Bone morphogenetic protein signaling pathway plays multiple roles during gastrointestinal tract development.

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ABSTRACT

The Bone Morphogenetic Protein (BMP) signaling pathway plays an essential role during gastrointestinal (GI) tract development in vertebrates. In the present study, we use an antibody that recognizes the phosphorylated and activated form of Smad1, 5 and 8 to examine (by immunohistochemistry) the endogenous patterns of BMP signaling pathway activation in the developing GI tract. We show that the endogenous BMP signaling pathway is activated in the mesoderm, the endoderm and the enteric nervous system (ENS) of the developing chick GI tract, and is more widespread than BMP ligand expression patterns. Using an avian specific retroviral misexpression technique to activate or inhibit BMP signaling pathway activity in the mesoderm of the gut, we show that BMP activity is required to the pattern, the development and the differentiation of all three tissue types of the gut: mesoderm (that forms the visceral smooth muscle), endoderm (that forms the epithelium), and ectoderm (that forms the ENS). These results demonstrate that BMP signaling is activated in all the tissue layers of the GI tract during the development and plays a role during interactions and reciprocal communications of these tissue layers.

INTRODUCTION

The gastrointestinal (GI) tract is a remarkably complex, three dimensional, specialized and vital organ system derived from a simple tubal structure. The vertebrate GI tract includes the lumenal digestive system of the esophagus, stomach, intestines, and colon (which we will designate as "gut") and the GI tract derivatives - thyroid, lungs, liver, and pancreas. The gut is composed of the three germ layers - mesoderm (which forms the visceral smooth muscle layer), endoderm (which forms the epithelial lining), and ectoderm (which includes the enteric nervous system). The basic function of the GI tract is to digest food, absorb nutriments and water and to eliminate processed lumenal contents. All these functions are assured by different levels and layers of the GI tract that for its need specific regionalization and specialization (Roberts, 2000).

Originally, the gut develops from two invaginations at the anterior (anterior intestinal portal, AIP) and posterior (caudal intestinal portal, CIP) end of the embryo, which elongate and fuse to form a straight tube. The primitive gut tube is initially patterned into three broad domains along its anterior-posterior (AP) axis: the fore-, mid- and hindgut. As they develop, each region of the gut is characterized by unique mesodermal and endodermal morphology, which can easily be discerned by gross and microscopic examination (de Santa Barbara et al., 2003). These tissues show regionally specific differentiation along the AP axis designating the pharynx, esophagus, and stomach (the foregut), small intestines (the midgut), and large intestines (hindgut). Stomach development illustrates this acquisition of highly specialized features. In the chick, the stomach is anatomically divided in 2 structures. The gizzard (muscular stomach) has a thick layer of smooth muscle that facilitates mechanical disruption of food. The proventriculus (glandular stomach), anterior to the gizzard, has a thick epithelial

layer with specialized cells for chemical disruption of food, but a very thin smooth muscle layer (Roberts, 2000). The intestines are specialized in digestion and absorption and show modest development of visceral smooth muscle layer. This regionalization that is maintained throughout life is essential and necessary for the normal adult gut function (de Santa Barbara et al., 2002a).

The GI tract must move luminal contents through the gut via a process of propulsion termed peristalsis. This process requires innervation of the visceral smooth muscle layers, which is provided by a specialized set of neural tissue, the enteric nervous system (ENS). The ENS arises from the neural crest cells in the dorsal region of the neural tube (Yntema and Hammond, 1954; Le Douarin and Teillet, 1973). Two specific regions of the neural tube provide the neural crest cells that colonize the vertebrate gut. The vagal neural crest originates from the dorsal neural tube between somite pairs 1-7 and colonizes the entire gut providing most of the ENS (Le Douarin and Teillet, 1973). This region of neural crest colonizes the gut earliest in development in a craniocaudal gradient. The neural tube caudal to somite pair 28 provides neural crest cells that colonize only the post-umbilical gut (mid-midgut to anus/cloaca). This sacral neural crest colonizes the post-umbilical gut in a caudocranial direction (Burns et al., 2000; Hearn and Newgreen, 2000; Kapur, 2000). After colonization, the ENS is patterned in two concentric rings of ganglia within the radial axis of the gut. The innermost ring is the submucosal plexus (or Meissner's plexus) and signals to the muscularis mucosa and specialized neuroendocrine cells of the epithelium (Debas and Mulvihill, 1991; Bjerknes and Cheng, 2001). The outermost ring, the myenteric plexus (or Auerbach's plexus), lies between the two smooth muscle layers of the gut (longitudinal and circular muscularis). Finally, the neural crest cells differentiate into subsets of specialized cell types including

ganglion cells and glia (Young and Newgreen, 2001). Interactions between visceral mesoderm and ENS are necessary to ensure normal development, but to date have been poorly studied.

Candidate factors for gut development include known pattern formation genes first identified in *Drosophila.* These include nuclear transcription factors (HOX and NKX factors) and secreted factors (BMP and Hedgehog) (Roberts et al., 1995; Roberts et al., 1998; Smith et al., 2000; Ramalho-Santos et al., 2000; de Santa Barbara and Roberts, 2002b). Bone morphogenetic proteins (BMP) are secreted signaling molecules that belong to the transforming growth factor β (TGFβ) superfamily. BMP ligands were initially identified as regulators of bone formation (Urist et al., 1979), but subsequent analyses have demonstrated that these ligands regulate a spectrum of developmental processes throughout embryogenesis and organogenesis (reviewed (Hogan, 1996; de Santa Barbara et al., 2003)). *Bmp4* is expressed in the mesoderm of the entire gut sparing the gizzard only (Roberts et al., 1998). When overexpressed, *Bmp4* causes a reduction in the thickness of the smooth muscle layer in the stomach demonstrating a regulatory role in gut muscular hypertrophy (Roberts et al., 1998). The homeotic gene *Bapx1* is only expressed in the chick gizzard mesoderm and acts as a repressor of *Bmp4* expression therefore regulating gizzard smooth muscle growth (Nielsen et al., 2001).

Functional implication of the BMP signaling pathway during gut development was supported by both examination of BMP ligand expression patterns and ectopic manipulation of the BMP pathway components (for review, see de Santa Barbara et al., 2002a). However, these studies did not address or analyze the endogenous BMP pathway activation during gut development. BMP signaling activity is controlled at many levels including ligand transcription, ligand-receptor interactions, and signal transduction (Faure et al., 2002). This complexity complicates the molecular dissection of the pathway and specific tissue patterning

function. Intracellular mediators Smad1, 5 and 8 transduce BMP2, 4, and 7 signals and are specifically phosphorylated by the same BMP-type I receptors at the last two serine residues in the carboxy-terminal SSVS motif (Kretzschmar et al., 1997). Anti-Phospho-Smad1 (anti-PSmad1) antibodies specifically detect phosphorylated and activated form of Smad1, 5 and 8 and constitute an important tool to measure endogenous cartography of BMP2, BMP4 and BMP7 activation in Xenopus (Faure et al., 2000) and chick (Faure et al., 2002) embryos. The results often can not be predicted from the mRNA or protein expression pattern of the ligands and/or receptors alone (Faure et al., 2002).

For this reason, we analyzed endogenous BMP pathway activation using the anti-PSmad1 antibodies during gut development and found activation and modulation of the BMP activity in all three tissues of the developing chick gut. Furthermore, using retroviral misexpression techniques, we showed that activation and inhibition of endogenous BMP activity result in multiple defects affecting mesenchyme-smooth muscle, epithelium and enteric nervous system of the gut. All these observations suggest that tightly regulated endogenous BMP activity is present and necessary to ensure normal gut development and differentiation.

RESULTS

Endogenous BMP signaling pathway activation in the chick developing gut

In order to examine BMP signaling activity during GI tract development in chick embryos, we used antibodies that specifically detected phosphorylated and active Smad1, 5 and 8 (referee as PSmad1). These antibodies, which constitute an unambiguous tool to measure activated BMP signaling pathway, were previously used to examine the endogenous pattern of BMP

signaling during both Xenopus and chick embryo development (Faure et al., 2000; Faure et al., 2002). Immunohistochemistry analyses were performed to localize BMP-activated cells in the developing chick guts (Figs. 1-3). At E7, the chick GI tract is morphologically distinct in the AP axis but remains immature at the histological level. At E7 PSmad1 immunoreactivity was found in the glands of the chick proventriculus (black arrow, Fig. 1B), as well as in the mesoderm near the connection with the esophagus (black arrowhead, Fig. 1B) and in the subserosal ENS of the gizzard (red arrowheads, Fig. 1B; see also red arrowhead in the insert). PSmad1 reactivity was not detected in the gizzard mesoderm or endoderm at this stage (large black arrows, Fig. 1B). At E7, PSmad1 was broadly localized in the midgut mesoderm that will give rise to the visceral smooth muscle layer (arrowhead, Fig. 1C) as well as in the undifferentiated endoderm (arrow, Fig. 1C). In the E7 hindgut, PSmad1 was detected in a restricted region within the mesoderm (red arrowhead, Fig. 1D), weakly in the presumptive lamina propria (black arrowhead, Fig. 1D) and in the Nerve of Remak (red arrow, Fig. 1D), but not in the endoderm (black arrow, Fig. 1D).

By E11, PSmad1 activity was present in the differentiating gut smooth muscles (red arrows, Fig. 2A,C,E,G). In addition, PSmad1 reactivity was now localized in the gizzard mesenchyme from E8 through to E18 (corresponding to decreased expression of *Bapx1* (Nielsen et al., 2001)) (red arrow, Fig. 2C). We also observed that PSmad1 immunoreactive cells were present in clusters of cells in the mesenchyme (black arrowheads, Fig. 2A,C,E,G) that colocalized with HNK-1 positive cells (black arrowheads, Fig. 2B,D,F,H). HNK-1 is an antibody that recognizes an epitope expressed on migratory and post-migratory neural crest cells and has been widely used to detect the ENS in chick tissues (Luider et al., 1992). By E10, the ENS is fully patterned into the two plexi (Doyle et al., 2003). The ENS at E11 is

patterned in two concentric rings: inner ring (submucosal plexus) and outer ring (myenteric plexus) (Fig. 2F,H). The exception to this is the stomach, which normally does not contain a submucosal plexus (Fig. 2B,D). We observed that BMP activation was present in the ENS cells (Fig. 2A-H). By E11, PSmad1 immunoreactivity was present at all levels along the AP axis of the gut epithelia (black arrows, Fig. 2A,C,E,G).

Before hatching by E17.5, PSmad1 reactivity was no longer detected in the differentiated visceral smooth muscles, but was present in the lamina propria (red arrows, Fig. 3B,E), ENS cells (arrowheads, Fig. 3D) and intestinal epithelia (Fig. 3A,B,D,E). The PSmad1 staining in the luminal part of the epithelium localizes with the apoptosis process as shown by TUNEL detection (Fig. 3C,F). In agreement with these observations, recently, Haramis et al. demonstrate that autocrine inhibition of BMP signaling pathways in adult intestinal epithelium leads to ectopic crypt formation that could lead to neoplasia (Haramis et al., 2004).

These observations highlight the complexity and the dynamic pattern of endogenous BMP activation present in the developing and differentiating gut and suggest multiple roles for BMP signaling during gut development.

Perturbations of the BMP signaling pathway activity in the stomachal mesoderm alter both mesoderm and ENS development

To study the role of BMP signaling pathway during gut development, we took advantage of the naturally occurring spatial expression pattern of the *Bmp4* and *Bapx1* in the foregut-midgut region. *Bapx1* is expressed in the developing gizzard from E2-E10 whereas *Bmp4* is not (Smith et al., 2000; Nielsen et al., 2001). In addition, misexpression techniques demonstrate that ectopic expression of *Bapx1* in the gut induces a robust down-regulation of *Bmp4* expression (Nielsen et al., 2001). In these studies, we found that BMP signaling

pathway activation reflects these observations (Figs. 1, 2). Using the avian specific retroviral expression system, ectopic expression of *Bmp4* in the gizzard mesoderm causes abnormal development of the stomach (Smith et al., 2000). The developing gizzard was infected at E1.5 and examined at E9 (Fig. 4). As we previously described and published, viral infection nearly infects only gut mesoderm (data not shown; Roberts et al., 1998; Smith et al., 2000; de Santa Barbara and Roberts, 2002b; Moniot et al., 2004). With ectopic *Bmp4* expression in the gizzard, the gizzard mesoderm develops abnormally with focally thin to absent smooth muscle (compare Fig. 4A with control Fig. 4H) and often ectopic cartilage structure is observed (black arrow, Fig. 4B). Using PSmad1 antibodies, we observed ectopic immunoreactivity in the mesenchyme around the metaplasic tissues, but weak activation of the BMP signaling pathway is found in the cartilage (Fig. 4C compare to control Fig. 4I). The normal gizzard ENS pattern at E9 shows small subserosal ganglia (black arrowhead, Fig. 4G). With ectopic *Bmp4* expression, this pattern is perturbed and giant ganglia are often ectopically positioned within the differentiating smooth muscle and submucosa (compare black arrows, Fig. 4D with control Fig. 4G). Using PSmad1 antibodies, we observed strong immunoreactivity in the ENS cells and in the mesenchyme around the ENS ganglia (compare Fig. 4F with control Fig. 4I).

To inhibit BMP activation, we chose to misexpress *Bapx1* in the gut mesoderm. Western-blot analyses using *Bapx1*-misexpressed proventriculus demonstrated strong down-regulation of PSmad1 expression (data not shown). When *Bapx1* is ectopically expressed in the developing gut mesoderm either anteriorly in the proventriculus (Nielsen et al., 2001) or posteriorly in the duodenum (Fig. 5), these experiments result in a dramatic gut phenotype (black arrows, Fig. 5D,E). The *Bapx1* misexpressed regions develop marked muscular hypertrophy resembling that seen in the gizzard associated with a duodenal stenosis (compare

Fig. 5D,E with control Fig. 5A). We found that PSmad1 immunoreactivity was absent in mesoderm and ganglia, but weak endodermal staining was still observed (compare Fig. 5F with control Fig. 5B). The HNK-1 + ganglia are increased in number, abnormally positioned and the normal concentric ring pattern is totally disturbed (compare Fig. 5G with control Fig. 5C and Table 1).

These results show that activation (mediated by ectopic *Bmp4* expression) and inhibition (mediated indirectly by ectopic *Bapx1* expression) of the endogenous BMP signaling activity result in both mesoderm and ENS defects.

Perturbations of the BMP signaling pathway activity in hindgut mesoderm alter the development of all tissue layers

By E10, *Bapx1* expression is also observed in the caudal hindgut mesoderm (Nielsen et al., 2001). Over-expression of *Bapx1* in the developing hindgut earlier in development from E2 results in perturbations of the ENS (Fig. 6). By inhibiting BMP signaling pathway activation in the developing hindgut mesoderm and in the developing Nerve of Remak, we produced a marked reduction (or absence) of ENS ganglia in the hindgut as observed by HNK-1 immunostaining analyses (compare Fig. 6B with control Fig. 6A). This was associated with abnormal morphology of the Nerve of Remak, larger in size or in multiple bundles instead of the usual single bundle (arrows, Fig. 6B). These multiple bundles of the Nerve of Remak resemble that seen normally in the cloacal region (Doyle et al., 2003). In addition to the neural phenotype, sustained *Bapx1* expression in the mesoderm of the hindgut was associated with poor differentiation and proliferation of undifferentiated endoderm resulting in stenosis of the lumen (compare Fig. 6B,D with control Fig. 6A,C). We found that PSmad1 immunoreactivity

was absent in mesenchyme and ganglia, but weak endodermal staining was still observed (compare Fig. 6D with control Fig. 6C).

Multiple attempts to directly overexpress *Bmp4* in the developing midgut or hindgut resulted in embryonic lethality before E5, and therefore ENS development could not be studied (data not shown). Instead overexpression of *Bmp4* was achieved indirectly, by expressing the reverse function of *Bapx1* (*Bapx1-VP16*) that can act as an activator of *Bapx1* repressor targets (Nielsen et al., 2001). We observed changes in epithelial differentiation, with the presence of broader colonic villi in the E14 *Bapx1-VP16* expressing hindgut compared to control hindgut (arrows, Fig. 7A with control Fig. 7B). Older survivors (E17) obtained with *Bapx1-VP16* expression in the hindgut showed viral infection in a patchy mesodermal distribution and often in the ENS (insert, Fig. 7C). The embryos strongly infected in the hindgut were lethal (as were the *Bmp4* overexpressing hindguts, data not shown). The hindguts of these patchy *Bapx1-VP16* expressing survivors show abnormalities in the mesoderm and ENS (Fig. 7). In highly expressing foci, the mesodermal cells appear histologically immature and show no smooth muscle differentiation, which should be easily morphologically identifiable by this time (compare Fig. 7C with control Fig. 7D). The *Bapx1-VP16* expressing hindguts develop with a decrease in detectable ENS ganglia often showing only small individual cells in the lamina propria or abnormal ganglia in the serosa (Fig. 7C). In addition, we found that PSmad1 immunoreactivity was present in the ectopic and isolated ENS cells (black arrows, Fig. 7E), and in the area of undifferentiated mesenchyme (red arrowhead, Fig. 7E). In conclusion, we observed that modulation of mesodermal BMP signaling pathway in the hindgut affected all tissues layers: mesenchyme-smooth muscle, ENS, and endoderm-epithelium.

DISCUSSION

Members of the BMP signaling pathway are known to be involved in different aspects of gut development: *Bmp4* is critical for development of the gut (Roberts et al., 1995; Roberts et al., 1998; Smith and Tabin, 1999; Smith et al., 2000; Nielsen et al., 2001; Moniot et al., 2004), *Bmp7* is involved in stomach gland formation (Narita et al., 2000), and *Bmp2* is involved in the maturation of the enteric neuronal cells in vitro (Lo et al., 1997; Pisano et al., 2000). In this study, we expand our understandings of the BMP signaling pathway role in gut development by detecting the endogenous BMP pathway activation. Our results showed that the BMP pathway is dynamically activated in the different tissue layers of the gut (mesoderm-mesenchyme, endoderm-epithelium, enteric nervous system) and plays active roles in gut layer interactions.

Role of BMP signaling pathway activity in the gut mesoderm and visceral smooth muscle layer

In this study, we show that activated BMP signaling is present in the all undifferentiated gut mesoderm with the exception of the gizzard mesoderm during the early gut development period (Fig. 1). Using retroviral misexpression techniques, we show that sustained activity via ectopic *Bmp4* or indirectly via *Bapx1-VP16* expression perturbed patterning and induced mesodermal grown and cell fate change (metaplasia) (Fig. 4, data not shown). We show that inhibiting the BMP signaling pathway activity via ectopic *Bapx1* expression leads to hypertrophy of the duodenal mesoderm (Fig. 5).

BMP signaling activity is present during the differentiation of the gut mesoderm into visceral smooth muscle. We show for the first time activity is present in the mesenchyme of the gizzard (Fig. 2). After smooth muscle cell differentiation is complete a pronounced down-regulation of BMP pathway activity occurs (Fig. 3). Sustained BMP signaling activity via *Bmp4* or *Bapx1-VP16* misexpression perturbed the differentiation leaving undifferentiated mesenchyme in place of smooth muscle (Figs. 4, 7). These experiments demonstrate that down-regulation of BMP signaling activity may be required for differentiation of the visceral mesoderm into smooth muscle.

These results show that regulation of the BMP signaling pathway activity is required to control pattern formation and correct differentiation of gut mesoderm.

Function of BMP signaling pathway activation in tissue interactions during the gut development and differentiation

We show that BMP signaling pathway is activated in the undifferentiated midgut endoderm during gut development (Figs. 1, 2). We also observed that BMP signaling pathway is not activated in the colon endoderm at E7, but is activated at E11 (Figs. 1, 2). These findings were not expected given BMP ligand expression which is restricted to the mesoderm at these stages. Mesoderm to endoderm signaling leading to activation of the BMP receptor expressed in the gut endoderm must be occurring (data not shown). The colonic delay in PSmad1 expression can be explained by the presence of *Bapx1*. *Bapx1* is expressed from E7-E10 in the mesoderm of the colon and after its expression is quickly down-regulated (Nielsen et al., 2001). We took advantages of the pattern expression of *Bapx1* in the colon in order to perturb the BMP signaling pathway activity. Sustained *Bapx1* expression in the hindgut mesoderm

resulted in persistence of undifferentiated endoderm and stenosis of the hindgut lumen (Fig. 6). A similar stenosis phenotype was recently observed with the misexpression of a dominant negative form of LEF1 (DN-LEF1) in the cecal mesoderm (Theodosiou and Tabin, 2003). These observations suggest a potential connection between BMP and WNT signaling pathways involving mesodermal-endodermal signaling. Activation of the BMP signaling pathway via *Bapx1-VP16* expression in the hindgut mesoderm also affects crypt/villous formation resulting in broader colonic villi (Fig. 7). We show that mesodermal perturbations of BMP activity (with activation or inhibition) in the colon perturb both the mesoderm patterning and/or differentiation and the epithelial development and differentiation (Figs. 6, 7).

The enteric nervous system (ENS) is closely associated with gut mesoderm and their reciprocal interaction is needed to ensure normal ENS colonization of the gut (Newgreen and Young, 2002a; Newgreen and Young, 2002b). In this study, we observe the activation of the BMP signaling pathway in the developing ENS cells (Figs. 2, 3). The morphogen BMP4 is the most likely early signal that can effect ENS pattern formation at migratory stages. Early in gut development, before colonization by the ENS precursors, *Bmp4* is the only member of the BMP family expressed in the mesoderm of the gut (Roberts et al., 1998). *Bmp4* is expressed before ENS colonization and continues to be expressed in the mesoderm adjacent to the migrating neural crest ENS precursors throughout early ENS patterning (E1.5-10). At later stages (E5-E18), *Bmp2* expression is detectable in the mesoderm and the ENS (data not shown), therefore BMP2 may be involved in other aspects of ENS development (differentiation or viability). In fact, BMP2 has been shown to function in the maturation process of enteric neurons in vitro (Lo et al., 1997; Pisano et al., 2000). In addition, receptors for BMP ligands (BMPR Ia, BMPR Ib and BMPR II) are all expressed in the developing gut

with expression early in the undifferentiated mesenchyme then, as they become identifiable, expression of at least one receptor is present in the ENS plexi, Nerve of Remak, and developing visceral smooth muscle (data not shown). Inhibition of the BMP signaling pathway activation in the mesoderm of the gut results in a prominent disorganization of the ENS pattern locally in the duodenum (Fig. 5) and distally in the hindgut, suggesting a block of ENS cell migration (data not shown). We also found that sustained expression of *Bapx1* in the hindgut mesoderm impairs the colonization of the hindgut by the ENS (Fig. 6). Others have found that inhibition of *Bmp4,* using *Noggin* as a BMP inhibitor, results in alterations of the ENS (Sukegawa et al., 2000; Goldstein et al., 2005). Chalazonitis *et al*. have also found aberrancies and increases in neuronal density in both myenteric and submucosal plexuses of the ENS of transgenic mice overexpressing *Noggin* directed by the neuron-specific enolase promoter (Guha et al., 2001; Chalazonitis et al., 2004).

Our studies do not directly address the mechanism by which BMP activation effects ENS development. In the inhibition studies, the anterior pattern alterations of the ENS are always accompanied by muscular hypertrophy, thus the ENS alterations may be secondary to the muscular hypertrophy. It is known that migration of the neural crest is affected by extracellular matrix proteins (Perris and Perissinotto, 2000). The marked increase in the mesodermal tissues may be affecting these proteins and the ENS may not receiving the correct signals for proper migration. In other experimentally manipulated guts, visceral muscle perturbations are associated with ENS patterning alterations. Hedgehog signal appears necessary for normal ENS pattern, either directly or via their effect on gut muscle development (Ramalho-Santos et al., 2000; Fu et al., 2004). Murine models show marked thinning of the gut smooth muscle but each demonstrate distinct ENS anomalies. These findings support a requirement of mesoderm/muscle signaling to normally pattern the ENS. It

is interesting to note that both Shh (in the gut, (Roberts et al., 1995)) and Ihh (in other systems, (Pathi et al., 1999)) have been shown to be activators of *Bmp4* and are expressed in the ganglia (data not shown; Fu et al., 2004). Further studies in which muscular hypertrophy is induced through other BMP unrelated pathways will help answer this question.

Human ENS disorders are fairly common and include Hirschsprung's disease, a pediatric dysmotility disorder associated with a varying amount of colonic agangliosis (Amiel and Lyonnet, 2001). It is speculative but interesting to suggest that defects in the BMP pathway may be associated with this human ENS disorder. Hirschsprung patients often have malformations of the gastrointestinal tract (Amiel and Lyonnet, 2001) and recently intestinal atresia has been reported in association with ENS colonization and development abnormalities (Khen et al., 2004). We can hypothesize that BMP signaling pathway deregulation in the visceral mesoderm can be responsible for some Hirschsprung's disease patients.

EXPERIMENTAL PROCEDURES

Chick embryos

Timed fertilized white Leghorn eggs (SPAFAS, CT) were incubated at 38°C in a humidified incubator (Kuhl,NJ) until used experimentally. Embryos were staged according to Hamburger and Hamilton (St.) (Hamburger and Hamilton, 1951) or by embryonic day (E). Whole embryos or dissected gut tissues were fixed in 4% paraformaldehyde for 4 (at room temperature) to 18 (at 4°C) hours before processing as described below.

Viral infection

This technique has been previously described by (Morgan and Fekete, 1996). Embryos at St. 6-10/E1-1.5 were used for experiments to target the proventriculus or duodenum. To target the hindgut, St.10-13/E1.5-2 embryos were injected. These times are prior to any regional ENS colonization (Newgreen and Young, 2002a; Newgreen and Young, 2002b; Burns and Le Douarin, 1998). Targets were approximated using published fate maps (Matushita, 1995) such that foregut/midgut injections were placed adjacent to somites 3-7 bilaterally and hindgut injections started adjacent to the last 2 somites and continued caudally in the presomitic mesoderm. Approximately 1-5μl of freshly defrosted virus dyed with fast green was injected per embryo. Eggs were then placed at 38°C until harvested. Injected viral constructs included *Bmp4* (Smith et al., 2000), *Bapx1*, the reverse function *Bapx1* (constructed with the activation domain VP-16) *Bapx1-VP16* (Nielsen et al., 2001), and, as control, *GFP* (de Santa Barbara and Roberts, 2002b). Viral infection was documented in all cases by either detection of the viral gag protein by immunohistochemistry or by in situ hybridization using the viral specific probe *Rcs* as previously published (de Santa Barbara and Roberts, 2002b).

Immunohistochemistry

Fixed chick tissues were embedded in paraffin. Paraffin blocks were cut into 3-5 μm sections and placed on super frost plus slides (Fisher). Immunohistochemical studies were performed using standard techniques. Endogenous peroxidase was blocked in 1.5% hydrogen peroxide for 30-60 minutes. Antigen unmasking step was performed in 0.1M Sodium Citrate solution by boiling the samples for 10 minutes. Samples were then left to cool for about forty-five minutes at room temperature. The sections were briefly washed in PBT. Blocking step was

performed in 10% chick serum plus 200 μl/ml Avidin D solution (Vector Laboratories). These incubations were 30 min at room temperature. Primary antibody solutions were made with 10% serum plus 200 μl/ml biotin solution (Vector Laboratories). The primary antibodies were applied at the following dilutions: 3C2 (1:5) (Morgan and Fekete, 1996), HNK-1 (NeoMarkers, 1:50) and PSmad1 (Cell signaling, 1:30 for paraffin fixed sections, 1:100 for frozen sections). The antibodies were incubated in a humidity chamber at 4^0C overnight. On the second day, sections were washed in PBT and were then re-blocked in 10% serum. Biotinylated goat anti-mouse IgM, biotinylated goat anti-mouse IgG, and biotinylated goat anti-rabbit IgG immunoglobulins (Vector) were all diluted 1:400 and incubated for 1 hour at room temperature. Sections were washed several times in PBS in room temperature, followed by incubation in ABC reagent according manufacturer instructions during 1 hour (Dako). Sections were washed again and then incubated in DAB solution (Sigma) up to 20 minutes. Samples were then washed several times in PBS and mounted with Paramount aqueous mounting medium (Dako).

In Situ Hybridization

In situ hybridization was performed on tissue embedded in paraffin. The samples were sectioned at 4-6 μm for in situ hybridization performed as previously described (Nielsen et al., 2001). All sections were hybridized for 18-24 hours; detection was performed using BM purple per manufacturer's instructions (Roche Molecular Biochemicals). Digoxigenin riboprobes were prepared as previously described (Riddle et al., 1993) and included: *Bapx1* (Nielsen et al., 2001), *Bmp4* (Zou and Niswander, 1996), *Bmp2* (Roberts et al., 1995), *Bmp7*

(Roberts et al., 1998), *BmpR Ia, Ib* and *II* (Smith et al., 2000), *Rcs* (Morgan and Fekete, 1996).

Photography

Photographs of whole-mounts were captured with either an Olympus SZX9 or a Nikon SMZ800 dissecting microscope using SPOT diagnostic RT color digital camera and PhotoShop software. Sections were photographed through a Nikon microphot FXA microscope using a SPOT diagnostics RT color digital camera and PhotoShop software.

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FIGURES

Fig. 1. Phosphorylated Smad1 expression in the E7 chick gut detected by immunohistochemistry. (A) E7 chick gut cartoon, ventral view, showing the three broad regions (Foregut, Midgut, Hindgut), subdivisions (proventriculus, gizzard, duodenum, cecum) and levels of sections for B-D. (B) Section through proventriculus/gizzard. PSmad1 protein is detected in the smooth muscle (large black arrowhead) and glands (small arrow) of the proventriculus and in cells at the periphery (small red arrowheads), but not the muscle and the endoderm of the gizzard (large black arrows). Higher magnification of the red arrowhead area in (B) showing peripheral PSmad1 positive cells with ENS cluster (outlined in red, red arrowheads). (C) Section through midgut detecting PSmad1 expression (arrow, marking endoderm; arrowhead at mesoderm). (D) Section through hindgut stained for PSmad1 expression. PSmad1 is present in developing smooth muscle (red arrowhead) and Nerve of Remak (red arrow), and weakly in the lamina propria (black arrowhead), but not detected in the epithelium (black arrow). Compass: A-anterior, P-posterior, R-right, L-left.

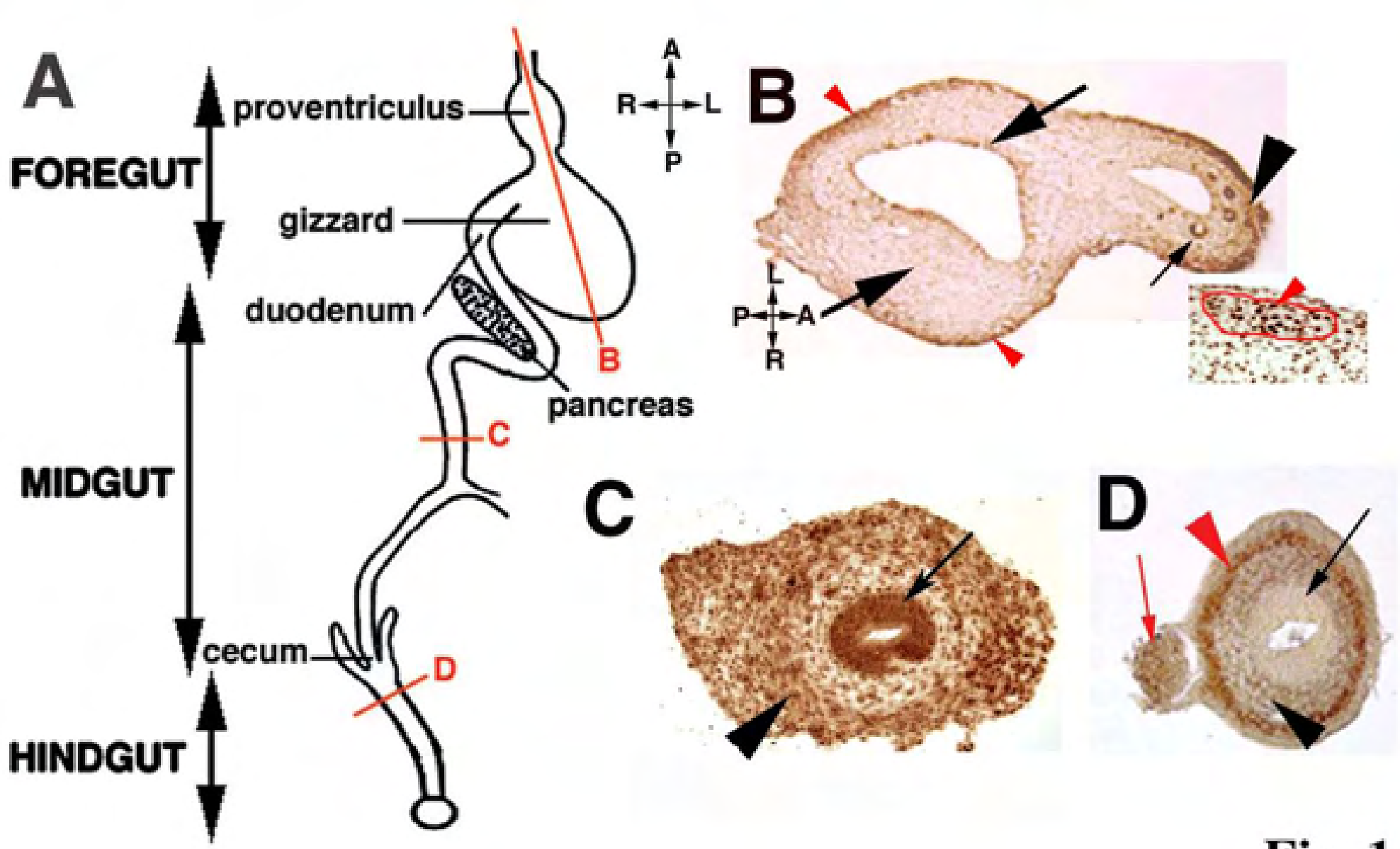
A
proventriculus
FOREGUT
gizzard
duodenum
B
pancreas
C
MIDGUT
cecum
D
HINDGUT
A
R
L
P
B
L
P
A
R
C
D

Fig. 1

Fig. 2. Expression of Phosphorylated Smad1 and HNK-1 in the E11 chick gut detected by immunohistochemistry. Sections of wild type E11 gut detecting PSmad1 (A,C,E,G) and HNK-1 (B,D,F,H). (A,B) proventriculus; (C,D) gizzard; (E,F) duodenum; (G,H) hindgut/midgut junction and ceca. Both PSmad1 and HNK-1 are detected in the ENS (black arrowheads). PSmad1 (red arrows) but not HNK-1 is detected in the smooth muscle layer. PSmad1 but not HNK-1 is detected in all epithelia (small black arrows). Red arrowheads in B and D show HNK-1 reactivity in neurites.

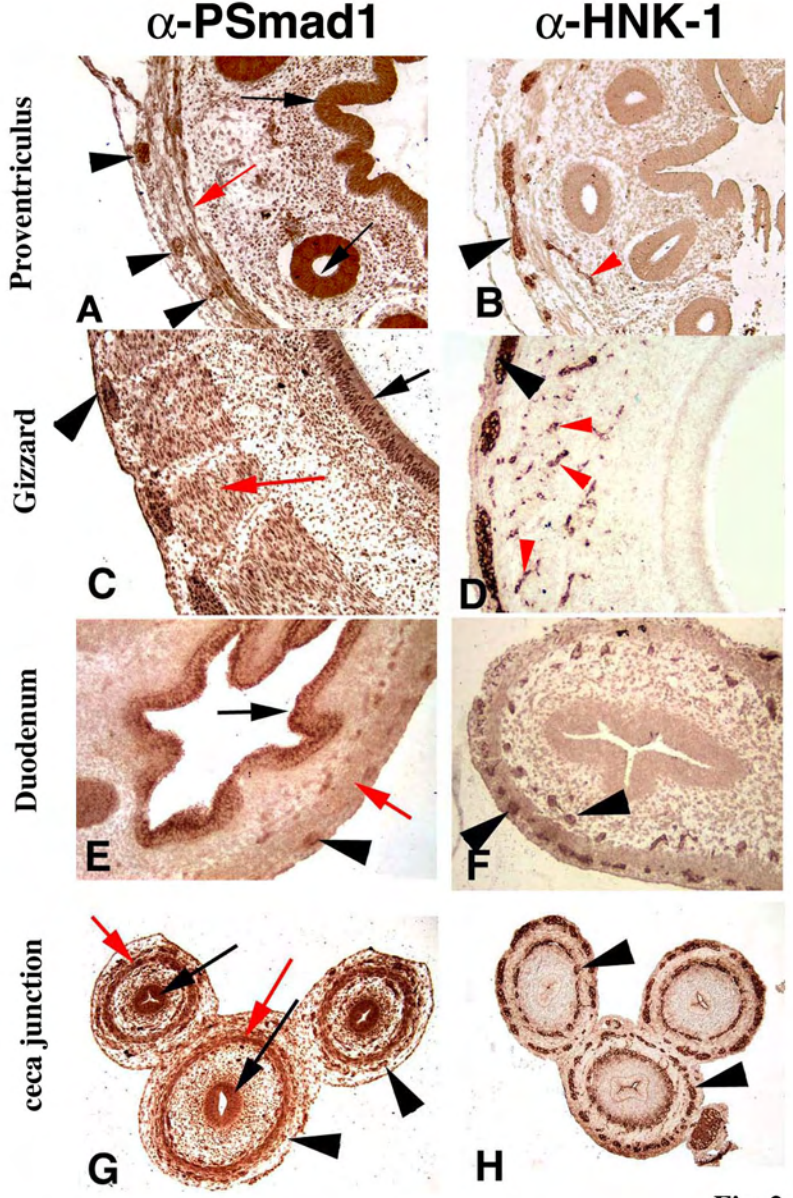
α-PSmad1
α-HNK-1
Proventriculus
Gizzard
Duodenum
ceca junction
A
B
C
D
E
F
G
H

Fig. 2

Fig. 3. Expression of Phosphorylated Smad1 in the E18 chick gut detected by immunohistochemistry. Sections of wild type E18 gut detecting PSmad1 (A,B,D,E). (A,B) small intestine; (D,E) colon. PSmad1 immunoreactivity are localized in the ENS cells (black arrowhead), lamina propria (red arrows) and intestinal epithelium (black arrows) (B,D,E). BMP signaling pathway is activated in the luminal part of the villi (black arrows, B,E), at lower level in the middle of the villi, and is not detected in the bottom of the villi. Sections of wild type E18 gut detecting TUNEL assays (C,F). Apoptotic cells detected by TUNEL assays are localized at the luminal part of the small intestine (black arrow, C) and colon (black arrow, F) villi.

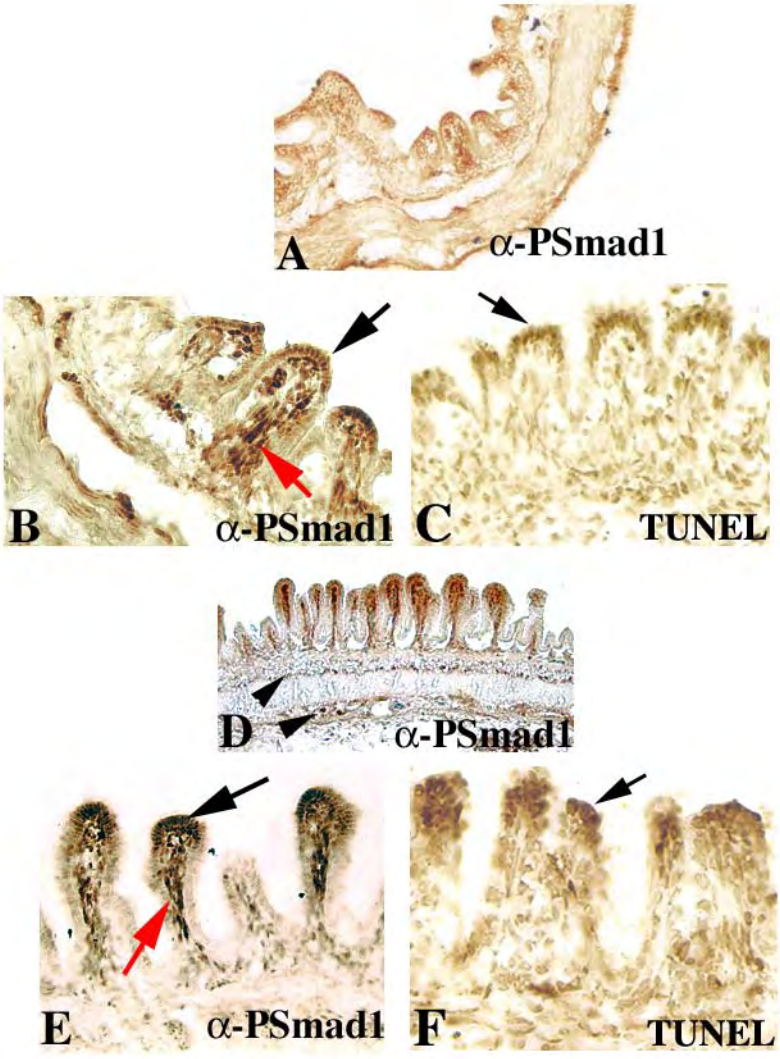

Fig. 3

Fig. 4. Interfering with the normal BMP signaling pathway by ectopically expressing *Bmp4* results in abnormalities of the mesoderm and ENS. (A, B) Histological stained section of E9 gizzard ectopically expressing *Bmp4* showing thinned smooth muscle (A, black arrows) and abnormal mesodermal development including cartilage formation (B, black arrow) and absent muscle (A, arrow) compare to normal E9 gizzard (H). HNK-1 antibody demonstrates abnormally large (D, arrow) and malpositioned (E, arrow) ganglia compare to normal ganglia observed on control E9 stomach (G, arrow). Sections of wild type (I) and *Bmp4* misexpressing (C, F) E9 stomach detecting PSmad1. PSmad1 immunoreactivity is present near cartilage metaplasic formation (C, red arrow), and in abnormal large ganglia (F, black arrow). (I) Red arrow indicates normal ganglia stained with PSmad1 antibody. Scale bars indicate 100 μm unless otherwise noted.

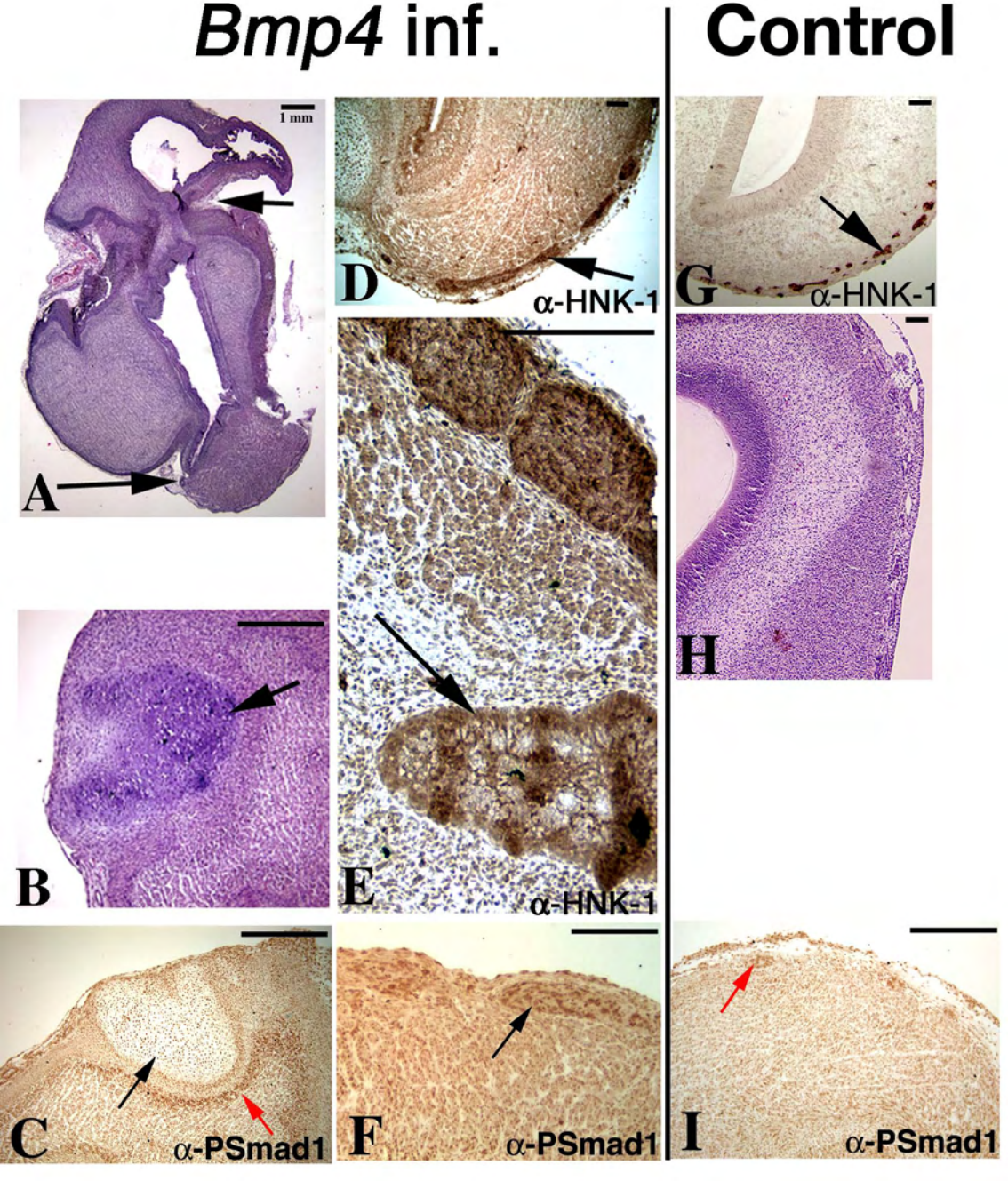
Bmp4 inf.
Control
1 mm
A
B
C
α-PSmad1
D
α-HNK-1
E
α-HNK-1
F
α-PSmad1
G
α-HNK-1
H
I
α-PSmad1

Fig. 4

Fig. 5. Interfering with the normal BMP signaling pathway by ectopically expressing *Bapx1* results in abnormalities of the mesoderm and ENS. (A-C) E9 wild type stomach complex. (A) Gross phenotype showing proventriculus-gizzard-duodenum. (B) Section of wild type duodenum showing PSmad1 expression in endoderm (arrowhead), ENS ganglia (black arrow), and differentiating smooth muscle (red arrow). (C) HNK-1 shows strong staining in two rings of ganglia in the longitudinal section of E9 duodenum. (D,E) Ectopic expression of *Bapx1* in the duodenum results in an expansion of the musculature (arrows in D,E). (F,G,H) Adjacent sections through expanded *Bapx1* expressing duodenum. Multiple lumens are present due to tortuosity of abnormal duodenum and plane of section (F,G,H). (F) PSmad1 immunoreactivity is absent in ganglia and mesoderm, but weak endodermal staining is present (arrows). (G) Abnormal position and smaller ganglia immunoreactive for HNK-1. (H) 3C2 antibody detecting viral infection throughout the mesoderm but sparing endoderm (arrows).

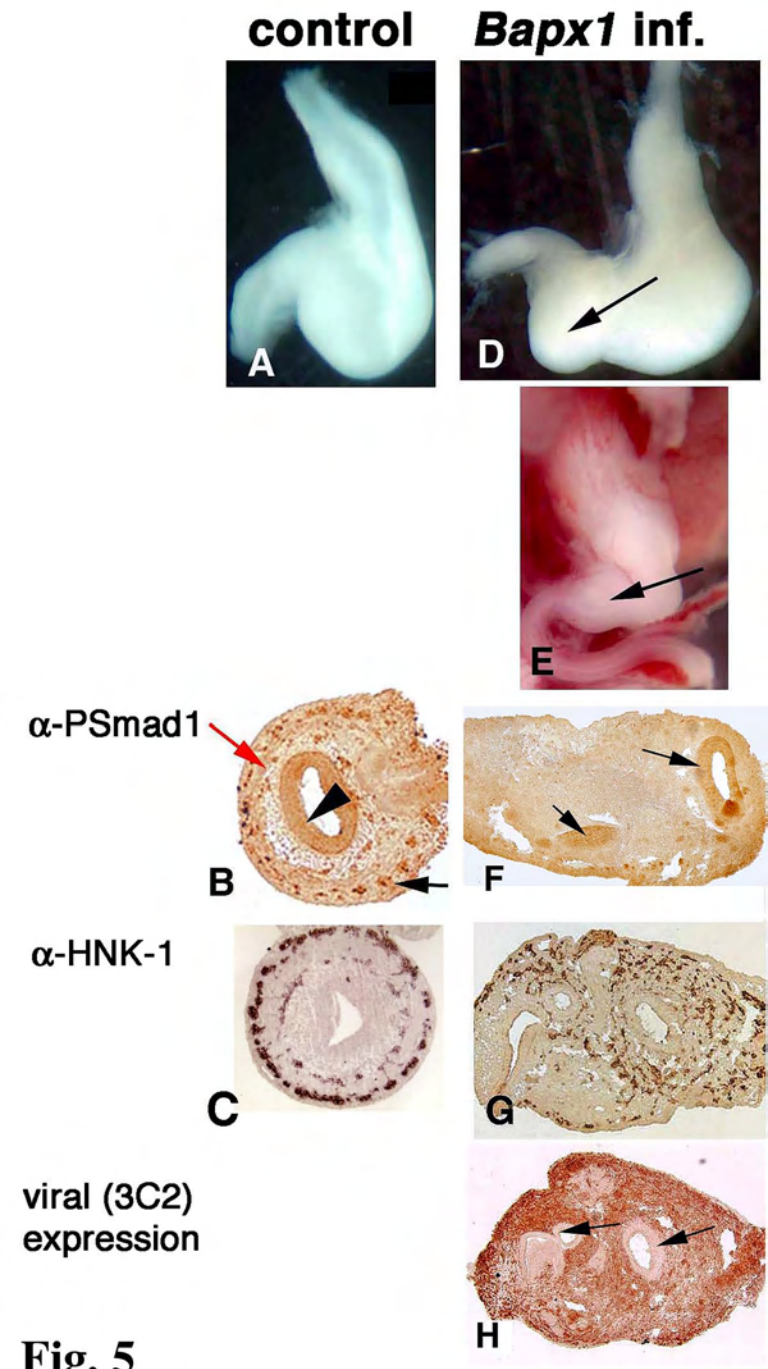
control
Bapx1 inf.
A
D
E
α-PSmad1
B
F
α-HNK-1
C
G
viral (3C2)
expression
H

Fig. 5

Fig. 6. All the hindgut tissue layers are affected by mesodermal *Bapx1* misexpression. ENS in wild type E12 (A) and *Bapx1* overexpressing (B) hindguts marked by HNK-1 immunoreactivity. The two concentric rings of ENS are well demonstrated in the wild type hindgut (A, black arrowheads) and a normal single round Nerve of Remak (A, red arrowhead). The *Bapx1* expressing hindguts show HNK-1 immunoreactivity only in the dysmorphic Nerve of Remak (B, black arrows) and do not demonstrate the normal two concentric rings of ENS (B). (C) Section of wild type hindgut showing PSmad1 expression in differentiated visceral smooth muscle (red arrowhead), ENS ganglia (black arrowhead), and endoderm (red arrow). (D) Section of *Bapx1* misexpressing hindgut. PSmad1 immunoreactivity is absent in ganglia and strongly decreases in the mesenchyme but weak endodermal staining is present (red arrow). Hindgut endoderm is also affected by mesodermal *Bapx1* misexpression as abnormal undifferentiated endoderm is observed and form stenosis of the hindgut lumen (B,D,E, red arrows) compare to control (A,C, red arrow). 3C2 demonstrates spatial location of retroviral expression of *Bapx1* (E). Viral expression was noted to include mesoderm and the Nerve of Remak (E).

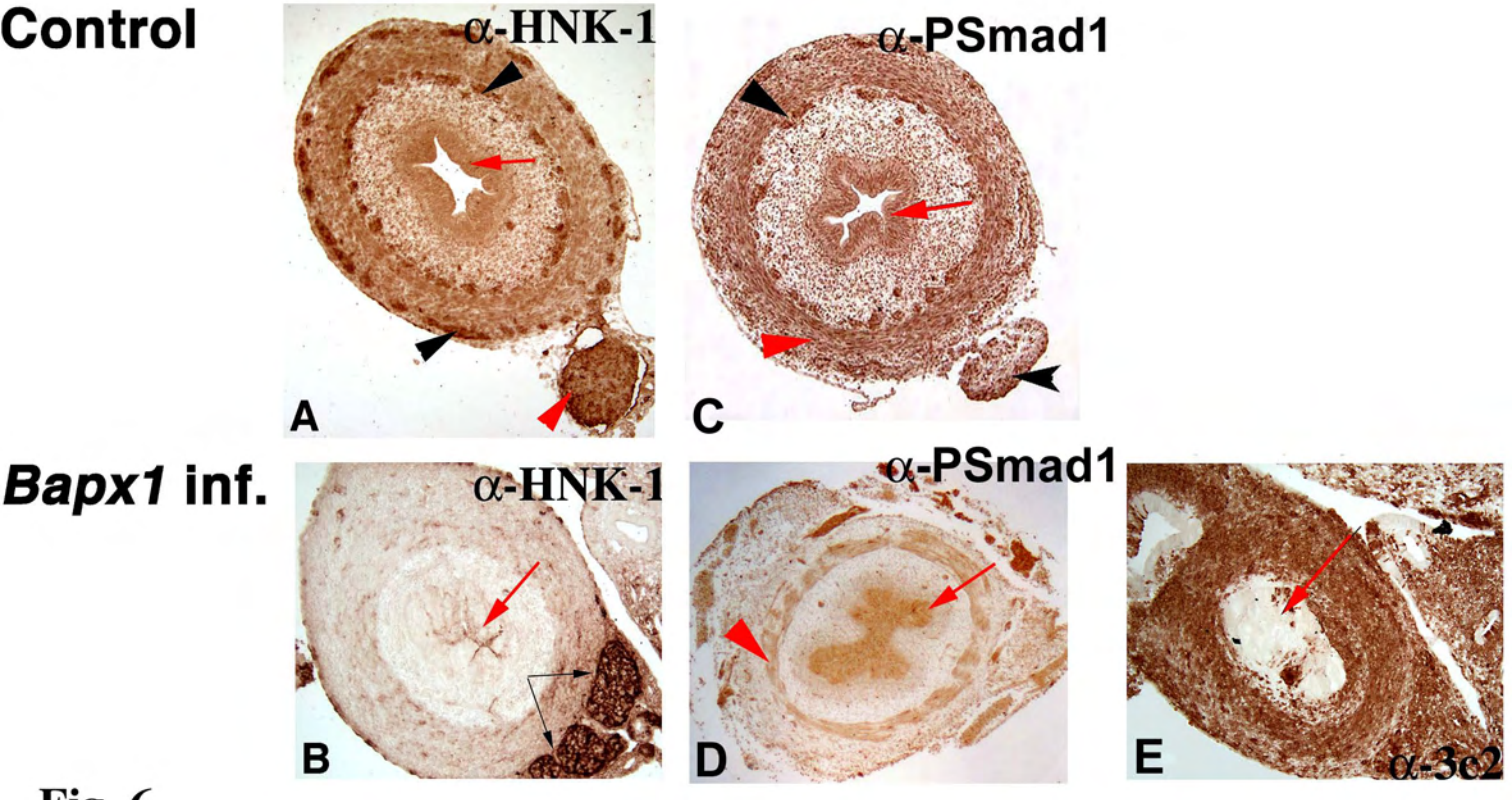

Fig. 6

Fig. 7. *Bapx1-VP16* expressing hindgut presents abnormalities in the mesenchyme, the epithelium and the ENS. (A) Histological staining of E14 hindgut expressing *Bapx1-VP16* shows poor muscle differentiation (black arrowhead) and broader villi formation (red arrow). (B) Histological staining of control E14 hindgut shows differentiation of the mesenchyme (black arrowhead) and normal villi formation (black arrow). (C) E17 hindgut expressing *Bapx1-VP16* stained for HNK-1 shows ectopic ENS ganglia or single cells in the submucosa/lamina propria (arrows), large ganglia are present abnormally in the serosa, and immature mesenchyme with poor muscle differentiation is observed (red arrowheads center and top inset). Bottom inset shows viral expression using 3C2 antibody present in mesoderm (black arrow) and ectopic serosal ganglia (red arrow). (D) Control E17 hindgut stained for HNK-1 shows myenteric and submucosal ganglia in the differentiated mesenchyme (black arrows). Sections of wild-type (F) and *Bapx1-VP16* misexpressing (E) E17 hindgut detecting PSmad1. PSmad1 immunoreactivity is present in the resulting immature mesenchyme (red arrowhead), in the ectopic and large ENS ganglia (black arrows), and in the lamina propria. (F) Black arrows indicate normal ganglia stained with PSmad1 antibody.

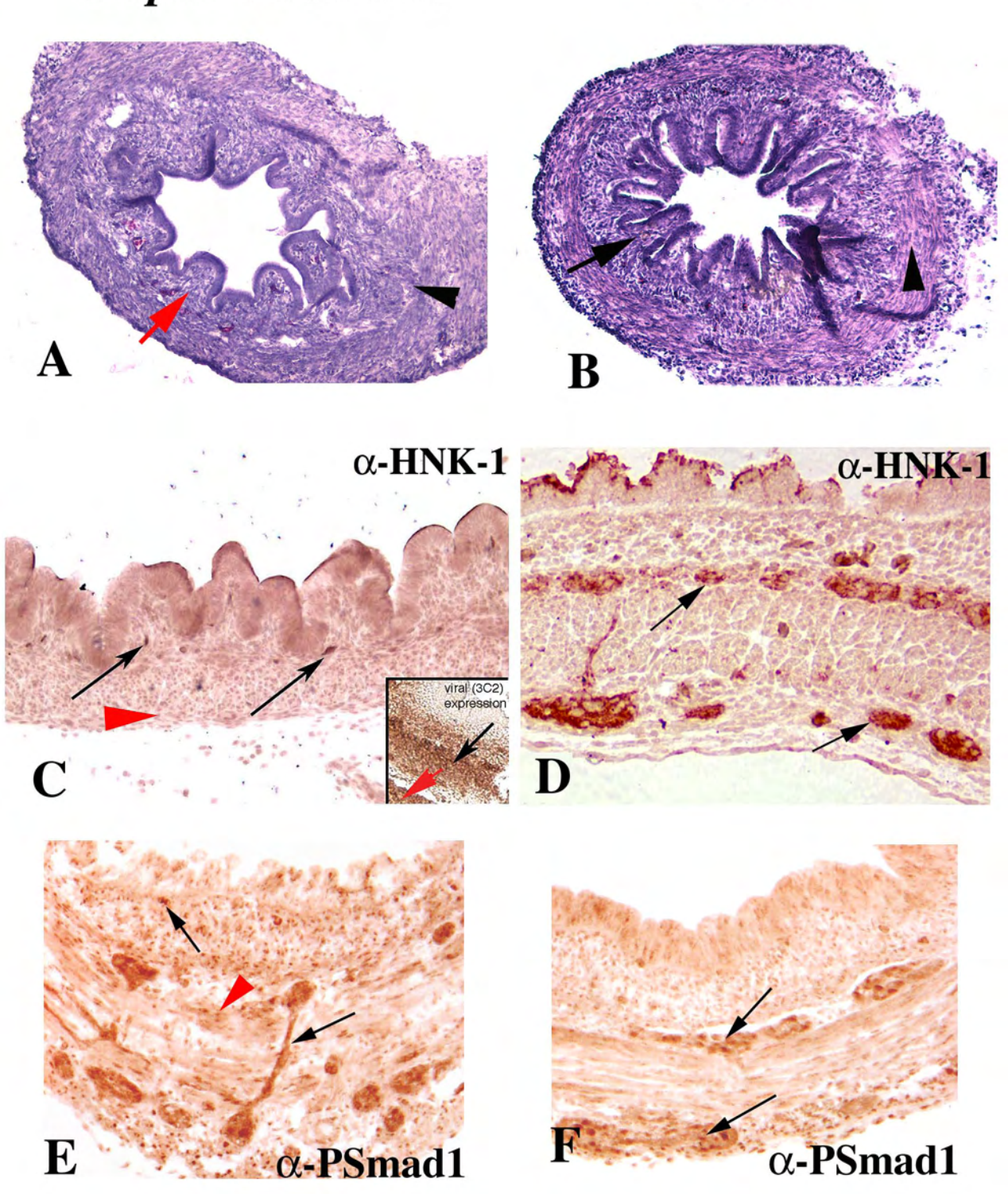
Bapx1-VP16 inf.
Control
A
B
α-HNK-1
α-HNK-1
viral (3C2) expression
C
D
E
α-PSmad1
F
α-PSmad1
Bapx1-VP16 inf.
Control

Fig. 7

Table 1. Number of HNK-1 positive cells in chick embryo duodena at E9.

GI tract	**HNK-1 positive cells**
Control	45
Bapx1-duodenal infection	95

Average on different cryostat plane sections counted in a 20X field in the duodenum level (n=4 each).