DLK1 and the Notch pathway in the liver
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INTRODUCTION PART II:

Bile duct development and involved transcription factors

CHAPTER 5
List of abbreviations
ED, embryonic day
STM, septum transversum mesoderm
IHBD, intrahepatic bile duct
EHBD, extrahepatic bile duct
PDS, primitive ductular structures
Introduction

Early liver and biliary development
The liver and biliary tract arise from the hepatic diverticulum at embryonic day (ED)9 in mice. The cranial portion of this endodermal diverticulum gives rise to the liver and all intrahepatic bile ducts (IHBDs), while the extrahepatic bile ducts (EHBDs) develop from the caudal portion (1, 2). At ED9.5, the hepatoblasts separate from the endodermal epithelium and invade the adjacent septum transversum mesenchyme (STM) to form the liver bud (1, 3). Hepatoblasts are bipotential and can either differentiate into hepatocytes or cholangiocytes. The cholangiocytes lining the EHBDs form directly from the endodermal cells of the caudal portion of the hepatic diverticulum (1, 2). The EHBDs and the developing intrahepatic biliary tree maintain luminal continuity throughout development (4, 5).

Intrahepatic bile duct (IHBD) morphogenesis
The morphogenesis of the IHBD is similar in humans, rats and mice proceeds stepwise (6). In ED 13.5-14.5 mice, the hepatoblasts around the large portal veins begin to express biliary-specific cytokeratins, which by ED15.5 transforms into the single-cell layered ductal plate at the boundary between the hepatocytes and the portal field. During the next day, the ductal plate becomes partly bilayered and then starts to remodel by forming focal dilations between the two cell layers to generate the lumina of “primitive ductular structures” (PDS) (7). The parts of the ductal plate which do not form patent ducts regress. In the final stage, which starts around birth, the ducts are incorporated into the portal mesenchyme (6). IHBD morphogenesis proceeds along a gradient from the hilum to the periphery of the liver, so that the smallest portal-vein branches in the liver periphery are still surrounded by ductal plates at birth. In humans, these peripheral ductal plates only develop into small portal ducts by 4 weeks after birth (2).

Transcription factors involved in early liver development
Foxa1-3
Fibroblast Growth Factor (Fgf) signals from the developing heart and Bone Morphogenetic Protein (Bmp) signals from the STM initiate liver development in the ventral foregut endoderm (8). The three members of the FoxA gene family of forkhead-box transcription factors (Foxa1-3) are present in the endoderm before the induction of the hepatic program by Fgf signals. Both single and double deletion of Foxa1 and Foxa3 resulted in embryonic lethality with histologically normal livers. However, Foxa1-2/Foxa3Cre embryos, where both Foxa1 and -2 are absent in the hepatic diverticulum, do not develop a hepatic bud and do not express α-fetoprotein in the ventral foregut (9) due to a loss of competence to respond to inductive signaling by FGF (9). Deletion of Foxa1 and -2 at ED11-12 with AlfpCre (Foxa1/2Cre/Foxa3Cre mice) resulted in viable mice with livers containing an increased amount of disorganized and dilated bile ducts surrounded by extensive extracellular matrix (10). Cholangiocytes in these mutant ducts showed increased proliferation compared to controls, probably due to increased IL-6 levels in serum and liver which has been
shown to stimulate cholangiocyte proliferation (10). These experiments show that the Foxa transcription factors determine liver specification and are required for normal bile duct morphogenesis.

Foxm1b
The Foxm1b (Forkhead box M1) transcription factor is expressed in actively dividing cells and critical for cell cycle progression. Its expression becomes highly upregulated during liver regeneration in mice (11). Foxm1b−/− mice are embryonic lethal between ED 15.5 and 18. Foxm1b−/− livers are disorganized, contain many polyploid hepatocytes, a diminished amount of large hepatic veins (11), and absent cholangiocyte differentiation, with decreased concentrations of HNF1β in the cytosol of periportal hepatoblasts.

Hnf1β
The Hnf1β (Tcf2) gene is essential for epithelial differentiation of the visceral endoderm. Deletion results in death during gastrulation. Tetraploid embryo complementation revealed that rescued Hnf1β-deficient embryos failed to develop a liver bud and expressed very low levels of Foxa1-3, Hhex and a-Fetoprotein in the ventral foregut at ED10.5 and ED11.5 (12).

Gata4 and Gata6
Mutation of the zinc-finger transcription factors Gata4 and Gata6 results in early embryonic lethality. Tetraploid embryo complementation, in which Gata6+/− embryos are provided with Gata6+/+ extra-embryonic endoderm, allowed survival till ED10.5 and showed that Gata 6 is essential for liver bud expansion and normal expression of Albumin, Hhex and Hnf4 in early hepatoblasts (13). The same approach for Gata4 yielded a similar phenotype with initial hepatic bud formation, but subsequent failure of hepatocytes to delaminate due to the absence of the STM (14). These results show that Gata transcription factors 4 and 6 are dispensable for hepatic specification, but indispensable for the maintenance of hepatic competence.

Hhex
The hematopoietically expressed homeobox gene (Hhex) is one of the earliest transcription factors expressed in the liver bud (15). Hhex−/− embryos die at ED10.5 (16). Although a hepatic diverticulum is present, proliferation of cells in the diverticulum is greatly reduced, resulting in a cystic primordium (15). Conditional deletion of Hhex in the hepatic diverticulum with Foxa3Cre caused death around ED18.5, with hypoplastic, cystic livers. In ED18.5 mutant livers, many CK-positive cysts developed in the absence of a portal vein branch. The embryos also lacked a gallbladder and EHBDs. Expression of Hnf4α and Hnf6 in Hhex+/−/Foxa3Cre mutants was 3 days delayed and appeared on ED16.5. After later deletion of Hhex with AlfpCre, ED18.5 livers also revealed many irregular duct-like structures and biliary cysts around portal vein branches, but also in the parenchyma. Hhex−/−/Foxa3Cre mice survived, but
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gradually developed polycystic liver disease, with all cysts being CK19-positive. These findings indicate that Hhex is indispensable for hepatoblast differentiation and for both IHBD and EHBD morphogenesis.

Prox1
The Prospero-related homeobox 1 (Prox1) transcription factor is already expressed in the hepatic epithelium by ED8.5 (17). In Prox1−/− embryos, a liver bud is formed, but fails to proliferate, because the basal lamina surrounding the bud is not degraded and disturbs invasion into the STM (18). The liver of a Prox1−/− liver is smaller than that of a wild-type embryo, and its hepatocytes remain clustered, even though the mesodermal components, including the definitive haematopoietic progenitors are present. The Tbox transcriptional repressor (Tbx3), which may act upstream of Prox1, also regulates delamination and expansion of the liver bud. Tbx3−/− hepatoblasts express reduced amounts of Hnfα, C/ebpα and Albumin, whereas Hnf6 and Hnf1β were increased. These findings indicate that Tbx3 promotes the hepatic fate and represses the cholangiocyctic fate in the liver bud (19).

Transcription factors involved in biliary development

Hnf6 and Hnf1β
The Hepatocyte Nuclear Factor 6 (Hnf6, Onecut1), is expressed in the liver bud and, at later stages, in hepatocytes and biliary epithelial cells of the IHBDs, EHBDs and gallbladder (20). Hnf6−/− mice livers have an abnormal morphology of IHBDs and EHBDs. Hnf6−/− fetuses at ED10 did not develop a gallbladder primordium. Furthermore, they did not show development of a proper ductal plate around ED15. The disorganized biliary structures with intrahepatic biliary cysts resulted neonatally in a cholestatic syndrome with a death rate of ~75% between postnatal day (P) 1 and P10. The EHBDs were enlarged. In Hnf6−/− mice, Hnf1β expression was virtually abolished between at ED12.5 and ED14.5, but was restored at P3 (20). Hnf1β, therefore, appears to be responsible for most of the effects of Hnf6 on bile-duct development. In agreement, liver-specific deletion of Hnf1β with AlfpCre results in a liver phenotype that resembles Hnf6−/− mice (21), while Hnf6 expression levels were unaffected in these mice.

C/ebpα
CCAAT/Enhancer Binding Protein alpha (C/ebpα) is one of the markers of hepatoblasts which becomes suppressed in biliary epithelial cells (22). In C/ebpα−/− mice, which die perinatally due to hypoglycemia and hyperammonemia, no bile ducts develop. Instead, the hepatoblasts develop into pseudoglandular structures, which interfere with the establishment of normal hepatic plates (22, 23). The cells lining the pseudoglandular structures have both biliary and hepatocyte characteristics, with upregulation of Hnf6 and Hnf1β expression (22). These results suggest that downregulation of Cebpα leads to improper commitment of hepatoblasts to the biliary and hepatocytic fate.
**Tgfβ**
Transforming Growth factor β (Tgfβ) acts, together with BMP, as a developmental timer during liver specification (24). Tgfβ signaling occurs as a gradient with high activity near the portal vein and lower activity in the parenchyma (1, 7). To circumvent the embryonic lethality of most Tgfβ-pathway knockouts (25), anti-Tgfβ antibodies were administered to pregnant mice. In the resulting embryos, the number of cytokeratin-positive cells was reduced and the biliary basal lamina absent (26). In vitro, TGFβ promoted cholangiocyte differentiation of hepatoblasts (7, 26). Tgfβ expression was increased in Hnf6 knockouts (26), suggesting that Hnf6 suppresses Tgfβ expression in the parenchyma and allows Tgfβ accumulation near the portal vein to facilitate segregation of the hepatocytic and biliary lineages.

**Sox9**
SRY-related HMG box transcription factor 9 (Sox9) is the earliest biliary marker known thus far (7). Expression is first detected in endodermal cells lining the hepatic diverticulum, but is absent during the invasion of hepatoblasts into the STM. Sox9 becomes re-expressed at ED11.5 in a subset of cells near the portal veins. Thereafter, its expression remains restricted to the biliary lineage (7). Liver-specific deletion of Sox9 with AlfpCre impairs the timing of the maturation of primitive ductular structures (PDS). PDS are normally asymmetrical, with Hnf4α being expressed on the parenchymal side and Sox9 on the portal side. By the end of gestation at ED18.5, all cells lining the matured bile ducts express Sox9 (and E-cadherin) and are negative for Hnf4α. In Sox9−/− livers, however, this phenotypic maturation of the bile ducts is delayed for one week, therefore it was suggested that biliary development proceeds according to a mode of tubulogenesis characterized by transient asymmetry, whose timing is controlled by Sox9. (7).

**The Notch pathway in biliary development**
The Notch signaling pathway is highly conserved throughout evolution and plays an important role in cell fate determination by way of cell-cell contacts. Mammals express four Notch receptors (NOTCH1-4) with five ligands (DLL1, DLL3, DLL4, JAGGED1 and JAGGED2). DNA binding of the Notch intracellular domain via Rbp-J leads to transcriptional activation of Notch effector genes, such as Hes1 (27). The best established role for the Notch pathway in liver development is the involvement of NOTCH2 in Alagille syndrome, a rare hereditary disorder, which is characterized by impaired differentiation of the IHBDs and chronic cholestasis, among with other developmental abnormalities (28). Liver-specific deletion of Notch2 is sufficient to induce the liver abnormalities seen in Alagille syndrome and results in livers with a reduced amount of mature bile ducts postnataally, with intact ductal plate formation (29, 30). From these observations it was concluded that Notch2 is mainly required for IHBD morphogenesis and indispensable for cholangiocyte fate determination. In agreement with this hypothesis, mice livers lacking the Notch downstream effector Hes1, show normal ductal plates without tubular structures late embryonically (31).
However, both Notch2 knockout studies were conducted with the Alb^Cre recombinase, which becomes expressed relatively late during embryonic development, thus the possibility remains that inactivation of Notch2 occurred after or during the time point of hepato-biliary specification, still allowing Notch2 to regulate biliary cell fate. Additionally, early liver-specific knockout of the Notch DNA-binding co-factor Rbpj, with Foxa3^Cre, led to a more severe, but comparable phenotype with a reduced number of ductal plate cells on ED16.5 and a significant decrease in the number of bile ducts at postnatal day 0, suggesting that Notch signaling could indeed regulate cholangiocyte cell fate (32).

In conclusion, during biliary development there is an intricate network of transcription factors regulating cholangiocyte lineage segregation and/or biliary morphogenesis, with Hhex, Hes1, Notch2 and Hnf6 on top of the signaling cascade and Hnf1β, Tgfβ, Sox9, Foxa1/2, C/ebpα as downstream factors, however, the interplay in time between these factors remains to be fully elucidated.
Reference list


