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GATA6 modulates the ductular reaction to bile duct ligation

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Abstract

Background GATA6, a transcription factor expressed in cholangiocytes, has been implicated in the response to liver injury. In biliary atresia, a disease characterized by extrahepatic bile duct obstruction, liver expression of *GATA6* increases with pathological bile duct expansion and decreases after successful Kasai portoenterostomy. The aim of this study was to garner genetic evidence that GATA6 is involved in ductular formation/expansion.

Methods The murine *Gata6* gene was conditionally deleted using *Alb-cre*, a transgene expressed in hepatoblasts (the precursors of hepatocytes and cholangiocytes) and mature hepatocytes. Bile duct ligation (BDL) was used to model biliary obstruction.

Results *Alb-Cre;Gata6^{fllox/fllox}* mice were viable and fertile. Cre-mediated recombination of *Gata6* in hepatocytes had little impact on cellular structure or function. GATA6 immunoreactivity was retained in a majority of biliary epithelial cells in adult *Alb-Cre;Gata6^{fllox/fllox}* mice, implying that surviving cholangiocytes were derived from hepatoblasts that had escaped biallelic Cre-mediated recombination. Although GATA6 immunoreactivity was preserved in cholangiocytes, *Alb-cre;Gata6^{fllox/fllox}* mice had a demonstrable biliary phenotype. A neutrophil-rich infiltrate surrounded newly formed bile ducts in neonatal *Alb-Cre;Gata6^{fllox/fllox}* mice. Foci of fibrosis/necrosis, presumed to reflect patchy defects in bile duct formation, were observed in the livers of 37% of adult *Alb-cre;Gata6^{fllox/fllox}* mice and 0% of controls ($p < 0.05$). Most notably, *Alb-cre;Gata6^{fllox/fllox}* mice had an altered response to BDL manifest as reduced survival, impaired bile ductule proliferation, increased parenchymal necrosis, reduced fibrosis, and enhanced macrophage accumulation in the portal space.

Conclusions GATA6 orchestrates intrahepatic biliary remodeling and mitigates liver injury following extrahepatic bile duct obstruction.

Graphic abstract

Keywords Biliary atresia · Biliary obstruction · Cholangiocyte · GATA factor · Gene expression · Hepatocyte · Mouse · Liver · Partial hepatectomy · Transcription factor

Marjut Pihlajoki and Tea Soini are co-first authors.

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Abbreviations

ALT	Alanine aminotransferase
BA	Biliary atresia
BDL	Bile duct ligation
BW	Body weight
CK	Cytokeratin
DR	Ductular reaction

E	Embryonic day
PFA	Paraformaldehyde
PHx	Partial hepatectomy
P	Postnatal day
R26R	<i>Rosa26</i> Reporter

Introduction

The two principal liver cell types, hepatocytes and cholangiocytes, derive from foregut (definitive) endoderm [7, 11]. At embryonic day (E) 9.0 in the mouse, hepatoblasts delaminate from foregut endoderm and invade the adjacent septum transversum, forming the liver bud [7]. Hepatoblasts proliferate and then differentiate into hepatocytes and cholangiocytes, a process that begins at ~E12.5 and continues through the balance of gestation [7]. Underscoring their bipotential nature, hepatoblasts express markers of both hepatocytes [albumin (ALB)] and cholangiocytes [cytokeratin 19 (CK19)] [7]. The differentiation of cholangiocytes initiates at the ductal plate, a monolayer of cells surrounding the portal vein [7]. Cholangiocyte precursors, which express higher levels of CK19 and SRY-related HMG box transcription factor 9 (SOX9) than adjoining hepatoblasts, bud from the ductal plate to form primitive tubular structures [12, 17]. Shortly after birth, these structures integrate with portal mesenchyme to form mature bile ducts [25]. The differentiation of intrahepatic bile ducts begins in the hilum of the liver and progresses toward its periphery [25]. Whereas cholangiocytes lining intrahepatic bile ducts derive from hepatoblasts, cholangiocytes in extrahepatic bile ducts arise directly from definitive endoderm through branching morphogenesis [25].

Although normally quiescent in adults, intrahepatic bile ducts can undergo adaptive remodeling following liver injury [9, 17]. A histopathological hallmark of this compensatory response is ductular reaction (DR), different forms of which occur in a range of hepatobiliary diseases [11, 20]. Extrahepatic biliary obstruction elicits a classic form of DR characterized by accumulation of primitive bile ductules, intermediate hepatobiliary cells, inflammatory cells, and collagen within the portal tract [1]. Ductular expansion serves to limit the toxic effects of excessive bile accumulation within the parenchyma as evidenced by the pronounced liver damage seen in mouse models with impaired DR [10, 17]. The cellular origin of reactive bile ductules is not fully understood [11]; some of the cholangiocytes in these new ductules arise from mitosis, but hepatocytes also can give rise to cholangiocytes via direct lineage conversion (metaplasia or transdifferentiation) [16, 21].

One of the transcription factors involved in liver cell differentiation and the response to injury is GATA6, a member of a family of zinc finger proteins that regulate cell fate

specification in many tissues [14]. *Gata6* is expressed in definitive endoderm and hepatoblasts of the liver bud [29]. *Gata6*^{−/−} mice die during gastrulation due to defects in primitive endoderm [29], so the role of this transcription factor in liver function cannot be deduced from these animals. Through tetraploid embryo complementation, a means of circumventing defects in primitive endoderm function, investigators have shown that GATA6 is essential for liver bud formation and expansion in the mouse [29]. Later in gestation, liver expression of *Gata6* becomes restricted to non-hepatocyte cell populations, mainly cholangiocytes, endothelial cells, and Kupffer cells [23, 30]. Viral-mediated silencing of *Gata6* in hepatocytes of the adult mouse has little impact on liver histology or gene expression, implying that GATA6 is dispensable in this cell type [30]. On the other hand, emerging evidence suggests that this transcription factor is important for cholangiocyte differentiation and bile duct development. Heterozygous loss-of-function mutations in human *GATA6* cause gallbladder agenesis and other extrahepatic biliary malformations [5]. In biliary atresia, a disease characterized by obstruction of extrahepatic bile ducts, liver *GATA6* expression increases with pathological intrahepatic bile duct expansion and decreases after successful Kasai portoenterostomy [23]. *Gata6* is upregulated during de novo transdifferentiation of hepatocytes into cholangiocytes in a mouse model of Alagille syndrome [21, 23], and forced expression of *GATA6* in cultured human hepatocytes induces expression of cholangiocyte lineage markers [23].

Although GATA6 has been implicated in cholangiocyte differentiation and bile duct proliferation, much of the evidence is circumstantial. Here, we use Cre-*LoxP* technology to probe the role of GATA6 in hepatobiliary pathophysiology in an animal model. We show that conditional deletion of the murine *Gata6* gene using *Alb-cre* disrupts intrahepatic bile duct development and the response to bile duct ligation (BDL).

Methods

Experimental animals and surgical procedures

Gata6^{fllox/fllox} mice [22] (Jackson Laboratory, 129.B6 background) were crossed with *Alb-cre* transgenic mice [19] (Washington University core, C57Bl/6 background) to generate *Alb-cre;Gata6*^{fllox/+} mice, which were then backcrossed to *Gata6*^{fllox/fllox} mice to yield *Alb-cre;Gata6*^{fllox/fllox} mice. Mice were genotyped using established PCR primers [19, 22]. Mice were euthanized by CO₂ asphyxiation. Serum samples were collected via cardiac puncture immediately after death. Two-thirds partial hepatectomy (PHx) was performed [8] on 2-months-old male *Gata6*^{fllox/fllox} and *Alb-cre;Gata6*^{fllox/fllox} mice. Tissue was harvested 36 h after surgery; 1 h prior to

harvest, mice were injected with 5-bromo-2'-deoxyuridine (BrdU, 100 mg/kg intraperitoneally) [12]. BDL or sham surgery (incision/suturing of the skin and peritoneum) was performed on 2-months-old *Gata6^{flx/flx}* and *Alb-cre;Gata6^{flx/flx}* mice [24]. Following surgery, mice were monitored by technicians who were blinded to genotype. Mice subjected to BDL or sham surgery were euthanized after 14 days; animals exhibiting signs of distress were euthanized earlier. At the conclusion of the experiment, mice were examined for gross anatomical evidence that BDL was a technical success [gallbladder enlargement, biloma formation, jaundice, and liver enlargement/texture], and tissue was harvested for analysis.

Histological analyses

Tissues were fixed overnight in 4% paraformaldehyde (PFA) in PBS. Paraffin-embedded tissue sections (5 µm) were prepared and stained with H&E, Masson's trichrome, or Sirius red. For histomorphometric analysis, images were generated using a 3DHISTECH Pannoramic 250 FLASH II digital slide scanner. Fibrosis was quantified as a percentage of Sirius red stained area compared with total section area. Areas of necrosis were quantified in trichrome-stained sections in a similar manner. Paraformaldehyde-fixed, paraffin-embedded tissue sections were immunostained using an avidin–biotin immunoperoxidase system (Vector Laboratories) or fluorescent antibodies [18]. The primary and secondary antibodies used for each antigen are listed in Table S1. BrdU labeling and CK19⁺ areas were quantified as described [10].

Electron microscopy

Mice were anesthetized and then perfused with modified Karnovsky fixative via a needle inserted into the left ventricle [18]. Tissue was harvested and processed for transmission electron microscopy [30].

Serum chemistry measurements

Serum alanine aminotransferase (ALT), total bilirubin (as diazo derivative), and albumin (as bromocresol green complex) were measured on a Roche c501 chemistry platform. Whole blood glucose levels were determined by handheld glucometer (Arkray).

Genomic PCR to assess the Cre-mediated recombination

DNA was extracted from liver and subjected to PCR using a set of primers that discriminate wild-type, floxed, and recombined alleles [22]. Hepatocytes were isolated as described [8].

Quantitative RT-PCR (RT-qPCR)

RNA was isolated from liver and subjected to RT-qPCR analysis [8] using the primers in Table S2. Expression was normalized to the housekeeping genes *Gapdh*, *Actb*, and *Ppig*.

Statistical methods

Data are expressed as mean ± SE. Statistical significance was determined using the Student's *t* test (two-tailed) except where otherwise noted.

Results

Generation of *Alb-cre;Gata6^{flx/flx}* mice

Consistent with prior reports [23, 30] we observed nuclear GATA6 immunostaining in cholangiocytes, as evidenced by co-localization with SOX9, within small and large intrahepatic bile ducts of both adult (Fig S1A, S2) and neonatal (Fig S3A, B) mice. Variable GATA6 immunoreactivity was seen in endothelial cells and Kupffer cells (Fig S3A, B) but hepatocytes were devoid of GATA6 staining (Fig S1A).

To investigate the role of GATA6 in hepatobiliary development and function, we generated liver-specific knockout mice using an *Alb-cre* transgene [19] and a floxed *Gata6* allele [22] (Fig S1B). Cre-mediated recombination of *Gata6^{flx}* deletes exon 2, yielding a functionally null allele (*Gata6^Δ*). *Rosa26* reporter (*R26R*) experiments have shown that *Alb-cre* is expressed stochastically in hepatoblasts, leading to recombination of floxed alleles in a subset of these bipotential precursors of hepatocytes and cholangiocytes [16]. *Alb-cre* expression persists in fetal and postnatal hepatocytes, resulting in extensive Cre-mediated recombination in this cell type by 6 weeks of age [28]. *Alb-cre* expression is extinguished in cholangiocytes, but *Alb-cre;R26R* lineage tracing studies have shown that a preponderance of cholangiocytes in adult mice derive from hepatoblasts that have undergone Cre-LoxP recombination [16, 28]. Therefore, *Alb-cre* is suitable for targeting gene deletion in both hepatocytes and cholangiocytes, although the extent of Cre-LoxP recombination in cholangiocytes varies depending on the floxed gene [16, 28]. *Alb-cre* is not active in other lineages such as sinusoidal endothelial cells or Kupffer cells.

Male and female *Alb-cre;Gata6^{flx/flx}* mice were born at the expected Mendelian frequencies in two separate crosses (Fig S1C). The conditional knockout mice appeared healthy and survived to adulthood with normal growth (Fig S4). When mated with proven breeders, male and female *Alb-cre;Gata6^{flx/flx}* mice produced normal sized litters (9–11 pups).

Fig. 1 *Gata6* expression is induced in cholangiocytes around the time of birth, and conditional deletion of *Gata6* triggers a periductal infiltrate in neonatal liver. Liver sections from E15.5 (a–d) or P2 (e–h) mice of the indicated genotypes were stained with antibody against SOX9 (a, b), GATA6 (c–f), or myeloperoxidase (MPO) (g, h). SOX9⁺ ductal plate cells (arrowheads in a) were evident in both *Gata6*^{flx/flx} and *Alb-cre;Gata6*^{flx/flx} mice. In contrast, there was no discernible GATA6 staining in the ductal plate cells of either strain. GATA6 staining in cardiac atrial cells served as an internal positive control (insets, c, d). At P2, GATA6⁺ cholangiocytes were observed in both *Gata6*^{flx/flx} and *Alb-cre;Gata6*^{flx/flx} mice. An inflammatory infiltrate composed mainly of MPO⁺ neutrophils (arrow in f) surrounded newly formed bile ducts in 4 of 4 (100%) *Alb-cre;Gata6*^{flx/flx} mice but was absent from 4 littermate *Gata6*^{flx/flx} mice ($p < 0.01$, two populations proportions testing). The insets in panels g and h show smaller bile ducts in the periphery. at atrium, b bile duct, v portal vein. Bars = 20 μ m

Extent of Cre-mediated recombination in hepatocytes vs. cholangiocytes

To confirm Cre-mediated deletion of *Gata6*, we subjected DNA isolated from liver extracts of adult mice to PCR using primers that distinguish intact and recombined (*Gata6* ^{Δ}) alleles (Fig S1B). We detected *Gata6* ^{Δ} in the livers of *Alb-cre;Gata6*^{flx/+} and *Alb-cre;Gata6*^{flx/flx} mice, but not *Gata6*^{flx/flx} mice (data not shown). PCR of DNA isolated from purified hepatocytes yielded similar results (Fig S5).

To assess the extent of Cre-mediated recombination in cholangiocytes, we performed immunostaining for GATA6 on liver tissue from 2-months-old *Alb-cre;Gata6*^{flx/flx} mice (Fig S6A, B). Adjacent tissue sections were stained with CK19 to highlight cholangiocytes (Fig S6C, D). Notably, GATA6 immunoreactivity was detected in the vast majority of cholangiocytes, including those in large ducts near the liver hilum and in smaller ducts near the periphery. Thus, most of the surviving cholangiocytes in adult *Alb-cre;Gata6*^{flx/flx} mice were derived from hepatoblasts that had escaped biallelic deletion of *Gata6* (Fig S6E). Although GATA6 immunoreactivity was preserved in the majority of cholangiocytes in *Alb-cre;Gata6*^{flx/flx} mice, there was nevertheless a demonstrable hepatobiliary phenotype in neonatal and adult *Alb-cre;Gata6*^{flx/flx} mice, as detailed in the following sections and summarized in Table S3.

Transient inflammation accompanies bile duct maturation in newborn *Alb-cre;Gata6*^{flx/flx} mice

At E15.5, SOX9 immunostaining delineated ductal plate cells (nascent cholangiocytes) in both *Gata6*^{flx/flx} and *Alb-cre;Gata6*^{flx/flx} liver (Fig. 1a, b). These SOX9-immunoreactive cells were GATA6-negative (Fig. 1c, d), suggesting that GATA6 may not be crucial for the earliest phases of cholangiocyte differentiation. By P2, GATA6 immunoreactivity was evident in cholangiocytes of *Gata6*^{flx/flx} (Fig. 1e), implying that this gene is upregulated in biliary epithelial

cells between E15.5 and P2, which coincides with bile duct formation/maturation.

Interestingly, a prominent infiltrate of inflammatory cells surrounded newly formed bile ducts in *Alb-cre;Gata6*^{flx/flx} mice but not littermate *Gata6*^{flx/flx} mice (Fig. 1e–g). The infiltrate, which contained many myeloperoxidase-positive neutrophils, was morphologically distinct from islands of extramedullary hematopoiesis that typify this stage of liver development. These findings suggest that when *Gata6* upregulation is impaired by gene targeting, inflammation ensues. Neonatal jaundice was not evident in the *Alb-cre;Gata6*^{flx/flx} mice, indicating that bile flow was not severely compromised in these animals. By P30, this periductal inflammatory infiltrate resolved (Fig S7), and at P75 serum total bilirubin levels were below the limit of detection (<0.1 mg/dL) in adult *Gata6*^{flx/flx} and *Alb-cre;Gata6*^{flx/flx} mice ($n = 10$ each).

Thus, mosaic inactivation of *Gata6* at the hepatoblast stage of development leads to transient inflammation in the bile ducts of newborn mice. The appearance of the neutrophil-rich infiltrate in *Alb-cre;Gata6*^{flx/flx} mice coincides with the normal upregulation of *Gata6* in maturing bile ducts, suggesting that GATA6 modulates intrahepatic biliary remodeling in newborn mice.

Focal histological abnormalities in the livers of 2-months-old *Alb-cre;Gata6*^{flx/flx} mice

In a majority of young adult *Alb-cre;Gata6*^{flx/flx} mice, the liver appeared normal when examined by H&E staining or trichrome staining (Fig. 2a–d). At the ultrastructural level (Fig. 2e–f), hepatocytes and cholangiocytes exhibited normal polarization and cell-to-cell contacts. The apical surfaces of adjacent hepatocytes formed bile canaliculi, structures that facilitate the transport of newly secreted bile to cholangiocyte-lined bile ducts. Along their basolateral surface, hepatocytes were separated from adjoining sinusoidal endothelial cells by the space of Disse, the site at which substances are exchanged between plasma and hepatocytes. The apical poles of cholangiocytes contained junctional complexes and primary cilia, structures that control mechanico- and chemo-sensing.

Two indicators of hepatocyte function, serum albumin (Fig. 2g) and whole blood glucose (Fig. 2h), were indistinguishable in young adult *Alb-cre;Gata6*^{flx/flx} and *Gata6*^{flx/flx} mice. There was no difference in expression of hepatocyte-specific transcripts (*Alb*, *Cebpb*) between the conditional knockout and control mice (Fig. 2i). Collectively, these results suggest that silencing of *Gata6* in hepatocytes has no substantial effect on basal function, in agreement with a previous report [30]. To extend the functional analysis of hepatocytes, we subjected 2-months-old *Gata6*^{flx/flx} and *Alb-cre;Gata6*^{flx/flx} mice to two-thirds

Fig. 2 Histological, ultrastructural, and biochemical analysis of livers from P75 *Alb-cre;Gata6^{flox/flox}* mice. **a–d** Liver sections from *Alb-cre;Gata6^{flox/flox}* mice were stained with H&E or trichrome. The majority (5/8=63%) of *Alb-cre;Gata6^{flox/flox}* mouse livers were indistinguishable from those of control mice (n=13); the remainder (3/8=37%) had isolated patches of portal fibrosis with cholestasis (arrows) or hepatocyte necrosis (*, inset). Abbreviation: v, portal vein. Bars=40 μ m. **e–f** Transmission electron microscopy of *Alb-cre;Gata6^{flox/flox}* mouse liver (n=3). Normal-appearing hepatocytes abut an endothelial cell-lined sinusoid (e). Note the presence of bile canaliculi (apical hepatocyte membrane) and the peri-sinusoidal space of Disse (basolateral hepatocyte membrane). Normal appear-

ing cholangiocytes line a bile duct (f). Junctional complexes between adjacent cholangiocytes and a primary cilium are highlighted. Bars=2 μ m (left), 1 μ m (right), 0.4 μ m (inset). **g** Serum albumin levels in P75 *Gata6^{flox/flox}* (n=11) and *Alb-cre;Gata6^{flox/flox}* mice (n=5 per group). **h** Blood glucose levels (n=5 per group). **i** RT-qPCR analysis of the hepatocyte markers *Alb* and *Cebpb*, the cholangiocyte marker *Ck19*, and the macrophage marker *Adgre* in P75 mice *Gata6^{flox/flox}* (n=11) and *Alb-cre;Gata6^{flox/flox}* mice (n=6). **j** Serum ALT levels. Dashed lines indicate the mean. None of these biochemical parameters differed significantly between conditional knockout and control mice

PHx, an established model of regenerative stress. Tissue was harvested 36 h after PHx (Fig S8A), a time point normally associated with a brisk hepatocyte proliferative response [8]. *Gata6^{flox/flox}* and *Alb-cre;Gata6^{flox/flox}* mice had similar

liver to body weight ratios at baseline and post-PHx (Fig S8B). There were no differences in post-PHx blood glucose levels between *Gata6^{flox/flox}* and *Alb-cre;Gata6^{flox/flox}* mice (Fig S8C). Moreover, hepatocyte BrdU labeling index, a

measure of proliferation, was similar in *Gata6^{flox/flox}* and *Alb-cre;Gata6^{flox/flox}* mice (Fig S8D–F). The cholangiocyte BrdU labeling index was reduced in the *Alb-cre;Gata6^{flox/flox}* mice, although the difference was not significant ($p = 0.27$, Student's *t*-test) (Fig S8F). The absence of a PHx phenotype in *Alb-cre;Gata6^{flox/flox}* mice serves as a counterpoint to the robust BDL phenotype described below.

Although adult liver function appeared intact in the aforementioned experiments, isolated foci of portal fibrosis, hepatocyte necrosis, or mild DR were observed in the livers of 3/8 (37%) young adult *Alb-cre;Gata6^{flox/flox}* mice (Fig. 2b, d; Fig S9). By comparison, no lesions were observed in 13 age-matched control (*Gata6^{flox/flox}*) mice ($p < 0.05$, two-populations-proportion-testing). Within affected livers, these foci of fibrosis/necrosis were rare, constituting $< 5\%$ of the liver cross sectional area. The focal liver lesions in adult *Alb-cre;Gata6^{flox/flox}* mice may reflect patchy defects in intrahepatic bile duct development. At 2 months of age, serum

ALT activity, an indicator of hepatocyte injury, did not differ significantly between *Gata6^{flox/flox}* vs. *Alb-cre;Gata6^{flox/flox}* mice (Fig. 2j).

Aberrant response to BDL in *Alb-cre;Gata6^{flox/flox}* mice

In patients with biliary atresia, liver *GATA6* expression correlates with pathological intrahepatic bile duct expansion [23]. Whether *GATA6* is essential for this process is unclear. To assess the impact of *Gata6* deletion on the response to extrahepatic bile duct obstruction, 2-months-old *Alb-cre;Gata6^{flox/flox}* or control (*Gata6^{flox/flox}*) mice were subjected to BDL. Tissue and blood samples were analyzed 14-days later (Fig. 3a). Studies of wild-type mice have shown that the acute cholestasis caused by BDL elicits a stereotypical response that includes necroinflammation, cholangiocyte proliferation, intermediate cell activation, and fibrosis [1,

Fig. 3 *Alb-cre;Gata6^{flox/flox}* mice exhibit increased hepatic necrosis and decreased survival following BDL. **a** Adult (2-months-old) *Gata6^{flox/flox}* or *Alb-cre;Gata6^{flox/flox}* mice were subjected to BDL or sham surgery and monitored for 14 days by technicians who were blinded to genotype. Mice exhibiting signs of distress were euthanized before day 14 and scored as non-survivors. **b, c** BDL resulted in gallbladder dilation (indigo arrows) and biloma formation in both *Gata6^{flox/flox}* and *Alb-cre;Gata6^{flox/flox}* mice, but only *Alb-cre;Gata6^{flox/flox}* mice had large patches of liver necrosis (white arrow) evident on gross examination at day 14. **d, e** Kaplan–Meier curves comparing post-BDL survival of *Gata6^{flox/flox}* (solid line) and *Alb-cre;Gata6^{flox/flox}* (dashed line) mice. Sham surgery survival was 100% for both strains (not shown).

The inset of panel **d** shows a dot plot of post-BDL serum total bilirubin levels for the *Gata6^{flox/flox}* and *Alb-cre;Gata6^{flox/flox}* mice that survived 14 days. Horizontal dashed lines in the inset indicate the mean serum total bilirubin levels. Some of the survivors had only modest elevations in serum total bilirubin levels (< 6 mg/dL, red circles), suggesting that BDL may have been incomplete in these animals. When these mice were censored in a post hoc analysis (**e**), there was still a marked difference in post-BDL survival between *Gata6^{flox/flox}* and *Alb-cre;Gata6^{flox/flox}* mice. Prepared using image vectors from Servier Medical Art (www.servier.com), licensed under the Creative Commons Attribution 3.0 Unported License (<http://creativecommons.org/licenses/by/3.0/>).

Fig. 4 Abnormal histological and biochemical response to BDL in *Alb-cre;Gata6^{fllox/fllox}* mice. Adult (2-months-old) *Gata6^{fllox/fllox}* or *Alb-cre;Gata6^{fllox/fllox}* mice were subjected to BDL or sham surgery, and tissue was harvested 14 days later for analysis. H&E (a), GATA6 (b), and CK19 (c) immunostaining demonstrated numerous reactive bile ductules (arrowheads) in the portal regions of *Gata6^{fllox/fllox}* but not *Alb-cre;Gata6^{fllox/fllox}* mice. Consistent with the immunostaining results, RT-qPCR demonstrated reduced expression of the cholangiocyte markers *Ck19* and *EpCAM* (e) in post-BDL liver of *Alb-cre;Gata6^{fllox/fllox}* mice ($n=7-11$ per group). Histomorphometric analysis (d) confirmed reduced CK19 immunoreactivity in *Alb-cre;Gata6^{fllox/fllox}* mice ($n=5$ per group). *b* bile duct, *n* necrosis, *v* portal vein. Bars = 50 μ m (a), 25 μ m (b), 75 μ m (c). (* $p<0.05$)

24]. Following BDL interlobular bile ducts undergo adaptive remodeling, wherein in the intraluminal surface area is expanded via corrugation and branching [27]. *Gata6* is expressed in these reactive bile ductules [23].

Consistent with published reports for wild-type mouse strains [1], *Gata6^{fllox/fllox}* mice had a 14-day post-BDL survival rate of 88% (Fig. 3d). In contradistinction, *Alb-cre;Gata6^{fllox/fllox}* mice had a 14-day post-BDL survival rate of only 50% (Fig. 3d, $p=0.014$, Gehan-Breslow-Wilcoxon test). Some of the surviving *Gata6^{fllox/fllox}* and *Alb-cre;Gata6^{fllox/fllox}* mice had only modest elevations in serum total bilirubin levels (<6 mg/dL), suggesting that BDL may have been incomplete in these animals (Fig. 3d, inset). When these mice were censored in a post hoc analysis (see legend), there was still a marked difference in post-BDL survival between *Gata6^{fllox/fllox}* and *Alb-cre;Gata6^{fllox/fllox}* mice (Fig. 3e, $p=0.009$, Gehan-Breslow-Wilcoxon test). As expected, BDL ligation resulted in gallbladder dilation $\pm$ biloma formation in both *Gata6^{fllox/fllox}* and *Alb-cre;Gata6^{fllox/fllox}* mice (Fig. 3b, c). Post-BDL liver to body weight ratios did not differ between *Gata6^{fllox/fllox}* and *Alb-cre;Gata6^{fllox/fllox}* mice ($7.60 \pm 0.29\%$ and $7.63 \pm 0.48\%$, respectively), but the conditional knockout mice had large patches of liver necrosis evident on gross examination (Fig. 3b vs. c). Massive areas of liver necrosis were observed in *Alb-cre;Gata6^{fllox/fllox}* mice that died within a week of BDL (Fig S10).

H&E, GATA6, and CK19 staining of post-BDL liver sections demonstrated an abundance of reactive bile ductules in the portal regions of *Gata6^{fllox/fllox}* mice (Fig. 4a–c). In contrast, there was a paucity of CK19⁺ neoductules in *Alb-cre;Gata6^{fllox/fllox}* mice (Fig. 4d), and the ductules that did form were irregular [N.B., we define neoductules newly formed ductular structures within the portal space that express CK19, SOX9, and proliferation markers (Ki-67 or BrdU)]. Reactive cholangiocytes in *Alb-cre;Gata6^{fllox/fllox}* mice had a reduced Ki67 labeling index (Fig. 5a, b), suggesting that GATA6 deficiency negatively impacts cholangiocyte proliferation in response to BDL. Following BDL, there was a prominent infiltrate of macrophages in the portal region of *Alb-cre;Gata6^{fllox/fllox}* vs. *Gata6^{fllox/fllox}* mice, as highlighted by F4/80 immunostaining (Fig. 5c), 1 μ m epoxy sections

(Fig. 5e), and transmission electron microscopy (Fig S11). Corroborating the immunohistochemical findings, RT-qPCR demonstrated reduced expression of two cholangiocyte markers (*Ck19* and *EpCAM*; Fig. 4e) and increased expression of the macrophage marker *Adgre* (encoding F4/80 antigen; Fig. 5d) in post-BDL liver of *Alb-cre;Gata6^{fllox/fllox}* vs. *Gata6^{fllox/fllox}* mice.

Next, we examined the post-BDL liver specimens for the presence of intermediate cells, which are defined as cells exhibiting both hepatocytic and biliary differentiation in diseased liver [11]. BDL is known to induce the expression of *Sox9*, a cholangiocyte marker, in periportal hepatocytes of wild-type mice [11]. SOX9 immunoreactivity was evident in periportal hepatocytes (arrowheads) of both *Gata6^{fllox/fllox}* and *Alb-cre;Gata6^{fllox/fllox}* mice (Fig S12). That *Gata6* targeting attenuates post-BDL CK19 immunoreactivity (Fig. 4c, d) but not SOX9 immunoreactivity is in keeping with an earlier report showing that forced expression of GATA6 in primary human hepatocytes induces expression of *CK19* but not *SOX9* [23].

To quantify the extent of baseline and reactive fibrosis, we performed Sirius red staining of collagen on sham and BDL mice (Fig. 6a–d). Consistent with published reports for wild-type mice [1, 24], BDL triggered a marked increase in fibrosis in *Gata6^{fllox/fllox}* mouse liver (Fig. 6e). On the other hand, BDL elicited no significant change in fibrosis in *Alb-cre;Gata6^{fllox/fllox}* mouse. Using histomorphometric analysis, we documented increased parenchymal necrosis in the livers of *Alb-cre;Gata6^{fllox/fllox}* mice, both pre- and post-BDL (Fig S13). The measured degree of necrosis in livers of *Alb-cre;Gata6^{fllox/fllox}* mice is an underestimate, because we intentionally did not make tissue sections through large, confluent areas of necrosis (Fig. 3c). Despite these histological differences, post-BDL serum total bilirubin levels were comparable in *Gata6^{fllox/fllox}* (11.7 ± 5.6 mg/dL) and *Alb-cre;Gata6^{fllox/fllox}* (11.1 ± 4.3 mg/dL) animals ($p=0.68$; Fig. 3d, inset).

In summary, we found that *Alb-cre;Gata6^{fllox/fllox}* mice have an altered response to BDL that includes decreased survival, reduced bile ductule proliferation, pronounced parenchymal necrosis, increased macrophage infiltration, and decreased fibrosis. Our analysis of the impact of BDL included both male and female mice. No significant sex variation was observed in the survival or histological response of *Alb-cre;Gata6^{fllox/fllox}* mice to BDL (data not shown).

Discussion

GATA6, a transcription factor expressed in definitive endoderm and hepatoblasts, is crucial for the earliest stages of liver development. GATA6 is known to bind and activate genes necessary for endoderm differentiation, and

Fig. 5 Impaired cholangiocyte proliferation and increased macrophage infiltration following BDL in *Alb-cre;Gata6*^{flox/flox} mice. Adult (2-months-old) *Gata6*^{flox/flox} or *Alb-cre;Gata6*^{flox/flox} mice were subjected to BDL or sham surgery, and tissue was harvested 14 days later for analysis. Reactive cholangiocytes in *Alb-cre;Gata6*^{flox/flox} mice had reduced Ki67 immunoreactivity compared to controls (**a**, **b**) ($n=5$ per group). F4/80 immunostaining (**c**) and 1 μ m epoxy sec-

tions (**e**) showed increased macrophages (arrows) in the portal region of *Alb-cre;Gata6*^{flox/flox} mice. Consistent with the immunostaining results, RT-qPCR demonstrated increased expression of the macrophage marker *Adgre* (**d**) in post-BDL liver of *Alb-cre;Gata6*^{flox/flox} mice ($n=7-11$ per group). *b* bile duct, *h* hepatocyte, *v* portal vein. Bars = 25 μ m (**a**, **c**), 20 μ m (**e**). (* $p<0.05$; *** $p<0.005$)

modulation of GATA6 expression has been shown to affect in vitro differentiation of human pluripotent stem cells into various endoderm derivatives, including liver parenchymal cells [6, 15, 26]. Tetraploid embryo complementation experiments have proven that GATA6 is required for liver bud formation and expansion [29], but the resultant embryos do not survive beyond ~E11, precluding an assessment of later aspects of hepatic development such as cholangiocyte differentiation and bile duct formation.

To investigate the role of GATA6 in postnatal liver development and function, we generated and analyzed *Alb-cre;Gata6*^{flox/flox} mice. Cre-mediated recombination in

hepatocytes of *Alb-cre;Gata6*^{flox/flox} mice had little impact on hepatocyte function in young adult mice, as evidenced by normal levels of serum albumin and an intact regenerative response to PHx. This confirms and extends an earlier report showing that viral-mediated silencing of *Gata6* in hepatocytes of the adult mouse had minimal effects on gene expression [30]. Although hepatocellular function was preserved in *Alb-cre;Gata6*^{flox/flox} mice, these animals had a demonstrable biliary phenotype first manifest at P2 as a neutrophil-rich inflammatory infiltrate surrounding newly formed bile ducts. Later, isolated foci of parenchymal fibrosis/necrosis, presumed to reflect patchy defects in the

Fig. 6 Decreased BDL-induced fibrosis in *Alb-cre;Gata6*^{flox/flox} mice. Adult (2-months-old) *Gata6*^{flox/flox} or *Alb-cre;Gata6*^{flox/flox} mice were subjected to sham surgery (a, b) or BDL (c, d). Tissue was harvested 14 d later and stained with Sirius red to quantify collagen content (e, $n=5-7$ per group). As expected, BDL induced fibrosis in *Gata6*^{flox/flox} mice. In contrast, BDL did not elicit fibrosis in *Alb-cre;Gata6*^{flox/flox} mice. Bars = 40 μ m. (* $p < 0.05$)

biliary tree, were observed in the livers of a subset of adult *Alb-cre;Gata6*^{flox/flox} mice. Most strikingly, the conditional knockout mice had an altered response to BDL that included an impaired DR, increased hepatic necrosis, and decreased survival. These findings are reminiscent of another genetically-engineered mouse model in which impaired DR was associated with submassive hepatic necrosis and increased mortality after BDL [10], supporting the premise that adaptive remodeling of bile ducts helps contain the accumulation of bile acids following acute biliary obstruction. We propose that upregulation of *Gata6* in neoductules is required to compensate for obstructed bile flow and minimize acute liver injury (inflammation and infarcts) and that impaired survival post-BDL reflects inadequate bile duct expansion to protect

the parenchyma. Taken together, our studies establish that GATA6 plays a role in intrahepatic biliary development and biliary epithelial tissue expansion/remodeling in response to injury. Our findings are in keeping with a recent analysis of tissue samples from patients with biliary atresia [23], wherein GATA6 was implicated in the reparative response to hepatobiliary damage. Although BDL is a widely used experimental model of biliary obstruction and fibrosis, it has limitations. Notably, the reactive cholangiocytes in this model derive mainly from proliferation of mature cholangiocytes in larger bile ducts rather than from activation of bipotential liver progenitor cell populations, and the latter is thought to underlie the pathobiology of many human hepatobiliary diseases. Moreover, focal abnormalities in bile duct development may exacerbate the post-BDL phenotype of adult *Alb-cre;Gata6*^{flox/flox} mice.

Analysis of mice harboring germline or conditional mutations in transcription factor genes has yielded insights into biliary morphogenesis, a complex process driven by coordinated cell–cell interactions [7]. Transcriptional regulators crucial for intrahepatic bile duct development or remodeling include HNF6, HNF1 β , C/EBP α , FOXM1, SOX4, SOX9, KLF5, and NF κ B [2, 4, 13, 17]. These factors work in concert with key signaling pathways such as the NOTCH, TGF β , and Hippo/YAP pathways [3, 10, 20]. Based on our findings, GATA6 can be added to this list of transcription factors, although it may not be crucial for the earliest aspects of biliary development given the limited expression of *Gata6* detected in the ductal plate. Interestingly, many of the growth factors and transcription factors involved in formation of the biliary epithelium during embryonic life are reactivated in adulthood in response to liver injury [11]. Our results suggest that GATA6 participates in the gene regulatory networks that underlie DR. Moreover, our findings reinforce the notion that transcription factors involved in injury repair recapitulate those required for biliary ontogenesis.

Although the findings presented here establish a role for GATA6 in biliary development and the response to extrahepatic biliary obstruction, the mechanistic underpinnings of the *Alb-cre;Gata6*^{flox/flox} phenotype are less clear. Potential mechanisms include: (1) a decrease in the number of proliferating hepatoblasts, (2) a cell-autonomous defect in the differentiation of GATA6-deficient hepatoblasts into cholangiocytes, (3) impaired survival of GATA6-deficient cholangiocytes, (4) a cell-autonomous defect in lineage conversion of GATA6-deficient hepatocytes into cholangiocytes, and (5) altered cell–cell interactions or impaired production of secreted factors by liver cells. Also, a haploinsufficiency effect is theoretically possible, but germline *Gata6*[±] mice do not have a known liver phenotype.

The cellular origin of reactive bile ductules remains an area of active investigation and controversy. Under certain circumstances, murine hepatocytes can undergo lineage

conversion into cholangiocytes [16, 21]. Previously we suggested that *GATA6*, which is upregulated in periportal hepatocytes of children with biliary atresia, may drive hepatocyte-cholangiocyte lineage conversion in humans [23]. Likewise, *Gata6* has been shown to be induced in periportal hepatocytes in mouse models of DR, including BDL [23]. A recent study showed that *Gata6* expression is upregulated 4.3-fold during de novo transdifferentiation of hepatocytes into hepatocyte-derived peripheral cholangiocytes in a mouse model of Alagille syndrome [21]. Therefore, the impaired DR seen in *Alb-cre;Gata6^{flox/flox}* mice post-BDL might be attributed to deletion of *Gata6* from hepatocytes undergoing lineage conversion. Arguing against this, a fate tracing study in mice showed that cholangiocytes generated in response to selective ligation of the left hepatic duct do not derive from hepatocytes [16].

Altogether, we conclude that GATA6, among other factors, orchestrates intrahepatic biliary remodeling and mitigates liver injury following extrahepatic bile duct obstruction.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest. All institutional and national guidelines for the care and use of laboratory animals were followed.

Ethical approval All procedures in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. This article does not contain any studies with human participants performed by any of the authors.

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