SOX9 specifies the pyloric sphincter epithelium through mesenchymal-epithelial signals

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Summary

Gastrointestinal (GI) development is highly conserved across 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 gizzard) and its derivate organs, the lung and pancreas. Moreover, SOX9 is also expressed at the mesoderm of the pyloric sphincter, a structure that demarcates the gizzard from the duodenum. Using retroviral misexpression technique, we show that *Sox9* expression in the pyloric sphincter is under the control of the BMP signaling pathway, known to play a key role in the development of this structure. By misexpressing *SOX9* in the mesoderm of the gizzard, 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 is necessary and sufficient to specify the pyloric sphincter epithelial properties.

Supplemental data available on-line

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 lumenal digestive system of the esophagus, stomach, intestines and colon (to which we will refer as gut), and the derivate organs, thyroid, lungs, liver and pancreas (Roberts, 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 anteroposterior (AP), dorsoventral (DV), left-right (LR) and radial (cross-sectional, crypt-villous) axes (de Santa Barbara et al., 2002). Specific regional differentiation along AP axis will give rise to the foregut (pharynx, esophagus and stomach), midgut (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, 2000) and in adulthood for epithelial regeneration (Clatworthy and Subramanian, 2001).

Several molecular factors involved during GI tract development have been identified (for reviews, 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, 2002). 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 have shown 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, which encode 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* has been shown to be necessary for the gut endoderm development (Kanai-Azuma et al., 2002). *SOX9* was initially identified as the gene responsible for campomelic dysplasia (CD) syndrome, an autosomic 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, individuals with CDs often display abnormalities in visceral organs and brain, suggesting a role for SOX9 in some aspects of the GI and central nervous system (CNS) development. 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). Reported GI tract anomalies include megacolon and intestinal malrotation (Houston et al., 1983). Owing to the high mortality, only a few studies have addressed GI diseases or malformations in individuals with CD (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 gizzard 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 stomach/duodenum 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 phosphate-buffered saline (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 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 ethic 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 staining was performed on cryostat sections as previously described (de Santa Barbara and Roberts, 2002) using standard techniques and the Vectastain ABC detection system (Vector Laboratories, CA) following the manufacturer's directions. Used anti-SOX9 (αSOX9) antibodies were raised against the transactivation 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 Figs S1 and S2 at http://dev.biologists.org/supplemental. Anti-avian retroviral GAG protein (α3C2) antibodies were used as previously described (de Santa Barbara and Roberts, 2002). Anti-HNK1 (αHNK1) antibodies were purchased from NeoMarkers and used diluted 1:400. These antibodies recognize neural crest-derived cells.

Antisense RNA probes were previously described for chick Bmp4 (Nielsen et al., 2001), chick Gremlin (Capdevila et al., 1999), chick Nkx2.5, chick Shh (Smith et al., 2000a), chick Sox8 (Bell et al., 2000), chick Sox9 (Healy et al., 1999), chick Sox10 (Cheng et al., 2000), chick Wnt11 (Theodosiou and Tabin, 2003), chick Pdx1 and chick Sox2 (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 (10 μm) were collected onto Superfrost Plus slides (Fisher Scientific), air dried for 4-18 hours and kept at –20°C until used. Fixed and stained embryos were embedded in paraffin wax and sectioned at 8 μm for histological analysis. Haematoxylin and Eosin staining was performed using standard techniques. In situ hybridization and immunohistochemistry on paraffin wax-embedded sections were performed as previously described (de Santa Barbara and Roberts, 2002).

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, 2002).

New constructs were produced and characterized in this study (see Figs S1 and S2 at http://dev.biologists.org/supplemental). The full-length and C-terminal deleted (ΔCter) human *SOX9* cDNAs were cloned into the shuttle vector Slax13 and then subcloned into RCAS(A) vector. Full-length and ΔCter *SOX9* RCAS vectors were transfected into chick embryonic fibroblasts, and virus harvested and titered using standard techniques (Morgan and Fekete, 1996). 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 μl 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 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-day-old (E5) dissected guts (stage 26-27). At this stage, *Sox9* expression was detected in different regions

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 Figs S1 and S2 at http://dev.biologists.org/supplemental) 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 was 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 mesentery (black arrow, Fig. 1E1). In addition, SOX9 expression was detected in the mesoderm of the posterior region of the stomach (Fig. 1C1). As ENS cells colonize the mesodermal layer at this stage of development to assure final gut innervation, we analyzed whether or not mesodermal SOX9 expression colocalized with ENS cells by immunohistochemical analyses using anti-HNK1 antibodies that recognize neural crest-derived cells (Fig. 1B2-F2). At E5, ENS cells are migrating along AP axis and are not yet well organized into plexi (Fig. 1D2). SOX9-positive cells detected in the lung, stomach and caeca mesoderm did not colocalize with HNK1-positive cells (compare Fig. 1B1,C1,E1 with B2,C2,E2, respectively). 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.

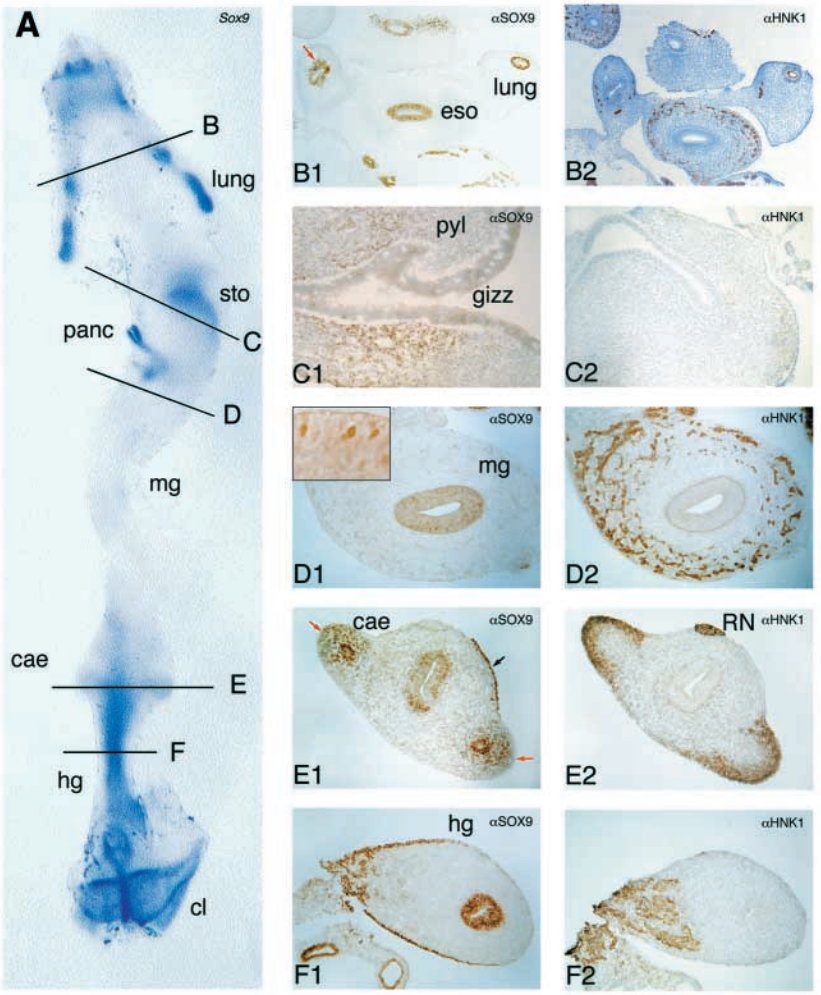

Fig. 1. Expression of *Sox9* mRNA and SOX9 protein in 5-day-old (E5) chick GI tract. (A) Whole-mount in situ hybridization of E5 dissected gut using antisense *Sox9* riboprobe. (B1-F2) Paraffin cross-sections along AP axis as indicated in A, stained with anti-SOX9 (B1,C1,D1,E1,F1) or anti-HNK1 (B2,C2,D2,E2,F2) antibodies. Cross-sections of the gut were taken at the following levels: esophagus and lung bud (B1,B2), posterior region of the stomach (C1,C2), midgut anterior to the umbilicus (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 expression is also detected in the lung and caeca (red arrows, B1,E1). SOX9 is strongly expressed in the endoderm of the esophagus, lung, pancreas and hindgut (A,B1,E1). Some SOX9-positive cells are present in the midgut epithelium (insert, D1). SOX9 expression is observed in the mesentery (black arrow, E1), but not in the Remark's nerve, which is essentially composed of neural crest-derived cells as shown by anti-HNK1 antibodies (E2). cae, caeca; cl, cloaca; eso, esophagus; gizz, gizzard; hg, hindgut; mg, midgut; panc, pancreas; pyl, pyloric sphincter; RN, Remark's nerve; sto, stomach.

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 regions, the proventriculus (avian glandular stomach) and the gizzard (avian muscular stomach),

which are distinct both morphologically and physiologically (Romanoff, 1960). The connection between the gizzard and the duodenum is demarcated by the pyloric sphincter. This structure is a mesodermal sphincter that allows for maintaining food in the stomach and controls the gastric content flow into the duodenum. *Sox9* expression was also detected in the distal stomach in a ring form corresponding to the pyloric sphincter (Fig. 2A). SOX9 protein is detected in the mesoderm of the pyloric sphincter (Fig. 2C1). No colocalization between SOX9- and HNK1-positive cells was observed (compare Fig. 2C1 with C2).

In order to determine whether SOX9 expression in the GI system is conserved in humans, 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 (Fig. 3A,B,D,E) and mesodermal SOX9 expression was detected 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 to 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 eight 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 SOX9 expression in the pyloric sphincter mesoderm is a common feature of all E subgroup Sox members or whether this expression pattern is specific to SOX9, we performed in situ hybridization experiments with *Sox8*, *Sox9* and *Sox10* riboprobes on chick E7 stomach. Expression of all three 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). Together, our results demonstrate that SOX9 is the only E subgroup SOX member expressed in the pyloric sphincter mesoderm in vertebrates.

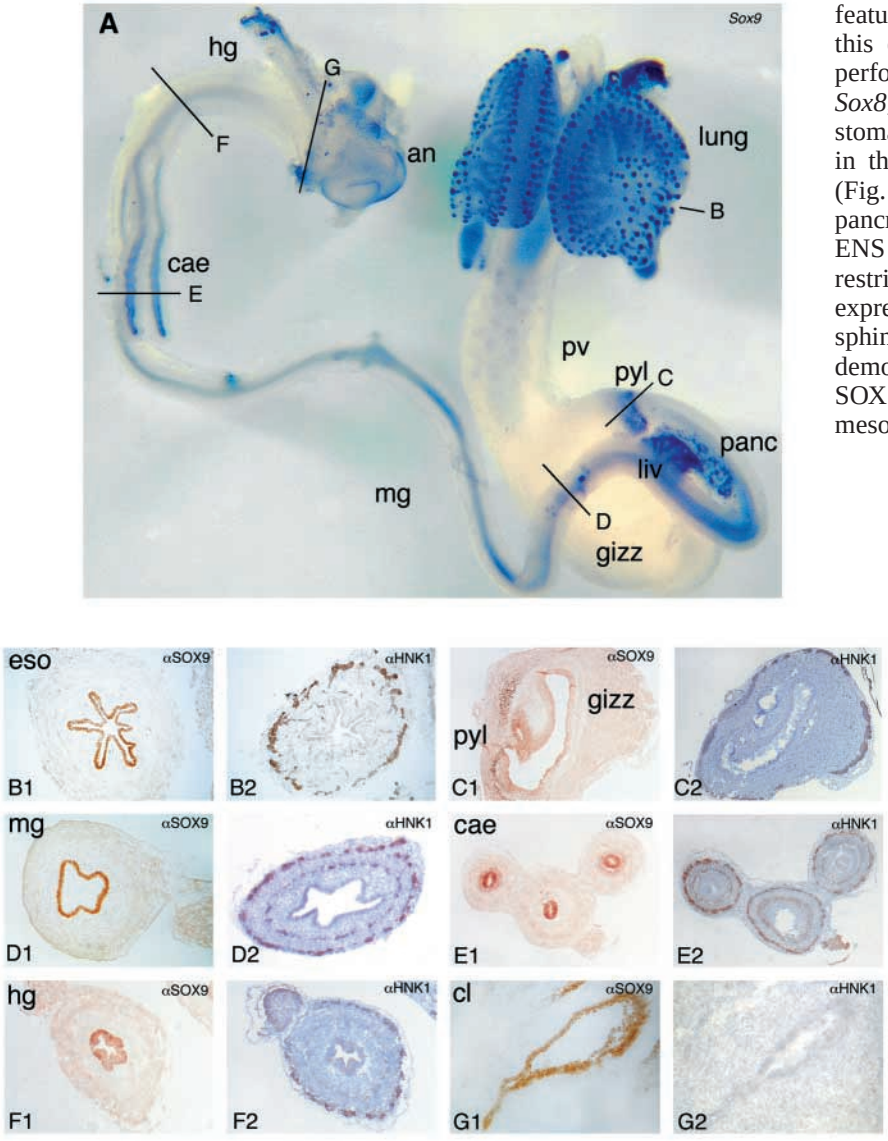

Fig. 2. Expression of *Sox9* mRNA and SOX9 protein in 9-day-old (E9) chick GI tract. (A) Whole-mount in situ hybridization of E9 dissected gut using antisense *Sox9* riboprobe. Paraffin cross-sections along AP axis as indicated in A, stained with anti-SOX9 (B1,C1,D1,E1,F1) or anti-HNK1 (B2,C2,D2,E2,F2) antibodies. Cross-sections of the gut were done at the following levels: esophagus (B1,B2), pyloric sphincter (C1,C2), midgut anterior to the umbilicus (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.

BMP signaling regulates *Sox9* expression in the stomach

Previous studies have shown that BMP signaling pathway is necessary for normal stomach development (Smith and Tabin, 1999). *Bmp4* is expressed in the mesenchyme of the entire gut with the exception of the gizzard (Fig. 4D). It regulates both muscular thickness and pyloric sphincter specification (Roberts et al., 1998; Smith and Tabin, 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).

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 technique to specifically misexpress *Bmp4*, *Nkx2.5* and the BMP-antagonist *Noggin* in the stomach 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 a control. Infection of mesoderm in the analyzed stomach was confirmed by immunohistochemistry using antibodies directed against the avian retroviral GAG protein (α3C2) (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, the domain of *Sox9* expression was extended anteriorly from the pyloric sphincter to the right part of the gizzard (double red arrows, Fig. 5B). This suggested that activation of BMP signaling pathway in the stomach mesoderm upregulates *Sox9* expression. Examination of cryostat sections also indicated that not all infected mesodermal cells express SOX9 in response to *Bmp4* misexpression, suggesting that only few competent cells are

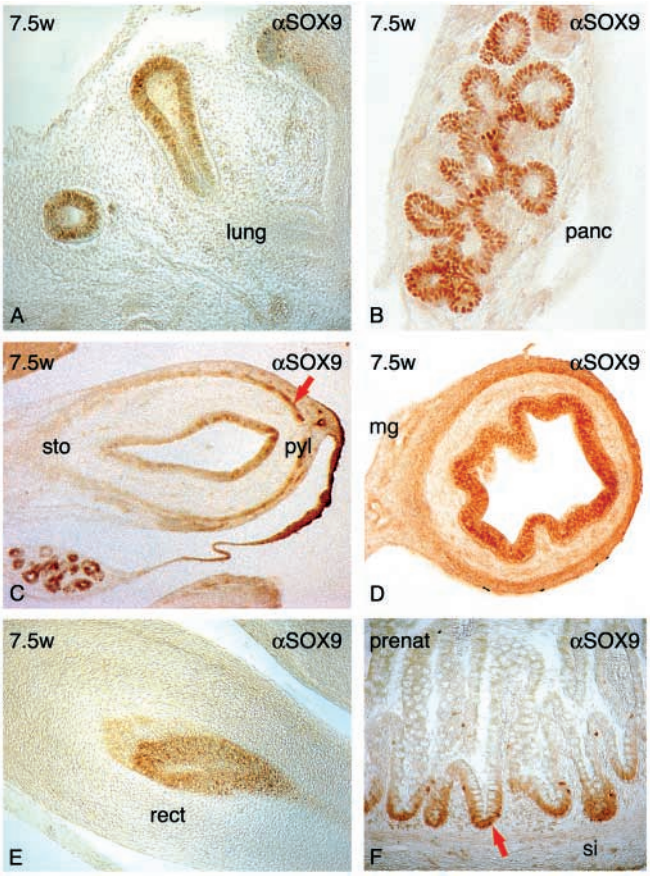

Fig. 3. SOX9 expression in human embryo GI system. (A-E) Immunohistochemistry analyses using anti-SOX9 antibodies on paraffin sections from a 7.5-week-old (7.5 w) human embryo. (A) Lung. (B) Pancreas. (C) Posterior region of the stomach. (D) Small intestine. (E) Rectum. Expression of SOX9 protein is found in human viscera epithelial layers and there is exclusive mesodermal expression of SOX9 protein at the pyloric level in the stomach (red arrow, C). (F) Immunohistochemistry with anti-SOX9 antibodies on paraffin wax sections from prenatal human small intestine (22 w). Note the expression of SOX9 in the proliferative compartment of the villi (red arrow, F). mg, midgut; panc, pancreas; prenat, prenatal; pyl, pyloric structure; rect, rectum; si, small intestine; sto, stomach.

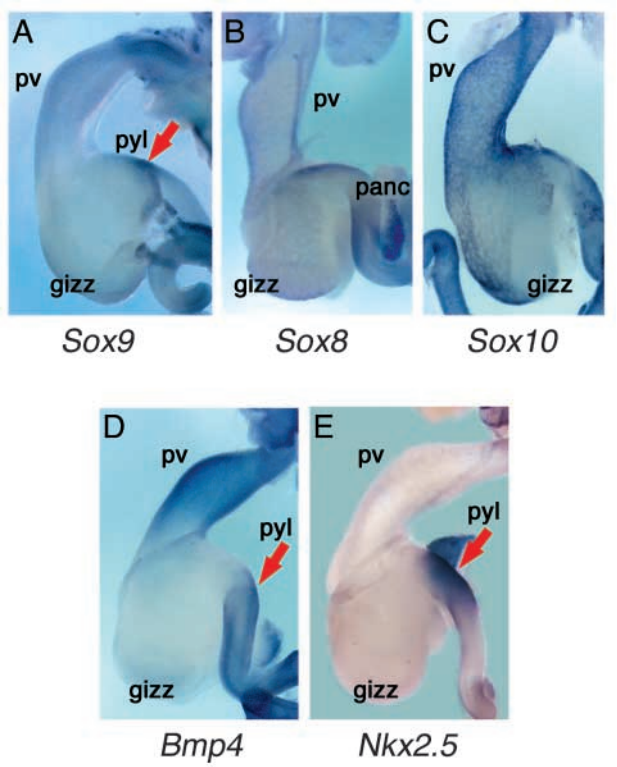

Fig. 4. Expression of E subgroup Sox genes, *Bmp4* and *Nkx2.5* in 7-day-old (E7) chick stomach. (A) Whole-mount in situ hybridization of E7 dissected gut using antisense *Sox9* riboprobe. *Sox9* expression strongly demarcates the stomach from the duodenum and is only observed in the mesoderm of the pyloric sphincter (red arrow, A). (B,C) Whole-mount in situ hybridization of E7 dissected gut, using antisense *Sox8* (B) and *Sox10* (C) riboprobes. *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 antisense *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 observed only in the pyloric mesenchyme (red arrow, E). gizz, gizzard; pv, proventriculus; pyl, pyloric sphincter.

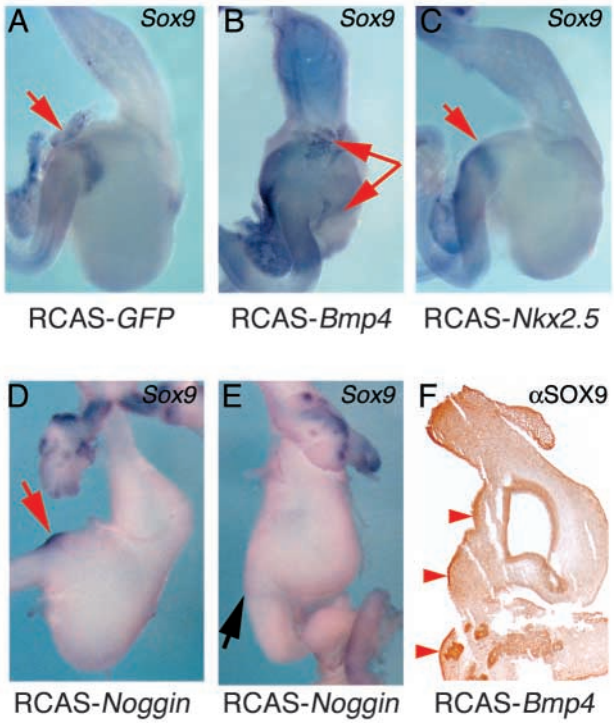

Fig. 5. Modulation of the BMP signaling pathway in the stomach affects *Sox9* expression. Whole-mount in situ hybridization using antisense *Sox9* riboprobe on *GFP*- (A), *Bmp4*- (B), *Nkx2.5*- (C) and *Noggin*- (D,E) misexpressing E8 stomachs. The morphological change of *Bmp4*-misexpressing stomach is characterized by a reduced musculature of the gizzard (compare B with A). *Noggin* misexpression in the stomach gives rise to a range of phenotypes (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). Neither *GFP* (A) as control nor *Nkx2.5* (C) misexpression affects stomach morphology. *Sox9* expression is upregulated in *Bmp4*-misexpressing stomach (B), strongly downregulated in *Noggin*-misexpressing stomachs (D,E) and not affected in *Nkx2.5*- (C) or *GFP*- (A) misexpressing stomachs. Red and black 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).

able to respond to BMP4 activation (red arrowheads, Fig. 5F). In similar experiments, Smith and Tabin (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 et al., 2000a), we observed that *Nkx2.5* misexpression in the stomach region did not affect the morphology of the stomach 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 5B). E8 *Noggin*-misexpressing stomachs develop a range of phenotypes from moderate to severe (Fig. 5D,E). Moderate phenotype was associated with a mild stomach/duodenum boundary perturbation associated with downregulation of *Sox9* expression (compare Fig. 5D with 5A). Severe phenotype was marked by a gross pyloric defect and correlated with the inhibition of *Sox9* expression in the malformed pyloric structure (compare Fig. 5E with 5A).

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 have epithelial morphologic differences. The gizzard epithelial cells have a keratin layer and long cilia allowing resistance to the abrasive grinding (Fig. 6A). The pyloric sphincter epithelial cells have bulbous cilia without the keratin layer seen in the gizzard (Fig. 6B). *SOX9* misexpression in the mesoderm of the gizzard modified the gizzard epithelium to a pyloric sphincter-like epithelium with bleb-like cilia (compare Fig. 6C with 6A). This epithelial transformation was not due to the expression of ectopic *SOX9* in the gizzard epithelium as α3C2 and αSOX9 detections demonstrated that retroviral infection was restricted to the gizzard mesoderm (Fig. 6C inset, see Figs S1 and S2 at http://dev.biologists.org/supplemental). 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) (P.d.S.B., unpublished). *Pdx1* is a marker of the duodenum, pancreas and pyloric epithelia, but *Pdx1* expression is not present in the stomach epithelium (Grapin-Botton et al., 2001) (Fig. 6E). Abnormal PDX1 expression in stomach has been described associated with the pathologic presence of pseudopyloric glands in humans (Sakai et al., 2004). *SOX9* misexpression in the gizzard did not modify the expression of *Shh*, *Sox2* and *Gata4* markers (data not shown). However, in *SOX9*-misexpressing gizzards, 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 misexpress a mutant form of *SOX9* deleted of this C-terminal domain (SOX9ΔCter). This deleted mutant form is the one found in human campomelic individuals (Sudbeck et al., 1996). We and others have shown previously that this mutant form is 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 behaves as a dominant-negative inhibitor of SOX9 action. Misexpression of SOX9ΔCter in the pyloric mesoderm showed no

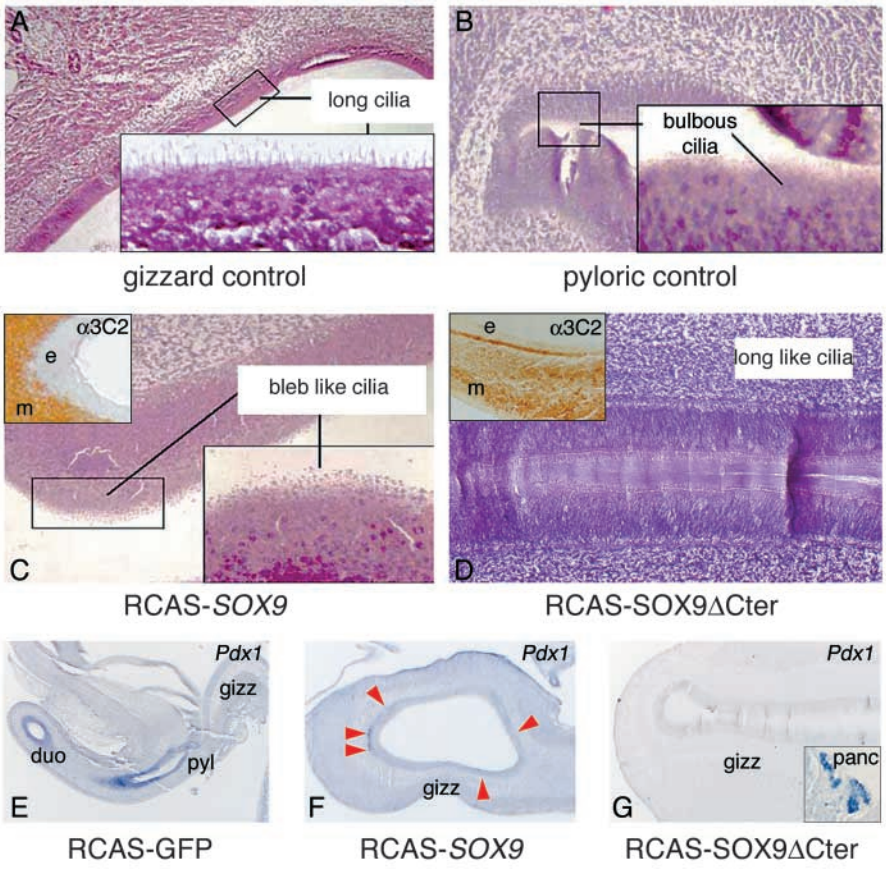

Fig. 6. Misexpression of *SOX9* in the gizzard mesoderm specifies the gizzard epithelium into pyloric epithelium. Histological sections of control E9 stomach (A,B), *SOX9*- (C) and SOX9ΔCter- (D) misexpressing E9 stomachs. Immunohistochemistry analyses with α3C2 antibodies confirmed that the infection was restricted to the mesoderm of the stomach (inset in 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). Upon mesodermal *SOX9* misexpression, the gizzard epithelial cells present bleb-like cilia (C), while after mesodermal SOX9ΔCter misexpression, the pyloric epithelial cells present keratin long like cilia (D). (E-G) Analyses of *Pdx1* expression by in situ hybridization on section of *GFP*- (E), *SOX9*- (F) and SOX9ΔCter- (G) misexpressing stomachs using antisense *Pdx1* riboprobe. There is normal expression of *Pdx1* in the pyloric endoderm. Ectopic *Pdx1* expression is detected in *SOX9*-misexpressing gizzard epithelium (red arrowheads, F). In SOX9ΔCter-misexpressing stomach, no *Pdx1* expression is detected in the gizzard epithelium and downregulation of *Pdx1* is observed in the pyloric epithelium (G). Note the expression of *Pdx1* in the pancreas in these SOX9ΔCter-misexpressing stomach (inset, G). duo, duodenum; e, endoderm; gizz, gizzard; m, mesoderm; panc, pancreas; pyl, pyloric structure.

phenotypic modification of mesoderm, but a clear perturbation of the pyloric epithelium (Fig. 6D). SOX9ΔCter misexpression in the mesoderm of the pyloric structure modified the pyloric epithelium to a gizzard-like epithelium with keratin long cilia (compare Fig. 6D with 6B). In addition and by contrast with the full-length *SOX9* misexpression, no obvious epithelial phenotype was observed in the gizzard epithelium, suggesting that the transactivation domain of SOX9 is required to transdifferentiate the gizzard epithelium. SOX9ΔCter misexpression in the stomach did not modify the expression of *Shh*, *Sox2* and *Gata4* markers (data not shown). However, in SOX9ΔCter-misexpressing stomach, we observed a significant decrease of epithelial *Pdx1* expression in the infected pyloric sphincter, indicating that the transformed pyloric epithelium lost pyloric epithelium features (Fig. 6G).

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 and Tabin, 1999). *Wnt11* expression, which is restricted to the pyloric sphincter mesoderm, was not affected in *SOX9* and SOX9ΔCter-misexpressing stomach (data not shown). We observed that *Gremlin*, a modulator of the BMP pathway, was expressed in the mesoderm of the pyloric sphincter (Fig. 7A,C). Anterior misexpression of *Gremlin* in the gizzard mesoderm induced no gross morphological phenotype, but, as does *SOX9*, did induce an epithelial phenotype characterized by the presence of pyloric bleb like cells in the gizzard epithelium (compare Fig. 7B with Fig. 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 SOX9ΔCter were misexpressed in the stomach and *Gremlin* expression was monitored by in situ hybridization (Fig. 7D,E). We observed an ectopic *Gremlin* expression in the mesoderm of the gizzard with full-length *SOX9* (red arrowheads, Fig. 7D). This was not detected in SOX9ΔCter-misexpressing stomach (Fig. 7E). Nevertheless, we observed a decrease of mesodermal *Gremlin* expression in the pyloric structure upon SOX9ΔCter misexpression (black arrowheads, Fig. 7E). Finally, we found that *Sox9* expression was not affected in *Gremlin*-misexpressing gizzard (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.

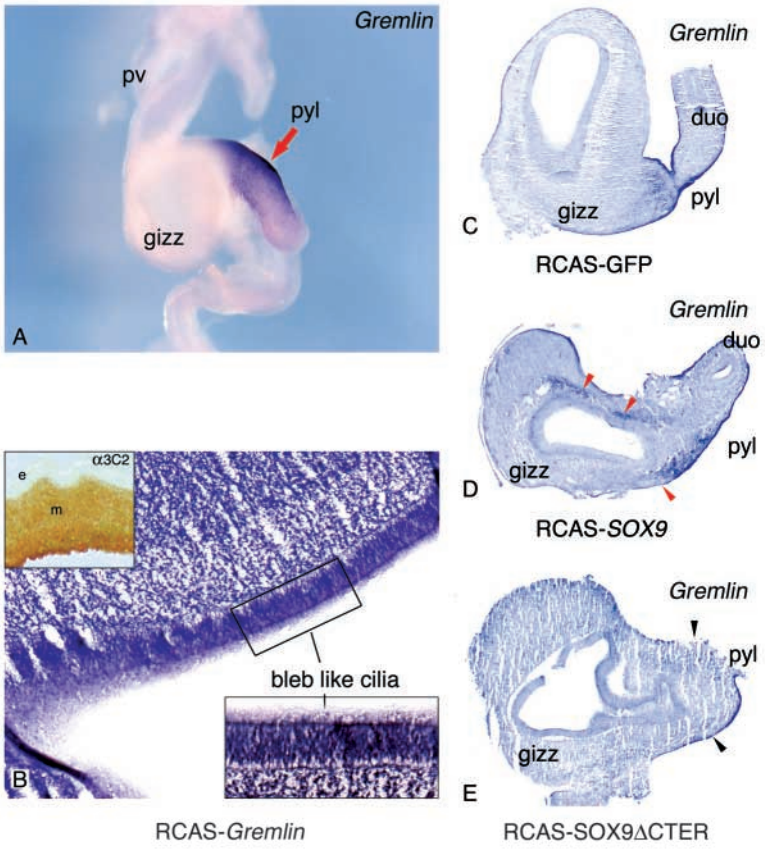

Fig. 7. *Gremlin* modulation of mesenchymal-epithelial interaction in the pyloric sphincter is under SOX9 control. (A) Whole-mount in situ hybridization of E7 dissected gut using antisense *Gremlin* riboprobe. *Gremlin* expression strongly demarcates the stomach from the duodenum and is present in the mesoderm of the pyloric sphincter (red arrow, A). (B) Histological section of *Gremlin*-misexpressing E9 gizzard. After mesodermal *Gremlin* misexpression, the gizzard epithelial cells present bleb like cilia (B). Immunohistochemistry with α3C2 antibodies show retrovirus infection restricted to the mesoderm of the gizzard (upper inset B). *GFP*- (C), *SOX9*- (D) and SOX9ΔCter- (E) misexpressing E7 stomach sections followed by detection of *Gremlin* expression by in situ hybridization using antisense *Gremlin* riboprobe. *Gremlin* is expressed 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 gizzard (red arrowheads, D). Downregulation of *Gremlin* expression is correlated with retroviral SOX9ΔCter misexpression in the pyloric sphincter (black arrowheads, E), while *Gremlin* expression is normal in the adventitia. duo, duodenum; e, endoderm; gizz, gizzard; m, mesoderm; pv, proventriculus; pyl, pyloric structure.

Table 1. Summary of SOX9 sites of expression in the developing chick GI tract

	Five days old		Nine days old	
GI level	Mesoderm	Endoderm	Mesoderm	Endoderm
Esophagus	–	+++	–	+++
Stomach	–	–	–	–
Pyloric sphincter	+++	–	+++	–
Midgut	–	+	–	+++
Caeca	+++	++	+	+++
Hindgut	–	+++	–	++

–, no detection; +, weak expression; ++, moderate expression; +++, strong expression.

Discussion

SOX9 expression in the gastrointestinal system is conserved during evolution

In early studies, SOX9 expression has been mainly observed in chondrocytes, neural crest cells and genital ridges (Healy et al., 1999; Spokony et al., 2002; de Santa Barbara et al., 2000). Here, we have 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). As SOX9 is later expressed in the proliferative compartment of the villi (Fig. 3), this suggests 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 structure that allows food to be grinded in the stomach before flowing in the small intestine. SOX9 strongly demarcates the nascent boundary between the stomach and the duodenum (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 Fig. 3; 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

Recently, chick embryos have widely been 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 development of the pyloric sphincter, we decided to use the specific avian retrovirus mediated gene expression technique, which we previously showed to be useful to target and express a transgene into the stomach mesoderm (Smith et al., 2000a; 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

(SOX9ΔCter), 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 the expression of *Pdx1*, an epithelial pyloric marker (Sakai et al., 2004), strongly suggesting that SOX9 specifies the pyloric sphincter epithelium (Fig. 6). Furthermore, we showed that this effect was mediated by an indirect mechanism using transcriptional regulation of diffusible factor 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 stomach/duodenum boundary involved a highly molecular regulated process as *Bmp4* (Smith and Tabin, 1999) and *Nkx2.5* (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 show herein that activation and inhibition of BMP signaling pathway by viral misexpression modulated *Sox9* expression in the stomach (Fig. 5). Gizzard retroviral misexpression of *Nkx2.5* leads to epithelial phenotype similar to *SOX9* misexpression [compare Smith et al., 2000 (Smith et al., 2000a) with Fig. 6]. Interestingly, misexpression of *SOX9* and SOX9ΔCter 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 two genes. We propose the following model in which BMP signaling pathway regulates *Sox9* and *Nkx2.5* expression in the pyloric sphincter (Fig. 8). These two transcription factors could act independently or interact to specify the pyloric sphincter epithelium. In support of this, genetic interactions between SOX and Nkx2 family genes were reported in *Drosophila* to promote neuroblast formation (Zhao and Skeath, 2002).

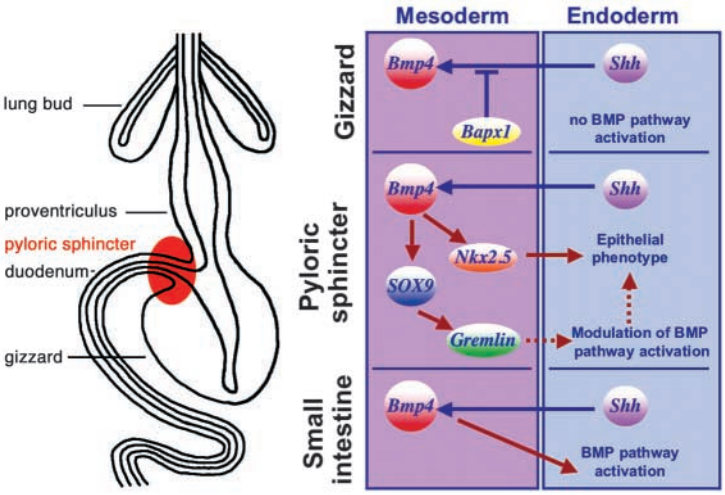

Fig. 8. Model of the molecular pathways and their potential interactions involved during the development of the pyloric sphincter. Schematic representations of avian stomach (left panel) and the molecular pathways involved (right panel). The avian stomach can be divided in proventriculus (glandular stomach) and gizzard (muscular stomach). The pyloric sphincter is a highly conserved structure present in all vertebrates, which (in avians) anatomically separates the gizzard from 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 (S.F. and P.d.S.B., unpublished). 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.

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 (S.F. and P.d.S.B., unpublished). Gremlin could act in this structure to modulate 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). Connections between BMP signaling pathway and SOX factors might be a reiterated process during development, as it was also demonstrated in vertebrate limb (Chimal-Monroy et al., 2003) and *Drosophila* CNS (Cremazy et al., 2000) development.

In summary, our work reveal new functions of the transcription factor SOX9 during the GI tract development. SOX9 patterns the pyloric sphincter and specifies the pyloric sphincter epithelium at least by regulating the expression of *Gremlin*, a diffusible factor that modulates BMP pathway. As we previously commented, molecular controls of GI development patterning events are remarkably conserved across species. We hypothesize that the described molecular mechanisms that control pyloric sphincter development are conserved in human and that alterations of these mechanisms may account for malformations such as hypertrophic pyloric stenosis (Ohshiro and Puri, 1998).

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