Roles of BMP signaling and Nkx2.5 in patterning at the chick midgut-foregut boundary

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Accepted 9 June; published on WWW 9 August 2000

SUMMARY

Patterning of the gut into morphologically distinct regions results from the appropriate factors being expressed in strict spatial and temporal patterns to assign cells their fates in development. Often, the boundaries of gene expression early in development correspond to delineations between different regions of the adult gut. For example, Bmp4 is expressed throughout the hindgut and midgut, but is not expressed in the early gizzard. Ectopic BMP4 in the gizzard caused a thinning of the muscularis. To understand this phenotype we examined the expression of the receptors transducing BMP signaling during gut development. We find that the BMP receptors are differentially expressed in distinct regions of the chicken embryonic gut. By using constitutively activated versions of the BMP type I receptors, we find that the BMP receptors act similarly to BMP4 in the gizzard when ectopically expressed. We show that the mesodermal thinning seen upon ectopic BMP signaling is due to an increase in apoptosis and a decrease in proliferation within the gizzard mesoderm. The mesodermal thinning is characterized by a disorganization and lack of differentiation of smooth muscle in the gizzard mesoderm. Further, ectopic BMP receptors cause an upregulation of Nkx2.5, the pyloric sphincter marker, similar to that seen with ectopic BMP4. This upregulation of Nkx2.5 is a cell-autonomous event within the mesoderm of the gizzard. We also find that Nkx2.5 is necessary and sufficient for establishing aspects of pyloric sphincter differentiation.

Key words: BMP4, BMP receptor, Nkx2.5, Gut, Gizzard, Pyloric sphincter, Chick

INTRODUCTION

The digestive system is critical for maintenance of all multicellular organisms. As the organism ingests food, it must be processed and essential nutrients removed and absorbed into the body prior to expulsion of the waste products. In higher organisms, these steps are handled by a series of specialized organs. In spite of its ultimate complexity, the vertebrate gut develops from a primitive tubular structure composed of two tissue types, an underlying endoderm and a surrounding splanchnic mesoderm. As development proceeds, the endoderm and mesoderm differentiate their organ-specific characteristics, producing the organs of the gastrointestinal tract. Although relatively little is known about the molecules that pattern the gut tube, experiments have shown the importance of epithelial-mesenchymal signaling in proper formation of the gut derivatives (Kedinger et al., 1986, 1990).

Epithelial-mesenchymal signaling is characterized by a cross-talk between the two tissue types, which results in patterning of the organ. Epithelial-mesenchymal signaling in the gut is characterized by a primary signal emanating from the endoderm that specifies the surrounding mesoderm to become gut mesoderm (Kedinger et al., 1986, 1990). A secreted molecule expressed throughout the gut endoderm, Sonic Hedgehog (SHH), has been implicated as this primary mesodermal specification signal (Roberts et al., 1995; Apelqvist et al., 1997). Sonic hedgehog (Shh) expression is seen as soon as the gut primordia begin to invaginate to form the gut tube at the anterior intestinal portal and the caudal intestinal portal. This expression is maintained and extends along the entire length of the gut endoderm (Roberts et al., 1995). Consistent with endodermally derived Shh mediating epithelial-mesenchymal interactions, the receptor for Shh is expressed in the underlying mesoderm (Marigo et al., 1996).

Following this primary specification of the visceral mesoderm, the mesoderm signals back to the endoderm providing anteroposterior cues that pattern the endoderm (Kedinger et al., 1986, 1990; Roberts et al., 1998). These secondary signals contribute to the specification of differential endodermal phenotypes along the gut tube. Hence, although the endoderm is very specialized in each organ of the gut, some of the signals that determine this specification emanate from the overlying mesoderm. Hox genes, a family of genes encoding homeodomain-containing transcription factors, have been found to play a regulatory role in the mesoderm to endoderm signaling (Kondo et al., 1996; Roberts et al., 1998). The Hox genes are expressed in an organ-specific
anteroposterior pattern in the mesoderm of the gut tube (Roberts et al., 1995; Yokouchi et al., 1995). For example, HoxD13 is expressed throughout the mesoderm of the cloaca and also in the endoderm of the large intestine in the chick embryo (Roberts et al., 1995; Yokouchi et al., 1995). When HoxD13 is ectopically expressed throughout the mesoderm of the small intestine, the endoderm of the small intestine is converted morphologically, and in its biochemical properties, to tissue more characteristic of the normal large intestine (Roberts et al., 1998). This organ-specific patterning downstream of Hox genes must involve secreted proteins, as the ectopic expression does not extend into the endoderm, however, no specific secreted factors have been identified that play a role in this specification. Hox genes have also been shown to play a role in the patterning of sphincters within the gut (Kondo et al., 1996; Zakany and Duboule, 1999).

Anteroposterior differences in the gut are reflected in differential gene expression and differential responsiveness to subsequent signals. BMP4, a member of the TGFβ family of secreted proteins, is expressed in the mesoderm of the gut tube adjacent to the Shh-expressing endoderm, but is absent from the mesoderm of the developing gizzard, or stomach, of the chicken embryo (Roberts et al., 1998). Members of the BMP family are often expressed in tissues next to those expressing hedgehog genes, reflective of an ancient epistatic relationship between these classes of molecules (Biggood and McMahon, 1995). In the gut, however, the regulatory relationship between those molecules is complex. In the hindgut (Roberts et al., 1995) and midgut (Roberts et al., 1998), SHH induces the expression of Bmp4. In the foregut-derived lung bud, both factors are similarly expressed in adjacent endodermal and mesodermal tissues, but the expression of each is independent of the others (Bellusci et al., 1996, 1997). Finally, in the stomach, ectopic production of SHH is unable to elicit Bmp4 expression (Roberts et al., 1998). Thus, differences in Shh responsiveness preexisting in the gut primordia lead to distinct domains where BMP4 is expressed or not expressed.

One of the key boundaries between BMP4-expressing and non-expressing tissue is at the gizzard-small intestinal border. Previous experiments demonstrated that BMP signaling from the small intestine induces the gizzard tissue at the border to be respecified into a pyloric sphincter fate (Smith and Tabin, 1999). This respecification includes a change in endodermal morphology as well as induction of expression of the sphincter-specific mesodermal marker Nkx2.5, a homeodomain gene related to the Drosophila gene Tinman (Fu et al., 1998). The pyloric sphincter exists in most vertebrates to retain food within the stomach lumen, but it varies in its morphology among species. Nkx2.5 is expressed in a temporally and spatially restricted manner in the gut (Buchberger et al., 1996; Smith and Tabin, 1999). In the chick, Nkx2.5 expression realizes a sharp boundary in the mesoderm of the pyloric sphincter, the junction between foregut and midgut (Buchberger et al., 1996; Smith and Tabin, 1999). The restriction of expression of Nkx2.5 to the pyloric sphincter suggests that it might be involved in the specification of that domain of the gut.

To further understand the genetic pathways leading to regionalized gut morphogenesis, we have further explored the roles of BMP signaling and its target, Nkx2.5, in chicken gut development. We find that BMP signaling plays multiple roles in gut morphogenesis. By regulating proliferation and apoptosis, it is involved in establishing the proper thickness of the mesodermal layers of different regions of the gut. It also regulates smooth muscle differentiation, perhaps repressing differentiation when the progenitors are undergoing early proliferative cycles and later repressing smooth muscle fate in particular mesodermal cell layers. Further, we find that activated BMP receptors in the chicken gizzard can function identically to the ligand BMP4. We utilized these reagents to test the ability of BMP signaling to induce Nkx2.5 expression in a cell-autonomous manner. Ectopic activation of Nkx2.5 is exclusively seen in cells infected with the activated BMP receptors. Finally, we tested the ability of Nkx2.5 to create sphincter-like morphology when misexpressed and find that Nkx2.5 is necessary and sufficient for aspects of pyloric sphincter patterning.

MATERIALS AND METHODS

Viruses and production

The following replication competent viruses were used: BMP4 (Düpere et al., 1995), and constitutively active versions of BMPR1B and BMPR1A (Zou and Niswander, 1996). The enrepNkx2.5 consists of the Nkx2.5 homeodomain attached in frame to the engrailed repressor (Fu et al., 1998). For the Nkx2.5 retroviruses, the full-length Nkx2.5 and enrepNkx2.5 constructs were cloned into the shuttle vector Slax and then cloned into RCAS using standard techniques (Logan and Tabin, 1998). Viral constructs were transfected into DF1 cells using Superfect (Qiagen). Cells were grown to confluency and supernatant was harvested. Viral concentration and titering were as described (Cepko, 1991).

Replication incompetent viruses were created by inserting the inserts out of the replication competent retroviral vectors and cloning these inserts into the replication incompetent viral vector pRAS. The retrovirus was packaged in DF1 cells utilizing the VSV-G protein as the ENV protein in this retrovirus (Chen et al., 1999). DF1 cells were transfected, and virus was harvested, concentrated and titered as described (Chen et al., 1999).

Embryonic injections

Fertile chicken eggs were obtained from SPAFAS (Connecticut) and incubated until the desired stages were obtained (Hamburger and Hamilton, 1951). Embryos were incubated at 37°C until embryonic day 1.5 (E1.5; stage 10) and injected as described (Roberts et al., 1995, 1998). Embryos were harvested at appropriate time points and placed into 4% paraformaldehyde.

In situ hybridization and histology

Whole-mount in situ hybridization was performed as described (Riddle et al., 1993). Section in situ hybridization was performed upon paraaffin sections using digoxigenin-labeled probes (Smith and Tabin, 1999). Probes used include: cBarxl (Barlow et al., 1999), cFKBP/SMAP (Fukuda et al., 1998), Nkx2.3 (Buchberger et al., 1996), Nkx2.5 (Buchberger et al., 1996), CdxA (Frumkin et al., 1996), Wnt5a, cBMP4, cBMPR1A, cBMPR1B (Zou and Niswander, 1997), Shh, HoxC8 (Burke et al., 1995) and Pdx1 (Hrebak et al., 1998).

Double labeling with a riboprobe and with an antibody was also performed. The section in situ hybridization was performed first followed by the antibody labeling with 3C2 (see below).

For histological staining, embryos sections on slides were deparaffinized and stained with Hematoxylin and Eosin following standard procedures. Photographs were taken from Zeiss Axiophot microscope using a digital camera.
3C2/BrdU/TUNEL staining

Embryos were harvested and placed into 4% paraformaldehyde overnight. Embryos were then washed 3x with PBS, dehydrated through an ethanol series and embedded in paraffin. Embryos were sectioned at 10 µm with every serial section being placed upon the next slide in succession. Slides containing serial sections were then treated with the appropriate reagents for either 3C2, BrdU or TUNEL analysis. 3C2 is an antibody against the GAG region of the Rous Sarcoma Retrovirus. Antibody staining was as described (Roberts et al., 1998). For analysis of proliferation, a sterile PBS solution containing BrdU (5mg/ml) was injected into the amniotic cavity of chick embryos (100 µl/embryo). Embryos were incubated for 2 hours, harvested and placed into 4% paraformaldehyde. BrdU staining was performed upon 10 µm paraffin sections using a kit according to manufacturer’s specifications (Oncogene Research Products). BrdU-positive nuclei as well as total nuclei were counted using a grid. Ten grid regions per section were counted on ten sections per slide. Ten slides for each embryo were counted giving the percentage labeled as the number of labeled nuclei over the total number of nuclei. TUNEL staining was performed using an apoptosis detection kit (Boehringer) according to manufacturer’s specifications. TUNEL-positive cells were counted per section with section volume normalized using a grid. Ten sections per slide were counted and ten slides per embryo were counted to give total number of apoptotic nuclei.

RESULTS

Expression pattern of BMP signaling components

Bmp4 has been previously noted as being expressed in the mesoderm of the developing gut, exclusive of the developing gizzard (Roberts et al., 1998). To get a more complete picture of BMP signaling during the development of the gut, we examined the expression pattern of Bmp4 over a range of stages of gut development. Bmp4 is expressed in the small intestine from E2.5 through at least E9 (Fig. 1A,B). Bmp4 expression was always limited to the mesodermal layer of the gut (Fig. 1C). Bmp4 was not expressed in the developing gizzard until E7, when it is expressed in the connective-tissue-containing submucosal layer of the gizzard (Fig. 1D,E). The exclusion of BMP4 from the gizzard early in gut development, and its subsequent expression in a distinct layer of the gizzard, is suggestive of the existence of multiple roles for this ligand in gut development. To get a fuller picture of this pathway, we also analyzed the expression pattern of the BMP4 type I receptors, BMPR1B, and BMPR1A, and the type two receptor BMPRII (Kawakami et al., 1996; Zou et al., 1997; Natsume et al., 1997). BMPR1B is expressed in the mesoderm and endoderm of the gizzard from E3 through early developmental stages (Fig. 1F,G). Expression is lost by E7. BMPR1A is expressed in the mesoderm of the small intestine and large intestine, as well as in the pancreas from E3 through E7, but is absent from the gizzard mesoderm (Fig. 1H). The expression pattern of BMPR1A places it in a position to transduce signals from BMP4. In addition, we analyzed the expression pattern of the type II receptor, BMPRII, and found it is localized to the mesoderm throughout the gut, although it is not expressed in the gizzard mesoderm (Fig. 1I). Other members of the BMP family examined, including Bmp2, Bmp5 and Bmp7 are not expressed in the gizzard or small intestine during these stages (data not shown), although Bmp2 and Bmp7 are expressed in the proventriculus later in development (Narita et al., 2000).

BMP signaling leads to thinning of gizzard mesoderm

To test the role of BMP signaling in gut development, replication competent retroviral vectors were used to misexpress various components of the BMP pathway. Previously, we have described a decrease in the thickness of the mesoderm of the gizzard when BMP4 was ectopically expressed (Roberts et al., 1998; compare control in Fig. 2A.
with injected embryo in Fig. 2B). To further understand the role of BMP signaling in embryos, we injected retroviruses containing the constitutively active type I receptors BMPR1A or BMPR1B cDNAs (Zou and Niswander, 1997). Due to the restricted expression patterns of these two type I receptors, we hypothesized that there might be functional differences between these two constitutively active receptors. Gizzards in injected embryos were much smaller than control gizzards (compare Fig. 2A with C,D). The gross phenotype observed in the gizzard due to misexpression of these two viruses containing the constitutively activated receptors resulted in a small gizzard that resembled the phenotype seen with ectopic BMP4 (Fig. 2B,D). In addition, there were no observable differences in the phenotypes seen in guts injected with either constitutively active BMP receptor-expressing virus.

To further characterize the phenotypes observed in the gizzard upon ectopic BMP signaling, sections of paraffin-embedded gizzards were stained with Hematoxylin and Eosin (Fig. 2E-H). Examination of sectioned tissue verified that the mesoderm was indeed much thinner than the surrounding mesoderm (Fig. 2F-H) when compared to controls (Fig. 2E). This phenotype is confined to the mesodermal layer, as no defects were seen in the endodermal layer (Fig. 2F-H). The mesoderm of infected gizzards resembled the mesoderm of the normal small intestine which has a thinner mesodermal layer and which is exposed to BMP4 during development.

**Mesodermal thinning is due to apoptosis and changes proliferation rates**

The decrease in mesodermal thickness seen in injected embryos could be due to either a decrease in proliferation of the mesoderm or an increase in mesenchymal cell death or some combination of the two. To differentiate between these possibilities, we performed an analysis of the mitotic activity and cell death that occur in the infected gizzard mesoderm. Alternate sections of injected guts were cut and slides were stained using 3C2 (anti-gag antibody) to visualize virally infected cells (Fig. 3C), an anti-BrdU antibody to visualize proliferating cells (Fig. 3A,B), or a TUNEL method to visualize cells undergoing apoptosis (Fig. 3D,E). The viral infection appeared to be fairly complete in the mesoderm of injected guts (Fig. 3C), and confined to the mesodermal layer, consistent with the previous suggestion that the basement membrane of the endoderm is relatively impervious to

![Fig. 2. Gross morphological defects in embryos injected with retroviruses containing BMP signaling components. (A) Control gut at E9.5. (B) E9.5 gut injected with RCAS-BMP4. (C) E9.5 gut injected with RCAS-CABMPR1A. (D) E9.5 gut injected with RCAS-BMPR1B. (E) 10 μm section of a control E9.5 gizzard. (F) 10 μm section of an E9.5 gizzard injected with RCAS-BMP4. (G) 10 μm section of an E9.5 gizzard injected with RCAS-CABMPR1A. (H) 10 μm section of an E9.5 gizzard injected with RCAS-CABMPR1B. Abbreviations: SI, Small intestine; Gizz, gizzard; Inj, injected with retrovirus.](image-url)

![Fig. 3. Detailed analysis of morphological defects seen with ectopic BMP signaling within the gizzard. (A-E) Analysis of proliferation rates in control embryo gizzards and in gizzards of embryos injected with BMP signaling components. Embryos stained for either BrdU labeling to mark proliferating cells (A,B), TUNEL analysis to identify cells undergoing apoptosis (D,E), or 3C2 to label cells infected with retrovirus (C). (A,D) Control embryos. (B,C,E) Embryos injected with RCAS-BMPR1A. Red arrowheads point to labeled nuclei. (F-K) The mesoderm of gizzards injected with BMP signaling components is disorganized with ectopic regions of cartilage. (F) 10 μm section through a control embryo gizzard. 10 μm sections through gizzards of embryos injected with either BMP4 (G) CABMPR1A (H) CABMPR1B (J,K) containing retroviruses. Red arrowheads in F-K, ectopic cartilage in the mesoderm; purple arrowheads, regions of differentiated muscle; green arrowheads, regions of undifferentiated mesoderm. Abbreviations: End, endoderm; Sub, submucosa; Mu, muscularis layer; Inj, injected with retrovirus.](image-url)
Ectopic BMP signaling leads to mesodermal disorganization

In addition to the effects that we observed on the thickness of the gizzard mesoderm, we noted in the histological sections of infected embryos that, at later stages, the smooth muscle in the mesoderm did not appear to differentiate properly. Instead of the well-organized and differentiated muscular and submucosal layers seen in the uninfected E9 gizzard (Fig. 3F), regions of the mesoderm infected with either the BMP4 virus or the activated receptor viruses remained mesenchymal, while other regions appeared to contain islands of differentiated muscle (Fig. 3G,K). In addition, we occasionally observed small patches of cartilage within the mesoderm of infected gizzards (Fig. 3H,J), similar to that which forms in the somatopleural mesoderm in response to BMP signaling (Murtaugh et al., 1999).

To verify that BMP signaling was having an effect on muscle differentiation, injected gizzard sections were stained with the early smooth muscle marker FKBP/SMAP (Fukuda et al., 1998). Expression of FKBP/SMAP was decreased in injected gizzards compared to controls (Fig. 4A,B). There were regions within the mesoderm where FKBP/SMAP was absent and other regions where the expression was spotty and disorganized. To further characterize the mesoderm and the smooth muscle of the gizzard, an antibody for smooth muscle actin, which marks mature smooth muscle, was used on sections. It was found that the control gizzard mesoderm uniformly expresses smooth muscle actin in an ordered pattern in the muscularis layer while, in injected gizzards, we find that some regions of the mesoderm do not express smooth muscle actin (Fig. 4C,D). In addition, the regions that do express smooth muscle actin appear disorganized. Finally, the enteric nervous system bundles in the injected gizzards appear disorganized in shape and size compared to controls (Fig. 4C,D).

Molecular characterization of the BMP response

Molecular markers were then tested to further characterize the phenotype seen with ectopic BMP signaling in the gizzard. To determine whether the restricted intestinal expression of BMP4 is important for the anteroposterior regionalization of the gut, we employed a series of markers that show borders of expression at the posterior gizzard. Markers for the gizzard mesoderm, gizzard endoderm, small intestinal mesoderm and small intestinal endoderm were used to assay for any molecular phenotype in these embryos. Barx1 is a marker of gizzard mesoderm (Barlow et al., 1999), while Sox2 is a marker of gizzard endoderm (Ishii et al., 1998), neither of which is normally expressed in the intestine. We find that there is no change in these markers when tested on gizzards of injected embryos in both whole-mount and section in situ hybridization (data not shown). This suggests that the infected gizzards retain gizzard properties. This lack of regulation of Barx1 by BMP4

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**Table 1. Proliferation and cell death rates**

<table>
<thead>
<tr>
<th>Manipulation performed upon embryo</th>
<th>% of cells labelled with BrdU</th>
<th>Number of apoptotic cells per section</th>
</tr>
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<tbody>
<tr>
<td>Control gizzard</td>
<td>30.0</td>
<td>5.1</td>
</tr>
<tr>
<td>Control small intestine</td>
<td>18.8</td>
<td>15.4</td>
</tr>
<tr>
<td>RCAS-BMP-4-injected gizzard</td>
<td>14.4</td>
<td>48.6</td>
</tr>
<tr>
<td>RCAS-CABMPR1A-injected gizzard</td>
<td>14.6</td>
<td>52.3</td>
</tr>
<tr>
<td>RCAS-CABMPR1B-injected gizzard</td>
<td>17.5</td>
<td>42.8</td>
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**Fig. 4.** Ectopic BMP signaling affects the gut smooth muscle. 10 µm sections through the gizzards of (A,C) control embryos, and (B,D) embryos injected with RCAS-BMP4, with (A,B) in situ hybridization using the riboprobe for the smooth muscle marker FKBP/SMAP and (C,D) staining with an antibody for smooth muscle actin. Red arrowhead, regions staining for smooth muscle markers; blue arrowhead, regions devoid of smooth muscle marker staining; green arrowhead, enteric nervous system neural bundles.
is in contrast to the repression of Barx1 by BMP4 seen in the developing tooth and craniofacial region (Tucker et al., 1998). Nkx2.3 (Buchberger et al., 1996) is a marker of small intestinal mesoderm, while pdx-1 (Hebrok et al., 1998) and CdxA (Frumkin et al., 1994) are markers of anterior small intestinal endoderm. No change in these markers was seen in embryos injected with virus containing BMP signaling components (data not shown). This suggests that the injected gizzard is also not gaining small intestinal qualities, which is consistent with the lack of small intestinal morphology seen in the injected gizzards (see above). This suggests that, although BMP4 is expressed specifically in the small intestine, it does not play a role in specification of this organ along the anteroposterior axis, at least not based upon the molecular markers used in these experiments.

However, the response to BMP signaling is anteroposterior restricted, as evidenced by the activation of Nkx2.5 exclusively in the gizzard. The pancreas forms by budding from the gut primordium near the small intestine-stomach border. We, therefore, decided to take advantage of the differential inductive responses to determine if the mesoderm of the pancreas originates from stomach or intestinal primordium. Others have shown that ectopic Shh in the pancreas transforms the pancreas into a duodenal type structure (Apelqvist et al., 1997). We know that this should also induce BMP4 in the mesoderm (Roberts et al., 1998). This assay allows us to test if ectopic Shh can activate Nkx2.5 in the transformed pancreas to test if the pancreas is more gizzard-like or intestinal-like in its responsiveness. To test this, we injected a retrovirus containing the Shh cDNA. We find that ectopic Shh can, indirectly presumably via BMP4, activate Nkx2.5 in the transformed pancreas (compare Fig. 5A,B with C). This upregulation of Nkx2.5 in the transformed pancreas was only seen in the distal parts of the pancreas suggesting that only the distal pancreas responds like the gizzard to ectopic Shh, while the proximal pancreas responds like the duodenum to Shh signaling. To confirm that the activation of Nkx2.5 by ectopic Shh is mediated by BMP4, we coinjected retroviruses containing Shh and Noggin, an antagonist of BMP signaling. Embryos (n=12) injected with these two retroviruses display a greatly thickened mesodermal layer, as previously described (Apelqvist et al., 1997; Roberts et al., 1998), but there is no ectopic activation of Nkx2.5 within the pancreas and gizzard (data not shown). Thus, Shh cannot induce Nkx2.5 in the absence of BMP signaling.

Cell autonomy of BMP signaling

The ability of Shh to induce Nkx2.5 is an indirect consequence of its upregulation of BMP4. We wanted to determine whether BMP4 is likewise acting indirectly via induction of yet another, unknown, intercellular signal or whether its effect upon Nkx2.5 is cell-autonomous. To test this we took advantage of the activated BMP receptor (BMPR1A and BMPR1B) viral constructs. Gizzards infected with replication competent viruses transducing these constructs induced ectopic Nkx2.5 in the gizzard, but not the small intestine (compare Fig. 5A,B with D), similar to the response to BMP4 misexpression (Smith and Tabin, 1999). To test whether this response was cell-autonomous, we placed the BMP4 and the constitutively active receptor constructs into a replication-incompetent retroviral vector. The replication-incompetent pseudotyped retrovirus (Chen et al., 1999) was injected into stage 10 chick embryos and embryos were harvested 3 days later (E4.5). Section in situ hybridization was performed with a riboprobe to Nkx2.5, followed by staining with the antibody 3C2, to identify the individual cells that had been infected (Fig. 5E). BMP4 was found to activate Nkx2.5 in cells adjacent to those infected with virus in a cell-nonautonomous manner (Fig. 5E), however, the two activated BMPR constructs activated Nkx2.5 in a strictly cell-autonomous manner (Fig. 5F,G). Thus, the activation of Nkx2.5 and the formation of the pyloric sphincter appear to be a direct response to BMP signaling within the mesoderm of the gizzard.

Nkx2.5 is involved in the specification of the pyloric sphincter

Since we know that Nkx2.5 is a marker for the pyloric sphincter and that Nkx2.5 expression is under the control of BMP signaling, we decided to test the role of Nkx2.5 in specifying the pyloric sphincter by ectopically expressing Nkx2.5 via a retroviral vector. Injected embryos display no gross phenotype; however, upon histological sectioning and staining, the gizzard epithelium reveals a pattern alteration. At E9.5, gizzard epithelium is normally characterized by long cilia extending into the lumen (Fig. 6A; Toner, 1966). Gizzards of infected embryos at the same developmental stage have epithelium bordered by small mound or bleb-like cilia (Smith and Tabin, 1999, Fig. 6B). These affected patches of epithelium in which the viral transgene is expressed are reminiscent of pyloric sphincter epithelium (Fig. 6C) and of the gizzard epithelium
when transformed following exposure to BMP4 (Smith and Tabin, 1999).

While BMP signaling affects epithelial differentiation within the gizzard, we found by molecular analysis above that it did not affect the regionalization of the gizzard versus small intestine. In situ hybridizations of Nkx2.5-injected tissue sections similarly show no alterations of the normal expression patterns of Bmp4 and HoxC8 in the intestinal mesoderm, Pdx1 in the anterior small intestinal endoderm, and Wnt5a in the proventricular mesoderm (data not shown). Control viral injections showed no phenotype either histologically or molecularly.

In situ hybridization of Nkx2.5-infected gut tissue shows that the spread of the viral Nkx2.5/RCAS construct is limited to the mesoderm. Injected gut tissue processed for detection of RCAS transcripts displays staining only in the mesoderm (Fig. 6D) and suggests that the endoderm is affected by injected Nkx2.5 only after a signaling event between the tissue layers.

Nkx2.5 is necessary to specify some aspects of the pyloric sphincter

To test whether Nkx2.5 is necessary for normal specification of the pyloric sphincter, we made use of a retroviral construct containing the Engrailed repressor domain attached in frame to the Nkx2.5 homeodomain (enrepNkx2.5). This construct can repress endogenous Nkx2.5 activity in a dominant-negative manner in the Xenopus system (Fu et al., 1998). When examined at a gross morphological level, enrepNkx2.5-infected embryos showed no anomalies in the region of the junction between the gizzard and small intestine. However, histological examination revealed a change in the endodermal phenotype of the pyloric sphincter. The pyloric sphincter normally has a layer of endodermal cells containing a single short microvillus with a bulbous tip with no keratin-like covering (Fig. 7B), while the gizzard endoderm has long thin microvilli covered with a thick keratin-like material, called koilen (Fig. 7A, purple arrowhead). In the injected guts, the endoderm of the pyloric sphincter is covered with a keratin-like material similar to that of the normal gizzard (Fig. 7C, purple arrowhead), although the microvilli retain the bulbous tip characteristic of the sphincter region.

In addition to the phenotype at the pyloric sphincter, we also observed changes in other regions of the gut, perhaps related to interference with other Nkx family members. Indeed, due to the highly conserved DNA-binding domain, it would be expected that enrepNkx2.5 would be able to affect the activity of other Nkx family members, and consistent with this idea a similar virus, enrepNkx2.3, acts identically to enrepNkx2.5 in gut mesoderm (data not shown). The gizzard and proventriculus of enrepNkx2.5-infected embryos appeared greatly distended and there was no clear distinction between the two organs (Fig. 7D). Hematoxylin and Eosin staining of sections revealed that there was a loss of proventricular glands in the enrepNkx2.5-infected embryos and that the gizzard domain seemed to have expanded into the region normally forming the proventriculus (Fig. 7E-H).

Among the other Nkx genes expressed in the gut mesoderm, Nkx2.3 is interesting in this regard (Buchberger et al., 1996). Nkx2.3 expression extends throughout the length of the hind and midgut (Buchberger et al., 1996), abutting but not overlapping that of Nkx2.5 at the pyloric sphincter (Fig. 7I,J). The expression of both of these genes is altered following enrepNkx2.5 infection. The boundary of Nkx2.5 expression is shifted anteriorly beyond the pyloric sphincter into the gizzard and proventriculus mesoderm (Fig. 7K,L). Nkx2.3 expression is similarly expanded anteriorly throughout the gizzard and proventriculus (Fig. 7M,N). While the phenotypic effects observed outside of the pyloric sphincter may thus relate to other endogenous Nkx genes, we nonetheless interpret the sphincter phenotype as a dominant negative effect upon the endogenous Nkx2.5, since it is the member of this gene family expressed specifically within the pyloric sphincter and the dominant negative form of Nkx2.5 (enrepNkx2.5) results in a loss of sphincter characteristics, while the wild-type form induces an ectopic sphincter phenotype.
DISCUSSION

The BMP family is a large family of signaling molecules that are critical for patterning numerous regions of the vertebrate embryo (Raftery and Sutherland, 1999). The BMP signal is transduced via a heterodimeric receptor complex composed of a type I and a type II receptor. The BMP receptors are distinct from those used by other TGFβ family members. Once the ligand binds to the type I receptor, the type II receptor dimerizes and phosphorylates the type I receptor activating it, which then transduces the signal into the cell. This study has focused on the role of BMP signaling in patterning the vertebrate gut.

BMP signaling is capable of activating Nkx2.5 within the gizzard mesoderm (Smith and Tabin, 1999). Nkx2.5 is a marker for the pyloric sphincter and ectopic BMP signaling leads to an ectopic sphincter phenotype within the gizzard (Smith and Tabin, 1999). This suggests that Nkx2.5 plays a role in gizzard differentiation. Here we have expanded these studies to include the role of Nkx2.5 in specifying the pyloric sphincter.

BMP signaling plays multiple roles in gut development

We have analyzed the expression pattern of members of the BMP family as well as some of the receptors for this family. We find that Bmp4 is the only member of this family expressed in the gut at the early stages that we examined. We find several roles for BMP signaling in the gut (Fig. 8). First, BMP signaling is responsible for mediating the thickness of the mesoderm in the small intestine, as previously noted (Roberts et al., 1998). Second, BMP signaling plays a role in regulating smooth muscle differentiation. Third, BMP signaling patterns the pyloric sphincter at the border between the gizzard and the small intestine. We also find that the two type I receptors are expressed in the mesoderm of the gut in complementary patterns to one another, while the type II receptor is expressed in the mesoderm exclusive of the gizzard.

BMP antagonists work in concert with BMP production to define domains of signaling in many important developmental processes (Vogt and Duboule, 1999). We examined the expression of two such antagonists, but were unable to detect...
the transcription of either Noggin or Chordin in the developing chicken gut at the stages that we analyzed (data not shown). There is, however, a growing list of BMP antagonists, so it remains plausible that a BMP antagonist does play a role in defining gut differentiation, proliferation and patterning.

We find that the BMP type I receptors act in identical ways to one another in the context of the gut. This is in contrast to the developing limbs where the same two activated receptors have been reported to trigger distinct developmental pathways (Zou and Niswander, 1997). This is likely due to the different downstream components of the BMP pathway, such as the SMAD family of transcription factors downstream of the BMP receptor complex (Raftery and Sutherland, 1999).

**Regulation of mesodermal thickness**

Previous studies of mesodermal patterning in the gut suggested a role for BMP4 in mediating both proliferation and differentiation of the splanchnic mesoderm. Ectopic BMP4 in the gizzard results in a thinning of the mesoderm (Roberts et al., 1998). Constitutively active versions of the BMP type I receptors give identical phenotypes to the BMP4 virus when misexpressed in the gizzard mesoderm. This thin mesoderm phenotype in the gizzard is due both to an increase in apoptosis and a decrease in cell proliferation. The same results were obtained using constitutively active BMP receptor type 1A/1B constructs. The resultant phenotypes were very similar to the apoptotic and proliferation rates seen in the wild-type small intestine. This suggests that BMP signaling regulates the thickness of the normal small intestinal mesoderm via BMP4 binding to BMPR1A expressed in the small intestinal mesoderm to affect proliferation and apoptosis.

**Patterning the radial axis of the gut**

In addition to the modulation of mesodermal thickness seen in the gizzard upon ectopic BMP signaling, we also observed a disruption of mesodermal patterning. The mesoderm of the gizzard is typically organized into two major layers, an inner submucosa of loose connective tissue and an outer muscularis layer of smooth muscle. In the gizzards with ectopic BMP4 signaling, we find that the smooth muscle is disorganized and regions of the mesoderm are undifferentiated. This supports our model in which BMP signaling regulates smooth muscle differentiation or prevents proliferation of the muscle precursor cells, such that few muscle cells are created via cell division. Since BMP4 is not expressed in the gizzard during its most active morphogenetic (hypertrophic) period, but becomes expressed at later stages only in the submucosa, similar to that seen in the small intestine, this could prevent smooth muscle differentiation from occurring in this specialized non-muscular portion of the mesoderm. In addition, the early expression of BMP4 in the small intestine could prevent premature differentiation of the intestinal mesoderm, which would allow the small intestine to elongate.

**Patterning the pyloric sphincter**

Previously, we showed that BMP4 expressed in the small intestinal mesoderm activates Nkx2.5 in the posterior gizzard mesoderm (Smith and Tabin, 1999). This is presumably via binding to BMPR1B, which is expressed in the gizzard mesoderm. We also found that Nkx2.5 is a marker for the pyloric sphincter (Smith and Tabin, 1999). In this study, we find that the constitutively active type I receptor constructs are both capable of activating Nkx2.5 in the gizzard mesoderm. It is interesting to note that, although we see upregulation of Nkx2.5 in the gizzard mesoderm, we do not see any upregulation of Nkx2.5 in the small intestinal mesoderm. There is, thus, a differential responsiveness to the signaling molecules such that only the gizzard mesoderm is competent to develop the pyloric sphincter. This segregation of the mesoderm responsiveness could be due to the early expression of other patterning factors, such as Hox genes (Roberts et al., 1995, 1998; Yokouchi et al., 1995).

We also tested whether the upregulation of Nkx2.5 by BMP signaling was a cell-autonomous or a cell-nonautonomous event. As would be expected, we found that BMP4 upregulation of Nkx2.5 was a cell-nonautonomous event, while the upregulation by the two constitutively active receptor constructs was a cell-autonomous event. This shows that the upregulation of Nkx2.5 by BMP signaling was due to a direct cell-signaling event in the gizzard mesoderm.

Nkx2.5 is a marker for the pyloric sphincter (Smith and Tabin, 1999). Nkx2.5 is upregulated by BMP signaling in the gizzard and ectopic BMP signaling can ectopically alter the gizzard endoderm to look sphincter-like (Smith and Tabin, 1999). We find that ectopic Nkx2.5 can ectopically specify the endodermal sphincter phenotype, while a dominant-negative Nkx2.5 causes the endoderm of the sphincter to lose some sphincter qualities, in particular the keratin-like coating. These data show that Nkx2.5 is both necessary and sufficient to pattern some of the aspects of the pyloric sphincter.

**Nkx genes are involved in regulating the gut boundaries**

In addition to their roles in phenotypic specification, Nkx2.5 and Nkx2.3 can be viewed as regional markers, with a mutual border at the junction of the small intestine with the pyloric sphincter. The mutually exclusive expression patterns are themselves dependent upon Nkx activity since misexpression
of the dominant-negative form of Nkx2.5 results in the exclusivity being released and the two genes are coexpressed through the pyloric sphincter and gizzard. We hypothesize that a third, unknown member of the Nkx family establishes the anterior border of the pyloric sphincter restricting the anterior boundary of normal Nkx2.5 expression, since the dominant-negative Nkx2.5 misexpression also results in the spread of the domains of Nkx2.3 and Nkx2.5 throughout the gizzard and proventriculus. This also results in the loss of a distinct proventriculus phenotype, including a common morphology with the gizzard and an absence of proventricular glands. It is unclear whether the gizzard-like phenotype in the proventriculus is attributable to the ectopic Nkx2.3 and Nkx2.5 in this region or to the enrepNkx2.5 misexpression blocking the activity of some other endogenous Nkx family member.

The enrepNkx2.5 construct has been shown to have a dominant-negative activity in Xenopus (Fu et al., 1999) and presumably is acting similarly here. This implies that Nkx genes function normally as transcriptional activators in the gut. Establishment and maintenance of boundaries between regions of the gut are important elements of normal gut patterning, and Nkx genes appear to be important for these patterning events in gut development. However, the lack of changes in the expression boundaries of regionally restricted Bmp4, Hoxc8, Pdx1 and Wnt5a in Nkx2.5-infected embryos indicates that not all aspects of gut regionalization are downstream of the Nkx genes.

Mesodermal-endodermal communication exists in tissue expressing Nkx2.5

Misexpression of Nkx2.5 during early gut development alters the epithelial phenotype of a foregut derivative. Misexpression is achieved using a microinjection technique that allows targeting of Nkx2.5 to specific tissue layers in specific embryonic regions. Nkx2.5 is normally expressed in the visceral mesoderm of the gut; further, injection of Nkx2.5 during early gut development alters boundary of normal Nkx2.5 through the pyloric sphincter and gizzard. We hypothesize that BMPR1B to cause upregulation of Nkx2.5 within the posterior gizzard mesoderm and to specify the phenotype of the pyloric sphincter. Nkx2.5, a marker for the pyloric sphincter mesoderm, plays a direct role in this process, patterning the endoderm of the pyloric sphincter via some unknown signal(s). Meanwhile, BMP4 expressed in the small intestinal mesoderm and later in the gizzard submucosa delays smooth muscle differentiation in these tissues. Hence, BMP signaling has many important developmental roles in gut patterning. The universality of this anatomic boundary in vertebrates, expression of the molecules delineating this region, and conservation of the signaling pathways involved underscores the importance of gut development as a model system for understanding development.

This research was supported by grants to D. J. R. (HD34448) and C. T. from the NIH. We thank the members of the Cepko/Tabin group for their help and advice throughout this work, especially Craig Nelson for help with nonradioactive in situ hybridization and Tiffany Heanue and Jeff Blenker for their help with technical aspects. We are grateful to Lee Niswander, Paul Brickell, Amy Chen, Susan Dymecki, Phillipa Francis-West, Abraham Fainsod, Sylvia Evans and Tsutumi Nogiol for reagents used in these studies.

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