# 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 $3 \times 10^5$ 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, $3 \times 10^5$ 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). HNF4$\alpha^+$/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.