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Article

Essential Role of BMP4 Signaling in the Avian Ceca in Colorectal Enteric Nervous System Development

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Abstract: The enteric nervous system (ENS) is principally derived from vagal neural crest cells that migrate caudally along the entire length of the gastrointestinal tract, giving rise to neurons and glial cells in two ganglionated plexuses. Incomplete migration of enteric neural crest-derived cells (ENCDC) leads to Hirschsprung disease, a congenital disorder characterized by the absence of enteric ganglia along variable lengths of the colorectum. Our recent data [1] support an essential role for the avian ceca, present at the junction of midgut and hindgut, in hindgut ENS development, since ablation of the cecal buds leads to incomplete ENCDC colonization of the hindgut. *In situ* hybridization shows bone morphogenetic protein-4 (BMP4) highly expressed in the cecal mesenchyme, leading us to hypothesize that cecal BMP4 is required for hindgut ENS development. To test this, we modulated BMP4 activity using embryonic intestinal organ culture techniques and retroviral infection *in ovo*. We show that overexpression or inhibition of BMP4 in the ceca disrupts hindgut ENS development, with GDNF playing an important regulatory role. Our results suggest that these two important signaling pathways are required for normal ENCDC migration and enteric ganglion formation in the developing hindgut ENS.

Keywords: enteric nervous system; neural crest; ceca; hindgut; hirschsprung disease; BMP4; Noggin; GDNF

1. Introduction

Neural crest cells (NCC) represent a transient population of multipotent stem cells capable of migrating along specific paths throughout the vertebrate embryo to differentiate into a variety of cell types, including the neurons and glial cells present in the gastrointestinal tract (GI). Neural tube ablation in early chick embryos, and neural tube transplantation experiments between chicken and quail embryos, proved that NCCs adjacent to somites 1-7 are the major source of enteric glial and neuronal precursors [2–4]. A small portion of NCCs from the sacral neural tube level (posterior to the 28th somite) were also found to contribute to the ENS, mostly in the hindgut [5–7]. Once NCCs enter the gut mesenchyme, at which point they are referred to as enteric neural crest-derived cells (ENCDCs), they migrate along the length of the entire GI tract to form the enteric nervous system (ENS)[3,4,6,8]. Abnormal ENS formation leads to a number of congenital neurointestinal disorders in humans, including intestinal neuronal dysplasia, hypoganglionosis, and Hirschsprung disease [9,10]. Hirschsprung disease is characterized by the absence of the ENS along a variable length of the distal GI tract, resulting in functional obstruction due to severely impaired gut motility.

Epithelium-mesenchyme recombination experiments demonstrated that the instructive role of the mesenchyme during the ontogeny of the intestine is essential in the formation and patterning of ENCDC-derived enteric plexuses [11–13]. For example, mesenchymal expression of BMP4 in the presumptive submucosal area is initiated by Sonic hedgehog (SHH) secreted from intestinal epithelial cells and has multiple effects during ENS development [14–16]. Developmental studies have also

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demonstrated a reciprocal interaction between FGF in the gut epithelium and BMP4 in the mesenchyme regulating the development of the cecal primordium in mice [17]. This region of the intestine has been proposed to have a key role in hindgut ENS formation. In both avian and rodent embryos, the cecal environment modifies the proliferation, differentiation and migratory properties of ENCDCs to promote their journey into the distal hindgut [1,18,19]. These results suggest that BMP4 produced by the cecal mesenchyme may influence ENCDCs as they colonize the distal gut.

Previous experiments demonstrated that BMP4 acts through BMP receptors on ENCDCs and regulates cell migration [20,21] and differentiation of enteric neurons [22] and glial cells [23]. BMP proteins also play an important role in determining the neuronal-to-glial cell ratio in the ENS, with Noggin-mediated inhibition of BMP increasing the total number of neurons [22] while decreasing the proportion of glia [23]. Transcripts encoding *BMP-2* and *BMP-4* were each detected in ENCDCs. Similarly, transcripts encoding *NOGGIN* and BMP receptors (BMPRII) and their signal transducers (pSMADs) were also detected in the developing ENS, especially after ENCDCs complete their colonization, suggesting a potential paracrine or autocrine role in ENS development [21,24,25]. BMPs also promote gangliogenesis when ENCDCs aggregate, which is associated with changes in the expression of neural cell adhesion molecule (NCAM) [20,21,26]. Neuron-specific overexpression of Noggin increased the number of neurons in both submucosal and myenteric plexuses of developing rat small intestine [27]. Blocking BMP signaling in the mesenchymal layer of the intestine inhibits the development of smooth muscle and results in abnormal patterning of the ENS [21,24,25,28]. However, experimental results from avian and mouse embryos have shown conflicting results. Noggin overexpression in chicken embryos, for example, leads to hindgut hypoganglionosis [21], while excess BMP4 in mouse organ cultures inhibits ENCDC migration [20]. BMP4 misexpression in the chicken gizzard mesenchyme conducts to hypertrophic ectopically positioned ganglia [25]. BMP2 promotes neuronal differentiation of mouse and rat ENCDCs [29], and also induces the expression of GDNF receptors on the surface of NCCs to promote GDNF responsiveness [30]. Finally, exposure of rat ENCDC cultures to GDNF in combination with high levels of BMP2 or BMP4 leads to significantly more neurons [27].

The present study aimed to characterize the expression of BMP4 during avian hindgut ENS development and to investigate the effect of BMP signaling on ENCDC migration and differentiation. We find that BMP4 is not expressed in the hindgut mesenchyme prior to the arrival of ENCDCs in the ceca. Interestingly, however, BMP4 is expressed in the ceca at least 1 day before ENCDC arrival, leading us to hypothesize that BMP4 may prepare the mesenchymal environment of the ceca ahead of their arrival. Using BMP4-encoding retrovirus and recombinant BMP4 protein, we show that modulating endogenous BMP activity disrupts normal hindgut ENS development. Our data support multiple essential roles for BMP signaling during hindgut ENS formation: i) BMP4 overexpression leads to large and ectopic enteric ganglia, while its inhibition with Noggin leads to hypoganglionosis; ii) BMP4 promotes enteric ganglion formation; iii) GDNF inhibits the response to BMP4, suggesting interactions between these signaling pathways in the regulation of distal ENS formation.

2. Results

2.1. BMP4 is an important signaling hub in the developing intestine.

The avian ceca provide signaling cues essential for ENCDC colonization of the distal hindgut [1]. To explore signaling hubs with the potential to regulate enteric ENCDC and development, we generated a PPI (protein-protein interaction) network based on RNA-seq data obtained from the embryonic (E5) ceca and "interceca," which represents the segment of intestine between the paired avian ceca The top ligand in the ceca, ranked by the number of potential interactions (degrees), was BMP4, which was upregulated 1.8 fold in the ceca and was predicted to interact with 24 DEGs (differentially expressed genes) in the dataset (Figure 1 C). This included interactions with WNT proteins and SHH, which were previously determined to be required for ENS colonization (Figure 1 D) [13]. Expression of genes involved in BMP signaling were further assessed in the ceca and interceca (Figure 1 E). BMP4 was the most highly expressed BMP ligand across the dataset. Receptors for BMP4 were detected in both the ceca and interceca regions, including ACVR1, ACVR2A and BMPRII.

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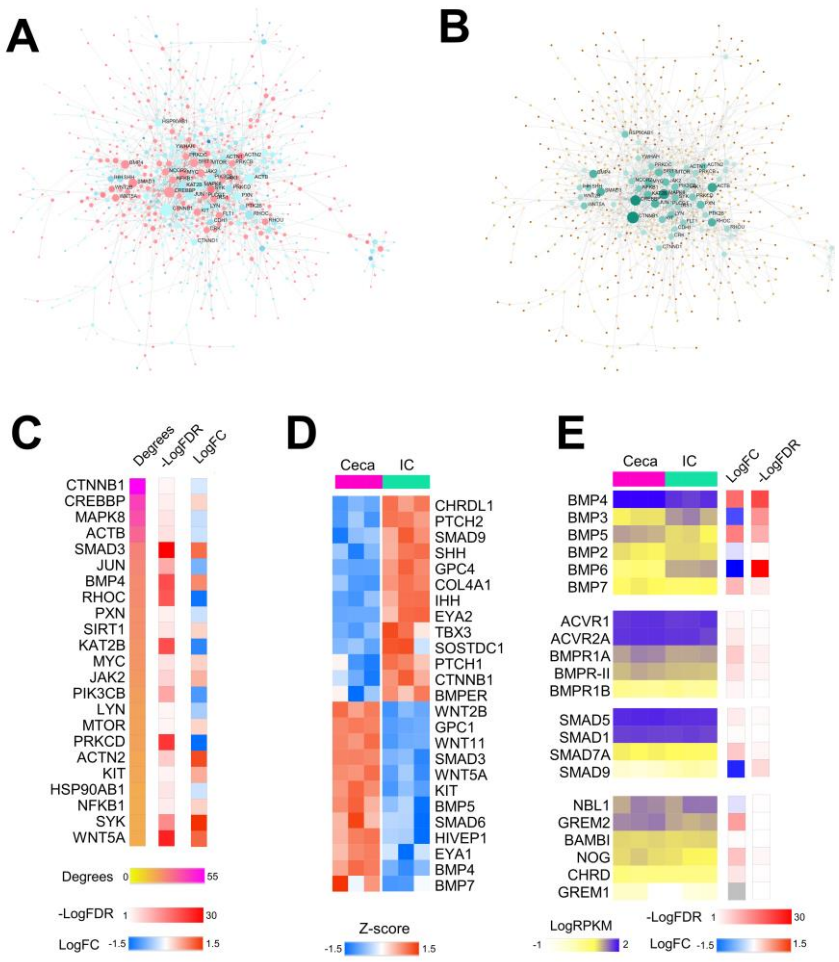

Figure 1. RNA-seq analysis of the BMP signaling network in the E5 ceca and interceca. A) Node-edge map visualization of PPI networks in DEGs upregulated in the ceca (Red) or the interceca (Blue). B) Network topology visualization of PPI interactions with node sizes and colors (brown – lowest, green - highest) representing the number of potential interactions between nodes (degrees). C) Heatmap representation of the top signaling hubs ranked by degrees with corresponding significance levels (-Log10FDR) and fold change (log2FC) for genes in the ceca relative to the interceca. D) Heatmap of BMP4 network interactors presented as Z-scores of RPKM values in the ceca and interceca. E) Expression levels (Log10RPKM) of ligands, receptors, SMADs and antagonist in the BMP pathway in the ceca and interceca with corresponding significance levels (-Log10FDR) and fold change (log2FC) for genes in the ceca relative to the interceca.

2.2. Expression of BMP4 signaling components supports a role during hindgut ENS formation

BMP4 expression has been previously described in the vertebrate GI tract [14,15,20,21,25,28,31], but its expression during development of the avian hindgut and formation of the ENS is not known. To examine the spatial pattern of BMP4 expression in the developing colorectum, wholemount *in situ* hybridization was performed at E5 (Figure 2A) and E6 (Figure 2C), shortly after ENCDCs colonize the post-umbilical midgut and ceca, respectively [1]. Undifferentiated ENCDCs were defined as those that express p75 (NGFR, nerve growth factor receptor), SOX10 (markers for ENCDCs and enteric glia), N-cadherin (CDH2) or HNK1. At E5, BMP4 is restricted to the ceca mesenchyme (Figure 2B), with expression in the hindgut mesenchyme starting at E6 (Figure 2D). Transverse sections at E6 and E7 confirm that BMP4 transcripts are specifically present in mesenchyme immediately adjacent to the hindgut epithelium (Figure 2D,E). In contrast to the hindgut, BMP4 is not expressed in the E5 or E6 midgut mesenchyme (Figure 2A,C). By E14, BMP4 expression is restricted to the prospective lamina propria (Figure 2F,G) and does not co-localize with p75-expressing ENCDCs [4] or SMA-expressing intestinal smooth muscle (Figure 2F,G-G").

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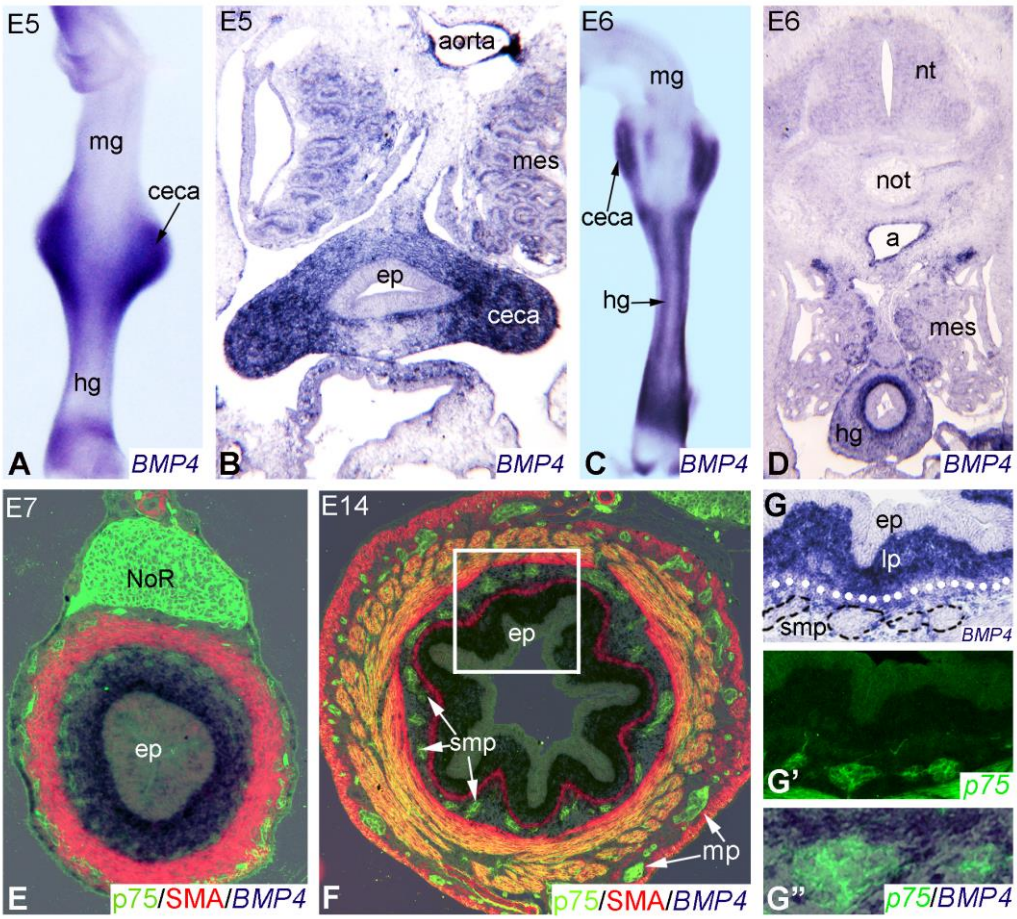

Figure 2. Expression of BMP4 during hindgut development. Whole-mount *in situ* hybridization of an E5 gut shows BMP4 expression primarily in the ceca (A,C). From E6 through E14, BMP4 is expressed in the inner mesenchyme along the entire hindgut and ceca, shown in transverse (B,D,E,F) sections. Double staining of E7 and E14 hindgut by BMP4 *in situ* (E,F) and p75 immunofluorescence (E,F,G) shows that BMP4 is not expressed by the p75+ ENCDCs. G is high magnification of submucosa/epithelium of F. Dotted lines denote location of lamina muscularis mucosae. a, aorta; *ep, epithelium; hg, hindgut; lp, lamina propria; mes, mesonephros; mg, midgut; mp, myenteric plexus; NoR, nerve of Remak; nt, neural tube; not, notochord; smp, submucosal plexus.*

In situ hybridization was also performed on hindgut sections at various developmental stages using a BMPRII riboprobe (Figure 3A,B). In addition, functional BMP activity was confirmed at the same stages with an antibody, recognizing the active, and phosphorylated form of SMAD 1,5, and 8 (namely pSMAD; Figure 3C,D). At E6, BMPRII is expressed in the nerve of Remak (Figure 3A,A'). Double staining of E8 hindgut for BMPRII transcript (Figure 3B) and p75 protein shows co-expression on ENCDCs (Figure 3B'). To define the relationship of BMP signaling in ENCDCs, immunostaining was performed for pSMAD and Ncadherin on longitudinal sections of E6 hindgut. pSMAD is specifically detected in the ceca mesenchyme (Figure 3C) at E6, with subsequent expression in the N-cadherin+ nerve of Remak, enteric ganglia, inner layer of the muscularis propria, and subepithelial mesenchyme (Figure 3D,D'). Interestingly, as shown in Figure 3C'-C", pSMAD expression is not present in the N-cadherin+ ENCDCs present at the migratory wavefront, suggesting that exclusion of BMP signaling in cells migrating through the ceca may help to prevent ganglion formation and maintain the migratory stream for hindgut colonization. At E6, the ENCDC wavefront is in the ceca, whereas at E8 ENCDCs have reached the distal hindgut [1]. At this stage, ENCDCs in the enteric ganglia express pSMAD (Figure 3D'). To confirm expression of BMPRII and pSMAD in the developing ENS, explanted E6 chick midguts were cultured with GDNF (10 ng/ml) for 24 hours, which leads to migration of ENCDCs onto the fibronectin coated surface (Figure 3E-F"). BMPRII was broadly expressed by the ENCDCs (Figure 3E-E"), whereas pSMAD immunoreactivity was heterogenous (Figure 3F-F"), confirming that ENCDCs possess the cellular machinery for BMP4 signaling.

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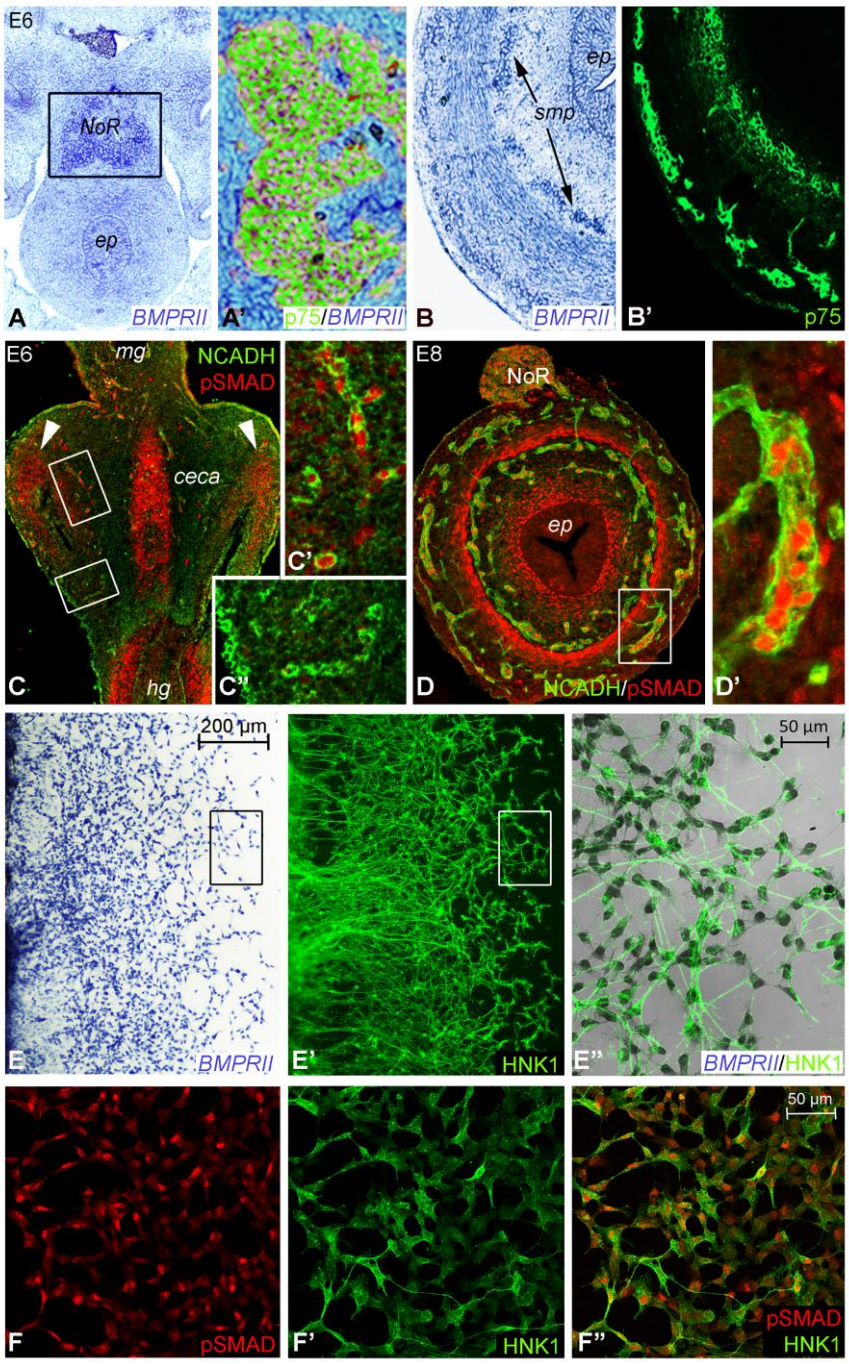

Figure 3. Expression of BMPRII and phosphoSMAD during hindgut development. *In situ* hybridization of an E6 gut shows BMPRII expression concentrated in the nerve of Remak (A). Boxed area in A is magnified in A'. At E8, BMPRII is expressed in the p75+ ENS (B,B'). Longitudinal section through the ceca and hindgut at E6 shows N-cadherin+ ENCDCs throughout the ceca mesenchyme. Arrowheads indicate localized pSMAD expression in ceca mesenchyme. Boxed areas are magnified in C' and C''. Double-immunofluorescence with the pSMAD antibody shows that the trailing cells shown in Figure C' co-express pSMAD, while the wavefront cells (Figure C'') defined as the distalmost N-cadherin expressing undifferentiated ENCDCs, show no expression of pSMAD. At E8 pSMAD is strongly expressed by the subepithelial mesenchyme, the inner layer of the tunica muscularis, as well as in the developing N-cadherin immunoreactive ENS (D; boxed area in D is magnified in D'). Explanted E6 chick midgut was cultured with GDNF, which induces robust ENCDC migration from the intestinal tissue. Both BMPRII (E) and pSMAD (F) are strongly expressed by the migrating HNK1+ ENCDCs (E'',F''). Boxed area in E and E' is magnified in E''. *ep, epithelium; hg, hindgut; mg, midgut; NoR, nerve of Remak; smp, submucosal plexus.*

2.3. Inhibition of BMP4 signaling leads to hindgut hypoganglionosis

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Given the observation that the ENS-containing E6 ceca mesenchyme expresses BMP4, whereas the hindgut has neither ENCDC nor BMP4 expression at this stage, we hypothesized that cecal BMP4 is required for hindgut colonization, as previously shown for other morphogens expressed in the ceca, like GDNF, Endothelin-3, and Wnt11 [1,19]. To test this, the E6 intestine was placed in catenary culture for 48h in the presence of recombinant BMP4 (200ng/ml) or Noggin (200ng/ml) (Figure 4). Tuj1 antibody was used to label neurons and SOX10 to mark all ENCDCs. Culturing E6 intestine for 2 days with no additive (DMEM) led to full ENCDC colonization of the hindgut, with Tuj1+ cells in both enteric plexuses along the length of the hindgut (Figure 4A). In BMP4-treated explants, Tuj1+ cells were present throughout the hindgut and were characterized by large aggregates, suggesting that BMP4 induces premature ganglion formation and hyperganglionosis (Figure 4B). To determine whether BMP4 enhances glial differentiation in addition to premature ganglion formation, enteric neuron specific anti-HU (ELAV-like protein 4) and enteric glia specific brain-fatty acid binding protein (BFABP; FABP7) double immunofluorescence was performed. After culturing E6 intestine for 2 days with no additive, BFABP+ cells are found distributed throughout the nerve of Remak and enteric plexuses (Figure S1A-A”). In BMP4-treated cultures, BFABP+ cells were present throughout the intestine in large ganglia, consistent with hyperganglionosis (Fig. 4B). Interestingly, the majority of the nerve of Remak cells expressed BFABP, suggesting that BMP4 promotes enteric glial differentiation (Figure S1B-B”), as previously reported[23]. In contrast, explants treated with Noggin are characterized by hindgut hypoganglionosis, with fewer Tuj1+ neurons present (Figure 4C). In Noggin-treated cultures, nerve of Remak cells do not express BFABP, suggesting that BMP inhibition inhibits glial differentiation (Figure S1 C-C”).

The size of enteric ganglia and length of colonized hindgut was also measured in each of the treatment conditions and shows statistically significant differences between CTRL vs. Noggin treated, CTRL vs. BMP4 and BMP4 vs. Noggin treated groups. (Figure 4D,E). Figure 4D shows that Noggin treatment accelerates hindgut colonization. Statistical analysis was performed using Kruskal–Wallis test with a post-hoc Dunn’s test showing significant differences in ganglion sizes between control vs. BMP4 treated ($p<0.01$) and BMP4 vs. Noggin treated guts ($p<0.001$). There is no difference in ganglion’s diameter between control and Noggin treated segments (Figure 4E). The opposite effect of BMP4 and Noggin on ganglion formation could result from changes in ENCDC proliferation. Therefore, we examined ENCDC proliferation by measuring EdU incorporation into SOX10+ cells in the presence of BMP4 or Noggin. In controls, EdU+ cells were primarily seen in the mesenchyme and in 20.9±5.7% of SOX10+ cells (Figure 4F). Interestingly, both treatments showed a trend toward decreased ENCDC proliferation, suggesting a potential role for BMP signaling in regulating ENCDC proliferation in the developing gut.

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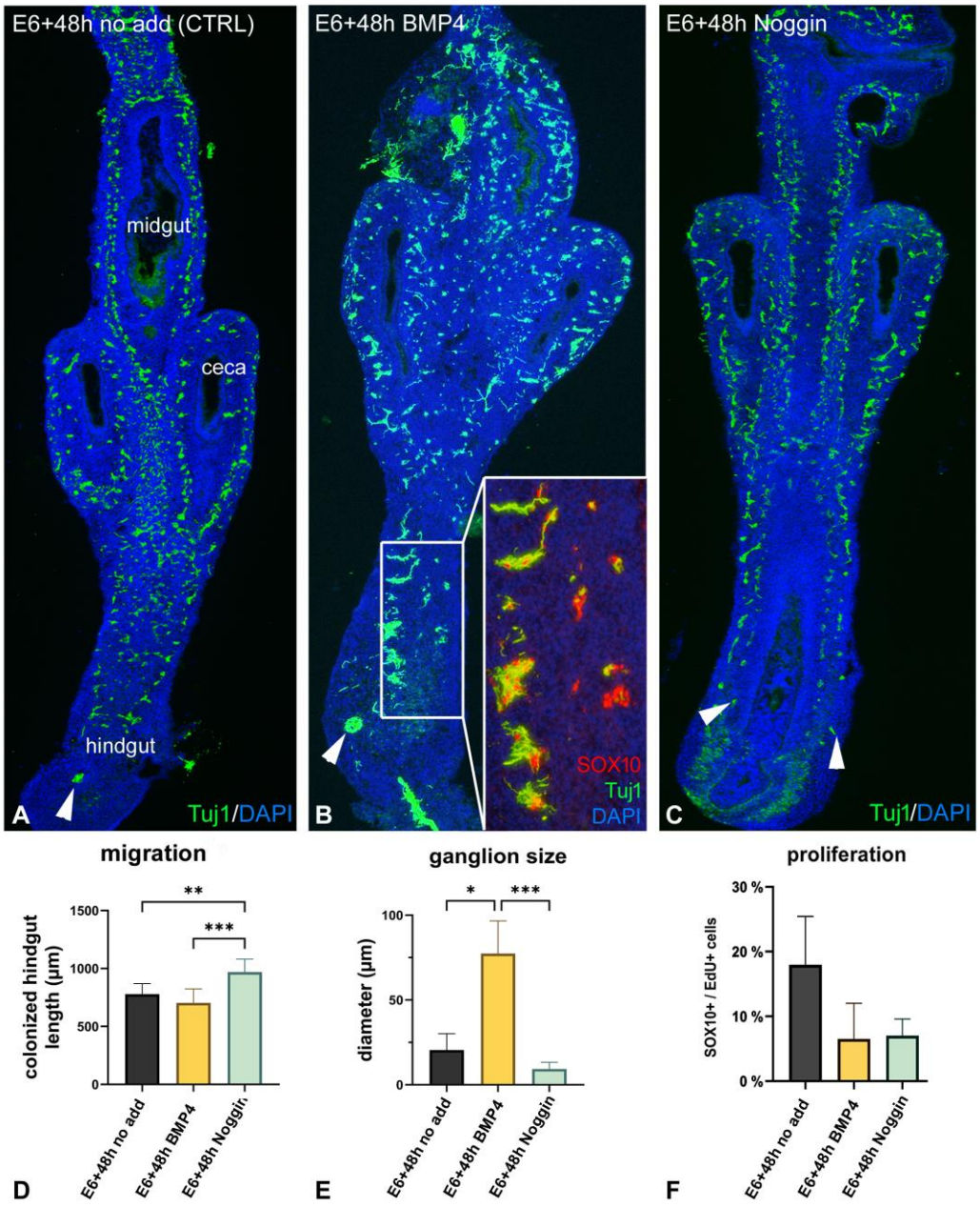

Figure 4. BMP4 signaling is required for hindgut colonization. E6 chick gut was cultured in catenary culture for 2 days in the absence of additives (A), with BMP4 protein (B), or with Noggin protein (C). Longitudinal sections are shown in A, B and C figures. Arrowheads indicate wavefront cells. Corresponding transverse section through the mid-hindgut used for quantification is shown beneath (E). Addition of BMP4 induce large ganglion formation in the hindgut (B, B inset), whereas inhibition of BMP4 signaling with Noggin leads to small ganglia formation (C). n=21. Cells in the ganglion express neuronal markers Tuj1, and SOX10, which labels ENCDCs and enteric glial cells (B, B inset). Quantification of the length of SOX10+ ENCDC colonization of the hindgut from the ceca (n=7 guts per group) (D). The average diameter of the SOX10+/TUJ1+ enteric ganglia in control DMEM-treated hindgut, compared to BMP4-treated and Noggin-treated hindgut ganglia. Data are mean ±s.d. n=9 (3 different areas were measured on 3 hindguts/group). (E). Following BMP4 and Noggin treatment, there was a tendency toward reduced ENCDC proliferation compared with no additive controls (F). Kruskal-Wallis with Dunn's multiple comparison test was used for statistical analysis. n=12. *** $p<0.001$, ** $p<0.01$, * $p<0.05$.

To further test the effect of BMP4 signaling on hindgut ENS development, E6 intestine was cultured for 2 days in the presence or absence of BMP4 or its antagonist Noggin. Normally, the E6 ENCDC wavefront is at the level of ceca [1], and in the distal hindgut by E8 (Figure 5A-C'). Similarly, E6 intestine cultured for 2 days demonstrates complete ENCDC colonization (Figs 4A, 5D-F').

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Addition of BMP4 to the culture media leads to large and ectopic ganglia formation in the midgut and hindgut (Figure 5G,H,I-I″), with no effect on ganglion size or ENS patterning in the ceca (Figure 5H). By contrast, addition of Noggin caused hindgut hypoganglionosis (Figure 5L,L′). Interestingly, addition of Noggin did not affect ENS formation in the ceca (Figure 5K), but had a significant effect on midgut ENS formation, with complete disruption of normal patterning into two plexuses. (Figure 5J). Staining for alpha-smooth muscle actin (SMA) is shown for comparison. (Figure 5C, F″,I″,L″).

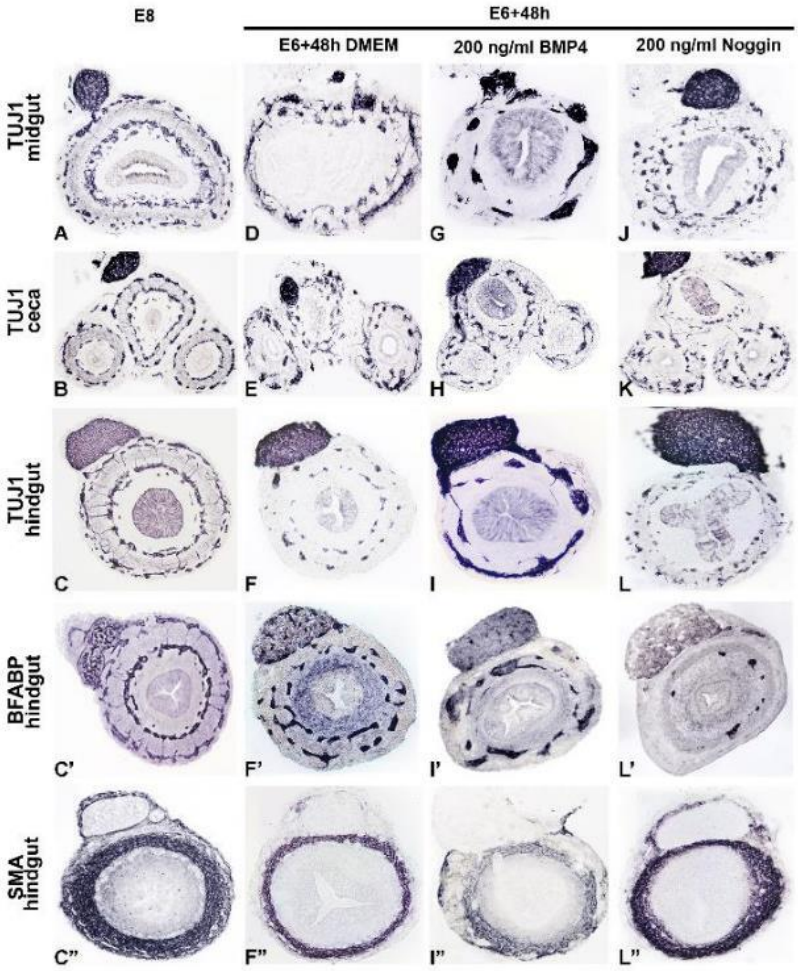

Figure 5. BMP4 overexpression leads to hyperganglionosis in chicken midgut and hindgut. Chick E6 gut was cultured in catenary culture for 2 days in the absence of additives (D-F″), with BMP4 protein (G-I″), or with Noggin (J-L″). Transverse sections are shown through the mid-hindgut. Addition of BMP4 induced ectopic and large ganglia in the midgut (G) and hindgut (I, I″), whereas inhibition of BMP4 signaling led to significant hindgut hypoganglionosis (L, L′). Noggin treatment induced the disorganization of the midgut ENS: (J) compared to control (D) and E6 gut cultured for 48h. Interestingly, BMP4 treatment did not alter significantly the ENS pattern at the level of the ceca, as seen by comparing ENS morphology of Tuj1 immunostained sections of BMP4 treated ceca (H) to control (B) and E6 gut cultured for 48h (E). Consecutive cross-sections of the hindgut show the presence of BFABP+ glia cells (C′, F′,I′,L′) and alpha-SMA+ smooth muscle layers (C″, F″,I″,L″).

2.4. Retrovirus-mediated overexpression of BMP4 induces robust gangliogenesis in vivo

The effect of BMP4 on the development of the hindgut ENS was also examined *in vivo* using a replication-competent retrovirus (RCAS) expressing the chicken BMP4 gene (Figure 6). As we previously described [13], RCAS virus was injected into the E6 chicken hindgut mesenchyme and then cultured on an E8 chick chorioallantoic membrane (CAM) for 9 days (Figure 6A). Trophism of avian retroviruses is specific to mesenchymal cells and does not directly target ENCDCs (Faure et al, Development, 2015). The 3C2 antibody recognizing the RCAS P19 gag protein shows successful and

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extensive viral replication in the intestinal wall (Figure 6F,F'). As shown in Figure 6G-H, RCAS-BMP4 leads to significant enteric hyperganglionosis, with large and disorganized ganglia throughout the gut wall, with loss of the distinct patterning into myenteric and submucosal plexuses observed in control intestine (Figure 6B-E). Smooth muscle morphology is similarly disturbed (Figure 6I), with no discernible separation into the three muscle layers (muscularis mucosae, circular muscle, longitudinal muscle) normally seen (Figure 6E).

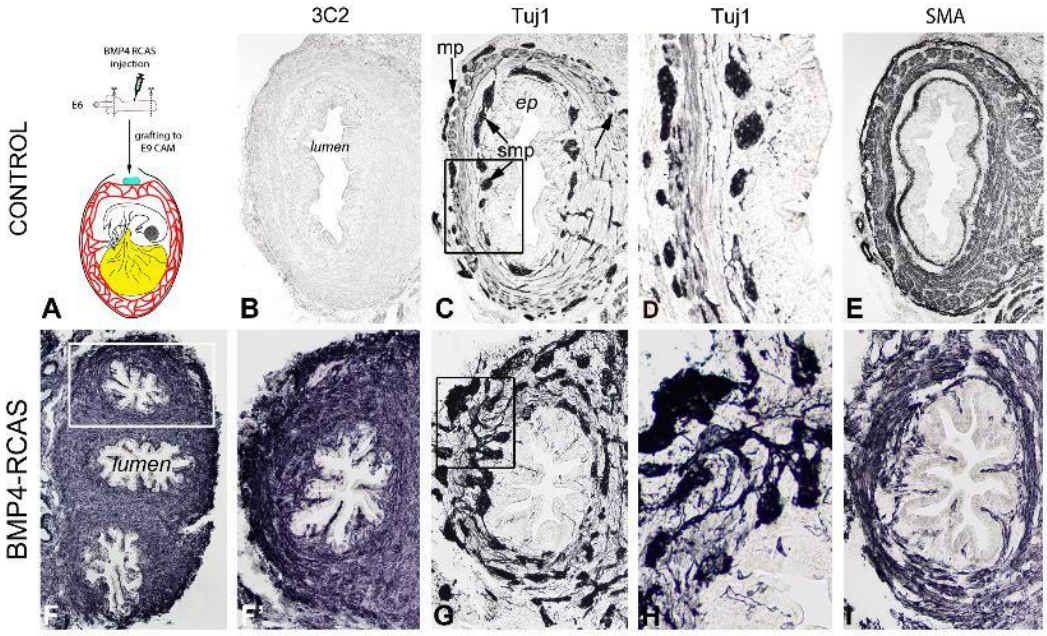

Figure 6. Retrovirus-mediated misexpression of BMP4 disrupts hindgut ENS development. Overexpression of BMP4 induced ectopic and large ganglia in the gut wall. Schematic illustration demonstrate ex vivo injection of dissected E5 hindguts with RCAS-BMP4 and cultured for 9 days on an E8 CAM (A). Immunostaining of consecutive sections obtained from control CAM grafts for 3C2 antibody specific for RCAS (B), Tuj1 (C, boxed area magnified in D) and alpha-SMA (E) is shown for comparison. RCAS was strongly expressed throughout the gut wall (F, boxed area magnified in F'), and associated with hyperganglionosis, abnormal radial patterning of enteric ganglia (G, boxed area magnified in H) and abnormal smooth muscle development (I). ep, epithelium; mp, myenteric plexus; smp, submucosal plexus.

2.5. GDNF inhibits BMP4 induced ENCDC aggregation

GDNF signaling is the most important pathway implicated in congenital neurointestinal pathogenesis, and like BMP4, it is spatially and temporally restricted to the cecal region just prior to the arrival of ENCDCs (Nagy and Goldstein, 2006). To test the effect of BMP4 on ENCDCs as they migrate through the cecal region to colonize the hindgut, explanted E6 ceca were cultured with BMP4 in the absence (Figure 7) or presence of GDNF (Figure 8). ENCDCs exhibit robust migration from ceca explants after 48 h of culture in response to GDNF, as previously reported [1], with the majority of ENCDCs expressing the neuronal marker Tuj1 (Figure 7A,A'). When GDNF was removed from the culture media after the first 24 h, and replaced with no additive morphogen, cell migration over the next 24 h was significantly reduced (Fig 7B) and ENCDCs aggregated into a uniform network of interconnected ganglia (Figure 7B'). When the GDNF was instead replaced after 24 hours with either BMP4 (Figure 7C) or Noggin (Figure 7D) for an additional 24 h period, major changes were observed. BMP4 treatment resulted in large ENCDC aggregates with extensive Tuj1 expression (Figure 7D') and markedly reduced ENCDC proliferation (Figure 7G). In contrast, Noggin prevented normal ganglion formation and disrupted the development of interganglionic connections (Figure 7D,D'). Noggin did not affect ENCDC migration (Figure 7E), or proliferation (Figure 7F), but significantly reduced the rate of neuronal differentiation.

Finally, we assessed the effect of BMP4 treatment in combination with GDNF in ceca explants (Figure 8). When the culture media contained both BMP4 and GDNF, large cell aggregates did not occur (Figure 8 B,B'). We therefore conclude that BMP4 acts directly on ENCDCs to reduce their proliferation and promote their aggregation into ganglia, but this effect is normally regulated by the

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presence of GDNF in the cecal mesenchyme, preventing premature ganglion formation so that the ENCDC wavefront can continue its migration into the hindgut.

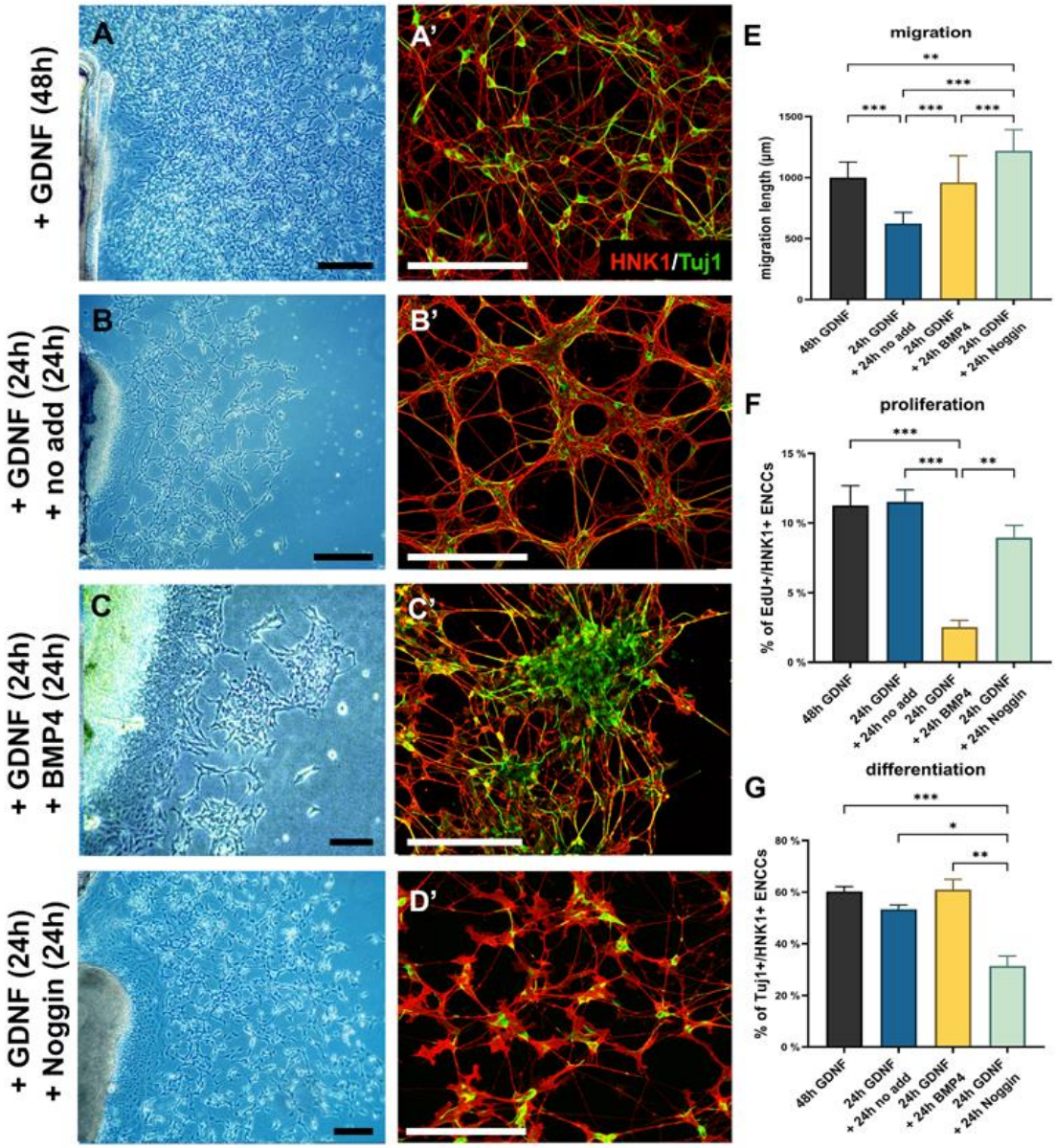

Figure 7. BMP4 induces in vitro gangliogenesis. E6 ceca were cultured on fibronectin-coated surface with GDNF for 48 h (A) or after 24 h the GDNF was removed and followed by 24 h with either no added factors (B) or addition of BMP4 (C) and Noggin (D) protein. Cell cultures were stained with HNK1 and Tuj1 antibodies (A'-D') to assess ENCDC migration distance and neuronal differentiation. (E) Addition of BMP4 did not significantly alter the migration compared to 48h GDNF cultures, but Noggin increased the migratory distance of the ENCDCs. (F) The addition of BMP4 protein significantly inhibited the proliferation of cultured ENCDCs. (G) There is no difference in percentage of Tuj1+/HNK1+ cells between (48h) GDNF, or 24h GDNF + 24h no additive. Excess of BMP4 not induced significant neuronal differentiation of ENCDCs but initiated their aggregation. In contrast, in presence of Noggin, neuronal differentiation and aggregation were markedly reduced. All scalebars represent 200 µm. n=7-10 cell cultures / experiment. Kruskal-Wallis with Dunn's multiple comparison test was used for statistical analysis. *** $p<0.001$, ** $p<0.01$, * $p<0.05$.

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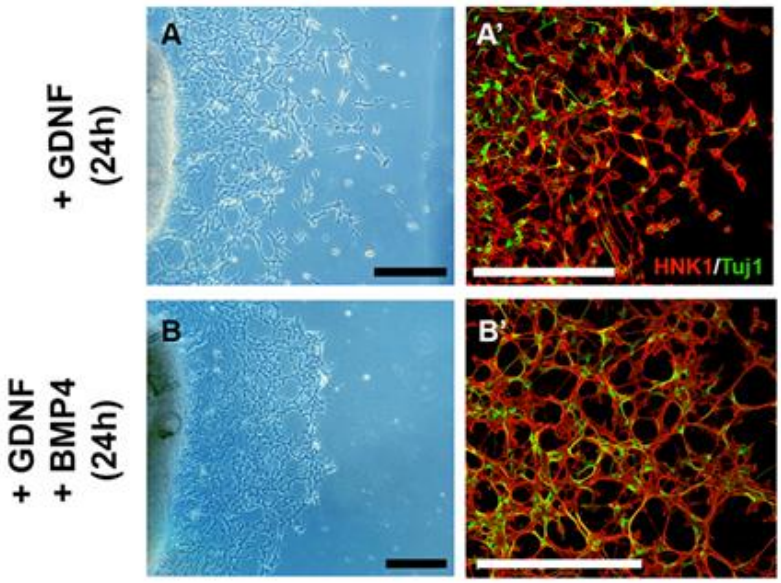

Figure 8. Concurrent administration of GDNF and BMP4 hinders the ganglion-forming effect of BMP4. E6 ceca were cultured on fibronectin-coated surface with GDNF for 24 h (A) or GDNF+BMP4 (B) for 24 h. Cell cultures were stained with HNK1 and Tuj1 antibodies (A'-B') to assess ganglion formation. GDNF-mediated ENCDC migration from E6 ceca is robust at 24 h, with the distance of cell migration shown (A). There is no significant difference between GDNF and GDNF+BMP4 groups, but the simultaneous administration of GDNF with BMP4 inhibited aggregation of Tuj1+/HNK1+ cells. n=7 cultures / experiment. All scalebars represent 200 μm.

3. Discussion

Among developmental anomalies of the neurointestinal system the most common are the neuronal dysplasias, characterized by ectopic ganglia associated with hypoganglionosis or hyperganglionosis, and Hirschsprung disease, which is characterized by colorectal aganglionosis. These congenital diseases is largely due to genetic changes occurring in the GDNF and endothelin-3 signaling pathways [19,32]. Several lines of evidence support the hypothesis that BMP-4, which, like GDNF and endothelin-3, is also secreted by intestinal mesenchymal cells, contributes to ENS development. However, experiments conducted using a variety of *in vivo* and *in vitro* systems and different model organisms have yielded conflicting results [16,18,20,21,27,28]. BMPs (BMP2, 4, and 7) bind and dimerize with type I (BMPR1A or BMPR1B) and type II (BMPRII) receptors to phosphorylate and activate the canonical SMAD1/5/8 signaling cascade [33]. BMP receptors and the BMP antagonists are all expressed in the developing ENS and regulate ENCDC migration [20,21,25] and differentiation into specific neurons and glia [27,29] by controlling the expression of genes involved in ENS specification and maturation. BMP signaling is also involved in the formation and patterning of the enteric ganglia, ensuring the proper distribution and connectivity of enteric neurons. Furthermore, transcription factor Smad-interacting protein 1 (also known as SIP1 or ZEB2), a negative regulator of BMP4 signaling [34], is involved in the specification, differentiation, and migration of neural crest cells [35] and mutations in this gene are associated with Hirschsprung disease [36–38]. Despite the widely recognized relationship between BMP4 and ENS development, the mechanism by which BMP4 controls hindgut colonization is unknown.

Our current study provides several new observations regarding the role of BMP4 on colorectal ENS development: (i) BMP4 expression is restricted to the cecal mesenchyme just prior to ENCDC arriving there; (ii) pSMAD is not expressed by wavefront ENCDCs as they migrate through the ceca, which may prevent premature ganglion formation and maintain the migratory stream for hindgut colonization; (iii). presence of excess BMP4 protein led to complete colonization of the hindgut and formation of large, ectopic ganglia while addition of Noggin to inhibit BMP signaling enhanced ENCDC migration into the hindgut, prevented ganglion formation, and reduced neuronal and glial differentiation; (iv) Presence of GDNF reverses the effect of BMP4 on ganglion formation. These

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results support the idea that ceca mesenchyme rich in BMP4 plays an important role in the colorectal colonization by ENCDCs during avian embryo development, and its interaction with GDNF.

BMP4 and its receptors have been previously identified during mammalian [20,27] and avian [21,25] ENS development. BMP signaling components have been identified throughout the mesenchyme of the developing chicken gut, except the stomach and hindgut [15,39]. According to Goldstein and his group [21], earliest BMP4 expression was seen at E8 in subepithelial mesenchyme of the ENCDC colonized hindgut. More recent studies shown that members of BMP family (BMP2, BMP4 and BMP7) were symmetrically expressed within the E12 chicken midgut and mesentery and BMP signaling, in addition to regulating ENS development, also controls intestinal villus formation, smooth muscle differentiation and intestinal lopping morphogenesis [24,40,41]. Our transcriptome analysis combined with wholemount in situ hybridization study extends previous observations demonstrating that BMP4 is produced first in the ceca mesenchyme at developmental stage just prior to the colonization by migrating ENCDCs. Early detection of BMP4 expression in E5 chicken ceca is consistent with wholemount in situ hybridization data observed in 10.5 dpc mouse embryo where first sign of BMP4 expression was also seen in the precolonized cecum bud as soon as the primordium begins to grow from the intestine [17].

The ceca is a structure at the junction of the small and large intestines. It has been proposed that exposure to the cecum is indispensable for the colonization of the hindgut by ENCDCs [1]. Expression of developmentally important molecules (GDNF, EDN3, or non-canonical Wnt proteins, including Wnt5a and Wnt11) are higher in the cecum than elsewhere in the hindgut [1,19]. Remarkably, it is the first site where defects in hindgut colonization are observed in EDN3 and EdnrB mutant mice [42–45]. At the level of the ceca, the mesenchymal derived EDN3 and Wnt11 growth factors are permissive for the chemoattractive function and inhibitory to the pro-neurogenic effects of GDNF, thereby promoting the migration of undifferentiated ENCDCs through the ceca and into the hindgut [1,19,46]. Our studies add BMP4 to this signaling complex, supporting a role for BMP4 signaling in colorectal ENS development. In contrast to the ceca, BMP4 expression is turned on in the hindgut mesenchyme after ENCDCs pass the ceca, and it continues to be expressed adjacent to the migrating and differentiating ENCDCs throughout early ENS patterning in the hindgut. This finding is consistent with previous observations that BMP4 signaling influences hindgut ENS development [20,21] and supports the idea that this protein promotes glial and neuronal differentiation and induces cell aggregation, all key steps for ENS formation.

To assess how BMP4 signaling regulates hindgut colonization and supports ENS development, we used virus-mediated overexpression of BMP4 in the precolonized hindgut mesenchyme and further cultured the hindgut on a CAM surface. We used the same avian specific retroviral expression system in gastrulating chicken embryo as previously reported [25] to induce ectopic and giant ganglion formation in the stomach. These embryos however are not viable and die around E5, prior to ENCDC arrival to the ceca. Similarly, in our experiments where RCAS-BMP4 infected embryonic intestine was combined with CAM grafting to extend the culture period, we find ectopic and large ganglia along the *in ovo* cultured hindgut. This is also consistent with BMP4 inactivation studies, where Noggin misexpression in early chicken embryo leads to hypoganglionosis and failure of enteric ganglia formation [21]. Catenary and cell culture results described here also show that Noggin based inhibition of BMP activity in the hindgut results in abnormal ENS development with variable phenotypic expression, including hindgut hypoganglionosis, and failure of normal neuronal and glial differentiation. When recombinant Noggin is added to intestinal explants, ENCDC migration is enhanced, ganglion formation is impaired and enteric neurons are distributed sporadically throughout the hindgut wall similar to previously reported mouse experiments [20]. Contradictory to these findings, misexpression of Noggin during early chicken embryo development produced significant delay in ENCDC migration and abnormally small ganglia formation [21]. This is consistent with experiments where inhibition of BMP4 activity by Noggin at the level of the neural tube inhibited emigration of neural crest cells [47], reducing the ENCDC pool colonizing the gut. The different effects of Noggin on ENCDC migration between *in ovo* RCAS-Noggin injected early chick embryo and recombinant Noggin protein treated intestine isolated from chicks and mice embryo could be due to different delivery methods.

To determine whether BMP4 can directly influence ENCDC migration and differentiation, we treated precolonized E6 ceca explants with GDNF, which promotes robust migration of ENCDCs out

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of the gut and onto the fibronectin-coated surface. Twenty-four hours later, after the ENCDCs had emigrated out of the ceca, BMP4 recombinant protein was added to the culture medium, where we find that, consistent with previous results in embryonic mouse gut [48], BMP4 alone does not affect ENCDC migration. Interestingly, BMP4 promotes cell aggregation in this system, similar to the phenotype observed in our RCAS-BMP4 infected hindguts, while Noggin prevents ENCDC aggregation in vitro, as reported by Chalazonitis and his colleagues [27]. In contrast, when BMP4 and GDNF are both added to the ceca explants prior to the onset of cell migration, no ENCDC aggregation occurs. This leads us to hypothesize that during ENS development the presence of high GDNF concentrations in the ceca prevents wavefront ENCDCs from responding to the aggregation-mediating effects of BMP4. This effect must be time-limited since enteric ganglia will eventually need to form in the ceca. We propose that the migrating cells in the cecum gradually deplete the GDNF reserve. Therefore, while the early arriving ENCDCs are pSMAD-negative and continue to migrate to the proximal hindgut, as the concentration of GDNF in the ceca declines, BMP4 begins to exert its effect on the trailing ENCDC population, which leads to cell aggregation and neuronal differentiation. Our results are consistent with prior studies where co-administration of BMP factors and GDNF enhance neuronal differentiation in zebrafish and murine ENS cells [49,50].

In summary, our results show that ENCDC colonization of the hindgut relies on interactions between BMP4 and GDNF signaling in the cecum, which leads to inhibition of premature gangliogenesis prior to hindgut colonization (Fig. 9). Accordingly, if BMP4 expression is increased in the hindgut by retroviral misexpression, ectopic ganglia form and hyperganglionosis results. Similarly, when recombinant BMP4 was added to cultured ENCDCs, the cells aggregated into large ganglion-like structures, as opposed to cultures supplemented with both BMP4 and GDNF, where ganglion formation did not occur. Consequently, wavefront ENCDCs reaching the cecum as a response to the chemoattractant effect of GDNF do not differentiate into enteric glia and neurons, and do not form enteric ganglia prematurely, which allows proper post-cecal colonization of the intestine. In addition to highlighting an important aspect of BMP4 signaling during ENS development, this work illustrates how molecular interactions in the cecal mesenchyme are involved in ENCDC colonization of the hindgut.

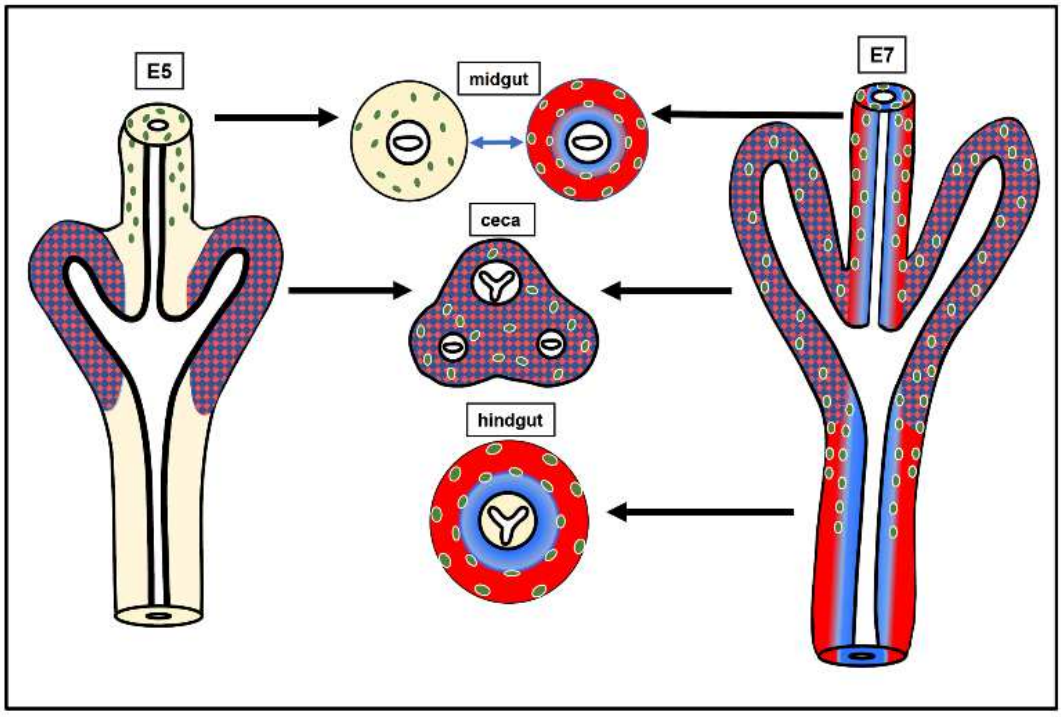

Figure 9. Model of hindgut ENCDC colonization and the roles of BMP4. BMP4 reduces ENCDC proliferation, promotes differentiation and induce gangliogenesis. GDNF signalling promotes ENCDC proliferation, migration and differentiation (Nagy and Goldstein, 2006). Expression of both growth factors is precisely localized to the cecal mesenchyme before the arrival of ENCDCs, which suggests that these molecules act locally to directly or indirectly influence the migration of ENCDCs. We hypothesize the overlapping cecal

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expression of BMP4 and GDNF is required for hindgut ENS formation: GDNF inhibits BMP4- from promoting gangliogenesis in the cecum, allowing wavefront cells to proceed to the hindgut and continue their journey in a BMP4-free, GDNF-rich outer mesenchyme to complete ENS formation in the colorectum. Enteric ganglia (green); mesenchymal cells (yellow); GDNF expression territory (red); BMP4 expression territory (blue).

4. Materials and Methods

4.1. Embryos

Fertilized white Leghorn chicken (*Gallus gallus domesticus*) eggs were obtained from commercial breeders (Prophyl-BIOVO, Mohács, Hungary) and maintained at 37.5 °C in a humidified incubator. Embryos were staged according to the number of embryonic days (E) or to Hamburger Hamilton (HH) tables [51,52]. Gut stages were referenced to the chick embryo gut staging table [53] and the ENS formation timetable [4].

4.2. RNA-seq

RNA-seq data from the ceca and interceca of 3 E5 chick embryos were obtained from the GEO database (GSE182783) [1]. This publicly available dataset was re-analyzed focusing on the BMP signaling module. Differential expression of transcripts between the ceca and interceca were determined using the R package edgeR as previously reported with a P-value less than 0.01 after FDR correction considered significant differentially expressed genes (DEGs) [54]. Genes with more than 1 Read Per Kilobase of transcript, per Million mapped reads (RPKM) in 3 samples were considered to be expressed in the dataset. To identify potential signaling hubs in the developing gut, protein-protein interaction networks generated from DEGs were visualized by the web-based tool NetworkAnalyst 3.0 [55] using the STRING functional protein association networks database with a high confidence (800) interaction score threshold. The top 20 (23 due to equal values) genes ranked by degrees (number of potential interactions) in the PPI network topology analysis were considered as potentially important signaling hubs. BMP signaling pathway genes were identified from the BMP component of the KEGG database pathway (TGF-beta signaling pathway: hsa04350) with the substitution of equivalent genes in the *Gallus gallus* genome.

4.3. Immunohistochemistry

Immunohistochemistry and immunofluorescence techniques were performed on frozen tissue sections and primary cell cultures. 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 (Merck, 106056). Frozen sections were cut at 12 µm, collected on poly-L-lysine-coated slides (Sigma-Aldrich, P-8920), and stained by immunocytochemistry using the primary antibodies listed in Table 1 as previously described [7]. For obtaining the best result, some primary antibodies needed prior Triton-X 100 treatment (e.g. SOX10, TUJ1, pSMAD). After primary antibody incubation, sections were incubated with the corresponding biotinylated or Alexa-conjugated fluorescent secondary antibodies. In case of chromogenic immunostaining the development of the signal was with 4-chloro-1-napthol. Section images were recorded using a Nikon Eclipse E800 fluorescence microscope and Zeiss LSM 710 confocal microscope, whole-mount images were recorded using a Nikon SMZ25 (with Prior L200/E unit) fluorescence stereomicroscope.

Table 1. Primary antibodies used.

Antibody (clone)	Host species	Antigen specificity	Dilution	Vendor (catalog number)
HNK-1	mouse	IgM	1:50	ThermoFisher (MA5-11605)
Tuj1 (AA10) [1]	mouse	IgG2a	1:100	Santa Cruz (sc-80016)
SMA (1A4)	mouse	IgG2a	1:400	Dako (M0851)
$p75^{NTR}$	rabbit (polyclonal)	IgG (H+L)	1:300	Promega (G3231)

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pSMAD1/5/9 [1]	rabbit (polyclonal)	IgG (H+L)	1:200	CellSignaling (#13820)
Bfabp	rabbit (polyclonal)	IgG (H+L)	1:50	kind gift of Dr. Thomas Müller
HuC/D (16A11)	mouse	IgG2b	1:100	Invitrogen (A-21271)
N-cadherin (GC-4)	mouse	IgG1	1:200	Sigma-Aldrich (C3865)
N-cadherin (6B3-c)	mouse	IgG1	1:5	DSHB
SOX10 [1]	mouse	IgG1	1:200	Santa Cruz (sc-365692)
RCAS gag protein (AMV-3C2)	mouse	IgG1	1:5	DSHB

[1] This target required 0.1 % Triton-X 100 permeabilization prior to labeling with primary antibodies.

Table 2. Biotinylated secondary antibodies used.

Host	Target	Specificity	Vendor (catalog number)
horse	Anti-Mouse	IgG(H+L)	Vector Laboratories BA-2000
goat	Anti-Rabbit	IgG(H+L)	Vector Laboratories BA-1000
goat	Anti-Mouse	IgM (μ chain)	Vector Laboratories BA-2020 [1]

All biotin conjugated secondary antibodies were diluted 1:200 in PBS-BSA prior to use.

Table 3. Fluorescent secondary antibodies used.

Host	Target	Specificity	Excitation wavelength	Vendor[1] (catalog number)
donkey	Anti-Mouse	IgG(H+L)	488 nm	A21202
donkey	Anti-Mouse	IgG(H+L)	594 nm	A21203
donkey	Anti-Mouse	IgG(H+L)	647 nm	A31571
donkey	Anti-Rabbit	IgG(H+L)	488 nm	A21206
goat	Anti-Mouse	IgG1	488 nm	A21121
goat	Anti-Mouse	IgG1	594 nm	A21125
goat	Anti-Mouse	IgG2a	488 nm	A21131
goat	Anti-Mouse	IgG2a	594 nm	A21135
goat	Anti-Mouse	IgG2b	594 nm	A21145
goat	Anti-Mouse	IgM	594 nm	A21044
goat	Anti-Mouse	IgM	633 nm	A21046

[1] All fluorochrome conjugated secondary antibodies were purchased from Invitrogen-Life Technologies and diluted 1:200 in PBS prior to use.

4.4. In situ hybridization on sections and whole-mount

4.4.1. Whole-mount in situ hybridization

Dissected gastrointestinal tracts were fixed in 4% paraformaldehyde (PFA), dehydrated in methanol, and stored at -20 °C until ready for processing [56,57]. Published chick probes were used:

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Bmp4 [14,58]. Digoxigenin riboprobe synthesis and whole-mount RNA in situ hybridization were performed as previously described [59,60].

4.4.2. In situ hybridization on FFPE tissue sections

For sections, GI tissues were fixed in 4% paraformaldehyde at room temperature for 1 h, washed in PBS, gradually dehydrated in ethanol and embedded in paraffin. Sections (10 µm) were cut using a microtome and collected on poly-L-lysine coated slides. *In situ* hybridization performed as previously described [61,62]. All sections were hybridized for 18-24 h. Detection was performed using BM purple according to manufacturer's instructions (Roche Molecular Biochemicals). Digoxigenin riboprobes were prepared as previously described [60] and included probes for BMP4 [14,58] and BMPRII [39].

4.5. Intestinal organ culture assay

Guts were removed from E5 (HH26) chick embryos, pinned down to silicone-coated tissue culture plates with insect pin as described previously [13], and allowed to float in DMEM (Sigma-Aldrich, D5429) cell culture media containing GDNF, and GDNF in combination with BMP4 or Noggin respectively.

4.6. Viral overexpression of BMP4 (+chorioallantoic membrane transplantation)

BMP4 overexpression in the developing chick gut was performed using a replication-competent retroviral vector (RCAS). The RCAS vector to produce replication-competent retroviruses (RCAS) has been previously described [56,57]. The full-length bmp4 construct [39] was cloned into the shuttle vector Slax as Morgan and Fekete (1996) [63] previously described and then cloned into RCAS using established techniques [64]. The DF-1 chicken fibroblast cell line (ATCC-LGC) was transfected with RCAS-based construct to produce retroviruses expressing BMP4, grown to confluence and the supernatant harvested. Viral harvesting, concentration and titering were performed described [65]. Approximately 1-5 µl of freshly defrosted virus dyed with fast green was injected per intestine. The gut segment was transplanted on the chorioallantoic membrane (CAM) of E8 chick embryos. Before transplantation, a small portion of the CAM was gently traumatized by laying a strip of sterile lens paper onto the surface of the epithelium and then removing it immediately. The gut segment was placed over a junction of blood vessels on the CAM and incubated for 9 days. The graft, together with the surrounding CAM was excised, fixed in 4% buffered paraformaldehyde, and embedded in gelatin for further immunohistochemistry. Viral infection was confirmed in all cases by 3C2 immunohistochemistry (1:5) against the viral gag protein. Controls were not injected, or injected with empty RCAS vector.

4.7. Cell migration assay

For ENCDC migration assay, distal midgut without ceca and cecal region was removed from E6 (HH29) chick embryos and cultured on 20 µg/ml fibronectin coated dishes with GDNF (10 ng/ml; R&D Systems, 212-GD-010; n=12) and GDNF in combination with recombinant BMP4 (200 ng/ml; R&D Systems; 5020-BP) and Noggin (200 ng/ml; R&D Systems, 6997-NG-025).

4.8. EdU labeling

Click-iT Plus EdU AlexaFluor 488 and 647 Imaging Kits (Thermo Fisher) were used according to the manufacturer's instructions to evaluate the cell proliferation rate either on tissue sections and ENCDCs migrated out to tissue culture surface. We developed the fluorescent signals of EdU labeling after immunofluorescent staining was performed.

4.9. Statistics

Data are expressed as mean±s.d. All statistical analysis were performed using GraphPad Prism version 10.0.2 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com. After Shapiro-Wilk normality test we used Kruskal-Wallis test with posthoc Dunn's multiple comparison test of differences. $p < 0.05$ was considered statistically significant.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: title; Table S1: title; Video S1: title.

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https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE182783.

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References

1. Nagy, N.; Kovacs, T.; Stavely, R.; Halasy, V.; Soos, A.; Szocs, E.; Hotta, R.; Graham, H.; Goldstein, A.M. Avian Ceca Are Indispensable for Hindgut Enteric Nervous System Development. *Dev. Camb. Engl.* **2021**, *148*, doi:10.1242/DEV.199825.
2. Yntema, C.L.; Hammond, W.S. The Origin of Intrinsic Ganglia of Trunk Viscera from Vagal Neural Crest in the Chick Embryo. *J. Comp. Neurol.* **1954**, *101*, 515–541, doi:10.1002/cne.901010212.
3. Burns, A.J.; Le Douarin, N.M. Enteric Nervous System Development: Analysis of the Selective Developmental Potentialities of Vagal and Sacral Neural Crest Cells Using Quail-Chick Chimeras. *Anat Rec* **2001**, *262*, 16–28.
4. Nagy, N.; Burns, A.J.; Goldstein, A.M. Immunophenotypic Characterization of Enteric Neural Crest Cells in the Developing Avian Colorectum. *Dev. Dyn. Off. Publ. Am. Assoc. Anat.* **2012**, *241*, 842–851, doi:10.1002/dvdy.23767.
5. Catala, M.; Teillet, M.A.; Le Douarin, N.M. Organization and Development of the Tail Bud Analyzed with the Quail-Chick Chimaera System. *Mech. Dev.* **1995**, *51*, 51–65, doi:10.1016/0925-4773(95)00350-a.
6. Burns, A.J.; Le Douarin, N.M. The Sacral Neural Crest Contributes Neurons and Glia to the Post- Umbilical Gut: Spatiotemporal Analysis of the Development of the Enteric Nervous System. *Development* **1998**, *125*, 4335–4347.
7. Nagy, N.; Brewer, K.C.; Mwizerwa, O.; Goldstein, A.M. Pelvic Plexus Contributes Ganglion Cells to the Hindgut Enteric Nervous System. *Dev. Dyn. Off. Publ. Am. Assoc. Anat.* **2007**, *236*, 73–83, doi:10.1002/dvdy.20933.
8. Le Douarin, N.M.; Teillet, M.A. The Migration of Neural Crest Cells to the Wall of the Digestive Tract in Avian Embryo. *J. Embryol. Exp. Morphol.* **1973**, *30*, 31–48.
9. Kapur, R.P. Colonization of the Murine Hindgut by Sacral Crest-Derived Neural Precursors: Experimental Support for an Evolutionarily Conserved Model. *Dev. Biol.* **2000**, *227*, 146–155, doi:10.1006/dbio.2000.9886.
10. Goldstein, A.M.; Hofstra, R.M.W.; Burns, A.J. Building a Brain in the Gut: Development of the Enteric Nervous System. *Clin. Genet.* **2013**, *83*, 307–316, doi:10.1111/cge.12054.
11. Roberts, D.J. Molecular Mechanisms of Development of the Gastrointestinal Tract. *Dev. Dyn. Off. Publ. Am. Assoc. Anat.* **2000**, *219*, 109–120, doi:10.1002/1097-0177(2000)9999:9999<::aid-dvdy1047>3.3.co;2-y.
12. Jiang, Y.; Liu, M.; Gershon, M.D. Netrins and DCC in the Guidance of Migrating Neural Crest-Derived Cells in the Developing Bowel and Pancreas. *Dev. Biol.* **2003**, *258*, 364–384, doi:10.1016/s0012-1606(03)00136-2.
13. Nagy, N.; Barad, C.; Graham, H.K.; Hotta, R.; Cheng, L.S.; Fejszak, N.; Goldstein, A.M. Sonic Hedgehog Controls Enteric Nervous System Development by Patterning the Extracellular Matrix. *Development* **2016**, *143*, 264–275, doi:10.1242/dev.128132.

14. Roberts, D.J.; Johnson, R.L.; Burke, A.C.; Nelson, C.E.; Morgan, B.A.; Tabin, C. Sonic Hedgehog Is an Endodermal Signal Inducing Bmp-4 and Hox Genes during Induction and Regionalization of the Chick Hindgut. *Dev. Camb. Engl.* **1995**, *121*, 3163–3174.
15. Roberts, D.J.; Smith, D.M.; Goff, D.J.; Tabin, C.J. Epithelial-Mesenchymal Signaling during the Regionalization of the Chick Gut. *Dev. Camb. Engl.* **1998**, *125*, 2791–2801.
16. Chalazonitis, A.; Kessler, J.A. Pleiotropic Effects of the Bone Morphogenetic Proteins on Development of the Enteric Nervous System. *Dev. Neurobiol.* **2012**, *72*, 843–856, doi:10.1002/dneu.22002.
17. Zhang, X.; Stappenbeck, T.S.; White, A.C.; Lavine, K.J.; Gordon, J.I.; Ornitz, D.M. Reciprocal Epithelial-Mesenchymal FGF Signaling Is Required for Cecal Development. *Development* **2006**, *133*, 173–180, doi:10.1242/dev.02175.
18. Kruger, G.M.; Mosher, J.T.; Tsai, Y.-H.; Yeager, K.J.; Iwashita, T.; Gariepy, C.E.; Morrison, S.J. Temporally Distinct Requirements for Endothelin Receptor B in the Generation and Migration of Gut Neural Crest Stem Cells. *Neuron* **2003**, *40*, 917–929, doi:10.1016/s0896-6273(03)00727-x.
19. Nagy, N.; Goldstein, A.M. Endothelin-3 Regulates Neural Crest Cell Proliferation and Differentiation in the Hindgut Enteric Nervous System. *Dev. Biol.* **2006**, *293*, 203–217, doi:10.1016/j.ydbio.2006.01.032.
20. Fu, M.; Vohra, B.P.S.; Wind, D.; Heuckeroth, R.O. BMP Signaling Regulates Murine Enteric Nervous System Precursor Migration, Neurite Fasciculation, and Patterning via Altered Ncam1 Polysialic Acid Addition. *Dev. Biol.* **2006**, *299*, 137–150, doi:10.1016/j.ydbio.2006.07.016.
21. Goldstein, A.M.; Brewer, K.C.; Doyle, A.M.; Nagy, N.; Roberts, D.J. BMP Signaling Is Necessary for Neural Crest Cell Migration and Ganglion Formation in the Enteric Nervous System. *Mech. Dev.* **2005**, *122*, 821–833, doi:10.1016/j.mod.2005.03.003.
22. Chalazonitis, A.; Pham, T.D.; Li, Z.; Roman, D.; Guha, U.; Gomes, W.; Kan, L.; Kessler, J.A.; Gershon, M.D. Bone Morphogenetic Protein Regulation of Enteric Neuronal Phenotypic Diversity: Relationship to Timing of Cell Cycle Exit. *J Comp Neurol* **2008**, *509*, 474–492, doi:10.1002/cne.21770.
23. Chalazonitis, A.; D'Autreaux, F.; Pham, T.D.; Kessler, J.A.; Gershon, M.D. Bone Morphogenetic Proteins Regulate Enteric Gliogenesis by Modulating ErbB3 Signaling. *Dev Biol* **2011**, *350*, 64–79, doi:10.1016/j.ydbio.2010.11.017.
24. Huycke, T.R.; Miller, B.M.; Gill, H.K.; Nerurkar, N.L.; Sprinzak, D.; Mahadevan, L.; Tabin, C.J. Genetic and Mechanical Regulation of Intestinal Smooth Muscle Development. *Cell* **2019**, *179*, 90-105.e21, doi:10.1016/j.cell.2019.08.041.
25. De Santa Barbara, P.; Williams, J.; Goldstein, A.M.; Doyle, A.M.; Nielsen, C.; Winfield, S.; Faure, S.; Roberts, D.J. Bone Morphogenetic Protein Signaling Pathway Plays Multiple Roles during Gastrointestinal Tract Development. *Dev. Dyn. Off. Publ. Am. Assoc. Anat.* **2005**, *234*, 312–322, doi:10.1002/dvdy.20554.
26. Faure, C.; Chalazonitis, A.; Rheaume, C.; Bouchard, G.; Sampathkumar, S.G.; Yarema, K.J.; Gershon, M.D. Gangliogenesis in the Enteric Nervous System: Roles of the Polysialylation of the Neural Cell Adhesion Molecule and Its Regulation by Bone Morphogenetic Protein-4. *Dev Dyn* **2007**, *236*, 44–59, doi:10.1002/dvdy.20943.
27. Chalazonitis, A.; D'Autréaux, F.; Guha, U.; Pham, T.D.; Faure, C.; Chen, J.J.; Roman, D.; Kan, L.; Rothman, T.P.; Kessler, J.A.; et al. Bone Morphogenetic Protein-2 and -4 Limit the Number of Enteric Neurons but Promote Development of a TrkC-Expressing Neurotrophin-3-Dependent Subset. *J. Neurosci. Off. J. Soc. Neurosci.* **2004**, *24*, 4266–4282, doi:10.1523/JNEUROSCI.3688-03.2004.
28. Sukegawa, A.; Narita, T.; Kameda, T.; Saitoh, K.; Nohno, T.; Iba, H.; Yasugi, S.; Fukuda, K. The Concentric Structure of the Developing Gut Is Regulated by Sonic Hedgehog Derived from Endodermal Epithelium. *Development* **2000**, *127*, 1971–1980.
29. Pisano, J.M.; Colón-Hastings, F.; Birren, S.J. Postmigratory Enteric and Sympathetic Neural Precursors Share Common, Developmentally Regulated, Responses to BMP2. *Dev. Biol.* **2000**, *227*, 1–11, doi:10.1006/DBIO.2000.9876.
30. Thang, S.H.; Kobayashi, M.; Matsuoka, I. Regulation of Glial Cell Line-Derived Neurotrophic Factor Responsiveness in Developing Rat Sympathetic Neurons by Retinoic Acid and Bone Morphogenetic Protein-2. *J. Neurosci. Off. J. Soc. Neurosci.* **2000**, *20*, 2917–2925, doi:10.1523/JNEUROSCI.20-08-02917.2000.
31. Ishizuya-Oka, A.; Hasebe, T. Sonic Hedgehog and Bone Morphogenetic Protein-4 Signaling Pathway Involved in Epithelial Cell Renewal along the Radial Axis of the Intestine. *Digestion* **2008**, *77 Suppl 1*, 42–47, doi:10.1159/000111487.
32. Amiel, J.; Lyonnet, S. Hirschsprung Disease, Associated Syndromes, and Genetics: A Review. *J. Med. Genet.* **2001**, *38*, 729–739, doi:10.1136/jmg.38.11.729.
33. Zhang, Y.; Que, J. BMP Signaling in Development, Stem Cells, and Diseases of the Gastrointestinal Tract. *Annu. Rev. Physiol.* **2020**, *82*, 251–273, doi:10.1146/annurev-physiol-021119-034500.
34. van Grunsven, L.A.; Taelman, V.; Michiels, C.; Verstappen, G.; Souopgui, J.; Nichane, M.; Moens, E.; Opdecamp, K.; Vanhomwegen, J.; Kricha, S.; et al. XSip1 Neuralizing Activity Involves the Co-Repressor CtBP and Occurs through BMP Dependent and Independent Mechanisms. *Dev. Biol.* **2007**, *306*, 34–49, doi:10.1016/j.ydbio.2007.02.045.

35. Wei, W.; Liu, B.; Jiang, H.; Jin, K.; Xiang, M. Requirement of the Mowat-Wilson Syndrome Gene Zeb2 in the Differentiation and Maintenance of Non-Photoreceptor Cell Types During Retinal Development. *Mol. Neurobiol.* **2019**, *56*, 1719–1736, doi:10.1007/s12035-018-1186-6.
36. Wakamatsu, N.; Yamada, Y.; Yamada, K.; Ono, T.; Nomura, N.; Taniguchi, H.; Kitoh, H.; Mutoh, N.; Yamanaka, T.; Mushiake, K.; et al. Mutations in SIP1, Encoding Smad Interacting Protein-1, Cause a Form of Hirschsprung Disease. *Nat. Genet.* **2001**, *27*, 369–370, doi:10.1038/86860.
37. Cacheux, V.; Dastot-Le Moal, F.; Kääriäinen, H.; Bondurand, N.; Rintala, R.; Boissier, B.; Wilson, M.; Mowat, D.; Goossens, M. Loss-of-Function Mutations in SIP1 Smad Interacting Protein 1 Result in a Syndromic Hirschsprung Disease. *Hum. Mol. Genet.* **2001**, *10*, 1503–1510, doi:10.1093/hmg/10.14.1503.
38. Nagaya, M.; Kato, J.; Niimi, N.; Tanaka, S.; Wakamatsu, N. Clinical Features of a Form of Hirschsprung's Disease Caused by a Novel Genetic Abnormality. *J. Pediatr. Surg.* **2002**, *37*, 1117–1122, doi:10.1053/jpsu.2002.34455.
39. Smith, D.M.; Nielsen, C.; Tabin, C.J.; Roberts, D.J. Roles of BMP Signaling and Nkx2.5 in Patterning at the Chick Midgut-Foregut Boundary. *Dev. Camb. Engl.* **2000**, *127*, 3671–3681, doi:10.1242/dev.127.17.3671.
40. Shyer, A.E.; Huycke, T.R.; Lee, C.; Mahadevan, L.; Tabin, C.J. Bending Gradients: How the Intestinal Stem Cell Gets Its Home. *Cell* **2015**, *161*, 569–580, doi:10.1016/j.cell.2015.03.041.
41. Nerurkar, N.L.; Mahadevan, L.; Tabin, C.J. BMP Signaling Controls Buckling Forces to Modulate Looping Morphogenesis of the Gut. *Proc. Natl. Acad. Sci. U. S. A.* **2017**, *114*, 2277–2282, doi:10.1073/pnas.1700307114.
42. Druckenbrod, N.R.; Epstein, M.L. Age-Dependent Changes in the Gut Environment Restrict the Invasion of the Hindgut by Enteric Neural Progenitors. *Dev. Camb. Engl.* **2009**, *136*, 3195–3203, doi:10.1242/dev.031302.
43. Wu, J.J.; Chen, J.X.; Rothman, T.P.; Gershon, M.D. Inhibition of in Vitro Enteric Neuronal Development by Endothelin-3: Mediation by Endothelin B Receptors. *Dev. Camb. Engl.* **1999**, *126*, 1161–1173, doi:10.1242/dev.126.6.1161.
44. Bondurand, N.; Natarajan, D.; Barlow, A.; Thapar, N.; Pachnis, V. Maintenance of Mammalian Enteric Nervous System Progenitors by SOX10 and Endothelin 3 Signalling. *Dev. Camb. Engl.* **2006**, *133*, 2075–2086, doi:10.1242/dev.02375.
45. Bondurand, N.; Dufour, S.; Pingault, V. News from the Endothelin-3/EDNRB Signaling Pathway: Role during Enteric Nervous System Development and Involvement in Neural Crest-Associated Disorders. *Dev. Biol.* **2018**, *444 Suppl 1*, S156–S169, doi:10.1016/j.ydbio.2018.08.014.
46. Mwizerwa, O.; Das, P.; Nagy, N.; Akbareian, S.E.; Mably, J.D.; Goldstein, A.M. Gdnf Is Mitogenic, Neurotrophic, and Chemoattractive to Enteric Neural Crest Cells in the Embryonic Colon. *Dev. Dyn. Off. Publ. Am. Assoc. Anat.* **2011**, *240*, 1402–1411, doi:10.1002/dvdy.22630.
47. Sela-Donenfeld, D.; Kalcheim, C. Regulation of the Onset of Neural Crest Migration by Coordinated Activity of BMP4 and Noggin in the Dorsal Neural Tube. *Dev. Camb. Engl.* **1999**, *126*, 4749–4762, doi:10.1242/dev.126.21.4749.
48. Fu, M.; Tam, P.K.H.; Sham, M.H.; Lui, V.C.H. Embryonic Development of the Ganglion Plexuses and the Concentric Layer Structure of Human Gut: A Topographical Study. *Anat. Embryol. (Berl.)* **2004**, *208*, 33–41, doi:10.1007/s00429-003-0371-0.
49. Huang, S.; Wang, Y.; Luo, L.; Li, X.; Jin, X.; Li, S.; Yu, X.; Yang, M.; Guo, Z. BMP2 Is Related to Hirschsprung's Disease and Required for Enteric Nervous System Development. *Front. Cell. Neurosci.* **2019**, *13*, 523, doi:10.3389/fncel.2019.00523.
50. Anitha, M.; Shahnavaz, N.; Qayed, E.; Joseph, I.; Gossrau, G.; Mwangi, S.; Sitaraman, S.V.; Greene, J.G.; Srinivasan, S. BMP2 Promotes Differentiation of Nitrergic and Catecholaminergic Enteric Neurons through a Smad1-Dependent Pathway. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2010**, *298*, G375-383, doi:10.1152/ajpgi.00343.2009.
51. Hamburger, V.; Hamilton, H.L. A Series of Normal Stages in the Development of the Chick Embryo. *J. Morphol.* **1951**, *88*, 49–92, doi:10.1002/jmor.1050880104.
52. Hamburger, V.; Hamilton, H.L. A Series of Normal Stages in the Development of the Chick Embryo. *Dev. Dyn.* **1992**, *195*, 231–272, doi:10.1002/aja.1001950404.
53. Southwell, B.R. Staging of Intestinal Development in the Chick Embryo. *Anat. Rec. A. Discov. Mol. Cell. Evol. Biol.* **2006**, *288A*, 909–920, doi:10.1002/ar.a.20349.
54. Robinson, M.D.; McCarthy, D.J.; Smyth, G.K. EdgeR: A Bioconductor Package for Differential Expression Analysis of Digital Gene Expression Data. *Bioinformatics* **2010**, *26*, 139–140, doi:10.1093/bioinformatics/btp616.
55. Zhou, G.; Soufan, O.; Ewald, J.; Hancock, R.E.W.; Basu, N.; Xia, J. NetworkAnalyst 3.0: A Visual Analytics Platform for Comprehensive Gene Expression Profiling and Meta-Analysis. *Nucleic Acids Res.* **2019**, *47*, W234–W241, doi:10.1093/nar/gkz240.
56. Moniot, B.; Biau, S.; Faure, S.; Nielsen, C.M.; Berta, P.; Roberts, D.J.; de Santa Barbara, P. SOX9 Specifies the Pyloric Sphincter Epithelium through Mesenchymal-Epithelial Signals. *Development* **2004**, *131*, 3795–3804, doi:10.1242/dev.01259.

57. Le Guen, L.; Notarnicola, C.; de Santa Barbara, P. Intermuscular Tendons Are Essential for the Development of Vertebrate Stomach. *Dev. Camb. Engl.* **2009**, *136*, 791–801, doi:10.1242/dev.029942.
58. Zou, H.; Niswander, L. Requirement for BMP Signaling in Interdigital Apoptosis and Scale Formation. *Science* **1996**, *272*, 738–741, doi:10.1126/science.272.5262.738.
59. Jowett, J.B.; Xie, Y.; Chen, I.S.Y. The Presence of Human Immunodeficiency Virus Type 1 Vpr Correlates with a Decrease in the Frequency of Mutations in a Plasmid Shuttle Vector. *J. Virol.* **1999**, *73*, 7132–7137.
60. Riddle, R.D.; Johnson, R.L.; Laufer, E.; Tabin, C. Sonic Hedgehog Mediates the Polarizing Activity of the ZPA. *Cell* **1993**, *75*, 1401–1416, doi:10.1016/0092-8674(93)90626-2.
61. Faure, S.; Georges, M.; McKey, J.; Sagnol, S.; de Santa Barbara, P. Expression Pattern of the Homeotic Gene Bapx1 during Early Chick Gastrointestinal Tract Development. *Gene Expr. Patterns* **2013**, *13*, 287–292, doi:10.1016/j.gep.2013.05.005.
62. Nielsen, C.; Murtaugh, L.C.; Chyung, J.C.; Lassar, A.; Roberts, D.J. Gizzard Formation and the Role of Bapx1. *Dev. Biol.* **2001**, *231*, 164–174, doi:10.1006/dbio.2000.0151.
63. Morgan, B.A.; Fekete, D.M. Manipulating Gene Expression with Replication-Competent Retroviruses. *Methods Cell Biol.* **1996**, *51*, 185–218, doi:10.1016/s0091-679x(08)60629-9.
64. Logan, M.; Tabin, C. Targeted Gene Misexpression in Chick Limb Buds Using Avian Replication-Competent Retroviruses. *Methods* **1998**, *14*, 407–420, doi:10.1006/meth.1998.0595.
65. Cepko, C.L. Transduction of Genes Using Retroviral Vectors. In *Current Protocols in Molecular Biology.*; Ausubel, F.M., Kingston, R.E., Moore, D.D., Seidman, J.G.,, Smith, J.A., Struhl, K., Eds.; John Wiley and Sons: New York, 1991; p. 9:9.10.11-19.14.13.