BASIC AND TRANSLATIONAL—LIVER

A Feedback Loop Between the Liver-Enriched Transcription Factor Network and Mir-122 Controls Hepatocyte Differentiation

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BACKGROUND & AIMS: Hepatocyte differentiation is controlled by liver-enriched transcription factors (LETFs). We investigated whether LETFs control microRNA expression during development and whether this control is required for hepatocyte differentiation. **METHODS:** Using in vivo DNA binding assays, we identified miR-122 as a direct target of the LETF hepatocyte nuclear factor (HNF) 6. The role and mechanisms of the HNF6–miR-122 gene cascade in hepatocyte differentiation were studied in vivo and in vitro by gain-of-function and loss-of-function experiments, using developing mice and zebrafish as model organisms. **RESULTS:** HNF6 and its paralog Onecut2 are strong transcriptional stimulators of miR-122 expression. Specific levels of miR-122 were required for proper progression of hepatocyte differentiation; miR-122 stimulated the expression of hepatocyte-specific genes and most LETFs, including HNF6. This indicates that HNF6 and miR-122 form a positive feedback loop. Stimulation of hepatocyte differentiation by miR-122 was lost in HNF6-null mice, revealing that a transcription factor can mediate microRNA function. All hepatocyte-specific genes whose expression was stimulated by miR-122 bound HNF6 in vivo, confirming their direct regulation by this factor. **CONCLUSIONS: Hepatocyte differentiation is directed by a positive feedback loop that includes a transcription factor (HNF6) and a microRNA (miR-122) that are specifically expressed in liver. These findings could lead to methods to induce differentiation of hepatocytes in vitro and improve our understanding of liver cell dedifferentiation in pathologic conditions.**

Keywords: Liver Development; Gene Regulatory Network; Biliary Hyperplasia; Embryonic.

Hepatocytes exert most homeostatic functions of the liver, including the secretion of bile, which is transported via a network of ducts lined by cholangiocytes. During development, hepatocytes and cholangiocytes differentiate from bipotential precursors, called hepatoblasts. Hepatoblasts express a number of genes (eg, *hepatocyte nuclear factor* [*HNF*] *4α*, *transthyretin* [*Ttr*], *albumin* [*Alb*]) that, at the stage of hepatobiliary segregation, are repressed in biliary cells and become restricted to the hepatocyte lineage. Following lineage segregation, hepatocytes mature to form cords and acquire metabolic properties, and the cholangiocytes form ducts.[1–4]

A network of liver-enriched transcription factors (LETFs) controls differentiation and maturation of hepatic cells.[5] HNF6 and Onecut (OC) 2 belong to the LETFs and control hepatobiliary segregation by modulating a gradient of transforming growth factor β/activin signaling. In their absence, hepatoblasts abnormally differentiate toward hybrid cells that display characteristics of both hepatocytes and biliary cells.[6–8]

MicroRNAs (miRNAs) are implicated in development and differentiation of several tissues. Depletion of miRNAs in liver resulting from deletion of Dicer did not uncover a role for miRNAs in hepatic development, because inactivation of Dicer1 only occurred postnatally.[9–11] However, analysis of miR-30a/30c and miR-23b/27b/24-1 revealed that they are required for bile duct development and for repression of biliary gene expression in hepatocytes, respectively.[12,13]

Several promoters of miRNAs enriched in adult liver contain putative binding sites for HNF4α and Forkhead box A2 (FoxA2).[14,15] In hepatocarcinoma cell lines, the expression of miR-122, the most abundant liver-specific miRNA,[16–18] positively correlates with the level of LETFs.[19,20] Moreover, the promoter of miR-122 was stimulated in vitro by HNF1α, FoxA2, HNF4α, and CCAAT/enhancer binding protein α.[21] However, whether LETFs control miRNA expression during normal hepatic cell

Abbreviations used in this paper: Alb, albumin; Apo, apolipoprotein; BMEL, bipotential murine embryonic liver; ChIP-Seq, chromatin immunoprecipitation/ultra-high throughput sequencing; dKO, double knockout; E, embryonic day; FoxA2, Forkhead box A2; HNF, hepatocyte nuclear factor; KO, knockout; LETF, liver-enriched transcription factor; miRNA, microRNA; OC, Onecut; Q-RT-PCR, quantitative reverse-transcription polymerase chain reaction; SOX, SRY-related HMG box transcription factor; Ttr, transthyretin; WT, wild-type.

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differentiation and how LETFs and miRNAs coordinately regulate hepatic differentiation remains unknown.

Here we investigated the role of LETF-miRNA cascades in hepatic cell differentiation during embryonic development. We show that HNF6 and OC2 redundantly control the expression of miRNAs during liver development, and we identify an HNF6–miR-122 positive feedback loop that drives hepatocyte differentiation.

Materials and Methods

Animals

Experiments were performed with approval of the University Animal Welfare Committee. *MiR-122* Tg mice were generated as described[22] with the Alfp/pre-miR-122 plasmid. HNF6 and OC2 knockout (KO) mice were described.[6,23]

Plasmids

Alfp/pre-miR-122 plasmid was generated by inserting the genomic sequence coding for pre-miR-122 plus 50 base pairs flanking each side into Alfp-pBluescript, containing the *albumin* promoter and enhancer and *α-fetoprotein* enhancers (gift from K. Kaestner). miR-122 prom/*Luc* was obtained by cloning the −300 to +327 sequence of miR-122 promoter into pGL3basic (Promega, Leiden, The Netherlands). Primers used to generate miR-122 prom/*Luc* were 5′ACGCGTATCAGAGTCCTGAGAGAAAATG3′ and 5′GTCGACATGTCTCTAGCCTTCCCCTT3′. In miR-122mut prom/*Luc*, the HNF6 binding site 5′-CAATCGATAA-3′ was mutated to 5′-CAGACGGGAA-3′.

Cell Culture, Antagomir Treatment, and Transfection

Hepatocyte-like differentiation of bipotent murine embryonic liver (BMEL)[7] cells was obtained by growing the cells as floating aggregates.[24]

Antagomirs and antagomir treatment were as described.[25] MiR-122 was overexpressed in BMEL cells cultured in monolayer by transfecting 3×10^5 cells using Lipofectamine 2000 (Invitrogen, Merelbeke, Belgium) and miR-122 mimics (miRIDIAN Mimic-122; Thermo Scientific, Tournai, Belgium). Total miR mimic amount was adjusted to 1 μg with the miR-ctl mimic (miRIDIAN Mimic-ctl#1; Thermo Scientific). RNA was extracted 48 hours after transfection.

To study the *miR-122* promoter, 3×10^5 BMEL cells in monolayer were transfected using Lipofectamine 2000 (Invitrogen) with 1 μg of pCMV-GFP or pCMV-HNF6 and 1 μg of miR-122 prom/*Luc* or miR-122mut prom/*Luc*. Forty-eight hours after transfection, RNA was extracted and luciferase activity was measured (Dual-Luciferase Reporter Assay System; Promega), using pCMV-*renilla luciferase* for normalization.

Microarray Analysis of miRNA Expression

Livers from WT or *H6/O2* double knockout (dKO) embryos at embryonic day (E) 15.5 were stored in RNAlater (Ambion, Lennik, Belgium). Total RNA was isolated using TriPure (Roche, Basel, Switzerland) and cleaned using the RNeasy MinElute Cleanup Kit (Qiagen, Venlo, The Netherlands). Two micrograms of RNA was labeled and hybridized to miRCURY LNA Arrays v8.1 by miRCURY LNA Array microRNA Profiling Services (Exiqon, Vedbaek, Denmark). Three biological samples of *H6/O2* dKO and wild-type (WT) livers labeled with Hy3 were analyzed against a common reference labeled with Hy5 and consisting of a pool of all samples. miRNAs with Student *t* test values at $P < .05$ were selected.

Microarray Analysis of Messenger RNA Expression

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). Two micrograms of RNA was hybridized to GeneChip Mouse Genome 430A 2.0 Array (Affymetrix, Wooburn Green, England), following the manufacturer's protocol. Two biological samples of BMEL Agg antagomir-122 and of BMEL Agg antagomir-mut were analyzed. Up-regulated or down-regulated genes in each BMEL Agg antagomir-122/BMEL Agg antagomir-mut pair were selected, and median fold change was calculated.

Reverse Transcription and Real-Time Quantitative Polymerase Chain Reaction

Quantitative reverse-transcription polymerase chain reaction (Q-RT-PCR) analyses were as described.[25] A specific stem-loop primer was used for reverse transcription of miRNAs, and quantitative polymerase chain reaction was performed using a specific forward primer and a common universal reverse primer (Supplementary Tables 1 and 2). Quantitative polymerase chain reaction was performed with KAPA SYBR FAST Bio-Rad iCycler qPCR Master Mix (Sopachem; Kapa Biosystems, Eke, Belgium) on an IQ cycler (Bio-Rad, Eke, Belgium). Quantification of messenger RNAs and miRNAs was normalized to â-actin and 18S RNA, respectively.

Morpholino Injection in Zebrafish Embryos

Knockdown of dre-miR-122 was performed with the morpholino oligonucleotide 5′-ATACAAACACCATTGTCACACTCCA-3′ (Gene Tools, Philomath, OR). Two nanograms was injected at the one-cell stage with 0.25% rhodamine dextran (Invitrogen).

Whole Mount In Situ Hybridization and Immunofluorescence Analysis of Zebrafish Embryos

Colorimetric whole mount in situ hybridization was performed with DIG-labeled dre-miR-122 RNA probe (Exiqon).[26] Whole mount immunohistochemistry[27] was performed using the antibodies indicated in Supplementary Table 3. Fluorescent images were captured with a Leica SP2 confocal microscope (Leica Microsystems, Groot-Bijgaarden, Belgium). HNF4α^+/2F11$^+$ cells were counted in the center of the liver excluding the peripheral cell layer, across 15 consecutive optical sections, and normalized to the total number of Prox1-positive hepatic cells.

Immunofluorescence Analysis of Mouse Embryos

Embryos were fixed at 4°C for 4 hours in 4% paraformaldehyde in phosphate-buffered saline, washed overnight in phosphate-buffered saline, and embedded in paraffin. Immunofluorescence[28] (9-μm sections) was performed using the antibodies indicated in Supplementary Table 4. Images were captured with a Cell Observer Spinning Disk (Zeiss, Zaventem, Belgium) confocal microscope.

Chromatin Immunoprecipitation/Ultra-High Throughput Sequencing

Chromatin immunoprecipitation/ultra-high throughput sequencing (ChIP-Seq)[29] was performed with anti-HNF6 antibody (sc-13050; Santa Cruz Biotechnology, Santa Cruz, CA) on adult liver. All sequencing reads were aligned using MAQ[30] with default parameters to the mouse NCBI37/mm9 genome assembly. Data are available on the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) (ArrayExpress accession no. E-MTAB-438). The HNF6 motif was determined using MEME,[31] 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 using TargetScan mouse 5.0 (http://www.targetscan.org), PicTar (http://pictar.mdc-berlin.de/), and MicroCosm Targets Version 5 (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/). MiR-122 was aligned to *Hnf6* gene using MultAlin (http://multalin.toulouse.inra.fr/multalin/) to predict target sequences. Ingenuity pathway analysis was used to search for molecular connections between miR-122 and HNF6, HNF4α, FoxA2, and OC2, with exclusion of all connections involving HNF1α and HNF1β.

Results

HNF6 and OC2 Control the Expression of miRNAs During Liver Development

To identify miRNAs regulating hepatic cell differentiation, we compared miRNA expression in WT embryos and in embryos KO for both *Hnf6* and *Oc2* (*H6/O2* dKO) livers at E15.5 by microarray analysis. Fifteen miRNAs were differentially expressed in WT and HNF6/OC2 dKO livers (Student t test, $P < .05$; Table 1). When correcting for multiple testing by applying a Bonferroni correction or by applying Simes' procedure, only miR-122 remains significantly inhibited in the absence of HNF6 and OC2 ($P < .05$; Supplementary Figure 1). The results for miR-122 were validated by Q-RT-PCR, but this also revealed minor but statistically significant differences at E15.5 and/or E17.5 between WT and HNF6/OC2 dKO livers for the expression of miR200a, miR-18, miR-107, miR-339, miR-337, and miR-200b (Supplementary Figure 1).

HNF6 and OC2 have similar DNA target sequences.[32] 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 maximally perturb 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 (Supplementary Figure 1).

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, with the strongest control being exerted on miR-122.

Table 1. Altered miRNA Expression in *H6/O2* dKO Livers.

microRNA	*H602* dKO/WT (Fold change)	*P* values
mmu-miR-22a	0.15	9.40E-05
mmu-miR-200a	1.88	6.70E-04
has-miR-491-3p	0.88	1.20E-03
mmu-miR-292-5p	1.30	3.00E-03
mmu-miR-744	1.26	3.40E-03
has-miR-193a-5p	1.17	4.20E-03
mmu-miR-689	1.28	8.60E-03
mmu-miR-18	1.09	1.30E-02
mmu-miR-107	0.91	1.60E-02
mmu-miR-341	1.26	2.10E-02
mmu-miR-710	0.92	2.20E-02
mmu-miR-329	1.17	2.40E-02
mmu-miR-337	1.12	3.40E-02
has-miR-200b[a]	0.92	3.50E-02
mmu-miR-143	1.32	3.80E-02

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 down-regulated in the *H6/O2* dKO livers (3.5- to 7-fold decrease; Table 1 and Supplementary Figure 1). Therefore, we further focused on the mechanism by which HNF6 and OC2 regulate miR-122.

The *miR-122* promoter contains a conserved sequence matching the HNF6 binding consensus upstream of the transcription start site (Figure 1*A*). To verify if HNF6 can stimulate the *miR-122* promoter, we cotransfected a reporter construct (miR-122 prom/*Luc*) with an HNF6 expression vector in BMEL cells, a cultured hepatoblast cell line.[24] The results showed that HNF6 stimulates the *miR-122* promoter (Figure 1*B*). When the HNF6 binding site was mutated in the miR-122 promoter, the latter was less stimulated by HNF6 (Figure 1*B*). Overexpression of HNF6 in BMEL cells also stimulated endogenous miR-122 levels (Figure 1*C*). Finally, ChIP-Seq experiments performed in liver showed that HNF6 binds to several sites within 10 kilobases encompassing the *miR-122* locus (Figure 1*D*), the strongest binding peak overlapping with the *miR-122* promoter, which was stimulated by HNF6 in cultured cells.

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

MiR-122 Stimulates the Expression of Hepatocyte-Specific Genes During Hepatocyte Differentiation In Vitro

Because HNF6 controls hepatic cell differentiation while stimulating expression of miR-122, we investigated the role of miR-122 during hepatocyte differentiation. We first cultured BMEL cells as floating aggregates in which the expression of hepatocyte-specific genes is known to be induced.[24] As shown in Figure 2*A*, miR-122 expression

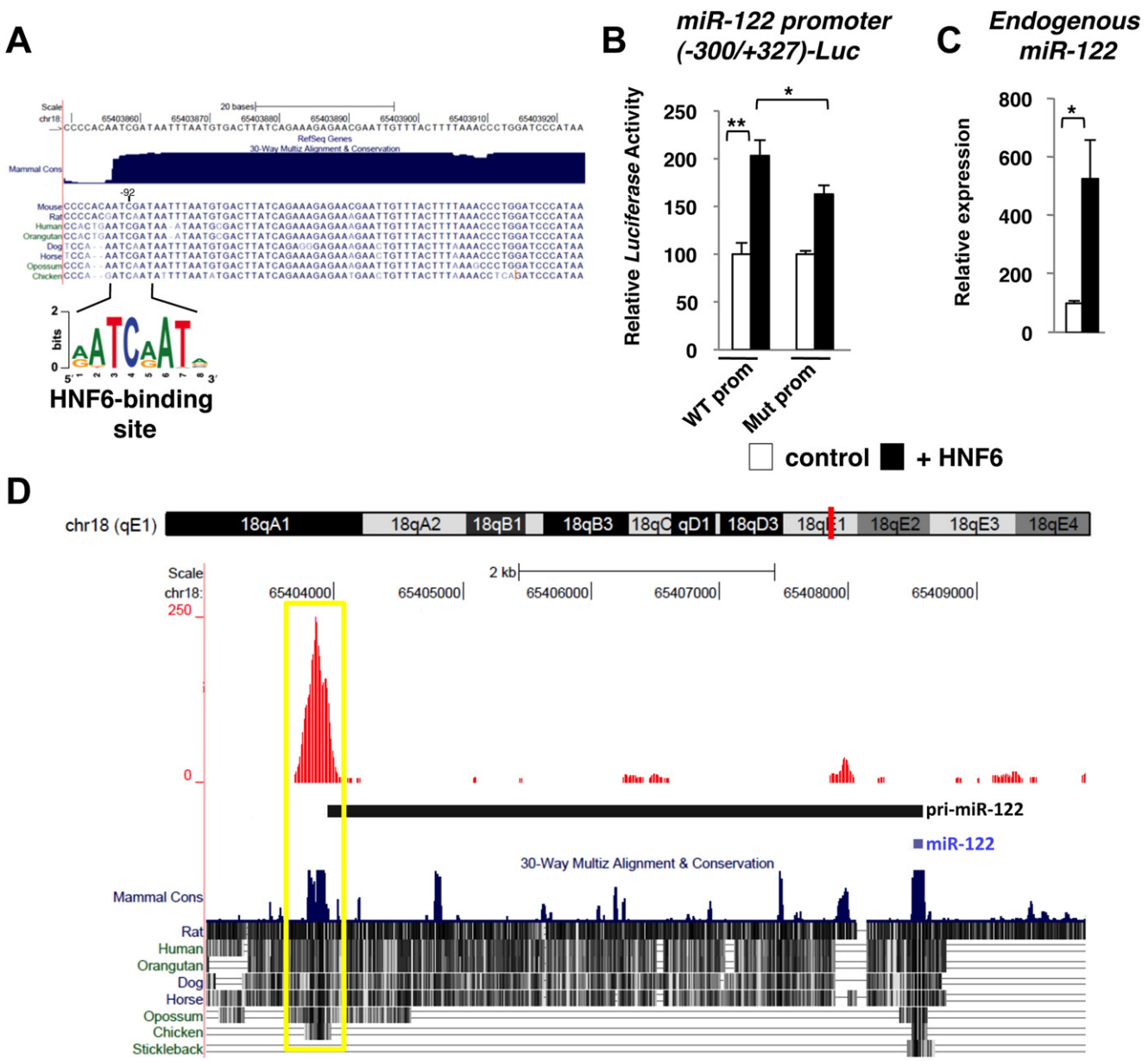
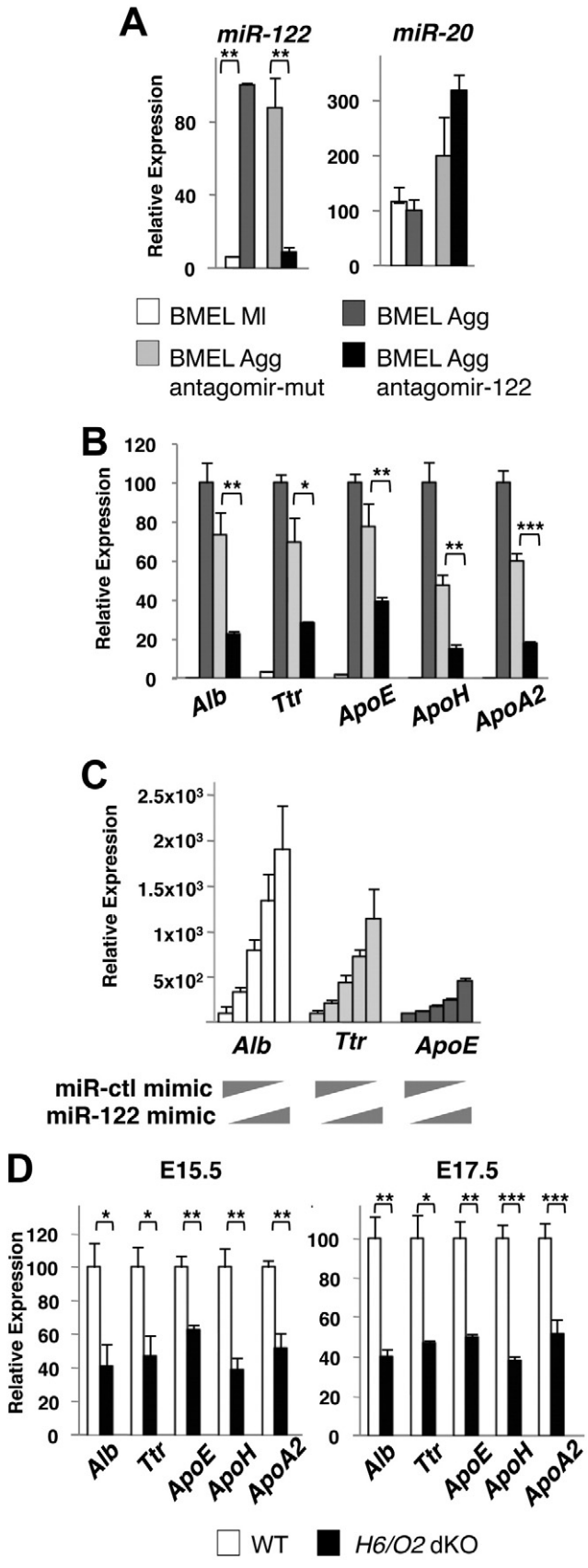


Figure 1. HNF6 binds to and stimulates the miR-122 gene promoter. (*A*) Alignment of the *miR-122* gene revealed evolutionary conservation of an HNF6 binding site in the promoter. (*B*) Overexpression of HNF6, but not of GFP (control), stimulated the expression of a miR-122 promoter-luciferase reporter construct in transfected BMEL cells. Mutation of the HNF6 binding site in the miR-122 promoter reduced the stimulation by HNF6. (*C*) Overexpression of HNF6 in BMEL cells stimulated expression of endogenous miR-122, as measured by Q-RT-PCR. (*D*) ChIP-Seq shows that HNF6 binds to *miR-122* in liver, with the strongest peak overlapping with the promoter region; the latter (*yellow box*) was stimulated by HNF6 in BMEL cells. Data are displayed using the UCSC Mouse Genome Browser (NCBI37/mm9) (http://genome.ucsc.edu/). Data in *B* and *C* are means ± SEM; n = 3; $^{*}P < .05$; $^{**}P < .01$.

was strongly up-regulated in aggregate-cultured BMEL cells (BMEL Agg) as compared with BMEL cells maintained in monolayer culture (BMEL Ml), thereby mimicking the increase in miR-122 occurring in vivo during hepatocyte differentiation. When anti–miR-122 antagomir was added to aggregate-cultured BMEL cells (BMEL Agg antagomir-122), the induction of miR-122 was abolished, as compared with cells treated with a mutated antagomir (BMEL Agg antagomir-mut; Figure 2*A*). The expression of the unrelated miR-20 was not significantly affected by anti–miR-122 antagomir, thereby showing the specificity of miR-122 inhibition (Figure 2*A*).

To investigate the function of miR-122, we compared the messenger RNA (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 (Supplementary Figure 2). 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 hepatocyte-specific genes (Supplementary Figure 2). The microarray data were validated by Q-RT-PCR analysis of 5 genes down-regulated by anti–miR-122 antagomir (Figure 2*B*).

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 *apolipoprotein* (*Apo*) *E* was increased in a dose-dependent way, thereby confirming that miR-122 stimulates hepatocyte-specific genes (Figure 2*C*).

Because miR-122 expression is down-regulated in *H6/O2* dKO liver (Table 1 and Supplementary Figure 1), 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 down-regulated in *H6/O2* dKO liver at E15.5 and E17.5 (Figure 2*D*).

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 HNF6 and OC2 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 is required for hepatocyte differentiation in vivo, we turned to the zebrafish. In this organism, miR-122 is specifically expressed in liver[33] (Supplementary Figure 3*A*). During development, all hepatic epithelial cells express Prox1 (Figure 3*A*). At 50 hours postfertilization, the hepatoblasts coexpressed 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 arrowheads* in Figure 3*A*, *upper panels*). Later, at 80 hours postfertilization, HNF4α and 2F11 were restricted to hepatocytes and cholangiocytes, respectively (Figure 3*A*, *middle panels*); only very few cells, localized in the peripheral layer of hepatic cells, still coexpressed HNF4α and 2F11 (*white arrowhead* in Figure 3*A*, *middle right panel*). Later in development, such HNF4α$^+$/2F11$^+$ cells were no longer found

Figure 2. miR-122 stimulates the expression of hepatocyte-specific genes during hepatocyte-like differentiation in vitro. (*A*) BMEL cells were cultured in monolayer (BMEL MI) or in floating aggregates (BMEL Agg) to induce hepatocyte-specific genes. After 72 hours 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) served as control. Expression of the unrelated miR-20 was unaffected by antagomir treatment. (*B*) *Alb*, *Ttr*, *ApoE*, *ApoH*, *and ApoA2* induction in aggregate BMEL cell cultures was down-regulated by miR-122 inhibition. (*C*) *Alb*, *Ttr*, and *ApoE* expression in BMEL cells (monolayer) was dose-dependently stimulated by transfected miR-122 mimic (0, 0.1, 0.25, 0.5, 1 μg). (*D*) Hepatocyte-specific genes targeted by miR-122 were down-regulated in *H6/O2* dKO livers at E15.5 and E17.5. Means ± SEM; n = 3; **P* < .05; ***P* < .01; ****P* < .001.

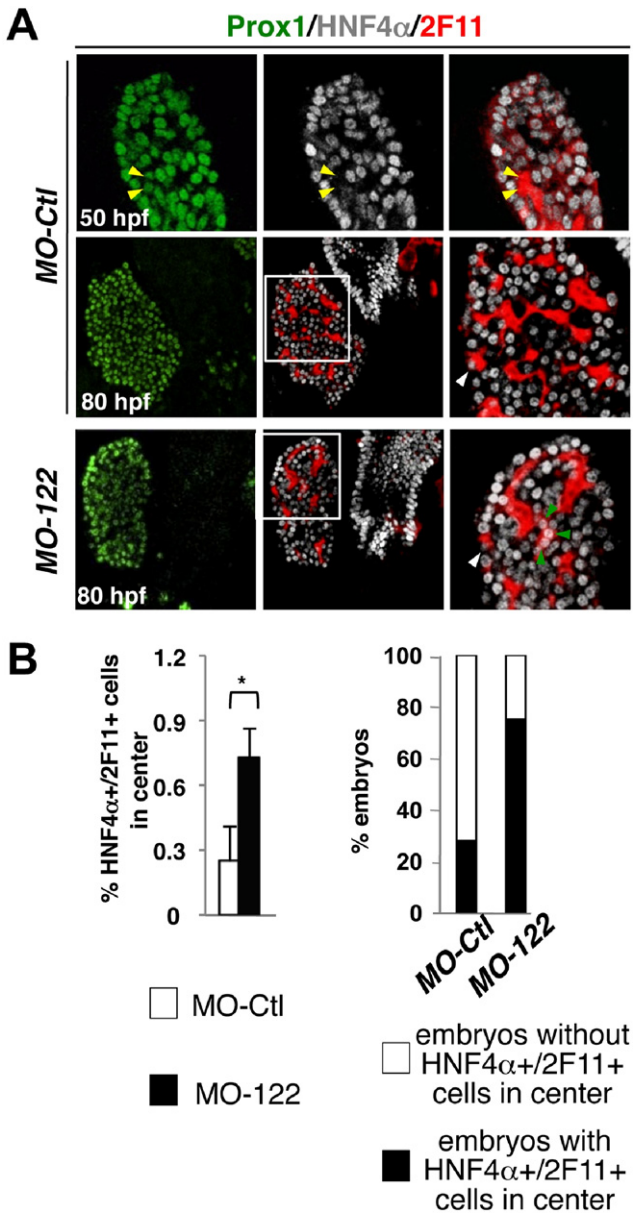


Figure 3. miR-122 is required for normal liver development in zebrafish embryos. (*A*) In zebrafish embryos at 50 hours postfertilization, hepatic cells (Prox1$^+$) committed to the hepatocyte lineage coexpressed HNF4α and low levels of 2F11, whereas cells committed to the cholangiocyte lineage (*yellow arrowheads*) expressed high levels of 2F11 and were HNF4α^-. At 80 hours postfertilization in control morpholino-injected embryos (MO-Ctl), hepatocytes were HNF4α^+/2F11$^-$, and a few cells localized at the periphery of the liver were still HNF4α^+/2F11$^+$ (*white arrowhead*). In anti–miR-122 morpholino-injected embryos (MO-122) at 80 hours postfertilization, HNF4α^+/2F11$^+$ were found in the peripheral layer of hepatic cells (*white arrowhead*) but also in the center of the organ (*green arrowheads*). (*B*) Proportion of HNF4α^+/2F11$^+$ cells in total number of hepatic cells (Prox1$^+$) in the center of the liver at 80 hours postfertilization (*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 in *B* are means ± SEM; n ≥ 7; *$P < .05$.

in control liver (data not shown). Therefore, in zebrafish, segregation of the hepatocyte lineage is characterized by transient coexpression of HNF4α^+ and 2F11$^+$, followed by differentiation toward HNF4α^+/2F11$^-$ hepatocytes.

In embryos injected with the anti–miR-122 morpholino-oligonucleotide (MO-122), miR-122 expression was knocked down (Supplementary Figure 3*A*) and HNF4α^+/2F11$^+$ cells were found not only in the peripheral layer of the liver (*white arrowhead* in Figure 3*A*, *lower panel*), but also in the center of the liver (*green arrowheads* in Figure 3*A*, *lower panels*). Quantification showed that such cells were more abundant and more frequently present in MO-122–injected embryos (Figure 3*B*) than in the controls. Later, 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). Deficient hepatocyte differentiation in miR-122 morphants at 80 hours postfertilization was not due to a global developmental delay. For instance, pancreatic acinar tissue, which undergoes key developmental events between 52 and 84 hours postfertilization, grew and differentiated normally (Supplementary Figure 3*B* and *C*). Hepatocyte proliferation, measured by incorporation of 5-ethynyl-2′-deoxyuridine (EdU), was unaffected by MO-122; 29.8% ± 2.3% (mean ± SD) and 28.3% ± 1.1% EdU$^+$/HNF4α^+ cells were found in control embryos and morphants, respectively. Rescue experiments by combining injection of anti–miR-122 morpholino and miR-122 duplex were not conclusive due to high toxicity of the miR-122 duplex. We concluded that miR-122 is specifically required for normal hepatic cell differentiation in vivo.

In Vivo Stimulation of Hepatocyte Differentiation by miR-122 Depends on HNF6

To study how miR-122 regulates hepatic cell differentiation in coordination with LETFs, 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) to E15.5 (1.7-fold) and then returned to normal values (Figure 4*A*). Interestingly, at E15.5, the hepatocyte differentiation markers that were stimulated by miR-122 in cultured cells (Figure 2) were also up-regulated in *miR-122* Tg livers (Figure 4*B*). Their expression levels at E15.5 in *miR-122* Tg livers were similar to those in WT livers at E17.5 (Supplementary Figure 4), indicating that miR-122 accelerates hepatocyte differentiation.

We next measured, in *miR-122* Tg mice, the expression of HNF1α, HNF1β, Foxa2, HNF4α, and HNF6/OC2, which constitute the core of the LETF network.[5] At E15.5, we found a 1.4-fold increase in Foxa2, HNF4α, and OC2 but a stronger 5-fold increase in HNF6 as compared with controls (Figure 4*C*). HNF6 expression was stimulated in *miR-122* Tg livers from E13.5 onward and paralleled that of miR-122 (Figure 4*D*). Immunostaining on sections of WT livers at E15.5 showed predominant expression of HNF6 in biliary cells forming the ductal plate and lower expression in hepatoblasts and developing hepatocytes[8,34,35] (Figure 4*F*). In E15.5 *miR-122* Tg livers, HNF6 was up-regulated in hepatocytes, thereby mimicking later

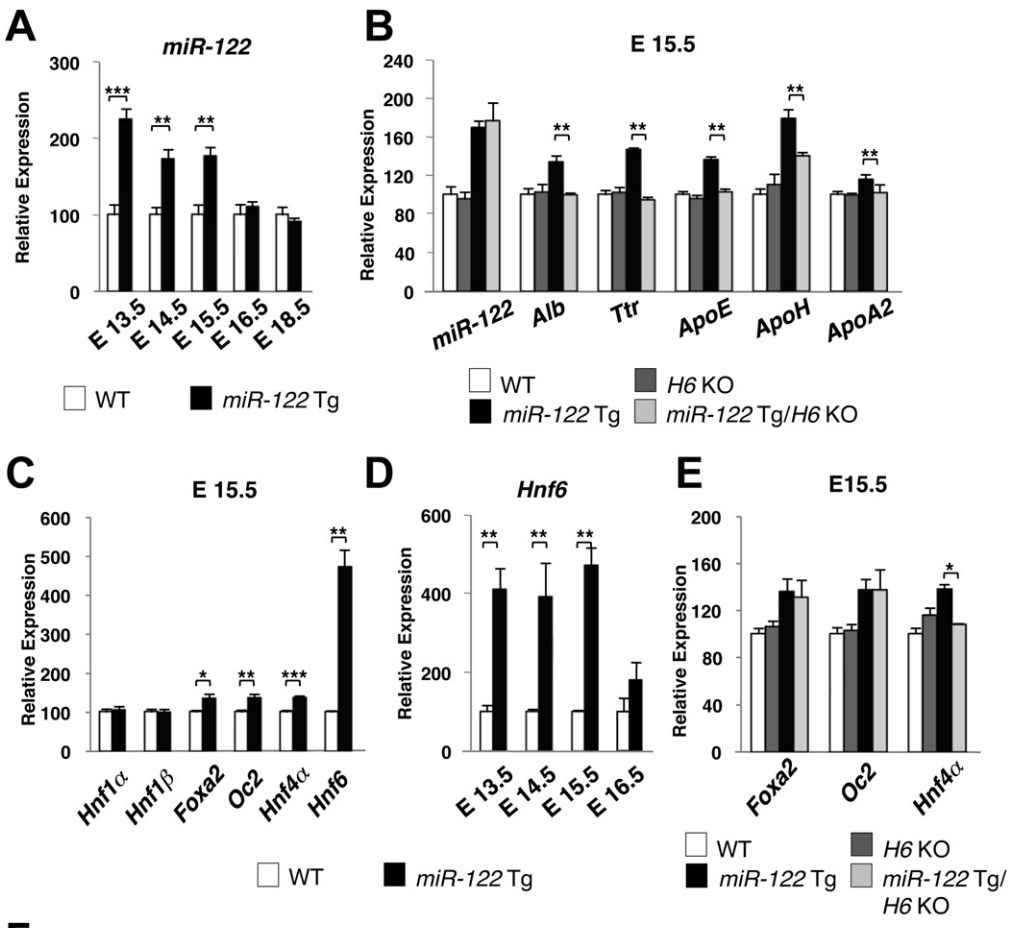


Figure 4. In vivo stimulation of hepatocyte differentiation by miR-122 depends on HNF6. (*A*) In livers from *miR-122* Tg mice, miR-122 was overexpressed from E13.5 to E15.5. (*B*) Overexpression of miR-122 stimulated expression of hepatocyte-specific genes in the presence (*miR-122* Tg) but not in the absence of HNF6 (*miR-122* Tg/*H6* KO). (*C*) Expression of the FoxA2, OC2, HNF4α, and HNF6, but not HNF1α and HNF1β, was stimulated in *miR-122* Tg. (*D*) Stimulation of *Hnf6* expression in *miR-122* Tg livers was detected from E13.5 to E15.5. (*E*) Stimulation of HNF4α, but not of Foxa2 and OC2, in *miR-122* Tg embryos depended on HNF6. (*F*) 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; *$P < .05$; **$P < .01$; ***$P < .001$. E-cad, E-cadherin; pv, portal vein. *Asterisk*, lumen of biliary structures; *dotted lines* delineate biliary structures.

stages of liver maturation[34] (Figure 4*F*). Therefore, overexpression of miR-122 induces a premature increase of *Hnf6* in differentiating hepatocytes while increasing 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 depends on HNF6. First, we verified by ChIP-Seq if HNF6 binds to the genes up-regulated by miR-122. This was the case (Supplementary Figure 5).

We then asked if HNF6 mediates the effects of miR-122 by overexpressing miR-122 in an 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 (Figure 4*B*). 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* (Figure 4*E*). Importantly, 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; Figure 4*B*). Therefore, we concluded that HNF6 is required for stimulation of hepatocyte differentiation by miR-122.

Overexpression of miR-122 Induces HNF6-Independent Biliary Differentiation

MiR-122 Tg embryos presented with biliary hyperplasia characterized by more numerous and larger ducts (Figure 4*F*). Because biliary hyperplasia did not result from proliferation (data not shown), we investigated bil-

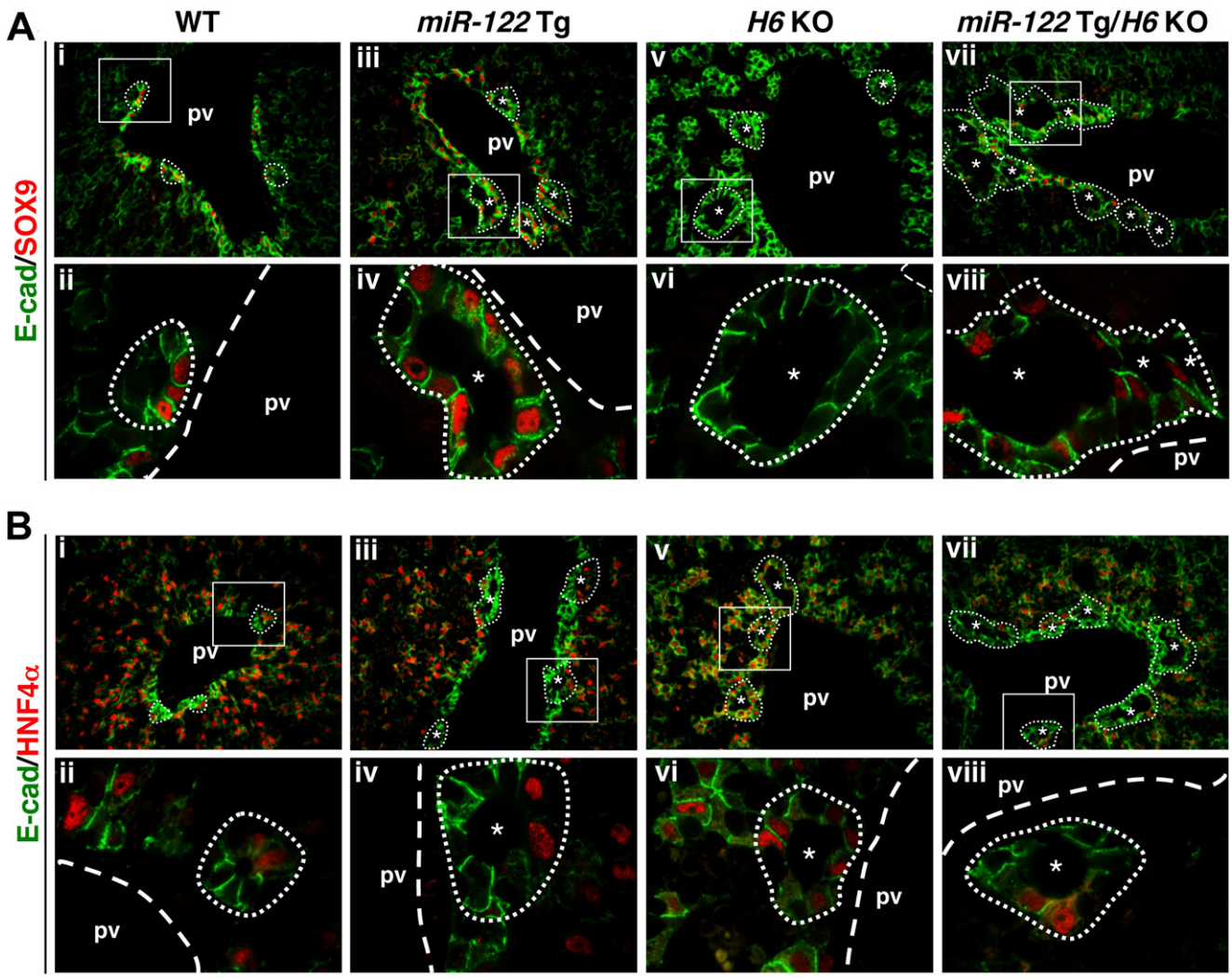


Figure 5. Abnormal biliary differentiation induced by overexpression of miR-122 is not dependent on HNF6. At the onset of bile duct development in WT livers at E15.5, SOX9 is expressed on the portal side of ducts (*A*, *i–ii*) and HNF4α on their parenchymal side (*B*, *i–ii*). In *miR-122* Tg mice, SOX9 is prematurely induced on the parenchymal side of developing ducts (*A*, *iii–iv*), whereas HNF4α is normally expressed on their parenchymal side (*B*, *iii–iv*). In *Hnf6*$^{-/-}$ mice (*H6 KO*), SOX9 is not expressed (*A*, *v–vi*), and HNF4α is found both on the parenchymal and portal side of ducts but also on their portal side (*B*, *v–vi*). 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 (*A*, *vii–viii*), and HNF4α is found exclusively on their parenchymal side (*B*, *v–viii*). E-cad, E-cadherin; pv, portal vein; *asterisk*, lumen of hyperplastic biliary structures; *dotted lines* delineate biliary structures.

iary differentiation and morphogenesis. Bile duct development normally starts with the formation at E15.5 of asymmetrical ductal structures lined on the portal side by SRY-related HMG box transcription factor (SOX) 9$^+$/HNF4α^- cells and on the parenchymal side by SOX9$^-$/HNF4α^+ cells (Figure 5*A* [*i–ii*] and *B* [*i–ii*]). 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 (Figure 5*A* [*iii–iv*]), thereby mimicking later stages of duct morphogenesis. Transforming growth factor β receptor II (TβRII) and HNF4α were expressed on the parenchymal side of biliary structures in *miR-122* Tg mice, which is the normal location at that stage (Supplementary Figure 6 [*i–ii* and *iii–iv*] and Figure 5*B* [*i–ii* and *iii–iv*]). The biliary hyperplasia in *miR-122* Tg livers was transient (Supplementary Figure 7). In conclusion, overexpression of miR-122 induces biliary hyperplasia and acceleration of SOX9 expression.

We next determined if the effects of miR-122 on biliary development were HNF6 dependent by comparing WT, *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 hyperplastic biliary structures devoid of SOX9 (Figure 5*A* [*v–vi*]) but ectopically expressing HNF4α and TβRII on both the parenchymal and portal side (Figure 5*B* [*v–vi*] and Supplementary Figure 6 [*v–vi*]). Importantly, when miR-122 was overexpressed in an HNF6-null background (*miR-122* Tg/*H6* KO), the phenotype did not significantly differ from that of livers overexpressing miR-122 in a *Hnf6*$^{+/+}$ background; biliary hyperplasia was still detected, most biliary cells expressed SOX9, and HNF4α and TβRII were

expressed asymmetrically, that is, on the parenchymal side of the biliary structures (Figure 5*A* [*vii–viii*] and *B* [*vii–viii*] and Supplementary Figure 6 [*vii–viii*]).

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

Hepatic gene expression is controlled by a network of core LETFs (HNF1α, HNF1β, FoxA2, HNF4α, HNF6, and Liver receptor homolog 1).[5,36] The stability and complexity of this network increases during hepatocyte maturation as a result of the progressive rise in concentration of the transcription factors.[5] Here we add a further layer of regulation by showing that HNF6 directly stimulates miR-122 expression and that miR-122 controls hepatocyte differentiation while stimulating the expression of core LETFs, including HNF6. The effect of miR-122 on hepatocyte differentiation is dependent on HNF6. Because HNF6 stimulates and binds directly to the hepatocyte-specific genes up-regulated by miR-122 (Supplementary Figure 5), we propose that miR-122 stimulates these genes via up-regulation of *Hnf6*. Expression of HNF4α, another regulator of hepatocyte differentiation,[37,38] is also dependent on miR-122 and HNF6, most likely via direct binding of HNF6 to the *Hnf4α* gene[5,36] (Supplementary Figure 5). Together, our data reveal that the positive feedback loop between HNF6 and miR-122 constitutes a regulatory mechanism driving progression of hepatocyte differentiation (Figure 6).

MiR-122 expression is regulated in part by CCAAT/enhancer binding protein α, HNF1α, FoxA2, and HNF4α, both in cultured hepatocellular cancer–derived cells[19–21] and in adult liver.[14] This raises the possibility that not only HNF6 but also other LETFs positively feedback on miR-122 expression to control hepatocyte differentiation.

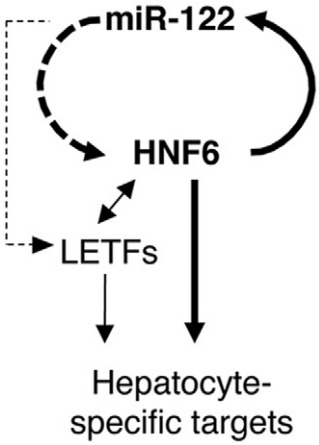


Figure 6. A positive feedback loop between HNF6 and miR-122 drives hepatocyte differentiation. HNF6 directly stimulates miR-122 gene expression, and the stimulation of HNF6 by miR-122 is direct or indirect. Both HNF6 and miR-122 stimulate the expression of LETFs, and HNF6 and LETFs coregulate expression of hepatocyte-specific genes. The effect of miR-122 on hepatocyte differentiation depends on HNF6.

An unresolved question is how miR-122 stimulates *Hnf6* expression. miRNAs can stimulate gene expression by binding to promoters[39] or to mRNAs in their 5′ untranslated region, 3′ untranslated region, or coding sequence.[40–42] However, using bioinformatic tools (TargetScan, PicTar, MicroCosm Target, MultAlin), no miR-122 site was found in the *Hnf6* gene or mRNA. Cotransfection of miR-122 mimics and luciferase reporters driven by *Hnf6* regulatory regions 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 target *Hnf6* mRNA, were not inhibited by miR-122 (data not shown). miR-122 could also inhibit a repressor protein of HNF6. Several transcriptional inhibitors were predicted to be targeted by miR-122,[21,43] but there is no evidence that they repress HNF6 during liver development. Finally, using Ingenuity (Ingenuity Sytems, Redwood City, CA) 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 stimulates HNF6.

A small increase in miR-122 (1.7-fold) is sufficient to 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 physiologic regulator of biliary development. However, they suggest that inaccurate levels of miR-122 can perturb hepatic cell specification.

miR-122 stimulates and represses gene expression 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 among 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 an enzyme that plays a key role in ketone body degradation. Because ketone bodies are synthesized in liver and provide a source of energy to several tissues during fasting, *Oxct1* must be repressed in liver to avoid hepatic catabolism of ketone bodies. Our work provides evidence that *Oxct1* is repressed during hepatocyte maturation and that this repression depends in part on miR-122.[25]

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.

Supplementary Material

Note: To access the supplementary material accompanying this article visit the online version of *Gastroenterology* at www.gastrojournal.org and at doi: 10.1053/j.gastro.2011.09.001.

References

1. Zhao R, Duncan SA. Embryonic development of the liver. Hepatology 2005;41:956–967.
2. Lemaigre FP. Mechanisms of liver development: concepts for understanding liver disorders and design of novel therapies. Gastroenterology 2009;137:62–79.
3. Si-Tayeb K, Lemaigre FP, Duncan SA. Organogenesis and development of the liver. Dev Cell 2010;18:175–189.
4. Simion A, Laudadio I, Prevot PP, et al. MiR-495 and miR-218 regulate the expression of the Onecut transcription factors HNF-6 and OC-2. Biochem Biophys Res Commun 2010;391:293–298.
5. Kyrmizi I, Hatzis P, Katrakili N, et al. Plasticity and expanding complexity of the hepatic transcription factor network during liver development. Genes Dev 2006;20:2293–2305.
6. Clotman F, Jacquemin P, Plumb-Rudewiez N, et al. Control of liver cell fate decision by a gradient of TGF beta signaling modulated by Onecut transcription factors. Genes Dev 2005;19:1849–1854.
7. Plumb-Rudewiez N, Clotman F, Strick-Marchand H, et al. Transcription factor HNF-6/OC-1 inhibits the stimulation of the HNF-3alpha/Foxa1 gene by TGF-beta in mouse liver. Hepatology 2004;40:1266–1274.
8. Antoniou A, Raynaud P, Cordi S, et al. Intrahepatic bile ducts develop according to a new mode of tubulogenesis regulated by the transcription factor SOX9. Gastroenterology 2009;136:2325–2333.
9. Hand NJ, Master ZR, Le Lay J, et al. Hepatic function is preserved in the absence of mature microRNAs. Hepatology 2009;49:618–626.
10. Sekine S, Ogawa R, Ito R, et al. Disruption of Dicer1 induces dysregulated fetal gene expression and promotes hepatocarcinogenesis. Gastroenterology 2009;136:2304–2315.
11. Sekine S, Ogawa R, McManus MT, et al. Dicer is required for proper liver zonation. J Pathol 2009;219:365–372.
12. Hand NJ, Master ZR, Eauclaire SF, et al. The microRNA-30 family is required for vertebrate hepatobiliary development. Gastroenterology 2009;136:1081–1090.
13. Rogler CE, Levoci L, Ader T, et al. MicroRNA-23b cluster microRNAs regulate transforming growth factor-beta/bone morphogenetic protein signaling and liver stem cell differentiation by targeting Smads. Hepatology 2009;50:575–584.
14. Gao Y, Schug J, McKenna LB, et al. Tissue-specific regulation of mouse MicroRNA genes in endoderm-derived tissues. Nucleic Acids Res 2011;39:454–463.
15. Tuteja G, White P, Schug J, et al. Extracting transcription factor targets from ChIP-Seq data. Nucleic Acids Res 2009;37:e113.
16. Lagos-Quintana M, Rauhut R, Yalcin A, et al. Identification of tissue-specific microRNAs from mouse. Curr Biol 2002;12:735–739.
17. Chang J, Nicolas E, Marks D, et al. 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 2004;1:106–113.
18. Landgraf P, Rusu M, Sheridan R, et al. A mammalian microRNA expression atlas based on small RNA library sequencing. Cell 2007;129:1401–1414.
19. Coulouarn C, Factor VM, Andersen JB, et al. Loss of miR-122 expression in liver cancer correlates with suppression of the hepatic phenotype and gain of metastatic properties. Oncogene 2009;28:3526–3536.
20. Zeng C, Wang R, Li D, et al. A novel GSK-3 beta-C/EBP alpha-miR-122-insulin-like growth factor 1 receptor regulatory circuitry in human hepatocellular carcinoma. Hepatology 2010;52:1702–1712.
21. Xu H, He JH, Xiao ZD, et al. Liver-enriched transcription factors regulate microRNA-122 that targets CUTL1 during liver development. Hepatology 2010;52:1431–1442.
22. Poll AV, Pierreux CE, Lokmane L, et al. A vHNF1/TCF2-HNF6 cascade regulates the transcription factor network that controls generation of pancreatic precursor cells. Diabetes 2006;55:61–69.
23. Jacquemin P, Durviaux SM, Jensen J, et al. Transcription factor hepatocyte nuclear factor 6 regulates pancreatic endocrine cell differentiation and controls expression of the proendocrine gene ngn3. Mol Cell Biol 2000;20:4445–4454.
24. Strick-Marchand H, Weiss MC. Inducible differentiation and morphogenesis of bipotential liver cell lines from wild-type mouse embryos. Hepatology 2002;36:794–804.
25. Thorrez L, Laudadio I, Van Deun K, et al. Tissue-specific disallowance of housekeeping genes: the other face of cell differentiation. Genome Res 2011;21:95–105.
26. Manfroid I, Delporte F, Baudhuin A, et al. Reciprocal endoderm-mesoderm interactions mediated by fgf24 and fgf10 govern pancreas development. Development 2007;134:4011–4021.
27. Dong PD, Munson CA, Norton W, et al. Fgf10 regulates hepatopancreatic ductal system patterning and differentiation. Nat Genet 2007;39:397–402.
28. van Eyll JM, Pierreux CE, Lemaigre FP, et al. Shh-dependent differentiation of intestinal tissue from embryonic pancreas by activin A. J Cell Sci 2004;117:2077–2086.
29. Schmidt D, Wilson MD, Spyrou C, et al. ChIP-seq: using high-throughput sequencing to discover protein-DNA interactions. Methods 2009;48:240–248.
30. Li H, Ruan J, Durbin R. Mapping short DNA sequencing reads and calling variants using mapping quality scores. Genome Res 2008;18:1851–1858.
31. Bailey TL, Elkan C. Fitting a mixture model by expectation maximization to discover motifs in biopolymers. Proc Int Conf Intell Syst Mol Biol 1994;2:28–36.
32. Jacquemin P, Lannoy VJ, Rousseau GG, et al. 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 1999;274:2665–2671.
33. Wienholds E, Kloosterman WP, Miska E, et al. MicroRNA expression in zebrafish embryonic development. Science 2005;309:310–311.
34. Clotman F, Lannoy VJ, Reber M, et al. The onecut transcription factor HNF6 is required for normal development of the biliary tract. Development 2002;129:1819–1828.
35. Beaudry JB, Pierreux CE, Hayhurst GP, et al. 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 2006;26:6037–6046.
36. Odom DT, Dowell RD, Jacobsen ES, et al. Core transcriptional regulatory circuitry in human hepatocytes. Mol Syst Biol 2006;2:2006.0017.
37. Battle MA, Konopka G, Parviz F, et al. 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 2006;103:8419–8424.

38. Parviz F, Matullo C, Garrison WD, et al. Hepatocyte nuclear factor 4alpha controls the development of a hepatic epithelium and liver morphogenesis. Nat Genet 2003;34:292–296.
39. Place RF, Li LC, Pookot D, et al. MicroRNA-373 induces expression of genes with complementary promoter sequences. Proc Natl Acad Sci U S A 2008;105:1608–1613.
40. Vasudevan S, Tong Y, Steitz JA. Switching from repression to activation: microRNAs can up-regulate translation. Science 2007; 318:1931–1934.
41. Orom UA, Nielsen FC, Lund AH. MicroRNA-10a binds the 5′UTR of ribosomal protein mRNAs and enhances their translation. Mol Cell 2008;30:460–471.
42. Henke JI, Goergen D, Zheng J, et al. microRNA-122 stimulates translation of hepatitis C virus RNA. EMBO J 2008;27:3300–3310.
43. Tsang JS, Ebert MS, van Oudenaarden A. Genome-wide dissection of microRNA functions and cotargeting networks using gene set signatures. Mol Cell 2010;38:140–153.

Reprint requests

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Conflicts of interest

The authors disclose no conflicts.

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