Sonic hedgehog controls enteric nervous system development by patterning the extracellular matrix

Nandor Nagya, b*, Csilla Baradb, Hannah Grahama, Ryo Hottaa, Lily Chenga, Nora Fejszakb, Allan M. Goldsteina

aDepartment of Pediatric Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA

bDepartment of Human Morphology and Developmental Biology, Faculty of Medicine, Semmelweis University, Budapest-1094, Hungary

* Corresponding Author:
Nandor Nagy
Semmelweis University
85 Tuzolto st
Budapest, Hungary 1094
Tel: 2156920, Fax: 2153064
Email: nagy.nandor@med.semmelweis-univ.hu

Keywords:
enteric nervous system; sonic hedgehog; patched; epithelium; extracellular matrix; enteric neural crest cells; Hirschsprung disease
Abstract
The enteric nervous system (ENS) develops from neural crest cells that migrate along the intestine, differentiate into neurons and glia, and pattern into two plexuses within the gut wall. Inductive interactions between epithelium and mesenchyme regulate gut development, but the influence of these interactions on ENS development is unknown. Epithelial-mesenchymal recombinations were constructed using avian hindgut mesenchyme and non-intestinal epithelium from the bursa of Fabricius. These recombinations led to abnormally large and ectopically positioned ganglia. We hypothesized that Sonic hedgehog (Shh), a secreted intestinal epithelial protein not expressed in the bursa, mediates this effect. Inhibition of Shh signaling, by addition of cyclopamine or a function-blocking antibody, resulted in large, ectopic ganglia adjacent to the epithelium. Shh overexpression, achieved in ovo using Shh-encoding retrovirus and in organ culture using recombinant protein, led to intestinal aganglionosis. Shh strongly induced the expression of versican and collagen type IX, whereas cyclopamine reduced expression of these chondroitin sulfate proteoglycans known to be inhibitory to neural crest cell migration. Shh also inhibited ENCC proliferation, promoted neuronal differentiation, and reduced expression of glial-derived neurotrophic factor, a key regulator of ENS formation. Ptc1 and Ptc2 were not expressed by ENCCs, and migration of isolated ENCCs was not inhibited by Shh protein. These results suggest that epithelial-derived Shh acts indirectly on the developing ENS by regulating the composition of the intestinal microenvironment.
Introduction

Gut development relies on interactions among cell types originating from the three germ layers: endoderm, which gives rise to epithelium; mesoderm, which forms the smooth muscle, endothelial cells, and connective tissues; and ectoderm, which gives rise to the enteric nervous system (ENS). Enteric neural crest-derived cells (ENCCs) emigrate from the vagal level of the neural tube, turn ventrally to colonize the foregut, and undergo massive proliferation as they continue caudally to populate the embryonic gut (Yntema and Hammond, 1954; Burns and Le Douarin, 2001). In the developing hindgut, vagal crest-derived cells join neural crest cells originating from the sacral level of the neural tube to form the ENS (Burns and Le Douarin, 1998; Nagy et al., 2007, 2012). The ENS consists of two ganglionated plexuses, myenteric and submucosal, composed of multiple types of neurons and glia arranged as concentric rings and responsible for regulating the function of the gut, including peristalsis. Congenital abnormalities of the ENS cause severe intestinal disorders, such as Hirschsprung disease (HSCR; Goldstein et al., 2013), characterized by the absence of enteric ganglia along a variable length of distal intestine. Other ENS abnormalities include hyperganglionosis, ectopic ganglia, and hypoganglionosis, which are often associated with intestinal dysmotility (Kapur, 2000).

ENS development relies on reciprocal interactions between ENCCs and their environment which is critically important for ENCC survival, migration, proliferation, patterning, and differentiation. Deficiency in the ENCC receptors Ret or Ednrb, for example, or their respective mesenchymal ligands, Gdnf or Edn3, leads to intestinal aganglionosis (Goldstein and Nagy, 2008; Lake and Heuckeroth, 2013). Previous studies have shown that extracellular matrix (ECM) proteins in the gut mesenchyme regulate ENCC migration and differentiation (Wu et al., 1999; Breau et al., 2009; Druckenbrod and Epstein, 2009; Nagy et al., 2009; Akbareian et al., 2013; Raghavan et al., 2013). This mesenchymal environment is highly influenced during gut organogenesis by the developing epithelium (Roberts, 2000). One would therefore expect that epithelial abnormalities could influence ENS development by altering these inductive interactions. Diffusible factors produced by gut epithelium, including Netrin and Hedgehog (Hh) proteins, are potential candidates. Netrin-1 promotes radial migration of ENCCs from myenteric to submucosal region and also prevents premature apoptosis of ENCCs (Jiang et al., 2003). The role of Hh proteins is less clear. A genome-wide association study on HSCR reported genetic variants in Ptc1, supporting a potential role for the Hh pathway in the etiology of aganglionosis (Ngan et al., 2011). Mice deficient in sonic hedgehog (Shh) have increased enteric neurons and abnormally distributed ganglia. Deletion of indian hedgehog (Ihh) leads to segmental aganglionosis in rodents (Ramalho-Santos et al., 2000). Shh:Ihh double mutants display a major reduction in neuronal numbers in the stomach (Mao et al., 2010). In addition, targeted deletion of the G-protein coupled receptor for Shh, Smoothened (Smo), decreases enteric neuronal number, while overexpression of the downstream transcription factor, Gli1, causes aganglionosis (Yang et al., 1997; Huang et al., 2013). In order to clarify how Shh signaling controls ENS patterning, we used the avian embryo and applied a variety of techniques, including tissue recombination, organ culture, retroviral-mediated gene overexpression, and cell migration assays, confirming our observations in the mouse embryonic gut. In the absence of Shh-expressing epithelium, large and ectopic enteric ganglia develop. When Shh is overexpressed, aganglionosis ensues. These phenotypes do not result from a direct effect of Shh on ENCCs since Ptc1 and Ptc2 receptors are not expressed on these cells. Rather, it appears that the effect of Shh on the ENS is mediated through the ECM, where Shh induces proteins that inhibit ENCC migration.
Results

Shh and Ptc1 are expressed in the developing gut during ENS development.

At E6 (HH28), the preganglionic hindgut epithelium expresses Shh, as shown by in situ hybridization (Fig. 1A) and by immunofluorescence (Fig. 1B). Fixation with Histochoice increased the intensity of staining, showing a gradient deposition of Shh protein in the subepithelial mesenchyme (Fig. 1B, inset). Adjacent sections were hybridized with the Shh receptor, Ptc1, and reveal Ptc1 transcript in the mesenchyme under the luminal epithelium (Fig. 1C). When migrating ENCCs reach the distal hindgut at E8 (HH34), Ptc1 is expressed underneath the epithelium as well as in a second circumferential ring (Fig. 1D). This outer mesenchymal Ptc1 expression appeared to overlap the presumptive submucosal plexus, but Ptc1 in situ (Fig. 1D,E) combined with p75 immunofluorescence to mark ENCCs (Fig. 1F; Young et al., 1998; Nagy et al., 2012) showed no overlap, and therefore no Ptc1 expression by ENCCs. Shh and Ptc1 expression were assessed at later stages of hindgut development. At E13 (HH39), Ptc1 continues to be expressed by the subepithelial mesenchyme (Fig. 1G), and not the epithelium itself, as shown in the magnified inset (Fig. 1G). Shh is expressed specifically by the gut epithelium (Fig. 1H) and not the epithelium of the bursa of Fabricius (Fig. 1H), which is positive for E-cadherin (Fig. 1I). Similarly, hindgut contains Hu+ enteric neurons, while the adjacent bursa does not (Fig. 1J). At E16, Ptc1 is restricted to the lamina propria, between the epithelium and muscularis mucosae, and is not expressed in the submucosa (Fig. S1A). After hatching, Ptc1 expression in the colon remains limited to the lamina propria (Fig. S1B,C). At no stages examined was Ptc1 expression seen in the ENS.

Expression of Ptc2 was also examined by in situ hybridization and expression did not overlap with p75+ ENCCs (Fig. S2).

Signaling from the hindgut epithelium is critical for ENS development.

Given the observation that the ENS-containing hindgut epithelium expresses Shh, while the bursa has neither an ENS nor Shh expression, we hypothesized that the gut epithelium is critically important for normal ENS development, as previously suggested in the stomach (Sukeygawa et al., 2000). To study the effect of hindgut and bursa epithelial signaling on ENS development, chick-quail tissue recombination experiments were performed. The epithelium of the bursa of Fabricius from E9 (HH35) chick embryos was separated from the underlying mesenchyme and recombined with E5 (HH27) quail hindgut mesenchyme (Fig. S3A). E9 bursa epithelium is used because the epithelial anlagen of this organ develops at E8, later than the hindgut epithelium. To ensure epithelial-mesenchymal adherence, recombinants were embedded into a three-dimensional collagen gel overnight (Fig. S3B; Nagy and Goldstein, 2006a). Staining with QCPN confirms the quail origin of the mesenchyme (Fig. S3C). Tissue recombinations were implanted into the coelomic cavity of E3 (HH19) chick embryos (Fig. S3D), a model that allows host neural crest cells to colonize the transplanted intestine (Nagy and Goldstein, 2006b). After 9 days, the graft is removed for immunohistochemistry, and results compared to control recombinations in which E5 quail hindgut mesenchyme was recombined with E5 chicken hindgut epithelium. QCPN immunostaining shows that the mesenchyme is derived from quail (Fig. 2A,E) and 8F3 staining confirms that both epithelium and ENS are chicken-derived (Fig. 2B,F).

In recombinations using bursa epithelium, large and ectopic Hu+ ganglia are located abnormally close to the epithelium, notably different than control (Fig. 2C,G). As shown in Fig. 2I, the distance from the epithelial basement membrane to the submucosal ganglia is significantly shorter in the presence of bursa epithelium (56±25µm) as compared to controls using gut epithelium (83±20µm). Submucosal ganglia are also markedly larger (92±41µm v.
35±17µm; Fig. 2J). Staining with alpha-smooth muscle actin shows a significant difference in muscle thickness (Fig. 2D,I). Total thickness of the gut wall and of the muscularis propria were both markedly increased in bursa recombinants (K,L), with the muscularis propria comprising a greater proportion of the total gut wall (M). In bursa epithelial recombinants, the muscularis mucosae is also located immediately under the epithelium (H), much closer than normal (D), suggesting loss of the normal lamina propria.

**Inhibition of Shh signaling leads to intestinal hyperganglionosis.**

Given the absence of Shh expression in bursa epithelium and the abnormal ENS development in bursa epithelial recombinants, we hypothesized that Shh is a critical epithelium-derived factor during ENS formation. To test this, we modulated Shh activity in explanted embryonic intestine. E5 chick gut was cultured 3 days in the presence of Shh protein (2 µg/ml) or cyclopamine (2 µM). Wholemount and longitudinal sections were stained with Hu antibody (Fig. 3). Normally, by E5 enteric neurons have only migrated to the level of the distal midgut (Fig. 3A, arrow), but reach the distal hindgut by E8 (Fig. 3B, arrow). Similarly, E5 intestine cultured for 3 days demonstrates full ENCC colonization of the hindgut (Fig. 3C). In contrast, when cultured in the presence of excess Shh protein, the hindgut remains aganglionic (Fig. 3D,I), whereas addition of cyclopamine to inhibit Shh signaling leads to complete colonization and the formation of large, ectopic ganglia (Fig. 3E,J).

The effect of Shh inhibition on ENCC migration could result from changes to ENCC proliferation, survival, or differentiation. We examined cell proliferation by measuring BrdU incorporation into p75+ ENCCs at the migratory wavefront in the presence of Shh protein or cyclopamine. In control, non-treated hindguts, BrdU+ cells are primarily seen in the submucosal mesenchyme and in 29.7% ± 8.7% of p75+ cells (Fig. 4A). Shh treatment increased the proliferation of mesenchymal cells (Fig. 4B), but had no effect on the rate of ENCC proliferation (22.9% ± 11.4%). In contrast, cyclopamine dramatically increased proliferation of p75+ ENCCs (Fig. 1C; 52.8% ± 14.8%) compared to both other groups (Fig. 4D), suggesting that inhibition of Shh signaling promotes ENCC proliferation in the hindgut.

To determine whether Shh induces ENCC apoptosis, we examined expression of activated caspase-3, a marker of programmed cell death. Caspase-3 expression was not present in any of the treatment groups (Fig. 4E-G). Apoptosis was occasionally identified in the gut mesenchyme, and was readily seen in the E8 dorsal sensory ganglia (Fig. 4D), which is known to contain apoptotic neural crest cells and therefore served as a positive control.

**Shh promotes neuronal differentiation in the hindgut.**

To determine whether Shh induces premature neuronal differentiation, thus accounting for the aganglionic phenotype, Tuj1 and neurofilament double-immunofluorescence was performed. In the chick, the distal gut is colonized by p75+ ENCCs at E8, and immediately followed by neuronal and glial differentiation. Tuj1 antibody marks early differentiating neurons, while neurofilament expression identifies later neurons (Nagy et al., 2012). After culturing E5 intestine for 3 days with no additive or with cyclopamine, Tuj1+ cells are found in both enteric plexuses along the length of the gut, but neurofilament is not yet expressed (Fig. 4H-I’). In Shh-treated cultures, Tuj1+ cells are present only at the level of the cecum, consistent with hindgut aganglionosis. Interestingly, the majority of these Tuj1+ cells co-express neurofilament (Fig. 4H-J), suggesting that Shh may induce premature differentiation of wavefront cells, leading to aganglionosis.
Misexpression of Shh changes extracellular matrix patterning in the hindgut.

While Shh has a major influence on ENS development, the absence of Shh receptor expression on ENCCs indicates that the effect is mediated indirectly via alterations in the microenvironment. We determined how modulating Shh signaling affects expression of ECM proteins known to be permissive or inhibitory to neural crest cell migration. We tested the following ECM proteins: collagen I, III, VI, IX, XVIII; laminin; fibronectin; versican; tenascin; agrin; heparan sulfate; and chondroitin sulfate proteoglycan (CSPG). Fibronectin is uniformly expressed throughout the gut mesenchyme, with intense fibrillar staining in the inner mesenchymal layer. Presence of Shh or cyclopamine did not significantly alter this expression pattern (Fig. 5A-D). Similarly, expression of collagen III, VI, and XVIII; laminin; agrin; and heparan sulfate were not significantly altered (data not shown). In E8 hindgut, collagen I is normally expressed in the outer mesenchyme (Fig. 5E,F). In contrast, E5 hindgut cultured for 3 days in the presence of Shh or cyclopamine shows an altered distribution pattern, with collagen I fibers uniformly expressed throughout the gut wall (Fig. 5G).

The most dramatic ECM effects, however, were observed with molecules in the CSPG family, known inhibitors of neural crest cell migration (Ring et al., 1996; Dutt et al., 2006). We observed significant Shh-induced changes in collagen IX, CS-56 (marks glycosaminoglycan portion of CSPG core protein), and versican isoforms (including anti-GAGβ or anti-GAGα protein domain-specific antibodies and an antibody that recognizes common epitopes on core protein in the V0 and V2 isoforms; Landolt et al., 1995; Zanin et al., 1999; Dutt et al., 2006; 2011). As shown in Fig. 5, collagen IX and versican V0/V2 are normally expressed strongly in the inner mesenchymal layer, just beneath the epithelium. CS-56 has a similar expression pattern (Fig. S4B). After Shh treatment, both collagen IX and versican V0/V2 were dramatically upregulated and distributed throughout the mesenchyme (Fig. 5K,O). The same pattern was observed with isoform-specific antibodies to versican (data not shown). In contrast, cyclopamine treatment markedly reduced collagen IX and versican, limiting it to a small ring of subepithelial mesenchyme (Fig. 5L,P).

To confirm the effect of Shh inhibition on ECM expression, Shh activity was also inhibited with a function-blocking antibody (5E1; Ericson et al., 1996) and results compared to control guts treated with a non-specific monoclonal antibody. p75 expression demonstrates large, ectopically positioned ganglia following 5E1 treatment (Fig. S4E), as observed with cyclopamine (Fig. 3J). Treatment with 5E1 reduced expression of CS-56, collagen IX, and versican V2 proteins, restricting them to the inner mesenchyme (Fig. S4F,G,H). The radial extent of versican expression was quantitatively analyzed (Fig. S4I) in guts treated with no additive, Shh protein, cyclopamine, and 5E1 and confirmed the immunohistochemical results shown in Fig. 5O,P and Fig. S4H. The extent of versican inhibition following 5E1 (Fig. S4H) is less pronounced than following cyclopamine (Fig. 5P), likely due to the more potent Shh inhibition achieved with cyclopamine, as noted previously (Liu et al., 2004).

Given the important role of mesenchymal Gdnf and Edn3 during ENS development, we used quantitative RT-PCR to determine their transcript levels in E5 guts cultured in the presence or absence of Shh. As shown in Fig. 6, Shh significantly reduced Gdnf expression compared to control, while Edn3 expression was unchanged.

Retrovirus-mediated overexpression of Shh induces aganglionosis in vivo.

To test whether the effect of Shh on ENS development could be recapitulated in vivo, Shh was misexpressed in avian embryos using a replication competent retrovirus (RCAS) expressing chick Shh. RCAS virus was injected into the presumptive hindgut mesoderm at E2 (HH10-12), as previously described (Goldstein et al., 2005). The gross morphology of RCAS-Shh infected embryos was markedly different by E5, when extra digits could be seen
in the hindlimb (Fig. 7A; normal embryos have 4 digits at this stage), as previously reported (Riddle et al., 1993). By E8, 3C2 antibody, which recognizes the gag protein P19 of RCAS (Potts et al., 1987), demonstrates extensive viral expression throughout the gut wall (Fig. 7B). Importantly, HNK1-positive ENCCs are absent from the hindgut mesenchyme where Shh is overexpressed (Fig. 7C), consistent with Shh-induced aganglionosis, as seen in organ culture (Fig. 3D,I). Since 80% of embryos die by E8 after RCAS-Shh infection, E5 midgut/hindgut explants were injected with RCAS-Shh ex vivo, and transplanted onto a chick chorioallantoic membrane (CAM) for 9 days (Fig. 7D,E). Successful viral replication was confirmed with 3C2 immunostaining (Fig. 7F), and robust Shh overexpression was confirmed using Shh-specific antibody (Fig. 7G). ENCC colonization of the Shh-overexpressing hindgut was markedly diminished, with severe hypoganglionosis in the proximal colon (Fig. 7H) and aganglionosis distally. Furthermore, enteric ganglia proximal to the aganglionic segment are small, sparse, and ectopic, with no organized patterning into discrete submucosal and myenteric plexuses (Fig. 7H), as compared to wild-type control (Fig. 7K). Also, Hu+ enteric neurons are seen immediately adjacent to the epithelium (Fig. H, arrow), where they normally do not occur. Smooth muscle development is severely disorganized (Fig. 7I), as compared to the normal patterning of the three muscle layers (muscularis mucosae, circular and longitudinal) at this stage (Fig. 7L). Interestingly, collagen IX expression is dramatically increased in the presence of Shh overexpression (Fig. 7J), as compared to wild-type control (Fig. 7M). Versican showed a similar change (data not shown).

Shh induces collagen IX expression.
Collagen IX is normally expressed by the gut, and not bursa, mesenchyme (Fig. 8A). Shh-expressing epithelium was isolated from E6 chick hindgut and recombined with E9 chick bursa mesenchyme (Fig. 8B). Recombinants were cultured in the E3 chick coelom for 7 days and collagen IX immunostaining performed. The presence of hindgut epithelium induced expression of collagen IX in the bursa mesenchyme (Fig. 8C). To test whether Shh is responsible for inducing this expression, Affi-gel beads soaked in Shh protein were implanted into E9 bursa primordia and cultured on a CAM for 9 days (Fig. 8D). Shh can be detected in the surrounding bursa mesenchyme (Fig. 8E), where a ring of collagen IX immunoreactivity is seen (Fig. 8F), suggesting that Shh induces expression of this ECM protein.

Shh does not act directly on ENCCs to inhibit their migration.
Given the significant changes in ECM expression induced by Shh, and the lack of Ptc1 expression by ENCCs, our results suggest that Shh does not directly affect ENCC development, but rather disrupts the microenvironment and indirectly leads to aganglionosis. To determine whether Shh has a direct effect on ENCC migration, E8 midgut was cultured with Gdnf (10 ng/ml) or with both Gdnf and Shh (2 μg/ml) for 48 hours (Fig. 9). As expected, Gdnf leads to extensive migration of ENCCs into the surrounding collagen gel (Fig. 9A), and Shh inhibits this Gdnf-mediated migratory effect (Fig. 9B). Cross-sections of the guts confirm the presence of Tuj1+ enteric neurons in the surrounding gel following Gdnf treatment (Fig. 9C), but not if Shh is added (Fig. 9D). Importantly, versican expression is significantly upregulated by Shh (Fig. 9E,F). To distinguish between a direct versus indirect effect of Shh on ENCCs, E6 midgut was cultured on a fibronectin-coated surface for 24 hours in the presence of Gdnf, which again led to robust ENCC migration onto the surrounding surface, extending approximately 150 μm (Fig. 9G). In situ hybridization with a Ptc1 riboprobe shows Ptc1 expressed in the gut, but not in the migrating HNK1+ neural crest cells (Fig. 9H). After 24 hours in the presence of Gdnf, soluble Shh protein was added to the media and cultures incubated for an additional 48 hours. As shown in Fig. 9I, robust ENCC migration continued in the presence of Shh, with cells migrating up to 500 μm (n=10). We
conclude that in the absence of the gut mesenchyme, Shh has no direct inhibitory effect on ENCC migration.

**Shh signaling has a similar effect on mouse ENS development.**

To determine whether the role of Shh on avian ENS development is conserved in mammals, we first characterized the temporal pattern of colorectal ENS development in the mouse using p75 antibody. At E12.5 the migratory wavefront is in the mid-hingut (Fig. 10A). Similar to the chick embryo, Ptc1 and p75 double-immunofluorescence staining at E12.5 shows Ptc1 expressed in the inner mesenchyme and not in p75+ ENCCs (Fig. 10B). When E12.5 hindguts were cultured for 48 hours with no additive (Fig. 10C) or with cyclopamine (Fig. 10D), ENCCs completed their migration into the distal hindgut. Expression of the CSPG marker, CS-56, is reduced in the outer mesenchyme of cyclopamine-treated guts compared to control (Fig. 10C,D). With addition of Shh protein, the distal hindgut remained aganglionic and exhibited expansion of the CS-56 domain (Fig. 10E), consistent with our avian results.

**Discussion**

Development of a normal ENS is important for gut function. In HSCR, ENCCs fail to complete rostrocaudal migration, leaving the distal intestine aganglionic. ENS development relies on migration, proliferation, differentiation, and patterning of ENCCs into two ganglionated plexuses within the submucosal and intermyenteric regions of the gut. These events require tightly regulated interactions between ENCCs and their microenvironment. While many factors involved have been identified (Goldstein et al., 2013), their precise mechanism of action remains unknown. In the stomach, tissue recombinations showed that removal of the gastric epithelium resulted in increased enteric neuronal density (Sukegawa et al., 2000), suggesting the epithelium inhibits ENCC proliferation. We tested this using epithelial-mesenchymal recombinations by replacing the normal hindgut epithelium with a non-intestinal epithelium from the bursa of Fabricius, a lymphoid organ derived from the cloaca. In these recombinations, ENCCs migrate close to the epithelium, forming ectopic and abnormally large ganglia adjacent to the epithelial basement membrane, demonstrating the importance of the hindgut epithelium during ENS patterning and suggesting that it may contain an inhibitory factor that is both chemorepulsive to prevent inward migration of ENCCs and anti-mitogenic to limit ganglion size.

We previously showed that Shh is expressed by the gut epithelium but not the bursa (Nagy and Olah, 2010). Sukegawa (2000) showed not only that removing the gastric epithelium results in hyperganglionosis, but also that inhibiting Shh leads to increased neuronal numbers and ectopic ganglia. We hypothesized that Shh is a candidate epithelium-derived factor responsible for patterning the hindgut ENS. In Shh−/− mice, the intestine contains increased numbers of neurons that differentiate ectopically under the epithelium and project abnormal extensions into the villi (Ramalho-Santos et al., 2000; Jin et al., 2015). Deletion of Gas1, a Hh cell-surface receptor, or Ganz1, an intracellular effector for Gas1, leads to similar phenotypes, with mislocalized enteric ganglia and increased enteric neuronal numbers (Biau et al., 2013; Jin et al., 2015). Conversely, overexpression of Gli1 leads to patchy absence of enteric ganglia in the gut (Yang et al., 1997). When Shh is added to intestinal explants, ganglion formation is impaired and enteric neurons are scattered sparsely throughout the gut wall (Fu et al., 2004). These data support an inhibitory role for Shh on ENCC proliferation, cell migration, ganglion formation, and axon extension. However, addition of Shh to mouse enteric neurospheres increased ENCC proliferation (Fu et al., 2004), and addition of cyclopamine to zebrafish embryos inhibited ENCC proliferation and
led to intestinal aganglionosis (Reichenbach 2008). These inconsistencies suggest a complex role for Hedgehog activity during ENS development.

We took advantage of the versatility of the avian embryo to test the role of Shh on ENS development using a variety of experimental approaches, including organ culture, tissue recombination, chick-quaill chimera, chorioallantoic membrane grafting, and retroviral-mediated gene overexpression in ovo. We find that inhibiting Hedgehog signaling, either with cyclopamine or with a function-blocking antibody, results in hyperganglionosis, while Shh overexpression causes aganglionosis. The failure of ENCCs to complete their colonization can result from abnormalities in cell migration, proliferation, or differentiation. We find that Shh overexpression significantly decreases ENCC proliferation and promotes their premature differentiation into neurons, accounting for the aganglionic phenotype. Retrovirus-mediated overexpression of Shh in vivo confirms these results, leading to distal aganglionosis with severe hypoganglionosis in the proximal colon. Our results corroborate previous observations and reveal an essential role for Shh signaling in regulating ENCC proliferation, differentiation, migration, and patterning (Fu et al., 2004; Biau et al., 2013).

The effects of a secreted ligand, like Shh, would presumably occur through a receptor expressed by the target cell, but whether or not ENCCs express Shh receptors has been unclear. Fu et al (2004) and Ngan et al (2011) both reported expression of Ptc1 by ENCCs, with Ngan (2011) showing that ENCC-specific deletion of Ptc1 reduces ENCC proliferation. Reichenbach (2008) similarly found Ptc1 expressed by Phox2b-expressing ENCCs in zebrafish. In contrast, however, reporter mice expressing β-galactosidase driven by Ptc1, Gli1, or Gli2 show no LacZ expression in ENCCs (Washington-Smoak et al., 2005; Kolterud et al., 2009), suggesting that these Hh pathway genes are not expressed. We performed Ptc1 and Ptc2 in situ hybridization in embryonic avian hindgut and also in cultured ENCCs and find both receptors expressed by the mesenchymal compartment of the gut, but not by the ENCCs. We found a similar expression pattern of Ptc1 in the mesenchyme, but not the ENS, in embryonic mouse hindgut. Importantly, we observed an analogous role for Shh in the intestines of both species. While we cannot explain the contradictory results obtained in these studies, our results suggest that inter-species differences, at least between avian and rodent, do not exist.

The lack of Shh receptor expression leaves two possibilities to explain the observed Shh-mediated effects on ENS development: (1) Shh acts directly on ENCCs via a Ptc-independent pathway or (2) the effect of Shh on the ENS is indirect via modification of the ENCC environment. To determine whether Shh can directly influence ENCCs, we treated intestinal explants with Gdnf, which promotes robust migration of ENCCs out of the gut and onto a fibronectin-coated surface. Twenty-four hours later, after the cells had emigrated out of the gut wall, Shh protein was added to the culture media, yet this did not affect continued ENCC migration. In contrast, when Shh and Gdnf are both added prior to the onset of cell migration, no migration occurs, consistent with previous results in embryonic mouse gut (Fu et al., 2004). This indicates that Shh does not directly inhibit ENCC migration, but rather must do so indirectly, presumably by altering the gut microenvironment and thus preventing their ability to respond to exogenous Gdnf.

We tested the effect of Shh overexpression and inhibition ex vivo and in ovo and observed that modulating Shh activity leads to dramatic changes in expression of ECM proteins that are known regulators of neural crest cell migration (Payette et al., 1988; Newgreen and Hartley, 1995; Nagy et al., 2009). While laminin, fibronectin, and collagen I are permissive to neural crest cell migration, the CSPG family, including versican and collagen IX, are inhibitory (Newgreen and Thiery, 1980; Bronner-Fraser, 1986; Oakley et al., 1994; Perris et al., 1996; Dutt et al., 2006). We found that Shh strongly induces expression of versican, collagen IX, and CS-56 in the intestine, while cyclopamine reduces their
expression. Furthermore, both the gut epithelium and a Shh-expressing bead are able to induce ectopic expression of collagen IX in the bursa of Fabricius, which normally does not express this. A similar induction of versican expression by Shh has been demonstrated in the developing trigeminal ganglia (Fedtsova et al., 2003). Induction of these factors that inhibit neural crest cell migration could account for the aganglionosis associated with Shh overexpression in both our cultured intestine and our in ovo model. We hypothesize that the gradient of epithelial-derived Shh protein in the lamina propria thus leads to creation of an inhibitory ECM environment that prevents ENCCs from migrating too close to the epithelium, counteracting the chemoattractive effect of Netrin (Jiang et al., 2003) and thus contributing to patterning the submucosal plexus.

In addition to its effect on ECM composition, Shh also decreases expression of Gdnf, a mesenchymal factor important for ENS development (Goldstein et al., 2013). Gdnf promotes ENCC proliferation and migration via a chemoattractive effect (Young et al., 2001; Nagy and Goldstein 2006; Mwizerva et al., 2011), and loss of Gdnf in rodents and avians leads to aganglionosis (Moore et al., 2006; Mwizerva et al., 2011). Gdnf downregulation is another example of Shh indirectly inhibiting ENS development. We noted that addition of exogenous Gdnf to cultured intestine does not alter versican expression. This suggests that Shh primarily acts to alter ECM patterning, which we hypothesize secondarily decreases Gdnf expression. Shh has previously been shown to directly induce versican in cranial mesenchyme (Fedstova et al., 2003) and collagen IX in somitic mesenchyme (Cairns et al., 2008), consistent with our observation of Shh-induced collagen IX in bursa mesenchyme. Further studies are needed to understand the mechanisms by which the microenvironment influences ENS development in normal and pathologic development.

Materials and Methods

Animals

Fertilized White Leghorn chicken (Gallus gallus) and quail (Coturnix coturnix japonica) eggs were incubated at 37°C in a humidified incubator. Embryos were staged according to Hamburger and Hamilton (HH; Hamburger and Hamilton, 1992) or number of embryonic days (E).

In vitro epithelium-mesenchyme recombination

Epithelium from E6 chick hindgut or E9 chick bursa was recombined with aganglionic E5 quail hindgut mesenchyme as described (Nagy and Olah, 2010). E6 chick hindgut epithelium was also recombined with E9 chick bursa mesenchyme. To separate epithelium from mesenchyme, tissue was incubated in DMEM (Sigma) containing 0.03% collagenase (Sigma) for 15 minutes. Tissue recombinants were embedded in a three-dimensional collagen gel overnight (BD Bioscience) as described (Nagy and Goldstein, 2006a), then harvested from the gel and implanted into E3 chick coelom (Nagy et al., 2005; Nagy and Goldstein, 2006b; Fig. S3). After 9 days, grafts were removed and immunohistochemistry performed. A total of 31 chimeric experiments were performed.

In the recombinants, measurements included: gut wall thickness (from epithelial basement membrane to outer edge of intestine) and muscle wall thickness (from inner to outer layer of muscularis propria). Both measurements were made at the bottom of the epithelial folds and included 3 measurements per gut and 3 guts per group. Additional measurements included submucosal ganglion diameter in the radial direction and distance of the inner edge of the submucosal enteric ganglia from the epithelial basement membrane (3 guts per group).
**Intestinal organ culture assay**

Gut was removed from E5 chick and embedded in collagen gel with recombinant mouse Shh protein (2 μg/ml; R&D Systems), monoclonal Shh-blocking antibody, 5E1 (50 μg/ml; DSHB, Iowa), normal mouse IgG (50 μg/ml; Invitrogen), or cyclopamine (2 μM; Toronto Research Chemicals, Canada) for 48–72 hours. For migration assays, midgut was removed from E8 chick embryos and cultured in collagen gel or fibronectin-coated dishes with GDNF (10 ng/ml; R&D Systems) or Shh protein (2 μg/ml). Mouse intestine was dissected from C57BL/6 mice at E12.5 (Jackson Laboratory), pinned to a silicone-coated petri dish, and cultured 48 hours with no additive, Shh protein (2 μg/ml), or cyclopamine (2 μM).

**In ovo Shh-RCAS viral misexpression**

Shh misexpression was achieved with the replication-competent retroviral vector, RCAS. DF-1 chicken fibroblast cells (ATCC) were transduced with RCAS-Shh construct (gift of Cliff Tabin). Viral harvesting, concentration, and titering were performed as described (Logan and Tabin, 1998). For in ovo infection, embryos were incubated until E2 (HH10-12) and, using a Hamilton syringe, 1 μl of virus was injected into the presumptive distal gut mesoderm, based on chick fate maps (Matsushita, 1995). Eggs were harvested 4–6 days later. Controls were uninjected or injected with empty RCAS vector.

**Chorioallantoic membrane transplants**

Hindgut, including ceca and cloaca, was dissected from E5 embryos and transplanted on the CAM of E9 chick. For retroviral experiments, the nerve of Remak was removed from E5 intestine and RCAS-Shh injected into the hindgut wall, which was cultured on the CAM for 9 days.

**Shh bead implantation**

Affi-Gel Blue agarose beads (70–150 μm diameter; BioRad) were soaked in 100 μg/ml Shh protein at 37°C for 2 hours (Tiecke and Tickle, 2007). Beads were inserted into the E9 bursa mesenchyme, which was cultured on the CAM for 7 days. PBS-soaked beads served as controls.

**Immunohistochemistry**

For cryosections, tissue was fixed in 4% formaldehyde for 1 h, then infiltrated with 15% sucrose overnight, followed by 7.5% gelatin in 15% sucrose for 1–2 h, then rapidly frozen at −60°C in isopentane (Sigma). For Shh immunostaining, guts were fixed in Histochoice (EMS, Hatfield, PA). Cryosections and wholemounts were stained using the primary antibodies listed in Table S1 as described (Nagy et al., 2007). Fluorescent secondary antibodies included Alexa Fluor 594 (goat anti-mouse IgG, IgM, and IgG1), Alexa Fluor 488 goat anti-mouse IgG2a, and Alexa Fluor 546 goat anti-rabbit IgG (Molecular Probes).

**BrdU labeling and apoptosis detection**

For cell proliferation, guts were incubated for 3 hours in BrdU (5 mg/ml; Roche). To detect apoptosis, sections were examined with anti-activated caspase-3 (Cell Signaling). Nuclei were labeled with Dapi (Molecular Probes).
**In situ hybridization**

*In situ* hybridization was performed for chick sonic hedgehog (Shh), Patched (Ptc1), and Ptc2 on paraffin sections using digoxigenin-labeled riboprobes (kindly provided by Cliff Tabin; Riddle et al., 1993; Roberts et al., 1995) according to standard protocols (Riddle et al., 1993; Acloque et al., 2008).

**Quantitative PCR**

Total mRNA was extracted using RNeasy Mini kit (Qiagen, Santa Clarita, CA, USA). cDNA was synthesized with the Superscript III Reverse Transcription Kit (Invitrogen). Gene expression levels were measured using quantitative PCR (qPCR) with Gapdh as the internal standard. Primers included: Edn3 forward, TGCGTCTACTACTGCCACCTC and Edn3 reverse CCAAACAAGAGCACCGAAAT; Gdnf forward, TCTCCACCTCACCCCTG and Gdnf reverse, CTGGGTAATCCTCTGGCATATTAG; Gapdh forward GTGCTAAGCGTGTTATCATCTC and Gapdh reverse GACAACCTTGGGCATTGTGGA. Relative expression was calculated by 2^-ΔCt.

**Statistical analysis**

Data were expressed as means ± SD. Paired t-tests were used to evaluate the statistical significance between groups. When more than two groups were compared, means were statistically compared by one-way ANOVA with Tukey’s post hoc multiple-comparison tests of differences. Statistical significance was considered at a cutoff of p<0.05.

**Acknowledgments**

This work was supported by NIH-NIDDK R01DK080914 and the American Pediatric Surgical Association Foundation. NN was supported by a Bolyai Fellowship of the Hungarian Academy of Sciences. Several antibodies in Table 1 were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by University of Iowa, Dept of Biological Sciences, Iowa City, IA 52242.

**Author contributions**

NN and AGM designed the experiments, wrote and edited the manuscript. NN, CB, HKG, RH, LC, and NF assisted with experiments and analyzed the data. All authors reviewed and approved the final manuscript.

**Competing interests**

The authors declare no competing or financial interests.
References


Figure 1: Expression of Shh and Ptc1 in the developing hindgut.
Expression of Shh and Ptc1 was determined in E6 (A-C) and E8 (D,E) hindgut. In situ hybridization shows Shh transcript (A) in the epithelium, with a gradient of protein expression seen by immunofluorescence extending into the subepithelial mesenchyme (B, magnified view in inset; dashed line marks epithelial basement membrane). Ptc1 in situ shows expression in the subepithelial mesenchyme (C). Double staining of E8 hindgut by Ptc1 in situ (D,E) and p75 immunofluorescence (F) shows that Ptc1 does not overlap with
p75-immunoreactive ENCCs (E, F, magnified from boxed area in panel D). Outlined areas in E mark p75+ submucosal ganglia in F. Cross sections of E13 bursa and hindgut show Ptc1 (G) and Shh (H) expressed only in the gut, whereas E-cadherin is expressed in epithelia of both organs (I). Bursa lacks enteric neurons (J).

ep, epithelium; mes, mesenchyme; NoR, nerve of Remak
Figure 2. Hindgut epithelium regulates enteric ganglion size and patterning. Chick-quail chimeras were generated by recombining E5 quail hindgut mesenchyme (HGmes) and E6 chick hindgut epithelium (HGep) (A-D; n=11) or E9 chick bursa epithelium (BFep) (E-I; n=11). Recombinants were implanted into E3 chick coelom for 9 days. QCPN confirms the mesenchyme is quail-derived (A,E), while 8F3 (B,F) reveals the chick origin of...
the epithelium, enteric ganglia, and blood vessels (B, arrowheads). When gut epithelium is replaced by bursa epithelium, enteric ganglia are larger and closer to the epithelium (F,G) than in control recombinations (B,C). This was confirmed quantitatively (I,J). The muscularis propria also appears thickened in bursa epithelium recombinations (H), confirmed by quantitative analysis showing increased thickness of gut wall (K) and muscularis propria (L), the latter comprising a greater proportion of the wall than in controls (M). Muscularis mucosae appears close to the epithelium in bursa recombinants (H) as compared to control (D, dotted line marks epithelial basement membrane).

ep, epithelium; mp, myenteric plexus; smp, submucosal plexus; SMA, alpha-smooth muscle actin.

*p<0.05; **p<0.01; ***p<0.001
Figure 3. Shh overexpression leads to hindgut aganglionosis.
At E5, the ENCC wavefront is in the distal midgut (A, arrow), while at E8 ENCCs have reached the distal hindgut (B, arrow). E5 gut was cultured in collagen gel for 3 days in the absence of additives (C), with Shh protein (D), or with cyclopamine (E). Longitudinal sections are shown (A-E), with corresponding cross sections through the mid-hindgut below each image (F-J). Addition of Shh inhibits ENCC colonization of the hindgut (D,J), whereas inhibition of Shh signaling leads to ectopic and large ganglia (E,J). n=27.
ep, epithelium; hg, hindgut; mg, midgut; NoR, nerve of Remak
Figure 4. Shh overexpression inhibits ENCC proliferation and promotes neuronal differentiation. E5 hindgut was cultured 3 days with or without Shh protein or cyclopamine. The rate of ENCC proliferation without additive (A, example of BrdU+/p75+ ENCC shown in inset) was 29%, and was markedly reduced by Shh protein (B) and increased by cyclopamine (C), as shown graphically in (D). Cultured guts were stained for activated caspase-3 and Tuj1, and no apoptotic enteric neurons were observed in control (E) or Shh-treated intestine (F). Apoptosis was seen in an E8 dorsal root ganglion (DRG), used as a positive control (G). Tuj1 and neurofilament, markers of early and late neuronal differentiation, respectively, show that Tuj1+ neurons are present in all treatment group (H-J), whereas neurofilament is increased following Shh treatment (H’-J’).

*p<0.002; **p< 0.0004.
Figure 5. Altering Shh expression in the gut modifies ECM patterning. Expression of several ECM proteins was examined in E8 hindgut (A,E,I,M) and compared to E5 guts cultured for 3 days in the presence of no additive (B,F,J,N), Shh protein (C,G,K,O), or cyclopamine (D,H,L,P). The expression of fibronectin (A-D), collagen I (E-H), collagen IX (I-L), and versican V0/V2 (M-P) through the mid-hindgut is shown. ep, epithelium; NoR, nerve of Remak.
**Figure 6. Shh overexpression reduces levels of GDNF transcript.** The relative expression of Edn3 and Gdnf genes in E5 guts cultured for 3 days in the presence of no additive or 2 μg/ml Shh recombinant protein was determined using quantitative RT-PCR. While Edn3 expression was not affected, the expression of Gdnf was markedly reduced (*p<0.01).
Figure 7. Virus-mediated in ovo misexpression of Shh disrupts ENS and ECM development. Injection of RCAS-Shh into the E2 presumptive hindgut mesoderm leads to hindlimb polydactyly (A, digits numbered) and tailbud malformation by E5 (A, arrow). By E8, RCAS is expressed throughout the hindgut, as detected by anti-3C2 antibody (B), and associated with severe ENS disruption (C). Since in ovo injection was lethal by E7-E9 (n=75), explanted E5 guts were infected with RCAS-Shh ex vivo (D, arrows denote fast green-labeled RCAS injection) and cultured for 9 days on an E9 CAM (E). RCAS (F) and
Shh (G) were strongly expressed throughout the gut, and associated with hypoganglionosis, abnormal radial patterning of ENCCs (H), and altered smooth muscle development (I). RCAS-Shh infection significantly expanded collagen IX (J). Immunostaining of control E14 colon with Hu (K), SMA (L), and collagen IX (M) antibodies is shown. ep, epithelium; mp, myenteric plexus; smp, submucosal plexus.
Figure 8. Shh signaling induces collagen IX expression. Collagen IX is expressed by the gut mesenchyme, but not the bursa (A). Intestinal epithelium from E6 hindgut was transplanted into E9 bursa mesenchyme from which the epithelium had been removed (B), and cultured for 10 days in an E3 chick coelom. Hindgut epithelium induces ectopic collagen IX expression in the surrounding bursa mesenchyme (C). Shh-coated beads were implanted into E9 chick bursa and cultured on the CAM for 7 days (D). Shh protein expression in and around the bead is observed (E), with induction of collagen IX in the mesenchyme (F, arrows).

BF, bursa of Fabricius; CAM, chorioallantoic membrane; ep, epithelium; hg, hindgut
Figure 9. **Shh does not act directly on ENCCs.** Explanted E8 midgut was cultured with Gdnf (A,C,E) or Gdnf and Shh (B,D,F) for 48 hours. Gdnf induces ENCCs to migrate out of the gut (A,C) whereas addition of Shh inhibits this effect (B,D). The presence of Shh induces a significant increase in versican expression (F), as compared to Gdnf alone (E). Gdnf-mediated ENCC migration from E6 midgut is robust at 24 hours, with the distance of cell migration shown (G). In situ hybridization for Ptc1 expression shows no staining of migrating HNK1+ neural crest cells (H). Addition of Shh at 24 hours, for an additional 48 hours, does not inhibit the continued migration of these cells (I).
Figure 10. Shh overexpression leads to aganglionosis and modifies CS-56 expression in the mouse hindgut. At E12.5, p75+ ENCCs have migrated into the mid-hindgut (A, panel 2), and the distal hindgut is still aganglionic (A, panel 3). ENCCs do not co-express Ptc1 (B). After 48 hours in culture (n=15), ENCCs complete their migration into the distal hindgut and CS-56 expression is observed (C). Addition of cyclopamine to the cultured gut does not inhibit ENCC migration, but reduces CS-56 expression in the outer mesenchyme (D). Addition of Shh inhibits ENCC colonization, leading to distal aganglionosis, and increases CS-56 expression throughout the mesenchyme (E).