CHAPTER TWO

Signals and forces shaping organogenesis of the small intestine

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Abstract

The adult gastrointestinal tract (GI) is a series of connected organs (esophagus, stomach, small intestine, colon) that develop via progressive regional specification of a continuous tubular embryonic organ anlage. This chapter focuses on organogenesis of the small intestine. The intestine arises by folding of a flat sheet of endodermal cells into a tube of highly proliferative pseudostratified cells. Dramatic elongation of this tube is driven by rapid epithelial proliferation. Then, epithelial-mesenchymal crosstalk and

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physical forces drive a stepwise cascade that results in convolution of the tubular surface into finger-like projections called villi. Concomitant with villus formation, a sharp epithelial transcriptional boundary is defined between stomach and intestine. Finally, flask-like depressions called crypts are established to house the intestinal stem cells needed throughout life for epithelial renewal. New insights into these events are being provided by *in vitro* organoid systems, which hold promise for future regenerative engineering of the small intestine.

1. Generation and growth of the intestinal tube

1.1 Embryonic origins of the murine small intestine

We focus this chapter primarily on development of the mouse small intestine, since the majority of work has been done in that system. Around embryonic day 6 (E6), the mouse embryo is cup-shaped and ready for gastrulation. The cup is composed of two cellular layers: (1) epiblast cells, which will give rise to the embryo proper, line the inner surface and are surrounded by (2) a layer of squamous cells known as the embryonic visceral endoderm (emVE). As gastrulation initiates, presumptive definitive endoderm (DE) cells travel through the primitive streak and intercalate into the emVE layer. Early lineage tracing using injected dyes or markers and histological analyses suggested that DE cells insert contiguously, displacing the emVE cells laterally, so that these extraembryonic cells are eventually completely eliminated from the portion of the developing endodermal sheet that will give rise to the embryonic gastrointestinal tract (reviewed in Viotti, Foley, & Hadjantonakis, 2014). In those studies, cell movements were inferred from analysis of multiple samples harvested at different times after labeling. However, more recent work, using broadly expressed lineage tracing tools, combined with live imaging of developing embryos, has revealed the actual dynamics of cellular movements and showed that the entering DE cells disperse rather than displace the emVE cells (Kwon, Viotti, & Hadjantonakis, 2008). Indeed, scattered emVE cells can be found in the completely formed gut tube as late as 15 somites (E8.75), and perhaps even later. However, it is not known whether these emVE-derived cells or their progeny have characteristics or roles that are distinct from DE cells, or how long they persist in the growing animal.

1.2 Shaping the intestinal tube

As the definitive endodermal sheet is being established (E7.0–8.0), the embryo is elongating along the anterior/posterior axis, head and cardiac regions are being defined and left-right asymmetry is initiating. Interestingly,

the developing endodermal layer influences all of these processes (Martinez Barbera et al., 2000; Nascone & Mercola, 1995; Saund et al., 2012; Schultheiss, Xydas, & Lassar, 1995; Viotti, Niu, Shi, & Hadjantonakis, 2012; Withington, Beddington, & Cooke, 2001). In the case of left-right patterning, two groups showed that loss of the transcription factor *Sox17* in the endoderm perturbs both gut development and the acquisition of left-right asymmetry (Saund et al., 2012; Viotti et al., 2012). In the endoderm of *Sox17* mutant mice, Connexin 43 (CX43), a gap junctional protein, is missing (Viotti et al., 2012), resulting in defective intercellular transport between endoderm cells. Further investigations led both groups to conclude that the planar transmission of signals across gap junctional connections within the endoderm acts as a conduit to transfer a left-right asymmetry signal, generated in the node, to the lateral plate mesoderm.

Around E8.0, the flat endodermal sheet becomes indented at its anterior end and, shortly thereafter, its posterior end, forming the anterior and caudal intestinal portals (Fig. 1). Careful cell tracing studies have revealed the intricate morphogenic movements of endodermal cells during the formation and extension of these portals as the endodermal sheet is wrapped into a tube between E8.0 and E9.0 (Franklin et al., 2008; Tremblay & Zaret, 2005). Even before this complex cellular choreography establishes the gut tube proper,

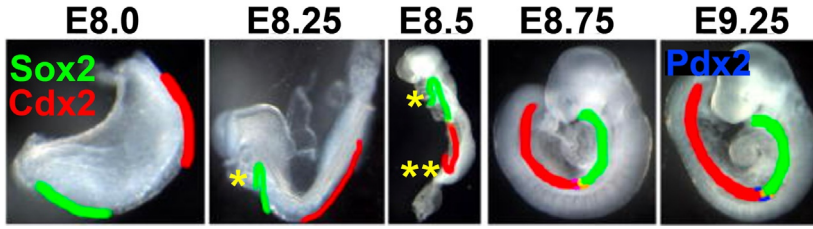

Fig. 1 Expression domains of CDX2, SOX2, and PDX1 during gut tube closure and formation of the anterior and caudal intestinal portals. At E8.0, SOX2 (green) and CDX2 (red) are expressed in the anterior- and posterior-most endoderm, respectively. At E8.25, the anterior intestinal portal is observed in the SOX2 domain (*) and at E8.5, the caudal intestinal portal (**) begins to form within the CDX2 domain. Between E8 and E8.75, the two domains extend toward each other as the endoderm wraps into a tube. The two domains meet at E8.5 and cells at the boundary co-express SOX2 and CDX2 (yellow). By E9.25, the boundary appears sharper in whole mount tissue (but see text) and PDX1 expression is seen in cells in the future pyloric region, extending from both sides of this boundary (blue). *Images were modified with permission from Sherwood, R. I., Chen, T. Y., & Melton, D. A. (2009). Transcriptional dynamics of endodermal organ formation.* Developmental Dynamics, 238, *29–42.*

anterior-posterior (A-P) patterning is initiating, regionalizing the gut into foregut, midgut and hindgut territories (Zorn & Wells, 2009). Soluble signals from surrounding mesodermal tissues are responsible, at least in part, for A-P patterning; an important role for FGF and WNT signals in posterior specification has been documented (Dessimoz, Opoka, Kordich, Grapin-Botton, & Wells, 2006; Sherwood, Maehr, Mazzoni, & Melton, 2011). By E7.75, the transcription factors *Sox2* and *Cdx2* are activated in the most anterior and most posterior endoderm, respectively (Fig. 1). As the portals move toward the middle of the embryo, the expression domains of these factors also move toward one another. At E9.5, after turning of the embryo completes gut tube formation, a distinct *Sox2-Cdx2* border is visible in whole mount tissue, in the region of the future pylorus (Sherwood, Chen, & Melton, 2009). Transcriptomic studies have revealed a number of additional transcription factors and cell surface proteins that show dynamic and region-specific expression patterns during this critical morphogenic process, and likely participate in establishment of the regional pattern (Sherwood et al., 2009).

1.3 Formation of the convoluted intestinal tract

The midgut first forms a hairpin loop at E10.5 (Fig. 2A, D, and J), which extends toward the ventral side of the embryo (Onouchi, Ichii, Otsuka, Hashimoto, & Kon, 2013) (Fig. 2B, E, E′, and K). As the gut tube actively elongates, it herniates into the umbilicus (Fig. 2B, E, and E′). At the same time, it also rotates 90° counterclockwise, around the axis of the superior mesenteric artery, so that its proximal end is to the right and distal end is to the left (Fig. 2B) (Kaufman, 1992; Kluth, Jaeschke-Melli, & Fiegel, 2003). A cecum bulge also forms more posteriorly, which separates the small and large intestine (Fig. 2K). The midgut continues to elongate rapidly while coiling into loops over the next 5 days outside of the body (Fig. 2E, E′, F, F′, G, G′, and L). Retraction of the gut back into the body cavity begins proximally, possibly driven by the muscular contraction of the duodenojejunal region (Cervantes, Yamaguchi, & Hebrok, 2009) (Fig. 2C and H). During this retraction, the gut undergoes a further counterclockwise rotation (Fig. 2C), which adjusts the position of the future small intestine with respect to the future large intestine (Davis et al., 2008). By E16.5, the entire gut has returned to the abdominal cavity with a properly convoluted pattern (Fig. 2I, I′, and M).

The complex process of herniation, looping of the growing intestine outside of the body and ordered return of the midgut to the body cavity

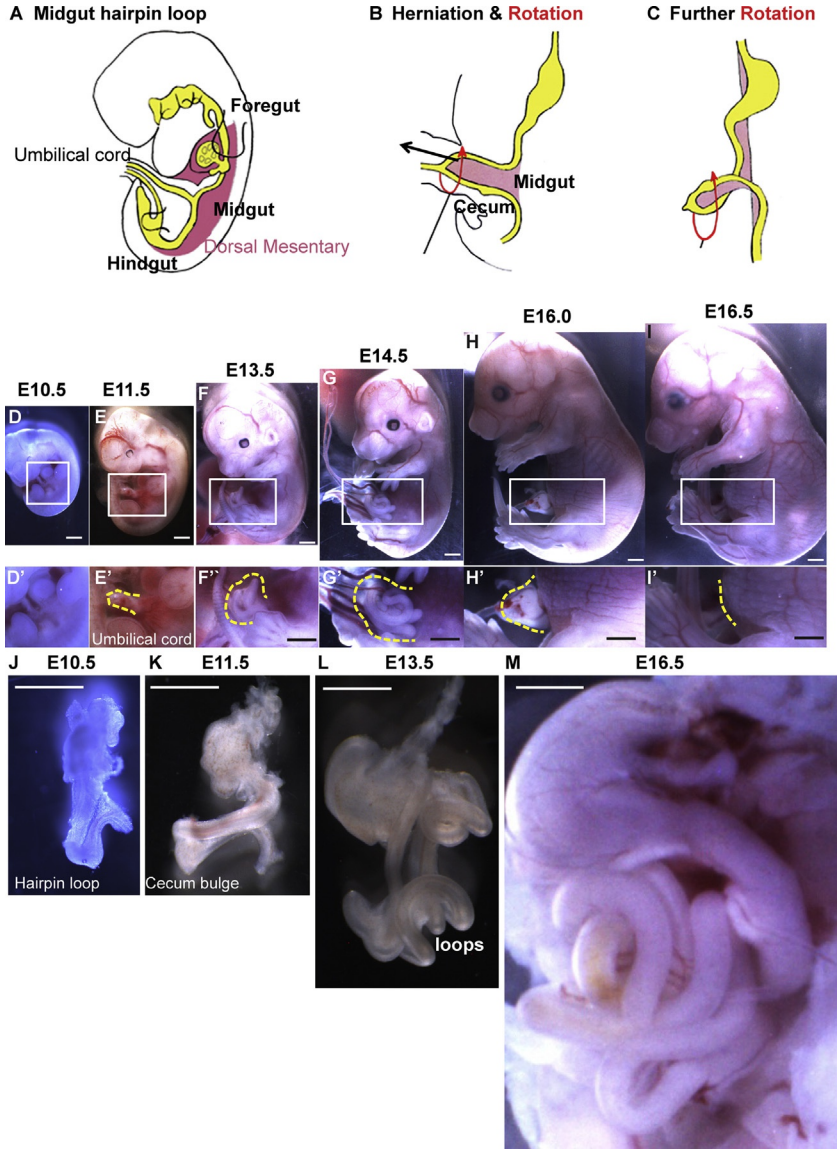

Fig. 2 Intestinal hernia, rotation, looping, and retraction during development. (A-C) Schematic illustration of intestinal hairpin loop formation (A), herniation (B) and rotation (B, C) in amniotes. As the midgut elongates, it protrudes into the umbilical cord and forms an intestinal hernia. As it protrudes, the midgut loop makes a counterclockwise rotation. Outside of the body, the gut elongates dramatically; looping/coiling occurs as the result of differential growth of the intestine and the attached mesentery. As the gut returns to the body cavity, it makes a further rotation. (D-I) Mouse embryos at E10.5–16.5. (D′-I′) Higher magnifications of the white boxed regions in (D-I), respectively (herniation and retraction). Yellow dashed line outlines the intestine located outside of the body cavity. (J-L) Dissected mouse GI tracts at E10.5 (hairpin loop), E11.5 (cecum bulge formation), E13.5 (looping). (M) Convoluted GI tract in an opened abdominal cavity of an E16.5 mouse embryo, showing intestinal loops. Scalebars are 1 mm.

are critical for proper intestinal function after birth. Intestinal malrotation, a congenital mis-positioning of the intestine within the peritoneal cavity (Applegate, Anderson, & Klatte, 2006), is a relatively common human birth defect (1 in 500 births) (Torres & Ziegler, 1993). Since the intestine is attached along its length to the dorsal mesentery, which provides the blood supply, abnormal rotation can cause ischemia of bowel segments, bowel necrosis or volvulus (Applegate et al., 2006).

The characteristic intestinal loops that form while the intestine is herniated allow intestinal length to extend well beyond the linear length of the embryo, while establishing an ordered configuration in the body cavity (Nerurkar, Mahadevan, & Tabin, 2017). Interestingly, looping is driven by mechanical buckling, due to elongation of the intestine against the constraint of the attached dorsal mesentery (Arnaout & Stainier, 2011; Savin et al., 2011). These two tissues have different growth rates, with the intestine outpacing the mesentery. As a result, the elongating intestine stretches the mesentery, which pulls against the intestine, compacting it into regular loops. The intestinal looping pattern (number and size of loops) is also species-specific and this observation led Nerurkar et al. to discover that BMP2, expressed by the dorsal mesentery, controls the rate of growth of this tissue (Nerurkar et al., 2017). Ectopic expression of BMP2 in the chick mesentery does not alter intestinal growth but further slows the growth of the mesentery, which enhances the growth rate gap between the two tissues and causes more tension on the mesentery, resulting in more and tighter loops (Nerurkar et al., 2017). Thus, through its differential effect on growth of two attached tissues, BMP signals tune the tensile forces that control looping patterns.

1.4 Intestinal elongation

One of the most amazing aspects of the small intestine is its length, a feature that contributes to the extensive absorptive surface area necessary for its function. The human intestine attains 40% of its adult length before birth (Weaver, Austin, & Cole, 1991). Failure of proper elongation of fetal intestine (congenital short bowel syndrome) is often fatal (Hasosah, Lemberg, Skarsgard, & Schreiber, 2008; van der Werf, Halim, Verheij, Alves, & Hofstra, 2015).

The intestinal tube begins to actively elongate immediately after its formation. Elongation can be divided into two distinct phases, based on the absence (Phase I) or presence (Phase II) of villi (Fig. 3). During Phase I (E10.5–14.5),

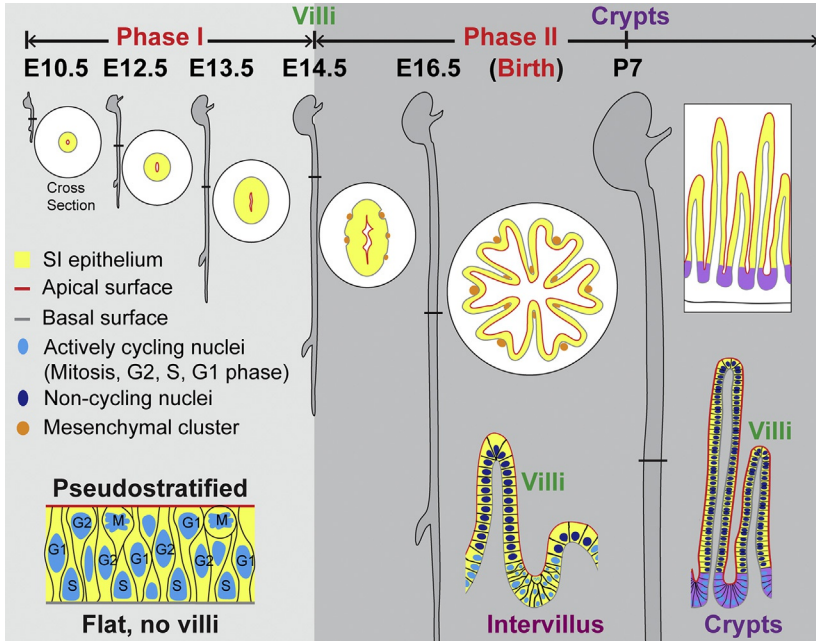

Fig. 3 Small intestinal elongation and its epithelial configuration in Phase I and Phase II. Schematics of intestinal lengthening (drawn to scale) are shown at top. Accompanying circular diagrams represent intestinal cross sections at the point of the horizontal line in top figures. Bottom diagrams show detail of cell shape in the epithelium. In Phase I, the epithelium is pseudostratified with a small flat apical surface (red line) and nearly all cells are actively cycling (blue nuclei, cell cycle phases are noted). In Phase II, beginning at E14.5, mesenchymal clusters form beneath the epithelium (orange) and villi begin to emerge. Cells on villi stop cycling (black nuclei) and change shape, becoming columnar. Cells in the intervillus regions remain proliferative (blue nuclei). Around P7, crypts (purple) form and house proliferating cells.

the epithelial tube is pseudostratified (Grosse et al., 2011) with a flat lumenal surface (Kohlnhofer, Thompson, Walker, & Battle, 2016; Walton, Freddo, Wang, & Gumucio, 2016). Phase II begins at E14.5. During this phase, finger-like villi emerge at the luminal surface, convoluting and expanding the apical surface area (Freddo et al., 2016; Walton, Freddo, et al., 2016; Walton et al., 2012). In addition to differences in epithelial structure, epithelial proliferation patterns differ in Phase I and II. Proliferating cells are distributed throughout the Phase I epithelium, but once villi emerge in Phase II, epithelial cells on top of the villi withdraw from the cell cycle and proliferation becomes confined to intervillus regions (Noah, Donahue, & Shroyer, 2011; Walton, Mishkind, Riddle, Tabin, & Gumucio, 2018).

During Phase I, the mouse small intestinal length, measured from pylorus (the opening from the stomach to the small intestine) to cecum (which marks the transition from small intestine to large intestine), increases more than 11-fold. Early studies led to the proposal that this rapid elongation was driven by convergent-extension movements, as multiple cell layers of a stratified epithelium converge into one layer, generating length by sacrificing girth (Cervantes et al., 2009; Matsumoto, Hashimoto, Yoshioka, & Otani, 2002; Reed et al., 2009). However, later work established that Phase I intestinal epithelium is composed of a single pseudostratified cell layer (Fig. 3) (Grosse et al., 2011; Wang, Cebrian, Schnell, & Gumucio, 2018). Additionally, the girth of the epithelial tube increases as it elongates as well, further ruling out convergent-extension as a mechanism of lengthening (Wang et al., 2018). Wang et al. found that all or nearly all epithelial cells are actively cycling, with cell cycle times of approximately 16 h. This uniformly rapid proliferation therefore appears to be the predominant driver for Phase I midgut growth.

As with other pseudostratified epithelia, the Phase I intestinal epithelium undergoes interkinetic nuclear migration (IKNM), in which nuclei synthesize DNA near the basal surface, migrate apically for mitosis and then return to basal to repeat the cycle (Fig. 4) (Grosse et al., 2011; Wang et al., 2018). Because of this constant nuclear motion, nuclei are staggered along the apical-basal axis. By combining high-resolution 3D confocal imaging and 2D live imaging, Wang et al. found that, as the nucleus moves apically to divide, the cell remains connected to the basal surface via a basal process. During mitosis, the basal process splits in a basal-to-apical direction. Subsequently, one of the two basal processes is often lost or retracted and the other is inherited by one of the two daughter cells. This means that one nascent daughter is born without a basal connection, though it does retain an apical link to its sister. During G1, the nuclei of both daughter cells return to the basal surface, but they do so in two distinct modes. In Mode I, one nucleus returns faster than the other. The daughter that inherits the basal process uses this basally connected process as a "conduit" to quickly return its nucleus to the basal side. In contrast, its sister actively projects a new process to establish a basal pathway and uses that path to return its nucleus; nuclear return by "pathfinding" takes twice as long as the "conduit" strategy. In Mode II, despite the fact that one daughter possesses a basal process, it does not use it to return its nucleus. Instead, both daughters utilize the "pathfinding" strategy, and in this case, both return their nuclei at a similar (slow) pace (Wang et al., 2018) (Fig. 4). Whether the mode of nuclear return or the

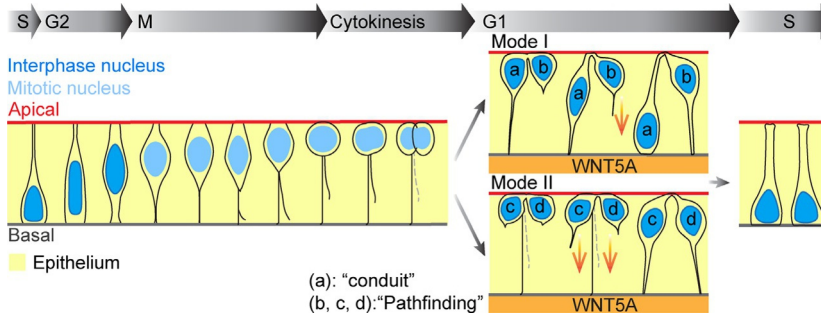

Fig. 4 The choreography of IKNM-associated cell division in Phase I small intestinal epithelium. As the nucleus migrates apically for mitosis, the cell maintains a basal connection *via* a thin filament, termed the basal process. This process splits into two, but only one of the two remains intact and is inherited by one daughter cell, leaving the other daughter disconnected basally. During G1, daughter pairs return their nuclei to the basal side in two distinct modes. In Mode I, the daughter with the basal process uses it as a "conduit" for a quick nuclear return; return of the other daughter's nucleus is slower as this cell must generate a filopodium to build a new pathway to return its nucleus—the "pathfinding" strategy. In Mode II, both daughters utilize "pathfinding" and return their nuclei basally at a similar pace. Pathfinding sometimes fails in the absence of the mesenchymal cue, WNT5A. The two daughters remain apically connected throughout mitosis. Finally, two daughters separate from each other (they are not side-by-side in the epithelium) and prepare for the next round of division. *Adapted from Fig. 7L from Wang, S., Cebrian, C., Schnell, S., & Gumucio, D. L. (2018). Radial WNT5A-guided post-mitotic filopodial pathfinding is critical for midgut tube elongation.* Developmental Cell*, 46, 173–188.e3.*

asymmetric inheritance of the basal process has any functional consequence, with respect to stem cell potential, is currently unknown.

The critical importance of the "pathfinding" strategy for rapid Phase I elongation is revealed by analysis of cell behavior in *Wnt5a* knockout mice (Wang et al., 2018) which have severely shortened intestines (Cervantes et al., 2009). During Phase I growth, WNT5A is expressed by the mesenchymal cells that underlie and surround the intestinal epithelium. In its absence, filopodial pathfinding is impaired. Though daughter cells that retain a basal process can still return their nuclei by the conduit strategy, "pathfinding" daughter cells are impaired in their ability to build a new filopodial path. Their nuclei often remain at the apical side for prolonged periods, and eventually, some of these cells undergo apoptosis. The continual loss of approximately 10% of epithelial cells during this phase is sufficient to explain the intestinal length deficit seen in these mice (Wang et al., 2018). Of note, without WNT5A, some "pathfinding" daughters do manage to

return basally, indicating the likely presence of other guidance cues, which could also play critical roles for Phase I midgut elongation.

ROR2 is a known WNT5A receptor and is expressed in both epithelial and mesenchymal cells in the developing intestine. Loss of *Ror2* also results in a shortened intestine, but to a lesser extent (by E13.5, $Wnt5a^{-/-}$ small intestines are only 28% of the wild-type length; $Ror2^{-/-}$ small intestines are about 63% of wild-type length) (Cervantes et al., 2009; Yamada et al., 2010). It is possible that ROR2 acts as a WNT5A sensor for "pathfinding" daughter cells to sense the gradient of WNT5A and extend filopodia toward to the basal surface. Also of note, mutations in FLNA (Filamin A), an actin-binding protein that acts downstream of WNT5A/ROR2 in filopodia formation (Nishita et al., 2006), have been identified in patients with congenital short bowel syndrome (van der Werf et al., 2013). A next important goal is to further probe the potential involvement of a WNT5A-ROR2-FLNA pathway in intestinal elongation, which might shed a light on the pathogenesis of congenital short bowel syndrome.

Several other genes and signaling pathways have also been reported to affect intestinal lengthening during Phase I, including GATA4 (Kohlnhofer et al., 2016), Hedgehog (Hh) (Mao, Kim, Rajurkar, Shivdasani, & Mcmahon, 2010), and FGF9 (Geske, Zhang, Patel, Ornitz, & Stappenbeck, 2008). GATA4 is an epithelially-expressed transcription factor required for epithelial cell proliferation, acting in part by regulating transcription of cell cycle mediators. Eliminating GATA4 from the midgut endoderm reduces intestinal length to 84% of control length by E14.5 (Kohlnhofer et al., 2016). In addition, perturbing the growth of the underlying mesenchyme can also hinder intestinal elongation, though in this case, it is hard to know whether simply reducing the amount of mesenchyme, or the perturbed signaling that necessarily results, is the cause (or both). Elimination of Shh and Ihh from the early intestinal endoderm (factors that signal from the epithelium to the underlying mesenchyme, in part to induce its proliferation) reduces intestinal length to approximately 10% of controls by E12.5 (Kim, Kim, Mao, Rowan, & Shivdasani, 2011; Mao et al., 2010; Ramalho-Santos, Melton, & McMahon, 2000). Similarly, loss of *Fgf9* limits mesenchymal fibroblast proliferation and reduces small intestinal length to 87% of its normal length by E14.5 (Geske et al., 2008).

Midgut elongation continues after villus initiation, but the underlying mechanisms are less well understood. Dissection of Phase II elongation is more complicated because the epithelium is no longer a set of relatively homogeneous proliferating pseudostratified cells. Cells at the tips of emerging villi become shorter and wider and cease cycling; thus, proliferative cells

are confined to intervillus regions. It is not clear how these changes in cell shape and proliferative patterns affect lengthening. The programs that regulate proliferation also differ in the two phases. Canonical Wnt signals are required for epithelial proliferation in Phase II, but not in Phase I, when canonical Wnt activity is suppressed by ID2 expression (Chin et al., 2016; Korinek et al., 1998; Nigmatullina et al., 2017). Indeed, loss of epithelial β-catenin does not affect lengthening during Phase I but reduces the intestinal length to about 83% of control length between E14.5 and E15.5, the beginning of Phase II (Chin et al., 2016). Further investigation is needed to dissect what additional factors may influence Phase II elongation.

2. Patterning and regionalization

2.1 The pylorus: The anterior intestinal boundary

Embryonically, the pyloric region is an amazing hub of organogenesis, since epithelial and mesenchymal cells of this region give rise to stomach, intestine, spleen, pancreas, liver and gall bladder; complex signaling crosstalk among all of these organs is critical for development of this region (reviewed in Udager, Prakash, & Gumucio, 2010). In the adult epithelium, the pyloric boundary between stomach and intestine is particularly sharp; stomach cells sit directly adjacent to intestinal cells. Establishment of this dramatic tissue-specific border occurs progressively during development.

As discussed above, by E9.5, a distinct SOX2/CDX2 boundary can be seen at the pyloric border by immunofluorescence, marking the boundary between the future gastric and intestinal domains (Sherwood et al., 2009). However, while this boundary appears sharp when whole mount tissue is examined, stained transverse sections of the pyloric border region, examined at higher resolution, reveal a somewhat diffuse boundary at the cellular level (Li et al., 2009). At E14.5, SOX2 expression is high in stomach and a gradient of expression fades off into intestinal territory; similarly, CDX2 is expressed robustly in the intestine and its expression declines a short distance into the stomach. Expression of the intestine-specific structural protein, VILLIN, shows a similar "fuzzy" border at E14.5, but at E16.5, the intestine-stomach boundary is precisely demarcated for both VILLIN and CDX2 (SOX2 expression recedes to the forestomach) (Li et al., 2009). Several additional intestine-specific transcription factors (*Hnf4γ*, *Creb3l3* and *Tcfec*) also exhibit sharp anterior expression borders in the E16.5 pyloric epithelium (Li et al., 2009). Thus, it appears that the formation of a very precise anterior

intestinal boundary occurs rather late in development, as the intestine begins to elaborate its characteristic finger-like villi.

Interestingly, at E14.5, transcriptomic analysis of developing gastric and intestinal epithelium and mesenchyme reveals only a handful of differences in gene expression (Li et al., 2009). These primarily represent clear regional patterning of *Sox2* and *Cdx2*, as well as several Hox and Parahox genes that are known to be regionally expressed. However, at E16.5, hundreds of intestinal genes are coordinately upregulated in the intestine and the vast majority of these are expressed in the epithelial, not the mesenchymal compartment. Gene ontology analysis indicates that the protein products of these genes have roles in metabolism and absorption, critical intestinal functions. Therefore, this transcriptional burst, which occurs concomitantly with villus development, appears to represent the acquisition of intestinal character or "intestinalization" (Li et al., 2009).

2.2 Signaling crosstalk: Endoderm/mesoderm interactions in gut tube patterning

Elegant tissue recombination studies performed over several decades by Dr. Nicole LeDouarin and colleagues produced solid evidence for the critical role of signaling crosstalk between the endoderm and its underlying mesoderm in patterning the intestinal domain. In practice, these experiments involve enzymatic separation of epithelium (prior to villus emergence) from mesenchyme and recombination with heterologous tissues from different regions of the GI tract or even with tissues from heterologous species (e.g., chick-quail). Grafts were derived from mouse, rat or chick intestine and were incubated in a vascular-rich environment such as under the skin of immunodeficient mice, under the rat kidney capsule, on the chick chorioallantoic membrane, or *ex vivo* in an appropriate culture system. The literature derived from these studies is extensive and thought-provoking. An excellent review interweaves the early literature with some of the more recent data (Grapin-Botton, 2005). Here, a few specific findings from these grafting experiments are highlighted.

First, pre-villus chick or rat endoderm cultured *in vitro* without underlying mesoderm will develop into a columnar epithelium with a well-polarized intestine-like apical surface that is studded with microvilli and express some intestine-specific enzyme markers (reviewed in Mizuno & Yasugi, 1990). While this suggests the presence of at least some "intestinal identity" of the endoderm, villi do not form in these cultures. In contrast, when endodermal pieces are recombined with isolated mesenchyme, villi are observed

(Duluc, Freund, Leberquier, & Kedinger, 1994). In fact, mesenchyme of many varieties will do. Even grafts composed of E14 rat intestinal endoderm and skin fibroblasts will form villi and express intestinal differentiation markers (Kedinger et al., 1998). However, the regional source of the endoderm exerts some dominance in the outcome. For example, when E14 rat endoderm from the proximal jejunum is combined with rat mesenchyme isolated from the distal ileum (or *vice versa*), the recombinants give rise to villus structures that express enzymes appropriate to the endodermal rather than the mesodermal portion of the graft (Duluc et al., 1994). Thus, while mesenchymal signals are critical for villus morphogenesis, the intestinal endoderm responds molecularly to mesenchymal signals by obeying a region-specific pre-pattern that is at least partially set before villi arise.

Second, small intestinal mesoderm has a powerful ability to instruct endoderm derived from other embryonic organ domains to become intestinal. When mesenchyme from pre-villus chick small intestine is combined with 5-day endoderm from esophagus, proventriculus, gizzard or colon, the grafts always take on an intestinal morphology (Duluc et al., 1994; Gumpel-Pinot, Yasugi, & Mizuno, 1978). In rodents, rat intestinal mesenchyme can instruct rat colon endoderm (but not lung or stomach endoderm) to form villi (Duluc et al., 1994). However, potentially due in part to its pre-patterned state, small intestinal endoderm tends to be rather resistant to heterologous instructions provided by mesoderm from other regions of the GI tract (Gumpel-Pinot et al., 1978).

Third, much of the mesenchymal instructional information appears to lie in a sub-epithelial fibroblast population. When 5.5-day chick gizzard endoderm (or E14 rat intestinal endoderm) is wrapped by a confluent sheet of pericryptal fibroblasts isolated from the intestine of postnatal rats and grafted into the chick coelomic cavity, the endoderm develops villi and expresses intestinal enzymes (Fritsch, Simon-Assmann, Kedinger, & Evans, 1997; Haffen, Lacroix, Kedinger, & Simon-Assmann, 1983). Some grafts also exhibited surrounding smooth muscle layers and express biochemical markers of intestine (Fritsch et al., 1997). Strikingly, a rat intestinal cell line, IEC-17, can be substituted for the endoderm; when wrapped with E14 rat intestinal mesenchyme and grafted under the rat kidney capsule, 10% of the grafts formed villi and exhibited an epithelium with typical intestinal cell types and enzymatic signatures of the intestine, with a well-developed surrounding muscular layer (Kedinger et al., 1986).

Finally, distinct molecular determinants seem to underlie structural morphogenesis and cytodifferentiation. For example, in grafts of E14 mouse

lung endoderm with mouse intestinal mesenchyme, villi form successfully, but the epithelial component expresses lung-specific surfactants instead of intestinal enzymes (Duluc et al., 1994). Nevertheless, the degree of morphogenic re-organization of both endoderm (villus formation) and mesoderm (intestinal muscle formation) in these recombination studies provides substantial evidence for bi-directional crosstalk between the two tissue layers. Indeed, the very fact that intestinal mesenchyme can instruct lung endoderm to generate villi (Duluc et al., 1994) supports a back and forth molecular cascade that is self-organizing at the local (villus) level. Newer molecular data that support this hypothesis are discussed below.

2.3 Regionalization within the small intestine: Duodenum, jejunum, and ileum

While a major job of the small intestine is absorption and metabolism, different parts of the intestine are specialized for distinct purposes. The duodenum secretes a variety of enzymes which, in association with pancreatic and bile secretions, serve to break down food so that nutrients can be absorbed by the jejunum, while the ileum specializes in vitamin B_{12} and bile acid absorption. The complete transcriptional network that controls these differences in gene expression has not been fully elucidated, but some important findings have emerged.

First, both the chick and the mouse have a well-organized Hox code throughout the gut, with overlapping domains from anterior to posterior (Sakiyama, Yokouchi, & Kuroiwa, 2001; Sekimoto et al., 1998). Most of these Hox genes are mesenchymally expressed in the intestinal domain, though a handful (e.g., *Hoxd13*) are epithelial. In general, while loss of some of these regionally-restricted Hox genes affects the development of the domain in which that gene is expressed, homeotic transformations of regional fates are not seen (Beck, Tata, & Chawengsaksophak, 2000; Boulet & Capecchi, 1996; Zacchetti, Duboule, & Zakany, 2007). Interestingly, the positions of the anterior borders of expression of several Hox genes seem to correspond to anatomical sphincters or distinctive regions in the gut and there is functional evidence that Hox genes play a role in the morphogenesis of the pyloric, ileocecal and anal sphincters (Kondo, Dolle, Zakany, & Duboule, 1996; Zakany & Duboule, 1999).

The dynamic expression pattern of the caudal transcription factor, CDX2, during establishment of the gut anlage is discussed above. Once the gut tube is completed, CDX2 marks the stomach-intestine border, but its expression in the intestine is graded, with highest levels in the

posterior ileum and colon (Silberg, Swain, Suh, & Traber, 2000). Indeed, loss of CDX2 early in development results in emergence of a squamous esophageal-like epithelium in the ileum and colon (Gao, White, & Kaestner, 2009). In *Cdx2* mutant tissue, a large number of intestinal genes are downregulated, while genes normally expressed in esophagus are upregulated, indicative of an anterior transformation. Interestingly, only subtle transient changes in the intestinal Hox code are noted in *Cdx2* mutants, reinforcing the idea that the Hox code may not be a primary determinant of regional patterning in the gut (Gao et al., 2009). Additionally, only minor morphological and transcriptional changes are seen in the duodenum of *Cdx2* null mice (Gao et al., 2009); thus, *Cdx2* function is not required to maintain intestinal character in the proximal intestine.

One of the most interesting regionalization phenotypes in Cdx2 mutants arises in the proximal colon of adult mice that are heterozygous for this gene. These animals develop polyps in tissue where the untargeted allele of *Cdx2* is ectopically silenced (Beck, Chawengsaksophak, Waring, Playford, & Furness, 1999; Chawengsaksophak, James, Hammond, Kontgen, & Beck, 1997). In the center of the polyp, the colonic epithelium takes on squamous, esophageal character. Amazingly, directly surrounding these lesions, a regular progression of regional gut identities can be seen, with concentric rings of tissue exhibiting gastric, followed by intestinal morphologies and gene expression patterns. Since analysis of *Cdx2* mutant ileum revealed dramatic changes in both Wnt and Hedgehog signaling (Gao et al., 2009), it will be interesting to determine whether such changes contribute to these interesting patterns.

Regionalization of the proximal duodenum is highly dependent upon proper expression of the Parahox factor, PDX1. *Pdx1* expression extends from the antral stomach to the mid-duodenum and includes the region of the developing pancreas and bile duct (Offield et al., 1996). In mutants lacking *Pdx1*, pancreatic buds form, but fail to grow out, and islets and acini are not established (Offield et al., 1996). In the proximal duodenum, villi do not form; instead, the surface epithelium is composed of cuboidal cells that resemble (morphologically and molecularly) bile duct epithelium. Finally, Brunner's glands, the submucosal glands characteristic of the duodenum, are reduced and structurally abnormal.

Regional patterning of the jejunum and ileum requires activity of GATA family transcription factors. GATA4 is expressed in the duodenum and jejunum, but absent from the ileum, while GATA6 is present throughout the intestine (Battle et al., 2008; Beuling et al., 2012; Bosse et al., 2006;

Fang, Olds, & Sibley, 2006). GATA4 is both necessary and sufficient to determine jejunal fate, since its loss results in expansion of ileal genes into the jejunal territory (Kohlnhofer et al., 2016; Walker, Thompson, Kohlnhofer, Faber, & Battle, 2014), whereas ectopic expression of GATA4 in the ileum (where it is normally absent) leads to activation of jejunal genes in the ileal domain (Thompson et al., 2017). On the other hand, *Gata6* loss has no effect on jejunal fate, but when this factor is deleted from the ileum, colonic markers are expressed in this domain (Beuling et al., 2011; Walker et al., 2014), consistent with an important role for GATA6 in suppressing colonic fate in the ileum.

3. Villus development

3.1 Signals and physical forces driving formation of the villi

Villus morphogenesis requires synchronized development of the epithelium and mesenchyme. This coordination is achieved by secreted signaling molecules as well as physical forces generated by tissue expansion and cell shape changes. At the top of the signaling hierarchy is the Hh signaling pathway which plays critical roles in multiple aspects of intestinal development in sea urchins, cnidarians, *Drosophila*, and vertebrates (Hoch & Pankratz, 1996; Matus, Magie, Pang, Martindale, & Thomsen, 2008; Sukegawa et al., 2000; Walton, Warner, Hertzler, & Mcclay, 2009). Loss of Hh signaling results in a plethora of intestinal malformations including duodenal stenosis, abnormal innervation, loss of smooth muscle, loss of villi, malrotation, reduced length, altered radial patterning and imperforate anus (Madison et al., 2005; Mo et al., 2001; Ramalho-Santos et al., 2000; Sukegawa et al., 2000).

In the small intestine, Hh signaling is paracrine. Shh and Ihh are produced and secreted from the nascent intestinal epithelium, even before gut tube closure (Bitgood & McMahon, 1995; Echelard et al., 1993). Hh signals are received by a variety of mesenchymal cell types including serosa, smooth muscle, nerves, endothelial cells, sub-epithelial mesenchymal cells, fibroblasts, and myofibroblasts (Kolterud et al., 2009). Constitutive activation of Hh signaling results in a massive expansion of the mesenchyme (Mao et al., 2010) and a large increase in smooth muscle differentiation (Zacharias et al., 2011), while conditional loss of embryonic Hh signaling, beginning at E10.5, results in reduced mesenchymal proliferation (Mao et al., 2010), especially of myofibroblasts and smooth muscle (Kosinski et al., 2010;

Madison et al., 2005; van Dop et al., 2009; Zacharias et al., 2011). Though the epithelium does not receive Hh signals, reduced Hh signaling causes spatial disruption of epithelial proliferative zones (normally restricted to intervillus regions during fetal stages). This indirect effect appears to be due to alterations in the signaling network downstream of Hh, including reduced Bmp signaling and increased Wnt signaling (Kosinski et al., 2010; Madison et al., 2005). Interestingly, a network of Hh responsive, FoxL1-positive subepithelial telocytes have recently been shown to secrete Wnt ligands and define the epithelial stem cell niche in adults (Aoki et al., 2016; Degirmenci, Valenta, Dimitrieva, Hausmann, & Basler, 2018; Shoshkes-Carmel et al., 2018). These cells may be, at least in part, the mesenchymal cells responsible for these epithelial alterations. It will be important to determine when these telocytes arise during development.

In mammals, villi emerge as domes from a flat epithelial tube and grow directly into finger-like projections (Fig. 5) (Walton et al., 2018). Villus emergence begins around E14.5 in mice when tight aggregations of Hh responsive mesenchymal cells aggregate into "clusters" (Karlsson, Lindahl, Heath, & Betsholtz, 2000; Walton et al., 2012). Such clusters were first noted in early electron microscopic studies of villus development (Mathan, Moxey, & Trier, 1976) and later shown to be hubs of PDGF signaling (Karlsson et al., 2000). Cluster cells express PDGFRα, while the epithelial cells above them express PDGFA ligand. Loss of either the receptor or the ligand does not affect the formation of the initial clusters but does suppress the proliferative activity of PDGFRα-positive mesenchymal cells so that formation of subsequent clusters is inhibited, leading to sparsely spaced and aberrantly shaped villi (Karlsson et al., 2000).

Since PDGF signaling does not affect cluster initiation, other signals are clearly necessary. Analysis of Hh signal transduction prior to villus emergence revealed that scattered sub-epithelial mesenchymal cells express Hh target genes (Walton et al., 2012). At E14.5, in the most proximal intestine, these mesenchymal cells begin to aggregate to form clusters next to the basement membrane of the epithelium. Hh signaling is critical for cluster formation since inhibition of this pathway abolishes cluster formation, while Hh pathway augmentation increases cluster size (Madison et al., 2005; Walton et al., 2012). Furthermore, cluster formation is required for villus emergence (Walton, Whidden, et al., 2016). Downstream from Hh, Bmp signals sent by and received by cells within the clusters themselves regulate cluster size and spacing through a self-organizing Turing field mechanism. Progressively reducing the level of Bmp signaling changes

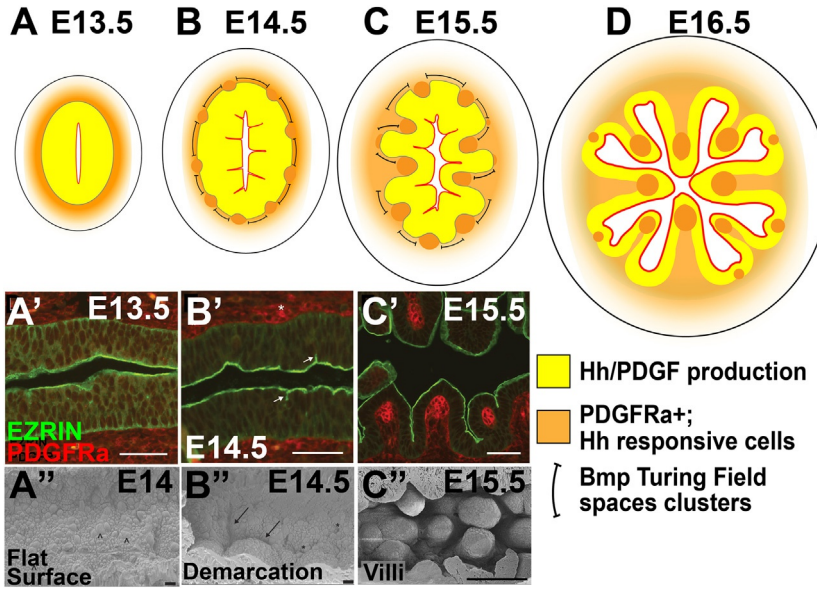

Fig. 5 Villus formation. (A) At E13.5, the intestinal epithelium (yellow) is a tube with a flat lumenal surface that uniformly secretes Hh and PDGF signals (yellow) to subepithelial mesenchymal cells (orange). (A′) The flat apical surface is highlighted by EZRIN staining (green) and the even distribution of PDGF responsive cells is noted by PDGFRa staining (red). (A″) SEM image showing the flat lumenal surface at E13.5. (B) By E14.5 in the anterior duodenum, Hh/PDGF responsive mesenchymal cells begin to aggregate to form clusters (orange dots) in a regularly spaced field; spacing is determined by a Bmp-dependent Turing field, in which formation of one cluster prevents the initiation of another cluster in the immediate area. (B′) Mesenchymal clusters result in uneven compression in the epithelium, leading to membrane invaginations that demarcate villi, here shown as T-shaped extensions of the apical surface (arrows). Apical demarcations (arrows) form between mesenchymal clusters. (B″) SEM reveals demarcations of initial villi at the apical surface (arrows). (C and C′) Villi emerge at the site of each cluster; clusters remain associated with tips of emerging villi. (C″) Villus domes are obvious in SEM. (D) By E16.5, villi are longer and additional rounds of cluster formation occur in intervillus regions; villi are present throughout the small intestine. *Panels (A′-C″): Reproduced from Freddo, A. M., Shoffner, S. K., Shao, Y., Taniguchi, K., Grosse, A. S., Guysinger, M. N., et al. (2016). Coordination of signaling and tissue mechanics during morphogenesis of murine intestinal villi: A role for mitotic cell rounding.* Integrative Biology: Quantitative Biosciences from Nano to Macro*, 8, 918–928 with permission from The Royal Society of Chemistry.*

the pattern of discrete clusters (spots) into a continuous line of connected clusters (stripes) (Walton, Whidden, et al., 2016).

Hh-responsive mesenchymal clusters are signaling centers that express a myriad of soluble factors, including BMP ligands and modifiers

(BMP2,4,5,7, BMP1, TSG1, Noggin, FLSTL1) (Walton, Whidden, et al., 2016) as well as WNT5A (Wang et al., 2018). In addition to serving as patterning signals for the clusters themselves, these molecules feed back to the overlying epithelium, causing cells directly above the clusters to change shape and withdraw from the cell cycle. At this time, it is not known which signal or combination of signals produces these effects. Nevertheless, epithelial cells above the clusters, initially tall and pseudostratified, shorten and widen (without changing volume), becoming more columnar in shape (Freddo et al., 2016; Walton, Whidden, et al., 2016). During initial epithelial cell shortening, the apical surface remains flat and the cluster, which is adhered to the basement membrane, is drawn up into a shallow alcove in the epithelium (Fig. 5C). The focal widening of epithelial cells over clusters that accompanies cell shortening appears to place pressure on surrounding epithelial cells located in regions between clusters (Freddo et al., 2016). Within these regions, specialized cell divisions cause formation of rapid, deep membrane invaginations (Fig. 5B and B′), effectively carving out the villus domains and allowing the first villus "bumps" to emerge (Fig. 5B″) (Freddo et al., 2016). A similar mechanism of rapid membrane invagination, triggered by dividing cells, is also seen during *Drosophila* tracheal invagination (Kondo & Hayashi, 2013). In both cases, intraepithelial physical forces act on a tall, pseudostratified epithelium to fold it quickly (in the scale of minutes) in a patterned manner.

As villi emerge, cell proliferation at the tips is suppressed, perhaps due to BMP signals from the underlying clusters, which remain closely associated with the tips of emerging villi. Thus, proliferation becomes restricted to the intervillus regions. Additional rounds of villus formation occur, with new clusters forming beneath the proliferative, expanding intervillus epithelium; in the mouse, four rounds of cluster-driven villus formation are detected between E14.5 and E18.5 (Walton et al., 2012).

Curiously, the mechanisms of villus formation are quite different in the chick, though similar signaling molecules are used. In the mouse, the signaling cascade downstream of Hh-driven cluster formation provides the patterning cue that sets the stage for villus demarcation (Freddo et al., 2016; Walton et al., 2012; Walton, Whidden, et al., 2016). In contrast, the chick uses physical forces for patterning, driven by the sequential formation of smooth muscle layers in the deep mesenchyme, which confine the growing epithelium (Shyer, Huycke, Lee, Mahadevan, & Tabin, 2015; Shyer et al., 2013). A step-wise progression of epithelial bending results, which starts with length-wise ridges caused by formation of the circular muscle and

progresses to compression of the ridges into zig-zags as the longitudinal muscle forms. The deep zig-zag folds are proposed to "trap" epithelial Hh ligands, resulting in the formation of clusters at the most compressed points (Shyer et al., 2015, 2013). These clusters express BMP ligands which serve to suppress WNT signaling in the epithelium overlying the clusters, simultaneously restricting WNT signals to the intervillus regions.

This coordination of muscle layer development with epithelial bending observed in the developing chick intestine is not observed in mouse or humans (Walton, Whidden, et al., 2016), though physical confinement and coordinated contractions may be important for villus outgrowth. Also, in the mouse, the pseudostratified epithelium does not transduce WNT signals prior to cluster-induced villus emergence. Rather, epithelial WNT signaling is activated in the intervillus regions as villi emerge (Chin et al., 2016; Nigmatullina et al., 2017). The signaling program that drives cell proliferation in the early pseudostratified mouse epithelium is unknown.

3.2 Mesenchymal factors controlling villus development

Several mesenchymally-expressed transcription factors have been shown to play a role in villus development. Genetic deletion of the homeodomain transcription factor *Nkx2–3* (a Hh target) results in reduced gut mesenchyme, impaired intervillus localization of epithelial proliferation and delayed villus formation, specifically in the jejunum (Pabst, Zweigerdt, & Arnold, 1999). The few villi that do form in these mutants are short and misshapen. Interestingly, at adult stages, proliferation is increased and both villus and crypt morphology are greatly disturbed; villi are abnormally long and branched, with increased villus core vasculature, while crypts extend deeper and are disorganized with increased fissioning. Mice mutant for *FoxL1/Fkh-6*, another direct target of Hh signaling (Madison, McKenna, Dolson, Epstein, & Kaestner, 2009), display a phenotype very similar to that of *Nkx 2–3* mutants (Kaestner, Silberg, Traber, & Schütz, 1997; Katz et al., 2004). Yet, *Nkx 2–3* mutants have normal levels of *FoxL1* expression and *FoxL1* mutants have normal levels of *Nkx 2–3* (Pabst et al., 1999). In both cases, no changes in *Shh* or *Ihh* expression are detectable, but marked downregulation of *Bmp2* and *Bmp4* is seen in the jejunum where villus defects are noted. Loss of *FoxF* genes ($FoxF2^{-/-}$ or the double heterozygote, $FoxF1^{+/-}$; $FoxF2^{+/-}$) also exhibits reduced Bmp4 expression, perturbed patterning of proliferative regions and ectopic WNT signaling along the villi (Ormestad et al., 2006). These findings underline the critically important

role of mesenchymal cells in the proper patterning and emergence of villi. Of note, all of these mesenchymal factors (*Nkx2–3, FoxF* and *FoxL*) are Hh target genes. Similar phenotypes are also observed in mice transgenically expressing low levels of a secreted form of Hhip, an inhibitor of Hh signaling (Madison et al., 2005). Thus, all of these transcription factors may function together downstream of epithelial Hh signals to control proper villus morphogenesis.

Epimorphin/Syntaxin2 is highly expressed in the sub-epithelial mesenchyme during villus morphogenesis. Epimorphin is a t-SNARE protein which is involved in the targeting and fusion of intracellular transport vesicles for exocytosis in neural and polarized epithelial cells. Thus, *Epimorphin* might play a role in regulating secretion of cluster morphogens (such as Bmps or Bmp antagonists). In support of this notion, increased expression of *Epimorphin* results in increased *Bmp4* expression (Fritsch et al., 2002) and genetic deletion of *Epimorphin* leads to decreased *Bmp4* expression (Wang et al., 2006). In addition, Bmps are known to inhibit Wnt signaling in intestinal crypts (He et al., 2004) and consistent with a loss of Bmp expression, expansion of epithelial Wnt signal transduction (increased nuclear β-catenin) is noted in *Epimorphin* null mice; villi are longer, crypts are deeper and increased crypt fissioning is observed (Wang et al., 2006). Co-culture of rat intestinal endoderm with myofibroblasts transfected with *Epimorphin* engrafted into the chick coelomic cavity results in ectopic formation of villi. This is Epimorphin dependent, since inhibition of Epimorphin expression in the myofibroblasts prevents villus morphogenesis. Finally, treatment of these co-cultures with the Bmp antagonist, Noggin, partially blocks the ability of Epimorphin to induce villus emergence, suggesting that a Bmp signaling feed-back loop may be at least partially responsible for the effects of Epimorphin on the epithelium (Fritsch et al., 2002).

3.3 Epithelial factors important in villus emergence

The initiation of villus emergence entails both morphological change and local cell differentiation. Morphologically, the epithelium is initially composed of tall, thin, uniformly proliferative pseudostratified cells. But as villi form, the epithelium directly above mesenchymal clusters transitions to a shorter, wider columnar shape. This localized shape change simultaneously decreases the stiffness of the epithelial layer and increases its area (the wider cells take up more space), resulting in a pattern of epithelial buckling and villus emergence over each cluster. Simultaneously, these same cells cease

to divide and begin to differentiate, while epithelial cells located between the clusters remain pseudostratified and highly proliferative.

In addition to Shh and Ihh secreted by the epithelium, multiple epithelial transcription factors have been shown to contribute to these events of epithelial differentiation, villus morphogenesis and homeostasis (Chin, Hill, Aurora, & Spence, 2017). However, the role of the KLF5-FOXA1-ELF3 transcriptional networks is particularly noteworthy, since it appears to control the transition from pseudostratified epithelium to villus emergence. *Klf5* (Kruppel-like factor 5) is expressed in the embryonic endoderm that gives rise to the intestinal epithelium and expression continues during and after villi emergence (Dong et al., 2009; McConnell et al., 2011). *Foxa1* is active in the pseudostratified epithelium, but suppressed at E14.5 when villi form (Bell et al., 2013), while *Elf3* (ETS-related Transcription Factor 3) is activated at E14.5 (Ng et al., 2002). Deletion of epithelial *Klf5* expression prior to villus morphogenesis results in decreased intestinal length, poor epithelial differentiation and dramatically impaired villus morphogenesis (Bell et al., 2013). Loss of *Elf3* strongly mirrors this *Klf5* phenotype (Ng et al., 2002). Importantly, in Klf5 mutants, expression of *FoxA1* is expanded and expression of *Elf3* is suppressed. *FoxA1* is a repressor of epithelial differentiation, while *Elf3* is an important driver of differentiation. Bell et al. determined that KLF5 is required to suppress *FoxA1* activity at E14.5, as villus formation initiates. In its absence, *FoxA1*, a repressor of *Elf3*, is overexpressed and this impedes villus formation and epithelial differentiation (Bell et al., 2013). This leaves one important conundrum: since KLF5 is present before and after villus formation, while FOXA1 is only suppressed at the point of villus formation and not before, what temporal trigger limits the repressive effect of KLF5 on FOXA1 to E14.5 and after?

Finally, it is important to consider the role that the basement membrane likely plays in villus development. Basement membranes in tissues help maintain tissue integrity and compartmentalization as well as promote differentiation and provide a scaffold for cell migration. These roles may be conveyed through physical forces or through cell signaling. Indeed, as villi form, mesenchymal clusters interact closely with the overlying basement membrane (Walton et al., 2012). Expression of several laminin isoforms is tightly controlled and developmentally regulated (Lefebvre, Sorokin, Kedinger, & Simon-Assmann, 1999; Miner, 1998; Perreault, Vachon, & Beaulieu, 1995; Simon-Assmann et al., 1998). Loss of laminin a5 from the intestine results in an increased production of laminin a1 and a4, laminins typically expressed at high levels in the colon. This switch in regional laminins

is accompanied by structural and fate alterations of the epithelium to resemble colon rather than small intestinal villi. Whether this effect is the result of structural differences, changes in cell signaling, or changes in localization of sub-epithelial mesenchymal cell types that affect positional information is unclear.

4. Making crypts

Crypts are flask-like indentations of the epithelium into the underlying mesenchyme which serve as critical architectural units for housing the stem cells that ensure the continuous renewal of epithelial cells. In both mouse and human, crypts form in the intervillus regions after villi have formed. Human crypts are established early in gestation (weeks 11–12) (Trier & Moxey, 1979), while in the mouse, crypts only begin to form in the first week after birth (Dehmer et al., 2011). After observing a relatively constant distance between the bottom of intervillus regions/crypts and the smooth muscle layer at E17, post-natal day 3 (P3) and P8, Calvert et al. proposed that crypt formation occurs by "zipping up" the villus base, from bottom to top, driven by mesenchymal cell movements. This hypothesis held until Sumigray et al. carefully characterized the formation of nascent crypt structures, using 3D confocal imaging coupled with transcriptomic analysis to reveal and describe a dynamic process of crypt invagination (Sumigray, Terwilliger, & Lechler, 2018).

Sumigray et al. showed that CD44v6 is robustly expressed in crypt progenitor units throughout the formation of crypts; thus, they used CD44v6 to trace the structure of developing crypts step by step (Fig. 6A-D). With this marker, they sorted developing crypt cells from villus cells between P0 and P10 and performed RNA-seq to identify key regulators of crypt morphogenesis. They observed that, at P0, the CD44v6 positive compartment is a flat sheet of intervillus cells. Interestingly, CD44v6 expression becomes restricted to a circular pattern within the intervillus area, even before crypt invagination begins, marking the nascent crypt region (Fig. 6A, inset). Invagination of this compartment generates a cup-like structure (Fig. 6B and F). By analysis of their transcriptomic datasets, the authors found that a large subset of myosin II-associated contractility genes are enriched during crypt initiation and confirmed that Myosin II-driven apical constriction initiates this invagination process. Additionally, the transcriptomic analysis revealed several novel crypt-enriched and villus-enriched markers that will be useful in future analyses.

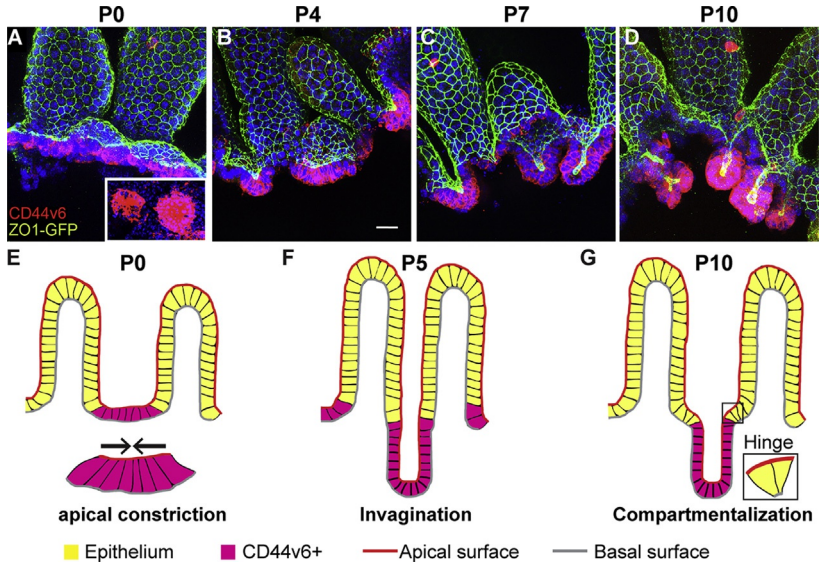

Fig. 6 Crypt development. (A-D) Whole mount staining of CD44v6 (magenta) in epithelium of ZO1-GFP labeled mouse intestines at P0, 4, 7 and 10. (E-G) Schematic summary of crypt morphogenesis. (E) The CD44v6+ compartment starts within the flat sheet of intervillus cells at P0. Myosin II-drives apical constriction of CD44v6 positive cells. (F) Forming crypts invaginate into the underlying mesenchyme. (G) Cells at the bend between the crypt and villus adopt a wedge-like shape—the "hinge" region—which morphologically separates crypts from villi. *Panels (A-D): Reproduced with permission from Sumigray, K. D., Terwilliger, M., & Lechler, T. (2018). Morphogenesis and compartmentalization of the intestinal crypt.* Developmental Cell*, 45, 183–197.e185 (Fig. B). Panels (E-G): Adapted with permission from the graphical abstract in Sumigray, K. D., Terwilliger, M., & Lechler, T. (2018). Morphogenesis and compartmentalization of the intestinal crypt.* Developmental Cell*, 45, 183–197.e185.*

By P3, the authors noted a clear bend between the emerging crypts and nearby villi (Fig. 6B and F). By P7, this defined "hinge" is formed from wedge-shaped cells that are expanded apically and constricted basally (Fig. 6C, D, and G). Genetic ablation of Rac1 impairs hinge formation and results in disorganized villus spacing. In part, Rac1 appears to function by downregulating α6/β4 integrins; these integrins make up hemidesmosomes, which attach these cells to the underlying basement membrane, perhaps so that the necessary basal constriction can be accomplished. In the future, it will be interesting to know how the CD44v6 population is first restricted to initiate crypt formation and what role the interactions between the epithelium and mesenchyme play in crypt formation, patterning, and maintenance.

5. Organoid systems to study intestinal development

The term "intestinal organoid" is a generic term for engineered intestinal tissues (epithelium only or epithelium with combinations of supporting mesenchyme) that are grown in a 3D matrix (Matrigel or various versions of hydrogels) and provided with essential factors to support intestinal stem cells and epithelial cell progeny. The first iterations of organoid culture were started from epithelial cells isolated from primary small intestinal tissue (Barker et al., 2010; Ootani et al., 2009; Sato et al., 2009); however, organoids can now be started from hESCs or iPSCs and driven toward an endodermal fate using specifically-timed treatments with combinations of growth factors (Spence et al., 2011). Many different forms/variations of engineered intestinal tissues using different starting material and different culture conditions have been developed. To further clarify these variations, a standard of nomenclature has been proposed by the National Institutes of Health Intestinal Stem Cell Consortium (Stelzner et al., 2012). "Enteroids" refer to multi-lobulated structures that develop from enterospheres, a spherical structure that forms from isolated epithelial cells that round off to form cysts, and contain only epithelium while organoids contain both epithelium and mesenchyme.

The ability to engineer 3D intestinal tissues in a dish holds great promise for future treatment of patients with shortened intestines or loss of absorptive surface area. However, currently, while organoids or enteroids readily generate crypt-like structures, finger-like villi only develop after transplantation into an animal host that provides additional growth factors, immune cells, vasculature, and perhaps nerve cells (Watson et al., 2014). Despite this deficit, organoids have been extremely useful for a variety of studies, including examining the role of specific genes in cell fate decisions and intestinal morphogenesis (Carulli et al., 2015; Chin et al., 2016), defining the role of the microbiome in maturation of the absorptive surface (Foulke-Abel et al., 2014; Hill et al., 2017), examining the role of specific cell types in intestinal morphogenesis (Schlieve et al., 2017; Workman et al., 2017), drug screening (Baumann, 2017; Liu et al., 2016), and studying patient specific diseases (VanDussen et al., 2015; Vlachogiannis et al., 2018). Two excellent recent reviews cover molecular and cellular development of intestinal organoids (Dedhia, Bertaux-Skeirik, Zavros, & Spence, 2016; Nakamura & Sato, 2018).

Interestingly, a 3D setting is not required to observe and investigate the organization of the crypt-villus axis. Two dimensional "enteroid monolayers," generated by plating the endodermal portion of isolated crypts

on Matrigel-coated plates, develop dense foci of proliferative cells that express stem cell markers encircled by non-proliferating cells that express markers of differentiated enterocytes, goblet cells and enteroendocrine cells (Thorne et al., 2018). Paneth cells are also present and are found in the center of the dense foci. In such a monolayer setting, ectopic addition of WNT or BMP has the expected effect of increasing or decreasing (respectively) the zone of proliferation. Importantly, the focal organization of cells within the monolayer sets up its own feedback controls: the main source of WNT is the Paneth population at the center of the foci, while the main source of BMP is the surrounding differentiated cells. Indeed, treatment of such cultures by addition of WNT3a expands the proliferative compartment initially, but the subsequent increase in differentiated cells leads to increased BMP production that then feeds back to suppress proliferation. Such feedback can be blocked by LDN, an inhibitor of BMP receptors (Thorne et al., 2018). Since *in vivo* loss of Paneth cells does not perturb the crypt proliferation or maintenance of stem cells (Durand et al., 2012), it is clear that these interepithelial signaling loops, while powerful, are not sufficient for normal homeostasis. Nevertheless, these findings point to a remarkable self-regulating activity of the epithelial cells, which is likely critical in maintaining an organized "crypt-villus" axis.

6. Summary

In 300 BC, Aristotle wrote the following about embryonic development: "*It is possible, then, that A should move B and B should move C, that, in fact, the case should be the same as with the automatic machines shown as curiosities. For the parts of such machines while at rest have a sort of potentiality of movement in them, and when any external force puts the first into motion, immediately the next is moved in actuality… in these automatic machines, the external force moves the parts in a certain sense (not by touching any part at the moment but by having touched one previously).*"

Certainly, establishment and regional patterning of the endodermal gut tube, its dramatic elongation and looping, and decoration of its lumenal surface with villi and then crypts, follows a type of "automatic" sequence suggested by Aristotle, which is rife with cascading inductions. Modifications of the tube anlage occur in a precisely timed temporal sequence, at a distinct regional address. Though we still know little about the temporal triggers, we are learning that the "external force" includes not only physical forces *per se*, derived from tissue growth, constraint, or cell shape change, but

soluble signals expressed by subsets of cells that act in an instructive manner on other cells. Together, these complex and precisely delivered stimuli result in a fully differentiated and functional intestinal tube. While development of the muscular features and enteric nervous system of the gut have not been discussed here in detail, recent studies provide more information about those aspects, which are also essential to adult intestinal function (Cotton et al., 2017; Gurdziel, Vogt, Walton, Schneider, & Gumucio, 2016; Huang et al., 2013; Kosinski et al., 2010; Mao et al., 2010; Nagy & Goldstein, 2017; Zacharias et al., 2011; reviewed in Heanue & Pachnis, 2007; Nagy & Goldstein, 2017; Obermayr, Hotta, Enomoto, & Young, 2013; Sasselli, Pachnis, & Burns, 2012; Uesaka, Young, Pachnis, & Enomoto, 2016). Importantly, secreted signals from endoderm are important for smooth muscle differentiation, while signals from the mesenchyme are believed to guide the influx of enteric nerves.

For the future, it remains clear that one of the most important clinical goals of research in this area is to learn how to produce or regenerate functional intestine that can successfully absorb nutrients in patients with intestinal failure or short bowel syndrome. Answers to several of the intriguing biological questions that are raised in this chapter will help move us closer to that goal by revealing key control factors. Further experimentation with organoid/enteroid systems holds promise for not only dissecting these critical signals but also for assembling functional villus/crypt units *in vitro*.

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