The septum transversum mesenchyme induces gall bladder development

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Summary
The liver, gall bladder, and ventral pancreas are formed from the posterior region of the ventral foregut. After hepatic induction, Sox17+/Pdx1+ pancreatobiliary common progenitor cells differentiate into Sox17+/Pdx1– gall bladder progenitors and Sox17–/Pdx1+ ventral pancreatic progenitors, but the cell-extrinsic signals that regulate this differentiation process are unknown. This study shows that the septum transversum mesenchyme (STM) grows in the posterior direction after E8.5, becoming adjacent to the presumptive gall bladder region, to induce gall bladder development. In this induction process, STM-derived BMP4 induces differentiation from common progenitor cells adjacent to the STM into gall bladder progenitor cells, by maintaining Sox17 expression and suppressing Pdx1 expression. Furthermore, the STM suppresses ectopic activation of the liver program in the posterior region of the ventral foregut following hepatic induction through an Fgf10/Fgfr2b/Sox9 signaling pathway. Thus, the STM plays pivotal roles in gall bladder development by both inductive and suppressive effects.

Introduction
The liver, gall bladder, and ventral pancreas emerge from the ventral foregut endoderm during development. Shortly after the liver begins to form, the gall bladder and ventral pancreas form from the remaining ventral foregut endoderm. During organogenesis, various genes expressed in the ventral foregut endoderm play important roles in a cell-autonomous manner. For instance, Pdx1 (Offield et al., 1996), Ptf1a (Kawaguchi et al., 2002; Krapp et al., 1998), Hhex (Bort et al., 2004; Hunter et al., 2007), Hnf6 (Clotman et al., 2002), Hes1 (Sumazaki et al., 2004), and Sox17 (Spence et al., 2009; Uemura et al., 2010) are important for the formation of the gall bladder and/or ventral pancreas. Sox17 functions as a master regulator of the differentiation of ventral foregut endodermal cells into gall bladder progenitor cells. In addition, interconversion between the gall bladder and the ventral pancreas, or between the ventral pancreas and the duodenum occurs in Sox17–, Hes1–, or Ptf1a-deficient mice (Kawaguchi et al., 2002; Spence et al., 2009; Sumazaki et al., 2004), indicating that these genes function cell-autonomously in the region-specific formation of individual organs.

In the absence of the cardiac mesoderm, the isolated mouse ventral foregut endoderm activates the pancreas marker, Pdx1, but not the liver marker, Alb (Deutsch et al., 2001), illustrating that not only cell-autonomous functions of genes expressed in the ventral foregut endoderm, but also interactions between the ventral foregut endoderm and the adjacent mesenchyme, play important roles in region-specific organogenesis. As mesenchyme-derived factors involved in these interactions, FGF from the cardiac mesoderm (Jung et al., 1999) and BMP from the STM (Rossi et al., 2001) play important roles in hepatic induction. After hepatic induction, pancreatobiliary common progenitor cells (Sox17+, Pdx1+) segregate into gall bladder progenitor (Sox17+, Pdx1–) and ventral pancreatic progenitor (Sox17–, Pdx1+) cells in a Sox17-dependent manner (Spence et al., 2009). However, it is unclear which extrinsic signals enable lineage segregation from pancreatobiliary common progenitor cells by regulating Sox17 and Pdx1 expression.

Pancreatic induction occurs in the region where FGF is either low or absent (Deutsch et al., 2001), that is, in the region separated from the cardiac mesoderm that expresses Fgf. In Hhex-null embryos, pancreas formation does not occur in the ventral foregut unless the ventral foregut is isolated from the adjacent mesenchyme (Bort et al., 2004). This is possibly because the nascent pancreatic region is not well separated from the cardiac mesoderm in Hhex-null embryos due to elongation defects in the ventral foregut resulting from decreased cell proliferation. These results indicate that organogenesis in the ventral foregut endoderm is induced when the appropriate region of the ventral foregut endoderm receives mesenchyme-derived signals at the proper time.

The STM originates from the lateral plate mesoderm (Douarin, 1975; Fukuda-Taira, 1981; Sherer, 1975) and is adjacent to the...
ventral foregut endoderm during ventral foregut-derived organogenesis. Gata4 (Watt et al., 2004) and Mab21l2 (Saito et al., 2012) are essential for STM formation. Gata4 and Mab21l2 are expressed in the STM, and in Gata4- and Mab21l2-deficient embryos, defective morphogenesis of the STM occurs. The STM is involved not only in hepatic induction, but also in the growth and survival of hepatoblasts, which are bipotential progenitors for hepatocytes and cholangiocytes (Zaret, 2002), demonstrating that the STM is important for liver development. However, it is not clear from previous studies using Gata4- or Mab21l2-deficient mice what roles the STM plays during gall bladder and ventral pancreas development. Therefore, the aim of this study was to determine at which point during development the STM is adjacent to the nascent gall bladder and/or ventral pancreas in the ventral foregut, and how the STM is involved in the development of these organs using Mab21l2-null embryos as a model for STM loss.

In this study, we show that the STM becomes adjacent to the nascent gall bladder region after embryonic day 9.0 (E9.0). This cellular event triggers the differentiation of pancreatobiliary common progenitor cells into gall bladder progenitor cells by maintaining Sox17 expression and suppressing Pdx1 expression. Moreover, after hepatic induction, STM-derived signals act on the nascent gall bladder and ventral pancreas regions to suppress ectopic induction of the liver program. Taken together, these observations indicate that following hepatic induction, the STM induces gall bladder development, while suppressing ectopic activation of the liver program in the posterior region of the ventral foregut.

Materials and Methods

Mice

The generation of the mutant mice used in this study has been reported previously (Yamada et al., 2004). Mice were backcrossed to the ICR strain. Knockout embryos are more readily obtained via the interbreeding of heterozygous mice using the ICR strain, because the litter size of the ICR strain is larger than that of the B6 strain. There are no differences in phenotype or lethality rates at each developmental stage between the ICR and B6 strains. Mice were maintained in accordance with protocols approved by the Animal Care and Use Committee of the University of Tokyo.

In situ hybridization

Whole-mount in situ hybridization was performed as described previously (Nieto et al., 1996) at 65˚C in 50% formamide containing 5×SSC. Paraffin sections were hybridized in situ at 65˚C in 50% formamide, 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 0.2% Sarkosyl, 1×Denhardt’s solution, 10% dextran sulfate, and 0.5 mg/ml yeast tRNA. All probes were labeled with digoxigenin using standard procedures. Details for probes will be provided upon request.

Histology

Embryos were dissected in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS overnight at 4˚C. Fixed embryos were dehydrated through a graded ethanol series. Sections were boiled in 10 mM citrate buffer (pH 6.0) for 10 min and washed with PBS. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in TBS for 10 min. After washing with PBS, sections were incubated with HRP-conjugated goat anti-rabbit IgG and 1% BSA in PBS for 1 hour. Immunoreactive sites were visualized with DAB and 0.5% H2O2. Sections were counterstained with hematoxylin.

Detection of proliferating or apoptotic cells

Paraffin sections were deparaffinized with xylene and dehydrated through a graded ethanol series. Sections were boiled in 10 mM citrate buffer (pH 6.0) for 10 min with washed with PBS. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in TBS for 10 min. After washing with PBS, sections were incubated with 1/400 anti-PHH3 antibody (CST) overnight. After washing with PBS, sections were incubated with HRP-conjugated goat anti-rabbit IgG and 1% BSA in PBS for 1 hour. Immunoreactive sites were visualized with DAB and H2O2. Sections were counterstained with hematoxylin.

TdT-mediated dUTP nick-end labeling (TUNEL) analysis was performed as follows. Paraffin sections (8 μm thick) of embryos were incubated in 3% H2O2 for 15 min, Proteinase K solution for 10 min, and then TdT reaction solution (0.2 mM fluorescein-12-dUTP (Roche), 0.2 mM dATP, 1 mM CoCl2, 30 mM Tris-HCl (pH 7.5), 140 mM sodium cacodylate, and 40 U terminal deoxynucleotidyl transferase (Roche)). Reactive sites were detected with an alkaline phosphatase-conjugated anti-fluorescein antibody (Roche) in a solution containing a phosphatase substrate (Fast Red Tablets, Roche). Sections were dehydrated via a graded ethanol series. Sections were boiled in 10 mM citrate buffer (pH 6.0) for 10 min and then TdT reaction solution (0.2 mM fluorescein-12-dUTP (Roche), 0.2 mM dATP, 1 mM CoCl2, 30 mM Tris-HCl (pH 7.5), 140 mM sodium cacodylate, and 40 U terminal deoxynucleotidyl transferase (Roche)). Reactive sites were detected with an alkaline phosphatase-conjugated anti-fluorescein antibody (Roche) in a solution containing a phosphatase substrate (Fast Red Tablets, Roche). Sections were counterstained with hematoxylin.

Whole-embryo culture

E9.0 embryos were dissected from the uteri for in vitro culture. All littermate embryos were then cultured for 6 hours in medium (DMEM, Gibco) containing 4 mM Noggin (Peprotech), 20 mM BMP4 (R&D Systems), and BSA at 37˚C in the presence of 5% CO2. Thereafter, embryos were fixed in 4% paraformaldehyde.

Results

After E9.0, the STM is adjacent to the Sox17, but not the Pdx1, expressing region of the foregut

During gall bladder and ventral pancreas development, pancreatobiliary common progenitor cells expressing both Sox17 and Pdx1 differentiate into Sox17+/Pdx1− gall bladder progenitor cells, and Sox17−/Pdx1+ ventral pancreatic progenitor cells (Spence et al., 2009). However, how this differentiation process is regulated is unknown. Interactions between the endodermal epithelium and adjacent mesenchyme are important during ventral foregut-derived organ development, and mesenchyme-derived signals regulate organ induction in the ventral foregut (Deutsch et al., 2001; Jung et al., 1999; Rossi et al., 2001). Therefore, we examined the relationship between the expression of Sox17 and Pdx1 and the position of the adjacent mesenchyme. At E8.5, pancreatobiliary common progenitor cells expressed both Sox17 and Pdx1 (Fig. 1A,B) (Spence et al., 2009); Sox17+/Pdx1− ventral foregut endodermal cells emerged at E9.0 (Fig. 1C,D, red arrowhead). At E9.5, Sox17 was expressed in the gall bladder primordium and the anterior region of the ventral pancreatic bud, and Pdx1 was expressed in the ventral pancreatic bud and the posterior region of the gall bladder primordium (Fig. 1E,F). At E10.5, Sox17 and Pdx1 were specifically expressed in the gall bladder primordium and in the ventral pancreatic bud, respectively (Fig. 1G,H). These results suggest that after E9.0, the expression of Sox17 and Pdx1 is differentially regulated in the posterior region of the ventral foregut, resulting in the tissue-specific separation of expression of these two factors between E9.5 and E10.5. Following hepatic induction from E8.0 to E8.5, the STM is adjacent to the ventral foregut endoderm (Rossi et al., 2001). We examined the positional relationship between the Sox17+ and Pdx1+ region and the STM. At E8.5, the region expressing Wt1, an STM marker, was separated from the Sox17-expressing region (Fig. 1J), suggesting that the STM is not adjacent to pancreatobiliary common progenitor cells, that is, the Sox17-expressing region. At E9.0, the Wt1-expressing region became juxtaposed to the Sox17-expressing region (Fig. 1K,L). At E9.25 and E9.5, Wt1 was expressed adjacent to the Sox17-expressing region (Fig. 1M–P), while Wt1 was not expressed near the Pdx1-expressing region (Fig. 1Q–T). These results show that after E9.0, the STM becomes adjacent to the Sox17-expressing region, but not the Pdx1-expressing region.

In Mab21l2-deficient embryos, complete loss of the STM occurs near the posterior region of the ventral foregut after E9.0

We have shown that Mab21l2 is expressed in the STM after E8.5. In E9.5 Mab21l2-deficient embryos, morphogenesis of the STM was defective, resulting in defective morphogenesis of the liver (Saito et al., 2012). However, it is unclear when defects in STM formation occur. We first examined Mab21l2 expression in the STM before E9.5. Mab21l2 was expressed in the Wt1-expressing...
region from E8.5 to E9.0 (Fig. 2A–D), showing that Mab21l2 is expressed in the STM after E8.5 and is involved in STM formation. We next examined the time at which defective morphogenesis of the STM occurred. At E8.5, Wt1 expression was detected near the nascent foregut in Mab21l2-deficient and wild-type embryos (Fig. 2E,F; red arrowheads), demonstrating that STM formation is normal at E8.5. At E9.0, Sox17 expression was detected near the posterior region of the ventral foregut in wild-type embryos, but not in Mab21l2-deficient embryos

(Fig. 2G,H; red arrowhead), showing that a complete loss of the STM occurs near the posterior region of the ventral foregut in Mab21l2-deficient embryos after E9.0. Near the ventral foregut, the STM and endothelial cells were present. At E9.5, Flk1, an endothelial cell marker, was expressed adjacent to the gall bladder primordium and the ventral pancreatic bud, indicating that endothelial cells are present next to these regions (supplementary material Fig. S1A). Moreover, Flk1-positive endothelial cells appeared normal in Mab21l2-deficient embryos.
compared to wild-type embryos at E9.0 (supplementary material Fig. S1B,C), indicating that the STM is only lost near the posterior region of the ventral foregut in Mab21l2-deficient embryos. Therefore, we examined the function of the STM in organogenesis in the posterior region of the ventral foregut after E9.0 using Mab21l2-deficient embryos as a model of STM loss.

Defective morphogenesis of the extrahepatic biliary system and gall bladder occurs in Mab21l2-deficient embryos
If the STM influences the differentiation of the pancreatobiliary common progenitor cells, then STM loss should affect the formation of the gall bladder and/or ventral pancreas. We examined the formation of the gall bladder and ventral pancreas in Mab21l2-deficient embryos. At E10.5, the formation of the liver, gall bladder, and ventral pancreas was detected morphologically in wild-type embryos (Fig. 3A). By contrast, the gall bladder primordium was absent in Mab21l2-deficient embryos, although a small liver and normal ventral pancreatic bud were observed (Fig. 3B). At E12.5, the formation of the extrahepatic biliary system, including the gall bladder, was observed in wild-type embryos (Fig. 3C), but not in Mab21l2-deficient embryos (Fig. 3D). We next examined gall bladder and pancreas formation in Mab21l2-deficient embryos using Sox17 as a marker of the gall bladder and Pdx1 as a marker of the pancreas.

Fig. 2. Mab21l2-deficient embryos exhibit defective morphogenesis of the STM after E9.0. In situ hybridization for the indicated transcripts was conducted using paraffin sections of wild-type and Mab21l2 mutant embryos. (A–D) Sagittal serial sections showing that at E8.5 (A,B), Mab21l2 was expressed in the Wt1-expressing STM near the nascent foregut, and at E9.0 (C,D), Mab21l2 was expressed in the Wt1-expressing STM near the ventral foregut. (E–H) Sagittal sections of wild-type and Mab21l2-deficient embryos showing that at E8.5 (E,F), Wt1 expression was detected near the nascent foregut in Mab21l2-deficient embryos, similarly to in wild-type embryos (red arrowheads). At E9.0 (G,H), Wt1 expression was detected in the STM near the ventral foregut in wild-type embryos, but not in Mab21l2-deficient embryos (red arrowhead). h, heart; a, atrium; v, ventricle; st, septum transversum mesenchyme; fg, foregut; sv, sinus venosus. Scale bars: 30 μm.

Fig. 3. The extrahepatic biliary system, including the gall bladder, is not formed in Mab21l2-deficient embryos. (A–D) Hematoxylin and eosin (H&E)-stained paraffin sections. (E–L) In situ hybridizations of paraffin sections. (A–D) Sagittal sections of wild-type and Mab21l2-deficient embryos at E10.5 (A,B), showing the gall bladder primordium and ventral pancreatic bud in wild-type embryos; the gall bladder primordium was lost in Mab21l2-deficient embryos. At E12.5 (C,D), the extrahepatic biliary system, including the gall bladder, and cystic and common bile ducts, was observed in wild-type embryos, but not in Mab21l2-deficient embryos. (E–H) Transverse sections of wild-type and Mab21l2-deficient embryos showing that at E9.5 (E,F), the Sox17-positive gall bladder primordium emerged from the hepatic diverticulum in wild-type embryos (red arrowhead), but not in Mab21l2-deficient embryos. At E10.5 (G,H), the Sox17-positive gall bladder primordium was observed in wild-type embryos (red arrowhead), but not in Mab21l2-deficient embryos. (I–L) Transverse sections of E10.5 wild-type and Mab21l2-deficient embryos showing that Pdx1 + ventral and dorsal pancreatic buds were observed in Mab21l2-deficient embryos, similarly to in wild-type embryos. gb, gall bladder primordium; vp, ventral pancreatic bud; m, midgut; d and du, duodenum; cd, cystic duct; cbd, common bile duct; hd, hepatic diverticulum; dp, dorsal pancreatic bud. Scale bars: 50 μm.
At E9.5, a Sox17+ gall bladder primordium arose from the hepatic diverticulum in wild-type embryos (Fig. 3E; red arrowhead), but not in Mab21l2-deficient embryos (Fig. 3F). Similarly, at E10.5, the Sox17+ gall bladder primordium was observed in wild-type embryos (Fig. 3G; red arrowhead), but not in Mab21l2-deficient embryos (Fig. 3H). Moreover, the formation of ventral and dorsal pancreas was normal in Mab21l2-deficient embryos at E10.5 (Fig. 3J,L), similar to wild-type embryos (Fig. 3I,K). These results reveal that the STM is required for formation of the gall bladder, but not the pancreas.

Reduced expression of Sox17 and ectopic expression of Pdx1 in Mab21l2-deficient embryos

Loss of the gall bladder in Mab21l2-deficient embryos suggested that defects occurred during the differentiation of pancreaticobiliary common progenitor cells into gall bladder progenitor cells. As Sox17 plays an essential role in this process (Spence et al., 2009), we examined the expression of Sox17 in the ventral foregut. At E8.5, the expression of Sox17 in wild-type embryos (Fig. 4A) was indistinguishable from that in Mab21l2-deficient embryos (Fig. 4B). At E9.0, the expression of Sox17 was significantly reduced in the ventral foregut in Mab21l2-deficient embryos (Fig. 4D; red arrowhead) compared to wild-type embryos (Fig. 4C). To examine whether reduced expression of Sox17 resulted from decreased expression of Sox17 in the ventral foregut endodermal cells, or from decreased proliferation and/or increased apoptosis of Sox17+ endodermal cells, proliferating and apoptotic cells in the ventral foregut were examined by immunohistochemistry using the mitosis marker phospho-histone H3 (PHH3) and a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay, respectively. At E9.0, there were no differences in PHH3-positive nuclei in the ventral foregut between wild-type (supplementary material Fig. S2A,C) and Mab21l2-deficient embryos (supplementary material Fig. S2B,C), indicating that the proliferation of ventral foregut endodermal cells was normal in Mab21l2-deficient embryos.

Similarly, the number of TUNEL-positive cells was unchanged in the ventral foregut in Mab21l2-deficient and wild-type embryos at E9.0 (data not shown). Moreover, elongation defects in the ventral foregut were not detected in Mab21l2-deficient embryos (Fig. 2H, Fig. 4D) compared to wild-type embryos (Fig. 2G, Fig. 4C) at E9.0, indicating that the reduced expression of Sox17 in the ventral foregut resulted from the reduced expression of Sox17 in endodermal cells, and that the STM is required for the maintenance of Sox17 in the ventral foregut. At E9.25, the expression of Pdx1 was unchanged in the ventral foregut in Mab21l2-deficient embryos (supplementary material Fig. S3B) compared to wild-type embryos (supplementary material Fig. S3A), demonstrating that the STM is not required for the maintenance of Pdx1 in the ventral foregut. We next examined the specificity of the Sox17- and Pdx1-expressing regions at E9.75 when Sox17 and Pdx1 are expressed in the gall bladder primordium and ventral pancreatic bud, respectively. In Mab21l2-deficient embryos, Sox17 was only expressed in the anterior region of the ventral pancreatic bud (Fig. 4G). Pdx1 was expressed in the ventral pancreatic bud and in the region where Sox17 was intrinsically expressed (Fig. 4H; red arrowhead) compared to wild-type embryos (Fig. 4E,F), showing that the STM is required for the suppression of Pdx1 in the presumptive gall bladder region. We next examined the expression of genes with important roles in gall bladder formation, which were also expressed in the ventral foregut endoderm. At E9.25, the expression of Hhex, Hnf6, and Hes1 in Mab21l2-deficient embryos was indistinguishable from that in wild-type embryos (supplementary material Fig. S3C–H). These results illustrate that gall bladder loss resulted specifically from defects in Sox17 and Pdx1 expression.

BMP4 influences the expression of Sox17 and Pdx1

Our results suggest that STM-derived signals regulate Sox17 and Pdx1 expression in the ventral foregut during differentiation of pancreaticobiliary common progenitor cells. However, the cell-extrinsic signals that regulate Sox17 and Pdx1 during this

Fig. 4. Defects in Sox17 and Pdx1 expression occur in Mab21l2-deficient embryos. **In situ** hybridization for the indicated transcripts was conducted using paraffin sections of embryos. (A–D) Sagittal sections of wild-type and Mab21l2-deficient embryos showing that at E8.5 (A,B), Sox17 expression was detected in the nascent foregut in Mab21l2-deficient embryos, similarly to in wild-type embryos. At E9.0 (C,D), Sox17 was expressed in the ventral foregut in wild-type embryos, but Sox17 expression was significantly reduced in the ventral foregut in Mab21l2-deficient embryos (red arrowhead). (E–H) Sagittal serial sections of an E9.75 embryo showing that in wild-type embryos (E,F), Sox17 was specifically expressed in the gall bladder primordium, and Pdx1 in the ventral pancreatic bud; in Mab21l2-deficient embryos (G,H), Sox17 was only expressed in the anterior portion of the ventral pancreatic bud, and Pdx1 was expressed not only in the ventral pancreatic bud, but also in the region where Sox17 was intrinsically expressed (red arrowhead). a, atrium; v, ventricle; fg, foregut; h, heart; st, septum transversum mesenchyme; gbp, gall bladder primordium; vp, ventral pancreatic bud; du, duodenum; dp, dorsal pancreatic bud. Scale bars: 30 μm (A–D), 50 μm (E–H).
Haploinsufficiency of FoxF1 results in defects in gall bladder formation because FoxF1 encodes a forkhead transcription factor, is expressed in the STM, and plays an important role in gall bladder formation. Previous studies have shown that expression of Bmp4 is regulated by FoxF1 (Mahlapuu et al., 2001), and that the BMP receptors BMPRIA, BMPRII, and ActRIIA are expressed in the ventral foregut endoderm (Mishina et al., 1995; Roelen et al., 1997). At E9.0, Bmp4 is expressed in the STM in wild-type embryos (Fig. 5A,B), but the expression of Bmp4 was not detected near the posterior region of the ventral foregut in Mab21l2-deficient embryos (Fig. 5C,D; red arrowhead), suggesting the possibility that STM-derived BMP4 may regulate the expression of Sox17 and Pdx1 in the ventral foregut endoderm. To test this hypothesis, we performed several experiments using whole-embryo cultures. We cultured E9.0 embryos for 6 hours in medium containing 4 nM Noggin, a BMP antagonist, and evaluated its effects on Sox17 and Pdx1 expression. Noggin had a tendency to reduce the expression of Sox17 compared with embryos cultured in control medium (Fig. 5E,F) and the size of the Sox17-expressing region were the same in control and Noggin-treated embryos. These results indicate that BMPs are involved in the regulation of Sox17 in the ventral foregut endodermal cells. Noggin did not influence Pdx1 expression (data not shown). We next cultured E9.0 Mab21l2-deficient embryos in medium containing 20 nM BMP4 for 6 hours, to determine whether the expression of Sox17 could be rescued by BMP4. In Mab21l2-deficient embryos cultured in control medium containing BSA, the expression of Sox17 was significantly reduced in the Hhex-positive foregut region (Fig. 5J). By contrast, in Mab21l2-deficient embryos cultured in medium containing BMP4, Sox17 expression had a tendency to be rescued in the Hhex-positive foregut region (n=3; Fig. 5K,L) and was similar to wild-type embryos cultured in control medium (Fig. 5F). These results suggest that BMP4 is involved in the maintenance of Sox17 in the ventral foregut. In E9.0 wild-type embryos cultured in medium containing 20 nM BMP4 for 6 hours, BMP4 had a tendency to increase Sox17 expression moderately (Fig. 5O) and to reduce Pdx1 expression (Fig. 5P) compared with embryos cultured in control medium (n=3; Fig. 5M,N). These results demonstrate that BMP4 maintains Sox17 expression and suppresses Pdx1 expression in the ventral foregut.

STM loss results in ectopic Alb expression and defects in the Fgfl0/Fgrf2b/Sox9 signaling pathway in the posterior region of the ventral foregut

Previous studies have shown that FGF from the cardiac mesoderm and BMP from the STM induce liver development (Jung et al., 1999; Rossi et al., 2001), while hepatic induction is suppressed in the region where FGF is either low or absent (Deutsch et al., 2001). However, the role of the STM in suppressing liver development is not known, and we therefore examined the function of the STM in the posterior region of the ventral foregut. At E9.25, the expression of Alb was only detected in the anterior region of the Hhex-positive ventral foregut (i.e.,...
the presumptive liver region) in wild-type embryos (Fig. 6A,B). By contrast, the expression of Alb in the Hhex-positive ventral foregut was detected not only in the anterior region but also in the posterior region (i.e., the presumptive gall bladder and ventral pancreas region) in Mab21l2-deficient embryos (Fig. 6C,D; red arrowhead). Moreover, in E9.75 Mab21l2-deficient embryos, Sox17 was only expressed in part of the anterior region of the ventral pancreatic bud (Fig. 6G). Alb was expressed not only in the nascent liver, as in wild-type embryos (Fig. 6E,F), but also in the posterior region of the ventral foregut including the ventral pancreatic bud and the region where Sox17 was intrinsically expressed (Fig. 6H; red arrowhead). These results suggest that STM loss results in ectopic activation of the liver program in the presumptive gall bladder and ventral pancreas regions. Previous studies have shown hepatic induction to occur in the ventral and dorsal foregut (Bossard and Zaret, 1998; Bossard and Zaret, 2000; Gualdi et al., 1996). During pancreas development, the FGF signaling pathway suppresses the liver program in the ventral and dorsal pancreas through an Fgf10/Fgfr2b/Sox9 pathway (Seymour et al., 2012). As Fgf10 is expressed in the STM at E9.0 (Berg et al., 2007), this signaling pathway may suppress the liver program during the initial stages of gall bladder and ventral pancreas development. To examine when Fgf10 was expressed in the STM, we examined the expression of the STM marker Wt1 and of Fgf10 before E9.0. At E8.5, Fgf10 expression was not detected in the Wt1-expressing region (Fig. 6I; red arrowheads). At E9.0, Fgf10 expression was observed in the Wt1-expressing STM near the ventral foregut (Fig. 6K,L), as previously reported. These results indicate that Fgf10 expression in the STM begins at E9.0. At E9.25, Fgf10 was expressed in the STM, and Fgfr2b, the main receptor for Fgf10, was expressed in the ventral foregut in the Sox17-expressing region (Fig. 6M–P). Sox17 was not expressed in the Alb+ presumptive liver region (Fig. 6Q,R), indicating that Fgfr2b is specifically expressed in the posterior region of the ventral foregut endoderm, that is, in the nascent gall bladder and ventral pancreas, but not in the nascent liver. These results indicate that after E9.0, the FGF signaling pathway functions specifically in the presumptive gall bladder and ventral pancreas regions through Fgf10/Fgfr2b. Sox9, a downstream target of Fgf10/ Fgfr2b signaling (Seymour et al., 2012), was mainly expressed in the presumptive gall bladder and ventral pancreas regions, including the Sox17-expressing region (Fig. 6S,T). Thus, the FGF signaling pathway may function through Fgf10/Fgfr2b/Sox9 to suppress the liver program in the nascent gall bladder and ventral pancreas shortly after liver formation begins (Fig. 6U). At E9.25, the expression of Fgf10 was lost near the ventral foregut in Mab21l2-deficient embryos (Fig. 6W; red arrowhead) compared to wild-type embryos (Fig. 6V), and Sox9 expression was also significantly reduced in the ventral foregut endoderm (Fig. 6Y; red arrowhead) compared to wild-type embryos (Fig. 6X). These results suggest that after E9.0 (i.e., after hepatic induction), the STM suppresses ectopic activation of the liver program in the posterior region of the ventral foregut through the Fgf10/Fgfr2b/Sox9 signaling pathway.

Discussion

The STM is required for induction of gall bladder development Mab21l2 is expressed in the STM, but not in endothelial and ventral foregut endodermal cells. In Mab21l2-deficient embryos, the extrahepatic biliary system including the gall bladder was completely lost. At E9.0, the STM was completely absent near the posterior region of the ventral foregut, but normal endothelial cells were present. Previous studies have shown that the blood vessel endothelium plays an important role in the induction of pancreatic differentiation (Lammert et al., 2001) and that Flik1, which is expressed in endothelial cells adjacent to the liver bud, is required to promote liver bud growth (Matsumoto et al., 2001). These studies demonstrated that endothelial cells are essential for organogenesis of ventral foregut-derived tissues. However, the expression of Flik1 was unchanged in Mab21l2-deficient embryos compared to wild-type embryos at E9.0, suggesting that the endothelial cells are normal. Thus, our results suggest that gall bladder loss resulted from loss of the STM, not from defects in the endothelial cells. Therefore, the STM is essential for the induction of gall bladder development.

STM-derived BMP4 regulates differentiation of pancreatobiliary common progenitor cells into gall bladder progenitor cells by regulating Sox17 and Pdx1 expression

This study showed that Sox17 and Pdx1 were expressed in the same region, in the pancreatobiliary common progenitor cells, at E8.5, and that Sox17+/−/Pdx1− ventral foregut endodermal cells began to emerge at E9.0. These results indicate that after E9.0, the expression of Sox17 and Pdx1 in pancreatobiliary common progenitor cells is differentially regulated. Moreover, between E9.5 and E10.5, Sox17 expression is specifically detected in the gall bladder primordium and Pdx1 expression in the ventral pancreatic bud, showing that between E9.5 and E10.5 the differential regulation is complete. Our study also showed that after E9.0, the STM is adjacent only to the Sox17+/−, Pdx1− region, and is involved in the differentiation of pancreatobiliary common progenitor cells, most likely by maintaining Sox17 expression and suppressing Pdx1 expression. A previous study showed that the regulation of Sox17 and Pdx1 plays an important role in the differentiation of pancreatobiliary common progenitor cells into gall bladder progenitor cells and ventral pancreatic progenitor cells. Sox17 is a master regulator of gall bladder formation (Spence et al., 2009). Sox17 expression in Mab21l2-deficient embryos was reduced in the ventral foregut and Pdx1 was ectopically expressed in the region where Sox17 was intrinsically expressed, indicating that gall bladder loss in Mab21l2-deficient embryos resulted from defects in the regulation of Sox17 and Pdx1. Thus, these results show that the STM is required to maintain Sox17 expression and suppress Pdx1 expression in the ventral foregut; the STM induces gall bladder development by differentially regulating Sox17 and Pdx1.

Using whole-embryo cultures, we showed that Noggin reduced the expression of Sox17 in the Hhex-positive ventral region of wild-type embryos, while Sox17 expression in endothelial cells did not differ from expression in embryos cultured in control medium. This result suggests that Noggin specifically reduces Sox17 expression in the foregut endodermal cells. Furthermore, Hhex expression in the foregut region was unchanged, suggesting that reduced expression of Sox17 is unlikely to result from secondary effects of Noggin addition. Therefore, our results suggest that BMPs function to maintain Sox17 expression in the ventral foregut. Several BMPs are expressed in the STM including BMP2, BMP4, BMP5, and BMP7 (Hogan, 1996; Zhao, 2003). Previous studies have shown that BMP4 is regulated by FoxF1, which is expressed in the STM and is important for gall bladder development (Kalinichenko et al., 2002; Mahlapuu
Fig. 6. Ectopic Alb expression and defects in the Fgf10/Fgfr2b/Sox9 signaling pathway occur in Mab21l2-deficient embryos. (A–T,V–Y) In situ hybridization for the indicated transcripts was conducted using paraffin sections of embryos. (A–D) Sagittal serial sections of E9.25 wild-type and Mab21l2-deficient embryos showing that Alb was expressed only in the anterior portion of the Hhex-positive ventral foregut in wild-type embryos, but was expressed in both the anterior and posterior regions of the Hhex-positive ventral foregut in Mab21l2-deficient embryos (red arrowhead). (E–H) Sagittal serial sections of E9.75 embryos showing that Sox17 was expressed in the gall bladder primordium and Alb was expressed in the nascent liver in wild-type embryos (E,F), but Sox17 was expressed only in the anterior portion of the ventral pancreatic bud, and Alb was expressed not only in the nascent liver, but also in the posterior region of the ventral foregut, including the Sox17+ region of the ventral pancreatic bud in Mab21l2-deficient embryos (red arrowhead). (I–L) Sagittal serial sections showing that at E8.5 (I, J), Fgf10 expression was not detected in the Wt1-positive STM (red arrowhead). At E9.0 (K,L), Fgf10 began to be expressed in the Wt1-positive STM. (M,N) Sagittal serial sections of an E9.25 embryo showing that Fgf10 was expressed in the STM near the ventral foregut and that Fgfr2b was expressed in the ventral foregut. (O,P) Sagittal serial sections of an E9.25 embryo showing that both Fgfr2b and Sox17 were expressed in the ventral foregut. (Q,R) Sagittal serial sections of an E9.25 embryo showing that Sox17 was expressed in the presumptive gall bladder and ventral pancreas, but not in the Alb+ hepatic region. (S,T) Sagittal serial sections of an E9.25 embryo showing that Sox9 was mainly expressed in the Sox17-expressing ventral foregut. (U) Diagrammatic summary of Fgf10, Fgfr2b, and Sox9 expression in the ventral foregut in an E9.25 embryo. Fgf10 is expressed in the STM, whereas Fgfr2b and Sox9 are expressed in the presumptive gall bladder and ventral pancreas, and not in the Alb-positive nascent liver. (V,W) Sagittal sections of E9.25 wild-type and Mab21l2-deficient embryos showing that Fgf10 was expressed in the STM near the ventral foregut in wild-type embryos, but Fgf10 expression was lost in Mab21l2-deficient embryos (red arrowhead). (X,Y) Sagittal sections of E9.25 wild-type and Mab21l2-deficient embryos showing that Sox9 was expressed in the ventral foregut in wild-type embryos, but Sox9 expression was significantly reduced in Mab21l2-deficient embryos (red arrowhead). a, atrium; gbp, gall bladder primordium; vp, ventral pancreatic bud. Scale bars: 50 μm (A–T,V–Y).
et al., 2001). These observations suggest that Bmp4 is involved in gall bladder development through regulation of Sox17 and Pdx1 expression. Bmp4 was expressed in the STM at E8.5 when hepatic induction occurred (Rossi et al., 2001). However, in Mab21l2-deficient embryos at E9.0, STM loss resulted in the loss of Bmp4 expression near the posterior region of the ventral foregut, including the presumptive gall bladder region, suggesting that reduced expression of Sox17 may be a consequence of the loss of BMP4 signaling. BMP4 rescued Sox17 expression in the foregut region of cultured Mab21l2-deficient embryos. A previous study showed that BMP4 promotes cell proliferation of Sox17-expressing cells (Sneddon et al., 2012). However, our study showed that Noggin did not influence the size of the Sox17-expressing region, suggesting that BMP4 is the primary regulator maintaining Sox17 expression at this developmental stage. Moreover, BMP4 reduced Pdx1 expression in the foregut of cultured wild-type embryos, suggesting that BMP4 also suppresses Pdx1 expression. Therefore, our results demonstrate that, during gall bladder development, STM-derived BMP4 induces differentiation of Sox17+/Pdx1+ pancreaticbiliary common progenitor cells adjacent to the STM into Sox17+/Pdx1− gall bladder progenitor cells by maintaining Sox17 expression and suppressing that of Pdx1. The positional relationship between the STM and the ventral foregut endoderm thus determines the boundary between the gall bladder and the ventral pancreas (supplementary material Fig. S5).

Previous studies have shown that Hgf is expressed in the STM surrounding the hepatoblasts (Schmidt et al., 1995), and that the HGF receptor c-met is expressed in hepatoblasts (Bladt et al., 1995). During liver development, the Hgf-c-met signaling pathway promotes cell proliferation and survival of hepatoblasts through pro-mitogenic and anti-apoptotic actions (Birchmeier et al., 2003; Schmidt et al., 1995), indicating that the STM is involved in liver differentiation and growth. At E9.5, Hgf was expressed in the STM surrounding the gall bladder primordium (supplementary material Fig. S4A,B), and c-met was expressed in the gall bladder primordium (supplementary material Fig. S4C,D), indicating that the STM promotes the proliferation and survival of gall bladder primordial cells. Therefore, the STM is involved in both differentiation and growth of the gall bladder.

The STM suppresses ectopic activation of the liver program in the presumptive gall bladder and ventral pancreas

Previous studies in chick and Xenopus suggest that during endoderm patterning, a concentration gradient of Wnt and FGF secreted from the adjacent mesoderm regulates the regional identity of the endoderm (foregut, midgut, or hindgut) (Dessimoz et al., 2006; McLin et al., 2007; Wells and Melton, 2000). Moreover, inhibition of the Wnt signaling pathway in the Xenopus posterior endoderm induces ectopic activation of the liver program in the intestine (McLin et al., 2007). These studies indicate that for each organ to develop in the appropriate position, not only inductive signals but also suppressive signals are required. After endoderm patterning, FGF from the cardiac mesoderm induces or suppresses liver development in the foregut endoderm, depending on the region (Deutsch et al., 2001; Jung et al., 1999). In Mab21l2-deficient embryos, in which the STM was completely lost near the posterior region of the ventral foregut, the expression of Alb was activated not only in the nascent liver but also in the remaining posterior region of the ventral foregut, suggesting that after hepatic induction, the STM normally suppresses ectopic activation of the liver program in the presumptive gall bladder and ventral pancreas regions.

Various genes that encode secreted ligands, including Fgf10, are expressed in the STM. During pancreas development, Fgf10 is expressed in the mesenchyme surrounding the pancreatic epithelium, and Fgf12b and Sox9 are expressed in the pancreatic epithelium. In addition, the Fgf10-Fgfr2b signaling pathway suppresses the liver program in the pancreatic epithelium by regulating Sox9 (Seymour et al., 2012), suggesting that FGF10 from the STM has similar functions. Here, we showed that Fgf10 is expressed in the STM at E9.0, and Fgfr2b and Sox9 were expressed in presumptive gall bladder and ventral pancreas regions, and not in the nascent liver. This expression pattern enables the Fgf10/Fgfr2b/Sox9 signaling pathway to function in a region-specific manner in the presumptive gall bladder and ventral pancreas. In Mab21l2-deficient embryos, STM loss resulted in the loss of Fgf10 expression near the ventral foregut. In addition, Sox9 expression in the ventral foregut was significantly reduced. These results suggest that FGF10 from the STM is involved in suppression of the liver program through the coordinated expression of Fgf10/Fgfr2b/Sox9 in the presumptive gall bladder and ventral pancreas and that the resulting expression pattern determines the boundary between hepatic and pancreaticbiliary common progenitors. Therefore, the STM functions not only in hepatic induction and liver growth, but also in suppression of the liver program in the nascent gall bladder and ventral pancreas (supplementary material Fig. S5).

Taken together, this study revealed that the establishment of an appropriate positional relationship between the STM and the ventral foregut endoderm is required for proper organogenesis in the ventral foregut, and that the STM induces the formation first of the liver and then of the gall bladder through the changes in the positional relationship of the STM to the ventral foregut at each developmental stage (supplementary material Fig. S5).

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Competing Interests

The authors have no competing interests to declare.

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