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Short Communication

Inflammation-Associated MicroRNA-130b Down-Regulates Cytochrome P450 Activities and Directly Targets CYP2C9

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ABSTRACT

Expression of genes involved in absorption, distribution, metabolism, and excretion (ADME) of drugs is impaired in pathophysiologic conditions such as cholestasis and inflammation. The mechanisms of ADME gene down-regulation remain unclear. In our previous study, strongly elevated levels of microRNAs (miRNA) miR-21, miR-34a, and miR-130b in cholestatic liver and of miR-21 and miR-130b during inflammation were observed. Using HepaRG cells, which retain many functional characteristics of human hepatocytes, we investigated the potential of these miRNAs to down-regulate ADME genes. Cells were transfected with the corresponding miRNA mimics, chemically modified double-stranded RNAs that mimic endogenous miRNAs, followed by mRNA profiling by quantitative reverse-transcription polymerase chain reaction. Activities of six cytochrome P450 enzymes (CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, and CYP3A4) were determined with a liquid chromatography with tandem mass spectrometric cocktail assay. Although miR-21 and miR-34a showed few effects, transfection of miR-130b led to significantly lower expression of nuclear receptors constitutive androstane receptor (CAR) and farnesoid X receptor (FXRα), the CYPs 1A1, 1A2, 2A6, 2C8, 2C9, and 2C19, as well as GSTA2. Furthermore, miR-130b negatively affected activity levels of all measured P450s by at least 30%. Reporter gene assays employing the CYP2C9 3′-untranslated region (3′-UTR) confirmed direct regulation by miR-130b. These data support miR-130b as a potential negative regulator of drug metabolism by directly and/or indirectly affecting the expression of several ADME genes. This may be of relevance in pathophysiologic conditions such as cholestasis and inflammation, which are associated with increased miR-130b expression.

Introduction

Cytochrome P450 (P450) enzymes and other drug metabolizing enzymes, drug transporters, and regulatory genes that are important for the absorption, distribution, metabolism, and excretion (ADME) of drugs and other xenobiotic substances are highly variable in expression and function (Sim et al., 2013; Zanger and Schwab, 2013). Numerous factors are known to influence expression and function of ADME genes. These include constant factors such as sex or genetic variation, and factors that change over time, including age, hormonal and circadian influences, or disease states, including cancer, cholestasis, and inflammation (Congiu et al., 2009; Zhang et al., 2011; Harvey and Morgan, 2014). Despite extensive research into the mechanisms of ADME variability, individualized prediction of pharmacokinetics as a cornerstone of personalized drug therapy remains difficult. Hence unrecognized regulating factors may exist that need to be identified.

A newer field of potentially high relevance for personalized medicine is the investigation of noncoding RNAs such as microRNAs (miRNAs) and their roles in disease pathology, regulation of gene expression, and drug toxicity. Generally, miRNA molecules are small noncoding RNAs that usually bind to recognition sites on target mRNAs, typically leading to translation inhibition and lower protein expression and/or to transcript degradation (Bartel, 2009). Several recent studies have shown that miRNAs play a role in the regulation of various ADME genes and as mediators of drug toxicity (Haenisch et al., 2011; Yu and Pan, 2012; Yokoi and Nakajima 2013; Lamba et al., 2014).

In our previous study we measured expression of 56 preselected miRNAs in a well-documented human liver tissue cohort (n = 92) followed by statistical analysis considering liver donor meta-data. This analysis revealed strongly elevated levels of miR-21, miR-34a, and miR-130b in cholestatic liver and of miR-21 and miR-130b during inflammation, as indicated by elevated C-reactive protein levels in the serum of the liver donors (Rieger et al., 2013). It is well known that during inflammatory conditions, cytokine signaling leads to a broad and effective down-regulation of ADME gene expression as well as P450 activities (Aitken et al., 2006; Klein et al., 2015). A number of studies have addressed potential mechanisms and hypotheses to explain the apparently coordinated response, although no single pathway could yet be elucidated that would be consistent with all experimental observations (Assenat et al., 2006 ; Morgan, 2009; Yang et al., 2010).

Here we hypothesized that miRNAs may have a role in down-regulating ADME genes during inflammation or other pathophysiologic conditions. To test our hypothesis, we investigated the impact of three previously identified disease-associated miRNAs, miR-21, miR-34a, and miR-130b, on the expression and function of a variety of ADME and other genes. As a hepatic cell model we used the HepaRG cell line, which is widely used as a surrogate for primary human hepatocytes because it retains regulated expression of many P450 and ADME genes and also inflammatory signaling (Rubin et al., 2015). Our data show that at least one of these miRNAs, miR130b, has the

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ABBREVIATIONS: ADME, absorption, distribution, metabolism, excretion; CAR, constitutive androstane receptor; HNF4α, hepatocyte nuclear factor α; LC-MS/MS, liquid chromatography with tandem mass spectrometry; miRNA, micro-RNA; P450, cytochrome P450; siRNA, small-interfering RNA; 3′-UTR, 3′-untranslated region.

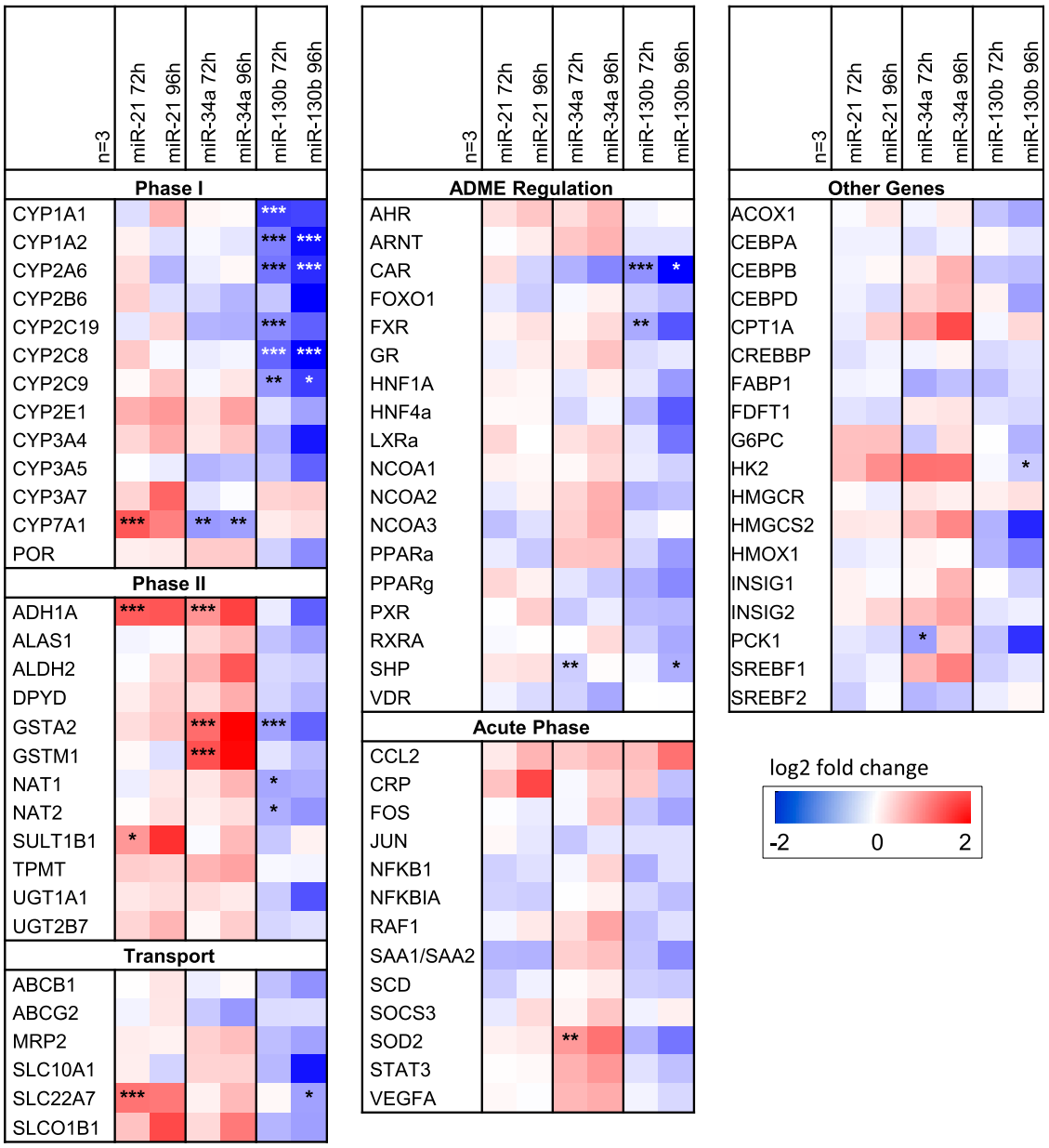

Fig. 1. Expression changes of selected genes induced by miRNA-21, miR-34a, and miR-130b in HepaRG cells. Fold changes are shown relative to control according to the indicated color key. All values are the means of three independent experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

potential to contribute to a marked down-regulation of certain P450s and other genes during inflammation.

Materials and Methods

Reagents. William's E medium was obtained from Invitrogen Life Technologies (Darmstadt, Germany); fetal bovine serum from PAA Laboratories GmbH (Pasching, Austria); human insulin from Sanofi (Frankfurt, Germany); hydrocortisone from Pfizer Pharma GmbH (Karlsruhe, Germany); HEPES, L-glutamine, minimum essential medium nonessential amino acids, penicillin/streptomycin, phosphate-buffered saline, and sodium pyruvate were purchased from GIBCO (Carlsbad, CA); bovine serum albumin, dexamethasone and dimethyl sulfoxide from Sigma-Aldrich (Steinheim, Germany). All TaqMan assays were purchased from Applied Biosystems (Foster City, CA). The miR-21, miR-34a, miR-130b, and the mirVana miRNA inhibitor miR-130b, as well as the Silencer Select Negative Control No. 2 small-interfering RNA (siRNA) (siCtr), and the mirVana Isolation Kit were purchased from Ambion (Austin, TX). The transfection agent Lipofectamine RNAiMax was from Invitrogen (Carlsbad, CA)

Cell Culture Differentiation and Transfection. HepaRG cells (batch HPR101007) were obtained from Biopredic International (Rennes, France) and cultured in HepaRG growth medium in accordance with the manufacturer's protocol. The mirVana miRNA mimic and Silencer Select Negative Control No. 2 siRNA (Ambion) were transfected using Lipofectamine RNAiMax (Invitrogen) according to manufacturer's protocol.

RNA Isolation and Quantification. Total RNA was extracted using the mirVana Isolation Kit (Ambion). The integrity and quantity of isolated and purified RNA were analyzed on the Bioanalyzer 2100 (Agilent Technologies) using the RNA 6000 Nano Kit (Agilent Technologies). RNA (0.1–1 μg) was reverse transcribed with TaqMan Reverse Transcription Reagents (Applied Biosystems). The cDNA was preamplified using TaqMan PreAmp Mastermix (2x) (Applied Biosystems) according to the manufacturer's guidelines from the Fluidigm Corporation (San Francisco, CA). All further steps were performed according to the manufacturer's guidelines for the 96:96 Dynamic Array Chip (Fluidigm Corporation). Relative quantification was calculated after normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression using the $\Delta\Delta$Ct method.

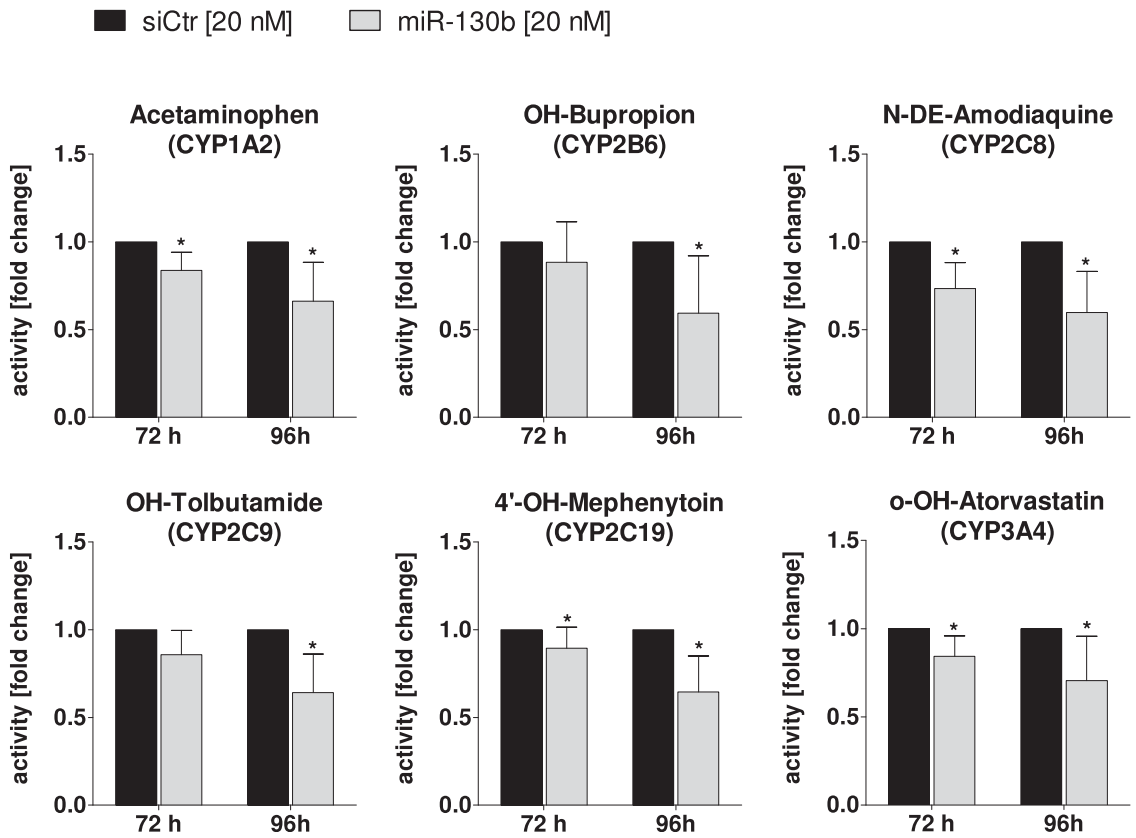

Fig. 2. Activity quantification of six cytochromes P450 (CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, and CYP3A4) in HepaRG cells after miRNA transfection. HepaRG cells were transfected with a nontargeting control siRNA (siCtr, black bars) or miR-130b (gray bars). Cytochrome P450 enzyme activities were measured by LC-MS/MS with a cocktail assay 72 and 96 hours after transfection in seven independent cultures.

Cytochrome P450 Enzyme Activity Quantification. Enzyme activities were determined using a previously established liquid chromatography with tandem mass spectrometry (LC-MS/MS) cocktail assay containing model substrates for CYP1A2 (phenacetin, 50 μM), CYP2B6 (bupropion, 25 μM), CYP2C8 (amodiaquin, 5 μM), CYP2C9 (tolbutamide, 100 μM), CYP2C19 (S-mephentoin, 100 μM), and CYP3A4 (atorvastatin, 35 μM; Feidt et al., 2010). Metabolites were quantified in supernatants of cultured cells mixed with 10% (v/v) formic acid and 10% (v/v) of deuterium-labeled internal standards for each metabolite as described elsewhere (Feidt et al., 2010).

Luciferase Reporter Gene Assay. Reporter plasmid pmiR-GLO-CYP2C9-3′UTR was constructed via insertion of the CYP2C9 full-length 3′-UTR (3-untranslated region) sequence into the Pme I/NotI restriction site 3′ to the luciferase gene in the pmiR-GLO plasmid (Promega) (see Fig. 3A). HuH7 cells were transfected in 96-well plates either with a pmiR-GLO-CYP2C9-3′-UTR construct and cotransfected with a siRNA control (siCtr), miR-130b mimic [2.5 nM-15 nM], or with a miR-130b mimic [2.5 nM] in combination with the inhibitor [10 nM] (Ambion). Lipofectamine RNAiMax (Invitrogen) was used as a transfection reagent according to the manufacturer's protocol. Cells were lysed in 50 μl of passive lysis buffer (Promega) 48 hours after transfection. For measurements the lysates were transferred to white OptiPlatesTM-96 (Perkin-Elmer, Norwalk, CT). Luciferase activities were determined using Beetle Juice (firefly luciferase) and *Renilla* Juice (p.j.k.-GmbH, Kleinblittersdorf, Germany) with the EnSpire Multimode Plate Reader (Perkin-Elmer). Three independent experiments were performed.

Western Blot Analysis. Cells were lysed with passive lysis buffer (Promega) 72 and 96 hours after transfection. Cell homogenates were analyzed by standard Western blotting procedures. The antibodies Cyp2C9abr against CYP2C9 (RDI, 1:1000) and A 5441 (1:5000; Sigma-Aldrich) were used as primary antibodies against CYP2C9 and against β-actin, respectively. Fluorescently labeled secondary antibodies goat-anti-rabbit-IRD800 (1:10,000, 926-32214; Li-COR Biosciences, Bad Homburg, Germany) and goat-anti-mouse-IRD680 (1:10,000, 926-32214, 926-68020; Li-COR Biosciences) were detected with a Li-COR Odyssey CLx fluorescence reader. Protein expression was calculated relative to β-actin.

In Silico Analyses. Our previously developed miRNA ranking tool, Mirna-Distiller, was used to collect and compare the prediction of putative miRNA binding sites from three miRNA databases (TargetScan, microCosm, miRDB) (Rieger et al., 2011).

Statistical Analysis. Statistical analyses for gene expression were performed using the ΔCt values by log2 transformation of data due to the considerably skewed symmetry of up- and down-regulation in the linear fold change. Statistical significance was determined by comparing the *t* ratio with the *t* distribution for the number of *df* calculated with a two-way analysis of variance (ANOVA) and applying the Bonferroni correction for multiple testing. Statistical differences were determined for luciferase assay results by a Student's *t* test, where $P < 0.05$, $P < 0.01$, and $P < 0.001$ were considered statistically significant.

Results and Discussion

Effects of miRNAs on mRNA Expression. To investigate effects of the selected miRNAs miR-21, miR-34a, and miR-130b, we used miRNA mimics, chemically modified double-stranded RNAs that mimic endogenous miRNAs and enable functional analysis by up-regulation of miRNA activity when transfected into cells. As shown in Fig. 1, miRNA 21- and 34a-mimics influenced only few genes significantly, including positive regulation of CYP7A1, ADH1A, SULT1B1, and SLC22A7 by miR-21, and of ADH1A, GSTA2, and GSTM1 by miR-34a, which also down-regulated CYP7A1 and small heterodimer partner (SHP). MicroRNA-21 plays a prominent role in diverse cellular processes and is associated with diseases such as cancer (Kumarswamy et al., 2011). Because it is involved in the regulation of peroxisome proliferator-activated receptor α (Kida et al., 2011), a transcription factor of genes involved in drug metabolism (Thomas et al., 2013), a negative association of ADME genes after overexpression of miR-21 was expected. Moreover, in our previous study we observed significant negative

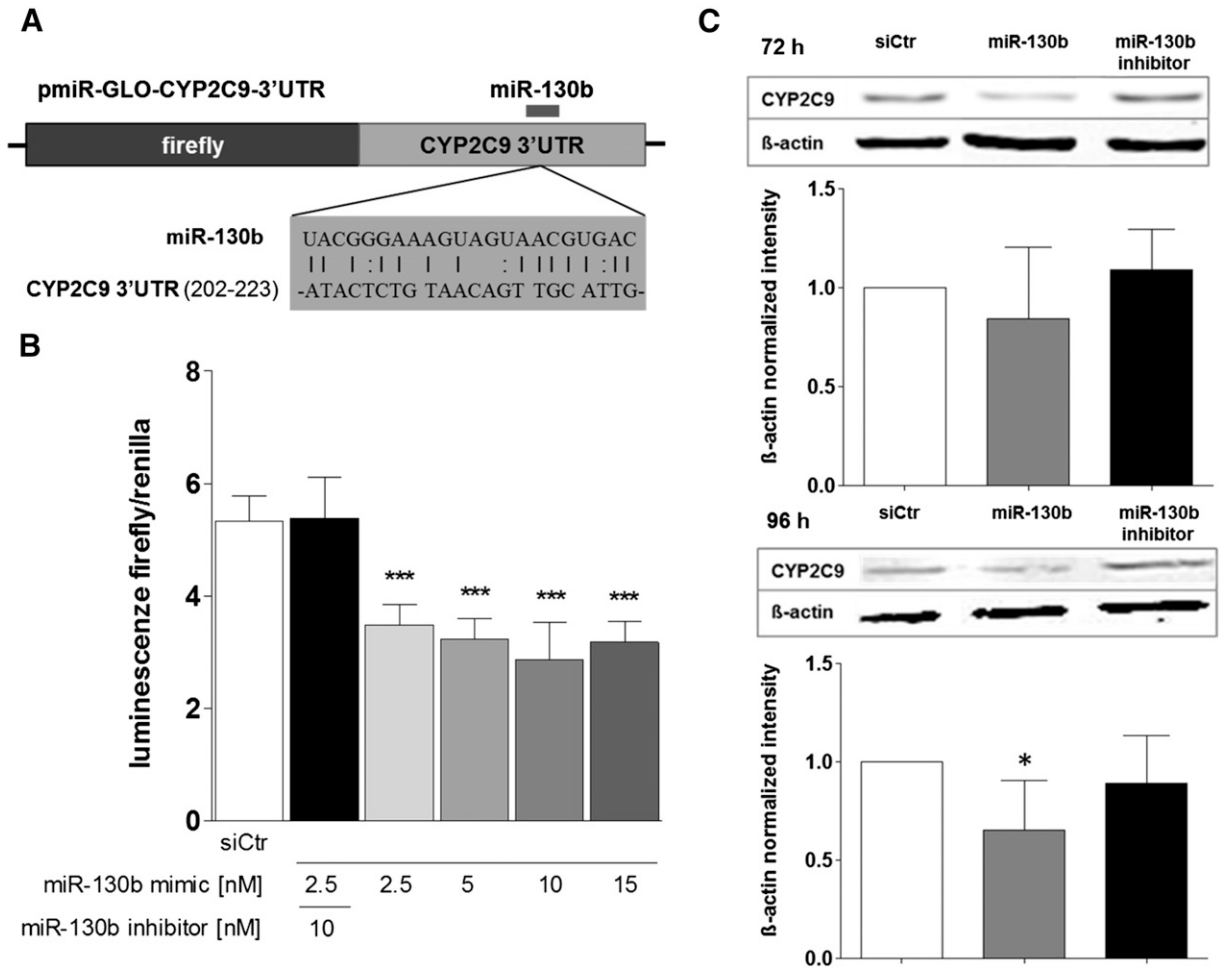

Fig. 3. MicroR-130b directly targets the 3′-UTR of CYP2C9. (A) Scheme of reporter plasmid pmiR-GLO-CYP2C9-3′-UTR containing the miR-130b binding site. The entire CYP2C9 3′-UTR (light gray, 362 base pairs) was inserted downstream of the firefly gene. The predicted binding site of miR-130b is indicated. (B) Cotransfection of reporter gene plasmid with control siRNA (siCtr), miR-130b mimic, or with a combination of miR-130b mimic and miR-130b inhibitor. Values are the mean of three independent experiments. $***P < 0.001$. (C) Western blot of CYP2C9 protein expression after miR-130b overexpression or inhibition. CYP2C9 protein was detected with a specific antibody 72 hours (mean of seven experiments) and 96 hours after transfection of miR-130b mimic, miR-130b inhibitor, or control siRNA (siCtr) in HepaRG cells. β-Actin was used as a loading control. $*P < 0.05$.

associations of miR-21 and ADME genes, such as CYP1A1, CYP1A2, and CYP2C19, which we could not confirm in HepaRG cells (Rieger et al., 2013). The positive regulations observed here thus need to be further investigated.

We could not identify a potential binding site for miR-34a in the negatively regulated genes CYP7A1 and SHP, but because the nuclear receptor hepatocyte nuclear factor α (HNF4α) is a known target of miR-34a and both CYP7A1 and SHP are regulated by HNF4α (Hwang-Verslues and Sladek, 2010; Takagi et al., 2010), the data may reflect indirect regulation. Lamba et al. (2014) showed negative correlations between miR-34a and constitutive androstane receptor (CAR) as well as CYP2C19 in human liver samples. This is in line with our findings in our human liver cohort of 92 samples (Rieger et al., 2013) and in this study, although the results presented here were not statistically significant. However, we were unable to confirm higher expression of miR-34a in males compared with females (Rieger et al., 2013; Lamba et al., 2014).

In contrast to miR-21 and miR-34a, transfection of the miR-130b mimic led to significant mRNA decreases of >25% after 72 hours and >55% after 96 hours for CYP1A1, 1A2, 2A6, 2C19, 2C8, and 2C9 (Fig. 1). Decreased expression was also found for several nuclear receptors and was significant for CAR, farnesoid X receptor, and SHP. In particular, the xenosensor CAR was pronouncedly decreased (83%) at 96 hours after transfection. In addition, phase II enzymes and transporters, including GSTA2, NAT1, NAT2, and SLC22A7, were also negatively influenced by miR-130b transfection.

These results indicated direct regulation by destabilization of mRNA, or indirect regulation by other factors such as nuclear receptors, or both in combination as potential mechanisms of the observed effects. Previously, it has been shown that miR-130b regulates the glucocorticoid receptor on protein level in multiple myeloma (Tessel et al., 2011). Furthermore, the glucocorticoid receptor has been shown to regulate the transcription of CAR (Pascussi et al., 2003). Because CAR lacks a predicted binding site for miR-130b, the dramatic down-regulation as observed here may be explained indirectly via the glucocorticoid receptor. As CAR is a prototypical xenosensor and constitutive regulator of P450s influencing the transcription of several P450s (Gao and Xie, 2010), this mechanism may contribute to coordinated ADME gene down-regulation under inflammatory conditions.

Effects of miR-130b on P450 Activity. Figure 2 summarizes the results obtained by measuring the activity of six expressed P450s (CYP1A2, 2B6, 2C8, 2C9, 2C19, and 3A4) in HepaRG cells transfected with miR-130b. CYP2D6 and CYP2E1 could not be measured due to their low expression. All measured P450s revealed statistically significant lower activities at both time points after transfection with miR-130b compared with

control, as shown in Fig. 2. Residual activities of CYP1A2, 2B6, 2C8, 2C9, 2C19, and 3A4 after 96 hours ranged between 60% and 70%.

CYP2C9 Is a Direct Target of miR-130b. The miRNA target prediction database microCosm (Griffiths-Jones et al., 2008) revealed a high-score miR-130b putative binding site in the 3′-UTR of CYP2C9 (Fig. 3A). Putative binding sites in other ADME genes were also predicted by this and other databases, although the predictions were inconsistent and the scores varied. To confirm direct targeting of CYP2C9 by miR-130b, we cloned the wild-type CYP2C9 3′-UTR into a luciferase reporter vector and measured the effects of miR-130b on the luciferase activity in HuH7 cells. As shown in Fig. 3B, miR-130b significantly suppressed luciferase activity in a dose dependent manner, and miR-130b inhibitor blocked this effect.

Western blot analysis of CYP2C9 protein 72 and 96 hours after transfection of miR-130b mimic or inhibitor in HepaRG cells confirmed the down-regulation by miR-130b overexpression, whereas inhibition of endogenous miR-130b led to slight up-regulation of CYP2C9 protein level (Fig. 3C).

Conclusions

Taken together, the results of this study show for the first time that CYP2C9 is directly and negatively regulated by miR-130b. This finding may be relevant for the dosing of warfarin, the most widely prescribed oral anticoagulant, which has a narrow therapeutic index and is metabolized by CYP2C9 (Jorgensen et al., 2012). Moreover, as miR-130b appears to influence additional drug metabolizing P450s at the transcriptional and functional level, and because miR-130b was reported to be also up-regulated in hepatocellular carcinoma (Wang et al., 2014) and in obesity (Wang et al., 2013), these data indicate the possible involvement of miR-130b as a negative regulator of ADME genes and as a potential biomarker under a variety of inflammatory conditions.

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Conducted experiments: Rieger, Reutter.
Contributed new reagents or analytic tools: Hofmann.
Performed data analysis: Rieger, Reutter, Hofmann.
Wrote or contributed to the writing of the manuscript: Rieger, Zanger.

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