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HEPATIC CELL DIFFERENTIATION REQUIRES A HNF6 – miR-122 POSITIVE FEEDBACK LOOP

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SUMMARY

Hepatocyte differentiation and maturation during embryogenesis is controlled by a network of liver-enriched transcription factors (LETFs). Whether LETFs control microRNA (miRNA) expression during development, and whether such control is required for hepatocyte differentiation has not yet been investigated.

Here we show that two LETFs, namely Hepatocyte Nuclear Factor (HNF) 6 and Onecut2 (OC2), redundantly stimulate the expression of several miRNAs during development, and that the hepatocyte-specific miR-122 is the most critically dependent on HNF6 and OC2. Using *in vivo* and *in vitro* gain- and loss-of-function experiments, we further demonstrate that accurate levels of miR-122 are required to ensure proper progression of hepatocyte differentiation and maturation from hepatoblasts: miR-122 stimulates the expression of hepatocyte-specific genes and of most LETFs, including HNF6. This reveals the existence of a HNF6 – miR-122 positive feedback loop. Moreover, stimulation of hepatocyte maturation by miR-122 is abolished in an HNF6-null background, revealing that a transcription factor can mediate microRNA function. Confirming direct regulation we provide evidence that all hepatocyte-specific genes whose expression is stimulated by miR-122 are occupied *in vivo* by HNF6.

MiR-122 also promotes hepatocyte maturation by directly repressing specific mRNAs. Mir-122 participates to liver-specific disallowance of the enzyme 2-oxoacid CoA transferase (OXCT1) during hepatocyte maturation. OXCT1 is involved in ketone body catabolism and needs to be repressed in liver, where ketone bodies are produced, in order to avoid hepatic catabolism of these metabolites. We show that OXCT1 mRNA is a direct target of miR-122 and that the rise in miR-122 level during hepatocyte maturation induces repression of OXCT1 protein production during liver development and in adulthood.

Together, our findings indicate that the expression level of the liver specific miR-122 is controlled by a HNF6 - miR-122 positive feedback loop and that miR-122 controls hepatocyte differentiation and maturation by indirect stimulation of hepatocyte-specific genes and by direct repression of an ubiquitous gene.

Table of content

ANNEXES

"Tissue-specific disallowance of housekeeping genes: the other face of cell differentiation"; Thorrez L., Laudadio I., Van Deun K., Quintens R., Hendrickx N., Granvik M., Lemaire K., Schraenen A., Van Lommel L., Lehnert S., Aguayo-Mazzucato C., Cheng-Xue R., Gilon P., Van Mechelen I., Bonner-Weir S:, Lemaigre F. and Schuit F.; *Genome Research*. 2011; 21(1):95-105.

"MiR-495 and miR-218 regulate the expression of the Onecut transcription factors HNF-6 and OC-2"; Simion A, Laudadio I, Prévot PP, Raynaud P, Lemaigre FP and Jacquemin P; *Biochem Biophys Res Commun*. 2010;391(1):293-8.

Abbreviations

Afp	α-fetoprotein
Alb	Albumin
Apo	Apolipoprotein
BMEL	Bipotential Murine Embryonic Liver
BMP	Bone morphogenetic proteins
C/EBP	CCAAT/enhancer binding protein
CAT1	Cationic amino acid transporter
CK	Cytokeratin
CYP7A	Cholesterol-7α-hydroxylase
EB	Embryoid body
ESC	Embryonic stem cell
FGF	Fibroblast growth factor
FoxA	Forkhead box A
G6pc	Glucose-6-phosphatase catalytic subunit
GRN	Gene regulatory network
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
Hes1	Hairy and enhancer of split homolog
Hex	Hematopoietically expressed homeobox
HGF	Hepatocyte growth factor
HMGCS	3-hydroxy-3-methylglutaryl-CoA synthase
HNF	Hepatocyte nuclear factor
LETF	Liver-enriched transcription factors
LRH1	Liver receptor homolog
miRNA	microRNA
NF-1	Nuclear factor 1
NICD	Notch intracellular domain
NKRF	NFκB repressing factor
OC	Onecut
Opn	Osteopontin
OSM	Oncostatin M

OXCT1	2-oxoacid CoA transferase 1
PDS	Primitive ductal structure
PEPCK	Phosphoenol-pyruvate carboxykinase
Prox1	Prospero-related homeobox 1
SOX9	SRY-related HMG box transcription factor 9
Tbx3	T-box transcription factor 3
TF	Transcription factor
TGFβ	Transforming growth factor β
Ttr	Transthyretin
TβRII	TGFβ receptor type II
Zhx2	Zinc-finger and homeobox factor 2

1. INTRODUCTION

1.1. Liver development

1.1.1. Architecture and functional organization of the mature liver

The liver is a large organ, with a mass that accounts 2% to 5% of the body weight. It plays a central role in the regulation of cholesterol metabolism and transport, glycogen storage, urea metabolism, drug detoxification, secretion of plasma protein such as Albumin and Apolipoproteins, and coagulation factors. The liver also exhibits endocrine and exocrine functions. Endocrine functions consist in secretion of several hormones (Insulin-like growth factors, Angiotensin and Thrombopoietin) in the bloodstream, whereas the exocrine function is the secretion of the bile to the gallbladder and small intestine. The secretion of bile acids by the liver is essential for emulsification and intestinal absorption of fats.

Histologically the liver is composed of hepatocytes, which account for ~78% of liver mass (Blouin et al. 1977), as well as cholangiocytes/biliary cells, endothelial cells, Kupffer cells (resident liver macrophages), hepatic stellate cells and pit cells (natural killer cells).

The basic architectural and functional unit of the liver is the hepatic lobule (Fig. 1). It consists of a roughly polygonal arrangement of hepatocyte plates, which are lined by sinusoidal capillaries, radiating from a central vein in the center. Sinusoidal cells are fenestrated endothelial cells which are separated from the hepatocytes by the space of Disse. Stellate cells are located in the space of Disse. Portal triads are distributed at the corners of the lobule; they contain a terminal branch of the hepatic artery and of the portal vein, and a terminal bile duct lined by cholangiocytes.

Hepatocytes secrete endocrine hormones into the sinusoids, whereas bile is secreted into biliary canaliculi, which are formed by the apical faces of adjacent hepatocytes welded together by junctional complexes. Bile flows from the canaliculi to the canals of Hering which form a link with the terminal bile ducts and are lined partly by hepatocytes and partly by cholangiocytes. Blood flows from the portal vein and the hepatic artery into the sinusoids, perfuses hepatocyte plates and flows out into the central vein reaching the systemic circulation. Bile flow is parallel to the sinusoids, but is opposite in direction to the blood flow. Indeed, bile is transported through the canaliculi into the bile ducts, and subsequently transported for storage in the gallbladder.

All hepatocytes appear similar when visualized by microscopy. However, there are functional differences between periportal hepatocytes located close to the portal vein, and pericentral ones located near the central vein. Indeed, periportal and pericentral hepatocytes express different gene sets and exert different metabolic functions, giving rise to the so-called metabolic zonation (Jungermann 1987; Gebhardt et al. 2007). Enzymes or other functional proteins (transporters, transcription factors, etc.) show decreasing or increasing gradients along the periportal to pericentral axis (Christoffels et al. 1999). Metabolic pathways performing opposing functions follow inverse gradients or compartments. Examples of the opposite functions are gluconeogenesis and glycolysis, ureogenesis and glutamine synthesis, or cholesterol biosynthesis and bile acid biosynthesis which, for each pair, are predominant in periportal and pericentral zones, respectively. As a consequence, each hepatocyte performs biochemical functions depending on its position along the periportal to pericentral axis.

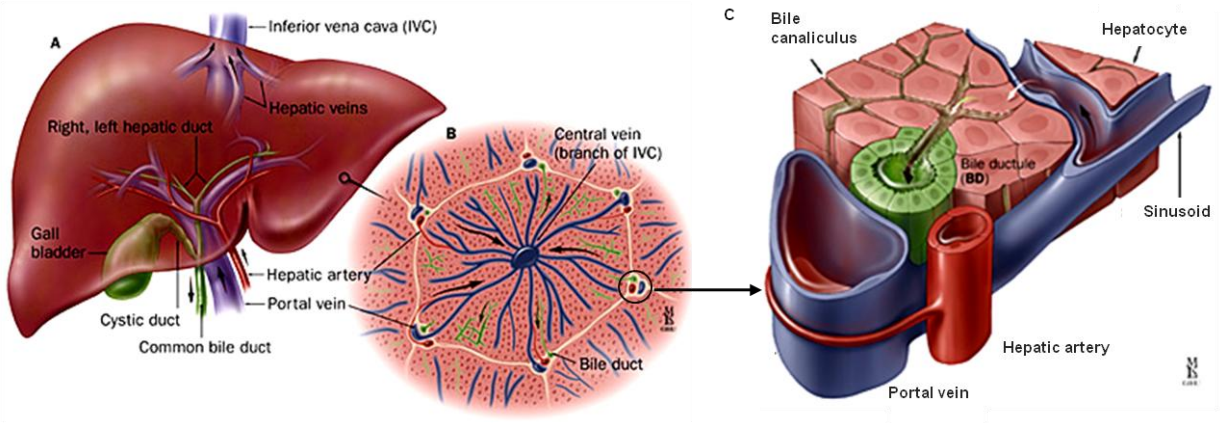

Figure 1. Architecture of the liver *(adapted from http://www.hopkins-gi.org).* Schematic representation of the liver (A), the hepatic lobule (B) and the portal triad (C).

1.1.2. Initiation of liver development

During embryonic development, hepatogenesis occurs as a series of developmental stages that progressively generate the complex cell composition and tissular architecture necessary for the execution of liver functions.

Accurate fate-mapping experiments have shown that the liver arises from 3 distinct domains of the anterior endoderm, that are located in the medial and lateral regions of the foregut (Tremblay and Zaret 2005; Zaret and Grompe 2008). During foregut closure these domains fuse

and generate a single prehepatic domain adjacent to the cardiac mesoderm and to the septum transversum mesenchyme (Fig. 2).

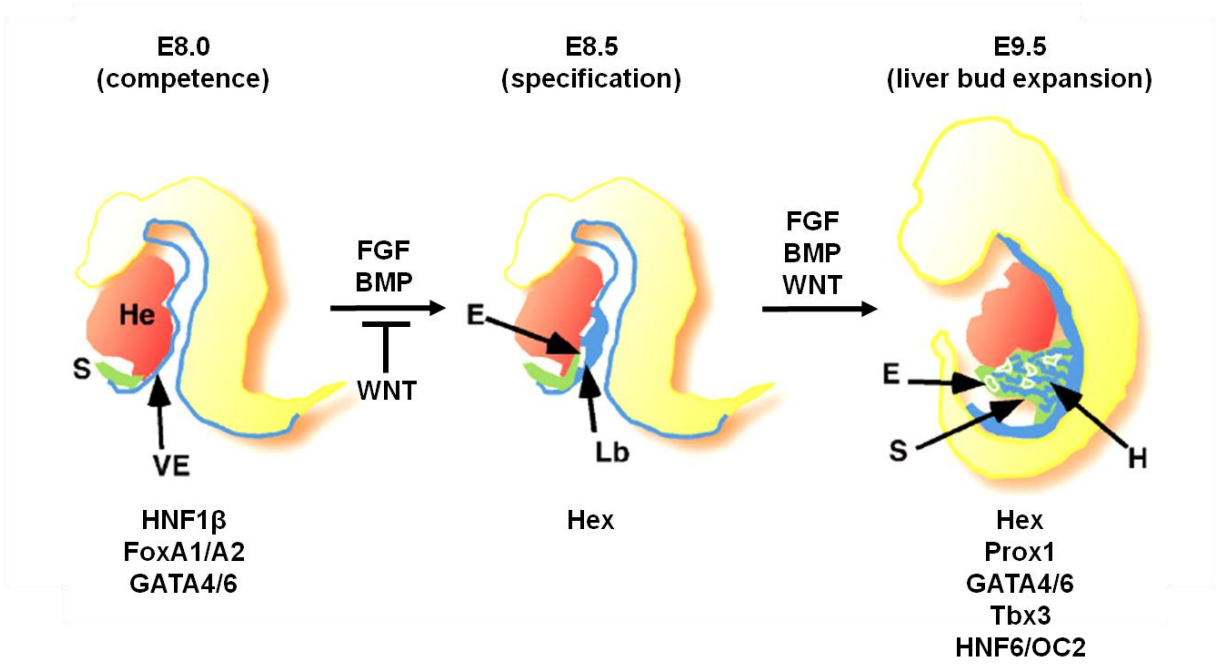

Figure 2. Early liver development in the mouse embryo. *(adapted from Zhao and Duncan 2005).* Illustration of initial steps of liver development and key transcription and extracellular factors acting at specific stages. *He*, heart; *S*, septum transversum; *VE*, ventral endoderm; *E*, endothelial cells; *Lb*, liver bud; *H*, hepatoblasts.

The first step in formation of the prehepatic domain is the acquisition of competence, which is defined by the capacity to respond to liver inducing-signals. Several studies have proposed Forkhead box (Fox) A and GATA transcription factors as “pioneer factors” in liver competence: they bind to liver-specific genes and open their chromatin, thereby rendering the cells responsive to transcription factors which will initiate liver-specific gene expression. Indeed, FoxA and GATA factors provide access to additional transcription factors, such as Nuclear Factor (NF)-1 and CCAAT-enhancer binding protein (C/EBP) β, thus resulting in activation of hepatic gene transcription (Gualdi et al. 1996; Bossard and Zaret 1998; Cirillo et al. 2002). When the hepatic genes become expressed, the endoderm cells are considered "specified", that is they are committed to become hepatic cells. Further confirmation of FoxA functions comes from a mouse model lacking FoxA1 and FoxA2, which are functionally redundant. These mice fail to initiate hepatic development and their endoderm is unable to respond to extracellular specifying-signals (Lee et al. 2005).

Hepatocyte Nuclear Factor (HNF) 1β is also critical for competence of ventral endoderm. Indeed, HNF1β was found to stimulate the expression of FoxA1 and FoxA2 in prehepatic

endoderm and HNF1β-deficient embryos fails to expressing liver-specific genes in developing liver (Lokmane et al. 2008). Taken together these data show that hepatic competence is controlled by a network involving HNF1β, FoxA and GATA factors.

Hepatic specification is induced by extracellular signals, such as Fibroblast growth factors (FGFs), Bone morphogenetic proteins (BMPs) and Wnt (Fig. 2). FGFs, such as FGF-1, -2, -8 and -10 are secreted by the cardiac mesoderm (Miller et al. 2000) and induce hepatic gene expression in competent endoderm through the activation of MAPK pathway (Calmont et al. 2006). FGF-mediated specification of hepatic cell fate is dependent on a well-defined FGF concentration, which depends on the distance separating the pre-hepatic endoderm from the cardiac mesoderm. Threshold concentrations of FGF indeed determine the patterning the foregut endoderm into distinct organ precursor regions (Serls et al. 2005). BMP-2 and BMP-4 are secreted by the septum transversum mesenchyme and are also required for liver specification as shown by delayed expansion of the liver bud in Bmp-4$^{-/-}$ mouse and by the *in vitro* repression of hepatic specification by BMP inhibitors (Rossi et al. 2001). Finally, to allow liver development from the anterior foregut endoderm, Wnt signaling must be repressed anteriorly (McLin et al. 2007) by endoderm secretion of Wnt inhibitors, such as Sfrp5 (Finley et al. 2003).

When endoderm cells are specified, they start expressing several hepatic genes such as Albumin (Alb), α-fetoprotein (Afp), Transthyretin (Ttr) and Hepatocyte nuclear factor (HNF) 4α, all of which are indicators of early hepatic fate. At embryonic day (E) 9 hepatic precursor cells, which at this stage are called hepatoblasts, become columnar and undergo a transition to a pseudostratified epithelium (Bort et al. 2006). This thickens the epithelium and induces formation of the hepatic diverticulum. The hepatoblasts proliferate and form a tissue bud that is surrounded by a basal membrane. Slightly later this basal membrane is degraded, hepatoblasts downregulate E-cadherin expression and migrate to invade the septum transversum (Fig. 2). This migration requires metalloproteinase activity (Margagliotti et al. 2008).

By analyzing mutant mouse models, several transcription factors were found to be implicated in the formation and expansion of the liver bud. The Homeobox transcription factor Hex promotes pseudostratification, hepatoblast proliferation and migration, but it is not required for hepatic specification (Keng et al. 2000; Martinez Barbera et al. 2000; Bort et al. 2004; Bort et al. 2006). In the liver bud, expression of Hex is regulated by FGF and BMP (Zhang et al. 2004). BMP signaling also controls GATA4 expression (Rossi et al. 2001), which in turn stimulates Hex expression (Denson et al. 2000). Moreover GATA4 and GATA6 are required for liver bud expansion (Zhao et al. 2005; Watt et al. 2007). Prospero-related homeobox (Prox) 1 promotes

proliferation and migration of hepatoblasts into the septum transversum. Indeed, hepatoblasts depleted in Prox1 are unable to degrade the surrounding basal membrane and to delaminate, probably as a result of the maintenance of high levels of E-cadherin (Sosa-Pineda et al. 2000). The expression of Prox1 is regulated by the T-box transcription factor (Tbx) 3, whose deletion impairs hepatoblasts proliferation and migration during liver bud expansion (Ludtke et al. 2009). A phenotype similar to that of Prox1-deficient mice is shown in the combined absence of the Onecut transcription factors HNF6, also called Onecut (OC) 1, and OC2. Indeed HNF6 and OC2 redundantly control degradation of basal membrane, hepatoblast migration and expression of genes involved in cell adhesion and migration such as thrombospondin-4 and osteopontin (Opn) (Margagliotti et al. 2007). All these data show that hepatoblast proliferation and migration is controlled by a network of genes involving Hex, GATA4/6, Prox1, Tbx3 and HNF6/OC2 (Fig.2).

Between E9.5 and E15.5, after invading the septum transversum, hepatoblasts continue to proliferate in order to expand the mass of the liver bud. This proliferation is controlled by multiple growth factors secreted by the septum transversum, such as Hepatocyte growth factor (HGF) (Schmidt et al. 1995; Monga et al. 2002), BMP (Rossi et al. 2001; Yanai et al. 2008), FGF (Jung et al. 1999; Calmont et al. 2006; Berg et al. 2007), Wnt (Monga et al. 2003; McLin et al. 2007), Transforming growth factor (TGF) β (Weinstein et al. 2001), and retinoic acid (Wang et al. 2006). Importantly, the liver bud is initially surrounded by endothelial cells, which also provide essential proliferation signals (Matsumoto et al. 2001; Matsumoto et al. 2008).

1.1.3. Segregation of hepatobiliary lineages

Hepatoblasts are bipotential precursors, having the ability to differentiate into either hepatocytes or biliary cells.

The timing of lineage segregation has not been determined. Indeed, the onset of hepatocyte differentiation cannot be defined with accuracy since it is a continuous process: between E9.5 and E12.5 hepatoblasts start expressing several proteins associated with hepatocyte functions such as HNF4α, Alb, Ttr and Afp. At E12 a subpopulation of hepatoblasts that has differentiated toward the hepatocyte lineage can be identified by electron micrography, showing an abundant rough endoplasmic reticulum and lipid vesicles. Beyond E12, hepatocyte-specific functions are gradually acquired. Otherwise the first indication of biliary differentiation is the expression of the transcription factors SRY-related HMG box transcription factor (SOX) 9 which

is detected at E11.5 in a subset of cells located near the branches of the portal vein. This factor remains restricted to the biliary lineage throughout development (Antoniou et al. 2009). Therefore, E11.5 is currently considered as the stage of biliary commitment (see also § *1.1.5 Transcriptional control of bile duct development*).

Several transcription factors have been described as making important contributions toward the decision of hepatoblasts to adopt a hepatocyte versus a biliary fate (Fig. 3).

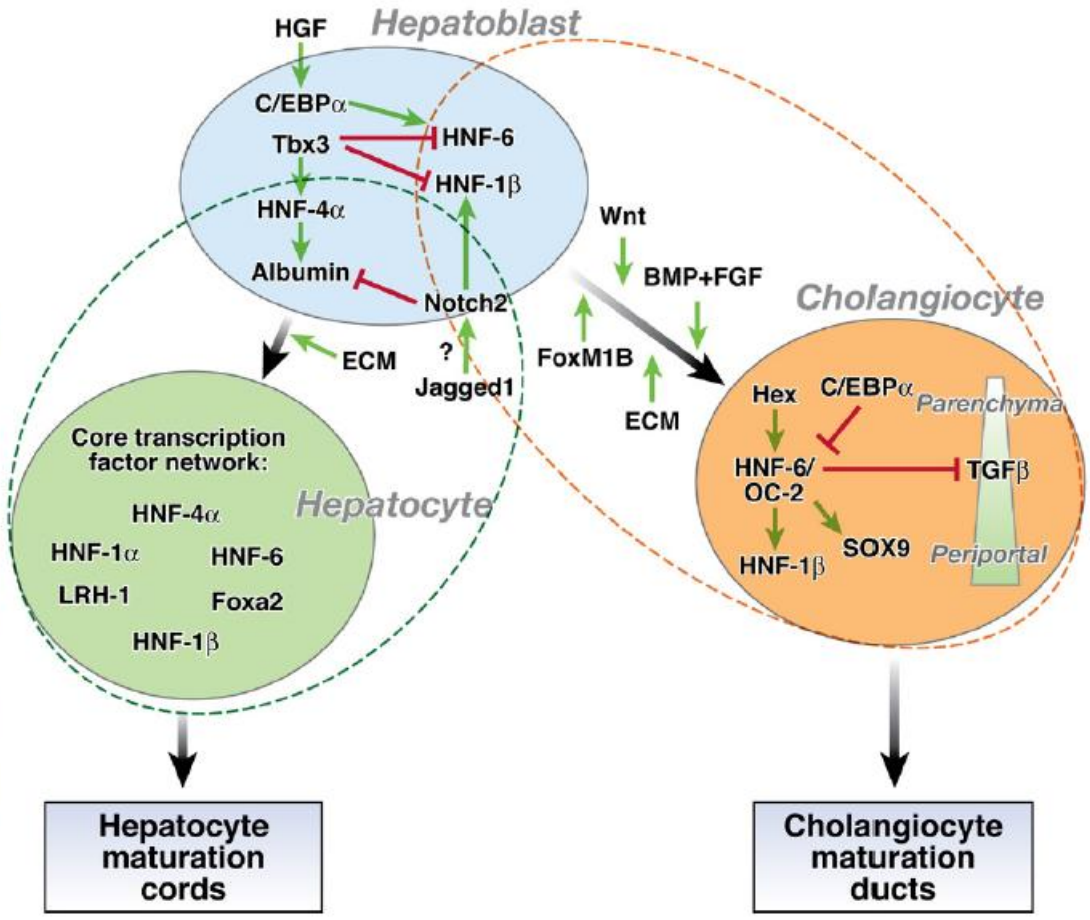

Figure 3. Mechanisms of hepatobiliary lineage segregation *(from Lemaigre 2009)*. Transcription factors and extracellular signals driving hepatoblast differentiation toward either the hepatocyte or the biliary lineage are illustrated. The *green dashed oval* delineates hepatocyte-inducing mechanisms, and the *orange dashed oval* integrates biliary differentiation mechanisms.

Appropriate levels of HNF6, which is expressed in hepatoblasts, hepatocytes and biliary cells, are required for the normal segregation of hepatoblasts into hepatocyte or biliary cells. Indeed, in the absence of HNF6 periportal hepatoblasts give rise to cyst-like structures that show a hybrid "hepatobiliary" phenotype, combining features of hepatocytes and biliary cells (Clotman et al. 2002). In the absence of both HNF6 and OC2, namely the double HNF6/OC2 knockout, this phenotype is aggravated, since hepatobiliary cells can be found not only in the

periportal region but also throughout the parenchyma (Clotman et al. 2005). This results from a severe anomaly in TGFβ signaling. TGFβ-2, TGFβ-3 and Activin A are produced by the periportal mesenchyme and stimulate biliary differentiation (Antoniou et al. 2009). TGFβ signaling occurs as a gradient with high activity near the portal vein and lower activity in the parenchyma (Clotman et al. 2005). In the double HNF6/OC2 knockout an excess of TGFβ signaling in the parenchyma is observed and this probably imposes a biliary differentiation program in cells normally fated to the hepatocyte lineage. This indicates that HNF6 and OC2 are required to accurately determine the slope of the TGFβ gradient, most likely by repressing the expression of TGFβ receptor type (TβR) II and by stimulating activin and TGFβ inhibitors, namely follistatin and α-2-macroglobuline (Clotman et al. 2002; Plumb-Rudewiez et al. 2004). Mice lacking the Hex transcription factor also show hybrid cells displaying hepatocyte and biliary features, and this is associated with decreased expression of HNF6 (Hunter et al. 2007). Livers depleted in C/EBPα are also characterized by the presence of hybrid cells, but in this case the phenotype is found in association with overexpression of HNF6 (Yamasaki et al. 2006). Moreover, in Tbx3-deficient mice, C/EBPα expression is repressed and in turn HNF6 is stimulated, resulting in an increase of the relative number of hepatoblasts that are committed to a biliary fate (Ludtke et al. 2009). Taken together these data suggest that for normal hepatoblast differentiation, Hex, C/EBPα and Tbx3 have to tightly control HNF6 expression levels (Fig. 3). Finally, a few other transcription factors are involved in the regulation of hepatobiliary segregation. These are FoxM1, which is critical for hepatoblast differentiation toward biliary cells (Krupczak-Hollis et al. 2004), and Sal4 which represses hepatocyte differentiation and enhances biliary differentiation (Oikawa et al. 2009).

Several signaling pathways have been implicated in lineage segregation (Fig. 3). Besides TGFβ signaling which has been previously described, Notch signaling pathway was also long suspected to be involved, based on the finding that patients affected with Alagille syndrome show bile duct paucity and mutations in the JAGGED1 ligand or NOTCH2 receptor genes (Li et al. 1997; Oda et al. 1997; McDaniell et al. 2006). *In vitro* experiments with purified hepatoblasts have suggested that the Notch pathway promotes biliary differentiation at the expense of hepatocyte differentiation (Tanimizu and Miyajima 2004). This has been confirmed *in vivo*. Inhibition of Notch pathway by inactivation of RBPJκ, a common transcriptional mediator of Notch, decreases the number of hepatoblasts differentiating to biliary cells, demonstrating a role for Notch signaling in biliary fate acquisition (Zong et al. 2009). These data have been further confirmed by the observation that activation of Notch signaling by overexpression of Notch

intracellular domain (NICD) induces biliary differentiation, even ectopically in the parenchyma (Tchorz et al. 2009; Zong et al. 2009).

Wnt signaling has also been investigated. *In vivo* experiments show that inactivation of Wnt signaling by ablation of its downstream effector β-catenin strongly reduces biliary differentiation (Tan et al. 2008), whereas stabilization of β-catenin by knocking out APC, which prevents nuclear activity of β-catenin, represses hepatocyte differentiation and enhances biliary differentiation (Decaens et al. 2008). These data suggest that Wnt signaling participates to the control of hepatobiliary segregation.

Finally, other signaling pathways such as HGF, FGF and BMP, and extracellular matrix component (Suzuki et al. 2003; Yanai et al. 2008) have a role in cell fate determination, yet their molecular mode of action remains to be determined.

1.1.4. Transcriptional control of hepatocyte maturation

Throughout embryonic development and during the perinatal period, cells that have differentiated to the hepatocyte lineage undergo a process of maturation that consists in the progressive acquisition of the gene expression profile, the morphology and the physiological functions of mature hepatic parenchymal cells (Jochheim et al. 2003; Petkov et al. 2004).

Hepatocyte-specific gene expression is controlled by a complex network of transcription factors, commonly referred to as liver-enriched transcription factors (LETFs) which organize to form a network of autoregulatory and cross-regulatory loops. For 12 LETFs, detailed expression profile and occupancy of regulatory regions within the promoters of other LETF genes have been analyzed *in vivo*. This study has shown that the number and complexity of interactions increase when hepatocyte maturation proceeds, following the progressive rise in concentration of the core transcription factors (Kyrmizi et al. 2006) (Fig. 4). Within this "dynamic transcriptional network" (Lemaigre 2009) a "core" of 6 transcription factors (HNF1α, HNF1β, FoxA2, HNF4α, HNF6 and Liver receptor homolog (LRH) 1) (Fig.3-4A), which occupy the regulatory regions of each others and of the peripheral LETFs (Kyrmizi et al. 2006), has been identified.

The expansion of cross-regulation between LETFs is likely to increase stabilization of the circuitry as development progresses to ensure terminal differentiation of the hepatocytes (Fig. 4A). Indeed, removal of a single LETF has much more impact when deleted in embryonic than in adult livers (Kyrmizi et al. 2006).

Interestingly LETFs synergistically and interdependently cooperate with other LETFs and cofactors to control the transcription of common target genes. HNF6 has been involved in two examples of this "synergistic interdependence" (Lemaigre 2009). Indeed, for the stimulation of glucose-6-phosphatase (G6pc) expression, HNF6 requires the presence of HNF4α and vice versa, and the synergy between these two transcription factors is dependent on the corecruitment of the coactivator PGC1α (Beaudry et al. 2006). In addition, HNF6 synergizes with C/EBPα to recruit the CBP coactivator protein and stimulate FoxA2 gene transcription (Yoshida et al. 2006). It is interesting to note that the synergistic interdependence only occurs when the concentrations of HNF6/HNF4α/PCG1α and HNF6/CEBPα reach threshold levels. Taken together these data suggest that corecruitment of coactivator and therefore time-specific activation of target genes is regulated by the increasing concentration of LETFs which reach threshold levels at a specific developmental stage (Fig.4B).

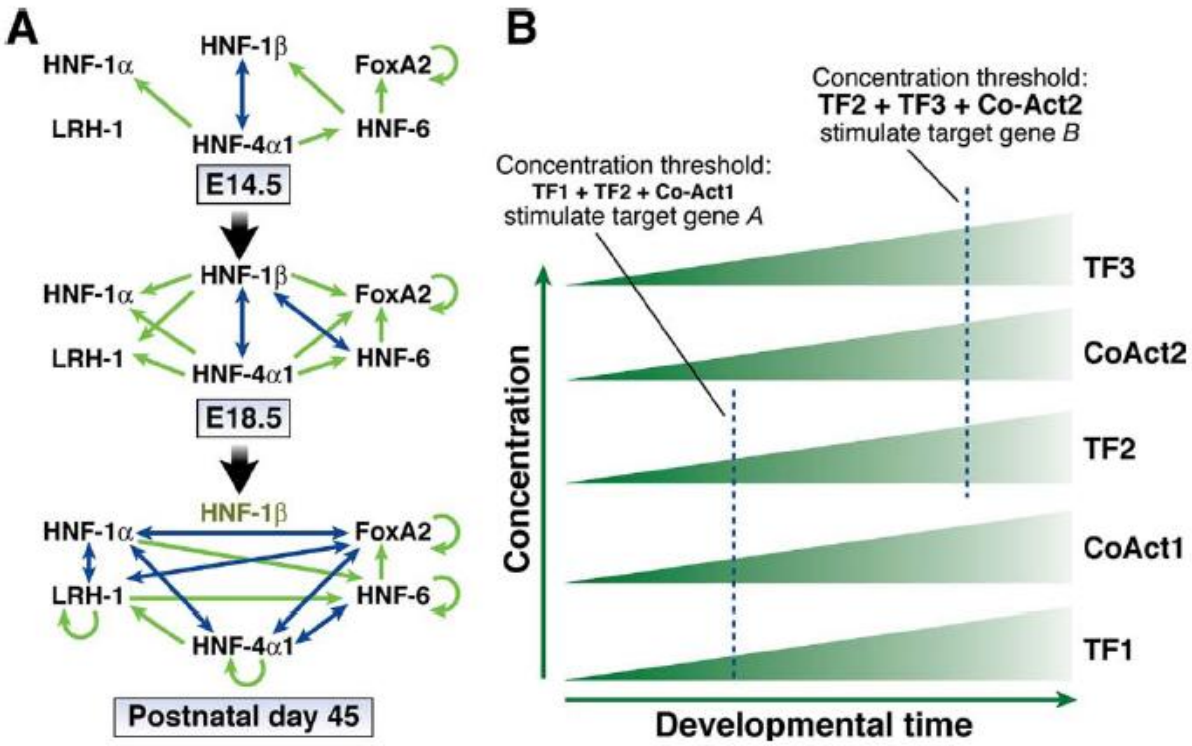

Figure 4. Dynamic organization of the liver-enriched transcription factors (LETF) network controlling hepatocyte maturation *(from Lemaigre 2009).* (A) Increase in the number and complexity of interactions between the core LETFs during development and in adulthood. *Green arrows*, unidirectional regulations; *blue double arrows*, bidirectional regulations. (B) Model for time-specific gene activation during development. The concentration of 3 transcription factors (TF1, TF2, and TF3) and of 2 coactivators (CoAct1 and CoAct2) increases during development. Stimulation of specific genes at specific time points during development only occurs when threshold levels are reached.

The role of core LETFs in the establishment and maintenance of hepatic functions has been proven *in vivo* by using knockout strategies in mouse.

Deletion of HNF4α results in embryonic mortality at E6.5 caused by severe extraembryonic endoderm defects preventing gastrulation (Chen et al. 1994). When the extraembryonic deficiency is rescued by tetraploid complementation, HNF4α$^{-/-}$ embryos develop until E12.5. At this stage these embryos show normal hepatic specification, but reduced levels of genes coding for several hepatocyte markers, such as apolipoproteins, metabolic proteins and serum factors (Li et al. 2000). When HNF4α is deleted specifically in hepatic cells by E15.5, the absence of HNF4α results in the decrease of genes involved in gluconeogenesis and glycogen synthesis (Parviz et al. 2003). Furthermore livers in which HNF4α has been deleted specifically in postnatal hepatocytes exhibit low levels of genes involved in lipid, cholesterol and bile acid homeostasis (Hayhurst et al. 2001). Therefore HNF4α is a key regulator of hepatocyte functions, but in addition it is also crucial for the control of the epithelial character of hepatocytes. Indeed in the absence of HNF4α, hepatocytes are small and fail to form cords (Parviz et al. 2003). This is due at least in part to the requirement of HNF4α for normal expression of an impressive set of genes coding for proteins involved in cell junction assembly and adhesion (Battle et al. 2006).

In HNF1α knockout mice livers develop normally, but they show complete extinction of the phenylalanine hydroxylase gene, which is involved in amino acid metabolism, and a partial reduction of Alb, α1-antitrypsin and fibrinogen (Pontoglio et al. 1996). This phenotype probably results from functional redundancy during hepatogenesis between HNF1α and HNF1β, two factors which display indistinguishable DNA binding sequence specificity (Cereghini 1996).

Besides its role in hepatic specification, HNF1β is also involved in the control of hepatic functions. Indeed, in the absence of HNF1β, expression of genes involved in bile acid sensing and fatty acid oxidation is decreased (Coffinier et al. 2002).

HNF6 is not only involved in hepatobiliary segregation but it also controls functions typical for mature hepatocytes. Indeed, it partially mediates the effects of growth hormone (Lahuna et al. 2000) and modulates glucocorticoid action in liver (Pierreux et al. 1999); it stimulates the expression of G6pc (Beaudry et al. 2006), phosphoenol-pyruvate carboxykinase (PEPCK) and 6-phosphofructo-2-kinase (Pierreux et al. 1999) which participate to gluconeogenesis, and of glucokinase, which catalyses the first reaction of glycolysis, and of the storage of glucose as glycogen (Lannoy et al. 2002). Moreover, HNF6 regulates hepatic cholesterol catabolism and bile acid synthesis through the transcriptional control of cholesterol-

7-hydroxylase (CYP7A1), the rate-limiting enzyme in the conversion of cholesterol to bile acids (Wang et al. 2004).

Deletion of FoxA2 specifically in hepatocytes provides evidence of the role of FoxA2 in the control of bile acid metabolism and gluconeogenesis. Indeed, mice lacking FoxA2 in hepatocytes display a complex alteration in the levels of transporters and modifying enzymes for bile acids, which results in increased intrahepatic bile acid levels (Bochkis et al. 2008). Moreover these livers fail to fully activate genes involved in gluconeogenesis upon fasting, such as PEPCK, tyrosine aminotransferase and insulin-like growth factor binding protein 1 (Zhang et al. 2005).

LRH1 knockout development is arrested prior to gastrulation, showing features typical of visceral endoderm dysfunction, such as in HNF4α knockouts. In contrast $LHR^{+/-}$ mice survive and show approximatively 40% of LHR1 levels found in wild type mouse livers. These mice display hypercholesterolemia and decreased expression of CYP7A1 (Pare et al. 2004).

All these data provide evidence that core LETFs control the acquisition of hepatic cell functions *in vivo* and are further supported by genomic studies using chromatin immunoprecipitation combined with DNA microarray or deep-sequencing, which show that LETFs bind to loci of numerous liver-specific genes (Odom et al. 2004; Odom et al. 2006; Bochkis et al. 2008).

Hepatocyte maturation also requires repression of a number of genes during prenatal and postnatal periods, such as Afp, H19 and Glypican 3 (Spear et al. 2006). Transcriptional repression of Afp seems to be controlled by the transcription repressor Zinc-finger and homeobox factor (Zhx) 2. Zhx2 is more strongly expressed in adult liver than in embryonic liver and insertion of a retrotransposon in the *Zhx2* locus increases Afp expression in adult livers (Perincheri et al. 2005). Further studies have also shown that Zhx2 represses not only Afp but also H19 and Glypican-3 (Perincheri et al. 2005; Morford et al. 2007). The Zinc-finger factor ZBTB20 is another repressor of Afp. It is expressed in liver only after birth, it binds to and represses the Afp promoter and, in the absence of ZBTB20, a strong increase in Afp expression is monitored in adult hepatocytes (Xie et al. 2008).

Finally, extracellular signals are also required for the progression of hepatocyte maturation. Oncostatin M (OSM) is produced by the hematopoietic precursor cells which reside in fetal liver, and stimulates the expression of hepatocyte specific genes (Kinoshita et al. 1999). HGF is secreted by endothelial cells and allows the organization of hepatocytes as cord-like

structures (Suzuki et al. 2006). Wnt, and in particular Wnt9a, is produced in chicken liver by sinusoidal cells and stimulates glycogen accumulation (Matsumoto et al. 2008).

1.1.5. Transcriptional control of bile duct development

Around E13 hepatoblasts differentiating to biliary cells form a near continuous monolayered ring of cells, called "ductal plate", which surrounds the periportal mesenchyme. The ductal plate must undergo a complex process of remodeling and morphogenesis that leads to the formation of mature bile ducts at birth. At E15.5, bilayered structures delineating a lumen become detectable at focal areas of the ductal plate. These structures are called "primitive ductal structures (PDS)" and mark the onset of bile duct morphogenesis. PDS have a unique asymmetric organization. They are lined on the portal side by biliary cells, which express typical biliary markers such as SOX9, Opn, cytokeratin (CK) 19 and high levels of E-cadherin. On the contrary the parenchymal side of PDS is composed of hepatoblasts which express HNF4α and low levels of E-cadherin. This asymmetry is transient and hepatoblasts on the parenchymal side undergo a radial and progressive differentiation into biliary cells, resulting in mature bile ducts entirely lined by biliary cells expressing SOX9, Opn, CK19 and high levels of E-cadherin, but not HNF4α (Antoniou et al. 2009) (Fig. 5). In parallel with acquisition of symmetry, the ducts become surrounded by the periportal mesenchyme. Ductal plate areas not involved in duct formation involute, possibly by undergoing apoptosis as shown in humans (Terada et al. 1995). The whole process of duct morphogenesis progresses in a hilum-to-periphery direction. It starts around the largest branches of the portal vein, close to the hilum of the liver and progressively extends toward the periphery of liver lobes (Van Eyken et al. 1988; Antoniou et al. 2009).

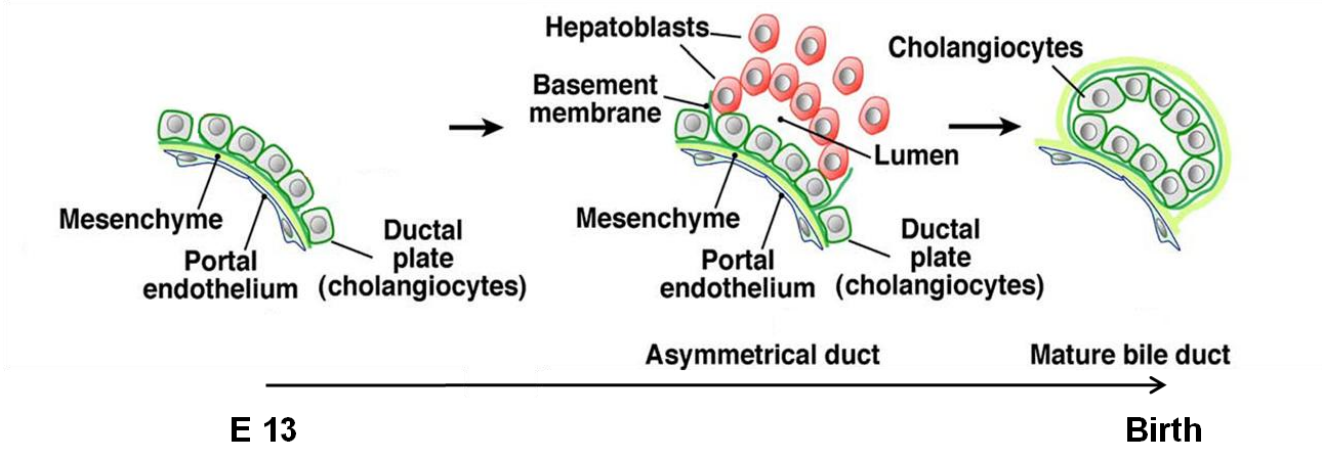

Figure 5. Bile duct morphogenesis *(adapted from Lemaigre 2009).* Schematic representation of the mechanism driving mature bile duct formation from a monolayered ring of biliary precursor cells.

Using mutant mouse models, several transcription factors have been implicated in the control of duct morphogenesis and a gene network regulating bile duct development can be proposed (Fig.3).

The first factors to be identified as transcriptional regulators of biliary development are HNF6 and HNF1β. In the absence of either factor, the ductal plate gives rise to cyst-like biliary structures of variable size and to postnatal persistence of ductal plate cells (Clotman et al. 2002; Coffinier et al. 2002). Moreover, HNF6 binds to *Hnf1β* gene promoter and controls its expression in fetal livers (Clotman et al. 2002). HNF6 also stimulates the biliary specific transcription factor SOX9, whose absence delays differentiation of the parenchymal layer of PDS during bile duct maturation (Antoniou et al. 2009). In zebrafish livers, the HNF6/HNF1β cascade controls the expression of *vps33b* (Matthews et al. 2005), a regulator of vesicular membrane fusion, which has been found mutated in humans affected with "Arthrogryposis-Renal Dysfunction and Cholestasis", a syndrome characterized by bile duct paucity (Gissen et al. 2004). Finally, since HNF6 and HNF1β control the expression of genes which regulate duct size and function in pancreas (*Cys1* and *Pkhd1*, (Pierreux et al. 2006)) and kidney (*Ift88*, *Pkd2*, *Pkhd1* and *Kif11*, (Gresh et al. 2004; Gong et al. 2009)), one can speculate that these genes are targeted by HNF6/HNF1β cascade also in liver.

Two transcription factors have been placed upstream of HNF6 and HNF1β in the biliary development cascade, namely Hex and C/EBPα. In mice in which Hex is ablated, cystic ducts can be identified with reduced levels of HNF6 and HNF1β (Hunter et al. 2007). On the other hand mice depleted of C/EPBα show abnormal biliary structures near the portal vein, which in this case are associated with high levels of HNF6 and HNF1β (Yamasaki et al. 2006). Interestingly like for hepatobiliary lineage segregation, experimental data support the notion that HNF6 expression levels must be tightly regulated to allow normal bile duct morphogenesis.

Since the absence of HNF6, Hex, and C/EBPα during liver development is not only associated with aberrant morphogenesis but also with abnormal biliary differentiation, it cannot be concluded if the role of transcription factors on duct morphogenesis is direct or a consequence of their control on biliary differentiation.

Bile duct maturation is also controlled by extracellular signals, such as TGFβ and Notch signaling pathways. TGFβ is likely to play a role in duct morphogenesis, based on the dynamic expression of its receptor TβRII. Indeed TβRII is expressed in all biliary precursor cells at the monolayered ductal plate stage, but in PDS it is absent from biliary cells on the portal side while

being detected in the hepatoblasts on the parenchymal side. In mature ducts, all biliary cells are negative for TβRII. Interestingly, delayed maturation of PDS in SOX9 knockout mice is associated with abnormal persistence of TβRII on the portal side. Moreover, TGFβ treatment of cultured hepatoblasts induces biliary marker expression and represses TβRII expression (Antoniou et al. 2009). Based on these data a model can be proposed in which TGFβ sequentially induces differentiation of the cells on the portal side and then on the parenchymal side of PDS. When cells have differentiated to biliary cells, TGFβ represses its own receptor by a negative feedback loop (Raynaud et al. 2010).

Beside its role in biliary cell commitment, Notch signaling is also required for duct morphogenesis, as shown by generation of mice showing loss-of-function of the Notch pathway. Inactivation of Notch signaling in the liver after the stage of ductal plate formation, either by combined deletion of Notch1 and Notch2 (Geisler et al. 2008), or RBP-Jκ (Zong et al. 2009) affected morphogenesis without influencing biliary differentiation. Mice knockout for the Notch effector Hairy and enhancer of split homolog (Hes) 1 (Kodama et al. 2004) or harboring a combination of the Notch ligand Jagged1 null allele and a Notch2 hypomorphic allele (Lozier et al. 2008) show the same phenotype of biliary paucity. Jagged1 is expressed by portal vein mesenchymal and endothelial cells, and later expands to biliary precursor cells (Louis et al. 1999; Loomes et al. 2002; Flynn et al. 2004; Zong et al. 2009; Hofmann et al. 2010). Interestingly during ductal plate induction the critical source of Jagged1 seems to be the portal vein mesenchyme. Indeed, specific ablation of Jagged1 in the portal vein mesenchyme, but not in endothelium, strongly perturbs morphogenesis, resulting in bile duct paucity (Hofmann et al. 2010). Finally Hes1 and Jagged1 are firstly detected on the portal layer and then on the parenchymal layer of PDS, thus suggesting that Notch pathway is sequentially activated on the two layers, following duct maturation (Antoniou et al. 2009; Zong et al. 2009).

1.2. MicroRNA function in the liver

1.1.1. General requirement of miRNAs in liver function

MicroRNAs (miRNAs) comprise a large family of small ~21-nucleotide-long noncoding RNAs that have emerged as key posttranscriptional regulators of gene expression in metazoan animals, plants and protozoa. In mammals, miRNAs are predicted to control the activity of more

than 60% of all protein-coding genes (Friedman et al. 2009) and they participate in the regulation of almost every cellular process investigated to date, such as signal transduction, cell cycle, apoptosis, pluripotency, differentiation, and transformation (Bushati and Cohen 2007; Bartel 2009).

MiRNA genes are transcribed by either RNA polymerase II or III into a long precursor, namely the primary miRNA (pri-miRNA). The pri-miRNA is endonucleolytically cleaved by the nuclear microprocessor complex formed by the RNase III enzyme Drosha and the Di George critical region (DGCR) 8 protein in the nucleus. The resulting precursor hairpin, namely the pre-miRNA, is exported from the nucleus by Exportin-5–Ran-GTP. In the cytoplasm, the RNase III enzyme Dicer1, in complex with the double-stranded RNA-binding protein TRBP, cleaves the pre-miRNA hairpin to mature miRNA. The functional strand of the mature miRNA is loaded together with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC), where, on the basis of sequence complementary, it guides RISC to target mRNAs, in order to inhibit their expression through mRNA cleavage, translational repression or deadenylation. The passenger strand is degraded (Bartel 2009; Winter et al. 2009; Fabian et al. 2010) (Fig 6).

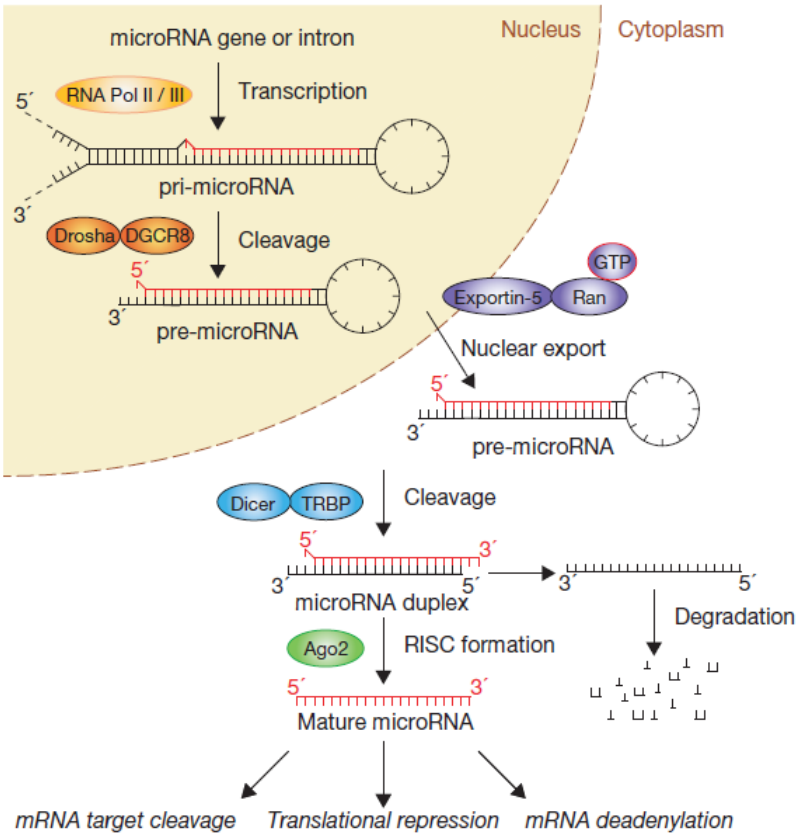

Figure 6. MiRNA biogenesis *(from Winter et al. 2009).* Representation of miRNA biogenesis pathway from transcription of miRNA genes in the nucleus to production of mature miRNAs that mediate post-transcriptional repression of target mRNAs in the cytoplasm.

To study the overall role of miRNA in mice, all mature miRNAs have been ablated via the disruption of the enzyme Dicer1. Constitutive loss of Dicer1 leads to early embryonic lethality at E7.5 (Murchison et al. 2005). Therefore, to circumvent this defect and study the role of miRNAs in specific tissues, conditional inactivation of Dicer1 has been performed by using the Cre-Lox system: loxP-flanked Dicer1 alleles are excised by the Cre recombinase, whose expression is driven by tissue-specific promoters. This strategy has been used for several tissues, including liver.

In liver, two conditional Dicer1 knockouts have been described, in which efficient deletion of Dicer1 and depletion of miRNAs occur only after birth. In both cases, loss of Dicer1 in the adult led to a higher rate of proliferation and apoptosis, and to reactivation of fetal stage-specific gene expression such as Afp, H19 and Insulin-like growth factor 2 (Hand et al. 2009b; Sekine et al. 2009a). Moreover Dicer1 knockouts display steatosis and depletion of glycogen storage in hepatocytes (Sekine et al. 2009a), as well as hepatocyte damage and inflammation (Hand et al. 2009b). Furthermore, with age, normal hepatocytes, which escaped Cre recombination, progressively replace apoptotic knockout cells (Hand et al. 2009b; Sekine et al. 2009a) and the surviving Dicer1-negative hepatocytes give rise to hepatocarcinoma in the majority of animals by the age of twelve months (Sekine et al. 2009a).

Interestingly deletion of Dicer1 impairs liver zonation. Periportal proteins such as PEPCK, E-cadherin, arginase 1 and carbamoyl phosphate synthethase 1 become expressed diffusely throughout parenchyma, whereas the expression of pericentral proteins is mostly preserved (Sekine et al. 2009b).

These data show that Dicer1 is involved in the control of hepatocyte survival, metabolism, fetal gene repression, tumor suppression and zonation in liver, but they do not rule out if all these effects are due to the loss of miRNA or to a direct involvement of Dicer1. Moreover it is interesting to note that three groups have recently provided evidence for an alternative miRNA biogenesis pathway which is Dicer-independent (Cheloufi et al. 2010; Cifuentes et al. 2010; Yang and Lai 2010), rising the possibility that deletion of Dicer1 could not be sufficient to completely deplete cells of all miRNAs.

1.2.2. Role of specific miRNAs in liver development

In previously described liver-specific Dicer1 knockouts, depletion of Dicer1 only occurs postnatally, hampering the exploration of the role of miRNAs in liver development.

Targeted approaches addressing the function of specific miRNAs revealed that the miR-30a/30c and miR-23b/27b/24-1 clusters were involved in developmental processes in liver. MiR-30a/30c are specifically expressed in biliary cells during the late stages of gestation and the early neonatal period. This biliary-restricted pattern is conserved in mouse, human and zebrafish. Specific downregulation of miR-30a and miR-30c in zebrafish larvae results in defective biliary morphogenesis, characterized by fewer terminal ductules. The mode of action of miR-30a/30c is still unclear, but it was predicted by bioinformatic tools that they modulated Epidermal growth factor receptor and Activin A, which control biliary proliferation and differentiation, respectively (Hand et al. 2009a). The miR-23b/27b/24-1 cluster is specifically expressed in hepatocytes at E17.5. Gain- and loss-of-function experiments with a cultured fetal liver stem cell line indicated that these miRNAs repress biliary gene expression by inhibiting TGFβ signaling via repression of Smad 2 and Smad 3 mRNAs. These data suggest a model in which high levels of miR-23b/27b/24-1 in differentiating hepatocytes inhibit TGFβ signaling and biliary gene expression, whereas low levels of the same miRNAs in cholangiocytes allow TGFβ signaling to stimulate biliary differentiation (Rogler et al. 2009). Therefore miR-23b/27b/24-1 cluster is likely to participate with HNF6 and OC2 (Clotman et al. 2005) in the establishment of TGFβ gradient in developing liver.

1.2.3. MiR-122: a liver-specific miRNA

MiR-122 is specifically expressed in the liver (Lagos-Quintana et al. 2002; Landgraf et al. 2007). It is detected in liver since E12.5 and increases until birth, (Chang et al. 2004; Rogler et al. 2009; Thorrez et al. 2011). From E18.5 onward, its expression is restricted to the hepatocyte compartment (Hand et al., 2009a), and in adult liver miR-122 is the most abundant miRNA, reaching approximately 70% of all expressed miRNAs (Chang et al. 2004).

MiR-122 is transcribed as a 4.7 kb-long pri-miRNA, which contains the pre-miR-122 sequence in its 3’ end (Chang et al. 2004; Gatfield et al. 2009). Examination of genomic DNA data for ortholog sequences in different animal genomes shows that best conservation in *miR-122* locus is observed in the regions spanning the promoter upstream of the transcriptional start site

and the pre-miR-122 (Fig. 7A). Within pre-miR-122, the sequence coding for mature miR-122 is perfectly conserved, whereas the rest of the sequence is significantly conserved, with variations almost exclusively present in the non-paired regions of the stem-loop structure (Chang et al. 2004) (Fig. 7B). These observations suggest that regulation of expression and function of miR-122 are similar in distinct organisms.

The regulation of miR-122 expression has been investigated. Several groups have provided evidence that LETFs (HNF1α, HNF4α, FoxA1, FoxA2, C/EBPα) bind to and stimulate miR-122 promoter in hepatocellular carcinoma (HCC)-derived cells or in adult liver (Coulouarn et al. 2009; Gao et al. 2010; Xu et al. 2010; Zeng et al. 2010). Moreover, the miR-122 promoter is repressed by the circadian clock component REV-ERBα, resulting in rhythmic pri-miR-122 transcription (Gatfield et al. 2009). Interestingly, miR-122 levels are also controlled by post-transcriptional events. In the liver, stabilization of the mature form of miR-122 requires 3'-adenylation by the cytoplasmic poly(A) polymerase GLD-2, after Dicer cleavage (Katoh et al. 2009).

Figure 7. Conservation of the *miR-122* gene among species. (A) Sequence corresponding to 4.7 kb of pri-miR-122. Regions showing highest conservation correspond to the promoter upstream the transcription start site and to the pre-miR-122, boxed in red and yellow, respectively. (B) Alignment of pre-miR-122 sequences shows that the mature miR-122 sequence (yellow box) is 100% conserved among species. Data are displayed with the help of the UCSC Genome Browser.

Several groups have assessed how miR-122 may affect adult liver biology by using *in vivo* and *in vitro* loss- and gain-of function approaches.

It has been shown that miR-122 is important for regulating liver lipid and cholesterol metabolism. Inhibition of miR-122 in adult mice by treatment with antisense oligonucleotide results in decreased expression of genes controlling cholesterol biosynthesis, whereas overexpression of miR-122 by injection of adenovirus coding for this miRNA increases their expression (Krutzfeldt et al. 2005; Davis et al. 2009). Furthermore, another group has also shown that repression of miR-122 decreases plasma cholesterol level, sterol and fatty acid synthesis, increases fatty acid oxidation and reduces liver steatosis in a diet-induced obesity mouse model (Esau et al. 2006). Interestingly, a study reveals that inhibition of miR-122 decreases plasma cholesterol levels without apparent liver toxicity in non-human primates (Elmen et al. 2008), suggesting that therapeutic approaches targeting miR-122 in humans could be feasible.

Expression of genes involved in lipid and cholesterol metabolism is under circadian control in the liver and miR-122 has been proposed as a regulator of their rhythmic expression. Indeed pri- and pre-miR-122, but not mature miR-122 are transcribed in a circadian fashion, by the circadian transcriptional repressor REV-ERBα. Depletion of miR-122 by *in vivo* injection of antisense oligonucleotides induces the alteration of rhythmic accumulation of several circadian mRNAs (Gatfield et al. 2009; Kojima et al. 2010). However, how mature miR-122, whose expression is constant over the day, can have an impact on the circadian regulation of its target remains unknown.

MiR-122 has been proposed as a tumor suppressor in the context of HCC. MiR-122 is downregulated in HCCs and in HCC-derived cells, and this correlates with aggressive phenotype and bad prognosis. Indeed miR-122 can suppress HCC cell proliferation by directly targeting Serum response factor and Insulin-like growth factor 1 receptor, which both stimulates cell cycle (Bai et al. 2009; Zeng et al. 2010). In addition, miR-122 targets Cyclin G1 (Gramantieri et al. 2007), resulting in increased p53 protein stability and transcriptional activity (Fornari et al. 2009). MiR-122 represses the expression of the anti-apoptotic protein Bcl-W, maintaining the integrity of apoptosis pathway in HCC derived cells, which is a barrier in malignant transformation (Lin et al. 2008). Moreover miR-122 suppresses intrahepatic metastasis of HCC by regulation of the disintegrin and metalloprotease family proteins ADAM10 and ADAM17 (Bai et al. 2009; Tsai et al. 2009). Finally, another study (Coulouarn et al. 2009) has shown that loss of miR-122 in HCC samples correlates with suppression of hepatocyte marker expression (such as several apolipoproteins, detoxification and coagulation factors and several LETFs) and

gain of metastatic properties. Furthermore *in vitro* overexpression of miR-122 in HCC-derived cells increases expression of CYP1A2, CYP2C9 and CYP7A1 (Xu et al. 2010). Taken together these data show that miR-122 controls many processes involved in development and progression of cancer, such as proliferation, apoptosis, metastasis and dedifferention.

A role of miR-122 in cellular stress response has also been suggested. A validated miR-122 target is the cationic amino acid transporter (CAT) 1, which facilitates uptake of arginine and lysine in mammalian cells (Chang et al. 2004). Maintenance of low CAT-1 activity in liver cells is important to avoid hydrolysis of the plasma arginine by arginase, which is highly expressed in hepatocytes. In response to different types of cellular stress, including amino acid deprivation, levels of CAT1 mRNA are upregulated, in order to sustain hepatocellular protein synthesis. This stress-induced upregulation is mediated by binding of HuR, an AU-rich element binding protein, to the 3'UTR of CAT1 mRNA, relieving miR-122-induced repression (Bhattacharyya et al. 2006).

Finally, miR-122 contributes to the liver tropism of Hepatitis C virus (HCV). MiR-122 binds to two closely spaced target sites in the 5'UTR of HCV genome, resulting in up-regulation of viremia, mainly by increasing translation (Jopling et al. 2005; Henke et al. 2008; Jopling et al. 2008). Ectopic expression of miR-122 in non hepatic-cells allows HCV replication and translation (Chang et al. 2008; Henke et al. 2008), whereas depletion of miR-122 in hepatic cells leads to rapid loss of viral RNA (Jopling et al. 2005; Randall et al. 2007; Henke et al. 2008). For all these reasons miR-122 can be considered an interesting target for antiviral intervention. This concept is recently substantiated by the treatment of chronically HCV-infected chimpanzees with anti-miR-122-oligonucleotides, which resulted in long-lasting suppression of viremia with no evidence of hepatic damage (Lanford et al. 2010).

1.3. References

Antoniou A, Raynaud P, Cordi S, Zong Y, Tronche F, Stanger B, Jacquemin P, Pierreux CE, Clotman F, Lemaigre FP. 2009. Intrahepatic Bile Ducts Develop According to a New Mode of Tubulogenesis Regulated by the Transcription Factor SOX9. *Gastroenterology* **136**: 2325-2333.

Bai S, Nasser MW, Wang B, Hsu SH, Datta J, Kutay H, Yadav A, Nuovo G, Kumar P, Ghoshal K. 2009. MicroRNA-122 inhibits tumorigenic properties of hepatocellular carcinoma cells and sensitizes these cells to sorafenib. *J Biol Chem* **284**: 32015-32027.

Bartel DP. 2009. MicroRNAs: target recognition and regulatory functions. *Cell* **136**: 215-233.

Battle MA, Konopka G, Parviz F, Gaggl AL, Yang C, Sladek FM, Duncan SA. 2006. Hepatocyte nuclear factor 4alpha orchestrates expression of cell adhesion proteins during the epithelial transformation of the developing liver. *Proc Natl Acad Sci U S A* **103**: 8419-8424.

Beaudry JB, Pierreux CE, Hayhurst GP, Plumb-Rudewiez N, Weiss MC, Rousseau GG, Lemaigre FP. 2006. Threshold levels of hepatocyte nuclear factor 6 (HNF-6) acting in synergy with HNF-4 and PGC-1alpha are required for time-specific gene expression during liver development. *Mol Cell Biol* **26**: 6037-6046.

Berg T, Rountree CB, Lee L, Estrada J, Sala FG, Choe A, Veltmaat JM, De Langhe S, Lee R, Tsukamoto H et al. 2007. Fibroblast growth factor 10 is critical for liver growth during embryogenesis and controls hepatoblast survival via beta-catenin activation. *Hepatology* **46**: 1187-1197.

Bhattacharyya SN, Habermacher R, Martine U, Closs EI, Filipowicz W. 2006. Relief of microRNA-mediated translational repression in human cells subjected to stress. *Cell* **125**: 1111-1124.

Blouin A, Bolender RP, Weibel ER. 1977. Distribution of organelles and membranes between hepatocytes and nonhepatocytes in the rat liver parenchyma. A stereological study. *J Cell Biol* **72**: 441-455.

Bochkis IM, Rubins NE, White P, Furth EE, Friedman JR, Kaestner KH. 2008. Hepatocyte-specific ablation of Foxa2 alters bile acid homeostasis and results in endoplasmic reticulum stress. *Nat Med* **14**: 828-836.

Bort R, Martinez-Barbera JP, Beddington RS, Zaret KS. 2004. Hex homeobox gene-dependent tissue positioning is required for organogenesis of the ventral pancreas. *Development* **131**: 797-806.

Bort R, Signore M, Tremblay K, Martinez Barbera JP, Zaret KS. 2006. Hex homeobox gene controls the transition of the endoderm to a pseudostratified, cell emergent epithelium for liver bud development. *Dev Biol* **290**: 44-56.

Bossard P, Zaret KS. 1998. GATA transcription factors as potentiators of gut endoderm differentiation. *Development* **125**: 4909-4917.

Bushati N, Cohen SM. 2007. microRNA functions. *Annu Rev Cell Dev Biol* **23**: 175-205.

Calmont A, Wandzioch E, Tremblay KD, Minowada G, Kaestner KH, Martin GR, Zaret KS. 2006. An FGF response pathway that mediates hepatic gene induction in embryonic endoderm cells. *Dev Cell* **11**: 339-348.

Cereghini S. 1996. Liver-enriched transcription factors and hepatocyte differentiation. *FASEB J* **10**: 267-282.

Chang J, Guo JT, Jiang D, Guo H, Taylor JM, Block TM. 2008. Liver-specific microRNA miR-122 enhances the replication of hepatitis C virus in nonhepatic cells. *J Virol* **82**: 8215-8223.

Chang J, Nicolas E, Marks D, Sander C, Lerro A, Buendia MA, Xu C, Mason WS, Moloshok T, Bort R et al. 2004. miR-122, a mammalian liver-specific microRNA, is processed from hcr mRNA and may downregulate the high affinity cationic amino acid transporter CAT-1. *RNA Biol* **1**: 106-113.

Cheloufi S, Dos Santos CO, Chong MM, Hannon GJ. 2010. A dicer-independent miRNA biogenesis pathway that requires Ago catalysis. *Nature* **465**: 584-589.

Chen WS, Manova K, Weinstein DC, Duncan SA, Plump AS, Prezioso VR, Bachvarova RF, Darnell JE, Jr. 1994. Disruption of the HNF-4 gene, expressed in visceral endoderm, leads to cell death in embryonic ectoderm and impaired gastrulation of mouse embryos. *Genes Dev* **8**: 2466-2477.

Christoffels VM, Sassi H, Ruijter JM, Moorman AF, Grange T, Lamers WH. 1999. A mechanistic model for the development and maintenance of portocentral gradients in gene expression in the liver. *Hepatology* **29**: 1180-1192.

Cifuentes D, Xue H, Taylor DW, Patnode H, Mishima Y, Cheloufi S, Ma E, Mane S, Hannon GJ, Lawson ND et al. 2010. A novel miRNA processing pathway independent of Dicer requires Argonaute2 catalytic activity. *Science* **328**: 1694-1698.

Cirillo LA, Lin FR, Cuesta I, Friedman D, Jarnik M, Zaret KS. 2002. Opening of compacted chromatin by early developmental transcription factors HNF3 (FoxA) and GATA-4. *Mol Cell* **9**: 279-289.

Clotman F, Jacquemin P, Plumb-Rudewiez N, Pierreux CE, Van der Smissen P, Dietz HC, Courtoy PJ, Rousseau GG, Lemaigre FP. 2005. Control of liver cell fate decision by a gradient of TGF beta signaling modulated by Onecut transcription factors. *Genes Dev* **19**: 1849-1854.

Clotman F, Lannoy VJ, Reber M, Cereghini S, Cassiman D, Jacquemin P, Roskams T, Rousseau GG, Lemaigre FP. 2002. The onecut transcription factor HNF6 is required for normal development of the biliary tract. *Development* **129**: 1819-1828.

Coffinier C, Gresh L, Fiette L, Tronche F, Schutz G, Babinet C, Pontoglio M, Yaniv M, Barra J. 2002. Bile system morphogenesis defects and liver dysfunction upon targeted deletion of HNF1beta. *Development* **129**: 1829-1838.

Coulouarn C, Factor VM, Andersen JB, Durkin ME, Thorgeirsson SS. 2009. Loss of miR-122 expression in liver cancer correlates with suppression of the hepatic phenotype and gain of metastatic properties. *Oncogene* **28**: 3526-3536.

Davis S, Propp S, Freier SM, Jones LE, Serra MJ, Kinberger G, Bhat B, Swayze EE, Bennett CF, Esau C. 2009. Potent inhibition of microRNA in vivo without degradation. *Nucleic Acids Res* **37**: 70-77.

Decaens T, Godard C, de Reynies A, Rickman DS, Tronche F, Couty JP, Perret C, Colnot S. 2008. Stabilization of beta-catenin affects mouse embryonic liver growth and hepatoblast fate. *Hepatology* **47**: 247-258.

Denson LA, McClure MH, Bogue CW, Karpen SJ, Jacobs HC. 2000. HNF3beta and GATA-4 transactivate the liver-enriched homeobox gene, Hex. *Gene* **246**: 311-320.

Elmen J, Lindow M, Schutz S, Lawrence M, Petri A, Obad S, Lindholm M, Hedtjarn M, Hansen HF, Berger U et al. 2008. LNA-mediated microRNA silencing in non-human primates. *Nature* **452**: 896-899.

Esau C, Davis S, Murray SF, Yu XX, Pandey SK, Pear M, Watts L, Booten SL, Graham M, McKay R et al. 2006. miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. *Cell Metab* **3**: 87-98.

Fabian MR, Sonenberg N, Filipowicz W. 2010. Regulation of mRNA translation and stability by microRNAs. *Annu Rev Biochem* **79**: 351-379.

Finley KR, Tennessen J, Shawlot W. 2003. The mouse secreted frizzled-related protein 5 gene is expressed in the anterior visceral endoderm and foregut endoderm during early post-implantation development. *Gene Expr Patterns* **3**: 681-684.

Flynn DM, Nijjar S, Hubscher SG, de Goyet Jde V, Kelly DA, Strain AJ, Crosby HA. 2004. The role of Notch receptor expression in bile duct development and disease. *J Pathol* **204**: 55-64.

Fornari F, Gramantieri L, Giovannini C, Veronese A, Ferracin M, Sabbioni S, Calin GA, Grazi GL, Croce CM, Tavolari S et al. 2009. MiR-122/cyclin G1 interaction modulates p53 activity and affects doxorubicin sensitivity of human hepatocarcinoma cells. *Cancer Res* **69**: 5761-5767.

Friedman RC, Farh KK, Burge CB, Bartel DP. 2009. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* **19**: 92-105.

Gao Y, Schug J, McKenna LB, Le Lay J, Kaestner KH, Greenbaum LE. 2010. Tissue-specific regulation of mouse MicroRNA genes in endoderm-derived tissues. *Nucleic Acids Res*.

Gatfield D, Le Martelot G, Vejnar CE, Gerlach D, Schaad O, Fleury-Olela F, Ruskeepaa AL, Oresic M, Esau CC, Zdobnov EM et al. 2009. Integration of microRNA miR-122 in hepatic circadian gene expression. *Genes Dev* **23**: 1313-1326.

Gebhardt R, Baldysiak-Figiel A, Krugel V, Ueberham E, Gaunitz F. 2007. Hepatocellular expression of glutamine synthetase: an indicator of morphogen actions as master regulators of zonation in adult liver. *Prog Histochem Cytochem* **41**: 201-266.

Geisler F, Nagl F, Mazur PK, Lee M, Zimber-Strobl U, Strobl LJ, Radtke F, Schmid RM, Siveke JT. 2008. Liver-specific inactivation of Notch2, but not Notch1, compromises intrahepatic bile duct development in mice. *Hepatology* **48**: 607-616.

Gissen P, Johnson CA, Morgan NV, Stapelbroek JM, Forshew T, Cooper WN, McKiernan PJ, Klomp LW, Morris AA, Wraith JE et al. 2004. Mutations in VPS33B, encoding a regulator of SNARE-dependent membrane fusion, cause arthrogryposis-renal dysfunction-cholestasis (ARC) syndrome. *Nat Genet* **36**: 400-404.

Gong Y, Ma Z, Patel V, Fischer E, Hiesberger T, Pontoglio M, Igarashi P. 2009. HNF-1beta regulates transcription of the PKD modifier gene Kif12. *J Am Soc Nephrol* **20**: 41-47.

Gramantieri L, Ferracin M, Fornari F, Veronese A, Sabbioni S, Liu CG, Calin GA, Giovannini C, Ferrazzi E, Grazi GL et al. 2007. Cyclin G1 is a target of miR-122a, a microRNA frequently down-regulated in human hepatocellular carcinoma. *Cancer Res* **67**: 6092-6099.

Gresh L, Fischer E, Reimann A, Tanguy M, Garbay S, Shao X, Hiesberger T, Fiette L, Igarashi P, Yaniv M et al. 2004. A transcriptional network in polycystic kidney disease. *EMBO J* **23**: 1657-1668.

Gualdi R, Bossard P, Zheng M, Hamada Y, Coleman JR, Zaret KS. 1996. Hepatic specification of the gut endoderm in vitro: cell signaling and transcriptional control. *Genes Dev* **10**: 1670-1682.

Hand NJ, Master ZR, Eauclaire SF, Weinblatt DE, Matthews RP, Friedman JR. 2009a. The microRNA-30 family is required for vertebrate hepatobiliary development. *Gastroenterology* **136**: 1081-1090.

Hand NJ, Master ZR, Le Lay J, Friedman JR. 2009b. Hepatic function is preserved in the absence of mature microRNAs. *Hepatology* **49**: 618-626.

Hayhurst GP, Lee YH, Lambert G, Ward JM, Gonzalez FJ. 2001. Hepatocyte nuclear factor 4alpha (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. *Mol Cell Biol* **21**: 1393-1403.

Henke JI, Goergen D, Zheng J, Song Y, Schuttler CG, Fehr C, Junemann C, Niepmann M. 2008. microRNA-122 stimulates translation of hepatitis C virus RNA. *EMBO J* **27**: 3300-3310.

Hofmann JJ, Zovein AC, Koh H, Radtke F, Weinmaster G, Iruela-Arispe ML. 2010. Jagged1 in the portal vein mesenchyme regulates intrahepatic bile duct development: insights into Alagille syndrome. *Development* **137**: 4061-4072.

Hunter MP, Wilson CM, Jiang X, Cong R, Vasavada H, Kaestner KH, Bogue CW. 2007. The homeobox gene Hhex is essential for proper hepatoblast differentiation and bile duct morphogenesis. *Dev Biol* **308**: 355-367.

Jochheim A, Cieslak A, Hillemann T, Cantz T, Scharf J, Manns MP, Ott M. 2003. Multi-stage analysis of differential gene expression in BALB/C mouse liver development by high-density microarrays. *Differentiation* **71**: 62-72.

Jopling CL, Schutz S, Sarnow P. 2008. Position-dependent function for a tandem microRNA miR-122-binding site located in the hepatitis C virus RNA genome. *Cell Host Microbe* **4**: 77-85.

Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P. 2005. Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. *Science* **309**: 1577-1581.

Jung HS, Oropeza V, Thesleff I. 1999. Shh, Bmp-2, Bmp-4 and Fgf-8 are associated with initiation and patterning of mouse tongue papillae. *Mech Dev* **81**: 179-182.

Jungermann K. 1987. Metabolic zonation of liver parenchyma: significance for the regulation of glycogen metabolism, gluconeogenesis, and glycolysis. *Diabetes Metab Rev* **3**: 269-293.

Katoh T, Sakaguchi Y, Miyauchi K, Suzuki T, Kashiwabara S, Baba T. 2009. Selective stabilization of mammalian microRNAs by 3' adenylation mediated by the cytoplasmic poly(A) polymerase GLD-2. *Genes Dev* **23**: 433-438.

Keng VW, Yagi H, Ikawa M, Nagano T, Myint Z, Yamada K, Tanaka T, Sato A, Muramatsu I, Okabe M et al. 2000. Homeobox gene Hex is essential for onset of mouse embryonic liver development and differentiation of the monocyte lineage. *Biochem Biophys Res Commun* **276**: 1155-1161.

Kinoshita T, Sekiguchi T, Xu MJ, Ito Y, Kamiya A, Tsuji K, Nakahata T, Miyajima A. 1999. Hepatic differentiation induced by oncostatin M attenuates fetal liver hematopoiesis. *Proc Natl Acad Sci U S A* **96**: 7265-7270.

Kodama Y, Hijikata M, Kageyama R, Shimotohno K, Chiba T. 2004. The role of notch signaling in the development of intrahepatic bile ducts. *Gastroenterology* **127**: 1775-1786.

Kojima S, Gatfield D, Esau CC, Green CB. 2010. MicroRNA-122 modulates the rhythmic expression profile of the circadian deadenylase Nocturnin in mouse liver. *PLoS ONE* **5**: e11264.

Krupczak-Hollis K, Wang X, Kalinichenko VV, Gusarova GA, Wang IC, Dennewitz MB, Yoder HM, Kiyokawa H, Kaestner KH, Costa RH. 2004. The mouse Forkhead Box m1 transcription factor is essential for hepatoblast mitosis and development of intrahepatic bile ducts and vessels during liver morphogenesis. *Dev Biol* **276**: 74-88.

Krutzfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M, Stoffel M. 2005. Silencing of microRNAs in vivo with 'antagomirs'. *Nature* **438**: 685-689.

Kyrmizi I, Hatzis P, Katrakili N, Tronche F, Gonzalez FJ, Talianidis I. 2006. Plasticity and expanding complexity of the hepatic transcription factor network during liver development. *Genes Dev* **20**: 2293-2305.

Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. 2002. Identification of tissue-specific microRNAs from mouse. *Curr Biol* **12**: 735-739.

Lahuna O, Rastegar M, Maiter D, Thissen JP, Lemaigre FP, Rousseau GG. 2000. Involvement of STAT5 (signal transducer and activator of transcription 5) and HNF-4 (hepatocyte nuclear factor 4) in the transcriptional control of the hnf6 gene by growth hormone. *Mol Endocrinol* **14**: 285-294.

Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A, Pfeffer S, Rice A, Kamphorst AO, Landthaler M et al. 2007. A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell* **129**: 1401-1414.

Lanford RE, Hildebrandt-Eriksen ES, Petri A, Persson R, Lindow M, Munk ME, Kauppinen S, Orum H. 2010. Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. *Science* **327**: 198-201.

Lannoy VJ, Decaux JF, Pierreux CE, Lemaigre FP, Rousseau GG. 2002. Liver glucokinase gene expression is controlled by the onecut transcription factor hepatocyte nuclear factor-6. *Diabetologia* **45**: 1136-1141.

Lee CS, Friedman JR, Fulmer JT, Kaestner KH. 2005. The initiation of liver development is dependent on Foxa transcription factors. *Nature* **435**: 944-947.

Lemaigre FP. 2009. Mechanisms of liver development: concepts for understanding liver disorders and design of novel therapies. *Gastroenterology* **137**: 62-79.

Li J, Ning G, Duncan SA. 2000. Mammalian hepatocyte differentiation requires the transcription factor HNF-4alpha. *Genes Dev* **14**: 464-474.

Li L, Krantz ID, Deng Y, Genin A, Banta AB, Collins CC, Qi M, Trask BJ, Kuo WL, Cochran J et al. 1997. Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. *Nat Genet* **16**: 243-251.

Lin CJ, Gong HY, Tseng HC, Wang WL, Wu JL. 2008. miR-122 targets an anti-apoptotic gene, Bcl-w, in human hepatocellular carcinoma cell lines. *Biochem Biophys Res Commun* **375**: 315-320.

Lokmane L, Haumaitre C, Garcia-Villalba P, Anselme I, Schneider-Maunoury S, Cereghini S. 2008. Crucial role of vHNF1 in vertebrate hepatic specification. *Development* **135**: 2777-2786.

Loomes KM, Taichman DB, Glover CL, Williams PT, Markowitz JE, Piccoli DA, Baldwin HS, Oakey RJ. 2002. Characterization of Notch receptor expression in the developing mammalian heart and liver. *Am J Med Genet* **112**: 181-189.

Louis AA, Van Eyken P, Haber BA, Hicks C, Weinmaster G, Taub R, Rand EB. 1999. Hepatic jagged1 expression studies. *Hepatology* **30**: 1269-1275.

Lozier J, McCright B, Gridley T. 2008. Notch signaling regulates bile duct morphogenesis in mice. *PLoS ONE* **3**: e1851.

Ludtke TH, Christoffels VM, Petry M, Kispert A. 2009. Tbx3 promotes liver bud expansion during mouse development by suppression of cholangiocyte differentiation. *Hepatology* **49**: 969-978.

Margagliotti S, Clotman F, Pierreux CE, Beaudry JB, Jacquemin P, Rousseau GG, Lemaigre FP. 2007. The Onecut transcription factors HNF-6/OC-1 and OC-2 regulate early liver expansion by controlling hepatoblast migration. *Dev Biol* **311**: 579-589.

Margagliotti S, Clotman F, Pierreux CE, Lemoine P, Rousseau GG, Henriet P, Lemaigre FP. 2008. Role of metalloproteinases at the onset of liver development. *Dev Growth Differ* **50**: 331-338.

Martinez Barbera JP, Clements M, Thomas P, Rodriguez T, Meloy D, Kioussis D, Beddington RS. 2000. The homeobox gene Hex is required in definitive endodermal tissues for normal forebrain, liver and thyroid formation. *Development* **127**: 2433-2445.

Matsumoto K, Miki R, Nakayama M, Tatsumi N, Yokouchi Y. 2008. Wnt9a secreted from the walls of hepatic sinusoids is essential for morphogenesis, proliferation, and glycogen accumulation of chick hepatic epithelium. *Dev Biol* **319**: 234-247.

Matsumoto K, Yoshitomi H, Rossant J, Zaret KS. 2001. Liver organogenesis promoted by endothelial cells prior to vascular function. *Science* **294**: 559-563.

Matthews RP, Plumb-Rudewiez N, Lorent K, Gissen P, Johnson CA, Lemaigre F, Pack M. 2005. Zebrafish vps33b, an ortholog of the gene responsible for human arthrogryposis-renal dysfunction-cholestasis syndrome, regulates biliary development downstream of the onecut transcription factor hnf6. *Development* **132**: 5295-5306.

McDaniell R, Warthen DM, Sanchez-Lara PA, Pai A, Krantz ID, Piccoli DA, Spinner NB. 2006. NOTCH2 mutations cause Alagille syndrome, a heterogeneous disorder of the notch signaling pathway. *Am J Hum Genet* **79**: 169-173.

McLin VA, Rankin SA, Zorn AM. 2007. Repression of Wnt/beta-catenin signaling in the anterior endoderm is essential for liver and pancreas development. *Development* **134**: 2207-2217.

Miller DL, Ortega S, Bashayan O, Basch R, Basilico C. 2000. Compensation by fibroblast growth factor 1 (FGF1) does not account for the mild phenotypic defects observed in FGF2 null mice. *Mol Cell Biol* **20**: 2260-2268.

Monga SP, Mars WM, Pediaditakis P, Bell A, Mule K, Bowen WC, Wang X, Zarnegar R, Michalopoulos GK. 2002. Hepatocyte growth factor induces Wnt-independent nuclear translocation of beta-catenin after Met-beta-catenin dissociation in hepatocytes. *Cancer Res* **62**: 2064-2071.

Monga SP, Monga HK, Tan X, Mule K, Pediaditakis P, Michalopoulos GK. 2003. Beta-catenin antisense studies in embryonic liver cultures: role in proliferation, apoptosis, and lineage specification. *Gastroenterology* **124**: 202-216.

Morford LA, Davis C, Jin L, Dobierzewska A, Peterson ML, Spear BT. 2007. The oncofetal gene glypican 3 is regulated in the postnatal liver by zinc fingers and homeoboxes 2 and in the regenerating liver by alpha-fetoprotein regulator 2. *Hepatology* **46**: 1541-1547.

Murchison EP, Partridge JF, Tam OH, Cheloufi S, Hannon GJ. 2005. Characterization of Dicer-deficient murine embryonic stem cells. *Proc Natl Acad Sci U S A* **102**: 12135-12140.

Oda T, Elkahloun AG, Pike BL, Okajima K, Krantz ID, Genin A, Piccoli DA, Meltzer PS, Spinner NB, Collins FS et al. 1997. Mutations in the human Jagged1 gene are responsible for Alagille syndrome. *Nat Genet* **16**: 235-242.

Odom DT, Dowell RD, Jacobsen ES, Nekludova L, Rolfe PA, Danford TW, Gifford DK, Fraenkel E, Bell GI, Young RA. 2006. Core transcriptional regulatory circuitry in human hepatocytes. *Mol Syst Biol* **2**: 2006 0017.

Odom DT, Zizlsperger N, Gordon DB, Bell GW, Rinaldi NJ, Murray HL, Volkert TL, Schreiber J, Rolfe PA, Gifford DK et al. 2004. Control of pancreas and liver gene expression by HNF transcription factors. *Science* **303**: 1378-1381.

Oikawa T, Kamiya A, Kakinuma S, Zeniya M, Nishinakamura R, Tajiri H, Nakauchi H. 2009. Sall4 regulates cell fate decision in fetal hepatic stem/progenitor cells. *Gastroenterology* **136**: 1000-1011.

Pare JF, Malenfant D, Courtemanche C, Jacob-Wagner M, Roy S, Allard D, Belanger L. 2004. The fetoprotein transcription factor (FTF) gene is essential to embryogenesis and cholesterol homeostasis and is regulated by a DR4 element. *J Biol Chem* **279**: 21206-21216.

Parviz F, Matullo C, Garrison WD, Savatski L, Adamson JW, Ning G, Kaestner KH, Rossi JM, Zaret KS, Duncan SA. 2003. Hepatocyte nuclear factor 4alpha controls the development of a hepatic epithelium and liver morphogenesis. *Nat Genet* **34**: 292-296.

Perincheri S, Dingle RW, Peterson ML, Spear BT. 2005. Hereditary persistence of alpha-fetoprotein and H19 expression in liver of BALB/cJ mice is due to a retrovirus insertion in the Zhx2 gene. *Proc Natl Acad Sci U S A* **102**: 396-401.

Petkov PM, Zavadil J, Goetz D, Chu T, Carver R, Rogler CE, Bottinger EP, Shafritz DA, Dabeva MD. 2004. Gene expression pattern in hepatic stem/progenitor cells during rat fetal development using complementary DNA microarrays. *Hepatology* **39**: 617-627.

Pierreux CE, Poll AV, Kemp CR, Clotman F, Maestro MA, Cordi S, Ferrer J, Leyns L, Rousseau GG, Lemaigre FP. 2006. The transcription factor hepatocyte nuclear factor-6 controls the development of pancreatic ducts in the mouse. *Gastroenterology* **130**: 532-541.

Pierreux CE, Stafford J, Demonte D, Scott DK, Vandenhaute J, O'Brien RM, Granner DK, Rousseau GG, Lemaigre FP. 1999. Antiglucocorticoid activity of hepatocyte nuclear factor-6. *Proc Natl Acad Sci U S A* **96**: 8961-8966.

Plumb-Rudewiez N, Clotman F, Strick-Marchand H, Pierreux CE, Weiss MC, Rousseau GG, Lemaigre FP. 2004. Transcription factor HNF-6/OC-1 inhibits the stimulation of the HNF-3alpha/Foxa1 gene by TGF-beta in mouse liver. *Hepatology* **40**: 1266-1274.

Pontoglio M, Barra J, Hadchouel M, Doyen A, Kress C, Bach JP, Babinet C, Yaniv M. 1996. Hepatocyte nuclear factor 1 inactivation results in hepatic dysfunction, phenylketonuria, and renal Fanconi syndrome. *Cell* **84**: 575-585.

Randall G, Panis M, Cooper JD, Tellinghuisen TL, Sukhodolets KE, Pfeffer S, Landthaler M, Landgraf P, Kan S, Lindenbach BD et al. 2007. Cellular cofactors affecting hepatitis C virus infection and replication. *Proc Natl Acad Sci U S A* **104**: 12884-12889.

Raynaud P, Carpentier R, Antoniou A, Lemaigre FP. 2010. Biliary differentiation and bile duct morphogenesis in development and disease. *Int J Biochem Cell Biol*.

Rogler CE, Levoci L, Ader T, Massimi A, Tchaikovskaya T, Norel R, Rogler LE. 2009. MicroRNA-23b cluster microRNAs regulate transforming growth factor-beta/bone morphogenetic protein signaling and liver stem cell differentiation by targeting Smads. *Hepatology*: Mar 23 Epub ahead of print.

Rossi JM, Dunn NR, Hogan BL, Zaret KS. 2001. Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. *Genes Dev* **15**: 1998-2009.

Schmidt C, Bladt F, Goedecke S, Brinkmann V, Zschiesche W, Sharpe M, Gherardi E, Birchmeier C. 1995. Scatter factor/hepatocyte growth factor is essential for liver development. *Nature* **373**: 699-702.

Sekine S, Ogawa R, Ito R, Hiraoka N, McManus MT, Kanai Y, Hebrok M. 2009a. Disruption of Dicer1 induces dysregulated fetal gene expression and promotes hepatocarcinogenesis. *Gastroenterology* **136**: 2304-2315.

Sekine S, Ogawa R, McManus MT, Kanai Y, Hebrok M. 2009b. Dicer is required for proper liver zonation. *J Pathol* **219**: 365-372.

Serls AE, Doherty S, Parvatiyar P, Wells JM, Deutsch GH. 2005. Different thresholds of fibroblast growth factors pattern the ventral foregut into liver and lung. *Development* **132**: 35-47.

Sosa-Pineda B, Wigle JT, Oliver G. 2000. Hepatocyte migration during liver development requires Prox1. *Nat Genet* **25**: 254-255.

Spear BT, Jin L, Ramasamy S, Dobierzewska A. 2006. Transcriptional control in the mammalian liver: liver development, perinatal repression, and zonal gene regulation. *Cell Mol Life Sci* **63**: 2922-2938.

Suzuki A, Iwama A, Miyashita H, Nakauchi H, Taniguchi H. 2003. Role for growth factors and extracellular matrix in controlling differentiation of prospectively isolated hepatic stem cells. *Development* **130**: 2513-2524.

Suzuki T, Kanai Y, Hara T, Sasaki J, Sasaki T, Kohara M, Maehama T, Taya C, Shitara H, Yonekawa H et al. 2006. Crucial role of the small GTPase ARF6 in hepatic cord formation during liver development. *Mol Cell Biol* **26**: 6149-6156.

Tan X, Yuan Y, Zeng G, Apte U, Thompson MD, Cieply B, Stolz DB, Michalopoulos GK, Kaestner KH, Monga SP. 2008. Beta-catenin deletion in hepatoblasts disrupts hepatic morphogenesis and survival during mouse development. *Hepatology* **47**: 1667-1679.

Tanimizu N, Miyajima A. 2004. Notch signaling controls hepatoblast differentiation by altering the expression of liver-enriched transcription factors. *J Cell Sci* **117**: 3165-3174.

Tchorz JS, Kinter J, Muller M, Tornillo L, Heim MH, Bettler B. 2009. Notch2 signaling promotes biliary epithelial cell fate specification and tubulogenesis during bile duct development in mice. *Hepatology*: April 27 Epub ahead of print.

Terada T, Okada Y, Nakanuma Y. 1995. Expression of matrix proteinases during human intrahepatic bile duct development. A possible role in biliary cell migration. *Am J Pathol* **147**: 1207-1213.

Thorrez L, Laudadio I, Van Deun K, Quintens R, Hendrickx N, Granvik M, Lemaire K, Schraenen A, Van Lommel L, Lehnert S et al. 2011. Tissue-specific disallowance of housekeeping genes: the other face of cell differentiation. *Genome Res*.

Tremblay KD, Zaret KS. 2005. Distinct populations of endoderm cells converge to generate the embryonic liver bud and ventral foregut tissues. *Dev Biol* **280**: 87-99.

Tsai WC, Hsu PW, Lai TC, Chau GY, Lin CW, Chen CM, Lin CD, Liao YL, Wang JL, Chau YP et al. 2009. MicroRNA-122, a tumor suppressor microRNA that regulates intrahepatic metastasis of hepatocellular carcinoma. *Hepatology* **49**: 1571-1582.

Van Eyken P, Sciot R, Callea F, Van der Steen K, Moerman P, Desmet VJ. 1988. The development of the intrahepatic bile ducts in man: a keratin-immunohistochemical study. *Hepatology* **8**: 1586-1595.

Wang M, Tan Y, Costa RH, Holterman AX. 2004. In vivo regulation of murine CYP7A1 by HNF-6: a novel mechanism for diminished CYP7A1 expression in biliary obstruction. *Hepatology* **40**: 600-608.

Wang Z, Dolle P, Cardoso WV, Niederreither K. 2006. Retinoic acid regulates morphogenesis and patterning of posterior foregut derivatives. *Dev Biol* **297**: 433-445.

Watt AJ, Zhao R, Li J, Duncan SA. 2007. Development of the mammalian liver and ventral pancreas is dependent on GATA4. *BMC Dev Biol* **7**: 37.

Weinstein M, Monga SP, Liu Y, Brodie SG, Tang Y, Li C, Mishra L, Deng CX. 2001. Smad proteins and hepatocyte growth factor control parallel regulatory pathways that converge on beta1-integrin to promote normal liver development. *Mol Cell Biol* **21**: 5122-5131.

Winter J, Jung S, Keller S, Gregory RI, Diederichs S. 2009. Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat Cell Biol* **11**: 228-234.

Xie Z, Zhang H, Tsai W, Zhang Y, Du Y, Zhong J, Szpirer C, Zhu M, Cao X, Barton MC et al. 2008. Zinc finger protein ZBTB20 is a key repressor of alpha-fetoprotein gene transcription in liver. *Proc Natl Acad Sci U S A* **105**: 10859-10864.

Xu H, He JH, Xiao ZD, Zhang QQ, Chen YQ, Zhou H, Qu LH. 2010. Liver-enriched transcription factors regulate microRNA-122 that targets CUTL1 during liver development. *Hepatology* **52**: 1431-1442.

Yamasaki H, Sada A, Iwata T, Niwa T, Tomizawa M, Xanthopoulos KG, Koike T, Shiojiri N. 2006. Suppression of C/EBPalpha expression in periportal hepatoblasts may stimulate biliary cell differentiation through increased Hnf6 and Hnf1b expression. *Development* **133**: 4233-4243.

Yanai M, Tatsumi N, Hasunuma N, Katsu K, Endo F, Yokouchi Y. 2008. FGF signaling segregates biliary cell-lineage from chick hepatoblasts cooperatively with BMP4 and ECM components in vitro. *Dev Dyn* **237**: 1268-1283.

Yang JS, Lai EC. 2010. Dicer-independent, Ago2-mediated microRNA biogenesis in vertebrates. *Cell Cycle* **9**: 4455-4460.

Yoshida Y, Hughes DE, Rausa FM, 3rd, Kim IM, Tan Y, Darlington GJ, Costa RH. 2006. C/EBPalpha and HNF6 protein complex formation stimulates HNF6-dependent transcription by CBP coactivator recruitment in HepG2 cells. *Hepatology* **43**: 276-286.

Zaret KS, Grompe M. 2008. Generation and regeneration of cells of the liver and pancreas. *Science* **322**: 1490-1494.

Zeng C, Wang R, Li D, Lin XJ, Wei QK, Yuan Y, Wang Q, Chen W, Zhuang SM. 2010. A novel GSK-3 beta-C/EBP alpha-miR-122-insulin-like growth factor 1 receptor regulatory circuitry in human hepatocellular carcinoma. *Hepatology* **52**: 1702-1712.

Zhang L, Rubins NE, Ahima RS, Greenbaum LE, Kaestner KH. 2005. Foxa2 integrates the transcriptional response of the hepatocyte to fasting. *Cell Metab* **2**: 141-148.

Zhang W, Yatskievych TA, Baker RK, Antin PB. 2004. Regulation of Hex gene expression and initial stages of avian hepatogenesis by Bmp and Fgf signaling. *Dev Biol* **268**: 312-326.

Zhao R, Duncan SA. 2005. Embryonic development of the liver. *Hepatology* **41**: 956-967.

Zhao R, Watt AJ, Li J, Luebke-Wheeler J, Morrisey EE, Duncan SA. 2005. GATA6 is essential for embryonic development of the liver but dispensable for early heart formation. *Mol Cell Biol* **25**: 2622-2631.

Zong Y, Panikkar A, Xu J, Antoniou A, Raynaud P, Lemaigre F, Stanger BZ. 2009. Notch signaling controls liver development by regulating biliary differentiation. *Development* **136**: 1727-1739.

2. AIM OF THE PROJECT

The aim of this project is to identify miRNAs which are regulated by the transcription factors HNF6 and OC2 during liver development and to study the role of these miRNAs in hepatic cell differentiation. This is performed by identifying miRNAs whose expression is affected in livers from embryos deficient in HNF6 and OC2, and by investigating the function of a selected miRNA through *in vitro* and *in vivo* gain-and loss-of-function experiments.

3. RESULTS

3.1. Control of liver differentiation by a positive feedback loop between a transcription factor and a tissue-specific microRNA

Manuscript submitted for publication

I performed the experiments of Fig. 1, Fig. 2 A,B,C, Fig.3, Fig.5 and Fig.6 as well as Supplementary Fig.1 and 2. I participated to the design of the project and to the writing of the manuscript.

Control of liver differentiation by a positive feedback loop between a transcription factor and a tissue-specific microRNA

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short title: HNF6 - miR-122 loop in hepatocytes

[Keywords: liver development, miR-122, Onecut, gene network]

Abstract

Transcription factors and microRNAs can constitute feedback or feedforward loops that modulate differentiation of cell types from precursors, yet how such regulatory loops drive tissue-specific gene expression during differentiation remains insufficiently characterized. Using liver differentiation as a well-characterized model, we identified miR-122 as a direct target of the transcription factor paralogs Hepatocyte Nuclear Factor (HNF) 6 and Onecut2 during mouse development. The two factors redundantly stimulate miR-122 expression. Conversely, using *in vivo* and *in vitro* gain- and loss-of-function experiments, we discovered that accurate levels of miR-122 are needed to stimulate expression of liver-specific transcription factors, and these include HNF6. Moreover miR-122 drives proper hepatocyte maturation from hepatoblasts, and this is abolished in an HNF6-null background, revealing that a transcription factor can mediate microRNA function. Confirming direct regulation, we found that that the promoters of the genes upregulated by miR-122 are occupied *in vivo* by HNF6. Together, our data reveal a developmental mechanism in which a transcription factor and microRNA, which are both specifically expressed in the same tissue, establish a positive feedback loop that is used to direct differentiation.

Introduction

The interactions between transcription factors and their targets are organized as complex networks which can be classified in distinct motifs (Balle et al. 1987; Christ et al. 1987). MicroRNAs (miRNAs) participate to such networks and, together with the transcription factors, constitute feedback or feedforward loops that control differentiation of cell types from their precursors (Bartels et al. 1987; Tsang et al. 2007; Martinez et al. 2008; Herranz and Cohen 2010). In this paper we investigate how transcription factors and miRNAs regulate tissue-specific gene expression during differentiation, and use liver development as a model in which the network of tissue-specific transcription factors is extensively characterized and in which miRNA expression profiles are well documented (Zhao and Duncan 2005; Landgraf et al. 2007; Hand et al. 2009a; Lemaigre 2009; Raynaud et al. 2010; Si-Tayeb et al. 2010).

During liver development, the hepatocytes and cholangiocytes differentiate from common bipotential precursors, usually referred to as hepatoblasts. Most hepatoblasts give rise to hepatocytes, and a smaller number of hepatoblasts generates biliary precursor cells. Hepatoblasts initially express a number of genes (*e.g. Hepatocyte Nuclear Factor* (HNF) *4α*, *Transthyretin* (*Ttr*), *Albumin* (*Alb*)) which later become restricted to the hepatocyte lineage and whose expression level rises during hepatocyte maturation. These hepatocyte-specific genes are used as markers of hepatocyte differentiation and maturation, and are repressed in developing biliary cells. The segregation of the hepatobiliary lineages starts around embryonic day (E) 11.5 in mice. It culminates around E13.5-E15.5, and is best monitored by the expression of biliary-specific differentiation markers such as the SRY-related HMG box transcription factor 9 (SOX9) in the biliary precursor cells (Antoniou et al. 2009). The latter are initially located around the branches of the portal vein to constitute a sleeve of cells called the "ductal plate". The segregation of hepatobiliary lineages is followed by the maturation phase which allows the hepatocytes to form cords and acquire their metabolic properties, and during which the cholangiocytes form ducts from the ductal plate. (Zhao and Duncan 2005; Lemaigre 2009; Raynaud et al. 2010; Si-Tayeb et al. 2010).

During development, a well-defined set of liver-enriched transcription factors (LETFs) is organized as auto- and cross-regulatory loops, and controls differentiation of hepatic cells (Kyrmizi et al. 2006). The Onecut transcription factors Hepatocyte Nuclear Factor (HNF) 6 and Onecut2 (OC2) belong to the LETFs and regulate hepatic cell fate determination (Clotman et al. 2002; Clotman et al. 2005). HNF6 and OC2 are expressed in hepatoblasts and in the hepatocyte and cholangiocyte lineages. They modulate a gradient of TGFβ/Activin signaling which

normally peaks around the portal vein and wanes into the parenchyma, thereby stimulating biliary differentiation of hepatoblasts near the portal vein (Plumb-Rudewiez et al. 2004; Clotman et al. 2005; Antoniou et al. 2009). In mice knockout for both HNF6 and OC2, the slope of the signaling gradient changes and TGFβ/Activin signaling is higher throughout the liver. This induces abnormal hepatoblast differentiation toward hybrid cells that display characteristics of both hepatocytes and biliary cells.

The role of miRNAs in liver has been studied by conditional inactivation of Dicer1, the type III endonuclease involved in miRNA maturation. This could not reveal a role for miRNAs in hepatic cell differentiation, since inactivation of Dicer1 only occurred postnatally (Hand et al. 2009b; Sekine et al. 2009a; Sekine et al. 2009b). However, targeted approaches addressing the function of specific miRNAs revealed that the miR-30a/30c and miR-23b/27b/24-1 clusters were involved in hepatic developmental processes. MiR-30a/30c are specifically expressed in cholangiocytes during the late stages of gestation and are required for normal bile duct development (Hand et al. 2009a). The miR-23b/27b/24-1 cluster is expressed in hepatocytes at E17.5 and experiments with a cultured hepatoblast line indicated that these miRNAs repress biliary gene expression by inhibiting TGFβ signaling via repression of Smad mRNAs (Rogler et al. 2009).

The expression of miRNAs and LETFs was shown to be correlated, yet how they coordinately control hepatic differentiation has not been investigated. Several promoters of miRNAs enriched in adult liver were shown to contain putative binding sites for HNF4α and Forkhead box A2 (FoxA2; formerly known as HNF3β) (Tuteja et al. 2009; Gao et al. 2011). In hepatocarcinoma lines the expression of miR-122, the most abundant liver-specific miRNA (Lagos-Quintana et al. 2002; Chang et al. 2004; Landgraf et al. 2007), positively correlates with the level of LETFs, and knockdown of HNF1α, FoxA1, FoxA2 or CCAAT-enhancer-binding protein (C/EBP) α reduced miR-122 expression in cultured cells (Coulouarn et al. 2009; Zeng et al.). Along the same lines, the promoter of miR-122 has been identified (Gatfield et al. 2009) and was bound and stimulated in transient transfection experiments by HNF1α, FoxA2, HNF4α and C/EBPα (Xu et al. 2010).

Therefore, to investigate how transcription factor – miRNA networks regulate tissue-specific expression of genes, we investigated the function of LETFs – miRNA networks in hepatic cell differentiation during embryonic development. Here we show that HNF6 and OC2 redundantly control the expression of miRNAs during liver development.

We identify a HNF6 <-> miR-122 positive feedback loop which stimulates differentiation of hepatoblasts toward hepatocytes, and which displays an undescribed topology in the control of cell-type specific gene expression.

Results

HNF6 and OC2 control the expression of several miRNAs during liver development

To identify miRNAs regulating liver-specific gene expression we resorted to mice that are deficient in both HNF6 and OC2 (*H6*/*O2* dKO) as a model for perturbed hepatic cell differentiation (Clotman et al. 2005). We compared the miRNA populations in wild-type (WT) and in *H6*/*O2* dKO livers at E15.5 by microarray analysis. Fifteen miRNAs were differentially expressed, 10 miRNAs being upregulated 1.1- to 2-fold in the *H6*/*O2* dKO mice and 5 miRNAs being downregulated 1.1- to 7-fold in the *H6*/*O2* dKO mice (Fig. 1A). These results were validated by measuring the expression of 5 miRNAs by Q-RT-PCR: differential expression of miR-122, miR-200a and miR-329 at E15.5 was in line with the microarray results, and differential expression of miR-122, miR-107, miR-200a, miR-329 and miR-337 at E17.5 further extended the microarray results (Fig. 1B).

HNF6 and OC2 have similar DNA-target sequences (Jacquemin et al. 1999). By comparing fetal livers from single HNF6 knockouts (*H6* KO) or single OC2 knockouts (*O2* KO), with livers from *H6*/*O2* dKO mice, we found that HNF6 and OC2 redundantly control the expression of miR-122, miR-107, miR-329 and miR-337. Indeed, the absence of both factors is necessary to obtain maximal perturbation of the expression of these 4 miRNAs. MiR-200a was preferentially controlled by HNF6 at E15.5, but was redundantly controlled by HNF6 and OC2 at E17.5 (Fig. 1B).

We concluded that abnormal hepatic cell differentiation in *H6*/*O2* dKO livers is associated with perturbed expression of miRNAs, and that HNF6 and OC2 redundantly control the expression of a subset of miRNAs.

A

microRNA	H6O2 dKO/WT (Fold change)	p-values
mmu-miR-122a	0.15	9.4E-05
mmu-miR-200a	1.88	6.7E-04
hsa-miR-491-3p	0.88	1.2E-03
mmu-miR-292-5p	1.30	3.0E-03
mmu-miR-744	1.26	3.4E-03
hsa-miR-193a-5p	1.17	4.2E-03
mmu-miR-689	1.28	8.6E-03
mmu-miR-18	1.09	1.3E-02
mmu-miR-107	0.91	1.6E-02
mmu-miR-341	1.26	2.1E-02
mmu-miR-710	0.92	2.2E-02
mmu-miR-329	1.17	2.4E-02
mmu-miR-337	1.12	3.4E-02
hsa-miR-200b*	0.92	3.5E-02
mmu-miR-143	1.32	3.8E-02

B

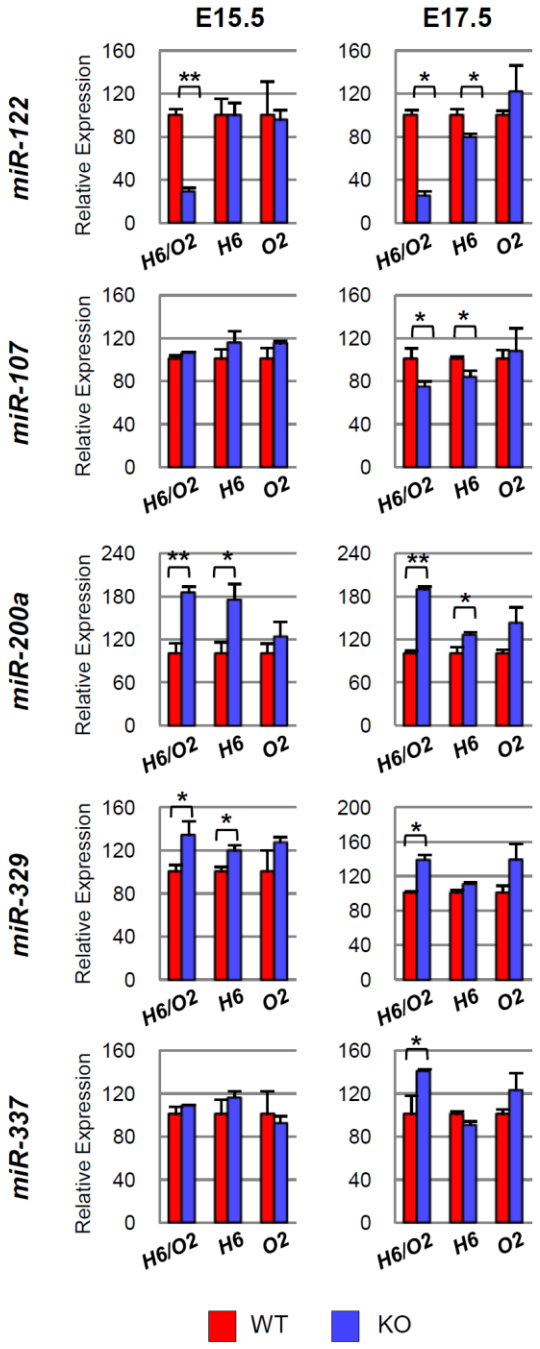


Figure 1. HNF6 and OC2 control the expression of miRNAs during liver development. *(A)* Microarray results showing the 15 miRNAs differentially expressed in wild type (WT) and *Hnf6*$^{-/-}$*/Oc2*$^{-/-}$ (*H6/O2* dKO) livers at E15.5. Data are means ± SEM; n=3; P<0.05.
(B) Expression of miR-122, miR-107, miR-200a, miR-329 and miR-337 by Q-RT-PCR in *H6/O2* dKO and in *Hnf6*$^{-/-}$*/Oc2*$^{+/+}$ (*H6* KO) and *Hnf6*$^{+/+}$*/Oc2*$^{-/-}$ (*O2* KO) mice at E15.5 and E17.5. MiRNA expression was in most cases redundantly controlled by HNF6 and OC2. Data are means ± SEM; n = 3; *P < 0.05; **P < 0.01.

HNF6 stimulates the expression of miR-122 by directly binding to its promoter

Among the miRNA targets of HNF6 and OC2, miR-122 was the most strongly downregulated in the absence of both transcription factors: a 3.5- to 7-fold decrease in its expression was detected in *H6*/*O2* dKO fetal livers (Fig. 1). Therefore, we further focused on the mechanism by which Onecut factors regulate miR-122.

The miR-122 promoter has been located earlier (Gatfield et al. 2009), and we found a sequence matching the HNF6 binding consensus approximately 90 bp upstream of the transcription start site. This site is well conserved among species (Fig. 2A). To verify if HNF6 can stimulate the miR-122 promoter, we cotransfected a reporter construct (miR-122 promoter (-300 to +327) – luciferase (*Luc*)) with an HNF6 expression vector in Bipotential Murine Embryonic Liver (BMEL) cells, a cultured hepatoblast cell line (Strick-Marchand and Weiss 2002). The results showed that HNF6 stimulates the miR-122 promoter (Fig. 2B). In addition, when HNF6 was overexpressed in BMEL cells, endogenous miR-122 levels increased, as measured by Q-RT-PCR (Fig 2C).

We further confirmed that HNF6 binds to the miR-122 promoter by performing chromatin immunoprecipitation/ultra-high throughput sequencing (ChIP-Seq) experiments *in vivo*. HNF6 binding sites were found in liver within 10 kilobases encompassing the miR-122 locus (Fig. 2D). The strongest binding peak overlapped with the promoter sequence which drove miR-122 expression in cultured cells and was stimulated by HNF6.

We concluded that HNF6 stimulates miR-122 expression by directly binding to and activating its promoter.

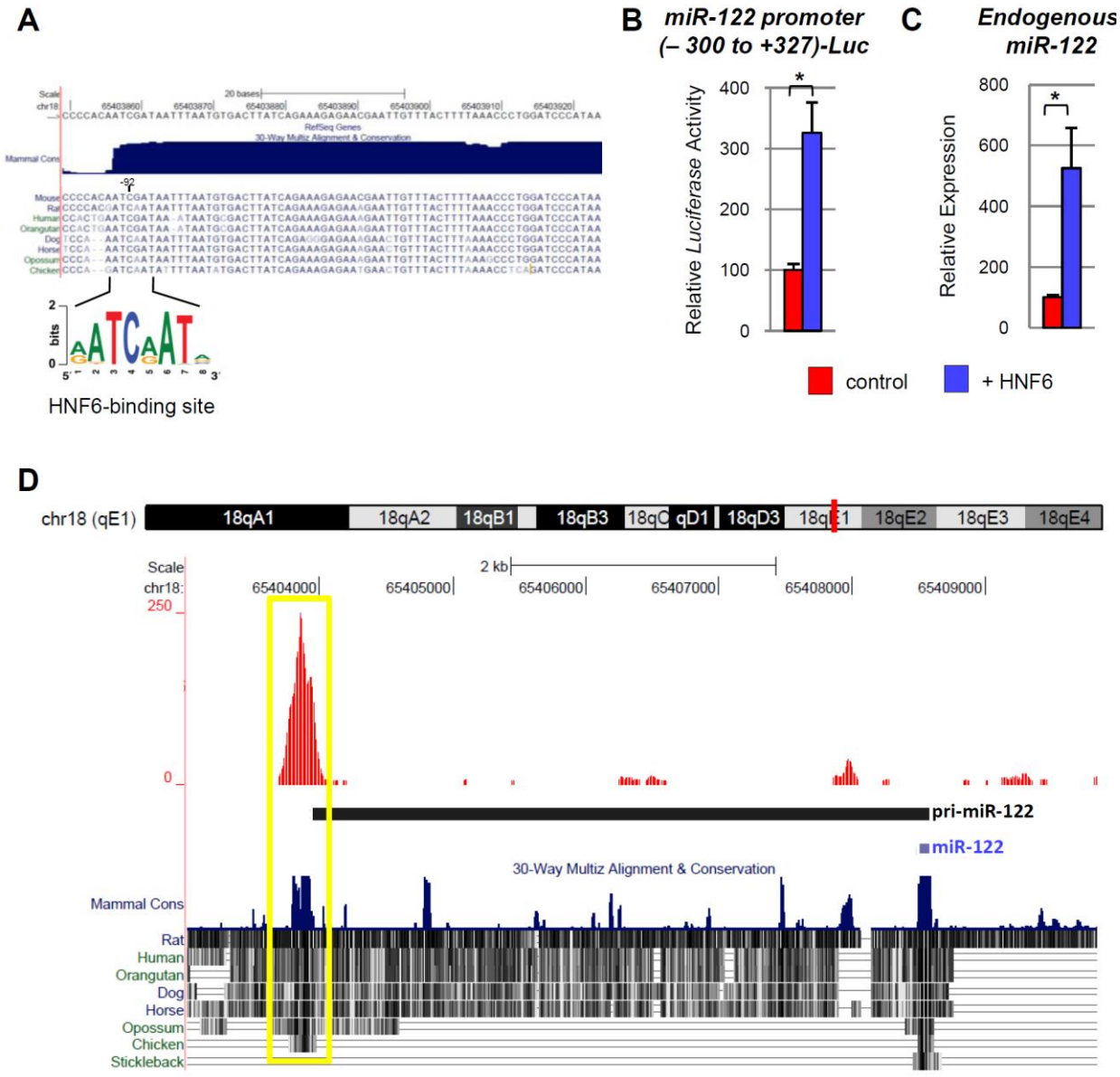


Figure 2. HNF6 binds to and stimulates the miR-122 gene promoter. *(A)* Sequence alignment of the miR-122 gene reveals high evolutionary conservation of the promoter and of a HNF6 binding site consensus. *(B)* 600 bp of the miR-122 promoter, encompassing the HNF6 binding site, were inserted upstream the *firefly luciferase* reporter gene and cotransfected with an HNF6 or GFP (as control) expressing-vector in BMEL cells. Overexpression of HNF6 stimulated luciferase activity. *(C)* Overexpression of HNF6 in BMEL cells also stimulated expression of endogenous miR-122, as measured by Q-RT-PCR. *(D)* Chip-Seq data showing that HNF6 binds to the miR-122 locus *in vivo* in liver, with the strongest peak overlapping with the promoter region; the latter (boxed yellow) was stimulated by HNF6 in BMEL cells. Data are displayed with the help of the UCSC Mouse Genome Browser (NCBI37/mm9). In *(B)* and *(C)* data are means ± SEM; n = 3; *P < 0.05.

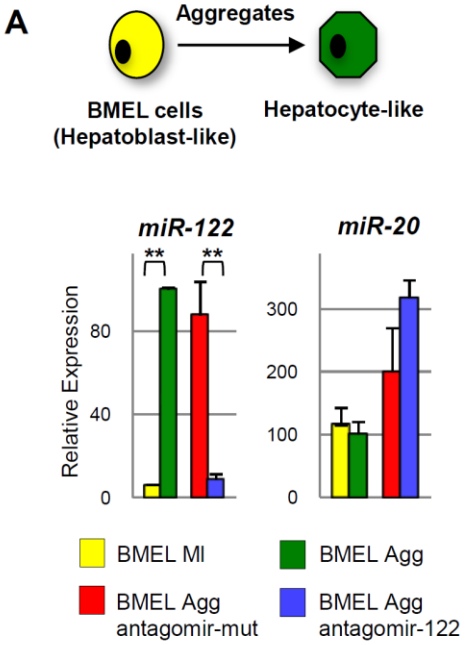
MiR-122 stimulates the expression of hepatocyte-specific genes during hepatocyte differentiation in vitro

Since HNF6 controls hepatic cell differentiation while stimulating expression of miR-122, we investigated the role of miR-122 during hepatocyte differentiation. In a first approach

we cultured BMEL cells in suspension for 72 hours to allow formation of aggregates in which the expression of hepatocyte-specific genes is induced (Strick-Marchand and Weiss 2002). Therefore, BMEL cells are a model to investigate *in vitro* the transition of hepatoblasts to maturing hepatocytes. As shown in Fig. 3A, miR-122 expression was strongly upregulated in BMEL cell aggregates (BMEL Agg) as compared to BMEL cells maintained in monolayer culture (BMEL Ml), thereby mimicking the increase in miR-122 occurring *in vivo* during hepatocyte differentiation (Chang et al. 2004). When anti-miR-122 antagomir was added during the aggregate BMEL cell culture (BMEL Agg antagomir-122), the induction of miR-122 was abolished. Cells cultured under the same conditions but treated with a mutated antagomir did not show decreased levels of miR-122 (BMEL Agg antagomir-mut; Fig. 3A). The expression of an unrelated miRNA, namely miR-20, was not significantly affected by anti-miR-122 antagomir, thereby demonstrating the specificity of miR-122 inhibition by anti-miR-122 antagomir (Fig. 3A).

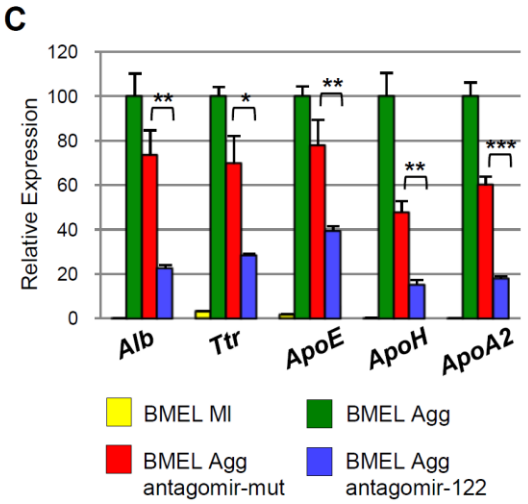
To characterize the consequences of miR-122 inhibition in BMEL cells undergoing hepatocyte-like differentiation, we compared the mRNA profiles in aggregate-cultured BMEL cells treated either with anti-miR-122 antagomir or with mutated antagomir. Microarray analysis revealed that miR-122 inhibition repressed the expression of 26 genes, indicating that miR-122 normally stimulates these genes, in a direct or indirect way. Twenty-four of these 26 genes coded for hepatocyte-specific proteins (Fig. 3B). Furthermore, treatment of BMEL cells with anti-miR-122 antagomir also stimulated expression of 22 genes. These 22 genes are therefore repressed, directly or indirectly, by miR-122; they did not belong to the category of hepatocyte-specific genes (Supp. Fig. 1). The microarray data were validated by Q-RT-PCR analysis of 5 genes downregulated by anti-miR-122 antagomir (*Alb*, *Ttr*, *Apolipoprotein E (ApoE)*, *Apolipoprotein H (ApoH)* and *Apolipoprotein A2 (ApoA2)*) (Fig. 3C).

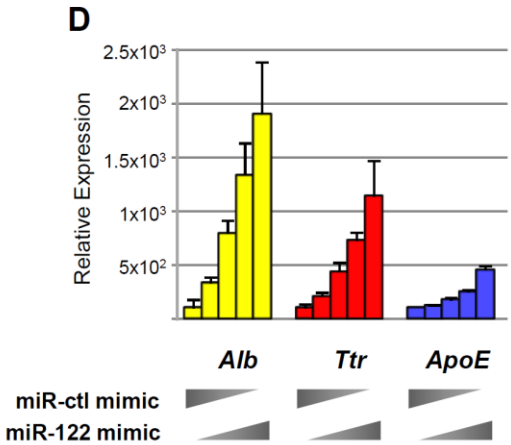
Figure 3. miR-122 stimulates the expression of hepatocyte-specific gene during hepatocyte differentiation *in vitro*. *(A)* BMEL cells were cultured in monolayer (BMEL Ml) or in suspension to allow the formation of floating aggregates (BMEL Agg) which express hepatocyte-specific genes. After 3 days of aggregate culture miR-122 expression was increased. This induction was abolished by antagomir-122 treatment (BMEL Agg antagomir-122); a mutated antagomir (BMEL Agg antagomir-mut) did not inhibit miR-122 expression and served as control. Expression of miR-20, a miRNA unrelated to miR-122, was unaffected by antagomir treatment. *(B)* Microarray analysis of BMEL Agg cells treated with antagomir-122 or mutated antagomir identified 26 genes repressed by miR-122 inhibition; 24 of these genes were hepatocyte-specific. *(C)* Validation of the microarray data by Q-RT-PCR. *Albumin* (*Alb*), *Transthyretin* (*Ttr*), *Apolipoprotein E* (*ApoE*), *Apolipoprotein H* (*ApoH*), *Apolipoprotein A2* (*ApoA2*) were downregulated by miR-122 inhibition. *(D) Alb*, *Ttr* and *ApoE* expression was stimulated by transfection of miR-122 mimic in BMEL cells, in a dose dependent manner. *(E)* Hepatocyte-specific genes targeted by miR-122 were downregulated in *H6*/*O2* dKO livers at E15.5 and E17.5. Data in *(A, C-E)* are means ± SEM; n = 3; *P < 0.05; **P < 0.01.

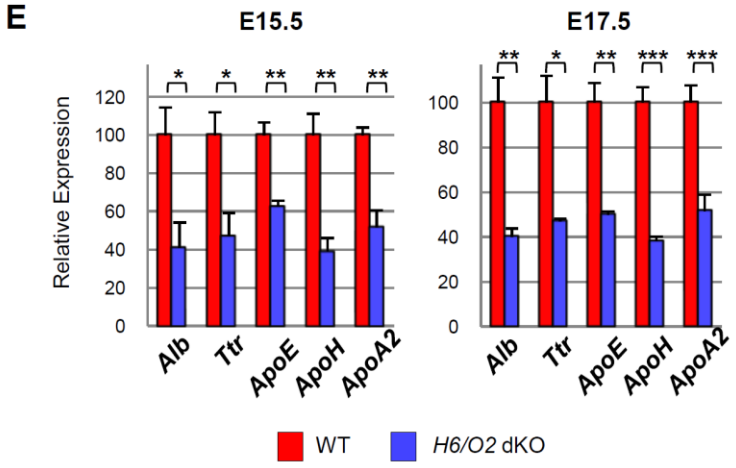


B

BMEL Agg antagomir-122/ antagomir-mut (Fold change)	Gene Symbol	Gene Title
0.51	Apoe	*apolipoprotein E*
0.53	Apoa2	*apolipoprotein A-II*
0.54	Alb	*albumin*
0.55	Apoh	*apolipoprotein H*
0.59	Itih2	*inter-alpha trypsin inhibitor, heavy chain 2*
0.64	Afm	*afamin*
0.64	Fgb	*fibrinogen, B beta polypeptide*
0.65	Itih4	*inter alpha-trypsin inhibitor, heavy chain 4*
0.66	Apoc1	*apolipoprotein C-I*
0.67	Pzp	*pregnancy zone protein*
0.71	Afp	*alpha fetoprotein*
0.71	Tff3	*trefoil factor 3, intestinal*
0.71	Apoa1	*apolipoprotein A-I*
0.71	Acat1	*acetyl-Coenzyme A acetyltransferase 1*
0.71	H19	*H19 fetal liver mRNA*
0.71	Gkn2	*gastrokine 2*
0.72	Fga	*fibrinogen, alpha polypeptide*
0.73	Ttr	*transthyretin*
0.73	Kng1	*kininogen 1*
0.73	Rdh7	*retinol dehydrogenase 7*
0.74	Gc	*group specific component*
0.74	Tff1	*trefoil factor 1 /// similar to pS2m*
0.77	Spp2	*secreted phosphoprotein 2*
0.78	Trf	*transferrin*







To strengthen these conclusions, we turned to gain-of-function experiments. MiR-122 mimics were transfected in BMEL cells, and we found that expression of *Alb*, *Ttr* and *ApoE* was increased in a dose-dependent way, thereby confirming that miR-122 stimulates hepatocyte-specific genes (Fig 3D).

Since miR-122 expression is downregulated in *H6*/*O2* dKO liver during development (Fig. 1B), we expected that hepatocyte-specific genes normally stimulated by miR-122 would be repressed in the absence of HNF6 and OC2. This was the case: *Alb*, *Ttr*, *ApoE*, *ApoH* and *ApoA2* were all downregulated in *H6*/*O2* dKO liver at E15.5 and E17.5 (Fig. 3E).

In conclusion, miR-122 directly or indirectly stimulates expression of genes coding for hepatocyte-specific functions during differentiation of cultured hepatoblasts toward the hepatocyte lineage. These genes are common targets of the Onecut factors and miR-122, possibly via a HNF6/OC2 -> miR-122 cascade.

MiR-122 is required for normal hepatic cell differentiation in developing zebrafish

To confirm that miR-122 controls hepatocyte differentiation using an *in vivo* model, we first turned to the developing zebrafish. In this organism, miR-122 is specifically expressed in liver ((Wienholds et al. 2005); Fig. 4A). During development, the liver is easily detected by staining for Prox1, which is expressed in all hepatic epithelial cells (Fig. 4B). At 50 hours post-fertilization (hpf) the hepatoblasts co-expressed the hepatocyte marker HNF4α and low levels of the biliary marker 2F11, whereas cells that had already initiated differentiation toward cholangiocytes expressed high levels of 2F11 and were devoid of HNF4α (yellow arrows in Fig. 4B, upper panels). Later, at 80 hpf, HNF4α was restricted to hepatocytes, whereas 2F11 expression became restricted to cholangiocytes (Fig. 4B, middle panels); only very few cells, localized in the peripheral layer of hepatic cells, still co-expressed HNF4α and 2F11 at that stage (white arrow in middle right panel of Fig. 4B). Later in development, such HNF4α+/2F11+ cells were no longer found in control liver (data not shown). Therefore, in zebrafish liver, segregation of the hepatocyte lineage is characterized by transient co-expression of HNF4α+ and 2F11+, followed by differentiation toward HNF4α+/2F11- hepatocytes.

MiR-122 expression can be inhibited by injection of an anti-miR-122 morpholino oligonucleotide at the 1-cell stage (Fig. 4A). This oligonucleotide overlaps the Dicer processing site of pre-miR-122 and inhibits miR-122 maturation. In embryos injected with the anti-miR-122 morpholino-oligonucleotide (MO-122) HNF4α+/2F11+ cells were found in the peripheral layer of the liver (Fig. 4B, white arrow in lower panel), like in embryos injected with the control

morpholino oligonucleotide (MO-Ctl). However, unlike in controls, embryos treated with MO-122 displayed immature HNF4α+/2F11+ cells in the center of the liver (green arrows in lower panels of Fig. 4B). Quantification showed that such cells were more abundant and more frequently present in MO-122-injected embryos (Fig. 4C) than in the controls. At a later stage immature HNF4α+/2F11+ cells were no longer found in MO-122-treated livers, indicating that maturation of the liver is not blocked but delayed (data not shown). We concluded that miR-122 is required for normal hepatic cell differentiation *in vivo.*

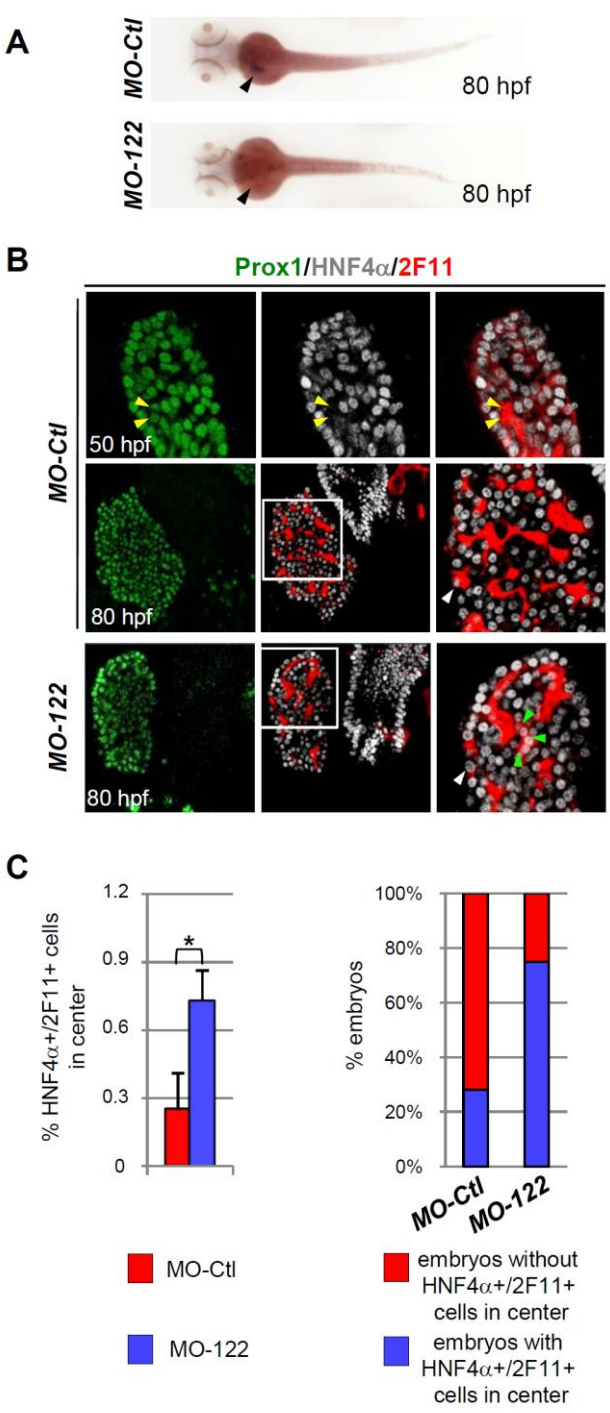


Figure 4. MiR-122 is required for normal liver development in zebrafish embryos. *(A)* In zebrafish embryos at 80 hpf miR-122 was specifically detected in liver (arrow) by *in situ* hybridization (arrow, upper panel). Injection of anti-miR-122 morpholino (MO-122) abolished miR-122 expression (arrow, lower panel). *(B)* In zebrafish embryos at 50 hpf hepatic cells (Prox1+) committed to the hepatocyte lineage co-expressed HNF4α and low levels of 2F11, whereas cells committed to the cholangiocyte lineage (yellow arrows) expressed high levels of 2F11 and were negative for HNF4α. At 80 hpf in a control-morpholino injected embryos (MO-Ctl), hepatocytes were HNF4α+/2F11-. Only very few cells localized at the periphery of the liver, were still HNF4α+/2F11+ (white arrow), and were considered as transient immature cells. In MO-122 injected embryos at 80 hpf, HNF4α+/2F11+ were found in the peripheral layer of hepatic cells, like in controls (white arrow). Unlike in controls, the center of the organ contained immature HNF4α+/2F11+ cells (green arrows). *(C)* Proportion of HNF4α+/2F11+ cells in total number of hepatic cells (Prox1+) in the center of the liver at 80 hpf (left), and frequency of embryos in which HNF4α+/2F11+ cells are found in the center of the liver (right). The center of the liver was defined as the area covering all hepatic cells, excluding the peripheral layer. Data are means ± SEM; n ≥ 7; *P < 0.05.

In vivo stimulation of hepatocyte differentiation by miR-122 depends on HNF6

To study the mode of action of miR-122 in liver development, and to investigate how its function is integrated with that of the LETF network, we turned to gain-of-function experiments in developing mouse liver. MiR-122 is normally expressed in hepatoblasts and hepatocytes, but not in developing biliary cells (Hand et al. 2009a). Therefore, we generated transgenic mice overexpressing miR-122 specifically in the liver under control of *Albumin* and *α-Fetoprotein* regulatory elements. MiR-122 expression was increased in the transgenic mice (*miR-122* Tg) from E13.5 (2.2-fold) until E15.5 (1.7-fold), and then returned to normal values (Fig. 5A). Interestingly, at E15.5 the hepatocyte differentiation markers which were stimulated by miR-122 in cultured cells (Fig. 3) were also upregulated in *miR-122* Tg livers (Fig. 5B). Their expression levels at E15.5 in *miR-122* Tg livers were similar to those in wild-type livers at E17.5 (Supp. Fig. 2), indicating that miR-122 accelerates hepatocyte differentiation.

We next measured, in *miR-122* Tg mice, the expression of the transcription factors *Hnf1α*, *Hnf1β*, *Foxa2*, *Hnf4α*, and *Hnf6/Oc2*, which constitute the core of the LETF network (Kyrmizi et al. 2006). At E15.5, we found a modest 1.4-fold increase in *Foxa2*, *Hnf4α* and *Oc2*, but a stronger 5-fold increase in *Hnf6*, as compared to controls (Fig. 5C). We then focused on HNF6 as it was the most strongly induced, and further showed that its expression was already stimulated in *miR-122* Tg livers from E13.5 onward (Fig. 5D). Immunostaining on sections of wild-type livers at E15.5 showed predominant expression of HNF6 in biliary cells forming the ductal plate, and lower expression in hepatoblasts and developing hepatocytes, confirming earlier findings ((Clotman et al. 2002; Beaudry et al. 2006; Antoniou et al. 2009); Fig. 5F). In E15.5 *miR-122* Tg livers HNF6 was upregulated in hepatocytes, thereby mimicking later stages of liver maturation ((Clotman et al. 2002); Fig. 5G). Therefore, overexpression of miR-122 induces a premature increase of HNF6 in differentiating hepatocytes, which is parallel with the premature increase of hepatocyte-specific gene expression.

The parallel increase in *Hnf6* and in hepatocyte-specific gene expression resulting from overexpression of miR-122, led us to hypothesize that the effect of miR-122 on gene expression depends on HNF6. To test this hypothesis, we first verified by Chip-Seq if HNF6 binds to the genes upregulated by miR-122. This was the case, as shown in Supplementary Figure 3. We then asked if HNF6 mediates the effects of miR-122 on hepatocyte gene expression by studying the effect of miR-122 overexpression in a HNF6-null background. *Hnf6*$^{+/-}$ mice were mated with *miR-122* Tg/*Hnf6*$^{+/-}$ mice and the resulting *miR-122* Tg/*Hnf6*$^{-/-}$ embryos (*miR122* Tg/*H6* KO)

were analyzed at E15.5. These embryos still showed a 1.7-fold overexpression of miR-122 as compared to wild-type embryos (Fig. 5B).

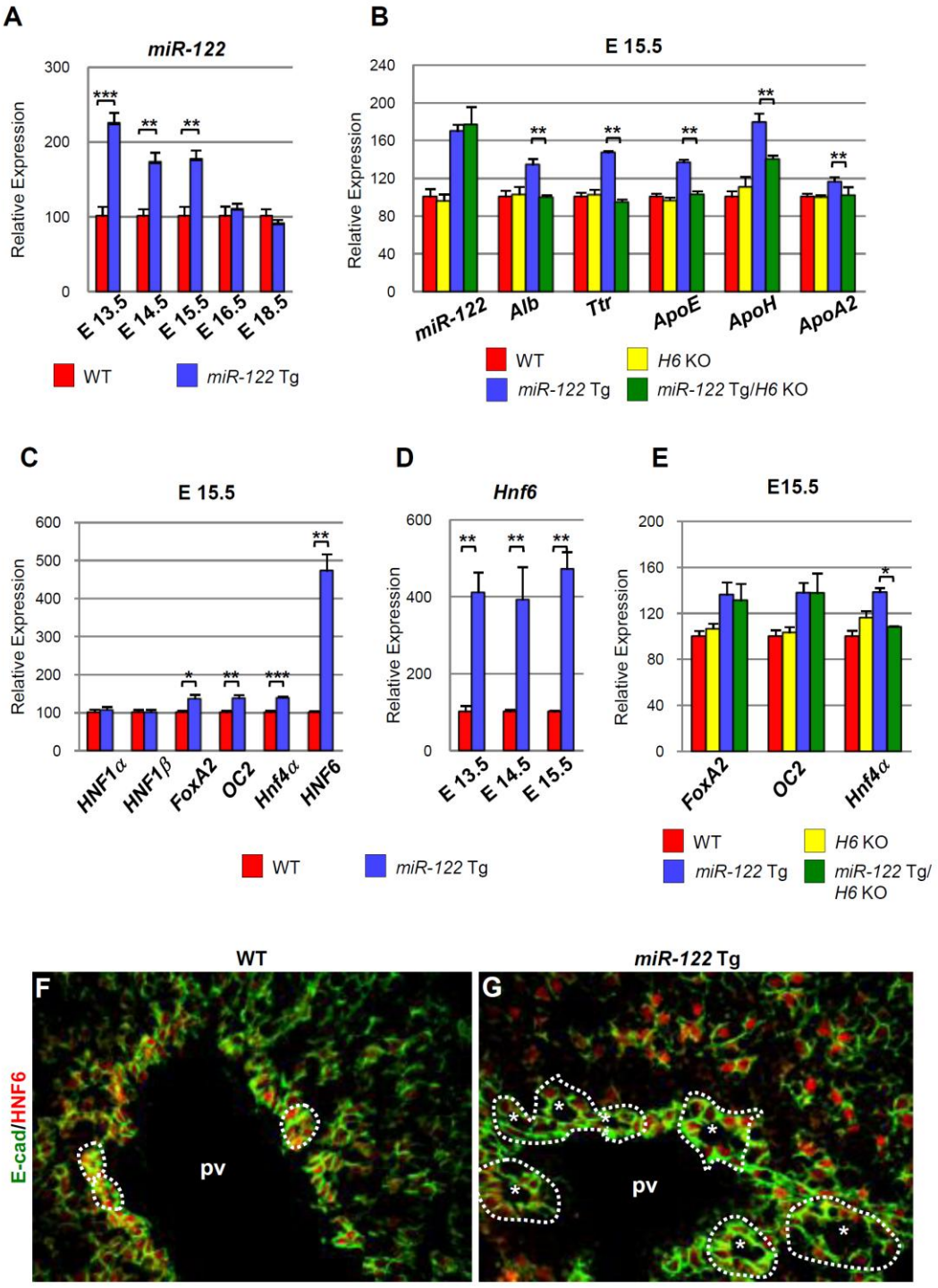


Figure 5. *In vivo* stimulation of hepatocyte differentiation by miR-122 is dependent on HNF6. *(A)* In livers from *miR-122* Tg mice, miR-122 was overexpressed from E13.5 to E15.5. *(B)* At E15.5 miR-122 overexpression stimulated *Alb*, *Ttr*, *ApoE*, *ApoH*, *ApoA2* in an *Hnf6*$^{+/+}$ background (*miR-122* Tg) but not in an *Hnf6*$^{-/-}$ background (*miR-122* Tg/*H6* KO). *(C)* Expression of the liver-enriched transcription factors *Foxa2*, *Oc2*, *Hnf4*α and *Hnf6*, but not *Hnf1*α and *Hnf1*β was stimulated in *miR-122* Tg at E15.5. *(D)* Stimulation of *Hnf6* expression in *miR-122* Tg livers was detected from E13.5 to E15.5. *(E)* Stimulation of *Hnf4*α, but not of *Foxa2* and *Oc2* in *miR-122* Tg embryos at E15.5 was dependent on HNF6. *(F-G)* Immunofluorescence analysis showing that HNF6 is prematurely expressed in hepatocytes of *miR-122* Tg livers at E15.5. Data in *(A-E)* are means ± SEM; n ≥ 4; *P < 0.05; **P < 0.01; ***P < 0.001. pv, portal vein; *, lumen of hyperplastic biliary structures; dotted lines delineate biliary structures.

Comparison of the *miR-122* Tg and *miR-122* Tg/*H6* KO embryos revealed that HNF6 is required for miR-122-induced stimulation of *Hnf4α*, but not of *Foxa2* and *Oc2* (Fig. 5E), indicating that the effects of miR-122 on LETF expression depend in part on HNF6. More important, the hepatocyte-specific genes stimulated by miR-122 (*miR-122* Tg) were no longer increased in the absence of HNF6 (*miR-122* Tg/*H6* KO; Fig. 5B). *ApoH* expression was still stimulated in *miR-122* Tg/*H6* KO embryos, but to a lesser extent than in *miR-122* Tg embryos. Therefore, we concluded that HNF6 is required for stimulation of hepatocyte differentiation by miR-122.

Overexpression of miR-122 induces HNF6-independent biliary differentiation

The study of *miR-122* Tg fetal livers also showed an unexpected biliary phenotype. *MiR-122* Tg embryos presented with biliary hyperplasia characterized by more numerous and larger ducts (Fig. 5F-G). Biliary hyperplasia in *miR-122* Tg mice did not result from proliferation (data not shown). To further characterize the biliary anomaly, we analyzed the expression of biliary differentiation and morphogenesis markers (Antoniou et al. 2009). Bile duct development normally starts in the ductal plate with the formation at E15.5 of asymmetrical ductal structures lined on the portal side by SOX9+/HNF4α- cells, and on the parenchymal side by SOX9-/HNF4α+ cells (Fig. 6A-A', I-I'). In *miR-122* Tg livers at E15.5 the biliary marker SOX9 was found on the portal side of developing ducts but it was also prematurely induced on their parenchymal side (Fig. 6B-B'), thereby mimicking later stages of duct morphogenesis. TGFβ receptor (TβR) II and HNF4α were expressed in cells on the parenchymal side of biliary structures in *miR-122* Tg mice, which is the normal location at that stage (Fig. 6E-E', F-F', I-I', J-J'). We concluded that overexpression of miR-122 induces biliary hyperplasia and acceleration of SOX9 expression in developing ducts.

We next determined if the effects of miR-122 on biliary development were HNF6-dependent, by comparing wild-type, *miR-122* Tg, *Hnf6*$^{-/-}$ (*H6* KO), and *miR-122* Tg/*H6* KO livers at E15.5. This comparison must take into account that the mere absence of HNF6 suffices to induce biliary anomalies (Clotman et al. 2002). Indeed, in *H6* KO livers biliary hyperplasia was detected. In these livers, the biliary structures did not show detectable SOX9 protein (Fig. 6C-C'). In addition, TβRII and HNF4α were detected on both the parenchymal and portal side of the *H6* KO biliary structures, unlike in wild-type developing ducts in which TβRII and HNF4α are found only on the parenchymal side (Fig. 6G-G', K-K'). Importantly, when miR-122 was overexpressed in a HNF6-null backrgound (*miR-122* Tg/*H6* KO), the phenotype did not

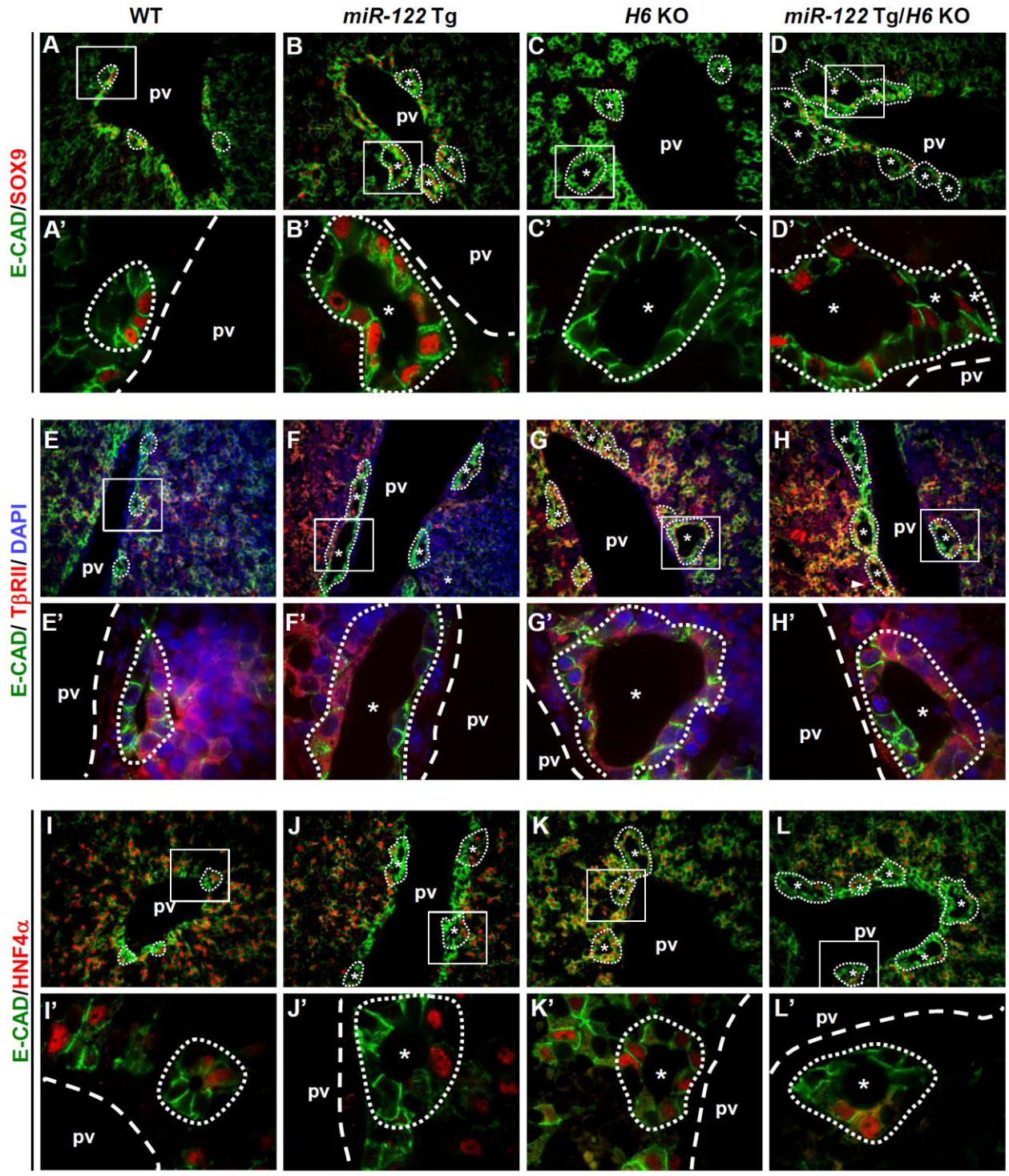


Figure 6. Abnormal biliary differentiation induced by miR-122 is not dependent on HNF6. At the onset of bile duct development in wild-type (WT) livers at E15.5, SOX9 is expressed on the portal side of ducts *(A-A')* and TβRII and HNF4α on their parenchymal side *(E-E', I-I')*. When miR-122 is overexpressed in *miR-122* Tg mice, SOX9 is prematurely induced on the parenchymal side of developing ducts *(B-B')*, whereas TβRII and HNF4α are normally expressed on their parenchymal side *(F-F', J-J')*. In *Hnf6*$^{-/-}$ mice (*H6* KO), SOX9 is not expressed *(C-C')*, and TβRII and HNF4α are found on the parenchymal side of ducts but also on their portal side *(G-G', K-K')*. The absence of HNF6 does not affect the biliary phenotype induced by overexpression of miR-122: *miR-122* Tg/*H6* KO livers show SOX9 expression on both the parenchymal and portal side of developing ducts *(D-D')*, and TβRII and HNF4α are found exclusively on their parenchymal side *(H-H', L-L')*. pv, portal vein; *, lumen of hyperplastic biliary structures; dotted lines delineate biliary structures.

significantly differ from that of livers overexpressing miR-122 in a *Hnf6*$^{+/+}$ background: biliary hyperplasia was still detected, most biliary cells expressed SOX9, and TβRII and HNF4α were expressed asymmetrically, *i.e.* on the parenchymal side of the biliary structures (Fig. 6D-D', H-H', L-L').

Both the absence of HNF6 and the overexpression of miR-122 induced biliary hyperplasia. Therefore, biliary hyperplasia in *miR-122* Tg/*H6* KO mice may result from the absence of HNF6, from overexpression of miR-122, or from both. Meanwhile, miR-122-induced premature expression of SOX9 is independent from HNF6.

Discussion

Cell differentiation is controlled both at the transcriptional level by sets of tissue-specific transcription factors, as well as at the post-transcriptional level by noncoding RNAs such as miRNAs. Recent studies have considered how these two levels of regulation can work together, and several interaction motifs have been described (Bartels et al. 1987; Tsang et al. 2007; Martinez et al. 2008; Herranz and Cohen 2010). Our data reveal a novel network topology in which a transcription factor directly regulates a miRNA which positively feedbacks on the transcription factor while stimulating gene expression in a way that is dependent on that same transcription factor (Fig. 7). In liver this network involves the tissue-specific factor HNF6 and the tissue-specific miR-122, and it drives hepatic cell differentiation during development.

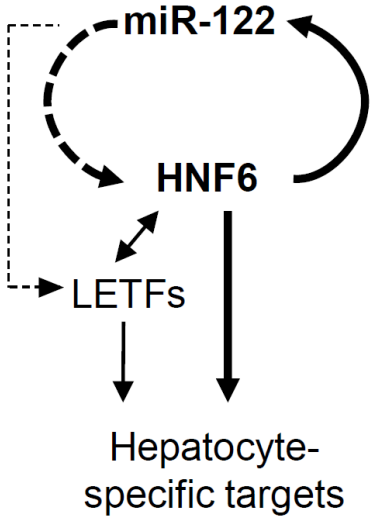


Figure 7. A positive feedback loop between HNF6 and miR-122 drives hepatocyte differentiation during development. HNF6 directly stimulates miR-122 gene expression, and the stimulation of HNF6 by miR-122 is direct or indirect. Both HNF6 and miR-122 stimulate the expression of liver-enriched transcription factors (LETFs), and HNF6 and LETFs co-regulate expression of hepatocyte-specific genes. Importantly the effect of miR-122 on hepatocyte differentiation is HNF6-dependent.

MiR-122 is stimulated by HNF6 and controls the network of core liver-enriched transcription factors

Gene expression in hepatocytes is controlled by a network of 6 core LETFs (HNF1α, HNF1β, FoxA2, HNF4α, HNF6 and Liver receptor homolog (LRH) 1), which are organized as autoregulatory and cross-regulatory loops (Beckh et al. 1987; Kyrmizi et al. 2006). The stability and complexity of this network increases during hepatocyte maturation, as a result from the progressive rise in concentration of the core transcription factors (Kyrmizi et al. 2006). In the present work we add a further layer of complexity by showing that HNF6 directly stimulates miR-122 expression and that miR-122 controls hepatocyte differentiation while stimulating the expression of core LETFs (*Foxa2*, *Hnf4α*, *Oc2* and *Hnf6*). HNF6 plays a major role in the miR-122-regulated LETF network. Among the LETFs it is the most strongly stimulated by miR-122, and the effects of miR-122 on hepatocyte differentiation are dependent on HNF6. HNF4α is another key regulator of hepatocyte differentiation (Parviz et al. 2003; Battle et al. 2006), and its expression is also dependent on miR-122 and HNF6, most likely via direct binding of HNF6 to the *Hnf4α* gene ((Beckh et al. 1987; Kyrmizi et al. 2006); Suppl. Fig. 3). Since HNF6 binds directly to the hepatocyte-specific genes upregulated by miR-122 (Suppl. fig. 3), we propose that miR-122 stimulates these genes via upregulation of HNF6.

Previous work has shown that miR-122 expression is regulated in part by C/EBPα, HNF1α, FoxA2, HNF4α, both *in vitro* in hepatocellular cancer-derived cells (Coulouarn et al. 2009; Xu et al. 2010; Zeng et al. 2010) and *in vivo* in adult liver. This raises the possibility that not only HNF6, but also other LETFs positively feedback on miR-122 expression.

Together, our data show that the topology of the positive feedback loop between HNF6 and miR-122 constitutes a core regulatory mechanisms which results in proper progression of hepatocyte differentiation, as shown by increased levels of hepatocyte-specific markers following overexpression of miR-122, or as shown by delayed hepatic differentiation in zebrafish morphants with downregulated miR-122 expression.

An unresolved question is how miR-122 stimulates *Hnf6* expression. Several scenarios were explored. MiRNAs can stimulate gene expression by binding to gene promoters (Place et al. 2008), or to mRNAs in their 5'UTR, 3'UTR or coding sequence (Vasudevan et al. 2007; Henke et al. 2008; Orom et al. 2008). However, using bioinformatic tools (TargetScan, Pictar, MicroCosm Target, MultAlin) no miR-122 site was found in the *Hnf6* gene or mRNA. Our analysis included the search for canonical, 3'compensatory and centered sites (Bartel 2009; Shin et al. 2010). Transient co-transfection of miR-122 mimics and luciferase reporter constructs driven by *Hnf6* regulatory regions (Poll et al. 2006) failed to reveal any effect of miR-122 in this

setting (data not shown). We envisaged that miR-122 represses miRNAs targeting *Hnf6*. However, we found that miR-9, miR-128, miR-218, and miR-383, which are predicted by TargetScan to inhibit HNF6 expression, were not inhibited by miR-122 (data not shown). MiR-122 could also inhibit a repressor protein of HNF6. Several negative regulators of transcription were recently predicted to be targeted by miR-122 (Tsang et al. 2010; Xu et al. 2010), but there is no evidence that they repress HNF6 during liver development. Finally, using Ingenuity Pathway Analysis, the search for a link between miR-122 and HNF6, HNF4α, FoxA2, and OC2, with exclusion of HNF1α and HNF1β, did not reveal any connection explaining how miR-122 might stimulate HNF6.

Accurate levels of miR-122 are required for normal hepatic cell differentiation

A small increase in miR-122 (1.7-fold) is sufficient to significantly perturb hepatic cell differentiation. Overexpression of miR-122 induces biliary hyperplasia, whereas no evidence was found that reduced levels of miR-122 perturb biliary development. The biliary hyperplasia does not depend on proliferation (data not shown), and is therefore likely to result from excessive differentiation of hepatoblasts toward the biliary lineage. Our overexpression data are not sufficient to claim that miR-122 is a physiological regulator of biliary development. However, they suggest that inaccurate levels of miR-122 can perturb hepatic cell specification.

Regulation of microRNAs by HNF6 and OC2

HNF6 and OC2 redundantly control the expression of several miRNAs during liver development. The two factors display 92% of amino acid identity in their DNA binding region, resulting in distinct but overlapping DNA binding sites (Jacquemin et al. 1999). Considering that Onecut factors are best known as transcriptional activators, miRNAs which are upregulated in the absence of HNF6 and OC2 are most likely indirectly regulated by the two transcription factors. MiR-200a is one of the upregulated miRNAs in *H6*/*O2* dKO. It controls cell-adhesion by inhibiting transcriptional repressors of E-cadherin (Gregory et al. 2008; Park et al. 2008). Upregulation of miR-200a may therefore contribute to the previously reported increase in E-cadherin expression in *H6*/*O2* dKO livers (Margagliotti et al. 2007).

MiR-122 stimulates hepatocyte differentiation by upregulating and downregulating gene expression

MiR-122 stimulates and represses expression of genes, in a direct or indirect way. Most stimulated genes code for hepatocyte-specific proteins. They are not predicted to be direct targets of miR-122 and we showed that they are indirectly regulated via HNF6. The repressed genes do not belong to the category of hepatocyte-specific genes, and amongst them only 3 (*Oxct1*, *Cdkn1a*, *Nfkbiz*) are predicted by TargetScan, PicTar or Microcosm Target to be direct miR-122 targets. Importantly, *Oxct1* repression by miR-122 contributes to hepatocyte differentiation. *Oxct1* is a housekeeping gene coding for 2-oxoacid CoA transferase, an enzyme that plays a key role in ketone body degradation. Since ketone bodies are synthesized in liver and provide an alternative source of energy to several tissues during fasting, *Oxct1* must be repressed in liver to avoid hepatic catabolism of ketone bodies. Our recent work provides evidence that *Oxct1* is repressed during hepatocyte maturation and that this repression depends in part on the rise in miR-122 (Thorrez et al. 2011).

We conclude that a positive feedback loop between HNF6 and miR-122 drives proper hepatocyte-specific gene expression. In this respect, monitoring or controlling the expression levels of HNF6 and miR-122 might help during programmed *in vitro* differentiation of stem cells toward hepatocytes for regenerative therapy of liver disease. In adult hepatocytes, maintaining the efficiency of this feedback loop may contribute to prevent dedifferentiation and malignant transformation.

Materials and Methods

Animals

Animals were raised and treated according to the principles of laboratory animal care of the University Animal Welfare Committee. *MiR-122* Tg mice were generated as described (Poll et al. 2006), with 2 ng/µl of purified Alfp/pre-miR-122 plasmid. HNF6 and OC2 knockout mice were as described (Jacquemin et al. 2000; Clotman et al. 2005).

Plasmid constructs

Alfp/pre-miR-122 plasmid was generated by inserting the genomic sequence coding for pre-miR-122 plus 50 bp flanking each side into the Alfp-pBluescript plasmid, containing the

Albumin promoter and enhancer and 3 *α-Fetoprotein* enhancers (kind gift from K. Kaestner). The miR-122 promoter/*firefly luciferase* (miR-122 prom/*Luc*) plasmid was obtained by cloning –300 to +327 nt sequence of miR-122 promoter into pGL3basic plasmid (Promega). The primers used to generate miR-122 prom/*Luc* were: 5'ACGCGTATCAGAGTCCTGAGAGAAAATG3' and 5'GTCGACATGTCTCTAGCCTTCCCCTT3'.

Cell culture, antagomir treatment and transfection

BMEL cells were described and cultured as described (Plumb-Rudewiez et al. 2004). Hepatocyte-like differentiation was obtained by growing the cells as floating aggregates (Strick-Marchand and Weiss 2002).

Antagomirs and antagomir treatment were as described (Thorrez et al. 2011b). MiR-122 was overexpressed in BMEL cells cultured in monolayer by transfecting 3 x 10^5 cells using Lipofectamin2000 (Invitrogen) and 0, 0.1, 0.25, 0.5 and 1 μg of miR-122 mimics (miRIDIAN Mimic-122, ThermoScientific). Total miR mimic amount was adjusted to 1μg with the miR-ctl mimic (miRIDIAN Mimic-ctl#1, ThermoScientific). RNA was extracted 48h after transfection.

To study the miR-122 gene promoter 3 x 10^5 BMEL cells in monolayer were transfected using Lipofectamin2000 (Invitrogen) with 1 μg of pCMV-GFP or pCMV-HNF6, and 1 μg of miR-122 prom/*Luc*. Forty-eight hours after transfection RNA was extracted and luciferase activity was measured using the Dual-Luciferase reporter assay system (Promega), using a CMV-driven *renilla luciferase*-expressing vector for normalization.

Microarray analysis of miRNA expression

Livers from wild-type or *H6*/*O2* dKO embryos at E15.5 were stored in RNAlater Solution (Ambion) at 4°C overnight and then at -80°C. Total RNA was isolated using TriPure (Roche) and cleaned using the RNeasy MinElute Cleanup Kit (Qiagen). RNA quality was assayed on an Agilent 2100 BioAnalyser. Two μg of RNA were labeled and hybridized to the miRCURY™ LNA (Locked nucleic acid) Array v8.1 by miRCURY™ LNA Array microRNA Profiling Services (Exiqon). Three biological samples of *H6*/*O2* dKO and WT livers labeled with Hy3 were analyzed against a common reference labeled with Hy5, and consisting of a pool of all samples.

Microarray analysis of mRNA expression

Total RNA was isolated from BMEL cells cultured in aggregates and treated with antagomir-122 or antagomir-mut, by using TriPure (Roche) and cleaned using the RNeasy Mini Kit (Qiagen).

RNA quality was assayed on an Agilent 2100 BioAnalyser. Two μg of RNA were labeled and hybridized to GeneChip® Mouse Genome 430A 2.0 Array (Affymetrix), following the manufacturer's protocol. Two biological samples of BMEL Agg antagomir-122 and of BMEL Agg antagomir-mut were analyzed. All genes found upregulated or downregulated in each BMEL Agg antagomir-122/BMEL Agg antagomir-mut pairs were selected and median fold change was calculated.

Reverse transcription (RT) and real-time quantitative PCR (Q-PCR)

Q-RT-PCR analysis of miRNAs and mRNAs was as described (Thorrez et al. 2011). A specific stem-loop primer was used for RT of miRNAs, and Q-PCR was performed using a specific forward primer and a common universal reverse primer (URP). Primer sequences are provided in Supplementary Tables 1 and 2. QPCR was performed with KAPA SYBR® FAST Bio-Rad iCycler™ qPCR Master Mix (Kapa Biosystems) on a IQ cycler (Bio-Rad). Quantification of mRNAs and miRNAs was normalized to β-actin and 18S RNA, respectively.

Morpholino injection in zebrafish embryos

Wild type AB adult fish and embryos were raised and maintained under standard conditions. Knock down of dre-miR-122 expression was performed with the morpholino oligonucleotide 5'-ATACAAACACCATTGTCACACTCCA-3' (Gene Tools, LCC). Two ng per embryo were injected at the 1-cell stage with 0.25% rhodamine dextran (Invitrogen).

Whole mount in situ hybridization and immunofluorescence analysis of zebrafish embryos

Colorimetric whole mount in situ hybridizations were performed with DIG-labeled dre-miR-122 RNA probe (Exiqon) (Manfroid et al. 2007). Whole mount immunohistochemistry was as described (Dong et al. 2007), using the following antibodies: polyclonal rabbit anti-Prox1 (1:1000, Chemicon), polyclonal goat anti-HNF4α (1:100, Santa Cruz), monoclonal mouse anti-2F11 (1:1000, kind gift from J. Lewis (Crosnier et al. 2005)) and fluorescently conjugated Alexa antibodies (Molecular Probes). Fluorescent images were captured with a Leica SP2 confocal microscope.

Immunofluorescence analysis of mouse embryos

Embryos were fixed at 4°C for 4 hours in 4% paraformaldehyde in phosphate-buffered saline (PBS), washed overnight in PBS, and embedded in paraffin. Immunofluorescence on 9 μm sections was as described (van Eyll et al. 2004) using anti-E-cadherin (1:500, 610182, BD

Biosciences), anti-HNF4α (1:50, sc-6556, Santa Cruz), anti-SOX9 (1:500, Chemicon), anti-HNF6 (1:50, sc-13050, Santa Cruz) and anti-TβRII (1:500, AB53168, Abcam) primary antibodies. Secondary antibodies were anti-goat/Alexafluor 594 (1:2000), anti-mouse/Alexafluor 488 (1:1000), and anti-rabbit/Alexafluor 594 (1:2500) (Invitrogen). Fluorescent images were captured with a Cell Observer Spinning Disk (Zeiss) confocal microscope.

Chromatin Immunoprecipitation sequencing

ChIP experiments were performed with an antibody against HNF6 (sc-13050, Santa Cruz) as described (Schmidt et al. 2009) on adult liver. Briefly, the immunoprecipitated material was end-repaired, A-tailed, ligated to the sequencing adapters, amplified by 18-cycles of PCR and size selected (200 - 300 bp) followed by single end sequencing on an Illumina Genome Analyzer according to the manufacturers recommendations. All sequencing reads were aligned using MAQ (Li et al. 2008) with default parameters to the mouse NCBI37/mm9 genome assembly. ChIP-seq data are displayed with the help of the UCSC Human Genome Browser (Christ and Jungermann 1987). Data are available on the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) (ArrayExpress accession: E-MTAB-438). The HNF6 motif was determined using MEME (Iwai and Jungermann 1987), with the default parameters. We selected the top 200 peaks ordered by SWEMBL score and used 24 bases around the peak summits as input for the motif discovery.

Bioinformatics

Prediction of miR-122 targets was performed by using TargetScan mouse 5.0 (Puschel et al. 1987), PicTar (Jungermann 1987) and MicroCosm Targets Version 5 (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/). The sequence of mature miR-122 was aligned to *Hnf6* gene using MultAlin (Hartmann et al. 1987) to predict target sequences for miR-122. Ingenuity Pathway Analysis was used to search for a molecular connections between miR-122 and HNF6, HNF4α, FoxA2, and OC2, with exclusion of all connections involving HNF1α and HNF1β.

Acknowledgments

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Supplementary Table 1: List of stem-loop primers used for reverse transcription of miRNAs

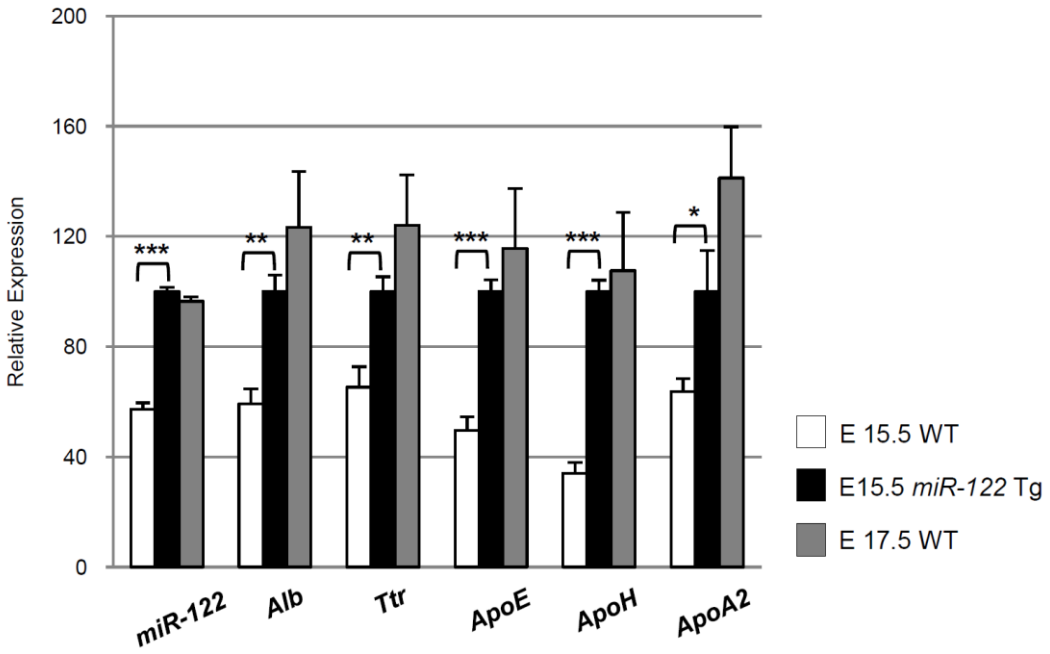
miRNA	*RT primer sequence (5'->3')*
miR-122	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACAAACAC
miR-107	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTGATAGCC
miR-200a	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACATCGTT
miR-329	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAAAAAGGT
miR-337	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAAAGGCAT
miR-20	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCTACCTGC

Supplementary Table 2: List of primers used for Q-PCR

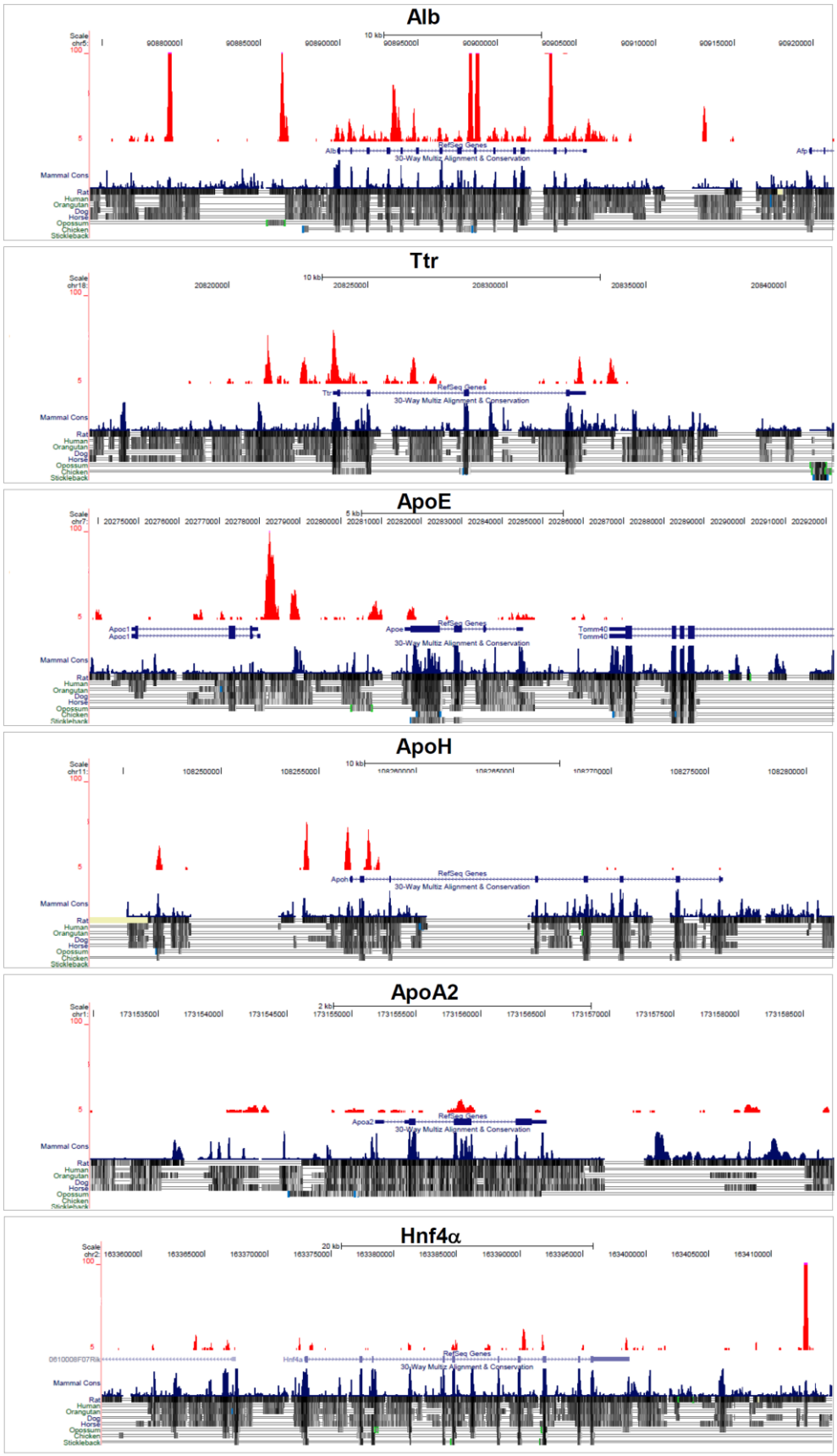
Gene	*Primer sequence (5'->3')*
18S	Fw GGCGCCCCCTCGATGCTCTTAG Rev GCTCGGGCCTGCTTTGAACACTCT
miR-122	Fw ACACTCCAGCTGGGTGGAGTGTGACAATGGT
miR-107	Fw ACACTCCAGCTGGGAGCAGCATTGTACAGGG
miR-200a	Fw ACACTCCAGCTGGGTAACACTGTCTGGTAA
miR-329	Fw ACACTCCAGCTGGGAACACACCCAGCTAAC
miR-337	Fw ACACTCCAGCTGGGTTCAGCTCCTATATGAT
miR-20	Fw ACACTCCAGCTGGGTAAAGTGCTTATAGTGC
URP	Rev GGTGTCGTGGAGTCGGCAA
β-actin	Fw TCCTGAGCGCAAGTACTCTGT Rev CTGATCCACATCTGCTGGAAG
Alb	Fw GCTGAGACCTTCACCTTCCA Rev TCTTCAGTTGCTCCGCTGTA
Ttr	Fw GGGCTCACCACAGATGAGAA Rev CAGAGTCGTTGGCTGTGAAA
ApoE	Fw ACAATTGCGAAGATGAAGGC Rev CCACTCGAGCTGATCTGTCA
ApoH	Fw TGTCCTTTCGCTGGAATCTT Rev GCTGGTCCCATTCAGAAAAA
ApoA2	Fw TTGATGGAGAAGGCCAAGAC Rev CGGTTTCTCCTCAAGGTTCA
HNF1α	Fw GGGCTTCACGAGCCACCCTCT Rev TGCTGGAGGACACTGTGGGACTG
HNF1β	Fw GAAAGCAACGGGAGATCCTC Rev GACTGCCCAGGCCCCTGGTTCTGT
FoxA2	Fw ATCCGACTGGAGCAGCTACTACG Rev CATGTATGTGTTCATGCCATTC
OC2	Fw GCGGGATTTCTTCTGCGAG Rev GCATGTCTGCCTTACGCCTG
HNF4α	Fw AGCTCGAGGCTCCGTAGTGTTT Rev GAAAATGTGCAGGTGTTGACCA
HNF6	Fw TTCCAGCGCATGTCGGCGCTC Rev GGTACTAGTCCGTGGTTCTTC

BMEL Agg antagomir-122/ antagomir-mut (Fold change)	Gene Symbol	Gene Title
3.19	**Saa3**	*serum amyloid A 3*
2.93	**Lcn2**	*lipocalin 2*
2.83	**Clca4**	*chloride channel calcium activated 4*
1.83	**Cebpd**	*CCAAT/enhancer binding protein (C/EBP), delta*
1.60	**Tnfaip2**	*tumor necrosis factor, alpha-induced protein 2*
1.57	**Ccl20**	*chemokine (C-C motif) ligand 20*
1.52	**Idi1**	*isopentenyl-diphosphate delta isomerase*
1.52	**Mmp13**	*matrix metallopeptidase 13*
1.49	**Klhl2**	*kelch-like 2, Mayven (Drosophila)*
1.44	**Vnn3**	*vanin 3*
1.44	**Spink4**	*serine peptidase inhibitor, Kazal type 4*
1.41	**Zc3h12a**	*zinc finger CCCH type containing 12A*
1.39	**Oxct1**	*3-oxoacid CoA transferase 1*
1.37	**Nfkbiz**	*nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta*
1.37	**Nfkbia**	*nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor.,alpha*
1.32	**Hk2**	*hexokinase 2*
1.27	**Cdkn1a**	*cyclin-dependent kinase inhibitor 1A (P21)*
1.27	**Acat2**	*acetyl-Coenzyme A acetyltransferase 2*
1.27	**Pmaip1**	*phorbol-12-myristate-13-acetate-induced protein 1*
1.25	**Ctsc**	*cathepsin C*
1.21	**Dmbt1**	*deleted in malignant brain tumors 1*
1.19	**Abcc3**	*ATP-binding cassette, sub-family C (CFTR/MRP), member 3*
0.51	**Apoe**	*apolipoprotein E*
0.53	**Apoa2**	*apolipoprotein A-II*
0.54	**Alb**	*albumin*
0.55	**Apoh**	*apolipoprotein H*
0.59	**Itih2**	*inter-alpha trypsin inhibitor, heavy chain 2*
0.64	**Afm**	*afamin*
0.64	**Fgb**	*fibrinogen. B beta polypeptide*
0.65	**Itih4**	*inter alpha-trypsin inhibitor, heavy chain 4*
0.66	**Apoc1**	*apolipoprotein C-I*
0.67	**Pzp**	*pregnancy zone protein*
0.71	**Afp**	*alpha fetoprotein*
0.71	**Tff3**	*trefoil factor 3, intestinal*
0.71	**Apoa1**	*apolipoprotein A-I*
0.71	**Acat1**	*acetyl-Coenzyme A acetyltransferase 1*
0.71	**H19**	*H19 fetal liver mRNA*
0.71	**Gkn2**	*gastrokine 2*
0.72	**Fga**	*fibrinogen, alpha polypeptide*
0.73	**Ttr**	*transthyretin*
0.73	**Kng1**	*kininogen 1*
0.73	**Rdh7**	*retinol dehydrogenase 7*
0.74	**Gc**	*group specific component*
0.74	**Tff1**	*trefoil factor 1 /// similar to pS2m*
0.77	**Spp2**	*secreted phosphoprotein 2*
0.78	**Trf**	*transferrin*
0.78	**Gstm1**	*glutathione S-transferase, mu 1*
0.78	**F10**	*coagulation factor X*

Supplementary Figure 1. List of genes differentially expressed in BMEL cells treated with antagomir-122 (BMEL Agg antagomir-122) or with antagomir-mutated (BMEL Agg antagomir-mut). Microarray result showed that miR-122 inhibition in BMEL cells differentiating to hepatocytes is associated with stimulation of 22 mRNAs and repression of 26 mRNAs.



Supplementary Figure 2. MiR-122 overexpression accelerates hepatocyte differentiation *in vivo*. In *miR-122* Tg livers at E15.5 the expression of *miR-122*, *Alb*, *Ttr*, *ApoE*, *ApoA2* and *ApoH* was increased as compared to WT livers at E15.5 and reached levels comparable to WT livers at E17.5. Data are means ± SEM; n ≥ 3; *P < 0.05; **P < 0.01;**P < 0.001.



Supplementary Figure 3. HNF6 binds to hepatocyte-specific genes whose expression is stimulated by miR-122. Chip-Seq data showed that HNF6 binds to the *Alb*, *Ttr*, *ApoE*, *ApoA2*, *ApoH* and *HNF4α* loci *in vivo* in adult liver. Data are displayed with the help of the UCSC Genome Browser (NCBI37/mm9).

References

Alon U. 2007. Network motifs: theory and experimental approaches. *Nat Rev Genet* **8**: 450-461.

Antoniou A, Raynaud P, Cordi S, Zong Y, Tronche F, Stanger B, Jacquemin P, Pierreux CE, Clotman F, Lemaigre FP. 2009. Intrahepatic Bile Ducts Develop According to a New Mode of Tubulogenesis Regulated by the Transcription Factor SOX9. *Gastroenterology* **136**: 2325-2333.

Bailey TL, Elkan C. 1994. Fitting a mixture model by expectation maximization to discover motifs in biopolymers. *Proc Int Conf Intell Syst Mol Biol* **2**: 28-36.

Bartel DP. 2009. MicroRNAs: target recognition and regulatory functions. *Cell* **136**: 215-233.

Battle MA, Konopka G, Parviz F, Gaggl AL, Yang C, Sladek FM, Duncan SA. 2006. Hepatocyte nuclear factor 4alpha orchestrates expression of cell adhesion proteins during the epithelial transformation of the developing liver. *Proc Natl Acad Sci U S A* **103**: 8419-8424.

Beaudry JB, Pierreux CE, Hayhurst GP, Plumb-Rudewiez N, Weiss MC, Rousseau GG, Lemaigre FP. 2006. Threshold levels of hepatocyte nuclear factor 6 (HNF-6) acting in synergy with HNF-4 and PGC-1alpha are required for time-specific gene expression during liver development. *Mol Cell Biol* **26**: 6037-6046.

Chang J, Nicolas E, Marks D, Sander C, Lerro A, Buendia MA, Xu C, Mason WS, Moloshok T, Bort R et al. 2004. miR-122, a mammalian liver-specific microRNA, is processed from hcr mRNA and may downregulate the high affinity cationic amino acid transporter CAT-1. *RNA Biol* **1**: 106-113.

Clotman F, Jacquemin P, Plumb-Rudewiez N, Pierreux CE, Van der Smissen P, Dietz HC, Courtoy PJ, Rousseau GG, Lemaigre FP. 2005. Control of liver cell fate decision by a gradient of TGF beta signaling modulated by Onecut transcription factors. *Genes Dev* **19**: 1849-1854.

Clotman F, Lannoy VJ, Reber M, Cereghini S, Cassiman D, Jacquemin P, Roskams T, Rousseau GG, Lemaigre FP. 2002. The onecut transcription factor HNF6 is required for normal development of the biliary tract. *Development* **129**: 1819-1828.

Coulouarn C, Factor VM, Andersen JB, Durkin ME, Thorgeirsson SS. 2009. Loss of miR-122 expression in liver cancer correlates with suppression of the hepatic phenotype and gain of metastatic properties. *Oncogene* **28**: 3526-3536.

Crosnier C, Vargesson N, Gschmeissner S, Ariza-McNaughton L, Morrison A, Lewis J. 2005. Delta-Notch signalling controls commitment to a secretory fate in the zebrafish intestine. *Development* **132**: 1093-1104.

Dong PD, Munson CA, Norton W, Crosnier C, Pan X, Gong Z, Neumann CJ, Stainier DY. 2007. Fgf10 regulates hepatopancreatic ductal system patterning and differentiation. *Nat Genet* **39**: 397-402.

Gao Y, Schug J, McKenna LB, Le Lay J, Kaestner KH, Greenbaum LE. 2011. Tissue-specific regulation of mouse MicroRNA genes in endoderm-derived tissues. *Nucleic Acids Res*. in press

Gatfield D, Le Martelot G, Vejnar CE, Gerlach D, Schaad O, Fleury-Olela F, Ruskeepaa AL, Oresic M, Esau CC, Zdobnov EM et al. 2009. Integration of microRNA miR-122 in hepatic circadian gene expression. *Genes Dev* **23**: 1313-1326.

Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G, Vadas MA, Khew-Goodall Y, Goodall GJ. 2008. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat Cell Biol* **10**: 593-601.

Hand NJ, Master ZR, Eauclaire SF, Weinblatt DE, Matthews RP, Friedman JR. 2009a. The microRNA-30 family is required for vertebrate hepatobiliary development. *Gastroenterology* **136**: 1081-1090.

Hand NJ, Master ZR, Le Lay J, Friedman JR. 2009b. Hepatic function is preserved in the absence of mature microRNAs. *Hepatology* **49**: 618-626.

Henke JI, Goergen D, Zheng J, Song Y, Schuttler CG, Fehr C, Junemann C, Niepmann M. 2008. microRNA-122 stimulates translation of hepatitis C virus RNA. *EMBO J* **27**: 3300-3310.

Herranz H, Cohen SM. 2010. MicroRNAs and gene regulatory networks: managing the impact of noise in biological systems. *Genes Dev* **24**: 1339-1344.

Jacquemin P, Durviaux SM, Jensen J, Godfraind C, Gradwohl G, Guillemot F, Madsen OD, Carmeliet P, Dewerchin M, Collen D et al. 2000. Transcription factor hepatocyte nuclear factor 6 regulates pancreatic endocrine cell differentiation and controls expression of the proendocrine gene ngn3. *Mol Cell Biol* **20**: 4445-4454.

Jacquemin P, Lannoy VJ, Rousseau GG, Lemaigre FP. 1999. OC-2, a novel mammalian member of the ONECUT class of homeodomain transcription factors whose function in liver partially overlaps with that of hepatocyte nuclear factor-6. *J Biol Chem* **274**: 2665-2671.

Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH, Zahler AM, Haussler D. 2002. The human genome browser at UCSC. *Genome Res* **12**: 996-1006.

Kyrmizi I, Hatzis P, Katrakili N, Tronche F, Gonzalez FJ, Talianidis I. 2006. Plasticity and expanding complexity of the hepatic transcription factor network during liver development. *Genes Dev* **20**: 2293-2305.

Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. 2002. Identification of tissue-specific microRNAs from mouse. *Curr Biol* **12**: 735-739.

Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A, Pfeffer S, Rice A, Kamphorst AO, Landthaler M et al. 2007. A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell* **129**: 1401-1414.

Lemaigre FP. 2009. Mechanisms of liver development: concepts for understanding liver disorders and design of novel therapies. *Gastroenterology* **137**: 62-79.

Li H, Ruan J, Durbin R. 2008. Mapping short DNA sequencing reads and calling variants using mapping quality scores. *Genome Res* **18**: 1851-1858.

Makeyev EV, Maniatis T. 2008. Multilevel regulation of gene expression by microRNAs. *Science* **319**: 1789-1790.

Manfroid I, Delporte F, Baudhuin A, Motte P, Neumann CJ, Voz ML, Martial JA, Peers B. 2007. Reciprocal endoderm-mesoderm interactions mediated by fgf24 and fgf10 govern pancreas development. *Development* **134**: 4011-4021.

Margagliotti S, Clotman F, Pierreux CE, Beaudry JB, Jacquemin P, Rousseau GG, Lemaigre FP. 2007. The Onecut transcription factors HNF-6/OC-1 and OC-2 regulate early liver expansion by controlling hepatoblast migration. *Dev Biol* **311**: 579-589.

Martinez NJ, Ow MC, Barrasa MI, Hammell M, Sequerra R, Doucette-Stamm L, Roth FP, Ambros VR, Walhout AJ. 2008. A C. elegans genome-scale microRNA network contains composite feedback motifs with high flux capacity. *Genes Dev* **22**: 2535-2549.

Odom DT, Dowell RD, Jacobsen ES, Nekludova L, Rolfe PA, Danford TW, Gifford DK, Fraenkel E, Bell GI, Young RA. 2006. Core transcriptional regulatory circuitry in human hepatocytes. *Mol Syst Biol* **2**: 2006 0017.

Orom UA, Nielsen FC, Lund AH. 2008. MicroRNA-10a binds the 5'UTR of ribosomal protein mRNAs and enhances their translation. *Mol Cell* **30**: 460-471.

Park SM, Gaur AB, Lengyel E, Peter ME. 2008. The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. *Genes Dev* **22**: 894-907.

Parviz F, Matullo C, Garrison WD, Savatski L, Adamson JW, Ning G, Kaestner KH, Rossi JM, Zaret KS, Duncan SA. 2003. Hepatocyte nuclear factor 4alpha controls the development of a hepatic epithelium and liver morphogenesis. *Nat Genet* **34**: 292-296.

Place RF, Li LC, Pookot D, Noonan EJ, Dahiya R. 2008. MicroRNA-373 induces expression of genes with complementary promoter sequences. *Proc Natl Acad Sci U S A* **105**: 1608-1613.

Plumb-Rudewiez N, Clotman F, Strick-Marchand H, Pierreux CE, Weiss MC, Rousseau GG, Lemaigre FP. 2004. Transcription factor HNF-6/OC-1 inhibits the stimulation of the HNF-3alpha/Foxa1 gene by TGF-beta in mouse liver. *Hepatology* **40**: 1266-1274.

Poll AV, Pierreux CE, Lokmane L, Haumaitre C, Achouri Y, Jacquemin P, Rousseau GG, Cereghini S, Lemaigre FP. 2006. A vHNF1/TCF2-HNF6 cascade regulates the transcription factor network that controls generation of pancreatic precursor cells. *Diabetes* **55**: 61-69.

Raynaud P, Carpentier R, Antoniou A, Lemaigre FP. 2010. Biliary differentiation and bile duct morphogenesis in development and disease. *Int J Biochem Cell Biol*. in press

Rogler CE, Levoci L, Ader T, Massimi A, Tchaikovskaya T, Norel R, Rogler LE. 2009. MicroRNA-23b cluster microRNAs regulate transforming growth factor-beta/bone morphogenetic protein signaling and liver stem cell differentiation by targeting Smads. *Hepatology* **50**: 575-584.

Schmidt D, Wilson MD, Spyrou C, Brown GD, Hadfield J, Odom DT. 2009. ChIP-seq: using high-throughput sequencing to discover protein-DNA interactions. *Methods* **48**: 240-248.

Sekine S, Ogawa R, Ito R, Hiraoka N, McManus MT, Kanai Y, Hebrok M. 2009a. Disruption of Dicer1 induces dysregulated fetal gene expression and promotes hepatocarcinogenesis. *Gastroenterology* **136**: 2304-2315.

Sekine S, Ogawa R, McManus MT, Kanai Y, Hebrok M. 2009b. Dicer is required for proper liver zonation. *J Pathol* **219**: 365-372.

Shin C, Nam JW, Farh KK, Chiang HR, Shkumatava A, Bartel DP. 2010. Expanding the microRNA targeting code: functional sites with centered pairing. *Mol Cell* **38**: 789-802.

Shoval O, Alon U. 2010. SnapShot: network motifs. *Cell* **143**: 326-e321.

Si-Tayeb K, Lemaigre FP, Duncan SA. 2010. Organogenesis and development of the liver. *Dev Cell* **18**: 175-189.

Strick-Marchand H, Weiss MC. 2002. Inducible differentiation and morphogenesis of bipotential liver cell lines from wild-type mouse embryos. *Hepatology* **36**: 794-804.

Thorrez L, Laudadio I, Van Deun K, Quintens R, Hendrickx N, Granvik M, Lemaire K, Schraenen A, Van Lommel L, Lehnert S et al. 2011b. Tissue-specific disallowance of housekeeping genes: the other face of cell differentiation. *Genome Res* **21**: 95-105.

Tsang J, Zhu J, van Oudenaarden A. 2007. MicroRNA-mediated feedback and feedforward loops are recurrent network motifs in mammals. *Mol Cell* **26**: 753-767.

Tsang JS, Ebert MS, van Oudenaarden A. 2010. Genome-wide dissection of microRNA functions and cotargeting networks using gene set signatures. *Mol Cell* **38**: 140-153.

Tuteja G, White P, Schug J, Kaestner KH. 2009. Extracting transcription factor targets from ChIP-Seq data. *Nucleic Acids Res* **37**: e113.

van Eyll JM, Pierreux CE, Lemaigre FP, Rousseau GG. 2004. Shh-dependent differentiation of intestinal tissue from embryonic pancreas by activin A. *J Cell Sci* **117**: 2077-2086.

Vasudevan S, Tong Y, Steitz JA. 2007. Switching from repression to activation: microRNAs can up-regulate translation. *Science* **318**: 1931-1934.

Wienholds E, Kloosterman WP, Miska E, Alvarez-Saavedra E, Berezikov E, de Bruijn E, Horvitz HR, Kauppinen S, Plasterk RH. 2005. MicroRNA expression in zebrafish embryonic development. *Science* **309**: 310-311.

Xu H, He JH, Xiao ZD, Zhang QQ, Chen YQ, Zhou H, Qu LH. 2010. Liver-enriched transcription factors regulate microRNA-122 that targets CUTL1 during liver development. *Hepatology* **52**: 1431-1442.

Zeng C, Wang R, Li D, Lin XJ, Wei QK, Yuan Y, Wang Q, Chen W, Zhuang SM. 2010. A novel GSK-3 beta-C/EBP alpha-miR-122-insulin-like growth factor 1 receptor regulatory circuitry in human hepatocellular carcinoma. *Hepatology* **52**: 1702-1712.

Zhao R, Duncan SA. 2005. Embryonic development of the liver. *Hepatology* **41**: 956-967.

3.2. MiR-122 participates to liver-specific disallowance of OXCT1

3.2.1. Introduction

In the previous chapter we showed that miR-122 stimulates hepatocyte differentiation during mouse development, by indirectly enhancing expression of genes that are specific to mature hepatic parenchymal cells (Jochheim et al. 2003; Petkov et al. 2004).

MiRNAs are mainly known as post-transcriptional repressors (Bushati and Cohen 2007; Bartel 2009). Therefore, we looked for genes that are repressed by miR-122 and whose expression needs to be downregulated to allow normal hepatocyte function. Amongst the genes identified in our experiments with BMEL cells in which miR-122 activity was inhibited, we identified 2-oxoacid CoA transferase (*Oxct1*), a housekeeping gene coding for an enzyme involved in ketone body metabolism (§ *3.1 Control of liver differentiation by a positive feedback loop between a transcription factor and a tissue-specific microRNA*; suppl. fig. 1). The expression of this gene was enhanced by inhibition of miR-122, indicating that it is negatively regulated by miR-122. The production of ketone bodies (ketogenesis) takes place in hepatocytes and requires the activity of the 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS) 2 enzyme. Conversely catabolism of ketone bodies provides an alternative energy source to many tissues during fasting, in particular to brain and muscles, and is dependent on OXCT1 (Cahill and Veech 2003) (Fig. 1A). *Oxct1* expression is specifically lower in adult mouse liver as compared to other tissues and this repression mirrors the expression of *Hmgcs2* (Fig. 1B). Therefore OXCT1 is specifically “disallowed” in liver and this is necessary to avoid hepatic catabolism of ketone bodies.

In an effort to understand the mechanisms which drive OXCT1 disallowance in liver, we investigated how miR-122 participates to OXCT1 repression during hepatocyte differentiation.

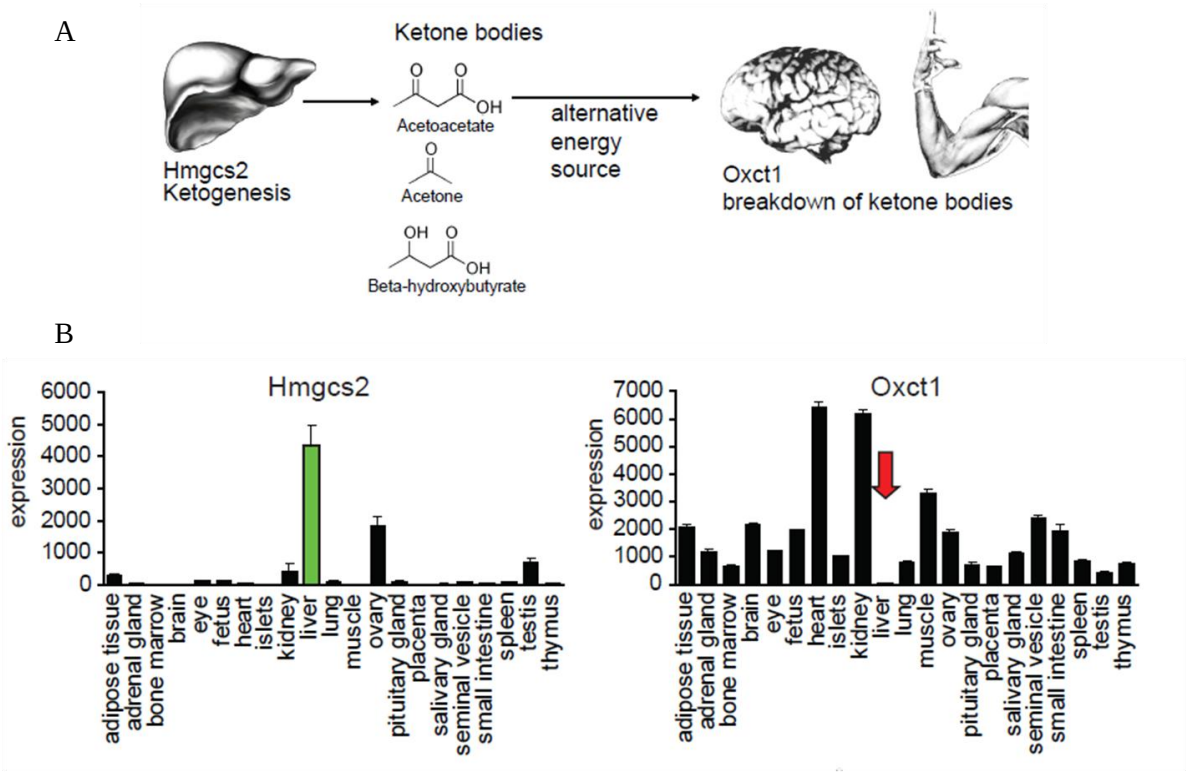


Figure 1. Liver-specific disallowance of OXCT1 (*from Thorrez et al. 2011).* (A) Schematic representation of ketone body metabolism. (B) Microarray expression profiles of 3-hydroxy 3-methylglutaryl-CoA synthase 2 (Hmgcs2) versus 2-oxoacid CoA transferase (Oxct1), which are, respectively, highly expressed and deeply repressed in adult liver.

3.2.2. Results and discussion

To analyzed whether tissue-specific disallowance of *Oxct1* was linked to liver development, we studied the expression profile of *Oxct1* and *Hmgcs2* at several developmental and adult stages. In mouse embryonic and neonatal liver, we observed progressive loss of expression of *Oxct1* and a parallel pronounced upregulation of *Hmgcs2* (Fig. 2). This suggests that during differentiation cell-specific genes are turned on while repressed housekeeping genes are turned off and that specific repression of *Oxct1* is a peculiar feature of hepatocyte differentiation and maturation.

In the previous chapter we showed that miR-122, which is specifically expressed in mouse liver and which is the most abundant liver microRNA species (Lagos-Quintana et al. 2002; Landgraf et al. 2007), enhances hepatocyte differentiation. By Q-RT-PCR we now show that its expression increases 12-fold between E15.5 and birth (Fig. 3A), and this parallels *Oxct1* repression (Fig. 2B). Moreover by analyzing the 3'UTR of mouse *Oxct1* mRNA by TargetScan Mouse 5.0 (http://www.targetscan.org/) we found a predicted target sequence for miR-122 (Fig.

3B). Taken together these data rise the hypothesis that miR-122 represses *Oxct1* mRNA during liver development.

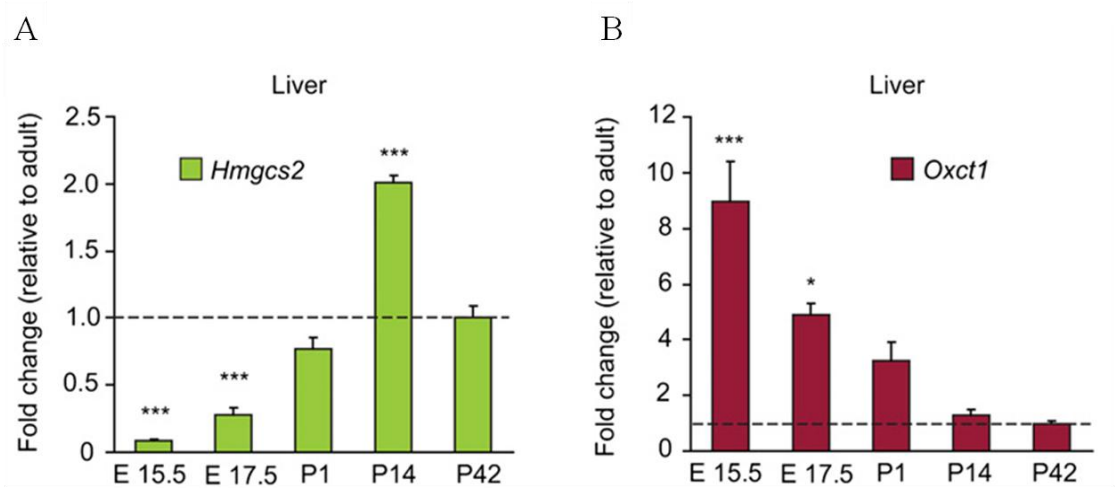


Figure 2. Tissue-specific gene expression and repression of housekeeping genes during mouse liver development (*from Thorrez et al. 2011).* mRNA expression level in extracts from mouse fetal and neonatal liver between E15.5 and 42 days after birth. A progressive up-regulation of Hmgcs2 (A) mirrors a progressive loss of expression of Oxct1 (B). Data are means ± SEM; n=3; *P < 0.05; **P < 0.01; ***P < 0.001

In order to verify this hypothesis we first reassessed in detail the effect of miR-122 antagomir on *Oxct1* expression in our *in vitro* model of hepatocyte differentiation, namely aggregate-cultured BMEL cells (see also § *3.1.*; Strick-Marchand and Weiss 2002). During hepatocyte-like differentiation of cultured BMEL cells, miR-122 expression increased and it could be specifically inhibited by treatment with miR-122 antagomir (α-miR-122; as shown previously (§ *3.1*)). Cells cultured under the same conditions but treated with a mutated antagomir (α-miR-122 mut) did not show decreased levels of miR-122 (Fig. 3C, left panel). In aggregate-cultured BMEL cells, *Oxct1* expression increased 1.6-fold by adding miR-122 antagomir (α-miR-122), while the mutated control antagomir (α-miR-122 mut) did not alter *Oxct1* expression (Fig. 3C, right panel).

To verify if miR-122 represses OXCT1 expression *in vivo*, we compared RNA and protein levels in wild-type (WT) and miR-122 transgenic (TG) mouse livers at E15.5. These mice are described in § *3.1*. Overexpression of miR-122 was associated with repression of OXCT1 RNA and protein, as determined by Q-RT-PCR and Western blotting (Fig. 3D). Therefore, we concluded that miR-122 targets and inhibits OXCT1 expression during hepatocyte differentiation *in vitro* and *in vivo*.

Upregulation and downregulation of miR-122 *in vivo* and *in vitro* induce an approximately 1.6-fold decrease and increase in OXCT1 expression, respectively. Although this effect is modest, it corresponds to what is expected as a result from the regulation by a single microRNA (Baek et al. 2008; Selbach et al. 2008). This repression may function as a secondary control level to further inhibit translation of a mRNA that is strongly repressed by other mechanisms. Indeed in Thorrez et al., we showed that *Oxct1* is also repressed at epigenetic level: nucleosomal chromatin around its promoter is more trimethylated and less acetylated in liver than in other tissues, such as in brain, which is indicative of low transcriptional activity (Thorrez et al. 2011).

In conclusion, we showed that during hepatocyte maturation the key enzyme for ketone body utilization (OXCT1) is strongly repressed, which is needed to allow synthesis and export of ketone bodies without their degradation. OXCT1 is selectively disallowed in liver as compared to all other tissues, and this repression is at least in part mediated by miR-122, which is specifically expressed in this organ.

Progressive repression of OXCT1 during hepatocyte maturation parallels the increase in miR-122, suggesting that miR-122 is a key determinant of the timing of OXCT1 repression.

Materials and methods are described in (Thorrez et al. 2011) (Annex).

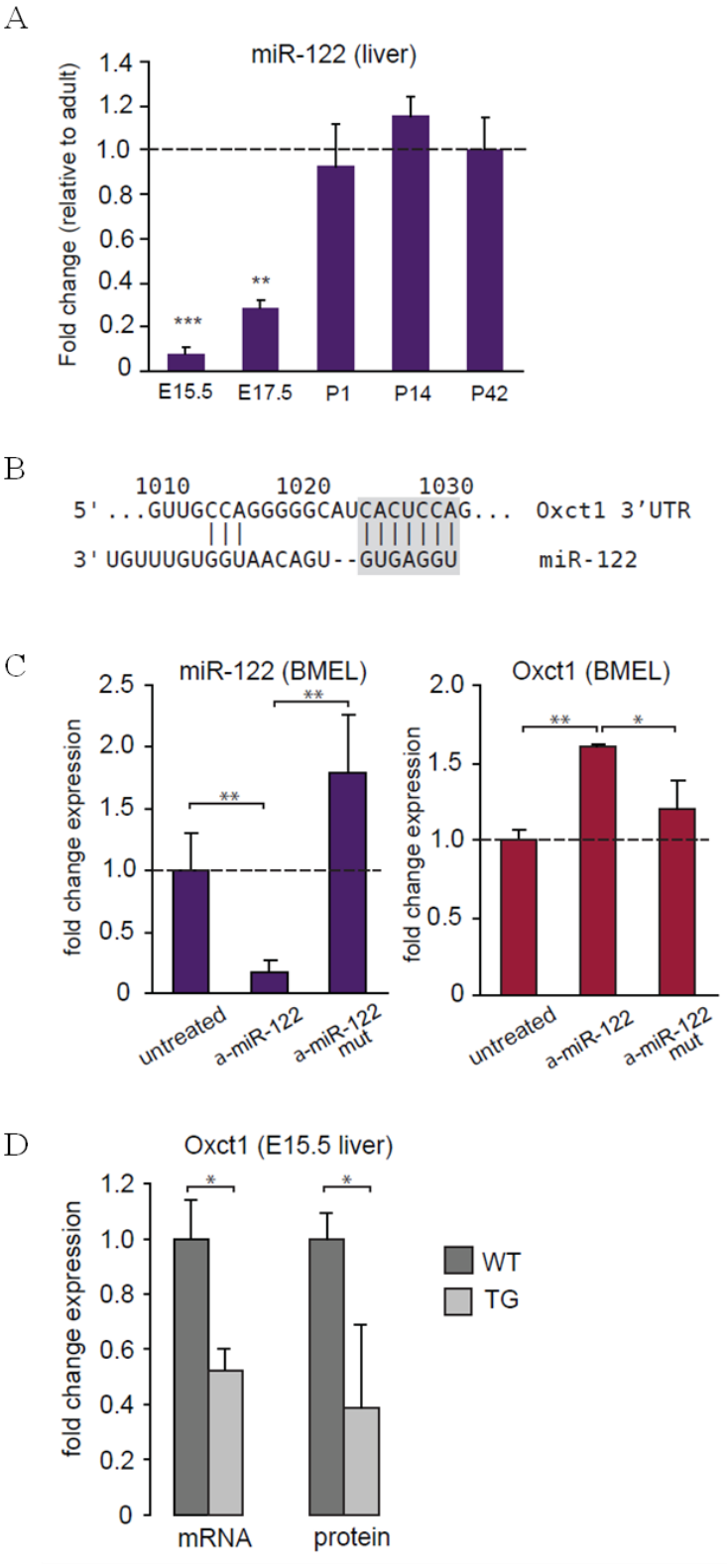


Figure 3. MiR-122 contributes to liver-specific silencing of Oxct1 expression (*adapted from Thorrez et al. 2011)*. (A) miR-122 expression level in extracts from mouse fetal and neonatal liver between E15.5 and 42 days after birth. Data are means ± SEM; n=3. (B) TargetScan predicted pairing of mouse Oxct1 3'UTR and miR-122. (C) Treatment of aggregate-cultured BMEL cells with antagomiR-122 (α-miR-122) induced repression of miR-122 (left panel) and upregulation of *Oxct1* mRNA (right panel), as compared to cells treated with a mutated antagomiR (α-miR-122 mut). Changes in miR-122 expression (left) and Oxct1 expression (right) were measured by Q-RT-PCR. Data are means ± SEM; n=3. (D) Oxct1 RNA and protein expression in livers at E15.5 of wild-type (WT) versus liver-specific miR-122 overexpressing transgenic mice (TG), as determined by Q-RT-PCR and Western Blotting respectively. Data are means ± SEM; n≥3. *P < 0.05; **P < 0.01; ***P < 0.001.

3.2.3. References

Baek D, Villen J, Shin C, Camargo FD, Gygi SP, Bartel DP. 2008. The impact of microRNAs on protein output. *Nature* **455**: 64-71.

Bartel DP. 2009. MicroRNAs: target recognition and regulatory functions. *Cell* **136**: 215-233.

Bushati N, Cohen SM. 2007. microRNA functions. *Annu Rev Cell Dev Biol* **23**: 175-205.

Cahill GF, Jr., Veech RL. 2003. Ketoacids? Good medicine? *Trans Am Clin Climatol Assoc* **114**: 149-161; discussion 162-143.

Jochheim A, Cieslak A, Hillemann T, Cantz T, Scharf J, Manns MP, Ott M. 2003. Multi-stage analysis of differential gene expression in BALB/C mouse liver development by high-density microarrays. *Differentiation* **71**: 62-72.

Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. 2002. Identification of tissue-specific microRNAs from mouse. *Curr Biol* **12**: 735-739.

Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A, Pfeffer S, Rice A, Kamphorst AO, Landthaler M et al. 2007. A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell* **129**: 1401-1414.

Petkov PM, Zavadil J, Goetz D, Chu T, Carver R, Rogler CE, Bottinger EP, Shafritz DA, Dabeva MD. 2004. Gene expression pattern in hepatic stem/progenitor cells during rat fetal development using complementary DNA microarrays. *Hepatology* **39**: 617-627.

Selbach M, Schwanhausser B, Thierfelder N, Fang Z, Khanin R, Rajewsky N. 2008. Widespread changes in protein synthesis induced by microRNAs. *Nature* **455**: 58-63.

Strick-Marchand H, Weiss MC. 2002. Inducible differentiation and morphogenesis of bipotential liver cell lines from wild-type mouse embryos. *Hepatology* **36**: 794-804.

Thorrez L, Laudadio I, Van Deun K, Quintens R, Hendrickx N, Granvik M, Lemaire K, Schraenen A, Van Lommel L, Lehnert S et al. 2011. Tissue-specific disallowance of housekeeping genes: the other face of cell differentiation. *Genome Res* **21**: 95-105.

4. GENERAL DISCUSSION

In multicellular organism, most genes need to be expressed in a specific spatiotemporal manner, in order to guide development and post-developmental physiology. Differential gene expression is a highly regulated process and is achieved through complex gene regulatory networks (GRNs) that are controlled in part by two types of *trans*-regulators: transcription factors (TFs) and miRNAs.

4.1. MiRNAs and gene regulatory networks

GRNs are made up of a small set of recurring circuits that occur more often in real networks than expected by chance. These circuits are called network motifs and were first identified in bacteria and yeast (Milo et al. 2002; Shen-Orr et al. 2002). GRNs of different organisms contain the same types of network motifs, suggesting that they constitute successful mechanisms for regulation of gene expression not only in one particular organism, but throughout evolution as well (Martinez and Walhout 2009). Network motifs can be subdivided into two broad categories: feedbacks and feed-forwards.

Feedback loops can be classified into coherent and incoherent loops (Fig. 1). In coherent loops the regulatory connections have the same effect (either repression or activation of the target). Negative coherent feedback loops can generate mutually exclusive expression of both miRNA and TF involved. This happens for miR-233 and nuclear factor 1-A (NF1-A) during granulocytic differentiation. In undifferentiated cells, miR-233 levels are low and NF1-A levels are high; however, upon retinoic acid signaling, miR-233 levels increase and NF1-A is repressed, facilitating differentiation to the myeloid lineage (Fazi et al. 2005). On the contrary positive coherent feedbacks amplify expression of both the miRNA and the TF involved. A positive feedback between miR-301a and NFκB is active in human pancreatic cancer and constitutes the driving force for constitutive NFκB activation. Indeed NFκB promotes miR-301a gene transcription, which in turn represses NFκB repressing factor (NKRF), resulting in further NFκB transactivational activity (Lu et al. 2011). In incoherent loops the regulatory connections have opposite effects. Incoherent feedback loops potentially function to fine-tune gene expression and to maintain precise steady state levels of components of the loop (Tsang et al. 2007). This is the case for miR-43 and LIN-26, which are both expressed in hypodermal seam cells during *C. elegans* development. LIN-26 stimulates miR-43 which in turn inhibits LIN-26 and this loop could be involved in epithelial differentiation (Martinez et al. 2008).

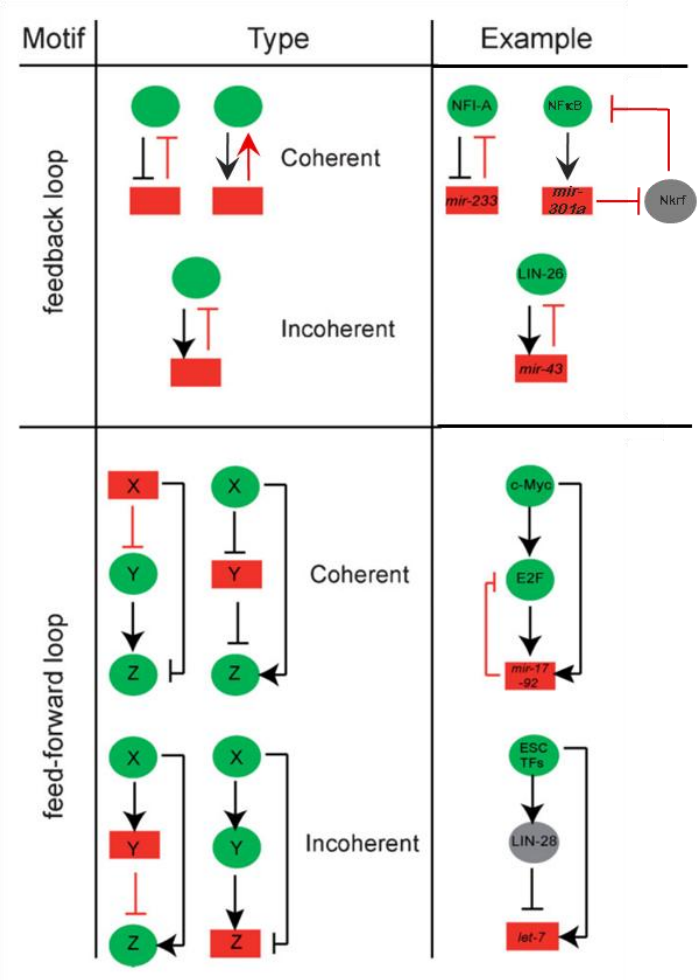


Figure 1. Coherent and incoherent feedback and feed-forward motifs involving miRNAs and TFs *(adapted from Martinez and Walhout 2009).* Green circles, TFs; red rectangles, miRNAs; gray circles, other protein coding genes; black/red arrows, activation; black/red blunted arrows, repression.

Feed-forward loops are made up of at least three elements: a regulator X regulates the expression of a target Z directly as well as indirectly via the second target Y. Therefore, feed-forward loops are more complex than feedbacks and their biological function is less intuitive. A feed-forward is called coherent when branches act in the same direction on Z, whereas it is called incoherent if branches act oppositely (Fig. 1). Coherent feed-forward loops involving TFs and miRNAs can function to maintain and tightly control target protein homeostasis (Tsang et al. 2007). The c-Myc/E2F/miR-17-92 circuit is an example of coherent feed-forward loop. The TF c-Myc simultaneously activates the expression of E2F and the miR-17-92 cluster. In addition, E2F promotes transcription of the miR-17-92 cluster. This coherent feed-forward loop is embedded in a more complex circuit, since miR-17-92 cluster negatively regulates E2F. Therefore c-Myc activates E2F transcription and limits its translation, through activation of miR-17-92 cluster, allowing a tightly controlled proliferative signal (O'Donnell et al. 2005; Sylvestre et al. 2007). Incoherent feed-forward loops involving miRNAs have been proposed to provide response acceleration upon signal detection (Tsang et al. 2007). For instance, the core embryonic stem cell (ESC) TFs, namely Oct4, Nanog, Sox2 and Tcf3, promote the transcription of the

miRNA let-7 and the RNA-binding protein LIN-28. LIN-28 blocks pri-let-7 maturation (Viswanathan et al. 2008), as a result pri-let-7 but not mature let-7 is abundant in ESC. On the contrary in differentiated cells mature let-7 is abundant. Upon differentiation signals, ESC TFs and subsequently LIN-28 decrease, allowing rapid maturation of pre-existing pri-let-7. Such a loop contribute to rapid cellular differentiation of ESCs (Marson et al. 2008).

Computational and experimental lines of evidence suggest that regulatory circuits involving miRNAs and TFs are not isolated instances but are in fact recurring mechanisms of gene expression at the genome-scale level. Indeed, it has been shown that the expression of miRNAs and their predicted targets tends to be positively or negatively correlated. That correlation or anti-correlation can be explained by the existence of feed-forward and feedback loops connecting miRNAs and TFs (Tsang et al. 2007). Moreover by predicting miRNA and TF binding sites, Shalgi and colleagues found that a considerable number of human genes (between ~1.000 and ~4.000) are under combined regulation of miRNA-TF pairs. They also observed that miRNA and TF in each pair are predicted to regulate each other more frequently than randomly picked pairs, suggesting again the existence of feed-forward and feedback loops (Shalgi et al. 2007). Finally genome-scale integration of experimentally mapped transcriptional TF-> miRNA interactions and computationally predicted miRNA->TF interactions show that miRNA <-> TF feedback loops occur more frequently than expected by chance in *C. elegans* (Martinez et al. 2008).

Both TFs and miRNAs can exert a widespread impact on gene expression. Indeed most, if not all, genes in the genome are controlled by TFs, and miRNAs are predicted to target approximately 60% of animal protein-coding genes (Friedman et al. 2009), with each miRNA repressing on average 200 transcripts (Lewis et al. 2005). This implies that the expression of not only the TF and the miRNA involved in loops, but also of their respective targets is tightly coordinated. This could be important in regulating large sets of genes, such as in different tissues or in response to developmental or environmental cues (Martinez and Walhout 2009).

In the present work by using hepatocyte differentiation as a well-established model, we described a developmental mechanism in which a transcription factor (HNF6) and a microRNA (miR-122), which are both specifically expressed in the same cell type, establish a positive feedback loop that is used to drive differentiation. The miR-122 - HNF6 loop and the recently published miR-301a - NFκB (Lu et al. 2011) loops are the only known examples of positive feedback loops involving a TF and a miRNA. Interestingly the miR-122 - HNF6 loop is based on transcriptional activation of genes coding for both TF and miRNA, whereas in the miR-301a - NFκB loop TF promotes miRNA transcription, but miRNA stimulates TF transactivational

activity on target genes (Lu et al. 2011). Taken together these data show that miRNAs contribute to feedback loops by modulating TF activity at several levels.

4.2. MiR-122, HNF6 and the network of liver-enriched transcription factors

As detailed in § *1.1.4. Transcriptional control of hepatocyte maturation*, hepatocyte differentiation is controlled by a network of 6 LETFs (HNF1α, HNF1β, FoxA2, HNF4α, HNF6 and LRH1), which organize as autoregulatory and cross-regulatory loops (Kyrmizi et al. 2006). Here we integrate the liver-specific miRNA miR-122 into this TF network, adding a further layer of complexity. Indeed we show that HNF6 directly stimulates expression of miR-122, which in turn reinforces expression of HNF6 and of other LETFs, namely FoxA2, OC-2 and HNF4α, through an unknown mechanism; this results in the stimulation of hepatocyte specific-gene transcription. Moreover, in previous work it has been shown that other LEFTs, namely HNF1α, HNF4α, FoxA1, FoxA2 and C/EBPα bind to and stimulate the miR-122 promoter (Coulouarn et al. 2009; Gao et al. 2010; Xu et al. 2010; Zeng et al. 2010). These data were obtained with hepatocellular carcinoma (HCC)-derived cells and their relevance for normal hepatocyte differentiation have not been proven, yet they suggest that not only HNF6, but also other LETFs positively feedback on miR-122 expression (Fig. 2).

The complexity of LEFT network increases during liver development, following the progressive rise in concentration of the core transcription factors (Kyrmizi et al. 2006). Interestingly, during liver development miR-122 expression also increases progressively (Chang et al. 2004; Rogler et al. 2009; Thorrez et al. 2011). This suggests that the miR-122 - LETFs positive feedback loop is the driving force for the rise in concentration, thereby ensuring proper progression of hepatocyte differentiation.

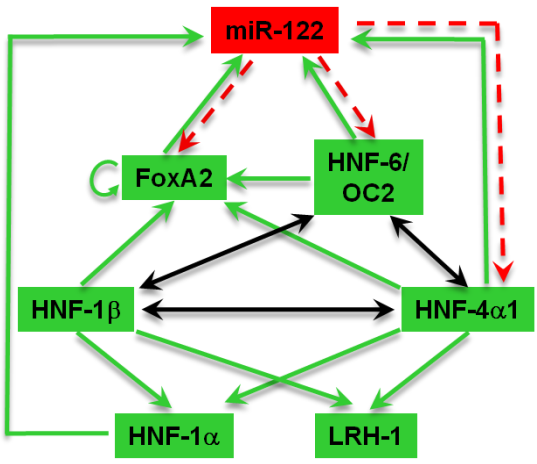


Figure 2. Integration of miR-122 in the LETF network during hepatocyte maturation.
Red rectangles, miRNAs; Green rectangles, TFs; red arrows, direct or indirect regulation via miR-122; green arrows, unidirectional transcriptional regulations; black arrows, bidirectional transcriptional regulations.

4.3 MiR-122, liver-enriched transcription factors and hepatocellular carcinoma

Monitoring miR-122 - LETFs positive feedback loops might be important in the study of HCC pathogenesis. HCC is the seventh most common cancer worldwide and is the third leading cause of cancer-related deaths (Parkin et al. 2005). Even if over the past decade, advances in molecular and cellular biology have increased our understanding of liver carcinogenesis, HCC still remains a major cause of morbidity and mortality. A feature for HCC diagnosis and staging is the grade of tumor cell differentiation. Indeed in most patients with chronic liver diseases, HCC usually arises as a small single nodule (up to 2 cm in diameter), which can be defined as either "indistinct" or "distinct". Indistinct nodules are well-differentiated, hypovascularized and with indistinct margins, whereas distinct nodules are moderately differentiated, hypervascularized, surrounded by a fibrous pseudocapsule, and frequently show portal invasion (Kojiro 2002). The first ones present a low degree of malignancy and a better prognosis (Nakashima et al. 1995). Interestingly when the small well-differentiated nodules increase in size, foci of moderately to poorly differentiated cancerous tissues arise within the tumor until totally replacing the well-differentiated HCC tissue (Kojiro 2004). There is clear evidence that the less differentiated the tumor, the faster its growth and the higher the risk of vascular invasion and metastases (Trevisani et al. 2008).

The grade of HCC differentiation correlates with LETF and miR-122 expression levels. Indeed, highly differentiated slow-growing mouse HCC, which rapidly gives rise *in vivo* to an invasive fast-growing dedifferentiated HCC, showed that HCC progression is characterized by downregulation of a broad spectrum of hepato-specific genes. This correlated with a decrease of LETF expression levels. Interestingly forced re-expression of one of these LETFs, namely HNF4α1, re-established the expression profile of LETFs and of hepatic genes that are associated with a differentiated hepatocyte phenotype (Lazarevich et al. 2004). In addition it has been demonstrated that loss of miR-122 in HCCs correlates with suppression of hepatocyte-specific genes, with low levels of LETFs and with aggressive phenotype and bad prognosis (Coulouarn et al. 2009). Therefore, in liver cancer, expression of miR-122, LETFs and hepatocyte genes seems to be strictly related. These data strongly support the idea that the miR-122 - LETFs positive feedback loops control hepatocyte differentiation not only during embryogenesis but also in adulthood. This is required to maintain the differentiated state and to protect from malignant transformation. Finally since miR-122 is involved in carcinogenesis, maintaining miR-122 levels could help to prevent not only dedifferentiation but also proliferation (Gramantieri et al. 2007;

Bai et al. 2009; Fornari et al. 2009; Zeng et al. 2010), apoptosis (Lin et al. 2008) and metastasis (Bai et al. 2009; Tsai et al. 2009).

4.4. MiR-122, liver-enriched transcription factors and programmed *in vitro* differentiation of hepatocytes

Embryonic stem cells (ESCs) can be isolated from the inner mass of the blastocyst, extensively proliferate *in vitro* and potentially differentiate into every cell type of the body, including hepatocytes. Therefore, ESCs hold great promise for therapeutic applications in the treatment of liver diseases and in the study of drug-induced liver toxicity. Indeed cell-based therapies, such as hepatocyte transplantation, are a potential way to replace orthotopic liver transplantation, which is increasingly facing a shortage of donor organs (Nussler et al. 2006). Hepatocyte transplantation has been performed to treat inherited disorders of liver metabolism. These diseases are ideally suited for cell therapy since a small number of functional transplanted hepatocytes is sufficient to correct the metabolic defect (Burlina 2004; Meyburg and Hoffmann 2010). In patients with acquired acute liver failure, cell therapy may help patients until a suitable liver becomes available for transplantation or until auto-recovery (Fisher et al. 2004; Schneider et al. 2006). In addition, *in vitro* cultured hepatocytes are used as model system in drug metabolism and toxicology studies for prediction of drug-induced hepatic side effects (Lake et al 2009; Greer et al 2009).

Human ESCs offer an alternative cell source to human hepatocytes, because of their ability to be expanded *in vitro*. A variety of approaches have been utilized for programming differentiation of mouse and human ESCs into functional hepatocyte-like cells. Current methods involve both spontaneous differentiation via the formation of embryoid bodies (EBs) and/or directed differentiation by using protocols recapitulating hepatic development. In the first instance, ESCs are differentiated in suspension cultures in aggregation, resulting in the formation of EBs, which exhibit regional differentiation into cell types of all three germ layers. EBs can be further induced to differentiate into hepatocyte-like cells in the presence of growth factors, cytokines, and hormones (Chinzei et al. 2002; Asahina et al. 2004). Differentiation via an EB-dependent stage is spontaneous and stochastic, and results in formation of mixed cell types originating from all three germ layers. On the contrary, directed differentiation resorts to extracellular signals that mimic the sequential developmental stages toward the hepatocyte lineage (Fig 3; (Dalgetty et al. 2009; Navarro-Alvarez et al. 2009). Cells are cultured in suspension to induce early endodermal development. This is followed by culture on plates in the

presence of FGF-2, TGFβ and Activin A in order to induce definitive endodermal cells. Hepatic specification can then be induced by treatment with FGF, BMP, and HGF, and hepatocyte maturation is promoted with the addition of Oncostatine M and dexamethasone. At each stage, hepatic differentiation is assessed by measuring the expression of a specific gene set (Fig. 3).

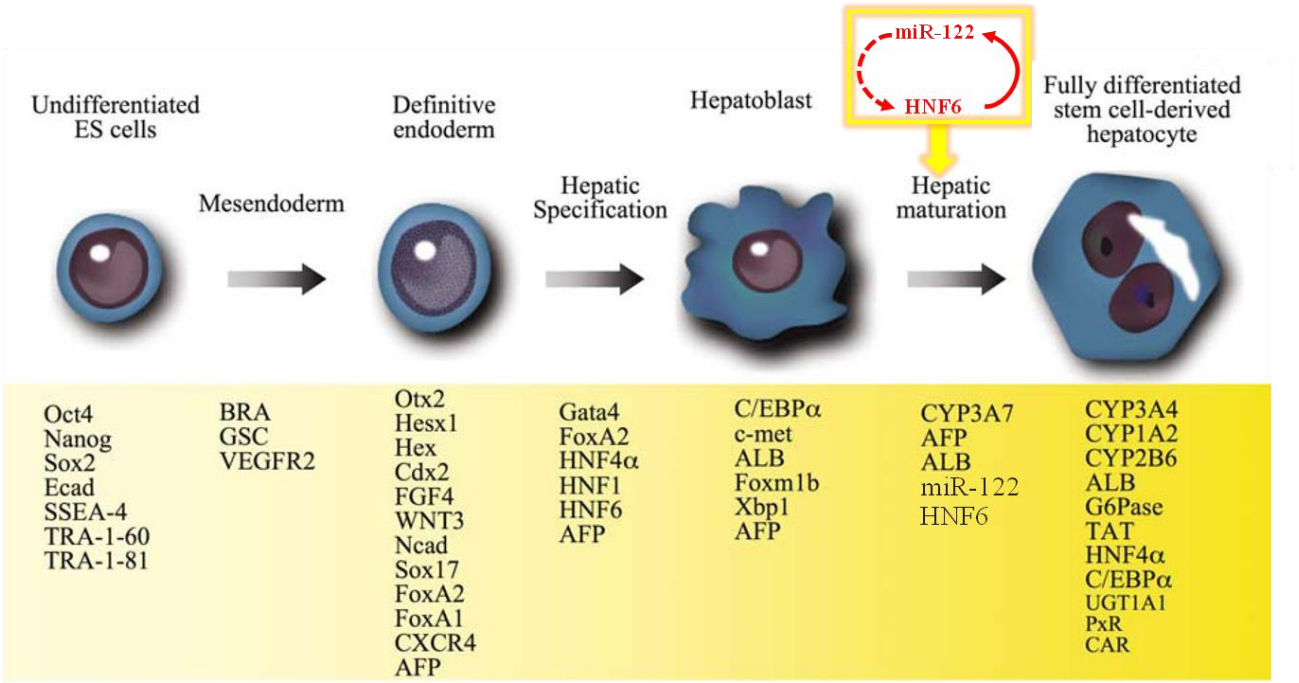


Figure 3. Directed differentiation of ESC cells to hepatocyte-like cells by mimicking embryonic development. *(adapted from Navarro-Alvarez et al 2009).* Key stages of hepatic differentiation stepwise differentiation of ES cells. Characteristic gene expression for each step of differentiation is shown.

Up to now these procedures still produce heterogeneous cell populations and rather immature hepatocytes. Monitoring the miR-122 - HNF6 positive feedback could increase our understanding of the maturation state of ESC-derived hepatocyte-like cells, by comparing their miR-122 and HNF6 expression levels to those in fetal and adult hepatocytes. Moreover, hepatocyte-like cells could be forced to further mature by increasing miR-122 expression. Overexpression of miR-122 in human ESCs has been reported: during sodium butyrate-induced differentiation of ESCs toward hepatocyte-like cells miR-122 expression increases, like in liver development, but stable transfection of human ESCs with a miR-122 expressing lentiviral vector had no obvious effects on hepatic differentiation (Tzur et al. 2008). In this study LETFs expression was not assessed, with exception of FoxA2, which was not influenced by miR-122 overexpression. This result indicates that miR-122 is on its own unable to drive hepatocyte differentiation from undifferentiated pluripotent stem cells, probably because its action depends on a LETF network in cells that are committed to the hepatocyte lineage. We suggest that

transient overexpression of miR-122 and/or of HNF6 during the late steps of ESC differentiation may trigger the endogenous miR-122 - HNF6 positive feedback, which in turn will allow proper progression of maturation (Fig. 3).

In conclusion in this work we show that tissue differentiation is controlled by tissue-specific miRNA and TF organized in a positive feedback motif, in which the TF directly regulates the miRNA which positively feedbacks on the TF while stimulating tissue-specific gene expression in a way that is dependent on that same TF. Therefore, miRNAs and TF expression is tightly connected and needs well-defined levels to fine-tune differentiation in health and disease.

4.5. References

Asahina K, Fujimori H, Shimizu-Saito K, Kumashiro Y, Okamura K, Tanaka Y, Teramoto K, Arii S, Teraoka H. 2004. Expression of the liver-specific gene Cyp7a1 reveals hepatic differentiation in embryoid bodies derived from mouse embryonic stem cells. *Genes Cells* **9**: 1297-1308.

Bai S, Nasser MW, Wang B, Hsu SH, Datta J, Kutay H, Yadav A, Nuovo G, Kumar P, Ghoshal K. 2009. MicroRNA-122 inhibits tumorigenic properties of hepatocellular carcinoma cells and sensitizes these cells to sorafenib. *J Biol Chem* **284**: 32015-32027.

Burlina AB. 2004. Hepatocyte transplantation for inborn errors of metabolism. *J Inherit Metab Dis* **27**: 373-383.

Chang J, Nicolas E, Marks D, Sander C, Lerro A, Buendia MA, Xu C, Mason WS, Moloshok T, Bort R et al. 2004. miR-122, a mammalian liver-specific microRNA, is processed from hcr mRNA and may downregulate the high affinity cationic amino acid transporter CAT-1. *RNA Biol* **1**: 106-113.

Chinzei R, Tanaka Y, Shimizu-Saito K, Hara Y, Kakinuma S, Watanabe M, Teramoto K, Arii S, Takase K, Sato C et al. 2002. Embryoid-body cells derived from a mouse embryonic stem cell line show differentiation into functional hepatocytes. *Hepatology* **36**: 22-29.

Coulouarn C, Factor VM, Andersen JB, Durkin ME, Thorgeirsson SS. 2009. Loss of miR-122 expression in liver cancer correlates with suppression of the hepatic phenotype and gain of metastatic properties. *Oncogene* **28**: 3526-3536.

Dalgetty DM, Medine CN, Iredale JP, Hay DC. 2009. Progress and future challenges in stem cell-derived liver technologies. *Am J Physiol Gastrointest Liver Physiol* **297**: G241-248.

Fazi F, Rosa A, Fatica A, Gelmetti V, De Marchis ML, Nervi C, Bozzoni I. 2005. A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPalpha regulates human granulopoiesis. *Cell* **123**: 819-831.

Fisher RA, Bu D, Thompson M, Wolfe L, Ritter JK. 2004. Optimization of conditions for clinical human hepatocyte infusion. *Cell Transplant* **13**: 677-689.

Fornari F, Gramantieri L, Giovannini C, Veronese A, Ferracin M, Sabbioni S, Calin GA, Grazi GL, Croce CM, Tavolari S et al. 2009. MiR-122/cyclin G1 interaction modulates p53 activity and affects doxorubicin sensitivity of human hepatocarcinoma cells. *Cancer Res* **69**: 5761-5767.

Friedman RC, Farh KK, Burge CB, Bartel DP. 2009. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* **19**: 92-105.

Gao Y, Schug J, McKenna LB, Le Lay J, Kaestner KH, Greenbaum LE. 2010. Tissue-specific regulation of mouse MicroRNA genes in endoderm-derived tissues. *Nucleic Acids Res*.

Gramantieri L, Ferracin M, Fornari F, Veronese A, Sabbioni S, Liu CG, Calin GA, Giovannini C, Ferrazzi E, Grazi GL et al. 2007. Cyclin G1 is a target of miR-122a, a microRNA frequently down-regulated in human hepatocellular carcinoma. *Cancer Res* **67**: 6092-6099.

Kojiro M. 2002. Pathological evolution of early hepatocellular carcinoma. *Oncology* **62 Suppl 1**: 43-47.

Kojiro M. 2004. 'Nodule-in-nodule' appearance in hepatocellular carcinoma: its significance as a morphologic marker of dedifferentiation. *Intervirology* **47**: 179-183.

Kyrmizi I, Hatzis P, Katrakili N, Tronche F, Gonzalez FJ, Talianidis I. 2006. Plasticity and expanding complexity of the hepatic transcription factor network during liver development. *Genes Dev* **20**: 2293-2305.

Lazarevich NL, Cheremnova OA, Varga EV, Ovchinnikov DA, Kudrjavtseva EI, Morozova OV, Fleishman DI, Engelhardt NV, Duncan SA. 2004. Progression of HCC in mice is associated with a downregulation in the expression of hepatocyte nuclear factors. *Hepatology* **39**: 1038-1047.

Lewis BP, Burge CB, Bartel DP. 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* **120**: 15-20.

Lu Z, Li Y, Takwi A, Li B, Zhang J, Conklin DJ, Young KH, Martin R. 2011. miR-301a as an NF-kappaB activator in pancreatic cancer cells. *EMBO J* **30**: 57-67.

Marson A, Levine SS, Cole MF, Frampton GM, Brambrink T, Johnstone S, Guenther MG, Johnston WK, Wernig M, Newman J et al. 2008. Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. *Cell* **134**: 521-533.

Martinez NJ, Ow MC, Barrasa MI, Hammell M, Sequerra R, Doucette-Stamm L, Roth FP, Ambros VR, Walhout AJ. 2008. A C. elegans genome-scale microRNA network contains composite feedback motifs with high flux capacity. *Genes Dev* **22**: 2535-2549.

Martinez NJ, Walhout AJ. 2009. The interplay between transcription factors and microRNAs in genome-scale regulatory networks. *Bioessays* **31**: 435-445.

Meyburg J, Hoffmann GF. 2010. Liver, liver cell and stem cell transplantation for the treatment of urea cycle defects. *Mol Genet Metab* **100 Suppl 1**: S77-83.

Milo R, Shen-Orr S, Itzkovitz S, Kashtan N, Chklovskii D, Alon U. 2002. Network motifs: simple building blocks of complex networks. *Science* **298**: 824-827.

Nakashima O, Sugihara S, Kage M, Kojiro M. 1995. Pathomorphologic characteristics of small hepatocellular carcinoma: a special reference to small hepatocellular carcinoma with indistinct margins. *Hepatology* **22**: 101-105.

Navarro-Alvarez N, Soto-Gutierrez A, Kobayashi N. 2009. Stem cell research and therapy for liver disease. *Curr Stem Cell Res Ther* **4**: 141-146.

Nussler A, Konig S, Ott M, Sokal E, Christ B, Thasler W, Brulport M, Gabelein G, Schormann W, Schulze M et al. 2006. Present status and perspectives of cell-based therapies for liver diseases. *J Hepatol* **45**: 144-159.

O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT. 2005. c-Myc-regulated microRNAs modulate E2F1 expression. *Nature* **435**: 839-843.

Parkin DM, Bray F, Ferlay J, Pisani P. 2005. Global cancer statistics, 2002. *CA Cancer J Clin* **55**: 74-108.

Rogler CE, Levoci L, Ader T, Massimi A, Tchaikovskaya T, Norel R, Rogler LE. 2009b. MicroRNA-23b cluster microRNAs regulate transforming growth factor-beta/bone morphogenetic protein signaling and liver stem cell differentiation by targeting Smads. *Hepatology* **50**: 575-584.

Schneider A, Attaran M, Meier PN, Strassburg C, Manns MP, Ott M, Barthold M, Arseniev L, Becker T, Panning B. 2006. Hepatocyte transplantation in an acute liver failure due to mushroom poisoning. *Transplantation* **82**: 1115-1116.

Shalgi R, Lieber D, Oren M, Pilpel Y. 2007. Global and local architecture of the mammalian microRNA-transcription factor regulatory network. *PLoS Comput Biol* **3**: e131.

Shen-Orr SS, Milo R, Mangan S, Alon U. 2002. Network motifs in the transcriptional regulation network of Escherichia coli. *Nat Genet* **31**: 64-68.

Sylvestre Y, De Guire V, Querido E, Mukhopadhyay UK, Bourdeau V, Major F, Ferbeyre G, Chartrand P. 2007. An E2F/miR-20a autoregulatory feedback loop. *J Biol Chem* **282**: 2135-2143.

Thorrez L, Laudadio I, Van Deun K, Quintens R, Hendrickx N, Granvik M, Lemaire K, Schraenen A, Van Lommel L, Lehnert S et al. 2011b. Tissue-specific disallowance of housekeeping genes: the other face of cell differentiation. *Genome Res* **21**: 95-105.

Trevisani F, Cantarini MC, Wands JR, Bernardi M. 2008. Recent advances in the natural history of hepatocellular carcinoma. *Carcinogenesis* **29**: 1299-1305.

Tsang J, Zhu J, van Oudenaarden A. 2007. MicroRNA-mediated feedback and feedforward loops are recurrent network motifs in mammals. *Mol Cell* **26**: 753-767.

Tzur G, Levy A, Meiri E, Barad O, Spector Y, Bentwich Z, Mizrahi L, Katzenellenbogen M, Ben-Shushan E, Reubinoff BE et al. 2008. MicroRNA expression patterns and function in endodermal differentiation of human embryonic stem cells. *PLoS ONE* **3**: e3726.

Viswanathan SR, Daley GQ, Gregory RI. 2008. Selective blockade of microRNA processing by Lin28. *Science* **320**: 97-100.

Xu H, He JH, Xiao ZD, Zhang QQ, Chen YQ, Zhou H, Qu LH. 2010. Liver-enriched transcription factors regulate microRNA-122 that targets CUTL1 during liver development. *Hepatology* **52**: 1431-1442.

Zeng C, Wang R, Li D, Lin XJ, Wei QK, Yuan Y, Wang Q, Chen W, Zhuang SM. 2010. A novel GSK-3 beta-C/EBP alpha-miR-122-insulin-like growth factor 1 receptor regulatory circuitry in human hepatocellular carcinoma. *Hepatology* **52**: 1702-1712.

ANNEXES

“Tissue-specific disallowance of housekeeping genes: the other face of cell differentiation”

Thorrez L., Laudadio I., Van Deun K., Quintens R., Hendrickx N., Granvik M., Lemaire K., Schraenen A., Van Lommel L., Lehnert S., Aguayo-Mazzucato C., Cheng-Xue R., Gilon P., Van Mechelen I., Bonner-Weir S:, Lemaigre F. and Schuit F.

Genome Research. 2011; 21(1):95-105.

Abstract

We report on a hitherto poorly characterized class of genes that are expressed in all tissues, except in one. Often, these genes have been classified as housekeeping genes, based on their nearly ubiquitous expression. However, the specific repression in one tissue defines a special class of ‘‘disallowed genes.’’ In this paper, we used the intersection-union test to screen for such genes in a multi-tissue panel of genome-wide mRNA expression data. We propose that disallowed genes need to be repressed in the specific target tissue to ensure correct tissue function. We provide mechanistic data of repression with two metabolic examples, exercise-induced inappropriate insulin release and interference with ketogenesis in liver. Developmentally, this repression is established during tissue maturation in the early postnatal period involving epigenetic changes in histone methylation. In addition, tissue-specific expression of microRNAs can further diminish these repressed mRNAs. Together, we provide a systematic analysis of tissue-specific repression of housekeeping genes, a phenomenon that has not been studied so far on a genome-wide basis and, when perturbed, can lead to human disease.

“MiR-495 and miR-218 regulate the expression of the Onecut transcription factors HNF-6 and OC-2”

Simion A, Laudadio I, Prévot PP, Raynaud P, Lemaigre FP and Jacquemin P.

Biochem Biophys Res Commun. 2010;391(1):293-8.

Abstract

MicroRNAs are small, non-coding RNAs that posttranscriptionally regulate gene expression mainly by binding to the 30UTR of their target mRNAs. Recent data revealed that microRNAs have an important role in pancreas and liver development and physiology. Using cloning and microarray profiling approaches, we show that a unique repertoire of microRNAs is expressed at the onset of liver and pancreas organo-genesis, and in pancreas and liver at key stages of cell fate determination. Among the microRNAs that are expressed at these stages, miR-495 and miR-218 were predicted to, respectively, target the Onecut (OC) transcription factors Hepatocyte Nuclear Factor-6 (HNF-6/OC-1) and OC-2, two important regulators of liver and pancreas development. MiR-495 and miR-218 are dynamically expressed in developing liver and pancreas, and by transient transfection, we show that they target HNF-6 and OC-2 30UTRs. Moreover, when overexpressed in cultured cells, miR-495 and miR-218 decrease the endogenous levels of HNF-6 and OC-2 mRNA. These results indicate that the expression of regulators of liver and pancreas development is modulated by microRNAs. They also suggest a developmental role for miR-495 and miR-218.