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Expression and function of mouse *Sox17* gene in the specification of gallbladder/bile-duct progenitors during early foregut morphogenesis

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ABSTRACT

In early-organogenesis-stage mouse embryos, the posteroventral foregut endoderm adjacent to the heart tube gives rise to liver, ventral pancreas and gallbladder. Hepatic and pancreatic primordia become specified in the posterior segment of the ventral foregut endoderm at early somite stages. The mechanisms for demarcating gallbladder and bile duct primordium, however, are poorly understood. Here, we demonstrate that the gallbladder and bile duct progenitors are specified in the paired lateral endoderm domains outside the heart field at almost the same timing as hepatic and pancreatic induction. In the anterior definitive endoderm, *Sox17* reactivation occurs in a certain population within the most lateral domains posterolateral to the anterior intestinal portal (AIP) lip on both the left and right sides. During foregut formation, the paired *Sox17*-positive domains expand ventromedially to merge in the midline of the AIP lip and become localized between the liver and pancreatic primordia. In *Sox17*-null embryos, these lateral domains are missing, resulting in a complete loss of the gallbladder/bile-duct structure. Chimera analyses revealed that *Sox17*-null endoderm cells in the posteroventral foregut do not display any gallbladder/bile-duct molecular characters. Our findings show that *Sox17* functions cell-autonomously to specify gallbladder/bile-duct in the mouse embryo.

Introduction

In mouse early embryogenesis, anterior definitive endoderm (i.e., foregut) first arises at the early- to mid-streak stages [1,2], and then its posteroventral segment gives rise to three different organs – namely, hepatic, pancreatic and gallbladder/bile-duct primordia at early somite stages [3,4]. Hepatic and pancreatic primordia have previously been shown to be specified by various signaling factors such as FGFs, BMPs and TGFβ emitted from the cardiac mesoderm and transverse septum in the anterior endoderm at early somite stages, following which organ-specific morphogenesis of the foregut endoderm is initiated under the heart field [5]. It has previously been shown that gallbladder formation is regulated by several factors such as *Foxf1* [6], *Hnf6* (*Onecut1*) [7], *Hes1* [8], *Hhex* [9] and *Lgr4* [10]. However, the mechanisms responsible for demarcating gallbladder/bile-duct progenitors during early organogenesis are poorly understood.

Sry-related HMG box gene-*17* (*Sox17*) has a conserved role in endoderm formation in various vertebrate species [11,12]. In mice, *Sox17*-null embryos show a drastic reduction in endodermal cell number throughout the anteroposterior (AP) axis, which leads to aberrant formation of a slender, primitive gut tube with reduced-diameter before their embryonic lethality at 10.5 dpc (day post coitum) [13]. In the definitive endoderm cell lineage, *Sox17* is first activated in the anterior definitive endoderm of mid-streak stage embryos (7.0 dpc). Its expression expands throughout the definitive endoderm by early-headfold stage (7.5 dpc), and then rapidly disappears in the anterior endoderm, becoming restricted to the posterior (i.e., hindgut) endoderm by late-headfold stage [13,14]. Since the fore, mid and hindgut endoderm are roughly generated in an anterior-to-posterior manner [1,2,15,16], this anterior-to-posterior pattern of *Sox17* expression may reflect its transient activation at the initial phase of definitive endoderm differentiation. However, *Sox17* is re-expressed during the development of the endoderm of the posteroventral foregut, where the progenitors of the gallbladder are localized during organogenesis (9.5 dpc) [17]. In *Sox17*-null embryos, marker expression shows that the hepatic primordium may be present but not of the pancreatic bud [13]. However, potential roles of *Sox17* in the initial specification and formation of the gallbladder remain unclear.

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In the present study, we examined the spatiotemporal pattern of *Sox17* expression during foregut development at early somite stages. Moreover, we analyzed the roles of *Sox17* activity in the formation of the gallbladder/bile-duct in *Sox17*-null embryos and chimeric embryos generated using *Sox17*-null ES cells.

Materials and methods

Animals and genotyping. Embryos at 8.25–10.0 dpc were obtained from pregnant wildtype (ICR strain) and *Sox17* heterozygous female mice mated with *Sox17* heterozygous male mice (ICR background). Chimeric embryos with *Sox17*-null mutant ES cells at 8.75–9.5 dpc were also generated by blastocyst injection of *Sox17*-null ES cells into C57BL/6-Tg (CAG–EGFP) mice (Green mice; SLC, Inc.), as described previously [13,18].

Organ culture. In order to analyze the defects in the *Sox17*-null foregut endoderm after embryonic death (possibly due to defective blood circulation [14,19]), we performed organ culture of the anterior trunk with the foregut–heart complex [20]. In brief, the foregut region including the heart field was isolated from the anterior trunk under a dissecting microscope at 15–20 somite stages. The foregut–heart complexes (without neural tube) from the *Sox17*-null and *Sox17*-heterozygote and wildtype littermates were placed onto an ISOPORE membrane filter (Millipore), and then cultured in 10% fetal calf serum-DMEM (Sigma) supplemented with EGF (10 ng/ml) and insulin (5 μg/ml) at 37°C for 12 to 72 h.

Whole-mount in situ hybridization. Whole-mount in situ hybridization was performed as previously described [21]. RNA probes for *Afp* [22], *Sox17* [13], *Hhex* [23] and *Pdx1* [24] were used in this study.

Histology, lectin histochemistry and immunohistochemistry. For whole-mount DBA lectin staining, the embryos were fixed in 4% PFA–PBS for 6 h at 4 °C, and then washed with TBST. For permeabilization, all embryos were dehydrated and stored in 70% methanol for several days. Then the embryos were incubated with Rhodamine-labeled DBA lectin (10 μg/ml) for 12 h at 4 °C.

For paraffin sections, the embryos were fixed in 4% PFA–PBS for 12 h at 4 °C, dehydrated, embedded in paraffin, and then serially sectioned (5 μm in thickness). For frozen sections, chimeric and mutant embryos were fixed in 4% PFA–PBS and then serially cryosectioned (7–8 μm in thickness). The sections were incubated with anti-SOX17 (10 μg/ml [25]), anti-PDX1 (10 μg/ml; Abcam, UK), anti-HNF4α (1/400 dilution; Upstate), or anti-HNF6 antibody (1/50 dilution; Santa Cruz). Finally, the immunoreaction was visualized by biotin-conjugated secondary antibody in combination with an ABC Kit (Vector labs; brown staining) or by secondary antibodies conjugated with alkaline phosphatase (ALP; purple staining)/Alexa-488/594 (green/red fluorescence).

Results and discussion

Sox17 is reactivated in the paired lateral domains of the foregut endoderm concurrently with hepatic and pancreatic marker expression

In order to determine the timing of the onset of *Sox17* reactivation in the anterior endoderm, we first examined developmental pattern of *Sox17* expression in wildtype embryos (Fig. 1A). In embryos before 8-somite stage, no *Sox17* signals are detected in the foregut endoderm, although weak signals are found in endothelial cells within the cardiac and dorsal aorta regions, as shown in a previous study [14]. In the anterior endoderm epithelia, *Sox17* signals were first detected in bilateral domains posterolateral to the anterior intestinal portal (AIP) lip at around 9–10 somite stages (approximately 8.5 dpc; "9s" in Fig. 1A). As foregut portal lengthens, these *Sox17*-positive domains extend ventromedially, and then fuse in the midline of the AIP lip ("11s", "15s" in Fig. 1A), culminating in their localization to the gallbladder primordium in the posteroventral foregut by 20-somite stage (approximately 9.5 dpc; "22s" in Fig. 1A). We further compared *Sox17* expression patterns with that of *Hhex*, a marker for hepatic buds, and *Pdx1*, an early marker for pancreatic buds (Fig. 1B). Interestingly, we found that *Sox17* is activated concurrently with *Hhex* and *Pdx1* (Fig. 1B), which is consistent with the onset of hepatopancreatic specification from 7 to 8 somite stages [26–29]. *Hhex* expression is initiated at the ventromedial region of the closed foregut tube at around 8-somite stage, and that its expression domain rapidly expands in the ventromedial direction by 11-somite stage (Fig. 1B). In contrast, both *Sox17* and *Pdx1* are upregulated at around 9–10 somite stages in the gut endoderm at the open foregut level located posterior to the *Hhex*-positive domain. *Sox17* expression was detected in the most lateral domain of the open foregut endoderm on both the right and left sides, while the *Pdx1*-positive domain is restricted to the ventromedial AIP lip adjacent to the *Hhex*-positive domain ("11s" in Fig. 1B and C). During foregut tube closure at 12–14 somite stages, the *Sox17*-positive domains expand and fuse to the AIP lip position defined between the *Hhex*- and the *Pdx1*-positive domains ("16s" in Fig. 1B and C).

Next, we examined SOX17 and PDX1 expression patterns in the ventral foregut by double-immunohistochemical staining (Fig. 1D). Anti-SOX17 signals were first detected in a certain cell population in the most lateral foregut endoderm juxtaposed to the cardinal vein (and partially vitellin vein) at around 10-somite stage ("10s" in Fig. 1D). SOX17-positive cells were located in the area defined between the PDX1-positive endoderm and the yolk-sac visceral endoderm at this stage. During tube closure, SOX17-positive cells relocate in a ventromedial direction (arrows in "12s" of Fig. 1D) in contrast to the posterior expansion of the PDX1-positive population toward the visceral endoderm side (right plates in "12s" of Fig. 1D). This appears to result in an intermingled pattern of the two populations at the ventromedial point at the just-closed foregut (AIP lip) level (broken arrow in "12s" of Fig. 1D). After 13–14 somite stages, SOX17-positive cells are restricted to the gallbladder/bile-duct area that is defined between the HNF4α-positive hepatic bud and the PDX1-positive pancreatic bud along the AP axis ("15s" and "24s" of Fig. 1D). Such expression and relocation profiles of SOX17-positive cells are clearly consistent with the present in situ hybridization analysis (lowest plates in Fig. 1B).

This expansion of paired lateral endoderm toward the medial AIP lip is similar to the relocation pattern of liver progenitors during 4–6 somite stages [30]. It has also been shown that the most posterior endoderm in the AIP lip (corresponding to the PDX-positive pancreatic domain) moves distally toward the visceral endoderm [16,30,31]. These cell marking data suggest that the tube-closing morphogenesis of the anterior endoderm directs the expansion and relocation of *Sox17*-positive gallbladder/bile-duct progenitors from the paired lateral domains into the midline of the AIP lip. This in turn suggests that the gallbladder/bile-duct progenitors are initially specified in the lateral endoderm region outside the heart field before their relocation to between the liver and pancreatic buds.

Defective formation of gallbladder and pancreatic regions in Sox17-null embryos

Next, we examined the expression pattern of markers for liver, pancreas and gallbladder/bile-ducts in *Sox17*-null and *Sox17*-heterozygote embryos. It was shown that *Hhex* expression was properly detected in both *Sox17*-null and *Sox17*-heterozygote liver buds at 11-somite stage, although defective expansion of the *Hhex*-positive area was seen in *Sox17*-null embryos after 18-somite stage

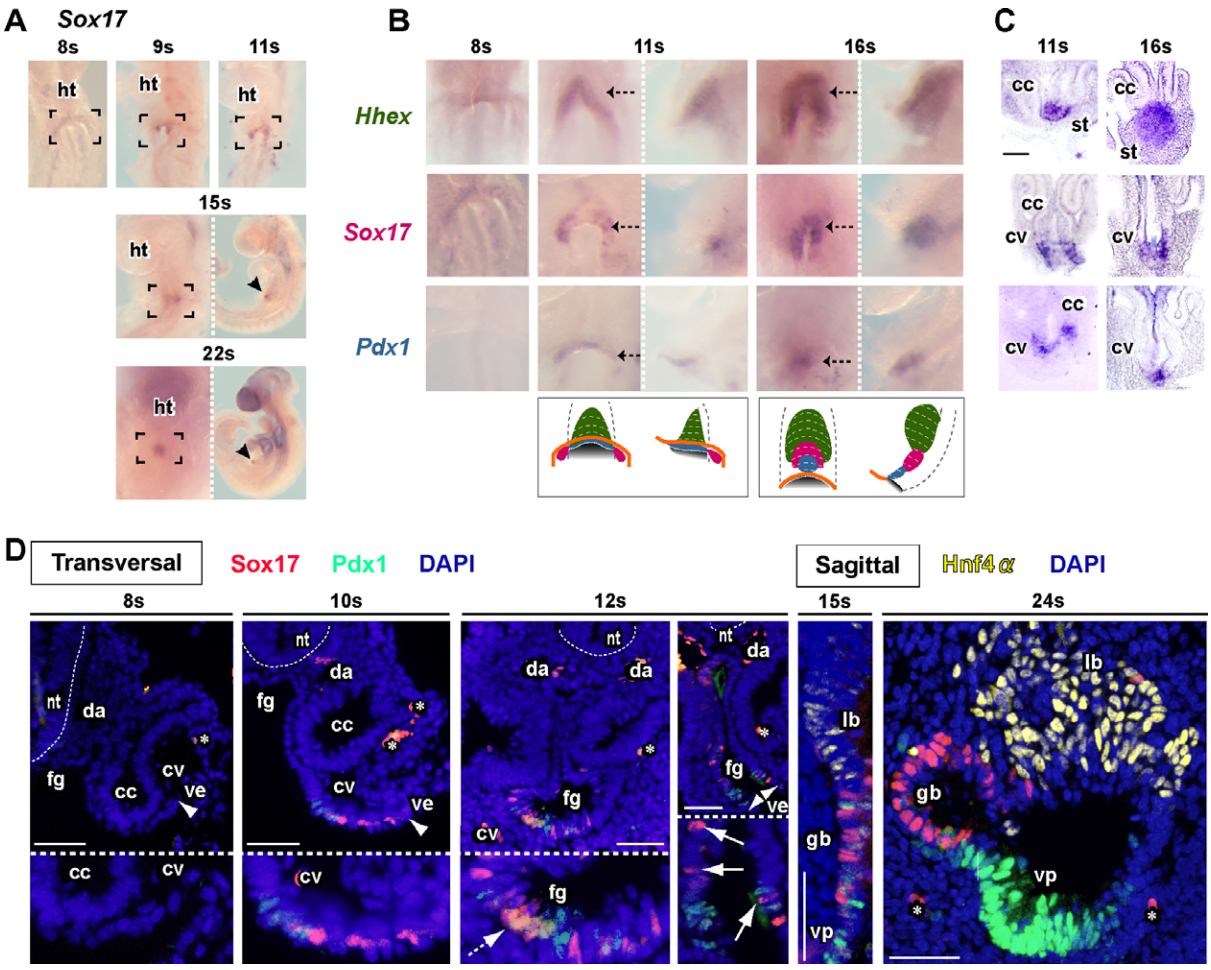

Fig. 1. Whole-mount in situ hybridization and immunohistochemical analyses showing spatiotemporal patterns of SOX17 expression during foregut morphogenesis. (A) *Sox17* expression profiles during 8.5–9.5 dpc (8–22 somite stages). In each plate, broken rectangles show ventral foregut areas (ventral view), while the arrowheads indicate *Sox17*-positive gallbladder primordium (lateral view). (B,C) Comparative expression analyses of *Hhex*, *Sox17* and *Pdx1* genes, showing in situ hybridization images (upper three rows in B), schematic illustration images (the lowest row in B [*Hhex*, green; *Sox17*, red; *Pdx1*, blue; visceral endoderm, orange]) and transversal sectioning images at the posteroventral foregut levels (C; the sectioning planes indicated by broken arrows in B). In some plates of (A) and (B), both ventral (left plate) and lateral (right plate) views of the same embryos are included. (D) Anti-SOX17 (red) and anti-PDX1 (green) double staining of the transversal (8, 10 and 12 somite stages) and sagittal (15 and 24 somite stages) sections at the posteroventral foregut levels. In "12s" plates of (D), left and right plates show two serial sections (separated by 5–6 section interval) at "closed" and "open" foregut levels of the same embryo, respectively. asterisk, background autofluorescence from blood cells; cc; coelomic cavity; cv, cardinal vein; da, dorsal aorta; fg, foregut; gb, gallbladder primordium; ht, heart tube; lb, liver bud; nt, neural tube; st, transverse septum; ve, visceral endoderm; vp, ventral pancreas. Bar, 50 μm. (For interpretation of color mentioned in this figure, the reader is referred to the web version of this article.)

(Fig. 2A). This finding suggests that there are no appreciable defects in the onset timing of *Hhex* expression in *Sox17*-null embryos. In contrast, no *Pdx1*-positive signals were detected in *Sox17*-null embryos throughout the developmental stages (Fig. 2B). Moreover, lectin histochemical analysis showed that, in *Sox17*-heterozygote embryos at 15-somite stage, positive signals for DBA lectin staining (a marker for epithelial cells of gallbladder/bile-ducts) are detected in the posteroventral foregut endoderm, in addition to the yolk-sac visceral endoderm (broken lines in upper plates of Fig. 2C). By 20-somite stage, DBA-positive signals are observed in the gallbladder/bile-duct and duodenum epithelia in *Sox17*-heterozygote and wildtype embryos. In *Sox17*-null embryos, however, DBA-positive signals are detected only in the visceral endoderm and not in the foregut endoderm inside the embryos (broken lines in lower plates of Fig. 2C).

Next, in order to exclude the possible influence of defective blood circulation in *Sox17*-null embryos [14,19], we isolated the anterior trunk with foregut and heart regions from *Sox17*-null embryos before embryonic death, and then cultured them for 12 to 72 h on a filter in the medium (Fig. 2D and E). Whole-mount in situ hybridization analysis of *Afp* probe revealed that proper expansion of liver primordium occurs even in anterior trunk explants of *Sox17*-null embryos (arrowheads in Fig. 2D and E). A weak *Pdx1*-positive region corresponding to the presumptive dorsal pancreas is also observed in some *Sox17*-null explants (arrowhead in right plate of Fig. 2D), although no *Pdx1* expression was detected in the presumptive ventral pancreatic region of all explants examined. In contrast to the rescue of liver expansion and dorsal pancreatic formation in *Sox17*-null anterior trunk explants, all *Sox17*-null explants showed defective formation of DBA-positive bile duct structures (Fig. 2D and E). All these findings strongly indicate defective formation of the gallbladder/bile-duct structure in the ventral foregut of *Sox17*-null embryos.

Defective formation of gallbladder and pancreatic primordia is likely due to a lack of progenitor tissues

Next, in *Sox17*-null embryos at 15–20 somite stages, we examined in detail the expression profiles of the bile duct markers, DBA lectin and HNF6 [7] using serial transverse sections at the postero-

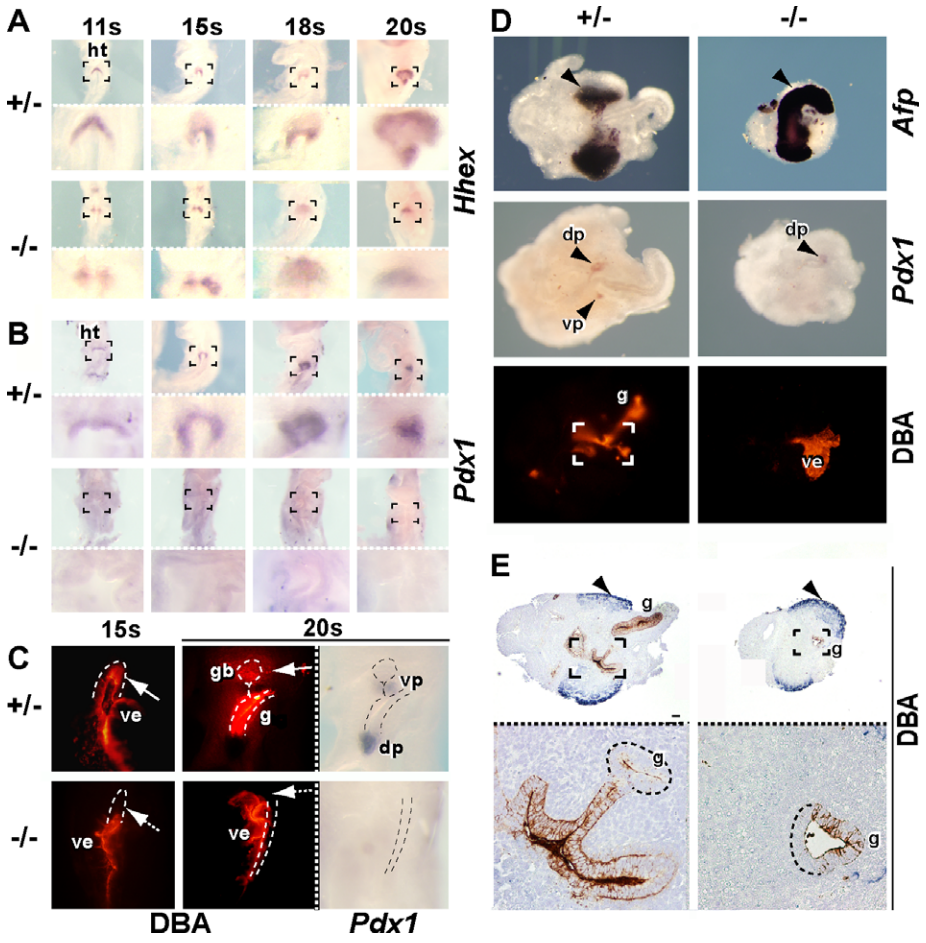

Fig. 2. Defective formation of DBA-positive gallbladder/bile-duct structures in *Sox17*-null embryos. (A,B) Whole-mount in situ hybridization (ventral views) showing expression profiles of *Hhex* (A) and *Pdx1* (B) in the ventral foregut endoderm of $Sox17^{+/-}$ and $Sox17^{-/-}$ embryos. (C) Whole-mount double staining of DBA lectin (red fluorescence) and *Pdx1* (in situ hybridization; purple), showing defective formation of DBA-positive ductal structures in the posteroventral foregut region (broken lines) of $Sox17^{-/-}$ embryos (broken arrows in C). In both $Sox17^{+/-}$ and $Sox17^{-/-}$ embryos at 20-somite stage, the images of *Pdx1* expression are also shown in the right plates. (D,E) The anterior foregut region with the heart and transverse septum was isolated from $Sox17^{+/-}$ and $Sox17^{-/-}$ embryos at around 8.75–9.0 dpc, and then cultured on a filter for 3 days. Whole-mount in situ hybridization (ventral views) showing the developmental profiles of *Afp*-positive liver bud (purple), *Pdx1*-positive pancreatic buds (purple) and DBA-positive gallbladder/bile-duct structures (red fluorescence) in anterior trunk explants. Figure (E) shows DBA lectin staining (brown) using sagittal sections of *Afp*-stained explants shown in (D). In (E), the broken rectangle encompasses the area magnified in the lower plate. dp, dorsal pancreas; g, gut; ht, heart tube; ve, visceral endoderm; vp, ventral pancreas. Bar, 50 μm. (For interpretation of color mentioned in this figure, the reader is referred to the web version of this article.)

ventral foregut level (Fig. 3). In the foregut region of normal *Sox17*-heterozygote embryos, strong DBA-positive reactions were detected in the gallbladder/pancreatic domain with cardinal veins in addition to the visceral endoderm at these stages (arrows and "ve" in "DBA" of Fig. 3A and B). HNF6 expression was also found in DBA-positive gallbladder and pancreatic domains of the ventral foregut endoderm (arrows in "Hnf6" of Fig. 3A and B), in addition to liver buds (Fig. 3C). In contrast, HNF4α expression was negative for these gallbladder and pancreatic regions, becoming restricted to liver buds and visceral endoderm cells at these stages ("lb" and "ve" in Fig. 3A–C). In *Sox17*-null embryos at both 15 and 18 somite stages, the DBA-positive/HNF6-positive endoderm domains adjacent to cardinal veins were completely lacking in posteroventral foregut epithelia, in which the ventral foregut domain was replaced by HNF4α/DBA-double-positive visceral endoderm in *Sox17*-null embryos (right plates in Fig. 3A and B). These findings suggest that the lateral (ventral) endoderm domains corresponding to gallbladder/bile-ducts as well as the pancreatic domain, are completely missing in *Sox17*-null embryos. This finding indicates defective positioning of the lateral/posterior leading edge of the endodermal epithelium beyond the heart field to allow gallbladder/pancreatic specification in *Sox17*-null embryos, rather than a defect in intrinsic competence of the mutant endoderm to initiate their programs. A similar aberrant endoderm patterning was previously reported in the ventral pancreatic domain of foregut endoderm in *Hhex*-null mutants [31]. In *Hhex*-null embryos at early somite stages, defective growth and positioning of foregut endodermal cells beyond the cardiogenic mesoderm does not allow pancreatic induction in the AIP lip region at 6–7 somite stages.

Defective expression of gallbladder/bile-duct markers in Sox17-null endoderm cells located in the posteroventral foregut epithelia of chimeric embryos

Finally we examined the potency of *Sox17*-null endoderm cells in ventral foregut epithelia using several chimeric embryos consisting of *Sox17*-null ES cells and EGFP-positive *Sox17*-wildtype blastocysts (around 8.75–9.5 dpc; $n = 3$, >90%; $n = 4$, 90–70%; $n = 4$, <50% EGFP-negative *Sox17*-null cells; total 11 embryos; Fig. 4A). Immunohistochemical analyses of serial sections of all moderate chimera embryos showed that *Sox17*-null ES cells were frequently found in hepatic buds (HNF4α in Fig. 4B) whereas only a few *Sox17*-null cells were found in the ventral foregut epithelia near SOX17-positive and PDX1-positive wildtype endoderm cells

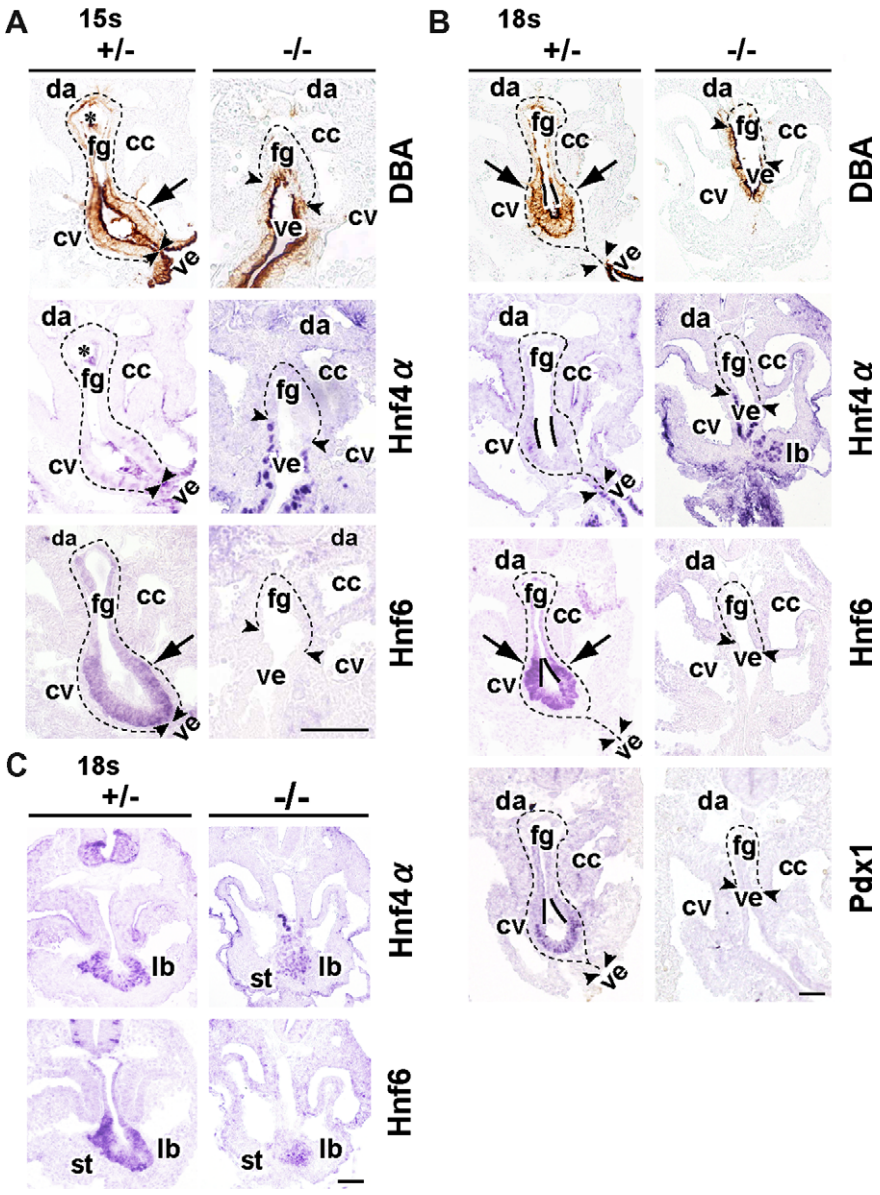

Fig. 3. Lack of DBA-/HNF6-positive domains corresponding to presumptive gallbladder/bile-duct and pancreatic primordia in posteroventral foregut endoderm of *Sox17*-null embryos. Comparative expression analysis of transversal serial sections at the posteroventral foregut level ((A,B) gallbladder/pancreatic levels; (C) liver bud level), showing that the DBA-/HNF6-positive domain corresponding to presumptive gallbladder/bile-duct and pancreatic primordia is missing in the ventrolateral foregut endoderm of *Sox17*$^{-/-}$ embryos at 15 (A) and 18 (B,C) somite stages. In each plate, broken lines mark the gut endoderm region, while small arrowheads indicate the border between the gut endoderm and visceral endoderm (ve). In figure (B), anti-PDX1-staining images are shown in the bottom row, while the presumptive gallbladder/bile-duct region (i.e., PDX1-negative, DBA/HNF6 double-positive domain) is marked by a solid line along the lumen. asterisk, non-specific staining; cc; coelomic cavity; cv, cardinal vein; da, dorsal aorta; fg, foregut; ht, heart tube; lb, liver bud; nt, neural tube; st, transverse septum; ve, visceral endoderm. Bar, 50 μm.

(broken lines in Fig. 4B and C), suggesting a reduced potency of *Sox17*-null endoderm cells to contribute to gallbladder/pancreatic domains in the developing posteroventral foregut. No PDX1-positive reactions were detected in *Sox17*-null endoderm cells in all chimera embryos used in this study. Most interestingly, in middle-to-high contribution chimeras, all *Sox17*-null foregut cells near the gallbladder/pancreatic regions showed a lack of DBA-positive and HNF6-positive staining abilities for gallbladder/bile-duct markers in the presumptive ventral foregut area (broken lines in Fig. 4B–D). This is in contrast to proper HNF6 expression in *Sox17*-null endoderm cells located in hepatic buds (upper plates in Fig. 4D). These findings, therefore, indicate that *Sox17* activity is cell-autonomously required for the specification and/or differentiation of the gallbladder/bile-duct system in the developing ventral foregut epithelia.

In conclusion, the present study demonstrates that *Sox17* activation in the posteroventral foregut endoderm is crucial for gallbladder/bile-duct formation during ventral foregut morphogenesis. Unfortunately, in this study, we could not demonstrate whether the first transient activation of *Sox17* at gastrulated stages or its subsequent reactivation after 9–10 somite stages (or both) are critically required for gallbladder/bile-duct formation. Most recently (during the preparation of this manuscript), an article using conditional *Sox17*-null mutant mice clearly demonstrated that the *Sox17* reactivation in the gallbladder/bile-duct primordia is essential for their specification at later stages [32]. The outcomes of that study are consistent with the findings of our present study which demonstrated a cell-autonomous crucial role of *Sox17* in the specification/differentiation of gallbladder/bile-duct progenitors during ventral foregut morphogenesis. Further analyses of *Sox17* upstream

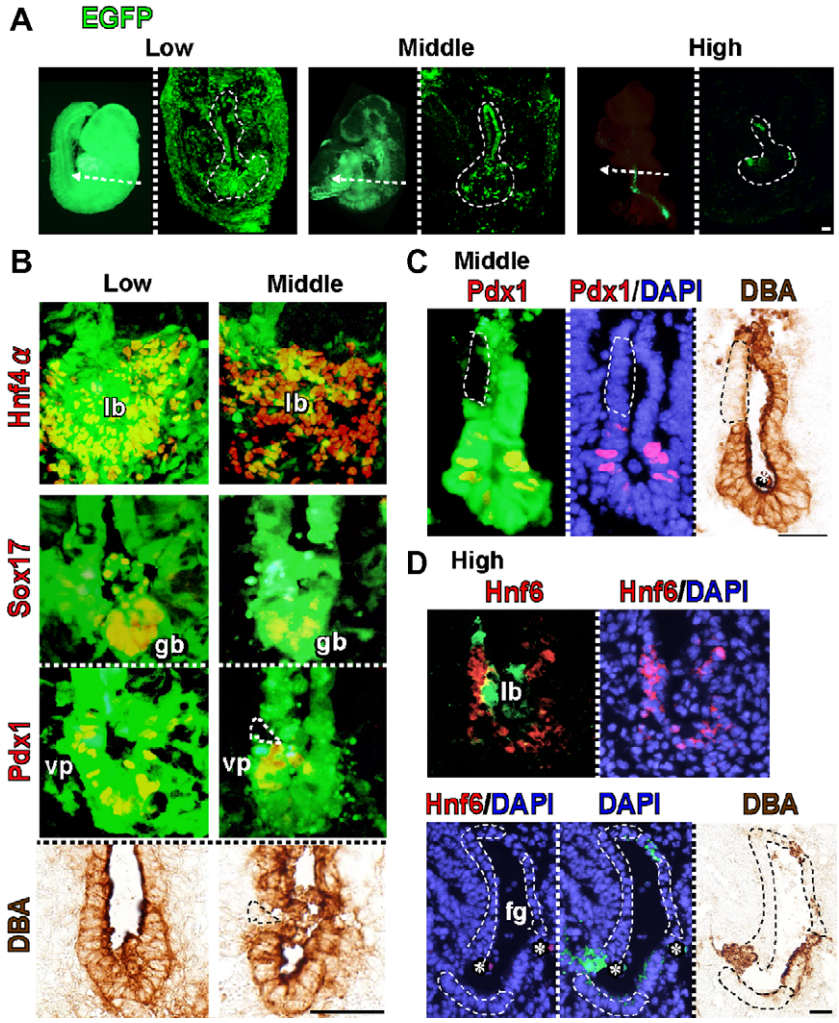

Fig. 4. Lack of gallbladder/bile-duct marker expression in *Sox17*-null endoderm cells located in the posteroventral foregut of chimeric embryos. (A) Three chimeric embryos (Low: ~40%; Middle: 70–75%; High: >90% in mutant ES cell contribution) at around 9.25 dpc. Chimeric embryos were generated by blastocyst injection of *Sox17*-null (EGFP-negative) ES cells into EGFP (green fluorescence)-positive wildtype mice (CAG–EGFP mice). Each plate includes both whole-mount (left plate) and sectioning (right plate) images. Broken arrows indicate the sectioning planes, while broken lines show the foregut endoderm. (B–D) Comparative expression analysis of the transversal serial sections of chimeric embryos with low-to-high ES cell contributions. Anti-HNF4α (red fluorescence in B), anti-SOX17 (red fluorescence in B), anti-PDX1 (red fluorescence in B and C)/DBA (brown staining)-double or anti-HNF6 (red fluorescence in D)/DBA (brown staining)-double staining of chimeric embryos with low (left column in B), middle (right column in B and C) and high (D) contributions. All *Sox17*-null foregut cells (indicated by broken lines) exhibit a lack of staining ability for DBA lectin (B–D) and anti-HNF6 antibody (D). gb, gallbladder/bile-duct region; fg, foregut; lb, liver bud; vp, ventral pancreas. Bar, 50 μm. (For interpretation of color mentioned in this figure, the reader is referred to the web version of this article.)

regulation in the gallbladder/bile-duct progenitors are required to gain a better understanding of the molecular mechanisms involved in the induction of the bile duct systems of the liver, pancreas, and gallbladder in mammalian organogenesis.

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