Phases of Canonical Wnt Signaling During the Development of Mouse Intestinal Epithelium

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Background & Aims: **Intestinal crypts constitute a niche in which epithelial progenitors respond to Wnt signals, replicate, and prepare to differentiate. Because mutations in Wnt pathway genes lead to intestinal cancer, the role of Wnt signaling in gut epithelial homeostasis is a subject of intense investigation. We studied how Wnt signaling is established during intestine development.** ***Methods:*** **We studied spatiotemporal features of Wnt signaling at formative stages in mouse embryos, when villous projections appear and crypt precursors occupy intervillus regions. We used TOP-GAL transgenic and Axin2^{LacZ} mice, which report faithfully on canonical Wnt activity, relevant molecular markers, and embryos with aberrant β-catenin activation.** ***Results:*** **Developing intestines first display evidence for Wnt signaling after appearance of villi. During villus morphogenesis, intervillus cells proliferate actively but lack signs of canonical Wnt signaling. Surprisingly, in late gestation and briefly thereafter, conspicuous Wnt activity is evident in differentiated, postmitotic villus epithelium. Neither Tcf4, a principal transcriptional effector of intestinal Wnt signals, nor candidate Wnt targets CD44 and cyclinD1 are expressed in late fetal villus cells that show high Wnt activity. Instead, those cells express the related factor Tcf3 and a different Wnt target, c-Myc. Premature and deregulated β-catenin activation causes severe villus dysmorphogenesis in transgenic mice.** ***Conclusions:*** **Relationships among Wnt signaling, epithelial proliferation, and tissue differentiation are reversed in the developing and adult gut. The canonical Wnt pathway has independent, albeit possibly overlapping, functions in early intestinal villi and adult crypts. These observations advance understanding of Wnt functions in intestinal development and disease.**

In the adult small intestine, monoclonal populations of epithelial stem cells reside in crypts of Lieberkühn and give rise to 4 daughter cell lineages: Paneth cells, which remain at the crypt base, and enterocytes, goblet, and enteroendocrine cells, which populate the villi.[1] Because gut mucosa is renewed every 3–5 days, intestinal stem cells or their immediate progeny replicate frequently,[2,3] and their behavior is regulated by canonical Wnt signals.[4] Crypt-villus junctions delimit a zone of active Wnt signaling and cell proliferation in crypts from that of postmitotic differentiation and absence of Wnt activity in villi.

Epithelial progenitors in the human colon are frequent targets of cancer. Somatic mutations that initiate colon tumorigenesis occur in the *adenomatous polyposis coli* (*APC*) or *β-catenin* (*CTNNB1*) genes and cause constitutive activation of the Wnt pathway.[4,5] β-catenin accumulates in affected cells and functions as a coactivator for transcription factors of the Tcf/LEF family, mainly Tcf4.[6,7] The resulting molecular derangements impose properties of crypt epithelial progenitors, including replication capacity, on transformed cells.[8] Acute APC loss in the adult gut rapidly expands the crypt compartment and impairs differentiation of secretory cell lineages,[9,10] whereas inhibition of Wnt signaling in adult mouse intestine reduces epithelial proliferation, coincident with loss of nuclear β-catenin and of crypt structures.[11] Less is known about the timing and manner in which a local niche for Wnt signaling is established in the developing gut.

Intestinal villus morphogenesis begins when mesenchymal aggregates impinge on the basal aspect of the epithelium to produce primitive folds. The pseudostratified squamous epithelium then converts into a single layer of columnar cells that line mesenchymal stalks or the lamina propria (Figure 1*A*); this occurs around embryonic day (E) 14 in the mouse, E18 in the rat, and between 9 and 10 weeks in human gestation.[12] Cells within the multilayered precursor tissue are joined by tight junctions and desmosomes, which rearrange during villus morphogenesis.[13,14] Rudimentary features of cell maturation, including microvilli and secretory granules,

Abbreviations used in this paper: Ab, antibody; BrdU, bromodeoxyuridine; E, embryonic day; GFP, green fluorescent protein.

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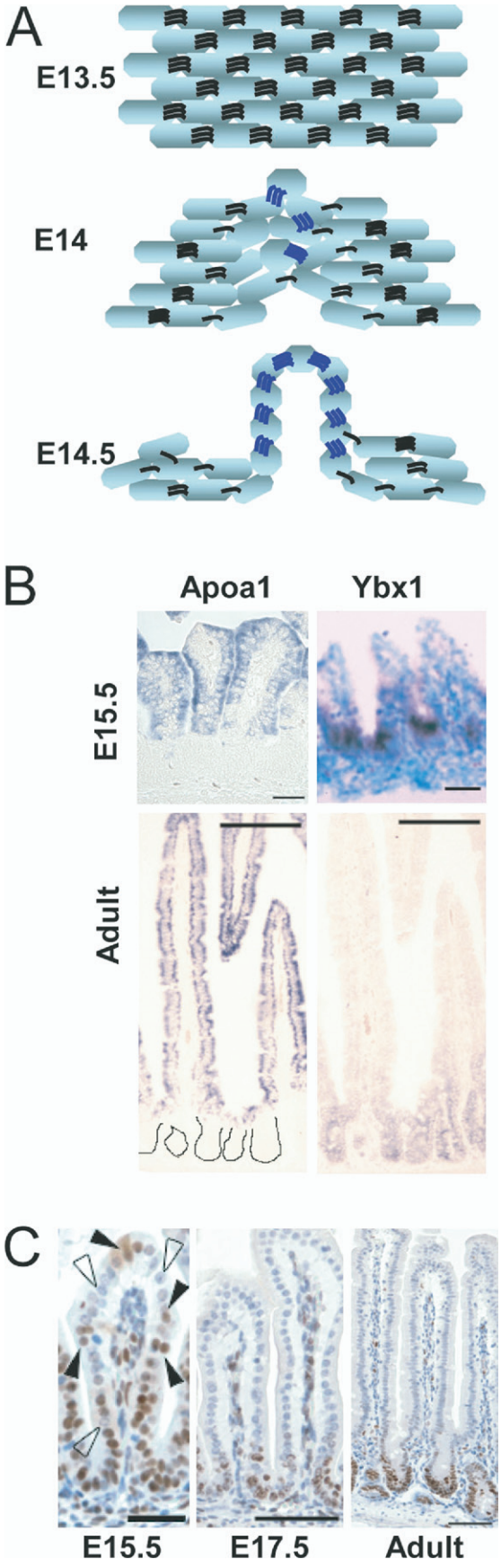


appear coincidentally, and cell numbers remain roughly constant, with attrition of a minority of superficial cells and virtually no dilution of bromodeoxyuridine (BrdU) or [^{3}H]-thymidine labels introduced earlier.[12–14] Thus, the earliest differentiated cells to populate intestinal villi are the same cells that recently were stacked in layers. By contrast, subsequent generations of villus epithelial cells originate in ordered differentiation and migration of intervillus or crypt progenitors.[2,3] To determine when the earliest Wnt-responsive stem/progenitor cells appear, we examined gene expression in relation to mouse gut epithelial development.

Materials and Methods

Experimental Animals

TOP-GAL transgenic mice and strain-matched (CD1) controls were purchased from Jackson Laboratories (Bar Harbor, ME). Sonic hedgehog (Shh)$^{+/Cre}$ mice originated by targeted insertion of a GFP-Cre fusion complementary DNA (cDNA) into the *Shh* locus, allowing expression of Cre recombinase under control of *Shh* regulatory sequences.[15] Catnb$^{+/lox(ex3)}$ mice carry an allele with LoxP sites flanking exon 3 of the β-catenin gene.[16] Axin2^{LacZ} mice have the β-galactosidase reporter cDNA embedded in the *Axin2* locus[25] and were kindly provided by Max-Delbrück Center, Berlin, Germany. Animals were housed and handled according to protocols approved by institutional committees. The morning of identification of a copulation plug was designated as day 0.5 of gestation.

β-Galactosidase Staining

Whole mouse embryos or organs were isolated in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (Invitrogen, Carlsbad, CA), fixed for 15 minutes with 4%

Figure 1. Early epithelial morphogenesis, differentiation, and proliferation in mouse gut development. (*A*) Schematic representation of the conversion of pseudostratified squamous endoderm to a columnar villous epithelium around the 14th day in mouse gestation, emphasizing that the first cells to line primordial villi arise by epithelial morphogenesis and not after programming in crypts or intervillus spaces. Cells with flat morphology become columnar and polarized, and the epithelial conversion involves extensive rearrangement of adherens junctions, represented in this diagram with *hooked bars*. The schema is based on results from Trier and Moxey,[12] Mathan et al,[13] and Madara et al.[14] (*B*) In situ hybridization results with markers of gut epithelial maturity, represented here by *Apoa1* and *Ybx1*, indicate that spatial segregation of gene expression in primordial villi and intervillus spaces occurs in apparent conjunction with epithelial conversion by E15.5, well before segregation of proliferating and postmitotic cells. *Scale bars*, E15.5, 40 μm; adult, 75 μm. Adult crypts lacking *Apoa1* expression are outlined manually for clarity. (*C*) Ki67 immunostaining in E15.5 embryos reveals proliferative activity in most (*solid arrowheads*) but not all (*open arrowheads*) epithelial cells, regardless of villus position. Proliferation is confined to intervillus regions by E17.5 and to the crypts of Lieberkühn in adults. *Scale bars*, embryos, 75 μm; adult, 150 μm.

paraformaldehyde in phosphate-buffered saline (PBS), washed 3 times in PBS, and incubated in staining solution (PBS, pH 7.2 containing 1 mg/mL 5-bromo-4-chloro-3-indoyl-β-D-galactoside, 5 mmol/L $K_3Fe(CN)_6$, 5 mmol/L $K_4Fe(CN)_63H_2O$, 1 mmol/L $MgCl_2$, 0.01% sodium deoxycholate, 0.02 % NP-40) for 9–10 hours at 37°C. Samples were photographed under a binocular microscope, postfixed in 4% paraformaldehyde for 1 hour at 4°C, dehydrated in ethanol, and embedded in paraffin. Ten-micrometer sections, counterstained with eosin or nuclear fast red, were examined by light microscopy, and images were captured with a CCD camera using QCapture and Adobe Photoshop 7.0 software.

Sample Preparation, Immunohistochemistry, and RNA in Situ Hybridization

Intestines were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, and sections of 5- to 6-μm thickness were prepared. For antigen retrieval, slides were incubated in a pressure cooker for 3 minutes in 10 mmol/L sodium citrate, pH 6.0. Samples were incubated for 24 hours at 4°C with one of the following mouse monoclonal antibodies (Ab): Tcf4 (1:500, Upstate Biotechnology, Charlottesville, VA), Tcf3/4 (1:250, Upstate), activated β-catenin (1:500, Upstate), CD44 (1:100, Becton Dickinson, San Diego, CA), BrdU (1:100, Developmental Studies Hybridoma Bank, University of Iowa), proliferating cell nuclear antigen (1:150, Zymed, San Francisco, CA), Cdx2 (1:20, Biogenex, San Ramon, CA; MU392A-UC) or with rabbit antisera against c-Myc (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), cyclin D1 (1:500, Santa Cruz), Lef-1 (1:1000, gift of Rudi Grosschedl), or Ki67 (1:2000, Vector Labs, Burlingame, CA). Samples were washed, incubated with biotinylated goat anti-mouse or anti-rabbit IgG, and treated with avidin-biotin-peroxidase complex (Vector Laboratories). The reaction was visualized with diaminobenzidine (DAB) hydrochloride (Sigma Chemical Co., St. Louis, MO). RNA in situ hybridization was performed as described previously[15] using digoxigenin-labeled Apoa1, Ybx1, or sense control riboprobes (1 μg/mL), alkaline phosphatase-conjugated anti-digoxigenin Ab (Roche, Indianapolis, IN), and nitroblue-tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Results

Regional Gene Expression in Relation to Early Villus Morphogenesis

Transcriptional profiles of the developing mouse gut reveal widespread activation of molecular markers of the mature epithelium between E13 and E15, coincident with early villus morphogenesis.[17] Even in the earliest villi, in situ hybridization localized many messenger RNA (mRNA) in patterns characteristic of adult intestine (Figure 1*B*). Transcripts that appear in differentiated cells in adults are confined to villi in E15 mouse embryos, whereas those restricted to adult crypts localize in intervillus cells. Thus, the earliest villus-lining cells, which converted from undifferentiated squamous epithelium, are readily distinguished from intervening crypt precursors at the level of gene expression; even in the absence of structured crypts, boundaries of gene expression between villi and intervillus regions appear at the same time that the epithelium is remodeled. This demarcation of gene expression domains precedes zonation of epithelial cell replication. Ki67 (Figure 1*C*) and proliferating cell nuclear antigen (data not shown) immunostaining of E15.5 gut identifies proliferation not only in intervillus cells but also in many cells that lie along the newly formed villi and express differentiation-related transcripts. Thus, gene expression in bowel mucosa is regionalized in conjunction with early villus morphogenesis.

Unexpected Regional Pattern of Wnt Activity in the Late Fetal Intestine

Mucosal Wnt signaling in adult intestine is confined to the crypt, including progenitor and Paneth cells.[4,18] To determine when prospective crypt cells first acquire this distinguishing feature, we assessed LacZ staining in TOP-GAL transgenic embryos, which carry the *Echerichia coli* β-galactosidase gene under the control of Wnt/Tcf-responsive *cis*-elements.[19] These animals report faithfully on canonical Wnt activity, as we reported previously[20] and verified in the vibrissae, brain, and tracheobronchial tree (Figure 2*A* and 2*B*), known sites of active Wnt signaling in midgestation embryos.[19,21,22] In adult gut epithelium, LacZ signal is confined to the crypt base (Figure 2*C*), corresponding to the location of Paneth cells, known recipients of intestinal Wnt signals.[18]

Surprisingly, LacZ staining in intervillus regions, the functional precursors of intestinal crypts, is prominent only on the third postnatal day (Figure 2*N*). Rather, it is the earliest intestinal villi that display strong β-galactosidase activity in late gestation and immediately after birth. On the CD1 genetic background, LacZ activity reproducibly appears first between E16.25 and E16.5 (Figure 2*G* and 2*H*). Prior to this period, β-galactosidase activity is detected with radial asymmetry in stomach endoderm (Figure 2*F*), as we reported previously,[20] but not at all in intestine (Figure 2*D* and 2*E*), including the pseudostratified squamous lining at E13 or E14 (data not shown). Villus LacZ staining increases steadily in intensity until E18.5 (Figure 2*J* and 2*M*) and persists until approximately 2 days after birth. Intervillus cells, which subsequently show strong β-galactosidase activity (Figure 2*N*), lack a signal for most of the duration that one is detected in villus surface cells. Brünner's glands, a submucosal network of branching secretory ducts[23] that is confined to the first portion of the duodenum in mice, also show strong LacZ staining in weanling TOP-GAL mice (Figure 2*P*). The embryos for our study were derived from crosses between wild-type and hemizygote trans-

genic mice so that only half the progeny carried the TOP-GAL reporter; we observed villus β-galactosidase activity in all transgenic embryos and never in nontransgenic littermates (Figure 2*I*).

β-galactosidase activity in any tissue likely correlates imperfectly with the duration of active Wnt signaling: the enzyme must first accumulate, and, because it is highly stable, its activity may outlast brief periods of Wnt signaling. Moreover, although differences in LacZ signal intensity likely reflect true variation in the degree of Wnt activity, there is probably a threshold below which Wnt signaling is undetected in TOP-GAL tissues. LacZ mRNA in situ hybridization confirmed exclusively villus expression in the fetal gut; we detected weak RNA expression at E16.0 (data not shown) and robust expression thereafter (Figure 3*A*). Our data hence reveal a perinatal window in which canonical Wnt signaling is active in newly formed villi but not in intervillus cells. Canonical Wnt signaling in the latter population first becomes evident on the day of birth in a fraction (~20%) of villus profiles; at this stage, the signal remains considerably more prominent in villi, and the majority of intervillus regions still lack β-galactosidase activity (green arrowheads in Figure 3*B*). Wnt signaling shifts completely from villi to intervillus regions over the next 2 days (Figure 2*N*). These staining patterns were unchanged when transgenic TOP-GAL mice were crossed into mixed CD1-C57BL/6 and CD1-129/Sv genetic backgrounds. Because intestinal epithelium differentiates in a proximal to distal wave, we surveyed the length of the gut to determine the timing of villus activation of the Wnt pathway. TOP-GAL mice showed the predicted proximal-to-distal gradient of intestinal LacZ staining (see Supplemental Figure 1 online at www.gastrojournal.org).

As an independent indicator of canonical Wnt signaling, we assessed fetal and postnatal mouse intestines for nuclear localization of β-catenin, a marker of Wnt pathway activation. As expected, immunohistologic detection of β-catenin is confined to the crypts of Lieberkühn in adult mice and especially to Paneth cells at the base (Figure 3*C*). In contrast, and in agreement with the β-galactosidase data shown in Figure 2, between E17 and 2 days after birth, nuclear β-catenin is found in cells lining

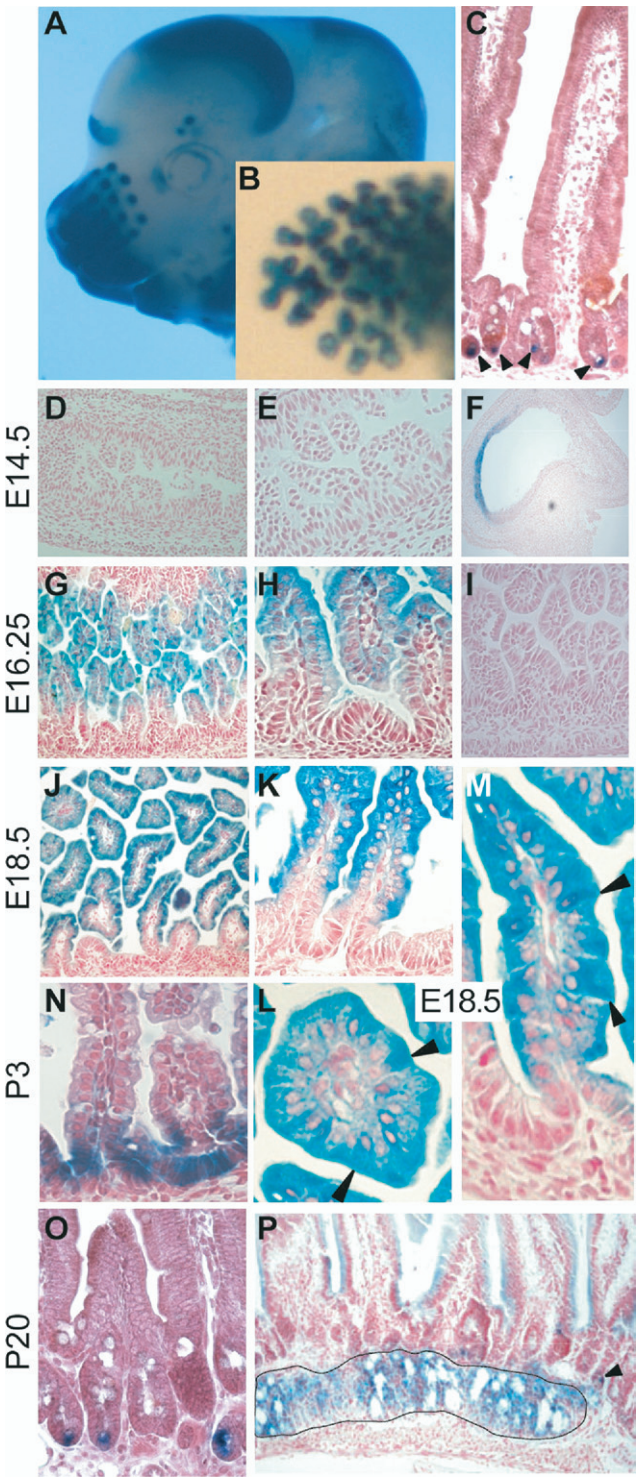


Figure 2. Sites of canonical Wnt signaling activity in gut development mapped in TOP-GAL reporter mice. (*A–C*) Whole-mount (*A* and *B*) and microscopic (*C*) evidence that TOP-GAL mice report faithfully on sites of high canonical Wnt signaling in embryos (*A* and *B*) and adults (*C*), as revealed in the follicles of vibrissae at E16,[19] points of bronchial branching at E16,[22] and near the base of adult intestinal crypts.[18] (*D–F*) At E14.5, there is no indication of canonical Wnt signaling in intestine (*D*, low magnification; *E*, high magnification), but asymmetric Wnt activity is detected in stomach endoderm (*F*), as reported previously.[20] (*G–I*) β-galactosidase staining is first detected along villi in fetal duodenum at E16.25 in TOP-GAL (*G*, low magnification; *H*, high magnification) embryos but not in nontransgenic littermate (*I*) controls. (*J–M*) β-galactosidase activity, a marker for Wnt signaling, peaks on the eighteenth gestational day (*J*, low magnification; *K*, high magnification) when it is still restricted to villous cells and excluded from intervillus regions. In these duodenal sections, scattered villous cells (*arrowheads* in *L* and *M*) show higher activity but do not correspond to discrete lineages. (*N–P*) Epithelial Wnt activity is fully shifted to intervillus spaces by the third postnatal day, P3, and confined to basal crypt cells (*O*; and arrowhead in *P*), probably of the Paneth lineage, and to duodenal Brünner's glands (outlined in *P*) in weanling (P20) mice.

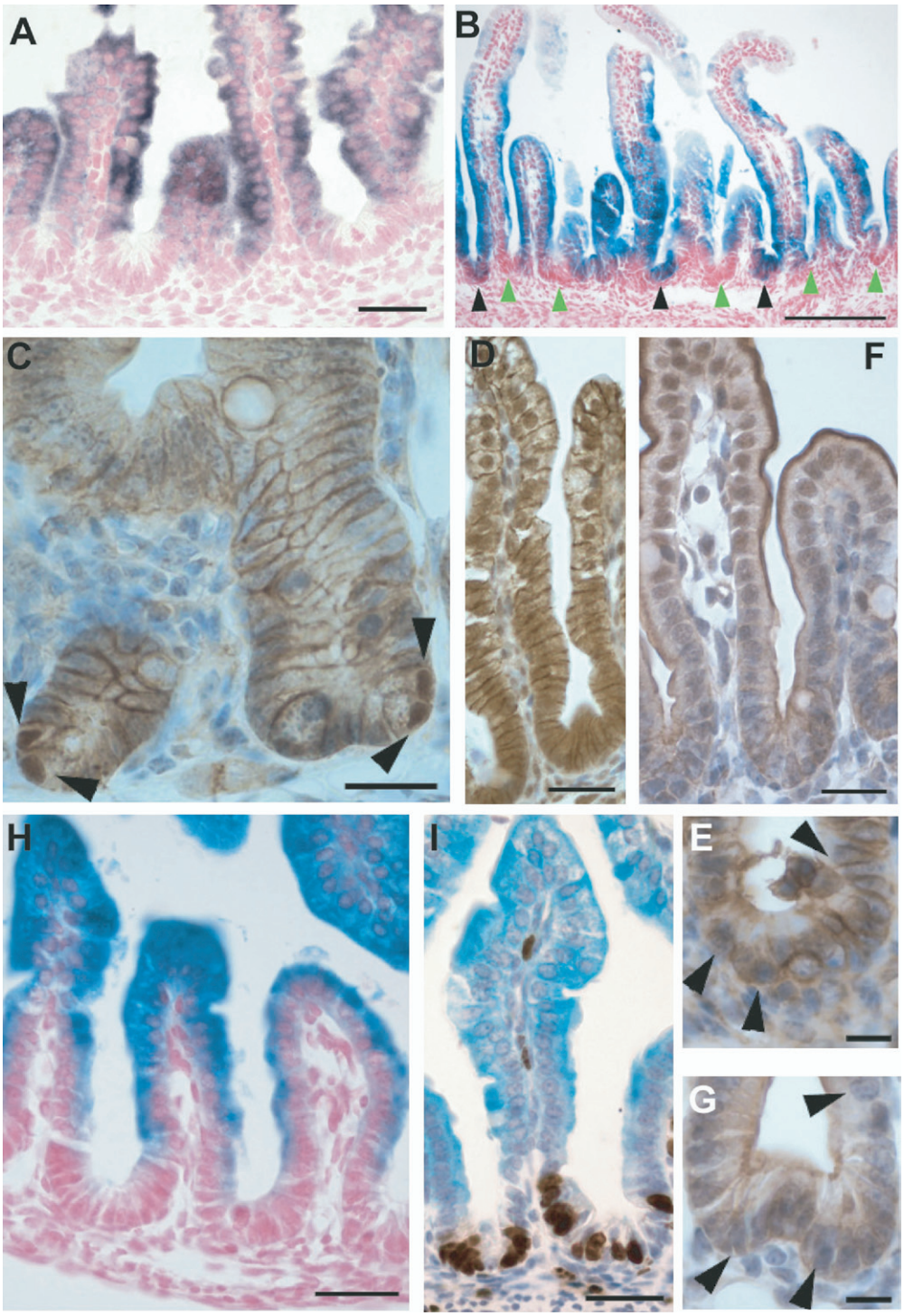
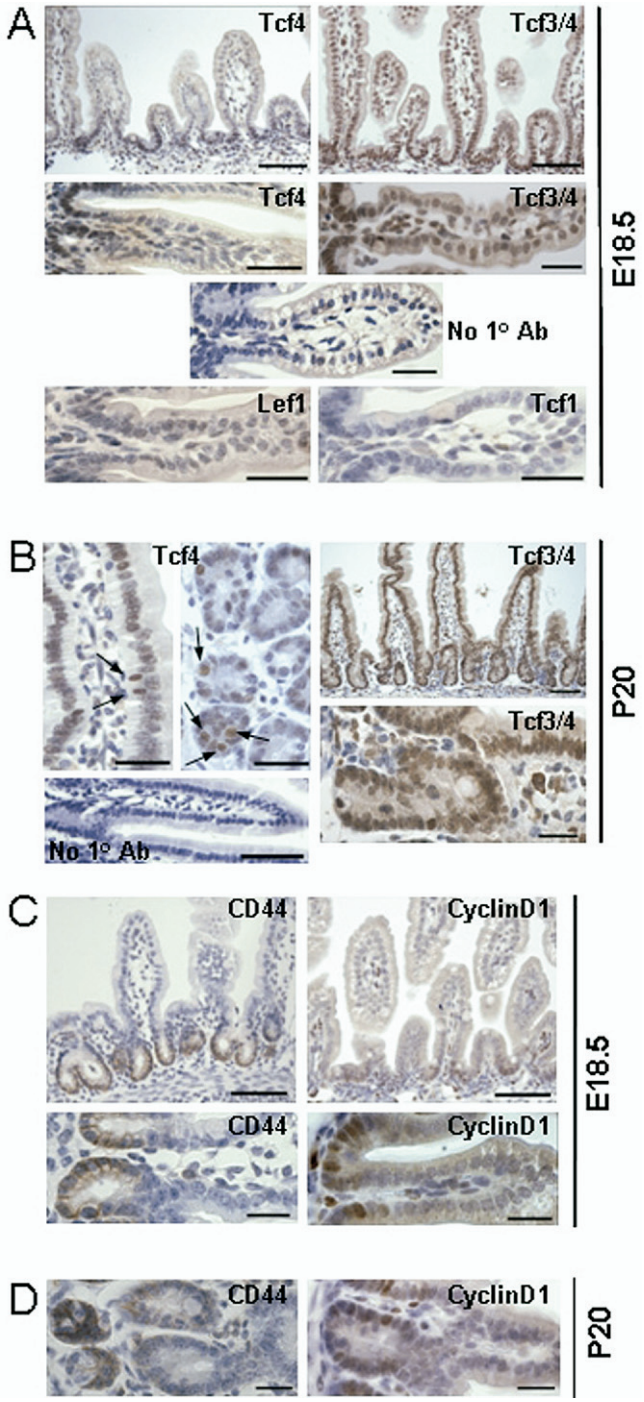


Figure 3. Relation of canonical Wnt signaling in perinatal intestinal villi to cell proliferation and to other markers of Wnt activity. (*A*) β-galactosidase in situ hybridization on E16.5 TOP-GAL mouse intestine, confirming mRNA expression in villus but not intervillus cells in prospective duodenum. (*B*) Transition from intestinal villus to intervillus concentration of Wnt activity in neonatal mice. The LacZ signal diminishes in TOP-GAL villus tips, and, although villus activity remains, β-galactosidase activity is detected for the first time in approximately 20% of intervillus regions (*black* compared with *green arrowheads*) in duodenum. (*C*) In older mice (P20), nuclear β-catenin is restricted to crypts, especially in Paneth cells at the base (*arrowheads*). (*D–G*) Immunohistochemistry for activated, nuclear β-catenin (*D* and *E*) and c-Myc (*F* and *G*) in E18.5 duodenum provides independent evidence for canonical Wnt signaling in villus (*D* and *F*) but not intervillus (*arrowheads* in *E* and *G*) cells in late fetal stages. (*H*) LacZ staining pattern in jejunum from E18.5 Axin2 LacZ embryos, again revealing Wnt activity in villi. (*I*) TOP-GAL dams were injected with BrdU on the eighteenth postcoital day; fetal duodeni were harvested 2 hours later and tested for β-galactosidase activity (*blue*) and BrdU immunostaining (*brown*). Absence of overlap between the 2 signals indicates that Wnt activity and epithelial cell proliferation are uncoupled at this stage. *Scale bars*, *A* and *B*, 300 μm; *C*, *E*, and *G*, 25 μm; *D* and *F*, 50 μm; *H* and *I*, 75 μm.

each villus (Figure 3*D*) but not in intervillus cells (detail in Figure 3*E*); it appears consistently in the intervillus zone only after the second postnatal day (data not shown). We also examined expression of c-Myc, one of the best characterized targets of canonical Wnt signaling.[8,11,24] c-Myc distribution is virtually identical to that of nuclear β-catenin, with highest expression in nuclei near villus tips, lower levels toward the villus base, and none in intervillus regions (Figure 3*F*, and detail in Figure 3*G*). Finally, we studied an independent murine reporter strain for Wnt activity, where LacZ cDNA replaces the coding sequence of the *Axin2/conductin* gene,[25] an established target of Wnt signaling in diverse tissues.[26] Just as in TOP-GAL embryos, the intestine in E18.5 Axin2^{LacZ} mice showed high β-galactosidase activity in villus epithelial cells and sparing of the intervillus regions (Figure 3*H*). Thus, 4 informative markers, reporter activity in TOP-GAL and Axin2^{LacZ} mice, nuclear β-catenin, and c-Myc expression, reveal canonical Wnt signaling over the villus surface in mouse gestation and earlier than its restriction in prospective crypt precursors.



Disparity Between Wnt Signaling and Intestinal Epithelial Proliferation in the Perinatal Period

Villus formation and early activation of intestinal differentiation genes both occur before the first signs of intestinal Wnt signaling in TOP-GAL embryos (Figures 1 and 2); accordingly, villus Wnt activity in the perinatal period must be associated with processes other than these. In adult gut, canonical Wnt signaling promotes crypt-specific behaviors and distinguishes proliferating cells from their differentiated progeny.[4] We evaluated intestines after treating pregnant dams with a 2-hour pulse of BrdU. By E17.5, when Wnt-responsive TOP-GAL expression is readily detected in mouse intestine, only rare proliferating (BrdU$^+$) cells are found in intestinal villi, the location of Wnt activity; epithelial proliferation is largely restricted to the intervillus regions, at which LacZ signals are absent (Figure 3*I*). Thus, Wnt signaling and epithelial cell proliferation are almost mutually exclusive in the late fetal gut. Wnt activity later localizes in

Figure 4. Transcriptional mediators and output of Wnt signaling in late fetal intestinal villi. (*A*) In E18.5 intestine, epithelial Tcf4 expression is detected exclusively in intervillus cells, which lack Wnt activity, and is absent from villus epithelial cells, in which Wnt activity is robust. Bispecific Tcf3/Tcf4 Ab stains the whole structure, in which stronger staining in intervillus regions likely represents Tcf4 expression, and the villus signal, absent with Tcf4-specific Ab, points to Tcf3 expression along perinatal villi. Low- and high-magnification images are shown in the *top* and *bottom panels*, respectively. Tcf1 and Lef1 are not detected in the epithelium (but strongly in lymphoid aggregates and hair follicles, respectively; data not shown). (*B*) In weanling mice, both Tcf3/Tcf4- and Tcf4-specific Ab react with nuclei along whole crypt-villus units; Tcf4 expression is highest in surface mucosal cells with basally displaced nuclei (*arrows* in the longitudinal villus section to the left) and in Paneth cells (*arrows* in the transverse crypt section on the right). (*C*) At E18.5, when canonical Wnt signals are prominent along villi and excluded from intervillus regions, expression of candidate Wnt targets, CD44 and cyclin D1, shows the opposite distribution. Their concentration in intervillus cells resembles their restriction to the crypts in older animals (*D*) and suggests that villus Wnt signaling activates a program distinct from that in adult epithelial progenitors. *Scale bars*, *A*, top 2 panels, 150 μm, bottom 5 panels, 75 μm. *B*, top left 2 panels, 75 μm; all others, 150 μm; bottom right, 50 μm. *C*, top 2 panels, 150 μm; bottom 2 panels, 50 μm. *D*, 50 μm.

the proliferative zone (Figure 2*C* and 2*N*, and data not shown), as expected.

Transduction of Perinatal Villus Wnt Signals by a Factor Other Than Tcf4

Canonical Wnt signals are transduced through Tcf/LEF family proteins. Tcf4 is especially abundant in, and virtually specific to, adult gut epithelium,[6,27] and Tcf4$^{-/-}$ mice die soon after birth from presumed complications of defects confined to the gut.[28] Intervillus cells in the Tcf4-null small bowel mucosa fail to proliferate; the capacity to populate villi is consequently reduced, and the defect is evident before birth.[28] Indeed, in E18.5 mouse embryos, near to term, epithelial Tcf4 protein expression is virtually restricted to cells in the intervillus space (Figure 4*A*).

The findings in Tcf4-null mice helped establish the idea that gut epithelial cell proliferation requires Wnt signals. However, we show above that intervillus Wnt signaling in reporter mice is at best patchy at birth (Figure 3*A*). This implies that early intervillus cells may not respond to Wnt or depend on levels that are below the detection threshold for β-galactosidase activity in TOP-GAL or Axin2^{LacZ} mice and of immunostaining for nuclear β-catenin in intervillus cells at E18.5 (Figure 3*D*–*G*) or 1 day after birth (data not shown). Later in life, Tcf4 expression occurs over the full surface of mouse intestinal villi (Figure 4*B*), similar to findings reported in the late human fetus,[27] although Wnt signaling is confined to crypts. In differentiated villus cells in adolescent mice, Tcf4 expression is highest in epithelial cells with basally displaced nuclei (Figure 4*B*, and data not shown), which seem to correspond to a subset of goblet cells.

Tcf3 and Tcf4 are the dominant Tcf/LEF family proteins present in the gut.[29] To assess developmental expression of Tcf3, we used an Ab that recognizes both Tcf3 and Tcf4;[27] when the results are interpreted in light of expression revealed by the Tcf4-specific reagent, reasonable inferences may be drawn about Tcf3 distribution. Unlike the pattern observed with Tcf4-specific Ab, which selectively stains intervillus cells in E18.5 mouse embryos, the bispecific Ab stains nuclei along the entire villus (Figure 4*A*). Immunostaining is most intense in intervillus nuclei, which may reflect abundance of Tcf4 protein in these cells, but Tcf3/4 is also readily detected in the differentiated cells that express β-galactosidase in TOP-GAL and Axin2^{LacZ} neonates. Taken together with absence of villus Tcf4 expression at perinatal stages, Tcf3 is a leading candidate effector of Wnt signaling in villi. Weak cytoplasmic background signal with anti-mouse secondary Ab was easily distinguished from specific nuclear staining, and we detected the 2 other members of the family, Tcf1 and Lef1, in lymphoid aggregates and hair follicles, respectively (data not shown), but hardly any in gut epithelial cells (Figure 4*A*).

Distinct Wnt-Induced Programs in Adult Crypts and Perinatal Villi

The unexpected pattern and timing of Wnt activity in the developing gut raise the question of whether the outcome of intestinal Wnt signaling differs according to developmental stage and cellular context. Pertinent transcriptional targets in adult intestinal epithelium include c-Myc, CD44, and possibly cyclinD1.[8,24,30] c-Myc immunostaining parallels that of β-catenin at E18.5 (Figure 3*F*) and provided one form of support for our conclusion that canonical Wnt signaling activity localizes in fetal villi. By contrast, at E18.5, both cyclinD1 and CD44 are restricted to intervillus regions and excluded from villi (Figure 4*C*), similar to the distribution observed in adult crypt-villus units (Figure 4*D*). These findings suggest that a part of the Wnt-associated intestinal gene expression program is initiated before reporter activity becomes evident in intervillus cells in TOP-GAL mice. They further imply that the 2 phases of gut epithelial Wnt activity result in distinct cellular outcomes.

Consequences of Premature β-Catenin Activation on Villus Morphogenesis

Tcf4$^{-/-}$ mice have a specific, late defect in gut epithelial proliferation,[28] but early demise of β-catenin-null embryos[31,32] and of other single and compound Tcf-deficient mice[33–35] precludes simple investigation of the role of canonical Wnt signaling in early intestinal villi. It is, however, possible to derive some insight into this role by evaluating the consequence of forced and premature β-catenin activation in gut endoderm. We studied embryos from crosses between mice that carry a floxed (Fl), activating β-catenin allele[16] and those expressing Cre recombinase under control of the endogenous *Shh* gene.[15] Because *Shh* is expressed throughout the gut endoderm before E9,[36] progeny from such a mating are expected to deregulate β-catenin in most gut epithelial progenitors, which should consequently express ligand-independent Wnt activity. As expected, significant β-catenin appears in nuclei in the presumptive intestinal mucosa (Figure 5*F* and 5*G*), although nonuniform Cre expression yielded regions with unequivocal nuclear localization (Figure 5*F* and 5*G*, solid arrowheads) and other areas in which cytoplasmic β-catenin was not stabilized and the signal appeared mainly in cell membranes (Figure 5*F* and 5*G*, open arrowheads). Such patchy distribution provided useful internal controls and also revealed apparent lack of clonality in the first villi, as predicted from the model for emergence of a columnar epithelium (Figure 1*A*).

Control (Shh$^{+/+}$Catnb$^{+/lox(ex3)}$) embryos showed intestinal villus architecture that is normal for E16.5 (Figure 5*A* and 5*C*), whereas villus formation was markedly abnormal in intestines from Shh$^{Cre/+}$Catnb$^{+/lox(ex3)}$ embryos of the same age. Histology revealed prominent structures that may represent merged villi or, more likely, a failure

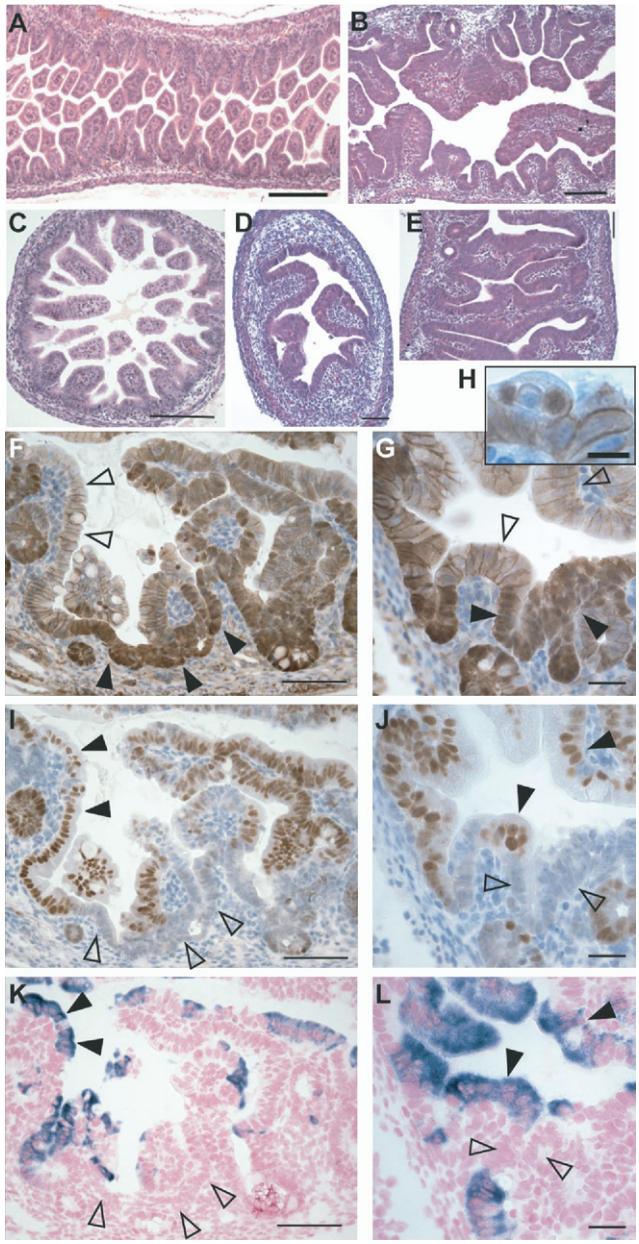


Figure 5. Severe villus dysmorphogenesis of fetal intestine upon premature β-catenin activation. (*A–E*) Longitudinal (*A*, *B*, and *E*) and transverse (*C* and *D*) sections of E16.5 small intestine from $Shh^{Cre/+}$ × $Catnb^{+/lox(ex3)}$ embryos. Representative tissues are shown from $Shh^{+/+}Catnb^{+/lox(ex3)}$ (*A* and *C*) and $Shh^{Cre/+}Catnb^{+/lox(ex3)}$ (*B*, *D*, and *E*) embryos; all samples were photographed at the same magnification, and all *scale bars* represent 300 μm. (*F–H*) Low- (*F*) and high- (*G* and *H*) magnification images of β-catenin immunostaining, which distinguishes epithelial cells with activated β-catenin (nuclear localization, *solid arrowheads*) from cells without activation, in which β-catenin is excluded from the nucleus and concentrated along cell membranes (*open arrowheads*). (*I–L*) Cdx2 immunostaining (*I* and *J*) and Apoa1 in situ hybridization (*K* and *L*) reveal expression of these markers in villi with normal morphology (*solid arrowheads*) or cells lacking nuclear β-catenin and absence from regions showing nuclear β-catenin and villus dysmorphogenesis (*open arrowheads*). Images in *F*, *I*, and *K* are taken from neighboring sections (*scale bars*, 75 μm). Images in *G*, *J*, and *L* are also taken from neighboring sections in a separate field and shown at higher magnification (*scale bars*, 50 μm). *Scale bar*, in *H*, 25 μm.

of individual villi to separate properly, and the gut lumen was accordingly narrowed (Figure 5*B*, 5*D*, and 5*E*). Villus morphogenesis requires formation of secondary lumina that separate villi from one another.[13,14] The latter process seems to be arrested when β-catenin is activated prematurely in gut epithelial progenitors, and the findings resemble those described in mice lacking *ezrin* gene function.[37] Mutant embryos retain 1 normal *Shh* allele, thus the defects cannot be attributed to *Shh* loss, which in any case does not cause similar defects.[38] Moreover, well-organized villi were evident in patches in which the floxed *Catnb* allele had escaped recombination (examples in Figure 5*B*, top right and bottom right). Epithelial cells with normal (membranous, nonnuclear) β-catenin distribution differentiated more fully, as judged by robust expression of the intestinal marker Cdx2 (Figure 5*H* and 5*I*; solid arrowheads) and expression of *Apoa1* mRNA in most $Cdx2^+$ cells (Figure 5*J* and 5*K*). These markers were absent from areas of premature β-catenin activation (open arrowheads [Figure 5F, 5G, 5I, 5J, 5K, and 5L]), whereas Ybx1, a marker of epithelial immaturity (Figure 1*B*), was expressed here widely (data not shown). These results imply that the developing gut is sensitive to the period when Wnt signaling occurs: premature, unregulated activity limits cell differentiation and formation of secondary lumina, leading to severe villus dysmorphogenesis.

Discussion

We have used reporter mice and histologic and molecular markers to examine the transition from pseudostratified squamous to columnar epithelium in the mouse fetal gut. Whereas Wnt pathway functions in adult intestinal crypt homeostasis are well recognized, our results uncover 2 new features. First, Wnt signaling in intervillus cells, the precursors of crypts, is barely evident at birth and localizes fully in these cells only approximately 2 days later. Crypt invagination in rodents occurs over the first several days of life; until crypts are established, the mucosa is replenished by intervillus cells, where proliferation becomes restricted as early as E16 in the mouse. TOP-GAL and Axin2-LacZ reporter mice thus reveal that the earliest columnar epithelial cells proliferate in the apparent absence, or in response to undetectable levels, of Wnt signaling. One corollary of this conclusion is that proliferation arrest and epithelial atrophy observed in $Tcf4^{-/-}$ mice[28] may reflect not only failure of physiologic Wnt signaling, as assumed, but possibly also Wnt-independent Tcf4 functions. Acute ablation of Wnt signaling in adult mice rapidly arrests crypt cell replication and affirms the role of Wnts in gut homeostasis[11]; determinants of adult mucosal renewal may thus differ from those in progenitors that initially populate the bowel epithelium. Alternatively, both fetal and adult gut epithelial progenitors may depend on levels of Wnt activity that are much lower than those observed in neigh-

boring areas such as in Paneth cells in adult small bowel and in differentiated villus cells in the perinatal period.

A second surprise is that canonical Wnt signals are transmitted in primordial intestinal villi, in differentiated cells that subsequently avoid Wnt signaling. We demonstrate this activity through both LacZ reporter activity and nuclear β-catenin localization and note that its properties differ notably from those encountered later in Wnt-dependent gut homeostasis. The zone of fetal intestinal Wnt signaling correlates closely with cell differentiation and expression of mature epithelial markers and not with cell proliferation. Although many villus cells continue to replicate, they do so for a limited number of cycles, and, after E16.5, most villus cells are postmitotic; these admixed populations show no differences at the level of nuclear β-catenin or of LacZ stain in TOP-GAL embryos. Consistent with the apparently different roles for Wnt signaling in perinatal villi and adult crypts, the corresponding gene expression programs are not identical. We identify c-Myc in E18.5 villi but not in intervillus cells, whereas other candidate Wnt targets (CD44 and cyclin D1; Figure 4*B*) are excluded from differentiated villus cells and confined to the intervillus compartment from the outset. Furthermore, the Wnt signal in perinatal villi is likely transduced by the transcription factor Tcf3, which is expressed in all epithelial cells, unlike Tcf4, which is restricted to intervillus nuclei at the time of villus Wnt activity. Expression domains of Wnt ligands and their receptors and regulators have been mapped in detail in adult mouse gut and developing chick embryos,[39–41] but there is limited appreciation of the ligand-receptor pairs responsible for epithelial homeostasis. The differences outlined here suggest that the earliest villi may respond to Wnt ligands different from those found in adult mucosal crypts.

Fetal villus morphogenesis is ostensibly normal in the absence of Tcf3, Tcf4, or both factors,[28,34] which implies that villus Wnt signaling elicits effects not readily discerned by histology. Although the purpose of the brief phase of villus Wnt signaling is hence unclear, our findings should be regarded in light of the transition between undifferentiated endoderm, which lacks Wnt signals, and the villous mucosa. It is during this transition that epithelial cells establish a lasting relationship with underlying mesenchyme, a tissue that profoundly influences mucosal properties.[42] One possibility is that epithelial cells that initially came in contact with Wnt ligands occupy the primordial villi before the mesenchyme demarcates pericryptal zones of Wnt release. Canonical Wnt signaling in these early villus cells may thus be a vestige of transient cellular contacts during villus morphogenesis, with responses different from those encountered in replication-competent epithelial progenitors. An alternative possibility is suggested by the observation that mucosal genes are activated in conjunction with the emergence of villi and in a distribution that unambiguously distinguishes villi from intervillus regions (Figure 1*B*). These early villus-lining cells do not originate in crypts, the niche in which programming for gut-specific differentiation occurs. If this programming depends on Wnts, then the transient phase of fetal villus Wnt activity might represent a crypt-independent mechanism to enable appropriate gene expression during development. In adult gut, Wnt signals regulate both epithelial cell proliferation and secretory cell differentiation,[9–11] indicating broad influence over crypt functions. Although Wnt signals do not coincide with conversion of squamous to a columnar epithelium, they may prime the gut mucosa for tissue-specific gene expression, and, until Wnt signaling centers form near intervillus regions after birth, this function may be assumed by cells in primitive villi.

Appendix

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1053/j.gastro.2007.04.072.

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Intestinal Development: The Many Faces of Wnt Signaling

See "Phases of canonical Wnt signaling during the development of mouse intestinal epithelium" by Kim B-M, Mao J, Taketo MM, et al on page 529; and "SOX9 is required for the differentiation of Paneth cells in the intestinal epithelium" by Mori-Akiyama Y, van den Born M, van Es JH, et al on page 539.

The mammalian intestinal epithelium perpetually renews itself every 3–5 days from a large reservoir of pluripotent stem cells in the lower crypt.[1] Intestinal stem cells divide and produce an actively proliferating population of transit amplifying cells that give rise to each of the 4 principal epithelial cell types in the small intestine. As immature cells migrate up the crypt–villus axis, they exit the cell cycle in the villus compartment and differentiate into either absorptive enterocytes, goblet cells, or enteroendocrine cells. By contrast, the Paneth cell lineage, which produces antimicrobial peptides, continues to proliferate and migrates downward to the crypt base. The crypt–villus unit is the basic developmental unit of the intestine where mature, terminally differentiated cells in the villi are topographically separated from immature proliferating cells in the crypts, making the small intestine an excellent model for studying development.

Small intestinal epithelial homeostasis, cell proliferation control, and cell lineage differentiation are regulated by a number of signaling pathways conserved throughout metazoan evolution. These pathways include the Wnt (wingless), Notch, hedgehog, and bone morphogenic protein (TGF-β/BMP) pathways. The potential importance of Wnt signaling in intestinal epithelial development was first recognized with the discovery that patients with familial adenomatous polyposis inherited one defective allele for the adenomatous polyposis (*APC*) gene. Mutations in the second APC allele resulted in abnormal Wnt signaling, leading to eventual development of colorectal cancer. Subsequent work has shown that sporadic colorectal cancers are frequently associated with somatic mutations that result in abnormal Wnt signaling.[2]

Studies in the fruit fly, *Drosophila melanogaster,* first revealed the importance of Wnt (Wingless) signaling in normal tissue development, regulating cell fate determination, cell proliferation, and cell polarity. A growing body of studies has confirmed the importance of Wnt signaling in normal mammalian development, especially with respect to the intestine. Two papers published in this issue of GASTROENTEROLOGY explore the role of Wnt signaling in intestinal cell differentiation and proliferation. Both studies utilize transgenic mouse models that provide new insight into multiple effects of the Wnt pathway in the intestine.

Wnt proteins, encoded by approximately 20 different genes, comprise an evolutionary conserved family of soluble, cysteine-rich glycoproteins[3] in mammals.[4] Initiation of canonical Wnt signaling occurs when Wnt proteins bind to Frizzled proteins, a family of 7 transmembrane receptors, and a transmembrane coreceptor, LRP5/6. Signals generated in the absence of the coreceptor do not involve β-catenin and are referred to as the noncanonical pathway.[5]

Although the canonical Wnt pathway is relatively quiescent in mature intestinal epithelial cells, it plays a critical role in both normal development and tumorigenesis. When Wnt signaling is not activated, β-catenin is phosphorylated by glycogen synthase kinase 3 (GSK3β) and casein kinase 1. Phosphorylated β-catenin is then recruited to a complex containing APC, Axin, and other proteins (Figure 1) that are targeted for rapid ubiquitination and proteosomal degradation. Binding of Wnt to its receptor, Frizzled, and LRP coreceptor leads to a signaling cascade involving the activation of Disheveled, which recruits Axin to the plasma membrane to inhibit phosphorylation of β-catenin. Unphosphorylated β-catenin is redistributed throughout the cytoplasm, and translocated into the nucleus. Nuclear β-catenin associates with one of several members of T-cell factor (TCF)/lymphoid enhancer (LEF) binding protein family of transcription factors.[6] This family of Wnt transcriptional effectors includes TCF1, TCF3, TCF4, and LEF-1, as well as isoforms arising from alternate promoter use and/or RNA splicing. Nuclear β-catenin serves as a coactivator for TCF/LEF proteins by displacing corepressor proteins and recruiting additional proteins including pygopus and Bcl9-2, resulting in increased transcription and expression of Wnt target genes, including a number of genes expressed in immature proliferating cells. Examples of Wnt target genes associated with cell proliferation include *c*-Myc, cyclin D, and Sox9.

Active Wnt signaling in the adult small intestine is normally confined to proliferating immature cells and Paneth cells in the lower crypt. A number of genetic models suggest a critical role for the Wnt pathway in maintaining proliferation required to support the high turnover rate of the intestinal mucosa. Mice with an inactivated TCF4 gene, the major intestinal TCF/LEF transcription factor, failed to develop crypts and several epithelial lineages in the intestine. The precursor to crypts, the intervillus zone cells, was devoid of proliferating cells. These observations indicated that TCF4 and canonical Wnt signaling were essential for maintenance of both stem cells and cell proliferation in crypts.[7]

At about embryonic day 14 in the mouse, the intestine undergoes major changes from a pseudostratified epithelium to a tube lined with a monolayer of columnar cells,

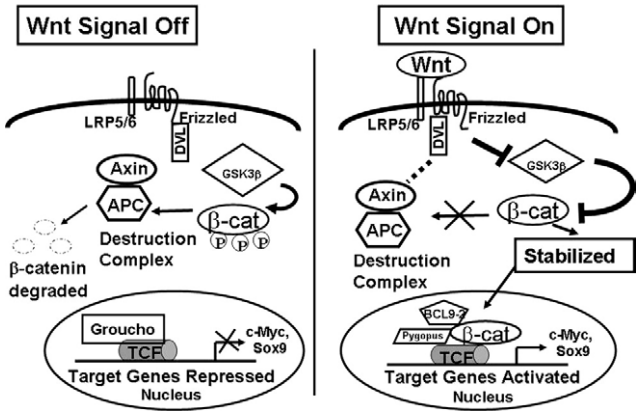

Figure 1. The canonical Wnt pathway and signaling through β-catenin. In the absence of Wnt ligand, β-catenin is phosphorylated by glycogen synthase kinase-3 (GSK3) and targeted for degradation through destruction complexes containing APC, Axin, and other proteins (*left*). As a result, Wnt target genes remain repressed. During active signaling, Wnt proteins bind to their receptors and inhibit phosphorylation of β-catenin by GSK3, thus preventing its degradation in APC complexes (*right*). As a result, stabilized β-catenin is redistributed from the cell membrane to the nucleus, where it interacts with TCF family transcription factors as a co-activator to activate target gene transcription. β-cat, β-catenin; APC, adenomatous polyposis coli; DVL, disheveled.

which undergo further morphogenesis to form villi. Crypts subsequently develop from cells in the intervillus zone. In this issue of GASTROENTEROLOGY, Kim et al[8] examined the developing mouse intestine to identify cells with active Wnt signaling. Their results demonstrate previously unappreciated differences in the location and components of Wnt signaling in the fetal intestine.

The first surprising observation by Kim et al was that proliferating intervillus cells in the fetal gut showed no evidence of canonical Wnt signaling. Instead, they found that postmitotic cells in developing villi were the major sites of Wnt activity. Unlike the adult intestine, the fetal intestine uses a different Wnt transcriptional effector, TCF3, rather than, TCF4, the major TCF/LEF family member expressed in the adult intestine. As a result, Wnt signals in the developing intestine appear to activate different Wnt target genes. Thus, Wnt signaling may serve a number of different functions in the intestine, depending on the developmental context and the specific pathway components involved. The importance of Wnt signaling for maintenance of cell proliferation during development requires additional study. The function of newly identified Wnt pathway activation in postmitotic cells remains to be elucidated.

The role of Wnt signaling in the specification and differentiation of Paneth cells has not been well established. The identification of nuclear β-catenin in Paneth cells suggests that Wnt signaling persists in this lineage. Activation of the Wnt pathway by conditional APC deletion in intestine resulted in increased numbers of Paneth cells, suggesting that this pathway is associated with Paneth cell development.[9] It has been more difficult to obtain direct proof that the Wnt pathway is required for Paneth cell differentiation. Mice lacking TCF4 failed to develop Paneth cells,[10] as did transgenic mice expressing the Wnt inhibitor Dkk1 throughout the intestine under control of the villin gene.[11] However, in these studies, mice failed to develop crypts and showed developmental defects in other lineages with the loss of Wnt function, raising the possibility that the loss of Paneth cells could have resulted from stem cell depletion rather than a specific requirement for Wnt signals. To address the role of the Wnt pathway in Paneth cell differentiation, van Es et al[10] generated mice lacking Frizzled5, which was believed to be the only Wnt receptor expressed in Paneth cells. *Frizzled5*-null mice developed Paneth cells that were mispositioned to the villi and upper crypt, leading to the conclusion that Wnt signals were required for Paneth cell maturation only, but not for their specification.[10] However, another study subsequently showed expression of 2 other *Frizzled* family genes, *Fz6* and *Fz7* throughout the crypt base that may compensate for the loss of *Fz5*.[12]

The findings reported in this issue of GASTROENTEROLOGY by Mori-Akiyama et al[13] in mice lacking intestinal expression of the transcription factor Sox9 firmly establish that canonical Wnt signaling is needed for Paneth cell specification. Recently, our group, using conditional deletion of β-catenin combined with recombination-based lineage tracing, reached a similar conclusion.[14]

Sox9 belongs to the Sox family of transcription factors related to gender-determining factor gene *SRY* and *TCF* family of transcription factors.[15] Sox family proteins contain highly conserved high mobility group domain that recognizes an A/T rich DNA binding motif: (A/T)(A/T)CAA(A/T)G. The roles of Sox proteins in the differentiation are well established for a number of tissues, including neural crest, heart, cartilage, and testes.[16] *Sox9* is expressed in the lower crypt region of the small intestine, overlapping areas with active Wnt signaling. It is also a direct transcriptional target of β-catenin–TCF4 complexes.[17] Mori-Akiyama et al[13] report in this issue of GASTROENTEROLOGY that Sox9 is required for Paneth cell differentiation by conditionally knocking out the *Sox9* gene in the intestine. In the absence of Sox9, mice fail to develop Paneth cells or to express early differentiation markers for this lineage. The other secretory lineages, goblet and enteroendocrine cells, were unaffected by the loss of Sox9, indicating that the loss of this Wnt target protein was specific for Paneth cells and did not disrupt stem cells.

A second important finding reported by Mori-Akiyama et al is that loss of Sox9 resulted in modest expansion of the proliferating crypt compartment in contrast to the failure to develop crypts seen with more generalized loss of Wnt function in TCF4-null mice. This important observation suggests that a different subset of Wnt target genes is important for stem cell maintenance and crypt progenitor cell proliferation. Recent work suggests *c*-Myc,

a well-defined target of the Wnt pathway, is the major effector in controlling Wnt-induced cell proliferation.[18,19]

Wnt signaling regulates many functions in different tissues. How some functions, but not others, are activated is not well understood. A very large number of genes are capable of participating in the Wnt pathway including >19 different ligands (Wnts), ≥8 different receptors (Frizzled), 2 coreceptors, a number of soluble inhibitors to their receptors and coreceptors, 4 different TCF transcription factors and their alternately spliced isoforms, and several β-catenin binding proteins (Pygopus, BCL9-2).[20] Thus, there are a potentially limitless number of combinations of Wnt ligands, receptors, and effectors to determine different subsets of functions through selective activation of some Wnt target genes but not others.

The 2 articles appearing in this issue of GASTROENTEROLOGY illustrate examples of how the outcomes of Wnt signaling may control a limited number of functions in the intestine that may depend in part on the tissue and developmental context. Mori-Akiyama et al show that one Wnt target gene, *Sox9*, controls specification of Paneth cells, with relatively little effect on other important Wnt functions, such as stem cell maintenance. Likewise, Kim et al show that the Wnt pathway is activated in distinct cell populations of the developing gut, utilizing a different set of effector transcription factors to activate expression of different Wnt target genes compared to the adult intestine. Both articles exemplify the kinds of new experimental approaches that will lead to a better understanding of roles of Wnt signaling in both normal gastrointestinal tract development and the development of common neoplasms like colorectal cancer.

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