Hepatitis B virus upregulates host microRNAs that target apoptosis-regulatory genes in an *in vitro* cell model

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**ARTICLE INFO**

**Keywords:** HBV, miR-192, miR-194, Non-coding RNA, Apoptosis, HepG2

**ABSTRACT**

Chronic hepatitis B (CHB) infection increases the risk of developing severe liver disease including cirrhosis and hepatocellular carcinoma (HCC). As microRNAs may modulate host – virus interactions, we here investigated if hepatitis B virus (HBV) infection modulate microRNA expression using an *in vitro* HepG2 cell model system with inducible HBV replication. We found that HBV replication was associated with upregulation of miR-192-5p, miR-194-5p and miR-215-5p, of which miR-192-5p and miR-215-5p have identical seed sequences. Bioinformatics analyses revealed a significant enrichment of potential target genes involved in apoptosis signaling of all three microRNAs. In line with this, transfection with a mimic of miR-192-5p suppressed the protein level of pro-apoptotic BIM and reduced endoplasmic reticulum (ER) stress-induced apoptosis in HepG2 cells. In contrast, transfection with a mimic of miR-194-5p downregulated the anti-apoptotic proteins SODD and cFLIP, and sensitized HepG2 cells to both ER stress- and cytokine-induced apoptosis. In conclusion, our study suggests that HBV upregulates the expression of miR-192-5p and miR-194-5p in the host cell. These microRNAs target important apoptosis-regulatory proteins, and may thus contribute to the development of HBV-related liver disease.

**1. Introduction**

The outcome of a hepatitis B virus (HBV) infection is age-dependent with chronic hepatitis B (CHB) developing predominantly after transmission perinatally from mother to child. The long term consequence is a 25% risk of developing severe liver disease such as cirrhosis and hepatocellular carcinoma (HCC) [1,2]. Current treatment options are insufficient, and a better understanding of the host – virus interactions is warranted.

MicroRNAs are small endogenous non-coding RNAs that function as important regulators of various cellular signal transduction pathways such as development, immune function and cell death [3]. They are believed to modulate host – virus interactions [4] and alterations in microRNA expression have been associated with disease [5,6]. HBV is considered a non-cytopathic virus as the damage inflicted on the liver after infection with a wildtype HBV strain is caused by the host immune response [7–9]. Immune cells can induce cell death by secretion of cytokines such as tumor necrosis factor alpha (TNF-α), which can trigger death receptor signaling in hepatocytes leading to apoptosis [10]. A key event in apoptosis signaling is the activation of the caspase family of cysteine proteases leading to the dismantling of the cell. The cellular FLICE-inhibitory protein (cFLIP) is an important negative regulator of death receptor signaling by inhibition of caspase 8 activation [11–13], while the pro-apoptotic BH3-only protein BIM facilitates...
apoptosis via mitochondrial outer membrane permeabilization (MOMP) [14].

In this study, we aimed to identify microRNAs differentially expressed in the presence of ongoing HBV replication. To further examine their biological functions and possible contribution to disease pathogenesis, we also investigated their putative roles as regulators of apoptotic cell death in a human liver cell line. Our data suggests that mir-192-5p and mir-194-5p are upregulated in the presence of HBV replication in vitro and that they have important regulatory functions in hepatocyte apoptosis. While mir-192-5p suppresses apoptosis, mirR-194-5p increases apoptosis via downregulation of cFLIP.

2. Materials and methods

2.1. Cell lines and cell culture

The Human Hepatoma HepG2 tet-on control cell line (HepG2 Tet-On Advanced cell line, #631150, CloneTech Laboratories, Mountain View, California, USA) containing the Tet-on inducible gene expression system [15,16] was stably transfected with the 3091 HBV genome (genotype D, serotype ayw3) [17].

Cells were maintained in Dulbecco Modified Eagle Medium (DMEM) (#41966-029, Gibco® by Life Technologies, Carlsbad, California, USA) supplemented with 10% Fetal Bovine Serum Premium (FBS) RNA interference [18]. Lysates were collected after 48 h and stored at −80 °C until RNA isolation using the mirNeasy Mini Kit (Qiagen, Hilden, Germany). RNA concentration was measured on a NanoDrop 6000 Nano-kit (Agilent Technologies, Santa Clara, California, USA). The Human BCL2L11 SMARTpool (Cat. No: L-004383-00), Human BAG4 SMARTpool (Cat. No: L-004379-00) and Human CFLAR SMARTpool (Cat. No: L-003772-00).

The HepG2 tet-on cells were reverse-transfected with 10 nM ON-TARGETplus SMARTpool siRNA (mixture of 4 siRNA) (Dharmacon inc.): Negative control 1 (Cat. No: D-001810-10-05), Negative control 2 (Cat. No: 479903), hsa-mir-122-5p (Cat. No: 471355) and hsa-mir-192-5p (Cat. No: 471895). ON-TARGETplus siRNA (Dharmacon inc.): Non-targeting pool (Cat. No: D-001810-10-05), Human BCL2L11 SMARTpool (Cat. No: L-004383-00), Human BAG4 SMARTpool (Cat. No: L-004379-00) and Human CFLAR SMARTpool (Cat. No: L-003772-00).

2.2. Generation of HBx-expressing clones

The HepG2 tet-on cell line was used to generate doxycycline-inducible HBx clones.

**Vector generation:** The complete open reading frame of HBx and the HBV polA was amplified from the 3091 full 1.1 HBV genome (genotype D) using primers with overlaps homologous to the CMV-Tet-on expression vector (Forw.: 5′-taagcttggtaccggatggctgctaggctgtgc-3′ and rev.: 5′-cttctcggggtcgcttgg-3′). The backbone of a pZDonor vector encoding a gene for puromycin resistance and a P-promoter (pZDonor vector encoding a gene for puromycin resistance and a P-promoter) was inserted into the pZDonor vector using the Clontech Laboratories. The sequence of the construct was verified by DNA sequencing before transfection into HepG2 tet-on cells using TurboFect Transfection Reagent (Thermo Fisher Scientific®). In parallel, HepG2 tet-on cells were stably transfected with an empty pZDonor-AAV1 Puromycin vector (#PZD0020, Sigma-Aldrich, St. Louis, Missouri, USA). Mock and HBx-expressing clones were picked and expanded after selection with puromycin for several weeks. HBx expression was verified by RT-qPCR (Forw.: 5′-ctttctgggtgcttg-3′, rev.: 5′-ccacaggctgcgaagttc-3′) and Western blotting. The Mock and HBx clones were maintained in DMEM with 10% FBS, 1% P/S, G418 (100 µg/ml) and Puromycin (0.5 µg/ml) at 37 °C with 5% CO2.

2.3. Transfection with siRNA and microRNA mimics

Cells were cultured in DMEM with 2% serum to reduce the risk of FBS RNA interference [18]. Lysates were collected after 48 h and stored at −80 °C until RNA isolation using the mirNeasy Mini Kit (Qiagen, Hilden, Germany). RNA concentration was measured on a NanoDrop 2000c spectrophotometer (Thermo Scientific) and RNA integrity (RIN > 1.8) was evaluated on an Agilent 2100 Bioanalyzer using RNA 6000 Nano-kit (Agilent Technologies, Santa Clara, California, USA).

The expression level of 752 human microRNAs was measured using the mirCURY LNA™ Universal RT microRNA PCR system (Exiqon) and microRnome panels (human panel I and II V4.M/R) (Exiqon). This screen includes microRNAs described by the manufacturer as likely to be differentially expressed in disease, highly expressed in general or often cited in the literature. The screen was performed on samples from three independent experiments. A blank sample was included to ensure optimal running conditions.

**Data analysis:** 322 microRNAs were left after excluding microRNAs that met the following criteria: 1) Ct > 35 in minimum two samples in both cell lines, 2) lacking melting temperature in minimum two samples in each cell line, 3) ≥ 1 degree difference in melting temperatures between samples, 4) more than one melting curve peak, and 5) a standard deviation > 1. MicroRNA expressions were normalized to the geometric mean of nine microRNAs (hsa-mir-92a-3p, hsa-mir-106a-5p, hsa-mir-339-5p, hsa-mir-18a-3p, hsa-mir-200c-3p, hsa-mir-574-3p, hsa-mir-25-3p, hsa-mir-328-3p) identified as the most valid combination of reference genes by ΔCt, geNorm [19] and NormFinder [20] analyses. The nine microRNAs used for normalisation were selected as the best compromises between the HBV cells and HepG2 tet-on cells. After correction for multiple testing (Benjamini-Hochberg, FDR < 0.1) 3 microRNAs were left: mir-192-5p (5′-cugaucaaguauagacac-3′), mir-194-5p (5′-uguaac-aggcagcagugga-3′) and mir-215-5p (5′-auguacuauagacacagac-3′).
2.5. Bioinformatics analysis

MicroRNAs significantly differentially expressed (absolute log2FC > 0.5) between HBV cells and HepG2 tet-on cells (miR-192-5p, miR-215-5p and miR-194-5p) were subjected to a bioinformatics analysis to identify potential target genes. All microRNA target genes with CLIP-seq overlap were retrieved from StarBase [21,22] and only unique target genes were retained for downstream analysis. The evidence of expression filter was applied on these to ascertain which of these target genes are actively recruited by the RNA-induced silencing complex (RISC) in HepG2 cells. Ago2 IP-based RNAseq data on a HepG2 cell line from a study by Furuta et al. [23] was used to confirm the loading of each target onto RISC using a cut-off RPKM > 1. The filtered target genes were subjected to Gene Ontology (GO) term and pathway evidence of expression determination and evaluated using the Panther database [24].

The mirSVR target scoring method [25] available at mirTarget.org [26] was used to identify the putative target site of miR-192-5p in cFLIP mRNA.

2.6. Western Blot analysis

Cells were lysed directly in wells using M-PER® Mammalian Protein Extraction Reagent (#78501, Thermo Scientific™) with Halt™ Protease & Phosphatase inhibitor Cocktail (#78440, Thermo Scientific™) following manufacturer's instructions. Lysates were centrifuged at 14,000 × g for 10 min and the supernatants (whole cell lysates) collected and stored at −80 °C. Protein concentrations were determined using the DC™ Protein Assay (BioRad, Hercules, California, USA) following the manufacturer’s instructions. Loading: 20 µg protein per lane (50 µg protein for HBx detection). Protein size was verified using MagicMark™ Prestained Protein Ladder (#10748-010, Invitrogen). Secondary HRP-conjugated antibodies were used and immune complexes were detected by chemiluminescence. Images were captured digitally using a FUJI LAS4000 imaging system (Fujifilm) and band intensities quantified using ImageQuant TL.

Primary antibodies: cFLAR (FLIP, #D5J1E, Cell Signaling Technology, Danvers, Massachusetts, USA), BAG4 (Silencer of Death Domain (EPR3596), ab108988, Abcam, Cambridge, United Kingdom), Caspase-8 (1C12) (#97465, Cell Signaling Technology), Caspase-7 (C7) (#9494S, Cell Signaling Technology), BIM (C34CS) (#2933S, Cell Signaling Technology), GAPDH (6CS) (#ab8245, Abcam), beta-Actin (AC-15) (#ab6276), Hepatitis B virus X Antibody (X36C) (#MA1–081, Thermo Scientific). Secondary antibodies: Anti-rabbit IgG, HRP-linked Antibody (#7074S, Cell Signaling Technology) and Anti-mouse IgG, HRP-linked Antibody (#7076S, Cell Signaling Technology).

2.7. RNA extraction and cDNA synthesis

microRNA; total RNA was extracted using the miRNeasy Mini Kit (Qiagen) to obtain enrichment of small RNAs. Manufacturer’s instructions were followed with minor modifications (200 µl chloroform was added to 700 µl lysate, and after enrichment were applied to miRNeasy Mini spin columns all centrifugation steps were performed at RT at 13,000 × g). RNA concentrations were determined and evaluated using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific™). RNA samples were stored at −80 °C. cDNA synthesis was performed using the universal cDNA Synthesis Kit II (Exiqon) in accordance with the manufacturer’s instructions on a GeneAmp PCR System 9700 (Applied Biosystems). Between 250 and 1000 ng total RNA was used for cDNA synthesis, and blank samples were included. cDNA was stored at −20 °C until further use.

mRNA: total RNA was extracted using RNeasy Mini Kit (Qiagen) following manufacturer’s instructions. RNA concentrations were determined and evaluated using the NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific™). RNA samples were stored at −80 °C. cDNA synthesis was performed using the iScript™ cDNA Synthesis Kit (BioRad) in accordance with the manufacturer’s instructions on a GeneAmp PCR System 9700 (Applied Biosystems). Between 250 and 1000 ng total RNA was used for cDNA synthesis, and blank samples were included. cDNA was stored at −20 °C until further use.

2.8. Real-Time qPCR

microRNA: Relative quantification of microRNA expression levels was performed by RT-qPCR using the miRCURY LNA™ Universal RT microRNA PCR system and specific microRNA LNA™ PCR primer sets (Exiqon): hsa-miR-192-5p (#204099), hsa-miR-194-5p (#204080), hsa-miR-215-5p (#204598), hsa-miR-93-5p (#204715), hsa-miR-151a-5p (#204007) and hsa-miR-245-5p (#204337). cDNA template corresponding to 1 ng RNA was used in each reaction and running performed in accordance with Exiqon’s instructions manual on a CFX384 Real-Time thermal cycler (Biorad). Each sample was run in duplicates, and negative controls i.e. no-template, blanks and a spike-in, UniSp6, were included in each run. The geometric mean of miR-93-5p, miR-151a-5p and miR-425-5p expression was used for normalisation. Fold changes were calculated using the 2ΔΔCt method.

mRNA: relative quantification of gene expression was performed using SsoFast™ EvaGreen Supermix (BioRad). cDNA template corresponding to 5 ng RNA was used in each reaction and running performed in accordance with Manufacturer’s instructions on a CFX384 Real-Time thermal cycler (Biorad). Primers: BCL2L11/BIM (#10025636, PrimePCR™ SYBR® Green Assay: BCL2L11, Human, BioRad), cFLAR/cFLIP (#10025636, PrimePCR™ SYBR® Green Assay: CFLAR, Human, BioRad), BAG4/SODD (#10025636, PrimePCR™ SYBR® Green Assay: BAG4, Human, BioRad), GAPDH exon 10–10 (Hs.PT.58.59810.g, Integrated DNA Technologies), ACTB exon 6–6 (Hs.PT.56a.4070309.g, Integrated DNA Technologies, Coralville, Iowa, USA).

2.9. Cell death measurement

Cell death was induced 24 h after transfection. Cell Death detection ELISAPLUS (#11920685001, Roche), determining the cytoplasmic fraction of mono- and oligonucleosomes in cell lysates, was performed 24 h after exposure to apoptotic stimuli following the instructions of the manufacturer. In short, cells were lysed directly in wells and lysates from duplicates collected and pooled. Each sample was measured in duplicates on a TECAN infinite M200PRO. The ELISA results were normalized to the DNA levels in the lysates measured after ultrasound sonication using the QuantFlour dsDNA System kit (E2670, Promega, Madison, Wisconsin, USA) following the manufacturer’s instructions. Caspase 3/7 activity as a measure of apoptosis was detected using the Caspase-Glo® 3/7 Assay (#G8093, Promega). Caspase 3/7 activity was measured in duplicates in 96-wells. Briefly, Caspase-Glo solution was added to the media in a 1:1 ratio, shaken for 30 s and incubated for 15 min at RT. Luminescence was measured on a TECAN, infinite M200PRO.

2.10. Statistics

Unless stated otherwise in Materials and Methods, the statistical analyses were performed in Prism version 7.02 (Graphpad Software Inc, La Jolla, California, USA). One-way ANOVA (no pairing) was used to analyze data from the three cell lines (HBV, HBs/HBx and HepG2 tet-on), while one-way ANOVA with repeated measures (RM) was used to analyze data from transfection experiments in the HepG2 tet-on cells. All ANOVA analyses were corrected for multiple comparisons (Bonferroni method). In case of comparison of only two conditions, two-tailed paired Student’s t-test was applied. All data is presented as means ± SEM. A p-value < 0.05 was considered statistically significant (p < 0.05, p < 0.01**, and p < 0.001****).
3. Results

3.1. HBV replication alters microRNA expression in a HepG2 cell model

To investigate if HBV replication modulates host cell microRNA expression, we used human liver HepG2 cells stably transfected with the tet-on system and a doxycycline-inducible HBV genome. In this system, the tetracycline-responsive promoter controls transcription of HBV pregenomic RNA, the preC/Core (including HBeAg) and polymerase genes, while HBs and HBx genes are controlled by their natural HBV enhancers/promoters. Thus, in the absence of doxycycline, only the viral surface proteins (HBs) and the HBx protein are expressed (HBs/HBx cells), whereas the addition of doxycycline to the culture medium induces generation of pregenomic HBV RNA, thus activating HBV replication (HBV cells). HepG2 tet-on cells were used as control (Fig. 1A-D and Suppl. Fig. 1A-B).

A real time qPCR-based microRNA screen was performed on total RNA enriched for smaller RNAs to detect differences in microRNA expression in the presence of HBV replication compared to the HepG2 tet-on control. The screen included 752 microRNAs, where 322 microRNAs were found to be expressed (Suppl. Table 1). MicroRNA expressions were normalized to the most stably expressed microRNAs and fold change (FC) calculated using the 2^ΔΔCt method.

A real time qPCR-based microRNA screen revealed 25 microRNAs differentially expressed between the HBV cells and HepG2 tet-on cells (Student's t-test, two-tailed, p ≤ 0.05). MicroRNA expressions were normalized to the most stably expressed microRNAs and fold change (FC) calculated using the 2^ΔΔCt method.

Table 1

| Target         | FC  | p-value |
|---------------|-----|---------|
| hsa-miR-192-5p | 1.69| 0.000   |
| hsa-miR-215-5p | 1.49| 0.000   |
| hsa-miR-194-5p | 1.54| 0.001   |
| hsa-miR-885-3p | 0.14| 0.002   |
| hsa-miR-33b-3p | 2.94| 0.003   |
| hsa-miR-196a-5p| 1.69| 0.004   |
| hsa-miR-885-5p | 0.48| 0.008   |
| hsa-miR-99a-5p | 0.51| 0.011   |
| hsa-miR-484    | 1.31| 0.013   |
| hsa-miR-1908-5p| 1.99| 0.014   |
| hsa-miR-1260a  | 0.79| 0.015   |
| hsa-miR-33b-3p | 1.57| 0.016   |
| hsa-miR-194-3p | 1.40| 0.017   |
| hsa-miR-139-5p | 0.44| 0.020   |
| hsa-miR-28-5p  | 1.11| 0.021   |
| hsa-miR-218-5p | 2.06| 0.028   |
| hsa-miR-192-3p | 1.35| 0.031   |
| hsa-miR-15b-5p | 1.44| 0.033   |
| hsa-miR-139a-5p| 0.50| 0.036   |
| hsa-miR-16-5p  | 1.32| 0.038   |
| hsa-miR-940    | 0.78| 0.039   |
| hsa-miR-200b-3p| 0.80| 0.047   |
| hsa-miR-1269a  | 0.66| 0.047   |
| hsa-miR-200b-5p| 0.72| 0.048   |
| hsa-miR-152-3p | 1.21| 0.049   |

Fig. 1. Verification of an HBV-replicating cell model system. A: Illustration of the HBV-replicating cell model system. The control cell line containing the tetracycline-regulated transactivator tet-on system (HepG2 tet-on cells) stably transfected with a doxycycline-inducible HBV genome. In the absence of doxycycline, only the viral surface proteins (HBs) and the HBx protein are expressed (HBs/HBx cells), whereas the addition of doxycycline to the culture medium induces generation of pregenomic HBV RNA, thus activating HBV replication (HBV cells). B: HBV DNA in growth medium detected with qPCR. Data are means ± SEM of 8 independent experiments. C: HBsAg secretion to the media detected by ELISA. Data are means ± SEM of 5 independent experiments. D: HBsAg secretion to the media detected by immunoassay giving sample to cut-off ratio (S/CO). Samples with S/CO > 1 is considered positive. Data are means ± SEM of 5 independent experiments. Statistics: One-way ANOVA, corrected for multiple comparisons (Bonferroni, p < 0.05*, p < 0.01** and p < 0.001***).
and are expected to target the same genes [27] (Fig. 2C). Further investigations, validating the transfection efficiency with microRNA mimics, revealed that the individual primer sets did not distinguish between the expression of miR-192-5p and miR-215-5p because of sequence homology (data not shown). Thus, it is not possible to differentiate between the expressions of these two microRNAs, and we refer to them as miR-192/215-5p when appropriate. Because miR-192-5p is most highly expressed in the liver compared to miR-215-5p [28], we focused on miR-192-5p and miR-194-5p in the following experiments.

3.2. HBx expression is sufficient to upregulate microRNA expression

The increased expression of miR-192-5p and miR-194-5p in the HBV cells compared to HepG2 tet-on cells were verified in individual RT-qPCR experiments (Fig. 3A). Interestingly, miR-192-5p and miR-194-5p were upregulated in the HBs/HBx cells to the same extent as in the HBV cells (Fig. 3A), indicating that the presence of HBs and HBx alone is sufficient to increase the expression of both microRNAs. Consistent with this, the HBx protein has previously been associated with changes in microRNA expression and disease progression [29-31]. To specifically investigate if HBx can upregulate miR-192-5p and miR-194-5p, we established clones of HepG2 tet-on cells with a doxycycline-inducible HBx expression. A dose-dependent doxycycline-induced expression of HBx was confirmed at the mRNA and protein levels (Fig. 3B and Suppl. Fig. 2A-B). However, even in the absence of doxycycline, low HBx expression was detected at the mRNA level (Fig. 3C). The expression levels of miR-192-5p and miR-194-5p were investigated under low and high HBx expression, i.e. without or with doxycycline, in two individual HBx-expressing clones, and compared to a mock clone stably transfected with an empty plasmid. Both low and high HBx expression induced miR-192-5p and miR-194-5p expression levels (Fig. 3D), however, not corresponding to the levels detected in the HBs/HBs and HBV cells. These results suggest that even a modest expression of HBx alone is sufficient to upregulate miR-192-5p and miR-194-5p expression in HepG2 cells.

3.3. MiR-192/215-5p and miR-194-5p have putative targets in the apoptosis signaling pathway

To gain insight into the putative biological functions of miR-192-5p and miR-194-5p, a bioinformatics-based target prediction and pathway analysis was performed. In this analysis, miR-215-5p was included to investigate the potential differences in target genes between miR-192-5p and miR-194-5p despite their strong sequence homology. All microRNA target genes with cross-linking immunoprecipitation-high-throughput sequencing (CLIP-seq) overlap of miR-192-5p, miR-215-5p and miR-194-5p were retrieved from StarBase [21,22] and filtered to ensure Ago2 association in HepG2 cells using online available Ago2 IP-based RNA-seq data from a HepG2 cell line [23]. In total, 1260 genes were identified, and the putative targets of miR-192-5p and miR-215-5p had more than 80% overlap (data not shown).

To identify pathways likely to be regulated by miR-192-215/215 and miR-194-5p, the potential target genes with Gene Ontology (GO) terms (1203 genes) were subjected to an enrichment analysis using the PANTHER database [24]. This bioinformatics analysis revealed both an over- and underrepresentation of target genes engaged in various biological processes (Suppl. Table 2). Two signaling pathways revealed a significant enrichment of potential target genes: the ubiquitin proteasome pathway with potential targets of miR-192-5p and the apoptosis signaling pathway with potential targets of all three microRNAs (Table 2). Notably, BIM, encoded by the BCL2L11 gene, was identified as a putative target of all three microRNAs. As the ubiquitin proteasome pathway also plays a central role in the regulation of apoptosis [32,33], these findings prompted us to investigate if miR-192-5p and miR-194-5p have apoptosis modulatory effects in HepG2 tet-on cells.

3.4. MiR-192-5p and miR-194-5p have anti- and pro-apoptotic effects, respectively

To directly examine whether miR-192-5p and miR-194-5p modulate apoptosis signaling, we transfected HepG2 tet-on cells with microRNA mimics of miR-192-5p and miR-194-5p or a non-targeting negative control mimic and measured cell death. To induce an apoptotic response, we treated cells with thapsigargin (TG), which inhibits the sarcoendoplasmic reticulum calcium transport ATPase (SERCA) pump causing endoplasmic reticulum (ER) stress, leading to apoptosis.
through the intrinsic apoptosis signaling pathway (Fig. 4A). While transfection with miR-192-5p mimic led to a significant reduction in TG-induced cell death, transfection with miR-194-5p mimic caused significant increased TG-mediated cell death (Fig. 4B). These results were confirmed at the level of caspase 3/7 activity verifying that the microRNAs interfered specifically with apoptosis signaling (Fig. 4C).

Notably, TG treatment was found to downregulate the expression of miR-194-5p and miR-192-5p in HepG2 tet-on cells (Fig. 4E), supporting a relationship between apoptosis signaling and these microRNAs.

We next measured if miR-192-5p and miR-194-5p also affect caspase 3/7 activity in the presence of a physiological stimulus using a cocktail of the pro-inflammatory cytokines TNF-α and interferon gamma (IFN-γ), which have been found increased in the serum of patients with acute hepatitis [34]. Interestingly, in cells transfected with negative control mimic, cytokines alone were insufficient to increase caspase 3/7 activity significantly, but transfection with miR-194-5p mimic strongly sensitized the cells to cytokine-induced apoptosis (Fig. 4D). Cytokine-induction did not affect the expression of miR-192-5p and miR-194-5p as seen for the more strong apoptosis-inducer TG (Fig. 4F).

Altogether, these data indicate that miR-192-5p and miR-194-5p have opposite regulatory effects on apoptosis in HepG2 cells.
Table 2  
Bioinformatics analysis on miR-192-5p, miR-194-5p and miR-215-5p. Target genes with CLIP-seq overlap of miR-192-5p, miR-194-5p and miR-215-5p were retrieved from StarBase and filtered for Ago2 binding in HepG2 cells using online available Ago2 IP-based RNA-seq data (RPKM > 1). Gene ontology (GO) and pathway analyses using the PANTHER database were performed with 1203 genes and two pathways with an enriched number of putative target genes of miR-192/215-5p and miR-194-5p were identified.

| Apoptosis signaling pathway | hsa-miR-192/215-5p | hsa-miR-194-5p |
|----------------------------|-------------------|---------------|
| TP7                        | x                 |               |
| TMBIM6                     |                   |               |
| CASP7                      |                   |               |
| CYCS                       | x                 |               |
| HSPA8                      |                   |               |
| MCL1                       |                   |               |
| BCL2L11                    | x                 | x             |
| MAPK1                      | x                 |               |
| XIAP                       | x                 |               |
| BAG4                       |                   |               |
| CFLAR                      |                   |               |
| CHUK                       |                   |               |
| MAP3K1                     | x                 |               |
| AKT2                       | x                 |               |
| ATF2                       |                   |               |
| RELA                       |                   |               |
| CASP8                      |                   |               |
| TNFRSF1A                   |                   |               |
| TP53                       | x (only 192-5p)   |               |

| Ubiquitin proteasome pathway | hsa-miR-192/215-5p | hsa-miR-194-5p |
|------------------------------|-------------------|---------------|
| UBE2E1                       | x                 |               |
| UBE2B                        |                   |               |
| PSMD12                       |                   |               |
| MDM2                         | x                 |               |
| UBA6                         | x                 |               |
| UBE2D3                       |                   |               |
| SMURF1                       | x                 |               |
| UBE2K                        | x                 |               |
| UBE3A                        | x                 |               |
| UB4A                         |                   |               |
| UBE2V2                       |                   |               |

3.5. MiR-192-5p and miR-194-5p downregulate the protein level of important regulators of apoptosis

To identify targets responsible for the observed effects of miR-192-5p and miR-194-5p on apoptosis, we investigated the protein level of some of the putative targets after transfection with miR-192-5p and miR-194-5p mimics. Based on the caspase 3/7 activity results, we selected two predicted targets of miR-192-5p known to be pro-apoptotic (BIM and CYCS encoding cytochrome c), and two predicted targets of miR-194-5p known to be anti-apoptotic (CFLAR encoding cFLIP and BAG4 encoding silencer of death domains (SODD)). The protein level of BIM was downregulated significantly by both miR-192-5p and miR-194-5p, verifying BIM as a target of both microRNAs (Fig. 5A), while cytochrome c was non-significantly affected by miR-192-5p (Fig. 5B). The anti-apoptotic proteins cFLIP and SODD were both downregulated in cells transfected with miR-194-5p mimic (Fig. 5C and D). Surprisingly, cFLIP was also suppressed in cells transfected with miR-192-5p mimic (Fig. 5C). The bioinformatics analysis only predicted cFLIP as a target of miR-194-5p, but a “low probability” target site of miR-192-5p was identified in the 3‘UTR of cFLIP mRNA using the mirSVR scoring method [25].

We next examined, whether the ~1.5-fold upregulation of miR-192-5p and miR-194-5p observed in HBs/HBx and HBV cells compared to the HepG2 tet-on cells (Fig. 3A) is sufficient to cause decreased protein levels of BIM, SODD and cFLIP. However, there were no significant differences in the expression of these proteins, suggesting that the ~50% increase in microRNA expression is insufficient to repress target protein expression under these (basal) conditions (Suppl. Fig. 3A-C).

Collectively, these results indicate that BIM and cFLIP are targeted by both miR-192-5p and miR-194-5p, while SODD is regulated solely by miR-194-5p.

3.6. Knockdown of cFLIP increases the sensitivity of HepG2 cells to induced apoptosis

To examine the contribution of microRNA-mediated downregulation of BIM, cFLIP and SODD to apoptotic cell death, we next measured caspase 3/7 activity after small interfering RNA (siRNA)-mediated knockdown of BIM, cFLIP and SODD followed by treatment with either TG or cytokines. Knockdown efficiency was confirmed after 48 h at both the mRNA (Fig. 6A) and protein levels (Fig. 6B). Knockdown of BIM or SODD affected neither basal nor TG- or cytokine-induced caspase 3/7 activity as compared to the siRNA control. However, knockdown of cFLIP strongly sensitized cells to both TG- and cytokine-induced apoptosis (Fig. 6C). These findings confirm an anti-apoptotic effect of cFLIP in HepG2 cells, and suggest that miR-194-5p-mediated suppression of cFLIP expression may contribute to increased levels of apoptosis.

4. Discussion

Chronic hepatitis B is associated with a high risk of developing HCC [1,2]. Therefore, a better understanding of the molecular mechanisms involved in HBV pathogenesis as well as identification of sensitive prognostic biomarkers is warranted. MicroRNAs play an important role in the host – virus interaction [35,36] and are potential biomarkers of disease [37]. In this study, we investigated the differential expression of microRNAs in an HBV-replicating human hepatoma cell line, and found an increased expression of miR-192/215-5p and miR-194-5p in the presence of HBV replication. Bioinformatics analyses revealed an enrichment of potential target genes in the ubiquitin proteasome pathway and the apoptosis signaling pathway. Furthermore, functional studies suggested opposite regulatory functions of miR-192-5p and miR-194-5p in apoptosis signaling.

The upregulation of miR-192/215-5p and miR-194-5p detected in response to HBV replication was only 1.5-fold. However, for highly expressed microRNAs even small changes in their expression levels could have an effect on target gene expression [38]. Interestingly, miR-192-5p constitutes 16.9% of the total microRNA pool in the liver [28] and in our experiments miR-194-5p came up only one Ct value later than miR-192-5p. We found that HBx expression was sufficient to upregulate miR-192-5p and miR-194-5p expression, but not corresponding to the levels detected in the HBs/HBx and HBV cells. The Tet-on inducible system has been found to be leaky [39] and a low level of HBV replication might be sufficient to alter microRNA expression in HBs/HBx cells. Alternatively, the activity of HBx could be different depending on the presence or absence of HBV replication.

The upregulation of miR-192-215p and miR-194-5p in response to HBV replication can either be HBV-mediated or a cellular response to the infection. We found that HBx upregulates miR-194-5p expression; thus, HBx could sensitize cells to TG- and cytokine-induced apoptosis via indirect downregulation of cFLIP. Interestingly, HBx expression has previously been found to sensitize cells to apoptosis [40,41]. HBV-mediated downregulation of cFLIP agrees with recent findings that the p22-cFLIP cleavage product produced upon TNF-α signaling inhibits HBV replication [42]. A direct interaction between HBx and cFLIP upon overexpression of HBx has also been reported [43]. Inferring these observations, it could be speculated that downregulation of cFLIP by miR-194-5p in response to HBV infection in vivo sensitizes hepatocytes to apoptotic signals leading to increased hepatocyte death thereby...
contributing to the development of severe liver disease.

Transfection with miR-194-5p mimic was found to downregulate cFLIP levels and increase both TG- and cytokine-induced apoptosis, as did siRNA-mediated knockdown of cFLIP. These results suggest a pro-apoptotic function of miR-194-5p through downregulation of cFLIP. An increase in TG-induced apoptosis upon knockdown of cFLIP was unexpected. Treatment with TG leads to ER stress and subsequent apoptosis via the intrinsic apoptosis signaling pathway, while cFLIP is an inhibitor of caspase 8, engaged in the extrinsic apoptosis signaling pathway [11–13]. However, it has been reported that TG-induced apoptosis partially depends on TNF-α signaling via enhancement of TNF-α expression [44]. An ER stress-induced autocrine TNF-α signaling could explain the increase in apoptosis that we detect after treatment with TG and transfection with siFLIP. Alternatively, cFLIP could have an inhibitory function in the intrinsic apoptosis signaling pathway, as has been suggested in mature mouse T lymphocytes [45].

Fig. 4. The effect of miR-192-5p and miR-194-5p on apoptosis signaling. A: Illustration of the extrinsic and intrinsic apoptosis signaling pathways. The extrinsic signaling pathway is illustrated with the TNF-α death ligand. HepG2 tet-on cells were transfected 24 h with microRNA mimics (5 nM) and apoptosis was induced with thapsigargin (TG 100 nM) or a cocktail of cytokines (10 ng/ml TNF-α and 10 ng/ml Interferon-γ (IFN-γ)) 24 h before harvest and apoptosis measurement. B: Cell death ELISA. Data are means ± SEM of 5 independent experiments. C: Caspase 3/7 activity, thapsigargin treatment. Data are means ± SEM of 4 independent experiments. The effect of (E) thapsigargin (100 nM), and (F) cytokines (10 ng/ml TNF-α + 10 ng/ml IFN-γ) on miR-192-5p and miR-194-5p expressions. Data are means ± SEM of 3 independent experiments. Statistics (B-D): RM one-way ANOVA on log transformed data, corrected for multiple comparisons (Bonferroni). Statistics (E-F): two-tailed, paired Student’s t-test. (p < 0.05*, p < 0.01** and p < 0.001***).
apoptosis by activation of BIM [49]. However, knockdown of BIM did not protect cells from TG-induced apoptosis. An explanation could be that the level of apoptosis caused by TG treatment was too harsh to be prevented by knockdown of BIM alone. Alternatively, other BH3-only proteins might substitute for the lack of BIM [50]. The inconsistency between the effect of transfection with miR-192-5p mimic and knockdown of BIM on TG-induced apoptosis indicates that the protective effect of miR-192-5p is not solely due to downregulation of BIM, but also regulation of other and hitherto unidentified target genes.

We found that miR-194-5p induced apoptosis while miR-192-5p suppressed apoptosis, although both microRNAs were found to target both pro- and anti-apoptotic regulators. It is known that microRNAs form complex regulatory networks, where a single microRNA can have several targets in the same pathway, but the regulation of individual targets often is minimal [51,52]. Hence, the phenotype is the sum of these regulations. This could explain the discrepancy between miR-192-5p-mediated regulation of anti-apoptotic cFLIP, but suppression of apoptosis, and miR-194-5p-mediated regulation of pro-apoptotic BIM, but induction of apoptosis. Furthermore, it could explain why we were not able to detect any change in the expression of cFLIP, BIM or SODD under “basal” conditions with 1.5-fold upregulation of miR-192/215-5p and miR-194-5p.

In this study, the cytokines TNF-α and IFN-γ were used as physiological apoptotic stimuli. These cytokines are increased in the serum of patients with acute hepatitis and are secreted by activated T cells [34], which are believed to be important immune mediators in HBV clearance [53]. Within the time and dose window used in our study, TNF-α and IFN-γ failed to induce apoptosis in HepG2 tet-on cells. An apoptotic response was only observed in cells transfected with miR-194-5p or siRNA against cFLIP, both of which strongly sensitized the cells. In agreement with our results, TNF-α and IFN-γ have previously been found to induce apoptosis in an HBV-expressing cell line, while having no effects on HepG2 cells [54]. Collectively, these data suggest that upregulation of miR-194-5p in response to HBV replication could cause
an increased apoptotic vulnerability of infected cells due to down-regulation of cFLIP.

miR-192-215-5p and miR-194-5p have previously been associated with cancer [64–69] and their levels in circulation have been found to correlate with HBV pathogenesis and the development of HBV-related HCC [55–61]. In addition, we recently found them differentially expressed in plasma from children with CHB [62], and during the different immunological phases of CHB [63]. In accordance with our results, others have found miR-192-5p and miR-194-5p upregulated in HBV-positive HCC cells [61,70,71]. Interestingly, Van der Ree and colleagues recently found miR-192-5p and miR-194-5p present in higher levels in the culture supernatant of HBV-positive HCC cells and present in both extracellular vesicles and HBsAg particles [61]. This may explain the upregulation of miR-192-5p and miR-194-5p found in the circulation of patients with CHB. However, the biological functions of these microRNAs in relation to HBV infection are largely unknown. In this study, we found a regulatory function of miR-192/215-5p and miR-194-5p in the apoptosis signaling pathway. Hence, even small changes in their expression level in CHB could contribute to the development of liver disease such as HCC over time. Altogether, this indicates that miR-192-215-5p and miR-194-5p have the potential to act as circulating biomarkers of CHB pathogenesis.

In conclusion, our findings suggest that HBV infection is associated with increased miR-192/215-5p and miR-194-5p expression in vitro. Further, these microRNAs were found to target important apoptosis-regulatory proteins. In particular, miR-194-5p-mediated suppression of cFLIP expression strongly sensitizes HepG2 cells to undergo apoptosis in response to a physiological apoptotic stimulus.

Acknowledgements

The excellent technical assistance of Ann-Sofie Bjern Hillese, Sigrun Broehl and Birgit Knudsen, and support in primer design from Pia Roppert are gratefully acknowledged. The input and advice from Maiken Worsøe Rosenstierne is highly appreciated. We are grateful to The Julie von Müllens Fond and The PA Messerschmidt and Wife Foundation for financial support. The work of DG was supported by a grant (SFB1021, B08) from the Deutsche Forschungsgemeinschaft (DFG).

Conflict of interest

The authors declare no competing financial interests.

Author contributions

KON, KSJ, TNW, JS, DG, FP and BH conceived and designed the study. DG provided the doxycycline-inducible HBV-replicating hepatoma cells. KON performed the experiments. KON, AHM, KSJ and JS analyzed the data. AHM performed the bioinformatics analysis. KON wrote the manuscript and revised it following significant scientific input from all authors.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.yexcr.2018.07.044.

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