Villification in the mouse: Bmp signals control intestinal villus patterning

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SUMMARY

In the intestine, finger-like villi provide abundant surface area for nutrient absorption. During murine villus development, epithelial Hedgehog signals promote aggregation of sub-epithelial mesenchymal clusters that drive villus emergence. Clusters arise first dorsally and proximally and spread over the entire intestine within 24-hours, but the mechanism driving this pattern in the murine intestine is unknown. In the chick, the driver of cluster pattern is tensile force from developing smooth muscle, which generates deep longitudinal epithelial folds that locally concentrate the Hh signal, promoting localized expression of cluster genes. In contrast, we show that in the mouse, muscle-induced epithelial folding does not occur and artificial deformation of the epithelium does not determine the pattern of clusters or villi. In intestinal explants, modulation of Bmp signaling alters the spatial distribution of clusters and changes the pattern of emerging villi. Increasing Bmp signaling abolishes cluster formation while inhibiting Bmp signaling leads to merged clusters. These dynamic changes in cluster pattern are faithfully simulated by a mathematical model of a Turing field in which an inhibitor of Bmp signaling acts as the Turing activator. In vivo, genetic interruption of Bmp signal reception in either epithelium or mesenchyme reveals that Bmp signaling in Hh-responsive mesenchymal cells controls cluster pattern. Thus, unlike the chick, the murine villus patterning system is independent of muscle-induced epithelial deformation. Rather, a complex cocktail of Bmps and Bmp signal modulators secreted from mesenchymal clusters determines the pattern of villi in a manner that mimics the spread of a self-organizing Turing field.
INTRODUCTION

Effective absorption of nutrients by the small intestine requires an enormous mucosal surface area. One adaptive mechanism for surface area amplification is the convolution of mucosal surface into a regular array of finger-like projections called villi. Villi arise at embryonic day (E)14.5 in mice (week 8-10 in humans), when the intestine undergoes a remarkable morphogenetic process to convert a pseudostratified epithelial tube surrounded by loose mesenchyme into a field of villi with mesenchymal cores, covered by columnar epithelium. Though the histological changes that accompany villus formation have been studied extensively (Dekaney et al., 1997; Dunn, 1967; Lacroix et al., 1984; Mathan et al., 1976; Matsumoto et al., 2002; Nakamura and Komuro, 1983; Sbarbati, 1982; Trahair and Robinson, 1986), the cellular and molecular drivers of this process are still incompletely understood, especially in mammals.

Recent progress has been made in deciphering the mode of villus emergence in the chick intestine. In that model, the initially flat epithelium gives way to longitudinal ridges, which evolve to regular zigzags and finally to villi and these sequential morphological stages correlate with maturation of three smooth muscle layers (Coulombre and Coulombre, 1958; Shyer et al., 2013). The deep and regular folding of the epithelium, imposed by mechanical forces from these developing muscles, creates periodic maxima of epithelially secreted Hh protein beneath the folded epithelium that direct villus emergence in a regular manner (Shyer et al., 2015).

In contrast, in many mammals, including mouse (Sbarbati, 1982), rat (Dunn, 1967; Trahair and Robinson, 1986), sheep (Trahair and Robinson, 1986), pig (Dekaney et al., 1997) and human (Lacroix et al., 1984; Matsumoto et al., 2002), ridges and zigzags never form and in several of these systems (including mouse, as shown herein), villus formation is not temporally coordinated with smooth muscle development (de Castro, 2001; Fekete et al., 1996; Georgieva
and Gerov, 1975; Kedinger et al., 1990; Keibel, 1910). Thus, localization of the Hh signal via smooth muscle-dependent epithelial deformation cannot explain the establishment of a uniform field of villi in many species; additional patterning forces are required.

In the mouse, villi arise as domes over condensed clusters of mesenchymal cells that express *Platelet derived growth factor receptor alpha* (*Pdgfra*) (Karlsson et al., 2000). We previously established that prior to villus formation, scattered mesenchymal cells express *Gli1*, *Ptc1* and *Pdgfra* (Karlsson et al., 2000) (Walton et al., 2012). Hh ligands from the epithelium cause agglutination of these cells into clusters beginning at E14.5 (Walton et al., 2012). In mice overexpressing the pan-Hh inhibitor *Hhip*, clusters and villi fail to form (Madison et al., 2005). Similarly, in cultured intestinal explants, inhibition of Hh signaling by cyclopamine or anti-Hh antibody (5E1) abolishes cluster formation and villus emergence, without altering smooth muscle (Fig.S1A,B,D,E) (Walton et al., 2012). In contrast, increasing the strength of the Hh signal increases cluster size, again without changing smooth muscle (Fig.S1C,F) (Walton et al., 2012), confirming that epithelial Hh controls a signaling cascade that drives mesenchymal cluster formation, independently of alterations in the surrounding smooth muscle layers.

Forming mesenchymal clusters are regularly positioned in the murine intestine, with an average cluster-to-cluster spacing of approximately 60-70 µm (Walton et al., 2012), suggesting that specific signals are required to generate such a well-patterned field. Though Hh signals control cluster agglutination and size, Shh and Ihh ligands are uniformly expressed by the pre-villus epithelium (Kolterud et al., 2009; Shyer et al., 2015); thus, it is unclear how epithelial Hh could establish the regular pattern of clusters, especially in species (e.g., mouse) where there is no epithelial folding to create local Hh maxima. Since Bmp signaling is known to control a variety of patterning fields in other contexts (Hogan, 1996) and mesenchymal clusters in
emerged villi express Bmp2 and Bmp4 (Karlsson et al., 2000), we examined the potential role of this pathway in cluster patterning. Here, we show that newly established clusters express multiple Bmp ligands and Bmp signal modifiers. Moreover, abolishing Bmp signal reception changes cluster pattern, causing rows of mesenchymal cluster “spots” to merge into “stripes”. Merged clusters can also be generated by conditional deletion of Bmpr1a in Hh-responsive cells of the mesenchyme, while epithelial Bmpr1a deletion has no effect. This “spots to stripes” pattern change is fully consistent with the mathematical predictions of a classical reaction-diffusion model, in which the overlapping activity domains of an activator and an inhibitor create a patterned field, as initially described by Turing (Maini, 2004; Meinhardt, 2012; Turing, 1952). In such a model, progressive saturation of the Turing activator (in this case, posited to be a Bmp inhibitor) causes spots to become stripes. Together our data establish that, in the mouse model, Bmp signaling controls cluster distribution. Patterning of clusters and emergence of villi in this model are not dependent upon muscular forces or epithelial deformation, but behave in accord with a Bmp-dependent self-organizing Turing field.
RESULTS

Villus patterning in the mouse is not driven by tensile forces

Villus emergence correlates with smooth muscle development in chick (Coulombre and Coulombre, 1958; Shyer et al., 2013); thus, we examined whether this is also true in mouse. Such an analysis requires that the same region of the intestine (here, jejunum) be compared at all time points since villus emergence propagates from proximal to distal intestine over a 24 hour period (Walton et al., 2012). At E13.0, 48 hours before villus morphogenesis initiates in the jejunum (Walton et al., 2012), a well-developed inner circular muscle (ICM) is already prominent (Fig. 1A) along the entire length of the intestine. A mature outer longitudinal muscle (OLM) is not seen until E16 (Fig. 1E), 24 hours after the first villi emerge in the jejunum. While a few scattered cells that are weakly positive for αSMA, a marker of mature smooth muscle, can be discerned at E15.0 (Fig. 1D), an organized OLM layer is absent at this time. Finally, the muscularis mucosa, immature at E16.5 (desmin positive, but αSMA negative, Fig. 1F), remains discontinuous at E18.5 (Fig. 1G). Thus, in the mouse, villus emergence is not temporally synchronized with maturation of any of the three muscle layers.

Since formation of the ICM precedes villus development, we further examined whether confinement forces mediated by this muscle might play a role in cluster formation or villus emergence. E14.0 intestines (prior to cluster formation) were opened longitudinally, interrupting the circularity of the ICM (Fig. 1H). Though the ICM might still impose some force on the overlying tissue, radial confinement is abolished; indeed, opened intestines tend to invert. After 20 hours in culture, clusters begin to form at the anterior end of the intestinal segment (Fig. 1I). By 38 hours, clear, well-patterned mesenchymal clusters and rudimentary villi are visible (Fig. 1J,K). Villus and cluster size is uniform, even at the cut edges of the intestine, where residual
strain is predicted to be lower (Fig. 1K, dashed line). Thus, in mouse, radial confinement from the ICM is not required for cluster formation, cluster patterning or initial villus emergence. We cannot, however, rule out the possibility that confinement from the ICM might facilitate the progression of villus outgrowth after initiation.

*Epithelial deformation does not determine cluster pattern in the mouse intestine*

In the chick, epithelial bending is an upstream driver of cluster formation and patterning. Thus, we examined the relationship between clusters and epithelial deformation in the mouse intestine. Thick (80-100 μm) vibratome sections of E14.5-E15.0 mouse intestine were stained for α-tubulin, a marker that reveals epithelial cell shape and is also enriched in clusters, and confocal z stacks were collected (Supplemental Movie S1). Individual sections of a representative stack, presented in Fig.2A-C, reveal that the apical surface of the epithelium is still flat when the first clusters form basally beneath the epithelium. Thus, deep folds of the entire epithelium, such as those observed in the chick, are not seen in the mouse. On the basal side, however, epithelial cells directly above nascent clusters are shorter, creating shallow but obvious epithelial deformations (arrows). No basal deformations are detected in regions lacking a mesenchymal cluster or after treatment with cyclopamine (Fig.S1B)(Walton et al., 2012), which abolishes clusters, consistent with the idea that basal shortening is driven by signals from these clusters.

Though the analysis above suggests that cluster formation is upstream rather than downstream of basal epithelial deformation in the mouse, we tested directly whether artificially imposed epithelial deformation can drive cluster and villus pattern in this model. Shyer et al. deformed the chick intestinal epithelium by placing a grid on the opened epithelial surface and observed precocious induction of villus cluster genes and emergence of single villi through the
holes of the grid (Shyer et al., 2015). To take this analysis one step further, we utilized grids of different mesh size, placing them on the opened epithelial surface of the E13.5 mouse intestine, prior to cluster or villus formation. Intestinal explants were observed daily for four days. We reasoned that, if epithelial deformation is a critical determinant of cluster and villus pattern in the mouse, grids of increasingly wider mesh size should produce increasingly wider clusters and villi. However, this was not seen (Fig.2D-F). Mesh sizes that approximate cluster size (55 μm aperture) allowed single villi to grow into the mesh spaces (Fig.2D), as seen previously (Shyer et al., 2015). However, in grids with larger mesh sizes (75 μm aperture and larger), multiple clusters (and villi), rather than larger clusters and villi, were observed (Fig.2E). Additionally, analysis of the boundary of the grid revealed that grid placement slowed rather than accelerated cluster formation and villus emergence (Fig.2F). Together, these experiments demonstrate that for the mouse intestine, epithelial deformation is not sufficient to impart patterning cues to the field of clusters and emerging villi. Rather, the presence and patterning of mesenchymal clusters determines the presence and pattern of the emerging villi. We therefore sought to identify the signal(s) downstream from Hh-mediated cluster aggregation that is responsible for cluster patterning.

**Mesenchymal clusters express multiple Bmp signaling molecules**

Bmps are secreted ligands responsible for patterning in many developmental contexts (Hogan, 1996) and several Bmp ligands are known targets of Hh signaling (Roberts et al., 1995). To better assess the potential involvement of Bmp signaling during cluster formation and patterning of nascent clusters, we examined the localization of RNA transcripts for several Bmp ligands and modifiers during the initial round of cluster formation as well as in clusters
associated with emerged villi (Fig.3). Prior to cluster formation, Bmp ligands 4, 5 and 7 are expressed in many cells of the subepithelial mesenchyme, while Bmp2 is primarily epithelial (Fig.3A-D). As clusters form (E14.5), Bmp2 expression is initiated in clustered cells (Fig.3A inset). As villi emerge (E15.5), all Bmp genes except Bmp7 are expressed robustly in mesenchymal clusters (Fig.3I-L); Bmp2 continues to be the most specific cluster marker (Fig.3I). Expression of the Bmp modifier, Twisted gastrulation (Fig.3E,M) is similar to the pattern of Bmp5, while the tolloid-like molecule, Bmp1, is weakly expressed at E14.5 (Fig.3F), but is high in mesenchymal clusters at E15.5 (Fig.3N). The Bmp inhibitor Noggin is expressed in mesenchymal clusters only after emergence (Fig.3G,O), while Follistatin-like1 is highly expressed throughout the mesenchyme at both stages (Fig.3H,P). Thus, multiple Bmp ligands and Bmp signaling modifiers are dynamically expressed both in unclustered mesenchyme and in nascent and mature mesenchymal clusters.

Modulation of Bmp signaling affects cluster formation and pattern

To determine whether Bmp ligands or signaling modifiers could modulate cluster pattern, intestines from E13.5 PtcLacZ/+ embryos (prior to cluster formation) were cultured on transwell filters in the presence of agarose beads soaked in bovine serum albumin (control) or recombinant Bmp2, Bmp4, Bmp5, Bmp7, or heterodimerized Bmp2/7 or Bmp4/7. After two days, clusters formed in the expected pattern near control beads (Fig.4A,D,G,J). However, all Bmp-soaked beads inhibited mesenchymal cluster formation and subsequent villus emergence in the region surrounding the bead, but not on the opposite side of the intestine (Fig.4B,C,E,F,H,I,K and Fig.S2). Lack of cluster formation near Bmp beads was confirmed by immunostaining for PDGFRα, a marker of mesenchymal clusters (Karlsson et al., 2000) (Fig.4K). Bmp2 was the
most potent ligand for inhibiting cluster formation near the beads, strongly inhibiting cluster formation beginning at 125 ng/ml (Fig.4L and Supplemental Fig.2E).

Similarly, we tested the effects of several of the Bmp signaling modulators that are expressed in clusters (Fig.5). The cluster patterning perturbations were more subtle, consisting of larger or merged clusters rather than clearing of clusters (Fig.5A-C). Pattern changes for Tsg1 were most obvious (Fig.5C), though cluster sizes for intestines treated with Bmp1, Noggin, or Tsg1 in the media (10 ng/ml) were all statistically different from clusters treated with BSA (Fig.5E). Clusters closest to Tsg1 soaked beads (250 ng/ml) placed on top of the intestine were significantly larger than clusters measured away from the bead (Fig.5D). Clusters in BSA treated intestines were not different from clusters away from Tsg1 beads (Fig.5E).

Next, we tested the effect of complete Bmp signal inhibition on cluster formation and villus emergence. Intestines were harvested prior to cluster formation and cultured for two days with a small molecule inhibitor of Bmp signaling, dorsomorphin (Fig.6). Inhibition of Bmp signaling in this manner alters cluster patterning dramatically. Clusters are 2-3 times larger and often connected, so that the “spot-like” distribution of clusters in control intestines (Fig.6A-C) becomes “striped” in the presence of dorsomorphin (Fig.6D-F). In cross-sectioned intestines, the larger merged clusters alter the shapes of associated villi (compare Fig.6G to 6J). Of note, these changes occur without altering smooth muscle (Fig.6H-L), again confirming that cluster pattern, not muscle tension, determines the pattern of villus emergence in the mouse.

Studies in other systems have shown that inhibition of Bmp signaling in the epithelium causes cells to shorten and become columnar or cuboidal (Eom et al., 2011; Gibson and Perrimon, 2005; Rajagopal et al., 2009; Shen and Dahmann, 2005). However, dorsomorphin treatment does not cause widespread columnar conversion; epithelium above the merged clusters
is columnar while epithelium between these clusters remains pseudostratified (Fig.6M-O). Doubling the dose of dorsomorphin generates larger clusters and larger villi, but does not convert all epithelial cells to a columnar shape (Fig.6O). Thus, inhibition of Bmp signaling throughout the intestine affects cluster pattern without altering cell shape in the epithelium.

We previously showed that increased Hh signaling also produces larger clusters (Walton et al., 2012), though a striped pattern was not seen. Here, dorsomorphin treatment does not appear to dramatically affect the expression levels of the Hh target gene, Ptc1 in these Ptc1\textsuperscript{LacZ/+} intestines. Lack of a significant effect of dorsomorphin on the Hh signaling pathway was further confirmed by Q-RT-PCR (Fig.S3).

*Genetic loss of Bmpr1a in mesenchymal clusters regulates cluster size and villus morphology*

While the experiments above reveal the importance of Bmp signaling in modulating cluster pattern, they do not establish which compartment (epithelial, mesenchymal or both) must transduce Bmp signals to control this process. Since Bmp signaling activity is detected in both the epithelium and mesenchyme (Fig.S4), we used a conditional Bmpr1a\textsuperscript{f/f} mouse model (Mishina et al., 2002), in combination with tissue-specific Cre drivers, to genetically delete Bmpr1a signaling in either the mesenchyme (using Gli1\textsuperscript{CreERT2/+})(Bai et al., 2002) or the epithelium (with Shh\textsuperscript{Cre}) (Harfe et al., 2004) prior to cluster formation. Reporter demonstration and Q-RT-QPCR of deletion efficacy of deletion is shown in Fig.S5.

Loss of Bmpr1a in the epithelium (Shh\textsuperscript{Cre}; activated by E9.5)(Harfe et al., 2004)) does not affect muscle development, cluster size, cluster distribution or epithelial morphology (Fig.7A,B). However, loss of Bmpr1a in Hh responsive mesenchymal cells results in larger clusters and wide villi, (Fig.7C,D), a phenotype closely resembling that seen in intestines treated with
dorsomorphin. Scanning electron microscopy confirms larger/merged villi in the Bmpr1a^{f/f}; Gli1^{CreER/+} mutants as compared to control littermates (Fig.S6). Indeed proliferation was increased with loss of Bmpr1a in the mesenchyme; however, neither the amount (Fig.S7) nor the pattern of proliferation was altered in the epithelium (Fig.S8). Together, these data demonstrate that the patterning of mesenchymal clusters is determined by the level of Bmp signal transduction in the hedgehog-responsive mesenchymal compartment.

*Cluster patterning resembles a spreading Turing field*

We previously showed that mesenchymal clusters are established in an anterior to posterior and dorsal to ventral wave, eventually generating a regular pattern along the length of the intestine (Walton et al., 2012). Turing first postulated that similar patterns can be generated by the reactions of diffusive chemicals; small perturbations in initially uniformly distributed chemicals can increase in amplitude over time, leading to a regular spatial pattern. The Turing system employs an activator and an inhibitor, both expressed by the same cell or group of cells. The inhibitor spreads farther than the activator and patterned spots become stripes when the activator is saturating (Turing, 1952).

Cluster patterning *in vivo* shares several features with the Turing system: a) An initially homogeneous state, lacking clusters and villi, evolves quickly (within 12 hours) to a patterned state that then spreads (Walton et al., 2012); b) The pattern can be altered by Bmp ligands, which inhibit formation of clusters where Bmp signaling is high (Fig.4, Fig.S2); thus, one or more Bmp ligands may act as Turing inhibitors; c) Dorsomorphin treatment changes the cluster pattern from spots to stripes (Fig.6) and beads soaked with several Bmp inhibitors/modulators cause cluster fusion into short stripes (Fig.5) suggesting that an inhibitor of Bmp signaling could be the Turing activator; d) Multiple Bmp ligands and signaling modulators are expressed by cluster cells
(Fig.3), coordinate with the requirement for expression of activator and inhibitor by the same cells; e) The genetic experiments above show that patterning requires the reception of Bmp signals in the mesenchymal compartment, including the cluster cells themselves (Fig.7).

To test the degree to which a mathematical model of a Turing system resembles the experimentally derived data, we constructed a Turing system comprised of two modulators of Bmp signaling following a “pure activator-inhibitor” system (Dillon and Othmer, 1993) and incorporating cell density (see Supplementary Methods for details). We propose that the Turing activator is a Bmp inhibitor and that the Turing inhibitor consists of one or more Bmp ligands. The evolution of the resulting pattern, represented as cell density, in the absence or presence of saturating activator (Bmp inhibition/dorsomorphin) is displayed in Figure 8A and 8B. Light green regions represent high concentrations of Bmp inhibitor (Turing activator), while the dark regions are low concentrations. In our model, mesenchymal cluster cells are assumed to produce these proteins, whose interactions give rise to these gradients, so that regions of high Bmp inhibitor correspond to the location of mesenchymal cells where clustering occurs. As predicted by the Turing system, spots are seen when the activator concentration is well below saturation, while saturation of the activator produces larger clusters and areas with stripy pattern. These patterns closely resemble those observed experimentally, including the 2-3 fold increase in cluster size with saturating activator (Bmp inhibitor) (plotted in Fig.8M,N).

We then used the model to predict the patterns that would evolve with intermediate levels of activator. At such intermediate levels, spots and stripes were mixed and stripes were predicted to be shorter and less frequent (Fig.8C). This was indeed borne out experimentally, after exposure of intestinal explants to an intermediate dose of dorsomorphin (Fig.8G). We also modeled a localized source of high concentration of Turing inhibitor (Bmp ligand) and found
that the resulting simulation (Fig. 8D) mirrored our experimental results of intestines cultured with Bmp ligand-soaked agarose beads (Fig. 4, 8H and S2E). Finally, we examined a scenario in which a spot-like pattern was allowed to evolve for 24 hours in the absence of saturated activator, and then activator concentration was computationally increased in a stepwise fashion. In this simulation, the initial spot-like pattern evolved to become more stripe-like (Fig. 9I, J; Supplemental Movie 2). Experimentally, we allowed E13.5 intestines to develop for 48 hours on transwells, until the spot-like pattern of clusters became apparent (Fig. 8K) and then added dorsomorphin to the culture for an additional 48 hours. The initial spot pattern filled in to become stripy, in a manner closely resembling the computed simulation (Fig. 8L). Overall, these results provide strong evidence that cluster patterning and subsequent villus emergence is controlled, at least in part, by Bmp signaling and that the patterning field evolves in a manner consistent with a self-organizing Turing field within the mesenchyme.
DISCUSSION

Optimal absorptive function by the small intestine depends upon the generation of a tightly packed and well-organized field of villi, a process that begins in fetal life. Substantial evidence over the past several decades has emphasized the role of complex epithelial-mesenchymal crosstalk in the process of villus formation (for a recent review, see [Wells and Spence, 2014]). Indeed, we previously established that one of the earliest steps in villus development takes place at E14.5 in the mouse, when Hedgehog signals, expressed uniformly from the epithelium (Kolterud et al., 2009) act on evenly distributed \( Ptc1/Gli1/Pdgfr\alpha \)-positive sub-epithelial mesenchymal cells, causing their aggregation into mesenchymal clusters (Walton et al., 2012). Here, we provide evidence that, downstream from this cluster-forming epithelial Hh signal, a Bmp signaling network that operates entirely within the mesenchyme is responsible for establishment of cluster spacing and pattern. We show that cluster pattern can be dynamically altered simply by modifying the concentration of Bmp ligands or Bmp signaling modifiers and that the pattern evolves in a manner consistent with a Turing activator/inhibitor field.

There is mounting evidence for the validity of Turing-based models to explain pattern evolution in several diverse biological systems, for example in feather bud arrangements (Baker et al., 2009), hair follicle spacing (Maini et al., 2006; Sick et al., 2006), palatal rugae distribution (Economou et al., 2012), tongue papilla patterning (Zhou et al., 2006), digit patterning (Raspopovic et al., 2014) and zebrafish mesodermal pigmentation (Eom et al., 2012; Kondo and Miura, 2010), and it is interesting that Bmp ligands appear to act as Turing inhibitors in several of these systems (Garfinkel et al., 2004; Harris et al., 2005; Mou et al., 2011). In the intestine, we have seen that several different Bmp ligands are expressed by clusters (Bmp2, 4, 5, 7) and functional assays indicate that high concentrations of all of these species act to inhibit the
formation of clusters (Fig. 4 and S2). Similarly, multiple Bmp signaling modifiers are expressed by clusters (Noggin, Twsg1, Bmp1, Follistatin) and several of these cause pattern perturbations in the explant agarose bead assay (Fig. 5). Thus, while we show that pattern formation in the intestine is faithfully modeled by a computational framework that embodies a two component system as originally described by Turing (Turing, 1952), it is highly likely that pattern establishment and maintenance \textit{in vivo} is actually a product of a much more complex combination of Bmp pathway molecules. In fact, in the introduction to his classical paper describing such patterning fields, Turing himself stated that his model is an idealization and simplification of reality (Turing, 1952).

In addition to their role in patterning, our data suggest that signals from mesenchymal clusters are responsible for the epithelial cell shape changes that initiate villus emergence. Epithelial cells begin to shorten apico-basally as clusters first form (Fig. 2). If clusters do not form (for example, after inhibition of Hh signaling (Madison et al., 2005; Walton et al., 2012) or in the vicinity of a Bmp-soaked bead), the epithelium remains pseudostratified. In contrast, induction of larger clusters (e.g., SAG or dorsomorphin treatment) results in larger villi, over which more epithelial cells take on a columnar shape (Walton et al., 2012). Since, in the fly wing disc, clonal loss of the Bmp receptor, \textit{thickveins}, causes cells to become columnar (Gibson and Perrimon, 2005), we predicted that epithelial Bmpr1a deletion or addition of dorsomorphin to cultured intestines would cause widespread conversion to epithelial columnar morphology. This was not observed. Thus, our studies suggest that Bmp signaling alone does not mediate the epithelial cell shape changes that occur over villus clusters. Determining the pathway(s) responsible for this morphogenic process, which likely provides an important part of the driving mechanism for villus outgrowth, remains an important future goal.
It is interesting that, while the use of intestinal villi to expand intestinal surface area is a well-conserved attribute in multiple species, divergent strategies for patterning of the villi have emerged during evolution. In the chick intestine, recent studies have shown that tensile forces from developing smooth muscles progressively deform the epithelium to create localized peaks of Hh protein underneath sharply bent epithelial alcoves; these Hh maxima seem to determine the location of the villi (Shyer et al., 2015). However, our data show that a different epistatic relationship between cluster formation, muscular forces and epithelial deformation portends in the mouse. Maturation of the various smooth muscle layers does not correspond temporally with the process of villus emergence in the murine intestine. Additionally, the epithelium is not remodeled into ridges or zigzags prior to villus formation; rather, villi arise as discrete domes directly from a flat epithelial surface. Though we did note soft basal epithelial deformations above nascent clusters, previous modeling studies suggest that such minimal bending is unlikely to create a substantial concentration of Hh signals (Shyer et al., 2015). Moreover, we never observed these soft basal deformations in the absence of a cluster; indeed, our evidence suggests that the deformations are a consequence of unknown signals from the underlying clusters. We provide extensive evidence that mesenchymal clusters and Bmp signaling in cluster cells control villus pattern in the mouse. Directly perturbing mesenchymal cluster pattern by altering Bmp signaling does not affect smooth muscle development (Fig.6,7) but does alter the pattern of clusters, thereby producing predictable changes in the pattern of the villi (Fig.9). In fact, dramatic changes in Bmp concentration can even alter established patterns (Fig.9I-L). Strikingly, however, despite the different modes of villification in the chick and mouse, conserved signaling pathways are involved: Hedgehog signals from the epithelium play central roles in the induction of cluster genes such as Bmp in both species.
Like the mouse, intestines of human, pig and rat lack the zigzag-like structures of chick intestine (Dekaney et al., 1997; Lacroix et al., 1984; Matsumoto et al., 2002; Nakamura and Komuro, 1983). Though the proximal human intestine may contain short ridge-like structures that are later broken up into individual villi (Johnson, 1910), the human distal small intestine develops villi directly, as in the mouse (Johnson, 1910). Additionally, only the ICM, which may play a confinement role, aiding but not initiating villus emergence, is formed prior to villus emergence and maturation of the remaining smooth muscle layers in the human (Fekete et al., 1996; Keibel, 1910), pig (de Castro, 2001; Georgieva and Gerov, 1975) and rat (Kedinger et al., 1990) occurs well after the initiation of villus formation, as in the mouse. It is therefore likely that these mammalian species also rely on a villification patterning process that is controlled by gradient fields of signaling proteins rather than the avian model of muscle-directed epithelial deformation. It is also important to note that several rounds of villus formation have been demonstrated in the mouse (Walton et al., 2012) and likely occur in all species. Once the initial pattern is set, a Turing-like patterning mechanism in a growing domain could act to establish the arrangement of subsequent mesenchymal clusters, thereby generating a field of uniformly patterned villi in the intestine of all these species, including the chick.
MATERIALS AND METHODS

Mice

Mice were handled humanely according to UCUCA guidelines. The following lines were used: C57BL6 and CD1 (Charles River); Rosa<sup>mt/mG</sup> (Muzumdar et al., 2007), PDGFRα<sup>EGFP/+</sup> (Hamilton et al., 2003), ShhEGFP<sup>Cre/+</sup> lines (Harfe et al., 2004), Ptc<sup>LacZ</sup> (Goodrich et al., 1997), Gli1<sup>CreERT2</sup> (Bai et al., 2002)(Jackson Labs); Bmpr1α<sup>f/f</sup> (Mishina et al., 2002)(Dr. Yuji Mishina).

Tamoxifen induction of recombination

Pregnant females were gavage fed daily from E12.5-14.5: 250 µl of tamoxifen dissolved in corn oil at a concentration of 20 mg/ml. Embryos were collected at E15.5.

Cultures, recombinant proteins & inhibitors

Embryonic intestines were harvested at E12.5 or E13.5 and grown in culture with protein-soaked agarose beads or dorsomorphin as described (Walton and Kolterud, 2014). Media was changed twice daily. Recombinant proteins were from R&D Systems.

Cluster area measurements

Using ImageJ software, ellipses were drawn around clusters to measure area. All statistical tests were done in excel or Prism. T-tests were two-tailed and non-parametric unless otherwise noted.

Mesh Screen cultures

E13.5 intestines were harvested from PDGFRα<sup>EGFP/+</sup> embryos, cut open lengthwise and placed on a transwell membrane to expose the luminal surface. Mesh screens (55 or 75 µm, the Mesh Company; #300 or #230, respectively) were cut to size and placed on top of the intestines to culture for 1 week with images acquired daily.

Tissue Fixation and Immunostaining
Tissues were fixed for 2hrs at room temperature or overnight at 4°C in 4% PFA. Vibratome, paraffin, and frozen sections were prepared as previously described (Walton and Kolterud, 2014; Walton et al., 2012). Antibodies used were: PDGFRα (Santa Cruz sc338), Ecadherin (BD 610181), αSMA(Sigma C6198), Desmin (Abcam ab8592), Ezrin (Sigma E8897), αTubulin (Sigma T6199).

**RNA in situ hybridization**

RNA *in situ* hybridization was performed on 8 µm frozen sections as described previously (Li et al., 2007).

**Turing Field simulations**

In our model, the activator-inhibitor interactions assume a saturating source of both the activator species \( u \) and the inhibitor species \( v \), the latter of which is altered by pharmacological inhibitors of Bmp signaling such as dorsomorphin (Gierer and Meinhard, 1972). The saturating source of \( u \) is also inhibited by the presence of \( v \), according to the classic Turing activator-inhibitor system. We include density-dependent cell proliferation, along with the diffusive and chemotactic movement of the mesenchymal cells. Additional details regarding the Turing model are provided in Supplemental Methods.
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AUTHOR CONTRIBUTIONS

We note the following author contributions: 1) Concepts and approaches, 2) performed experiments, 3) analyzed data, 4) prepared manuscript, 5) edited manuscript
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Figure 1. Smooth muscle development does not correlate with mouse villus emergence; tensile forces are not required for cluster patterning. Alpha smooth muscle actin (αSMA) staining (red) marks mature muscle cells in the developing muscle layers at E13 (A), E14 (B), E14.5 (C), E15 (D), E16 (E), E16.5 (F), E18.5 (G). The ICM is already mature at E13 (A), but a mature OLM is not present until E16 (outer circle, arrow, E). Scattered αSMA positive cells at E14.5 (arrows, C) and E15 (arrows, D) are not organized into a continuous muscle. (F,G) Muscularis mucosa is not present at E16.5 (F) and is still incomplete at E18.5 (G, arrows). Desmin (green) marks smooth muscle precursor cells (F, arrows). DAPI (blue) stains nuclei. H-K) An E13 intestine from a
PdgfrαEGFP/+ mouse was opened longitudinally and cultured for 2 days. No clusters were present initially (H). Clusters began to develop by 20 hours (I, near arrow). By 38 hours, well-patterned clusters were visible (J) and small villi were emerging (magnified in K). Dashed line in K indicates cut edges of the intestine, where it has rolled back after cutting. Clusters and villi were observed to form in intestines cut open prior to cluster formation and grown in culture for 2 days in more than 25 independent samples from at least 8 separate experiments. Scalebars are 50 µm in A-G and 100 µm in H-K.
Figure 2. Epithelial deformation does not determine cluster pattern in the mouse intestine. A-C) Single slices from top (A), middle (B) and bottom (C) of a 50 µm thick z-section of an E14.5 intestine stained with α-tubulin (red) to outline epithelial cells and mark clusters (outer ring marks the ICM); DAPI (blue) marks nuclei. White arrows indicate the position of two clusters. Note basal epithelial deformation with no apical deformation. Supplemental Movie (S1) steps through the entire z-stack. D-F) Mesh screens force deformation of the epithelium in E13.5 PdgfrαEGFP/+ intestines that were cut open lengthwise and cultured under a mesh screen. D) Individual villi with single mesenchymal clusters develop under a screen with a 55 µm aperture. E) Multiple villi with a single cluster per villus develop under a mesh screen with a 75 µm aperture. F) Note that cluster formation and villus development are delayed in tissue under a mesh screen (anterior, left side) as compared to the posterior side (right side) that was not under the screen. n > 12 in four separate experiments for each of the mesh screen cultures. Scalebars are 30 µm for A-C and 50 µm for D-F.
### Figure 3

RNA in situ localization of Bmp pathway ligands and modifiers at E14.5 just prior to cluster formation and villus emergence (A-H), and at E15.5 once villi have begun to emerge (I-P). Inset in panel A shows cluster-specific expression of Bmp2 in nascent clusters at a slightly later stage when expression is switching from epithelial to mesenchymal. Scalebars are 100 μm.
Figure 4. Bmp ligand-soaked agarose beads inhibit cluster formation and villus emergence. A-I) E13.5 Ptc<sup>LacZ/−</sup> intestines were harvested prior to cluster formation and cultured for 2 days with agarose beads soaked in BSA or recombinant protein, as indicated on each panel. X-gal staining
shows the pattern of Ptc\textsuperscript{LacZ/+} clusters that form in whole (A-F) or sectioned (G-I) intestines. Hatched circles in B,C,E,F outline the clearance of Ptc\textsuperscript{LacZ/+} clusters around the Bmp soaked bead; faint clusters that appear within the hatched circle are on the transwell/opposite side of the intestine, as seen in the cross sections in H and I. J,K) Anti-PDGFR\textalpha (green) marks clusters formed near a BSA soaked bead (J) which are absent around the Bmp soaked bead (K). Inhibition of cluster formation near Bmp soaked beads was observed in at least 100 beads placed on more than 25 intestines. (L) Scatterplot of the mean of the distances from the edge of Bmp2 soaked beads (circles) or Bmp4 beads (square) to the nearest clusters measured in a sampling from those experiments (at least 6 beads placed on at least 3 different intestines). Error bars are SD. See also Supplemental Figure 2 for measurements of clearing around other Bmp ligand soaked beads. Scalebars are 50 μm.
Figure 5. Bmp modifiers alter cluster pattern and villus shape. E13.5 Ptc\textsuperscript{LacZ/+} intestines were harvested prior to cluster formation and cultured for 2 days with 10 ng/ml of recombinant protein, as indicated on each panel (A-C) or 250 ng/ml Tsg1 on agarose beads (D, hatched circle). A-D) X-gal staining shows the pattern of Ptc\textsuperscript{LacZ/+} clusters that form in whole intestines. Note merging of clusters is most dramatic in Tsg1 treated intestines in C and near the Tsg1 beads in D. E) Box and whisker plots of cluster areas showing the largest, smallest, median (center line) and mean (+) for each treatment. Cluster areas for Bmp1 and Noggin treated intestines were significantly different from BSA treated intestines and Tsg1 treated intestines (p < 0.0001), but were not different from each other. Tsg1 treated intestines were significantly different from BSA treated intestines (p < 0.0001). Clusters immediately surrounding Tsg1 soaked beads were significantly different from clusters away from the beads (p < 0.0001), but not from BSA treated intestines (p= 0.689). Scalebars are 250 µm in A and B and 100 µm in C and D.
Figure 6. Inhibition of Bmp signaling alters cluster pattern and villus size. Ptc\(^{LacZ/+}\) intestinal pieces were harvested at E13.5 and cultured with DMSO (A-C,G,H,K,M), 20 \(\mu\)M dorsomorphin (N,O), or 20 \(\mu\)M dorsomorphin (M).
(D-F,I,J,L,N), or 40 μM dorsomorphin (O). Treatments were performed in at least 25 control and 35 dorsomorphin treated intestines; representative images of duodenum (A,D), jejunum (B,E,G-O), and ileum (C,F) are shown. After 2 days, intestines were fixed and Xgal stained to show the pattern of PtcLacZ/+ clusters (blue) in whole intestines (A-F) and 100 μm sections (G,J). Arrows in D,E mark areas where clusters merge to stripes with dorsomorphin. Arrowheads in J mark large fused clusters and large villi. H, I, K, L) Dorsomorphin treatment does not alter smooth muscle (αSMA, red). Sections are shown with (K,L) and without (H,I) DAPI staining. M-O) Epithelial cells (outlined by green anti-Ecadherin) remain pseudostratified in intervillus regions (red arrowheads) when Bmp signaling is inhibited (N, O) similar to controls (M). Scalebars are 100 μm for A-F, 50 μm for G-N.
Figure 7. Conditional loss of Bmp signaling in Hh responsive mesenchymal cells, but not in epithelial cells, results in fused clusters and wide villi. A,B) Bmpr1a$^{fl/f}$ mice were mated to Shh$^{Cre}$ mice. Shh$^{Cre}$ is activated by E10 in the intestinal epithelium (Kolterud et al., 2009). No changes in epithelial (Ecadherin, blue) or mesenchymal pattern (PDGFRα, green) were noted; muscle was unchanged (αSMA, magenta). C,D) Bmpr1a$^{fl/f}$ mice were mated with Gli1$^{CreERT2/+}$ mice. Recombination was induced with 3 doses of tamoxifen, beginning at E12.5 prior to cluster formation and tissues were harvested at E15.5. Large clusters (PDGFRα, green) and wide villi (E-cadherin, red) are seen in Bmpr1a$^{fl/f}$; Gli1$^{CreER}$ mutant intestines (D). Eight mutant and eight control littermate intestines from three separate litters were analyzed for Bmpr1a$^{fl/f}$; Shh$^{Cre}$. Twenty mutant and twenty-one control littermate intestines from six separate litters were analyzed for Bmpr1a$^{fl/f}$; Gli1$^{CreER}$. Scalebars are 50 μm.
Figure 8. A Turing field model of cluster cell patterning recapitulates experimental data. A-D) Still images of the patterns predicted by the simulations (further details are described in Supplemental Methods). A regularly spaced pattern of clusters (spots) is predicted in control (A) Turing simulations. The pattern is altered to stripes (B) when the Turing activator (Bmp inhibitor) is saturated. An intermediate level of activator results in shorter stripes (C, lower dose of dorsomorphin) while a localized increase in Turing inhibitor (excess Bmp ligand at center) prevents cluster formation near the source (D). E-H are representative images of experimental results done under treatment conditions that match the Turing simulations. Treatments were as follows: control (E), 20 μM dorsomorphin (F), 10μM dorsomorphin (G) and Bmp soaked bead (250 ng/ml) (H). I-L) Dynamic changes in Bmp inhibitor concentration change patterns in simulations and experimental tests. A developed spot pattern (I) evolved toward stripes (J) when inhibitor concentration was computationally increased. Experimentally, established cluster spots (K) merge to form short stripes (L) when dorsomorophin (20 μM) is added (N= 18 intestines). Scalebars are 100 μm. M) Box and whisker plots show the largest, smallest, median (middle
line), and mean (+) cluster area for intestines treated for two days with vehicle (DMSO) or increasing doses of dorsomorphin. N= 5 fields for at least 5 different intestines for DMSO, 10 μM, 20 μM, 40 μM; N= 5 fields for 3 different intestines for 1 μM. N) Scatter plot comparing the average cluster area at increasing concentrations of dorsomorphin (experimental) verses the simulated model. Error bars are SE. SD increases with increasing concentrations of Bmp inhibitor due to striped patterns and boundary conditions (N=73, 75, 74, 34, 15, 3).
REFERENCES


