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REVIEW

Twists and Turns in the Development and Maintenance of the Mammalian Small Intestine Epithelium

Andrew L. Hauck, Kelly S. Swanson, Paul J. A. Kenis, Deborah E. Leckband, H. Rex Gaskins and Lawrence B. Schook*

Experimental studies during the last decade have revealed a number of signaling pathways that are critical for the development and maintenance of the intestinal epithelium and that demonstrate the molecular basis for a variety of diseases. The Notch-Delta, Wnt, Hedge Hog, TGF-β, and other signaling pathways have been shown to form and steadily maintain the crypt-villus system, generating the proper quantities of highly-specialized cells, and ultimately defining the architectural shape of the system. Based on the characterized phenotypes and functional defects of mice resulting from various targeted knockouts, and overexpression and misexpressions of genes, a picture is emerging of the sequence of gene expression events from within the epithelium, and in the underlying mesenchyme that contribute to the regulation of cell differentiation and proliferation. This review focuses on the contributions of multiple signaling pathways to intestinal epithelial proliferation, differentiation, and structural organization, as well as the possible opportunities for cross-talk between pathways. The Notch pathway's potential ability to maintain and regulate the intestinal epithelial stem cell is discussed, in addition to its role as the primary mediator of lineage specification. Recent research that has shed light on the function of Wnt signaling and epithelial-mesenchymal cross-talk during embryonic and postnatal development is examined, along with data on the interplay of heparan sulfate proteoglycans in the signaling process. **Birth Defects Research (Part C) 75:58–71, 2005.**

OVERVIEW OF DEVELOPMENT

The surface of the small intestine (SI) is covered with a simple columnar epithelium exhibiting invaginations, known as the crypts of Liberkühn, which are comprised predominately of proliferating cells, and finger like projections called villi, that contain the majority of differentiated absorptive cells (Young and Heath, 2000). This architecture is established with contributions from cells in the underlying mesenchymal connective tissue, such as fibroblasts, which deposit gradients of extracellular matrix components into the basement membrane of the epithelium along the crypt-villus axis (Kedinger et al., 1998a,b; Karlsson et al., 2000). A population of specialized cells, the intestinal subepithelial pericryptal myofibroblasts (ISEMFs), are concentrated beneath crypts, and believed to assist in their formation (Madison et al., 2005). The mesenchyme, or lamina propria, contains blood vessels, lymphatics, immune cells, enteric nerves, and some smooth muscle fibers, which participate in the digestive process (Young and Heath, 2000; Brittan and Wright, 2004). The villi are supplied with cells from 6 to 10 crypts that migrate towards the villus tip in ordered columns, until they commit apoptosis and are reabsorbed (Schmidt et al., 1985; Potten and Loeffler, 1990; Hall et al., 1994). Relatively undifferentiated stem cells, residing near the crypt base, perpetually give rise to all of the differentiated cell lineages, are maintained throughout life, and have the ability to restore damaged epithelium (Booth and Potten, 2000; Marshman et al., 2002). In the process of migrating from the crypt to the villus, the proliferative cells differentiate into one of four principle types: absorptive enterocytes, mucus-secreting goblet cells, hormone-secreting enteroen-

Andrew Hauck is from the Department of Cell and Structural Biology, University of Illinois, Urbana, Illinois. **Kelly Swanson** is from the Department of Animal Sciences, Institute for Genomic Biology, University of Illinois, Urbana, Illinois. **Paul J.A. Kenis** and **Deborah E. Leckband** are from the Department of Chemical and Biomolecular Engineering, and the Regenerative Biology and Tissue Engineering Theme Institute for Genomic Biology, University of Illinois, Urbana, Illinois. **H. Rex Gaskins** and **Lawrence B. Schook** are from the Department of Animal Sciences, and the Regenerative Biology and Tissue Engineering Theme Institute for Genomic Biology, University of Illinois, Urbana, Illinois.
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*Correspondence to: Lawrence B. Schook, 382 ERML, 1201 West Gregory Dr., University of Illinois, Urbana, IL 61801.
E-mail: schook@uiuc.edu

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docrine cells, and antimicrobial Paneth cells (Marshman et al., 2002, Fig. 1). Paneth cells do not proceed up the axis, but rather migrate to the base of the crypt, where they survive for about three weeks (Bry et al., 1994). The stem cells generally divide asymmetrically, producing one self-like cell that remains near the crypt base and retains all of the chromosomes with the original template strands, and a daughter cell that participates in clonal amplification and migrates up the axis towards the villus tip (Marshman et al., 2002; Potten et al., 2002). The rates of cell proliferation and migration are tuned to balance the continual loss of cells further up the axis, and are highly responsive to signals from nutrition, stress, and infection.

The vertebrate gut develops from the ventral endoderm, which is specified along the anterior-posterior axis into the foregut, midgut, and hindgut early in development. In murine development, the endoderm invaginates anteriorly and posteriorly, forming pockets in the foregut and hindgut around ED 7.5 to ED 8.0. This invagination continues from the pockets along the midline, eventually meeting and fusing into a tube at ED 8.5 to ED 9.0. Splanchnic mesoderm, which will give rise to the muscle and mesenchyme, surrounds the endo derm, and neural crest cells migrate to develop the enteric nervous system (Rossant and Tam, 2002). Inductive interactions between the gut endoderm and mesoderm have been reviewed (Haffen et al., 1987). Hox genes are expressed from ED 8.5 to ED 12.5, and are involved in regionalizing the gut (Beck et al., 2000). During the period from ED 14.5 to ED 18.5, the undifferentiated pseudostratified endoderm is progressively converted along the proximal-distal axis into a simple columnar epithelium, and accompanied with villus outgrowth. The proliferative intervillus epithelium converts into crypts within the first two postnatal weeks, and this process increases rapidly during the third week (Calvert and Pothier, 1990). Paneth cell markers appear in a sequence beginning in late embryogenesis, with the majority of these markers localizing to cells with characteristic Paneth cell morphology by P7. The population of Paneth cells expands greatly from P14 to P28, coincident with increases in crypt quantity (Bry et al., 1994).

POSITIONING ALONG THE STARTING GATE

A variety of mechanisms were initially postulated in controlling the position of cells along the crypt-villus axis (Heath, 1996). Phenotypic analysis of mice with various knockouts of Ephrin ligands and their Eph receptors indicate that segregation of proliferating and differentiation populations, as well as positioning of the Paneth cell lineage, depend heavily on the interpretation of these cell surface molecular gradients along the crypt-villus axis.

The Eph tyrosine kinase receptors and Ephrins are membrane bound surface molecules, known for their role in morphogenesis and neural guidance. Generally, the A and B forms of these receptors are specific to their respective A or B Ephrins. Direct contact between adjacent cells generates uni- or bi-directional signaling that can result in repulsion or attraction, often for the purpose of guiding cells to target locations. The cytoplasmic domain of these proteins mediates interactions with the actin remodeling effectors, such as JNK and the Rho GTPase family (Klein, 2004; Tanaka et al., 2004).

In newborn mice, EphB2 and EphB3 are expressed in the intervillus pockets, with Ephrin B1 expression overlapping at the top of the pockets, and continuing up the villus. In adults, EphB2 is expressed in base columnar, but not Paneth cells, and continues up the crypt in a decreasing gradient, while EphB3 expression is restricted to the cells of the crypt base. Ephrin B1 and B2 expression is observed at the crypt-villus boundary and the lower villus, but declines to no presence above and below the boundary. Disruption of these interactions in knockout and dominant negative experiments resulted in improper cell localization (Table 1). In the case of EphB2 and EphB3 double-null mice, cells expressing a marker of differentiation mixed with those expressing a marker of proliferation (Batlle et al., 2002). Expression of Eph receptors is dependent on Tcf-4 mediated Wnt signaling (discussed below), and experiments either abrogating Tcf-4, or over inducing it, have resulted in mislocalized Paneth cells (Pinto et al., 2003; Sansom et. al, 2004).

DOES CELL POSITION DIRECTLY AFFECT DIFFERENTIATION?

The possibility that Eph/Ephrin expression assists in differentiation through regulation of the actin cytoskeleton is intriguing. Positional assisted differentiation could be associated with interactions with the extracellular matrix (ECM), as there are gradients of laminins along the crypt-villus axis and domains of expression of different integrins (Kedinger et al., 1998a, 1998b; Teller and Beaulieu, 2001). In vitro experiments with intestinal cell lines have indicated that laminin induces differentiation (Lorentz et al., 1997). Recently, a system of generating surfaces with overlapping gradients of collagen and laminin has been used to further clarify the effects of ECM proteins on intestinal cell proliferation and differentiation (Gunawan et al., 2005). Cytoskeleton remodeling associated with RhoA-Rock signaling has been shown to affect stem cell differentiation choices between osteoblasts and adipocytes (McBeath et al., 2004). Activation of RhoA-Rock is known to induce retraction and growth inhibition of neurites, while activation of Rac1 induces elongation (Tanaka et al., 2004). Expression of Rac1 was detected in the embryonic and adult small intestine, and by immunohistochemistry, Rac1 protein was identified in Paneth cells and villi. Targeted expression of a constitutively active form of Rac1 induced a premature differentiation of intervillus cells into Paneth and enterocyte lineages. In contrast, an equivalently

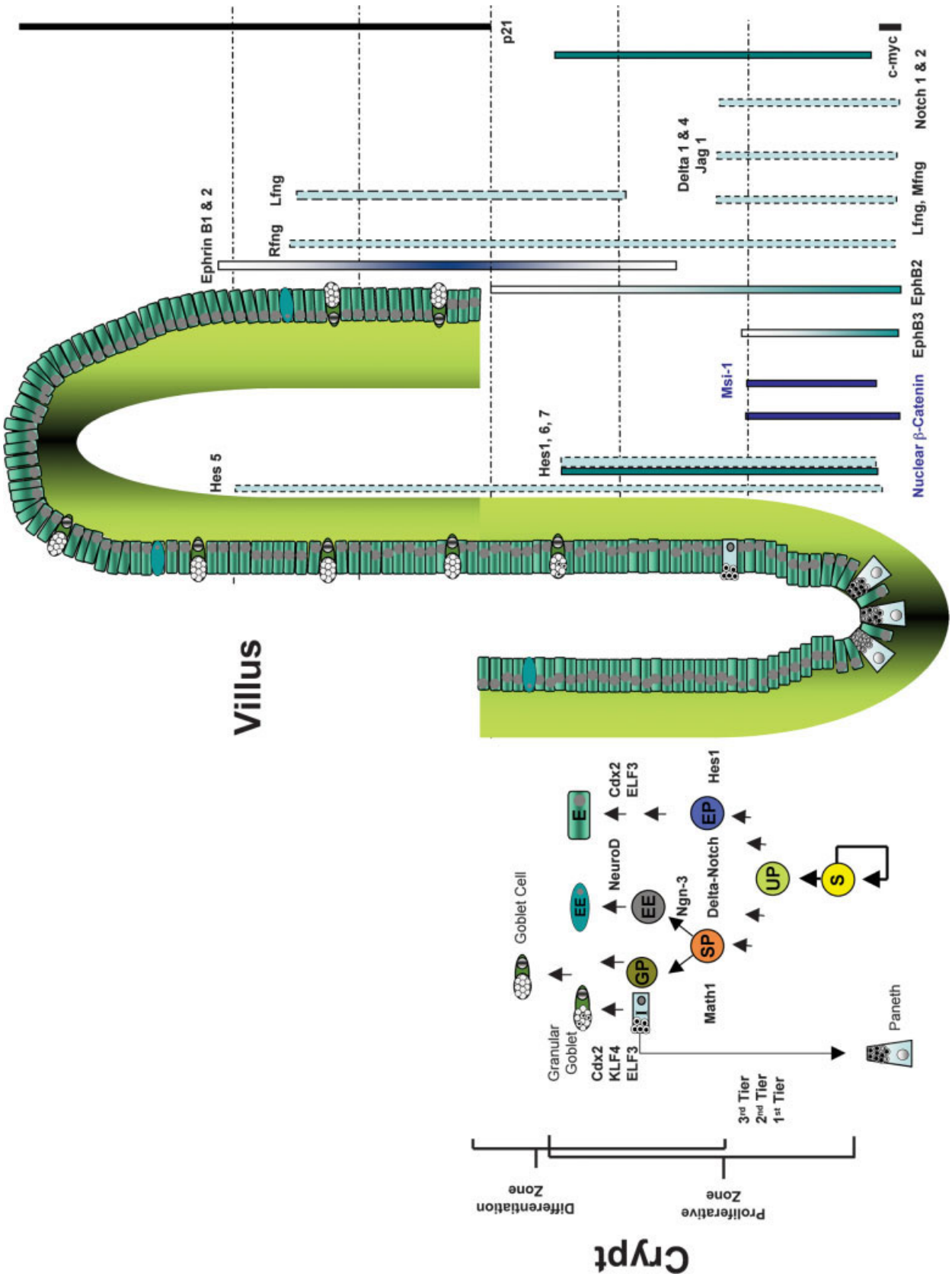
Villus
Crypt
Ephrin B1 & 2
Rfng
Lfng
Hes 5
p21
Hes1, 6, 7
Delta 1 & 4
Jag 1
Msi-1
c-myc
Notch 1 & 2
Lfng, Mfng
EphB2
EphB3
Nuclear β-Catenin
Differentiation Zone
Proliferative Zone
3rd Tier
2nd Tier
1st Tier
Granular
Goblet
Cdx2
KLF4
ELF3
Goblet Cell
Paneth
Math1
Ngn-3
Delta-Notch
Hes1
Cdx2
ELF3
NeuroD
S
UP
EP
SP
GP
EE
E

Figure 1.

targeted dominate negative Rac1 reduced the goblet, and to a lesser extent, Paneth cell lineages (Stappenbeck and Gordon, 2000). A subsequent study showed that constitutively active Rac1 affected the localization of p-JNK and Pak1, in addition to increasing crypt cell proliferation (Stappenbeck and Gordon, 2001).

Recently, a guanine-nucleotide exchange factor of Rac1, Tiam1, in response to EphB2, was shown to activate Rac1 in cells expressing Ephrin B-1 (Tanaka et al., 2004). This is particular interesting, considering that there is a zone along the crypt-villus axis at which EphB2 and Ephrin B-1 expression overlaps, which is also near the location of Rac1 activation. Expression of Tiam1 has been noted in the intestinal epithelium, and appears to be modulated by the presence of proliferation (Sansom et al., 2004). Although no morphological abnormalities were reported in the mislocalized cells of Eph mutant mice (Batlle et al., 2002), Eph signaling may participate in the process of differentiation.

The cytoskeletal remodeling that accompanies intestinal epithelial cell polarity has been attributed to LKB1, a Ser/Thr protein kinase. When activation of LKB1 is induced by STRAD in cultured intestinal epithelial cells, a polarization program ensues that generates microvilli, proper sorting of apical and basal proteins, and localization of junctional proteins (Baas et al., 2004a). Expression of LKB1 is observed in the cytoplasm of villus cells, increasing in a gradient up the axis and peaking at the tip, but it is also seen in the nucleus of cells as far down as the crypt base (Karuman et al., 2001). While the specific roles of the matrix and the cytoskeleton in determination of cell fate remains to be elucidated, there is a substantial body of evidence that Notch-Delta signaling is critical to the process.

CELL FATE DETERMINATION

The Notch-Delta signaling pathway has been shown to guide neuronal cell fate decision-making, and maintain the proliferative potential of stem or progenitor cells through members of the basic helix loop helix (bHLH) transcription factor family (Lee, 1997; Nakamura et al., 2000). Intensive investigations into neurogenesis have elucidated the mechanisms of these processes, and recent experiments have demonstrated that these pathways are also highly relevant to endocrine and intestinal systems as well. The Notch-Delta lateral inhibition mechanism describes a method by which heterogeneous populations of differentiated cells arise from a single ancestor (Lewis, 1998). The process involves contact activation of Notch receptors on one cell by the Delta (Delta-like, DLL) or Serrate (Jagged, Jag) ligands on another cell. Notch activation generates a rapid feedback amplification process that differentially regulates transcription in each cell, ensuring differences in identity (Lewis, 1998). In the case of neurogenesis, proneural early determination bHLH factors, Math1 (Atonal) and neurogenin (Ngn), begin commitment, while late cell differentiation factors, such as NeuroD, complete the process. These factors form heterodimers with the ubiquitously expressed bHLH factor, E12/E47 (daughterless), when activating transcription (Lee, 1997). Upon Notch signaling, the proteolytically cleaved intracellular Notch domain cooperates with RBP-Jk (suppressor of Hairless) to transcriptionally activate members of the Hes family, which are bHLH repressors that prevent the proneural differentiation program specified by the bHLH activators (Jarriault et al., 1995; Apelqvist et al., 1999). The presence of Notch, Hes, Math1, Ngn, and NeuroD factors in the intestine and pancreas, and their subsequent investigation, has revealed the importance of this pathway in specifying cell fates in these systems.

An earlier study in pancreatic development showed that removal of Delta 1 or RBP-Jk, overexpression of Ngn-3, or the presence of a Notch repressor, resulted in a depletion of progenitors, a drop in Hes expression, and an increase in the endocrine lineage at the expense of the exocrine population (Apelqvist et al., 1999). A follow-up study with Hes1 null mice corroborated the results of the previous study, confirmed the presence of Notch pathway components in gastrointestinal tissues, and demonstrated that the phenotype included perturbated intestinal cell fate decisions as well (Jensen et al., 2000). In particular, Hes1 null mice had a reduced enterocyte population, characterized by increases in the enteroendocrine and goblet lineages (Jensen et al., 2000). These imbalanced populations provided strong indication that Hes1 was also involved in early intestinal cell fate determination, and led to the investigation of the other members of the signaling pathway. The retained presence of enterocytes in Hes1 mice may be due to compensation from the observed presence of other Hes family members. Indeed, a severe Hes phenotype in neural stem cells required a triple knockout (Hatakeyama et al., 2004). Hes1 has been observed in most of the proliferating cells of crypts, but not in the enteroendocrine, Paneth, and goblet lineages, and it is absent in cells located in the villi. (Jensen et al., 2000; Kayahara et al., 2003). In the embryonic intestine, Hes1 was initially identified in the enterocytes of villi, but later

Figure 1. The ultimate stem cells reside above the Paneth compartment and commit apoptosis in response to low levels of DNA damage. Their immediate daughter cells are potential stem cells and comprise three tiers of repair capability. If the ultimate stem cells are lost, the potential stem cells can regenerate the system and take their place (Marshman et al., 2002). The factors involved with specification of the four intestinal epithelial lineages are listed next to the fate decision. Zones of expression for various factors are shown with respect to the crypt-villus axis (not to scale). The expression patterns of Hes 5, 6, and 7, Notch, Delta, Jag1, as well as radical, maniac, and lunatic fringe (Rfng, Mfng, Lfng), are estimated from the in situ hybridizations in Schroder and Gossler (2002). Other factors are discussed in the text. S: ultimate stem cell; UP: uncommitted progenitor/potential stem cells; SP: secretory progenitor; EP: enterocyte progenitor; E: enterocyte; EE: enteroendocrine cell; GP: goblet progenitor; I: intermediate cell).

TABLE 1. Small Intestinal Phenotypes Induced by Targeted Genetic Manipulation

Genetic manipulation	Phenotype characteristics	Reference
EphB2 and B3 double null	Altered morphology of intervillus pockets, mislocalized Paneth and undifferentiated cells as far up as villi, substantial mixing of Ephrin B positive and negative cell populations	Batlle et al. (2002)
EphB3 null	Mislocalized Paneth cells	
EphB2 null	Abnormal positioning of Ephrin B positive cells in the crypt and Ephrin B negative cells in the junction and lower villus	
Dominate negative EphB2	Abnormal positioning of Ephrin B positive cells in the crypt and Ephrin B negative cells in the junction and lower villus, mislocalized Paneth cells	
Constitutively active Rac1	Premature differentiation of intervillus cells into Paneth cells and (markers for) enterocytes. Increase in crypt proliferation, wide villi	
Dominate negative Rac1	Longer crypts, which sometimes penetrate into villus cores, wider villi, inhibits expression of enterocyte markers at junction, increase in intermediate cells (have smaller Paneth granules and some mucin), decrease in goblet cells, decrease in migration rate	Stappenbeck and Gordon (2000)
bHLH factors		
Hes1 null	Fewer enterocytes, more goblet cells, increased hormone expression and apoptosis, increased expression of math1, nkx2-2, Pax4, Pax6, Delta1, Delta3, Ngn-3, NeuroD; increased Hes3 and Hes5 expression. Villi were less organized	Jenson et al. (2000)
Math1 null	Lack secretory cells	Yang et al. (2001)
Ngn-3 null	Lack enteroendocrine cells Intestinal metaplasia of the stomach	Jenny et al. (2002) Lee et al. (2002)
Wnt pathway		
Tcf-4 null	Lack of proliferating cells, reduction in quantity of villi, absence of enteroendocrine cells; Intervillus cells have markers of differentiation.	Korinek et al. (1998a); Wetering et al. (2002)
Constitutively active β-catenin in chimeras	Villi branching, delay in migration, substantial increase in proliferation which is offset by apoptosis	Wong et al. (1998)
Constitutively active Lef-1/β-catenin fusion protein in chimeras	Increased apoptosis of transgenic cells during development, by adult age, crypts lack transgenic cells	Wong et al. (2002)
Tissue specific removal of β-catenin gene in adult mice	Loss of crypts and goblet lineage, increased apoptosis, loss of attachment of epithelium from villus mesenchyme	Ireland et al. (2004)
Deletion of APC gene in adult mice	Crypts are lengthened greatly, enhanced proliferation, retention of older villus cells, loss of Paneth localization, loss of goblet cells, decrease in enteroendocrine cells, inhibition of migration, increased apoptosis, increased expression of EphB2 and 3, ECM remodeling, lack of expression of EphrinBs and villus markers in affected regions	Sansom et al. (2004)
Tissue specific Dkk1 expression	One copy: some Paneth cells lacking EphB3 are mispositioned Two copies: severe reduction in the quantity and size of villi and crypts, lack of proliferation, nuclear β-catenin, c-myc, Enc1; loss of secretory lineages and Math1 expression. Expression of p21 in the crypt less regions.	Pinto et al. (2003)
Adenovirus delivery of Dkk1	Dose dependent effects: initially more severe in proximal small intestine with loss of crypts, decrease in villi quantity, mesenchyme disruption by ulceration and inflammation. Later, effect is stronger on the colon with the onset of colitis	Kuhnert et al. (2004)
Wnt1 mis-expressed under Pdx-1 promoter	Duodenum becomes more like stomach, expression of intestinal markers moved distally	Heller et al. (2002)
Lef-1/β-catenin fusion protein mis-expressed to lung endoderm	Conversion of lung morphology to simple cuboidal epithelium with microvilli and markers of intestinal differentiation	Okubo and Hogan (2004)
Mesenchyme factors		
PDGF-A null	Reduced proliferation, substantial loss of mesenchyme, fewer goblet cells, loss of pericryptal fibroblasts with age. Villi are greatly reduced in quantity, inconsistent in terms of width, height, and spacing.	Karlsson et al. (2000)
PDGFR-α null chimeras	Lethal by E16.5; villi reduced in quantity, shorter, and wider, reduced mesenchyme, increased smooth muscle.	
Target expression of pan-Hedge Hog inhibitor, Hhip	High transgene expression: early post natal lethality (P0), flattened, pseudostratified, hyper proliferative epithelium; disrupted formation of microvilli, reduced muscularis externa. Lower transgene expression: ectopic crypts in thick, extensively branched villi, inhibition of enterocyte differentiation, nuclear β-catenin positive cells and ISEMFs as far up as the villus tip, pervasive expression of Wnt target genes decrease in BMP expression, increased mesenchymal smooth muscle cell population.	Madison et al. (2005)
Shh null	Increased proliferation, extra innervation, intestinal metaplasia of the stomach, gut malrotation, decrease in smooth muscle, imperforate anus	Ramalho-Santos et al. (2000)
Ihh null	Reduced proliferation, decreased innervation and duodenal enteroendocrine population, gut malrotation, decrease in smooth muscle	
Foxl1 null	Embryonic: delay of mesenchymal condensation and villus formation Postnataly: over proliferation of stomach and small intestine, increase of goblet cells, clustering of goblet cells in crypts, mucin filled cysts, increased apoptosis, increased HSPGs, nuclear β-catenin positive cells	Kaestner et al. (1997) Perreault et al. (2001)
Nkx2-3 null	Embryonic: reduced proliferation, shorter and fewer villi, reduced mesenchyme Adult survivors: disordered hyperproliferative crypts, irregular, thicker, longer, branched Villi, increased migration rate, intestinal circumference, and vascularization, lack of leukocytes in the villus cores, spleen defects.	Pabst et al. (1999)
Targeted expression of BMP inhibitor noggin	Villi are wider, blunted, contain invaginations of proliferative cells, ectopic crypts expressing c-myc, ki-69, EphB3, lysozyme; Crypt-villus axis specification is lost.	Haramis et al. (2004)

TABLE 2. Disease Phenotypes Resulting from Signaling Pathway Mutations

Factor/pathway	Disease phenotypes	Reference
Wnt pathway ligand/receptor	Intestinal tumorigenesis	Holcombe et al. (2002)
	Mammary tumorigenesis, Syndecan-1 mediates process	Alexander et al. (2000)
Wnt pathway APC	Familial adenomatosis polyposis (FAP)	Kinzler et al. (1991); Su et al. (1992)
	Relationship to EphB Expression	Batlle et al. (2002)
	Relationship to KLF4 expression	Dang et al. (2000)
	Other intestinal genes that affect the phenotype	Cormier et al. (1997); Oshima et al. (1996)
	Intestinal tumorigenesis	Kinzler and Vogelstein (1996); Morin et al. (1997); Samowitz et al. (1999)
	Relationship to SMAD4 of BMP pathway	Takaku et al. (1998)
Wnt pathway β-Catenin	Intestinal tumorigenesis	Sparks et al. (1998); He et al. (1998); Morin et al. (1997); Samowitz et al. (1999)
LKB1	Peutz-Jeghers (PJ) syndrome	Hemminki et al. (1998); Jenne et al. (1998); Karuman et al. (2001)
BMP pathway	Juvenile Polyposis (JP) syndrome	Howe et al. (1998); Takaku et al. (1999)
Hedge hog pathway	Shh null mice have gut abnormalities similar to holoprosencephaly and Pallister-Hall syndrome; Ihh null mice have a phenotype similar to Hirschsprung's disease.	Ramalho-Santos et al. (2000)
	Intestinal tumorigenesis	Madison et al. (2005)
Cdx1	Intestinal tumorigenesis	Soubeyran et al. (2001); Domon-Dell et al. (2003)
Cdx2	Intestinal tumorigenesis	Kedinger et al. (1998b); Chawengsaksophak et al. (1997); da Costa et al. (1999)
Hath1 (Math1)	Intestinal tumorigenesis	Leow et al. (2004)
NeuroD	Diabetes	Malecki et al. (1999)

studies localized the transcript and protein to the intervillus cells (Jensen et al., 2000; Schroder and Gossler, 2002; Kayahara et al., 2003).

The localization of Math1 was subsequently determined using Math1$^{+/\beta\text{-gal}}$ transgenic mice. Embryonic expression of Math1 occurs in cells in the intervillus and villus regions, but in the adult, is observed in cells with Paneth and goblet morphologies, and expression is limited to a subset of cells in the midcrypt region. Math1$^{-/\beta\text{-gal}}$ mice suffered from early postnatal lethality, but analysis of ED 18.5 embryos indicated the absence of goblet and enteroendocrine cells, as well as the presumptive Paneth lineage (Yang et al., 2001). Consistent with its function as an early commitment factor, the loss of all secretory populations provided good evidence that Math1 was required to generate secretory lineage progenitors. The expression of Math1 in enteroendocrine cells may be similar to that of neuronal populations, in which Math1 is downregulated after downstream factors, such as Ngns and NeuroD (Beta2), exert their effects (Lee, 1997).

The role of NeuroD in the intestine had actually been explored earlier than that of Hes1 or Math1. Targeted ablation of NeuroD had resulted in the loss of CCK and secretin-secreting enteroendocrine cells (Naya et al., 1997). A further analysis reported the colocalization of a reporter for NeuroD with all chromogranin A positive cells, suggesting that it is present in all enteroendocrine cells, but that it is not required for expression of all types of hormones. Combined with the finding that selective destruction of secretin-producing enteroendocrine cells resulted in a differential loss of other members of the enteroendocrine population, this has led to the hypothesis that there are multiple enteroendocrine progenitors, each supplying a subset of the total enteroendocrine population, and expressing various combinations of hormones (Rindi et al., 1999). Thus, the final hormone produced by any particular enteroendocrine cell is likely determined by cooperation with other factors. This is supported by several lines of evidence. NeuroD increases expression of the homeodomain protein, Pdx-1 (Sharma et al., 1997), which has a role in the production of specific populations of enteroendocrine cells (Offield et al., 1996, Ramalho-Santos et al., 2000). Mice null for Pax4 lose most enteroendocrine cells of the duodenum and jejunum, while Pax6 null mice lose the GIP enteroendocrine cells of the duodenum (Larsson et al., 1998). Pax6 was shown to be required for production of the GLP-1 and GLP-2 enteroendocrine secretory products (Hill et al., 1999).

As for early specification of the enteroendocrine lineage, experiments have indicated Ngn-3 is the likely culprit. Ngn-3 expression in the gut was detected as early as ED 12.5, preceding that of NeuroD. In adults, Ngn-3 localized to the crypt

compartment, mostly to proliferating cells, and did not stain with the pan-endocrine marker, chromogranin A. In contrast, NeuroD positive cells were seen in the villus, co-expressing chromogranin A. Ngn-3$^{-/-}$ mice lack expression of enteroendocrine hormones, Chromogranin A, and NeuroD, but Math1 expression is retained. A tissue grafting experiment where Ngn-3$^{-/-}$ anlagen was implanted and allowed to develop to adult maturity, evaluated the role of Ngn-3 in adult intestine. The Ngn-3 graft also lacked enteroendocrine cell markers, and lineage-tracing experiments confirmed that the descendants of Ngn-3 expressing cells are enteroendocrine (Jenny et al., 2002). These results indicate that the early and late proneural bHLH factors, Ngn-3 and NeuroD, respectively, have a similar function in determining the enteroendocrine cells of the intestine.

The expression of a putative notch signaling regulator, Msi-1, has been observed in intervillus cells and in the base of crypts (Kayahara et al., 2003, Potten et al., 2003b), but is not detected in Paneth cells (Kayahara et al., 2003). The combination of Msi-1 localizing to the putative stem cell compartment (Potten et al., 2003b), the fact that Msi-1 was a neural stem cell marker (Kaneko et al., 2000) important for the maintenance of neural stem cells (Sakakibara et al., 2002), and the thought that it was involved in asymmetric divisions (Okabe et al., 2001), quickly led to the hypothesis that Msi-1 might also be a stem cell marker for the intestinal epithelia (Okabe et al, 2001; Kayahara et al., 2003; Potten et al., 2003b). However, the expression of Msi-1 is broader than would be expected for a specific marker of a stem cell lineage, suggesting that its expression may be retained in the immediate descendants of the mammalian small intestinal stem cells (Potten et al., 2003b).

Msi-1 has been shown to inhibit m-Numb, a suppressor of Notch signaling, by binding to a site on Numb transcripts conserved in vertebrates. The presence of Msi maintained expression of Hes1, while the overexpression of m-Numb induced differentiation (Imai et al., 2001). Other reports indicated that activation of Hes1 by Notch signaling maintains stemness in neural progenitors, and that other Hes members are functionally redundant, but not functionally equivalent (Ohtsuka et al., 1999; Nakamura et al., 2000). That is, members of the Hes family have different abilities in maintaining stemness (Ohtsuka et al., 1999). Thus, there may be a mechanism of graded preservation of proliferative potential, based on differential expression of Hes family members. A role for stem maintenance by Hes has also been implicated by the increased expression of Msi-1 in certain tumors (Kanemura et al., 2001; Potten et al. 2003b). While a stem cell maintenance function for Notch signaling is not out of the question for the intestinal stem cell, it remains to be proven.

ADDITIONAL EVIDENCE SUPPORTING A LATERAL INHIBITION MECHANISM IN THE INTESTINAL EPITHELIA

Notch signaling between cells can be highly complex, involving many different forms of Notch receptors, ligands, and other modifying factors, as well as layers of intricate positive and negative feedback loops. Lack of data on Notch signaling in the intestinal epithelium prevents a definitive explanation of the mechanism of lineage specification, but there are indications as to how Notch signaling may contribute to specification of lineages. The default regulatory mechanism of the bHLH factors involves autoregulation and Notch signaling. The bHLH factors bind a consensus site known as an E-box (Lee, 1997). Analysis of Math1's transcriptional regulatory element shows that, in addition to having a Hes preferred site for repression, it also has an E-box, indicating that Math1 can activate its own transcription (Helms et al., 2000). Math1 has also been shown to participate in its own downregulation, probably by activating transcription of Hes5, and can result in either balanced or complete loss of Math1 expression. The interaction between Math1 and Notch effectors of the Hes family was investigated after Math1 was determined to be involved in the expression of multiple Notch receptors and ligands (Gazit et al., 2004). During inner ear development, the presence of Math1 is associated with increased expression of Notch ligands, while in adjacent cells where Notch signaling has been activated, Math1 is repressed, and the expression of Notch receptors is increased (Zine and de Ribaupierre, 2002). In a simple model of Notch mediated lateral inhibition, loss of Hes would be expected to result in increased expression of Math1 and Notch ligands (Delta), while loss of Math1 should decrease expression of Notch ligands. That is the case in the intestine; Hes1 null mice have increased expression of delta 1 and 3, while in Math1 null mice, delta 3 expression is halved (Jensen et al., 2000; Yang et al., 2001).

Cell clustering is another indication of a perturbation in lateral inhibition. While enteroendocrine cell clustering was not observed in Hes1 null mice (Jensen et al., 2000), the Math1 null mice had an interesting presence of dark clusters of LacZ positive cells in the intervillus region, which stain strongly for the proliferative marker Ki67 (Yang et al., 2001). This indicates that the absence of the Delta family of ligands is preventing the expression of Hes, which leads to an accumulation of transcription factors on Math1's promoter, and is resulting in vigorous transcription of the lacZ reporter gene. A study by Schroder and Gossler (2002) specifically identified the localization of Notch signaling ligands, receptors, and Hes family members in the intestine. The expression of some Notch pathway components, in specific tissues and others over diffuse areas, suggests that notch signaling in the intestine is complex enough to fulfill many functions, such as maintenance of stem cells and

guiding multiple differentiation regimes, simultaneously.

The group of factors responsible for specifying enterocyte and goblet lineages are less defined, but there are some candidates, such as the intestinal homeobox gene Cdx2 and the transcription factor ELF3, which were shown to be important for in vitro or in vivo differentiation into both of those lineages (Suh and Traber, 1996; Ng et al., 2002). Laminin-1 appears to upregulate Cdx2 expression, which activates a differentiation program (Lorentz et al., 1997). Whether Cdx2 induces enterocyte or goblet differentiation could depend on the presence of previously generated factors from earlier specification, such as Hes1, Math1, or their effectors. Cdx2 is known to bind and activate the promoter of the goblet specific gene MUC2 (Yamamoto et al., 2003). KLF4, implicated as a terminal differentiation factor for goblet cells in the colon, also has Cdx2 binding sites in its promoter (Katz et al., 2002). Microarray analysis of intestinal culture cells, after induction of KLF4, showed downregulation of cell cycle promoters and up regulation of cell cycle inhibitors, structural proteins, such as villin 2, and a single colonic mucin (Chen et al., 2003). KLF4 transcripts are present in the small intestine, increasing along the proximal-distal axis (Shields et al., 1996), but it is not clear if the factor is required for goblet differentiation in the small intestine. Intermediate cells of the small intestine have granules similar to those of Paneth cells and granule-containing goblet cells, as well as some mucin (Throughton and Trier, 1969; Garabedian et al., 1997). Intermediate cells are so termed because their granules are smaller than those of Paneth cells, but larger than those observed in the granule goblet cells. Intermediate cells are observed predominately in the crypt above the Paneth cells, while the subpopulation of granule goblet cells are seen further up the axis, but the granules are lost as differentiation and migration proceeds (Throughton and Trier, 1969; Garabedian et al., 1997). A study in which the Simian virus 40 large T antigen was expressed under a cryptidin promoter resulted in ablation of the Paneth lineage, but increased the population of intermediate and granule goblet cells (Garabedian et al., 1997). In the previously mentioned study with dominant negative Rac1, increased numbers of intermediate cells were observed at higher locations along the axis (Stappenbeck and Gordon, 2000). It is currently thought that intermediate cells can give rise to Paneth or goblet cells, but whether this role is exclusive is not clear (Garabedian et al., 1997).

REGULATION OF PROLIFERATION

The Wnt signaling pathway has been found to play a critical role in the proliferative maintenance of the small intestine epithelium. In the absence of a Wnt signal, axin binds GSK3, APC, and β-catenin together, allowing GSK3 to phosphorylate the N-terminus of β-catenin, resulting in its ubiquitination and destruction. The binding of Wnt ligand to its receptor Frizzled (Frz) causes Disheveled to bind axin, disrupting the complex and preventing phosphorylation of β-catenin. Accumulation of β-catenin results in its translocation to the nucleus, where it interacts with members of the Lef/Tcf family to regulate transcription (Bienz and Clevers, 2000). Both Tcf-4 and Lef-1 are expressed in the intestinal epithelium during development. Whereas Lef-1 expression ceases postnatally, Tcf-4 expression is maintained throughout life (Korinek et al., 1998b; Wong et al., 2002).

The importance of β-catenin/Tcf-4 in maintenance of the intestinal epithelium was demonstrated in Tcf-4 null mice, which lack any proliferating intervillus cells after ED 16.5, and instead express markers of differentiation (Korinek et al., 1998a; van de Wetering et al., 2002). Nuclear localization of β-catenin is observed throughout the bottom third of the crypts, which includes base columnar, Paneth, and putative stem cells, but only membrane associated β-catenin persists further up the axis (Batlle et al., 2002). Evidence for the locations of nuclear β-catenin is controversial (Booth et al., 2002). The proliferative response initiated by Tcf-4 appears to be maintained further up the axis by its target c-myc, a repressor of the cell cycle inhibitor p21 (van de Wetering et al., 2002). In vitro experiments with intestinal cells have suggested that cell cycle arrest is initiated by p21 and maintained by p27 (Tian and Quaroni, 1999; Quaroni et al., 2000), but expression of p21 has been observed in differentiated villus and Paneth cells (Pinto et al., 2003). The presence of nuclear β-catenin in both cell cycle arrested Paneth cells and proliferating crypt cells indicates that a differential response to β-catenin signaling is established during differentiation. The proper distribution of β-catenin along the axis remained in Eph receptor double-knockout mice, despite the displacement of Paneth cells further up the axis, implicating a cell nonautonomous process, where Wnt signaling occurs at the base of the crypt, or possibly, that it is repressed elsewhere (Batlle et al., 2002). The regulation of the cell cycle by β-catenin/Tcf and the differentiation factors may be related through the factor p300/CBP. p300/CBP is known to be a coactivator of Tcf at high concentrations of β-catenin, and a repressor at low levels (Bienz and Clevers, 2000). KLF4 cooperates with p300/CBP to activate p21 transcription, in addition to repressing cyclin D1 expression (Bieker, 2001). NeuroD also interacts with p300/CBP to activate p21 expression in enteroendocrine cells (Mutoh et al., 1998). Thus, the repressive activities of differentiation factors may combine with the p300/CBP to interpret the β-catenin gradient along the crypt-villus axis and mediate cell cycle control.

In an attempt to better define the role of Wnt signaling and β-catenin, numerous genetic modifications of the system have been targeted to the small intestine epithelium. Expression of a constitutively active β-catenin gene in chimeras resulted in equally increased proliferation and apoptosis of the transgenic ep-

ithelium (Wong et al., 1998). In contrast, cells expressing a β-catenin/Lef-1 fusion protein were highly apoptotic, and transgenic crypts were lost during maturation (Wong et al., 2002). Using the *cre-lox* system, the β-catenin gene was removed, resulting in loss of crypts (Ireland et al., 2004). Use of the same system to remove the APC gene resulted in longer crypts comprised of cells containing nuclear β-catenin, an inhibition of migration up the crypt-villus axis, retention of villus cells that should have been lost, and a substantial increase in apoptosis (Sansom et al., 2004). Other recent studies have perturbed the Wnt pathway at the ligand-receptor level, using the extracellular Wnt antagonist Dkk1 to bypass redundancy. Pinto et al. (2003) misexpressed two copies of Dkk1, resulting in inhibition of proliferation and the substantial loss of crypts and villi. A similar effect was achieved following adenoviral-mediated expression of Dkk1 in adult mice (Kuhnert et al., 2004).

These gene knockout, misexpression, and overexpression studies affected lineage commitment to varying degrees. Tcf-4 null mice lack the enteroendocrine lineage (Jensen et al, 2000), removal of β-catenin (Ireland et al., 2004) or APC (Sansom et al., 2004) resulted in the loss of the goblet lineage, while misexpression of Dkk1 in the epithelium eliminated the secretory lineages and abrogated Math1 expression. However, loss of secretory lineages was not observed in the adenoviral-mediated delivery of Dkk1 (Kuhnert et al., 2004). It is unclear whether the loss of these lineages results from perturbation of developmental processes, reception of contradictory signals along the crypt-villus axis, or if Wnt signaling is inherently tied to lineage specification in adults.

A role for the Wnt signaling pathway in specification of the gut has been implicated by expression and misexpression studies. Some studies have identified regional and tissue specific expression of Wnt pathway components along the mouse and chick gut during development (Lickert et al., 2001; Theodosiou and Tabin, 2003). Heller et al. (2002), misexpressed Wnt1 under the control of the Pdx-1 promoter, resulting in the conversion of the proximal duodenum into stomach-like tissue, and movement of intestinal markers posteriorly. The expression of a constitutively active Lef-1-β-catenin fusion protein in developing lungs prevented the formation of the typical lung cell types, and resulted in a cuboidal epithelium with microvilli (Okubo and Hogan, 2004). Additionally, microarray analysis indicated the upregulation of many markers of the small intestine, including those specific to Paneth and goblet cells. The predominance of secretory gene expression was attributed to high levels of Math1 and, interestingly, there was also increased expression of the Notch ligand Delta 3 (Okubo and Hogan, 2004). The exact role of Lef-1 is unclear, since Lef-1 null mice were reported to lack gross intestinal defects (van Genderen et al., 1994). Tcf-4 may also have a role in patterning endoderm, as injection of murine Tcf-4 mRNA into *Xenopus* embryos resulted in ectopic expression of intestinal epithelium markers (Lee et al., 1999). Additional experiments are needed to further define the role of Wnt signaling in patterning the endoderm of the gut.

Taken together, this evidence suggests that Wnt signaling is required for the maintenance of the epithelial stem cell, is the normal means of initiating proliferative response in the epithelium, participates in endodermal specification, and may have an intrinsic role in lineage specification. The varying levels of overgrowth and apoptosis in the mutant studies also suggest that aberrant signaling is occurring with different levels of severity, or can be interpreted in a variety of ways. A study to determine the effects of these signaling changes on the epithelial stem cell population, using the known marking methods (Potten et al., 2002), has not been reported.

The frequency and distribution of normal apoptosis along the crypt-villus axis has been well documented (Hall et al., 1994; Potten et al., 1997). Apoptosis is observed predominately in the villus, beginning in the lower third and increasing towards the tip, but is also observed in cells near the crypt base (Hall et al., 1994). A consistent level of apoptosis in the putative stem cell location has been postulated to be a mechanism of preventing overproliferation by removal of excess stem cells (Potten et al., 1997). Interestingly, there is strong evidence that LKB1 regulates the p53 dependent apoptosis of mature villus cells (Karuman et al., 2001). LKB1 was originally identified as a tumor suppressor gene that is mutated in the majority of Peutz-Jeghers (PJ) cancer syndrome patients, and is downregulated in intestinal epithelial cancer lines (reviewed in Baas et al., 2004a, 2004b). Strong cytoplasmic expression of LKB1 is observed in apoptotic crypt and villus cells, but is absent in PJ polyps (Karuman et al., 2001). The exact contribution of the apoptotic and cell polarity functions of LKB1 to PJ polyp formation and progression remains unclear, but Baas et al. (2004b) suggest that loss of both functioning alleles is associated with malignancy, as PJ polyps remain differentiated and polarized.

Reciprocal signaling between the epithelium and underlying mesenchyme can provide important spatially coordinated proliferative instructions to cells of each tissue, during development and maintenance phases. Platelet-derived growth factor A (PDGF-A) is observed in the embryonic intervillus epithelium, while its receptor, PDGF-Rα, is expressed in the underlying mesenchyme (Karlsson et al., 2000). The interactions between PDGF-A and PDGFR-α ensures the survival and proper expansion of the pericryptal fibroblasts, as loss of PDGF-A results in massive defects of villi structure, and with time, the loss of the fibroblast community (Karlsson et al., 2000). Mesenchymal fibroblasts secrete keratinocyte growth factor (KGF), which stimulates the receptor expressing epithelium and enhances the number of goblet cells

by acting on their lineage progenitor (Bjerknes and Cheng, 2001). The importance of the fibroblast population has been greatly expanded in recent groundbreaking work by Madison et al. (2005). Expression of sonic hedge hog (Shh) and indian hedge hog (Ihh) was localized to the intervillus epithelium, and that of the receptor patched (Ptch) predominately to the putative fibroblasts beneath, but Ptch was also observed in the inner muscle layer, and in some cells of the mesenchyme of the villus. Transgenic mice with a pan hedge hog (Hh) inhibitor targeted to the intestinal epithelium were created. Mice with high levels of transgene suffered early postnatal lethality, and had a flat, pseudostratified, hyperproliferative epithelium. Mice with lower transgene levels had extensively branched villi with ectopic crypts containing proliferative cells, instead of differentiated enterocytes as far up as the villus tip. Nuclear β-catenin was observed in proliferative regions throughout the crypt-villus axis, and Tcf-4 target genes such as Cdx-1, normally restricted to the crypts, were highly expressed as well. Since ISEMFs were mislocalized to the villus, and were adjacent to the aberrantly proliferating villus epithelium, they are implicated as the source of the Wnt ligand. In vitro experiments with ISEMF cell lines demonstrated their ability to respond to Hedge Hog signals. The authors conclude that Hedge Hog signaling is required for embryonic villus formation, and for restricting ISEMF cells to the proper location and establishing crypt-villus polarity. A role for Ihh and Shh signaling in the small intestine was previously revealed by the developmental defects of Shh and Ihh null mice. The phenotypes indicated that Ihh signaling was important for generating a critical epithelial stem cell maintenance or proliferative signal not dependent on Tcf-4 activity, at least during later stages of development, and that Shh signaling restricted the ability of the epithelium to proliferate in the duodenum (Ramalho-Santos et al., 2000). However, there were a number of phenotypic differences between the hh inhibitor transgenic mice and the null mice.

Components of the TGF-β signaling pathway are also implicated in reciprocal signaling, appearing to prevent improper proliferation. TGF-βII receptors, which bind the ligand, localize to villus enterocytes, especially at the tip (Winesett et al., 1996), while BMP-4 protein is observed in the mesenchyme of the villi and activates TGF-β effectors and phospho-Smads 1, 5, and 8 in the adjacent differentiated villus cells. (Haramis et al., 2004). In another study, a TGF-β receptor, Bmpr1a, was observed in a gradient along the crypt-villus axis and in stem cells, but not in proliferative cells. Transient Noggin expression was seen in some stem cells, along with the phospho-Smads 1, 5, and 8 (He et al., 2004). Mice with knockouts of mesenchymal factors, Foxl1 or Nkx2-3, were both noted to have decreases in BMP expression, developmental delays from a lack of epithelial proliferation, and adult survivors that developed extended hyperproliferative crypts and large villi (Kaestner et al., 1997; Pabst et al., 1999).

The targeted expression of the BMP antagonist, *Xenopus* Noggin, did not result in pervasive epithelial proliferation, but generated scattered incidences of ectopic crypts in the villi, complete with proliferation and differentiated lineages, including Paneth. The presence of inflammation, branching villi, and cystic crypts early on, along with a later onset of polyp formation, phenocopied the cancer predisposition syndrome Juvenile Polyposis, which has been previously associated with mutations in BMP type IA receptors and Smad4 (Haramis et al., 2004). Recent experiments on the role of BMP and Wnt signaling in stem cell renewal have generated new insights. Transient Noggin expression near stem cells is believed to inhibit their reception of BMP signals, causing phosphorylation and inactivation of PTEN, allowing Phosphatidylinositol-3 kinase (PI3K), through Akt, to regulate nuclear β-catenin localization and transcriptional activity (He, et al. 2004). Madison et al. (2005) identified the presence of BMP-7 in the epithelium, and BMP-2 in both the epithelium and mesenchyme. In the transgenic mice with the Hedge Hog inhibitor, the expression of BMP-2 and 4 was reduced, but BMP-7 was severely decreased. The authors also exposed intestinal mesenchyme to a Shh ligand and observed the induction of BMP-4. Thus, the BMP signaling also appears to involve cross-talk with the Hedge Hog pathway, and functions to maintain the proper polarity of the crypt-villus axis by inhibiting inappropriate epithelial proliferation in the villus. The importance of BMP signaling in the underlying mesenchyme remains elusive.

A follow-up study on the Foxl1 null mice revealed that the heparan sulfate proteoglycans (HSPGs), Perlecan and Sydnecan-1, which are normally restricted to the proliferative compartment, were greatly increased along the crypt axis, coinciding with an increase in the zone of nuclear β-catenin staining (Perreault et al., 2001). HSPGs are a class of cell surface and ECM proteins with sulfated polysaccharide branches expressed differentially in tissues and through time. HSPGs are known for their ability to bind a wide variety of extracellular ligands, including BMPs 2 and 4, Shh, and the Wnts. The functional interactions can be quite strong and specific, and are based on various sulfate modifications produced by sulfotransferases. Many of the interactions between HSPGs and ligands have been shown to be important for proper signal transduction, as mutants with defects in the enzymes that produce HSPGs can phenocopy mutants for signaling ligands (Bernfield et al., 1999). HSPGs are suspected to modify signal transduction through a number of ways, such as transport, sequestration, or internalization of ligands, acting as a cell surface coreceptor, stabilizing the ligand receptor interaction, or by facilitating dimerization of receptors (Bernfield et al., 1999; Nybakken and Perrimon, 2002). Recent experiments in *Drosophila* indicate that HSPGs inhibit high strength local

Wnt signaling, but facilitate Wnt signaling at greater distances where the concentration of ligand is low, and that Wnt signaling is further modified by variable expression of Frz receptors to form distinct boundaries of signal transduction (Baeg et al., 2004; Kirkpatrick et al., 2004). The probability that similar kinds of mechanisms may be used to coordinate signal transduction of multiple pathways in the intestinal epithelium seems high, given the presence of signaling across the ECM and the Foxl1 phenotype. Microarray analysis of crypts enriched for progenitor cells by selective killing of Paneth cells results in upregulation of a cell surface HSPG, one of the biosynthetic enzymes, and another proteoglycan-related factor (Stappenbeck et al., 2002).

A number of correlations have been made between these signaling pathways and diseases (Table 2). Given the critical role of the pathways in regulating proliferation and apoptosis, it is not surprising that neoplastic transformation often results from perturbation of key regulatory domains. However, tumorigenesis in the small intestine is actually quite low, and most of the transformation resulting from mutations in these pathways occurs in the colon, which appears to be similarly regulated. The difference in tumorigenic frequency between the small and large bowel is attributed to the small intestinal epithelial stem cell's higher propensity to commit apoptosis when damaged, and the higher genetic fidelity achieved by regular retention of the template strands of DNA (Potten et al., 2003a).

DISCUSSION AND PERSPECTIVES

The last decade of research has generated a wealth of information on the regulation of the intestinal epithelium, but the functions of many pathways and their mechanisms remain unclear. However, there may be hints in other more highly defined systems. The generation of the mechanosensory bristle from the sensor organ precursor cell in *Drosophila* utilizes Notch signaling, and the presence or absence of Numb in successive cell divisions to specify each of the final four types of cells (Greenwald, 1998). Thus, Notch signaling in the intestinal epithelium has the potential to regulate differentiation choices between more specialized secretory cell types. A role for Notch signaling in lineage specification and stem cell maintenance is not mutually exclusive. Two types of Numb have been identified in humans, one of which functions to induce neural proliferation, while the other induces differentiation (Verdi et al., 1999).

The use of Notch signaling in conjunction with Wnt signaling in stem cell maintenance and proliferative responses has also been described. Wnt signaling is required for the survival of hematopoietic stem cells, and stimulates their entry into the cell cycle, probably through Lef-1/Tcf, but Notch signaling is required for the maintenance of their undifferentiated state (Reya et al., 2003; Duncan et al., 2005). In addition, there appears to be cross-talk between the pathways, since Wnt signaling increases Notch1 and Hes1 expression (Reya et al., 2003; Duncan et al., 2005). A similar mechanism may be in place in the intestinal epithelium.

The intestinal epithelial stem cell divides at a periodic rate (Potten et al., 2003a), but whether this process is regulated intracellularly or intercellularly is unknown. The presence of such factors such as Hes1, Hes5, Hes7, and Lunatic Fringe (Lfng) is curious, given their known roles in regulating timed events. Hes 1, 3, and 5 have also been shown to be required for the proper timing of neural stem cell differentiation (Hatakeyama et al., 2004). The specification of the presomitic mesoderm into segmented somites occurs at precise intervals of time, is autoregulated, and depends on the expression of Notch pathway components Hes1, Hes7, and Lfng (Bessho and Kageyama, 2003). In this system, Wnt signaling is also cyclic, and its disruption abolishes the oscillation of Lfng (Bessho and Kageyama, 2003). Further experiments revealed additional interactions between the Notch and Wnt factors, indicating how they might cooperate to form a timing mechanism (Ishikawaa et al., 2004).

The presence of an intestinal epithelial stem cell niche has not been confirmed, but interactions with ECM components and mesenchymal factors are known to be critical for maintenance of the system. Coculture of neural stem cells with endothelial cells has been reported to result in Hes1 upregulation, and the maintenance of stemness (Shen et al., 2004). The intestinal epithelium is situated very close to blood vessels, neurons, and lymphocytes, which are in constant communication. Signals from these tissues are known to regulate the epithelium, but they may be directly involved in stem cell maintenance. Finally, HSPGs have properties that make them excellent candidates for being the primary regulators of ligand diffusion, and HSPGs may be able to mediate the activities of many pathways by generating gradients of ligands along the crypt-villus axis, resulting in the synthesis of a large amount of positional information.

Future work in the intestinal epithelium is needed to clarify the exact functions of the various signaling pathways, describe the precise mechanisms of signaling, identify additional downstream effectors, and define how cross-talk coordinates the whole system.

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