DLK1 AND THE NOTCH PATHWAY IN THE LIVER

In this thesis we tried to unravel the role of DLK1 and the NOTCH2 receptor during liver development. DLK1 is a ligand and NOTCH2 a receptor in the Delta-Notch signaling pathway, which is involved in developmental cell-fate decisions.

CHAPTER 1 provides an introduction into the developmentally conserved Delta-Notch signaling pathway, with a special focus on DLK1. DLK1 is considered a noncanonical ligand, due to its atypical protein structure compared to canonical ligands. Dlk1 mRNA was shown to be highly expressed in a variety of embryonic organs, but only in few neuro(endocrine) organs after birth. This expression pattern suggest that DLK1 is involved in embryonic growth and differentiation of these organs. Furthermore, DLK1 becomes re-expressed in several pediatric tumors.

CHAPTER 2 describes the protein expression pattern of DLK1 during mouse embryonic development in daily intervals between embryonic day 12 and birth. DLK1 showed abundant expression in liver, lung, muscle, vertebrae, pancreas, pituitary and adrenal gland(s), but this expression declined rapidly in the fetal period. DLK1 expression was absent in heart, stomach, intestine, kidney, epidermis and CNS. DLK1 expression, therefore, correlates well with the reported Dlk1 mRNA expression pattern, which demonstrates that its expression is mainly regulated at the pre-translational level. DLK1 seems to be involved in developmental processes such as branching morphogenesis (lung, pancreas) and terminal differentiation (adipose tissue, muscle, liver, pituitary), with as common features among organs, stimulation of growth and inhibition of differentiation. Based on its expression pattern during development, its behavior during adipogenesis, and its effects upon experimental interventions, DLK1 appears to function as an inhibitory modulator of Notch signaling, either by competing with canonical ligands or by direct interaction with the NOTCH1 receptor, or both.

Since DLK1 expression is high during embryonic liver development whereas expression is absent after birth and becomes re-expressed in hepatoblastoma, a pediatric liver tumor, we hypothesised that DLK1 could be involved in its pathogenesis. We further hypothesised that the soluble form of DLK1 might be an additional serum marker for the diagnosis of hepatoblastoma in young infants, where the established serum marker α-fetoprotein (AFP) is not always a reliable marker due to physiologically high serum levels in this age group.

In CHAPTER 3 we explored the suitability of DLK1 as a serum marker for hepatoblastoma in young infants. We measured the concentration of DLK1 and AFP in serum of 48 pediatric control and 7 hepatoblastoma patients. Like AFP, DLK1 levels decline with age, but even in young patients, DLK1 serum levels were were ~10-fold higher than controls if a hepatoblastoma was present. This finding makes DLK1 a candidate serum marker to diagnose hepatoblastoma in the young infant age group.
In CHAPTER 4 the generation and characterization of a transgenic mouse that overexpresses Dlk1 in the liver (Alfp-Dlk1 mice) is described. These mice express high DLK1 levels in liver en plasma, but unexpectedly did not develop any phenotype and, in particular, no signs of spontaneous tumor formation. Since DLK1 also inhibits adipogenesis in a dose-dependent way, with soluble and membrane-bound DLK1 having mostly inhibitory and enhancing effects, respectively, we assessed lipogenesis and adipogenesis in adult Alfp-Dlk1 mice. The mice were fed either a high-fat or low-fat semi-synthetic diet for 4 weeks. Collectively, this dietary challenge revealed that during a low-fat diet, DLK1 overexpression led to inhibition of Notch1 and Sox9 mRNA expression in male liver, while a high-fat diet led to increased expression of Notch signaling and lipogenic genes in both liver and adipogenesis in Alfp-Dlk1 females and only in liver in Alfp-Dlk1 males. These findings show that DLK1 overexpression influences lipogenesis in both liver and adipose tissue in a sex- and diet dependent fashion.

Notch pathway expression has been described during mouse liver development and showed mainly expression of the Notch1 and Notch2 receptor, with peak expression just prior to birth. Furthermore, NOTCH2 mutations are described in human Alagille syndrome, a developmental disorder with heart, eye and kidney abnormalities together with bile duct paucity. Transgenic mice with liver-specific knockout of the Notch1 receptor do not show an obvious phenotype. However, transgenic mice with late-embryonic knockout of the Notch2 receptor showed neonatal bile duct paucity with postnatal cholestasis and cholangiocyte proliferation.

CHAPTER 5 provides an introduction in normal bile duct development and the involved transcription factors. Biliary differentiation and morphogenesis depend on a Hhex-Hnf6-Hnf1β transcriptional cascade, with the Tgfβ pathway and Foxa transcription factors mainly involved in biliary morphogenesis. The exact position of the Notch2 receptor in the transcriptional cascade is not completely elucidated and probably regulates both biliary differentiation and morphogenesis.

Therefore, in CHAPTER 6 we describe the characterization of mice with an early liver-specific deletion of the Notch2 receptor (Notch2-cKO mice). Embryonic Notch2-cKO mice did not show signs of cholangiocyte differentiation with cytokeratin stainings, whereas hepatocyte morphology was normal. Neonatal Notch2-cKO mice were severely jaundiced and their livers were completely devoid of bile ducts, demonstrating complete absence of cholangiocyte differentiation. Despite extensive cholestatic necrosis, mortality in the first 6 weeks of life was only ~15%. Unexpectedly, a slow process of newly formed (aberrant) bile ductular structures developed after weaning. Despite extensive liver fibrosis, jaundice had disappeared in ~30% of Notch2-cKO mice at 6 months of age. Newly formed ducts varied in morphology and histologically resembled the atypical ductular reaction, seen after massive hepatic necrosis. Notch2 and Hnf6 mRNA levels were permanently decreased in Notch2-cKO livers, indicating that Hnf6 acts downstream from Notch2 in cholangiocyte differentiation.
Summary and conclusion

Transcription factors involved in cholangiocyte differentiation and bile duct morphogenesis (Foxa1, Foxa2, Hhex, Hnf1β, Cebpα and Sox9) were all expressed at significantly lower than control levels in the perinatal period, but all mRNA levels except Foxa2 were expressed at normal or even higher levels after weaning, coincident with the secondary formation of bile ducts. These findings show that Notch2 deficiency causes bile duct agenesis, yet allows for a slow, de novo secondary bile duct formation after weaning that resembles atypical ductular reaction. Notch2 knockout did not affect hepatocyte differentiation.

Since DLK1 was shown to act, depending on sex and diet, either as an inhibitor or a stimulator of Notch signaling (Chapter 4) and Notch signaling has been shown to be associated with postnatal cholangiocyte proliferation, in CHAPTER 7, we explored the effects of DLK1 overexpression in Dlk1/Mdr2−/− double transgenic mice that had been fed a cholate diet (from which it is known to enhance cholestatic liver injury) for 4 weeks. Dlk1 overexpression in Mdr2-deficient mice resulted in mitigation of bile duct proliferation and downregulation of the expression of Notch1, Notch2, its downstream factors Hes1 and Sox9, and biliary transcription factor Hhex, showing that Notch signaling plays a central role in postnatal ductular proliferation and that, in this context, DLK1 acted as an inhibitor of Notch signaling. Dlk1 overexpression alone did not affect liver proliferation.

Conclusion
The main conclusion of the current thesis is that the NOTCH2 receptor, a member of the developmentally conserved Notch signaling pathway, is indispensible for cholangiocyte differentiation, since its absence leads to bile duct agenesis. Another important finding is the de novo secondary bile duct formation, which recapitulates most of the regulatory steps seen in embryonic bile duct development. Furthermore, the noncanonical Notch ligand DLK1, which is most highly expressed during embryonic development and in pediatric tumors, is not involved in the pathogenesis of hepatoblastoma, but seems to function as a conditional regulator of Notch signaling.