DBZ) resulted in loss of proliferating cells and increased goblet cell number[79]. A different study using DBZ treatment in rats reported increased numbers of all secretory cell types[80]. Conversely, activation of the Notch pathway using constitutively active NICD transgenic mice resulted in expansion of the proliferative zone and loss of secretory cells[81, 82]. Mice deficient

Table 1-1. Intestinal Phenotypes of Core Notch Pathway Mutants

Component Function	Gene(s)*	LOF/ GOF	Intestinal Phenotype	Reference
Receptor	Notch1[a]	LOF	Normal	Riccio et al., 2008
	Notch2[a]	LOF	Normal	Riccio et al., 2008
	Notch1, Notch2[a]	LOF	↑ goblet cells, ↓ proliferation, ↑ p21 and p27 expression linked to loss of Hes1 repression on these promoters	Riccio et al., 2008
Ligand	DeltaD[d]	LOF	↑ secretory cells in zebrafish[d]	Crosnier et al., 2004
Nuclear Effectors	RBP-J[a]	LOF	↑ Math1, ↑ goblet cells, ↓ proliferation	Van Es et al., 2005
	MAML[a,b]	LOF	↑ secretory cells	Maillard, unpublished
	NICD[a]	GOF	↑ Hes1, ↓ goblet and endocrine cells, ↑ progenitor cells and proliferation, ↑ apoptosis	Fre et al., 2005
	NICD[a]	GOF	↓ goblet and endocrine cells, ↑ proliferation, no change in apoptosis	Stanger et al., 2005
Receptor Proteolysis	ADAM10[a,b]	LOF	↑ secretory cells, ↓ proliferation	Dempsey, unpublished
	ADAM17[b]	LOF	Normal	Dempsey, unpublished
	γ-secretase*[,a]	LOF	↑ Math1, ↑ goblet cells, ↓ proliferation	Van Es et al., 2005
	γ-secretase*[,a]	LOF	↑ Rath1 (rat homolog of Math1), ↓ Hes1, ↑ goblet and endocrine cells, ↑ apoptosis in rat	Milano et al., 2004
Glycosyl-transferase Modifiers	Pofut1[c]	LOF	↓ Hes1 and Hes5, ↑ Math1, ↑ secretory cells (restricted to crypts), ↓ proliferation with dispacement towards top of crypt	Guilmeau et al., 2008
Membrane Trafficking Regulators	Mindbomb[d]	LOF	↑ secretory cells in zebrafish	Crosnier et al., 2004
	Mindbomb[c]	LOF	↑ secretory cells, mislocated Paneth cells on villi, ↓ proliferation	Koo et al., 2009
Canonical bHLH Target	Hes1[d]	LOF	↑ goblet and endocrine cells, no change in proliferation, ↑ apoptosis in intervillus zone, ↑ Math1, ↑ Hes5	Jensen et al., 2000
	Hes1[d]	LOF	Precocious differentiation of Paneth cells	Suzuki et al., 2005

Phenotypes reported in mouse models unless otherwise stated.

*Pharmacological inhibitor; LOF, Loss-of-function; GOF, gain-of-function

[a] conditional mutant, adult analysis; [b] conditional mutant, developmental analysis

[c] constitutive mutant, adult analysis; [d] constitutive mutant, developmental analysis

in the Notch1 or Notch2 alone did not exhibit a gut phenotype; however, compound Notch1/Notch2 receptor KO mice exhibited a similar phenotype to the intestine-specific RBP-J knockout mouse suggesting that these genes are redundant and that together they likely transmit all of the epithelial Notch signal[83].

Hes1-deficient embryos exhibited gut phenotypes similar to the Notch disruption models suggesting that Notch signaling exerts most of its effects through Hes1[68]; however, the phenotype of these mice was not as severe as complete loss-of-function mutants suggesting that other Hes proteins may convey Notch signaling effects as well. In support of possible redundancy among Hes genes, Hes1, Hes5, Hes6 and Hes7 have all been reported to be expressed in the intestine epithelium[68, 75]. In particular, Hes5 is known to be expressed in the crypt in a similar pattern to Hes1 (Chapter 4) and Hes5 expression was found to be up-regulated in the gut of Hes1-deficient embryos[68]. Another report on Hes1-deficient embryos showed that these mice also exhibited precocious differentiation of Paneth cells located primarily in the intervillus zone and that this was associated with increased expression of Paneth cell genes[84].

Mind bomb-1 (Mib1) is an E3 ubiquitin ligase that is important for Notch signaling purportedly due to its role in endocytosis of Notch ligand and receptor complexes upon their interaction[85, 86]. Mib1 loss-of-function mutations in both zebrafish and mouse intestine resulted in increased intestinal secretory cells similar to other Notch disruption phenotypes[86, 87]. Decreased proliferation was also reported in the mouse model, suggesting that mindbomb is important for Notch signaling[86]; however, these mice were examined at 2-4 weeks of age, and the secretory conversion was milder than complete Notch signaling loss-of-function models, suggesting that some Notch signaling still occurs in the absence of mindbomb activity in mice. Interestingly, in the mouse Mib-1 mutant, Paneth cells were occasionally mislocated to the villus, a phenotype reminiscient of the EphB3$^{-/-}$ mutant that will be discussed in more detail in a later section.

Finally, the phenotype of a mouse model with deficiency in Protein O-fucosyl-transferase (Pofut1), an ER chaperone protein that can post-translationally modify the Notch receptor, also demonstrated a phenotype

reminiscent of Notch disruption models with increased secretory cells and decreased proliferation, although the phenotype was less severe than total loss-of-function mutants and mice survived through adulthood[88]. Studies in Drosophila and mammals have suggested that the essential role of the fucosyl transferases is ER chaperone activity and not enzymatic activity; non-fucosylated Notch receptors were able to reach the cell membrane, bind ligand and signal, and overexpression of unrelated ER chaperone proteins was able to rescue the phenotype in Pofut1-deficient cells[89-93]. Although fucosylation is not required for signaling, it may affect which ligands bind the receptor[94] and, therefore, is a mechanism that could affect overall Notch signaling in a context-dependent manner.

γ-Secretase Inhibitors are Pharmacological Inhibitors of Notch Signaling

The γ-secretase complex has the ability to cleave more than 30 types of transmembrane proteins[49], including the Notch receptor[95], Notch ligands Dll1 and Jag2[96], ErbB4[97], CD44[98], and E-cadherin[99]. There does not appear to be a specific consensus sequence to determine whether a protein is cleaved by γ-secretase; instead, the main prerequisite for a potential substrate appears to be removal of the ectodomain by sheddases[49] such as removal of the ectodomain of the Notch receptor by the ADAM protease. A class of pharmacological reagents known as γ-secretase inhibitors (GSIs) have been developed as inhibitors of γ-secretase-mediated cleavage and downstream signaling events. These inhibitors have been actively studied for decades because they have potential to block the generation of Aβ peptide generation that is associated with Alzheimer's disease[100]. More recently, there has been interest in using GSIs as anti-cancer drugs because they could potentially block hyperactive Notch signaling driving cancer progression[101]. Unfortunately, a side effect of these drugs is often gastrointestinal toxicity due to Notch's key role in intestinal cell homeostasis. Rodents treated with the GSI benodiazepine (BZ), DBZ, or LY-411,575 exhibited intestinal goblet cell hyperplasia and epithelial degeneration reminiscent of the phenotype observed in Notch disruption models[79, 80, 102]. Thus, while use of

these drugs is useful for the study of Notch disruption, such as was done in Chapter 4, modifications to the timing of repeated dosing and/or drug specificity are being explored to make these drugs suitable for Alzheimer's disease treatment or cancer therapy[103, 104].

Expression of Math1 in the Intestine

Math1 is a basic helix-loop-helix transcription factor involved in cell fate determination of intestinal secretory cells as well as several neuronal cell types important for the proprioceptive system including spinal cord interneurons[105], inner ear hair cells[106], and subsets of cerebellar and brainstem neurons[107, 108]. Currently, it is not well understood how Math1 expression is activated in the intestinal epithelium, but once activated, it binds to its own 3' enhancer to perpetuate its own expression[109]. One report suggests that Cdx2 is directly upstream of Math1[110]. Math1 was down regulated in Foxa3-Cre;Cdx2$^{flox/flox}$ intestine[10]; however, this could be due directly to loss of Cdx2 itself or indirectly to loss of normal intestinal patterning. Since Cdx2 is generally expressed in the intestinal epithelium, it is very likely that it would act in conjunction with other factors to activate Math1. Studies in other tissues have indicated that Wnt signaling may be important for up-regulating Math1 expression. Overexpression of β-catenin in neural progenitor cells increased the activity of a GFP reporter under the control of a Math1 enhancer[111] and hyperactive Wnt signaling in lung progenitors activated Math1 along with intestinal secretory cell development in the lung *in vivo*[112]. Notch signaling is known to repress Math1 transcription via Hes1[113]. Accordingly, models of Notch disruption exhibited increased Math1 expression[68, 79]. A recent study has shown that increased Math1 expression after Notch inhibition depends on formation of a DNA-binding complex that contains the Wnt pathway co-activators β-catenin and Tcf/Lef[114], further supporting the role of Wnt signaling in activation of Math1 expression.

Use of a mouse model in which lacZ was inserted into the endogenous Math1 locus (Math1$^{lacZ/+}$) and immunohistochemistry have allowed for visualization of the Math1 expression pattern. In the intestine, Math1 expression

is restricted to the epithelium by E16.5, near the time of the initiation of epithelial cytodifferentiation[115]. Consistent with its role in cell fate determination, Math1-expressing cells are found in the progenitor zones of both embryonic and adult intestine. Furthermore, a portion of the lacZ-positive cells co-express proliferative markers such as Ki67[37, 115] suggesting that secretory cell fate specification occurs early in the differentiation process and these specified cells continue to divide. In addition, Math1 also continues to be expressed in all mature secretory cells[37, 115]. Analysis of Dll1 expression in the intestine by *in situ* hybridization or with a $Dll1^{lacZ}$ reporter mouse showed that Dll1 is expressed in a very similar pattern to the $Math1^{lacZ/+}$ mouse, suggesting that mature secretory cells and progenitor cells specifying to the secretory lineage express Notch ligand[87, 116]. These expression patterns are consistent with Notch-mediated lateral inhibition contributing to secretory cell fate selection in the intestine.

The Role of Math1 in Intestinal Cell Differentiation and Adaptive Response

The studies presented in this thesis and others point towards Notch-regulation of Math1 as the key mechanism by which Notch signaling affects cell fate determination. Math1 is required for intestinal secretory cells in both developing and adult intestine. Intercrossing of the $Math1^{lacZ/+}$ mice described above generated a Math1-null mouse ($Math1^{lacZ/lacZ}$). These mice lacked all intestinal secretory cells and died upon birth, presumably from a respiratory defect resulting from loss of certain neuronal populations, which prevented studies in adult mice[117]. A second study utilized a Cre-lox approach to specifically ablate Math1 expression in the intestinal epithelium ($Math1^{\Delta intestine}$). To produce mice that lived to adulthood, a mosaic Cre transgenic mouse, $Fabpl^{4X\ AT-132}$, was used to activate recombination of the $Math1^{flox/lacZ}$ allele in the intestinal epithelium[118]. Loss of secretory cells was observed specifically in the recombined $Math1^{\Delta intestine}$ crypts while non-recombined crypts retained normal secretory cell number. These results demonstrated that Math1 has a similar role in both developing and adult intestine[118]. The clinical relevance of this phenotype was indicated by the report of a patient who presented with severe diarrhea,

enterocolitis and loss of all intestinal secretory cell types whose pathology did not improve with immunosuppressive therapy, suggesting that the patient had a developmental block in secretory cell differentiation and not an autoimmune disease[119]. Math1 expression was not assessed in this case report; however, it is tempting to speculate that Math1-deficiency was involved in this pathology. Nonetheless, it is likely that secretory cell differentiation in humans closely parallels that in rodents.

Since Math1 is expressed in mature secretory cells in addition to cells in the progenitor zone, one could argue that Math1 may actually be required for secretory cell maintenance rather than cellular differentiation. While Math1 may also be important for maintenance of the secretory cell identity, many studies indicate that Math1 actively promotes secretory cell fate during cellular differentiation and is important for adaptive and disease response mechanisms. Small bowel resection (SBR) in mice causes an adaptive response in which there is increased epithelial proliferation and sustained increases in secretory cell number and mucosal surface area, including increased crypt depth and villus height[120]. Math1$^{\Delta intestine}$ mice had a blunted response to SBR with smaller increases in crypt depth[118]. This demonstrated the importance of Math1 for intestinal remodeling. Increased secretory cell number is also observed in mice infected with the parasitic nematode *Trichinella spiralis* (*T. spiralis*)[121, 122]. It was recently described that Math1 transcript levels are more than 2-fold higher in *T. spiralis* infection, suggesting that the increase in secretory cells may be due to induction and differentiation of a Math1-expressing progenitor cell[123]. Thus, regulation of Math1 expression may be mechanistically important for the ability of the intestine to respond to adaptive and environmental stimuli.

The Potential Role of Math1 as a Tumor Suppressor

As a pro-differentiation transcription factor, recent studies have explored whether Math1 functions as a tumor suppressor gene in the gut[124-127]. This is of great interest because colorectal cancer is among the top three types of cancer found in men and women of the United States population[128]. Colon

adenocarcinomas are characterized by expansion of proliferative progenitor cells, inhibited cellular differentiation (including loss of the very abundant goblet cell population in affected regions of the colon) and inhibited apoptosis. Importantly, comparison of colon adenocarcinoma samples to surrounding normal tissue from human patients showed that expression of Hath1, the human ortholog of Math1, was down-regulated in the carcinoma samples[125]. Compared to control mice, enhanced polyp and tumor formation was observed in Math1$^{\Delta intestine}$ mice treated with the carcinogen azoxymethane (AOM) and in Math1$^{\Delta intestine}$ mice crossed to the APC^{min} mouse model that spontaneously develops adenomas due to hyperactive Wnt signaling[124]. Moreover, when Hath1 was transfected into the colorectal cancer cell line HT29, proliferation of the cells decreased and expression of the differentiated goblet cell marker Muc2 increased[125], thus suggesting that loss of Hath1 expression might contribute to neoplastic growth. Aberrant Wnt signaling is highly associated with development of colorectal adenocarcinoma[129], with mutations in the APC gene that lead to overactive Wnt signaling present in 80% of sporadic colorectal tumors[130]. Experiments in colon cancer cell lines have demonstrated that Wnt signaling can target Hath1 for proteasomal degradation via glycogen synthase kinase 3β (GSK3β)-mediated phosphorylation[126, 127]. This suggests that Wnt signaling maintains the undifferentiated state of adenocarcinoma through down-regulation of Math1/Hath1. In addition, human tumors with loss-of-function mutations and epigenetic silencing of the Hath1 gene were identified in one study, suggesting that loss of Math1 expression could also be a primary event in oncogenic progression[124].

Characterization of the Secretory Progenitor Cell

At this time, the presence and character of "intermediate-stage" progenitors that have been specified to the secretory fate is under debate. Data from Math1 loss-of-function mouse models and from the Math1 gain-of-function model described in this thesis suggest that Math1 may be expressed in a common secretory progenitor cell capable of differentiating into any of the three

mature secretory cell types[115, 118] (also see VanDussen, Chapter 2). Alternatively, each lineage could have its own progenitor, each of which shares a common requirement for Math1. Two studies that followed the progeny of single, randomly-labeled cells in the intestine determined the existence of short-lived progenitors that can yield just one or two cell types and long-lived progenitors that yield only enterocytes, only goblet cells, only endocrine cells, enterocytes and goblet cells, enterocytes and endocrine cells, or a mix of all epithelial cell types[78, 131]. These data demonstrated the presence of long-lived progenitors that have been restricted to a particular lineage. They also demonstrate the presence of a progenitor that yields progeny belonging to one of the secretory cell lineages or to the enterocyte lineage, which suggests that selection of a particular secretory cell fate occurs prior to Notch-mediated lateral inhibition. Finally, clones that contain all epithelial cell types are likely derived from a multipotent stem cell. Due to their slow turnover rate, Paneth cell clones have not been examined. Of note, clones containing multiple secretory cell types and no enterocytes have not been observed suggesting that a common secretory progenitor does not exist or is extremely short-lived[78, 131].

Specification of the Secretory Lineage

Differentiation of an intestinal progenitor to a mature secretory cell must involve the progenitor or a daughter cell becoming competent for the secretory cell fate, specification to a specific secretory lineage, and finally terminal differentiation. Although reports from others and the findings presented in this thesis suggest that Math1 is the critical factor for specification to the secretory lineage, it is not currently well understood how progenitor cells become competent for secretory cell fate or how they are further specified to a specific secretory cell lineage. Some recent studies have begun to elucidate transcription factors that are critical for specific secretory lineages and, from these data, a model of epithelial cell differentiation can be assembled (Figure 1-3).

In accordance with a possible role in activation of Math1 expression, Wnt signaling can affect secretory cell differentiation and may be involved in making

intestinal progenitor cells competent for secretory cell fate[1]. Tcf4 null mice and Villin-Dkk1 transgenic mice, both of which have loss of Wnt signaling, showed loss of goblet and endocrine cells[35, 37]. Furthermore, Math1 expression was lost in the villin-Dkk1 mice[37]. The Notch ligands Dll1 and Jag1 have been reported to be downstream targets of Wnt signaling[132-135]. Thus, Wnt signaling appears to have a strong interaction with the Notch signaling pathway and, therefore, can potentially influence secretory cell differentiation through Notch-dependent or Notch-independent mechanisms.

Shared Mechanisms of Lineage Allocation to Goblet and Paneth Cell Lineages

There is growing evidence that suggests that Paneth and goblet cells may share a common progenitor or, at least to some extent, a common mechanism of differentiation. There are many genes in the intestine that are expressed highly in both Paneth and goblet cells including SAM pointed domain ETS factor (Spdef), serine/threonine kinase 11 (STK11; also called Lkb1), cAMP responsive element binding protein 3-like 4 (Creb3l4), chemokine (C-C motif) ligand 6 (Ccl6), kallikrein 1 (Klk1), hepatocyte growth factor activator (Hgfac), serine peptidase inhibitor, Kazal type 4 (Spink4), and tryptase gamma 1 (Tpsg1)[116, 136, 137]. Mutation of some of these genes has been shown to alter Paneth and/or goblet cell differentiation (described below). The transcription factors growth factor independent 1 (Gfi1) and Spdef have been identified as downstream targets of Math1[138] with mutation of Gfi1 specifically affecting allocation to the goblet and Paneth cell lineage at the expense of the endocrine lineage and mutation of Spdef affecting allocation between the goblet and Paneth cell lineages[116, 136, 138]. These data suggest that Math1, Gfi1, and Spdef affect lineage selection at distinct points in the differentiation process and function in a step-wise fashion.

Gfi1 is a zinc-finger transcriptional repressor that is important for control of proliferation and differentiation in hematopoietic stem cells, immune cells, and sensory hair cells of the inner ear[139]. In the intestine, Gfi1 is expressed in Math1-positive progenitor cells and endocrine progenitor cells in the crypts as well as a

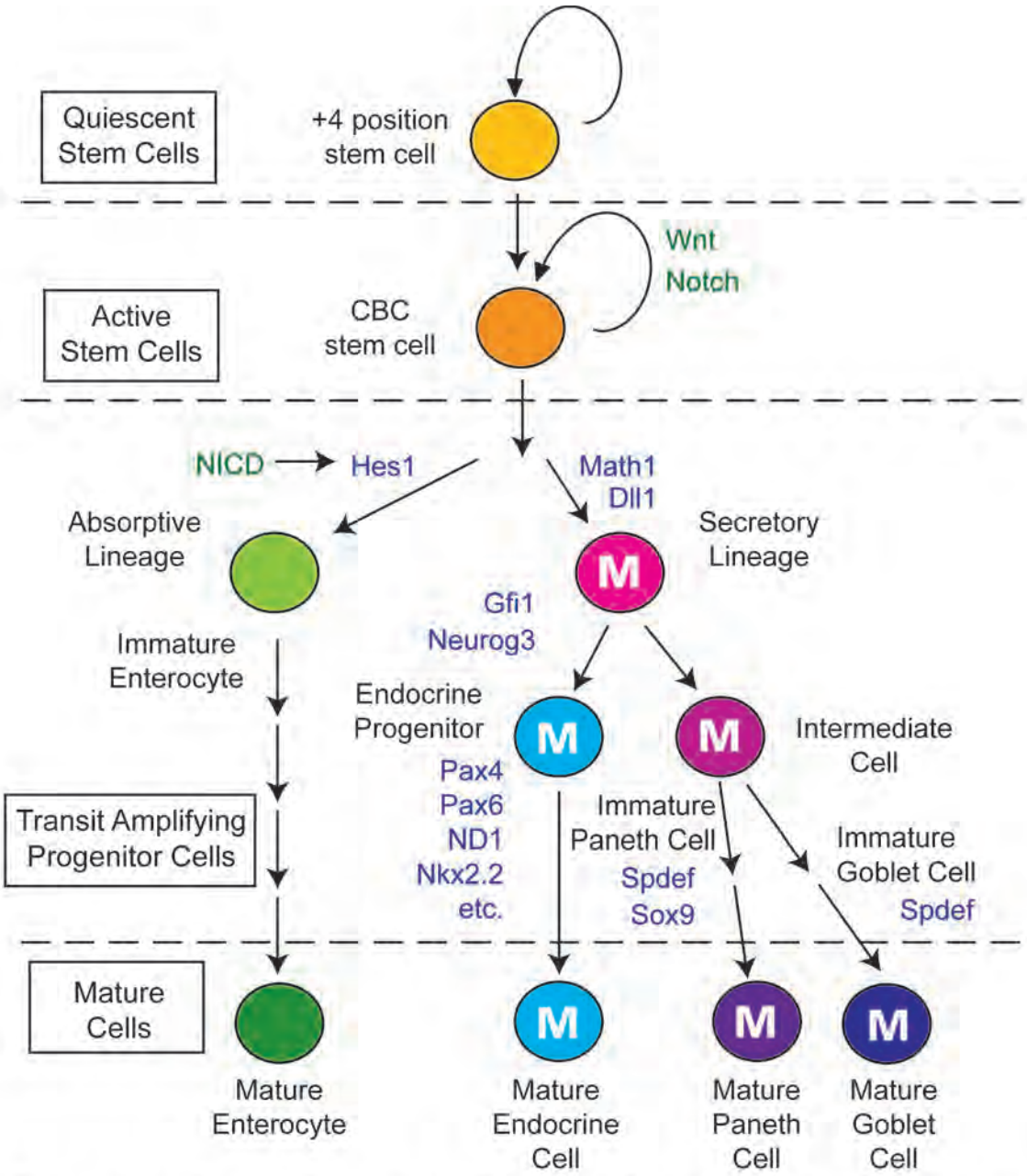

Figure 1-3. Model of Intestinal Epithelial Cell Differentiation. Stem cell renewal is regulated by Wnt and Notch signaling. Early in the differentiation process, Notch signaling selects the absorptive cell fate over the secretory fate through the process of lateral inhibition. The Notch target Hes1 inhibits expression of Math1 in the absorptive lineage which undergoes ~4 rounds of cell division prior to terminal differentiation. Math1 is expressed in all progenitors and mature cells of the secretory lineage (designated by **M**). Differentiation of the secretory progenitor may involve a primary selection of endocrine or Paneth/goblet lineage followed by secondary selection to the goblet or Paneth lineage. Critical transcription factors are listed in blue. TA, transit-amplifying; CBC, crypt base columnar.

subset of mature endocrine cells[138]. Gfi1-deficient intestine exhibited loss of Paneth cells, decreased goblet cells and increased endocrine cells[138] suggesting that Gfi1 functions to select the goblet/Paneth cell fate over endocrine cell fate or that Gfi1 regulates the proliferation of a subset of endocrine cell types.

Intestinal expression of Spdef is dependent on Math1 and Gfi1[136]. Spdef is found mainly in post-mitotic goblet cells and Paneth cells, although it appears to also be present in a small population of progenitors in the crypt[116, 136]. A mouse model deficient in Spdef expression (Spdef$^{-/-}$) had impaired maturation of both goblet and Paneth cells; this impairment was thought to lead to accumulation of secretory progenitors that were unable to terminally differentiate (as determined by a larger number of Dll1-positive cells in the crypt)[116]. Expression of early goblet cell markers including Tff3 and goblet-5 was normal, but there was loss of period acid Schiff (PAS) staining, which stains the mucin normally present in goblet cells[116]. Accordingly, many goblet cells presented with abnormal ultrastructure typical of immature goblet cells including poorly defined vacuoles in their cytoplasm, a clear brush border and a pronounced ER[116]. Paneth cells were also affected with reduction in the relative size of the Paneth cell compartment at the crypt base and in the cellular granule content[116]. Therefore, loss of Spdef affects terminal differentiation of both goblet and Paneth cells. Endocrine cell number was reported to be unchanged in the Spdef$^{-/-}$ model, but, by microarray, the expression of the endocrine cell markers Ghrelin and Peptide YY (PYY) were increased suggesting that there could be increases in specific endocrine cell populations in this model[116].

Inducible transgenic expression of Spdef in the intestinal epithelium resulted in expansion of goblet cell number and increased goblet cell gene expression at the expense of other secretory cell types[136]. Decreased epithelial proliferation without apoptosis was also observed suggesting that Spdef inhibits proliferation of intestinal secretory progenitors and promotes the terminal differentiation of goblet cells via activation of goblet cell associated genes to the detriment of the Paneth cell gene program. Additionally, due to an affect on the

endocrine cell lineage, Spdef may also be able to redirect endocrine progenitors to the goblet/Paneth lineage.

A microarray approach was combined with *in situ* hybridization analysis to determine the expression pattern of downstream targets regulated by Spdef and whether these targets were affected in Paneth cells, goblet cells, or both[116]. Spdef was found to be important for activating multiple genes expressed in goblet cells including Creb3l4, Ccl6, Klk1, mucin 2 (Muc2), Hgfac, and Tpsg1. Spink4 is normally expressed highly in Paneth cells and to a lesser extent in goblet cells; Spink4 expression was specifically lost in Paneth cells of the Spdef$^{-/-}$ intestine[116]. Other genes expressed in Paneth cells, Defcr21 and Nupr1, were also reduced in the Spdef$^{-/-}$ mouse[116] suggesting that Spdef expression is required to activate expression of these genes. In contrast, other Paneth cell genes, including matrix metallopeptidase 7 (MMP7) and angiogenin 4 (Ang4), were highly up-regulated due to their ectopic expression in the goblet cells of Spdef$^{-/-}$ intestine[116] suggesting that Spdef normally functions to repress expression of these genes in goblet cells. The majority of this data indicates that Spdef does indeed function to promote goblet cell differentiation and goblet cell gene activation and to repress the Paneth cell gene program in terminally differentiated goblet cells. Taken together, analysis of Gfi1 and Spdef mouse models putatively suggest the presence of a common Paneth/goblet progenitor cell.

Of note, Spdef also plays an important role in goblet cell differentiation in the lung which highlights the parallels between intestinal secretory differentiation and secretory differentiation in other tissues. Spdef expression has been shown to increase in the lung in response to allergens and transgenic expression of Spdef throughout the lung or specifically in Clara cells, the goblet cell precursors resulted in goblet cell hyperplasia without proliferation[140, 141]. In contrast, goblet cell differentiation was prohibited in the Spdef$^{-/-}$ model following allergen exposure[141] demonstrating that Spdef is required for goblet cell hyperplasia that occurs in response to allergens in the lung.

Additional Factors Important for Goblet Cell Differentiation

Intestinal goblet cells secrete mucin glycoproteins, the major component of which is Muc2, that function to protect the intestinal epithelium and as a medium for transport between the luminal contents and epithelial cells. The number of goblet cells in neonatal and adult intestine increases along the anterior-posterior axis with the highest number of goblet cells located in the colon (which correlates with the anterior-posterior distribution of Math1 expression)[142] (see also Chapter 2). In addition, the mucin content of goblet cells is larger in the distal intestine compared to the proximal intestine[143], possibly related to the larger bacterial population in the distal intestine. The intestinal mucins are mostly acidic and so stain blue with periodic acid Schiff-alcian blue (PAS-AB) (compared to the stomach which has neutral mucins that stain pink with PAS-AB); the acidic mucins are further characterized by the presence of sulfated (sulfomucins) or non-sulfated (sialomucins) groups with sulfated mucins being the predominant group present after birth in rodents[142]. Interestingly, the rate of mucus secretion and the type of mucins expressed can be modulated by host-microbe interactions[142]. Increased number of goblet cells is found in a variety of intestinal diseases and adaptive responses including enteric infections, inflammatory bowel disease, colon cancer, small bowel resection, and total parenteral nutrition[120, 122, 142, 144, 145] (and VanDussen unpublished).

Kruppel-like factor 4 (KLF4) is a zinc finger transcription factor that is expressed in terminally differentiated cells of intestinal epithelium[146-148]. Recently, KLF4 expression has been shown to be regulated by Notch signaling with increased KLF4 expression observed when Notch signaling is inhibited with DBZ and decreased KLF4 expression when NICD1 is over-expressed in HT29 colon cancer cells[149]. This inverse relationship of KLF4 expression and Notch activity fits with the findings that KLF4 inhibits progression of the cell cycle[150, 151] while Notch is important for maintaining progenitors in a proliferative state. KLF4 also appears to be important for goblet cell differentiation because KLF4 homozygous null mice displayed a 90% loss of goblet cells in their colons[152].

Thus, KLF4 is an important regulator of proliferation and of goblet cell differentiation in the intestine.

The Foxa1 and Foxa2 transcription factors are expressed in the intestinal crypts and also appear to regulate differentiation of goblet cells[153]. In the Foxa1/2 intestine-specific double mutant, there is decreased goblet cell number throughout the intestine and colon and reduced density of the mucus granules[153]. It was determined that Foxa1 can bind directly to the Muc2 promoter *in vivo* and, accordingly, Foxa1/2 mutants exhibited a switch in their mucin profile with decreased expression of Muc2 and Muc6 and increased expression of Muc5b and Muc5ac in the colon[153].

Anterior gradient 2 (Agr2) is a member of the ER protein disulfide isomerase (PDI) family that functions to isomerize specific protein substrates into their bioactive confirmations while they are trafficking through the ER. If PDI substrates are not isomerized, they accumulate in the ER and initiate ER stress, cell cycle arrest, and apoptosis[154, 155]. Muc2 has been shown to be a specific substrate of Agr2 with disruption of Agr2 resulting in destabilized Muc2 protein and decreased mucus production in mice[156]. Another Agr2-deficient mouse model also showed decreased expression of Muc2 along with decreased alcian blue-positive staining; however Tff3 was unaffected[157]. Interestingly, an expansion of Paneth cells was also observed in this mouse model with some of the Paneth cells mislocated to the villi[157]. Using an inducible Agr2-deficient model, the Paneth cell expansion was shown to occur within 24 hours, prior to the goblet cell defects[157]. Of note, inflammation was observed in the terminal ileum and colon of Agr2-deficient mice[157] highlighting the role of goblet cell mucins in epithelial protection.

Paneth Cells in the Small Intestine

Like the other secretory cell types, Paneth cells increase in number along the anterior-posterior axis of the intestine. In contrast to goblet and endocrine cells, Paneth cells are absent from the colon under normal conditions, but they can be induced in the colon in disease states including adenocarcinoma[32, 158, 159].

Paneth cells produce several proteins associated with host defense including lysozyme, cryptdins, MMP7, and TNFα [160]. MMP7 is required for processing procryptdin precursors to active cryptdin proteins[161]. Accordingly, a mouse model with deficient MMP7 expression lacks mature cryptdins and is defective in clearing intestinal infections[162].

Morphologically, Paneth cells appear the same throughout the small intestine; however there is at least some degree of heterogeneity based on the distribution of the Paneth cell cryptdin genes. For example, cryptdin-4 is only in Paneth cells of the distal intestine whereas cryptdins-1 and -5 are expressed throughout the intestine[163]. This heterogeneity may be related to host defense since there is a greater population of bacteria in the distal gut and cryptdin-4 has very potent bactericidal activity against *E. coli* and *S. aureas* compared to other cryptdins[164].

Mature Paneth cells appear in the small intestine at 2-3 weeks of age in mice, concomitant with crypt formation. Of note, multiple Paneth cell genes including cryptdins, MMP7 and lysozyme are expressed prior to appearance of recognizable Paneth cells or crypts. In neonatal mice, the cells that stain for cryptdin and MMP7 are scattered throughout the villi[160] (also see Chapter 2). Paneth cells are thought to initially differentiate at the top of the Paneth cell compartment and then migrate downward to the base of the crypt as they mature[165]. Supporting this downward migration, lineage tracing of the CBC marker Leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) (discussed more fully in a subsequent section) showed labeled Paneth cells first appearing at cell position 5 at the top of the Paneth cell compartment and then labeled cells moved nearer to the base of the crypts over time[166].

Paneth Cell Differentiation Requires Wnt Signaling

In addition to its major role in promoting proliferation in the intestinal crypts, Wnt signaling is very important for Paneth cell maturation and distribution. Indeed, approximately 20% of downregulated genes in Tcf4$^{-/-}$ embryos encoded for Paneth cell genes, including some genes that appear to be direct Wnt targets

such as MMP7 and EphB3[158]. Moreover, APC^{min} mice with overactive Wnt signaling exhibit a general loss of differentiation, but have excessive expression of Paneth cell markers[32, 33].

SRY-Box9 (Sox9) is a Wnt target gene that is essential for Paneth cell differentiation and has been implicated in regulation of crypt proliferation. Nuclear Sox9 expression is localized to Paneth cells and, additionally, almost perfectly overlaps with the proliferative zone in both the intervillus zone of the developing intestine and crypts of the adult intestine[167, 168]. Sox9 is typically thought to function as an activator and indeed its expression increases expression of a number of Paneth cell genes including lysozyme, MMP7 and Ang4[169]. It has been shown to repress expression of the differentiation genes Cdx2 and Muc2, but this likely occurs through an intermediate repressor[167]. Two models of Sox9-deficiency have been described; each utilized a Cre-lox approach in which the second and third exons of the Sox9 gene were floxed and recombination was targeted specifically to the intestinal epithelium using a Vil-Cre driver[168, 169]. In both models, Paneth cells were found to be absent and crypt dimensions were increased along with enhanced proliferation[168, 169]. Reduced goblet cell number and formation of villus-like structures in the colon was reported in one model[169] while goblet cell number was reported to be unchanged in the other[168]. This difference may have been due to use of different Vil-Cre lines; comparison of these lines is discussed in Chapter 3. Together, these data show that Sox9 is essential for Paneth cell differentiation and also suggest a role for Sox9 in regulation of proliferation. Sox9 is also a likely candidate for activation of ectopic Paneth cells in intestinal diseases; indeed, Sox9 expression has been documented in adenomas[167]. Recently, a $Sox9^{EGFP}$ reporter mouse was used to show that high EGFP/Sox9 expression occurs in post-mitotic endocrine cells and low EGFP/Sox9 expression occurs in CBCs[170] suggesting that Sox9 might perform some function in stem cells. Interestingly, overexpression of Sox9 in the IEC-18 enteroendocrine cell line inhibited proliferation[170].

Paneth Cell Distribution Along the Crypt-Villus Axis is Regulated by Wnt and Eph/Ephrin Signaling

The Ephs are receptor tyrosine kinases whose ligands are the ephrins. Both Ephs and ephrins are membrane-bound proteins so signaling occurs between neighboring cells. Upon ligand engagement, a bi-directional signaling event is activated that frequently results in repulsion of the signaling cells[171]. In the developing intestine, EphB2 and EphB3 are expressed in the intervillus zone and EphrinB1 is expressed on the villi[44]. In the adult intestine, this general pattern holds true, but it is a little more complicated with EphB2 and EphB3 both expressed in Paneth cells while EphB2 is also expressed more widely throughout the lower portion of the crypt. EphrinB1 and EphrinB2 have low expression in the mid-crypt and this level gradually increases toward high expression on the villi. Canonical Wnt signaling through Tcf4 and β-catenin has been shown to increase expression of Eph receptors and decrease expression of EphrinB1 ligand[44]. EphB3 is a critical regulator of Paneth cell distribution along the crypt-villus axis; EphB3$^{-/-}$ mice have Paneth cells scattered among the crypt and villus[44]. Reinforcing the importance of Wnt in Paneth cell distribution, the same phenotype was achieved in a different mouse model in which the Wnt receptor Frizzled5 (Fzd5) was disrupted in the intestinal epithelium[44]. The mislocated Paneth cells were devoid of EphB3 expression, nuclear β-catenin expression, and cryptdin-1 whereas Paneth cells that remained at the base of the crypt retained these characteristics[44]. This suggests that at least a portion of the Paneth cell program is cell non-autonomous and instead depends on the position of the cell along the crypt-villus axis.

The Eph genes also appear to promote proliferation in the crypts and to separate the proliferative and post-mitotic compartments. EphB2/B3$^{-/-}$ compound mutant mice displayed intermingling of proliferative and differentiated cells with EphrinB1-positive cells, which are normally excluded from the crypts, now found scattered throughout the crypt[44]. In addition, acute inhibition of EphB2 via a blocking antibody resulted in overall decreased proliferation while, conversely, overexpression of EphB2 resulted in increased epithelial proliferation[172].

Other Factors Affecting Paneth Cell Differentiation

Other factors have been shown to function in Paneth cell differentiation including peroxisome proliferator-activated receptor β (PPARβ), colony stimulating factor 1 (Csf1) and fibroblast growth factor receptor 3 (FGFR3). Deficiency of PPARβ impairs postnatal development of Paneth cells; these mice do not have expansion of the Paneth cell lineage between 2 and 3 weeks compared to control mice that have a 6-fold increase in Paneth cells during this time period[173]. In addition, mouse intestine deficient in Csf1 or FGFR3 exhibits decreased Paneth cell marker expression[174, 175]. Together, these data suggest that complex interactions of signaling pathways and compartments direct Paneth cell differentiation, maintenance and distribution.

Intermediate Cells: A Rare Intestinal Cell Population

A rare cell population in the intestinal crypts was identified and characterized by electron microscopy many decades ago. These cells had some characteristics of an undifferentiated cell including granular ER that is not well developed with many free ribosomes in the cytoplasm and some very small secretory granules. Additionally, other electron-dense secretory granules of intermediate size between those of young Paneth cells and immature goblet cells (called granule goblet cells) and some granules containing small amounts of mucin were observed; thus, these cells were dubbed “intermediate cells”[165, 176]. Intermediate cells comprise ~1% of the crypt cell population and are most often located on the lateral side of the crypts and very rarely at the base of the crypts between the mature Paneth cells[176]. Intermediate cells stain positively with PAS-AB and have been shown to co-express genes that are normally expressed in Paneth cells including cryptdins and phospholipase A2 (Pla2g2a), and MMP7 and genes that are normally expressed in goblet cells including Muc2[121, 165] (also VanDussen, unpublished). Of note, these cells do not express the full goblet or Paneth cell gene program; these cells do not contain sulfomucins, the predominant mucin form in the adult intestine, nor are they immunoreactive for

Tff3 or lysozyme suggesting they are functionally distinct from either differentiated lineage[121, 177]. Consequently, intermediate cells have been proposed to be goblet cells that are trans-differentiating to the Paneth cell lineage or vice versa or, alternatively, the shared precursor of these lineages.

Early studies of intermediate cells have demonstrated that intermediate cells are capable of proliferating (or arise shortly after a previous cell division event) in accordance with their putative precursor identity. Injection of Thymidine-^{3}H into mice resulted in labeled intermediate cell nuclei[176]. Studies in a mouse model with Paneth cell ablation have also supported the idea that intermediate cells may be precursors to Paneth and goblet cells. The cryptdin2 (CR2) promoter is expressed in Paneth cells, intermediate cells, and immature goblet cells; use of the CR2 promoter to drive expression of diptheria toxin resulted in reduced numbers of these cells, but most markedly a decrease in Paneth cells[165] likely due to the highest CR2 expression in this cell type. Following this study, CR2 was used to drive expression of the SV40 TAg in mice which blocks differentiation of progenitor cells and thus, would block differentiation to Paneth cells in this model due to their high CR2 expression. The CR2-SV40 TAg intestines exhibited loss of mature Paneth cells as expected; the Paneth cell compartment was instead filled with undifferentiated CBCs[165]. Additionally, amplification of the goblet cell lineage and intermediate cell number was observed, supporting the idea that since intermediate cells were unable to differentiate to the Paneth cell lineage, they were redirected to the goblet cell lineage[165]. The expanded populations of intermediate cells and immature goblet cells were located throughout the lower two-thirds of the crypt and upper crypt/lower villus, respectively[165]. EM showed that the diameter of the electron-dense granule cores was diminished and the mucin-occupied area was increased in the intermediate cells near the upper portion of the crypts compared to those near the bottom[165], suggesting that transformation of intermediate cells to immature goblet cells to mature goblet cells is a bona fide mechanism of cellular differentiation in the intestine.

One report has placed STK11 as a critical regulator of intermediate cell differentiation[137]; however, the data presented do not fit with many other reports of intermediate cell characteristics. STK11 has been demonstrated to inhibit cell proliferation in a number of tumor cell lines including the intestinal colorectal adenocarcinoma line SW480[178]. It is widely expressed in the intestine but has especially high protein expression in Paneth and goblet cells[137]. Inducible deficiency of STK11 in the intestinal epithelium resulted in increased Muc2 expression and enlarged mucus droplets in goblet cells[178]; these goblet cells purportedly also expressed lysozyme, but this data was not shown[178]. The Paneth cells at the base of crypts in STK11-deficient intestine were abnormal and contained mucin droplets[178]. The authors concluded that there was emergence of intermediate cells in their model; however, since these cells were lysozyme-positive and mostly located at the base of the crypts[178], these cells may not be equivalent to the intermediate cells described in other studies[165, 176, 177]. Perhaps STK11 instead functions as a negative regulator of the goblet cell mucin genes or in maintenance of the mature Paneth cell program.

Intermediate cells have been implicated in the physiological response to infection; their numbers are increased in mice infected with the nematode *T. spiralis*[121]. The expanded intermediate cell population was localized to the lateral sides of the crypts and extended onto the lower villi[121]. The increase in intermediate cell number was accompanied by other epithelial cell changes in Paneth cells, goblet cells, and proliferation[121, 123]. *T. spiralis* infection led to increased epithelial proliferation within 2 days, leading to crypt hyperplasia by day 6, the day of maximal worm burden[123]. The numbers of Paneth cells, goblet cells, and intermediate cells were increased by 6 days with maximal increase by 12 days (the time at which worm burden starts to decline) and a slow decline through days 18-27 when the worms are fully cleared[123]. In accordance with increased secretory cell number, expression of Math1 was upregulated on days 6-18 of the study[123]; however, it is not clear if this was due to its expression in the increased number of mature secretory cells or if the increased expression of Math1 induced the expansion of secretory cells or both. In accordance with

increased differentiation of progenitor cells, the number of proliferating cells declined over time, being comparable to uninfected controls over days 6-12, and being lower than controls on days 18-27[123]. These data suggest that the progenitor cells are being influenced more towards differentiation than self-renewal during the response to *T. Spiralis* infection. The intermediate cell response in this infection model is thought to be regulated by the host immune cell response, specifically, an unidentified population of T-cells[121]. In Chapter 4, I show that a dramatically increased population of intermediate cells emerges in the small intestine subsequent to inhibition of Notch signaling via GSI treatment. In addition, induction of intermediate cells was observed in the colons of DBZ-treated mice and throughout the gut of Vil-Math1 transgenics (Chapter 4 and VanDussen, unpublished). Together, these data suggest that the host immune response is able to modulate intestinal Notch signaling which, in turn, affects intermediate cell differentiation and/or proliferation.

Factors Controlling Differentiation to the Endocrine Lineage

Endocrine cells comprise only ~1% of intestinal epithelial cells yet these cells play a very important role in communication between the organs, especially those involved in digestion and metabolism. Many important transcription factors regulating endocrine cell differentiation in the intestine have been identified because they perform a similar function in pancreatic endocrine differentiation. Neurogenin 3 (Neurog3) is expressed in the endocrine progenitor cells of both the pancreas[179] and intestine[131, 180, 181]. Its expression is required and sufficient for endocrine cell differentiation in the intestine[180, 181]. The endocrine progenitor cell is a transient cell type present in the intestinal crypt that soon begins to express the pan-endocrine marker chromogranin A, turns off Neurog3 expression, and then terminally differentiates to a specific hormone-producing cell type[131]. During its short period of expression, Neurog3 functions as a master regulator of the endocrine gene program and activates a network of pro-endocrine transcription factors that likely push the differentiating cell toward a particular hormone-expressing endocrine cell type. Transcription factors that

have been shown to affect differentiation of specific endocrine cell types or groups of endocrine cells include Pax4[182], Pax6[182], NeuroD1[183], and Nkx2.2[184]. For example, Pax4 expression is required for many endocrine cell types including duodenal serotonin, secretin, cholecystokinin (CCK), gastric inhibitory peptide (GIP) and PYY cells[182] while NeuroD1 expression is required just for CCK and secretin cells[183]. Also, in addition to effects on goblet cell differentiation as described above, Foxa1 and Foxa2 are important for differentiation of L-cells (GLP-1, GLP-2, PYY) and D-cells (somatostatin) and expression of the pro-endocrine transcription factors Pax6 and Isl-1, demonstrating that some intestinal transcription factors may affect distinct steps of the terminal differentiation of secretory cells[153]. All in all, approximately 15 types of mature endocrine cells are found in the intestine; these cells are differentially distributed along the anterior-posterior axis of the gut as well as the crypt-villus axis[185] suggesting that a complex signaling network facilitates proper differentiation and distribution of the hormone-producing cells in the intestine.

The Location, Number and Nature of Intestinal Stem Cells

The ability to replace epithelial cells and regenerate intestinal tissue following damage is essential for proper maintenance of the intestine. It has long been recognized that immature, undifferentiated cells exist near the base of the intestinal crypts, nestled between the Paneth cells; these cells are the CBCs[186, 187]. Early investigation of CBCs determined that they are radiosensitive and that they possess phagocytic capacity; subsequent to radiation, labeled phagosomes were generated[186, 187]. The labeled phagosomes were first observed in the crypt base and later on the villus and could be traced to all mature epithelial cell types, suggesting that there were multipotential cells in the crypt base that could give rise to all epithelial cell types[186, 187]. Since these seminal observations, many studies have been undertaken to further our understanding of the intestinal stem cell and the signals that are critical for maintaining its renewal properties and directing its differentiation. To demonstrate true “stemness” in a population of cells, two criteria must be met: 1) the stem cell population must be able to be

maintained over long periods of time and 2) the stem cell population must be able to generate all of the differentiated cell types that comprise the organ/system.

Currently, there is a debate about the exact location of the intestinal stem cell (schematic in Figure 1-4). Cellular position within the crypt is counted starting from the middle of the crypt base. This position is position 1. Studies that assessed the position of label-retaining cells identified the +4 position, the position just above the Paneth cell compartment, as being occupied by a putative stem cell[188-190]. Label-retention assumes that only stem cells will retain the label and is based upon Cairn's hypothesis that stem cells will retain the template DNA strand during cell division to protect against DNA replication errors[191]. It is of note that in the previously mentioned studies, label-retaining cells were not only found at the +4 position, but distributed throughout the crypt with highest concentration in the first nine positions. This wide distribution and high number of label-retaining cells suggests that stem cells may be located at other positions than the +4 position and that non-stem cells may also be retaining label[192]. Thus, it is currently not known if the so-called "+4 stem cell" that resides above the Paneth cell compartment or the CBC that resides at the base of the crypt is the "true" stem cell. Furthermore, it is not known whether these putative stem cell populations are equivalent or if they serve distinct functions in regeneration of the intestinal epithelium.

Experimental evidence from analysis of chimeric mice or mice heterozygous for an X-linked gene mutation suggests that neonatal crypts are polyclonal in nature and then, by an unknown mechanism, one stem cell is "selected" for each crypt[193, 194]. After the selection process, each crypt remains monoclonal throughout adulthood[193, 194] with 4-6 stem cells per crypt during steady-state conditions[195-197]. However, 30-40 cells in the crypt (which contains ~250 cells total) appear to possess capability to regenerate the crypt if a damaging event occurs[166]. This suggests that there is flexibility in the self-maintenance properties of the intestinal stem cell that allow it to adapt to the needs of the tissue.

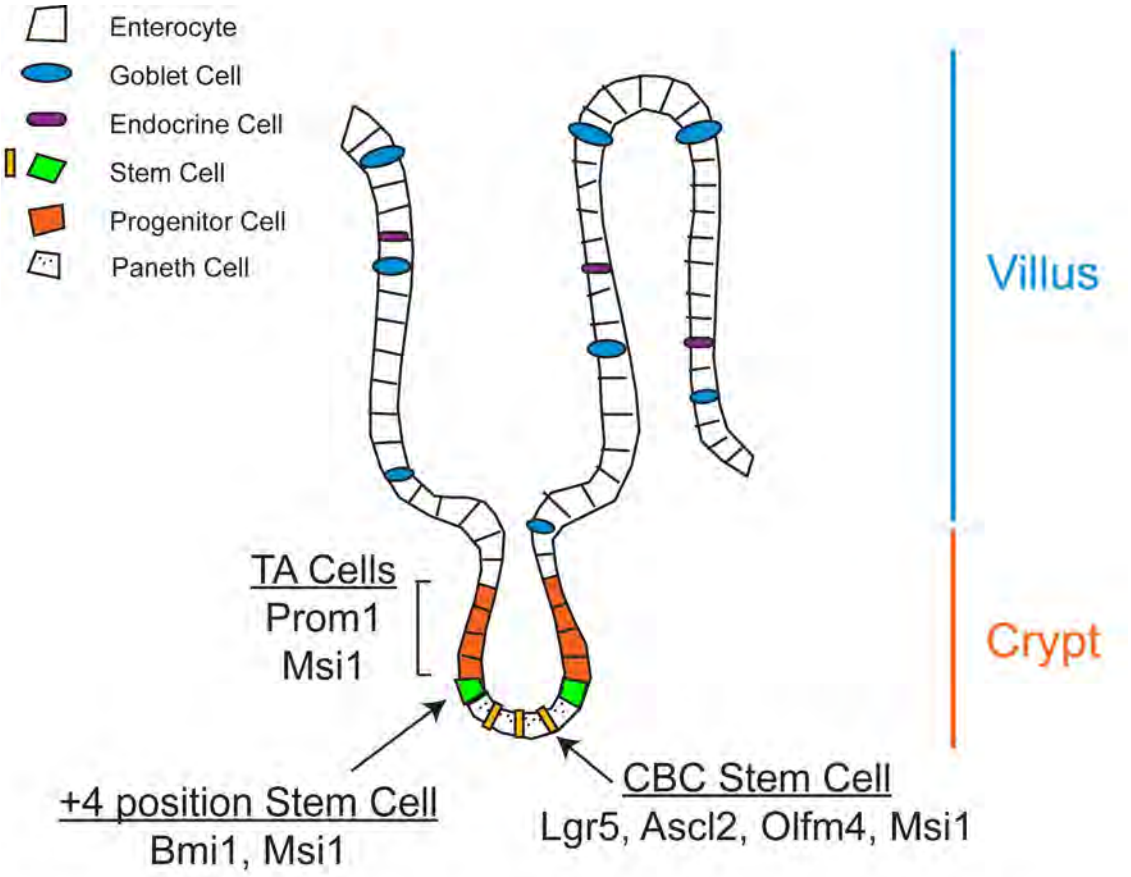

Figure 1-4. Location of Epithelial Stem and Progenitor Cell Populations in the Adult Intestine. The intestinal stem and progenitor cells are located exclusively in the crypts. There are thought to be two stem cell populations in the adult intestine, a long-term label-retaining cell at the so called "+4 position" and an actively cycling crypt base columnar (CBC) stem cell that resides between the Paneth cells at the base of the crypts. In addition, there is a highly proliferative population of progenitor cells called transit amplifying (TA) cells which are located above the Paneth cell compartment. Markers for stem/progenitor cell populations are indicated.

Signaling between the epithelium and mesenchyme is vital for epithelial stem cell function and for control of epithelial cell turnover rate. The intestinal mesenchyme is thought to be important for creation and maintenance of the intestinal stem cell "niche", the specialized microenvironment in which the stem cells reside. Mesenchymal cells residing in or near the stem cell niche may regulate stem cell function through secreted products or direct interaction with the stem cells. It is thought that if stem cells are removed from their niche, they lose their stemness and become committed to differentiation[198]. Migration of differentiating cells toward the top of the crypts would remove them from the influence of the stem cell niche and is likely an important step in the maturation process of most intestinal epithelial cells.

Identification of Intestinal Stem and Progenitor Cell Markers

Recent advances in stem cell research have brought renewed vigor into identification and characterization of the intestinal stem cell. Many new intestinal stem cell markers have been identified (Figure 1-4) and the first *in vitro* cultures of the intestinal stem have been achieved. A summary of putative stem and progenitor cell markers, the cell population(s) they are thought to mark, and a description of their reported properties is included in Table 1-2. It is also noted if the stem cell marker has been described as a contributing factor to adenoma progression.

Markers of the Crypt Base Columnar Stem Cell

Since mouse models had demonstrated the importance of Wnt signaling for intestinal stem cell proliferation, it was hypothesized that Wnt-regulated genes would be expressed in stem and/or progenitor cell populations. Screening of gene expression in colorectal cancer cells that had been transfected with a dominant-negative Tcf4 construct to block Wnt signaling identified ~80 Wnt-regulated genes[40]. Most of the genes were expressed in transit-amplifying cells or Paneth cells; however, expression of some genes, including Lgr5, was restricted to a limited number of cells in the base of the crypt, the CBC cell

Table 1-2. Gene Markers of Intestinal Stem Cells

Gene	+4	CBC	TA	Longevity	Multilineage Differentiation	Adenoma Formation	Reference(s)
Ascl2		✓			✓	✓	Van der Flier et al., 2009
Bmi1	✓	✓		Long	✓	✓	Sangiorgi et al., 2009
DCAMKL1	✓						May et al., 2009
Lgr5		✓		Long	✓	✓	Barker et al., 2007 Barker et al., 2009
Msi1	✓	✓	✓				Kayahara et al., 2003 Potten et al., 2003 Gregorieff et al., 2005
Olfm4		✓					Van der Flier et al., 2009
Prom1		✓	✓	Short	✓	✓	Snippert et al., 2009 Zhu et al., 2009
PTEN	✓						He et al., 2004

Descriptions of reported expression pattern (+4, +4 cell position stem cell; CBC, crypt base columnar stem cell; TA, transit amplifying progenitor cell) is provided for each marker along with length of longevity (self-renewal capacity), capacity to form all major epithelial lineages, and role in adenoma formation of the cell labeled by each marker.

described by Cheng and LeBlond[199]. Lgr5 was also expressed at the base of the colonic crypts and the base of the antral glands of the stomach where the stem cells for these tissues are thought to reside[199]. Lgr5 encodes an orphan G-protein coupled receptor with high similarity to the peptide hormone receptors for luteinizing hormone, follicle-stimulating hormone and thyroid-stimulating hormone[200]. Using an Lgr5-lacZ knock-in transgenic mouse and BrdU-labeling studies, it was determined that Lgr5-positive cells are actively cycling cells with an average of one round of cell division per day[199]. A separate knock-in transgenic model in which a GFP-CreERT2 fusion protein was inserted into the Lgr5 locus allowed for lineage tracing when crossed to the Cre-activated Rosa26-lacZ reporter mouse[199]. Lineage-tracing of the progeny of Lgr5-positive cells labeled all epithelial cell types with a time period of ~5 days for a migrating "ribbon" of labeled cells to reach the top of the villus, consistent with the rate of epithelial migration from crypt to the villus tip. The labeling is long-lived, with labeled ribbons still maintained 14 months after induction of the lineage tracing[201]. Thus, the Lgr5-positive cells fulfill both characteristics of stemness: the ability to form all epithelial cell types and longevity. Although it is accepted that Lgr5 is expressed in an intestinal stem cell population, the function of Lgr5 in the stem cell has remained more elusive. Deficiency of the Lgr5 gene caused neonatal lethality presumably due to fusion of the tongue to the floor of the mouth which prevented normal nursing and caused the newborns to swallow air[202]. The intestinal phenotype of these mice was described in a later study. Although Lgr5 was found to be expressed in the proliferative intervillus zone at this developmental stage, no affect on proliferation or migration of epithelial cells was observed in $Lgr5^{-/-}$ embryos[203]. $Lgr5^{-/-}$ embryos had normal distribution of enterocytes, goblet cells, and endocrine cells, but they exhibited premature differentiation of Paneth cells which normally mature 2-3 weeks after birth[203]. Thus, at this point, Lgr5 has been accepted as a CBC marker, but a function for Lgr5 in stem cell maintenance has not been demonstrated although it does appear that Lgr5 can affect cellular differentiation.

Analysis of genes enriched in Lgr5-positive cells identified two additional genes, Achaete scute-like 2 (Ascl2; also called Mash2) and Olfmactomedin4 (Olfm4), that were expressed specifically in the CBC by *in situ* hybridization[204]. Ascl2, itself a Wnt target gene, was shown to direct the expression of a number of other Wnt target genes, including Lgr5[204]. Transgenic expression of Ascl2 in the intestinal epithelium via the villin promoter resulted in crypt hyperplasia and disorganized and branched villi with ectopic proliferative crypts at 2 weeks of age. In contrast, conditional deletion of Ascl2 in the intestine led to disappearance of Lgr5-positive stem cells within 5 days as shown by loss of Olfm4 expression (although proliferation was not dramatically different compared to controls). Thus, although Ascl2 is essential for CBC maintenance, other proliferating cells must be able to compensate for the loss of Lgr5-positive CBCs. Interestingly, over 15 days CBCs gradually reappeared; the authors attributed the regeneration due to increased crypt fission events of the crypts with stem cells that had escaped the Cre-mediated recombination event[204]. Alternatively, a stem cell population that is Ascl2-negative could be able to rescue the Ascl2 loss-of-function phenotype; this stem cell population potentially could be the +4 position cell.

Bmi1 is a Potential Marker of the "+4 position" Stem Cell

Bmi1 is a member of the Polycomb group gene family that is involved in chromatin silencing[205]. It has been shown to participate in self-renewal of neuronal, hematopoietic and and leukemic cells[206-208]. Bmi1-expressing cells were found to be located in the intestinal crypts, most often at the +4 position above the Paneth cells and sometimes in the CBC, both positions where stem cells are thought to be located[209]. In accordance, Bmi1 expression was enriched in Lgr5-positive sorted cells[204]. Of note, Bmi1-positive cells were not found in every crypt suggesting that separate stem cells are proliferating in the crypts without a Bmi1 cell or that Bmi1 expression is not constitutively expressed or both. Lineage tracing of the progeny of the Bmi1-positive cell resulted in labeling of all mature epithelial cell types, a similar result to Lgr5 lineage tracing, with

ribbons of labeled cells persisting for up to 12 months[209]. The first labeled cells in the Bmi1 lineage tracing study were observed 20 hours after induction of labeling and these cells were usually located at the +4 position. After 5 days, some crypts remained with only a single labeled cell suggesting that the Bmi1-positive cell may cycle more slowly than the Lgr5-positive cell. Therefore, Bmi1 cells may represent a more quiescent state of the intestinal stem cell while Lgr5 cells are an actively cycling stem cell population that supplies the transit amplifying progenitor population. Ablation of the Bmi1-cell in 3-week old mice was achieved by activating diptheria toxin specifically in Bmi1-expressing cells using the Bmi1-CreER transgenic model. Injection of tamoxifen on 3 consecutive days resulted in death of the mouse while a single injection of tamoxifen produced intestinal regions that were devoid of crypts which were gradually regenerated over 9 months through crypt fission or some other mechanism. This suggests that Bmi1 is essential for stem cell function and epithelial cell maintenance. Whether Bmi1-positive and Lgr5-positive cells represent the same cell population or distinct populations (or states) of stem cells remains a hotly debated and open issue in the field.

Prominin1 Marks Short-Lived Progenitor Cells

Prominin1 (Prom1; also called CD133) has been used as a surface marker for isolation of stem cells in many tissues[210-214]. Two independently generated knock-in transgenic mouse models reported differing expression patterns of Prom1; both studies demonstrated that Prom1 is co-expressed in Lgr5-positive cells[215, 216] while Snippert et. al. also showed Prom1 mRNA and protein expression was expressed in the transit-amplifying cell population above the Paneth cell compartment[215]. Lineage tracing of the progeny from Prom1-expressing cells showed that cells from all epithelial lineages were labeled and that labeling persisted for at least 60-75 days[215, 216]. Interestingly, the frequency of tracing events observed 7 days after induction of labeling was only 10% of that observed 1 day after induction of labeling suggesting that many of the Prom1-positive cells are short-lived progenitor cells and not stem cells; the remaining

labeling could potentially arise from the Prom1-positive cells that are also Lgr5-positive[215].

Musashi-1 is a General Marker of Stem and Progenitor Cells

Musashi-1 (Msi1) is an RNA binding protein that is thought to function in asymmetric division in neural stem cells by binding to and repressing m-Numb, an inhibitor of Notch signaling[217, 218]. Msi1 is able to upregulate Hes1 expression in neural stem cells[219] which is essential for neural stem cell self-renewal and suppression of differentiation[113, 220, 221]. In the intestine, Msi1 is expressed in the CBC and in the +4 position cell[222, 223]. Interestingly, a similar pattern of expression was observed for Hes1 suggesting that Msi1 could also positively regulate Hes1 in the intestine[222]. Another study has shown that Msi1 is a more general marker with expression in many cells of the crypt proliferative compartment[43]. Indeed, it has been noted that Msi1 appears to be expressed in a greater number of cells per crypt than the 4-6 proposed stem cells per crypt[223]. Thus, based on the current data, Msi1 appears to be a more general marker of crypt stem and progenitor cells.

Controversial Putative Stem Cell Markers

Other markers potentially expressed in stem cells include phosphorylated phosphatase and tensin homolog (P-PTEN) and double cortin and calcium/calmodulin-dependent protein kinase-like-1 (DCAMKL1). P-PTEN was described as an intestinal stem cell marker based on its expression in BrdU label-retaining cells[26]; PTEN is linked to regulation of cell proliferation through PI3K and Akt signaling making it a provocative marker of intestinal stem cells. However, another study showed that P-PTEN-positive cells in the intestinal crypts always co-expressed the pan-endocrine marker chromogranin A[224], suggesting that P-PTEN is actually expressed in differentiated endocrine cells that are present in the crypts. DCAMKL1 was originally identified as a putative gastric and intestinal stem cell marker based on its expression in cDNA libraries generated from laser-captured tissue from regions where adult progenitor cells

reside[225]. To facilitate the sample collection process, mice with a genetic mutation resulting in Paneth-cell ablation were used which may have influenced the results of the study. Immunostaining for DCAMKL1 showed that it was expressed in the +4 position and not co-expressed with markers for any of the major epithelial lineages[225], a finding confirmed by another study that analyzed DCAMKL1 expression in normal and irradiated intestine[226]. DCAMKL1 did not co-localize with proliferation markers such as PCNA, but was found to be co-expressed with BrdU in a label retention assay[227]. Of note, DCAMKL1-positive cells are also present on the villus[226, 228]. It has since been demonstrated that most, if not all, DCAMKL1-positive cells express markers of a minority cell lineage in the intestine, the tuft cell, including Cox1/2 and high levels of villin and α-tubulin[228]. Tuft cells are a post-mitotic, differentiated cell type that is thought to function as a chemosensory cell type in the hollow organs of the gastrointestinal tract and respiratory system[229]. Thus, at this time, both P-PTEN and DCAMKL1 remain extremely controversial stem cell markers.

***In Vitro* Culture of Intestinal Stem Cells**

Methodologies for long-term culture of intestinal tissue and stem cells have recently been developed and described[230, 231]. Previous attempts to develop *in vitro* growth systems had been restricted by the rapid initiation of apoptosis after cells were removed from the basement membrane[232, 233]. Culturing of intestine from neonatal and adult mice was achieved using a 3D culturing technique with collagen gel to support the tissue at the air-media interface[230]. The cultures formed spheroid structures with proliferative and differentiated zones. The differentiated zones contained all major epithelial cell types. Many of the spheroids produced crypt structures within 2 weeks of culture that contained the proliferative compartment, Lgr5- and Bmi1-expressing cells. Growth of the crypts could be inhibited by addition of recombinant Dkk1 and enhanced by addition of the Wnt agonist R-spondin, which had previously been shown to promote intestinal proliferation *in vivo*[230, 234]. Finally, the differentiation of the spheroids could be driven to the secretory or endocrine lineage via

treatment with GSIs or adenoviral Neurog3, respectively, suggesting that the stem cells in these structures were able to respond to normal differentiation cues[230]. Single Lgr5 stem cells have also been cultured long-term; these cells form organoids with crypt-villus structures that contain all the major epithelial cell types and form normal crypt-villus patterning with Paneth cells and Lgr5 cells situated at the base of the crypts as *in vivo*[231]. The ability to sustain long-term culture from single intestinal stem cells that had been removed from their niche was somewhat surprising; however, a look at the culturing conditions reveals that many components normally provided by mesenchymal cells are required, including Noggin and a Wnt agonist (R-spondin was used). In addition, Matrigel was used to provide a laminin-rich support and it is known that Matrigel also contains many growth factors. EGF, which has been associated with intestinal proliferation[235], and Jagged 1, a Notch ligand and agonist, were also present in the culturing media. Together, this shows that the physical presence of mesenchymal cells may not be needed to maintain stem cells, but an extracellular matrix component and other secreted components from mesenchymal cells are essential. These exciting advances in methodology for studying intestinal stem cells will be important for understanding stem cell identity, maintenance and differentiation.

The Role of Intestinal Stem Cells in Progression to Adenoma

Current cancer theory proposes that the same stem cells that maintain the epithelium during steady state can accumulate mutations that may lead to malignancy; these stem cells are unable to undergo normal differentiation and instead sustain cancerous growth. Thus, identification of adult stem cells is an important step towards generation of therapeutic agents that can counteract aberrant stem cell activity. Consistent with this theory of cancer, many of the recently identified intestinal stem cell markers are expressed in intestinal adenoma including Lgr5, Ascl2, Olfm4, Msi1 and Prom1[199, 204, 215, 223, 236]. Furthermore, only a small proportion of the cells within the adenoma expressed Lgr5 and Olfm4 making it tempting to suggest that these cells represent

malignant stem cells driving formation and progression of the adenoma[204, 236]. In contrast, markers such as Prom1 were expressed widely throughout the adenoma[215]. Inducing overactive Wnt signaling *in vivo* via APC loss-of-function or β-catenin gain-of-function mutants specifically in the Lgr5-cell, Bmi1-cell, Ascl2-cell or Prom1-cell led to development of hyperproliferative crypts followed by progression to adenoma suggesting that these cells can function as the driving force behind tumor development when they acquire certain mutations[204, 209, 216, 236].

Thesis Overview

For my thesis research, I have investigated the role of the Notch signaling pathway in directing intestinal cellular differentiation. Notch signaling is the critical pathway controlling the bimodal switch between the absorptive and secretory cell lineages through regulation of a network of bHLH transcription factors. In particular, Notch signaling regulates the pivotal balance between the transcriptional repressor Hes1 and its target, the pro-secretory transcription factor Math1. My work and that of others has shown that Math1 is the key transcription factor regulating intestinal secretory cell differentiation. I have also explored how Notch signaling affects intestinal stem cells and have identified the crypt base columnar stem cell as a direct target of this signaling pathway.

In Chapter 2, I demonstrate that Math1 is sufficient to induce secretory cell differentiation to the detriment of the absorptive lineage during late fetal development. Transgenic expression of Math1 was targeted to the intestinal epithelium via the villin promoter (the Vil-Math1 transgenic model). Transgenic founders analyzed at E18.5 were found to have increased goblet and endocrine cells throughout the intestine with the most severely affected mice having almost the entire epithelium converted to goblet cells. Interestingly, precocious activation of Paneth cell markers was observed in the small intestine of Vil-Math1 founders along with ectopic activation of Paneth cell markers in the transgenic colon. Epithelial proliferation was decreased and displaced out of the intervillus zone and onto the villi; surprisingly, mesenchyme proliferation was greatly

increased leading to expansion of the mesenchyme compartment. Accordingly, multiple mesenchyme cell types were increased in number including neurons, myofibroblasts, myoblasts and smooth muscle cells. Since transgenic Math1 expression was limited to the epithelium, this suggests that the epithelial changes in transgenic mice altered epithelial-mesenchymal signaling to remodel the mesenchyme and suggests that Math1 is a novel regulator of "cross-talk" signaling pathways.

Chapter 3 attempts to expand on the findings presented in Chapter 2 through the generation of an inducible Math1 transgenic model (called VZM) which would be used to generate stable transgenic lines and to study the affect of Math1 expression in adult mice (stable lines of Vil-Math1 mice were not able to be generated presumably due to neonatal lethality of expressing founders). As part of this project, the recombination pattern of five Cre transgenic mouse models was analyzed to determine the best model for activation of the inducible Math1 transgene. This work is presented in Chapter 3-A. Chapter 3-B discusses the establishment and initial characterization of seven VZM transgenic lines. Unfortunately, due to lack of increased Math1 protein expression after induction of the transgene, this model was unsuitable for examining the role of Math1 overexpression in adult mice.

Chapter 4 discusses the results of two models of Notch disruption to determine which stem/progenitor cells are responding to Notch signaling and also to carefully analyze the induction of secretory cell lineages. I have confirmed that Math1 expression and goblet cells are increased in adult mice treated with the GSI dibenzapine (DBZ). In addition, I have shown that endocrine cells and Paneth cell markers are increased after GSI-treatment which agrees with a study in rat intestine reported by Milano et al[80] and with our understanding that increased Math1 leads to increased numbers of all secretory cell types. These cell lineage changes were also observed in GSI-treated embryonic intestine organ cultures treated with DAPT, a GSI. Interestingly, I made a novel observation that a population of intermediate cells that express markers of both goblet and Paneth cells emerges in both the small intestine and colon of GSI-

treated mice. Decreased proliferation in the crypts of GSI-treated mice has been previously reported suggesting that Notch signaling regulates intestinal stem and/or progenitor cells. We analyzed expression of intestinal stem and progenitor cell genes and found that expression of the CBC marker Olfm4 was dramatically down-regulated in both GSI-treated adult mice and embryonic organ cultures, suggesting that this gene may be directly regulated by Notch signaling. Transcriptional regulation of the Olfm4 gene was analyzed using promoter sequence analysis and luciferase assays to identify key regulatory regions of the Olfm4 promoter within the proximal 500 base pairs of the Olfm4 5' promoter. These studies have further supported that Notch can directly regulate Olfm4 gene transcription and have identified the CBC stem cell as a target of Notch signaling.

Finally, Chapter 5 will summarize the conclusions of this dissertation research and discuss the implications of the results and how they have contributed to the field of intestinal Notch signaling and secretory cell fate selection. Future directions for investigation will be presented and expanded upon.

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CHAPTER 2

MOUSE ATONAL HOMOLOG 1 DIRECTS INTESTINAL PROGENITORS TO SECRETORY CELL RATHER THAN ABSORPTIVE CELL FATE

SUMMARY

BACKGROUND & AIMS: The Notch-regulated transcription factor mouse atonal homolog 1 (Math1) is required for the development of intestinal secretory cells, as demonstrated by the loss of goblet, endocrine and Paneth cell types in null mice. However, it was unknown whether Math1 is sufficient to induce the program of secretory cell differentiation. **METHODS:** Intestinal morphology and epithelial and mesenchymal cell fate were examined by histological staining and marker gene expression in transgenic mice expressing a villin-regulated Math1 transgene. Cellular proliferation was assessed by Ki67 immunostaining. **RESULTS:** Late prenatal transgenic founders exhibited a gross cellular transformation into a secretory epithelium. The expansion of secretory cells coupled with the almost complete loss of absorptive enterocytes suggested reprogramming of a bipotential progenitor cell. Moreover, Math1 expression inhibited epithelial cell proliferation, as demonstrated by a marked reduction in Ki67 positive cells and blunted villi. Unexpectedly, the transgenic mesenchyme was greatly expanded with increased proliferation. Several mesenchymal cell types were amplified, including smooth muscle and neurons, with maintenance of basic radial patterning. Since transgenic Math1 expression was restricted to the epithelium, these findings suggest that epithelial-mesenchymal signaling is altered by the cellular changes induced by Math1. **CONCLUSIONS:** Math1 is a

key effector directing multipotential precursors to adopt secretory and not absorptive cell fate, and is a novel regulator of epithelial-mesenchymal crosstalk.

INTRODUCTION

The intestine is composed of a simple columnar epithelium overlying a complex multi-layered mesenchyme. The tissue is organized into villi that protrude into the lumen and crypts that invaginate into the mesenchyme. Stem and progenitor cells in the crypts replenish the epithelium at a rapid rate throughout the lifespan of the organism. The multipotential progenitor cells differentiate into two general cell lineages. The absorptive (columnar) cell lineage forms enterocytes, which are responsible for absorption of nutrients and constitute the majority of the epithelium. The secretory (granulocytic) cell lineage is composed of goblet, endocrine and Paneth cells, which secrete mucus, hormones and antimicrobial peptides, respectively. Epithelial cytodifferentiation first occurs during fetal development; the various mature cell types are established by birth, with the exception of Paneth cells, which are formed postnatally with the emergence of crypts. Although the mechanisms regulating epithelial cell fate choice have not been fully elucidated, multiple signaling pathways, including Notch, Wnt and Hedgehog, have been shown to influence progenitor cell proliferation and epithelial cell specification.[1, 2]

Animals with perturbations in intestinal Notch signaling remodel their epithelium in a manner suggesting that activation of this pathway directs multipotential precursors toward an enterocyte fate at the expense of secretory fates.[3-9] For example, transgenic mice expressing a constitutively active form of the Notch 1 receptor (NotchICD) in the intestinal epithelium exhibited a loss of secretory lineage cells, including goblet and endocrine cells as well as Paneth cell markers.[3, 4] Conversely, blockade of Notch signaling in rodent intestine with gamma secretase inhibitors or by deletion of either the Notch pathway transcription factor CSL/RBP-J or both Notch1 and Notch2 receptors promoted

excessive differentiation of secretory cell types.[5-7, 9] A similar phenotype was observed in zebrafish with disrupted Notch signaling,[8] suggesting that Notch is a fundamental pathway regulating the specification of absorptive versus secretory cells in the vertebrate gut.

A cascade of Notch-regulated basic-helix-loop-helix (bHLH) transcription factors have been identified as critical effectors directing intestinal epithelial cell fate decisions. In particular the bHLH transcriptional repressor Hairy and enhancer-of-split 1 (Hes1) has been determined to regulate the choice between absorptive and secretory cell differentiation. Hes1 is known to be a transcriptional target of Notch signaling, with expression localized to the epithelial progenitor zone of the intestine.[10, 11] Importantly, mice with a Hes1 loss-of-function mutation exhibited a phenotype similar to models of Notch disruption, with excessive differentiation of secretory cell types, suggesting that Notch signaling primarily exerts its influence on intestinal progenitor cell fate choice through Hes1.[12] In turn, Hes1 acts, at least in part, by repressing transcription of the bHLH activator atonal homolog 1 (Atoh1).[13] Accordingly, Notch disruption results in decreased expression of Hes1 and increased expression of Atoh1.[7, 12] Mouse Atoh1 (Math1) loss-of-function mutants have a phenotype similar to Notch gain-of-function models, with loss of goblet, endocrine and Paneth cells, demonstrating that Math1 is required for intestinal secretory cell development.[14, 15] Additional bHLH transcription factors have been shown to function downstream of Math1, including Neurogenin 3 (Neurog3), which is instructive for endocrine cell development, as demonstrated by loss of enteroendocrine cells in null mice[16] and enhanced endocrine cell development in transgenic mice expressing Neurog3 in the developing intestinal epithelium.[17]

Since Math1 expression is required for intestinal secretory cell development, it has been hypothesized to be the key effector regulated by Notch signaling that controls the bimodal switch between absorptive and secretory lineages. However, since Math1 is expressed in mature secretory cells in addition to cells in the progenitor zone,[14, 18] it is not clear whether Math1 functions to initiate the secretory cell program of differentiation or to maintain the

differentiated phenotype. In this study we tested whether Math1 expression promotes the program of secretory cell development with a transgenic mouse model that overexpressed Math1 in the developing intestinal epithelium.

MATERIALS AND METHODS

Generation of Vil-Math1 Transgenic Mice

The Vil-Math1 transgene contained the mouse cDNA under the control of the mouse villin enhancer/promoter. The transgene was prepared by isolating Math1 from pCS2+Math1[19] after *Cla*I/*Kpn*I digestion and shuttling into pBluescript SK (+/-) (Stratagene) before cloning into the villin expression plasmid pBSII-p12.4KVillΔATG.[20] Following verification by sequencing, the 15 kb transgene was excised with *Pme*I and microinjected into F2 zygotes from C57BL/6 X SJL parents by the University of Michigan Transgenic Animal Model Core. Potential founders were harvested at E18.5 and screened by polymerase chain reaction (PCR) amplification of a 270 bp product using the following primers: V1S 5'-GTA ACA GGC ACT AAG GGA GCC AAT GTA GAC; CM, 5'-TTA CCT CAG CCC ACT CTT CTG CAT GCA GCA. Mouse use was approved by the University of Michigan Committee on Use and Care of Animals.

Tissue Morphology and Immunohistochemistry

Intestines were dissected from potential E18.5 Vil-Math1 transgenic founders, and the proximal region (one cm distal to the pylorus), distal region (one cm distal to the midpoint), and colon (one cm distal to the cecum) were paraffin embedded after fixing overnight in 4% paraformaldehyde. Adjoining intestinal segments were processed for RNA. Sections (5 μm) were stained with H&E to assess cellular morphology and Periodic-acid Schiff (PAS)/Alcian blue (Newcomer Supply) to visualize mucin-containing goblet cells. Staining to visualize enterocytes was performed with the Alkaline Phosphatase Substrate Kit I (Vector Laboratories). For immunostaining, the following primary antibodies

were used: rabbit anti-chromogranin A (1:500; 94188/5 gift from J. F. Rehfeld), rabbit anti-Muc2 (1:500; Santa Cruz), rabbit anti-Ki67 (1:500; Novacastra), mouse anti-a SMA conjugated to Cy-3 (1:500; Sigma), rabbit anti-desmin (1:500; Abcam), and rabbit anti-neurofilament (1:500; Zymed), followed by appropriate secondary antibodies conjugated to Cy2 or Cy3 (1:400; Jackson ImmunoResearch Laboratories), AlexaFluor488 (1:500; Invitrogen) or biotin (1:200; Vector Laboratories), as described.[21] Co-staining for desmin and Ki-67 (1:100; BD Pharmigen mouse monoclonal) used Trilogy antigen retrieval (Cell Marque). Staining for Neurog3 (1:4000, F25A1B3 concentrated mouse monoclonal, Developmental Studies Hybridoma Band, University of Iowa) used tyromide signal amplification (TSA kit #2, Molecular Probes-Invitrogen), as previously described.[17] TUNEL staining was performed as previously described.[22] Microscopy was performed with either a Nikon E800 or Olympus BX-51 equipped with a SPOT or Olympus DP70 digital camera, respectively, or a Zeiss LSM 510 confocal microscope.

Quantitation of mRNA Abundance

Gene expression was measured by quantitative reverse transcription-PCR (qRT-PCR) analysis of transgenic (Tg) founders and nontransgenic (Ntg) littermate controls using the distal intestinal segment unless otherwise noted. RNA was isolated, DNase-treated and purified using the RNeasy Mini kit (Qiagen). RT reactions (50 μl) used 1 μg RNA and the Iscript cDNA synthesis kit (Bio-Rad), as recommended by the manufacturer. qRT-PCR was performed as described[23] with SYBR green dye and the primers listed in Table 2-1. Expression levels were determined for individual embryos with triplicate assays per sample and normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (Gapdh), which remained the same in Tg and Ntg controls.

Morphometric Analysis

Table 2-1. Oligonucleotide Primers for Quantitative Reverse Transcription Polymerase Chain Reaction.

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Amplicon (bp)
Cryptdin*	AGGAGCAGCCAGGAGAAG	ATGTTCAGCGACAGCAGAG	192
CCK	TACATCCAGCAGGTCCGCAAAG	CGATGGGTATTCGTAGTCCTCGG	153
Gapdh	TCAAGAAGGTGGTGAAGCAGG	TATTATGGGGGTCTGGGATGG	350
Glucagon	CGCTGATGGCTCCTTCTCTGAC	CAAGTGACTGGCACGAGATGTTG	156
Hes1	GCTCACTTCGGACTCCATGTG	GCTAGGGACTTTACGGGTAGCA	144
Lactase	CAGCGATGCCCACAGGAAAG	ACGGAGCCCTTGACGAGAG	144
Math1	GCCTTGCCGGACTCGCTTCTC	TCTGTGCCATCATCGCTGTTAGGG	116
Muc2	AGAACGATGCCTACACCAAG	CATTGAAGTCCCCGCAGAG	132
Neurog3	ACCCTATCCACTGCTGCTTGTC	CGGGAAAAGGTTGTTGTGTCTCTG	136
SM22α	CTCTAATGGCTTTGGGCAGTTTGG	GCTCCTGGGCTTTCTTCATAAACC	90
Vimentin	ACTGCTGCCCTGCGTGATGTG	GGTACTCGTTTGACTCCTGCTTGG	163

* Amplifies Cryptdins 1, 3, 6-12, 14, 15

Image J (1.34s by Wayne Rasband, NIH, USA, http://rsb.info.nih.gov/ij/) was used to calculate epithelial or total tissue area from 10-20 contiguous field views of an intestinal region (representing the whole tissue segment), except for goblet cell analysis which included 3 contiguous field views. For specific epithelial cell populations, the number of positive cells was counted and data were expressed as number of positive cells/area of epithelium (μm^2). To determine proportional tissue areas, the measured epithelial area (μm^2) was divided by the measured total tissue area (μm^2) to calculate the epithelial component, while the remainder was attributed to mesenchyme.

Statistics

Quantitative data were presented as mean ± SEM and analyzed by ANOVA followed by a Dunnett post test to compare data from each Vil-Math1 Tg founder to Ntg littermate controls. $P < 0.05$ was considered significant.

RESULTS

Increased Secretory Cell Development in Vil-Math1 Transgenics

To test the relationship between Math1 and secretory cell differentiation, we used the mouse villin promoter[20] to target Math1 expression in transgenic mice to all intestinal epithelial cells, including stem and progenitor cells (Fig. 2-1). Stable Vil-Math1 transgenic lines were unable to be generated due to the lack of recovery of founder mice with effective transgene expression. Thus we analyzed prenatal transgenic founders to avoid the lethality resulting from the dramatic cellular changes induced by Math1. Interestingly, the intestines of Vil-Math1 transgenics were distended, translucent and fluid-filled (Fig. 2-1B), possibly due to decreased fluid absorption or increased secretion resulting from the epithelial cell remodeling caused by Math1 expression (described below). Total Math1 mRNA was measured in proximal small intestine, distal small intestine, and colon

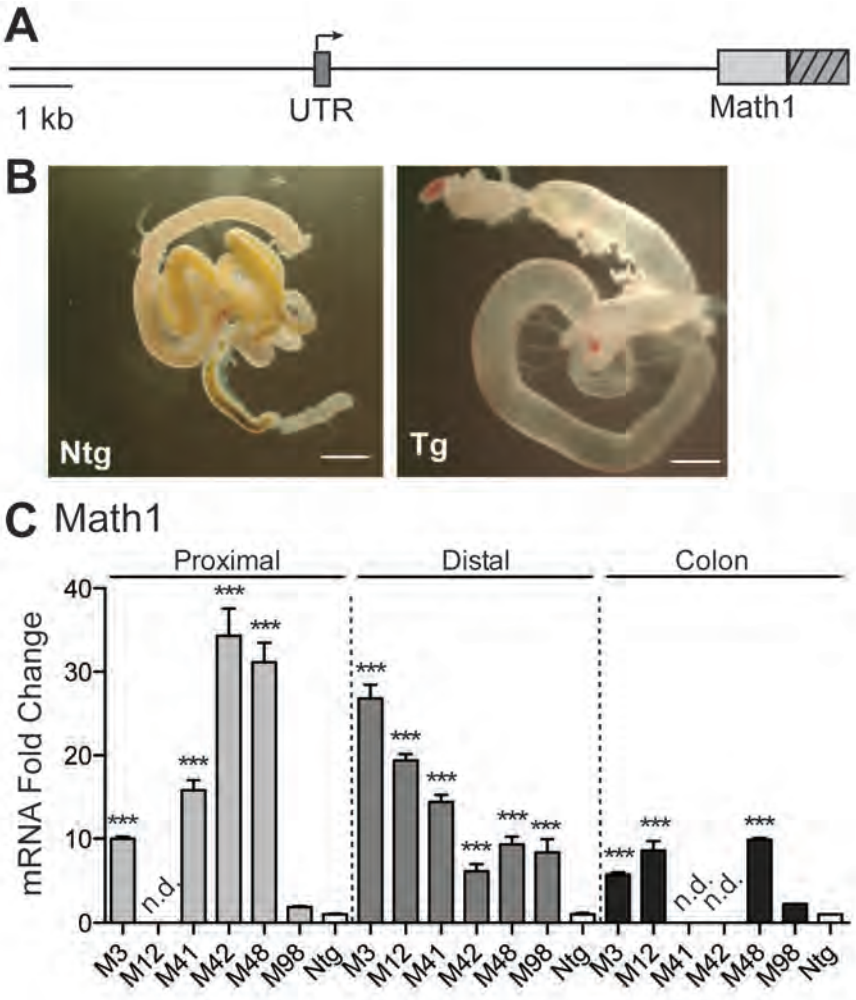

Figure 2-1. Abnormal Intestinal Morphology in Vil-Math1 E18.5 Transgenic mice. (A) The Vil-Math1 transgene contained mouse villin sequences, including 5' flanking sequence, the first untranslated exon (UTR) and intron, to regulate expression of the mouse Math1 cDNA, and SV40 sequences (hatched) to provide a polyA site. (B) Transgenic (Tg) intestines were fluid-filled and translucent (observed in M12, M41, and M48) compared to Nontransgenic (Ntg) controls. Bars: 2 mm. (C) Total Math1 mRNA (transgene plus endogenous) was measured by quantitative reverse transcriptase-PCR (qRT-PCR) analysis of proximal intestine, distal intestine, and colon RNA. Six Vil-Math1 founders exhibited significantly higher Math1 mRNA levels in at least one region. Values were normalized to Gapdh expression and reported as fold-change relative to the corresponding region of Ntg littermate controls (***$p<0.001$). n.d., not determined.

intestinal segments and six Vil-Math1 founder embryos with increased Math1 were analyzed. Increased Math1 mRNA was generally observed throughout the gut of Vil-Math1 mice with increases up to 34-fold in the proximal intestine, 27-fold in the distal intestine, and 10-fold in the colon (Fig. 2-1C).

Histological analysis demonstrated a complex, disorganized morphology in transgenic intestine with stunted villi, hypocellular epithelium and expanded mesenchyme (Fig. 2-2). Analysis of the cellular changes induced by Math1 included three separate regions, proximal and distal small intestine, and colon, to account for the normal regional variation in secretory cell numbers along the anterior-posterior gut axis. Analysis of Ntg controls demonstrated that Math1 expression increases in the posterior intestine in strong correspondence to goblet cell numbers (Supplementary Fig. 2-1). Thus, analysis of Tg phenotypes strictly compared outcomes with the corresponding region in Ntg controls. H&E staining showed increased numbers of goblet-like cells throughout the gut, which was confirmed by staining with PAS/Alcian blue (Fig. 2-2). The epithelium of some Vil-Math1 transgenics appeared to be almost completely transformed to the goblet cell lineage (eg. Fig. 2-2F, J). Morphometric analysis demonstrated significant increases in goblet cell number along the entire length of the small intestine and colon (Fig. 2-2G, K, O). For example, Tg M12 exhibited greater than 10-fold increases in goblet cell numbers in both proximal and distal small intestine, and a smaller, yet significant, increase in colon.

Next we examined endocrine cells, another cell type belonging to the intestinal secretory lineage. Similar to the goblet cell findings, a general expansion of endocrine cells was observed in all three regions of the Vil-Math1 intestine. Immunostaining for the pan-endocrine marker chromogranin A (CgA) revealed 2- to 8-fold increased endocrine cell numbers in Vil-Math1 transgenics compared to Ntg littermates (Fig. 2-3A-D). Normally, endocrine cells are distributed in the epithelium as single, scattered cells surrounded by enterocytes. In contrast, some CgA-positive cells were found in juxtaposition in the transgenics (Fig. 2-3C, inset). Goblet cell juxtaposition was also commonly observed in the transgenic intestine (eg. Fig. 2-2F, J). The observation of

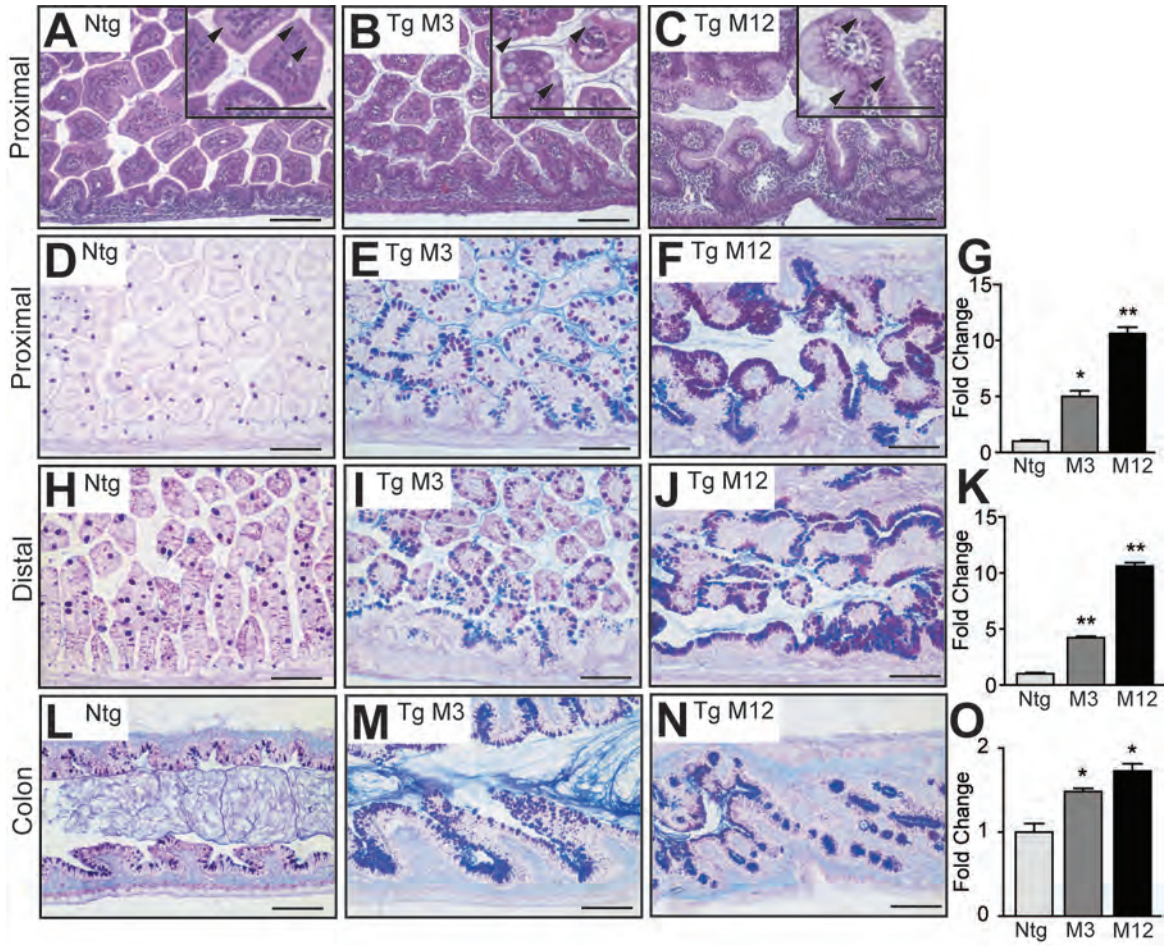

Figure 2-2. Math1 Promotes the Development of Goblet Cells Throughout the Intestine. Histological analysis of Ntg (A, D, H, L) and Tg (B, C, E, F, I, J, M, N) intestines from E18.5 founders. Transgenics M3 and M12 are shown to demonstrate the range in phenotypes. (A-C) H&E staining of proximal intestine showed increased numbers of goblet-like cells in Tg mice, apparent in the higher magnification insets (arrowheads), as well as mesenchymal tissue expansion. (D-N) PAS/Alcian blue staining demonstrated increased goblet cells in Tg mice in proximal intestine (D-F), distal intestine (H-J) and colon (L-N). (G, K, O) Quantification of PAS/Alcian blue-positive cells showed increased goblet cell number throughout the intestine of Tg founders compared to Ntg controls. Goblet cell numbers were normalized to epithelial area (μm^2) and reported as fold change relative to Ntg (*$p < 0.05$; **$p<0.01$). Bars: 100 μm.

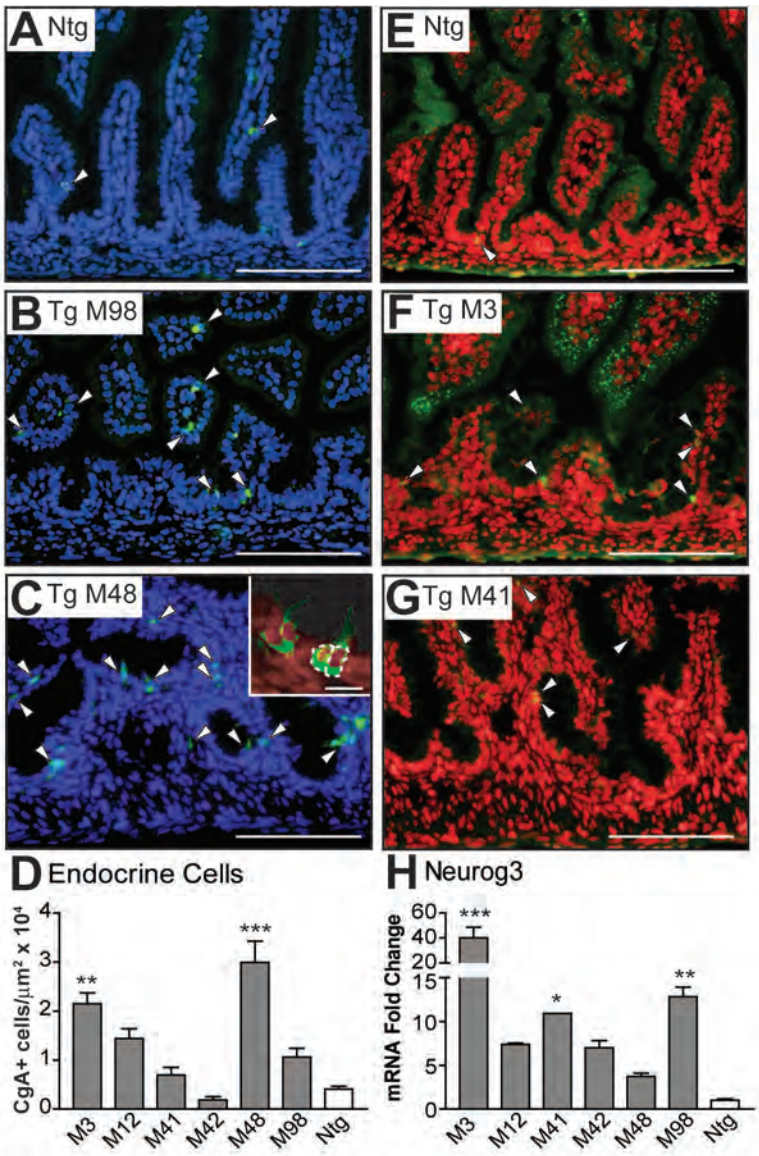

Figure 2-3. Math1 Stimulates the Endocrine Cell Differentiation Program. (A-C) Immunostaining for chromogranin A (CgA; green) showed increased endocrine cell numbers (arrowheads) in proximal Tg intestines, which was associated with loss of lateral inhibition (C, inset shows confocal image of 2 cell doublets with one doublet outlined). (D) Quantification of endocrine cells from CgA-stained sections. Values were normalized to epithelial area (μm^2) and statistical significance was determined by comparison to Ntg. (E-G) Immunostaining proximal intestines for Neurogenin 3 (Neurog3; green nuclear stain) demonstrated increased numbers of Neurog3-positive cells (arrowheads) in Vil-Math1 transgenics, which were often mislocated outside of the intervillus zone. DAPI (blue/red) nuclear stain. Bars: 100 μm; 10 μm (inset). (H) qRT-PCR analysis of Neurog3 mRNA abundance in distal intestine. Values were normalized to Gapdh expression and reported as fold-change relative to Ntg (*p<0.05; **p<0.01; ***p<0.001).

enteroendocrine and goblet cells clustering in the Vil-Math transgenic mice suggested that increased Math1 expression disturbed the process of epithelial cell distribution, which is thought to be orchestrated through lateral inhibition regulated by Notch signaling.[24, 25]

The endocrine cell expansion encompassed both mature endocrine cells and endocrine progenitor cells, which are marked by the transcription factor Neurog3.[16, 17, 26, 27] In Ntg controls, Neurog3-positive cells were observed as very rare single cells located in the intervillus progenitor cell zone (Fig. 2-3E). Increased numbers of Neurog3-positive cells were observed in Vil-Math1 transgenic founders and these cells were often observed on the villus, displaced from their normal location (Fig. 2-3F-G). Correlating with the increased numbers of Neurog3-positive cells, Neurog3 mRNA levels were increased up to 40-fold in Vil-Math1 Tg founders (Fig. 2-3H). Tg M3, M41, and M98, which exhibited the greatest increase in Neurog3 mRNA levels, also had the highest number of Neurog3-positive staining cells (data not shown).

Co-immunostaining for the endocrine hormone products cholecystokinin (CCK) and serotonin showed increased numbers of cells expressing a single hormone product (a pattern characteristic of mature endocrine cells) and did not reveal cells expressing multiple hormones (data not shown). Analysis of CCK and glucagon mRNA abundance showed that hormone expression was increased in all three regions of the Vil-Math1 intestines (Supplementary Fig. 2-2). Furthermore, expression patterns for these two hormones generally followed the normal regional patterning, with greater expression observed in small intestine than in colon. These data suggest that forced Math1 expression in the developing intestinal epithelium increased the formation of Neurog3-positive endocrine progenitor cells that further differentiated to form expanded numbers of mature endocrine cells. Moreover, the regulatory network that influences differentiation of endocrine cells to specific hormone-producing subtypes was conserved.

Paneth cells, the third secretory cell type, are not mature at E18.5; however, expression of Paneth cell anti-microbial genes is observed in the

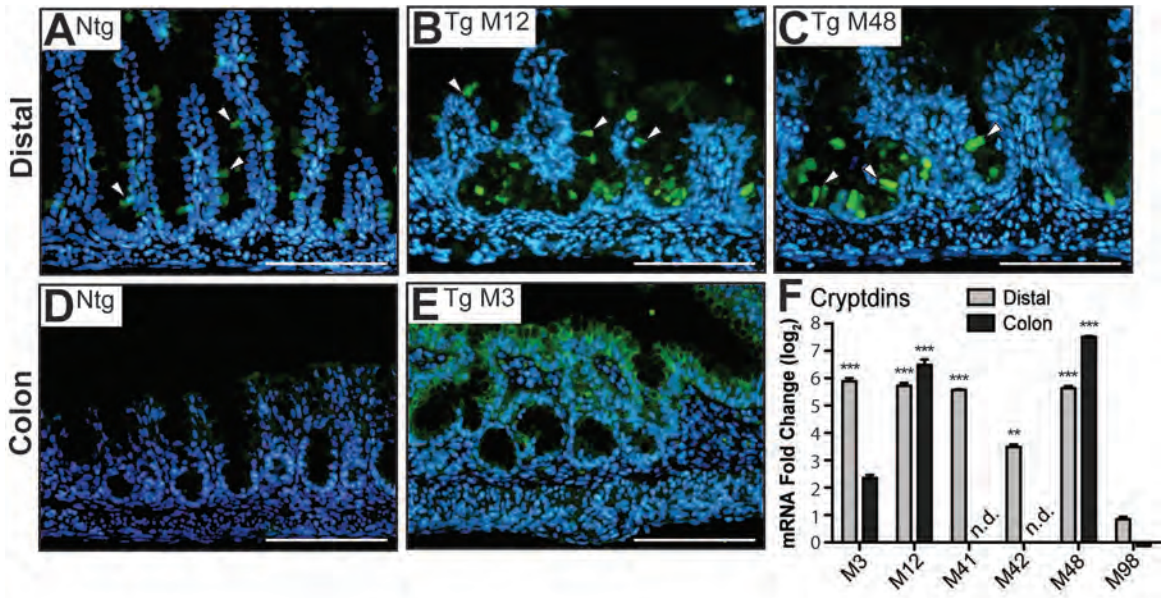

Figure 2-4. Emergence of a Paneth-like Cell in the Intervillus zones and Colons of Vil-Math1 transgenic Intestine. (A-E) Immunostaining for lysozyme (green) showed faintly-staining cells scattered in Ntg distal intestine (A) and no positive-staining in the Ntg colon (D). Tg founders had increased numbers of lysozyme-positive cells predominantly in the intervillus zone of the distal intestine Arrowheads denote some lysozyme-positive cells (B, C). Lysozyme-positive cells were also apparent in the Tg colon (E). DAPI (blue) nuclear stain. Bars: 100 μm. (F) qRT-PCR analysis of cryptdin mRNA abundance in distal intestine and colon. Values were normalized to Gapdh expression and reported as fold-change relative to Ntg (**p<0.01; ***p<0.001).

prenatal intestine. Staining distal intestine from control mice for the Paneth cell markers lysozyme (Fig. 2-4A) and MMP7 (not shown) showed weakly immunopositive cells scattered on the villus epithelium. In contrast, Vil-Math1 transgenic founders had increased numbers of intensely-stained cells, which were commonly found in or near the intervillus zone. In accordance with the expansion of Paneth-like cells, cryptdin mRNA levels were increased in the distal intestines of Vil-Math1 transgenics, with levels up to 80-fold greater than in Ntg controls (Fig. 2-4F). Unexpectedly, lysozyme staining, which is never observed in the Ntg colon, was widespread in Vil-Math1 Tg colon together with increased levels of colonic cryptdin mRNA (Fig. 2-4D-F), suggesting that increased Math1 expression in the colon shifted the normal pattern of secretory cell differentiation to include Paneth cell genes. Together, these findings demonstrate that Math1 is sufficient to trigger the program of intestinal differentiation to goblet, endocrine and Paneth cells throughout the intestine and colon.

Loss of Enterocytes in Vil-Math1 Transgenics

We tested the absorptive cell lineage in the Vil-Math1 transgenics to determine if the increase in secretory cells was associated with a corresponding reduction in enterocyte differentiation. A striking decrease in enterocytes was demonstrated in both proximal and distal small intestine by staining for the brush border enzyme alkaline phosphatase (Fig. 2-5). The Vil-Math1 transgenics exhibited decreased intensity of staining with an almost complete loss of staining in the most severely affected transgenics. Transgenics with the greatest increase in secretory cell number exhibited the most dramatic loss of enterocytes. Thus, excessive differentiation of the secretory lineage occurs to the detriment of the absorptive lineage in Vil-Math1 mice. Analysis of the enterocyte brush border enzyme lactase showed a dramatic reduction in mRNA abundance, with greater than 2000-fold decreases, consistent with the observed loss of enterocytes (Fig. 2-5G). Similar reductions in mRNA abundance were seen for the enterocyte marker intestinal fatty acid binding protein (data not shown). These results

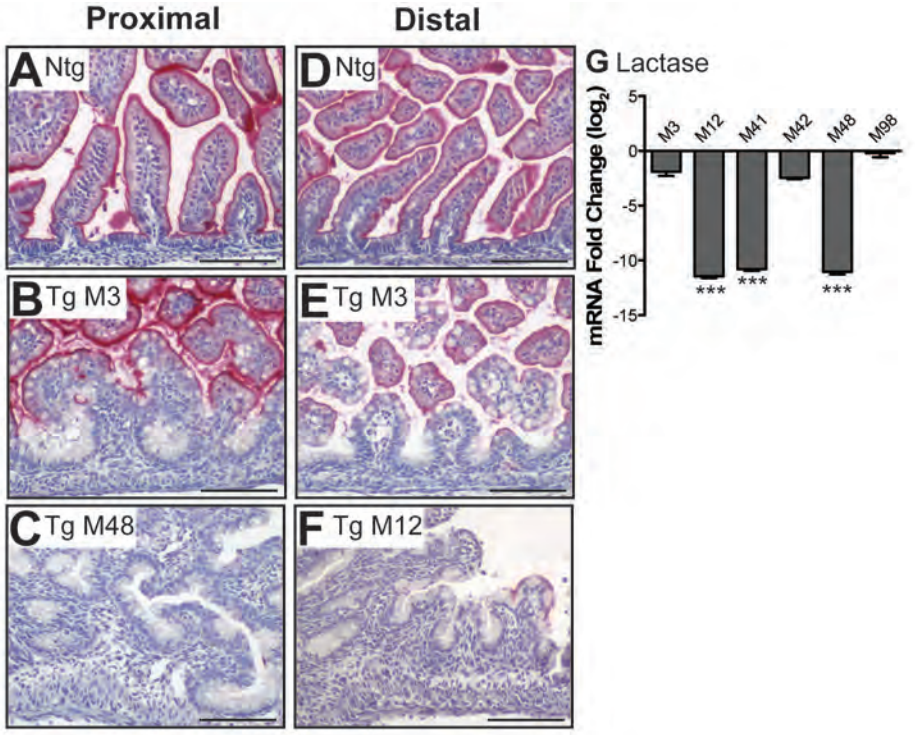

Figure 2-5. Loss of the Absorptive Lineage in E18.5 Vil-Math1 Transgenics. Ntg (A, D) and Tg (B, C, E, F) proximal (A-C) and distal (D-F) intestine sections were stained for the enterocyte brush border enzyme alkaline phosphatase (red) and counterstained with hematoxylin (blue). Bars: 100 μm. (D) Lactase mRNA abundance was measured by qRT-PCR analysis of distal intestine. Values were normalized to Gapdh expression and reported as fold-change relative to Ntg (***$p<0.001$).

suggest that Math1 is capable of directing multipotential progenitor cells to adopt the secretory program of differentiation and not absorptive cell fate.

Reduced and Displaced Epithelial Cell Proliferation in Vil-Math1 Transgenics

The epithelium in Vil-Math1 transgenics comprised a significantly smaller proportion of the intestinal tissue area than that of nontransgenics, consistent with the hypocellular appearance of the epithelium (Fig. 2-2 and Supplementary Fig. 2-3). This phenotype led us to examine whether Vil-Math1 transgenics had alterations in cellular proliferation or apoptosis. Analysis of apoptosis by TUNEL staining showed no change in apoptotic rate (data not shown). However, striking changes in cellular proliferation were observed. Normally, proliferating cells are restricted to the intervillus zone of the E18.5 intestinal epithelium as well as scattered cells in the mesenchyme, as shown by immunostaining for the Ki67 antigen (Fig. 2-6A). In contrast, epithelial proliferation in Vil-Math1 transgenics was displaced and significantly decreased (Fig. 2-6B-D). An overall 2-fold reduction in epithelial proliferation in Vil-Math1 transgenic mice was shown by morphometric analysis of Ki67-positive cells (Fig. 2-6E). All 6 transgenic founders exhibited displacement of Ki67-positive cells from the intervillus zone to the villus (Fig. 2-6B-D and data not shown) suggesting that the progenitor cell “niche” was displaced towards the villus tips. Our finding that Neurog3-positive cells were also displaced to the villus, strengthens this conclusion. Moreover, in contrast to Ntg controls, Vil-Math1 transgenics had many cells in the intervillus zone that stained with PAS/Alcian blue or lysozyme suggesting that differentiated cells occupied the intervillus zone (Fig. 2-2 and 2-4).

Mesenchymal Remodeling in Vil-Math1 Transgenics

Signaling between the intestinal epithelium and mesenchyme is thought to play a critical role in the regulation of intestinal differentiation and proliferation.[28] Although Math1 transgene expression was limited to the epithelium, we observed marked changes in the mesenchyme. In contrast to the overall reduction in epithelial cell proliferation, there was a robust increase in Ki67-positive cells in

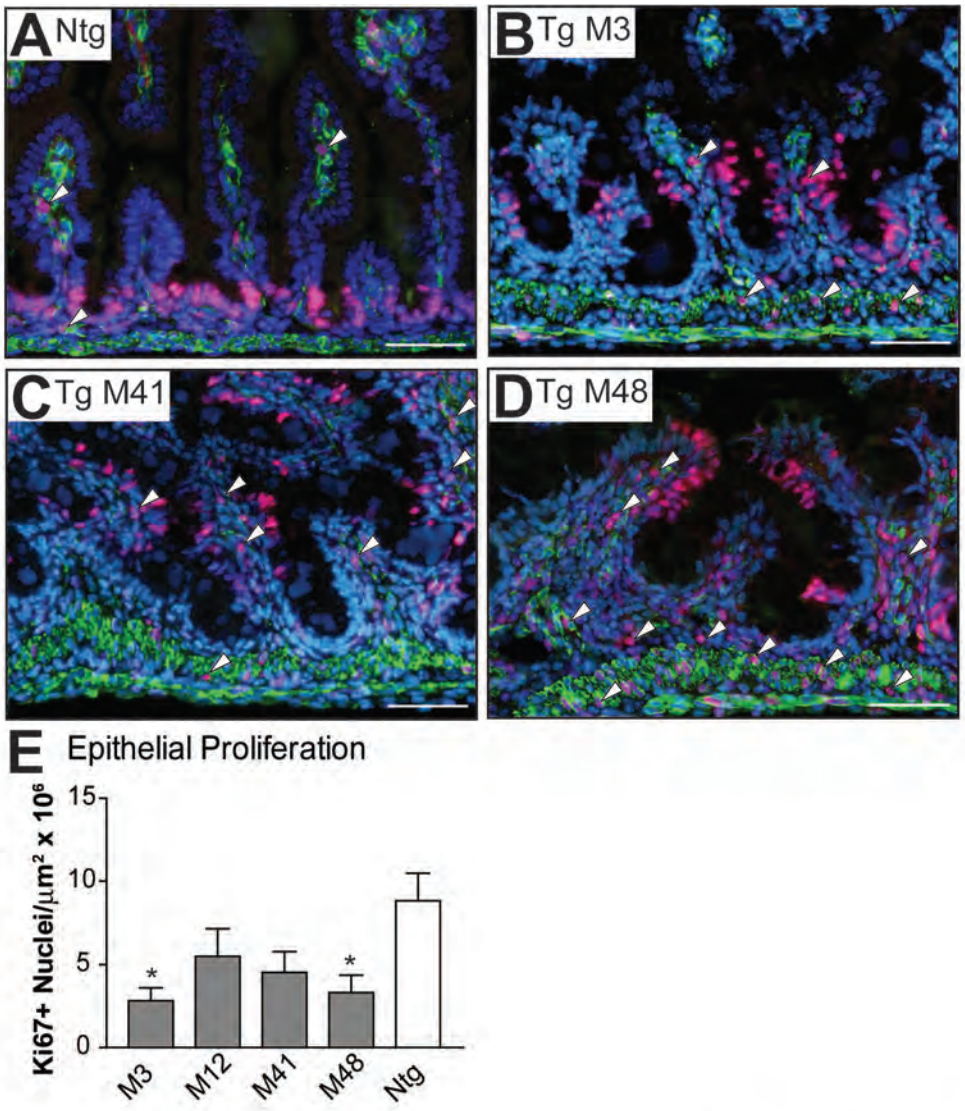

Figure 2-6. Altered Epithelial and Mesenchymal Cell Proliferation in E18.5 Vil-Math1 Intestine. (A-D) Proliferation was assessed in Ntg (A) and Tg (B-D) proximal intestine by Ki67 immunostaining (red) co-immunostained with desmin (green) to mark mesenchymal cells and DAPI (blue) nuclear stain. Arrowheads identify some proliferating cells in the mesenchyme. Bars: 50 μm. (E) Quantification of epithelial cell proliferation by morphometric analysis of Ki67 immunostaining of proximal intestine. Data are presented as Ki67-positive nuclei per epithelial area (μm^2) (*$p<0.05$ compared to Ntg).

the mesenchyme of Vil-Math1 transgenics (Fig. 2-6A-D, arrowheads), accounting for the thickened mesenchyme layer seen in H&E stained sections (Fig. 2-2). Mesenchymal expansion was observed in the villus core regions as well as the submucosa (Fig. 2-6 and data not shown).

The mesenchymal expansion was substantiated by increased expression of the general mesenchyme marker vimentin, with 2 to 4-fold increased expression (Fig. 2-7A). The cellular basis for the expansion was determined by immunohistochemical analysis. Co-staining with antibodies to α-smooth muscle actin (SMA) and desmin enabled identification of three different mesenchymal cell types: myofibroblasts (SMA-positive), myoblasts (desmin-positive) and differentiated smooth muscle (SMA- and desmin-positive) (Fig. 2-7C-E). Vil-Math1 transgenics exhibited an expansion of both SMA-positive myofibroblasts and desmin-positive smooth muscle precursors. In addition, there was a substantial increase in double-stained, differentiated smooth muscle. The myocyte expansion was verified by analysis of SM22α, a differentiated smooth muscle marker (Fig. 2-7B). SM22α expression was increased as much as 3.7-fold in Vil-Math1 transgenics, similar to the overall expansion in mesenchyme shown by increased vimentin expression (Fig. 7A, B). Immunostaining for the neuronal marker neurofilament demonstrated that enteric neurons were also expanded in the transgenic mesenchyme (Fig. 2-7F-H). Although Vil-Math1 transgenics exhibited increased numbers of numerous mesenchymal cell types, the basic radial patterning was maintained, with myofibroblasts more closely associated with the epithelium and enteric neurons situated between the circular and longitudinal smooth muscle. These data suggested that the epithelial cell changes induced by forced Math1 expression altered signaling to the mesenchyme to affect proliferation and differentiation, with resulting expansion of several mature cell types.

DISCUSSION

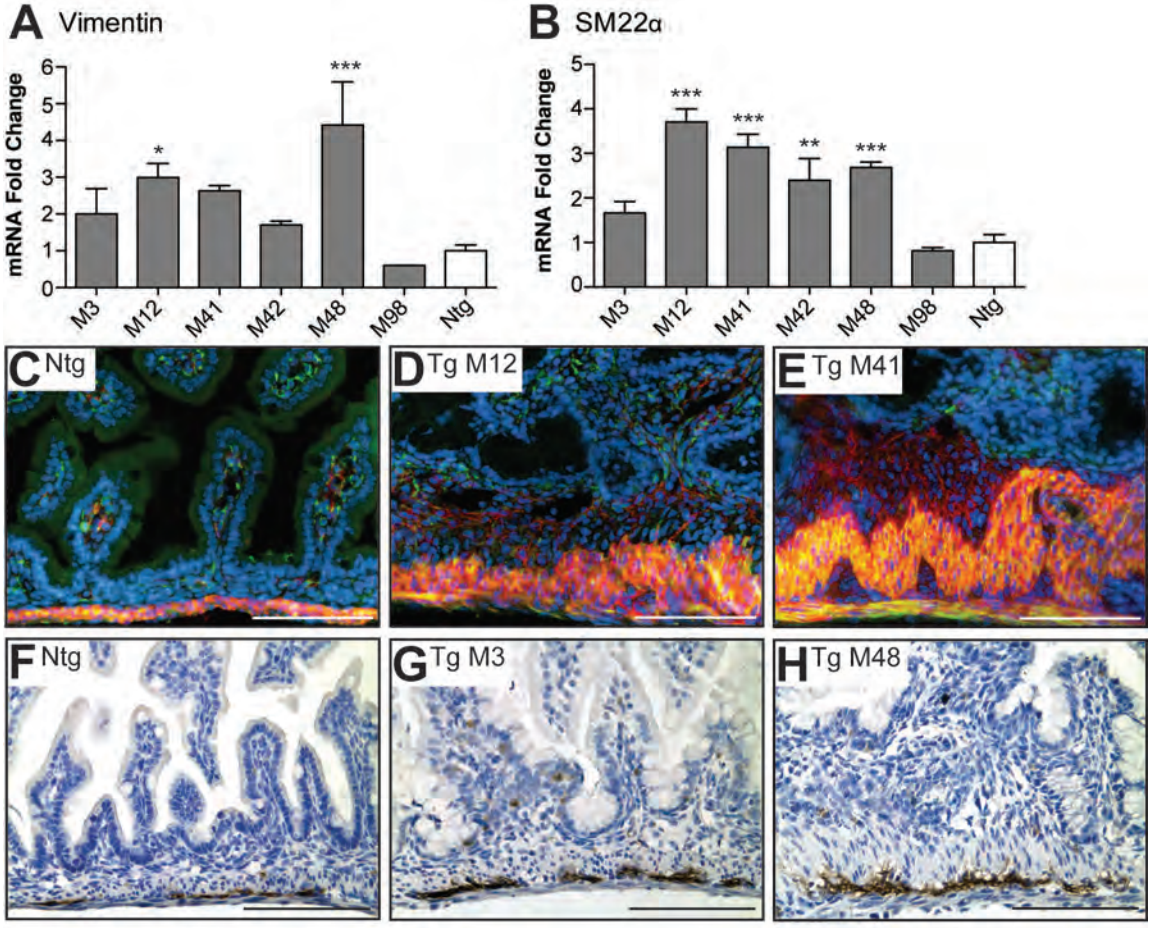

Figure 2-7. Remodeled Mesenchyme in E18.5 Vil-Math1 Intestine. (A, B) qRT-PCR analysis of vimentin (A) and SM22α (B) mRNA abundance was performed with distal small intestine RNA. Values were normalized to Gapdh expression and reported as fold-change relative to Ntg (*p<0.05; **p<0.01; ***p<0.001). (C-E) Co-immunostaining of proximal intestine sections with α-smooth muscle actin (red) and desmin (green) with DAPI (blue) nuclear stain. Differentiated smooth muscle is yellow due to co-expression of these two markers. (F-G) Immunostaining for the neuronal marker neurofilament (brown), with hematoxylin counterstain (blue) shows expansion of the enteric nervous system in Vil-Math1 transgenics. Bars: 100 μm.

This study has established that Math1 is sufficient to induce the program of secretory cell development in the mouse intestine. Transgenic expression of Math1 resulted in almost complete transformation of the intestine into a secretory epithelium. The loss of enterocytes and expansion of the secretory cell lineage suggested that Math1 regulates the cell fate choice of a bipotential progenitor. The importance of Math1 for intestinal lineage determination had previously been suggested from the loss of secretory cell types in Math1-deficient mice.[14, 15] Since Math1 is expressed in mature secretory cells as well as in cells in the progenitor zone,[14, 18] the question remained whether its primary role was maintenance of the differentiated secretory cell phenotype or induction of differentiation. Our study showed that Math1 expression can override the normal developmental program to induce secretory cell differentiation, suggesting that a primary role of this transcription factor is determination of cell fate choice. Interestingly, expression of Math1 stimulated apparently normal terminal differentiation processes since we observed expression of differentiation markers specific for each secretory cell type, including Muc2 (goblet cells), hormones (enteroendocrine cells), and cryptdins (Paneth cells). Accordingly, the expression of transcription factors downstream of Math1 that are known to be important for specific secretory cell types were increased robustly in Vil-Math1 transgenic mice, including Neurog3 (Fig. 2-3)[16, 17] and Gfi1 (data not shown).[29]

Notch signaling has been shown to play a primary role in the regulation of cell lineage determination in the intestinal epithelium. Our study and others point to Notch regulation of Math1 transcription as the critical determinant of cell fate. Treatment with γ-secretase inhibitors to block Notch signaling has been shown to induce intestinal phenotypes similar to those evident in the Vil-Math1 transgenic mice.[5, 7, 30] Similarly, genetic models with disrupted Notch signaling exhibited a generalized secretory cell expansion together with loss of enterocytes.[7-9, 12] Math1 expression was increased in these models of Notch disruption, consistent with our finding that Math1 expression controls absorptive versus secretory cell fate. Accordingly, genetic models with increased Notch signaling exhibited decreased Math1 and loss of intestinal secretory cell types.[3, 4] To determine if

increased Math1 mRNA had an effect on the Notch signaling pathway in the Vil-Math1 transgenics, we analyzed expression of the Notch target gene Hes1. No change in Hes1 mRNA levels were observed in Vil-Math1 transgenic founders (Supplementary Fig. 2-4), suggesting that the epithelial changes that we observed in our studies were due to increased levels of Math1 and not due to inhibition of Notch signaling. Together these studies and our data point to Math1 as the key effector regulating the choice of secretory versus absorptive cell fate choice in the intestine.

Transgenic Math1 expression affected intestinal proliferation in complex ways. We observed an overall reduction in epithelial cell proliferation, which suggested that Math1 stimulated differentiation and withdrawal from the cell cycle. Loss of Notch signaling in the intestine, and thus increased Math1, had previously been shown to convert proliferating crypt progenitors into post-mitotic cells with derepression of the cyclin-dependent kinase inhibitors $p27^{Kip1}$ and $p57^{Kip2}$.[9] Increased cell-cycle inhibitor expression was proposed to result from loss of Hes1 repressor action on the promoters of these genes.[9] However, our observations suggest that increased Math1 may also contribute to the Notch affect on proliferation. Although Vil-Math1 transgenics exhibited decreased epithelial cell proliferation, intestinal Hes1 mRNA expression was not changed, suggesting that the reduced proliferation and increased differentiation of progenitors was likely due to changes in Math1 and not Hes1.

In addition to the profound changes to the epithelium, there were marked changes to the mesenchyme in Vil-Math1 transgenic mice, with a general cellular expansion and increased proliferation observed in both submucosa and villus cores. Since Math1 expression in our transgenic model was limited to epithelial cells, the mesenchymal changes were indirect, suggesting that the epithelial cell changes altered signaling to control the differentiation of the mesenchyme. The epithelial cell changes were multifaceted, thus signaling changes could result from changes to the intervillus progenitor zone, the loss of enterocytes, and/or expansion of secretory cell types. It is likely that multiple signaling pathways were affected by these cellular changes, including Wnt and Hedgehog.

In conclusion we have demonstrated that Math1 is a key transcriptional determinant of cell fate choice in the intestine. Proper Math1 levels are required for the normal patterning of epithelial cell proliferation as well as the distribution of secretory versus absorptive cells. The associated expansion of secretory cell types and loss of enterocytes observed when Math1 levels were increased suggests that this transcription factor directs the cell fate choice of a bipotential progenitor. Furthermore, the epithelial cell changes induced by increasing Math1 had consequences for the mesenchyme due to alterations in epithelial-mesenchymal crosstalk. Although radial patterning was essentially preserved, the mesenchyme of Vil-Math1 transgenic mice was grossly expanded, with increased proliferation and differentiation of several mesenchymal cell types. Thus, Math1 expression is critical for coordinated differentiation and morphogenesis of both epithelium and mesenchyme.

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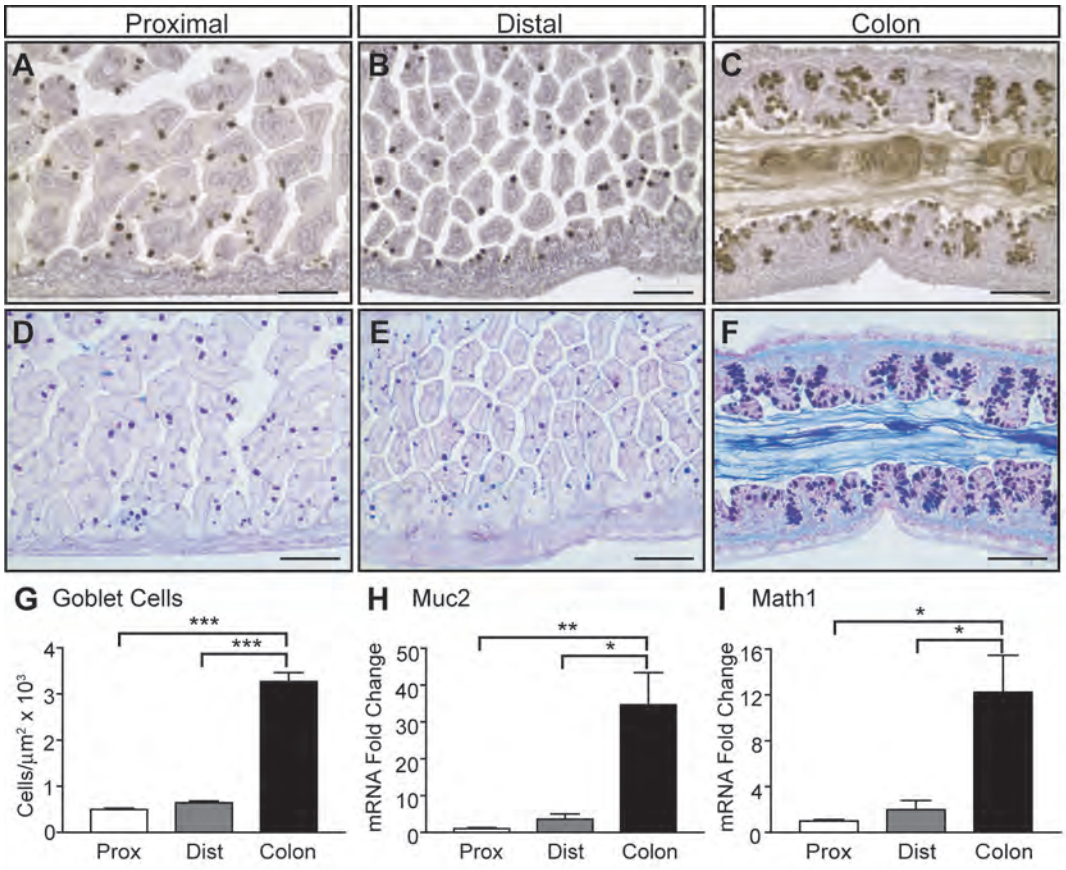
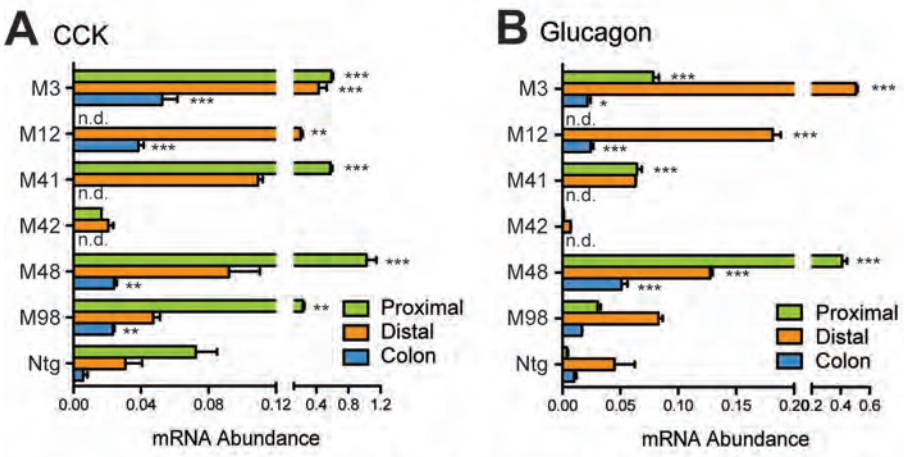
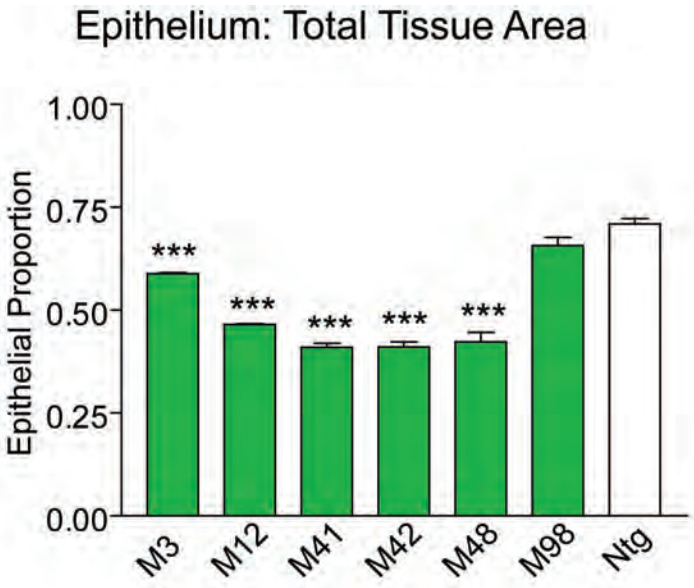


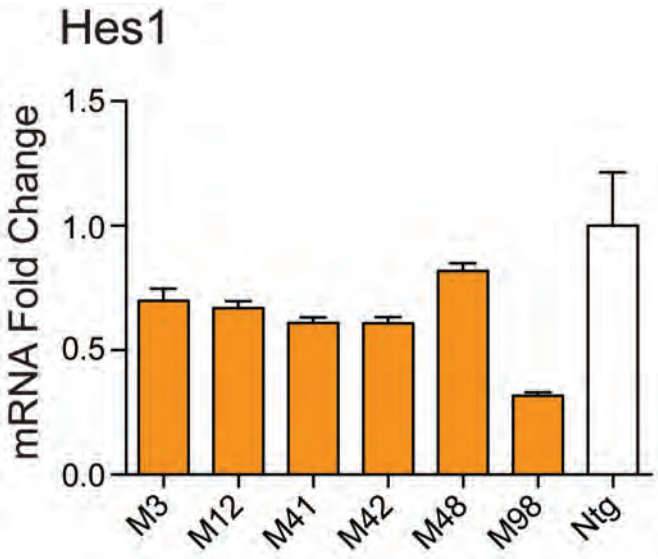
Supplementary Figure 2-1. Corresponding Pattern of Goblet Cell Number and Math1 Expression in the Mouse E18.5 Intestine. Goblet cells were detected in control mice by Muc2 immunostaining (A-C) and Periodic-acid Schiff (PAS)/Alcian blue staining (D-F) of paraffin sections from proximal (A, D) and distal (B, E) intestine and colon (C, F). (G) Quantification of PAS/Alcian blue-stained goblet cells per epithelial area (μ m^2) showed increased cell numbers in the posterior gut, with a 7-fold increase in goblet cell number in the colon compared to proximal intestine. (H,I) Quantitative reverse transcription- polymerase chain reaction (qRT-PCR) analysis of Muc2 (H) and Math1 (I) mRNA abundance along the anterior-posterior axis showed increased expression in the posterior gut, with Math1 mRNA abundance increased 12-fold in colon compared to proximal intestine. Values were normalized to Gapdh expression and reported as fold change relative to levels in the proximal intestine (Prox). Dist, distal intestine. Quantitative data for all figures are presented as means ± SEM with statistical signficance determined by 1-way ANOVA followed by a Tukey post test (*p<0.05; **p<0.001; ***p<0.0001). n=3 mice/region. Bars:100 μm.



Supplementary Figure 2-2. Increased Enteroendocrine Hormone Gene Expression in Vil-Math1 Transgenic Intestine. qRT-PCR analysis of (A) cholecystokinin (CCK) and (B) glucagon mRNA expression in proximal intestine (green bars), distal intestine (orange bars), and colon (blue bars) of transgenic founders and nontransgenic (Ntg) littermate controls. There was increased mRNA expression of both hormones in transgenic intestine with many transgenics retaining normal proximal-distal endocrine hormone distribution. For example, CCK is expressed the highest in proximal intestine of both Ntg and transgenics M3, M41, M48 and M98 with lowest expression seen in colon. In contrast, glucagon was expressed the highest in distal intestine of both Ntg and transgenics M3, M12 and M98. Values are presented as mean ± SEM. Statistical significance was determined by comparing expression levels in each transgenic founder region to the similar region in Ntg controls using 1-way ANOVA followed by a Dunnett post test (**p<0.001, ***p<0.0001).



Supplementary Figure 3. Decreased Epithelial Tissue Area in E18.5 Vil-Math1 Transgenics. Epithelial area and total tissue area were calculated from H&E-stained paraffin sections as described in Materials and Methods. Data are reported as the epithelial proportion of total tissue area (epithelial area (μm^2)/total area (μm^2)). The epithelium occupies ~70% of the total tissue area in nontransgenics (Ntg) while this was decreased to as little as 41% in Vil-Math1 transgenics. Values are presented as mean $\pm$ SEM (***p<0.0001 compared to Ntg).



Supplementary Figure 2-4. Normal Notch Signaling in Vil-Math1 Transgenics. qRT-PCR analysis of Hes1 mRNA abundance in distal intestine showed normal Hes1 expression levels in Vil-Math1 transgenics compared to Ntg littermate controls. Values were normalized to Gapdh expression and reported as fold change compared to Ntg. Values are presented as mean ± SEM. Statistical analysis performed using 1-way ANOVA followed by a Dunnett post test showed that Hes1 expression was not significantly changed in Vil-Math1 transgenic mice.

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CHAPTER 3

DEVELOPMENT OF AN INDUCIBLE TRANSGENIC MOUSE TO OVEREXPRESS MATH1 IN THE INTESTINAL EPITHELIUM

OVERVIEW

Differentiating intestinal progenitor cells face a binary switch in which they are selected to differentiate into an absorptive or secretory cell type. The cell fate selection process is regulated by Notch signaling primarily through control of Mouse atonal homolog 1 (Math1) transcription. Active Notch signaling represses Math1 expression through the Notch target hairy and enhancer of split 1 (Hes1). Math1 is required for intestinal secretory cell differentiation during development and adulthood. However, since Math1 continues to be expressed in mature secretory cells, it is unclear whether Math1 is needed to specify the secretory cell fate or to maintain it. Using a transgenic mouse (Vil-Math1) approach, I have shown that Math1 is sufficient to promote secretory cell differentiation during development. Embryonic Vil-Math1 transgenic founders had increased numbers of goblet, Paneth and endocrine cells throughout their intestines and a loss of absorptive cells suggesting that Math1 can drive differentiating progenitor cells to the secretory lineage at the expense of the absorptive lineage. Due to the dramatic remodeling of the intestinal epithelium in the Vil-Math1 transgenic founders, transgenic mouse lines could not be established. To circumvent this limitation, I developed a new transgenic model (VZM) utilizing the Cre-lox system in which the timing of intestine-specific Math1 over-expression can be controlled by activation of Cre-mediated recombination.

For Part A of this chapter, I analyzed the intestinal recombination pattern of five mouse lines that express Cre recombinase in the intestine in either a constituitive or tamoxifen-inducible manner. This analysis was used to determine which Cre line(s) would be best suited for recombination of the VZM transgene to activate Math1 expression. In Part B of this chapter, I characterized seven VZM lines that were generated and determined that lines VZM1 and VZM7 would be used for further experiments. VZM1 and VZM7 transgenics were crossed to 12.4kbVil-Cre, 9kbVil-Cre, and Shh-EGFP-Cre mice to induce recombination of the transgene. VZM1;12.4kbVil-Cre transgenic mice exhibited increased Math1 mRNA expression, but, surprisingly, Math1 protein was unchanged. Thus, secretory cell differentiation was not changed in this model and the proposed experiments could not be completed. Interestingly, this study suggests that Math1 protein is regulated by an unknown mechanism.

PART A: ANALYSIS OF CRE TRANGENIC MOUSE LINES WITH INTESTINAL EXPRESSION

INTRODUCTION

Complex interactions between the intestinal epithelium and mesenchyme are necessary for proper steady state and adaptive response mechanisms that maintain the epithelium throughout the lifetime of an organism. The pathways that govern intestinal development and maintenance such as Notch, Wnt, Hedgehog and BMP are fundamental for many bodily processes. To circumvent lethality, the ability to alter gene expression *in vivo* in an intestine-specific or cell type-specific manner is critical for studies in this field.

The Cre-lox system is a powerful tool that can be utilized in mouse genetic studies to target alterations in gene expression to a specific cell population. The bacterial Cre recombinase protein recognizes a specific 24-bp loxP DNA sequence. When two loxP sequences are present, Cre excises the "floxed" DNA sequence contained within the two sites[1]. Accordingly, Cre-lox technology has provided a means to conditionally delete specific transgene sequences *in vivo* to control gene expression. For example, a Cre transgenic line with an intestine-specific promoter is used in conjunction with a floxed sequence-containing transgenic line to study the effects of gene alteration in the intestine. This approach can be used to circumvent early embryonic lethality as well as to study specific cell populations. Therefore, the choice of Cre transgenic line is intimately related to the output of a transgenic mouse experiment.

The use of the villin promoter to drive Cre expression in transgenic mice has been instrumental for many intestinal studies; however, there are two reported villin promoters used for Cre transgenic lines and each has distinct advantages and disadvantages[2, 3]. In addition, other Cre transgenic lines have been developed as more widely expressed drivers and their intestinal expression

pattern has not yet been described. Thus, a direct comparison of these Cre transgenic lines will be beneficial for choosing the best strain for a particular study. Here we directly compare the intestinal recombination patterns of 5 Cre transgenic lines crossed to the Cre-inducible Rosa26 lacZ reporter mice[4] to aid in the choice of Cre transgenic line for intestinal studies: 12.4kbVil-Cre[3], 9kbVil-Cre[2], 9kbVil-CreER^{T2} [2], CAGG-CreER^{TM} [5], and Shh-EGFP-Cre[6]. In addition, we describe the stomach expression pattern of CAGG-CreER^{TM} and Shh-EGFP-Cre transgenic lines because very few Cre drivers have been identified for studies in the stomach.

MATERIALS AND METHODS

Mice

All experiments were performed according to protocols approved by the University of Michigan Committee on Use and Care of Animals. Mice were maintained in a specified-pathogen-free barrier facility under a 12-hour light cycle. Mice were generated as described previously: Rosa26-lacZ reporter mice (R26R)[4] (Jackson Laboratories; stock #003474), 12.4kbVil-Cre [3](Jackson Laboratories; stock #004586), 9kbVil-Cre[2] (gift from S. Robine), 9kbVil-CreER^{T2} [2] (gift from S. Robine), Shh-EGFP-Cre[6] (Jackson Laboratories; stock #005622), CAGG-CreER^{TM} [5] (Jackson Laboratories; stock #004682).

Administration of Tamoxifen

To make a 10 mg/mL tamoxifen solution, tamoxifen (Sigma) was first suspended in 500 μL mL of 100% ethanol and then added to 9.5 mL corn oil (Sigma) and vortexed until completely dissolved. For vehicle controls, 500 μL of ethanol was added to 9.5 mL corn oil. Both solutions were aliquoted and stored at -20°C in the dark. For adult mice, 4 mg tamoxifen per 40 g body weight (10 μL of 10 mg/mL tamoxifen solution per gram body weight) was injected i.p. daily for 5 days. Mice were sacrificed 5 days after the last injection. For fetal tissue

collection, timed pregnant females were injected at embryonic day 16.5 of their pregnancy with one i.p. injection of 4 mg tamoxifen per 40 g body weight and embryos were collected 48 hours later, at E18.5.

Collection of Tissues and Histological Analysis

Intestines were dissected from adult (1 month old) or fetal (embryonic day 18.5) Cre transgenic (Tg) or Cre Tg; $R26^{+/-}$ littermates. For adult intestine, regions were identified as follows: duodenum (1 cm segment distal to the pylorus), jejunum (1 cm segment at the midpoint), ileum (1 cm segment proximal to the cecum) and colon (1 cm segment distal to the cecum). For fetal intestine, regions were identified as proximal (1 cm segment distal to the pylorus), distal (1 cm distal to the midpoint) and colon (1 cm distal to the cecum). For adult stomach dissections, the stomach was opened along the greater curvature, pinned flat on dental wax. Tissues were prepared for cryosectioning by fixing in 4% paraformaldehyde for one hour and incubating overnight at 4°C in 30% sucrose solution in 1X PBS before embedding in OCT (TissueTek) and freezing on dry ice.

Cryosectioning and Staining for β-galactosidase

Tissue blocks were warmed to -20°C and 8 μM sections were generated on a Microm HM 500M cryostat. Slides were dried at room temperature for 5 minutes, fixed for 5 minutes in 4% paraformaldehyde and then washed 3 times in X-gal wash buffer (0.1 M Sodium Phosphate pH 7.3 containing 2 μM $MgCl_2$ and 0.02% NP-40). Slides were placed overnight in X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactosidase) staining solution (1 mg/mL X-gal in N,N-dimethylformamide, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$-$3H_2O$ in X-gal wash buffer) at 37°C and protected from light. Stained tissues were washed 3 times in X-gal wash buffer, counterstained with 0.5% neutral red (Sigma), and dehydrated with a series of ethanol washes followed by xylene washes. Slides were mounted with Permount. Microscopy was performed with either a Nikon E800 or Olympus BX-51 equipped with a SPOT or Olympus DP70 digital camera,

respectively. Negative controls including R26R transgenic intestine or Cre transgenic intestine did not have positive staining for β-galactosidase (data not shown).

RESULTS AND DISCUSSION

The 12.4kbVil-Cre and 9kbVil-Cre Constitutive Drivers

The villin promoter has been used extensively for transgenic mouse studies in the intestine. The endogenous villin gene is expressed in the yolk sac, the proximal tubules of the kidney, and in the intestinal epithelium[7-9]. Additionally, the villin promoter has been reported to be active in a rare subpopulation of cells in the gastric antrum[10]. Two villin promoters that can be used to direct transgene or Cre expression have been described, one containing 12.4kb[3] and the other containing 9kb[2] of mouse villin sequence. We crossed the 12.4kbVil-Cre or the 9kbVil-Cre transgenics with the Rosa26-lacZ (R26R) reporter mouse[4] to induce β-galactosidase expression in cells that expressed Cre recombinase and their progeny (Fig. 3-1).

We observed homogenous β-galactosidase expression throughout the epithelium of the small intestine of adult Vil-Cre;R26R transgenics with positively-labeled cells throughout the crypt-villus axis (Fig. 3-1A-C, E, F). Each of these Cre transgenics appeared to have a similar expression pattern in the small intestine; however, the colonic β-galactosidase expression pattern was very different. The 12.4kbVil-Cre;R26R was expressed in a mosaic pattern in the colon while the 9kbVil-Cre;R26R had strong, homogenous staining throughout the colonic epithelium (Fig. 3-1D, G). The patchy staining suggests that the 12.4kbVil-Cre transgene was expressed in a mosaic fashion in the colonic stem cells. The 12.4kbVil-Cre promoter has been reported to be expressed most highly in the proximal intestine with lower expression in the ileum and colon[3], consistent with our observed results. In addition, staining for β-galactosidase was slightly less intense in the more undifferentiated cells of the crypts compared

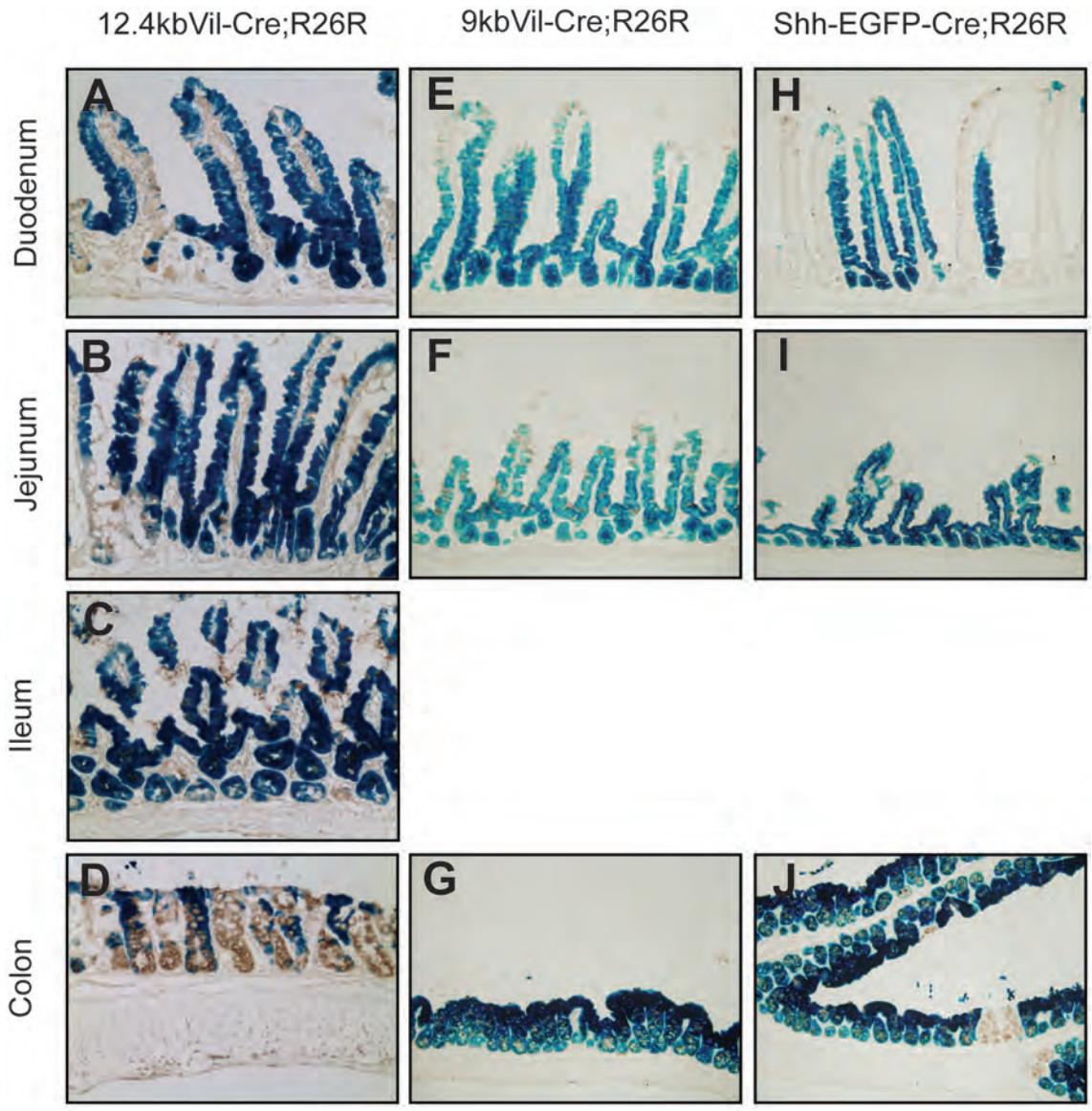

Figure 3-1. Intestinal Recombination of Constitutive Cre Transgenic Lines. X-gal staining of 12.4Vil-Cre;R26R transgenics (A-D), 9kbVil-Cre;R26R transgenics (E-G), and Shh-EGFP-Cre (H-J) in the duodenum (A, E, H), jejunum (B, F, I), ileum (C), and proximal colon (D, G, J) of adult mice.

to the mature villus cells, also consistent with previous reports[3, 9] (Fig. 3-1, see also VZM transgene expression in Fig. 3-5). The 9kbVil-Cre;R26R was previously reported to have strong β-galactosidase staining in the ileum and colon; however, Western blot for the Cre protein showed Cre expressed in a gradient with highest expression in proximal intestine and low expression in the ileum and colon[2]. Thus, it may be that β-galactosidase expression and Cre protein expression are not always coupled in this transgenic line. Of note, however, it is thought that very little Cre protein expression is necessary to facilitate loxP site recombination[1].

β-galactosidase staining in the kidney was not assessed in the Vil-Cre models in this study, the endogenous villin gene is known to be expressed in this tissue[9]. Previous studies of the 12.4kbVil-Cre transgene promoter was never found to direct β-galactosidase expression in the kidney[3]. In contrast, the 9kbVil-Cre;R26R transgenic had strong expression in the kidney[2]. Kidney phenotypes have been observed in studies using the 9kbVil-Cre transgenic and, in fact, it has been used to alter gene expression in the kidney[11], suggesting that while Cre protein expression may be minimal, the Cre protein levels in the kidney are capable of initiating transgene recombination. Thus, it is important to consider whether recombination of a particular floxed DNA sequence using the 9kbVil-Cre transgenic will cause a deleterious side-affect in the kidney.

The recombination pattern of 12.4kbVil-Cre;R26R and 9kbVil-Cre;R26R mice were also examined at E18.5, during late fetal development (Figure 3-2). The 12.4kbVil-Cre line has been reported to have onset of expression at ~E12.5 while the 9kbVil-Cre appears to activate at least 2 days earlier, at ~E10.5[2, 3]. Thus, both Vil-Cre transgenics are expressed prior to the major morphogenesis events of the intestine. Similar to the findings in adult mice, the 12.4kbVil-Cre showed a gradient of β-galactosidase, staining with the strongest staining in the proximal intestine and diminished staining in the distal small intestine and in the colon (Fig. 3-2A-C). The 9kbVil-Cre showed strongest β-galactosidase staining in the colon, similar to its adult staining pattern (Fig. 3-2F). Neither of the Cre

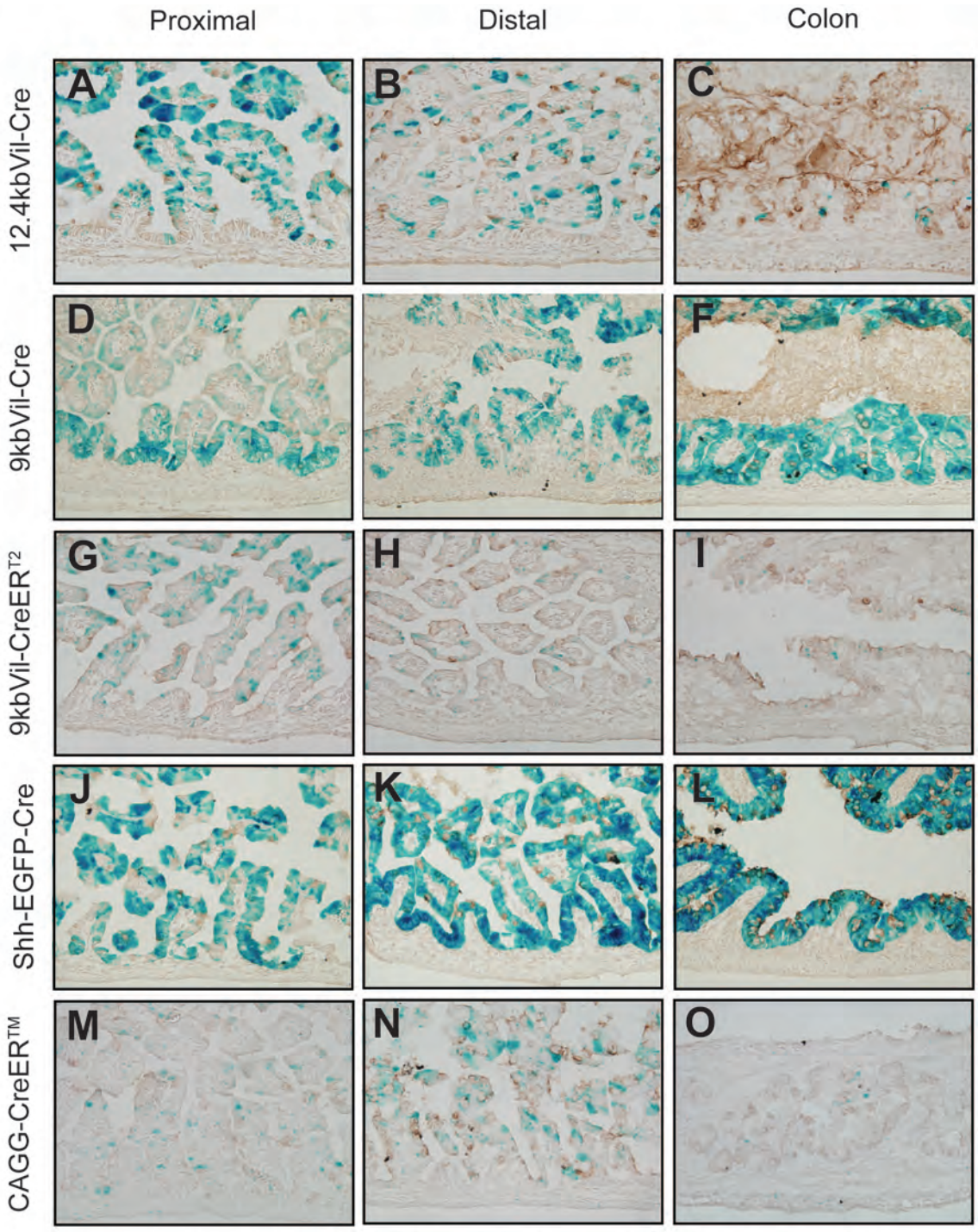

Figure 3-2. Fetal Intestinal Recombination of Cre Transgenic Lines. X-gal staining of 12.4kbVil-Cre;R26R (A-C), 9kbVil-Cre;R26R (D-F), Vil-CreER^{T2};R26R (G-I), Shh-EGFP-Cre;R26R (J-L), and CAGG-CreER^{TM};R26R (M-O) transgenics in the proximal (A, D, G, J, M) and distal (B, E, H, K, N) small intestine and in the colon (C, F, I, L, O) of E18.5 fetal mice. Tamoxifen was administered to pregnant females at E16.5 of pregnancy for inducible Cre trangenic lines Vil-CreER^{T2} and CAGG-CreER^{TM}.

drivers had β-galactosidase staining that was as homogenous as that observed in adults (Fig. 3-1 and Fig. 3-2).

Intestinal Expression of the 9kbVil-CreER^{T2} Inducible Driver

The 9kb villin promoter was also used to drive expression of an inducible form of the Cre recombinase gene, CreER^{T2} [2]. The CreER^{T2} construct generates a fusion protein with Cre and a mutated form of the ligand-binding domain of the human estrogen receptor[12, 13]. This fusion protein is sequestered in the cytoplasm by heat shock protein chaperones until binding of the estrogen analog tamoxifen allows translocation of the Cre protein to the nucleus where it can initiate recombination of floxed DNA sequences[12, 13]. Expression of this transgene was reported to not be found in the kidney upon tamoxifen induction, suggesting that the expression of the 9kb villin promoter in the kidney may depend on positional effects from the transgene insertion site[2]. Injection of 4 mg/40g body weight tamoxifen on 5 consecutive days followed by 5 days of "chase" and sacrifice of the Vil-CreERT2;R26R transgenic resulted in mosaic recombination throughout the small and large intestines (Fig. 3-3). In addition, ribbons of labeled cells were apparent suggesting that recombination occurred in a stem or progenitor cell located in the intestinal crypts (Fig. 3-3E, arrow). This finding is consistent with labeled cells persisting up to 2 months in Vil-CreERT2;R26R transgenics that were injected daily for 5 days with 1mg tamoxifen[2]. However, expression was patchy suggesting that the transgene is not activated uniformly in the tissue. A single injection of 4mg/40g body weight into pregnant females at stage E16.5 with fetuses analyzed at E18.5 showed weak recombination in the intestinal epithelium with the most β-galactosidase staining in the proximal small intestine and very little staining in the distal small intestine or colon (Fig. 3-2G-I). Thus, if high recombination efficiency is desired, either a more concentrated dose of tamoxifen or multiple injections might be necessary. Moreover, if experimental outcomes are being examined in the developing intestine, more efficient recombination would be achieved using a constitutive Vil-Cre model.

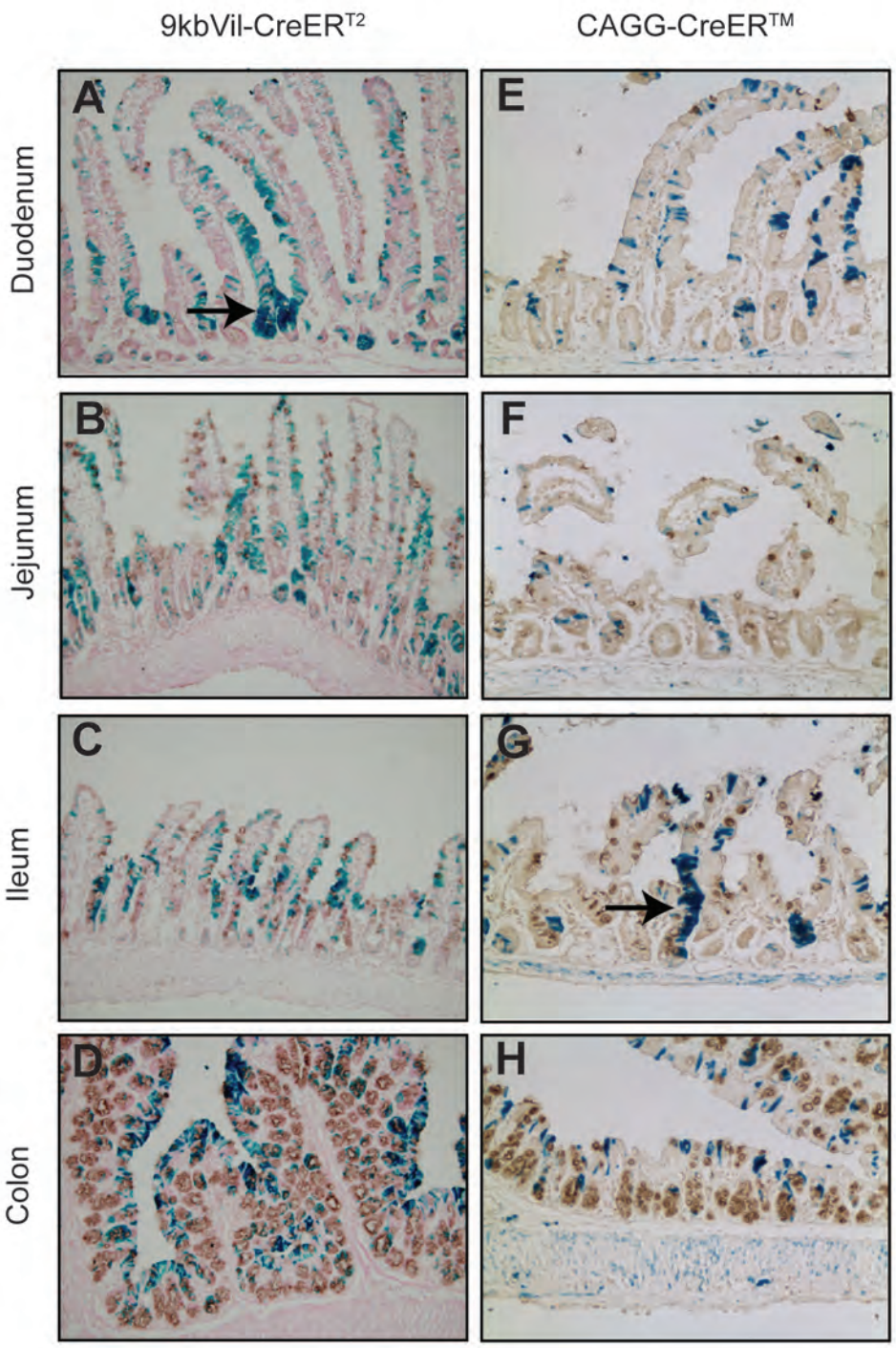

Figure 3-3. Intestinal Recombination of Taxoxifen-Inducible Cre Transgenic Lines. X-gal staining of 9kbVil-CreER^{T2};R26R transgenics (A-D) and CAGG-CreER^{TM};R26R transgenics (E-H) in the duodenum (A, E), jejunum (B, F), ileum (C, G), and proximal colon (D, H) of adult mice. Arrows in (A) and (C) denote ribbons of labeled cells likely generated from a stem or progenitor cell.

The Shh-EGFP-Cre Driver Line

The Shh-EGFP-Cre line was developed for studies in the limb bud[6]; however, many other tissues also express Sonic hedgehog (Shh), including the intestine[14-16]. Heterozygous mice do not exhibit any apparent phenotype from the loss of one Shh allele due to knock-in of the EGFP-Cre cassette and thus should be suitable for directing Cre-mediated recombination[6]. Hh signaling in the intestine is strictly paracrine with the Hh ligands, including Shh, secreted from the epithelium and acting on cells within the mesenchyme[15]. Accordingly, we observed strong β-galactosidase staining in the adult intestinal epithelium of Shh-EGFP-Cre;R26R transgenics (Fig. 3-1H-J). Endogenous Shh is expressed prior to intestinal morphogenesis, with robust endodermal expression throughout the midgut and hindgut apparent by ~E10.5[14, 15]. Strong β-galactosidase expression was observed in the Shh-EGFP-Cre;R26R E18.5 intestine, demonstrating that this Cre driver is suitable for studies of developing intestine (Fig. 3-2J-L). Of note, β-galactosidase was observed in a mosaic pattern with ribbons of cells being generated from some crypts while others were devoid of β-galactosidase-positive cells (Fig. 3-1H, J). The mosaicism was the most apparent in the adult duodenum, but was present to some extent in the adult colon (Fig. 3-1J) and the E18.5 proximal intestine (Fig. 3-2J). Since Shh becomes restricted to the proliferative zones during intestinal morphogenesis[15], the β-galactosidase-postive cells on the villi were likely derived from a labeled stem or progenitor cell.

We also examined the gastric recombination pattern in Shh-EGFP-Cre;R26R adult mice (Fig. 3-4). As in the intestine, recombination appeared to be specific for the stomach epithelium and was not observed in the mesenchyme (Fig. 3-4). In the corpus, a mosaic pattern was observed with occasional staining of entire glands (Fig. 3-4A). This pattern is consistent with transgene expression in all major epithelial lineages in the corpus as reported for a Shh reporter mouse in Waghray et al.[17] and/or in occasional stem cells. Although positive staining was much less frequent, recombination was also observed in the antrum (Fig. 3-4B) consistent with less expression of a Shh reporter transgene in the antrum[17] and with antral Shh expression being detected in a study by Kolterud et. al.[15].

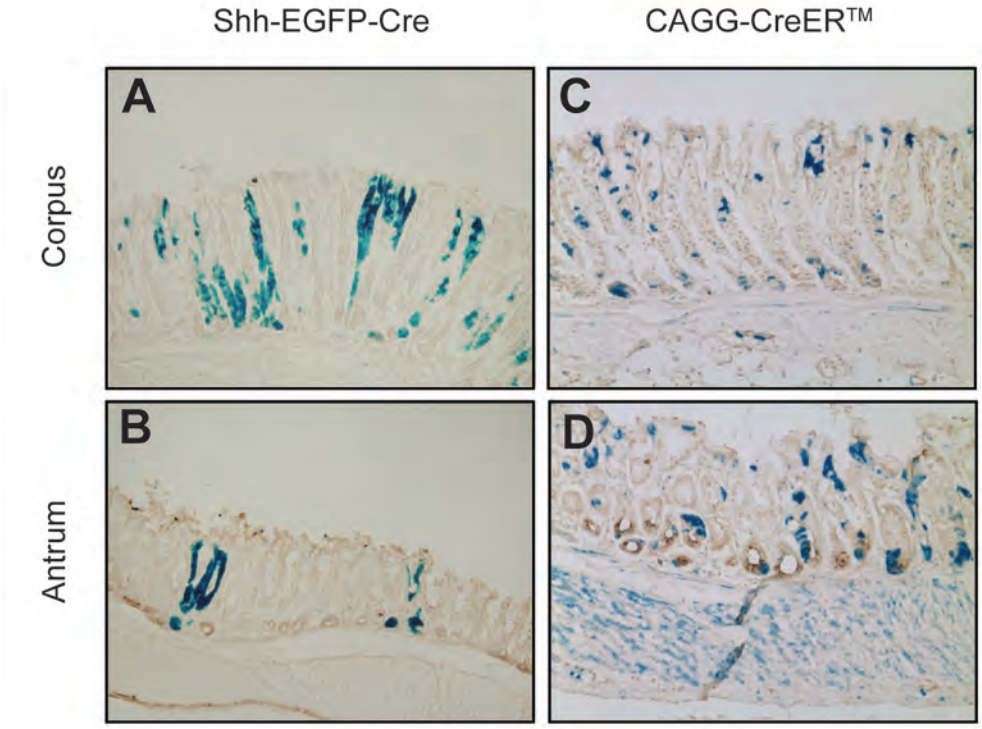

Figure 3-4. Cre Driver Line Expression in the Stomach. X-gal staining of adult Shh-EGFP-Cre;R26R (A, B) and CAGG-CreER™;R26R transgenic (C, D) stomach, including corpus (A, C) and antrum (B, D).

The CAGG-CreER^{TM} Driver Line

The CAGG promoter is a chimeric promoter of the cytomegalovirus immediate-early enhancer and the chicken β-actin promoter, which direct widespread expression of transgenes in mice[5]. For the CAGG-CreER^{TM} transgenic, the CAGG promoter was used to drive expression of a CreER^{TM} fusion protein[12, 18, 19]. CreER^{TM} is an earlier version of CreER^{T2}; the CreER^{T2} has been further optimized for tamoxifen sensitivity and specificity[20]. Similar to the CreER^{T2}, CreER^{TM} is a fusion protein that sequesters CreER^{TM} to the cytoplasm until translocation to the nucleus is induced by administration of tamoxifen[5].

Although the CAGG-CreER^{TM} driver line was reported to be expressed in the intestine, this data was not shown in the original description of the expression pattern for this line[5]. Therefore, we examined β-galactosidase expression in CAGG-CreERTM;R26R transgenics after 5 consecutive daily i.p. injections of 4mg/40g body weight tamoxifen followed by 5 days of chase (Fig. 3-3). A very patchy staining pattern was observed throughout the small and large intestine with labeling occurring in scattered cells of the intestinal epithelium and mesenchyme (Fig. 3-3A-D). Occasionally, we observed ribbons of labeled cells emanating from intestinal crypts, demonstrating that recombination occurred in a stem cell population (Fig. 3-2C, arrow). CAGG-CreER;R26R reporter expression was also examined in the adult mouse stomach. β-galactosidase-positive staining was observed in a patchy distribution throughout the epithelium and mesenchyme of the gastric corpus (Fig. 3-4C) and antrum (Fig. 3-4D).

A very weak, mosaic recombination was observed in the intestines of E18.5 embryos from pregnant mothers who were injected at E16.5 with a single dose of 4mg/40g body weight tamoxifen (Fig. 3-2M-O). This Cre driver has been shown to be dose-dependent[5], therefore, it is possible that multiple injections of tamoxifen or a higher dose of tamoxifen would increase the recombination efficiency. Of note, Hayashi et. al. reported embryonic lethality as a result of high doses of tamoxifen; most embryos, including controls and CAGG-CreER^{TM} embryos, developed normally when pregnant mothers were injected at E10.5

with 3mg/40g body weight tamoxifen while many embryos from pregnant mothers injected similarly with a 6-9 mg/40g body weight tamoxifen dose survived past E13.5[5]. This lethality effect appeared to not simply be tamoxifen dose-dependent, but also dependent on the mass of the pregnant female[5]. I also observed loss of litters after a single tamoxifen injection in both CAGG-CreER^{TM};R26R and Vil-CreER^{T2};R26 transgenics. The loss of our litters appeared to be alleviated when the tamoxifen dose was delivered via multiple injection sites suggesting that the injected volume and not necessarily the tamoxifen dose caused lethality.

Concluding Remarks on Cre Driver Selection

Direct comparison of the 12.4kbVil-Cre, 9kbVil-Cre, 9kbVil-CreER^{T2}, Shh-EGFP-Cre, and CAGG-CreER^{TM} Cre drivers has contributed to our knowledge of intestinal and gastric recombination patterns that can be achieved with these lines. This information will aid researchers who are using Cre-lox transgenic mouse technology to alter gene expression in these tissues. Based on our results and the results of previous studies, the authors make the following suggestions:

1. To alter gene expression in the developing intestine, use a constitutive Vil-Cre driver rather than the inducible 9kbVil-CreER^{T2} driver.
2. When using the 9kbVil-Cre transgenic, the potential development of a phenotype in the kidney must be assessed.
3. For studies in the colon, more recombination may be achieved with the 9kbVil-Cre than the 12.4kbVil-Cre. However, the mosaic recombination pattern in the 12.4kbVil-Cre colons may be useful for side-by-side comparison of normal and mutant crypts.
4. The Shh-EGFP-Cre driver may be useful for inducing epithelial gene alterations in the intestine and gastric corpus. In the intestine, recombination is more complete in the distal intestine whereas in the duodenum and gastric corpus, normal and mutant crypt-villus units/glands could be studied side-by-side.

5. The tamoxifen treatment method used in this study would need to be modified to induce more efficient recombination with the CAGG-CreER^{TM} driver to make it useful for intestinal or gastric studies.

PART B: AN INTESTINE-SPECIFIC INDUCIBLE MATH1 TRANSGENIC MOUSE MODEL

SUMMARY

Mouse atonal homolog 1 (Math1) is the critical transcription factor promoting secretory cell fate over absorptive cell fate in the intestine. A fetal mouse model with complete loss of Math1 and an adult mouse model with intestine-specific loss of Math1 both exhibited loss of the intestinal secretory lineage. In contrast, the Vil-Math1 transgenic model, in which Math1 expression was forced throughout the developing intestinal epithelium, exhibited increased secretory cell differentiation and loss of absorptive cells (Chapter 2). Likely due to the extreme epithelial remodeling, stable transgenic Vil-Math1 lines were unable to be generated. To generate stable transgenic lines that could be used to further study how Math1 in adult intestine, a new intestine-specific inducible transgenic mouse model was generated. Cre-mediated activation of the transgene resulted in as much 19-fold increased expression of Math1 mRNA compared to controls. However, immunostaining for Math1 revealed that protein levels remained unchanged. Thus, this model could not be used to study the affect of intestinal remodeling in response to increased secretory cell differentiation. However, these data suggest that Math1 protein stability is regulated by some unknown mechanism.

INTRODUCTION

Notch signaling regulates cell fate selection of absorptive lineage cells over secretory lineage cells in the intestine. It is thought that Notch controls the cell fate selection process primarily by transcriptional regulation of Math1 via the

repressor bHLH transcription factor Hes1. Cells receiving the Notch signal have increased expression of Hes1, which represses Math1 expression[21]. In contrast, Math1 continues to be expressed in the progenitors and mature cells belonging to the secretory lineage[22, 23]. Math1 is both required and sufficient for secretory cell differentiation[23, 24] (Chapter 2). Lack of Math1 during development or in adult intestine results in absence of all secretory cells, including goblet, Paneth, and endocrine cells[23, 24]. Conversely, forced expression of Math1 in the intestinal epithelium during late development in the Vil-Math1 transgenic model resulted in almost complete conversion of the intestinal epithelium to secretory cells with a corresponding loss of absorptive enterocytes suggesting that Math1 can direct differentiation of a bipotential progenitor cell to the secretory lineage (Chapter 2). Interestingly, Vil-Math1 founders also had an expanded mesenchyme compartment with increased proliferating cells and increased differentiated smooth muscle and neurons. Transgenic Math1 expression was targeted to the intestinal epithelium, so these affects on the mesenchyme were indirect, suggesting that the cellular changes induced by Math1 affected signaling between the epithelium and mesenchyme (Chapter 2).

Likely due to the extreme remodeling of the intestinal epithelium, Vil-Math1 transgenic founders with high transgene expression could not be recovered after birth and thus could not be maintained as stable transgenic lines. Thus, in order to study the affects of Math1 expression in mature intestine, an inducible transgenic model was required. In addition, to study the complex question of how Math1 affects epithelium-mesenchyme signaling in the intestine, the generation of stable transgenic lines would be necessary. Thus, in this study, we generated an intestine-specific inducible transgenic mouse model in which Math1 expression could be activated by Cre recombinase to study the affects of Math1 expression in adult intestine.

MATERIALS AND METHODS

Transgene Construction and Generation of Transgenic Lines

The VZM transgene was prepared in a three step process. First, the multiple cloning site from *SacII* to *XhoI* of pBluescript SK (+/-) (Stratagene) was shuttled into pVFnLZ+Hhip[25] (gift from D.L. Gumucio) to remove Hhip sequences and to generate the plasmid pVFnLZ+MCS containing a unique *XhoI* site. Second, Math1 was shuttled to pBluescript SK (+/-) after addition of *XhoI* sites to the 5' and 3' ends of Math1 by polymerase chain reaction (PCR) using pCS2+Math1[26] as a template and the primers 5Math1XhoI-F 5'- GGG CTC GAG CGA ATT CCG CCA TGT CC and 3Math1XhoI-R 5'- GGG CTC GAG TTC TAG ACT AAC TGG CC. Third, Math1 was isolated by *XhoI* digestion from the shuttle vector and cloned into the *XhoI* site in pVFnLZ+MCS to generate the pVFnLZ+Math1 (VZM) transgene (Figure 3-5A). The VMZ transgene contained a floxed nuclear lacZ-STOP cassette followed by the mouse cDNA under the control of the 12.4 kb mouse villin enhancer/promoter[3]. Following verification by sequencing, the 19.3 kb transgene was excised with *Pme*I and microinjected into F2 zygotes from C57BL/6 X SJL parents by the University of Michigan Transgenic Animal Model Core.

Seven transgenic founders were obtained and used to establish stable transgenic lines (VZM1-7), which were maintained on a C57BL/6 genetic background. Transgenic founders and progeny were identified by multiplex PCR amplification of a 374 bp WT Math1 and a 525 bp transgenic product using the following primers: M1-F 5'-TGC TGC ATG CAG AAG AGT GGG CTG AGG TAA; M1-R, 5'-TCA GCT TGC ACA GCT GTT CCC GTA CTT TGA; pVFnLZ(17367)-F 5'- TCG ACC TGC AGC CCA AGC TGA TCC TCT AGT. For Cre-mediated recombination of the VZM transgene, VZM1 or VZM7 Tg were crossed to 12.4kbVil-Cre[3], 9kbVil-Cre[2], or Shh-EGFP-Cre[6]. More information is provided and intestinal recombination patterns of these strains are compared in Chapter 3A. Mouse use was approved by the University of Michigan Committee on Use and Care of Animals.

Collection of Tissues and Histological Analysis

Intestines were dissected from adult (1-4 month old) or developing (embryonic day 18.5-3 weeks) VZM transgenic (Tg) and non-transgenic (Ntg) littermates. For adult mice, the duodenum (2 cm segment distal to the pylorus), jejunum (2 cm segment at the midpoint), ileum (2 cm segment proximal to the cecum) and colon (2 cm segment distal to the cecum) were collected for histological analysis. For fetal intestine, proximal intestine (2 cm segment distal to the pylorus), distal intestine (2 cm segment distal to the midpoint), and colon (2 cm segment distal to the cecum) were collected. The proximal 1 cm segment of each intestinal region was paraffin embedded after fixing overnight in 4% paraformaldehyde (Pfa). The distal 1 cm region was prepared for cryosectioning by fixing for 1 hour in 4% Pfa followed by overnight incubation in a 30% sucrose solution in 1X PBS, embedding in OCT (TissueTek), and freezing on dry ice. Adjoining intestinal segments were processed for RNA. Paraffin sections (5 mm) were stained with H&E to assess cellular morphology, Periodic-acid Schiff (PAS)/Alcian blue (Newcomer Supply) for mucin-containing goblet cells, rabbit anti-chromogranin A (1:500; 94188/5 gift from J. F. Rehfeld) for enteroendocrine cells, and rabbit anti-lysozyme (1:100; Zymed) for Paneth cells. For immunostaining, the appropriate secondary antibodies conjugated to Cy2 (1:400; Jackson ImmunoResearch Laboratories) or AlexaFluor488 (1:400; Invitrogen) were used. For Math1 immunostaining, following rehydration, slides were boiled 25 minutes in Trilogy Solution (Cell Marque), cooled 25 minutes on ice and placed in 3% hydrogen peroxide in methanol for 60 minutes to block endogenous peroxidase activity. Tissues were blocked using 1% BSA + 10% goat serum in 0.3% Triton X-100 in PBS followed by incubation with rabbit anti-Math1 (1:50; gift from J. Johnson) for 3 days overnight at 4°C and biotinylated goat anti-rabbit IgG (1:200 Vector Laboratories) secondary antibody. Staining was visualized with the DAB kit (Vector Laboratories) and counterstaining with hematoxylin. Cryosectioning and staining for β-galactosidase was performed as described in Chapter 3A. Microscopy was performed with either a Nikon E800 or Olympus BX-51 equipped with a SPOT or Olympus DP70 digital camera, respectively.

Analysis of Transgene and Cellular Marker Gene Expression

Gene expression was measured by quantitative reverse transcription-PCR (qRT-PCR) analysis of VZM Tg and VZM;12.4kbVil-Cre Tg. RNA was isolated using Trizol (Invitrogen) according to the manufacturer's directions. RNA was then DNase-treated and purified using the RNeasy Mini kit (Qiagen). RT reactions (50 µl) used 1 µg RNA and the Iscript cDNA synthesis kit (Bio-Rad), as recommended by the manufacturer. qRT-PCR was performed as described[27] with SYBR green dye and the primers listed in Table 2-1 for cell lineage markers and Math1. Expression of the VZM-lacZ transcript was determined using the primers Vill-F 5'-CAA CTT CCT AAG ATC TCC CAG GTG and lacZ-R2 5'-GGA TGT GCT GCA AGG CGA TTA AG. Expression levels were determined with triplicate assays per sample (n = 2-8) and normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (Gapdh), which remained the same in all samples.

Statistical Analysis

Quantitative data were presented as mean ± SEM and analyzed by a Student's t-test to compare data from VZM;Cre Tg to VZM Tg with $p < 0.05$ considered significant.

RESULTS

Generation of VZM Transgenic Lines

Constitutive expression of Math1 in the developing intestine causes dramatic epithelial remodeling that is not compatible with maintenance of stable transgenic lines with high transgene expression. Thus, in order to generate stable transgenic lines, we utilized an inducible transgenic approach. The VZM transgene contained the 12.4 kb villin promoter driving a floxed nuclear lacZ cassette with STOP and polyA sequences[3, 25] and the Math1 cDNA (Fig. 3-5A). In the absence of Cre expression, the villin promoter drives expression of a lacZ

transcript (VZM-lacZ). In the presence of Cre expression, recombination of the loxP sites excises the lacZ and STOP sequences so that the villin promoter now drives expression of Math1 (VZM-Math1). Seven independent VZM transgenic founder lines (VZM1 through VZM7) were generated.

Regional expression of the VZM-lacZ transcript was determined in adult mice from each VZM line by qRT-PCR gene expression analysis and staining for β-galactosidase (Fig. 3-5,C). Line VZM1 highly expressed the transgene throughout the intestine with strong lacZ expression localized to most if not all of the nuclei of the intestinal epithelium. VZM1 also expressed the transgene in the colon, although the expression was more patchy than in the intestine, consistent with the expression pattern of the villin promoter[3] (Fig. 3-1 and data not shown). Also consistent with the expression of the villin promoter, the transgene appeared to be expressed more strongly in mature cells on the villus than in the less-differentiated cells of the crypts[3] (Fig. 3-5C). Compared to VZM1, the other 6 lines expressed the transgene at relatively low levels. Lines VZM2, VZM3, VZM4, and VZM7 had similar β-galactosidase staining patterns with most villus epithelial cells being lacZ-positive and very little if any staining in the crypt cell nuclei (Fig. 3-5C and data not shown). VZM5 and VZM6 transgenics expressed the transgene in only a few scattered epithelial cells (data not shown). Based on this initial characterization, the high-expressing line VZM1 and one low-expressing line, VZM7, were kept for further analysis.

Fetal Onset of VZM Transgene Expression

The timing of transgene activation was determined in VZM1 and VZM7 lines by examining transgene expression and β-galactosidase staining at fetal and perinatal (P0) stages. Contrary to the adult expression levels, VZM7 had stronger fetal and perinatal transgene expression than VZM1 (Fig. 3-6). The villin promoter has been reported to activate ~E12.5[3]; however, the timing of promoter activation appears to vary among different transgenes, likely due to positional insertion affects and depending on the linked trangsgene (D.L. Gumucio, personal communication). Low levels of VZM-lacZ transcript were

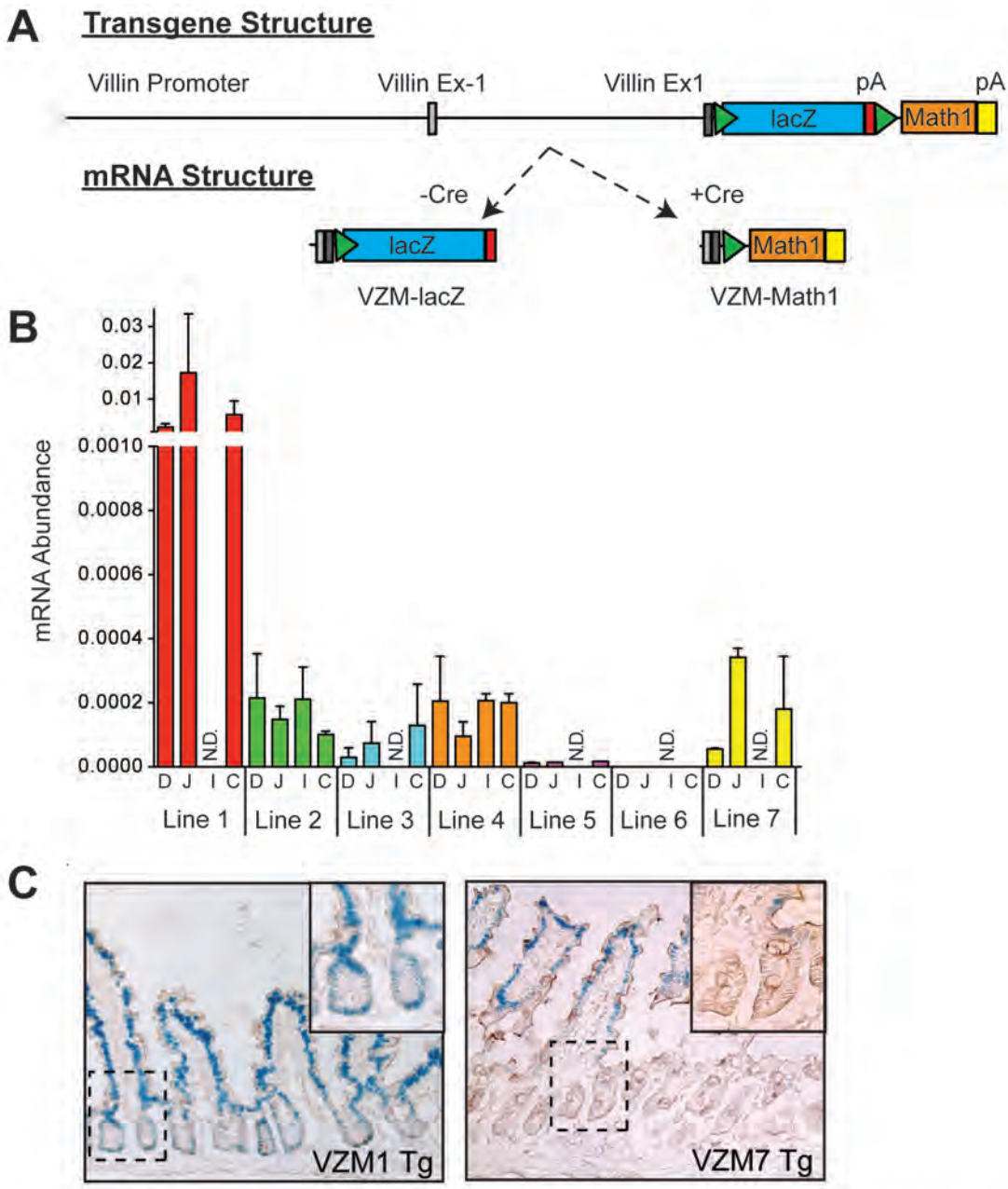

Figure 3-5. Characterization of VZM Transgenic Lines. (A) VZM transgene structure with green triangles representing loxP sites, a red box indicating a STOP and polyA (pA) sequences, and a yellow box indicating SV40 and pA sequences. (B) Expression of the VZM transgene was determined by qRT-PCR in duodenum, jejunum, ileum, and colon of adult mice from each of the 7 generated lines. (C) Staining of duodenal tissue sections for β-galactosidase in VZM1 and VZM7 transgenics. N.D., not determined; D, duodenum; J, jejunum; I, ileum; C, colon; Tg, transgenic. Data were normalized to Gapdh and presented as mean ± SEM.

detected in VZM7 at E15.5 by qRT-PCR and this expression increased steadily through E17.5 and P0 time points with higher expression of the transgene in the small intestine compared to the colon (Fig. 3-6A). Accordingly, the number of lacZ-positive epithelial cells was higher in duodenal tissue sections from P0 VZM7 mice compared to E17.5 VZM7 mice (Fig. 3-6B). Expression levels of the VZM-lacZ transcript in VZM1 mice at P0 was comparable to those of VZM7 mice at E15.5 (Fig. 3-6A). This corresponded to patchy expression of the transgene as visualized by β-galactosidase staining, with a small proportion of nuclei intensely-expressing lacZ (Fig. 3-6B). Thus, line VZM1 had the strongest adult transgene expression, but line VZM7 had the more robust transgene expression during intestinal development.

Cre-induced Activation of Math1 mRNA Expression, but not Math1 Protein

Due to its strong expression of the transgene, the VZM1 line was used first for induction of transgene recombination by intercrossing this line with 12.4Vil-Cre model. 12.4Vil-Cre specifically expresses Cre recombinase in the intestinal epithelium[3] (see Chapter 3A). As a note, this specific Cre expression was not required since the VZM transgene is also intestine-specific; however, the 12.4kbVil-Cre transgenic model is very robust and has been used in numerous intestinal studies. The extent of transgene recombination was monitored by examination of VZM-lacZ transcript expression and Math1 expression as well as loss of lacZ-positive staining in tissue sections (Fig. 3-7). VZM1;12.4kbVil-Cre mice were examined at E18.5, 2 weeks, 3 weeks, 4 weeks, and 4 months of age. Transgene recombination was not detectable at E18.5 by analysis of VZM-lacZ transcript abundance or β-galactosidase staining, however total Math1 expression (endogenous Math1 plus VZM-Math1) was significantly increased 2.6-fold compared to VZM1 transgenic controls, suggesting that some transgene recombination had occurred. Complete transgene recombination was evident by 2 weeks of age with loss of VZM-lacZ transcript expression and corresponding loss of lacZ-positive staining and increased total Math1 mRNA expression (Fig. 3-7). The villin promoter is active in fetal mice, but is patchily expressed (Figure

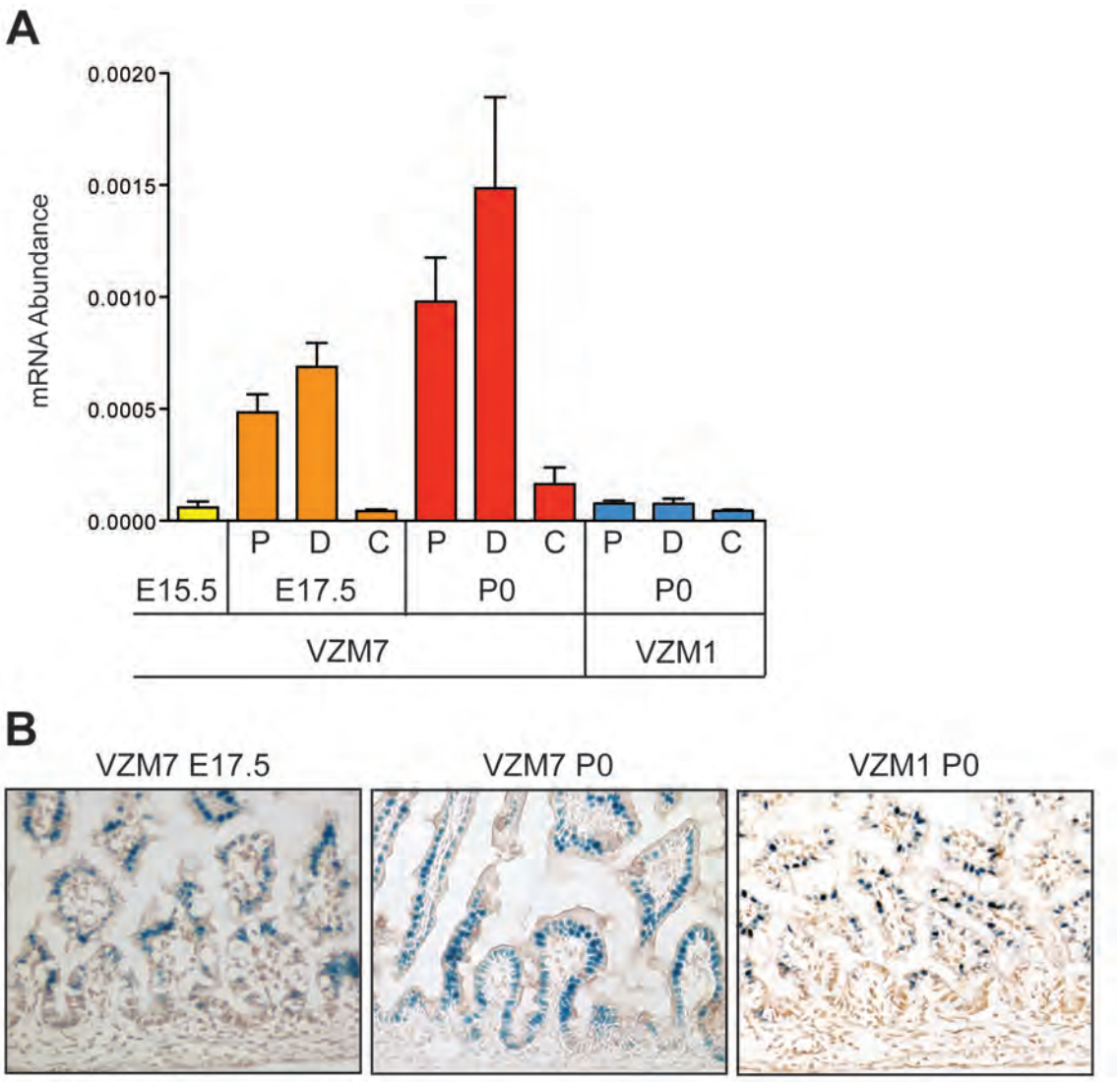

Figure 3-6. Fetal and Perinatal Expression of VZM Transgene. Expression of the VZM transgene was determined by qRT-PCR in fetal and perinatal mice from lines VZM1 and VZM7 in proximal and distal intestine and colon (B). Regional expression was not determined in E15.5 intestine. Staining of duodenal tissue sections for β-galactosidase in VZM1 and VZM7 transgenics (C). E, embryonic day; P0, day of birth; P, proximal; D, distal; C, colon. Data were normalized to Gapdh and presented as mean ± SEM.

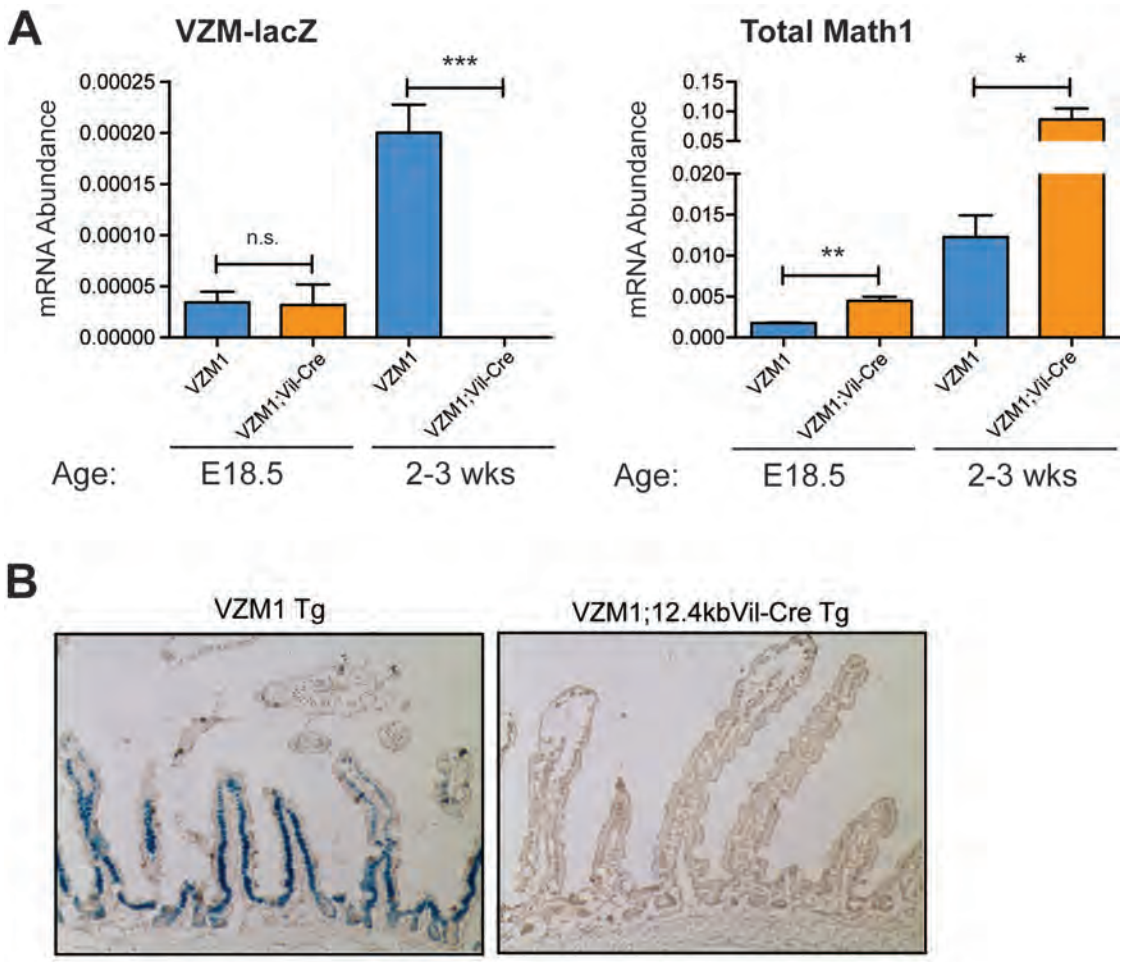

Figure 3-7. 12.4kbVil-Cre-mediated Recombination of the VZM Transgene Occurs by 2 Weeks of Age. Recombination of the VZM transgene was induced by intercrossing VZM1 transgenics (Tg) with 12.4kbVil-Cre Tg to generate VZM1;12.4kbVil-Cre Tg. (A) Duodenal expression of the VZM-lacZ transcript and total Math1 mRNA was determined by qRT-PCR in E18.5 and 2-3 week old VZM1 Tg and VZM1;12.4kbVil-Cre Tg. (B) Staining of duodenal tissue sections for β-galactosidase in 2 week old VZM1 and VZM1;Vil-Cre Tg shows loss of lacZ-positive staining in the VZM1;12.4kbVil-Cre intestine. Data were normalized to Gapdh and presented as mean ± SEM. E, embryonic day; *p<0.05, **p<0.01, ***p<0.0001 by student's t-test.

3-2). Therefore, transgene recombination may not be complete until after birth due to the expression pattern of the 12.4kbVil-Cre transgenics, specific details relating to the VZM1 transgene, its insertion site or timing of activation. Despite late recombination, a 7-fold increase in Math1 expression was observed at 2 weeks and as high as 19-fold increased Math1 expression was observed in the duodenum of 4 week old VZM1;12.4Vil-Cre transgenics compared to VZM1 controls (data not shown). Math1 mRNA levels were similar between VZM1 and 12.4kbVil-Cre control mice demonstrating that increased Math1 mRNA levels were specific to mice with recombination of the VZM transgene (data not shown).

Math1 protein is found in progenitor cells in the crypts as well as in mature secretory cells[22]. Immunostaining for Math1 protein in VZM1 and VZM7 mice showed a normal distribution of Math1-positive cells (Fig. 3-8A, C). Surprisingly, a normal distribution of Math1-positive cells was also present in VZM1;12.4kbVil-Cre transgenics, demonstrating that Math1 protein is not accumulating in recombined VZM transgenics (Fig. 3-8B). A similar finding was made in VZM1 and VZM7 mice that were crossed to other Cre transgenics including VZM7;9kbVil-Cre transgenics (Fig. 3-8D) and VZM1;Shh-EGFP-Cre transgenics (data not shown). Sequencing of the recombined VZM-Math1 transcript showed that the complete Math1 protein coding region was present as well as an intact Kozak consensus sequence, suggesting that the proper elements for transgenic Math1 protein expression are present. These data suggest that Math1 protein is being regulated by some unknown mechanism in the recombined VZM transgenics.

Secretory Cell Differentiation is Normal in VZM1;12.4KBVil-Cre Transgenics

In accordance with normal expression of Math1 protein, no changes in secretory cell differentiation were observed in VZM1;12.4kbVil-Cre transgenics (Fig. 3-9). Intestinal morphology remained normal in all ages examined with similar numbers of goblet cells stained by the mucus-specific stain Periodic acid Schiff-Alcian blue (PAS-AB) stain (Fig. 3-9A-D and data not shown). Additionally, expression of cellular markers including Mucin2 (Muc2) for goblet

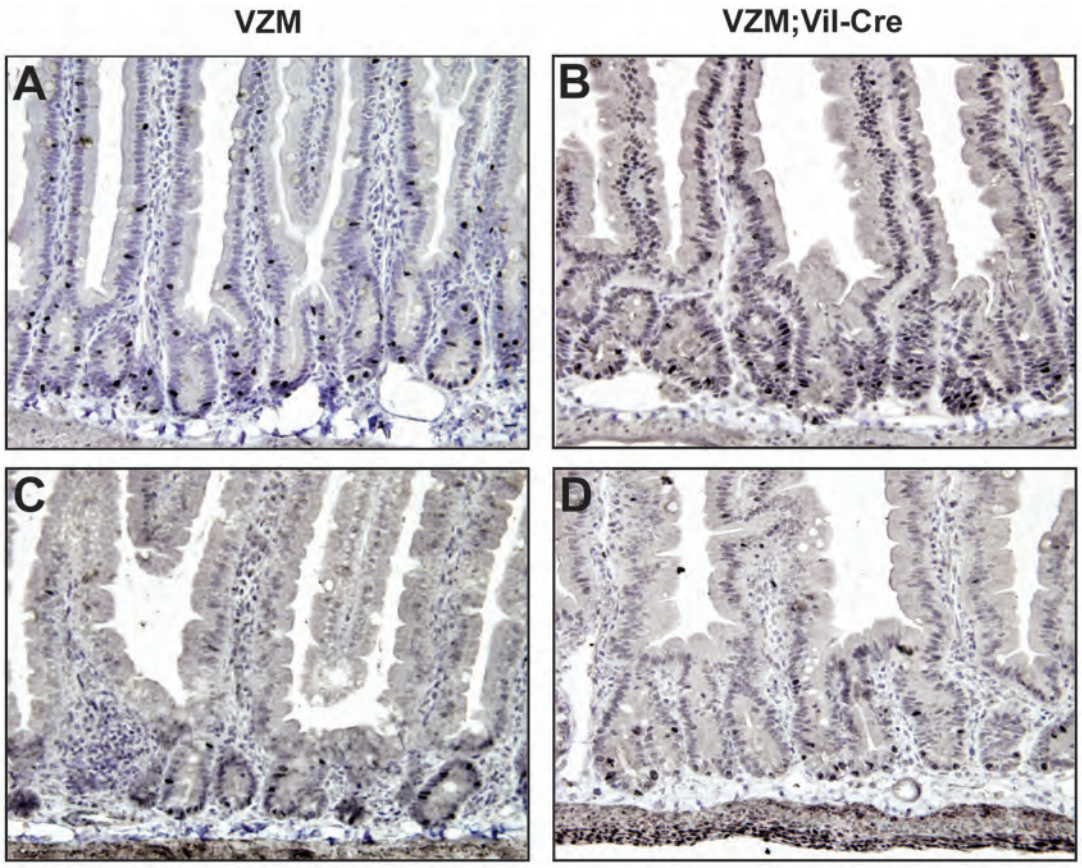

Figure 3-8. Math1 Protein is Not Increased in Recombined VZM Transgenics. Math1 immunostaining of adult duodenal sections (A-D) appears similar in all groups: VZM1 Tg (A), VZM1;12.4kbVil-Cre (B), VZM7 Tg (C) and VZM7;9kbVil-Cre (D). Some background staining can be observed in the smooth muscle layer.

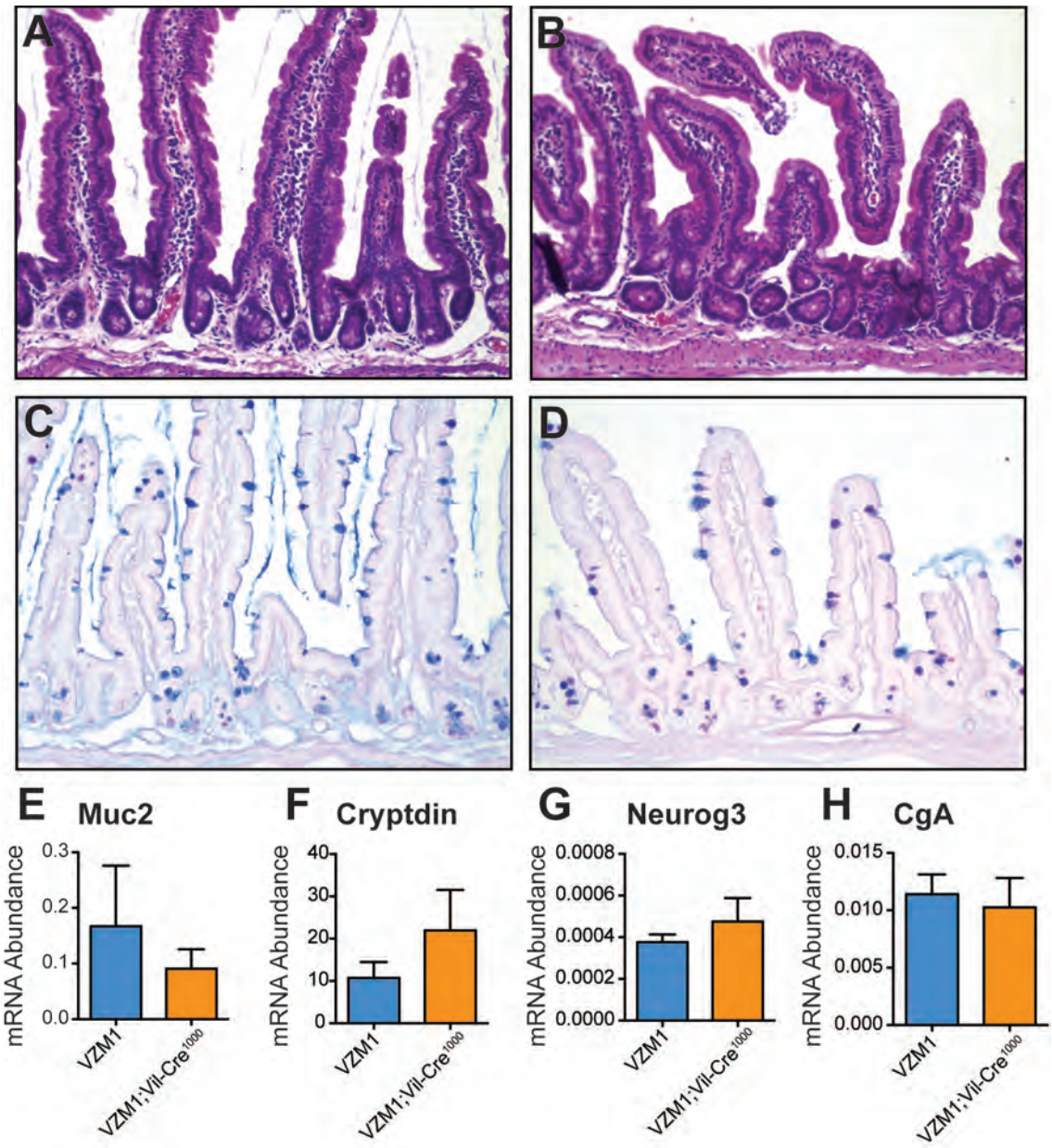

Figure 3-9. Secretory Cells are Not Induced in VZM1;12.4kbVil-Cre Transgenic Mice. Analysis of VZM1 (A, C) and VZM1;12.4kbVil-Cre (B, D) duodenal sections from 4 month old mice. H&E staining (A, B) shows similar crypt-villus morphology in both groups of mice. Similar numbers of goblet cells are present in both groups as shown by PAS-AB staining (C, D). qRT-PCR for cellular markers including Mucin2 (Muc2) for goblet cells (E), cryptdin for Paneth cells (F), Neurogenin3 (Neurog3) for endocrine cell precursors (G), and chromogranin A (CgA) for mature endocrine cells. Data were normalized to Gapdh and presented as mean ± SEM.

cells, cryptdin for Paneth cells, Neurogenin 3 (Neurog3) for endocrine progenitor cells, and Chromogranin A (CgA) for mature endocrine cells was similar between VZM1 and VZM1;12.4kbVil-Cre transgenics (Fig. 3-9). Similar results were observed in other recombined transgenics including adult VZM1;Shh-Cre-EGFP mice and E18.5 and adult VZM7;9kbVil-Cre mice (data not shown).

DISCUSSION

In this study we generated a novel transgenic mouse model in which Math1 expression could be activated in the intestinal epithelium with expression of Cre recombinase. Despite increased Math1 mRNA with Cre-mediated recombination of the VZM transgene, Math1 protein was found to be unchanged compared to non-recombined controls. Accordingly, secretory cell number was unchanged in VZM;Cre transgenic mice. Use of transgenic mouse models is complicated by unique transgene insertion sites that cause variability in transgene expression levels and timing of activation between founders/lines; therefore, we analyzed the phenotype of two independent transgenic lines, VZM1 and VZM7. Neither of these lines exhibited changes in secretory cell number when combined with multiple Cre driver lines, suggesting that effective Math1 protein levels were never achieved.

We do not currently understand why Math1 protein was not induced in the VZM;Cre transgenics. Sequencing of the recombined VZM-Math1 transcript showed a complete Math1 protein coding region and an intact Kozak consensus sequence suggesting that the elements necessary for Math1 protein production are present. Indeed the recombined VZM-Math1 transcript is very similar to the Vil-Math1 transgene transcript that produced a secretory cell differentiation in E18.5 transgenic founders (Chapter 2). In addition, strong epithelial phenotypes were observed in Vil-Math1 transgenic founders with 7- to 10-fold increased expression of Math1 (Chapter 2), similar to the level of Math1 mRNA abundance in the VZM1;12.4kbVil-Cre mice by 2 weeks of age; this suggests that effective

Math1 mRNA abundance was achieved in the VZM;12.4kbVil-Cre. Of note, we have not determined the localization of increased Math1 mRNA in the VZM;12.4kbVil-Cre transgenics. It is likely that Math1 mRNA can only affect the differentiation of progenitor cells and not mature cells on the villus. The villin promoter is less active in undifferentiated epithelial cells compared to mature cells and, therefore, the VZM transgene was expressed more weakly in the crypts (Fig. 3B-1). Thus, to confirm that Math1 mRNA is being induced in the crypt compartment of VZM;Cre transgenics, Math1 mRNA should be analyzed by *in situ* hybridization or with a Math1 reporter mouse[23, 28].

The difference in timing of transgene activation might be an important contributing factor to the lack of phenotype in the VZM;Cre transgenics. Efficient recombination in the VZM1;12.4kbVil-Cre transgenics was not observed until 2 weeks of age, an age when the mouse intestine is reaching maturity with emergence of crypts and Paneth cells. The VZM1;12.4kbVil-Cre did not have increased Math1 mRNA expression during the developmental stages that the Vil-Math1 trangenic founders were examined. Thus, perhaps there are key differences in regulation of Math1 protein in mature compared to developing intestine. The VZM7 transgenics, which had early transgene activation (~E15.5), were crossed with 9kbVil-Cre and Shh-EGFP-Cre mice in an attempt to activate transgene recombination at an earlier, developmental age, but increased secretory cells were still not observed in these mice at E18.5. Increased secretory cells were not observed in either of these compound transgenics at E18.5, but Math1 mRNA abundance has not been analyzed in these mice, so it is unknown if Math1 expression was comparable to Vil-Math1 transgenic founders.

Recent reports in the literature have demonstrated regulation of Math1 transcription and protein stability by Wnt-regulated proteins GSK3β and β-catenin[29-32]. Active Wnt signaling inactivates GSK3β kinase, which results in accumulation of unphosphorylated β-catenin that translocates to the nucleus and activates gene transcription. A nuclear complex of stabilized β-catenin and Tcf/Lef factors on the Math1 3' enhancer has been shown to be important for induction of Math1 transcription at baseline conditions and after Notch inhibition

in the neuroblastoma cell line Neuro2a and also in neural progenitor cells derived from embryonic stem cells[29]. Conversely, active Notch signaling increased GSK3β activity via phosphorylation at Y216, a critical activating modification, therefore decreasing stabilized nuclear β-catenin and decreasing Math1 transcription[29]. Accordingly, disruption of Notch signaling resulted in a relative decrease in the amount of phospo-Y216 GSK3β and both increased nuclear β-catenin and Math1 transcription[29]. Thus, the authors of this study proposed a regulatory hierarchy such that Notch signaling must first be turned down and then β-catenin can initiate Math1 transcription[29]. Furthermore, GSK3β appears to be a key point of convergence of the Notch and Wnt signaling pathways[29, 33] (Fig. 3-10A). While these studies suggest that Wnt signaling is important for activating Math1 expression, transgenic Math1 expression in our system is not regulated by endogenous enhancers. Thus, it is more likely that Math1 protein is being regulated in our system.

Wnt signaling and GSK3β have also been implicated in regulation of Math1 protein stability. Colon cancer cells maintain their proliferative, undifferentiated state due to hyperactive Wnt signaling and down-regulation of Math1 protein[31, 32, 34]. In addition, active Notch signaling appears to be important for tumor progression[35]. While some human colon cancer samples had down-regulated HATH1 (human ortholog of Math1) mRNA, others had HATH1 mRNA abundance that was comparable to healthy neighboring tissue[32], suggesting that Math1 protein levels are negatively regulated in colon cancer. Treatment of the colon cancer cell line SW480 with proteasome inhibitors resulted in increased expression of HATH1 protein, suggesting that HATH1 protein is normally targeted for degradation by the proteasome in cancer cells[30]. Finally, Wnt-dependent and GSK3β-mediated phosphorylation at HATH1 S54 and S58 was determined to target HATH1 protein for degradation[30]. Thus, in cancer cells, GSK3β appears to be a key switch regulating stability of β-catenin versus HATH1 (Fig. 3-10B). Furthermore, because forcing stable HATH1 expression in colon cancer cell lines slows cellular growth and activates differentiation genes,

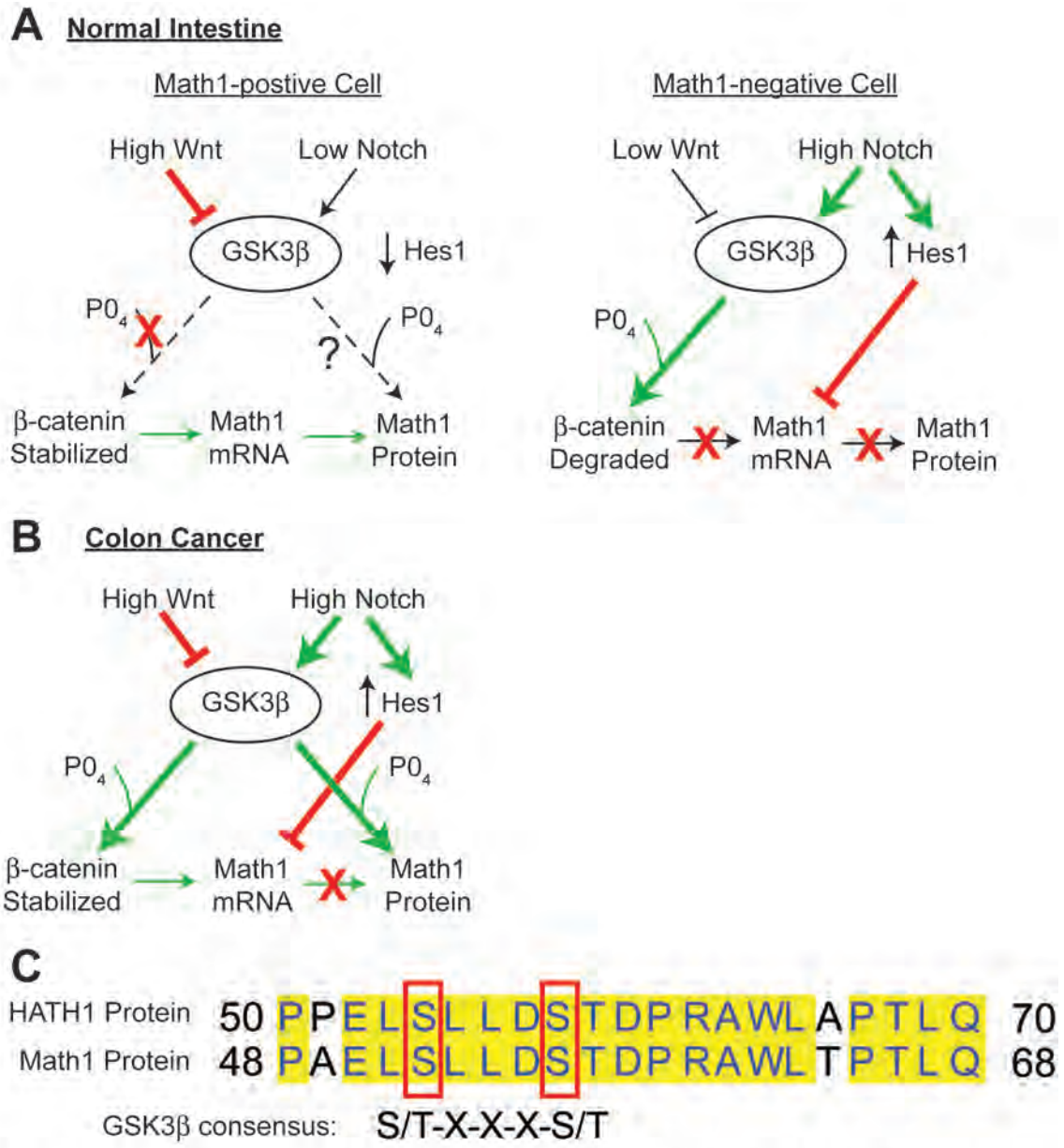

Figure 3-10. Model of Math1 mRNA and Protein Regulation in the Intestine. Notch and Wnt signaling pathways regulate GSK3β activity in opposing manners. When Wnt signaling is high and Notch signaling is low, unphosphorylated β-catenin is stable and translocates to the nucleus where it participates in activation of Math1 transcription (A). It is unknown whether GSK3β regulates Math1 protein levels during baseline conditions. When Wnt signaling is low and Notch signaling is high, β-catenin is phosphorylated and degraded; additionally, Math1 transcription is repressed by Hes1 (A). In colon cancer, both Wnt and Notch signaling are hyperactive, which provides possible mechanisms for blocking Math1 transcription as well as targeting Math1 for proteasome-mediated degradation by GSK3β phosphorylation at serine 52 and serine 56 (B). Alignment of the mouse Math1 protein and its human ortholog, HATH1, showing conservation of the GSK3b phosphorylation site (C).

including Mucin2[31, 32], more knowledge of mechanisms regulating Math1 protein stability could contribute to development of colon cancer therapeutics.

Alignment of Math1 and HATH1 amino acid sequence shows that the GSK3β consensus site is conserved between both species (Fig. 3-10C). Therefore, Math1 protein in the VZM;Cre trangenics could be degraded in a Wnt-dependent manner via GSK3b phosphorylation at Math1 S52 and S56. Accordingly, experiments that tested whether Math1 protein accumulated in VZM;Cre transgenic intestine treated with proteasome inhibitor could differentiate between the two possibilites that 1) Math1 protein is not being made, and 2) Math1 protein is being made but being degraded in a proteasome-dependent manner. Finally, GSK3β inhibitors or DNA constructs in which the S52 and S56 sites were mutated could be used to determine if Math1 protein is targeted for degradation due to phosphorylation by the GSK3β kinase.

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CHAPTER 4

NOTCH SIGNALING DIRECTLY TARGETS THE CRYPT BASE COLUMNAR STEM CELL

SUMMARY

Notch is a critical pathway regulating proliferation and differentiation in the intestine. Blocking Notch signaling by genetic or pharmacologic means results in decreased epithelial cell proliferation and altered cell fate, suggesting that a stem or progenitor cell is targeted by Notch; however, the identity of this target is unknown. This study sought to identify Notch-responsive cells by analysis of stem and progenitor markers in fetal and adult mouse intestine after Notch inhibition. Notch signaling was disrupted in two mouse models by treatment with a γ-secretase inhibitor (GSI): dibenzapine (DBZ) in adult mice and DAPT in fetal intestinal organ cultures. Cell lineages were measured by histological analysis and marker gene expression. Consistent with other studies of Notch disruption both GSI-treated models exhibited reduced proliferation and increased secretory cell types, including goblet, Paneth and endocrine cells, demonstrating effective Notch inhibition. Interestingly, intermediate cells that possess characteristics of both goblet and Paneth cells were observed in the crypts of adult ileum and colon, suggesting that Notch may regulate cell specification and maturation in distinct ways. Stem and progenitor cell marker expression was measured by qRT-PCR, including genes specific for the +4 quiescent stem cell, the active crypt base columnar (CBC) stem cell and progenitor cells. We found that disruption of Notch signaling affected the CBC stem cell specific transcript olfmactomedin 4 (Olfm4), while the other markers were unchanged. Olfm4 was reduced 40-fold in DAPT-treated fetal intestine cultures and 13-fold in DBZ-

treated adult mouse intestine, suggesting that Notch activates transcription of this gene. The mechanism of Notch regulation of Olfm4 gene expression was further examined by transient transfection studies in the human colon cancer cell line LS174T. An OLFM4-luciferase construct containing 427 base pairs of human 5' proximal promoter sequence had increased activity with constitutive Notch signaling and decreased activity in the presence of GSI or dominant negative mastermind, a key component of the Notch transcriptional complex, consistent with OLFM4 expression being activated by Notch signaling. Analysis of the OLFM4 5' proximal promoter revealed three consensus binding sites for the essential Notch co-activator, RBP-J. The most upstream RBP-J site was not required for Notch-mediated regulation of OLFM4-luciferase activity; however, deletion of the region containing the other two binding sites resulted in loss of responsiveness to constitutive Notch signaling. These studies have shown that Notch signaling targets the intestinal CBC stem cell to induce transcription of the secreted protein OLFM4. In addition, disruption of Notch signaling induces formation of intermediate cells in crypts of fetal and adult mouse intestine.

INTRODUCTION

The intestinal epithelium is arranged into finger-like villi that extend into the lumen and invaginations into the mesenchyme called crypts. The mature epithelium is comprised of four differentiated cell types including enterocytes that belong to the absorptive (columnar) lineage and goblet, endocrine and Paneth cells that belong to the secretory (granulocytic) lineage. The villi contain mature enterocytes, goblet cells, and endocrine cells while the crypts contain the Paneth cells and the proliferative stem and progenitor cells. All of these cell types are derived from stem cells that replenish the epithelium throughout the lifespan on an organism. Notch signaling regulates homeostasis of the intestinal epithelium through maintenance of the progenitor cell pool and through binary cell fate selection between the two differentiated lineages of the intestinal epithelium[1].

Notch signaling occurs between neighboring cells; both the Notch receptors and ligands are transmembrane proteins (see Fig. 1-2, reviewed in [2]). Engagement of Notch receptor by ligand initiates a series of receptor cleavages mediated by the A Disintegrin and Metalloprotease (ADAM) and the γ-secretase complex, ultimately resulting in Notch Intracellular Domain (NICD) release from the membrane and translocation to the nucleus. In the nucleus, NICD interacts with its co-activators RBP-J (CSL) and mastermind (MAML) to activate gene transcription of target genes including Hairy and enhancer of split 1 (Hes1). Localization of NICD or Hes1 protein shows that active Notch signaling occurs in the crypts, suggesting that Notch may target the intestinal stem cells[3, 4]; however, the cellular identity of Notch signaling targets is unknown.

Rodent models with alterations in intestinal Notch signaling have demonstrated the importance of Notch pathway regulation of stem or progenitor cell populations. Disruption of Notch signaling in mice by genetic depletion of RBP-J, Hes1, or both Notch1 and Notch2 receptors resulted in depletion of proliferating cells[3, 5, 6]. Conversely, constitutively active Notch signaling results in expansion of the proliferative zone[7, 8] and hyperactive Notch signaling is associated with colorectal cancer formation[9]. These data demonstrate that Notch signaling can control the balance of differentiated versus undifferentiated cells in the intestinal epithelium; it is unknown if Notch achieves this control through general affects on all cell populations or if it can directly regulate a specific progenitor cell population.

In addition to regulation of epithelial proliferation, Notch signaling controls selection of the absorptive cell lineage over the secretory lineage. Disruption of Notch signaling leads to increased differentiation to the secretory lineage[3, 5, 6, 10] while activated Notch signaling results in loss of secretory cells[7, 8]. Notch signaling controls lineage selection primarily through regulation of the basic helix-loop-helix transcription factor Mouse atonal homolog 1 (Math1); Math1 is both required and sufficient for differentiation of all secretory cells[11] (Chapter 2). Notch signaling acts to repress Math1 expression via the transcriptional repressor Hes1[12]. Accordingly, when Notch signaling is disrupted, there is

increased expression of Math1[3, 6]. The use of γ-secretase inhibitors (GSIs) to block Notch signaling in rodents resulted in similar phenotypes to genetic models of Notch inhibition[6, 10]. However, there are conflicting reports in the literature in regard to the cell changes induced by GSI treatment. One study in rat reports GSI-induced increases in all secretory cell lineages[10] while a study in mouse reports that only goblet cells were induced by GSI[6]. Thus, some discrepancy about the secretory cell phenotype resulting from Notch inhibition exists in the literature and, therefore, this phenotype should be carefully reexamined.

Recently, many advances have been made in the intestinal stem cell field, including identification of mRNA markers that are expressed in specific stem and progenitor cell populations[13]. It is thought that two stem cell populations are present in the intestine, one of which is located at the so-called "+4 position" above the Paneth cell compartment at the crypt base and marked by Bmi1 expression[14] and the crypt base columnar stem cells which are interspersed among the Paneth cells and marked by leucine-rich-repeat-containing G-protein-coupled-receptor 5 (Lgr5), Achaete scute-like 2 (Ascl2), and Olfmactomedin 4 (Olfm4; also called GW112 and hGC-1)[15, 16]. The +4 position stem cell likely represents a quiescent stem cell population while the CBC has been shown to be actively cycling[17]. Intestinal stem cells supply the population of short-lived transit amplifying progenitors, likely marked by Prominin1 (Prom1)[18, 19]. Mushashi1 (Msi1) appears to be expressed in both of the stem cell populations and may also be expressed in at least some transit amplifying progenitor cells, thus, making it a more general progenitor cell marker[20-22]. Identification of these markers has made it possible to distinguish distinct populations of stem and progenitor cells in the intestine.

Since pharmacological agents disrupting Notch signaling throughout the body, including GSIs, are currently under study for treatment of Alzheimer's disease and T-cell leukemia[23, 24], it is important to characterize the intestinal response to Notch inhibition. In this study, we sought to determine which stem cells and/or progenitor cell populations are responding to active Notch signaling by examining cellular markers of stem and progenitor cells in mouse models of

GSI-mediated Notch disruption. This study led us to identify a stem cell gene that is likely a direct target of Notch signaling, Olfm4. In addition, we further characterized the expansion of secretory cells that accompanies the loss of proliferation in Notch disruption models in response to increased Math1 expression.

MATERIALS AND METHODS

Mice

Experiments were done using wild type C57BL/6 mice unless otherwise noted. Math1$^{lacZ/lacZ}$ mice were obtained from Jackson Laboratories (stock # 005970) and genotyped by polymerase chain reaction (PCR) as described[11, 25]. Hes1-GFP and Hes5-GFP mice[26] were genotyped by PCR of a ~250 base pair product with the following primers: GFP_1 5'- GCA CGA CTT CTT CAA GTC CGC CAT GCC; GFP_2 5'- GCG GAT CTT GAA GTT CAC CTT GAT GCC. Mice were maintained in a specified-pathogen-free barrier facility under a 12-hour light cycle. All experiments were performed according to protocols approved by the University of Michigan Committee on Use and Care of Animals.

Intestinal Organ Culture

To stage pregnancies, mice were mated and the morning of the vaginal plug were considered embryonic day (E) 0.5. Embryos were collected from pregnant females at E15.5 and the gastrointestinal tract was dissected out from each embryo and temporarily kept in PBS on ice. The small intestine from each embryo was split into four segments of equal length (referred to as A, B, C, and D from proximal to distal). Once all dissections were complete, intestinal segments were transferred onto a transwell membrane in a 6-well tissue culture plate format (Costar 3428) containing 950 μL BGJ/b media (Gibco 12591) with 0.1 mg/mL ascorbic acid (Sigma A-4544) and 1X Pen-Strep (Gibco 15070). DAPT (gamma-Secretase Inhibitor IX in Solution; Calbiochem 565784) or an

equivalent volume of DMSO (vehicle) was added to the media to a final concentration of 10 μM, 20 μM, or 40 μM. A small number of organ cultures were treated with 300 μM DBZ to confirm that a similar phenotype was observed after treatment with a different γ-secretase inhibitor (GSI). Organ cultures were incubated at 37°C with 5% CO_2 for 1-3 days with media changed daily. Segments A and D were processed for histology of proximal and distal intestine, respectively. Segments B and C were pooled for RNA analysis.

Administration of dibenzapine (DBZ) and bromodeoxyuridine (BrdU)

Administration of DBZ was performed using a method similar to previously published studies[6, 10]. To make a 0.3 M stock solution of DBZ, 0.01389 g of DBZ (Syncom) was dissolved in 100 μL DMSO and stored at 4°C in the dark. To make 3 mL of injection solution, 30 μL of 0.3 M DBZ stock solution was finely suspended in 1.5 mL 1% (w/v) hydroxypropyl methylcellulose (Methocel E4M) and 0.6 mL 0.5% (w/v) Tween80 (Sigma P-1754) in H_20. Vehicle injection solution contained 30 μL of DMSO in place of DBZ stock solution.

DBZ injection solution or an equivalent volume of vehicle injection solution was injected i.p. into 7-8 week old mice at a dose of 30 μmol/kg (10 μL/g body weight) or 10 mmol/kg (3.3 μL/g body weight) daily for 5 days. Mice were fasted overnight on the 5th day and sacrificed for tissue collection on day 6.

For adminstration of BrdU (Sigma B-9285), a 5 mg/mL BrdU solution was made fresh in phophate buffered saline (PBS). A 50 mg/kg (10 μL/g body weight) dose of BrdU solution was delivered by i.p. injection 2 hours prior to sacrifice.

Collection of Tissues and Histological Analysis

For adult mice, the intestine was dissected out and regions were identified as follows: duodenum (4 cm segment distal to the pylorus), jejunum (4 cm segment at the midpoint), ileum (4 cm segment proximal to the cecum) and colon (4 cm segment distal to the cecum). The proximal 1 cm of each tissue segment was processed for paraffin embedding after fixation in 4% paraformaldehyde at

4°C overnight. Paraffin processing of segments A and D from fetal organ cultures was performed similarly. Paraffin sections (5 μm) were stained with H&E to assess cellular morphology and Periodic-acid Schiff (PAS)/Alcian blue (Newcomer Supply) to stain mucin-containing goblet cells. Immunostaining was performed as previously described[27] using the following primary antibodies: rabbit anti-lysozyme (1:100, Zymed), rabbit anti-chromogranin A (1:500, 94188/5 gift from J. F. Rehfeld), rabbit anti-Mucin2 (1:200, Santa Cruz), rat anti-MMP7 (1:100, gift from P. J. Dempsey), mouse anti-BrdU (1:50, Dako), and chicken anti-GFP (1:500, Aves Laboratories). For immunofluorescence, the appropriate secondary antibodies conjugated to AlexaFluor488, AlexaFluor555 (1:400; Invitrogen), or FITC (1:400) were used along with a DAPI nuclear stain (ProLong Gold, Invitrogen). For BrdU immunostaining, biotinylated goat anti-mouse IgG (1:200 Vector Laboratories) secondary antibody was used and staining was visualized with the DAB kit (Vector Laboratories) and hematoxylin nuclear stain. Microscopy was performed with either a Nikon E800 or Olympus BX-51 equipped with a SPOT or Olympus DP70 digital camera, respectively. Confocal microscopy was performed with an Olympus FV500.

Analysis of Gene Expression

RNA was isolated from the distal 2 cm of each region of adult intestine using Trizol (Invitrogen) according to the manufacturer's directions. RNA was then DNase-treated and purified using the RNeasy Mini kit (Qiagen). For intestinal organ cultures, B and C segments were pooled from two embryos for each sample and RNA was isolated with the RNeasy Mini Kit according to the manufacturer's directions. Gene expression was determined by quantitative reverse transcription-PCR (qRT-PCR). RT reactions (50 μl) used 1 μg RNA and the Iscript cDNA synthesis kit (Bio-Rad), as recommended by the manufacturer. qRT-PCR was performed as described[28] with SYBR green dye and the primers listed in Table 4-1. Expression levels were determined with triplicate assays per sample and normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (Gapdh), which remained the same in all samples.

Luciferase Constructs and Generation of Olfm4 Promoter Mutants

The human OLFM4 expression constructs 2039OLFM4-luciferase and 427OLFM4-luciferase were generated as described[29] (gift from G.P. Rodgers). The dominant negative mastermind (dnMAM) construct MCSV-dnMAM was a gift from I. Maillard[30]. The Notch pathway activator 3XNICD1 was a gift from R. Kopan[31]. Deletion and mutation of the -251 RBP-J site in the OLFM4 promoter used the QuikChange Mutatgenesis Kit (Stratagene) and the primers listed in Table 4-2 according to manufacturer's directions. Generation of the OLFM4 promoter deletion series was done with an overlap extension PCR method using the primers listed in Table 4-2 and 427OLFM4-luciferase as a template. Briefly, the 5' promoter segment and 3' promoter segment flanking each deletion were amplified using the outside primers pGL3-RVprimer3 and pGL3-GLprimer2 and a corresponding set of deletion-specific primers. The two promoter segments were then ligated together by a second PCR step with the outside primers only. The deletion-containing PCR products were cloned into 427OLFM4-luciferase subsequent to digestion with *KpnI* and *NheI*. Constructs were verified by analysis of digested products and sequencing.

Cell Culture, Transient Transfections and Luciferase Assays

LS174T colon cancer cells (ATCC #CL-188) were grown in MEM media (Gibco 11090) containing 10% fetal calf serum (Atlanta Biologicals), sodium pyruvate (Gibco 11360), non-essential amino acids (Gibco 11140), and Pen-Strep-Glut (Gibco 10378) at 37°C and 5% CO_2. The afternoon before transfection, cells were split into 24-well plates at $5x10^5$ cells/well so that they would be ~90% confluent at the time of transfection. Equivalent amounts of luciferase contructs and co-factors were transfected per well using Lipofectamine2000 (Invitrogen) according to manufacturer's instructions (0.8 μg DNA total). In cases where co-factors were not transfected, an equivalent amount of promoterless DNA (pBluescript SK) was added to maintain 0.8 μg total DNA. Cell lysates were collected 24-48 hours post-transfection after a brief wash

Table 4-1. qRT-PCR Primer Sequences			
Gene	Forward Primer	Reverse Primer	Amplicon (bp)
Ascl2	5'-CCT CTC TCG GAC CCT CTC TCA G	5'-GCA TGG AAG CAC ACC TTG ACT G	145
Bmi1	5'-TAT AAC TGA TGA TGA GAT AAT AAG C	5'-GAC ATA CCC AAT ACT TTC CAG	205
Gapdh	5'-TCA AGA AGG TGG TGA AGC AGG	5'-TAT TAT GGG GGT CTG GGA TGG	350
Gfi1	5'-TCG TCC GAG TTC GAG GAC TT	5'-CAG AGA GCG GCA CAG TGA CTT	77
Hes1	5'-GCT CAC TTC GGA CTC CAT GTG	5'-GCT AGG GAC TTT ACG GGT AGC A	75
Hes5	5'-GCT CGC TAA TCG CCT CCA GAG	5'-CTG CTC TAT GCT GCT GTT GAT GC	143
Hey1	5'-GCC GAC GAG ACC GAA TCA ATA AC	5'-CCC AAA CTC CGA TAG TCC ATA GCC	199
Hey2	5'-TTG AAG ATG CTC CAG GCT ACA GG	5'-CAT TGG ATT CCG AGA GTG CTT GAC	281
HeyL	5'-AGA GAC CGC ATC AAC AGT AGC C	5'-TCA GTG AGG CAT TCC CGA AAC C	215
Lgr5	5'-GAC AAT GCT CTC ACA GAC	5'-CCA TAA TAA TAG AAT CCA CTC C	143
Math1	5'-GCC TTG CCG GAC TCG CTT CTC	5'-TCT GTG CCA TCA TCG CTG TTA GGG	116
Msi1	5'-AAC CAC CAA CAG GCA CAG AGG	5'-ATG AAG GCA TCC ATT CCG TAG GG	201
Neurog3	5'-ACC CTA TCC ACT GCT GCT TGT C	5'-CGG GAA AAG GTT GTT GTG TCT CTG	136
Olfm4	5'-GCC ACT TTC CAA TTT CAC	5'-GTG TAT GAG AAG AGG CTC	199
Prom1	5'-CAC TCC TGA CTG AAA CAC CAA AGC	5'-TGC CAT CCA GGT CTG AGA ATG C	90
Spdef	5'-GGA CGG ACG ACT CTT CTG ACA G	5'-GCT CCT GAT GCT GCC TTC TCC	166

Table 4-2. Primer Sequences Used for Plasmid Construction		
OLFM4 Mutation	Primer Sequence	Deletion Size (bp)
RBPJa-deletion	5'-CTC TTT CAA GGA AGT ACC AAG ACC AAA TAT TGG GCT TTA CAA	6
RBPJa-mutation	5'-CAT CTC TTT CAA GGA AGT ACC AAG TGT TTA ACC AAA TAT TGG GCT TTA CAA AGC A	N/A
D427-306	5'-TGT GCT GGA ATT CGC CTC TGT GTC ATC CAC	122
D356-234	5'-GGA GAG CAC TAG AAG GGG CTT TAC AAA GCA C	123
D290-161	5'-CTC TGT GTC ATC CAC CCT CAC TGA CAA GGT	130
D217-98	5'-GGG CTT TAC AAA GCA CTC ACA CGT TCC CTG G	120
D145-56	5'-CCT CAC TGA CAA GGT CAT GGG GAA ATC ACC	90
D217-161	5'-GGG CTT TAC AAA GCA CCC TCA CTG ACA AGG T	57
D145-98	5'-CCT CAC TGA CAA GGT TCA CAC GTT CCC TGG	48
D82-56	5'-TCA CAC GTT CCC TGG CAT GGG GAA ATC ACC	27

in PBS followed by a 5 minute lysis with 100 μL passive lysis buffer (Promega). Luciferase activity was assayed in triplicate using 20 μL of cell lysates with Dual Luciferase reagents (Promega) and a PerkinElmer Victor^3 luminometer. Luciferase activity was normalized to total protein content, which was similar between samples. Samples for protein quantification were prepared by diluting cell lysates 1:10 in PBS and measuring protein content with a BCA Protein Assay kit (Thermo Scientific) according to the manufacturer's directions using 25 μL samples and running assays in duplicate.

Olfm4 Promoter Sequence and Transcription Factor Binding Site Analysis

Mouse and human Olfm4 promoter sequences were obtained from the UCSC genome database (www.genome.ucsc.edu) and aligned using Lasergene8 MegAlign software. Transcription factor binding sites were identified using the matrices defined by Genomatix software and default stringency parameters.

Statistical Analysis

Quantitative data were presented as mean ± SEM and analyzed by a 1-way ANOVA followed by a Tukey post test to compare data from GSI-treated samples to vehicle-treated controls with $P < 0.05$ considered significant.

RESULTS

Increased Differentiation of All Secretory Lineages in GSI-treated Adult Mice

Transmission of Notch signaling through the cytoplasm requires a γ-secretase-mediated cleavage event to release the bioactive Notch intracellular domain (NICD) from the membrane (see Fig. 1-2). We utilized the γ-secretase inhibitor (GSI) dibenzapine (DBZ) to inhibit Notch signaling in adult mice *in vivo.* Effective inhibition of Notch signaling was achieved in mice treated with a 30 μmol/kg dose of DBZ, as shown by decreased intestinal expression of the Notch

target genes hairy and enhancer of split related with YRPW motif (Hey) 1, Hey2, and HeyL compared to vehicle-injected control mice (Fig. 4-1). Interestingly, Hes1 and Hes5 were not significantly changed by GSI treatment (Fig. 4-1).

Notch signaling primarily regulates secretory cell differentiation through control of the bHLH transcription factor Mouse atonal homolog 1 (Math1)[11, 32] (Chapter 2). We observed a robust 9-fold increase in Math1 expression in GSI-treated mice[3, 6]. We also observed 2-fold and 8-fold increased expression of Math1 target genes growth factor independent 1 (Gfi1) and SAM pointed domain containing ets transcription factor (Spdef), respectively, (Fig. 4-2)[33-35]. These data show that Notch signaling was significantly inhibited and that the pro-secretory differentiation program had been activated.

Changes in secretory cell number were observed in both mice treated with 10 μmol/kg (data not shown) and 30 μmol/kg DBZ, with a much more dramatic increase in secretory cell number in the mice treated with the higher DBZ dose (Fig. 4-2). A dramatic expansion of goblet-like cells was observed by H&E staining (Fig. 4-2A, B) and by increased numbers of cells staining for mucin production with periodic acid Schiff-alcian blue (PAS-AB) staining (Fig. 4-2D, E). Increased numbers of endocrine cells were observed by immunostaining for the pan-endocrine marker chromogranin A (CgA). The expansion of goblet and endocrine cells was present throughout the intestine and colon (Fig. 4-2 and data not shown). Additionally, there was expansion of the third secretory cell type, Paneth cells, in the duodenum and jejunum. Expansion of the Paneth cell compartment at the base of the crypts was shown by increased staining with the Paneth cell marker lysozyme (Fig. 4-2 and data not shown). Thus, all secretory cell lineages were expanded in response to DBZ-treatment.

GSI-treatment Leads to Expansion of Intermediate Cells in the Distal Intestine

Intermediate cells are a rare epithelial cell type most often located on the lower lateral sides of the crypts and are named for their intermediate-sized secretory granules compared to those of immature goblet and immature Paneth cells[36, 37]. Intermediate cells have been reported to express some Paneth cell

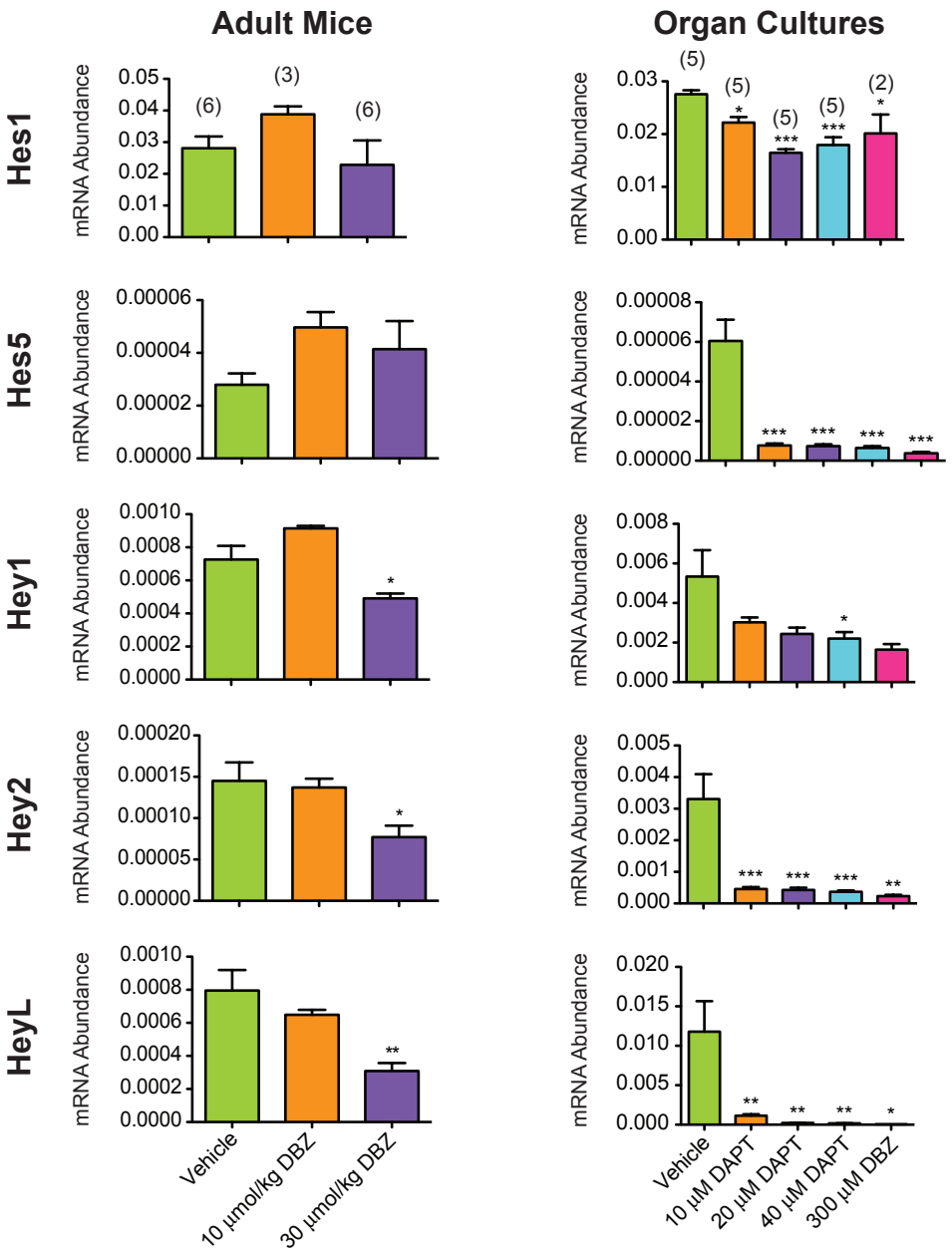

Figure 4-1. Effective Notch Disruption in GSI-treated Intestine. Expression of the Notch signaling target genes Hes1, Hes5, Hey1, Hey2, and HeyL was analyzed by qRT-PCR to show that Notch signaling is effectively inhibited in both adult mice treated with DBZ and in intestinal organ cultures treated with DAPT or DBZ. Data were normalized to Gapdh and presented as mean ± SEM (n for each group shown above bar in parentheses, *p<0.05; **p<0.001; ***p<0.001 by 1-way ANOVA followed by a Tukey post test).

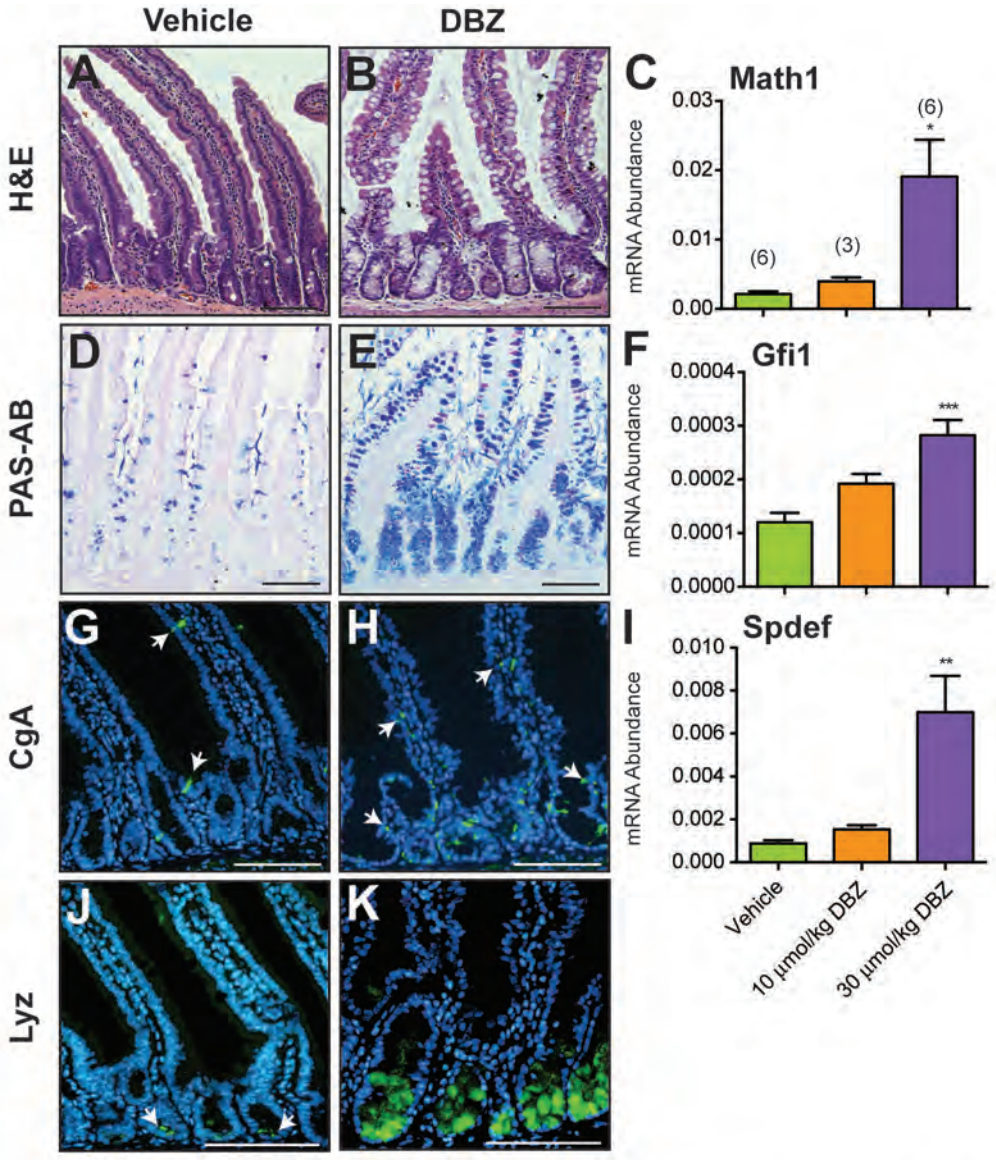

Figure 4-2. GSI Treatment Increases Math1 Expression and Induces Secretory Cell Differentiation. Secretory cell populations were analyzed in the duodenum of vehicle-treated (A, D, G, J) and 30 µmol/kg DBZ-treated (B, E, H, K) adult mice. Increased goblet cell number in DBZ-treated mice was apparent by hematoxylin and eosin (H&E) staining (A, B) and periodic acid Schiff-alcian blue (PAS-AB) staining (D, E). Immunostaining for chromograninA (CgA) demonstrated increased endocrine cells (arrows) in DBZ-treated mice compared to controls (G, H). Immunostaining for the Paneth cells (arrows) with lysozyme showed expansion of the Paneth cell compartment in the DBZ-treated intestine (J, K). Duodenal mRNA expression of Math1 (C) and its targets, Gfi1 (F) and Spdef (I). Data were normalized to Gapdh and presented as mean ± SEM (n is in parantheses above bars, *p<0.05, **p<0.001, ***p<0.001 by 1-way ANOVA followed by a Tukey post test).

markers including MMP7 and phospholipase A2 (Pla2g2a), but not lysozyme[36, 38]. Goblet cells markers such as Mucin2 (Muc2) are also present in intermediate cells due to small amounts of mucus in their cytoplasm, which will also stain with PAS-AB; however, other differentiated goblet cell markers such as trefoil factor 3 (Tff3) are not present in intermediate cells[39]. Thus, intermediate cells may represent a bipotential progenitor cell shared by the goblet and Paneth cell lineages[36, 37]. Surprisingly, we observed a dramatic expansion of intermediate cells in the ileums of DBZ-treated mice. Co-immunostaining for Muc2 and MMP7 showed significant expansion of cells co-expressing these markers located primarily in the mid crypt to lower villi (Fig. 4-3A, B). Paneth cells were still present in the GSI-treated ileum as evidenced by lysozyme-positive cells located at the base of the crypts and exclusion of Muc2 from this region (Fig. 4-3D). In contrast to the more proximal regions of the intestine, the Paneth cell compartment in the ileum appeared to be of roughly the same size in vehicle-treated and GSI-treated crypts. However, all intestinal regions had a similar change in the cellular localization of lysozyme protein, which is normally present as a punctate stain due to localization in secretory granules, but presented as a diffuse cytoplasmic stain in GSI-treated intestine suggesting that secretory granules may be altered in the Paneth cells of GSI-treated intestine (compare Fig. 4-3C, D to Fig. 4-2J, K and data not shown). Together, these data suggest that Notch signaling regulates specification to the Paneth/goblet lineage and maturation of these cell types in distinct ways.

The colon is normally devoid of Paneth cells or Paneth cell marker expression. Surprisingly, expression of the Paneth cell marker MMP7 was observed in scattered clusters of crypts in DBZ-treated colon and it was found to colocalize with Muc2, suggesting that an intermediate cell population had emerged in the colon (Fig. 4-3E, F). Lysozyme-positive cells were never observed in GSI-treated colon (data not shown) supporting the notion that the Muc2/MMP7-positive cells represented intermediate cells and not fully differentiated Paneth cells.

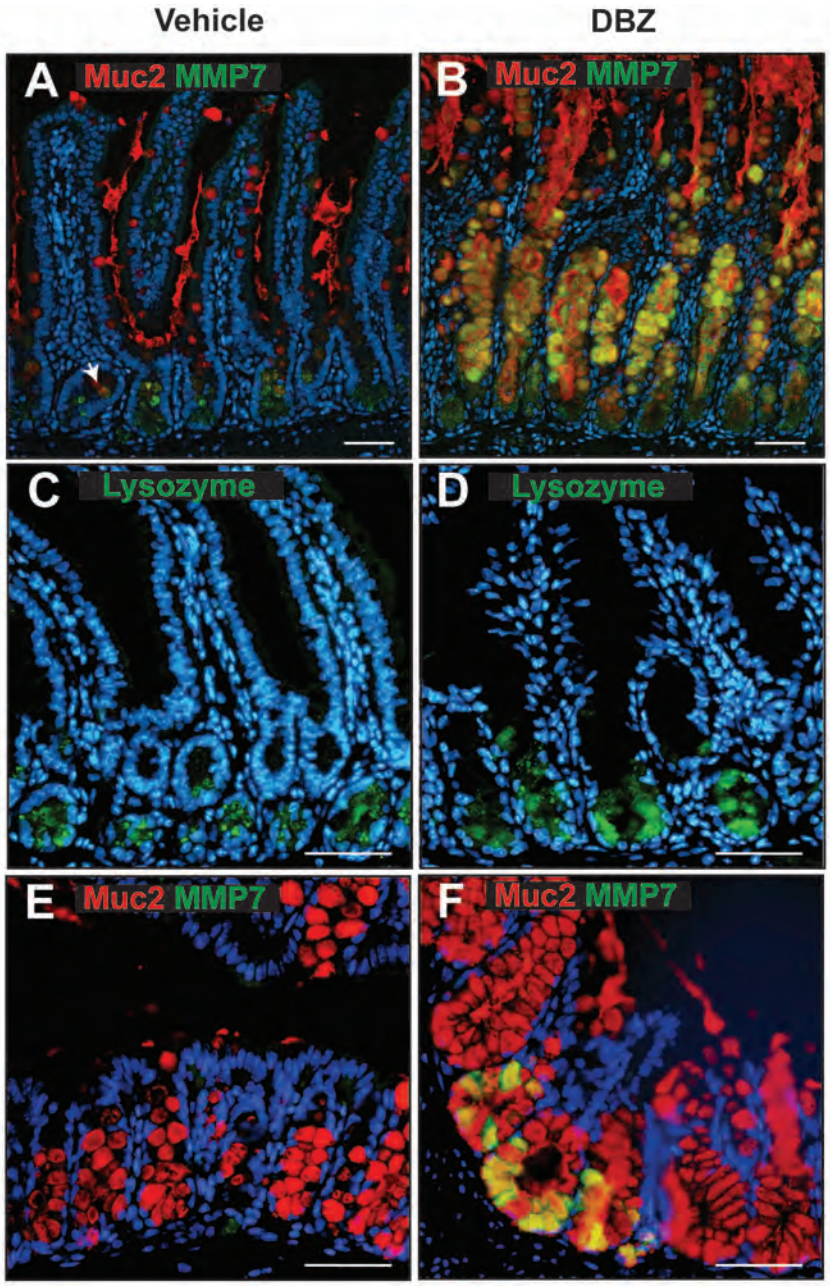

Figure 4-3. Expansion of Intermediate Cells in GSI-treated Ileum and Colon. Co-immunostaining for the goblet cell marker Muc2 (red) and the Paneth cell marker MMP7 (green) was used to identify intermediate cells (A, B, E, F). Rare intermediate cells were observed in vehicle-treated ileum (A, arrow) with a dramatic expansion of intermediate cell number observed in DBZ-treated ileum (B). Immunostaining for lysozyme (C, D) showed that Paneth cells are present in roughly equivalent numbers in the crypt base of vehicle-treated and DBZ-treated ileum; however, there is increased intensity and altered cellular distribution of staining in DBZ-treated intestine (E, F). Colons of DBZ-treated mice showed patches of MMP7/Muc2 co-stained intermediate cells (F) while no MMP7-positive cells were observed in vehicle-treated colon (E). Scale bars, 50 μm.

Increased Secretory Cell Differentiation Depends on Math1

We also explored the affect of Notch inhibition in the developing gut with a fetal organ culture system. Small segments of E15.5 intestine were cultured with media containing vehicle or the GSI DAPT for 3 days. Fetal intestine cultured with 40 μM DAPT had increased numbers of goblet and endocrine cells, the secretory cell types normally present before birth, compared to vehicle-treated cultures (Fig. 4-4). We observed increased mRNA expression of the endocrine progenitor cell marker Neurogenin3 (Neurog3) in DAPT-treated cultures, supporting increased differentiation to the endocrine lineage (Fig. 4-4N) [40-42]. Increased secretory cell differentiation was also observed in cultures treated with 10 and 20 μM DAPT, with a milder secretory cell phenotype at 3 days of culture compared to 40 μM DAPT-treated samples which became similar to 40 μM DAPT-treated samples by 6 days of culture (data not shown).

In addition to increased goblet and endocrine cell numbers, premature activation of Paneth cell markers was observed by immunostaining for lysozyme in DAPT-treated intestine (Fig. 4-4D, H); these cells tended to be located near the base of the intervillus zones (Fig. 4-4H). Interestingly, most cells of the intervillus zones stained with PAS-AB as well (Fig. 4-4F). Thus, it is very likely that the lysozyme and PAS-AB staining are co-labeling a subset of cells in the intervillus zones, although these Paneth-like cells are different than the intermediate cells observed in the ileum of adult DBZ-treated mice based on their lysozyme immunoreactivity.

Similar to the studies in adult mice, 6-fold increased expression of Math1 and 3-fold increased expression of Gfi1 were observed in 40 μM DAPT-treated organ cultures (Fig. 4-4). The expression of these genes increased in a dose-dependent manner in 3 day organ cultures treated with 10 μM, 20 μM, or 40 μM DAPT or 300 μM DBZ (Fig. 4-4L, M) confirming that the expression of these genes is inversely correlated to the degree of Notch inhibition and positively correlated with the degree of secretory cell differentiation (Fig. 4-1 and data not shown). Since Math1 has been shown to be required for and sufficient for intestinal secretory cell differentiation at steady state conditions[11, 32] (Chapter 2),

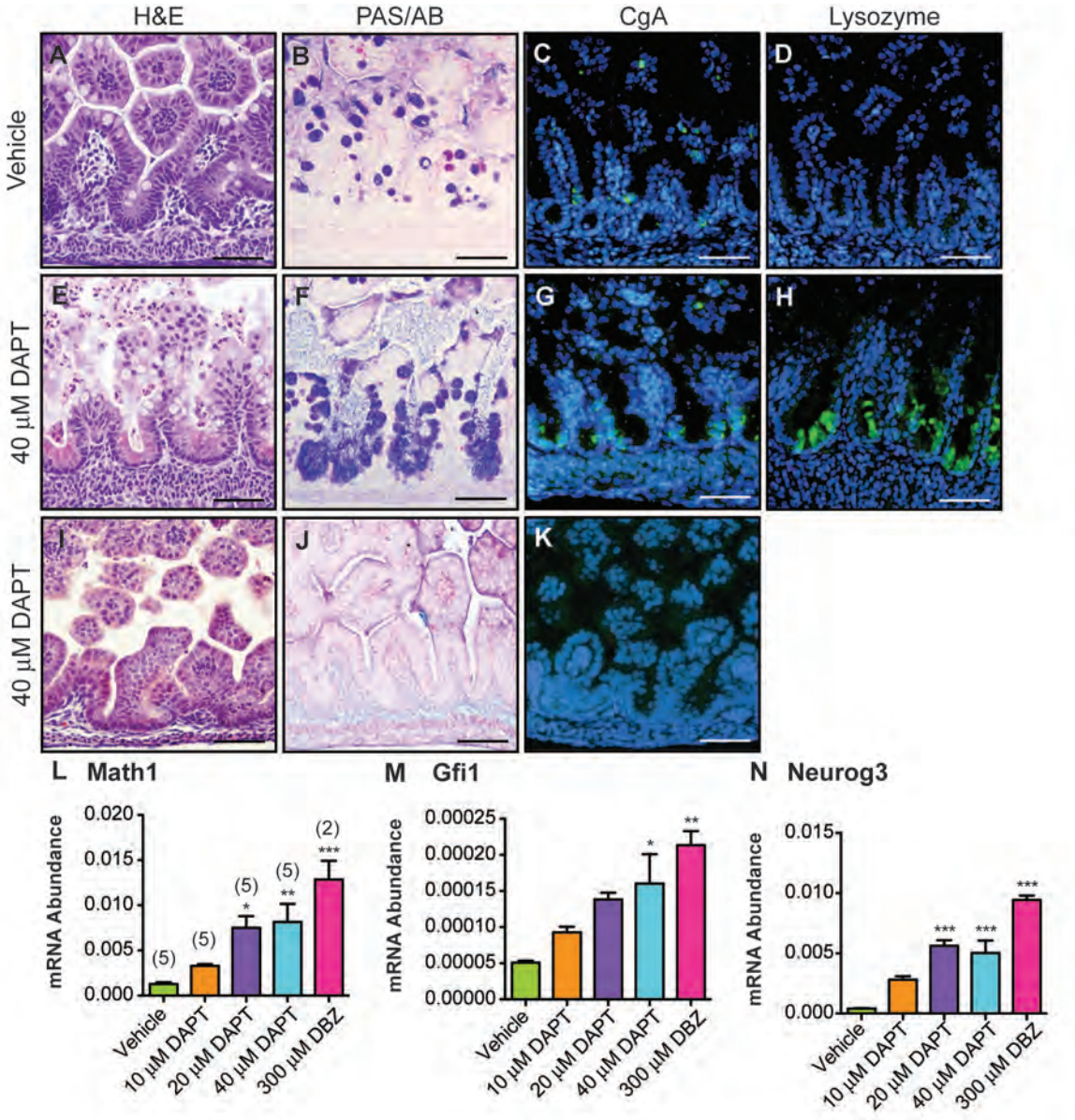

Figure 4-4. GSI-mediated Induction of Secretory Cell Differentiation is Dependent on Math1. Fetal intestinal organ cultures were treated with vehicle or 40 μM DAPT for 3 days and secretory cells were analyzed in the distal intestine by H&E staining for morphology (A, E, I), PAS-AB staining for goblet cells (B, F, J), CgA immunostaining for endocrine cells (C, G, K), and lysozyme immunostaining for Paneth-like cells (D, H). DAPT-treated intestine (E-H) had increased secretory cells compared to vehicle-treated intestine (A-D). Math1-deficient ($Math1^{lacZ/lacZ}$) intestine treated with 40 μM DAPT did not have any secretory cells (I-K). Expression of pro-secretory transcription factors Math1 (L), Gfi1 (M) and Neurog3 (N). Data were normalized to Gapdh with data presented as mean ± SEM (n was the same for each assay as shown above bars in (L) in parantheses, *p<0.05, **p<0.001, ***p<0.001 by 1-way ANOVA followed by a Tukey post test).

we sought to determine if Math1 is also required for the secretory cell induction subsequent to Notch inhibition by treating a mouse model deficient in Math1 (Math1$^{lacZ/lacZ}$) with GSI. The Math1$^{lacZ/lacZ}$ intestine lacks all secretory cells and dies shortly after birth purportedly due to the lack of certain respiratory neurons[11, 43] and, therefore, was unable to be examined during adulthood. Math1$^{lacZ/lacZ}$ intestinal organ cultures treated with DAPT were devoid of secretory cells (Fig. 4-4I-K), an identical phenotype to vehicle-treated Math1$^{lacZ/lacZ}$ intestine, demonstrating that Math1 is required for both normal secretory cell development and the induction of secretory cell differentiation subsequent to Notch inhibition.

The Crypt Base Columnar Stem Cell is a Cellular Target of Notch Signaling

Decreased proliferation was observed in DBZ-treated adult intestine, as demonstrated by decreased bromodeoxyuridine **(**BrdU) incorporation (Fig. 4-5)[5, 6]. This finding suggests that Notch signaling targets a stem cell and/or progenitor cell population; however, the identity of cellular targets of Notch signaling was unknown. Expression of a GFP reporter construct driven by a Hes1 or Hes5 promoter[26] is localized to the intestinal crypts (Fig. 4-6) suggesting that Notch signaling is normally acting in the progenitor cell zone.

To identify which progenitor cell populations are directly responding to Notch signaling, we examined mRNA expression of specific markers of stem and progenitor cell populations in Notch disruption models. Bmi1, which is expressed primarily in the "+4 position" stem cell[14], was unchanged in either GSI-treated adult intestine or fetal organ cultures (Fig. 4-7). The actively cycling CBC stem cell expresses Ascl2, Lgr5, Olfm4 genes[15, 16]. Lgr5 and Ascl2 were not significantly changed in either GSI-treated model; however, these markers trended towards being down-regulated in the DBZ-treated adult intestine (Fig. 4-7), suggesting that the CBC is still present in GSI-treated intestine, but the number of cells may be decreased slightly. Expression of Olfm4, however, was dramatically decreased in both adult intestine and organ cultures treated with GSI; the loss of Olfm4 gene expression occurred in organ cultures treated even with a very low 10 μM dose of DAPT (Fig. 4-7). These data suggest that the

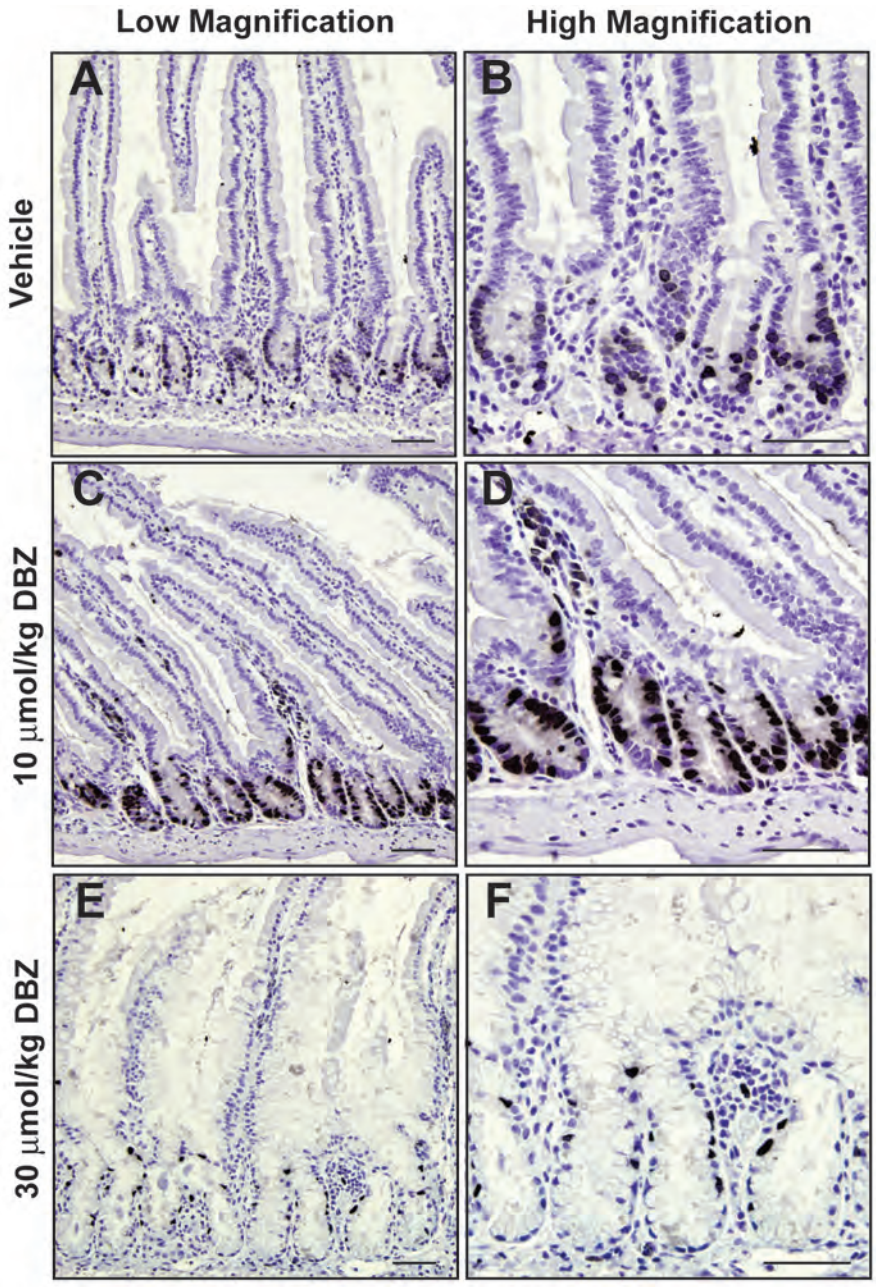

Figure 4-5. Altered Proliferation in DBZ-treated Intestine. Adult mice were administered BrdU 2 hours prior to sacrifice. Immunostaining for BrdU incorporation in vehicle-treated (A, B), 10 μmol/kg DBZ-treated (C, D) and 30 μmol/kg DBZ-treated (E, F) duodenum showed loss of proliferation in 30 μmol/kg DBZ-treated crypts compared to controls. Crypts from mice treated with 10 μmol/kg DBZ appeared to have increased BrdU incorporation compared to controls. (B, D, F) are higher magnification of (A, C, E). Scale bars, 50 μm.

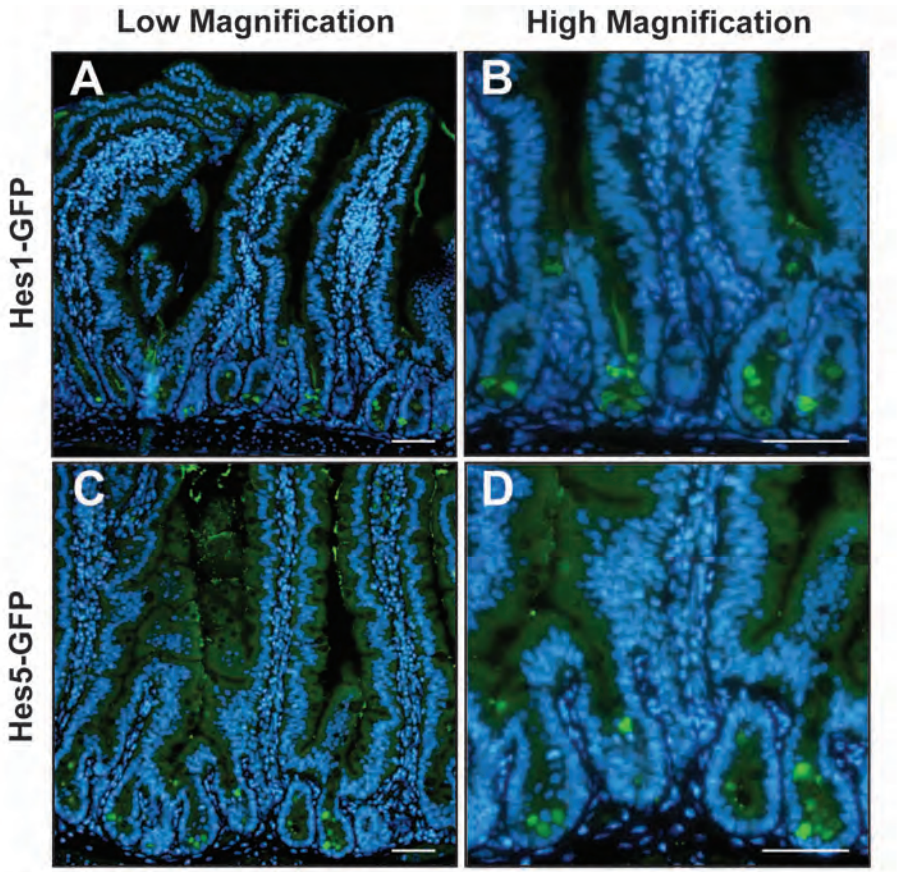

Figure 4-6. Active Notch Signaling in the Intestinal Crypts. The duodenal expression patterns of transgenic Hes1-GFP (A, B) and Hes5-GFP (C, D) reporter mice were analyzed in adult mice by immunostaining for GFP. GFP reporter expression was primarily localized to the lower portion of the crypts with a similar population of cells being labeled in each model. These data suggest that Notch signaling is active in the intestinal crypts. Similar expression patterns were observed in the jejunum and ileum (data not shown). (B) Higher magnification of (A). (D) Higher magnification of (C). DAPI, nuclear stain. Scale bars, 50 μm.

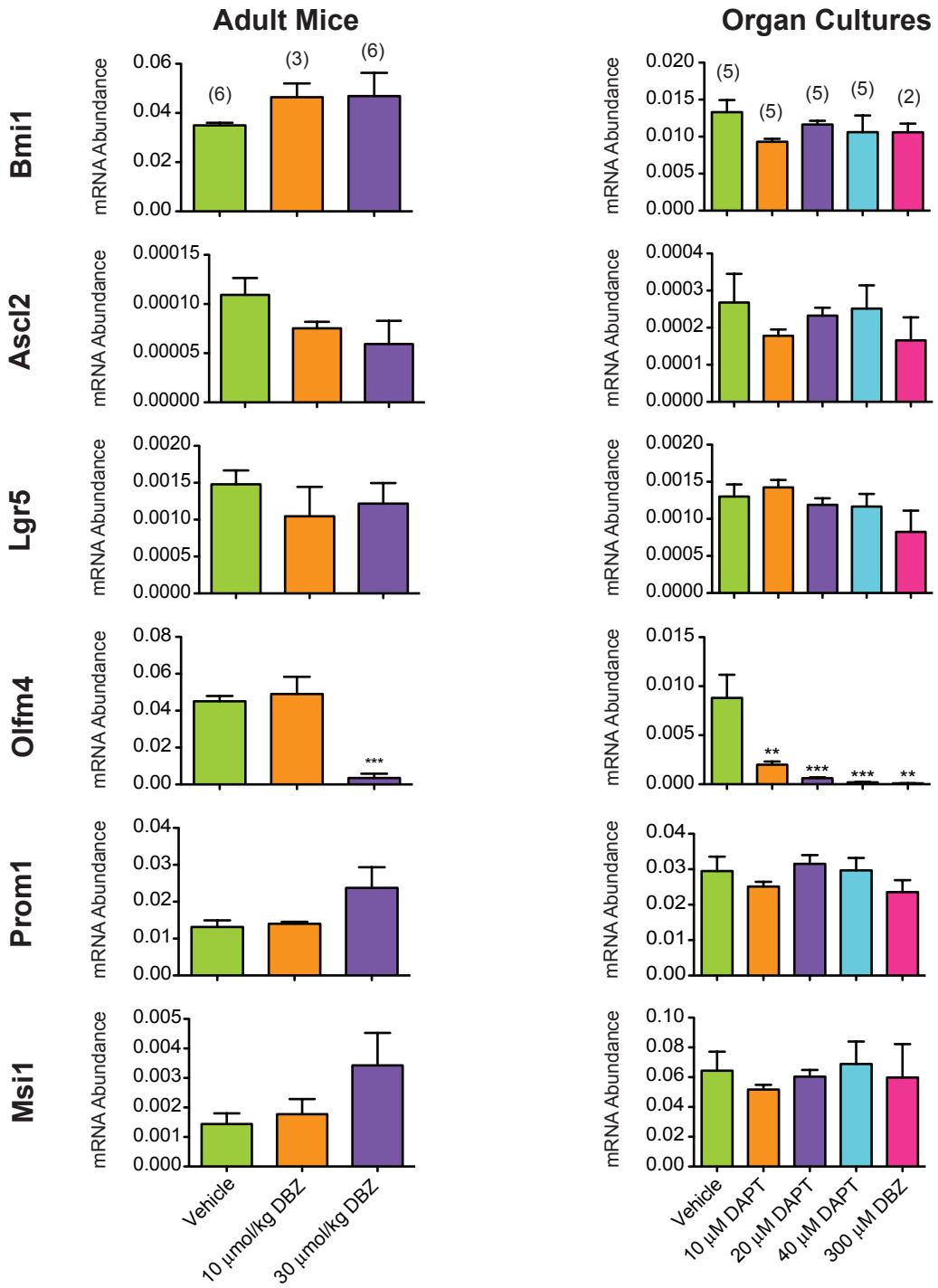

Figure 4-7. Olfm4 Expression is Decreased in GSI-treated Intestine. Intestinal stem cell marker expression was analyzed by qRT-PCR in GSI-treated intestinal organ cultures and adult mouse duodenum. +4 position marker (Bmi1); crypt base columnar stem cell markers (Ascl2, Lgr5, Olfm4). Transit amplifying progenitor marker (Prom1). General stem cell marker (Msi1). Data were normalized to Gapdh and presented as mean ± SEM, (n is shown above bars in parantheses, *p<0.05; **p<0.001; ***p<0.001 by 1-way ANOVA followed by a Tukey post test).

CBC is a cellular target of Notch signaling and Notch signaling activates Olfm4 gene expression in the CBC cell.

Finally, the transit amplifying progenitor cell marker Prom1[18, 19] and the general stem cell marker Msi1[20-22] were not significantly changed in either adult or developing intestine, but did trend towards being increased in DBZ-treated adult intestine (Fig. 4-6). The results from adult mice presented in Figure 4-6 are from duodenum, however, similar trends were also observed in the jejunum and ileum (data not shown). These data point towards Notch disruption leading to premature differentiation of CBCs which leads to increased numbers of transit amplifying progenitor cells and premature differentiation to the secretory lineage.

OLFM4 Promoter Activity is Regulated by Notch Signaling

To explore the relationship between Notch signaling and Olfm4 gene expression, we analyzed the expression of Hes1, Hes5, Math1, and Olfm4 after 1, 2, and 3 days of intestinal organ culture with vehicle- or 40 μM DAPT-treated media (Fig. 4-8). Expression levels of Hes1, Hes5, and Olfm4 were decreased in GSI-treated samples while expression of Math1 was increased. Hes1 was not down-regulated as quickly as Hes5, potentially due to other signaling pathways being involved in maintenance of Hes1 gene expression[44-47]. Loss of Olfm4 expression in embryonic organ cultures treated with 40 μM DAPT was significant by t-test ($p<0.0001$) at 1 day (Fig. 4-8D). Interestingly, the pattern of Olfm4 expression exactly mirrored the pattern of Hes5 expression, with the expression of each gene increasing in the vehicle-treated samples over the 3 days of the experiment and consistently low expression of each gene in the DAPT-treated samples. Moreover, the loss of Olfm4 expression occurred prior to a change in Math1 expression, suggesting that premature secretory cell differentiation is not responsible for the loss of Olfm4 gene expression.

To further test Notch-regulation of Olfm4 expression, we analyzed OLFM4 promoter activity with an *in vitro* luciferase assay. Luciferase constructs that contained either 2039 (2039OLFM4-Luc) or 427 (427OLFM4-Luc) base pairs (bp) of the human 5' OLFM4 promoter[29] had increased activity when co-

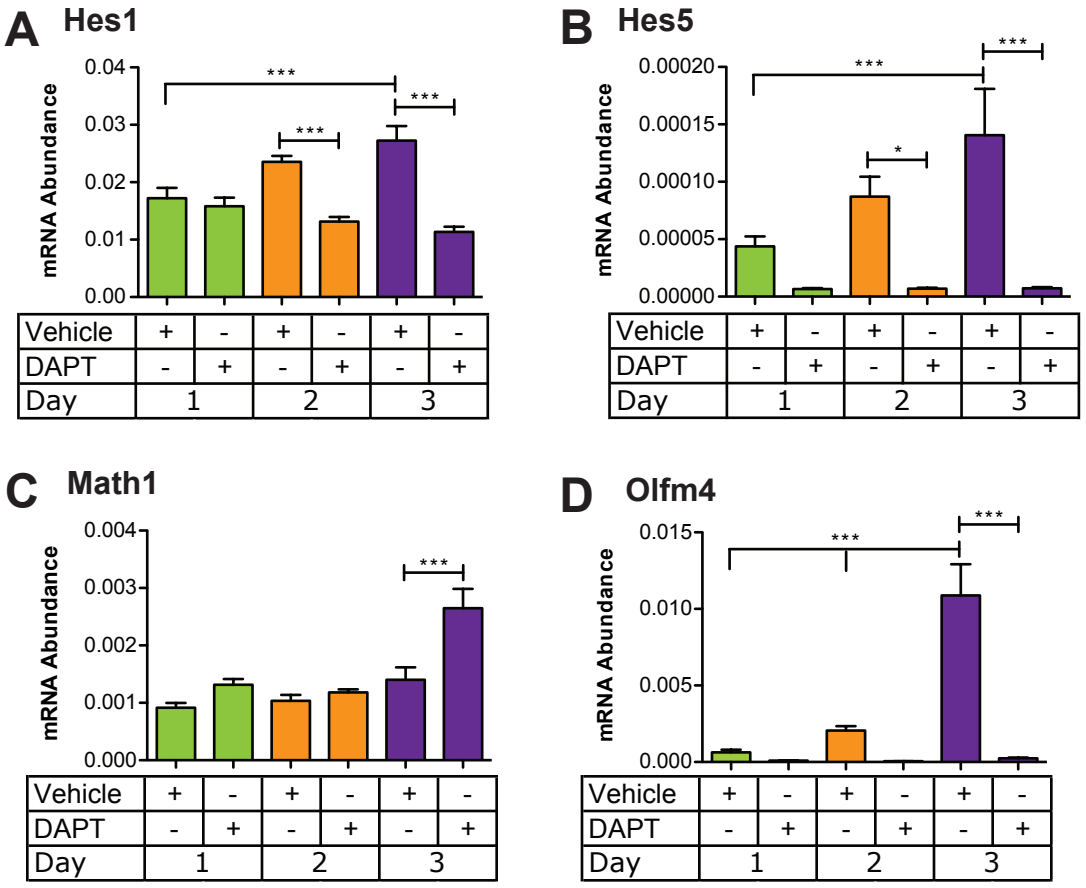

Figure 4-8. Olfm4 Expression Rapidly Decreases in Response to GSI Treatment. Embryonic intestinal organ cultures were treated with vehicle or 40 mM DAPT for 1, 2, or 3 days. Expression of Hes1 (A), Hes5 (B), Math1 (C), and Olfm4 (D) mRNA were measured by qRT-PCR. Olfm4 mNRA abundance was consistently lower in DAPT-treated samples compared to vehicle-treated controls. Data were normalized to Gapdh and presented as mean ± SEM (n=5 for all groups, *p<0.05; ***p<0.001 by 1-way ANOVA followed by a Tukey post test).

transfected with NICD1, a constitutively active form of the Notch1 receptor NICD (Fig. 4-9). The increase in activity was attenuated when a dominant-negative form of mastermind (dnMAML), an essential co-activator of Notch signaling, was added. Thus, activity of the OLFM4 promoter correlates to the amount of Notch signaling within the cell, suggesting that Notch signaling functions as an activator of OLFM4 expression and critical elements for Notch signaling control of OLFM4 expression are located within the 427 bp of 5' proximal promoter.

The -145 to -56 region of the OLFM4 5' Promoter Contains Critical Notch Regulatory Elements

The human proximal OLFM4 promoter sequence was analyzed for putative transcription factor binding sites that could confer Notch-mediated regulation of this gene (Fig. 4-10). This analysis revealed an optimal RBP-J binding site at position -251 upstream of the transcriptional start site (labeled RBP-Ja; Fig. 10). Deletion or mutation of the RBP-Ja consensus site responded to Notch activation in a similar manner as did the WT construct, showing that this site is not required for Notch regulation of the OLFM4 promoter (Fig. 4-9). Indeed, deletion of the region containing the site (Δ356-234 or Δ290-234) did not affect the ability of this construct to respond to Notch signaling (Fig. 4-11). Moreover, removal of a region containing a consensus N-box sequence (Δ427-306), the binding site for Hes1, had no affect (Fig. 4-11), suggesting that this region is also not required for Notch regulation of OLFM4 gene expression.

To identify the potential region by which Notch signaling controls OLFM4 promoter activity, a series of deletions were made in the promoter of 427OLFM4-Luc ranging from ~25 bp to ~120 bp. Luciferase assays of these constructs determined that loss of the sequence between -145 and -56 resulted in decreased ability of the promoter to respond to NICD1 (Fig. 4-11) suggesting that this region contains critical Notch signaling regulatory *cis*-elements.

In silico analysis using Genomatix identified two additional RBP-J consensus sites within the -169 to -80 region that were present in both the mouse and human Olfm4 promoters. One of these sites (RBP-Jb) was aligned in

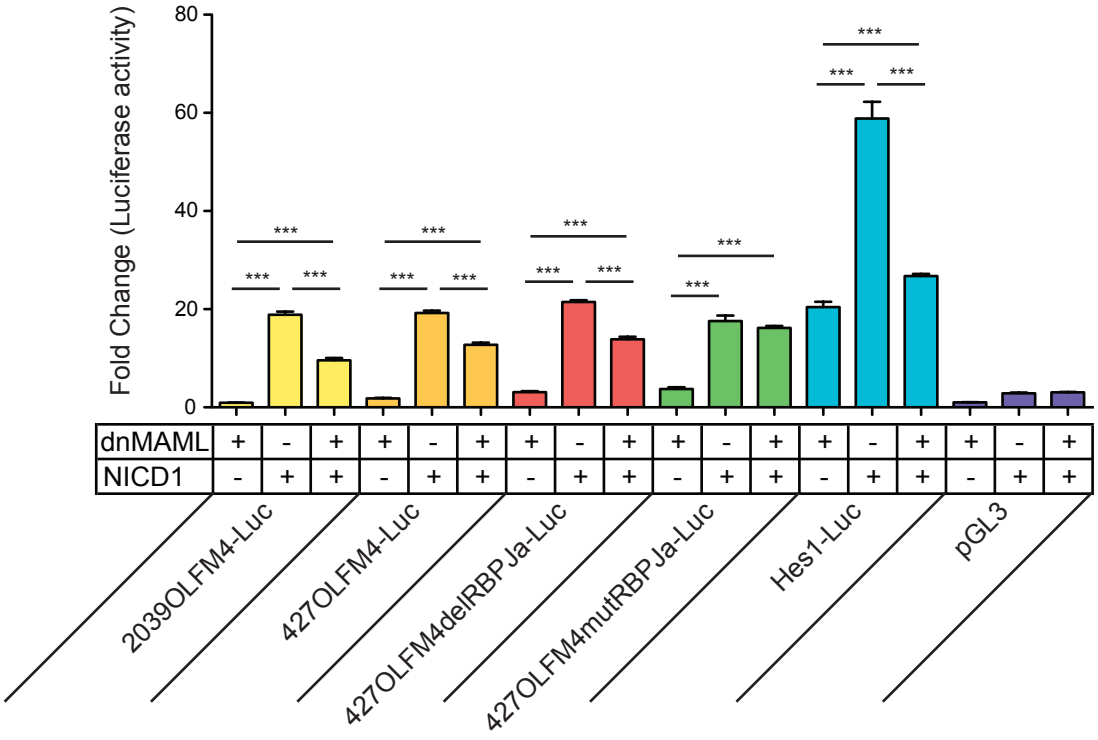

Figure 4-9. OLFM4 Promoter Activity is Regulated by Notch Signaling. Human OLFM4 luciferase constructs were transfected into LS174T cells along with dominant-negative MAML (dnMAML), constitutively active Notch1 receptor intracellular domain (NICD1) or both. OLFM4 promoter activity increased in response to NICD1 and decreased when Notch signaling was blocked with dnMAML. An RBP-J consensus site was identified at position -251 in the OLFM4 5' promoter (named RBP-Ja site in Figure 4-10). Constructs with deletion of this site (427HOLFM4delRBPJa-Luc) or mutation of this site (427HOLFM4mutRBPJa-Luc) still responded to activation and inhibition of Notch signaling, demonstrating that this site is not required for Notch- regulated OLFM4 promoter activity. Hes1-luciferase and pGL3 were used as positive and negative controls, respectively. Cell lysates were collected 48 hours post-transfection. Data were normalized to protein and presented as fold change compared to pGL3 + dnMAM (mean $\pm$ SEM; n=9; ***p<0.001 by 1-way ANOVA followed by a Tukey post test).

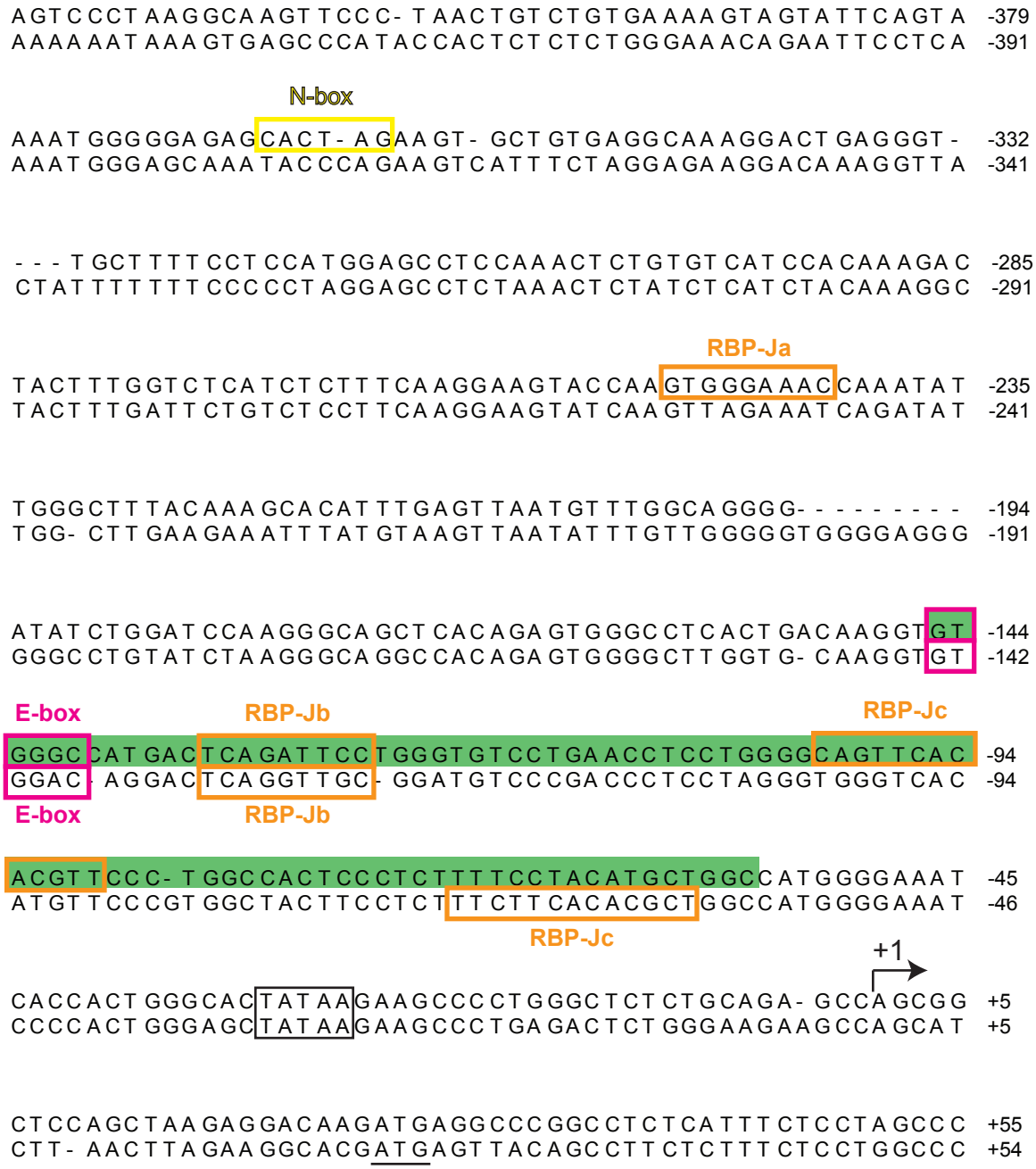

Figure 4-10. Alignment of Human and Mouse 5' Olfm4 Promoter Sequence and Positions of Selected Transcription Factor Consensus Binding Sites. Human (top) and mouse (bottom) Olfm4 promoter sequences were aligned and key *cis*-element seqeunces were annotated as follows: N-box (yellow box), RBP-J consensus (orange box), E-box (pink box), TATA box (black box), transcriptional start site (arrow, +1), translational start site (ATG underlined), -145 to -56 region (shaded green).

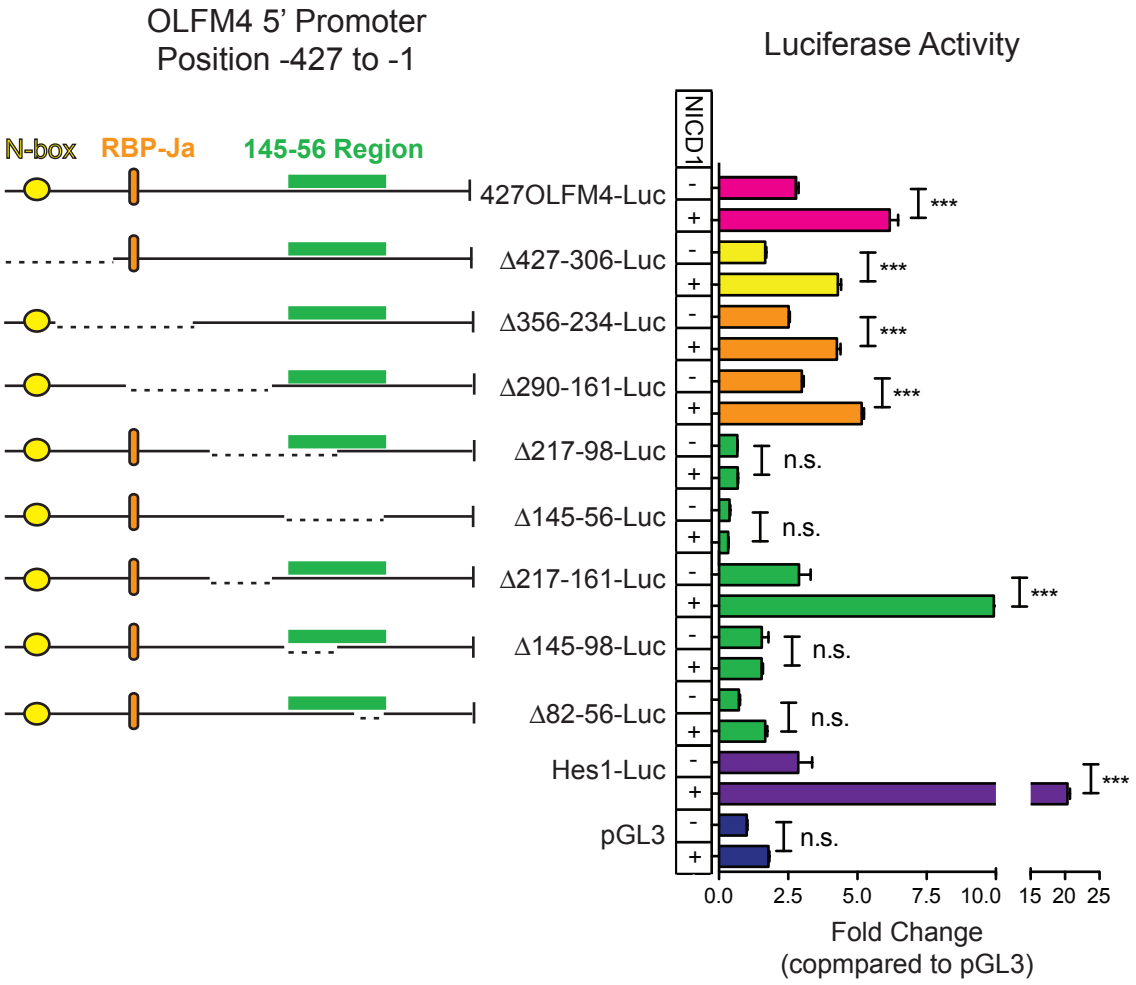

Figure 4-11. Critical Notch Regulatory *cis*-elements are Located in the -145 to -56 Region of the 5' OLFM4 Promoter. Regional deletions were made within the 5' promoter sequence of 427OLFM4-Luc and tested for their ability to respond to constitutively active Notch signaling. Constructs were transfected into LS174T colon cancer cells with or without the addition of NICD1. Hes1-Luc and pGL3 were used as positive and negative controls, respectively. A schematic of the OLFM4 promoter contained within each construct is shown on the left with the corresponding luciferase activity on the right. Deletion of a consensus N-box site is shown in yellow. Deletion of a consensus RBP-J site at position -251 (RBP-Ja) is shown in orange. Deletion of all or a portion of the -145 to -56 region is shown in green. Deletion contruct bar colors correspond to region deleted. Cell lysates were collected 24 hours post-transfection. Data were normalized to protein and and presented as fold change compared to pGL3 (mean ± SEM, n=9, ***p<0.001 by 1-way ANOVA followed by a Tukey post test).

the two species and near an aligned E-box consensus site, the binding site for bHLH transcription factors (Fig. 4-10). A second RBP-J consensus site (RBP-Jc) within the -145 to -56 region was observed in both human and mouse in close, but slightly different locations. These sites have a weaker overall consenus compared to the RBP-Ja site; however, these sites could be functional, especially if a nearby co-factor helped strengthen affinity for the site. Ascl2 is a bHLH transcription factor known to be expressed in the CBC and, additionally, many other bHLH transcription factors are regulated by Notch signaling in the intestine; thus, the presence of paired RBP-J and E-box consensus sites is very intriguing and could contribute to Notch regulation of OLFM4 expression. Additionally, many consensus binding sites for transcription factors that could potentially be important for intestinal Olfm4 gene expression regulation were identified including binding sites for the KLF family, HNF1 and HNF4, Gfi1 and Pax4 in both the human and mouse promoters (data not shown).

DISCUSSION

Notch signaling regulates the binary cell fate selection between absorptive and secretory lineages in the intestine through control of the balance between Hes1 and Math1[3, 6, 11, 32]. My studies and others have shown that Math1 is the key factor promoting differentiation of secretory cells. Forced expression of Math1 in the developing intestinal epithelium resulted in increased differentiation of all secretory cell types (Chapter 2). Despite reported increases in Math1 expression in models of Notch disruption, there has been some discrepancy as to which secretory cell lineages are expanded in GSI-treated rodents[6, 10]. We report here that all secretory cell types are expanded in GSI-treated mouse intestine, consistent with Math1 expression being sufficient for induction of all secretory cell types (Fig. 4-2) (Chapter 2). In addition, we show that Math1 expression is required for the increased secretory cell differentiation because

GSI treatment was unable to induce secretory cell differentiation in Math1$^{lacZ/lacZ}$ mice.

We made the novel observation that intermediate cells accumulate in the ileums and emerge in the colons of DBZ-treated intestine. Evidence points towards intermediate cells being a shared precursor cell of the goblet and Paneth cell lineage that is capable of proliferating[36, 37]. Paired with a loss of proliferative capability due to Notch inhibition, the intermediate cells observed in the ileum of DBZ-treated mice could be blocked from terminal differentiation to the Paneth or goblet cell lineage, resulting in their accumulation. Interestingly, there was regional heterogeneity in the response to GSI-treatment with increased Paneth cell differentiation in the proximal intestine and increased intermediate cells in the distal intestine. The Paneth cell compartment in GSI-treated intestine had increased number of cells, yet retained normal position near the base of the crypts suggesting that crypt-villus patterning signals were maintained. Of interest, the Paneth-like cells present in the DAPT-treated fetal organ cultures occurred in the intervillus zone, suggesting that patterning is established independently of Notch signaling at early developmental stages and during adulthood.

The Paneth-like cells that arose in the DAPT-treated organ cultures appeared to be distinct from intermediate cells due to their positive immunoreactivity for lysozyme. Similar cells emerged in the intestine of Vil-Math1 transgenic founders in which Math1 expression was forced throughout the intestinal epithelium (Chapter 2 and data not shown). These data suggest that immature intestine is not able to support the terminal differentiation of Paneth cells as was observed in the GSI-treated adult intestine. In the Vil-Math1 founders, Notch signaling was unchanged as assessed by Hes1 mRNA expression, suggesting that the premature activation of the Paneth cell gene program may be a byproduct of increased Math1 expression (Chapter 2). Accordingly, Hes1 deficient mice, which exhibited increased Math1 expression, also had premature differentiation of Paneth-like cells[48].

Very little has been reported about intermediate cell regulation and the mechanism behind the regional difference in intermediate cell formation is currently unknown; however, a number of factors could be contributing. First, Paneth cells of the distal ileum, although morphologically similar to those in the proximal intestine, express high levels of cryptdin-4 whereas those in the proximal intestine do not[49]. Thus, not all Paneth cells are equal and there must be signaling pathways regulating this difference. Second, Notch signaling activity does not appear to be uniform throughout the intestine; following Notch signaling by a modified lineage tracing mouse model in which Cre recombinase expression was activated by ligand-induced Notch1 receptor proteolysis (NIP-Cre) showed that higher frequency of labeled crypts was observed in the duodenum and jejunum than the ileum, which contained very few labeled crypts[4]. Thus, Notch signaling may be more active in the proximal intestine than the distal intestine and this could lead to differential sensitivity to GSI treatment. Third, active Notch signaling, as demonstrated by NIP-Cre labeling and NICD immunostaining, has been reported to be present in some differentiated goblet cells suggesting that Notch may play a role in terminal differentiation of these secretory cells[4]. Fourth, alterations in Wnt signaling are very likely to be involved because downstream targets of Wnt signaling, including Sox9, are essential for Paneth cell differentiation; mice lacking intestinal Sox9 expression have loss of Paneth cells[50, 51]. Multiple Paneth cell differentiation genes, including MMP7 and cryptdins, are Wnt target genes[52, 53] and are expressed in intermediate cells[36, 38] whereas lysozyme is neither a Wnt target nor expressed in intermediate cells[38, 53]. Furthermore, hyperactive Wnt signaling in APC^{min} mice has been shown to activate Paneth cell genes in the colon and to result in ectopic Paneth-like cells on the villus[52-54]. Interestingly, the induced Paneth cells in the APC^{min} mouse model did not have typical secretory granules or express UEA-1 lectin suggesting that they might not be fully differentiated Paneth cells[52]. Thus, although Wnt signaling is essential for Paneth cell differentiation, hyperactive Wnt signaling might be detrimental to the full terminal differentiation of Paneth cells. Indeed, the pattern of lysozyme staining was more diffuse throughout the cytoplasm of

Paneth cells in the GSI-treated intestine, suggesting that a change in secretory granule structure had occurred; however, more work is needed to determine if the Paneth cells in GSI-treated intestine are normal. Together, the increased Paneth cells versus intermediate cells result demonstrates that Notch may function in distinct ways in each intestinal region and/or that interacting factors may be regionally distributed along the length of the intestine. In addition, there is likely interaction of the Notch pathway with the Wnt signaling pathway to regulate Paneth and intermediate cell differentiation in the intestine.

Here we show that the CBC stem cell is a direct cellular target of Notch signaling and, moreover, that the gene marker of CBC cells Olfm4 appears to be directly regulated by Notch signaling. To our knowledge, this is the first specific cellular target of Notch signaling identified in the intestinal crypt. Analysis of stem cell markers in GSI-treated adult intestine and fetal organ cultures revealed a marked loss of Olfm4 mRNA expression. The γ-secretase complex can regulate cleavage of many signaling proteins other than Notch[55-59] so we have confirmed our findings in a genetic model of Notch disruption in which an activating cleavage of the Notch receptor is blocked due to deletion of the ADAM10 protease gene in the intestine (Vil-ADAM10 KO) (VanDussen and Dempsey, data not shown).

In this study, Olfm4 was identified as a putative direct target of Notch signaling. Transcriptional studies showed that OLFM4 promoter activity increased with constitutively active Notch and decreased with addition of dnMAML. Currently, the intestinal function of Olfm4 is unknown; however, reports from other studies in other tissues have suggested that Olfm4 has functions associated with stem cell properties. Murine Olfm4 was originally cloned from hematopoietic precursor cells[60] and has since been characterized as a secreted extracellular matrix glycoprotein that can facilitate cell adhesion and can bind to cell surface cadherins and lectins[61]. Olfm4 has been shown to attenuate apoptosis at the level of cytochrome *c* release and caspase activation, potentially through an inhibiting interaction with GRIM-19, a known pro-apoptotic protein[62]. In addition, overexpression of Olfm4 promoted tumor growth of

TRAMP-C1 prostate cancer cells that were injected into syngeneic C57BL/6 mice[62]. Along with many studies showing high expression of Olfm4 in breast, lung, pancreatic, colorectal and gastric cancer and inflamed colonic mucosa, these results suggest that Olfm4 may be an important regulator of tissue homeostasis and tumor progression[63-68]. Olfm4 has also been suggested to have a role in the promotion of proliferation; siRNA against Olfm4 in the human pancreatic cancer cell line PANC-1 resulted in cell cycle arrest during early S-phase[63]. In addition, Olfm4 expression was noted to fluctuate throughout the cell cycle of PANC-1 cells with highest expression during S-phase[63]. Thus, Olfm4 may mediate important pro-proliferation and anti-apoptotic functions required for proper stem cell activity.

In the intestine, Olfm4 mRNA expression is specifically expressed in the CBC stem cells[16]. Additionally, Olfm4 mRNA is found in a subset of colon cancer cells suggesting that it may also be a marker of malignant stem cells[67]. Of interest, overactive Notch signaling is also important in cancer development[9]; thus, Notch signaling could contribute at least in part to tumor progression in the intestine through dysregulation of Olfm4 gene expression. The precise localization of Olfm4 protein is not well understood. One study shows diffuse staining of Olfm4 throughout the intestinal and colonic epithelium[69] while another study shows a more limited protein expression pattern with expression limited to the proliferative zones the intestinal crypts and base of the colonic crypts[65]. Thus, more work is needed to understand Olfm4 protein expression and function in the intestine.

A series of promoter deletions in the OLFM4 5' proximal promoter identified the -145 to -56 region as the region likely containing Notch-regulatory *cis*-elements; the -145 to -98 region is especially attractive due to complete loss of increased promoter activity in the presence of active NICD. Sequence analysis has identified two RBP-J sites (RBP-Jb and RBP-Jc) within this region that are present in both mouse and human Olfm4 promoters. These sites have a weaker consensus sequence than the RBP-Ja site (Fig 4-10); however, they may still be functional, and potentially strengthened by the addition of co-factors.

Future studies will address whether Notch signaling regulates OLFM4 promoter activity through RBP-J binding at these sites.

Finally, many other potential regulators of Olfm4 activity were identified by sequence analysis which may be important for regulation of Olfm4 expression in the intestine. KLF4 and KLF5 have been shown to be important for regulation of proliferation in the intestine[70]. Additionally, KLF4 is regulated by Notch signaling. HNF1α and HNF4α are downstream targets of Cdx2 and function along with Cdx2 to activate the intestinal differentiation program[71]. Thus, these binding sites could be generally important for activation of Olfm4 in the intestine. Finally, Pax4 and Gfi1 have been shown to be important for cell lineage differentiation in the intestine[35, 72] and may function as tumor suppressors in some contexts[73, 74]; thus, cis-elements like these could be important for turning off Olfm4 gene transcription in differentiated cells. Future studies will need to address whether Olfm4 is functionally important for CBC stem cell activity and cellular differentiation. These studies will be important for understanding the role of Notch signaling and Olfm4 in intestinal maintenance and progression to disease.

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

The Role of Math1 in Secretory Cell Fate Selection

This thesis has furthered our understanding of Notch signaling as a critical regulator of stem cell maintenance and cell fate selection in the intestine. First, I have shown that the Notch-regulated transcription factor mouse atonal homolog 1 (Math1) is the key regulator of secretory cell differentiation in the developing and adult intestine. Transgenic expression of Math1 in the intestinal epithelium of fetal mice resulted in a conversion of the intestinal epithelium to secretory cells to the detriment of absorptive cells, suggesting that Math1 can redirect a bipotential progenitor cell to the secretory lineage (Chapter 2). To further investigate the intestinal function of Math1, an inducible Math1 transgenic mouse, the VZM model, was generated and characterized (Chapter 3). Cre-mediated induction of VZM transgene recombination resulted in increased expression of Math1 mRNA; however, Math1 protein levels were not changed. This finding suggests that Math1 protein may be regulated in the intestine by some unknown mechanism. Consequently, we were not able to determine whether Math1 expression is sufficient for secretory cell differentiation in the mature intestine with the VZM transgenic model so it is currently unknown whether Math1 performs a similar role in the developing and adult intestine.

Studies using the γ-secretase inhibitor (GSI) dibenzepine (DBZ) to inhibit Notch signaling in adult mouse intestine are consistent with my conclusion from Chapter 2 that Math1 is the key regulator of secretory cell induction. DBZ-treated intestine had increased Math1 mRNA and increased differentiation of all

secretory cells, including goblet, Paneth, and endocrine cells (Chapter 4). These data complement my studies of fetal cell lineage determination. The results are consistent with the conclusion that Math1 controls cell fate in developing and mature intestine. Previously, a discrepancy in the literature existed because DBZ-treated rats were reported to have increased numbers of all secretory cells[1] while DBZ-treated mice were reported to only have increased goblet cells[2]. Our results agree with those from the study in rats and have reconciled this discrepancy in the literature. Thus, Math1 expression appears to be sufficient for inducing differentiation of all secretory cells in both developing and adult intestine.

Presently, the mechanism of Math1-mediated regulation of secretory progenitor cells is not well understood, although recent studies have identified two downstream targets of Math1: Growth factor independent 1 (Gfi1) and SAM pointed domain containing ets transcription factor (Spdef), which both function in secretory lineage allocation and terminal differentiation of secretory cells[3-5]. In addition to its role in secretory lineage selection, Math1 may also be important for maintenance of the secretory cell gene program since it continues to be expressed in mature secretory cells. Future studies will need to determine the function of sustained Math1 expression in mature secretory cells. In addition, the identity of progenitor cells that express Math1 need to be further characterized. As a starting point, planned future studies will examine co-localization of Math1 in leucine g-protein coupled receptor 5 (Lgr5)-positive crypt base columnar (CBC) stem cells using the Math1$^{lacZ/+}$ and Lgr5-GFP-CreER reporter mice[6, 7]. This study will determine if Math1 expression is activated in the CBC stem cell as a possible initiating event in the process of cellular differentiation. In addition, co-localization of Math1 and hairy and enhancer of split 1 (Hes1) or Hes5 using Math1$^{lacZ/+}$ and Hes1-GFP or Hes5-GFP reporter mice[8] may help to distinguish progenitor cells undergoing Notch signaling and fated to enterocyte differentiation versus those fated by Math1 to undergo secretory cell differentiation. Since Hes1 is known to repress Math1 transcription[9], this study will provide clues about how progenitor cells are regulated.

Regulation of Intermediate Cells

Interestingly, we observed regional differences in the DBZ-induced secretory cell phenotype. Lysozyme-positive Paneth cells were expanded in number in the duodenum and jejunum, but did not appear to be expanded in the ileum of DBZ-treated mice. Instead, we observed a marked increase in the number of intermediate cells in the ileum and colon that shared characteristics of both Paneth and goblet cells in DBZ-treated mice. Ongoing studies are aimed at analysis of the ultrastructure of crypt cells in vehicle- versus GSI-treated intestine to definitively identify intermediate cells[10, 11]. Preliminary results have identified crypt cells in DBZ-treated ileum that contain both electron-dense secretory granules and mucin droplets within their cytoplasm, characteristic of intermediate cells. In addition, despite our observation that lysozyme staining is present in the cells at the base of the GSI-treated ileal crypts, none of these cells appear to be normal Paneth cells with normal electron-dense secretory granules. Thus, the diffuse lysozyme staining that we observed throughout the small intestines of DBZ-treated mice may be indicative of abnormal Paneth cell ultrastructure in all regions of the intestine upon Notch disruption. This notion will be explored by further ultrastructural analysis of more proximal intestinal regions. In addition, ultrastructural analysis after a shorter DBZ treatment (e.g. 2-3 days instead of 5 days) may provide information about how the ultrastructural remodeling occurs. For instance, do we observe this phenotype because mature cells are being reprogrammed or because there is induction of new cells? Finally, this analysis may also be able to confirm loss of undifferentiated cells in the crypts based on morphological properties.

It is not currently understood why there are regional differences in secretory cell induction in the GSI model of Notch inhibition. To probe the ileal intermediate cell induction further, it will be beneficial to first determine if this phenotype is observed in other models of Notch disruption such as the inducible RBP-J-deficient mouse intestine (see Table 1-1 for description). Since Wnt signaling is critical for Paneth cell differentiation[12-14], experiments that explore

differences in Wnt signaling between vehicle- and DBZ-treated mice as well as DBZ-treated duodenum versus DBZ-treated ileum may illuminate Wnt signaling as a contributor to the formation of intermediate cells.

A mouse model with simultaneous loss of the three proliferation-promoting CDC25 family genes, CDC25a, b, and c, had loss of intestinal proliferation with increased expression of Wnt target genes and increased nuclear accumulation of β-catenin, including at the +4 stem cell position[15]. The authors suggested that enhanced Wnt signaling was induced to preserve the +4 position stem cells in which proliferation was blocked. In contrast, ultrastructural analysis suggested that the CBC stem cells underwent terminal differentiation upon blockade of the cell cycle[15]. Thus, since I hypothesize that a similar situation is occurring in the DBZ-treated intestine, I would predict that Wnt signaling is hyperactive in the DBZ-treated intestine as a compensatory mechanism due to loss of crypt cell proliferation. However, since the cell cycle blockade with DBZ-treatment is accompanied by Notch inhibition and increased Math1, there is a second aspect of the phenotype, induction of secretory cell differentiation, which is not present in the CDC25 gene family mutant. Therefore, if hyperactive Wnt signaling is induced by blockade of the cell cycle, the same Wnt signal is also likely to affect the cellular differentiation process.

Notch Signaling Targets the CBC Stem Cell

Notch signaling is important for progenitor cell maintenance in the intestine; however, relatively little is known about which specific stem or progenitor cell populations are responding to Notch signaling or how these cell populations are affected by gain or loss of Notch function. My thesis work begins to address this knowledge gap by analyzing stem and progenitor cell markers in Notch loss-of-function mutants (Chapter 4). Previous studies have shown that disruption of Notch signaling leads to loss of proliferating cells while hyperactive Notch signaling leads to increased numbers of proliferating cells and has been associated with cancer[2, 16-18]. My studies strongly suggest that Notch signaling is targeting the CBC stem cell based on Notch-regulation of the CBC cell-specific

gene Olfmactomedin 4 (Olfm4) (Chapter 4). However, more experiments are needed to definitively show that Notch signaling is active in the CBC cell.

As a starting point, microarray revealed that Notch1 is enriched in high expressing Lgr5 CBC stem cells[19]. Following up on this observation, future studies should determine if Notch target genes are expressed in the CBC cell. Due to poor antibody reagents, the use of *in situ* hybridization and/or transgenic reporter mice will be necessary. As one possible approach, breeding of the Lgr5-lacZ reporter mice[6] with the Hes1-GFP or Hes5-GFP reporter mice[8] could be used to show co-localization of known Notch target genes in the CBC stem cell. Future studies will also determine if alteration of Notch signaling specifically in the CBC cell mimics the phenotypes of whole intestinal epithelium alterations. To do this, the phenotypes of Cre-inducible RBP-J deficiency[2] and Cre-inducible constitutive NICD1 activation[16] using the CBC Cre driver Lgr5-EGFP-CreER[6] or the intestinal epithelium Cre driver Vil-CreER^{T2} [20] will be compared.

Identification of which stem cell populations are affected by Notch signaling will be important for understanding the role of Notch signaling in the formation and progression of cancer. Accordingly, a future direction for this project will be to quantitate CBC cell number in mouse models with Notch signaling alterations using the Lgr5-EGFP-CreER reporter mouse[6] in which the CBC stem cells are labeled with GFP and by *in situ* hybridization for CBC markers including Ascl2 and Olfm4. Profiling of stem cell gene expression may also provide some clues about how the various stem and progenitor cell populations are affected by alterations in Notch signaling. A pilot study in perinatal 9kbVil-Cre;NICD1 mice showed a 2-fold increase in Olfm4 expression despite down-regulation of other CBC markers that suggest that CBC number may be decreased (VanDussen, unpublished). This result supports the finding that Notch signaling activates Olfm4 gene expression *in vivo*, but complicates the role of Notch signaling in maintaining the CBC. Furthermore, this result emphasizes the need to examine progenitor cell populations by multiple approaches.

Notch Regulation of Olfm4 Gene Expression

The results presented in this thesis have also suggested that active Notch signaling occurs in the CBC stem cell. Disruption of Notch signaling in fetal organ cultures or adult intestine resulted in a striking decrease in the CBC stem cell transcript Olfm4, suggesting that Notch signaling normally activates Olfm4 expression in the CBC cell (Chapter 4). Transcriptional studies of the human OLFM4 promoter have supported the results of the *in vivo* studies and suggest that Notch signaling activates OLFM4 promoter activity. A series of OLFM4 promoter deletions identified that critical *cis*-elements for Notch regulation of the OLFM4 promoter are located in an 89 base pair region (-145 and -56) upstream of the transcription start site. Of interest, 2 DNA binding consensus sites for the Notch co-activator RBP-J lie within this region; these sites are conserved between mouse and human, with one site being paired to an E-box (Figure 4-10). Mutation of a third RBP-J site 5' to the critical -145 to -56 region did not have an affect on Notch-regulation of OLFM4 promoter activity. Future studies will determine if one or a combination of these RBP-J sites are required for Notch activation of OLFM4 transcription.

In addition, the importance of the conserved E-box will be explored. If the E-box consensus site is important for OLFM4 expression, the identity of the bHLH that binds there would be of great interest since it is a half helix turn away from and would likely interact with the nearby putative RBP-J/NICD1 DNA-binding complex. A provocative bHLH protein candidate for binding to this site is Ascl2, a CBC cell-specific gene that is a target of Wnt signaling and functionally important for CBC stem cell activity[19]. Olfm4 was identified as a putative Ascl2 target gene by microarray comparison of Ascl2-deficient and control intestine[19]; however, since CBC cells were temporarily lost in Ascl2-deficient mice, it is not clear whether Olfm4 gene expression was down-regulated due to loss of Ascl2-mediated activation or due to loss of CBC cells. Hes1 is also a bHLH transcription factor that is likely to be expressed in the CBC cell, if Notch signaling is indeed active in this cell population; however, traditionally, Hes1

functions as a repressor protein and thus would not be likely to participate directly in activation of Olfm4 expression.

Other transcription factors with consensus binding sites that were identified within the critical -145 to -56 region have been reported to interact with Notch signaling, including CREB (located at -143 to -123) and TEF-1 (located at -133 to -121). Of interest, these sites overlap the paired E-box and RBP-J consensus sites. In mature CD8+ T-cells, CREB forms a DNA-binding complex with NICD and p300 to activate gene expression of granzyme B, an important contributor to the cytotoxicity of CD8+ T-cells[21]. Thus, potential CREB binding on the OLFM4 promoter could link transcription of this gene to signaling from secondary messengers such as cAMP or calcium or activation of the MAPK pathway. TEF-1 is part of the TEA domain (TEAD) family of transcription factors. TEAD transcription factors interact with nuclear yes-associated protein (YAP), a strong co-activator that promotes cell growth and is inhibited by the Hippo pathway[22]. Activation of the Hippo pathway sequesters YAP in the cytoplasm, preventing its association with nuclear TEAD transcription factors, to limit organ size in flies and mammals[22]. Overexpression of YAP or absence of the Hippo pathway gene WW45 in mice produces intestinal phenotypes that are reminiscient of Notch gain-of-function mutants with loss of differentiated cells and increased proliferation[23, 24]. Furthermore, the YAP overexpression phenotype can be blocked by administration of DBZ, suggesting that YAP indeed can modulate Notch signaling or the affects of Notch signaling in the intestine[23]. Thus, the Hippo pathway may be an important regulator of intestinal stem cell activity through modulation of Notch signaling itself or of expression of Notch target genes, potentially including OLFM4.

Currently, very little is known about the Olfm4 protein other than it is a secreted glycoprotein and that its expression levels appear to correlate with progession of tumors[25, 26]. However, some recent studies in cell lines have suggested that Olfm4 may be possess properties that would be important in stem cell function, including promotion of proliferation and inhibition of apoptosis[27, 28].

Therefore, future studies should address the function of Olfm4 in the CBC to determine if it is required or sufficient for stem cell activity.

The Effect of Notch Inhibition on Intestinal Stem and Progenitor Cells

Based on the data presented in this thesis and in previous studies, I am proposing the model presented in Figure 5-1 as my current hypothesis on the effects of disruption of Notch signaling in the intestine. Notch functions to maintain the CBC cell pool in an active, cycling state. After asymmetric division, the newly formed undifferentiated cells migrate away from the base of the crypt and are exposed to changes in the signaling niche that start the differentiation process and these cells become the transit amplifying progenitor (TA) cells. At this point, Notch signaling now directs the process of cell fate selection with cells responding to active Notch signals with cells expressing Hes1 being directed to the absorptive lineage and cells expressing Notch ligand and Math1 being directed to the secretory lineage.

When Notch signaling is disrupted, CBC cells prematurely differentiate into TA cells, which are then directed almost exclusively to the secretory lineage due to high Math1 expression and low Hes1 expression. This data is supported by consistent decreases in CBC cell gene expression and increases in TA cell gene expression in models of Notch disruption (Chapter 4). Furthermore, if this model holds true, one would expect to see an initial surge of proliferation after Notch inhibition due to increased numbers of highly proliferative TA cells; indeed, 10 μmol/kg DBZ treated adult mice, which likely develop the Notch inhibition phenotype more slowly, exhibited increased crypt proliferation. A direct time course study of DBZ treatment is needed to more accurately test this hypothesis. After 5 days of 30 μmol/kg DBZ treatment, proliferating cells were mostly absent from the intestinal crypts suggesting that cycling cells were either 1) still present but not cycling or 2) post-mitotic differentiated cells. Due to the increased presence of markers of differentiated cells in the crypts, the latter possibility seems more likely.

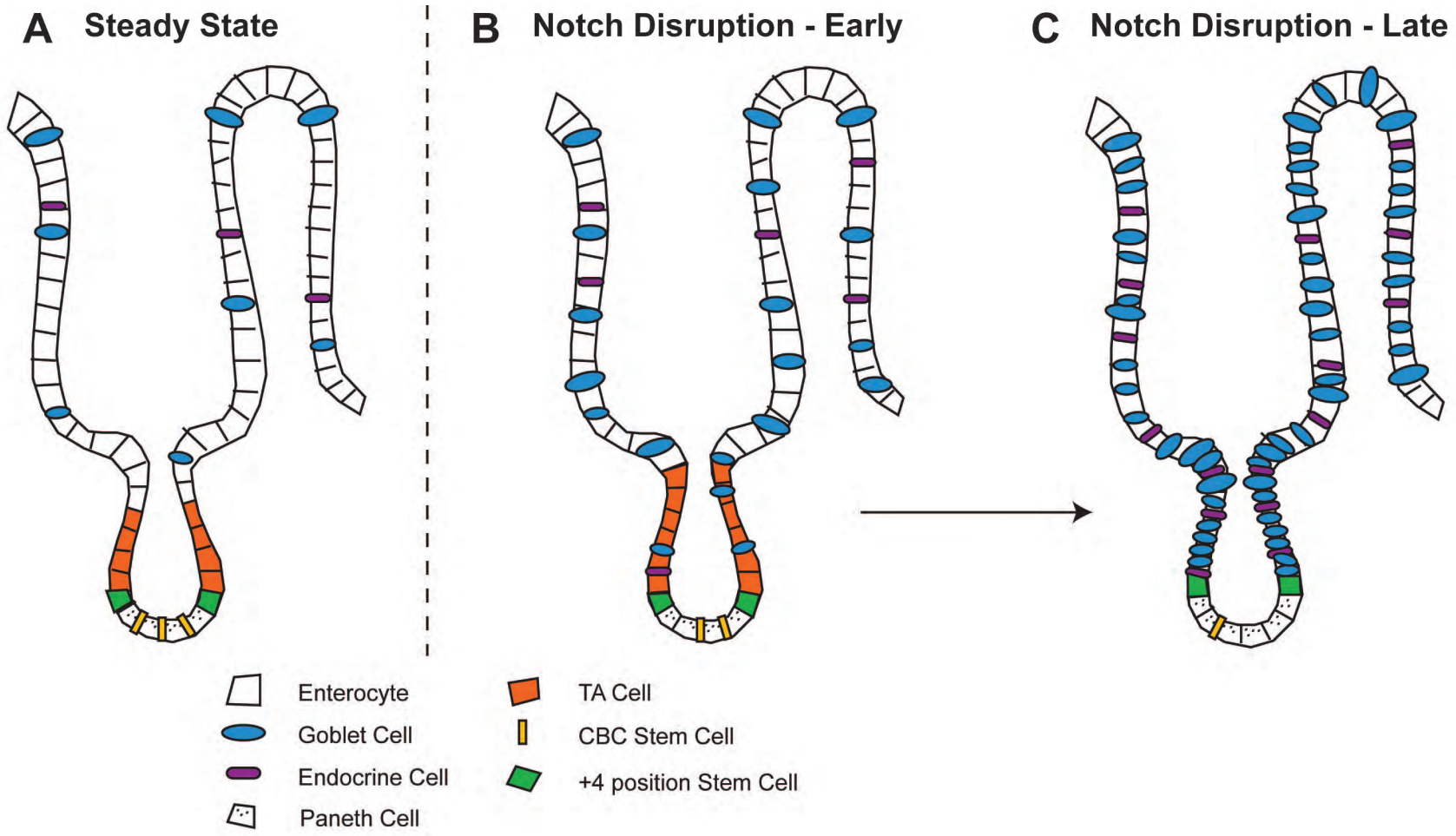

Figure 5-1. Model of Stem Cell Population Alterations in GSI-treated Intestine. Schematic of the adult intestinal epithelium at steady state (A) and the proposed cellular changes that occur early (B) and late (C) after Notch inhibition. TA Cell, transit amplifying progenitor cell; CBC stem cell, crypt base columnar stem cell.

The contribution of premature cellular differentiation to loss of proliferation in models of Notch disruption is also not fully understood. Stated another way, is an increase in Math1 required for premature cellular differentiation and loss of proliferation? Initial studies in fetal organ cultures demonstrated that Math1 is required for increased secretory cell differentiation in GSI-treated intestine (Chapter 4). Future studies will address whether proliferation is affected in this model of developing intestine and whether Math1-deficiency attenuates the loss of proliferation observed in other models of Notch disruption.

Remaining Questions in the Intestinal Stem Cell Field

The intestinal stem cell field has made many recent advances that have rapidly increased our knowledge of the intestinal stem cell, our ability to define where stem cells exist and which cells in the crypt actually are stem cells; however, many questions still remain to be definitively addressed. The precise number and location of stem cells is still not entirely known. Estimates have placed the number at 4-6 cells per crypt; if this is true, what controls this number and does it change in adaptive response compared to steady state? Many stem cell markers have been identified but it is not well understood if these markers are expressed in similar or distinct cell populations or whether these markers perform any functional role contributing to stemness. In addition, markers of progenitor cells that are not long-lived but do produce all epithelial lineages such as Prom1 suggest that there must be distinguishing characteristics between the stem cells and their progeny. More research is needed to determine the sequence of events that occurs during stem cell differentiation and what signaling pathways might act directly on the intestinal stem cell to influence this process.

The ability to isolate and culture Bmi1-positive and Lgr5-positive cells is an exciting advancement that will be important for controlled experiments that will help to define whether stem cell characteristics are intrinsic or whether they are determined by the niche[29]. Currently, conditions for culturing isolated stem cells includes many media components such as Noggin, the Wnt agonist R-spondin, laminin- and growth factor-rich Matrigel, and the Notch agonist Jagged 1[29]. With

many of the required factors in the culturing media used for these experiments coming from the mesenchyme *in vivo*, it suggests that the niche serves an essential role in stem cell function and regulation. Defining this niche and its interaction with the intestinal stem cell will greatly contribute to our ideas on how epithelial maintenance is preserved.

Lastly, with the discovery that CBC cells are dividing so rapidly, mechanisms must be in place to ensure that it remains a long-lived cell without accumulating mutations that cause disease. It remains to be understood how these cells rise to the challenge of replenishing the majority of the intestinal epithelium every few days. Two potential stem cell populations reside in the intestinal crypts with the more quiescent Bmi1-cell functioning to replenish and maintain healthy CBC cells[30]. Thus, studies in the field need to determine if indeed two stem cell populations exist and to define the relationship between these cell populations.

Some Remaining Questions about Intestinal Notch Signaling

The body of work represented by the work of several laboratories and many mouse models with alterations in Notch signaling (Table 1-1) has definitively established the Notch signaling pathway as a fundamental pathway regulating epithelial proliferation and differentiation in the intestine. This function appears to be conserved among vertebrates as recent studies in zebrafish and fruit fly have shown phenotypes in Notch pathway mutants that are similar to those observed in mice[31, 32]. Despite the importance of Notch signaling for development and maintenance of the intestinal epithelium, there remains a large knowledge gap about the basic components involved in intestinal Notch signaling.

For instance, the critical ligands that activate intestinal Notch signaling have not been described. Targeted mutation of Delta-like 1 (Dll1)[33] or Jagged 1 (Jag1)[34] mice results in early embryonic lethality (~E12) prior to intestinal cellular differentiation. Delta-like 4 (Dll4)-deficient[35] and Jagged 2 (Jag2)-deficient[36] mouse mutants have been generated and survive to at least neonatal stages, but

have not been analyzed for an intestinal phenotype. Conditional mutants have been generated for analysis in other tissues and could be utilized for intestinal studies[37-40]. Thus, it is currently unknown which Notch ligands are functionally important for intestinal Notch signaling *in vivo*. It is also not known whether Notch signaling control of secretory cell differentiation occurs entirely within the epithelium or whether it cooperates with ligand- or receptor-expressing cells in the adjacent mesenchyme. Thus, identification of the signal sending and signal receiving cells in the intestine will be critical for understanding Notch function in the regulation of proliferation and cell fate selection. It will also be important to understand the composition of the γ-secretase complex in the intestine because this likely has effects on its cleavage specificity and efficiency. As a starting point, mouse mutants for presenillin1[41] and presenillin 2[42] have been generated for study in other systems and could be used for intestinal studies.

Many intestinal diseases, disorders, and adaptive responses display changes in proportions of progenitors or secretory cells such as colorectal cancer, parasitic infection, post-small bowel resection, and leukocyte adhesion deficiency type II[43-48] which may depend on Notch signaling. For example, there are increased proliferative cells and increased numbers of undifferentiated cells in colorectal cancer[47]. Thus, a more detailed and complete knowledge of Notch signaling will be critical for understanding intestinal homeostasis, repair, and progression to disease. In addition, identification of each of the intestinal components of the Notch signaling pathway will be critical for developing treatments for T-cell leukemia, Alzheimer's disease, and potentially certain cancers because drugs that can be delivered systematically with minimal intestinal side effects are greatly needed for these diseases[49-51]. Thus, identification of therapeutics that target critical Notch targets in diseased tissues without severely affecting intestinal Notch signaling would be of great benefit.

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