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From Definitive Endoderm to Gut—a Process of Growth and Maturation

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The intestine and colon carries out vital functions, and their lifelong maintenance is of the upmost importance. Research over the past decades has carefully addressed bowel function, how it is maintained and begun to unravel how disorders such as cancer and inflammatory bowel disease form. In contrast, very little is known about the molecular mechanisms that trigger tissue maturation during development. With this review, our aim is to carefully provide a critical appraisal of the literature to give a state-of-the-art view of intestinal development. Starting from definitive endoderm at gastrulation to the emergence of a structure with mature properties, the tissue undergoes complex morphogenetic processes that rely on both biophysical changes and secreted signaling molecules. We will also discuss how new and exciting developments using in vitro models are likely to provide new insights into this process and potential therapeutic strategies for gastrointestinal disorders.

Introduction

Adult tissues are maintained by stem cells throughout life. Under normal steady state conditions, positive and negative feedback mechanisms ensure balanced replenishment of dead cells through proliferation [1]. The gastrointestinal (GI) tract is responsible for processing our diet and for nutrient uptake. The GI tract constitutes an excellent organ to study stem cell behavior due to its well-organized structure and well-known morphology. The tube-like structure extends from the mouth to the anus and can be divided into the esophagus, stomach, and intestine in its adult form.

The intestine is responsible for digestion and absorption of nutrients. Due to the physicochemical nature of these processes, the well-organized epithelial surface that faces the lumen and therefore the harsh environment require constant cellular replenishment from resident intestinal stem cells (ISCs) [2]. The surface of the adult small intestine is organized into crypts and villi, which increases its surface area significantly, whereas the surface epithelium in the colon is organized into crypts that form a continuum with a smooth surface. The lifelong supply of new intestinal cells is governed by ISCs that are located at the bottom of crypts [3]. On cell division in the ISC compartment, progeny will leave the stem cell niche and become transiently amplifying cells destined to shortly differentiate into absorptive enterocytes or secretory goblet cells, tuft cells, Paneth cells, Microfold cells, or enteroendocrine cells. The regional identity of the intestinal tract predetermines the differentiation patterns with Paneth cells restricted to the small intestine [4,5] and a greater proportion of goblet cells in the distal part of the small intestine and colon [6,7]. Moreover, depending on their localization along the intestine, cells of the enteroendocrine lineage will secrete specific types of hormones and growth factors [8].

ISCs have been described as either highly proliferative or quiescent and depending on their localization express specific markers. Leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5) [3], Olfactomedin4 (Olfm4) [9], and Achaete scute-like2 (Ascl2) [10] are specifically associated with proliferative crypt base columnar (CBC) cells that are intercalated between Paneth cells at the bottom of the crypt. Quiescent or reserve ISCs have only been reported for the small intestine and have traditionally been associated with the +4 position [11]. Cells in this position are predominantly positive for Green Fluorescent Protein (GFP), when expressed from minimal promoters for B-cell-specific Moloney murine leukemia virus integration site 1 (Bmi1) [11] and mouse Telomerase reverse transcriptase (mTert) [12,13]. The expression of Bmi1 and mTert as exclusive markers of +4 position remains highly controversial, since several reports demonstrate promoter activity, protein, and mRNA in all cells located at the crypt bottom [11,12,14,15]. Recent evidence unequivocally demonstrates that quiescent cells in the small intestine are committed to a secretory fate unless the tissue is severely damaged in a way that eliminates CBC cells [16,17]. Musashi-1 [18], Prominin-1 [19], Ephrin type-B receptor 2 (EphB2) [20], CD44v6 [21], and Leucine-rich repeats, and immunoglobulin-like domains 1 (Lrig1) [22,23] display a broader and graded expression pattern with the highest expression at the bottom of the crypt. A current model therefore poses that a continuum of cellular fates exists in the

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crypts with the highest self-renewal capacity residing in cells found at the very crypt bottom [24].

The intestine develops from the ensheathing of the early endodermal structure by mesoderm-derived cells. After regionalization, the different organs are specified along the gut tube and these will reach their mature state either in late fetal development/postnatal stages in mice or at the end of the second trimester in humans. The intestine reaches its mature state around 2 weeks after birth in mice, [25,26] whereas adult characteristics can be observed as early as gestational 22 weeks in human fetuses (Fig. 1) [27]. Before the adult state, where cell loss and gain are carefully balanced, intestinal cells are continuously proliferating to increase both the length and girth of the intestine. The constant expansion allows the simple epithelium to fold into an undulating structure with villi and intervillus regions in both small intestine and colon. The appearance of crypts, which in the adult epithelium contains the proliferative compartment, is a relatively late event that marks the final step in tissue maturation. The observed transition from an immature fetal into an adult mature epithelium is tightly regulated by mechanical forces and signals from the surrounding environment. To understand this complex process, we need insights into how heterogeneous populations of cells in the epithelium, mesenchyme, and muscle interact during intestinal development.

The adult intestine has been extensively characterized phenotypically. In contrast, the mechanisms leading to the appearance of the adult epithelium have gained little attention. This review will focus on how the adult intestinal epithelium arises from the primitive gut.

Fetal Intestinal Development

E1-E9.5 Gut tube formation and patterning

Morphological events. Symmetric and asymmetric cell divisions, cell reorganization, and cell specification are part of orchestrating the transition from a one-cell zygote to a fetus with a properly formed primitive gut tube [28] (Table 1). This is initiated at embryonic day 6.0 (E6.0) in mice and at day 12 in humans by the onset of gastrulation. During this process, pluripotent stem cells in the epiblast transit into a three-dimensional structure that drives the specification of cells into the three germ layers: ectoderm, mesoderm, and endoderm. The early fate decision in the gastrula provides the foundation for the adult organism.

Lineage specification in the gastrula follows an invariable pattern, which is conserved across species. The first cells with endodermal identity form in a defined region within the gastrula. As cells of endodermal origin accumulate, a sheet of cells develop with pockets at both the anterior (foregut) and posterior (hindgut) ends. The pockets join in a folding wave, where the foregut meets the hindgut, leading to the formation of the primitive gut-tube [29]. Regional specification subsequently ensures that the different segments of the tube will develop into esophagus, thyroid, trachea, lungs, stomach, liver, biliary system, and pancreas from the foregut; small intestine from the mid-gut; and colon from the hindgut.

Molecular events. Distinct molecular signatures characterize the foregut, mid-gut, and hindgut [30]. Despite these obvious differences, a limited number of transcription factors have proved causal for cellular identity and regionalization. Sex determining region Y-box 2 (Sox2) and the caudal type homeobox factors Cdx1, 2, and 4 are expressed in the anterior and posterior part of the developing endoderm, respectively, where they have dominant effects on cell fate (Fig. 2A). Specific foregut identities are acquired via the dose-dependent action of Sox2 in combination with NK2 homeobox 1 (Nkx2.1), Hematopoietically expressed homeobox protein (Hhex), and Pancreatic and duodenal homeobox 1 (Pdx1) [31–36]. Cdx2, on the other hand, controls mid- and hindgut regionalization [37–40].

Sox2 is expressed in precursors of the esophagus and stomach, while Nkx2.1 is restricted to the trachea. On loss of Sox2, segment identity is lost and the esophagus begins to resemble the trachea accompanied by increased Nkx2.1 expression [35]. In *Hhex* mutants, the liver is absent and thyroid is either hypoplastic or not formed [33]. Pdx1, on the other hand, is required for specification of the distal foregut as demonstrated by the lack of pancreas and the presence of an abnormal pylorus in *Pdx1*-null animals [31,34]. In contrast to the caudalization of *Pdx1*-null animals, the intestinal epithelium in *Cdx2*-deficient mice expresses markers that are usually associated with the stomach and esophagus [38,40]. It is likely that Wnt signaling plays a very important role in the early patterning, since *Tcf1/Tcf4* mutant mice have an abnormal intestine that at E13.5 has characteristics

Table 1. Gut Tube Formation and Patterning

Mutant gene	*Type of mutant*	*Phenotype*	*References*
Cdx2	Fox3 Cre Villin CreER^{T}	Hindgut identity is lost.	[38,40]
Hhex	KO	Liver not formed and thyroid hypoplasia.	[33]
Hoxa13/ Hoxd13	Double KO	Hindgut patterning defects. No separation of urogenital sinus and rectum.	[47]
Hoxa3	KO	Foregut pattering defects in thymus and thyroid morphogenesis.	[45]
Hoxa5	KO	Foregut pattering defects in tracheal and lung morphogenesis.	[43]
Hoxc4	KO	Esophagus lumen blockage.	[44]
HoxD	HoxD Del (1–10)	Defective caecum budding in distal mid-gut.	[46]
Pdx1	KO	Lack of pancreas and stomach differentiation defects.	[31,32,34]
Sox2	Sox2$^{EGFP/f}$(Sox2 KD)	Esophagus identity lost, resembles trachea.	[35]
Tcf1/Tcf4	KO	Expression of Sox2 and downregulation of Cdx2 in hindgut.	[41]

KO, conventional knockout; Cre, conditional; KD, knockdown.

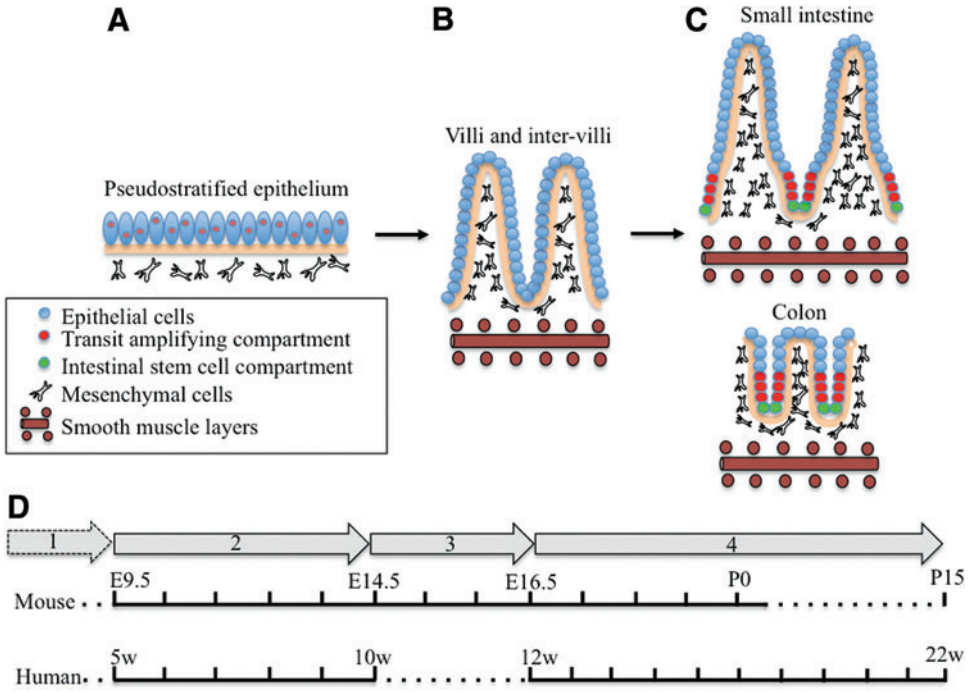

FIG. 1. Human and mouse intestinal development at the morphological level. At early developmental stages, the intestine is a pseudostratified epithelium **(A)** that transits toward a simple epithelium composed of villi and inter-villi regions **(B)**. This will eventually give rise to the crypt-villus structure in the small intestine and crypts connected to the smooth surface in the colon **(C)**. The developmental timing is indicated in **(D)**. This process can be summarized in four main stages: (1) Pseudostratified epithelium formation and intestinal segment patterning. (2) Intestinal growth and elongation. (3) Epithelial reorganization and villification. (4) Crypt formation and acquisition of adult properties. Mutations affecting these individual stages have been identified and are summarized in Tables 1–4. Color images available online at www.liebertpub.com/scd

of the stomach epithelium with ectopic Sox2 expression and low levels of Cdx2 (Fig. 2A) [41].

Hox genes encode transcription factors that are involved in patterning along the anteroposterior axis during embryonic and fetal development [42]. *HoxA* and *HoxB* (groups 4–8) genes are expressed in the foregut, *HoxA*, *HoxB* and *HoxC* (except *Hoxc-4*), and *HoxD* (only in distal part) in the mid-gut, and *Hoxd-8*, *Abd-B* (except Hoxb-9), and *HoxD* in the hindgut (Fig. 2A). Importantly, *Hoxa3*, *Hoxc4*, and *Hoxa5* mutant mice have defective foregut patterning [43–45]. Moreover, in *HoxD* mutants (Del 1–10), *Hoxd11* and *Hoxd12* are ectopically expressed, leading to defective caecum budding in the distal mid-gut [46]; whereas *Hoxa13 - Hoxd13* double mutant animals have defects in distal

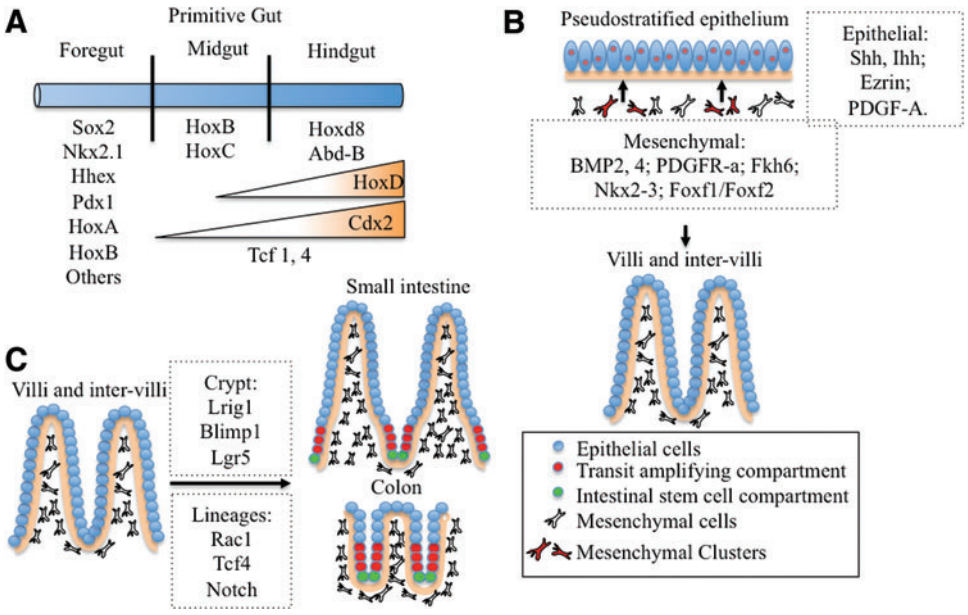

FIG. 2. Key players in intestinal maturation. Specific molecular pathways drive the patterning of the primitive gut **(A)**. From E9.5 to E14.5, the pseudostratified epithelium transits into a simple epithelium organized into villi and inter-villi regions and cells acquire polarity. This process requires a tight molecular regulation. Underneath the epithelium, clusters of mesenchymal cells drive the villi emergence **(B)**. Finally, crypts and cell lineages that are characteristic of the adult epithelium are formed **(C)**. Color images available online at www.liebertpub.com/scd

Table 2. Gut Tube Growth and Elongation

Mutant gene	*Expression*	*Type of mutant*	*Phenotype*	*References*
Shroom3	E	KO	Disorganization and stratification of intestinal epithelium.	[48]
Fgf9	E	KO	Shortened intestine.	[54,86]
Wnt5a	M	KO	Shortened intestine.	[55]

E, epithelial; M, mesenchymal; KO, conventional knockout.

hindgut patterning [47]. The impact of many individual Hox genes on segment identity still remains largely unexplored apart from their mechanism of action.

E9.5-E14.5 Gut tube growth and elongation

Morphological events. After the gut tube is fully formed, the simple epithelium condenses to form a seemingly uniformly proliferative pseudostratified epithelium (Fig. 1A, D and Table 2). During this process, the gut tube expands in length and girth by a combination of proliferation and dynamic acquisition of epithelial polarity [48,49]. Controlled proliferation of cells within the sub-mucosa and muscle layer carefully balance the expansion of the epithelial compartment.

Molecular events. The most prominent molecular change is the polarization of the epithelium. Shroom3, which is expressed at the apical membrane, tightly regulates the apicobasal elongation of cells within the pseudostratified epithelium [48]. Mechanistically, Shroom3 acts via controlling actinomyosin signaling and γ-Tubulin organization [50–53]. Loss of *Shroom3* results in a disorganized and stratified epithelium [48], but it is unclear how this affects subsequent developmental stages such as villification.

The elongation of the forming intestine is mediated via a number of different pathways that are most likely organized in complex regulatory networks. Evidence suggests that longitudinal and lateral extensions are regulated via cross-talks between the epithelium and the underlying mesenchyme implicating both Fibroblast growth factor 9 (Fgf9) and Transforming growth factor beta (TGFβ) signaling [54]. Similarly, Wnt5a, which stimulates the noncanonical Wnt pathway, is expressed in the mesenchyme from E9.5 [54,55]. However, although it is important for gut elongation, the effect of Wnt5a is somewhat unclear since loss of Wnt5a leads to an increased girth of the intestine, and postmitotic cells do not intercalate properly into the epithelium. This results in a stratified phenotype, but it is currently unclear whether the observed effect on the epithelium is a direct consequence of Wnt5a deletion or compromised expansion of the intestinal tube.

E14.5–16.5 Epithelial reorganization and villification

Morphological events. The pseudostratified epithelium in the mid-gut and hindgut transitions into a simple columnar epithelium at E14.5 in mice and around gestational week 9 in human fetuses (Fig. 1A, B, D and Table 3) [56,57]. During the transition toward a columnar epithelium, transient cavities are formed, which most likely fuse to the lumen as villi form in a rostral to caudal wave [56–60]. It has been proposed that this process is tension driven by the muscles in the outer layer of the intestine [61]. In this model, ridges form along the longitudinal axis by contraction of the circular muscle fibers. The subsequent contraction of longitudinal muscle fibers converts the ridges into a zig-zag pattern that will break up into singular villi as tension increases. The result of the tension-mediated folding of the epithelium is evident at E16 in mice, when villification is complete. The simple epithelium is at this point composed of a continuum of cycling cells in the intervillus regions with interspersed villi covered by noncycling cells. Interestingly, although the structure of the adult colonic epithelium is distinct from the small intestine, the hindgut adopts transient villus-like

Table 3. Epithelial Reorganization and Villogenesis

Mutant gene	*Expression*	*Type of mutant*	*Phenotype*	*References*
Shh	E	KO	Smooth muscle reduction and defective enteric nervous system.	[67]
Ihh	E	KO	Smooth muscle reduction and defective enteric nervous system.	[81]
Hhip	M	Villin-Hhip	Compromised smooth muscle formation and vilification.	[73]
Pdgf-A *Pdgfr-α*	E (Pdgf-A) M (Pdgf-α)	KO	Fewer and thicker villi, smooth muscle reduction.	[69]
Fkh6	M	KO	Delayed villi formation, increased epithelial proliferation coupled to longer villi.	[70,127]
Nkx2-3	M	KO	Delayed villi formation, increased epithelial proliferation coupled to longer villi.	[78,128]
Ezrin	E	$Ezrin^{f/f}$/ EIIa-Cre	Villi disorganization and abnormal cell polarization.	[80]
Foxf1/Foxf2	M/M	KO	Megacolon, Smooth muscle hypoplasia, epithelial depolarization.	[84,85]
β-Catenin activation	E	$Catnb^{+/f(ex3)}$/ Shh-Cre	Villi disorganization.	[82]
EGF-R	nd	KO	Delayed vilification, shorter and thinner villi. Thinner smooth muscle layer.	[79]

E, epithelial; M, mesenchymal; nd, not determined; KO, conventional knockout; f: loxP sites; KI, knockin.

structures before being converted into its adult form [62,63]. The significance of this transient structure and its putative role in ISC specification remains unknown.

Villification follows a similar series of events in humans starting from gestational week 9 in a cranio-caudal direction [59,64,65]. Interestingly, smooth muscle formation occurs at gestational week 8 [66], thereby preceding villification and the mechanism mentioned earlier could, therefore, potentially be conserved through evolution.

Molecular events. Cross-talk between the epithelium and mesenchyme is crucial for the continuous morphogenesis of the intestine. It has been proposed that clusters of mesenchymal cells underneath the forming villi, which express Hedgehog pathway components [67,68], members of the Bone morphogenetic protein (BMP) family [69], Alpha-type platelet-derived growth factor receptor (PDGFR-α) [69], and Forkhead 6 (Fkh6) [70], play a key role during the process of villification (Fig. 2B).

The Hedgehog family is involved in many developmental processes [71], and the two ligands Sonic and Indian Hedgehog (Shh and Ihh) are expressed in the developing intestinal epithelium [67]. In both Hedgehog ligand mutant mice, the circular smooth muscle layer is reduced in thickness without affecting villification [67]. In contrast, double-knockout animals have defective villification and a reduced number of mesenchymal progenitors. This strongly indicates that these ligands have redundant functions [72]. In agreement with this observation, exogenous expression of the pan-Hedgehog inhibitor Hedgehog interacting protein (Hhip) in all intestinal epithelial cells compromises muscle formation as well as villification [73].

BMP4 is downstream of the Hedgehog pathway and is similar to BMP2 and PDGFR-α that are expressed in the mesenchyme underneath nascent villi [69,74,75]. The expression pattern within the villus clusters suggests that the BMP pathway has a role in vilification. Although genetic evidence supports an important function for BMP receptor signaling in regulation of adult stem cell behavior, there is little evidence for its involvement in vilification [73,76,77]. Moreover, it is unclear whether BMP2, BMP4, and PDGFR-α are expressed by the same or neighboring cells. Interestingly, disruption of *PDGFR-α* and *PDGF-A* expression in mice results in fewer and thicker villi without affecting proliferation, suggesting that the signaling interface between the epithelium and villus cluster affects morphogenesis. In these mutant mice, the premature formation of the smooth muscle could be a confounding explanation for the abnormal vilification [69].

At E14.5 mesenchymal clusters are only detected in the proximal part of the intestine and spread progressively in a rostral to caudal wave completing at E16.5, thereby preceding villification [68]. The correlation between the morphological defects in PDGF-A and Hedgehog mutant mice and their association with the mesenchymal cell clusters suggests that the clusters play a role in villification. The inductive capacity of the mesenchyme in villification is further substantiated by the defects observed in the *Fkh6* knockout mice. In this case, loss of Fkh6 leads to reduced levels of *BMP2* and *BMP4*, pronounced delay in the transition from a pseudostratified to columnar epithelium, and retarded formation and growth of nascent villi [70]. Again, it remains unclear whether Fkh6, PDGF-A, and Hedgehog ligands are co-expressed by the same cells. The homeobox transcription factor Nkx2-3 is another putative regulator of BMP2 and BMP4 as mutant mice express reduced levels of intestinal BMP and phenocopy Fkh6 mutant animals [78]. Interestingly, *Fkh6* levels are not affected in *Nkx2-3* mutants, suggesting that *Nkx2-3* is downstream or in a genetically independent pathway. In addition to the traditional pathways affecting morphogenesis, signaling via the Epidermal Growth Factor Receptor (EGFR), which usually affects proliferation, has important roles in a number of tissues, including the intestinal epithelium. Villus formation is severely affected on loss of *EGFR*; it is, however, not clear whether an epithelial, mesenchymal, or muscle defect causes these defects to occur [79].

Altogether, the multitude of signaling pathways important for early villification and epithelial organization clearly illustrates the intimate relationship between the muscle layer, the submucosa, and the epithelium that drives morphogenesis in the intestine. The specific role of the different component, in particular the mesenchyme, remains to be explored using genetic models that specifically target the submucosa rather than relying on complete knockout animal models. This will help define the role of the mesenchyme and the importance of the villus clusters in the process of villification as well as their homogeneity within the same and different intestinal segments.

Epithelial polarity. During remodeling toward a simple columnar epithelium, polarization provides the remits for generating distinct apical and basolateral cell surfaces. In this process, the cytoskeleton plays a major role. Ezrin proteins are present at the apical surface in the fetal intestine, where they provide links between membrane proteins and the actin cytoskeleton. Their importance is evident from studies of Ezrin mutant animals, where nascent villi at E15.5 are fused together via regions of disorganized and stratified epithelial cells (Fig. 2B) [80]. A similar phenotype is detected in mutants expressing a stabilized version of β-catenin, which via its important role at the Adherens Junctions and interaction with Ezrin is involved in establishing cellular polarity [80–83]. Downstream of the Hedgehog pathway, the forkhead transcription factors Foxf1 and Foxf2 are also involved in establishing epithelial polarization (Fig. 2B) [84,85]. In mutants of these transcription factors, villi and inter-villi patterns are not properly formed, which is associated with altered deposition of extracellular matrix molecules and depolarization of E-Cadherin at the basolateral membrane [85]. Considering that misregulation of E-Cadherin in these mutant also causes smooth muscle hypoplasia, the observed phenotype could be driven by compromised function of both the epithelial and lacking tension in the muscle layer [61]. A further dissection of the phenotypic aberrance in these mutants is required to bring resolution to the specific effects of many of these components.

E16.5-P15. Crypt of Lieberkühn formation and cell lineage specification

Morphological events. From E16.5 to birth, the intestine is organized into villi and intervillus regions. In the rodent small intestine, distinct parts of intervillus regions are transformed during the first 2 postnatal weeks into crypts of Lieberkühn (Fig. 1B–D and Table 4) [25,61]. Measurements of the distance between the inner circular smooth muscle layer and the

Table 4. Crypt of Leiberkühn Formation and Cell Lineage Generation

Mutant gene	*Expression*	*Type of mutant*	*Phenotype*	*References*
Lrig1	E	KO	Enlarged crypts and increased ErbB signaling.	[23]
Blimp1	E	Villin-Cre Cyp1a1-Cre	Intestinal maturation is accelerated.	[105,106]
Rac1	E	Rac1Leu61 (activation)	Precocious Paneth cell differentiation.	[109]
		Rac1Asn17 (inhibition)	Reduction of the secretory goblet cells and Paneth cells.	[109]
Lgr5	E	KO	Precocious Paneth cell differentiation.	[110]
Tcf4	E, M	KO	Abolished proliferation in inter-villi region and reduced amounts of enteroendocrine cells.	[111]

E, epithelial; M, mesenchymal; KO, conventional knockout; Cre, conditional knockout.

inter-villus epithelium or the bottom of the crypts strongly suggest that crypts do not develop as down growths of the epithelium, but that the crypt-villus junction via expansion of the mesenchymal cell populations moves upward and encapsulates epithelial cells within the intervillus region [25]. Two weeks after birth, the intestine reaches its mature form [25,26]. In humans, crypts form in gestational weeks 11–12 (Fig. 1B–D) [65] and the intestine reaches maturity around 22 weeks of gestation [86]. In terms of the origin of the crypts, early lineage analysis demonstrates that they form as polyclonal entities, but rapidly become monoclonal most likely due to the same mechanisms that operate in the adult epithelium [87–89]. The growth of the intestine from this point onward is driven by crypt fission, whereby crypts duplicate by bifurcation at the crypt bottom [90]. Crypt fission is maintained during adulthood, but the frequency is drastically reduced in mice older than 10 weeks of age [91].

The role of microbiota and the symbiosis with the epithelium and immune system has gained attention during the past years because of its involvement in normal intestinal function [92] and impact on disease (reviewed in Sartor [93]). The GI tract is believed to be sterile before birth, and then, it is subsequently colonized by microorganisms [94–96]. The role of microbiota as a driver of tissue maturation remains largely unexplored. The observation that humans acquire mature crypt-villus architecture before birth under sterile conditions implies that the microbiota has little influence on this process.

Late fetal development is associated with a shift in lineage specification. Villi are in the immature form of the intestine covered primarily by enterocytes. Maturation is associated with the emergence of secretory lineages where goblet and enteroendocrine cells precede the other secretory lineage [97]. After crypt morphogenesis, Paneth cells form initially halfway up the rudimentary crypts [98–100]. It is subsequently believed that Paneth cells migrate to the bottom of the crypt and intercalate between CBC cells [101,102]. In humans, mucins, which mark goblet cells in the adult epithelium, are detected as early as gestational week 9–10 concomitantly with the emergence of immature absorptive enterocytes [27,31,65,103]. It is, however, not until gestational week 12 that Paneth cells, goblet cells, and endocrine cells are detected [65].

Molecular events. A number of proteins have been implicated in the transition of the fetal epithelium from an exponentially expanding structure to homeostatic conditions where cell loss and gain are balanced (Fig. 2C).

Lrig1 is a negative regulator of the EGFR family of growth factor receptors. In the adult epithelium, it is expressed in the lower one-third of the crypt, including the Lgr5 expressing stem cell compartment [23,104]. Interestingly, after the first postnatal week and concomitantly with the expression of markers of the adult stem cell niche such as Olfm4, loss of *Lrig1* causes pronounced crypt enlargement accompanied by increased ErbB activation and c-Myc expression [23]. B-lymphocyte-induced maturation protein 1 (Blimp1), which is a negative transcriptional regulator of *c-Myc* and other transcription factors, prolongs the transition from the fetal to adult intestine [105,106]. Specifically, Blimp-1 is expressed in the developing and postnatal epithelium. Interestingly, the expression is almost completely lost weaning. In conditional knockouts for *Blimp-1* in the intestine, the maturation process is accelerated. This is evident by increased crypt length at P7, presence of serrated villi (characteristic of the adult intestine), increased expression of metabolic pathways associated with the adult rather than the fetal state, increased epithelial turnover, and premature generation of Paneth cells. The molecular mechanism for Blimp-1 in tissue maturation remains unknown. Considering that crypt formation in humans occurs during the second gestational trimesters, and therefore the neonates already have rudimentary crypts and Paneth cells, it is relevant to elucidate whether Blimp-1 regulation is exclusive to mice or whether it is functional in humans at earlier stages.

The small GTPase Rac1 has important functions in adult stem cell maintenance in a number of tissues [107,108]. Interestingly, activating mutations in the small GTPase Rac1 (Rac1Leu61) causes premature Paneth cells differentiation as early as E18.5, whereas expression of a dominant-negative Rac1 mutant (Rac1Asn17) reduces goblet and Paneth cells numbers [109]. Similarly, loss of *Lgr5*, a Wnt target gene, co-receptor for the Wnt agonists R-spondins, and a marker of CBC cells, is associated with precocious Paneth cell differentiation [110]. Since both Lgr5 and Rac1 have been implicated in both Wnt signaling and planar cell polarity pathways, this could imply common effects on early fate decisions in fetal tissues.

Tcf4 is expressed from E16.5 and onward by cycling cells in the inter-villus regions. It is one of the transcription factors that acts as a co-receptor for β-catenin and mediates Wnt-dependent transcription. Loss of *Tcf4* abolishes proliferation and is accompanied by a reduction in the number of enteroendocrine cells [111]. Wnt reporter activity is not detected in the inter-villus regions until postnatal day P2,

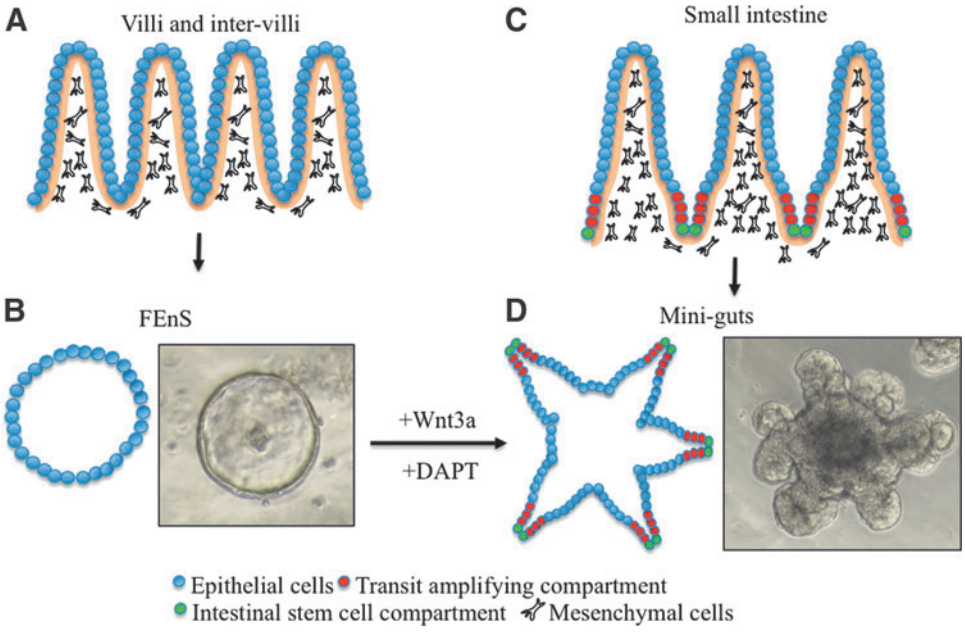

FIG. 3. Fetal progenitors and adult intestinal stem cells. The establishment of villi and inter-villi pattern **(A)** coincides with the possibility to culture intestinal fetal enterospheres (FEnS) **(B)**. In contrast, the adult small intestine **(C)** containing adult intestinal stem cells gives rise to branching structures, called mini-guts **(D)** when propagated as in vitro cultures. Color images available online at www.liebertpub.com/scd

and it is, therefore, unclear whether Tcf4 at these stages is important as a Wnt mediator or whether it has alternative transcriptional functions [82]. Moreover, cells expressing a constitutively active TCF-β-catenin fusion protein in chimeric mice are selectively lost during development, suggesting a deleterious effect of aberrant canonical Wnt signaling [112]. The effect of positional strength and context dependency in signaling is likely to prove vital during these stages of development, and the effects of signaling might, therefore, be difficult to modulate.

From fetal progenitors to the adult ISCs

It is clear and well documented that the structure and composition of the immature fetal intestine is distinct from its adult mature counterpart. There is, however, a number of unresolved questions related to whether predefined stem cells exist in the fetal intestinal epithelium and await a maturation stimulus or whether all epithelial cells that proliferate are equipotent. Immature intestinal progenitors can be isolated from mouse and human fetuses [97,113]. Fetal intestinal progenitors maintain their distinct properties in vitro, which reflect the immature differentiation status of the tissue from which they are derived. While immature intestinal epithelium in vitro grows as seemingly homogenous spheres (Fetal enterospheres - FEnS) (Fig. 3A, B) [97,113], cells from the adult epithelium give rise to branching structures or ''mini-guts'' (Fig. 3C, D) [114]. FEnS are characterized by the absence of secretory lineages and can be isolated from mouse small intestine until P2. Importantly, FEnS retains their function to mature as has been demonstrated by their ability to engraft into the adult colon [97].

The transition from an immature to a mature epithelium assessed by the capacity to generate FEnS or mini-guts in vitro follows a caudal to rostral wave [97]. This wave is contrary to the reported tissue folding and the emergence of Paneth cells [98]. Paneth cells constitute part of the adult ISC niche and a source of intestinal Wnt [115,116]. However, stimulation with Wnt is required to induce Paneth cell differentiation, and this, therefore, represents a conundrum in the maturation of the epithelium [82]. This is also interesting from a developmental point of view, since Wnt stimulates the transition of FEnS into mini-guts [97]. It remains to be elucidated how the Wnt pathway operates in this process and within the epithelium and whether the induction of Paneth cells by Wnt is secondary to the emergence of adult ISC. Inhibition of the Notch pathway is an additional trigger of the transition of FEnS into mini-guts (Fig. 3B, D) [113]. This supports early observations where Notch signaling via Hes1 controls secretory cell fate [117]. Interestingly, Notch activation is also important in the developing intestinal mesenchyme, suggesting multiple layers of reciprocal modulation of Notch components in tissue patterning [118]. In the adult intestine, the Notch pathway is vital for balancing the formation of absorptive enterocytes and secretory cells [119,120]. It remains to be elucidated whether high levels of Notch signaling are prohibiting secretory lineage production during fetal stages, as well as a putative cross-talk with the Wnt pathway.

Future Perspectives and Final Remarks

Our current understanding of late developmental transition within the intestine highlights key morphological events and pathways. However, the mechanisms that drive transition remains poorly understood. To address these questions, it will be instrumental to identify specific markers that define cellular heterogeneity within the developing epithelium. This will provide a handle on the fetal states, facilitate fate mapping, and provide us with the ability to modulate specific molecular pathways in the developing epithelium. Once we understand the process in rodents, it will be important to assess whether the molecular program driving intestinal maturation is similar in humans. In this context, in vitro cultures of intestinal fetal progenitors represent a crucial tool to address these questions.

The insights gained from the in vivo analysis is also bound to inform the directed differentiation of intestinal epithelial cells from both pluripotent stem cells and potentially patient-derived somatic cells. Current tools are limited in their ability to generate cells with adult characteristics [14,17,121]. Exciting recent developments have revealed that the gut epithelium derived from pluripotent stem cells, when transplanted with nonepithelial cells, transits from an immature to an adult epithelium, thereby recapitulating the behavior of fetal-derived intestinal epithelium [97,122,123]. The mechanism leading to maturation still remains unknown but is likely a reflection of our poor understanding of the process that promotes normal tissue maturations. With these developments, we now have the tools to be able to dissect both murine and human gut development. Enhanced resolution and insights into the molecular mechanisms that drive physiological intestinal maturation are crucial to establish platforms for intestinal neonatal disease modeling. This will notably contribute to the design of therapeutic strategies to several disorders such as neonatal IBD, Microvillus inclusion disease, or tufting enteropathy. Moreover, the establishment of human and fetal intestinal cultures as well as the possibility to transplant such cells could provide new therapeutic options based on, for example, in vitro gene correction. Here, genome editing using technologies such as guide RNA-directed CRISPR-Cas9 editing will likely play a major role [124]. Importantly, these technologies have been successfully adapted to studies for human adult intestinal organoids [125,126]. Altogether, this is likely to pave the way for future cell therapies.

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Author Disclosure Statement

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