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# BMP signaling is necessary for neural crest cell migration and ganglion formation in the enteric nervous system

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**Abstract**

The enteric nervous system (ENS) is derived from neural crest cells that migrate along the gastrointestinal tract to form a network of neurons and glia that are essential for regulating intestinal motility. Despite the number of genes known to play essential roles in ENS development, the molecular etiology of congenital disorders affecting this process remains largely unknown. To determine the role of bone morphogenetic protein (BMP) signaling in ENS development, we first examined the expression of bmp2, bmp4, and bmprII during hindgut development and find these strongly expressed in the ENS. Moreover, functional BMP signaling, demonstrated by the expression of phosphorylated Smad1/5/8, is present in the enteric ganglia. Inhibition of BMP activity by noggin misexpression within the developing gut, both in ovo and in vitro, inhibits normal migration of enteric neural crest cells. BMP inhibition also leads to hypoganglionosis and failure of enteric ganglion formation, with crest cells unable to cluster into aggregates. Abnormalities of migration and ganglion formation are the hallmarks of two human intestinal disorders, Hirschsprung's disease and intestinal neuronal dysplasia. Our results support an essential role for BMP signaling in these aspects of ENS development and provide a basis for further investigation of these proteins in the etiology of neuro-intestinal disorders.


*Keywords:* Enteric nervous system; BMP; Noggin; Hirschsprung's disease; Chick; Gut development

## 1. Introduction

Neural crest cells (NCC) comprise a population of pluripotent cells capable of migrating along specific pathways throughout the developing embryo to generate a wide variety of cell types. Among these cell types are the neurons and glia of the gastrointestinal (GI) tract, which form a network of ganglion cells that regulate critical functions in the mature intestine, including motility, secretion, and absorption. The enteric nervous system (ENS) arises primarily from vagal NCC that populate the intestine in an antero-posterior wave of migration. A smaller contribution of cells arises from the sacral neural crest and migrates in the opposing direction to contribute neurons and glia to the post-umbilical gut (Burns and Douarin, 1998). Developmental defects of the ENS lead to a variety of disorders in humans. The most common of these is Hirschsprung's disease (HSCR), a frequent congenital cause of intestinal obstruction affecting 1 in 5000 births (Newgreen and Young, 2002). Approximately, 80% of affected individuals have the sporadic, 'short-segment' form of the disease, characterized by the absence of ganglion cells along a variable length of the terminal colon. In the less common 'long-segment' form, a familial inheritance is often seen and the aganglionosis extends further anteriorly.

Spontaneous and targeted mutations in mice have identified genes critical for ENS formation (Gershon, 1999). Null mutations in the Ret signaling pathway, including the tyrosine kinase receptor c-ret, its ligand glial

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cell line-derived neurotrophic factor (gdnf), and the co-receptor gfrα1, prevent enteric NCC migration and lead to aganglionosis of the small and large intestine (Enomoto et al., 1998; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Schuchardt et al., 1994). Expression of GDNF in the gut mesenchyme and Ret/GFRα1 in enteric crest-derived cells has been shown to be required for proliferation, migration, differentiation, and survival of enteric neurons (Durbec et al., 1996; Hearn et al., 1998; Natarajan et al., 2002; Young et al., 2001).

Enteric crest cell colonization of the distal hindgut requires a group of genes that, when mutated, lead to aganglionosis of variable lengths of the colorectum, mimicking the phenotype most often seen in HSCR. One example of this is the endothelins. Expression of endothelin-3 (edn3) in the intestinal mesenchyme and its G protein-coupled receptor, endothelin receptor B (ednrB), in the enteric NCC is required for their proliferation and migration (Barlow et al., 2003; Kruger et al., 2003; Lahav et al., 1996; Lee et al., 2003). Homozygous deletion of either gene leads to distal aganglionosis (Baynash et al., 1994; Hosoda et al., 1994; Wu et al., 1999). Heterozygous mutation of the transcription factor Sox10 produces distal aganglionosis in the Dom mouse model (Herbarth et al., 1998; Kapur et al., 1996; Southard-Smith et al., 1998) by a mechanism that may involve dysregulation of ednrB activity (Zhu et al., 2004). Similarly, targeted deletion of the Ret9 isoform of the Ret receptor leads to absence of ganglion cells in the distal three-fourths of the colon (de Graaff et al., 2001). While these genes are essential for formation of the colonic ENS, additional genes are likely to be involved. Coding sequence mutations in ret are the most commonly identified mutation in HSCR, and yet occur in only 20–35% of short-segment cases (Kusafuka and Puri, 1998). Mutations of edn3 and endrB account for an additional 3% of cases (Kusafuka and Puri, 1998), leaving the molecular cause unknown in the majority.

Bone morphogenetic proteins (BMP) comprise a subgroup of the TGF-β family of secreted signaling molecules. BMP 2, 4, and 7 transduce their signal by binding to a heterodimer consisting of a type I receptor, BMPRIA or BMPRIB (or in the case of BMP7, activin receptor-like kinase (Alk)-2), and a type II (BMPRII) receptor. Receptor activation leads to phosphorylation and activation of the Smad signaling cascade (Zwijsen et al., 2003). BMPs regulate multiple critical functions during organogenesis, including the epithelial–mesenchymal interactions that underlie specification, regionalization, and differentiation within the developing gut (Faure et al., 2002; Roberts et al., 1998; Smith et al., 2000). BMP signaling is also critical in early neural crest cell induction and migration (LaBonne and Bronner-Fraser, 1999; Sela-Donenfeld and Kalcheim, 1999), with a later role in promoting differentiation to an autonomic neuronal fate (McPherson et al., 2000; Shah et al., 1996). The role of BMP signaling in enteric neuronal differentiation is less clear. BMP4 has been shown to either promote (Kruger et al., 2003) or inhibit (Sukegawa et al., 2000) enteric neuronal differentiation, while BMP2 promotes neuronal differentiation of post-migratory enteric crest cells (Lo et al., 1997; Pisano et al., 2000). The link between BMP signaling and ENS development is further supported by the identification of a mutation in the transcription factor Smad interacting protein 1 (Sip1) in several individuals with a syndromic form of HSCR (Cacheux et al., 2001; Nagaya et al., 2002).

We hypothesize that BMP activity is essential for normal development of the ENS. To clarify the role of BMP signaling in ENS development, we have examined the normal expression pattern of bmp2, bmp4, bmprII, and activated Smad in the developing chick gut and find these proteins expressed in the submucosal and myenteric plexuses, as well as the nerve of Remak (an avian structure analogous to the pelvic plexus in humans). Targeted inhibition of intestinal BMP activity in ovo using the BMP inhibitor, noggin (Balemans and Van Hul, 2002; Smith and Harland, 1992), leads to impaired migration of enteric crest cells by interfering with their responsiveness to GDNF. Secondly, in the absence of BMP activity, fewer numbers of neural crest cells reach the hindgut and subsequently fail to aggregate normally into clusters to form ganglia. These findings establish an essential role for BMP signaling in ENS development, specifically in the regulation of key steps in crest cell migration and patterning.

## 2. Results

### *2.1. Bmp4 expression in the gut is consistent with a role in ENS development*

To explore the potential role of bmp4 in ENS patterning, we first examined the expression and activity of bmp4 mRNA in the avian hindgut at developmental stages corresponding to the arrival of enteric neural crest cells, from E8 to E14. We find bmp4 expressed along the length of the hindgut, excluding the cloacal epithelium, at all stages examined, from E7 to E14, as shown by whole-mount in situ hybridization at E12 (Fig. 1M). Transverse sections of the hindgut demonstrate bmp4 expression within the mesenchyme immediately adjacent to the epithelium (Fig. 1A–C), as previously shown by others (Bitgood and McMahon, 1995; Sukegawa et al., 2000). This expression is limited to a narrow portion of subepithelial mesenchyme at E8 (Fig. 1A) and progressively broadens to encompass the entire region of non-smooth muscle mesenchyme by E14 (Fig. 1C). Expression of bmp4 transcript is also seen in the ganglia of the ENS and within the nerve of Remak at E14 (Fig. 1C). BmprII is expressed in the non-smooth muscle mesenchyme of the hindgut (Fig. 1D–F). Expression of bmprII is also noted throughout all components of the ENS, with strongest expression in the submucosal plexus and nerve of Remak (Fig. 1D–F). BmprII is also present in the epithelium,

indicative of the known role of BMP4 in mesenchymal to epithelial signaling (Roberts et al., 1998).

The expression pattern of bmp4 and its receptor is not necessarily indicative of its sites of activity, particularly given the large number of regulatory molecules that modulate BMP signaling (Balemans and Van Hul, 2002; Faure et al., 2002). Therefore, to examine the endogenous spatiotemporal pattern of BMP signaling, we have used an antibody that detects the phosphorylated form of Smads 1, 5, and 8 (P-Smad). These Smads are phosphorylated following activation of BMP receptors by BMP 2, 4, or 7, and therefore reflect the activity of all three ligands (Zwijsen et al., 2003). P-Smad expression is first detected at E8 in the nerve of Remak (Fig. 1G, arrowhead) and in the submucosal

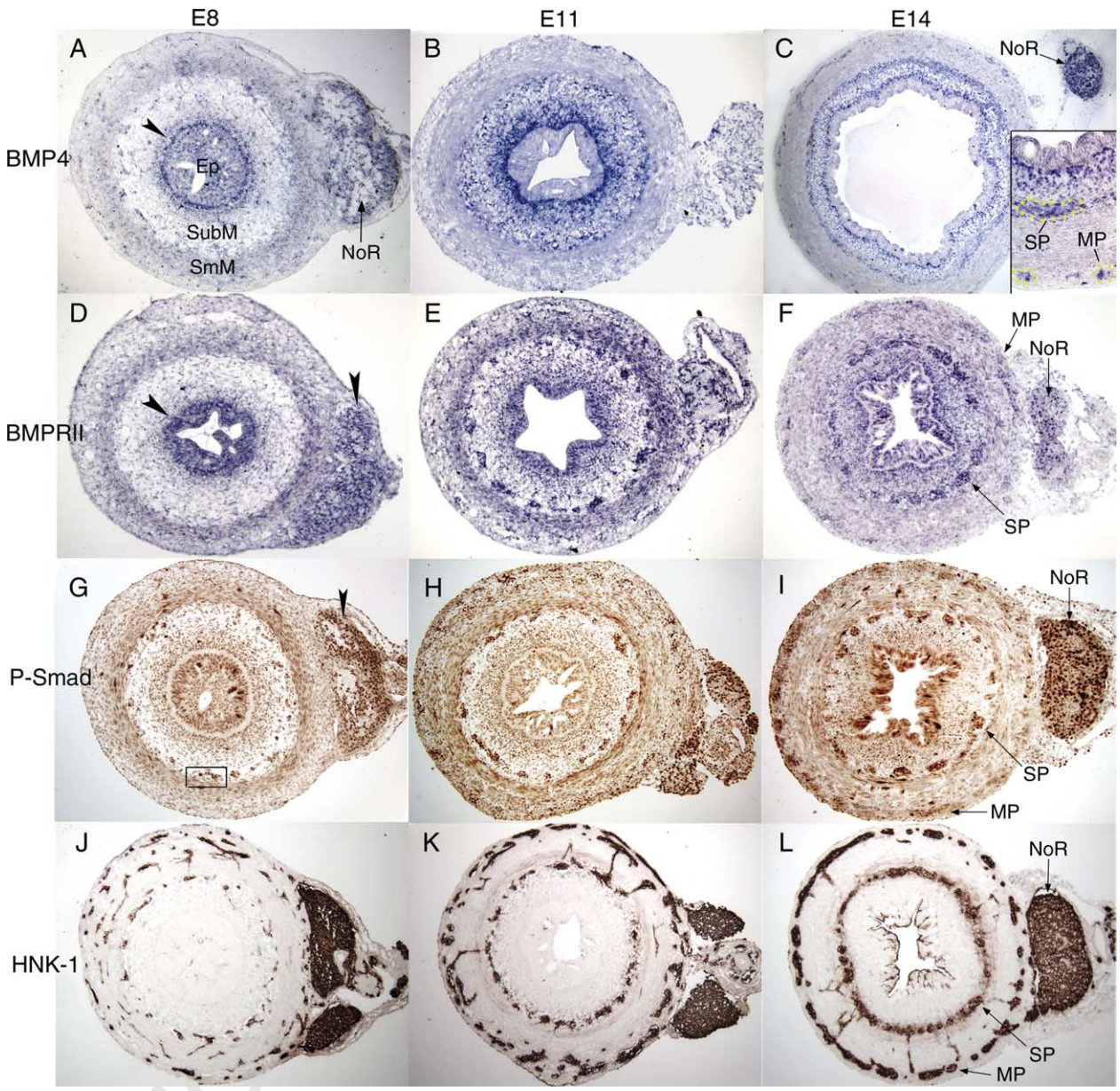


Fig. 1. BMP4 signaling is present in the enteric nervous system. Sections through the hindgut at E8–E14 show BMP4 expression initially limited to the submucosa immediately adjacent to the epithelium (A, arrowhead) and later spreading throughout the non-smooth muscle mesenchyme (B,C). BMP4 is expressed in a subgroup of cells in the Nerve of Remak at all stages shown, and within the ganglionated plexuses at later stages (C; magnified inset shows expression in the submucosal and myenteric ganglia [dotted outlines]). BMPRII is initially expressed in the epithelium and nerve of Remak (D, arrowheads). Strong expression is later evident throughout the ENS (E,F), as well as the submucosa (F). P-Smad expression, an indicator of active BMP signaling, is expressed in the nerve of Remak (G, arrowhead) and submucosal plexus (G, boxed area) at E8, with strong expression evident in all layers of the ENS at E11 and E14 (H,I). HNK-1 immunoreactivity demonstrates normal development of the hindgut ENS, with the earliest neural crest cells present at E8 (J) and later forming well-developed ganglia within the two plexuses (K,L). Whole-mount BMP4 in situ hybridization at E12 is shown to demonstrate its expression throughout the length of the hindgut, excluding the cloacal epithelium (M, arrow). Ep, epithelium; SubM, submucosa; SmM, smooth muscle; SP, submucosal plexus; MP, myenteric plexus; NoR, nerve of Remak.

Fig. 1 (*continued*)

plexus (Fig. 1G, boxed area). By E11, strong immunoreactivity to P-Smad is evident throughout the ENS—in both submucosal and myenteric plexuses and in the nerve of Remak (Fig. 1H,I). HNK-1 immunoreactivity is shown at similar stages to compare the pattern of BMP activity with the appearance of neural crest-derived cells (Fig. 1J–L). All immunohistochemistry experiments included a negative control with no primary antibody (not shown).

The expression of bmp4 was compared with that of bmp2 and bmp7 in the E14 hindgut (Fig. 2). Bmp2 is expressed throughout all layers of the intestinal wall, including epithelium, mesenchyme, and ENS (Fig. 2A). Strongest expression is seen in the submucosal layer, which is also the region of bmp4 expression (Fig. 2B). Ganglion cells in both plexuses express bmp2 and 4, though expression of the former is more difficult to see because of the presence of ubiquitous mesenchymal staining (Fig. 2A,B). Bmp7 expression is limited to the epithelial layer, with no ENS staining detected (Fig. 2C).

### 2.2. Noggin overexpression downregulates P-Smad

We do not detect expression of noggin in the wild-type avian hindgut prior to E10. At later stages, however, noggin

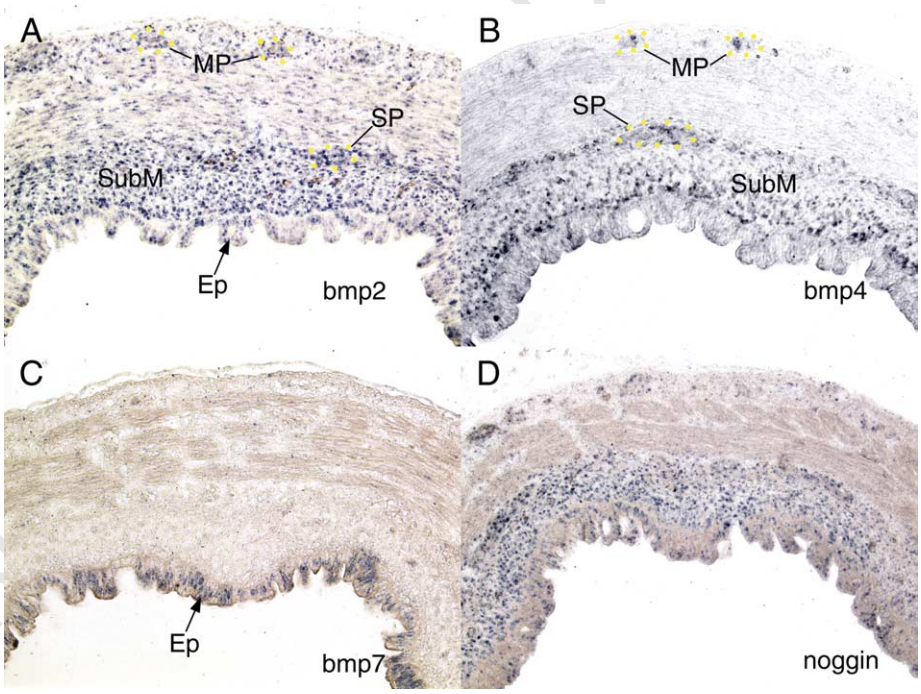


Fig. 2. Expression of BMP ligands and noggin in the avian hindgut. Cross-sections of an E14 hindgut demonstrate BMP2 mRNA expression throughout all layers, including the epithelium, mesenchyme, and enteric ganglion cells (A). BMP4 expression is limited to the submucosa and ENS (B), while BMP7 is only expressed in the epithelium (C, arrow). Noggin expression is strongest in the submucosa, with weak expression in the epithelium and ENS (D). Ep, epithelium; SubM, submucosal; SP, submucosal plexus; MP, myenteric plexus.

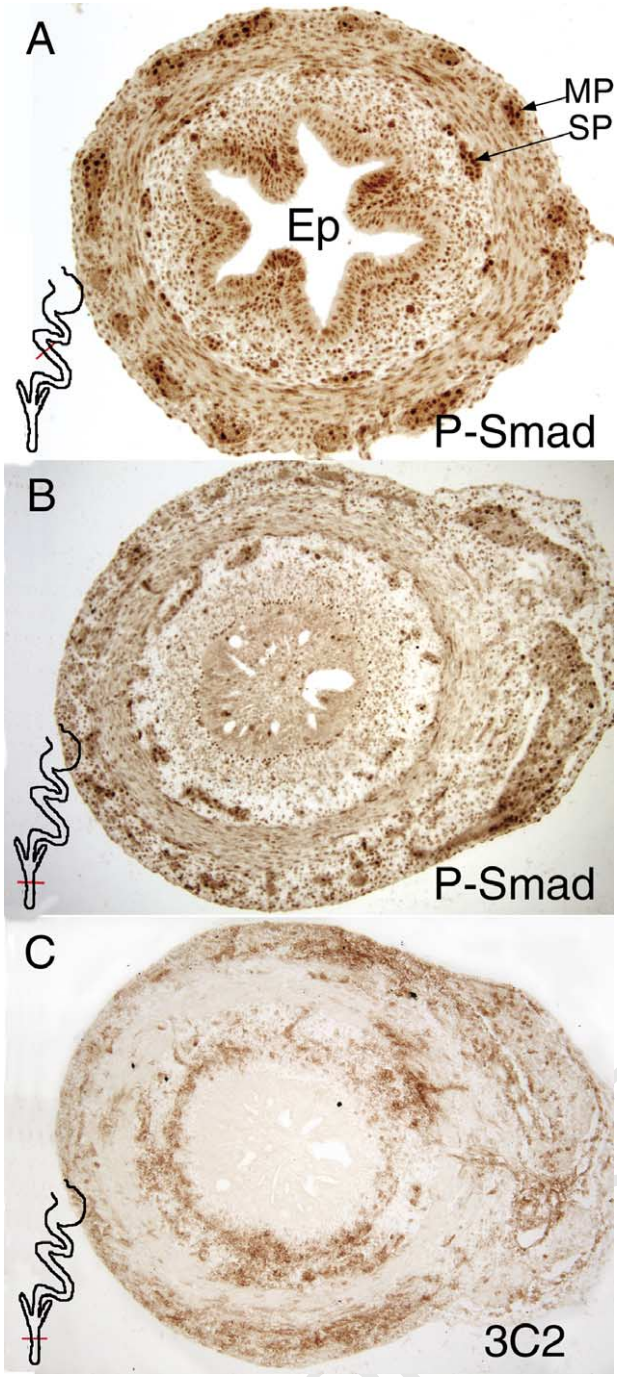


Fig. 3. Noggin overexpression downregulates expression of P-Smad. The hindgut was targeted with RCAS-noggin at E2 and the gut removed at Ell. (A) P-Smad immunoreactivity in a section of proximal midgut, a region uninfected by virus, shows normal expression of P-Smad throughout all layers of the bowel wall, including the ENS. This section was negative for 3C2, not shown. (B) P-Smad expression is significantly reduced in a section of hindgut from the same embryo as a result of noggin-overexpression. Some expression is still evident in cells of the ENS. (C) A section, adjacent to that shown in B, stained with the RCAS-specific antibody, 3C2, shows patchy expression throughout the mesenchyme. Occasionally, more confluent 3C2 staining was seen, but the epithelium was never infected. All sections are from the same embryo and stains were developed for an equal amount of time. Insets to the bottom left show the approximate level of the section. Ep, epithelium; SP, submucosal plexus; MP, myenteric plexus.

is strongly expressed in the submucosal layer of the intestine, with light expression detectable in the ENS and epithelium as well (Fig. 2D). RCAS-noggin injection into the presumptive hindgut mesoderm at E2 therefore leads to both spatial and temporal misexpression, with noggin expressed throughout the intestinal mesenchyme and at a much earlier embryonic stage than normal. Viral expression in the experimental embryos is confirmed by detection of the retroviral coat using the 3C2 antibody. As shown in Fig. 3C, 3C2 immunoreactivity is present in nearly the entire mesenchymal layer in an injected hindgut. The absence of retroviral expression in the intestinal epithelium has been previously described (Roberts et al., 1998). In order to confirm successful production and function of the viral noggin protein, expression of P-Smad was determined. Fig. 3B confirms a significant reduction in P-Smad expression resulting from noggin-overexpression as compared to control (Fig. 1H). P-Smad expression in a section of uninfected midgut (confirmed by absence of 3C2 staining, not shown) from the same embryo is shown as a control (Fig. 3A).

### *2.3. Inhibition of BMP activity delays enteric neural crest cell migration in vivo*

RCAS-noggin vector was injected at E2, targeting the presumptive intestinal mesoderm. These injections resulted in an overall mortality of about 20% by E9. Remaining embryos were allowed to develop to the appropriate stage. Successful targeting and replication of the virus was confirmed by immunohistochemistry with 3C2, as discussed above.

Enteric neural crest cell migration was significantly delayed in noggin-injected embryos as compared to controls. At E7, ENS precursors had only migrated halfway along the preumbilical gut in noggin-overexpressing embryos, as compared to control guts where the neural crest cells had reached the end of the midgut (data not shown). At E8, neural crest cells in noggin$^+$ embryos had only reached the distal midgut, while the hindgut of control embryos was almost entirely populated (Fig. 4A,B). By E9, injected embryos still remained aganglionic in the distal 25% of the hindgut. ENS migration was completed by E10, a full 1.5 days after the control embryos (Doyle et al., 2004). Immunohistochemistry with HNK-1 antibody (Fig. 4C,D), which detects all neural crest-derived cells, confirmed the Hu-immunoreactivity results, demonstrating that there is a delay in crest cell migration, not simply a delay in neuronal differentiation.

To test whether the delayed migration was a result of increased cell death, we compared hindgut sections through the neural crest migratory wavefront in noggin-injected and control embryos using immunohistochemistry for activated caspase-3, a well-known marker for apoptosis. Apoptotic cells were rarely identified in either control or

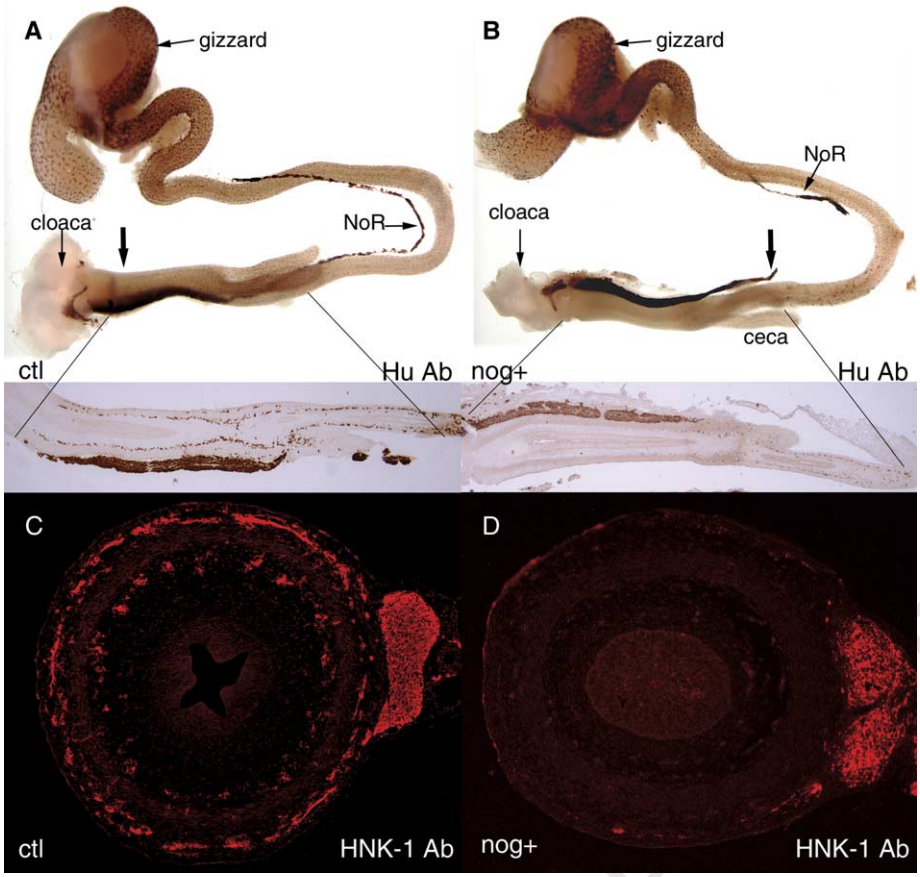


Fig. 4. Noggin overexpression delays ENS migration. Whole-mount immunohistochemistry of an E8 control gut with Hu antibody demonstrates enteric neurons extending along the length of the gastrointestinal tract all the way to the distal hindgut (A; heavy arrow). Below the whole-mount is a longitudinal section stained with Hu, demonstrating clearly the presence of crest-derived cells throughout the length of the hindgut. Hu immunoreactivity in a noggin-injected E8 gut (B) shows the migratory wavefront only at the level of the distal midgut (heavy arrow), with a gradual diminution in crest cell density at that level. The longitudinal section of hindgut below the whole-mount shows this failure of crest cell migration in greater detail. A histologic cross-section through the hindgut of an E9 embryo following Hu whole-mount immunohistochemistry confirms normal HNK-1-immunoreactive enteric crest cells in the control (C) and aganglionosis in the nog+hindgut (D). Note that the Nerve of Remak is partially missing in B. ctl, control; nog+, noggin-overexpressing gut; NoR, nerve of Remak; Ab, antibody.

noggin-injected guts (Fig. 5A,B), while cell death was readily seen in the dorsal root ganglion (Fig. 5C), which is known to contain apoptotic cells. Double-immunohistochemistry with HNK-1 and activated caspase-3 never showed apoptosis in the cells of the ENS, an observation previously made by others (Gianino et al., 2003; Kruger et al., 2003). Occasional cell death was identified in the epithelial cord of the distal hindgut (Fig. 5B, arrow) in both control and noggin$^+$ embryos. Apoptosis is known to occur during vacuolization of this portion of the distal gut (Miller et al., 1998). We could therefore find no indication that the delayed ENS migration was associated with increased cell death.

### 2.4. Noggin reduces the migratory response of enteric neural crest cells to GDNF

The migratory delay observed in noggin-injected embryos suggested that BMP signaling may have a role in neural crest cell migration along the gastrointestinal tract. To test this hypothesis, we cultured wild-type E8 avian guts in collagen gel in the presence of various additives. BMP4 alone did not promote migration of enteric neural crest cells out of the gut. In the absence of a direct effect, BMP4 could modulate the migratory effect of another factor, such as GDNF. As shown in Fig. 6, a significant number of cells migrate away from the gut into the collagen gel in the presence of 10 ng/ml GDNF (Fig. 6B), but not in its absence (Fig. 6A). These cells are neural crest-derived, as confirmed by their immunoreacitivity to HNK-1 (not shown) and Hu (Fig. 6C). In the presence of 400 ng/ml Noggin, the migratory effect of GDNF is significantly reduced (Fig. 6D), with the neuronal processes migrating a much shorter distance. The maximum distance migrated by cells in response to GDNF alone was compared to GDNF with Noggin. The inhibitory effect of Noggin was highly significant ($P<0.0001$), as shown in Fig. 6F.

To confirm that BMP signaling within the gut is required for normal GDNF-stimulated neural crest cell migration, embryos were injected with RCAS-noggin and allowed to

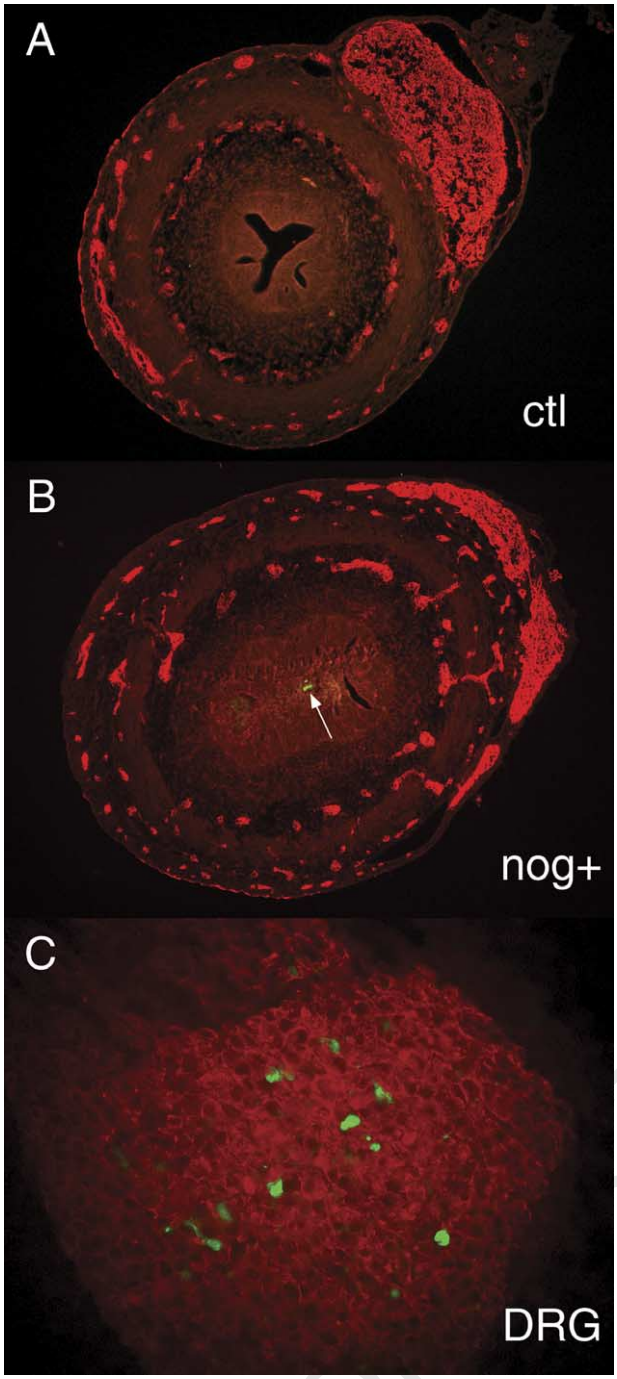


Fig. 5. Delayed migration is not associated with increased cell death. Sections of E9 hindguts from control (A) and noggin-injected (B) embryos were stained with antibody to activated caspase-3 (green) to detect apoptotic cells and antibody to HNK-1 (red) to detect enteric neural crest cells. Apoptotic cells were never identified within the ENS and rarely were they found within the gut, except for occasional cells in the epithelium (B, arrow). Cell death was detected in the dorsal root ganglion of an E6 embryo (C), which is known to contain apoptosis. ctl, control; nog+, noggin-overexpressing gut; DRG, dorsal root ganglion.

develop to E8. Noggin-injected guts were dissected and cultured in collagen gels in the presence of GDNF. Neuronal migration was significantly decreased in these guts (Fig. 6E). Following organ culture, these guts were processed for whole-mount in situ hybridization using a noggin riboprobe, confirming overexpression of the gene (not shown).

### 2.5. Inhibition of BMP signaling leads to hindgut hypoganglionosis and the formation of smaller enteric ganglia

In control embryos, neural crest cells aggregate into clusters to form discreet ganglia. Within the normal hindgut, this process is first noted at E9 (Doyle et al., 2004). Noggin-overexpressing embryos were allowed to develop to E12, at which point the entire hindgut is colonized with neural crest cells. As shown in Fig. 7, the ganglia of noggin-overexpressing E12 guts are much smaller than their control counterparts. Whole-mount immunohistochemistry with anti-Hu antibody shows smaller groupings of neuronal cells in the noggin$^+$ (Fig. 7B) as compared to the control embryo (Fig. 7A). HNK-1 immunoreactivity in sectioned guts confirms the dramatic difference in ganglion size (Fig. 7C,D). This difference is present in both plexuses, although more significant in the myenteric plexus.

In order to characterize the difference in ganglion size, we counted the number of HNK-immunoreactive cells present per myenteric ganglion in control and in noggin-overexpressing guts. Fig. 8 graphically depicts the average number of cells per ganglion ($x$-axis) against the number of ganglia containing that density of cells ($y$-axis). The data demonstrate that noggin$^+$ guts have significantly fewer cells per ganglion, accounting for their smaller size.

Not only are there fewer HNK-1-immunoreactive cells per ganglion, but also the total number of cells present per hindgut section is significantly reduced in the noggin-overexpressing gut. The number of HNK-1 cells was counted in representative sections from four control and nine noggin$^+$ embryos. Controls had an average of $424 \pm 71$ ENS cells per section, while noggin$^+$ guts had $153 \pm 60$ cells ($P < 0.05$ by Student's $t$-test), 64% fewer than controls. The reduced number of ENS cells in noggin$^+$ guts was not related to premature neurogenesis, as the appearance of Hu-immunoreactivity in more proximal sections was unchanged compared to controls. In addition, as discussed above, no neural crest cell death was detected proximally or at the migratory wavefront (Fig. 5).

### 2.6. Enteric crest cell differentiation is unaffected by noggin overexpression

Neural crest cells normally arrive in the hindgut at E8 and undergo neuronal and glial differentiation immediately thereafter (Connor et al., 2003; Doyle et al., 2004). Noggin overexpression, while delaying the timing of migration, did not alter the relative timing or patterning of differentiation. As shown in Fig. 9, differentiation of neural crest cells (Fig. 9A,B) to neurons (Fig. 9C) and glia (Fig. 9D) appeared to occur normally in these noggin-overexpressing guts, as

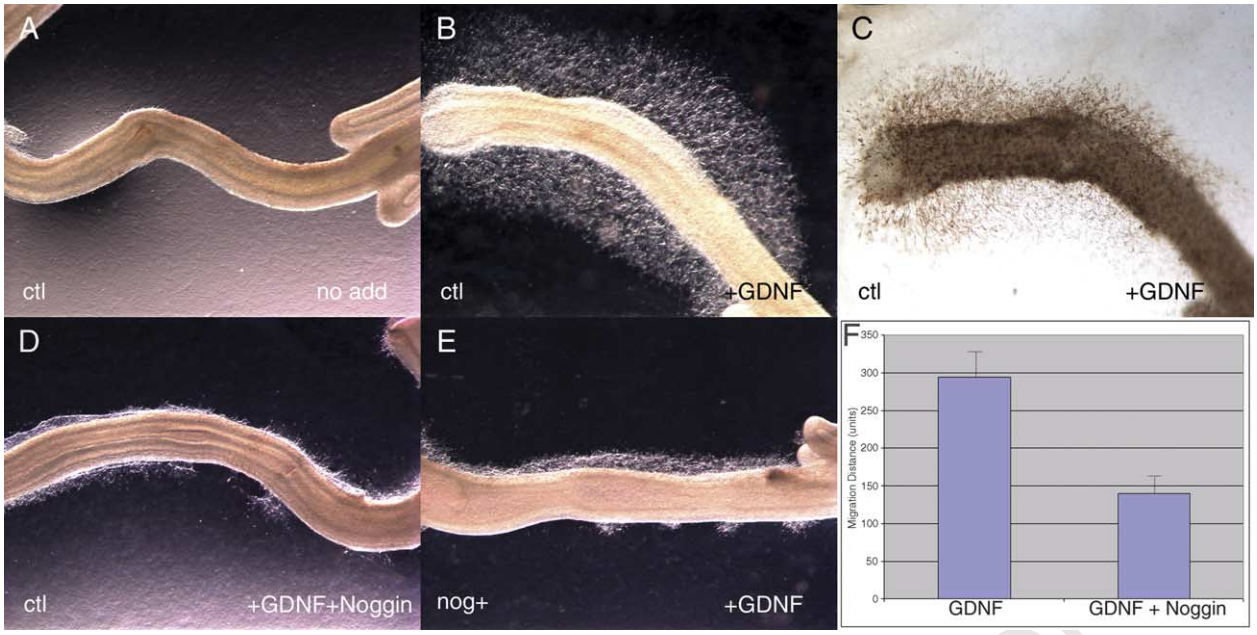


Fig. 6. Noggin is required for GDNF-stimulated neural crest cell migration. E8 wild-type gut was isolated and grown in collagen gel in the absence (A) or presence (B) of 10 ng/ml GDNF. The addition of GDNF produces a dramatic stimulation of cell migration out of the gut following 24–48 h in culture. To confirm that the migrating cells represent neurons, whole-mount immunohistochemistry was performed with the Hu antibody (C). The addition of 400 ng/ml Noggin abrogated the GDNF response (D). Similarly, GDNF did not promote significant migration of cells from noggin$^+$ gut (E). (F) The maximum distance migrated by neural crest cells in the presence of GDNF alone or GDNF+Noggin was measured in pixels using Spot 3.3.1 software ($n=6$ in each group). The data were log-transformed prior to performing a two-way analysis of variance. The ratio of the maximum distance migrated in the presence of GDNF compared to GDNF+Noggin was $1.9\pm0.2$ ($P<0.0001$). ctl, control; nog+, noggin-overexpressing gut.

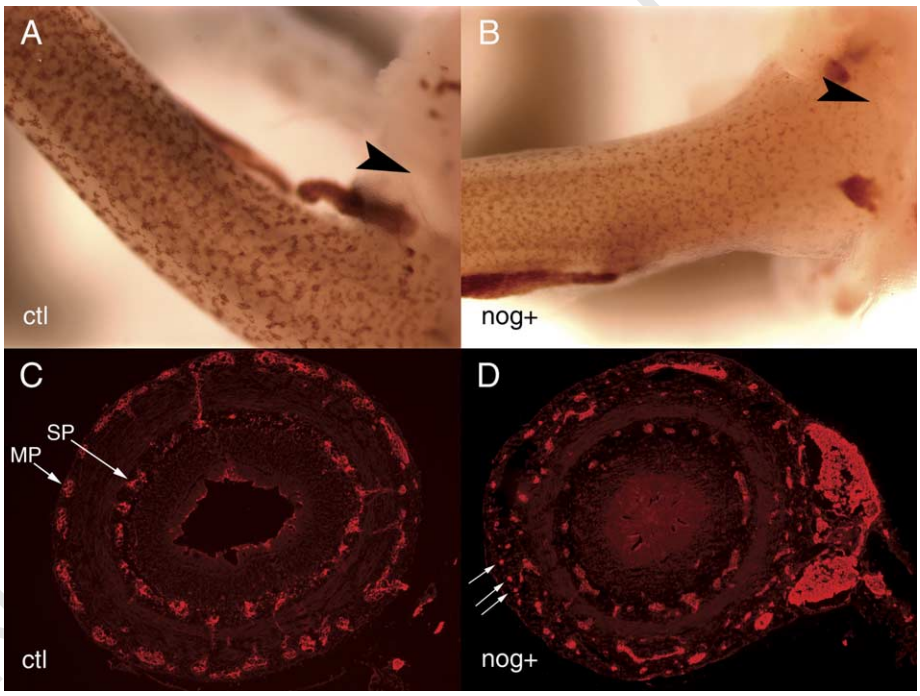


Fig. 7. Inhibition of BMP impairs ganglion formation. Control (A) and noggin-injected (B) guts were dissected at E12 and whole-mount immunohistochemistry performed with Hu antibody. The distal hindgut is shown, with the cloaca to the right (arrowhead). The control exhibits well-developed clusters of enteric neurons forming mature ganglia, whereas the noggin$^+$ gut shows much smaller ganglia. (C) A cross-section of control gut stained with HNK-1 and a fluorescent secondary antibody shows the two plexuses populated with neural crest cells that have formed dense clusters of ganglia. (D) Noggin-overexpressing gut shows abnormally small ganglia consisting of few HNK-1-immunoreactive cells (small arrows). ctl, control; nog+, noggin-overexpressing; SP, submucosal plexus; MP, myenteric plexus.

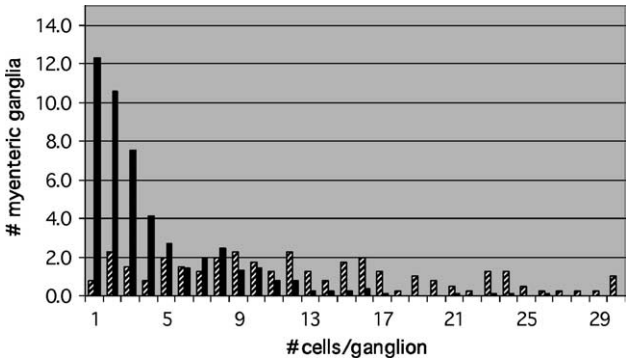


Fig. 8. Noggin dramatically reduces the number of crest cells per ganglion. The number of HNK-immunoreactive cells per myenteric ganglion was counted in cross-sections of control ($n=4$) and noggin-overexpressing ($n=9$) E10 and E11 hindguts. The *x*-axis represents the average number of cells/ganglion while the *y*-axis represents the number of ganglia containing that density of cells. Noggin$^+$ guts (solid boxes) demonstrate a significant reduction in the density of cells per ganglion as compared to control embryos (hatched boxes).

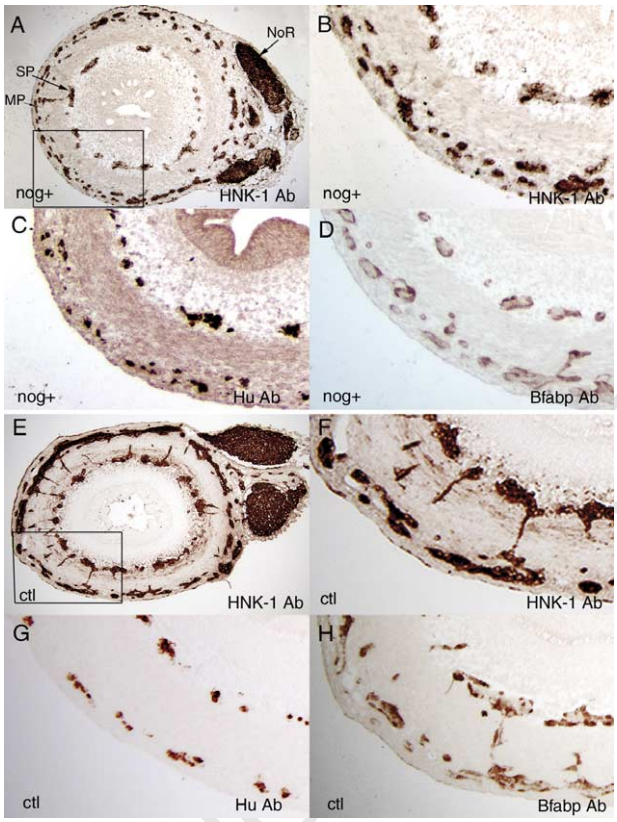


Fig. 9. Noggin does not alter neural crest cell differentiation. Cross-section of an E11 noggin-overexpressing hindgut stained with HNK-1 antibody (A) with boxed area magnified in (B), demonstrating again (as in Fig. 5) abnormally small ganglia. Hu-immunoreactivity of an adjacent section of the same specimen (C) shows normal neuronal differentiation. (D) Glial differentiation, as detected with Bfabp antibody, is also normal. A control hindgut (E–H) is shown for comparison. Note that (F) is a magnified view of the boxed area in (E). ctl, control; nog+, noggin-overexpressing; Ab, antibody; SP, submucosal plexus; MP, myenteric plexus; NoR, nerve of Remak.

demonstrated by immunoreactivity to the neuronal marker, Hu, and the glial differentiation marker, brain fatty acid binding protein (Bfabp) (Woodhoo et al., 2004). While Noggin delays migration, the timing of differentiation was normal relative to the appearance of neural crest cells in the hindgut. The pattern of HNK-1, Hu, and Bfabp immunoreactivity in a control hindgut is shown for comparison (Fig. 9E–H). The ganglia in noggin$^+$ guts are again noted to be significantly smaller than normal (Fig. 9B,F), as described above and shown in Figs. 7 and 8.

## 3. Discussion

The purpose of this study was to determine the role of BMP signaling in ENS development. We first investigated the expression patterns of bmp4, bmprII, and P-Smad in the developing hindgut. We find these strongly expressed early during the development of both ganglionated plexuses and within a subpopulation of cells in the nerve of Remak (Fig. 1). The timing and location of these expression patterns strongly support a potential role for BMP signaling in development of the ENS. Chalazonitis et al. (2004) recently demonstrated the presence of bmp2 and bmp4, their receptors, and antagonists (gremlin, noggin, chordin, follistatin) by RT-PCR in fetal rat gut in both crest-derived and noncrest-derived cells during the earliest stages of ENS formation, further supporting a formative role in this process.

To test whether BMP signaling is essential for normal ENS development, we used in ovo viral overexpression of noggin in the presumptive gut mesoderm. Noggin overexpression has previously been shown to lead to ectopic neurons in cultured gizzard explants, suggesting a role for BMPs in patterning the ENS (Sukegawa et al., 2000). In our in ovo system we do not see the occurrence of ectopic ganglion cells. We do find, however, that noggin overexpression within the gut mesoderm produces a significant delay in enteric crest cell migration (Fig. 4), with cells failing to reach the distal hindgut until well beyond the stage at which this normally occurs. Delayed crest cell migration is observed in several mouse models of Hirschsprung's disease, including mutations of Ret (Natarajan et al., 2002), ednrB (Lee et al., 2003), and Sox10 (Maka et al., 2005). In these mice, not only is a variable length of the distal gut aganglionic, but also the rate of migration of the neural crest cells that are present is delayed. Others have hypothesized that this delayed migration leads to distal aganglionosis because the arriving crest cells encounter an intestinal environment that is more mature, and therefore less permissive to migration. Interestingly, our noggin-overexpressing model demonstrates that neural crest cell migration can be delayed without resulting in distal aganglionosis. This observation suggests that a migratory delay alone may not be adequate to account for the Hirschsprung's-like phenotype seen in these mouse models,

since the distal gut remains competent to accept neural crest cells for some period of time.

Multiple factors can contribute to a delay in migration, including increased cell death, premature neuronal differentiation, or a direct effect on migration. Using activated caspase-3 immunoreactivity (Fig. 5) and a panel of differentiation markers (Fig. 9) we found no change in the rate of cell death or in the timing of neuronal and glial differentiation. We studied the effect of Noggin on migration itself by establishing ex vivo gut explant cultures. In this assay, we find that BMP4 alone is not sufficient to promote migration of crest-derived cells away from the gut. In the absence of a direct chemoattractive effect, BMP4 could modulate the effect of a known migratory factor, namely GDNF. As previously shown (Young et al., 2001), we found that crest cell migration from the explant is GDNF-dependent. Our results clearly demonstrate a role for BMP in this process, since the addition of Noggin significantly reduces the migration of crest-derived cells in response to GDNF (Fig. 6). An interaction between GDNF and BMP4 in the ENS was recently proposed with the demonstration that the combination of the two proteins synergistically enhances enteric neuronal development (Chalazonitis et al., 2004). Our data extend this observation to include a role for these two factors acting together in crest cell migration. The mechanism through which GDNF promotes migration remains poorly understood. The migratory response of ENS cells to GDNF is known to require activation of the Ret receptor, with downstream activation of the phosphatidylinositol-3 kinase and mitogen-activated protein kinase signaling pathways (Natarajan et al., 2002). A direct interaction between GDNF and neural cell adhesion molecule (NCAM), which is expressed on the surface of neural crest cells, has also been implicated (Fu et al., 2004; Paratcha et al., 2003). In addition, both the edn3 and sonic hedgehog pathways appear to be involved since the presence of either protein reduces the migration of crest-derived cells in response to GDNF in vitro (Barlow et al., 2003; Fu et al., 2004; Kruger et al., 2003). Since BMP4 is expressed in both crest-derived and noncrest-derived cells within the gut, its influence on migration may be either a direct effect on the crest cell itself or an indirect one through the intestinal microenvironment. Current efforts are focused on understanding the interactions between GDNF, BMP, and edn3 signaling in regulating migration.

Following completion of hindgut colonization, noggin-overexpressing hindguts demonstrate hypoganglionosis. Moreover, the ganglia in these guts are significantly smaller in size as compared to controls (Figs. 7 and 8). The smaller ganglia may simply result from the fact that there are fewer neural crest cells present to aggregate. However, it is also possible that BMP signaling is directly required for neural crest cells to cluster to form discreet ganglia. This possibility is supported by the observation that BMP2 and BMP4 are required in vitro to promote the aggregation of isolated enteric neurons to form clusters of cells, simulating the appearance of enteric ganglia in vivo (Chalazonitis et al., 2004). Ganglion formation also requires GDNF, which increases the number of neurons per cluster (Chalazonitis et al., 1998). Ganglion size has significant clinical relevance, particularly in intestinal neuronal dysplasia (IND), a severe motility disorder characterized by abnormally large 'giant' ganglia (Kapur, 2000). Understanding the regulation of ganglion size, which likely depends partly on neural crest cell density and partly on specific molecular regulation, may offer new insights into this poorly understood entity.

Noggin non-selectively inhibits BMP2, 4, and 7 (Balemans and Van Hul, 2002). While we find bmp4 expressed in the cells of the enteric nervous system, inhibition of other BMP ligands may be responsible for the ENS abnormalities induced by noggin. For example, we find bmp2 expressed in all layers of the developing hindgut, including the ENS, whereas bmp7 expression is limited to the epithelium alone (Fig. 2). Additional experiments allowing more specifically targeted inhibition, perhaps using antisense oligonucleotides, will allow us to distinguish among these BMP ligands.

In conclusion, our results support an essential role for BMP signaling in ENS development, both in modulating the responsiveness of enteric neural crest cells to GDNF-mediated migration and in the formation of enteric ganglia. Further investigations are ongoing into understanding the mechanism by which BMP influences these morphogenetic events and how the various signaling pathways interact to pattern the mature ENS. A thorough molecular dissection of ENS formation is essential to enhancing our understanding of neuro-intestinal disorders and ultimately improving their treatment.

## 4. Experimental procedures

### 4.1. Embryos

Fertilized white leghorn chicken eggs (Spafas, CT) were incubated at 37 °C with 50% humidified air and staged by day of incubation (E) and/or according to Hamburger and Hamilton stage (Hamburger and Hamilton, 1951).

### 4.2. Organ culture

The gastrointestinal tract was removed from E8 or E9 chick embryos and placed in a three-dimensional collagen gel matrix. Gels were prepared by adding 6 mM NaOH and 1 mg/ml type I rat tail collagen (BD Biosciences) to DMEM medium (Gibco) supplemented with 1% penicillin–streptomycin antibiotic mixture. GDNF (10 ng/ml; RandD Systems), Noggin (400 ng/ml; R&D Systems), or BMP4 (20–200 ng/ml; R&D Systems) protein was added to the collagen gel as described. After 24–48 h the gut

was removed and processed for whole-mount immunohistochemistry.

### 4.3. In situ hybridization

Whole embryos or dissected gastrointestinal tracts were fixed in 4% formaldehyde, dehydrated in methanol, and stored at −20 °C until ready for processing. Riboprobe synthesis and whole-mount RNA in situ hybridization was performed as previously described (Jowett, 1999). Dixoginenin-labeled RNA probes were generated from the chick BMP2, BMP4, BMP7, BMPRII, and noggin genes, kind gifts of Cliff Tabin. In situ hybridization was performed on 6 μm sections from paraffin-embedded tissue, as described (Riddle et al., 1993).

### 4.4. Immunohistochemistry

Immunohistochemistry was performed as described (Faure et al., 2002), with minor adjustments. Sodium citrate antigen unmasking was performed following incubation with hydrogen peroxide. Signal was detected with DAB (Sigma). The following primary antibodies were used: HNK-1 (NeoMarkers, CA), which recognizes an epitope on the surface of migratory and postmigratory neural crest-derived cells (Young and Newgreen, 2001); anti-HuC/D (Molecular Probes, OR), which recognizes a neuron-specific RNA-binding protein; P-Smad 1/5/8 (Cell Signaling, MA), recognizing the phosphorylated form of Smads 1, 5, and 8; BMP4 (Novocastra Laboratories, UK); Bfabp (brain fatty acid binding protein; kind gift of Carmen Birchmeier); 3C2, an antibody against the GAG region of the Rous Sarcoma Retrovirus, recognizing the RCAS viral coat, and cleaved caspase-3 (Cell Signaling). Biotinylated secondary antibodies included goat anti-mouse IgM or IgG, and goat anti-rabbit IgG (Vector Laboratories, CA). Fluorescent secondary antibodies included Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 594 goat anti-mouse IgM, and Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes).

Whole-mount immunohistochemistry was performed using a modification of the protocol previously described (Burke et al., 1995). Methanol-dehydrated guts were rehydrated in PBS, bleached in 15% $H_2O_2$ in Dent's fix (20% DMSO in methanol) for 3 h at room temperature, washed in methanol, and fixed overnight in Dent's fix. Guts were washed in five 1-h washes of PBS, then labeled with primary antibody overnight at 4 °C in a blocking solution of 5% goat serum and 20% DMSO in PBS. PBS washes were performed, followed by overnight incubation at 4 °C with secondary antibody in the same blocking solution. PBS washes were followed by the addition of ABComplex/HRP (Dako). Antibody detection was with 0.05 mg/ml diaminobenzidine and 0.02% $H_2O_2$ in 0.1 M Tris, pH 7.0.

### 4.5. Viral vectors

Noggin misexpression in the developing chick gut was performed using a replication-competent retroviral vector (RCAS). The full-length noggin construct was cloned into the shuttle vector Slax (Morgan and Fekete, 1996) and then cloned into RCAS using established techniques (Logan and Tabin, 1998). DF1 cells transfected with the viral construct were grown to confluence and the supernatant harvested. Viral harvesting, concentration, and titering were performed as described (Cepko, 1991). Control infections were performed using an RCAS—green fluorescent protein (GFP) viral construct.

### 4.6. In ovo viral infection

Embryos were incubated until E2 (approximately stage 12), windowed, and viewed under a Nikon SMZ800 dissection microscope. Using a Hamilton syringe, pulled glass needle, and micromanipulator (Narishige), approximately 1 μl of virus ($1\times10^5$ cfu) was injected into each side of the embryo, directed at the presumptive gut mesoderm, based on established chick fate maps (Matsushita, 1995). The eggs were then sealed, returned to the incubator, and harvested at various time-points, 10–12 days after infection. Controls consisted of both uninjected and GFP-injected embryos.

### 4.7. Tissue processing

Gastrointestinal tracts of experimental and control embryos were examined grossly, removed, fixed in 4% formaldehyde, and dehydrated. Paraffin embedding was as described (Faure et al., 2002). General cytoarchitecture was examined using hematoxylin and eosin (H&E), prepared using standard procedures (Bancroft and Stevens, 1990).

### 4.8. Photography

Sections were examined under a Nikon Microphot FXA microscope and digital images captured with a Spot camera and software version 3.3.1 (Diagnostic Instruments). Images were compiled using Adobe Photoshop.

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