Cytokine-mediated modulation of the hepatic miRNome: miR-146b-5p is an IL-6-inducible miRNA with multiple targets

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Abstract
Interleukin-6 (IL-6)-type cytokines play important roles in liver (patho-)biology. For instance, they regulate the acute phase response to inflammatory signals and are involved in hepatocarcinogenesis. Much is known about the regulation of protein-coding genes by cytokines whereas their effects on the miRNome is less well understood. We performed a microarray screen to identify microRNAs (miRNAs) in human hepatocytes which are modulated by IL-6-type cytokines. Using samples of 2 donors, 27 and 68 miRNAs (out of 1,733) were found to be differentially expressed upon stimulation with hyper-IL-6 (HIL-6) for up to 72 h, with an overlap of 15 commonly regulated miRNAs. qPCR validation revealed that miR-146b-5p was also consistently up-regulated in hepatocytes derived from 2 other donors. Interestingly, miR-146b-5p (but not miR-146a-5p) was induced by IL-6-type cytokines (HIL-6 and OSM) in non-transformed liver-derived PH5CH8 and THLE2 cells and in Huh-7 hepatoma cells, but not in HepG2 or Hep3B hepatoma cells. We did not find evidence for a differential regulation of miR-146b-5p expression by promoter methylation, also when analyzing the TCGA data set on liver cancer samples. Inducible overexpression of miR-146b-5p in PH5CH8 cells followed by RNA-Seq analysis revealed effects on multiple miRNAs, including those encoding IRAK1 and TRAF6 crucial for Toll-like receptor signaling. Indeed, LPS-mediated signaling was attenuated upon overexpression of miR-146b-5p, suggesting a regulatory loop to modulate inflammatory signaling in hepatocytes. Further validation experiments suggest DNAJC6, MAGEE1, MPHOSPH6, PPP2R1B, SLC10A3, SNRNP27, and TIMM17B to be novel targets for miR-146b-5p (and miR-146a-5p).

KEYWORDS
hepatocarcinogenesis, IL-6-type cytokines, liver, microRNAs, miR-146a-5p

1 | INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver cancer, accounting for 85–90% of primary hepatic malignancies. Over the last decades, its incidence rate has increased in Western countries, reflected by the situation in the United States where the rate raised from 2.6 per 100,000 in 1975 to 8.5 per 100,000 in 2014. This cancer is heavily linked to the inflammatory status of the liver and arises usually in patients with advanced chronic liver diseases.

Interleukin-6 (IL-6) is a pleiotropic cytokine mainly secreted by immune cells such as macrophages and lymphocytes and is involved in various processes including immune regulation, hematopoiesis, inflammation, and cancer. IL-6 stimulates the acute phase response, regulates the acute phase response to inflammatory signals, and is involved in hepatocarcinogenesis. The mechanism of IL-6 action is complex and involves the activation of multiple signaling pathways including the nuclear factor kappaB (NF-κB) and Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways.

Interleukin-6-type cytokines (HIL-6, OSM) are important mediators of inflammation and hepatocarcinogenesis. They play a role in the acute phase response, regulate the inflammatory response, and are involved in the development of various liver diseases, including hepatocellular carcinoma. The role of interleukin-6-type cytokines in the modulation of the miRNome is less well understood. This study aimed to identify microRNAs (miRNAs) in human hepatocytes which are modulated by IL-6-type cytokines. Using samples of 2 donors, 27 and 68 miRNAs (out of 1,733) were found to be differentially expressed upon stimulation with hyper-IL-6 (HIL-6) for up to 72 h, with an overlap of 15 commonly regulated miRNAs. qPCR validation revealed that miR-146b-5p was also consistently up-regulated in hepatocytes derived from 2 other donors. Interestingly, miR-146b-5p (but not miR-146a-5p) was induced by IL-6-type cytokines (HIL-6 and OSM) in non-transformed liver-derived PH5CH8 and THLE2 cells and in Huh-7 hepatoma cells, but not in HepG2 or Hep3B hepatoma cells. We did not find evidence for a differential regulation of miR-146b-5p expression by promoter methylation, also when analyzing the TCGA data set on liver cancer samples. Inducible overexpression of miR-146b-5p in PH5CH8 cells followed by RNA-Seq analysis revealed effects on multiple miRNAs, including those encoding IRAK1 and TRAF6 crucial for Toll-like receptor signaling. Indeed, LPS-mediated signaling was attenuated upon overexpression of miR-146b-5p, suggesting a regulatory loop to modulate inflammatory signaling in hepatocytes. Further validation experiments suggest DNAJC6, MAGEE1, MPHOSPH6, PPP2R1B, SLC10A3, SNRNP27, and TIMM17B to be novel targets for miR-146b-5p (and miR-146a-5p).
inflammatory processes as well as the modulation of cellular growth and apoptosis (reviewed in Garbers et al.6 and Guo et al.5). In the liver, IL-6 triggers liver regeneration, e.g. after partial hepa
tectomy (reviewed in Taub6) and protects the liver against apoptosis follow-
ing viral infection or ingestion of chemicals2,8 and necrosis (reviewed in Schmidt-Arras and Rose-John9). IL-6 is important for tumor deve-
lopment, regulating angiogenesis as well as migration, invasion, pro-
liferation, and apoptosis of tumor cells (reviewed in Fisher et al.10).

Its action is mainly mediated by the Janus kinase (JAK)/signal trans-
ducer and activator of transcription 3 (STAT3) signaling pathway. Upon phosphorylation, STAT3 homodimers are formed and translocate to the nucleus where they modulate the expression of many genes includ-
ing several cytokines (e.g., EGF, HGF, and VEGF), apoptosis inhibitors such as Bcl-2, Bcl-xl, survivin, Mcl-1, and XIAP,11,12 cell-cycle-related
genes such as cyclin D1,13 metastasis-related genes matrix metallo-
protease 1 (MMP1), MMP2,14 and MUC1,15 and acute phase pro-
teins (APP) specifically in the liver.16

Both IL-6 and STAT3 have been shown to promote HCC develop-
ment in mouse models. While 100% of male mice develop liver
tumors upon N,N-diethylnitrosamine (DEN) treatment, only 20% of IL-6−/−
males were treated with DEN develop HCC.17 Similarly, a liver-
specific knock-out of STAT3 in mice results in a significant decrease in HCC prevalence and smaller tumor size.18 In addition, deletion of STAT3 in cultured DEN-induced mouse hepatocytes leads to cell
death whereas cells with only partial reduction are viable but with a
senescent phenotype and without capacities to form subcutaneous
tumors, showing that activated STAT3 is required for survival of HCC
cells.18 Finally, aberrant activation of this pathway, e.g. due to gain-of-
function mutations affecting STAT3 or gp130 (as observed in inflam-
atory hepatocellular adenomas),19,20 or the constitutive release of
cytokines which activate STAT3 (mainly IL-6),21 has also been shown to
occur in cancer cell lines and solid tumors (reviewed in Guo et al.5
Subramaniam et al.22).

MicroRNAs (miRNAs) are small non-coding RNAs characterized by their length, between 20 and 30 nucleotides, and their association with Argonaute (AGO) proteins. They function as guides during the RNA interference process by binding to complementary sequences, mainly in the 3’UTR of mRNAs, leading to the cleavage of the target (in case of perfect complementarity), its translational repression or its deadenylation (in case of imperfect complementarity) (reviewed in Gu and Kay23). In humans, most of miRNAs are intergenic or located in introns of both non-coding and coding transcripts. However, a small portion can also be found inside exon sequences (reviewed in Olena and Patton24). When primary-miRNA (pri-miRNA) sequences are located inside coding mRNAs, they usually share the host promoter and are thereby co-transcribed. In some cases, the miRNA promoter can be distinct from the one of the mRNA host gene, allowing for an independent expression.25,26

While the regulation of the mRNA transcriptome mediated by IL-6 has been well studied, the modulation of the miRNome remains more elusive. So far, few miRNAs have been identified as being regulated by IL-6 and/or STAT3 (reviewed in Cao et al.27). Let-7 family members,28
miR-17–92 cluster,29 miR-181b-1–30 miR-21,30,31 and also miR-146b-5p.32 Therefore, we decided to further investigate the impact of IL-6 on the miRNome of both primary hepatocytes and liver-derived cell lines.

In the current study, we show that in both primary hepatocytes and non-neoplastic liver-derived cell lines miR-146b-5p is induced upon IL-6 stimulation whereas this is only the case in 1 out of 3 HCC cell
lines. In contrast to the situation in breast cancer,32 the methylation of specific CpG islands in the promoter of pri-miR-146b does not correlate with its inducibility in cell lines. In addition, miR-146b-5p does not seem to be differentially expressed in tumor samples in compari-
sion to the matched healthy adjacent tissue. Finally, we identified new target candidates of this miRNA, which may be related to its sug-
gested tumor suppressor role in cancer, and found evidence for a
regulatory feedback loop involving IL-6, miR-146b-5p, and the NFκB
signaling pathway.

2 MATERIALS AND METHODS

2.1 Ethical approval

Human primary hepatocyte collection was approved by the respective ethics committees in Germany (Ethik-Kommission der Aerztekammer des Saarlands, 79/12) and Luxembourg (Comité National d’Éthique de Recherche, 201309/07), and signed statements of informed consent were obtained from all patients.

2.2 Materials and cell culture

All cells were grown at 37°C in a water-saturated atmosphere at 5% CO2. The hepatoma cell lines Hep3B (bought from DSMZ, Braunschweig, Germany) and Huh-7 (bought from JCRB, Ibaraki city, Osaka, Japan) as well as the immortalized non-neoplastic PH5CH8 cells (kindly provided by Prof. N. Kato, University of Okayama, Japan) were maintained in DMEM medium (Lonza, Basel, Switzerland) supplemented with 10% FBS (Gibco, Thermo Fisher Scientific, Erembodegem-Aalst, Belgium), 25 nM HEPES (Lonza, Basel, Switzerland) and 100 μg/ml normocin (InvivoGen, Toulouse, France). HepG2 cells (bought from ATCC, Manassas, Virginia, USA), were cultured in DMEM:F12 medium (1:1 mixture with HEPES and L-glutamine, Lonza, Basel, Switzerland) supplemented with 10% FBS, 25 nM HEPES, and 100 μg/ml normocin. SV40 large T antigen-immortalized non-neoplastic THLE2 cells (bought from ATCC, Manassas, Virginia, USA) were maintained in LHC-8 medium (Thermo Fisher Scientific, Erembodegem-Aalst, Belgium) supplemented with 10% FBS, 70 ng/ml O-phosphoethanolamine (Sigma-Aldrich, Diegem, Belgium), 5 ng/ml epidermal growth factor (PeproTech, Tebu-Bio, Boechout, Belgium) and 100 μg/ml normocin. Before seeding of THLE2 cells, flasks and plates were coated with 1 mg/ml bovine collagen (Corning, Amsterdam, The Netherlands) and 3.1 mg/ml fibronectin (Corning, Amsterdam, The Netherlands) for at least 1 h at 37°C. Primary healthy liver
cells (named HepLux 5, 6, 7, and 8), derived from patients with colon cancer who underwent liver metastasis surgery, were received from the Department of Surgery of the Saarland University Hospital (Homburg, Germany). Directly after surgery, hepatocytes were isolated,
plated in collagen type I precoated flasks (Corning, VWR International, Leuven, Belgium) and incubated overnight at 37°C. The next day, the flasks were transferred to the Luxembourgh laboratory where the cells were immediately trypsinized (Trypsin/EDTA, Lonza, Basel, Switzerland), counted and plated in 6-well plates coated with 1 mg/ml bovine collagen. Depending on the isolation, different densities of cells were used in order to have duplicates or triplicates for all the conditions. Cells were maintained in HCM medium (HCM BulletKit Medium, Lonza, Basel, Switzerland) supplemented with 10% FBS and 100 µg/ml normocin. Six hours before the first stimulation, HCM medium was replaced by serum-free hepatocyte high performance medium (Upcyte Technologies, Hamburg, Germany).

2.3 Generation of PH5CH8 cells inducibly overexpressing miR-146b-5p

To further investigate the effects of miR-146b-5p, PH5CH8 cells were transduced with lentiviral particles (Dharmacon, GE Healthcare, Diegem, Belgium) containing a TurboGFP reporter followed by a SMARTvector universal scaffold containing pre-mir-146b. Both the expression of TurboGFP and of miR-146b-5p were induced upon doxycycline treatment (1 µg/ml Dox, Sigma-Aldrich, Diegem, Belgium). Briefly, cells were transduced with lentiviral particles at a multiplicity of infection of 0.3 and 4 µg/ml polybrene (Sigma-Aldrich, Diegem, Belgium) following the manufacturer’s instructions and grown under normal conditions. Three days after transduction, the antibiotic selection (0.3 µg/ml puromycin, InvivoGen, Toulouse, France) started and was maintained for 1 week.

2.4 Cytokine and LPS stimulation

Cytokines were used at the following concentrations: 5 ng/ml for TNFα (PeproTech, Tebu-Bio, Boechout, Belgium), 20 or 40 ng/ml for HIL-6 (a “designer cytokine” comprising IL-6 bound to the extracellular domain of IL-6Rα, a kind gift of Prof. Stefan Rose-John, University of Kiel, Germany), 20 ng/ml for OSM (PeproTech, Tebu-Bio, Boechout, Belgium) and 50 ng/ml for IFNγ (PeproTech, Tebu-Bio, Boechout, Belgium). LPS (Sigma-Aldrich, Diegem, Belgium) was used at 100 µg/ml. Briefly, cells were seeded and either kept untreated (Ctrl) or stimulated with HIL-6 for 6, 24, 48, and 72 h (time-course experiments, Fig. 1) or with TNFα, HIL-6, OSM, or IFNγ for 72 h (Fig. 2). To compare the effects of miR-146b-5p on LPS- and TNFα-induced genes (MCP1 and IL-6), PH5CH8-146b cells were treated with Dox for 48 h and then stimulated either with LPS or TNFα for 4 h (Fig. 4D). For RNA extraction, all cells were harvested at the end of the experiment.

2.5 Flow cytometry

Huh-7 and Hep3B cells were resuspended in cold PBS supplemented with 5% FBS and 0.1% sodium azide and incubated with a mouse antibody specific for OSM-R (sc-9992, Santa Cruz, Heidelberg, Germany) or the corresponding IgG control antibody (21275534, Immunotools, Friesoythe, Germany) for 1 h at 4°C. Cells were washed with cold PBS/azide and incubated with a secondary antibody against mouse IgG and coupled with R-phycocerythrin (1:100, 12-4015-82, eBioscience, Vienna, Austria) for 1 h at 4°C. Cells were then washed twice with cold PBS/azide and analyzed on a FACSCanto II flow cytometer using FACSDiva (BD Biosciences, Erembodegem, Belgium) software. Overlays were created using FlowJo software (Ashland, Oregon, USA).

2.6 Transfection of miR-146a-5p and miR-146b-5p mimics

Cells were reverse-transfected with 20 nM of negative mimic control 1 (NCM1, CN-001000-01-05), miR-146a-5p (C-300630-03-0005) or miR-146b-5p (C-300754-03-0005) mimics (miRIDIAN miRNA mimics from Dharmacon, GE Healthcare, Diegem, Belgium), using HiPerFect transfection reagent (Qiagen, Venlo, The Netherlands) according to the manufacturer’s instructions. After 72 h, cells were harvested and RNA was extracted.

2.7 Dual luminescence reporter assay

Hek293T cells were seeded in 96-well plates and co-transfected the next day with 20 nM of NCM1, miR-146a-5p or miR-146b-5p mimics, and 100 ng of PEZX-MT05 vector (GeneCopoeia, Tebu-Bio, Boechout, Belgium) containing the gene of the secreted Gaussia luciferase (GLuc) followed by the 3'UTR of a specific candidate target gene (synthesized and cloned by GeneCust, Ellange, Luxembourg) using DharmaFECT Duo transfection reagent (Dharmacon, GE Healthcare, Diegem, Belgium) following the manufacturer’s instructions. The following 3'UTR sequences were analyzed: CPA4 (NM_016352.3, 1504 bp), DNAJC6 (NM_01256864.1, 2844 bp), MAGEE1 (NM_020932.2, 562 bp), MPHOSPH6 (NM_005792.2, 569 bp), PPP2R1B (NM_002716.4, 3715 bp), PTX3 (NM_002852.3, 666 bp), SLC10A3 (NM_019848.4, 219 bp), SNRNP27 (NM_006857.2, 942 bp), or TIMM17B (NM_001167947.2, 299 bp). After 2 days of transfection, cell supernatants were harvested for subsequent measurements. GLuc activity was assayed in 10 µl of supernatant by adding 100 µl of GLS buffer preincubated with the substrate (LF033, GeneCopoeia, Tebu-Bio, Boechout, Belgium). Secreted alkaline phosphatase activity was measured in 10 µl of preheated supernatant (10 min at 65°C) by adding 100 µl of AP buffer preincubated with the substrate. Both activities were determined with the CLARIOstar plate reader (BMG Labtech, ISOGEN Life Science, PW De Meern, The Netherlands) with a gain set to 3000 to enable comparison of experiments. For each condition, at least 3 independent biological replicates were performed, including 3 technical replicates each.

2.8 Demethylation studies

Briefly, cells were seeded at day 0 and 8 h later treated with the vehicle alone (0.001% acetic acid, Carl Roth, Karlsruhe, Germany) or 1 µM S-aza-2’-deoxycytidine (5’AZA, Sigma-Aldrich, Diegem, Belgium). At days 1 and 3, fresh medium with 1 µM 5’AZA (or the vehicle) as well as 40 ng/ml HIL-6 were added to the cells. For RNA extraction, all cells were harvested at day 5.
miR-146b-5p is up-regulated upon HIL-6 stimulation in primary human hepatocytes. (A) In total, 27 and 68 miRNAs were significantly differentially expressed (FDR < 0.05), considering the whole time course upon stimulation with HIL-6 in HepLux5 and HepLux6 (primary hepatocytes isolated from 2 patients with colon cancer who underwent liver surgery to remove metastasis), respectively, including 15 common miRNAs. The indicated log2FC values were calculated for the 72 h time point with respect to the untreated control (Ctrl) sample. (B) HepLux5 and HepLux6 cells were stimulated for the indicated periods of time with HIL-6. Lysates were analyzed by Western blot using antibodies against pSTAT3 and total STAT3. Vinculin was detected as loading control. No Western blot analysis could be performed for HepLux7-8 due to limited amount of primary cells. (C) Primary hepatocytes from 4 patients (HepLux5-8) were left unstimulated (Ctrl) or stimulated with HIL-6 for 6 up to 72 h. Expression levels of miR-146b-5p were measured by qPCR and normalized to the control. Error bars represent the standard deviation of 2 (6, 24, 48 h) or 3 (Ctrl, 72 h) technical replicates (i.e., experiments done in parallel) (for HepLux8, 1 of the 2 "24 h samples" showed aberrant profiles and had to be removed with only 1 remaining sample depicted).

2.9 Total RNA isolation

Depending on the application and sample material, total RNA was extracted using different kits: the miRNeasy kit with an additional on-column DNase I digestion (Qiagen, Venlo, The Netherlands) for miRNA microarrays, the Quick-RNA MiniPrep Kit (Zymo Research, Laborimpex, Brussels, Belgium) for qPCR validation as well as for RNA-Seq analysis, and the Column-zol RNA isolation kit (Enzymax LLC, Lexington, Kentucky, USA) for patient-derived primary hepatocytes, according to the respective manufacturer’s instructions.

2.10 miRNome profiling

Microarray experiments were performed at the Proteome and Genome Research Unit at the Luxembourg Institute of Health (Strassen, Luxembourg). Extracted total RNAs were used for downstream microarray studies using Affymetrix GeneChip miRNA 3.0 Arrays (based on miRBase version 17, Affymetrix, Thermo Fisher Scientific, Santa Clara, California, USA) according to the manufacturer’s instructions. Results were analyzed as previously published by our group.34 Here, among all features available at Affymetrix miRNA
miR-146b-5p is up-regulated in liver cell lines upon stimulation with various cytokines. (A) Alignment of hsa-miR-146a-5p and hsa-miR-146b-5p sequences with seed sequences in bold and mismatches in gray. (B–F) miR-146a-5p and miR-146b-5p expression levels upon cytokine stimulation in non-neoplastic (B) PH5CH8 and (C) THLE2 cells as well as in neoplastic (D) Huh-7, (E) HepG2, and (F) Hep3B cells. Cells were left unstimulated (Ctrl) or stimulated with HIL-6, OSM, IFNγ, or TNFα for 72 h. Isolated RNA was analyzed by qPCR and normalized to the control. Error bars represent the standard deviation of at least 3 biological replicates. (G) Western blot analysis of the phosphorylation status of STAT1 and STAT3 in lysates of the various hepatic cell lines stimulated for 72 h with HIL-6, OSM, IFNγ, or TNFα. Detections with antibodies against total levels of STAT1 and STAT3 are also shown. Vinculin was used as loading control.

2.11 Gene expression analysis

Five hundred nanograms of total RNA was reverse-transcribed with the miScript II RT kit (Qiagen, Venlo, The Netherlands) in a volume of 10 μl according to the manufacturer’s instructions. Quantitative real time PCR (qPCR) was carried out on a CFX384 Detection System (BioRad, Temse, Belgium) using 5 (miRNA detection) or 50 ng (mRNA detection) RNA input in a 10 μl reaction volume, 2X iTaq SYBR Green Supermix (BioRad, Temse, Belgium) and 1 μl of 10X miRNA-specific primers (Qiagen, Venlo, The Netherlands) or either 2.5 pmol gene-specific primer pairs (sequences available in Table 1, Eurogentec, Liège, Belgium) or 1 μl of 10X QuantiTech primers (Qiagen, Venlo, The Netherlands). miRNAs and mRNAs of interest as well as small RNA used as miRNA normalizers (3 out of RNU1A, RNU5A, SCARNA17, and SNORD95) and reference genes used for RNA normalization (HRPT, PPIA, and TBP),
TABLE 1  Primer sequences used in qPCR analysis

| Gene   | Primer Sequence Forward | Primer Sequence Reverse | Size (bp) |
|--------|-------------------------|-------------------------|-----------|
| HPRT   | 5′-GGGACGAGGCGGTCACTA-3′ | 3′-GAGGACGACAGAGGCTCAG-5′ | 77        |
| IL-6   | 5′-TTGTTGATTGGTAAATTG-3′ | 3′-GCACATTATGATGAGAATC-5′ | 132       |
| IRAK1  | 5′-GGGACGAGGCGGTCACTA-3′ | 3′-CGCTGGAACTGCTAAGCAG-5′ | 92        |
| MCI1   | 5′-TTGTTGATTGGTAAATTG-3′ | 3′-GGGACGAGGCGGTCACTA-5′ | 85        |
| PPARG  | 5′-GCACATTATGATGAGAATC-3′ | 3′-GGGACGAGGCGGTCACTA-5′ | 27        |
| TBP    | 5′-TTGTTGATTGGTAAATTG-3′ | 3′-GGGACGAGGCGGTCACTA-5′ | 127       |
| TRAF6  | 5′-GGGACGAGGCGGTCACTA-3′ | 3′-GGGACGAGGCGGTCACTA-5′ | 88        |

were assessed in parallel for each sample and run in triplicates. Calculations were carried out by using the TCGA data analyses.

### 2.12 Analysis of protein expression

Cultured cells were lysed on the plate with ice-cold Laemmli 1x buffer and kept at ~20°C. Before separation by SDS-PAGE and blotting onto an Immobilon-FL PVDF membrane (Millipore, Overijse, Belgium), protein extracts were heated for 10 min at 96°C. Blots were incubated overnight with antibodies against human STAT1 (9172, Cell Signaling, Boike, Leiden, The Netherlands), phosphoSTAT1 (pSTAT1, 512233, BD Biosciences, Erembodegem, Belgium), STAT3 (610189, BD Biosciences, Erembodegem, Belgium), phosphoSTAT3 (pSTAT3, 9145, Cell Signaling, Boike, Leiden, The Netherlands), TSP4 (8028, Cell signaling, Boike, Leiden, The Netherlands), or Vinculin (13901, Cell Signaling, Boike, Leiden, The Netherlands). After washing steps, membranes were incubated for 1 h at room temperature with horseradish peroxidase-labeled secondary antibodies (Cell Signaling, Bioke, Leiden, The Netherlands, or Vinculin (13901, Cell Signaling, Boike, Leiden, The Netherlands)).

### 2.13 RNA-Sequencing

Library preparation for sequencing was done with 1 μg of total RNA using the TruSeq mRNA Stranded Library Prep Kit (Illumina, San Diego, California, USA) according to the manufacturer’s protocol. Briefly, the mRNA pull-down was performed using the magnetic beads with oligo dT primer. To preserve the strand information, the second strand synthesis was done with incorporation of dUTP so that during PCR amplification only the first strand was amplified. The libraries were quantified using the Qubit dsDNA HS assay kit (Thermo Fisher Scientific, Erembodegem-Aalst, Belgium) and the size distribution was determined using an Agilent 2100 Bioanalyzer. Pooled libraries were sequenced on NextSeq500 using the manufacturer’s instructions.

Following sequencing, reads were processed on the High Performance Computer of the University of Luxembourg. Reads quality was assessed using FastQC (v0.11 http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Due to the presence of adapter sequences in some reads, reads were trimmed using AdapterRemoval (v2.2.37) within the paleomix framework (v1.2.1228). Alignment was performed against the human genome hg38 (GRCh38.p1) with the Gencode annotation release 20 using the STAR aligner (v2.5.2b39). Mapping was then performed with the following command, using tweaked options suggested by Baruzzo et al. (see Supplementary Table 1 for mapping statistics): STAR –twopassMode Basic –outSAMmunnapped Within –limitOutSJcollapsed 1000000 –limitSJdbInsertNsj 1000000 –outFilterMultimapNmax 100 –outFilterMismatchNmax 33 –outFilterMismatchNoverLmax 0.3 –seedSearchStartLmax 12 –alignSJoverhangMin 15 –alignEndsType Local –outFilterMatchNminOverLread 0 –outFilterScoreMinOverLread 0.3 –winAnchorMultimapNmax 50 –alignSJDBoverhangMin 3 –outFilterType BySlack –outSAMtype BAM SortedByCoordinate

Raw counts were obtained by the R package Rsubread (v1.2.841 using R (v3.442)). Next, computation and visualization were performed with R and Rstudio. The differential expression analysis was performed with the R package DESeq2 (v1.18.144). Transcript annotations were retrieved with the R bioconductor package AnnotationDbi (v1.4045). Raw FASTQ files have been deposited in the European Nucleotide Archive (ENA accession number: PRJEB25247).

### 2.14 Pyrosequencing of selected CpG islands

Genomic DNA of non-neoplastic (PH5CH8 and THLE2) and cancerous (Hep3B, HepG2, and Huh-7) liver cells was extracted with the kit NucleoSpin Blood following the manufacturer’s instructions (Macherey-Nagel, Düren, Germany). Pyrosequencing experiments on extracted gDNA as well as the analysis were performed by the company Varionostic (Ulm, Germany).

### 2.15 TCGA data analyses

Datasets from the liver hepatocellular carcinoma cohort of The Cancer Genome Atlas (TCGA LIHC), which are freely available on the Xena Functional Genomics Explorer browser, were analyzed. miR-146b-5p expression levels (level 3 mRNA mature strand expression generated by Illumina HiSeq sequencing) as well as methylation state data for specific CpG islands located around the 1st nucleotide of pre-mir-146b (level 3 DNA methylation generated by Illumina Infinium...
HumanMethylation450 array) were downloaded on June 2017. Methylation data are presented as Tukey box plots with values higher or lower than 1.5 times of either the upper or lower quartile considered as outliers and depicted as dots.

2.16 Statistical analyses

For the statistical analysis of the normalized and prefiltered expression data of the microarrays, we used the limma package of R/Bioconductor, as previously described.\(^4^{4}\) False discovery rates (FDR) were assigned based on F-test P-values in order to identify miRNAs with non-random profiles. Features with FDR < 0.05 were considered significant.

Non-parametric one-way Anova (Kruskal–Wallis test) followed by Dunn’s multiple comparisons test were performed to assess the statistical significance between the untreated/unstimulated cells (Ctrl) and the different stimulations/treatments (Figs. 2B–F, 3C, 5, and 6).

Two-tailed paired t-test was used to assess statistical significance of the effects of miR-146b-5p overexpression (with and without Dox) on the expression levels of specific mRNA genes (Fig. 4A, B, and D). All comparisons were carried out with the statistical program Prism 7 (GraphPad Software, La Jolla, California, USA).

3 RESULTS

3.1 miR-146b-5p is an IL-6-regulated miRNA in primary human hepatocytes

We stimulated human primary hepatocytes isolated from 2 donors ("HepLux5", "HepLux6") with HIL-6 for different periods of time (0 [Ctrl], 6, 24, 48, and 72 h, see Fig. 1B for control Western blots monitoring stimulation-dependent pSTAT3 signals) and performed a microarray analysis of the miRNome. The maximum miRNA regulation was observed after 72 h of stimulation (in HepLux5) or after 48 h (in HepLux6, with slightly less miRNAs regulated at 72 h, data not shown). When considering the whole time course, 27 and 68 miRNAs (out of 1,733 miRNAs) were significantly differentially expressed (FDR < 0.05), including 15 miRNAs which were regulated in both sets, with miR-146b-5p showing the strongest increase in expression (log\(_2\)FC of 1.13 and 1.25 after 72 h, corresponding to an up-regulation of more than 2-fold, Fig. 1A). In subsequent qPCR analyses, additionally including RNA from 2 other donors, we could validate that expression levels of miR-146b-5p increased following HIL-6 stimulation and over time, with a more pronounced up-regulation (up to 8-fold) at 72 h in HepLux7 and HepLux8 samples compared to HepLux 5 and 6 (Fig. 1C).

3.2 Expression of miR-146b-5p, but not miR-146a-5p, is induced by HIL-6 and Oncostatin M in non-transformed liver cell lines

miR-146b-5p and miR-146a-5p, the other member of the miR-146 family, are located on different chromosomes (10 and 5, respectively) and differ by only 2 nucleotides at the 3’end (letters in gray, Fig. 2A), i.e. the seed region is shared by both miRNAs (in bold, Fig. 2A). We investigated the cytokine-mediated regulation of these miRNAs in 2 non-neoplastic immortalized cells lines (PH5CH8 and THLE2) and 3 HCC cell lines (Huh-7, HepG2, and Hep3B). As additional stimuli, another STAT3-activating cytokine, the IL-6-type cytokine OSM, as well as the STAT1-activating cytokine IFN\(_\gamma\) and an activator of the NFkB pathway, TNF\(\alpha\), were included. The activation was monitored by Western blot analysis (Fig. 2G). HIL-6 and OSM led to increased levels of pSTAT3 (except OSM-treated Hep3B cells which lack the OSM receptor, Supplementary Fig. 1A), and IFN\(_\gamma\) led to increased pSTAT1 levels. TNF\(\alpha\) did not affect phosphorylation of STATs. In PH5CH8 cells, the miR-146b-5p expression level was induced 3-fold after 72 h stimulation with the IL-6-type cytokines and ~2-fold upon IFN\(_\gamma\) stimulation in comparison to the unstimulated control (Ctrl, Fig. 2B). A slightly lower induction was observed in the THLE2 cell line upon HIL-6 and OSM treatment, whereas no up-regulation was observed after IFN\(_\gamma\) stimulation (Fig. 2C). In Huh-7 cells, the expression level of the miRNA increased upon stimulation although there was significant variability between the biological replicates (Fig. 2D). In the 2 other hepatoma cell lines tested, miR-146b-5p expression was not modified upon stimulation (HepG2 and Hep3B, Figs. 2E and F). Expression of miR-146a-5p was only increased in TNF\(\alpha\)-treated PH5CH8 cells (Fig. 2B). Taken together, these data show that in hepatocytes STAT1- and STAT3-activating cytokines (IFN\(_\gamma\), HIL-6, OSM) lead to an induction of miR-146b-5p but not of miR-146a-5p.

3.3 Promoter methylation does not account for lower induction of miR-146b-5p

In primary hepatocytes, the basal expression of this miRNA was already higher than in liver-derived cell lines (Supplementary Table 2) and we also observed a stronger up-regulation upon cytokine stimulation. To further address this difference in expression, we investigated the methylation state of the pri-mir-146b promoter. Specifically, we measured the methylation percentage of 4 CpG islands (at positions –63, –56, –26, and +70 relative to the 1st nucleotide of pre-mir-146b, Fig. 3A), which were shown to be crucial for the reduced miR-146b-5p expression in breast cancer cells\(^32\) (Fig. 3B). Interestingly, CpG island methylation for all 4 investigated sites was lower in the non-responsive HepG2 cells (Fig. 3B), while in the responsive Huh-7 and PH5CH8 cells, the percentage of methylation for each CpG island was higher (Fig. 3B). In addition, we performed 5-AZA demethylation studies in Hep3B, Huh-7, and PH5CH8 cells (showing a high degree of CpG island methylation, Fig. 3B). However, neither basal nor inducible miR-146b-5p expression was affected by the demethylation agent (Fig. 3C). To ensure the effectiveness of the 5-AZA treatment, we checked the expression level of SOCS3 in Hep3B and Huh-7 cells, for which it has been described that the promoter is subject to CpG island modification.\(^46\) Indeed, upon 5-AZA treatment, we observed an increased expression of this gene (Supplementary Fig. 1B). To study if other CpG islands located in the presumed promoter region of pri-mir-146b (Fig. 3A) could be implicated, we analyzed the methylene data from the Liver Cancer cohort of The Cancer Genome Atlas (TCGA LIHC) available for 50 HCC tumor tissues and the matched solid “normal” tissues (black and gray boxes, respectively, Fig. 3D). The
FIGURE 3 DNA methylation does not account for basal and HIL-6-induced miR-146b-5p expression. (A) Scheme of the genome landscape surrounding the pre-mir-146b genomic sequence (in gray, 73 base pairs long) as depicted in the NCBI database ref. NC000010.10. The methylation sites are shown with their respective positions from the 1st nucleotide of the pre-mir-146b sequence, the transcription starting site (TSS) and the consensus STAT binding motifs according to Xiang et al. are indicated in dark gray. (B) Methylation percentage found by pyrosequencing in liver-derived cell lines. (C) Expression of mature miR-146b-5p was assessed in non-neoplastic PH5CH8 cells, Huh-7 and Hep3B hepatoma cells after 5 days of incubation with the demethylation agent 5-aza-2′-deoxycytidine (5-AZA), with or without 72 h of HIL-6 stimulation (5-AZA + HIL-6). Acetic acid was used as vehicle control. Error bars represent standard deviation of at least 3 biological replicates. (D, F, G, H) pri-mir-146b promoter methylation was assessed in 50 (D) or 40 (F, G, H) matched HCC tumor and surrounding (“normal”) tissues. (D) Methylation data of 9 different CpG islands in 50 matched HCC tumor (in black) and normal (in gray) tissues were extracted from the TCGA Liver Cancer cohort. (E) Three different groups, based on the expression levels of miR-146b-5p presented as a log2 (reads/million + 1), were defined. In red: increased expression (14 pairs), in gray: none or a slight change in expression (6 pairs), and in blue: decreased expression in the tumors (20 pairs). (F–G) Methylation status for each group was re-analyzed. N: Normal tissue, T: Tumor tissue.
FIGURE 4 Effects of inducible overexpression of miR-146b-5p in PH5CH8-146b cells. (A) Normalized qPCR expression levels of miR-146b-5p upon treatment with 1 μg/ml Dox for 72 h. (B) Normalized qPCR expression levels of potential miR-146b-5p mRNA targets, as found by RNA-Seq analysis. (C) Comparison of computationally predicted and/or experimentally validated miR-146b-5p target genes and mRNAs significantly reduced in PH5CH8-146b cells upon Dox treatment (found by RNA-Seq analysis). The Venn diagram shows the distribution of the numbers of gene targets predicted by 2 algorithms as indicated, experimentally validated targets from miRTarBase and the RNA-Seq data. (D–E) miR-146b-5p modulates NF-κB signaling. (D) Normalized expression levels of IRAK1, TRAF6, MCP1, and IL-6 mRNAs. (E) Western blot analysis of TRAF6 with vinculin as loading control. Error bars represent standard deviation of at least 3 biological replicates. *: \(P\)-value (\(P\)) < 0.05, **: \(P\) < 0.01, ***: \(P\) < 0.001, ****: \(P\) < 0.0001

methylation of these CpG islands was overall lower in the tumor tissues than in the matched adjacent tissues (Fig. 3D), with a higher variability between patients in the tumor samples than in the adjacent tissues, suggesting a deregulation of the methylation status in the tumors, as already reported for HCC.47–49 To complement these results, miR-146b-5p expression levels of 40 HCC tumor tissues, for which both information on the miR-146b-5p expression levels and the methylation status was available, were compared to the corresponding adjacent “normal” tissues (Fig. 3E). A lower CpG islands methylation in the tumors than in the surrounding tissues was observed throughout,
irrespective of an increased, unchanged, or decreased miR-146b-5p expression in the corresponding samples (Fig. 3F, G, and H). Taken together, these results indicate that the methylation status of the tumor tissues was different from the one in the surrounding tissues but this was not reflected in the miR-146b-5p expression levels.

3.4 | miR-146b-5p affects expression of multiple genes, including IRAK1 and TRAF6 involved in TLR signaling

We showed that miR-146b-5p was reproducibly responding to HIL-6 stimulation, hence we investigated potential target genes of this miRNA. To do so, we engineered PH5CH8 liver cells, which inducibly overexpressed this miRNA, named PH5CH8-146b cells. Upon Dox treatment, a strong induction could be observed when miR-146b-5p levels were monitored by qPCR (Fig. 4A). Its induction affected the expression of several mRNAs, as revealed by RNA-Seq analysis. Expression of 79 genes was significantly (adjusted P-value < 0.05) changed in miR-146b-5p overexpressing cells: 45 (57%) were down-regulated and 34 (43%) were up-regulated; the top 20 most down-regulated ones are listed in Table 2. These included known targets of miR-146b-5p such as IRAK1, CARD10, and ZNRF3. TRAF6, another well-described target, was also down-regulated, however, with a lower log2|FC| (0.38) than the above-mentioned ones (complete lists of all significantly up- and down-regulated mRNAs can be found in Supplementary Table 3). As we were mainly interested in direct targets of miR-146b-5p, we focused on the down-regulated mRNAs found by RNA-Seq. Out of the top 20 of the down-regulated mRNAs, 16 as well as TRAF6 could be validated in PH5CH8-146b cells (Fig. 4B) (the 4 others were not further analyzed due to bad qPCR melt curves). Additionally, we compared the list of targets predicted by the algorithms MicroT-CDS (from DianaTools) and TargetScan as well as the experimentally validated targets in the miTarBase database, with the list of significantly differentially down-regulated genes (Fig. 4C). Indeed, CARD10, IRAK1, NRAS, SLC10A3, TRAF6, and ZNRF3, predicted targets by 2 algorithms, were down-regulated by overexpression of miR-146b-5p.

Reduced mRNA levels of IRAK1 and TRAF6, part of a complex involved in NFκB activation upon TLR4 triggering, were confirmed by qPCR following miR-146b-5p overexpression (Fig. 4D and E) as well as a reduced protein level of TRAF6 (Fig. 4E). We also monitored the expression of downstream targets of NFκB signaling, the genes encoding the chemokine MCP1 (also named CCL2) and IL-6, and found both to be induced by TNFα and LPS (Fig. 4D). Importantly, only the LPS-mediated up-regulation of MCP1 and IL-6 mRNA expression was attenuated when miR-146b-5p was overexpressed (Fig. 4D).

3.5 | miR-146a-5p and miR-146b-5p regulate similar mRNA target genes

miR-146a-5p and miR-146b-5p differ only by 2 nucleotides but have the same seed region (Fig. 2A). Therefore, we next investigated whereas they indeed regulate the same genes by transiently transfecting liver-derived cells (PH5CH8 and Huh-7) with a negative mimic control (NCM1), miR-146a-5p, or miR-146b-5p mimic and quantified the expression levels of the previously validated mRNA candidates (Fig. 5A and B, respectively). In contrast to the induction in stable PH5CH8-146b cells, transient transfection of 20 nM mimic led to a huge overexpression (more than 5,000 times in both cell lines, Supplementary Fig. 1C) of the corresponding miRNA. Not surprisingly, cross-reactivity of both primer pairs was observed under these conditions of strong miRNA overexpression. All 17 mRNA candidates identified by RNA-Seq of miR-146b-5p overexpressing PH5CH8 cells were confirmed following transient transfection of PH5CH8 and Huh-7 cells. Some candidate genes like FOSL1 and SHISAL1 could not be confirmed in Huh-7 cells. Importantly, both miR-146a-5p and miR-146b-5p showed very similar effects on the various potential targets, confirming the expected redundancy due to their identical seed regions.

To investigate if the effects observed after mimic transfection were due to a direct interaction between the miRNAs and the 3′UTR of potential targets (CPA4, DNAJC6, MAGEE1, MPHOSPH6, PPP2R1B, PTX3, SLC10A3, SNRNP27, and TIMM17B), we performed dual luminescence reporter assays. These mRNAs were selected as they were the only unknown targets of miR-146b-5p or miR-146a-5p. Hek293T cells were co-transfected with the vector containing the gene of the secreted Gaussia luciferase following by the 3′UTR of the candidate gene and with NCM1, miR-146a-5p, or miR-146b-5p mimics. We observed similar results for both miRNAs with a reduction of 30–40% in luciferase activity upon mimic transfection for the 3′UTRs of TIMM17B and DNAJC6, 40–50% for the 3′UTRs of MAGEE1, MPHOSPH6, and SNRNP27, and more than 60% for the 3′UTRs of PPP2R1B and SLC10A3 (Fig. 6 and Table 2). These data confirmed a direct binding of the miRNAs to the 3′UTRs of the selected mRNAs. While we observed a reduction of CPA4 expression at the mRNA level in PH5CH8 cells (Fig. 5A), only a moderate diminution of luciferase activity was measured. The data suggested an indirect effect of the miRNAs or that the binding sites were not only localized in the 3′UTR of the CPA4 mRNA, but rather elsewhere (5′UTR or coding sequence). Surprisingly, PPP2R1B showed the strongest effect on luciferase activity even though at the mRNA level the down-regulation was moderate in both cell lines (Fig. 5). Interestingly, most of the 3′UTRs further studied were not predicted as potential miR-146b-5p targets, highlighting the fact that experimental screenings and validation are necessary to identify new miRNA targets.

4 | DISCUSSION

Hepatocellular carcinoma most often develops in an inflamed liver, and the inflammatory cytokine IL-6 plays a role in hepatocarcinogenesis, as demonstrated in the DEN-induced HCC mouse model. In order to elucidate the role of IL-6 in the modulation of the hepatic miRNome, we performed microarray analyses on primary hepatocytes stimulated with HIL-6 and identified miR-146b-5p as being the most robustly induced miRNA.

This miRNA, belonging to the miR-146 family along with miR-146a, was shown to have a potential tumor-suppressive role in HCC development and to be differentially expressed in inflammatory liver


| Gene Symbol | log 2FC | Adj P-value | Description | miRTar Base | MiRTar CDS | TargetScan | PicTar | qPCR Validation | Mimic PH5CH8 | Mimic Huh-7 | 3'UTR Lucif. |
|-------------|---------|-------------|-------------|-------------|------------|------------|--------|----------------|----------------|---------------|--------------|
| IRAK1       | -1.64   | 8.24E-70    | Interleukin 1 Receptor Associated Kinase 1 | x           | x          | x          | x      | 73.69          | 53.02          | 65.00         |
| CARD10      | -0.95   | 1.02E-18    | Caspase Recruitment Domain Family Member 10 | x           | x          | x          |        | 67.37          | 60.59          | 73.16         |
| TIMM17B     | -0.88   | 2.88E-16    | Translocase Of Inner Mitochondrial Membrane 17B | x           | [47x728] | x          |        | 63.83          | 62.48          | 67.49         | 32.19        |
| SLC10A3     | -0.80   | 4.94E-14    | Solute Carrier Family 10 Member 3 | x           | x          | x          |        | 58.74          | 62.54          | 66.46         | 60.64        |
| SHISA1      | -0.63   | 5.22E-09    | Shisa like 1 |             |            | [47x728]  | [47x728] | 21.90          | 30.54          | 10.34         |
| DDAH1       | -0.56   | 6.50E-09    | Dimethylarginine Dimethylaminoimidohydrolase 1 |             |            |            |        | 44.99          | 36.78          | 24.35         |
| PPP2R1B     | -0.56   | 8.65E-08    | Protein Phosphatase 2 Scaffold Subunit Abeta |             |            |            |        | 40.84          | 27.86          | 23.72         | 68.64        |
| FOSL1       | -0.54   | 1.34E-04    | FOS Like 1, AP-1, Transcription Factor Subunit |             |            |            |        | 47.04          | 26.14          | 22.52         |
| SIKE1       | -0.52   | 3.57E-07    | Suppressor of IKBKE 1 |             |            |            |        | 34.40          | 18.68          | 19.44         |
| ZNRF3       | -0.5    | 3.13E-04    | Zinc and Ring Finger 3 | x           | x          | x          |        | 39.14          | 26.05          | 33.71         |
| SNRNP27     | -0.49   | 2.43E-05    | Small Nuclear Ribonucleoprotein U4/U6.U5 Subunit 27 | x           |            |            |        | 38.78          | 38.95          | 38.82         | 49.91        |
| SRPRB       | -0.49   | 1.62E-05    | Signal Recognition Particle Receptor B Subunit | x           |            |            |        | 40.74          | 28.65          | 11.50         |
| PHKB        | -0.48   | 1.56E-06    | Phosphorylase Kinase Regulatory Subunit Beta | x           |            |            | x      |                |                |               |
| MPHOSPH6    | -0.48   | 1.65E-04    | M-Phase Phosphoprotein 6 |             |            |            |        | 43.22          | 35.76          | 43.41         | 41.71        |
| MAGEE1      | -0.46   | 4.58E-03    | MAGE Family Member E1 |             |            |            |        | 52.31          | 42.74          | 42.00         | 43.12        |
| CPA4        | -0.45   | 3.13E-04    | Carboxypeptidase A4 |             |            |            |        | 38.33          | 41.20          | 17.64         | 12.02        |
| PTX3        | -0.45   | 5.80E-03    | Pentraxin 3, TNF-inducible gene 14 protein | x           |            |            | x      | 38.78          | 38.95          | 38.82         | 49.91        |
| ROR1        | -0.44   | 2.89E-04    | Receptor Tyrosine Kinase Like Orphan Receptor 1 | x           |            |            |        |                |                |               |
| DNAJC6      | -0.43   | 1.02E-04    | DnaJ Heat Shock Protein Family (Hsp40) Member C6 |             |            |            |        | 41.14          | 39.99          | 33.65         | 36.08        |
| FBXL3       | -0.43   | 7.00E-05    | F-Box And Leucine Rich Repeat Protein 3 |             |            |            |        |                |                |               |

Target predictions are indicated (performed with 3 different algorithms: MicroT-CDS, TargetScan, and PicTar) as well as results from miRTarBase researches and compared to our data for miR-146b-5p (qPCR validation, mimic transfection in PH5CH8, and Huh-7 cells as well as 3'UTR luciferase assay) representing the percentage of reduction in comparison to the respective controls.

diseases.\textsuperscript{51–53} Furthermore, Xiang et al. have demonstrated a cytokine-mediated induction of miR-146b-5p in healthy but not in cancerous breast cells.\textsuperscript{32} We therefore inspected various liver-derived cell lines regarding their ability to respond to cytokine stimulation with an increased expression of miR-146b-5p. We have shown that miR-146b-5p expression was up-regulated by STAT-activating cytokines (HIL-6, OSM, and to a lower extent IFN\(_\gamma\)) in 2 non-neoplastic immortalized hepatocyte cell lines (PH5CH8, THLE2), more variably in the cancerous Huh-7 cells and not at all in the 2 other neoplastic cell lines (Hep3B, HepG2), without a differential methylation of the pri-mir-146b promoter being involved. An induction by STAT-activating cytokines could previously be shown for IL-6- and IFN\(_\gamma\)-treated untransformed breast cells,\textsuperscript{32} for IL-6-stimulated mature adipocytes\textsuperscript{54} and for IL-10-treated monocytes.\textsuperscript{55} However, in airway smooth muscle cells, inducible miR-146b-5p expression necessitated a combination of IFN\(_\gamma\), TNFA, and IL-1\(\beta\) to be effective.\textsuperscript{56} Similarly, in retinal pigment epithelial cells TNFA and/or IL-1\(\beta\) strongly increased IFN\(_\gamma\)-mediated effects on miR-146b-5p expression,\textsuperscript{57} indicating that TNFA and/or IL-1\(\beta\) could potentiate the regulatory effects of STAT-activating cytokines on miR-146b-5p. TNFA is also able to induce the expression of miR-146b-5p in mature human adipocytes,\textsuperscript{54} although this cytokine is more generally...
miR-146a-5p and miR-146b-5p modulate the expression of common targets. (A) PH5CH8 and (B) Huh-7 cells were kept untreated (Ctrl) or transiently transfected with negative control mimic 1 (NCM1), miR-146a-5p or miR-146b-5p mimics for 3 days. (A and B) Normalized expression levels of mRNA targets, found by RNA-Seq analysis. Error bars represent standard deviation of at least 3 biological replicates. *: Adjusted $P$-value (adj. $P$) < 0.05, **: adj. $P$ < 0.01, #: adj. $P$ = 0.0525, +: adj. $P$ = 0.064, &: adj. $P$ = 0.078, $:$ adj. $P$ = 0.094.

FIGURE 6 miR-146a-5p and miR-146b-5p have similar effects on the 3′ UTR of target candidates. Hek293T cells were co-transfected with pEZx-MT05 vector containing the gene of the secreted Gaussia luciferase (GLuc) followed by the 3′ UTR of interest, as well as the negative control mimic (NCM1), miR-146a-5p or miR-146b-5p mimic or left untreated (Ctrl). Secreted-pair luminescence assays were carried out 48 h after transfection and GLuc was normalized to the activity of the secreted alkaline phosphatase and divided by the ratio obtained for the NCM1 control. Error bars represent standard deviation of at least 3 biological replicates with 3 replicates each. *: Adjusted $P$-value (adj. $P$) < 0.05, **: adj. $P$ < 0.01, #: adj. $P$ = 0.054, +: adj. $P$ = 0.067, $:$ adj. $P$ = 0.099.

Known to regulate miR-146a-5p expression, however, in none of our liver cell lines, TNFα stimulation led to an increased expression of these miRNAs (except for miR-146a-5p in PH5CH8 cells), pointing at a tissue- and/or cell type-specific regulation.

To further investigate the functional role of this miRNA, we engineered stable PH5CH8 cells to inducibly overexpress miR-146b-5p upon Dox treatment and identified by RNA-Seq 79 significantly differentially expressed mRNAs (45 down- and 34 up-regulated). We could validate 16 mRNAs (out of the top 20 down-regulated) as being affected by miR-146b-5p, comprising known targets (IRAK1, CARD10, and ZNRF3) as well as newly discovered candidates. Interestingly, Chou et al. have also identified CARD10, IRAK1, MAGEE1, SLC10A3, and TIMM17B by microarray experiment as being down-regulated in human BCPAP thyroid cancer cells upon miR-146b mimic transfection.

From this list, at least 3 so far unknown target candidates were interesting in the context of HCC: DNAJC6 (DnaJ heat shock protein family (Hsp40) Member C6), PPP2R1B (protein phosphatase 2 scaffold
This study provides evidence of (i) cytokine-inducible miR-146b-5p expression (Figs. 1 and 2), (ii) effects of (overexpressed) miR-146b-5p on expression of well-known targets IRAK1 and TRAF6 (Figs. 5 and 4E), and (iii) effects of (overexpressed) miR-146b-5p on LPS-regulated target genes (Fig. 4D). STAT3 binding to the pri-miR-146b-5p promoter has been shown for mammary cells.32

As the major mature forms of miR-146a/b (-5p) have very similar sequences and identical seed regions, they are predicted to target the same subset of genes. Therefore, we transiently transfected PH5CH8 and Huh-7 cells with the corresponding mimics to compare their effects on previously validated targets and obtained comparable results to RNA-Seq data for both mimics. In addition, 3’UTR reporter assays confirmed 7 mRNAs (DNAJC6, MPHOSPH6, PPP2R1B, SLC10A3, SNRNP27, and TIMM17B) from 9 tested ones as being post-transcriptionally regulated by miR-146a/b-5p. Regarding DNAJC6, MPHOSPH6, PPP2R1B, SNRNP27, or TIMM17B, this is, to the best of our knowledge, the first evidence that they may be direct targets of miR-146 miRNAs. For SLC10A3, interaction with both, miR-146b-5p and miR-146a-5p, was found by an AGO-PAR-CLIP approach using prostate cancer cells.70 While several studies have identified targets for miR-146a-5p or miR-146b-5p (reviewed in Paterson and Kriegel71), we show here that a broad set of genes is demonstrated to be modulated by both miRNAs in the same cells in a very similar way. Our results and the literature suggest that even if the targets are redundant, their miRNA-mediated modulation could still show specificity, depending on external stimuli and the cell-type (reviewed in Paterson and Kriegel71).

As 4 differentially regulated genes found by RNA-Seq were involved in the NFκB signaling pathways, including the well-known miR-146b targets IRAK1 and TRAF6,55,58,72 we stimulated cells with LPS or TNFα and monitored downstream regulated genes (mcp1 and il6). In Fig. 7, we schematically show a potential negative regulatory crosstalk in which LPS- (but not TNFα-) mediated signaling is inhibited by the IL-6-induced miR-146b-5p, as previously suggested for mammary cells.32 Our results obtained with cells overexpressing miR-146b-5p (Fig. 4D) are in line with the fact that TNFα, in contrast to LPS, does not utilize IRAK1 and TRAF6 for NFκB activation. It is, however, well possible that under physiological conditions a single miRNA plays only a role in fine modulation of complex signaling networks. It would therefore be interesting to test whether exogenous administration of miR-146b-5p could have beneficial effects in diseases involving IL-6, as shown in high-fat diet-induced non-alcoholic steatohepatitis in rats.73

IL-6, as well as other cytokines, has been reported to play a pivotal role in inflammatory events in the context of fatty liver diseases (reviewed in Hassan et al.74) which are known to increase the risk of...
cirrhosis and HCC development.\textsuperscript{75} Furthermore, autocrine IL-6 stimulation of liver cancer progenitor cells has been described to promote malignant progression of HCC.\textsuperscript{76} Interestingly, the IL-6-induced miR-146b-5p was found to be up-regulated during progression to steatosis and steatohepatitis in rats\textsuperscript{52} as well as in liver diseases (e.g., in obese subjects with non-alcoholic fatty liver diseases (NAFLD)\textsuperscript{53} and in NASH patients\textsuperscript{51}). On the contrary, it has been shown that this miRNA (as well as miR-146a-5p\textsuperscript{77}) was reduced in HCC tumor tissues compared to the surrounding tissues, indicating a tumor-suppressive role for this miRNA.\textsuperscript{50} Hepatoma most often arises in the context of chronic inflammation and cirrhosis.\textsuperscript{1} Thus, the “normal” tissues surrounding hepatic tumor tissues may reflect a wide range of inflammatory states. It can be envisaged that the discrepancy between our results on the TCGA HCC cohort (Fig. 3) and the findings of Li et al.\textsuperscript{50} may be due to heterogeneous disease backgrounds of the patients in the analyzed cohorts. In addition, a recent study on DEN-induced hepatoma in mice showed that expression of miR-146b-5p is stronger in the highly inflammatory para-tumor tissue compared to normal and tumor tissue,\textsuperscript{78} in line with a considerable impact of the tumor environment on the modulation of miR-146b-5p expression.

For the first time and in side-by-side experiments, we have shown that transient transfection of miR-146a-5p and miR-146b-5p leads to the regulation of the same subset of mRNAs which could be corroborated by luciferase-3′UTR reporter assays. Further studies investigating the cytokine-mediated regulation of both miRNAs as well as their target genes/proteins need to be performed to better define the regulatory loops, which could be targeted in inflammatory liver diseases and liver cancer.

AUTHORSHP

I.B. and S.K. conceived the study, I.B., F.A.S., M.K., M.H., and S.K. designed the research. F.A.S., M.K., M.H. performed experiments. F.A.S. and M.K. analyzed the data. R.H. prepared the library for sequencing. P.V.N. and A.G. did bioinformatics analysis of the microarray and RNA-Seq data, respectively. S.K. and L.V. gave conceptual advice. F.L., M.G., and M.K. analyzed the data. R.H. prepared the library for sequencing. I.B. and S.K. conceived the study, I.B., F.A.S., M.K., M.H., and S.K. wrote the manuscript. All authors revised and approved the final draft of the manuscript. M.K. and F.A.S. contributed equally to this work.

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DISCLOSURES

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

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