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SOX9 specifies the pyloric sphincter epithelium through mesenchymal-epithelial signals

Brigitte Moniot^{1,*}, Sandrine Biau^{1,*}, Sandrine Faure², Corinne M. Nielsen³, Philippe Berta¹, Drucilla J. Roberts³, and Pascal de Santa Barbara^{1,‡}

¹Institut de Génétique Humaine, UPR 1142 CNRS, 141 rue de la Cardonille, 34396 Montpellier, Cedex 5, FRANCE

²Centre de Recherche en Biologie Macromoléculaire, CNRS FRE 2593, 1919 Route de Mende, 34293 Montpellier, Cedex 5, FRANCE

³Department of Pathology, Massachusetts General Hospital, Harvard Medical School, Fruit Street, Boston, MA 02114, USA

*These authors contributed equally to this work

‡Author for correspondence (e-mail: desanta@igh.cnrs.fr)

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SUMMARY

Gastrointestinal (GI) development is highly conserved across the vertebrates. Although several transcription factors and morphogenic proteins are involved in the molecular controls of GI development, the interplay between these factors is not fully understood. We report herein the expression pattern of *Sox9* during GI development and provide evidence that it functions in part to define the pyloric sphincter epithelium. SOX9 is expressed in the endoderm of the GI tract (with the exclusion of the stomach) and its derivate organs, lung and pancreas. Moreover, SOX9 is also expressed in the mesoderm of the pyloric sphincter, structure that demarcates the stomach from the duodenum. Using viral misexpression technique, we show that *Sox9* expression in the pyloric sphincter is under the control of the BMP signaling pathway, which plays key role in the development of this structure. By misexpressing *SOX9* in the mesenchymal stomach, we show that SOX9 is able to transdifferentiate the adjacent gizzard epithelium into pyloric sphincter-like epithelium through the control of mesodermal-epithelial signals mediated in part by *Gremlin*, a modulator of the BMP pathway. Our results suggest that SOX9 acts in the regionalization of the foregut/midgut boundary and is necessary and sufficient to specify the pyloric sphincter epithelial properties.

Key words: SOX; SOX9; BMP; Gremlin; stomach; pyloric sphincter; differentiation; chick.

INTRODUCTION

The vertebrate gastrointestinal (GI) tract is a remarkably complex, three dimensional, specialized and vital organ system derived from a simple tubular structure. The GI tract includes the luminal digestive system of the esophagus, stomach, intestines, and colon (which we will refer as gut) and the derivate organs, thyroid, lungs, liver, and pancreas (Roberts et al., 2000). The gut is composed of the three germ layers, endoderm (which forms the epithelial layer), mesoderm (which forms the smooth muscle layers and myofibroblast), and ectoderm (which includes the enteric nervous system, and most anterior and posterior luminal epithelia). Originally, the gut develops from two invaginations at the anterior (anterior intestinal portal, AIP) and posterior (caudal intestinal portal, CIP) ends of the embryo, which elongate and fuse to form a straight tube. During development the gut becomes patterned along the anterior-posterior (AP), the dorsoventral (DV), the left-right (LR), and the radial (cross-sectional, crypt-villous) axis (de Santa Barbara et al., 2002a). Specific regional differentiation along AP axis will give rise to the foregut (pharynx, esophagus, and stomach), midgut (small intestines), and hindgut (colon, and rectum). During adult life, the gut epithelium is constantly regenerating and its epithelial pattern must be maintained throughout life (de Santa Barbara et al., 2003). In addition, the gut needs reciprocal signals between the mesoderm and endoderm during the patterning process during development (Roberts et al., 1998) up to adulthood for epithelial regeneration (Clatworthy and Subramanian, 2001).

Over the past years, several molecular factors involved during GI tract development have been identified (for review, see (Roberts, 2000; de Santa Barbara et al., 2002a)). We and others have shown that *Hox* genes play important and active roles in patterning the gut along AP axis and controls normal gut epithelial differentiation (Roberts et al., 1998; Zakany and Duboule, 1999; Aubin et al., 2002; de Santa Barbara and Roberts, 2002b). Morphogenic factors also play key roles during gut development and differentiation. Endodermal *Shh*

expression was described to regulate morphogenesis and mesenchymal differentiation through the induction of *Bmp4* in the adjacent mesoderm (Roberts et al., 1995; Sukegawa et al., 2000). Ramalho-Santos and colleagues showed more generally that *Hedgehog* genes play important roles during GI organogenesis, enteric nervous system (ENS) development, epithelial proliferation and differentiation (Ramalho-Santos et al., 2000). *Bmp4*, which is expressed in the gut mesoderm, is involved in controlling growth and differentiation of the GI musculature (Roberts et al., 1998; Smith et al., 2000a; Nielsen et al., 2001). The importance of all these factors in gut patterning is highlighted by their remarkable conservation across vertebrate species (Smith et al., 2000b).

SOX genes, encoding high-mobility group (HMG) domain-containing transcription factors, have been identified as key players in numerous developmental processes including sex determination, neurogenesis, muscle differentiation, chondrogenesis, and endoderm specification (Wegner, 1999). Recently, *Sox17* was shown to be necessary for the gut endoderm development (Kanai-Azuma et al., 2002). Among them, *SOX9* was initially identified as the gene responsible for campomelic dysplasia (CD) syndrome, an autosomal dominant disease characterized by skeletal malformations associated with sex reversal (Cameron and Sinclair, 1997). Studies on *SOX9* mainly focused on its role in skeletal and gonadal development. However, CD patients often display abnormalities in visceral organs and brain, suggesting a role of *SOX9* in some aspects of the GI and central nervous system development. Indeed, dysmorphogenesis have been described in CD affected individuals in their GI tract, tracheopulmonary system, urinary tract, and heart (Maroteaux et al., 1971; Houston et al., 1983). GI tract anomalies are characterized by megacolon and intestinal malrotation (Houston et al., 1983). Due to the high mortality, only few studies have addressed GI diseases or malformations in CD patients (Piper et al., 2002).

In this study, we have investigated the expression and function of SOX9 in visceral pattern formation using the chick embryo as a model system. We found that SOX9 was expressed throughout the gut endoderm sparing the gizzard during both avian and human GI development. Mesodermal expression is mainly restricted to the pyloric sphincter mesoderm. We provide evidence that *Sox9* expression in the pyloric sphincter structure is under control of the BMP signaling pathway. Ectopic expression of *SOX9* through retroviral misexpression technique in the stomach mesoderm induces the transdifferentiation of the adjacent gizzard epithelium into a pyloric sphincter-like epithelium, whereas *SOX9* loss-of-function expression in the pyloric mesoderm affects the differentiation of the pyloric epithelium. Taken together, our results show that SOX9 patterns the foregut/midgut boundary and is necessary and sufficient to induce the differentiation of the pyloric epithelium.

MATERIALS AND METHODS

Chick and Human embryos

Timed fertilized white Leghorn eggs (Haas Farm, France) were incubated at 38°C in a humidified incubator (Coudelou, France) until used experimentally. Staged-embryos (Hamburger and Hamilton, 1951) were harvested, washed in fresh PBS and then fixed in freshly made 4% paraformaldehyde in PBS for 2 hours. Fixed-embryos were washed in PBS and processed further either through a graded series of methanol-PBS to 100% methanol and kept at -20°C until used for whole-mount *in situ* hybridization studies, or frozen in cryomount (Fisher Scientific) for cryosectioning and immunostaining. Human embryonic tissues were obtained from surgical abortions as part of a program approved by both ethics committees from CNRS and French National Ethic Committee. Embryos were staged according to the Carnegie stages (O’Rahilly, 1983).

Immunohistochemistry and in situ hybridization on gastrointestinal system

Immunohistochemical stainings were performed on cryostat sections mainly as previously described (de Santa Barbara and Roberts, 2002b) using standard techniques and the Vectastain ABC detection system (Vector Laboratories, Inc, CA) following the manufacturer's directions. Used anti-SOX9 (α SOX9) antibodies were raised against the transactivation (TA) domain of human SOX9 protein and used for immunohistochemistry analyses diluted 1:100 in TBST (de Santa Barbara et al., 1998; de Santa Barbara et al., 2000). A full characterization in chick both by Western blot and immunohistochemistry is available in Supplemental data. Anti-avian retroviral GAG protein (α 3C2) antibodies were used as previously described (de Santa Barbara and Roberts, 2002b). Anti-HNK-1 (α HNK-1) antibodies were purchased from NeoMarkers and used diluted 1:400. These antibodies specifically recognize migrating neural crest-derived cells.

Antisense RNA probes were previously described for *cBmp4* (Nielsen et al., 2001), *cGremlin* (Capdevila et al., 1999), *cNkx2.5*, *cShh* (Smith et al., 2000a), *cSox8* (Bell et al., 2000), *cSox9* (Healy et al., 1999), *cSox10* (Cheng et al., 2000), *cWnt11* (Theodosiou and Tabin, 2003), *cPdx1* and *cSox2* (Grapin-Botton et al., 2001). DIG labeled riboprobes were made following manufacturer's instructions (Roche). Whole-mount *in situ* hybridization experiments were performed using a standard protocol (Roberts et al., 1998). Cryosections at 10 μ m onto Superfrost Plus slides (Fisher Scientific) were air dried for 4-18 hours and kept at -20°C until used. Fixed and stained embryos were embedded in paraffin and sectioned at 8 μ m for histologic analysis. Haematoxylin and eosin staining was performed using standard techniques. *In situ* hybridization and immunohistochemistry on paraffin sections were performed as previously described (de Santa Barbara and Roberts, 2002b).

Constructs and viral infection in the chick gastrointestinal tract

The viral constructs that we used were previously described, including vectors transducing *Bmp4* (Roberts et al., 1998), *Noggin* (Smith and Tabin, 1999), *Nkx2.5* (Smith et al., 2000a), *Gremlin* (Capdevila et al., 1999) and *GFP* (de Santa Barbara and Roberts, 2002b).

New constructs were produced and characterized in this study (see Supplemental data). The full-length and C-terminal deleted (Δ Cter) human *SOX9* cDNAs were cloned into the shuttle vector Slax13 and then subcloned into RCAS(A) vector. Full-length and Δ Cter *SOX9* RCAS vectors were transfected into chick embryonic fibroblasts, and virus harvested and tittered using standard techniques (Morgan and Fekete, 1996). Only high infectious viruses were used in the experiments described. In order to target the presumptive stomach mesoderm, misexpression experiments were performed on stage 10 embryos according to the published fate map (Matsushita, 1995). Approximately 1-5 μ l of freshly thawed virus, dyed with 1% fast green, were injected per embryo. Eggs were then placed at 38°C until harvested.

Photography

Images were collected in whole-mount under a Nikon SMZ1000 scope and in section under Zeiss Axiophot microscope, both using Nikon DXM1200 camera.

RESULTS

Spatial and temporal expression pattern of SOX9 in the developing GI system

To examine the expression pattern of *Sox9* during chick GI development, we first performed whole-mount *in situ* hybridization on 5 days old (E5) dissected gut (stage 26-27). At this stage, *Sox9* expression was detected at different sites of the GI system, in the stomach, the hindgut and derivate organs such as lung, and pancreas (Fig. 1A).

To define more in details this complex expression pattern, we took advantage of anti-SOX9 antibodies that we previously developed and used on human and mouse embryo tissues (de Santa Barbara et al., 1998; de Santa Barbara et al., 2000; Gasca et al., 2002). We first confirmed the specificity of these antibodies on chick embryo extracts and tissues (see Supplementary data) and then examined the expression pattern of SOX9 during GI development. At E5, SOX9 expression was detected in different epithelia of the gut, at high levels in the esophagus (Fig. 1B1), hindgut (Fig. 1F1) and cloaca (data not shown), and at lower levels in the midgut epithelium where its expression is detected in isolated positive epithelial cells (insert, Fig. 1D1). In addition, SOX9 expression was strong in derivate organ epithelia: lung (Fig. 1B1), pancreas, and liver (data not shown). We also found discrete areas of SOX9 expression in the lung mesoderm that we were unable to detect by *in situ* hybridization (red arrow, Fig. 1B1). At E5, the development of the long bilateral caeca has just begun and SOX9 protein was detected in the endoderm, but also in the mesoderm of the caecal tips (red arrows, Fig. 1E1) and in the mesenteric structures (black arrow, Fig. 1E1). In addition, SOX9 expression was detected in the mesoderm of the posterior region of the stomach (Fig. 1A,C1). Since ENS cells colonize the mesodermal layer to assure gut innervation and at this stage of development are part of this layer, we analyzed whether or not mesodermal SOX9 expression co-localized with ENS cells by immunohistochemical analyses using anti-HNK-1 antibodies (Fig. 1B2-F2). The anti-HNK-1 antibodies specifically recognize migrating neural crest-derived cells. At E5, ENS cells are migrating along AP axis and are not yet organized into plexi (Fig. 1D2). SOX9 stainings detected in the lung, stomach and caeca mesoderm did not co-localize with HNK-1 (compare respectively Fig. 1B1,C1,E1 with B2,C2,E2). These data demonstrate that SOX9 is expressed at different level of the GI system, in the endoderm and in the mesoderm and that SOX9 expressing mesodermal cells are not ENS cells.

The function of SOX9 was recently shown to be dependent and regulated by its nuclear-cytoplasmic translocation in the developing gonad (de Santa Barbara et al., 2000; Gasca et al., 2002). Only nuclear staining of SOX9 protein was observed in the developing GI tract (Fig. 1B1-F1), whereas we observed SOX9 cytoplasmic expression in the developing chick gonad before sexual differentiation, suggesting that SOX9 regulation might be context-dependent (data not shown).

At E9 (stage 35), *Sox9* expression was strongly detected in GI derivate organs (such as lung, pancreas and liver), in the hindgut and in the midgut (Fig. 2A). At this stage, SOX9 expression in the esophageal epithelium is high at the base of the villi and lower in the apex (Fig. 2B1). Strong nuclear expression of SOX9 protein was detected in the midgut and caeca epithelia (Fig. 2D1,E1), as well as in the hindgut and the cloaca epithelia (Fig. 2F1,G1) and the Fabricius bursa epithelium (data not shown). Mesodermal expression of SOX9 was observed at the caecal tips (Fig. 2E1). In chick, the stomach consists of two portions, the proventriculus (avian glandular stomach) and the gizzard (avian muscular stomach), which are distinct both morphologically and physiologically (Romanoff, 1960). In addition, the connection between the gizzard and the duodenum is demarcated by the pyloric sphincter. This structure is composed of mesodermal restriction that allows to maintaining food in the stomach and later to flowing gastric contents into the duodenum lumen. *Sox9* expression was also detected in the stomach in a ring form demarcating the boundary between the stomach and the duodenum (Fig. 2A). SOX9 protein is present in the pyloric sphincter mesenchyme (Fig. 2C1). We also noticed that no co-localization between SOX9 and HNK-1 positives cells was observed (compare respectively Fig. 2C1 with C2).

In order to determine whether SOX9 expression in the GI system is conserved in human, we examined the expression of SOX9 protein in human embryonic tissues. At 7.5 weeks gestational age, SOX9 expression was similar to that seen in the chick. Epithelial SOX9

expression was detected in the lung, pancreas, small intestine, and rectum (respectively, Fig. 3A,B,D,E) and mesodermal SOX9 expression in the posterior region of the stomach at the pyloric area (red arrow, Fig. 3C). Our results also indicate that SOX9 expression changes during the differentiation state of the small intestine epithelium; its expression is restricted at the proliferative compartment of the villi, suggesting potential function for SOX9 during the epithelial differentiation process (red arrow, Fig. 3F).

Taken together, our data indicate that SOX9 exhibits a restricted expression pattern in the GI system. SOX9 is expressed in different GI epithelia and in the mesoderm of the pyloric sphincter in vertebrates.

E subgroup *Sox* gene expression in the chick stomach

SOX proteins constitute a large family of transcription factors characterized by the presence of a HMG domain. Sequence homology outside of HMG domains allowed distinguishing 8 different groups (Schepers et al., 2002). *Sox9* belongs to the E subgroup and shares strong homology with *Sox8* and *Sox10*, the other members of this subgroup. In addition, overlapping functions and expressions of these E subgroup genes have been previously described (Montero et al., 2002; Schmidt et al., 2003).

In order to determine whether stomachal SOX9 expression is a common feature of all E subgroup *Sox* members or whether this expression pattern is specific to SOX9, we performed *in situ* hybridization with *Sox8*, *Sox9* and *Sox10* riboprobes on chick E7 stomach. Expression of all 3 genes was detected in the stomach, with specific expression patterns (Fig. 4A-C). *Sox8* expression was detected in the pancreas and weak expression was also observed in ENS cells (Fig. 4B). *Sox10* expression was restricted to ENS cells (Fig. 4C) and *Sox9* expression was exclusively observed in the pyloric sphincter mesoderm (Fig. 4A). All together, our

results demonstrate that SOX9 is the only E subgroup SOX member expressed in the pyloric sphincter mesoderm in vertebrates.

BMP signaling regulates *Sox9* expression in the stomach

Previous studies showed that BMP signaling pathway is necessary for normal stomach development (Smith et al., 1999). *Bmp4* is expressed in the mesenchyme of the whole gut with the exception of the gizzard (Fig. 4D). *Bmp4* regulates both muscular thickness and pyloric sphincter specification (Roberts et al., 1998; Smith et al., 1999). *Bmp4* function is mediated in part by the homeobox-containing gene *Nkx2.5*, which is expressed in the pyloric mesoderm (Fig. 4E) and is necessary and sufficient to control epithelial pyloric differentiation through mesenchymal-epithelial interactions (Smith et al., 2000a).

Then overlapping expression patterns in the stomach suggest a potential connection between BMP signaling pathway and SOX9. We thus investigated whether BMP pathway could regulate *Sox9* expression. We used the avian retroviral system to specifically misexpress *Bmp4*, *Nkx2.5*, and the BMP-antagonist *Noggin* in the stomachal mesoderm (Roberts et al., 1998; Nielsen et al., 2001) and monitored *Sox9* expression by *in situ* hybridization experiments (Fig. 5). *GFP* misexpression was used as control. Retrovirus presence in the analyzed stomach was confirmed by immunohistochemistry using antibodies directed against the avian retroviral GAG protein (α 3C2) (data not shown). As previously reported, *Bmp4* misexpression in the stomach induced a gross phenotype characterized by a small gizzard with thin musculature (Roberts et al., 1998), whereas the morphology of the pyloric sphincter was normal (Fig. 5B). However, we observed a broad domain of *Sox9* expression that extends from the pyloric sphincter to the right part of the gizzard (double red arrows, Fig. 5B), suggesting that activation of BMP signaling pathway in the stomachal mesoderm upregulates *Sox9* expression. Examination of cryostat sections also indicated that

not all stomachal cells are expressing SOX9 in response to *Bmp4* misexpression, suggesting that only few competent cells are able to respond to BMP4 activation (red arrowheads, Figs. 5F). In similar experiments, Smith and Tabin (1999) reported an extension of the expression domain of *Nkx2.5* in the stomach in response to *Bmp4* misexpression. As previously described (Smith and Tabin, 1999), we observed that *Nkx2.5* misexpression in the stomachal region did not affect the morphology of the stomach (Fig. 5C) and whole-mount *in situ* hybridization analyses revealed that *Sox9* expression pattern was not affected in *Nkx2.5*-misexpressing stomach (Fig. 5C). Misexpression of *Noggin*, a specific secreted antagonist of the BMP signaling pathway, in the stomach induced the predicted phenotype of muscular hypertrophy (compare Fig. 5D,E with B). E8 *Noggin*-misexpressing stomachs develop a range of phenotypes from moderate to severe (Fig. 5D,E). Moderate phenotype was associated with a mild foregut/midgut boundary perturbation associated with down-regulation of *Sox9* expression (compare Fig. 5D with A). Severe phenotype was marked by an increase of the muscular mass associated with pyloric morphologic defect, and correlated with the inhibition of *Sox9* expression in the malformed pyloric structure (compare, Fig. 5E with A).

Taken together, these results demonstrate that the BMP pathway regulates *Sox9* expression in the pyloric sphincter.

SOX9 is necessary and sufficient to specify the pyloric sphincter epithelium

In order to investigate more directly the role of SOX9 in pyloric sphincter development, we used the avian retroviral system to specifically misexpress full-length *SOX9*. Anterior misexpression of *SOX9* into the gizzard mesoderm did not lead to morphological change, but histological analyses demonstrated an effect of SOX9 on gizzard epithelium (Fig. 6C). In addition to morphological and mesodermal differences, the gizzard and the pyloric sphincter present different epithelial specificities. Indeed the gizzard epithelium harbors cells with

keratin long cilia allowing resistance to the abrasive grinding (Fig. 6A), and the pyloric sphincter presents epithelial cells with bulbous cilia (Fig. 6B). *SOX9* misexpression in the mesoderm of the gizzard modified the adjacent epithelium properties, as this epithelium had pyloric sphincter-like features showing bleb like cilia at the epithelial level (compare Fig. 6C with A). This epithelial transformation was not due to the expression of ectopic *SOX9* in the gizzard epithelium, since $\alpha 3C2$ detection demonstrated that retroviral infection was restricted to the gizzard mesodermal (insert, Fig. 6C). In order to characterize the epithelial transformation observed upon *SOX9* misexpression, we analyzed by *in situ* hybridization the expression of different endodermal markers (such as *Shh*, *Sox2*, *Gata4* and *Pdx1*). *Shh* is a general marker of the gut endoderm (Roberts et al., 1995), *Sox2* and *Gata4* are expressed in the stomach endoderm (Ishii et al., 1998, unpublished data). *Pdx1* is a marker of the duodenum epithelium, the pancreas epithelium and the pyloric gland, but *Pdx1* expression is not present in the stomach epithelium (Grapin-Botton and Melton, 2000; Fig. 6E). PDX1 expression is also associated to the abnormal presence of pseudopyloric glands in human patient stomach (Sakai et al., 2004). *SOX9* misexpression in the stomach did not modify the expression of *Shh*, *Sox2* and *Gata4* markers (data not shown). However, in *SOX9* misexpressing stomach, we observed an ectopic expression of *Pdx1* in the gizzard epithelium, indicating that the transformed gizzard epithelium exhibited characteristics of pyloric epithelium (red arrowheads, Fig. 6F).

We also used the avian retroviral system to specifically a mutant form of *SOX9* deleted of this C-terminal domain (*SOX9* Δ Cter). This deleted mutant form is the one found in human campomelic patients (Sudbeck et al., 1996). We and others showed previously that this mutated form was present in the nuclear compartment of the cell, able to bind DNA, yet unable to activate transcription and finally able to form heterodimer complex with endogenous *SOX9* protein (de Santa Barbara et al., 1998; Bernard et al., 2003; data not

shown). This suggests that this deleted form might behave as a dominant negative of SOX9 action. Misexpression of SOX9 Δ Cter in the mesodermal stomach showed no phenotypic modification of mesoderm, but clear perturbation of the pyloric epithelium (Fig. 6D). SOX9 Δ Cter misexpressing stomach showed gizzard-like features with keratin long cilia at the pyloric epithelial level (compare Fig. 6D with B). In addition and by contrast with the full-length SOX9 misexpression, no obvious epithelial phenotype was present in the gizzard epithelium, suggesting that the transactivation domain of SOX9 is required to transdifferentiate the stomachal epithelium. SOX9 Δ Cter misexpression in the stomach did not modify the expression of *Shh*, *Sox2* and *Gata4* markers (data not shown). However, in SOX9 Δ Cter misexpressing stomach, we observed a decrease of epithelial *Pdx1* expression in the infected pyloric sphincter, indicating that the transformed pyloric epithelium lost pyloric epithelium features (Fig. 6G).

All together, our data show that the transcription factor SOX9 is necessary and sufficient to specify the pyloric sphincter epithelium through mesenchymal-epithelial signals.

***Gremlin* mediated SOX9 mesenchymal-epithelial function in the pyloric sphincter**

Our results show that SOX9, which is expressed in the mesoderm of the pyloric sphincter, specifies the adjacent epithelium. As SOX9 is a transcription factor, we hypothesized that it may regulate the expression of diffusible ligands, such as Wnts, BMPs or their related inhibitors, which were described to be essential to establish pyloric epithelium phenotype through mesenchymal-epithelial interaction (Theodosiou and Tabin, 2003; Smith et al., 2000a). *Wnt11* expression, which is restricted to the mesenchymal pyloric sphincter, was not affected in SOX9 and SOX9 Δ Cter misexpressing stomach (data not shown). We observed that *Gremlin*, a modulator of the BMP pathway, was expressed in the mesenchymal pyloric sphincter (Fig. 7A,C). Anterior misexpression of *Gremlin* in the mesenchymal stomach

induced no obvious morphological phenotype, but, as *SOX9*, an epithelial phenotype characterized by the presence of pyloric bleb like cells in the gizzard epithelium (compare Figs. 7B with 6A). These data suggested that *Gremlin* might be a potential candidate for a downstream gene regulated by *SOX9* in the pyloric sphincter. To test this hypothesis, *SOX9* and *SOX9* Δ Cter were misexpressed in the stomach, and *Gremlin* expression was monitored by *in situ* hybridization (Fig 7C,D,E). We observed *Gremlin* ectopic expression in the mesenchymal gizzard with full-length *SOX9* (red arrowheads, Fig. 7D), which was not detected in *SOX9* Δ Cter misexpressing stomach (Fig. 7E). Nevertheless, we observed a decrease of mesodermal *Gremlin* expression in the pyloric structure upon *SOX9* Δ Cter misexpression (black arrowheads, Fig. 7E). Finally, we found that *Sox9* expression was not affected in *Gremlin* misexpressing stomach (data not shown).

All these data point to a function of *SOX9* in the stomach in specifying the pyloric sphincter epithelium through its expression in the adjacent mesenchyme, and suggest that *SOX9* might function in part by modulating *Gremlin* expression.

DISCUSSION

***SOX9* expression in the gastrointestinal system is conserved during evolution**

In early studies, *SOX9* expression has been mainly observed chondrocytes, neural crest cells and genital ridges (Healy et al., 1999; Spokony et al., 2002; de Santa Barbara et al., 2000). Here, we found that *SOX9* was specifically expressed in some restricted areas during GI tract development in chick (summarized in Table 1). *SOX9* is present throughout the gut endoderm with the exception of the gizzard endoderm (Fig. 1). We also observed expression of *SOX9* in the endoderm of organs derived from the gut tube, the pancreas, liver, and the lung (Figs. 1,2). *SOX9* expression is present in the gut endoderm from early stage of development and is maintained until adulthood (data not shown). Since *SOX9* is later expressed in the

proliferative compartment of the villi (Fig. 3), we might hypothesize a role for SOX9 in stem cell maintenance. In our study, we also observed mesodermal expression of SOX9 in the pyloric sphincter structure (Figs. 1,2). The pyloric sphincter is an anatomical sphincter that allows food to be grinded in the stomach before flowing in the small intestine. SOX9 strongly demarcates the nascent boundary between foregut/midgut (Fig. 1) and its early expression pattern let us hypothesize a function of SOX9 in the establishment of this boundary (Fig. 2). We also demonstrated that SOX9 expression in the pyloric sphincter is a specific feature of SOX9 and not a common propriety of the E subgroup SOX factors (Fig. 4). This pattern of SOX9 expression in chick GI system is very similar in human and mouse (compare Figs. 1,2 with 3, and data not shown). In addition, *Sox100B*, the *Drosophila* gene related to vertebrate *SOX9*, is expressed in the early hindgut, late midgut endodermal cells, and the anal plates (Hui Yong Loh and Russell, 2000). Thus, SOX9 expression pattern in the developing gut was conserved during evolution, suggesting that SOX9 might exhibit similar functions in this tissue in vertebrates and invertebrates.

SOX9 specifies the pyloric sphincter epithelium

These last years, chick embryo has been widely used as a model to study the function of transcription factors mainly expressed in the mesoderm layer during GI tract development (Roberts et al., 1998; Nielsen et al., 2001). In order to investigate the function of *SOX9* during the formation and the development of the pyloric sphincter, we decided to use the specific avian virus mediated gene expression technique which we previously showed to be useful in targeting different part of the GI tract and expressing a transgene into the stomach mesoderm (Smith et al., 2000b; Nielsen et al., 2001). We showed that mesodermal gizzard *SOX9* misexpression was sufficient to induce the transformation of the gizzard epithelium into pyloric sphincter like epithelium (Fig. 6). When we used virus expressing a mutant form of

SOX9, *SOX9* deleted of the C-terminal domain (*SOX9*ΔCter), we observed perturbations in the differentiation of the pyloric epithelium and gizzard like epithelium phenotype (Fig. 6). All these epithelial changes were associated with perturbations of an epithelial pyloric *Pdx1* marker expression, strongly suggesting that *SOX9* specifies the pyloric sphincter epithelium (Fig. 6). Furthermore, we proved that this effect was mediated by an indirect mechanism using transcriptional regulation of diffusible factors expressed specifically into the pyloric sphincter mesoderm, acting by diffusion to target the pyloric sphincter epithelium (see below).

Relationship between *SOX9* and BMP signaling pathway during the specification of the pyloric sphincter epithelium

Establishment and differentiation of the foregut/midgut boundary involved a highly molecular regulated process as *Bmp4* (Smith and Tabin, 1999) and *Nkx2.5* (Smith and Tabin, 1999; Smith et al., 2000a). *Bmp4* activates *Nkx2.5* and alter the epithelial differentiation to a pyloric sphincter type (Smith and Tabin, 1999; Smith et al., 2000a). We showed herein that activation and inhibition of BMP signaling pathway by viral misexpression modulated *Sox9* expression in the stomach (Fig. 5). Stomachal viral misexpression of *Nkx2.5* leads to epithelial phenotype very similar to *SOX9* misexpression (compare (Smith et al., 2000b) and Fig. 6). Interestingly, misexpression of *SOX9* and *SOX9*ΔCter in the stomach had no effect on *Bmp4* or *Nkx2.5* expression (data not shown), suggesting that *SOX9* does not regulate the expression of these 2 genes. We propose the following model, in which BMP signaling pathway activates both expressions of *Sox9* and *Nkx2.5* in the pyloric sphincter (Fig. 8). These two transcription factors could act independently or could interact to specify the pyloric sphincter structure. In support of this, genetic interactions between *SOX* and *NK2* family genes was reported in *Drosophila* to promote neuroblast formation (Zhao and Skeath, 2002).

SOX9 action in this mesenchymal-epithelial mechanism was investigated deeper in order to identify the signal activated by SOX9 in the pyloric mesenchyme and responsible to the pyloric epithelium differentiation. We identified *Gremlin* as a target gene of SOX9 in this process (Fig. 7). During chondrogenesis, the BMP pathway induces *Gremlin* expression and Gremlin modulates the BMP activation (Capdevilla et al., 1999). In addition, BMP activation is tightly controlled in the pyloric sphincter epithelium (our unpublished observations). Gremlin could act in this structure to diminish and modulate the BMP activity induced by BMP4; it is noteworthy that pyloric epithelium patterning needs low levels of BMP activity whereas the gizzard is associated with the absence of BMP activity (Fig. 8). Cross-regulatory interactions between BMP signaling pathway and SOX factors might be a reiterated process during development, since it was also demonstrated in vertebrate limb (Chimal-Monroy et al., 2003) and in *Drosophila* central nervous system development (Cremazy et al., 2000).

In summary, our work revealed new functions of the transcription factor SOX9 during the GI tract development. SOX9 patterns the pyloric sphincter and specifies the pyloric sphincter epithelium through regulation of Gremlin, a diffusible factor modulating BMP pathway. As we previously commented, molecular controls of GI development patterning events are remarkably conserved across species. We might hypothesize that the described molecular controls of pyloric sphincter formation are conserved in human and that alterations in the normal molecular controls of this region are associated with malformations such as hypertrophic pyloric stenosis (Ohshiro and Puri, 1998). Expressional analyzes of these factors on human fetal normal and malformed pyloric sphincter samples will be performed in order to address this hypothesis.

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FIGURE LEGENDS

Fig. 1. Expression of *Sox9* mRNA and SOX9 protein in 5 days old chick GI tract. (A) Whole-mount *in situ* hybridization of E5 dissected gut using antisens *Sox9* riboprobe. (B1-F2) Paraffin cross-sections along AP axis as indicated in (A), stained with anti-SOX9 (B1,C1,D1,E1,F1) or anti-HNK-1 (B2,C2,D2,E2,F2) antibodies. Cross-sections of the gut were done at the following levels: esophagus and lung bud (B1,B2), stomach (C1,C2), midgut anterior to the umbilic (D1,D2), caeca (E1,E2), and hindgut (F1,F2). Note SOX9 expression in the mesoderm of the posterior region of the stomach (C1). Mesodermal SOX9 expressions is detected in the lung and caeca (red arrows, B1,E1). SOX9 is strongly expressed in the endoderm of the esophagus, lung, pancreas and hindgut as shown in (A,B1,E1). Some SOX9 positive cells are present in the midgut epithelium (insert, D1). SOX9 expression is observed in the mesentere (black arrow, E1), but not in the Remark's nerve, which is essentially composed of neural crest-derived cells as shown by anti-HNK-1 antibodies (E2). Abbreviations: cae, caeca; cl, cloaca; eso, esophagus; gizz, gizzard; hg, hindgut; mg, midgut; panc, pancreas; pyl, pyloric sphincter; RN, Remark's nerve; sto, stomach.

Fig. 2. Expression of *Sox9* mRNA and SOX9 protein in 9 days old chick GI tract. (A) Whole-mount *in situ* hybridization of E9 dissected gut using antisens *Sox9* riboprobe. Paraffin cross-sections along AP axis as indicated in (A), stained with anti-SOX9 (B1,C1,D1,E1,F1) or anti-HNK-1 (B2,C2,D2,E2,F2) antibodies. Cross-sections of the gut were done at the following levels: esophagus (B1,B2), pyloric sphincter (C1,C2), midgut anterior to the umbilic (D1,D2), caeca (E1,E2), hindgut (F1,F2), and cloaca chamber (G1,G2). Note that mesodermal SOX9 expression strongly demarcates the pyloric sphincter (A,C1). SOX9 is strongly expressed in the epithelia of the esophagus, the small intestine and the cloaca chamber (B1,D1,G1), but faintly in the hindgut (F1). Abbreviations: an, anus; cae, caeca; cl, cloaca; eso, esophagus;

gizz, gizzard; hg, hindgut; liv, liver; mg, midgut; panc, pancreas; pv, proventriculus; pyl, pyloric sphincter.

Fig. 3. SOX9 expression in human embryo GI system. (A-E) Immunohistochemistry with anti-SOX9 antibodies on paraffin sections from a 7.5 weeks old human embryo. (A) Lung. (B) Pancreas. (C) Posterior region of the stomach. (D) Small intestine. (E) Rectum. Note the expression of SOX9 protein in human viscera epithelial layers and the exclusive mesodermal expression of SOX9 protein at the pyloric level in the stomach (red arrow, C). (F) Immunohistochemistry with anti-SOX9 antibodies on paraffin sections from prenatal human small intestine. Note the expression of SOX9 in the proliferative compartment of the villi (red arrow, F). Abbreviations: mg, midgut; panc, pancreas; prenatal, prenatal; pyl, pyloric structure; rect, rectum; si, small intestine; sto, stomach.

Fig. 4. Expression of E subgroup *Sox* genes, *Bmp4* and *Nkx2.5* in 7 days old chick stomach. (A) Whole-mount *in situ* hybridization of E7 dissected gut using antisens *Sox9* riboprobe. Note that *Sox9* expression strongly demarcates the stomach from the duodenum and is only found in the mesoderm of the pyloric sphincter (red arrow, A). (B,C) Whole-mount *in situ* hybridization of E7 dissected gut, using antisens *Sox8* (B) and *Sox10* (C) riboprobes. Note that *Sox8* and *Sox10* expression in the stomach is restricted to ENS cells (B,C). (D,E) Whole-mount *in situ* hybridization of E7 dissected gut using antisens *Bmp4* (D) and *Nkx2.5* (E) riboprobes. Note that *Bmp4* is expressed in the mesenchyme of the duodenum and the pylore (red arrow, D), but not in the gizzard. *Nkx2.5* expression is only present in the pylore mesenchyme (red arrow, E). Abbreviations: gizz, gizzard; pv, proventriculus; pyl, pyloric sphincter.

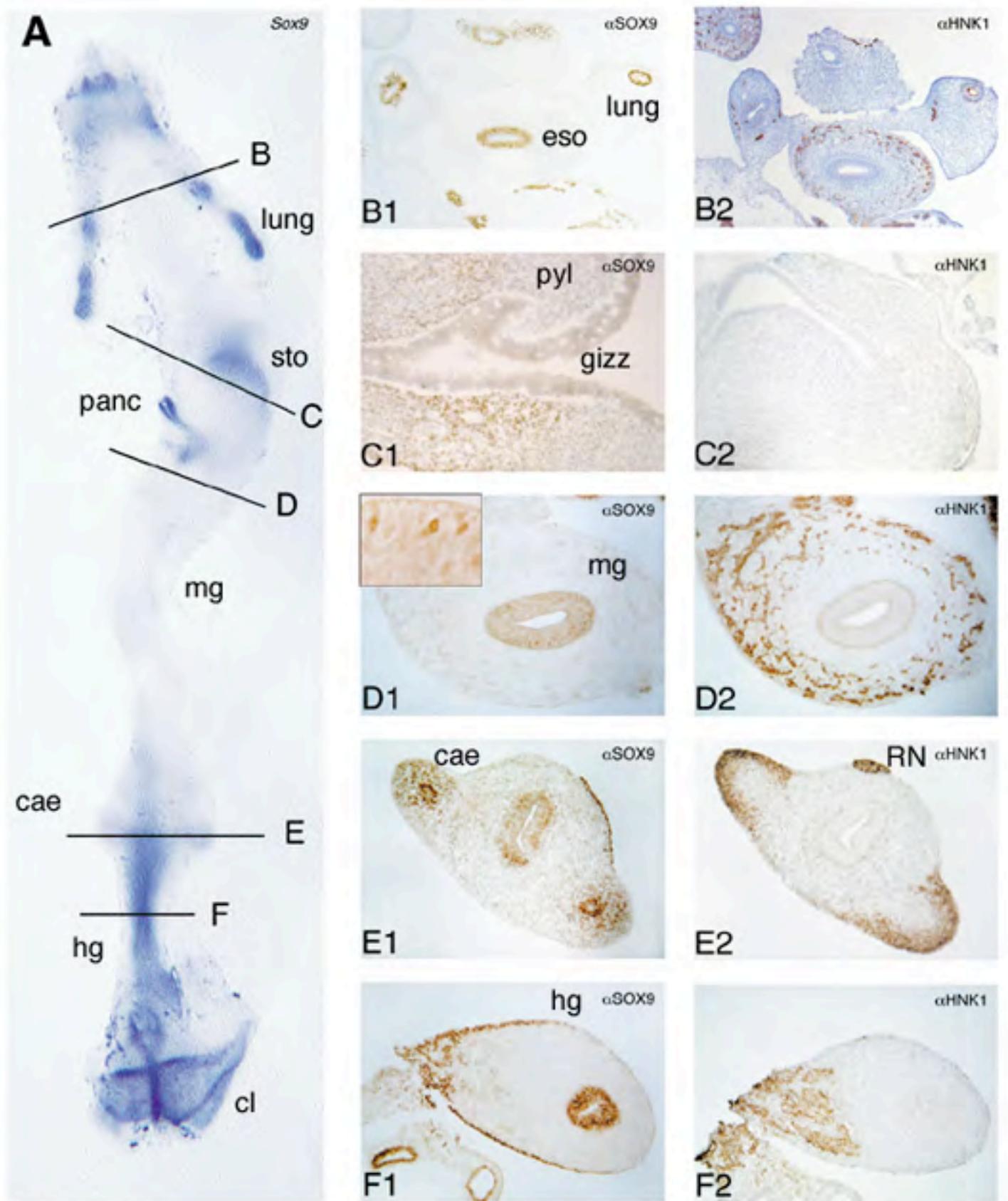


Fig.1.

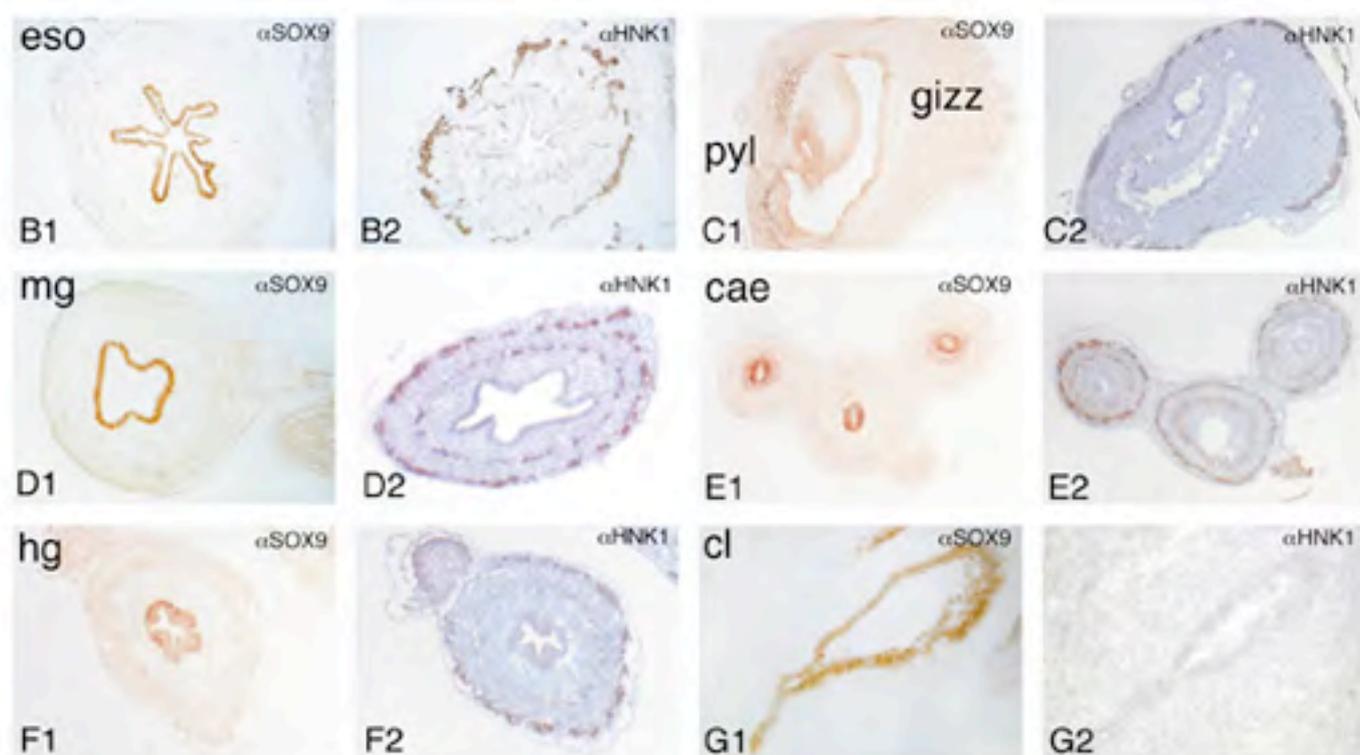
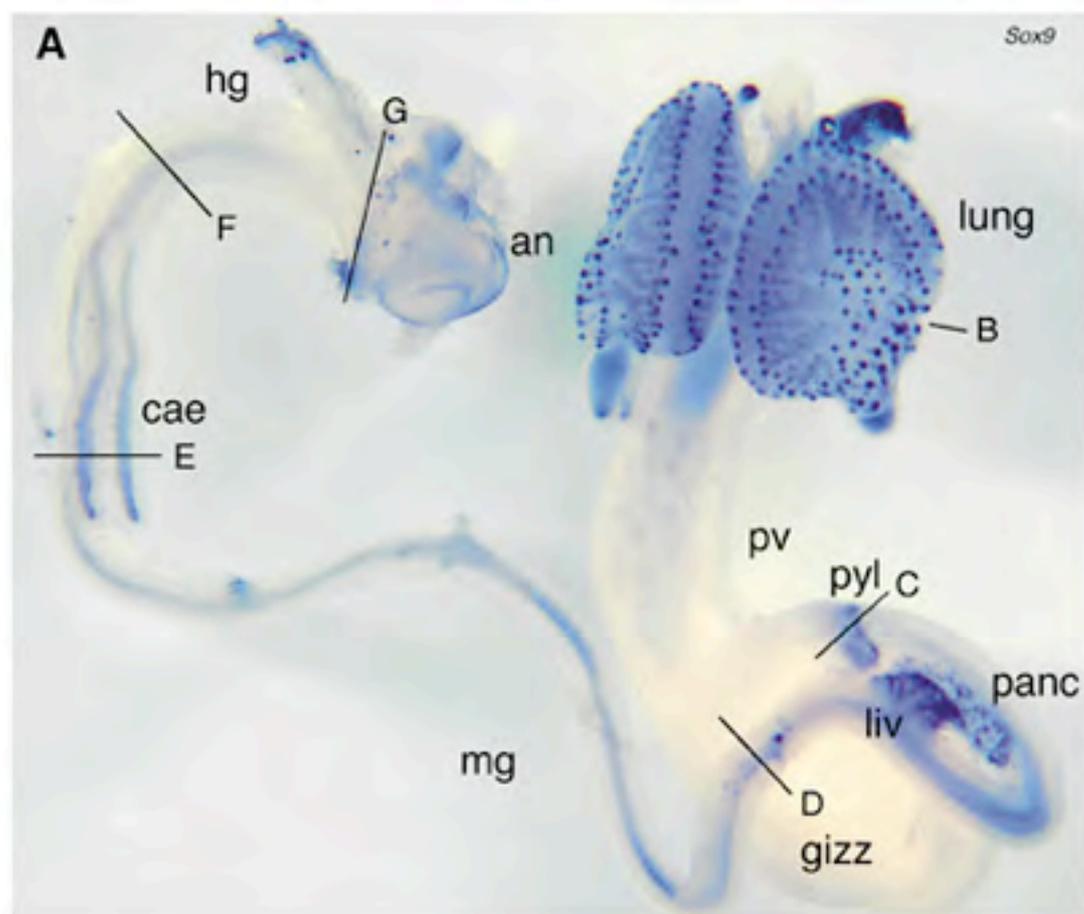


Fig.2.

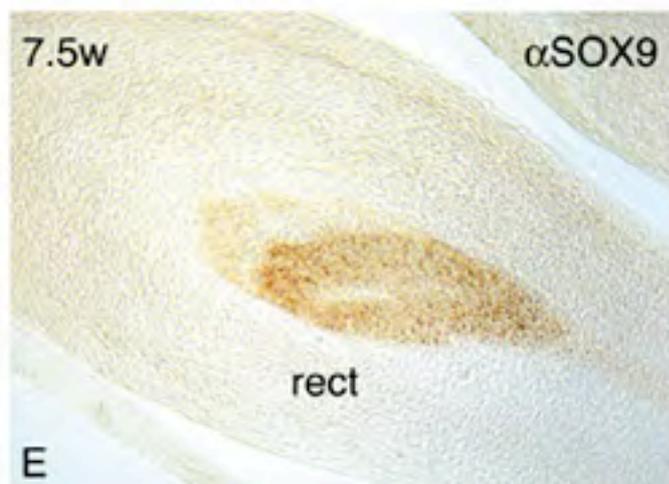
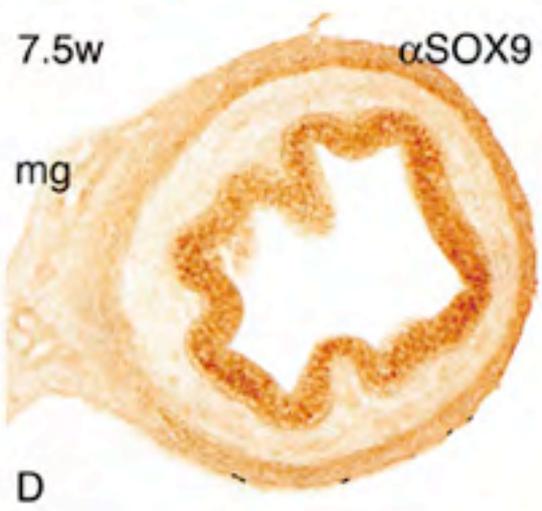
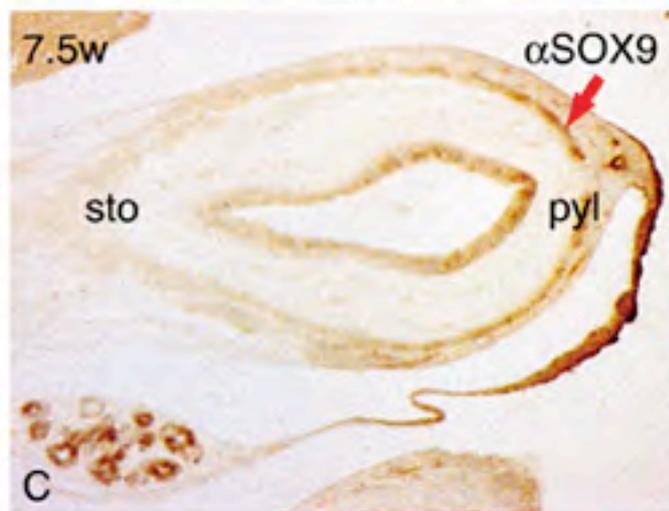
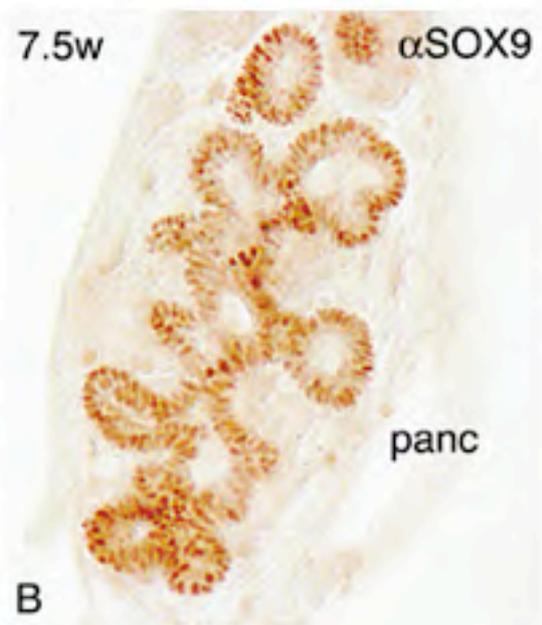


Fig.3.

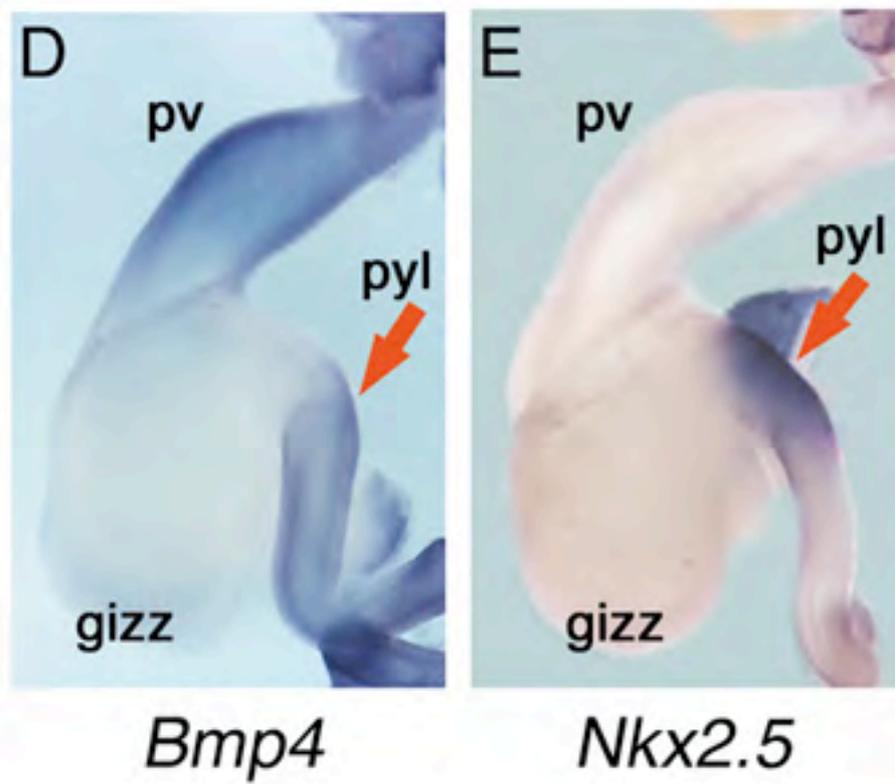
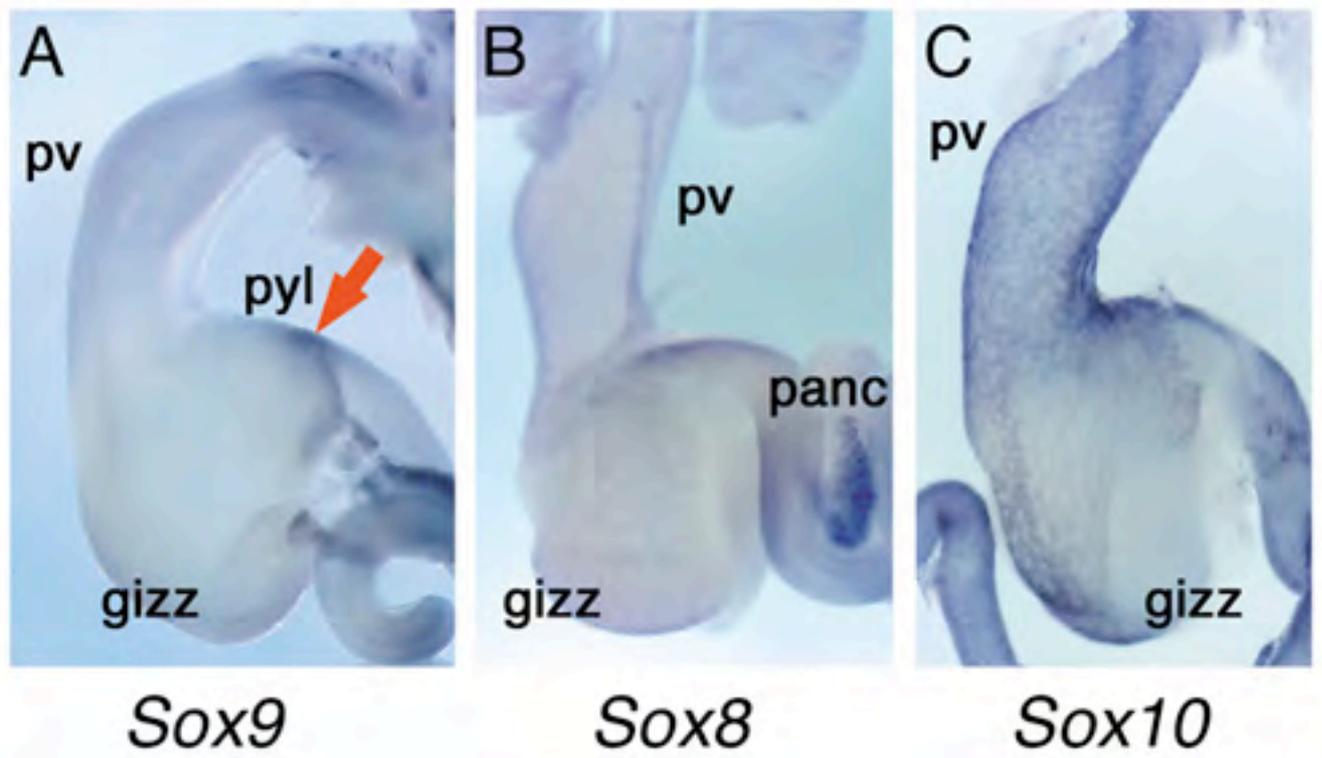


Fig.4.

Fig. 5. Modulation of the BMP signaling pathway in the stomachal mesoderm affects *Sox9* expression. Whole-mount *in situ* hybridization using antisense *Sox9* riboprobe upon *GFP* (A), *Bmp4* (B), *Nkx2.5* (C), and *Noggin* (D,E) misexpressing E8 stomach. Note morphological change of *Bmp4* misexpressing stomach characterized by gizzard musculature mass decrease (compare B with A). *Noggin* misexpression in the stomach gives rise to moderate to severe phenotype (D,E). Moderate phenotype present proventriculus fate change with gland formation inhibition and size increase (D). Severe *Noggin* phenotype is mainly characterized by stomach/duodenum connection defect and gizzard like phenotype found in the whole stomach (E). Nor *GFP* (A) as control and *Nkx2.5* (C) misexpression affects stomach morphology. *Sox9* expression is upregulated in *Bmp4* misexpressing stomach (B), strongly downregulated in *Noggin* misexpressing stomach (D,E) and unchanged in *Nkx2.5* (C) or *GFP* (A) misexpressing stomachs. Arrows indicate pyloric sphincter area. (F) *Bmp4* misexpressing stomach was sectioned and probed with anti-SOX9 antibodies and revealed SOX9 ectopic expression in the gizzard (red arrowheads, F). Abbreviations: gizz, gizzard; panc, pancreas; pyl, pyloric structure.

Fig. 6. Misexpression of *SOX9* in the stomachal mesoderm specifies the stomachal epithelium into pyloric epithelium. Histological sections of control E9 stomach (A,B), *SOX9* (C) and *SOX9* Δ Cter (D) misexpressing E9 stomachs. Immunohistochemistry with α 3C2 antibodies was used to show that the infection was restricted to the stomachal mesoderm, (insert in panels C and D) and clearly excluded from the endoderm. Control gizzard epithelium cells present keratin long cilia (A). Control pyloric sphincter epithelium cells are characterized by bulbous cilia (B). After mesodermal *SOX9* misexpression, the gizzard epithelial cells present bleb like cilia (C), while after mesodermal *SOX9* Δ Cter misexpression, the pyloric epithelial cells present keratin long like cilia (D). (E-G) Analyses of *Pdx1* expression by *in situ*

hybridization on section of control stomach (E), *SOX9* (F) and *SOX9* Δ Cter (G) misexpressing stomachs using antisens *Pdx1* riboprobe. Note the normal expression of *Pdx1* in the pyloric endoderm. Ectopic *Pdx1* expression is detected in *SOX9* misexpressing gizzard epithelium (red arrowheads, F). In *SOX9* Δ Cter misexpressing stomach, no *Pdx1* expression is detected in the gizzard epithelium, but downregulation of *Pdx1* is observed in the pyloric epithelium (G). However, note the expression of *Pdx1* in the pancreas in these *SOX9* Δ Cter misexpressing stomach (insert, G). Abbreviations: duo, duodenum; e, endoderm; gizz, gizzard; m, mesoderm; panc, pancreas; pyl, pyloric structure.

Fig. 7. *Gremlin* modulation of mesenchymal-epithelial interaction in the pyloric sphincter is under *SOX9* control. (A) Whole-mount *in situ* hybridization of E7 dissected gut using antisens *Gremlin* riboprobe. Note that *Gremlin* expression strongly demarcates the stomach from the duodenum and is present in the mesoderm of the pyloric sphincter (red arrow, A). (B) Histological section of *Gremlin* misexpressing E9 stomach. After mesodermal *Gremlin* misexpression, the gizzard epithelial cells present bleb like cilia (B). Immunohistochemistry with α 3C2 antibodies show retrovirus infection restricted to the mesoderm of the stomach (insert B). *GFP* (C), *SOX9* (D), and *SOX9* Δ Cter (E) misexpressing E7 stomach sections followed by detection of *Gremlin* expression by *in situ* hybridization using antisens *Gremlin* riboprobe. Note the expression of *Gremlin* in the mesoderm and adventitia of the pyloric sphincter (C). Ectopic expression of *Gremlin* in the mesodermal stomach is observed after retroviral *SOX9* misexpression in the stomach (red arrowheads, D). Downregulation of *Gremlin* expression is correlated with retroviral *SOX9* Δ Cter misexpression in the pyloric sphincter (black arrowheads, E), while *Gremlin* expression is normal in the adventitia. Abbreviations: duo, duodenum; e, endoderm; gizz, gizzard; m, mesoderm; pv, proventriculus; pyl, pyloric structure.

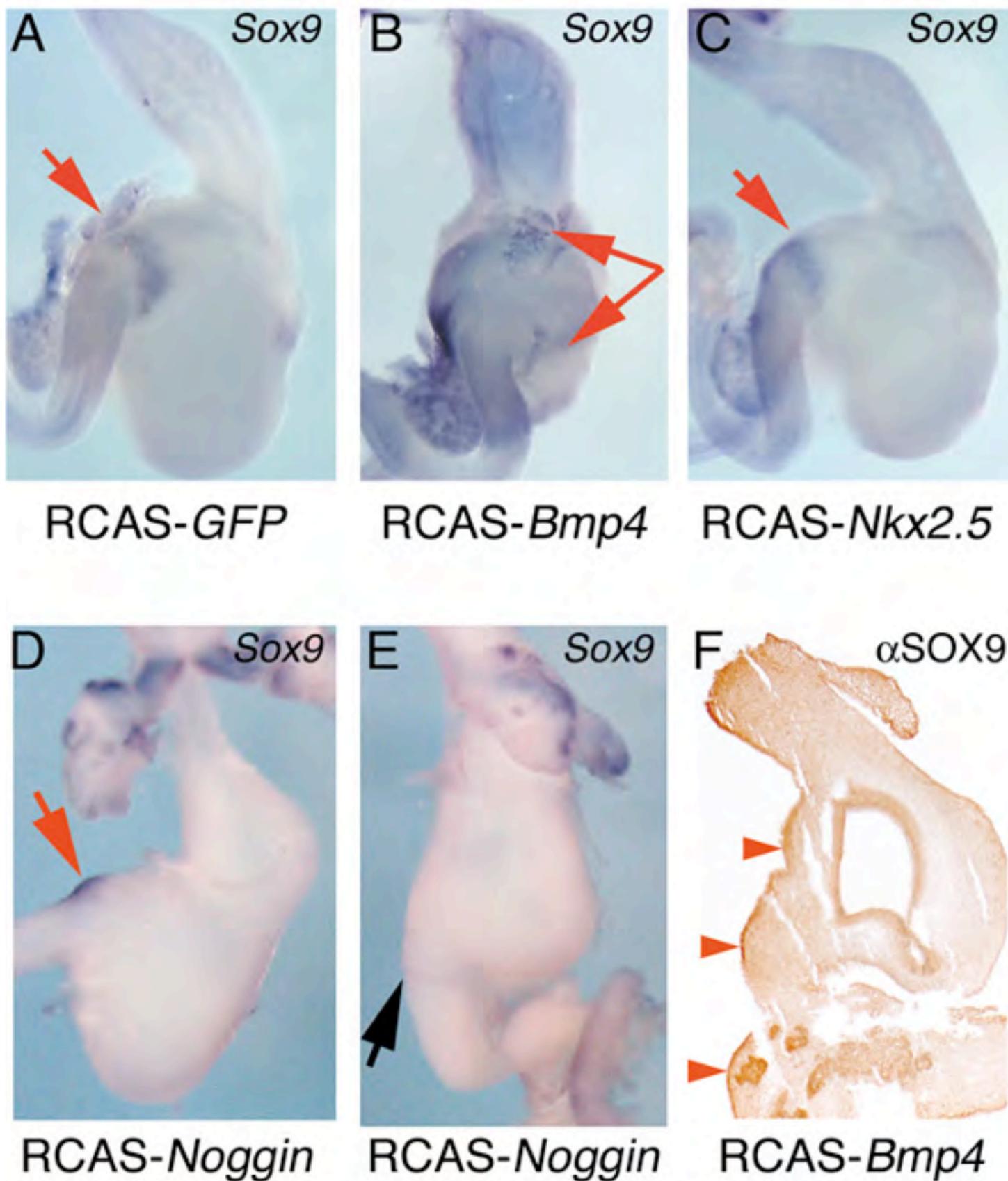


Fig.5.

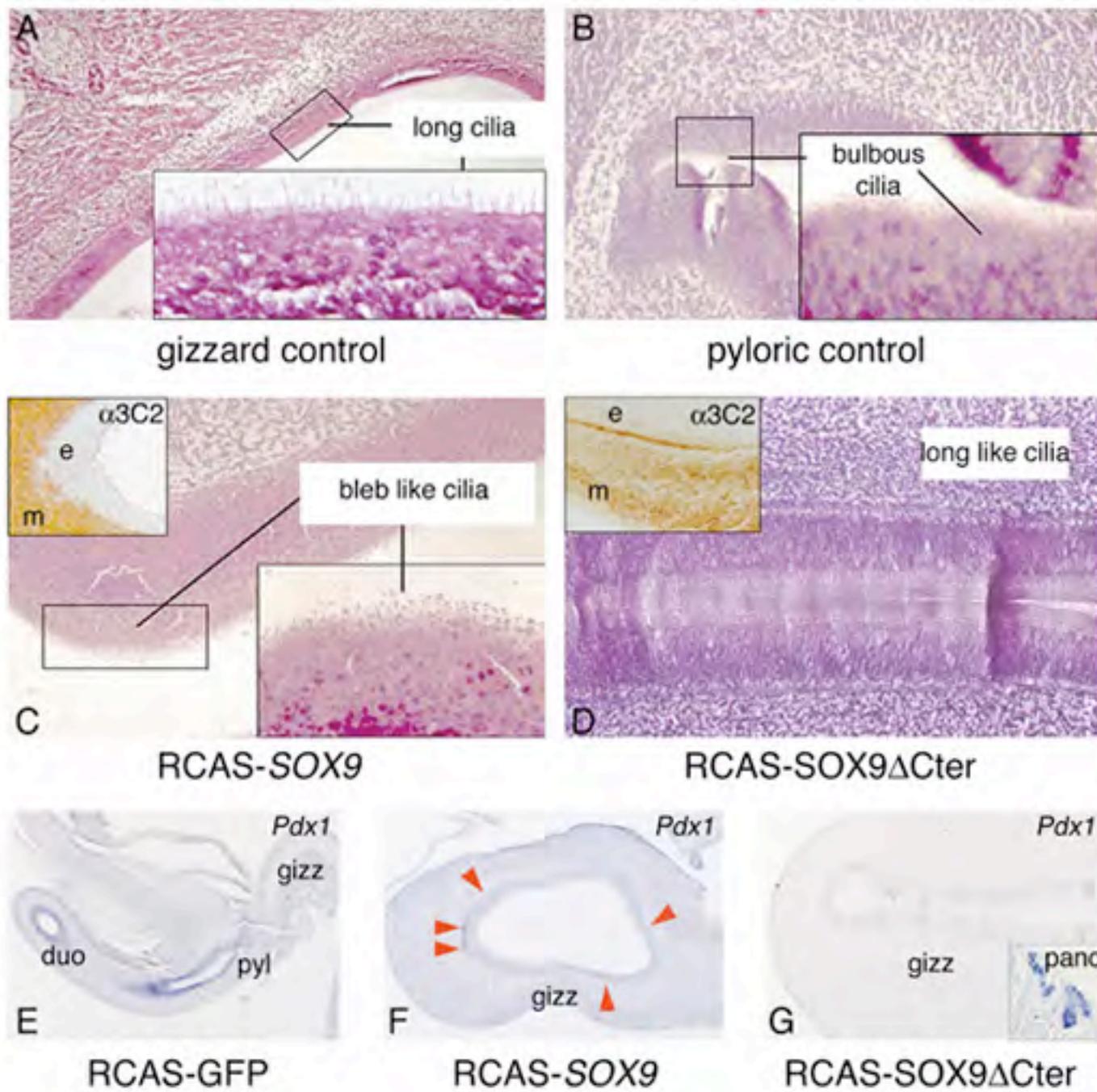
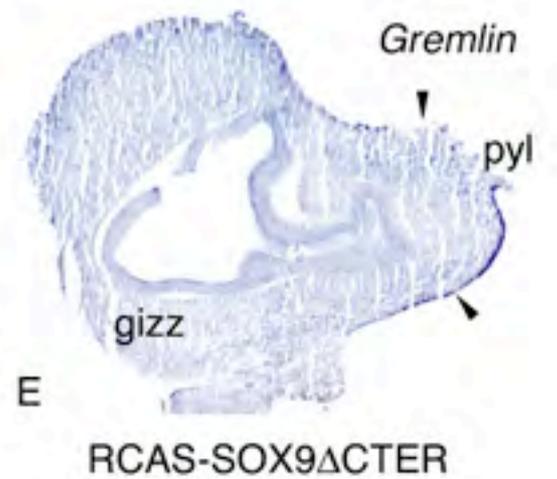
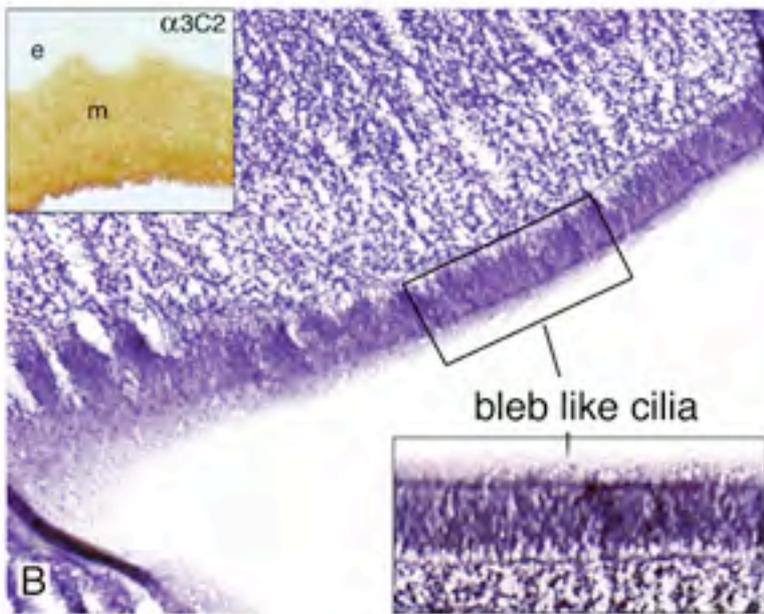
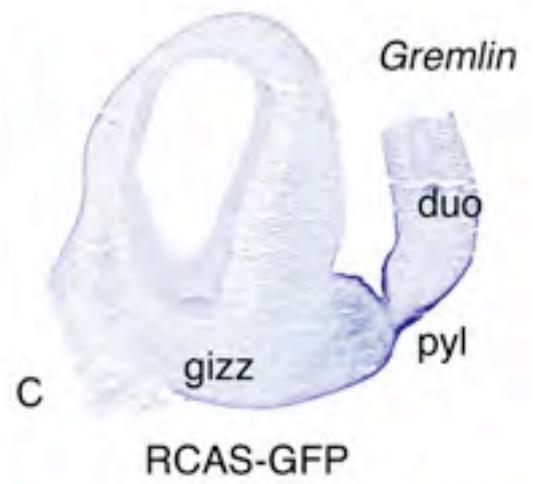
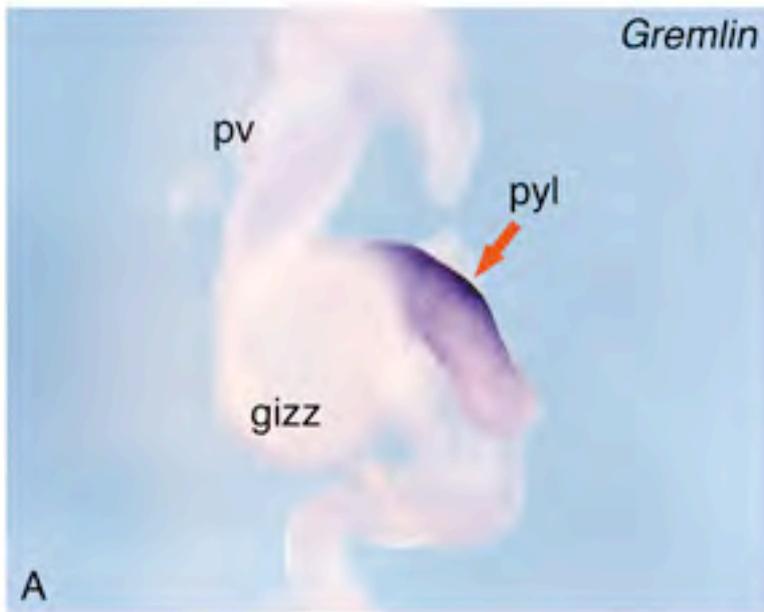


Fig.6.



RCAS-Gremlin

FIG.7.

Fig. 8. Model of the molecular pathways and their potential interactions involved during the development of the pyloric sphincter. Schematic representations of avian stomach (left panel) and the molecular pathways involved (right panel). The avian stomach can be divided in proventriculus (glandular stomach) and gizzard (muscular stomach). The pyloric sphincter is a highly conserved structure found in all vertebrates and anatomically separates the gizzard and the duodenum. *Shh* from epithelium induces *Bmp4* expression in the adjacent mesenchyme, except in the gizzard where *Bapx1* prevents *Bmp4* expression. In the small intestine, *Bmp4* activates the BMP signaling pathway in the mesoderm and endoderm (unpublished data). In the pyloric sphincter, *Bmp4* is able to activate the expressions of *Nkx2.5* and *Sox9*, which are both sufficient to induce pyloric epithelial phenotype through mesenchymal-epithelial signal modulation. Importantly, our data show that there is no cross-regulation between *Sox9* and *Nkx2.5* at the transcriptional level. SOX9 is able to control *Gremlin* expression in the pyloric sphincter mesenchyme. Gremlin, a diffusible factor, could modulate endodermal BMP pathway activation, in order to induce specific pyloric epithelium differentiation.

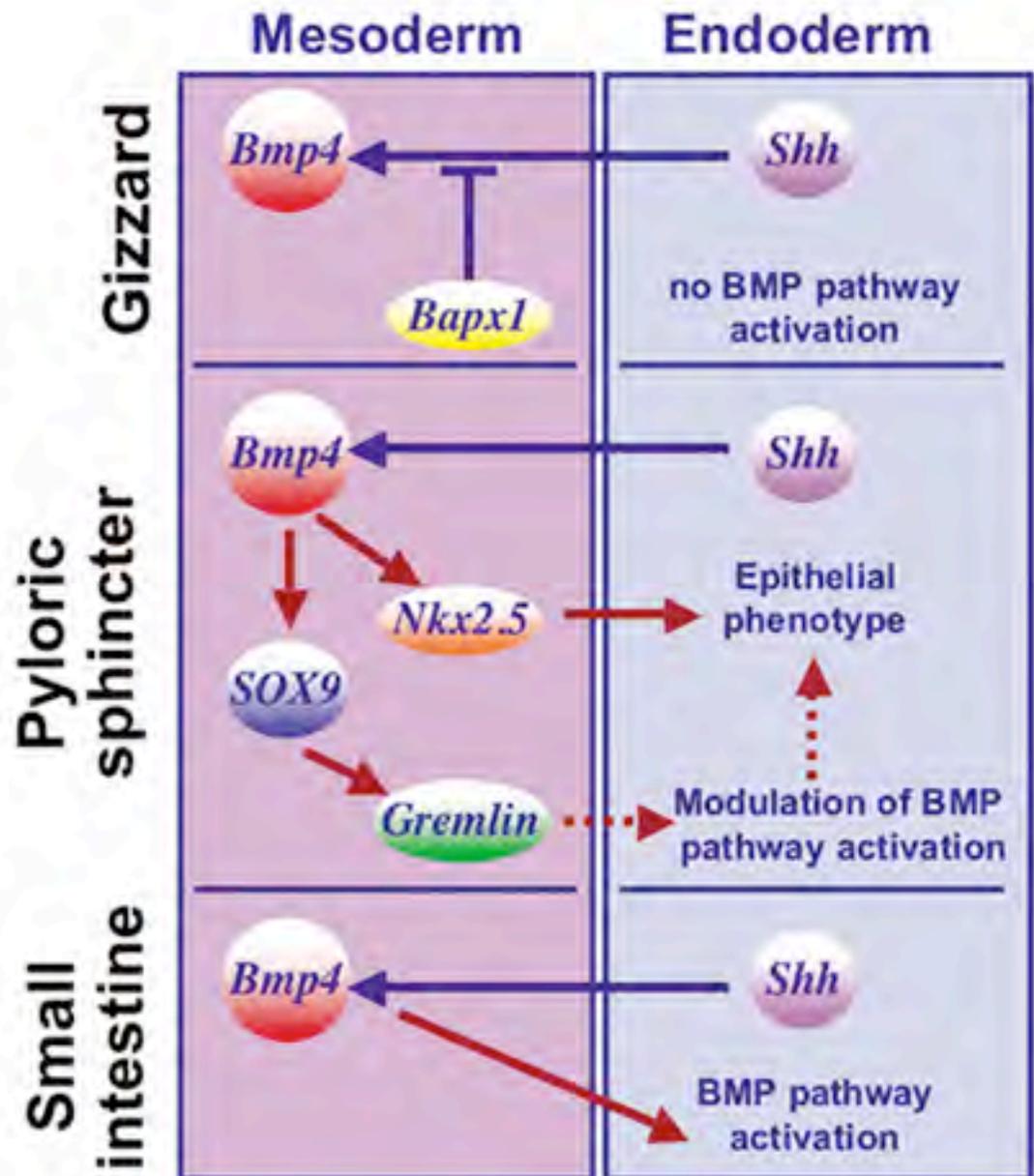


Fig.8.

TABLE 1

Summary of SOX9 sites of expression in the developing chick GI tract

GI level	5 days old		9 days old	
	Mesoderm	Endoderm	Mesoderm	Endoderm
Esophagus	-	+++	-	+++
Stomach	-	-	-	-
Pyloric sphincter	+++	-	+++	-
Midgut	-	+	-	+++
Caeca	+++	++	+	+++
Hindgut	-	+++	-	++

Note. Abbreviations: -, no detection; +, weak expression; ++, moderate expression; +++, strong expression.

Supplementary data

Fig. S1. Anti-SOX9 antibodies are specific for the chick SOX9 protein. (A) Western-blot analyses using anti-SOX9 (top panel) and anti-tubulin (bottom panel) antibodies. Cell lysates were prepared from human NT2/D1 (N-Tera2, clone D1, a human pluripotent embryonic carcinoma cell line) (1), chick embryo harvested at stage 16 (2), guts dissected from 5 days-old embryos (3). Anti-SOX9 antibodies detect a single band corresponding to the chick SOX9 protein both in embryo and gut lysates, which migrates with a lower calculated molecular weight compared to the human SOX9 protein as previously reported (Kamachi et al., 1999). (B-J) Immunohistochemical analyses on chick stage 26 cryosections. Specific detection of nuclear SOX9 staining into the prevertebrate structure (B, H) and the neural tube (E). (C,F,I) Addition of GST peptide antigen (α -SOX9 + GST peptide) does not affect anti-SOX9 immunoreactivity. (D,G,J) Addition of SOX9 TA domain peptide antigen in fusion with GST (α -SOX9 + SOX9 peptide) at the same concentration eliminates anti-SOX9 immunoreactivity.

Fig. S2. Characterization of RCAS-SOX9 constructs. (A-C) Immunohistochemistry detection using anti-SOX9 antibodies on RCAS-GFP (A), RCAS-SOX9 (B) transfected CEF cells and RCAS-SOX9 infected E7 stomach (C). Note the expression of SOX9 protein in RCAS-SOX9 transfected CEF cells (red arrow, B) and ectopic SOX9 expression in the mesenchyme of the gizzard (red arrow, C). Abbreviations: CEF, chick embryonic fibroblast; e, endoderm; gizz, gizzard; m, mesoderm.

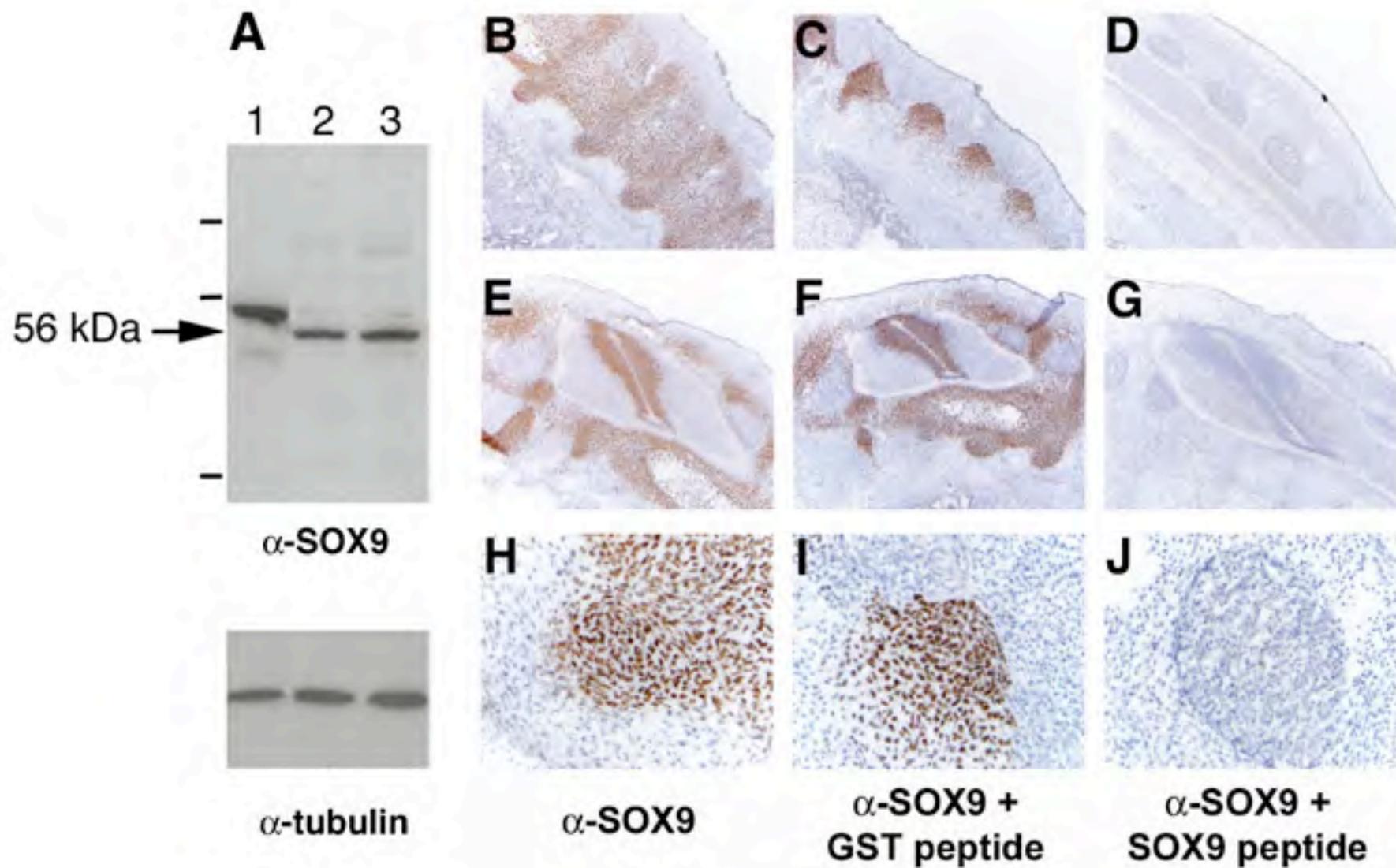
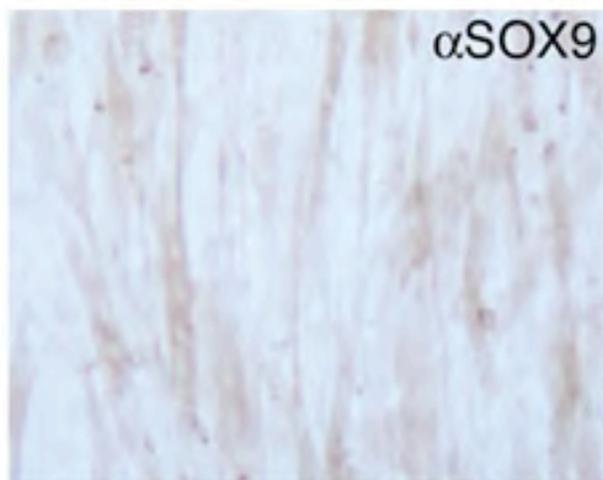


Fig.S1.



CEF+RCAS-GFP



CEF+RCAS-SOX9



RCAS-SOX9

Fig.S2.