Regulation of UDP-Glucuronosyltransferases UGT2B4 and UGT2B7 by MicroRNAs in Liver Cancer Cells[§]

Dhilushi D. Wijayakumara, Peter I. Mackenzie, Ross A. McKinnon, Dong Gui Hu, and Robyn Meech

Department of Clinical Pharmacology and Flinders Centre for Innovation in Cancer, Flinders University School of Medicine, Flinders Medical Centre, Bedford Park, South Australia, Australia

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ABSTRACT

The transcriptional regulation of UDP-glucuronosyltransferases *UGT2B4* and *UGT2B7* has been well studied using liver cancer cell lines, and post-transcriptional regulation of these two *UGTs* by microRNA (miRNA/miR) miR-216b-5p was recently reported. This study describes novel miRNA-mediated regulation of *UGT2B4* and *UGT2B7* in liver cancer cells. Bioinformatic analyses identified a putative miR-3664-3p binding site in the *UGT2B7* 3'-untranslated region (UTR) and binding sites for both miR-135a-5p and miR-410-3p in the *UGT2B4* 3'-UTR. These sites were functionally characterized using miRNA mimics and reporter constructs. A miR-3664-3p mimic induced repression of a luciferase reporter carrying the *UGT2B7* 3'-UTR in liver cancer cell lines; mutation of the miR-3664-3p site abrogated the response of the reporter to the mimic. Similarly, mutation of the miR-135a-5p site or miR-410-3p site in a luciferase reporter

bearing *UGT2B4* 3'-UTR abrogated the ability of miR-135a-5p or miR-410-3p mimics to reduce reporter activity. Transfection of miR-3664-3p mimics in HepG2 liver cancer cells significantly reduced mRNA and protein levels of *UGT2B7*, and this led to reduced enzymatic activity. Transfection of miR-135a-5p or miR-410-3p mimics significantly decreased *UGT2B4* mRNA levels in Huh7 liver cancer cells. The expression levels of miR-410-3p were inversely correlated with *UGT2B4* mRNA levels in The Cancer Genome Atlas cohort of liver hepatocellular carcinoma (371 specimens) and a panel of ten normal human tissues. Similarly, there was an inverse correlation between miR-135a and *UGT2B4* mRNA levels in a panel of 18 normal human liver tissues. Together, these data suggest that miR-135a and miR-410 control *UGT2B4* and that miR-3664 controls *UGT2B7* expression in liver cancer and/or normal liver cells.

Introduction

Glucuronidation refers to the covalent attachment of glucuronic acid to a variety of endogenous and exogenous small lipophilic compounds; glucuronidated products are generally biologically inactive and more water soluble, thus facilitating their excretion from the body (Mackenzie et al., 1997, 2005). There are four human UDP-glucuronosyltransferase (UGT) families (Mackenzie et al., 2005); however, glucuronidation is primarily carried out by members of the *UGT1A* and *UGT2B* subfamilies. Substrates for these enzymes include endogenous bioactive molecules (e.g., steroids, thyroid hormones, bilirubin, bile acids, retinoids, and fatty acids), carcinogens, environmental pollutants, and therapeutic drugs (Guillemette,

2003; Mackenzie et al., 2005). It was estimated that UGTs are involved in the metabolism of 35% of clinically administered drugs (Guillemette, 2003).

The liver is the primary site of glucuronidation; thus, the control of hepatic UGT expression and activity is of significant biologic and pharmacological interest (Hu et al., 2014b). *UGT2B4* and *UGT2B7* are both highly expressed in the liver (Congiu et al., 2002; Court, 2010). *UGT2B7* has high activity toward various endogenous compounds, including bile acids, retinoic acids, steroids, mineralocorticoid and glucocorticoid hormones, and fatty acids (Hu et al., 2014b), as well as exogenous compounds, including carcinogens, therapeutic drugs such as morphine, codeine (Coffman et al., 1997), epirubicin (Innocenti et al., 2001), valproic acid (Argikar and Remmel, 2009), and nonsteroidal anti-inflammatory drugs (Jin et al., 1993). Around 35% of the therapeutic drugs that are glucuronidated by UGTs are *UGT2B7* substrates (Williams et al., 2004). In addition to hepatic expression, *UGT2B7* is expressed in the small intestine, colon, kidney, and breast, where it may also play important roles in drug metabolism and homeostasis of endogenous bioactive molecules (Ohno and Nakajin, 2009; Hu et al., 2014b). *UGT2B4* is also abundantly expressed in many extrahepatic tissues and organs

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D.G.H. and R.M. are co-last authors.

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ABBREVIATIONS: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HNF, hepatocyte nuclear factor; HPLC, high-performance liquid chromatography; LIHC, liver hepatocellular carcinoma; miR, microRNA; miR-neg, negative control miRNA; miRNA, microRNA; PCR, polymerase chain reaction; RNAseq, RNA sequencing; RT-q, reverse-transcriptase quantitative real-time; TCGA, The Cancer Genome Atlas; UGT, UDP-glucuronosyltransferase; UTR, untranslated region.

(e.g., kidney and esophagus) (Ohno and Nakajin, 2009) and has an important role in the glucuronidation of bile acids (Fournel-Gigleux et al., 1989), catechol-estrogens (Ritter et al., 1992), androgen metabolites (Lévesque et al., 1999; Turgeon et al., 2001; Lépine et al., 2004), and phenols (Guillemette, 2003). UGT2B4 is involved in the glucuronidation of hydroxylated bile acids (Barre et al., 2007), as well as a subset of drugs and toxins including deoxynivalenol (a mycotoxin) (Maul et al., 2015), hydroxymidazolam (Seo et al., 2010), lorazepam (Uchaipichat et al., 2013), codeine (Raungrut et al., 2010), eslicarbazepine (an antiepileptic) (Loureiro et al., 2011), and carvedilol (a β -adrenoceptor blocker) (Ohno et al., 2004). However, in general, UGT2B4 is considered to play a more minor role in overall drug metabolism than UGT2B7.

As recently reviewed (Hu et al., 2014b), the transcriptional regulation of *UGT2B4* and *UGT2B7* has been well investigated using hepatic models such as liver cancer cell lines, human primary hepatocytes, and transgenic mice. The transcription factors found to be involved in controlling *UGT2B7* expression include hepatocyte nuclear factor (HNF) 1 α (Ishii et al., 2000), HNF4 α , caudal related homeobox factor 2 (Cdx2) (Gregory et al., 2006), nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (Nakamura et al., 2008), farnesoid X receptor (Lu et al., 2005), p53 (Hu et al., 2014c), and activator protein 1 (Hu et al., 2014a). *UGT2B4* is known to be transcriptionally regulated by the farnesoid X receptor, retinoid X receptor, and peroxisome proliferator-activated receptor α (Barbier et al., 2003a,b). MicroRNAs (miRNAs/miRs) are noncoding small RNAs (approximately 21–25 nucleotides) that mediate post-transcriptional gene regulation through translational repression and/or mRNA degradation (Macfarlane and Murphy, 2010). The first identification of a miRNA involved in *UGT2B4* and *UGT2B7* regulation (miR-216b-5p) in liver cancer cell lines was recently reported (Dluzen et al., 2016). Our study provides in-depth characterization of the roles of three additional miRNAs (miR-3664-3p, miR-135a-5p, and miR-410-3p) in the regulation of *UGT2B4* and *UGT2B7* in liver cancer cell lines.

Materials and Methods

miRNA Mimics and Human Tissue Total RNA. Mimics of hsa-miR3664-3p, hsa-miR-135a-5p, hsa-miR-410-3p, hsa-miR-1266-5p, hsa-miR-4483, hsa-miR-4317, hsa-miR216b-5p, hsa-miR-489-3p, hsa-miR-4691-5p, hsa-miR-101-3p, and a negative control miRNA (termed miR-neg) were purchased from Shanghai GenePharma (Shanghai, China). A miRNA inhibitor of miR-3664-3p and a negative control were purchased from Ambion/Applied Biosystems (Foster City, CA). Total RNA samples from a panel of human tissues were purchased from Ambion/Applied Biosystems (FirstChoice Human Total RNA Survey Panel). The normal human liver tissues were previously reported (Hu et al., 2014a).

Cell Transfection, RNA Extraction, and Reverse-Transcriptase Quantitative Real-Time Polymerase Chain Reaction. HepG2 cells were obtained from the American Type Culture Collection (Manassas, VA) and HuH7 cells were obtained from Dr. Jillian Carr (Flinders University, Adelaide, SA, Australia). Both cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum at 37°C in a 5% CO₂ atmosphere. Cells were plated in six-well plates and cultured overnight, reaching 60%–70% confluence prior to transfection. Transfections were performed with miRNA mimics, miRNA inhibitor, or miR-neg in triplicate at 30 nM using 8 μ l Lipofectamine 2000 (Invitrogen, Carlsbad, CA) per well. Total RNA was extracted after 24 hours using TRIzol reagent according to the manufacturer's protocol (Invitrogen). The

expression levels of target genes [*UGT2B7*, *UGT2B4*, 18S rRNA, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)] were quantified using reverse-transcriptase quantitative real-time (RT-q) polymerase chain reaction (PCR) in a RotorGene 3000 instrument (Corbett Research, Mortlake, NSW, Australia) as previously reported (Hu and Mackenzie, 2009, 2010; Hu et al., 2010). Quantification of miRNAs (miR3664-3p, miR-135a-5p, miR-410-3p, miR-1266-5p, miR-4483, miR-4317, miR216b-5p, miR-489-3p, miR-4691-5p, miR-101-3p) and RNU6-2 (miR-U6 small nuclear-2 RNA as a housekeeping miRNA) was performed as previously reported (Balcels et al., 2011; Wijayakumara et al., 2015). The expression levels of target genes relative to 18S rRNA were quantified using the 2⁻(Delta Delta C(T)) method (Livak and Schmittgen, 2001). The expression levels of individual miRNAs were presented relative to that of RNU6-2 (set as a value of 100%). Primers for RT-qPCR of miRNAs are listed in Table 1.

Generation of Luciferase Reporter Constructs and Mutagenesis.

The *UGT2B7* mRNA (NM_001074.3) contains a 251-nucleotide 3'-untranslated region (UTR), whereas the *UGT2B4* mRNA (NM_021139.2) contains a 469-nucleotide 3'-UTR. The full-length *UGT2B7* 3'-UTR region between the stop codon (TAG) and the poly(A) tail was amplified from human genomic DNA (Roche Diagnostics, Indianapolis, IN) using Phusion Hot-Start High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Pittsburgh, PA) and cloned into the XbaI restriction site of the pGL3-promoter vector (Promega, Madison, WI), generating the reporter construct pGL3/2B7/UTR. Similarly, the full-length *UGT2B4* 3'-UTR region between the stop codon and the poly(A) tail was amplified and cloned into the pGL3-promoter vector, generating the reporter construct pGL3/2B4/UTR. Primers used for amplifying the 3'-UTR of *UGT2B4* or *UGT2B7* are listed in Table 1.

Using the pGL3/2B7/UTR construct as a template and the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), the *UGT2B7* miR-3664-3p seed site (5'-UCCUGAG-3'), which is complementary to the miR-3664-3p seed sequence (3'-AGGACUC-5'), was changed to 5'-UAGCUAG-3', producing the mutated construct pGL3/2B7/UTR/miR-3664/MT. Similarly, using the pGL3/2B4/UTR construct as a template, the *UGT2B4* miR-135a-5p seed site was mutated from 5'-AAAGCCAU-3' to 5'-AAAUGACU-3', generating the mutated construct pGL3/2B4/UTR/miR-135a/MT; the *UGT2B4* miR-410-3p seed site was mutated from 5'-GUUAUAU-3' to 5'-GUCCGCU-3', generating the mutated construct pGL3/2B4/UTR/miR-410/MT. The identities of all constructs were confirmed by DNA sequencing. Primers used for mutagenesis are listed in Table 1.

Luciferase Reporter Assays. HepG2 and HuH7 cells were plated in 96-well plates and cultured overnight prior to transfection. Transfection was conducted in quadruplicate for each condition; each well was transfected with 100 ng of each UTR luciferase reporter, 0.8 ng pRL-null vector, and 30 nM miRNA mimics of each miRNA or miR-neg as the negative control. Twenty-four hours after transfection, cells were lysed in passive lysis buffer and then subjected to the dual-luciferase reporter assay according to the manufacturer's instructions (Promega). Firefly and Renilla luciferase activities were measured using a Packard TopCount luminescence and scintillation counter (PerkinElmer Life and Analytical Sciences, Waltham, MA). Firefly luciferase activity was first normalized to Renilla activity and the reporter activity was then presented relative to that of the control pGL3-promoter vector (set at a value of 100%).

Western Blotting. HepG2 cells were transfected with miRNA mimics (miR-3664-3p or miR-neg) at 30 nM; 72 hours post-transfection, whole cell lysates were prepared with radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 2 mM EDTA, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate). Protein concentrations of cell lysates were measured using the Bradford protein assay (Bio-Rad, Hercules, CA). Fifty micrograms of each cell lysate was subjected to SDS-PAGE on 12% acrylamide gels and transferred onto nitrocellulose membranes. The anti-UGT2B7 antibody used in Western blotting was developed in our laboratory as previously reported (Hu et al., 2014c). After the membranes were probed with primary antibodies (anti-UGT2B7 or

TABLE 1
Primers used for cloning, site-directed mutagenesis, and RT-qPCR

Primer	Sequence (5'–3')
Cloning	
UGT2B7_3'-UTR_F	CCGCTCTAGATTATATCTGAGATTTGAAGC
UGT2B7_3'-UTR_R	CCGGTCTAGACCGTAGTGTCTTCTTCATTG
UGT2B4_3'-UTR_F	CCGGTCTAGATTACGTCTGAGGCTGGAAGC
UGT2B4_3'-UTR_R	CCGATCTAGAGCTTCCTCAACAACAGTTAA
Site-directed mutagenesis	
2B7_miR3664_MT_F	AGATTTCTTTCTTAGCTAGACAAAAAAGAAAG
2B7_miR3664_MT_R	TTTTTTTTTTTGTCTAGCTAAGAAAGAAATCTTG
2B4_miR135a_MT_F	CAAAAATGATATAAATGACTATGAGGTTATATTG
2B4_miR135a_MT_R	CAATATAACCTCATAGTCATTTATATCATTTTTTG
2B4_miR410_MT_F	AAAGCCATATGAGGTCCGCTTGAAATGTATTGAG
2B4_miR410_MT_R	CTCAATACATTTCAAGCGGACCTCATATGGCTTT
RT-qPCR	
miR3664-3p_qPCR_F	GCAGTCTCAGGAGTAAAGACA
miR3664-3p_qPCR_R	AGGTCCAGTTTTTTTTTTTTTTTAACTC
miR-1266-5p_qPCR_F	GCC TCA GGG CTG TAG AAC A
miR-1266-5p_qPCR_R	GTC CAG TTT TTT TTT TTT TTT AGC CC
miR-4483_qPCR_F	GCA GGG GGT GGT CTG T
miR-4483_qPCR_R	GGT CCA GTT TTT TTT TTT TTT TCA ACA
miR-4317_qPCR_F	GCA GAC ATT GCC AGG GA
miR-4317_qPCR_R	CAG GTC CAG TTT TTT TTT TTT TTT AAA C
miR-216b-5p_qPCR_F	GCA GAA ATC TCT GCA GGC A
miR-216b-5p_qPCR_R	GGT CCA GTT TTT TTT TTT TTT TTT TTA TT
miR-135a_qPCR_F	CGC AGT ATG GCT TTT TAT TCC T
miR-135a_qPCR_R	GGT CCA GTT TTT TTT TTT TTT TTC ACA T
miR-410_qPCR_F	CGC AGA ATA TAA CAC AGA TGG C
miR-410_qPCR_R	AGG TCC AGT TTT TTT TTT TTT TTA CAG
miR-489_qPCR_F	CGC AGG TGA CAT CAC ATA TAC
miR-489_qPCR_R	CCA GTT TTT TTT TTT TTT TGC TGC C
miR-4691-5p_qPCR_F	GGT CCT CCA GGC CAT GA
miR-4691-5p_qPCR_R	AGT TTT TTT TTT TTT TTC CGC AGC
miR-101_qPCR_F	CGC AGT ACA GTA CTG TGA TA
miR-101_qPCR_R	GGT CCA GTT TTT TTT TTT TTT TTT CAG T

F, forward; R, reverse.

anti-Calnexin [Sigma-Aldrich, St. Louis, MO]), the membranes were probed with horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody (NeoMarkers, Fremont, CA). The SuperSignal-West Pico chemiluminescent kit (Thermo Fisher Scientific) and the LAS4000 luminescent image analyzer (GE Healthcare Life Sciences, Piscataway, NJ) were used for visualizing immunosignals. Band intensity was quantified using Multi Gauge version 3.0 image software (FUJIFILM, Tokyo, Japan).

Morphine Glucuronidation Assay. HepG2 cells were plated in six-well plates and cultured overnight before the transfection with mimics (miR-neg or miR-3664-3p) at 30 nM. Whole cell lysates were prepared after 24 hours post-transfection with 80 μ l Tris-HCl/EDTA buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.6) and the protein concentrations of the lysates were determined as described above. A 200- μ l reaction of each sample containing 100 mM potassium phosphate, pH 7.4, 4 mM MgCl₂, 5 mM morphine, 125 μ g lysate protein, and 5 mM UDPGA was incubated for 2 hours at 37°C in a shaking water bath. The glucuronidation reaction was terminated by the addition of 2 μ l 70% (v/v) perchloric acid and samples were kept on ice for 30 minutes before centrifugation at 5000g for 10 minutes at 4°C. The supernatant fraction (approximately 40 μ l) was analyzed by high-performance liquid chromatography (HPLC) using an Agilent 1100 series instrument (Agilent Technologies, Sydney, NSW, Australia) as previously described (Uchaipichat et al., 2004). The HPLC column eluent was monitored by fluorescence detection at an excitation wavelength of 235 nm and an emission wavelength of 345 nm. Quantification of morphine-3-glucuronide concentrations in the samples were calculated by comparing peak areas of the samples to those of standard curves prepared over the concentration ranges of 0.5 to 40 μ M morphine-3-glucuronide. Morphine-6-glucuronide concentration was not detected in the samples.

Data Analyses of Liver Hepatocellular Carcinoma. Transcriptome profiling data of RNA sequencing (RNAseq) and miRNA

sequencing of liver hepatocellular carcinoma (LIHC) were downloaded from The Cancer Genome Atlas (TCGA) data portal (<https://gdc-portal.nci.nih.gov/>). The LIHC RNAseq expression data from 371 samples were represented in the form of high-throughput sequencing counts. Genes (protein coding and noncoding) with a mean of less than 10 counts were discarded; the counts of the remaining genes were normalized using the upper quantile normalization method. Correlation analyses between the expression levels of UGT and miRNA gene sets were conducted using the Spearman rank method and plots were drawn using the R statistical package (<https://cran.r-project.org/>; R Foundation for Statistical Computing, Vienna, Austria).

Statistical Analysis. Statistical analyses of all data were performed using GraphPad Prism 6 software (GraphPad Inc., La Jolla, CA), with a two-tailed independent *t* test. Correlation analyses between expression levels of a UGT gene and a miRNA in the panel of liver tissues or the panel of human tissues were conducted by Spearman correlation. A *P* value of <0.05 was considered statistically significant.

Results

The 3'-UTRs of UGT2B7 and UGT2B4 Contain Putative miRNA Target Sites. Our sequence analysis of the UGT2B7 3'-UTR using TargetScan software (<http://www.targetscan.org>; Whitehead Institute for Biomedical Research, Cambridge, MA) identified putative target sites for five miRNAs: namely, miR-3664-3p, miR-1266-5p, miR-4483, miR-4317, and miR-216b-5p (Supplemental Fig. 1A). To test the potential functionalities of these sites, we cloned the UGT2B7 3'-UTR into the pGL3 vector downstream of the luciferase gene, generating the UGT2B7 3'-UTR luciferase reporter (termed

pGL3/2B7/UTR). Cotransfection of pGL3/2B7/UTR with miRNA mimics targeting the five predicted binding sites in HepG2 cells showed that two of the five mimics (namely, miR-3664-3p and miR-216b-5p) significantly reduced the reporter activity by 51% and 22%, respectively (Fig. 1A).

Our sequence analysis of the UGT2B4 3'-UTR using the TargetScan software predicted putative target sites for six microRNAs: namely, miR-135a-5p, miR-410-3p, miR-489-3p, miR-4691-5p, miR-216b-5p, and miR-101-3p (Supplemental Fig. 1B). We cloned the UGT2B4 3'-UTR into the pGL3 vector downstream of the luciferase gene, generating the UGT2B4 3'-UTR luciferase reporter (termed pGL3/2B4/UTR). Cotransfection of pGL3/2B4/UTR with miRNA mimics targeting the six predicted binding sites in HepG2 cells showed that the reporter activity was significantly reduced by 39%, 30%, 22%, 24%, and 24% by miR-135a-5p, miR-410-3p, miR-489-3p, miR-4691-5p, and miR-216b-5p, respectively (Fig. 1B). miR-101-3p mimics did not alter the reporter activity (Fig. 1B). The expression levels of the above-mentioned 10 miRNAs in HepG2 cells were measured by quantitative real-time PCR. As shown in Fig. 1C, miR-135a-5p and miR-101-3p were highly expressed; however, four miRNAs (miR-3664-3p, miR-1266-5p, miR-4317, and miR-410-3p) were expressed at relatively low levels, and the remaining four miRNAs (miR-4483, miR-216b-5p, miR-489-3p, and miR-4691-5p) were not detectable. The downregulation of *UGT2B4* and *UGT2B7* by miR-216b-5p in liver cancer cell lines was recently reported (Dluzen et al., 2016). In our study, we define the roles of miR-3664-3p, miR-135a-5p, and miR-410-3p in the regulation of *UGT2B4* and *UGT2B7* in liver cancer cell lines.

Regulation of UGT2B7 by miR-3664-3p in Liver Cancer Cell Lines. The predicted miR-3664-3p binding site in the *UGT2B7* 3'-UTR is located at nucleotides 77–99 downstream of the *UGT2B7* stop codon (Fig. 2A). This site is defined as a 8mer site by TargetScan (pairing to the 2- to 7-nucleotide miRNA seed and nucleotide 8 plus an A at nucleotide 1) and also shows 3'-sequence pairing of nucleotides 11–15 to miR-3664-3p. A 3'-sequence pairing at nucleotides 12–17 of miRNAs has been shown to enhance the efficacy of miRNA targeting (Grimson et al., 2007). To show a direct role of this site in the miR-3664-3p mimic-mediated reduction in pGL3/2B7/UTR reporter activity, the miR-3664-3p site in the pGL3/2B7/UTR reporter was mutated, generating the construct pGL3/2B7/UTR/miR3664/MT (Fig. 2B). miR-3664-3p mimics were cotransfected with wild-type pGL3/2B7/UTR or the pGL3/2B7/UTR/miR3664/MT reporter into HepG2 and HuH7 cells. The miR-3664-3p mimics significantly reduced the activity of the wild-type reporter in both cell lines; this reduction was abolished in HepG2 cells (Fig. 2C) or significantly abrogated in HuH7 cells (Fig. 2D) by a mutation of the miR-3664-3p site. These results indicate that *UGT2B7* is a direct target of miR-3664-3p in HepG2 and HuH7 cells.

The impact of miR-3664-3p on the expression and activity of UGT2B7 was tested in HepG2 cells using miRNA mimics and inhibitors. As expected, the miR-3664-3p mimics significantly reduced the UGT2B7 mRNA levels and the miR-3664-3p inhibitor significantly increased the UGT2B7 mRNA levels in HepG2 cells (Fig. 3A), while GAPDH mRNA levels were not significantly altered (Fig. 3B). Western blotting with a UGT2B7-specific antibody (Hu et al., 2014c) showed that UGT2B7 protein levels were reduced by 28% in HepG2 cells

Fig. 1. Effects of miRNA mimics on a luciferase reporter containing the UGT2B7 3'-UTR or UGT2B4 3'-UTR and expression of miRNAs in HepG2 cells. (A and B) HepG2 cells were cotransfected with 30 nM miR-neg or miRNA mimics targeting individual miRNAs, Renilla reporter vector as a control, and a luciferase reporter containing UGT2B7 3'-UTR (A) or UGT2B4 3'-UTR (B). Luciferase activity assays were performed as described in the *Materials and Methods*; the luciferase activity of the reporter cotransfected with miRNA mimics is presented relative to that of the reporter cotransfected with miR-neg (set as a value of 100%). Data shown are means $\pm$ S.E.M from four (A) or two (B) independent experiments performed in quadruplicate. ** $P < 0.005$; *** $P < 0.0005$. (C) The expression levels of miRNAs in HepG2 cells were measured using RT-qPCR, normalized to RNU6-2, and then presented relative to that of miR-410-3p (set at a value of 1). Data shown are means $\pm$ S.E.M from a single experiment in triplicate.

transfected with miR-3664-3p mimics relative to the miR-neg control (Fig. 3C). UGT2B7 glucuronidation activity was tested by HPLC assay using morphine as a substrate. There was a significant 41% reduction in morphine glucuronidation in

Fig. 2. A functional miR-3664-3p target site in the UGT2B7 3'-UTR. (A) Shown are the position of a miR-3664-3p target site in the UGT2B7 3'-UTR and the sequence complementarity between this site and miR-3664-3p, including seed pairing (highlighted in gray) and substantial 3'-sequence pairing. The nucleotides of the miR-3664-3p sites are numbered relative to the stop codon (TAG with G positioned as -1) (NM_001074.3). (B) The mutation of CCTC to AGCT (boxed) that abolished the seed pairing between the miR-3664-3p target site and miR-3664-3p is shown. (C and D) Cotransfection of the UGT2B7 3'-UTR luciferase reporter carrying either the wild-type or mutated miR-3664-3p site, 30 nM miR-neg or miR-3664-3p mimics, and Renilla reporter vector as the control in HepG2 (C) and HuH7 (D) cells; 24 hours post-transfection, cells were harvested for luciferase activity assays as described in the *Materials and Methods*. The luciferase activity of the reporter cotransfected with miR-3664-3p is presented relative to that of the reporter cotransfected with miR-neg (set as a value of 100%). Data shown are means $\pm$ S.E.M from three (C) or two (D) independent experiments performed in quadruplicate. * $P < 0.01$; ** $P < 0.005$; *** $P < 0.0005$.

Fig. 3. miR-3664-3p mimics reduce the expression and activity of endogenous UGT2B7, and miR-3664 levels are negatively correlated to UGT2B7 mRNA in a tissue RNA panel. HepG2 cells were transfected with miR-3664-3p mimics (or miR-3664-3p inhibitor) or miR-neg. (A, C, and D) Cells were harvested for 1) analysis of UGT2B7 mRNA levels by RT-qPCR (A), 2) analysis of UGT2B7 protein levels by Western blotting (C), and 3) analysis of UGT2B7 activity via morphine glucuronidation assays (D) as described in the *Materials and Methods*. (A and B) UGT2B7 and GAPDH mRNA levels in cells transfected with miR-3664-3p mimics (or miR-3664-3p inhibitors) are presented relative to that in cells transfected with the negative control miR-neg. Data shown are means $\pm$ S.E.M. from three independent experiments performed in triplicate. *** $P < 0.0005$. (C) UGT2B7 and calnexin protein immunosignals from a representative Western blot experiment (top); quantification of UGT2B7 protein levels (means $\pm$ S.E.M. from two independent experiments performed in triplicate) in cells transfected with miR-3664-3p mimics relative to cells transfected with miR-neg (bottom). * $P < 0.05$. (D) Morphine-glucuronidation activity in miR-3664-3p mimic-transfected cells relative to that of miR-neg-transfected cells (set as a value of 100%). Data shown are means $\pm$ S.E.M. from two independent experiments performed in triplicate. ** $P < 0.005$. (E) miR-3664 and UGT2B4 mRNA levels in a panel of 10 normal tissues were measured using RT-qPCR as described in the *Materials and Methods*. Shown is a Spearman correlation analysis between the expression levels of miR-3664-3p and UGT2B4 mRNA in this human tissue panel.

HepG2 cells transfected with miR-3664-3p mimics relative to the miR-neg control (Fig. 3D). Collectively, these data define a novel miR-3664-3p target site in the UGT2B7 3'-UTR that negatively regulates UGT2B7 expression and activity in liver cancer cell lines.

To assess whether miR-3664-3p might play a role in the expression of UGT2B7 in the normal liver, we measured the expression levels of miR-3664-3p and UGT2B7 in a panel of 18 normal human liver tissues using RT-qPCR. Consistent with previous reports (Congiu et al., 2002; Court, 2010), UGT2B7 mRNA levels were very high in liver tissues. In contrast, levels of miR-3664-3p were extremely low or undetectable (data not shown). An analysis of RNAseq data from a TCGA cohort of 371 LIHCs also revealed high levels of UGT2B7 mRNA and extremely low levels of miR-3664 (data not shown). These observations suggest a negative correlation between miR-3664 and UGT2B7 mRNA levels in both normal and cancerous liver tissues. Although an overall correlation analysis between miR-3664 and UGT2B7 mRNA levels in liver cancer/liver tissue was not possible due to the lack of miR-3664 expression in most samples, we did measure a significant negative correlation between the expression levels of UGT2B7 and miR-3664-3p in a panel of 10 normal human tissues that did express both UGT2B7 and miR-3664-3p (liver, kidney, colon, small intestine, ovary, testis, trachea, lung, thyroid, and placenta) (Pearson $r = -0.081$, $P < 0.01$) (Fig. 3E).

Regulation of UGT2B4 by miR-135a-5p and miR-410-3p in Liver Cancer Cell Lines. The predicted miR-135a-5p binding site in the UGT2B4 3'-UTR is located at nucleotides 202–224 downstream of the stop codon and is defined as an 8mer site by TargetScan (Fig. 2B). In addition to the seed pairing, the miR-135a-5p binding site has 3'-sequence pairing to miR-135a-5p (Fig. 4A). The predicted miR-410-3p binding site is located at nucleotides 213–235 downstream of the UGT2B4 stop codon and is defined as a 7mer-m8 site (pairing to the 2- to 7-nucleotide miRNA seed and nucleotide 8). The miR-410-3p binding site also has substantial 3'-sequence pairing to miR-410-3p including nucleotides 13–17 (Fig. 4A). Of note, the 5' 11 nucleotides (5'AUAAAGCCAUA3') of the miR-135a-5p target site overlap with the 3' 11 nucleotides of the miR-410-5p target site in the UGT2B4 3'-UTR (Supplemental Fig. 1B). To investigate the regulation of UGT2B4 by miR-135a-5p and miR-410-3p through these two predicted sites, we mutated the sites in the pGL3/2B4/UTR reporter construct to generate the pGL3/2B4/UTR/miR135a/MT and pGL3/2B4/UTR/miR410/MT constructs (Fig. 4B). Cotransfection of miR-135a-5p mimics with the reporter constructs showed that miR-135a-5p significantly decreased the activity of the wild-type reporter but did not significantly alter the activity of pGL3/2B4/UTR/miR135a/MT in both HepG2 (Fig. 4C) and HuH7 (Fig. 4D) cells. Cotransfection of miR-410-3p mimics with the reporters showed that miR-410-3p significantly reduced the activity of the wild-type reporter but not that of pGL3/2B4/UTR/miR410/MT in HepG2 cells (Fig. 4D).

To assess these miRNAs in the regulation of endogenous UGT2B4 mRNA levels, we transfected miRNA mimics targeting miR-135a-5p or miR-410-3p in HuH7 cells; these cells were used because they had higher levels of UGT2B4 expression relative to HepG2 cells (see Supplemental Fig. 2). Both miR-135a-5p and miR-410-3p mimics significantly reduced UGT2B4 mRNA levels (Fig. 5A) but did not change GAPDH mRNA levels (Fig. 5B). Collectively, these results indicate that

UGT2B4 is a direct target of miR-135a-5p and miR-410-3p in liver cancer cells. However, the potential impact of these miRNAs on UGT2B4 protein level or enzymatic activity could not be assessed due to the lack of a UGT2B4-specific antibody or a probe substrate that would be specific for UGT2B4 in HepG2 or HuH7 cells.

The relationship between miR-135a-5p and UGT2B4 expression levels was assessed in a panel of 18 normal human liver tissues using quantitative real-time PCR, revealing a significant negative correlation (Spearman $r = -0.47$, $P = 0.047$) (Fig. 5C). We also measured miR-135a-5p and UGT2B4 levels in HepG2 and Huh7 cells and found that miR-135a-5p levels are significantly lower in Huh7 cells than in HepG2 cells; conversely, UGT2B4 levels are significantly higher in Huh7 cells than in HepG2 cells (Supplemental Fig. 2), suggesting that negative correlation extends to these liver cancer cell lines. In contrast, when we examined miR-135a and UGT2B4 levels in the TCGA-LIHC RNAseq dataset containing 371 hepatocellular carcinoma specimens, we saw no correlation (Supplemental Fig. 3). Finally, we measured miR-135a-5p and UGT2B4 levels in a panel of 10 normal human tissues (liver, kidney, colon, small intestine, ovary, testis, trachea, lung, thyroid, and placenta). Both genes varied widely in expression but were uncorrelated (data not shown).

No correlation was observed between UGT2B4 and miR-410-3p expression levels in the normal human liver tissue panel (data not shown). However, analysis of the TCGA-LIHC RNAseq dataset (371 hepatocellular carcinoma specimens) revealed a significant negative correlation between miR-410 and UGT2B4 mRNA levels (Spearman $r = -0.15$, $P = 0.002$) (Fig. 5D). Furthermore, we also observed a significant negative correlation between the expression levels of UGT2B4 and miR-410-3p in the panel of 10 normal human tissues (liver, kidney, testis, lung, thyroid, placenta, prostate, heart, and cervix) (Pearson $r = -0.76$, $P = 0.010$) (Fig. 5E).

Discussion

UGT2B4 and UGT2B7 are highly expressed in the liver. While their transcriptional regulation has been well studied (Hu et al., 2014b), to date there has been only one report of their post-transcriptional regulation by miR-216b-5p in liver cancer cell lines (Dluzen et al., 2016). We used bioinformatics analysis to predict multiple potential miRNA binding sites in the UGT2B7 and UGT2B4 3'-UTRs, and we were able to confirm a subset of these experimentally. In particular, we used reporter analysis, mutagenesis, and endogenous gene expression analysis to show that miR-3664-3p negatively regulates the UGT2B7 mRNA via a binding site in the 3'-UTR. We also showed inhibition of UGT2B7 at both protein and activity levels using a specific antibody and probe substrate. Similarly, we found that miR-135a-5p and miR-410-3p bind and regulate the UGT2B4 mRNA. Both miR-410 (Wang et al., 2014; Marrone et al., 2016) and miR-135a (Liu et al., 2012; Zeng et al., 2016) are reported to be upregulated in liver cancer and are considered as liver onco-miRs. By contrast, these two miRNAs are frequently downregulated in some other cancers, including breast, prostate, and pancreatic cancer (Chien et al., 2011; Gattolliat et al., 2011; Tang et al., 2014; Theodore et al., 2014). Overexpression of miR-3664 in the peripheral blood of patients with glioblastoma (Dong et al., 2014) and individuals with gastric cancer (Liu et al., 2014) was

Fig. 4. A functional miR-135a-5p site and a functional miR-410-3p in the UGT2B4 3'-UTR. (A) Shown are the positions of the miR-135a-5p and the miR-410-3p target site in the UGT2B4 3'-UTR and the sequence complementarity between these two sites and their cognate miRNAs, including seed pairing (highlighted in gray) and 3'-sequence pairing. The nucleotides of the two miRNA sites are numbered relative to the stop codon (TAG with G positioned as -1) (NM_021139.2). (B) The mutation of GCCA to TGAC (boxed) that abolishes the seed pairing between the miR-135a-5p target site and miR-135a-5p, and the mutation of TATA to CCGC (boxed) that abolishes the seed pairing between the miR-410-3p target site and miR-410-3p is shown. (C and D) Cotransfection of a UGT2B4 3'-UTR luciferase reporter carrying either a wild-type or mutated miR-135a-5p target site, 30 nM miR-neg or miR-135a-5p mimics, and Renilla reporter vector as the control in HepG2 (C) and HuH7 (D) cells. (E) Cotransfection of a UGT2B4 3'-UTR luciferase reporter carrying either a wild-type or mutated miR-410-3p target site, 30 nM miR-neg or miR-410-3p mimics, and Renilla reporter vector as the control in HepG2 cells. Twenty-four hours post-transfection, cells were harvested for luciferase activity assays as described in the *Materials and Methods*. The luciferase activity of the reporter cotransfected with miRNA mimics is presented relative to that of the reporter cotransfected with miR-neg (set as a value of 100%). Data shown are means $\pm$ S.E.M from two independent experiments performed in quadruplicate. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$. n.s., not significant.

recently reported; however, the biologic role of miR-3664-3p in liver cancer remains to be investigated.

miRNAs generally repress gene expression through mRNA degradation or translational repression depending on the degree and nature of sequence complementarity between the miRNA guide and mRNA target (Macfarlane and Murphy, 2010). Extensive base-pairing between the miRNA guide and mRNA target permits mRNA cleavage by Argonaute (Ago2) (Macfarlane and Murphy, 2010); expression levels of such

miRNAs and their targets may thus be negatively correlated due to miRNA-mediated mRNA degradation. The three functional miR-binding sites (UGT2B4 miR-135a-5p, UGT2B4 miR-410-3p, and UGT2B7 3664-3p) reported in our study showed (Figs. 2A and 4A) extensive seed pairing and additional 3'-sequence pairing(s) with their targets, which would be expected to favor the mRNA degradation pathway.

Given the predicted role of each of the miRNAs that we studied in target mRNA degradation, we further predicted

Fig. 5. miR-135a-5p and miR-410-3p mimics reduce UGT2B4 mRNA levels in HuH7 cells, and correlation analyses between UGT2B4 and miR-135a-5p (or miR-410-3p) levels in human tissue panels and liver cancer specimens are presented. (A and B) HuH7 cells were transfected with miR-neg, miR-135a-5p, or miR-410-3p mimics. UGT2B4 and GAPDH mRNA levels were measured using RT-qPCR. The UGT2B4 mRNA levels in miRNA mimic-transfected cells are presented relative to that of cells transfected with miR-neg (set at a value of 100%). Data shown are means $\pm$ S.E.M. from two independent

that negative correlations might be observed between miRNA levels and target mRNA levels in various tissues. Although this was the case for some of our miRNA-mRNA pairings, it was not necessarily seen in all of the contexts examined. Moreover when interpreting our data, it is important to consider that correlation alone does not imply causation, and many regulatory factors, both transcriptional and post-transcriptional, are involved in the regulation of a given gene. In the case of miR-3664 and UGT2B7, levels were negatively correlated in both normal and cancerous liver tissues with high expression of UGT2B7 and low (or absent) expression of miR-3664. One reasonable interpretation of this result is that the absence (or very low level) of miR-3664 permits a high steady-state level of UGT2B7 mRNA to be maintained. However, this causal relationship would need to be explicitly tested to state definitively that miR-3664-3p is an important endogenous regulator of basal UGT2B7 levels. Interestingly, in the analysis of the 10-tissue panel (including nine extrahepatic tissues), while UGT2B7 and miR-3664-3p levels were still negatively correlated overall, there were some tissues with low or absent UGT2B7 levels (ovary, trachea, and testis) that also had very low miR-3664-3p levels. This may be explained by considering the role of transcriptional (promoter level) regulation in determining the overall level of UGT2B7 expression. The key positive transcriptional regulators that activate the *UGT2B7* promoter in the liver (e.g., HNFs) have little activity in tissues such as the trachea, ovary, and testis; hence, we expect low or absent *UGT2B7* transcription. In this context, post-transcriptional regulation may have little relevance.

We found a negative correlation between miR-410 and UGT2B4 mRNA levels in the liver cancer TCGA cohort of 371 specimens as well as in the panel of 10 normal human tissues, but unexpectedly, not in the panel of 18 normal livers. This discrepancy remains to be resolved and may be clarified in the future by examining larger cohorts of normal livers and liver cancers.

The relationship between miR-135a and UGT2B4 mRNA levels in the various samples that we analyzed was also complex. Although there was a negative correlation in the panel of 18 normal livers, there was no correlation between the levels of these genes in the liver cancer TCGA cohort. This could be related to deregulation of other miRNAs and transcription factors that are involved in controlling *UGT2B4* expression in liver cancer (Negrini et al., 2011; Callegari et al., 2015). Of note, Dluzen et al. (2014) reported the regulation of three *UGT1A* genes (1A1, 1A3, 1A6) by miR-491-3p in liver cancer cells and a significant inverse correlation between miR-491-3p and mRNA levels of UGT1A3 and UGT1A6 (but not UGT1A1) in a panel of normal human liver specimens.

Several studies have investigated the cooperative regulation of single-target mRNAs by multiple miRNAs and revealed that the efficacy and cooperativity between miRNA target sites is predominantly determined by the interval spacing

between seed sites (Doench and Sharp, 2004; Grimson et al., 2007; Saetrom et al., 2007; Pasquinelli, 2012). Specifically, the most effective cooperative downregulation is seen when the distance of two seed sites is between 13 and 35 nucleotides; however, this cooperativity becomes less likely when the internal spacing between miRNA seed sites is over 70 nucleotides (Saetrom et al., 2007). The seed site is defined as nucleotides 2–8 from the 3' end of a miRNA target site that are normally complementary to the seed sequence of the cognate miRNA (Bartel, 2009). Our findings, together with the recent report by Dluzen et al. (2016), indicate that *UGT2B4* is a direct target of three miRNAs (miR-135a-5p, miR-410-3p, and miR-216b-5p). As mentioned earlier, the 5' 11 nucleotides of the miR-135a-5p site overlap with the 3' 11 nucleotides of the miR-410-3p site in the UGT2B4 3'-UTR (Supplemental Fig. 1B). The seed sites of these two miRNAs are separated by 12 nucleotides, which falls just outside of the proposed optimal spacing range; whether miR-135a-5p and miR-410-3p could cooperatively regulate UGT2B4 remains to be investigated in future studies. The miR-135a-5p site (or miR-410-3p site) and the miR-216b-5p site are separated by 137 nucleotides (or 149 nucleotides) in the UGT2B4 3'-UTR (Supplemental Fig. 1B), and the miR-3664-3p site and the recently reported miR-216-5p site (Dluzen et al., 2016) are 73 nucleotides apart in the UGT2B7 3'-UTR (Supplemental Fig. 1A). Therefore, the cooperativity between these miRNA target sites is predicted to be less likely; however, this also remains to be tested.

When we initially screened the predicted miRNA binding sites using luciferase reporters, we also found repression of the UGT2B4 3'-UTR reporter by miR-4691-5p and miR-489-3p (Fig. 1B). The putative binding sites for miR-4691-5p and miR-489-3p in the UGT2B4 3'-UTR include overlapping seed sequences (Supplemental Fig. 1B). Thus, miR-4691-5p and miR-489-3p could possibly inhibit each other's activity based on a model of competitive loading of miRNA-activated miRNA-induced silencing complexes (Macfarlane and Murphy, 2010). In contrast, cooperative regulation between the miR-135a-3p and miR-4691-5p (or miR-489-3p) target sites may be possible, as the spacing between their seed sites is within the optimal range (13–35 nucleotides) for cooperativity. Again, future studies are required to test these possibilities.

In summary, this study demonstrates that UGT2B4 is a direct target of miR-135a-5p and miR-410-3p and that UGT2B7 is a direct target of miR-3664-3p in liver cancer cells. Combined with other recent reports of miRNA-mediated UGT regulation (Dluzen et al., 2014, 2016; Wijayakumara et al., 2015; Margaillan et al., 2016), our observations suggest that post-transcriptional regulation of UGTs by miRNAs may have an important role in fine-tuning glucuronidation activity in the liver as well as extrahepatic tissues. Although none of the miRNAs characterized in this study are predicted to have a role in cancer-specific UGT regulation, further analysis of the functional significance of the miRNA-regulatory network is ongoing. In particular, it is interesting to consider whether

experiments performed in triplicate. * $P < 0.05$; ** $P < 0.005$. (C and E) The expression levels of UGT2B4 mRNA, miR-135a-5p, and miR-410-3p in a panel of normal human liver tissues (C) and a human tissue panel (E) were quantified using RT-qPCR followed by correlation analyses between miR-135a-5p (or miR-410-3p) and UGT2B4 mRNA levels using GraphPad Prism 6 software as described in the *Materials and Methods*. (D) Transcriptome profiling data (RNAseq and miRNA sequencing) from a cohort of LIHCs were downloaded from the TCGA data portal. Data were normalized and a correlation analysis between miR-410 and UGT2B4 mRNA levels was conducted using the Spearman rank method; the graph was drawn using the R statistical package as described in the *Materials and Methods*.

deregulation of miRNA expression due to cellular stress or inflammation could alter UGT levels and hence the capacity for detoxification, which in turn may affect cancer risk.

Authorship Contributions

Participated in research design: Wijayakumara, Mackenzie, Hu, Meech.

Conducted experiments: Wijayakumara.

Performed data analysis: Wijayakumara, Mackenzie, Hu, Meech.

Wrote or contributed to the writing of the manuscript: Wijayakumara, Mackenzie, McKinnon, Hu, Meech.

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Address correspondence to: Dr. Robyn Meech, Department of Clinical Pharmacology, Flinders University School of Medicine, Flinders Medical Centre, Bedford Park SA 5042, Australia. E-mail: robyn.meech@flinders.edu.au or Dr. Dong Gui, Department of Clinical Pharmacology, Flinders University School of Medicine, Flinders Medical Centre, Bedford Park SA 5042, Australia. E-mail: donggui.hu@flinders.edu.au

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