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Down-Regulation of ATP-Binding Cassette C2 Protein Expression in HepG2 Cells after Rifampicin Treatment Is Mediated by MicroRNA-379[S]

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ABSTRACT

microRNAs (miRNAs), which contribute to the post-transcriptional processing through 3′-untranslated region-interference, have been shown to be involved in the regulation of ATP-binding cassette (ABC) membrane transporters. The aim of this study was to investigate whether ABCC2, an important efflux transporter for various endogenous and exogenous compounds at several compartment barriers, is subject to miRNA-mediated post-transcriptional gene regulation. We screened the expression of 377 human miRNAs in HepG2 cells after 48 h of treatment with 5 μM rifampicin [a pregnane X receptor (PXR) ligand] or vehicle using reverse transcription-polymerase chain reaction-based low-density arrays. Specific miRNA, ABCC2 mRNA, and protein expression were monitored in HepG2 cells undergoing rifampicin treatment for 72 h. Loss- and gain-of-function experiments and reporter gene assays were performed for further confirmation. Highly deregulated miRNAs compared with in silico data revealed miRNA (miR) 379 as candidate miRNA targeting ABCC2 mRNA. Under rifampicin treatment, ABCC2 mRNA increased significantly, with a maximal fold change of 1.56 ± 0.43 after 24 h. In addition, miR-379 increased (maximally 4.10 ± 1.33-fold after 48 h), whereas ABCC2 protein decreased with a maximal fold change of 0.47 ± 0.08 after 72 h. In contrast, transfection of miR-379 inhibitor led to an elevation of ABCC2 protein expression after rifampicin incubation for 48 h. We identify a miRNA negatively regulating ABCC2 on the post-transcriptional level and provide evidence that this miRNA impedes overexpression of ABCC2 protein after a PXR-mediated external transcriptional stimulus in HepG2 cells.

Introduction

MicroRNAs, which contribute to post-transcriptional processing through 3′-UTR-interference, have been demonstrated to be involved in the regulation of ABC membrane transporters. In cancer cell lines, miR-451 (Kovalchuk et al., 2008) and miR-326 (Liang et al., 2010) negatively regulate ABCB1 (multidrug resistance protein 1, P-glycoprotein) and ABCC1 (multidrug resistance associated protein 1), respectively. miR-328 (Pan et al., 2009) and miR-519c (To et al., 2008) were shown to affect post-transcriptionally the expression of ABCG2 (breast cancer resistance protein). An impaired expression of these miRNAs might be one of the underlying mechanisms of drug resistance against anticancer agents in neoplastic cells. So far, no information is available concerning the role of microRNAs in the post-transcriptional regulation of ABCC2 (multidrug resistance associated protein 2), another important efflux transporter. This drug efflux transporter not only eliminates exogenous compounds but also is involved in the elimination of conjugated and unconjugated endogenous substances (Jemnitz et al., 2010). The aim of this study was to identify miRNAs targeting the ABCC2 3′-UTR and to determine their role in ABCC2 protein expression.

On the basis of the hypothesis that ABCC2 transcriptional processing could be accompanied by an altered expression of miRNAs targeting ABCC2, we performed an array-based miRNA screen of HepG2 cells preincubated with either the PXR-ligand rifampicin or a vehicle. After in silico analysis of differentially regulated miRNAs and subsequent loss and

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ABBREVIATIONS: ABC, ATP-binding cassette; DMSO, dimethyl sulfoxide; miRNA or miR, microRNA; PXR, pregnane X receptor; UTR, untranslated region; PCR, polymerase chain reaction; rtPCR, real time PCR.

gain-of-function experiments and reporter gene assays, we identified a miRNA that negatively regulates ABCC2 protein expression by direct RNA interference. The identified miRNA seems to counteract overexpression of ABCC2 protein in HepG2 cells in presence of the transcriptional stimulus rifampicin.

Materials and Methods

Cell Culture. Human hepatoblastoma HepG2 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in a 5% CO_2 atmosphere. Cell culture medium and supplements were purchased from PAA Laboratories (Pasching, Austria). HepG2 cells were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany).

miRNA Expression Profiling. HepG2 cells were seeded in 10-cm dishes (10^5 cells/ml). After 24 h, the medium was replaced by medium supplemented with 5 μM rifampicin or vehicle [dimethyl sulfoxide (DMSO), 1:6000], both purchased from Sigma-Aldrich (Steinheim, Germany). Cells were incubated for 48 h. In all experiments, fresh medium supplemented with rifampicin or vehicle was replaced every 24 h, simulating a daily dosage. RNA was isolated using the mirVana miRNA isolation kit (Ambion, Heppenheim, Germany) and miRNAs contained in 800 ng of total RNA were reverse transcribed using Megaplex Primer Pools, Human Pools A version 2.1 and the TaqMan microRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). cDNAs and Universal Master Mix (Applied Biosystems) were loaded on TaqMan Array Human MicroRNA A cards version 2.0 (Applied Biosystems) in a volume of 100 μl per port, according to the manufacturer's recommendations. Quantitative real-time PCR (rtPCR) reactions were conducted using an ABI Prism 7900HT (Applied Biosystems). Data were analyzed by performing the $2^{-\Delta\Delta C_t}$ method. Mammalian U6 small nuclear RNA present in quadruplicate on the array was used as endogenous control. MicroRNAs were considered to have a strong impact if they exhibited a more than 8-fold altered expression level under rifampicin treatment compared to vehicle. For in silico identification of miRNAs predicted to target ABCC2, a search using the microCosm miRNA target data base (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) was performed.

miR-379, mRNA, and Protein Quantification. Effects of the PXR-ligand rifampicin were determined after 24 h incubation in normal medium followed by 72 h of incubation in medium supplemented with 5 μM rifampicin or vehicle (DMSO). Total RNA and protein were isolated after 0, 8, 24, 48, and 72 h of incubation. Two hundred nanograms of total RNA were reverse transcribed in a duplex approach using specific reverse transcription primers for miR-379 and RNU6B. RNU6B, one of the least variable small nuclear RNAs in human cell lines, was used as internal control. cDNA quantification was performed with TaqMan microRNA assays (for miR379 and RNU6B) in singleplex PCR reactions using Universal Master Mix (Applied Biosystems).

ABCC2 mRNA quantification was performed after reverse transcription of 200 ng of total RNA (Transcriptor First Strand cDNA Synthesis kit; Roche, Mannheim, Germany) using Universal Master Mix and the TaqMan assay (ID Hs00166123_m1). 18S rRNA was measured as internal reference using TaqMan assay ID Hs99999901_s1.

All rtPCR reactions were performed on an ABI Prism 7900HT (Applied Biosystems) in duplicate. Data were calculated using the $2^{-\Delta\Delta C_t}$ method.

Proteins were isolated using the Qproteome Mammalian Protein Prep Kit (QIAGEN, Hilden, Germany) and quantified with the a protein assay (Bio-Rad Laboratories, Munich, Germany). For Western blotting, proteins were separated on Nu-PAGE Novex 10% bis-Tris Gels (Invitrogen, Karlsruhe, Germany) and transferred to polyvinylidene fluoride microporous membranes (Millipore, Schwalbach, Germany) using a tank blotting system (Bio-Rad Laboratories). Membranes were blocked in 5% (w/v) nonfat dried milk in Tris buffered saline-T buffer [0.27 mM KCl, 13.7 mM NaCl, 5 mM Tris-HCl, pH 7.4, 0.1% (w/v) Tween 20] and incubated overnight with the primary antibodies MRP2 M2III-6 (1:500; Alexis, San Diego, CA) or anti-P-glycoprotein (ABCB1) (dilution 1:500; Enzo Life Sciences, Inc., Farmingdale, NY) and anti-β-actin as internal standard (1:10,000; Sigma-Aldrich). Horseradish peroxidase-labeled rabbit anti-mouse IgG (1:10,000; Sigma-Aldrich) was used as secondary antibody. Detection was performed by chemiluminescence with ECL Western Blotting Detection Reagents (GE Healthcare, Munich, Germany). Blots were quantified by densitometry using Quantity One 1-D Analysis Software 4.6.3 (Bio-Rad Laboratories). miR-379/RNU6B, ABCC2 mRNA/18S rRNA, and ABCC2 protein/β-actin protein ratios were normalized to values under respective vehicle-stimulated conditions. Data were obtained from three identical experiments.

Transient Transfection with miR-379. To investigate the post-transcriptional influence of selected miR-379 on ABCC2 mRNA and protein expression, HepG2 cells were reverse-transfected in six-well plates by covering 600 μl of transfection complex with 2.4 ml of cell suspension (10^5 cells/ml) per well. siPORT *NeoFX* Transfection Agent (Ambion) was diluted in Opti-MEM I Medium (Invitrogen) to a final dilution of 0.3% (v/v). In addition, dilutions of miR-379 miRNA precursor in final concentrations of 5, 20, 50, and 100 nM, miR-379 miRNA inhibitor (50 and 100 nM), and miRNA precursor (Pre-miR miRNA Precursor Molecules, Negative Control 1; 20 nM) and miRNA inhibitor (Anti-miR miRNA Inhibitors, Negative Control 1; 50 nM) negative controls were prepared from the respective stock solutions (6.25 μM) in Opti-MEM I medium. All were purchased from Ambion. After 10 min of incubation, transfection complexes were obtained by mixing equal parts of the diluted transfection agent and the respective diluted miRNA precursors or miRNA inhibitors. Optimization experiments were performed according to the manufacturer's recommendations using miR-1 precursor (Pre-miR hsa-miR-1 miRNA Precursor Positive Control, down-regulating PTK9 mRNA) and let-7c inhibitor (up-regulating HMG2A mRNA) as positive controls. Minimal effective transfection concentrations were 5 to 20 nM for miRNA precursor and 50 nM for miRNA inhibitor molecules, respectively (data not shown). Moreover, no evidence of cytotoxicity was observed for miRNA precursor and miRNA inhibitor negative controls at concentrations of 5 to 100 nM. The cytotoxicity factor, expressed as ratio of C_t value of 18S rRNA of transfected cells to C_t value of 18S rRNA of nontransfected HepG2 cells, was 1.0 for all tested concentrations. Therefore, all further transfection experiments used 20 nM of miRNA precursor negative control and 50 nM of miRNA inhibitor negative control.

Twenty-four hours after transfection, medium was replaced by fresh normal growth medium. After an additional 24 h of incubation, protein and total RNA were isolated. ABCC2 protein was quantified from three identical experiments and ABCC2 mRNA from two, as described above.

In Vitro Investigation of the ABCC2 3′-UTR as a Target of miR-379. To validate the ABCC2 3′-UTR as a target of miR-379, in vitro assays using the miTarget microRNA 3′-UTR target sequence expression clones (GeneCopoeia, Rockville, MD) were performed. These miRNA target clones consist of the pEZX-MT01 vector containing the coding sequences of firefly luciferase and *Renilla reniformis* luciferase under the control of the simian virus 40 enhancer and the cytomegalovirus promoter, respectively. The miRNA ABCC2 3′-UTR target clone exhibits the ABCC2 3′-UTR (accession number: NM_000392.2) inserted downstream of the firefly luciferase sequence. According to the Sanger miRBase (Kozomara and Griffiths-Jones 2010), the binding site of miR-379 (5′-U**GGUAGAC**U**AU**G-**GA**ACGUAGG; nucleotides of seed sequence are bold) is predicted at positions 180 to 200. The vector pEZX-MT01 exhibiting no 3′-UTR insert downstream of the firefly luciferase was used as a positive

control. After heat-shock transformation in competent *Escherichia coli* cells (One Shot TOP 10 competent cells; Invitrogen), plasmids were amplified in Luria-Bertani medium supplemented with 50 μg/ml kanamycin (Sigma-Aldrich). Plasmid DNA was purified by QIAprep spin miniprep kit (QIAGEN). Correctness of amplified plasmids was confirmed by capillary sequencing (GATC Biotech, Konstanz, Germany), using the sequencing primers 5′-GATCCGCGAGATCCTGAT (forward) or 5′-TTGGCGTTACTATGGGAACAT (reverse).

Cotransfection of 100 ng/well of plasmid DNA and precursor miRNA or miRNA inhibitor molecules was performed essentially as described above in a 96-well plate. The activities of both firefly and the internal control *R. reniformis* luciferases were determined 48 h after transfection with the dual-luciferase reporter assay system (Promega, Mannheim, Germany) and analyzed on a Veritas microplate luminometer (Tuner Biosystems, Sunnyvale, CA). Seven identical cotransfections were conducted.

Mutations within the potential miR-379 binding site were introduced by site-directed mutagenesis of the ABCC2 3′-UTR using appropriate primer pairs (Sigma-Aldrich, Munich, Germany), PfuUltra Hotstart DNA Polymerase (Agilent Technologies, Waldbronn, Germany), QIAquick PCR Purification Kit (QIAGEN), DpnI (New England Biolabs, Frankfurt, Germany), and DH5α competent cells. The first, third, and fifth position (bold) within the seed sequence of the predicted miRNA (underlined) binding site was changed as follows: wild type, 5′-GAGAAACCCCTCGATTGTCTACCTCGATCGTACTTCCTTGC-3′; mutant, 5′-GAGAAACCCCTCGATT**CTGTTCC**TCGATCGTACTTCCTTGC-3′. All reporter gene assays with mutated target clones were performed in quadruplicate.

Statistical Analysis. Mann-Whitney *U* tests were performed to compare the various effects of rifampicin on miR-379, ABCC2 mRNA, ABCC2, and ABCB1 protein expression and to test for effects of miR-379 precursor or inhibitor on ABCC2 mRNA and protein expression as well as on reporter gene activities using SPSS 17.0 (SPSS Inc., Chicago, IL). Values are given as means ± S.D. A p value ≤0.05 was considered to be statistically significant.

Results

Screen of Deregulated miRNA Expression after Rifampicin Treatment. Screening of HepG2 cells for differentially expressed microRNAs after rifampicin incubation revealed that 111 of 377 human miRNAs were not expressed at all or showed expression in only one of the different treatment conditions. After $\log_2$ transformation of fold changes, 11 miRNAs were identified as having a more than 3-fold altered expression (Fig. 1). miR-31 was 5.4-fold down-regulated, whereas all others (i.e, miR-193a-5p, miR-296-5p, miR-324-5p, miR-379, miR-411, miR-489, miR505, miR-519a, miR-545, and miR-548b-5p) exhibited up to 5.8-fold higher expression levels. Of these deregulated miRNAs, only miR-379 matched with in silico-predicted miRNAs (microCosm miRNA target data base) potentially targeting ABCC2.

Time-Dependent Impact of Rifampicin on ABCC2 and miR-379 Expression. To further explore the coregulation of miR-379 with ABCC2 under rifampicin treatment, we simultaneously investigated the time-dependent expression of miR-379, ABCC2 mRNA, and ABCC2 protein under rifampicin (5 μM) treatment up to 72 h in HepG2 cells. After normalizing the data to respective values of DMSO-treated cells compared to time point 0 h, the relative miR-379 expression showed significant changes after 48 h (4.10 ± 1.33-fold, $p = 0.04$). The relative ABCC2 mRNA was maximally up-regulated after 24 h (1.56 ± 0.43-fold, $p = 0.04$), whereas the relative protein content was down-regulated with a minimum after 72 h (0.47 ± 0.08-fold, $p = 0.04$) (Fig. 2).

Induction of ABCB1 Protein Expression by Rifampicin. As proof of principle, the influence of rifampicin on ABCB1 expression in HepG2 cells at the protein level was determined after stimulation with 5 μM rifampicin or DMSO as vehicle at different time points. After 72 h, ABCB1 protein was maximally induced and 3.4 ± 0.1 times higher compared to the unstimulated control (Supplemental Figure).

Impact of miR-379 on ABCC2 mRNA and Protein Expression. The influence of miRNA-379 on ABCC2 gene expression at the mRNA and protein level was determined after transfection of increasing concentrations of miR-379 precursor and inhibitor, as well as their respective negative controls. The mean relative ABCC2 mRNA expression decreased slightly to a minimal level of 72.0% at 50 nM of miR-379 precursor. The densitometric analysis of Western blots revealed significantly decreased mean relative ABCC2 protein amounts with a minimum of 46.2 ± 12.1% ($p = 0.04$), also at 50 nM of miR-379 precursor. Transfection of 50 or 100 nM miR-379 inhibitor did not affect ABCC2 mRNA expression, but 100 nM led to a significant up-regulation (143.7 ± 34.8%; $p = 0.04$) of ABCC2 protein as compared to cells transfected with miRNA inhibitor negative control (Fig. 3).

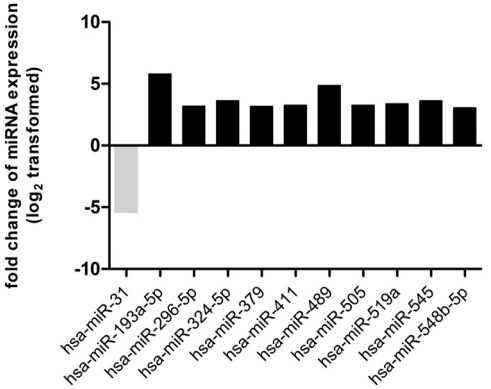

Fig. 1. Effects of 5 μM rifampicin incubation for 48 h on miRNA expression in HepG2 cells as compared to DMSO. MicroRNAs shown have more than 3-fold deregulation after $\log_2$- transformation.

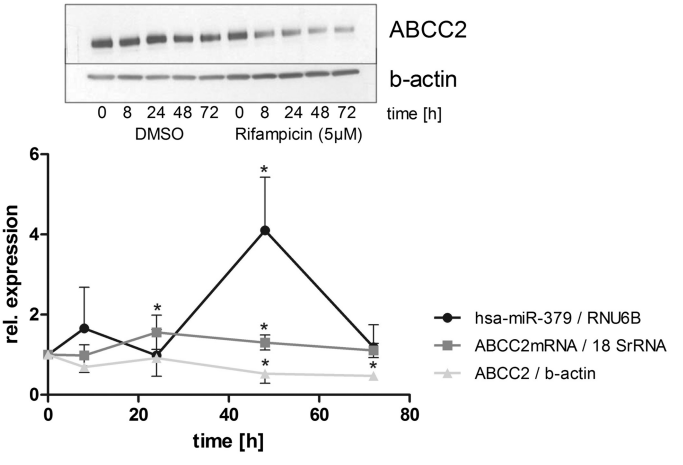

Fig. 2. Effects of incubation with 5 μM rifampicin or a vehicle in HepG2 cells up to 72 h on ABCC2 protein expression (Western blot with β-actin protein expression as loading control) and on relative expression of miR-379, ABCC2 mRNA and ABCC2 protein (analyzed densitometrically from three Western blots). *, $p < 0.05$, Mann-Whitney *U* test, $n = 3$.

ABCC2 3′-UTR as Target of miR-379. For confirmation that ABCC2 3′-UTR sequence represents a target site for miR-379, we also determined the reporter gene activities of the miRNA ABCC2 3′-UTR target clone after cotransfection with increasing concentrations of miR-379 precursor and inhibitor, as well as their respective negative controls in HepG2 cells. Transfection of miR-379 precursor led to a highly significant decrease of relative luciferase activities with a minimum of 39.6 ± 5.1% ($p = 0.001$) at a concentration of 50 nM compared with cells cotransfected with miRNA precursor negative control. In contrast, cells cotransfected with the target clone and 50 or 100 nM miR-379 inhibitor did not show any changes in luciferase activities compared to cells cotransfected with miRNA inhibitor negative control (Fig. 4A). A further proof of principle was performed by transfecting an ABCC2 3′-UTR target clone exhibiting a

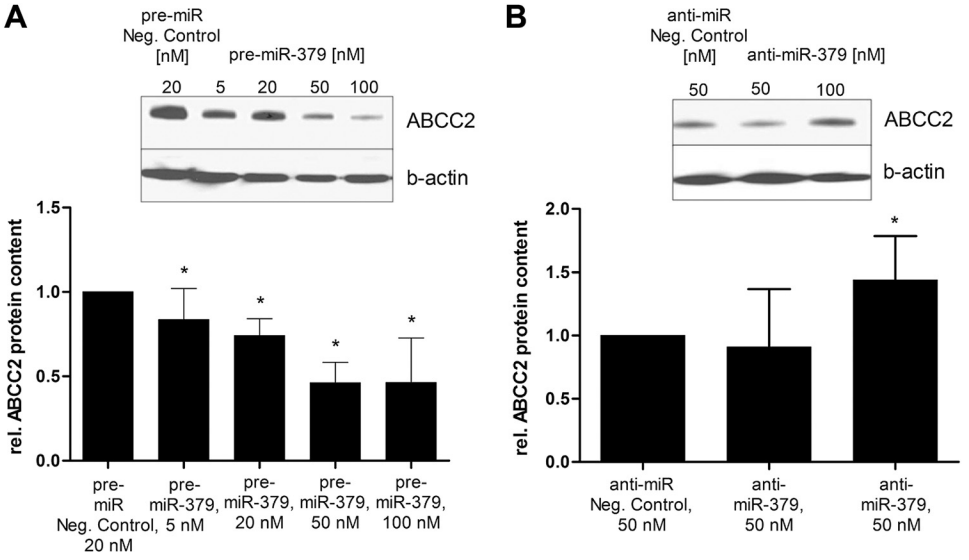

Fig. 3. Effects of miRNA-379 precursor (A) and inhibitor on ABCC2 expression in HepG2 cells (B) 48 h after transfection. ABCC2 protein expression relative to β-actin was determined by Western blot and subsequent densitometry. *, $p < 0.05$, Mann-Whitney-U test, $n = 3$.

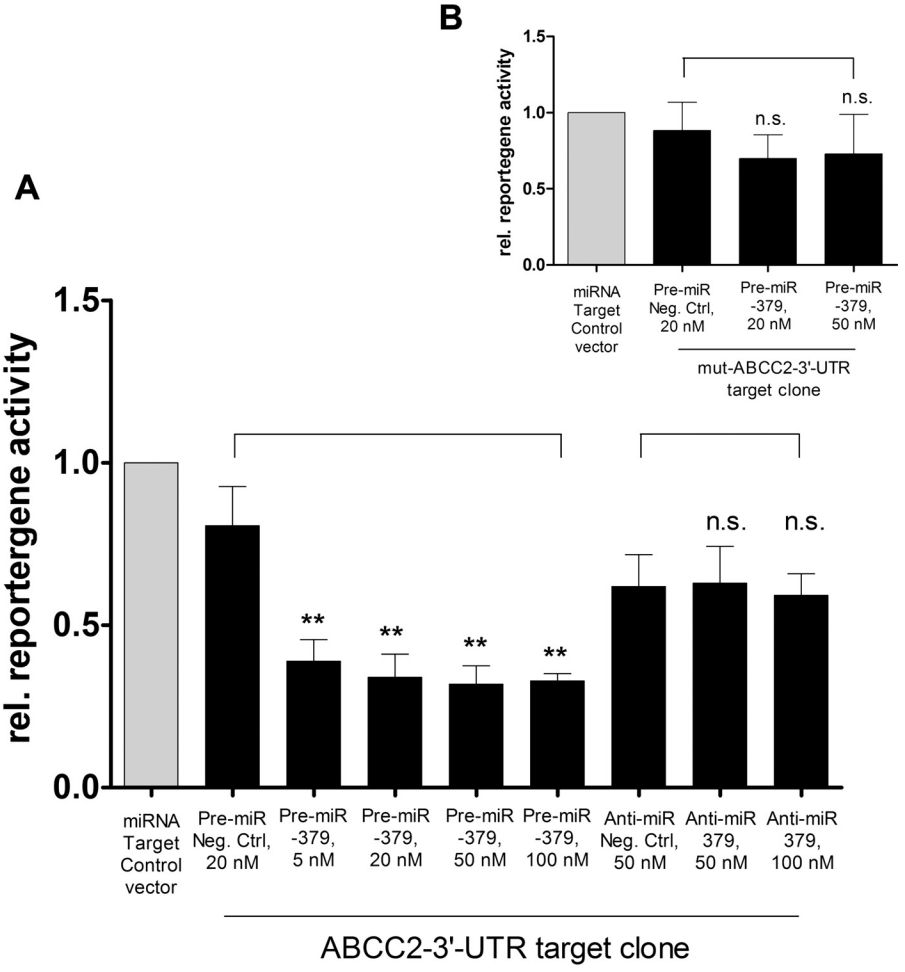

Fig. 4. Relative reporter gene activities 48 h after cotransfection of ABCC2 3′-UTR target clone ($n = 7$) (A) and mutated ABCC2 3′-UTR target clone ($n = 4$) (B) and increasing concentrations of miR-379 precursor (PremiR-379) and miR-379 inhibitor (Anti-miR-379) in HepG2 cells. MicroRNA target clone control vector was transfected as positive control. **, $p < 0.01$, Mann-Whitney-U test.

mutated seed sequence. In this case, cotransfection of miR-379 precursor did not lead to significantly lowered reporter gene activities compared with cells cotransfected with miRNA precursor negative control (Fig. 4B), indicating the significance of the miR-379 binding site at position 180 to 200 in the 3′-UTR.

Interplay of miR-379 and Rifampicin in ABCC2 Protein Expression. Because rifampicin led to a down-regulation of ABCC2 protein, we investigated whether inhibition of miR-379 expression could diminish this effect. Therefore, we transfected HepG2 cells with miR-379 inhibitor (50 nM) or miRNA inhibitor negative control (50 nM). Subsequently, cells were either treated with 5 μM rifampicin or vehicle for 48 h. As expected, cells transfected with the negative control vector exhibited a significantly lower ABCC2 protein expression after rifampicin incubation (78.6 ± 17.9%, $p = 0.04$, $n = 3$). In contrast, cells transfected with miR-379 inhibitor showed a significantly up-regulated ABCC2 expression after rifampicin treatment (187 ± 32.1%, $p = 0.04$, $n = 3$) (Fig. 5).

Discussion

The aim of our study was to investigate whether ABCC2 is a target of miRNA mediated post-transcriptional gene regulation. Our major results disclose that ABCC2 is targeted by miR-379 in a concentration dependent manner slightly on mRNA but more pronounced and significant on the protein level. However, we cannot exclude a contribution of further miRNAs, putatively by an indirect mechanism, to ABCC2 expression. Treatment with the PXR ligand and ABCC2 inducer rifampicin results in a concurrent increase in both ABCC2 mRNA and miR-379 levels and a significant down-regulation of ABCC2 protein.

Because there was no microRNA that had been shown to affect ABCC2 mRNA at the beginning of our study, we attempted to confine those miRNAs potentially targeting ABCC2 mRNA. We assumed that a stimulus inducing the transcription of ABCC2 could be accompanied by altered expression of miRNAs controlling ABCC2 gene expression on the post-transcriptional level. We therefore treated HepG2 cells, which provide readily detectable ABCC2 expression under normal growth conditions, with the PXR ligand and inducer of ABCC2 transcription rifampicin (Gerk and Vore, 2002; Kast et al., 2002; Oscarson et al., 2007). As proof of concept, we could detect a significant up-regulation of ABCB1 protein (Martin et al., 2008). After applying an rtPCR-based low-density array, which was composed of 377 of the most common human microRNAs, we could identify 11 more than 8-fold deregulated miRNAs, from which only miR-379 was predicted to target ABCC2 mRNA in an in silico alignment. miR-379 was then confirmed to down-regulate ABCC2 in HepG2 cells, only slightly on the mRNA level but significantly on ABCC2 protein level, with a maximal decrease of 54% at 50 nM of transfected miRNA precursor. These observations suggest that miR-379 is predominantly inhibiting the translation of ABCC2 mRNA rather than contributing to its degradation as a result of imperfect matching of the miRNA to the 3′-UTR. Using a vector construct exhibiting the ABCC2 3′-UTR target sequence downstream of the firefly luciferase sequence revealed an even more pronounced decrease of reporter gene activity, with a maximal decrease of 60% at a concentration of 50 nM of the cotransfected miRNA precursor. Moreover, after mutagenesis of the seed sequence in the predicted miR-379 binding site (position 180–200), the reporter gene experiments performed support not only the finding of direct interference of miR-379 and ABCC2 3′-UTR but also provide evidence about the exact location of the miRNA binding site.

In contrast, transfection experiments with 50 nM miR-379 inhibitor showed no increasing effect on ABCC2 mRNA or reporter gene activity of the ABCC2 3′UTR vector construct. However, miR-379 inhibitor concentrations of 100 nM led to a significant increase of ABCC2 protein expression, again giving evidence for the important role of miR-379 for the regulation of ABCC2. One reason for the necessity to apply relatively high concentrations of the miRNA inhibitor to diminish the basal miR-379 expression and to obtain increasing ABCC2 protein expression might be the relatively weak inhibiting effect of the miRNA inhibitor on the mature miR-379. More likely, the observation could also be explained by a relatively low basal miRNA-379 expression under normal growth conditions in HepG2 cells.

There are certain ways to identify miRNAs targeting genes of interest. Wang et al., (2009) determined the correlation between miRNA and genome-wide mRNA expression in 90

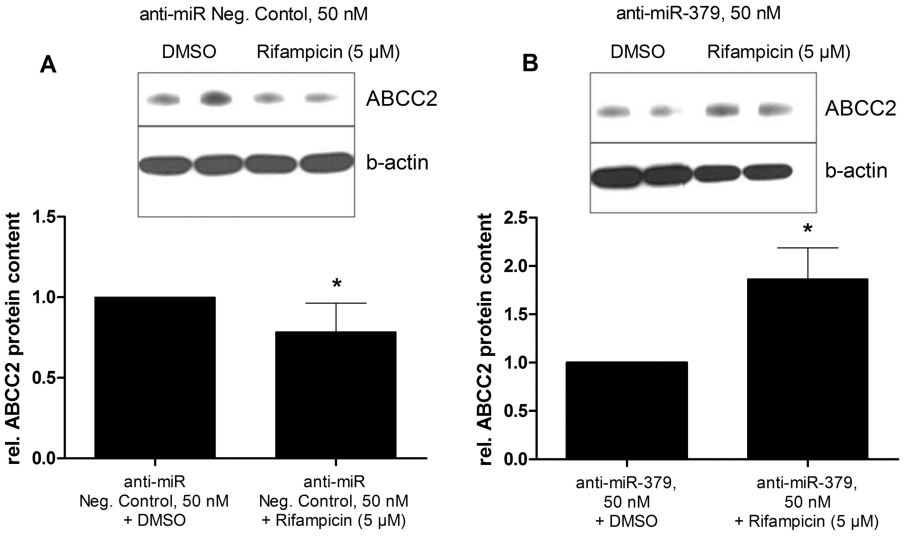

Fig. 5. Relative ABCC2 protein content (mean optical density of three Western blots) in HepG2 cells after 48 h of treatment with 5 μM rifampicin or vehicle and 72 h after transfection of miRNA inhibitor (anti-miR) negative control (A) and miR-379 inhibitor (anti-miR-379) (B), respectively. β-actin protein expression was used as internal standard. *, $p < 0.05$, Mann-Whitney-U test, $n = 3$.

lymphoblastoid cell lines. This approach revealed 7207 inversely or coregulated miRNA-mRNA pairs, presuming a false discovery rate of $q = 0.01$. The drawback of this approach is that an inverse correlation of mRNA and miRNA depends on the mechanistic assumption that miRNAs degrade their target mRNAs. However, because of imperfect matching of many miRNAs with the 3′-UTR, impairment of protein translation is more likely than destabilization of the targeted mRNA. Moreover, miRNA-mRNA pairs need to be confirmed by loss- and gain-of-function experiments. A further approach would be to systematically screen all in silico-predicted miRNAs for effects on the gene of interest in vitro. Our method of using a stimulus to alter the target gene transcription could demonstrate a more simplified and potentially time-saving approach to identify miRNAs.

Rifampicin incubation for 72 h caused an up-regulation of both ABCC2 mRNA and its targeting miRNA, indicating concurrent transcription. At the same time, however, a continuous decrease in total ABCC2 protein content could be observed. These findings suggest a miRNA-mediated negative feedback mechanism impeding ABCC2 overexpression by the transcriptional stimulus rifampicin. This hypothesis was substantiated by the observation that the presence of miR-379 inhibitor caused an increase in ABCC2 protein expression in HepG2 cells after rifampicin treatment.

Therefore, the question arises of whether ABCC2 and miRNA-379 are both regulated by PXR. A retinoid X receptor/PXR consensus sequence has been identified in the rat MRP2 promoter (Kast et al., 2002). Although human ABCC2 is located on chromosome 10, such a consensus sequence could also be located on chromosome 14 upstream of the predicted DNA coding region of the miR-379 precursor at position 101488403 to 101488469 (Sanger miRBase). It is noteworthy that another miRNA, miR-411, was found to be highly up-regulated after rifampicin treatment in our study. miR-411 precursor is encoded by a sequence located less than 1200 bp downstream of the miR-379 precursor at position 101489662 to 101489757 (Sanger miRBase). Therefore, it may be speculated that both miRNAs could originate from a single polycistronic primary transcript (pri-miRNA). Furthermore, we also found that one microRNA, miR-31, is highly down-regulated. Reportedly, the expression of this microRNA is inversely associated with the metastatic capacity of 15 different breast epithelial cell lines, as well as human primary breast tumors (Valastyan et al., 2009a,b). Whether rifampicin treatment thereby bears tumor-promoting effects, however, remains currently open. The results of our study contribute to the explanation of the frequently observed weak correlation between mRNA and protein expression (Maier et al., 2009), especially for ABC transporter proteins exhibiting long turnover times, such as ABCB1, which has a half-life of 3.7 to 5 days (Kipp et al., 2001; Petriz et al., 2004). In contrast, the half-life of ABCC2 mRNA is 11 to 15 h, as we showed in an earlier study (Laechelt et al., 2011).

As reviewed by Kipp and Arias (2002), ABC-transporters are subject to a cycling between intracellular pools and the cell membrane . Under physiological conditions, the intracellular stored fraction of the total transporter protein amount is several times higher than the membrane expressed fraction. Triggered by an external stimulus, transporter proteins can rapidly traffic from these reservoirs into the membrane without necessarily increasing transcription or translation of the protein ("transporter on demand"). In this context, the negatively regulating effect of miR-379 on ABCC2 protein expression probably does not affect the final membranal transporter function, but may be considered an attenuator between transcription, translation, and function of the transporter.

It is currently unclear whether our results on miR-379 in the human hepatoblastoma cell line HepG2 can be extrapolated to healthy tissues. Fromm et al. (2000) showed an increase of ABCC2 mRNA and protein after rifampicin treatment in duodenal epithelium, an observation that was confirmed by our group (Haenisch et al., 2008). The length of 3′-UTRs can vary in different tissue, possibly enabling rapidly proliferating tissues to exhibit a higher gene expression (Zhang et al., 2005; Sandberg et al., 2008). Consequently, the regulating effects of miRNAs in various tissues may be impaired or disrupted. In addition, some in vitro studies have reported an up-regulation of ABCC2 protein in HepG2 cells 48 h after rifampicin treatment (Schrenk et al., 2001; Kauffmann et al., 2002; Martin et al., 2008). Although we cannot clarify this discrepancy, high numbers of cell passages may also cause a selection of a cell population with truncated 3′-UTRs to contribute to the varying results in rifampicin-mediated ABCC2 induction by loss of miRNA binding sites. Such an effect was described for the occurrence of drug resistance in S1 colon cancer cells as a result of ABCG2 overexpression. Investigations of the ABCG2 3′-UTR revealed a truncated 3′-UTR and loss of the binding site of the ABCG2 inhibiting microRNA miR-519c (To et al., 2008).

Although miRNA encoding regions seem to be highly conserved (Saunders et al., 2007), a further parameter contributing to alteration of miRNA function may be genetic variants in the 3′-UTR or further variants that alter the secondary structure of the mRNA transcript. In a recent study, we identified different ABCC2 mRNA secondary structures for different ABCC2 haplotypes (Laechelt et al., 2011). These different structures may cause different folding of the 3′-UTR and consequently miRNA binding sites, possibly contributing to the observation of marked interindividual differences of ABCC2 expression and noncorrelation of duodenal mRNA and protein expression after rifampicin treatment (Haenisch et al., 2008).

In conclusion we could identify—to our knowledge, for the first time—a microRNA that negatively regulates the translation of the ABCC2 efflux transporter. Moreover, miR-379 is coinduced by rifampicin, impeding the overexpression of ABCC2 protein after treatment with this PXR ligand in HepG2 cells.

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Authorship Contributions

Participated in research design: Haenisch, Laechelt, Noack, Bruhn, Remmler, and Cascorbi.

Conducted experiments: Haenisch, Laechelt, Bruckmueller, Werk, and Remmler.

Contributed new reagents or analytic tools: Haenisch and Noack.

Performed data analysis: Haenisch, Bruckmueller, Werk, and Bruhn.

Wrote or contributed to the writing of the manuscript: Haenisch, Laechelt, Remmler, and Cascorbi.

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