



## *Sox9* and *Nkx2.5* determine the pyloric sphincter epithelium under the control of BMP signaling

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### Abstract

The organs of the digestive tract are specified by coordinated signaling between the endoderm and mesoderm during development. These epithelial–mesenchymal interactions lead to the organ-specific morphogenesis and differentiation of regions along the gut tube. In this paper, we show that in the chick, the SRY-related transcription factor *Sox9* is a marker for the posterior gizzard. Viral misexpression of *Sox9* in the gizzard mesoderm is sufficient to specify epithelium characteristic of the pyloric sphincter. *Sox9* expression is normally limited to the region of the posterior gizzard under the regulation of BMP signaling from the adjacent midgut. Misexpression of an activated form of *BMPRIb* in the gizzard upregulates *Sox9* expression, while the BMP antagonist *noggin* down-regulates *Sox9* expression in the gizzard mesoderm. Previously, *Nkx2.5* was identified as a marker for the mesoderm of the pyloric sphincter. As with *Sox9*, BMP signaling appears to regulate *Nkx2.5* and its ability to determine the pyloric epithelium. Despite these similarities, our evidence suggests that *Sox9* and *Nkx2.5* are regulated independently by BMP signaling, and act coordinately to specify the pyloric sphincter.

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### Introduction

The digestive tract initially forms as a simple tube that undergoes regional specialization followed by morphogenesis and differentiation, creating the different organs of the digestive tract. The primitive gut tube is composed of an inner luminal lining of endoderm-derived epithelium surrounded by an outer layer of splanchnic mesoderm. As development progresses, the splanchnic mesoderm forms radial layers and undergoes smooth muscle differentiation. Concurrently, the uniform luminal epithelium becomes specified into distinct regions and these regions subsequently undergo organ-specific differentiation (Kedinger et al., 1988). The formation of organs along the gut tube thus requires that smooth muscle regionalization of the meso-

derm along the anterior–posterior (A–P) axis be coordinated with epithelium differentiation (for review see (Roberts, 2000)). This coordination is necessary for later physiological function. For example, the thick muscle walls of the stomach physically grind food that is being chemically broken down by enzymes secreted from the gastric epithelium. Digested food then enters the duodenum through the pyloric sphincter, where the epithelium contains bulbous microvilli required for the absorption of nutrients which is pushed through the tube by peristaltic movements of the thin circular muscles of the small intestine.

Coordination of epithelial and mesodermal differentiation is achieved by molecular signaling between the two layers. Signals from the endoderm first act to allow the epithelial lumen to be surrounded by mesoderm specified to form visceral mesoderm. Sonic Hedgehog (SHH) has been implicated as one of the inductive signals from the endoderm that specifies and patterns the overlying mesoderm, both promoting increased cell proliferation of gut mesoderm and smooth muscle specification (Apelqvist et al., 1997; Roberts

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et al., 1995, 1998). The mesoderm in turn signals back to the endoderm to specify the A–P position of the endoderm and to consequently define organ fate and subsequent function (Haffen et al., 1983, 1987; Kedinger et al., 1986, 1988). For example, when stomach endoderm is grafted to small intestine mesoderm, the resulting epithelium contains microvilli indicative of the small intestine (Kedinger et al., 1986). Further communication between the endoderm and mesoderm direct organ-specific differences such that as development proceeds, the layers of tissue across the radial axis form distinct patterns at different A–P levels. Again, these differences are exemplified by the distinct features of the organs along the digestive tract; the large muscles for grinding food found in the chick gizzard (or posterior stomach), which are absent from the rest of the tract. While many of the tissue interactions which direct gut organogenesis have thus been defined, the molecular nature of the signals responsible for these inductive events are only starting to be elucidated.

In addition to SHH, several other families of secreted molecules have been implicated as putative inductive signals communicating between the endoderm and mesoderm. The Wnt family of signaling molecules has been shown to be involved in defining regionalization along the A–P axis, as well as defining organ-specific features of the inner epithelium (McBride et al., 2003; Theodosiou and Tabin, 2003). Components of the Wnt signaling pathway are expressed in very discreet domains along the A–P axis corresponding to organ boundaries. In addition, overexpression and gene knock-out studies in chick and mouse have begun to reveal roles for Wnt signaling molecules in determining organ-specific structural features during gut development (Heller et al., 2002; Korinek et al., 1998; McBride et al., 2003; Theodosiou and Tabin, 2003; Wang et al., 2001). Due to the large number of components and the complexity of Wnt signaling, much more work remains in elucidating the role of Wnt signaling and the possible interaction of Wnts with other signaling pathways during gut development.

Bone Morphogenetic Protein (BMP) signaling has also been implicated in specifying regionalization of the gut during development. A number of BMP molecules have been shown to be expressed in the developing gut including *BMP2*, *4*, and *7* (Narita et al., 2000; Roberts et al., 1998; Smith and Tabin, 1999). *BMP2* is expressed in the proventriculus mesoderm and is necessary for stomach gland formation (Narita et al., 2000). *BMP4* is induced by SHH and is expressed throughout the developing gut, except for the stomach. Studies in chick have shown that *BMP4* limits proliferation of mesodermal cells in the gut, hence its absence in the stomach primordial contributes to the thick muscle wall of that organ (Roberts et al., 1995, 1998). *BMP4* signaling also regulates expression of the pyloric sphincter marker, *Nkx2.5* (Smith and Tabin, 1999; Smith et al., 2000). *Bmp4* is expressed adjacent to but not within the gizzard, while *BMP receptor1B* is specifically expressed in

the gizzard. The location where *BMP4* diffusion overlaps with *BMPR1b* expression correlates with the location of the future pyloric sphincter. *Bmp4* misexpression induces expression of the pyloric sphincter marker *Nkx2.5* in the gizzard mesoderm (Smith and Tabin, 1999). Moreover, viral misexpression of *Nkx2.5* in the mesoderm leads to transformation of the gizzard epithelium to a pyloric-like epithelium, suggesting mesodermal *Nkx2.5* expression is indeed responsible for inducing aspects of pyloric sphincter differentiation.

In this paper, we present a second transcription factor that serves as a marker for the pyloric sphincter, *Sox9*. *Sox9* is an SRY-related transcription factor originally described in the context of its role in testes determination (da Silva et al., 1996; Kent et al., 1996; Vidal et al., 2001). The Sox family of transcription factors has since been shown to be involved in a number of developmental processes. *Sox9* has specifically been linked to cartilage development (Bi et al., 1999; Healy et al., 1999) as well as pancreas development (Lee and Saint-Jeannet, 2003; Lioubinski et al., 2003; Piper et al., 2002). In the chick gut, *Sox9* is expressed in the mesoderm of the pyloric sphincter and later in the ceca, as well as the intestine endoderm during development. As was shown in cartilage formation (Healy et al., 1999; Semba et al., 2000; Zehentner et al., 1999), we demonstrate that *Sox9* expression in the pyloric sphincter is regulated by BMP signaling. *Sox9* and *Nkx2.5* expression appear to be regulated independently by BMP signaling, suggesting they act together in regulating the formation of the sphincter.

## Materials and methods

### Expression analysis

Fertilized white Leghorn chick eggs were obtained from SPAFAS (Norwich, CT) and staged according to Hamburger and Hamilton (1951). Expression analysis was performed by whole-mount in situ hybridization using digoxigenin-labeled riboprobes (Riddle et al., 1993) (Burke et al., 1995). Embryos were fixed in 4% paraformaldehyde/PBS pH 7.4 for 5–12 h at 4°C. Embryos destined for whole mount in situ hybridization were dehydrated to 100% methanol and stored at –20°C until used. For section in situ hybridization, embryos were dehydrated to 100% ethanol and embedded in paraffin (Allen, 1994) (Murtaugh et al., 1999). Probes used in this study include *Sox9* (Healy et al., 1999), *Nkx2.5* (Buchberger et al., 1996), *Wnt11* (Tanda et al., 1995), and *Fgf10* (Ohuchi et al., 1997).

### Retroviral misexpression

The replication-competent retroviral vectors RCASBP(A) and RCASBP(B) were used for misexpression studies. Constructs carrying *Sox9* (Healy et al., 1999), *Nkx2.5* (Smith et al., 2000), *enRepNkx2.5* (Smith et al., 2000), *noggin*

(Capdevila and Johnson, 1998), and the constitutively active form of *BMPRIb* (Zou and Niswander, 1996) were made and viruses were generated as previously described (Hughes et al., 1987; Logan and Tabin, 1998; van de Westering et al., 2002). Retroviral particles were injected into the intra-coelomic cavity at HH stage 12, enabling viral particles to infect the splanchnic and somatic mesoderm (Hamburger and Hamilton, 1951). In order to confirm that the rate of viral infection was early enough to insure BMP signaling is required for induction of *Sox9* expression and not maintenance of expression, HH stage 12 embryos were injected with *RCAS-noggin* and harvested embryos at HH stage 22, well before endogenous *Sox9* expression is induced. Embryos were sectioned and stained with 3C2 antibody to detect viral infection. Indeed, by HH stage 22, there is clear infection and expression of the *gag* viral gene in expected tissues, such as the gut mesoderm and somites (data not shown). Viruses *RCASBP(A)Sox9* and *RCASBP(B)Nkx2.5* were simultaneously injected to study any synergistic relationship between the genes. Embryos were harvested at HH stage 26 for gene expression analysis by fixing in 4% paraformaldehyde/PBS pH 7.4 and dehydrating to 100% methanol in preparation for whole mount in situ hybridization. For histologic analysis, embryos were allowed to develop and harvested at HH stage 35. Guts were dissected from harvested embryos, fixed with 4% paraformaldehyde/

PBS pH 7.4 for 2 h at 4°C, and dehydrated to 100% ethanol prior to processing for histologic analysis.

#### *Histologic and immunohistochemistry analysis*

Infected guts were embedded in paraffin and cut into 8 µm sections. Adjacent sections were collected on sequential slides for direct comparison of areas of viral infection with gut histology. Sections were stained with Hematoxylin and Eosin following standard procedures (Allen, 1994). Regions of viral infection were detected on adjacent sections with the 3C2 antibody against the *gag* viral protein, and visualized by staining with DAB. As a negative control, *RCAS-GFP* was injected into HH stage 12 embryos and harvested at HH stage 35. Infected guts were sectioned and stained with Hematoxylin and Eosin and for viral infection by 3C2. There were no histological gut abnormalities associated with viral infection.

## Results

### *Sox9 expression in the developing gut*

In the course of other studies, we fortuitously noted *Sox9* expression within the developing gut. In an effort to identify

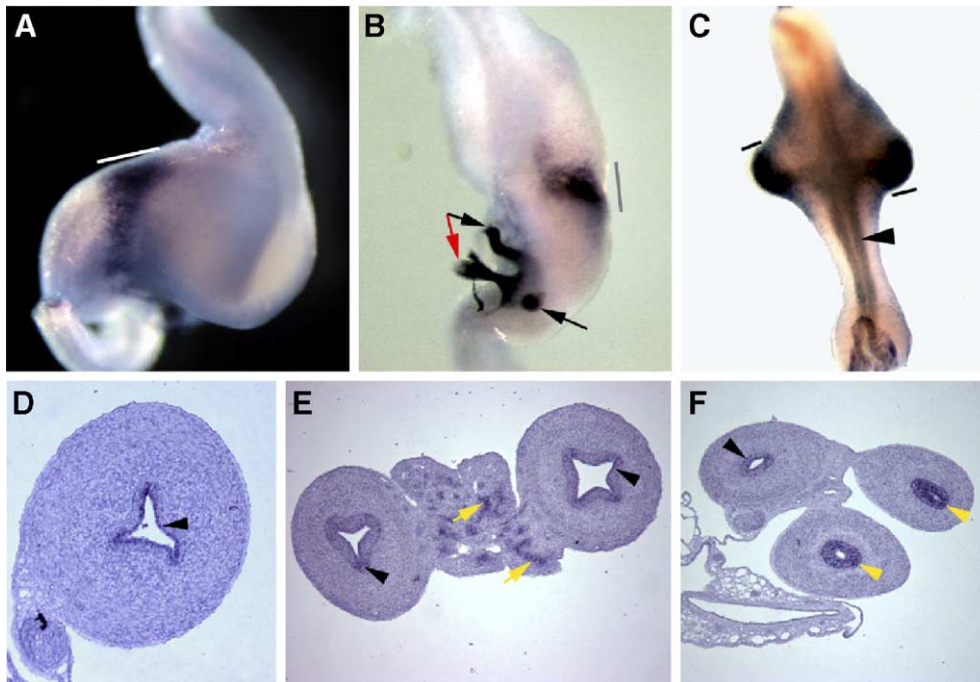


Fig. 1. *Sox9* expression in the developing digestive tract. (A–C) Whole mount in situ hybridization of regions of the gut from a HH stage 26 chick embryo. (A) *Sox9* expression in the posterior gizzard in the region of the pyloric sphincter (white bar). (B) Dorsal–lateral view of the gizzard–duodenal junction, expression of *Sox9* in the ducts of the liver (red arrow), pancreas (black arrows), and posterior gizzard (bar in (B) shows relative orientation in relation to panel (A)). (C) In the ceca, expression is observed in the mesoderm of the horns of the ceca (bars) as well as the endoderm of the hindgut (arrowhead). (D–F) Section in situ hybridization with the *Sox9* probe in HH stage 37 chick embryo gut sections. *Sox9* expression is found in the endoderm of the duodenum (D), ileum (E) and cecum (F). Expression in these sections appears to be restricted to the apical tip of the epithelium (black arrowheads in D–F), while stronger, more evenly distributed *Sox9* expression is found in the endoderm of the horns of the cecum (F, yellow arrowheads). (E) Expression is also observed in the ducts of the pancreas, as also seen in (B).

new organ-specific markers during development of the gut tube, we more closely examined *Sox9* expression in the developing chick gut by whole mount in situ hybridization. Expression was detected in both mesoderm and endoderm-derived structures of the gut. *Sox9* expression was detected in the pyloric sphincter and ceca mesoderm at HH stage 26 (Figs. 1A–C). *Sox9* expression is restricted to the tips of the ceca horns in the mesoderm (Fig. 1C, bars), and is also detected in the endoderm of the hindgut (Fig. 1C, arrowhead). To confirm endoderm expression, section in situ hybridization was performed. *Sox9* expression was detected throughout the small intestine (Figs. 1D, E), as well as the ceca and hindgut endoderm (Fig. 1F). Interestingly, *Sox9* expression appeared more strongly and evenly distributed in the epithelium of the horns of the ceca, than in the epithelium of the small intestine or hindgut (compare Figs.

1D, E, F black arrowheads to Fig. 1F yellow arrowheads). In addition to the inner epithelial lining of the gut, *Sox9* expression was also found in the ducts of the liver and pancreas (Figs. 1B and E) (Lee and Saint-Jeannet, 2003; Lioubinski et al., 2003; Piper et al., 2002). These discreet expression domains suggest important and varying roles for *Sox9* during gut development.

*Sox9* specifies the pyloric epithelium and increases cell number in the gut endoderm

To determine the role for *Sox9* during gut development, we virally misexpressed *Sox9* in the early splanchnic mesoderm. Two distinct phenotypes were observed associated with viral infection. In a normal gut, the gizzard epithelium is covered in hair-like microvilli (Fig. 2A). In

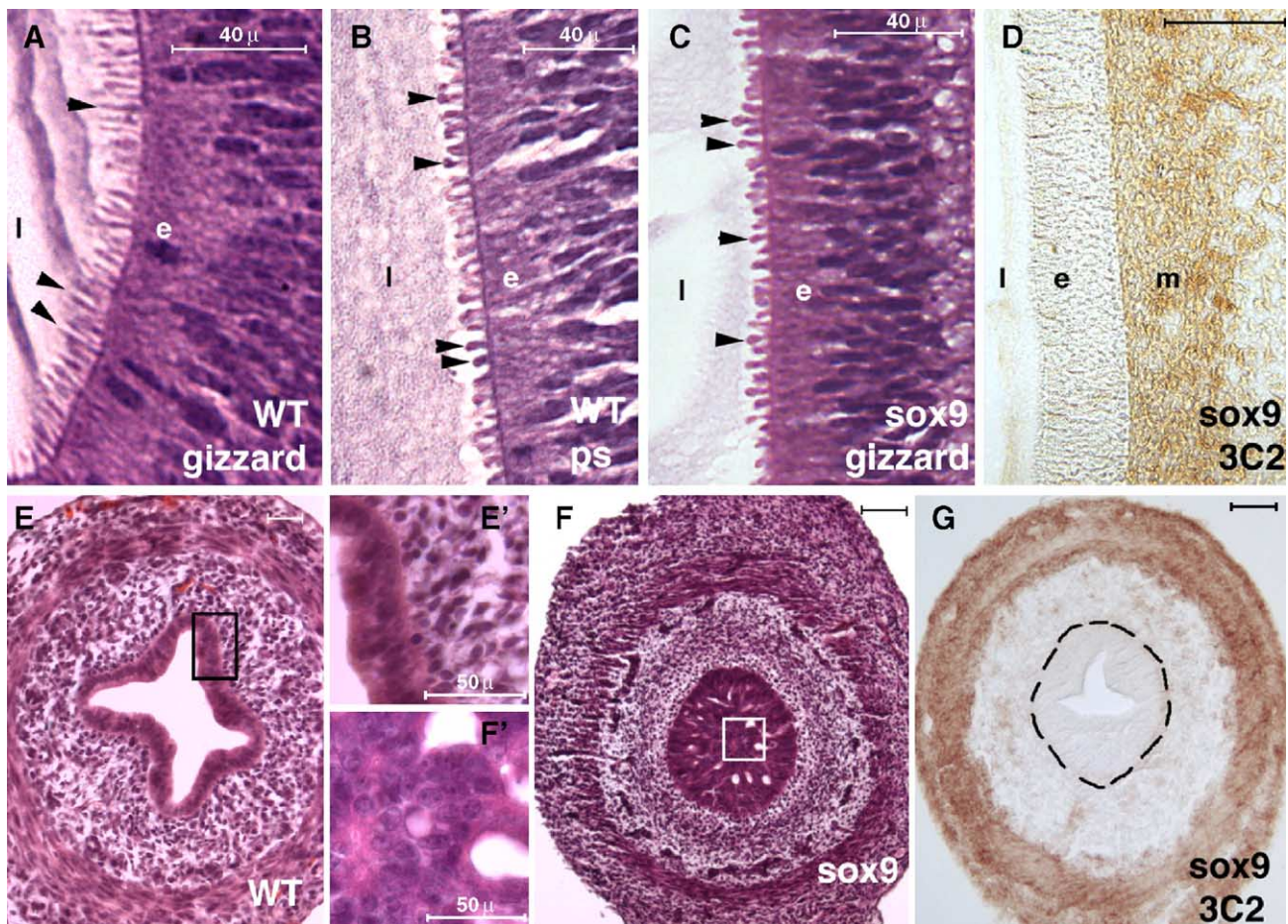


Fig. 2. Misexpression phenotypes of *Sox9* in the chick gut. Sections (8 $\mu$ m) through gut tissue at the gizzard and pyloric sphincter (A–D), and ileum (E–G). Sections (A–C, E, E', F, F') are stained with hematoxylin and eosin, while sections (D and G) are stained with the 3C2 antibody against the *gag* viral protein in order to detect regions of viral infection. Sections through a wild type gizzard show the hair-like microvilli of the organ (A, arrowheads) compared to the bulbous microvilli of the pyloric sphincter (B, arrowheads). (C) Viral misexpression of *Sox9* leads to transformation of the gizzard microvilli, into bulbous microvilli reminiscent of the pyloric sphincter (arrowheads). (D) Lower magnification view of the region in (C) in an adjacent section, showing viral misexpression of the *Sox9* retrovirus occurred in the mesoderm layer, the endoderm remains uninfected. Misexpression of *Sox9* also leads to an increase in cell number of the epithelium in the ileum (F) compared to wild type (E). (E' and F') correspond to the boxed areas in (E) and (F), respectively. (F') The lack of a lumen in (F) is due to the presence of more cells denoted by the high density of nuclei when compared to the simple single cell-layer epithelium in wild type (E'). (G) A non-adjacent section of the intestine in (F). Viral infection is observed in the mesoderm and is absent from the endoderm (outlined by dashed line), demonstrating an increase in cell number in the intestine epithelium corresponds with viral infection. Scale bars indicate 100  $\mu$ m unless otherwise noted. e, epithelium, l, lumen, m, mesoderm.

contrast, the epithelial lining of a normal pyloric sphincter contains bulbous microvilli involved in the absorption of nutrients (Fig. 2B). Ectopic expression of *Sox9* in the gizzard led to transformation of the microvilli of the gizzard epithelium to more pyloric-like, bulbous microvilli ( $n = 6/6$ ) (Fig. 2C). The transformation of the gizzard endoderm in response to *Sox9* infection suggests that a secondary signal downstream of *Sox9* is responsible for specification of the gizzard epithelium (Fig. 2D). Misexpression of *Sox9* in the mesoderm also led to an increase in the number of cells in the intestinal ( $n = 5/6$ ) (compare number of nuclei in Fig. 2E versus F), often leading to complete stenosis of the lumen (Figs. 2E–G). Together, these results suggest a dual role for *Sox9* in specifying the pyloric epithelium in the posterior gizzard, and regulating cell numbers in the small intestine and hindgut endoderm. We were particularly intrigued by the apparent patterning role of *Sox9* in determining the pyloric epithelium.

#### *BMP signaling regulates Sox9 expression in the pyloric sphincter*

Since prior studies had shown that other markers of the pyloric sphincter mesoderm are regulated by BMP signaling, we tested whether BMP signaling also regulated *Sox9* expression in the developing gizzard. Indeed, we observed that viral misexpression of the constitutively active form of the receptor *BMPRIb* led to an expansion in the

domain of *Sox9* expression in the gizzard ( $n = 8/13$ ) (compare Figs. 3A and B). The smaller size HH stage 26 gizzard in (Fig. 3B) compared to wild type (Fig. 3A) is due to the decrease in smooth muscle development attributed to by an increase in BMP signaling (Fig. 4H) (Smith et al., 2000). Conversely, misexpression of the BMP antagonist *noggin* resulted in a dramatic decrease of *Sox9* expression in the gizzard ( $n = 14/21$ ) (Fig. 3C). This apparent decrease in *Sox9* expression is not an artifactual consequence of the decrease in the thickness of the mesoderm following misexpression of the constitutively active *BMPRI* which causes up-regulation of *Sox9* expression. Thus, it appears that BMP signaling is both necessary and sufficient for *Sox9* expression in the gizzard mesoderm.

#### *Sox9 and Nkx2.5 both regulate the pyloric epithelium downstream of BMP signaling*

Like *Sox9*, *Nkx2.5* is expressed in the pyloric sphincter during gut development (Smith and Tabin, 1999). We compared the early expression pattern of *Nkx2.5* with *Sox9*, and found that *Nkx2.5* is expressed earlier in the posterior gizzard and at higher levels than *Sox9* (Figs. 4A and B). *Nkx2.5* expression in the pyloric sphincter is detected as early as HH stage 21, while low levels of *Sox9* in the pyloric sphincter are detectable for the first time

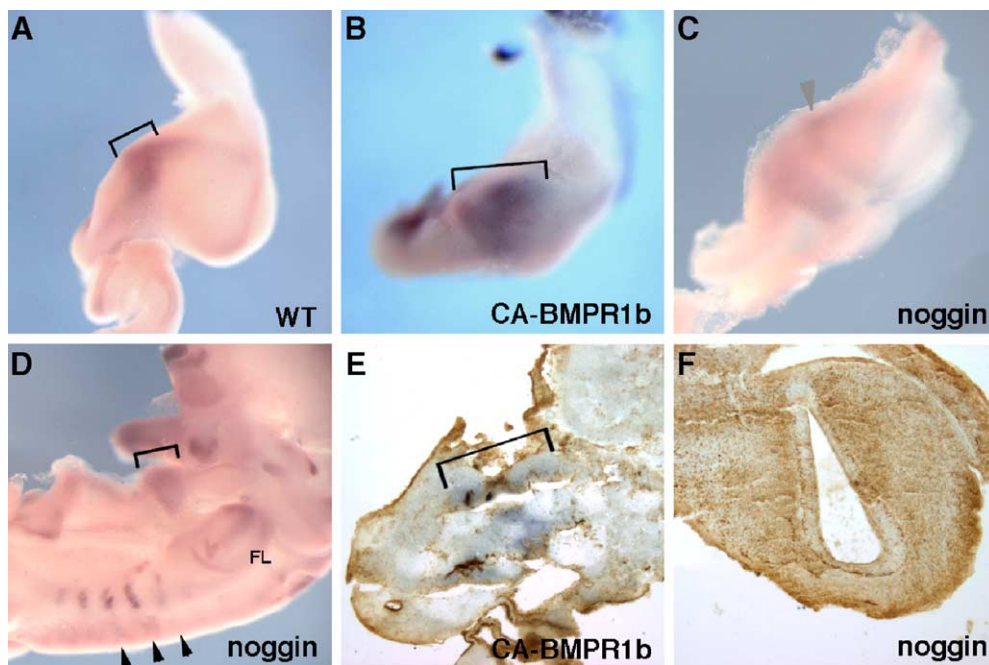


Fig. 3. *Sox9* expression in the posterior stomach is regulated by BMP signaling. (A–D) Whole mount in situ hybridization with *Sox9* probe on HH stage 26 gizzards. (E and F) Staining with 3C2 antibody in sections through whole mounts from (B and C), respectively. (B) Misexpression of a retrovirus containing the constitutively activated form of *BMPRIb* results in an expansion in the domain of *Sox9* expression in the gizzard (bracket in (B) compared to (A)). (C, arrowhead) Viral misexpression of *noggin* results in dramatic down regulation of *Sox9* expression compared to the wild type control (A). (D) The flank region of an embryo infected with *noggin*-expressing retrovirus. Note the decrease in *Sox9* expression in the somites (arrowheads) and forelimb (FL). Forelimb and gizzard (bracket) are also diminished in size. (E and F) Sections through (B and C), respectively, stained with 3C2 antibody demonstrating that changes in the expression domain of *Sox9* in the gizzard are associated with viral infection.

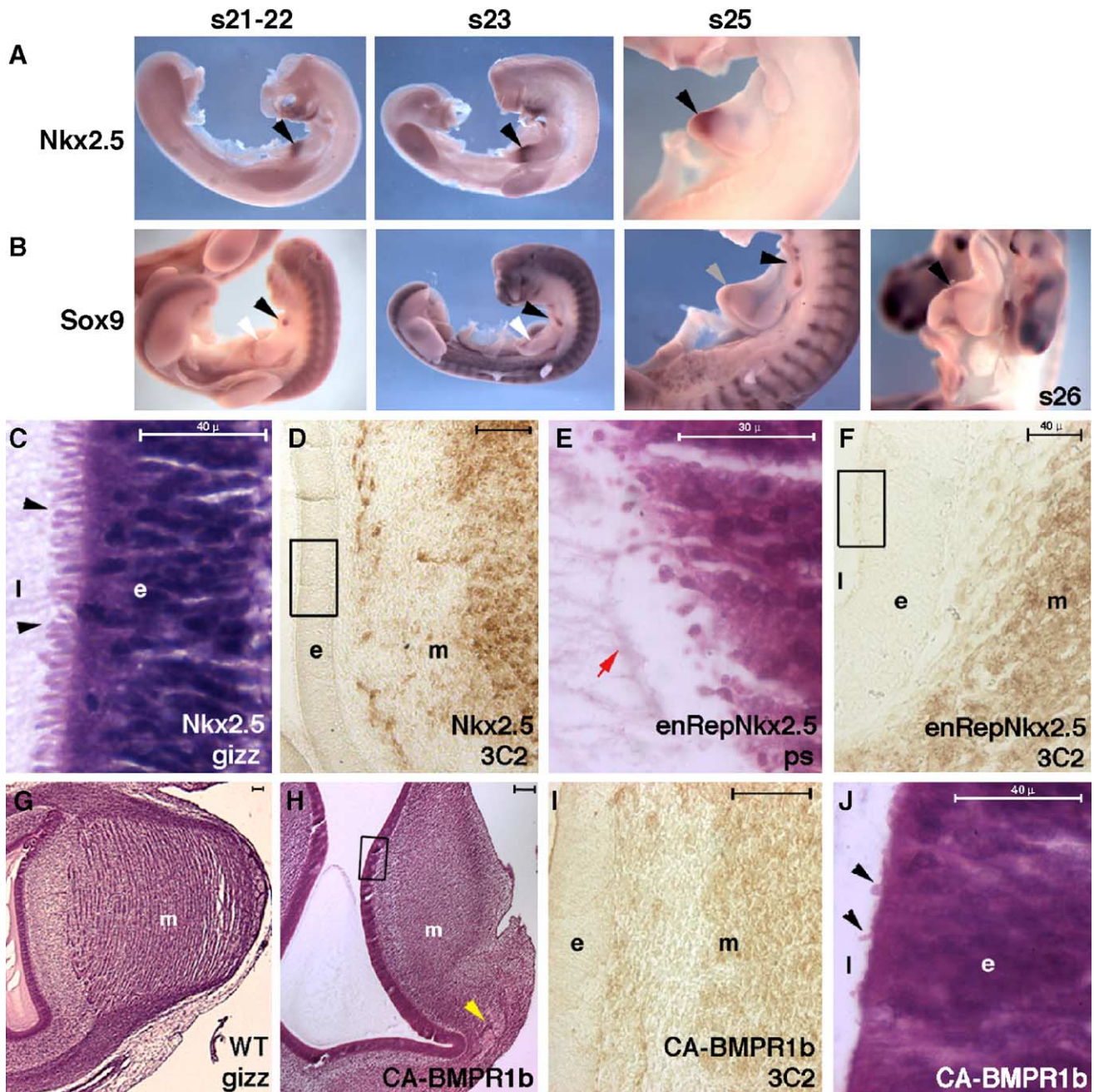


Fig. 4. Relationship of *Sox9* and *Nkx2.5* in regulating the pyloric epithelium. (A and B) Whole-mount expression profiles of *Nkx2.5* and *Sox9*, respectively, in developmental stage-matched embryos. Shade of the arrowheads in (A) and (B) correspond to relative levels of *Nkx2.5* and *Sox9* expression in the gut, respectively. (A) High levels of *Nkx2.5* expression are detectable in the posterior gizzard mesoderm at HH stage 21 (arrowhead) prior to closure of the gut tube at the midgut. *Nkx2.5* expression persists at high levels through the onset of *Sox9* expression in (B). (B) *Sox9* expression at early stages (HH stage 22) is not detectable in the gizzard (white arrowhead) although expression can be detected in the lung, a gut-derived tissue (black arrowhead). By HH stage 25 low levels of *Sox9* expression are detectable (grey arrowhead) and have increased by HH stage 26 (black arrowhead). (C–J) Sections through the gizzard and pyloric sphincter of *Nkx2.5* (C and D), *enRepNkx2.5* (E, F), wild type (G) and *CA-BMPR1b* (H–J) injected guts at HH stage 35. (C) Viral misexpression of *Nkx2.5* in the gizzard mesoderm (D) leads to transformation of the gizzard epithelium to a pyloric-like epithelium (boxed region in (D) magnified in (C)). (E and F) Misexpression of the dominant negative construct *enRepNkx2.5* in the gut mesoderm (F) does not block formation of bulbous microvilli in the pyloric sphincter epithelium (box in (F) magnified in (E)). Expression of *enRepNkx2.5* led to production and secretion of koilen in the pyloric sphincter lumen (arrows in (E) and (F)), normally found in the gizzard lumen. (H–J) Gizzard infected with virus expressing *CA-BMPR1b* has a smaller and less differentiated muscular layer than found in wild type (G). Infected gizzards also exhibit regions of cartilage deposits (arrowhead). (I) Region boxed in (H) demonstrating viral infection in the gizzard mesoderm but absent from the endoderm. (J) A close-up of the epithelium in (I) reveals a dearth of bulbous microvilli reminiscent of the microvilli found in the pyloric sphincter epithelium. Scale bars indicate 100 μm unless otherwise noted.

at HH stage 24 and are clearly visible by HH stage 26. The gizzard endoderm is transformed by ectopic expression of either *Sox9* or *Nkx2.5* to a phenotype characterized by microvilli typical of pyloric epithelium (Figs. 2C and 4C) (Smith and Tabin, 1999). Since both these factors induce a pyloric epithelial phenotype, and since both factors are themselves induced by BMP signaling, we expected that BMP activity would itself lead to a similar transformation. Indeed, following misexpression of constitutively active *BMPRIb*, the microvilli of infected gizzards adopted a more pyloric-like morphology most likely due to the upregulation of both *Sox9* and *Nkx2.5* expression (Fig. 4J, arrowheads). In addition, as was previously observed (Smith et al., 2000), we found a diminished region of smooth muscle differentiation as well as cartilage deposits within the gizzard mesoderm (Fig. 4H, arrow).

The correlation between the *Sox9* and *Nkx2.5* expression patterns, and the similarity of their misexpression phenotypes, suggested that *Sox9* and *Nkx2.5* might be part of a linear pathway downstream of BMP signaling, specifying the pyloric sphincter epithelium. To examine this possibility, the expression of each of these transcription factors was monitored after misexpression of the other in the gizzard. No change in *Nkx2.5* expression was observed with viral misexpression of *Sox9* in the gizzard mesoderm ( $n = 14$ ). Likewise, viral misexpression of *Nkx2.5* did not alter *Sox9* expression ( $n = 12$ ). Since *Nkx2.5* is expressed prior to *Sox9* (Figs. 4A and B), it was also possible that it might negatively regulate *Sox9* in its early phase. To test if *Nkx2.5* represses *Sox9* expression in the gizzard, we therefore misexpressed the dominant negative *enRep-Nkx2.5* retrovirus with still no effect on *Sox9* expression ( $n = 12$ ). Thus, there does not appear any regulatory interaction between *Nkx2.5* and *Sox9*. Misexpression of the dominant negative *enRep-Nkx2.5* retrovirus, while not having a phenotype in the gizzard or pyloric sphincter microvilli, did, as previously reported (Smith et al., 2000) result in the secretion of the keratin-like substance koilen from the pyloric epithelium into the lumen (Fig. 4E), suggesting that *Nkx2.5* alone is not necessary to specify the pyloric epithelium.

Along with *Sox9* and *Nkx2.5*, *Wnt11* is expressed in the posterior gizzard (Fig. 5C) (Smith et al., 2000; Theodosiou and Tabin, 2003). Additionally, *Fgf10* is initially expressed in a broad domain that becomes restricted to a discreet region in the posterior gizzard later in development (Figs. 5D, E). However, neither *Wnt11* misexpression ( $n = 14$ ) nor *Fgf10* misexpression ( $n = 9$ ) had any effect on expression of *Sox9* in the gut mesoderm (data not shown). Conversely, neither misexpression of *Sox9* ( $n = 12$ ) nor *Nkx2.5* ( $n = 18$ ) nor the co-misexpression of both of these transcription factors ( $n = 14$ ) had any effect on *Wnt11* expression (data not shown). Ectopic expression of *Wnt11* and *Fgf10* did result in phenotypes in the gizzard and lung, respectively, verifying that these viruses were active (data not shown). Viral misexpression of *Wnt11* leads to a loss of microvilli in

the gizzard epithelium. Misexpression of *Fgf10* results in ectopic contra-lateral branching of the early lung bud.

## Discussion

In an effort to identify new organ-specific markers during development of the gut tube, we discovered *Sox9* expression early in the posterior gizzard and ceca mesoderm as well as in the endoderm layer of the intestines (Fig. 1) (Lee and Saint-Jeannet, 2003; Lioubinski et al., 2003; Piper et al., 2002). *Sox9* expression in the gizzard corresponds with the expression of another marker, *Nkx2.5* at the pyloric sphincter (Figs. 4A, B). In this study, we examined the role of *Sox9* during development in the gizzard, its relationship to *Nkx2.5* and the BMP signaling pathway, and other signaling markers known to be expressed in the gizzard.

### *Sox9 is sufficient to determine the pyloric sphincter*

Viral misexpression studies revealed two roles for *Sox9* during gut development. First, misexpression in the gizzard mesoderm led to transformation of the gizzard endoderm to contain pyloric-like microvilli (Figs. 2A–D). This result demonstrated that *Sox9* is sufficient to determine the pyloric epithelium, and in addition requires a secondary secreted signal from the mesoderm to the endoderm. The identity of this second signal is unclear. Second, misexpression of *Sox9* in the mesoderm of the intestines led to an increase in cell number in the endoderm resulting in stenosis of the lumen (Figs. 2E–G). Thus, *Sox9* appears to have dual roles during gut development: to specify the pyloric epithelium and regulate epithelial cell numbers in the intestines.

The transcription factor *Nkx2.5* is also expressed in the pyloric sphincter during gut development, however, its expression appears earlier than *Sox9* (Figs. 4A, B). Furthermore, viral misexpression of *Nkx2.5* in the gizzard mesoderm leads to transformation of the gizzard endoderm to contain pyloric-like microvilli (Figs. 4C, D) (Smith et al., 2000). Misexpression of *Nkx2.5* in the developing gut does not appear to have any other phenotypes including proliferation of the intestine epithelium (data not shown), suggesting differences in the roles for *Nkx2.5* and *Sox9* during gut development. The correlation in both expression patterns and misexpression phenotypes suggested a possible inter-regulatory role for *Sox9* and *Nkx2.5* in specifying the pyloric sphincter epithelium.

### *BMP signaling regulates Sox9 and Nkx2.5 markers in the pyloric sphincter*

Studies conducted in cartilage have demonstrated that *Sox9* expression is regulated by BMP signaling during limb development (Healy et al., 1999; Semba et al., 2000; Zehentner et al., 1999). We found that as with cartilage

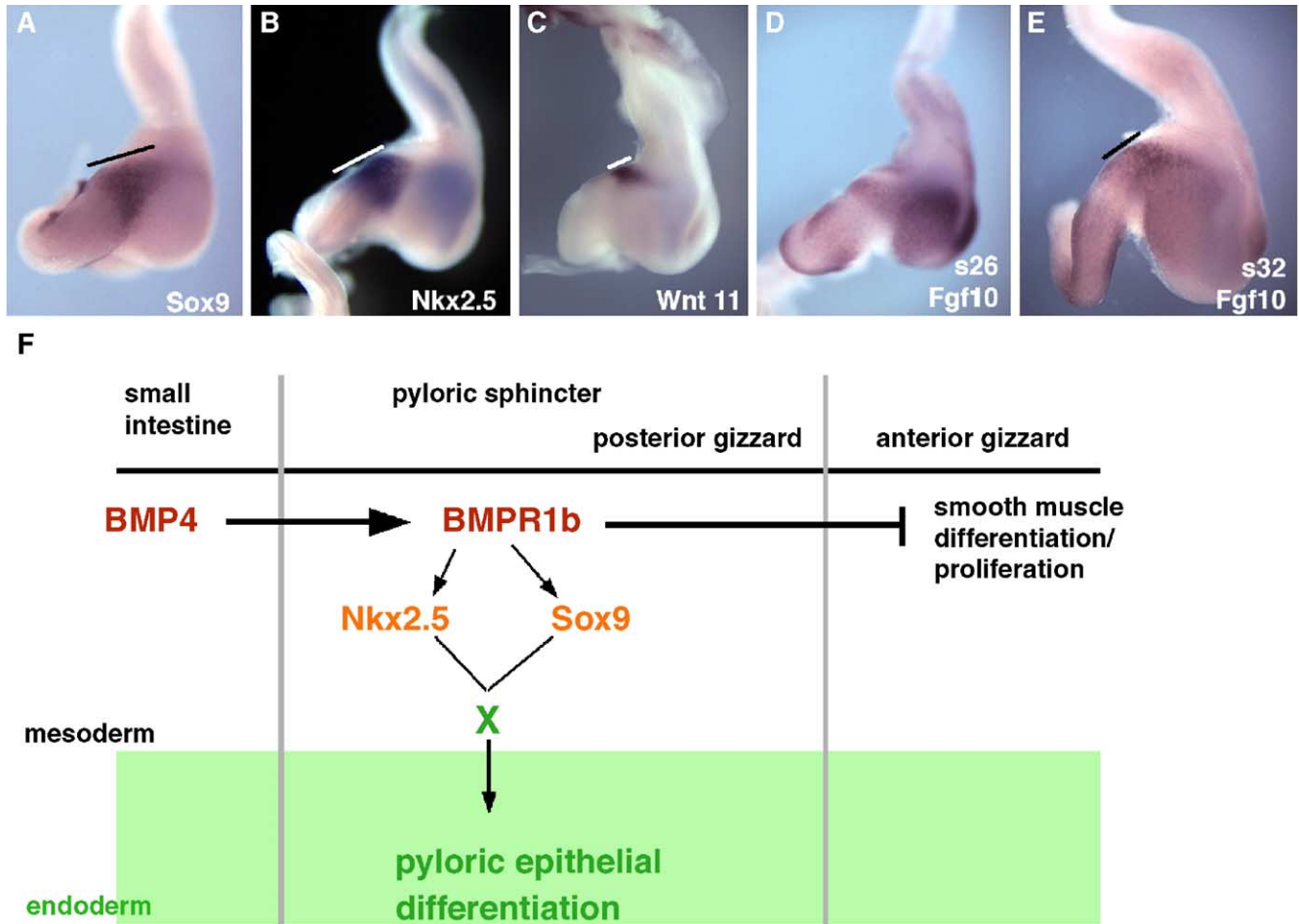


Fig. 5. Relative expression patterns of genes expressed in the posterior gizzard. (A–D) Whole mount in situ hybridization with probes at HH stage 26 of development. Probes used included *Sox9* (A), *Nkx2.5* (B), *Wnt11* (C), *Fgf10* (D). (D) *Fgf10* is expressed broadly in the gizzard and small intestine at HH stage 26. (E) By HH stage 32, expression of *Fgf10* is restricted to the posterior gizzard. Bars highlight domains of expression in the posterior gizzard. (F) Model illustrating that BMP4 in the small intestine mesoderm signals through its receptor BMPR1b in the adjacent pyloric and gizzard mesoderm to inhibit smooth muscle differentiation and proliferation. In the pyloric sphincter, signaling through BMPR1b leads to induction of *Nkx2.5* and *Sox9* expression. The activity of the two transcription factors is coordinated to possibly induce expression of a downstream, secreted signal that then instructs the epithelium to take on a pyloric morphology.

development, *Sox9* expression in the posterior gizzard also appeared to be regulated by BMP signaling. Activation of BMP signaling by misexpression of the constitutively active form of the receptor *BMPR1b* led to an increase in the domain of *Sox9* expression in the gizzard (compare Figs. 3A and B). Furthermore, *Sox9* expression was down-regulated by the misexpression of the BMP antagonist *noggin* (Fig. 3C). Thus, as seen with cartilage, *Sox9* expression in the pyloric sphincter appears to be regulated by BMP signaling (Healy et al., 1999).

Along with BMPs, FGFs have also been implicated in regulating *Sox9* expression (Murakami et al., 2000). In addition, Wnt-signaling has been found to interfere with Sox transcription factors (Takash et al., 2001; Zorn et al., 1999). Both *Fgf10* and *Wnt11* are expressed in regions proximal to *Sox9* expression (Figs. 5A, C, E). In misexpression studies, we found no effect of *Fgf10* or *Wnt11* on *Sox9* expression, confirming that regulation of *Sox9* expression by BMP signaling is specific.

The regulation of *Sox9* expression by BMP signaling is reminiscent of previous work on *Nkx2.5* in the sphincter (Smith and Tabin, 1999; Smith et al., 2000). Like *Sox9*, *Nkx2.5* expression in the posterior gizzard is upregulated by misexpression of *CA-BMPR1b*, and down-regulated by misexpression of *noggin* (Fig. 3). In addition, we show that ectopic expression of *CA-BMPR1b* in the gizzard mesoderm leads to a decrease in the number of microvilli as well as transformation of the microvilli to a more pyloric-like morphology (Fig. 4J). Thus, the expression of both pyloric sphincter markers is regulated by BMP signaling.

*Sox9* and *Nkx2.5* act coordinately to specify the pyloric sphincter

The apparent parallels between *Sox9* and *Nkx2.5* in the posterior gizzard during gut development are striking; however, subtleties in their regulation suggest independent roles in determining the pyloric sphincter. *Nkx2.5* expres-

sion is induced earlier than *Sox9* expression in the posterior gizzard. Moreover, misexpression of *Nkx2.5* did not lead to alteration of *Sox9* expression in the gizzard and vice versa. Despite the fact that the expression patterns of *Nkx2.5* and *Sox9* are both regulated by BMP signaling, these results suggest that there is no regulatory interaction between *Nkx2.5* and *Sox9*.

Viral misexpression of *Sox9* leads to transformation of the gizzard epithelium to pyloric-like bulbous microvilli as observed with *Nkx2.5* (Figs. 2A–D and Figs. 4C, D). Concurrent viral misexpression of *Nkx2.5* and *Sox9* in the gizzard mesoderm exhibited the same phenotype as misexpression of *Nkx2.5* or *Sox9* alone. Thus, either transcription factor on its own appears to be sufficient to specify the epithelial phenotype. Interestingly, a dominant-negative form of *Nkx2.5* does not disrupt the epithelial morphology of the pyloric sphincter, although it does lead to the inappropriate expression of a keratin-like material called koilen, normally produced by the non-sphincter epithelium (Figs. 4E, F) (Smith et al., 2000). Thus, it was concluded that *Nkx2.5* expression was sufficient but not necessary for formation of sphincter specific microvilli. The data presented here may explain that finding, as *Nkx2.5* and *Sox9* appear to be coexpressed, are each capable of inducing pyloric-specific epithelial morphology, and hence play at least partially redundant functions in formation of the pyloric sphincter (Fig. 5F).

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