Haploinsufficiency of the Mouse Forkhead Box fl Gene Causes Defects in Gall Bladder Development*

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The forkhead box fl (Foxfl) transcription factor is expressed in the visceral (splanchnic) mesoderm, which is involved in mesenchymal-epithelial signaling required for development of organs derived from foregut endoderm such as lung, liver, gall bladder, and pancreas. Our previous studies demonstrated that haploinsufficiency of the Foxfl gene caused pulmonary abnormalities with perinatal lethality from lung hemorrhage in a subset of Foxfl+/− newborn mice. During mouse embryonic development, the liver and biliary primordium emerges from the foregut endoderm, invades the septum transversum mesenchyme, and receives inductive signaling originating from both the septum transversum and cardiac mesenchyme, respectively (1, 2). This hepatic specification is associated with expression of the Foxa2 (HNF-3β) and Gata4 transcription factors (3). Liver morphogenesis involves a proliferative expansion period and development of the bipotential hepatoblasts that begin to differentiate into hepatocytes and bile duct epithelial cells of the intrahepatic bile ducts (IHB) at 13.5 dpc (3, 4). Interestingly, targeted disruption of either the homeodomain Hex gene or the Hgf gene allows normal development of the mouse hepatic diverticulum, but these cells fail to migrate into the septum transversum and undergo liver morphogenesis (5–7).

In the adult liver, bile is synthesized in hepatocytes from cholesterol, secreted into the bile canaliculi and transported through the intrahepatic and extrahepatic biliary system to the gall bladder, where it is stored for secretion into the digestive tract to emulsify lipids (8). The gall bladder and extrahepatic bile ducts (EHBD) develop from the caudal portion of the liver primordium at 10 dpc of mouse embryogenesis (9). However, little is known regarding visceral (splanchnic) mesoderm transcription factors that regulate expression of genes involved in mesenchymal-epithelial induction of gall bladder development from the liver primordium. Visceral mesenchymal expression of the homeodomain Hlx gene is required for the proliferative expansion required for mouse liver and intestine morphogenesis, but it is not essential for normal specification of the liver primordium (10).

The forkhead box (Fox) proteins are an extensive family of transcription factors that share homology in the winged helix/fork head DNA binding domain (11) and play important roles in regulating expression of genes involved in cellular proliferation, differentiation, and transformation, and in metabolic homeostasis (3, 4, 12–19). Expression of Foxf1 is restricted to the visceral (splanchnic) mesoderm, which provides mesenchymal-epithelial signaling for gut-derived organs such as liver, gall bladder, lung, stomach, pancreas, and intestine (20, 21). The Foxf1 gene is required for mesodermal differentiation and cell adhesion and its disruption results in embryonic lethality at midgestation (22). Interestingly, haploinsufficiency of the Foxf1 gene in heterozygous mice causes perinatal lethality from pulmonary hemorrhage with severe defects in formation of alveolar sacs and capillaries and fusion of the lung lobes (23–25). Lung hemorrhage was observed in a subset of newborn Foxf1+/− mice that displayed an 80% reduction in pulmonary Foxf1 levels. This pulmonary defect is associated with diminished expression of Bmp-4, vascular endothelial growth factor (VEGF) receptor type 2 (Flk-1), and the transcription factors of the brachyury T-box family (Tbx2-Tbx5) and lung Kruppel-like factor (23).

In this study, we show that Foxf1 is expressed in mouse embryonic septum transversum and that Foxfl haploinsuffi-

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The abbreviations used are: dpc, days postcoitum; Foxfl, forkhead box fl; HNF-3β, hepatocyte nuclear factor 3 β; HGF, hepatocyte growth factor; CK, cytokeratin; RT-PCR, reverse transcriptase-polymerase chain reaction; HGF, hepatocyte growth factor; PDGFRα, platelet-derived growth factor receptor α; PDGFRβ, platelet-derived growth factor receptor β; Dpc4, bone morphogenetic protein 4; Fgf2, fibroblast growth factor 2; Fg, foregut; GB, gall bladder; Ah, antibody; PV, portal vein; V, vessel; CD, cysctin duct; L, lumen; β-gal, β-galactosidase; BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium.
MATERIALS AND METHODS

Foxf1+/− Mice—We previously described the generation of heterozygous (+/−) Foxf1 mice in which the Foxf1 allele was disrupted by an in-frame insertion of a nuclear-localizing β-galactosidase (β-gal) gene, and thus staining for β-gal enzyme activity allowed identification of Foxf1-expressing cells (23). Foxf1+/− mice used in the gall bladder analysis were bred for three or four generations into the Black Swiss mouse genetic background. Both male and female Foxf1+/− mice exhibited similar defects in gall bladder formation.

Immunohistochemical and β-Galactosidase Enzyme Staining—Paraffin sections were stained with hematoxylin and eosin for morphological examination or used for immunohistochemistry as described previously (23, 26). Rat monoclonal PDGF receptor β-chain antibody (Ab; clone APA5; BD PharMingen) was used at 1:500 dilution, and immunoreactivity was detected using biotinylated anti-rat Ab, avidin-alkaline phosphatase complex, and BCIP/NBT substrate (all from Vector Laboratories, Burlingame, CA). Tissue was stained with mouse monoclonal α-smooth muscle actin Ab (clone 1A4; Sigma), and immunoreactivity was detected using an anti-mouse Ig protein conjugated to alkaline phosphatase with BCIP/NBT substrate as described (26). To stain for biliary epithelial cells, we used mouse monoclonal cytokeratin Ab (clone PCK-26; Sigma), which was detected by anti-mouse Ig protein conjugated with TRITC (Dako Corp, Carpinteria, CA), and endothelial cells were visualized using fluorescein isothiocyanate-conjugated isoelectric B4 from Griffonia Simplicifolia (GS; Vector Laboratories) as previously described (23). To develop a Foxf1 Ab, rabbits were immunized with mouse Foxf1 RHYESQSPSMDKRE peptide (Foxf1 amino acids 327–340) conjugated to keyhole limpet hemocyanin (KLH) protein using Sigma Genosys custom peptide antisera service. The Foxf1 peptide Ab was affinity-purified using the same peptide coupled to hydroxysuccinimide-activated Sepharose 4 Fast Flow beads (Amersham Biosciences, Inc.) and eluted from the column as described previously (27). For immunohistochemical staining with the Foxf1 peptide Ab, paraffin was removed from the tissue section, was rehydrated, and was then incubated for 15 min at room temperature with phosphate-buffered saline containing 20 μg/ml of proteinase K (Roche Molecular Biochemicals, Indianapolis, IN). The Foxf1 peptide Ab was used at 1:100 dilution and visualized using biotinylated anti-rabbit Ab (BD PharMingen), avidin-alkaline phosphatase complex, and BCIP/NBT substrate (Vector Laboratory). To determine Foxf1-expressing cells, Foxf1+/− embryos or liver tissue containing gall bladder (15 dpc and adult) were stained for β-gal activity with 5-bromo-4-chloro-3-indoyl β-D-galactosynirinoside substrate and paraffin-embedded and sectioned as described previously (23, 26). Nuclear fast red (Vector Laboratories) was used as a counterstain.

RNA Isolation, RT-PCR, and RNase Protection Assay—Total RNA from the gall bladder was prepared using RNA-STAT-60 (Tel-Test B Inc, Friendswood, TX). We pooled RNA from either three wild type or eight Foxf1+/− 18-dpc embryonic gall bladders for RT-PCR. RNA was prepared individually from the gall bladders of adult mice, RT-PCRs were performed using SUPERSCRIPT™ One-Step RT-PCR with PLATINUM® M™Taq kit (Invitrogen, CA) according to the manufacturer’s recommendations following an initial DNase digestion step as described by Duncan et al. (28, 29). The following forward and reverse primers were used for specific amplification: Foxf1, 5′-gcagacatctacaaacaaac and 5′-ccagtgg-aggcttatact; cyclophilin, 5′-atcctgactgagaggaa and 5′-ttctagcag-agaaggacctgacag; HGF, 5′-cagcaaaaacgactcttaccaagg and 5′-gcagccataccttcaccaaaac; VCAM-1, 5′-agtctgctg-cacttgctg-ctcctgg; Hex, 5′-caaaaagaggcctggtgctg-ctg-ctctgactgtagtgctgt-tg; Hex, 5′-caaaaagaggcctggtgctg-ctg-ctctgactgtagtgctgt-tg. Two different RNA concentrations were used for RT-PCR reactions to ensure that RT-PCR conditions were in the linear range. Quantitation of expression levels was determined with Tiff files of ethidium bromide-stained gels by using the BioMax 1D program (Kodak).

RESULTS

Foxf1+/− Mice Exhibit a Wide Range of Abnormalities in Gall Bladder Structure—Foxf1+/− mice either lacked a discernible gall bladder or possessed significantly smaller gall bladders with severe structural abnormalities. Examination of 25 adult Foxf1+/− mice revealed that 49% of adult Foxf1+/− mice lacked a discernible gall bladder (Fig. 1, A and B), another 49% of the heterozygous mice possessed a rudimentary gall bladder (Fig. 1, and C–E) or a collection of extraluminal bile ducts (EHBD; E and F). Box in E shows area magnified for F. Magnification: A and B, ×10; C–E, ×50; F, ×200.

Western Blot Analysis—Total protein extracts were prepared from gall bladder for Western blot analysis (23, 26) using the following antibodies for immune detection: rat monoclonal PDGFRe (clone APA5; BD PharMingen; 1:500 dilution), mouse monoclonal α-smooth muscle actin (clone 1A4; Sigma; 1:1000 dilution), mouse monoclonal β-actin (clone AC-15; Sigma; 1:3000 dilution), and rabbit polyclonal CDK2 (BD PharMingen; 1:1000 dilution). Detection of the immune complex was accomplished by using secondary Ab directly conjugated with horseradish peroxidase followed by chemiluminescence (ECL plus, Amersham Biosciences, Inc.). Quantitation of expression levels was determined with Tiff files from scanned films by using the BioMax 1D program. The CDK2 expression signal was used for normalization control between different protein samples.

Foxf1+/− Mice—Foxf1 Is Essential for Gall Bladder Formation

The gall bladders of Foxf1+/− mice exhibited malformation of the external smooth muscle cell layer, reduction in mesenchymal cell number, and in some cases, lack of a discernible biliary epithelial cell layer. Abnormalities in gall bladder formation are associated with diminished expression of vascular cell adhesion molecule 1 (VCAM-1), α5 integrin, platelet-derived growth factor receptor α (PDGFRα), and HGF. Normal development of the liver and intrahepatic bile ducts was found in Foxf1+/− mice, and this was associated with compensatory increase in hepatic Foxf1 levels.

Foxf1+/− mice exhibit abnormalities in gall bladder formation. A and B, subset of Foxf1+/− mice lacking a gall bladder. Liver and gall bladder were dissected from either adult wild type (A) or Foxf1+/− (B) mice and photographed using Nikon digital camera. Wild type mice possess a visible gall bladder (GB), which continues in the cystic duct (CD). Foxf1+/− mice lack a gall bladder (white arrow). C–F, structural defects in gall bladder formation. Liver tissue (containing the gall bladder) from adult wild type and Foxf1+/− mice was paraffin-embedded, sectioned, and stained with hematoxylin and eosin (HE). Foxf1+/− mice possess small rudimentary gall bladders with either partially closed lumen (D) or consisting of extraluminal bile ducts (EHBD; E and F). Box in E shows area magnified for F. Magnification: A and B, ×10; C–E, ×50; F, ×200.

FIG. 1. Foxf1+/− mice exhibit abnormalities in gall bladder formation. A and B, subset of Foxf1+/− mice lacking a gall bladder. Liver and gall bladder were dissected from either adult wild type (A) or Foxf1+/− (B) mice and photographed using Nikon digital camera. Wild type mice possess a visible gall bladder (GB), which continues in the cystic duct (CD). Foxf1+/− mice lack a gall bladder (white arrow). C–F, structural defects in gall bladder formation. Liver tissue (containing the gall bladder) from adult wild type and Foxf1+/− mice was paraffin-embedded, sectioned, and stained with hematoxylin and eosin (HE). Foxf1+/− mice possess small rudimentary gall bladders with either partially closed lumen (D) or consisting of extraluminal bile ducts (EHBD; E and F). Box in E shows area magnified for F. Magnification: A and B, ×10; C–E, ×50; F, ×200.
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In 15-dpc Foxf1+/− mouse embryos, β-gal staining was observed in the gall bladder mesenchyme surrounding the biliary epithelial cell layer and in mesenchymal cells of the liver, but Foxf1 expression was not detected in the vessel mesenchyme (Fig. 2G). In adult Foxf1+/− mice, β-gal staining was found in mesenchymal cells of the gall bladder (Fig. 2I), extrahepatic bile duct (Fig. 2J), and hepatic sinusoids (Fig. 2K), but no Foxf1 expression was detected in either heterozygous (Fig. 2K) or wild type (data not shown) IHBD.

Although embryonic 13-, 15-, and 18-dpc Foxf1+/− mice displayed variation in severity of their gall bladder defects, all of them exhibited significant reduction in the mesenchymal cell layer (Fig. 2, E, G, H, M, and N) compared with that of wild type gall bladder (Fig. 2, D, F, and L). The entire mesenchymal layer displayed Foxf1-dependent β-gal enzyme staining in 15-dpc Foxf1+/− gall bladders (Fig. 2, G and H). We identified a subset of Foxf1+/− mouse gall bladders that lacked the biliary epithelial cell layer (Fig. 2, H and N) as evidenced by undetectable immunohistochemical staining with cytokeratin (Fig. 3C, CK) compared with either wild type (Fig. 3A) or less severely affected Foxf1+/- gall bladder (Fig. 3B). The large vessels adjacent to the Foxf1+/− gall bladder exhibited normal development of the endothelial cells as evidenced by staining with fluorescein isothiocyanate-conjugated isolectin B4 (Fig. 3, A–C, GS), a finding consistent with the lack of Foxf1 expression in large vessels (Fig. 2, G–H). Furthermore, RT-PCR expression analysis of three discernible adult Foxf1+/− gall bladders revealed a 90% decrease in expression of the HNF6, HNF3β, and Hex transcription factors, suggesting that they lacked a normal epithelial cell layer (Fig. 4A). These results suggest that Foxf1+/− mice exhibit defects in the development of epithelial cells in the gall bladder, possibly due to reduced mesenchymal signaling.

Even in the severely affected Foxf1+/− gall bladders, the heterozygous liver architecture (data not shown) as well as the cytokeratin staining pattern (Fig. 3, E–F) and structure of intrahepatic bile ducts (Fig. 3H, IHBD) was indistinguishable from that of wild type liver (Fig. 3, D and O). Furthermore, Foxf1+/− mice displayed wild type liver function as determined by normal serum levels of liver aminotransferases, alkaline phosphatase enzymes, glucose, bilirubin, and albumin (Table I). Interestingly, RNase protection assays demonstrated that 18-dpc Foxf1+/− liver displays a 3-fold compensatory increase in Foxf1 mRNA levels compared with wild type livers (Fig. 3F).

Defects in Foxf1+−/− Gall Bladder Mesenchyme Are Associated with Significant Decreases in Foxf1, VCAM-1, α5 integrin, and HGF Expression—RT-PCR analysis of Foxf1+/− gall bladder RNA showed that defective gall bladder formation was associated with a 70% reduction in Foxf1 levels (Fig. 4, A and B). This finding is consistent with Foxf1+/− pulmonary defects, which were also associated with 50% reduction in Foxf1 mRNA levels (23). Because expression of the cell adhesion α5...
Foxf1 Is Essential for Gall Bladder Formation

![Figure 4](image_url)

**A** RT-PCR Adult GB

|                | +/+ | +/− |
|----------------|-----|-----|
| Foxf1          | 1.0 | 0.2 |
| cycl.          | 1.0 | 0.3 |
| VCAM           | 1.0 | 0.1 |
| HGF            | 1.0 | 0.3 |
| α5 integrin    | 1.0 | 0.3 |
| HNF6           | 1.0 | 0.1 |
| HNF3β          | 1.0 | 0.1 |
| Hex            | 1.0 | 0.1 |

**B** RT-PCR 18 dpc GB

|                | +/+ | +/− |
|----------------|-----|-----|
| Foxf1          | 1.0 | 0.3 |
| cycl.          | 1.0 | 0.9 |
| VCAM           | 1.0 | 0.05|
| HGF            | 1.0 | 0.3 |
| α5 integrin    | 1.0 | 0.1 |

**FIG. 4.** Defects in Foxf1 +/− gall bladder are associated with decrease in Foxf1, VCAM-1, α5 integrin, and HGF levels. RT-PCR reactions were performed with the indicated primers using either 18-dpc (B) or adult (A) gall bladder (GB) RNA. RNA was pooled from either three wild type or eight Foxf1 +/− 18-dpc embryonic gall bladders, or RNA was prepared individually from the gall bladder of adult mice. RNA levels were normalized to cyclophilin, and the numbers below each panel represent the average of expression levels in Foxf1 +/− gall bladder with respect to the wild type gall bladder (± S.D.).

integran and VCAM-1 genes is essential for mesoderm formation (22, 30), we examined their levels in Foxf1 +/− gall bladders. RT-PCR analysis showed a drastic reduction in VCAM-1 and α5 integrin expression in Foxf1 +/− gall bladders, suggesting that their diminished levels contribute to the observed mesenchymal defect (Fig. 4, A and B). Furthermore, reduced expression of HGF was found in Foxf1 +/− embryonic and adult gall bladders (Fig. 4, A and B). Because HGF plays a critical role in liver and bile duct morphogenesis (7, 9, 10, 31), the decline in HGF expression may explain defective formation of Foxf1 +/− gall bladders.

We generated an affinity-purified Foxf1 peptide antibody to determine the Foxf1 expression pattern in gall bladder using immunohistochemical staining. Western blot analysis demonstrated that the Foxf1 antibody detects an appropriate 39-kDa protein using protein extracts prepared from lung, a tissue that expresses high levels of Foxf1 (Fig. 5A). Immunohistochemical staining of 18-dpc wild type gall bladder with this affinity-purified Foxf1 peptide antibody demonstrated that mesenchymal Foxf1 staining gradually decreased toward the smooth muscle cell layer (Fig. 6A), and its expression continued in the submucosa and mucosa mesenchyme of the adult gall bladder (Fig. 6C). Interestingly, this Foxf1 protein expression pattern was significantly diminished in embryonic and adult Foxf1 +/− gall bladders (Fig. 6, B and D), confirming the reduction in Foxf1 mRNA levels.

**DISCUSSION**

The Foxf1 protein is a potent transcriptional activator and its expression is restricted to the visceral (splanchnic) mesoderm, which expresses genes involved in mesenchymal-epithelial signaling required for development of foregut-derived organs (20, 21). Our previous studies demonstrate that haploinsufficiency of the Foxf1 gene caused lung hemorrhage and perinatal lethality in a subset of Foxf1 +/− newborn mice that exhibited an 80% reduction in wild type pulmonary Foxf1 levels (23). Abnormalities in pulmonary alveolarization and vascularization and increased apoptosis of mesenchymal cells were associated with the severe Foxf1 +/− phenotype (23). In this study, we find that Foxf1 haploinsufficiency also resulted in defective formation of the gall bladder, which was associated with a 70% reduction in wild type Foxf1 mRNA levels. Similar to many heterozygous phenotypes, Foxf1 +/− embryos show differences in the severity of the gall bladder defect, and its severe phenotype does not exhibit total penetrance. The Foxf1 +/− mice either lacked an appreciable gall bladder or possessed a smaller rudimentary structure with severe abnormalities. All of the Foxf1 +/− gall bladders displayed a significant reduction in cell numbers within the mesenchymal layers, paucity of smooth muscle cells, and in some cases, lack of a discernible biliary epithelial cell layer. These gall bladder defects are associated with reduced expression of cell adhesion molecules VCAM-1 and α5 integrin as well as diminished levels of signal transduction PDGFRα and HGF proteins. Collectively, our data demonstrate that Foxf1 regulates expression of mesenchymal genes required for proper gall bladder development and function.

We used Foxf1 +/− embryos with a β-gal knock-in gene to demonstrate that Foxf1 is expressed in septum transversum mesenchyme during formation of the hepatic primordium and induction of gall bladder development. Although Foxf1 expression continues in the mesenchymal cells of the liver sinusoids, we found no defects in hepatic architecture, and serum analysis demonstrated that liver function was normal in Foxf1 +/− mice. Interestingly, unlike the gall bladder, we found that Foxf1 +/− livers exhibited an increase in Foxf1 mRNA, suggesting the possibility that its compensatory increase prevents defects in liver development. Moreover, Foxf1 is not expressed in the mesenchyme of the IHBD, and we found that Foxf1 +/− liver exhibited no defects in IHBD.

**In vivo** transplantation studies indicate that bile ducts differentiate abundantly when fetal liver tissue was placed adjacent to mesenchyme (9), suggesting that bile duct development requires mesenchymal signaling. Moreover, the mesenchyme-derived HGF has been shown to induce lumen formation in cultures of epithelial cell lines and to promote cyst maturation and proliferation of gall bladder epithelial cells (9, 31). Our data show that HGF expression is significantly reduced in Foxf1 +/− gall bladders and may therefore contribute to the mesenchymal and epithelial cell defects found in Foxf1 +/− gall bladder. Our finding that the HGF promoter region contains potential binding sites for the Foxf1 transcription factor (12)
Mouse serum was isolated from either wild type (WT) or Foxf1+/− mice, and the Biological Research Laboratories (BRL) at University of Illinois at Chicago performed all of the serum measurements. The serum measurements are expressed as the mean ± S.D. from three to six different mice. Abbreviations: aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP).

| Mouse strain | AST (units/l) | ALT (units/l) | ALP (units/l) | Glucose (mg/dl) | Bilirubin (mg/dl) | Albumin (g/dl) |
|--------------|--------------|---------------|---------------|-----------------|------------------|---------------|
| WT           | 175 ± 74     | 28 ± 4        | 85 ± 22       | 185 ± 41        | 0.7 ± 0.3        | 3.2 ± 0.5     |
| Foxf1+/−     | 94 ± 19      | 30 ± 5        | 103 ± 20      | 229 ± 55        | 0.6 ± 0.3        | 3.3 ± 0.2     |
| Reference    | 72–288       | 24–140        | 45–222        | 124–262         | 0.1–0.9          | 2.6–4.6       |

**FIG. 5.** Defects in Foxf1+/− gall bladder are associated with decrease in αSM actin and PDGFRα levels. A, specificity of Foxf1 peptide antibody. One hundred micrograms of total lung protein extract was used for Western blot analysis with affinity-purified Foxf1 peptide antibody. Indicated on the blot is the position of the 39-kDa Foxf1 protein and the nonspecific 50-kDa protein (NS) that is found with the secondary antibody control. B, Foxf1+/− gall bladder exhibit diminished αSM and PDGFRα protein levels. Western blot analysis of adult gall bladder protein extracts with αSM and PDGFRα antibodies. CDK2 and β-actin Western blots were used as controls. Protein levels were normalized to CDK2, and the numbers below each panel represent the average of expression levels in Foxf1+/− gall bladder with respect to the wild type gall bladder.

**FIG. 6.** Reduced number of smooth muscle cells in Foxf1+/− gall bladder is associated with diminished expression of PDGFRα. Mouse embryonic (18 dpc) or adult wild type or Foxf1+/− gall bladder sections were used for immunohistochemical staining with either Foxf1 (A–D), αSM (E–H), or PDGFRα (I–L) antibodies (purple) and then counterstained with nuclear fast red (red). The specificity of the Foxf1 antibody was verified by Western blot analysis using lung protein extracts (see the legend to Fig. 5). Magnification: A, B, E, F, I, J, ×100; C, D, G, H, K, L, ×200.

Table I

| Mouse strain | AST (units/l) | ALT (units/l) | ALP (units/l) | Glucose (mg/dl) | Bilirubin (mg/dl) | Albumin (g/dl) |
|--------------|--------------|---------------|---------------|-----------------|------------------|---------------|
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