Hedgehog signaling controls homeostasis of adult intestinal smooth muscle

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A B S T R A C T

The Hedgehog (Hh) pathway plays multiple patterning roles during development of the mammalian gastrointestinal tract, but its role in adult gut function has not been extensively examined. Here we show that chronic reduction in the combined epithelial Indian (Ihh) and Sonic (Shh) hedgehog signal leads to mislocalization of intestinal subepithelial myofibroblasts, loss of smooth muscle in villus cores and muscularis mucosa as well as crypt hyperplasia. In contrast, chronic over-expression of Ihh in the intestinal epithelium leads to progressive expansion of villus smooth muscle, but does not result in reduced epithelial proliferation. Together, these mouse models show that smooth muscle populations in the adult intestinal lamina propria are important link between Hh signaling and intestinal visceral myopathy.

Introduction

Hedgehog (Hh) signaling is a vital regulator of developmental patterning and adult homeostasis in many organ systems (reviewed in Varjosalo and Taipale, 2008). In the embryonic small intestine and colon, Hh signaling throughout life is paracrine, driven by the interaction of epithelia produced Shh and Ihh ligands with the mesenchymally expressed Patched (Pch) receptor and signal transduction machinery (Kolterud et al., 2009). Prenatally, experimental reduction of the Hh signal in the GI tract affects the expansion of the mesenchymal compartment (Mao et al., 2010), perturbs development of the muscularis externa (Kosinski et al., 2010; Madison et al., 2005; Ramalho-Santos et al., 2000; Sukegawa et al., 2000), alters the localization of pericryptal myofibroblasts (Madison et al., 2005) and disrupts villus patterning (Kosinski et al., 2010; Madison et al., 2005; Wang et al., 2002). Through tissue cross-talk, the mesenchymal compartment, perturbed by lowered epithelial Hh levels, emits signals that feed back on the epithelium. Several investigators have observed increased epithelial Wnt signaling, enhanced progenitor cell proliferation and altered location of the proliferative compartment (Kosinski et al., 2010; Madison et al., 2005; Wang et al., 2002) upon reduction of Hh signaling.

Hh signals continue to direct epithelial/mesenchymal cross-talk in adult life. Conditional reduction of Hh levels in adult animals has been accomplished through expression of a pan-Hh inhibitor (Zacharias et al., 2010) and by epithelial-specific knockout of Ihh (van Dop et al., 2010). In both cases, mesenchymal changes included progressive myeloid inflammation, loss of differentiated smooth muscle cells (SMC) and alterations in the number and position of myofibroblasts. This mesenchymal pathology is accompanied by a robust increase in epithelial proliferation, with deep crypts but blunted villi, a histological picture that, in some but not all aspects, resembles human celiac disease. These studies indicate that Hh sits atop a signaling cascade that directs both mesenchymal and epithelial homeostasis.

In all of these studies, the two most obvious and consistent consequences of reduced Hh signals are the loss of smooth muscle differentiation in the mesenchymal compartment and the increased proliferation in the epithelial compartment. Indeed, it has been suggested that these two phenotypes could be causally connected, in that smooth muscle cells of the muscularis mucosae may comprise an important part of the epithelial stem cell niche, acting to regulate the
number or fate of epithelial stem cells by sensing Hh levels (Kosinski et al., 2010). The idea that a negative feedback loop exists by which epithelial Hh ligands control the expression of some mesenchymal signal(s) that then feeds back to control small intestinal epithelial proliferation in a homeostatic manner had been proposed on the basis of earlier studies in the colon (van den Brink et al., 2004; van Dop et al., 2009). Though the vast majority of extant data from reduced Hh signaling models in the fetus and adult support this idea as discussed above (epithelial proliferation increases), only one previous study has examined the effects of increased Hh signal transduction on epithelial stem cell dynamics. In that study, ubiquitous activation of the Hh pathway through conditional deletion of the Ptc1 gene using a Rosa26CreERT2 driver resulted in accumulation of cSMa positive cells and crypt hypoplasia (de Santa Barbara et al., 2002). Importantly, the phenotype of this model was only studied in the colon.

The studies above have utilized conditional deletion of Ihh or of Ptc1 to study Hh signaling in the adult intestine and colon. The complete loss of Ihh or Ptc1 results in dramatic swings in Hh signal transduction that are likely seldom encountered homeostatically. To gain a better understanding of the role of Hh signaling in small intestinal homeostasis, we performed a careful analysis of two mouse models recently generated in our laboratory. Both models involve subtle alterations in Hh signaling levels. In one (Villin-Cre × Villin-Fl ox-Stop-Fl ox-Hhip, hereafter called VFhip), Hh signaling is reduced by over-expression of a solubile version of the pan-Hh inhibitor, Hhip, while in the other (Villin-Ihh, hereafter called Vihh), Hh signaling is increased by over-expression of Ihh in the intestinal epithelium. These models modulate Hh signaling by 3–5 fold. Analyses of mice between the ages of 3 and 11 months reveal progressive pathology that includes loss of villus smooth muscle and muscularis mucosa in the context of reduced Hh signaling and significant gain of smooth muscle in the presence of increased Ihh. Thus, villus smooth muscle is exquisitely sensitive to levels of Hh ligand. Additionally, we find that Hh can drive cell-autonomous SMC differentiation in vitro. Further analysis of the pathways involved reveals that Myocardin, a master regulator of smooth muscle, is up-regulated by Hh, with kinetics similar to that seen for known Hh target genes. Additionally, as seen previously, Hh down-regulation results in increased epithelial proliferation. Surprisingly, proliferation is initially unchanged, but later increased, rather than decreased, in the context of increased Ihh.

**Materials and methods**

**Transgenic animals**

**Reduced Hh signaling; the VFhip model**

These animals carry two transgenes: 12.4KviI-Cre, in which Cre recombinase is driven by the mouse villin promoter (Madison et al., 2002), and 12.4KviI-fox-lacZ-fox-HhipΔTM. The latter construct was created by the addition of the following components (from 5 to 3) into the pUC18 vector: the 12.4 kb Villin promoter (Madison et al., 2005), available at www.addgene.org, #19358); a floxed LacZ cassette constructed from the pFl vector (from Dr. Jamesy Marsh) containing a nuclear LacZ CDNA, pnaacZ [from Dr. Richard Palmiter] and bovine growth hormone polyA; HhipΔTM cDNA (Madison et al., 2005); and the SV40 late polyA/intron (from the pGL2-basic vector, Promega).

Transgenic mice expressing this construct exhibit LacZ expression in intestinal epithelium (Supplemental Figs. 1A, B) and HhipΔTM CDNA is held out of frame by the floxed-LacZ cassette until excision by Cre.

For generation of transgenic mice, the vector backbone was removed and transgenic animals were created by injection into C57Bl/6 × SJL/J oocytes by the University of Michigan Transgenic Animal Core. Founders were genotyped using primers spanning the promoter/insert border (Supplemental Table 1). Five founders were utilized for the creation of transgenic lines; the two lines with the best LacZ expression without Cre were selected for further analysis. Lines were maintained on the C57Bl/6 background and generations F1 to F4 were used here. Bi-transgenic animals carrying 12.4KviI-Cre and 12.4KviI-fox-lacZ-fox-HhipΔTM are designated VFhip.

**Increased Hh signaling: the 12.4KviIh model**

The full length Ihh cDNA was amplified from newborn jejunal cDNA, and cloned into the 12.4 kb VillinAAGT vector (Addgene #19358). Transgenic animals were generated by the University of Michigan Transgenic Animal Core. Founders were genotyped using primers spanning the promoter/insert border (Supplemental Table 1). Two lines (generations F1 to F3) were analyzed. These transgenic mice, maintained on a C57Bl/6 background, are designated Vihh.

**Tissue preparation, histology, and immunofluorescence**

Adult whole small intestine and colonic tissue were dissected in ice-cold PBS, opened transversely, washed, fixed overnight in 4% paraformaldehyde (PFA) at 4 °C, dehydrated, infiltrated with paraffin, and sectioned at 5 μm. For frozen sectioning, intestines were fixed for 30 min in 4% PFA at 4 °C, washed in PBS, soaked overnight in 30% sucrose in PBS, embedded in OCT, and sectioned at 6–8 μm. H&E staining was performed using standard methods. Alkaline phosphatase staining was performed using the Alkaline Phosphate Substrate Kit (Vector) and PAS/Alcian Blue staining was performed using the PAS Stain Kit (Newcomer Supply). Immunofluorescence was performed as described (Kolterud et al., 2009) using the following antibodies: mouse anti-α smooth muscle actin (Sigma, 1:500), anti-desmin (Abcam 1:500), rabbit anti-lysozyme (Zymed, 1:500), and anti-chromogranin A ( Lopez-Diaz et al., 2007). All secondary antibodies used were AlexaFlour (Invitrogen, 1:500). Quantification of epithelial lineage allocation was accomplished by counting a total of 10 random 20× fields for cell number, and normalizing to the linear length of epithelium per field in microns.

**RNA preparation and quantitative RT-PCR**

RNA was prepared from whole adult jejunum, cultured intestinal mesenchyme, or 10T1/2 cells using Trizol (Invitrogen). cDNA was prepared using the iScript cDNA Synthesis Kit (Biorad), and quantitative PCR was performed using a Biorad iCycler. Data analysis was performed using the ΔΔCt method, and fold changes were normalized to WT or vehicle treatment, as applicable. P values were calculated using Students t-test. Primers used in Q-RT-PCR are provided in Supplementary Table 1.

**Small intestinal mesenchyme culture**

Whole small intestine was harvested from E18.5 C57Bl/6 animals, cleaned of connective and pancreatic tissue, opened lengthwise, treated for 6–8 h with Cell Recovery Solution (BD Biosciences) at 4 °C to separate epithelium and mesenchyme, followed by shaking on ice as previously described (Madison et al., 2005). Separated mesenchyme was washed in sterile PBS, minced and plated in six-well plates coated with rat tail collagen (BD Biosciences) with DMEM and 10% FBS, 1 mM Heps, and Penicillin/Streptomycin at 37 °C in 5% CO2. Media was changed at 24 h. After 48 h, adherent mesenchyme was treated with 2.5 μg/ml recombinant mouse Shh or Ihh (R&D Systems), with or without 1 μg/ml recombinant noggin (R&D Systems) for 24 h, for a total culture of time 72 h. For RNA analysis, cells were lysed in 1 ml of Trizol (Sigma), and total RNA was prepared following the manufacturer’s protocol. For immunofluorescence, mesenchyme cultured on collagen-coated coverslips was fixed for 30 min in 4% PFA at 4 °C and staining was performed utilizing antibodies described above. For quantification of IF results, 10 fields per coverslip were randomly selected, imaged and counted by three blinded observers.
Smooth muscle induction in C3H/10T1/2 cells

C3H/10T1/2 cells (clone 8) were purchased from ATCC (www.atcc.org) and maintained in DMEM with 10% FBS at 37° in 5% CO₂. Cells were passaged at 30–50% confluency to prevent differentiation. For SMC development, 6 well plates containing coverslips were seeded with 2 × 10^5 cells per well. Cells were cultured for 24 h in maintenance media (above), transitioned to induction media (DMEM with 0.5% FBS) for 12 h, followed by addition of TGF-β1 (10 ng/ml final), Shh (2.5 μg/ml, R&D Systems) or Ihh (2.5 μg/ml, R&D Systems), with or without noggin (1 μg/ml, R&D Systems) in induction media for 24 h. For transfection, cells were cultured to 30–50% confluency, transfected with 2 μg/well of Gli2ΔN (Roessler et al., 2005) and induced to differentiate as described above. RNA preparation, QPCR, and immunofluorescence were performed as described above.

Identification of Gli binding sites, assessment of Gli binding site enrichment, and Luciferase assays

Please see the Supplementary Methods for details of these experiments.

Statistical analysis

Statistics were calculated using the t-test and ANOVA functions of GraphPad Prism (www.graphpad.com). P values and test used are indicated in the corresponding figure legends.

Results

VFHhip mice: a bitransgenic model of reduced Hh signaling in postnatal intestine

To evaluate the role of Hh signaling during postnatal intestinal development and homeostasis, we created a bi-transgenic model of Hh inhibition utilizing a modified version of the previously described 12.4KVillin-ΔHhip transgene (Madison et al., 2005). This modified transgene, 12.4KVillin-ΔLacZ-ΔHhipΔTM, uses the intestine-specific 12.4KVillin promoter (Madison et al., 2005) to drive expression of LacZ in the absence of Cre. When these mice are mated to mice carrying the intestine-specific 12.4KVillin-Cre transgene (Madison et al., 2005), excision of the LacZ cassette results in expression of HhipΔTM, a modified form of Hhip that lacks the transmembrane domain and acts as a secreted inhibitor of Hh signaling (Madison et al., 2005). We developed two independent transgenic lines of 12.4KVillin-ΔLacZ-ΔHhipΔTM with strong expression of LacZ in the small intestine, and bred each to 12.4KVilCre, which led to ablation of LacZ expression in the small intestine (Supplementary Fig. 1A, B) and caused mRNA expression of the recombined transgene VFHhip cDNA product by 1 month of age (Supplementary Fig. 1C). Expression of Gli1 and Ptc1, both direct target genes of Hh signaling, were significantly reduced in response to Hhip expression at 1 month (data not shown) and 3 months of age (Supplementary Fig. 1D), confirming that this strategy leads to effective postnatal inhibition of Hh signaling.

Chronic reduction in Hh signals causes crypt hyperplasia, expansion of SMC precursors, reduced villus SM and progressive inflammation

At birth, bi-transgenic VFHhip animals appear normal and no histological changes are notable in the small intestine (data not shown). This differs from the earlier direct transgenic overexpression of Hhip, in which a robust phenotype was seen at E18.5 (Madison et al., 2005); this difference is likely due to the later activation and lower levels of Hhip in the VFHhip model compared to the previous 12.4KVil-ΔHhip model (data not shown). Over the next few months of post-natal life, however, crypts deepen progressively in VFHhip animals. At 3 months, crypt depth is nearly twice that of single transgenic (ST) littermates (Figs. 1A, B). Ki67 staining reveals a hyperproliferative crypt compartment; proliferative epithelial cells are detectable even above the crypt opening in VFHhip animals (Figs. 1C, D). Our previous model of Hh inhibition in the fetus also exhibited ectopic epithelial proliferation and in that model, mis-localized myofibroblasts (normally confined to crypt regions) were seen to underlie ectopic pockets of epithelial proliferation on the villi (Madison et al., 2005). Thus, we examined myofibroblast positioning in the VFHhip model. These αSMA⁺ cells are confined to the crypt area in ST animals (Fig. 1E), but can be observed on villi proper in VFHhip animals (Fig. 1F).

Notably, as was observed in the fetus (Madison et al., 2005), the location of ectopic Ki67 positive epithelial cells in adults is very highly correlated with the presence of sub-epithelial myofibroblasts, suggesting a key role for myofibroblasts in controlling epithelial cell proliferation. Additionally, as in the fetus, the data suggest that Hh signals serve to restrict adult myofibroblasts to the crypt region.

![Fig. 1](image-url)
Like myofibroblasts, SMC precursors and differentiated SMC cells are direct cellular targets of Hh signaling in both fetal and adult mice (Kolterud et al., 2009; Madison et al., 2005). We therefore investigated whether postnatal inhibition of Hh signaling impacts these SMC populations. In VFHhip animals, the muscularis externa, muscularis mucosa, and villus core SMC appear normal at one month of age. However, at three months, SMC is reduced in the cores of the villi and the muscularis mucosa (Figs. 2A, B), although the muscularis externa is unaffected (not shown). In villus cores, αSMA⁺;desmin⁺ cells (differentiated SMC) are nearly absent, and αSMA⁺;desmin⁻ SMC precursor cells are expanded (Figs. 2A–D). In addition, breaks and gaps appear focally in the muscularis mucosa (arrows in Fig. 2E). These findings suggest that Hh signals are required for maintenance of villus and muscularis mucosa SMC populations during intestinal homeostasis.

Myeloid cells also transduce Hh signals and a previous study from our laboratory found a microarray signature consistent with changes in myeloid cell inflammatory phenotype in response to Hh administration in cultured intestinal lamina propria (Zacharias et al., 2010). Those studies suggested that Hh signals are required for tolerogenic myeloid cell function and that a reduction in Hh signaling leads to a pro-inflammatory phenotype in Cd11b⁺ and Cd11c⁺ myeloid populations. In the VFHhip mouse model, inflammation was indeed detected consistently in animals starting at 6 months of age and became progressively more prominent (Zacharias et al., 2010). Thus, it is possible that inflammatory cytokines also contributed to later expansion of the crypt zone in VFHhip animals.

Increased expression of \textit{lhh} drives smooth muscle differentiation

Since the phenotype of VFHhip animals indicates that decreased Hh signaling compromises SMC development, we tested whether increased Hh ligand production would promote SMC formation in vivo. We recently described Vlhh transgenic founders, generated by linking the 12.4 kb Villin promoter to a full length lhh cDNA (Kolterud et al., 2009). Here, we derived two transgenic lines carrying this transgene, and examined them over time for postnatal phenotypes. Effective transgene-mediated increases in the target genes \textit{Ptc1} and \textit{Gli1} are detectable in these mice by 3 months of age (Supplemental Fig. 2).

At birth, Vlhh mice appear normal. However, analysis of villus histology by H&E reveals that by 3 months of age, villi are broad, with clefted surfaces, but no notable alteration in villus architecture (Figs. 3A–B). In animals between 1 month and 1 year old, staining with αSMA and desmin reveals very few αSMA⁻;desmin⁺ SMC precursors, but confirms a progressive increase in differentiated (αSMA⁺;desmin⁻) SMC that, by 11 months, focally distort the villus architecture (Figs. 3C–G). The expansion of smooth muscle noted in Vlhh animals is confined to the villus smooth muscle population; no changes were noted in muscularis mucosa and muscularis externa smooth muscle populations at any timepoint examined.

Ki67 staining of Vlhh intestines reveals that epithelial proliferation is not changed in the first three months of life (Figs. 3H–I) and is not obviously changed even later in life in areas where villus architecture is largely preserved (Fig. 3J). However, in older Vlhh animals, in areas where villus morphology is disrupted by networks of interdigitated muscle, Ki67 staining demonstrates increased proliferation in adjacent epithelium (Fig. 3K). Ectopic myofibroblasts (αSMA⁻;desmin⁻) were not detectable in these regions, suggesting that signals for epithelial proliferation arose from a different cell type, possibly the amplified ectopic SMC themselves. Taken with previous findings that SMC precursors and differentiated SMC respond directly to Hh signals (Kolterud et al., 2009), these data indicate that Hh signaling drives SMC differentiation in the adult small intestine. Indeed, the adult SMC differentiation program appears to be quite responsive to the level of Hh ligand.

\textit{Hh} signal modulation does not affect epithelial lineage allocation

Hh signaling in the fetal and adult intestine is paracrine, from epithelium to underlying stroma (Kolterud et al., 2009). However, epithelial characteristics may be affected indirectly and it has been proposed that altered Hh signaling can affect Paneth cell census (Varnat et al., 2006). We therefore compared Vfhhip and Vlhh models, assessing the differentiation of the absorptive surface and quantitating the number of Paneth cells, goblet cells and enteroendocrine cells. As shown in Fig. 4, alkaline phosphatase staining is decreased, especially at the tops of the crypts/base of the villi in Vfhhip animals, suggesting that these cells are not fully differentiated, as predicted by the Ki67 results, above. In Vlhh mice, alkaline phosphatase staining extends to the crypt top as in WT mice. Epithelial cell lineage allocation was not visibly altered in the context of either decreased or increased Hh signaling. Careful quantification of Paneth, goblet and enteroendocrine lineages in our models demonstrates that there is no increase or decrease in any epithelial population per micron of epithelial surface (Supplemental Fig. 3). There is an absolute increase in the number of enteroendocrine cells per villus in Vlhh animals due to an increase in overall villus size, but no increase after normalization. Additionally, there are occasional Paneth cells found higher in the crypt of Vfhhip animals and a small absolute increase in the Paneth cell population in the crypt, but normalization to crypt depth demonstrates no increase compared to wild type animals. Therefore, we conclude that that, in the context of 3–5 fold modulation of Hh signals, as in these models, Hh signals do not play a major role in controlling lineage allocation in the intestinal epithelium.

\textit{Hh} signaling drives SMC differentiation in \textit{E18.5} intestinal mesenchyme

To confirm the role of Hh in SMC differentiation and to interrogate the pathway downstream of Hh, we examined the effect of Hh on
cultured intestinal mesenchyme from E18.5 WT and Gli1+/LacZ embryos. Before crypt formation, intestinal epithelium and mesenchyme can be cleanly separated; mesenchyme can be cultured for several days on a collagen matrix. Because the intestinal epithelium is the dominant source of Shh and Ihh (Kolterud et al., 2009), Hh signal transduction in isolated mesenchyme is severely reduced after 48 h in culture (Fig. 5A) as measured by lack of Xgal staining in cultures derived from Gli1+/LacZ animals (Bai et al., 2002). After treatment with Hh ligand for 24 h, strong LacZ expression is detectable, indicating robust Hh pathway activation (Figs. 5B, C). SMC precursor cells, myofibroblasts, and differentiated SMC are all present in vehicle treated culture after 72 h (Fig. 5D), confirming that this system is appropriate to examine the process of SMC differentiation in response to Hh pathway activation ex vivo.

We therefore treated isolated mesenchyme with vehicle, Shh, or Ihh (N = 4 wells per treatment) and quantified SM precursors, myofibroblasts, and differentiated SMC populations after 24 h. Vehicle treated mesenchyme contains a large population of αSMA−;desmin+ SM precursor cells (Fig. 5E). After treatment with Shh or Ihh, this smooth muscle precursor population is reduced by 50%, and the number of myofibroblasts and SMCs increase (Fig. 5E). These data suggest that Hh signaling promotes SMC differentiation in isolated mesenchyme, mirroring the findings seen in Vlhh animals.

Myocardin, a myogenic transcription factor, is downstream of Hh signaling

The fact that SM precursors and differentiated SMC express Gli1 (Kolterud et al., 2009) suggests that the effect of Hh on these cells might be direct. To understand the molecular pathway downstream of Hh signaling, we surveyed known intracellular inducers of SMC differentiation, focusing especially on transcriptional regulators of SMC fate. Myocardin is a crucial regulator of smooth muscle differentiation (Du et al., 2003). Therefore, we examined the expression of Myocd in VFHhip and Vlhh mice. Similar to the known Hh target genes, Bmp4 (Fig. 6A), Gli1 and Ptch1 (Supplemental Fig. 1D; Supplemental Fig. 2), Myocd expression was increased in Vlhh animals and decreased in VFHhip mice at 3 months (Fig. 6A). The decrease in Myocd expression seen in VFHhip animals, though not statistically significant, was reproducible. We suspect that the smaller response in these animals is due to the fact that the majority of SMC in this model is muscularis externa and this layer was not affected by downregulation of Hh signaling in VFHhip animals.

We next confirmed that Myocd is activated by Shh and Ihh in isolated intestinal mesenchyme (Figs. 6B–D). Kinetic studies performed on cultured intestinal mesenchyme confirmed that Myocd is clearly activated within 16 h (Figs. 6C, D). Importantly, the induction of Myocd by Ihh or Shh occurs with similar kinetics to the induction of the known Hh targets Gli1 and Ptch1, indicating that regulation could be direct (Figs. 6C, D). Another SMC expressed factor that has been shown to be involved in SMC differentiation, Igf1, did not respond acutely to Hh ligand addition in these cultures (Figs. 6C, D), suggesting that the response of Myocd is secondary to Hh induction rather than to SMC differentiation.

Hh signaling is sufficient to activate smooth muscle differentiation of C3H10T1/2 cells

Because the mesenchymal culture system is complex and contains many cells that do not respond to Hh signaling, we sought to examine the process of Hh-mediated SMC differentiation in a more homogeneous in vitro system. C3H10T1/2 is a mouse embryonic pluripotent mesenchymal cell line that expresses Ptch1 and responds to Hh
These cells have been shown to differentiate into αSMA+ SMC in response to treatment with Tgfβ1 in the presence of low serum (Lien et al., 2006). We confirmed that, in our hands, 10T1/2 cells express Ptch1 and Gli1 RNA as well as desmin but not αSMA protein (data not shown), suggesting that they are similar in phenotype to the smooth muscle precursor population of the mouse intestine.

Low passage, non-confluent 10T1/2 cells were treated with Shh, Ihh, Tgfβ1, or vehicle in low serum media and SMC differentiation was examined. As previously reported, Tgfβ1 treatment activates the SMC differentiation program as assessed by morphological change and by robust αSMA expression in the majority of cells (~70%, N=10 wells), whereas serum withdrawal alone causes very little SMC differentiation (Figs. 7A, B). Treatment with either Shh or Ihh also causes robust SMC differentiation (N=10 wells) (Figs. 7C, D), confirming the results seen in mesenchymal cultures.

Q-RT-PCR was used to further query the SMC differentiation program 24 h after induction with Ihh, Shh or Tgfβ1. These studies confirm that all three soluble factors induce SMC genes αSMA, SM22α, as well as Myocd (Fig. 7E). Notably, Shh or Ihh upregulates Myocd nearly two fold more robustly than Tgfβ1, though the overall degree of apparent SMC differentiation, assessed morphologically or by SMC gene expression, is similar for all three treatments.
A large number of Gli binding sites are present in intron 1 of mammalian Myocardin genes

The data above suggest that Hh activates SMC differentiation and that the SMC master regulator, Myocd, responds to Hh signals. To identify a region of the Myocd gene that might be responsible for this activity, we surveyed 100 kb of genomic sequence containing and surrounding the Myocd locus on mouse chromosome 11 for potential Gli binding sites (Supplementary Fig. 4 and Supplemental Methods). A 12 kb sequence, extending from just upstream of the transcriptional start site into intron 1, exhibited a statistically significant enrichment of Gli binding sites (Supplementary Fig. 4B). To investigate whether this region is responsible for the activation of Myocd expression downstream of Hh signaling, we cloned the Gli-enriched regions of this statistically predicted putative regulator element into Luciferase vectors, and tested whether these regions are sufficient to induce Luciferase expression in vitro (Supplementary Fig. 4). However, none of the cloned regions exhibited statistically significant Gli-dependent up-regulation of reporter activity (experimental details are presented in Supplementary Materials). Thus, we conclude that, though enriched in Gli sites, this region is not sufficient to drive the Hh-dependent expression of Myocd.

Hedgehog signals induce SMC differentiation in C3H10T1/2 cells independent of BMP and in a cell-autonomous manner

It has been suggested previously that Hh influences SMC differentiation indirectly, through the activation of Bmp4 (Caubit et al., 2008). As we were unable to directly identify a genomic enhancer through which Hh regulates Myocd, we sought to investigate whether BMP signaling is required for the activation of the SMC program downstream of Hh in C3H10T1/2 cells. We therefore treated cells simultaneously with Hh and the Bmp inhibitor, noggin. Noggin treatment did not inhibit the ability of Hh to promote SMC differentiation, indicating that the mechanism of SMC differentiation downstream of Hh ligand does not require active BMP signaling (Figs. 8A–B).

Next, we asked whether Gli factors can cell autonomously drive SMC differentiation. To that end, we utilized transfection with Gli2ΔN to robustly activate the Hh pathway in specific cells in culture. We transfected C3H10T1/2 cells with Gli2ΔN, then withdrew serum to induce SMC differentiation and assessed the correlation between SMC differentiation and expression of Gli2ΔN, as detected by its Myc epitope tag (Figs. 8C–E). A total of 63% of cells transfected with Gli2ΔN expressed αSMA and took on the morphology of SMCs, compared to 8% of cells transfected with GFP alone (N=4 independently transfected wells, Fig. 8F). In addition, non-expressing cells adjacent to Gli2ΔN-expressing cells did not develop into SMCs (Figs. 8C–E), further supporting the idea that transduction of Hh signals drives SMC differentiation in a direct, cell-autonomous manner, rather than through induction of a secondary secreted signal.

Discussion

These data demonstrate that Hh signaling is an important regulator of adult small intestinal homeostasis, with a critical role in the maintenance of smooth muscle populations in the villus core and muscularis mucosa. We show that Hh signaling is necessary and sufficient for villus smooth muscle differentiation in vivo and demonstrate in two in vitro models that Hh signaling can drive smooth muscle differentiation in a cell autonomous manner. Importantly, we show that
relatively modest alterations in Hh ligand concentration result in profound changes in villus smooth muscle mass.

In both of the mouse models described here, Hh-dependent alterations in SMC differentiation are accompanied by changes in the expression of the SMC master regulator, Myocardin. Kosinski et al. (2010) also observed that Myocd is among the prominently down-regulated genes in colon following conditional deletion of Ihh using a Villin-Cre driver. Additionally, Myocd expression was found to be reduced in the lungs of Shh null animals (Miller et al., 2004), indicating that the regulation of Myocd by Hh signaling may also occur outside the GI tract. However, these results do not clearly distinguish between modulation of Myocd as a part of the SMC differentiation program and direct transcriptional regulation of Myocd by Hh. Indeed, as shown in Fig. 7, Myocd is up-regulated (in the absence of Hh) as part of the SMC differentiation program and direct transcriptional regulation of Myocd by Hh. Indeed, as shown in Fig. 7, Myocd is up-regulated (in the absence of Hh) as part of the SMC differentiation program and direct transcriptional regulation of Myocd by Hh. In both of the mouse models described here, Hh-dependent alterations in SMC differentiation are accompanied by changes in the expression of the SMC master regulator, Myocardin. Kosinski et al. (2010) also observed that Myocd is among the prominently down-regulated genes in colon following conditional deletion of Ihh using a Villin-Cre driver. Additionally, Myocd expression was found to be reduced in the lungs of Shh null animals (Miller et al., 2004), indicating that the regulation of Myocd by Hh signaling may also occur outside the GI tract. However, these results do not clearly distinguish between modulation of Myocd as a part of the SMC differentiation program and direct transcriptional regulation of Myocd by Hh. Indeed, as shown in Fig. 7, Myocd is up-regulated (in the absence of Hh) as part of the SMC differentiation program and direct transcriptional regulation of Myocd by Hh. In those settings, high levels of Hh and the ensuing high levels of Bmp are inhibitory to smooth muscle development, a characteristic that was thought to be required for the correct concentric patterning of the muscularis externa on the outside of the intestinal tube, where Hh (and Bmp) levels are low (Sukegawa et al., 2000; Yu et al., 2002). However, in our Ihh over-expressing mice, postnatal Hh signaling is increased by as much as 5 fold (as assessed by Gli1 activity, Supplemental Fig. 2). Such high Hh signals do not inhibit, but in fact robustly promote SMC development, even in areas directly adjacent to the Ihh-expressing epithelium. Moreover, even though Bmp levels are increased in Vlhh intestinal mesenchyme, our in vitro studies indicate that Hh signals can, at least in some contexts, induce Myocd and promote SMC differentiation independently of Bmp signaling. Of course, it is also possible that Bmp and Hh signaling pathways are used in parallel. Indeed, in the chick heart, both Myocd and Nkx2.5 are activated during heart development and both are important for...
and show that intestinal SMC populations are very sensitive to
proper control of smooth muscle populations is required for proper GI
expression and cell shape change in a manner similar to Tgf-
expression, though dependent upon an unknown endodermal signal,
is independent of Bmp signaling (Ueyama et al., 2003; Warkman et al.,
2008). It will be important to test whether the maintenance of
intestinal villus smooth muscle is truly independent of Bmp signals in
vivo.

Smooth muscle dysfunction is seen in several disorders and
malformations of the gastrointestinal tract, including VACTERL (Kim
et al., 2001; Rittler et al., 1996), irritable bowel syndrome (Choudhury
et al., 2009), visceral myopathies (potentially leading to chronic
pseudo-obstruction) (Antonacci et al., 2008) and mesenchymal
tumors (Miettinen and Lasota, 2006), reaffirming the notion that
proper control of smooth muscle populations is required for proper GI
function. Our data implicate Hh and Myocardin directly in this control
and show that intestinal SMC populations are very sensitive to
changes in Hh levels. In this regard, it is interesting that SMC are
plastic in that they can reversibly switch between a proliferative and
differentiated phenotype. In mouse models of intestinal obstruction,
SMC anterior to the obstruction become proliferative and later,
hypertrophic (Chen et al., 2008). Potentially relevant is the fact that
Myocd expression is reduced in the proliferative phase and activated
during hypertrophy and differentiation of these cells (Chen et al.,
2008). An important future goal will be to examine whether changes
in Hh signal transduction are responsible for driving these adapta-
ations. Additionally, since Hh signaling is also important in urinary,
respiratory, and vascular smooth muscles, our data linking Hh to
Myocardin induction may have implications for smooth muscle
development and pathology in multiple organs.

In the mesenchymal compartment of the intestine, the cellular
targets of Hh signals (e.g., smooth muscle precursors, differentiated
smooth muscle cells, myofibroblasts) are similar in fetus and adult
(Kolterud et al., 2009). As was seen in the fetus (Madison et al.,
2005), we observed that reduced Hh signaling in the adult leads to
mislocalization of myofibroblasts, cells that are thought to produce
Wnt ligands and/or proteins that potentiate the Wnt signal, which are
important in the maintenance of epithelial proliferation (Powell et al.,
1999). Interestingly, ectopic crypt pockets and branched villi,
characteristic aspects of the pathology associated with reduced Hh
signaling in the fetus (Kosinski et al., 2010; Madison et al., 2005;
Wang et al., 2002), are not seen when Hh signaling is disrupted in the
adult (van Dop et al., 2010) and this study). Indeed, crypts are
expanded in Vfhhip animals and areas of ectopic epithelial prolifer-
ation are seen above mislocalized myofibroblasts, but overall crypt/
villus polarity is maintained. These findings indicate that villus
polarity is established, in a Hh-dependent manner, in fetal life and
that later reduction in Hh signaling does not disrupt polarity.
However, in 11–12 month old Vfh animals, focal areas of distorted
crypt architecture and branched villi are also accompanied by hyper-
proliferative epithelium. These areas are surrounded by extensively
hypertrophied SMC (Fig. 3G), but not by myofibroblasts, suggesting
that, in this setting, the signal for epithelial proliferation may emanate
from the ectopic SMC and/or associated extracellular matrix. These
findings suggest that two different cell types impacted by Hh signals
(myofibroblasts and SMC) may be important in tightly tuning the
proliferative level of the epithelial stem cell niche; either too much or
too little Hh appears to promote crypt proliferation through SMC and
myofibroblasts, respectively, in the adult intestine. Additionally, we
and others have seen that reduced Hh signals contribute to
progressive inflammation (Kosinski et al., 2010; van Dop et al.,
2010; Zacharias et al., 2010). Though inflammatory infiltrates are first
clearly visible in Vfhhip animals at 6 months, while crypt deepening
is detectable much earlier, it is not possible to rule out a role for
cytokine signaling in progressive crypt expansion. These data could
have important implications for colon cancer since studies in human
tissues and cell lines suggest that increased Hh signaling may drive
tumor growth and metastasis in late stage colon cancer (Varnat et al.,
2009). Such studies provide justification for ongoing phase II clinical
trials for the treatment of metastatic colorectal cancer patients with

It has been proposed that Hh participates in a negative feedback
loop whereby differentiated epithelial cells (which express the
majority of the Ihh ligand in the adult) use Hh signals to report
their census and/or integrity to surrounding mesenchymal cells.
When Hh levels are lowered, as in injury, the mesenchymal
compartment responds by promoting epithelial proliferation via
elaboration of its own soluble signals (van den Brink et al., 2004).
Conversely, increases in epithelial Hh signaling are posited to instruct
the mesenchyme to restrict epithelial proliferation (van den Brink
et al., 2004; van Dop et al., 2009). We (and others) have confirmed
that reduced Hh signal transduction results in increased proliferation,
both in the fetus and in the adult (Kosinski et al., 2010; Madison et al.,
2005).
2005; van Dop et al., 2010; Wang et al., 2002). But the studies here, which are the first to examine the consequences of increased Hh signaling in the small intestine, do not support the idea that augmented Hh signaling dampens epithelial proliferation. Indeed, in older VIhh mice, chronically increased Hh ligands seem to promote, rather than inhibit, the expansion of the proliferative compartment, though this occurs in association with, and may be secondary to, severe alterations in mesenchymal patterning. The negative feedback model is also difficult to reconcile with the observation that, as villi emerge in the fetus, Shh and to some extent Ihh, are concentrated in intervillus regions where epithelial proliferation is also most prominent (Kolterud et al., 2009; Madison et al., 2005). We argue that our findings, observed after modest perturbations of Hh signaling levels (augmentation and reduction), are likely relevant to normal homeostatic regulation in the small intestine. The data presented here are most consistent with the idea that epithelial Hh signaling is being used simultaneously in (at least) two different ways in a homeostatic setting: it is necessary and sufficient for promoting SMC differentiation (a reduced Hh signal decreases and an increased Hh signal increases the amount of differentiated SMC) and it indirectly limits the extent of epithelial proliferation (too little Hh allows expansion of this compartment, but increased Hh does not further dampen proliferation).

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Appendix A. Supplementary data

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References