

MicroRNA-328 Negatively Regulates the Expression of Breast Cancer Resistance Protein (BCRP/ABCG2) in Human Cancer Cells

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ABSTRACT

Breast cancer resistance protein (BCRP/ABCG2) is a molecular determinant of pharmacokinetic properties of many drugs in humans. To understand post-transcriptional regulation of ABCG2 and the role of microRNAs (miRNAs) in drug disposition, we found that microRNA-328 (miR-328) might readily target the 3'-untranslated region (3'-UTR) of ABCG2 when considering target-site accessibility. We then noted 1) an inverse relation between the levels of miR-328 and ABCG2 in MCF-7 and MCF-7/MX100 breast cancer cells and 2) that miR-328 levels could be rescued in MCF-7/MX100 cells by transfection with miR-328 plasmid. Luciferase reporter assays showed that ABCG2 3'-UTR-luciferase activity was decreased more than 50% in MCF-7/MX100 cells after transfection with miR-328 plasmid, the activity was increased over 100% in MCF-7 cells transfected with a miR-328 antagomir, and disruption of miR-328 response element within ABCG2 3'-UTR led to a 3-fold

increase in luciferase activity. Furthermore, the level of ABCG2 protein was down-regulated when miR-328 was over-expressed, and the level was up-regulated when miR-328 was inhibited by selective antagomir. Altered ABCG2 protein expression was associated with significantly declined or elevated levels of ABCG2 3'-UTR and coding sequence mRNAs, suggesting possible involvement of the mechanism of mRNA cleavage. Finally, miR-328-directed down-regulation of ABCG2 expression in MCF-7/MX100 cells resulted in an increased mitoxantrone sensitivity, as manifested by a significantly lower IC₅₀ value ($2.46 \pm 1.64 \mu\text{M}$) compared with the control ($151 \pm 32 \mu\text{M}$). Together, these findings suggest that miR-328 targets ABCG2 3'-UTR and, consequently, controls ABCG2 protein expression and influences drug disposition in human breast cancer cells.

Breast cancer resistance protein BCRP/ABCG2 is an ATP-binding cassette membrane transporter expressed ubiquitously in humans, controlling the absorption, distribution and clearance of numerous xenobiotics, including pharmaceutical agents, dietary carcinogens and conjugated metabolites (Mao and Unadkat, 2005; van Herwaarden and Schinkel, 2006; Vore and Leggas, 2008). In addition, overexpression of ABCG2 and other drug transporters in tumorigenic stem cells represents an important mechanism for multidrug resistance (Dean et al., 2005). Because ABCG2 was discovered in drug-resistant human cancer cells (e.g., MCF-7/AdrVp and S1MI80),

these cell lines have been widely used for studying the function and regulation of ABCG2 and defining its role in drug disposition and multidrug resistance. In particular, gene amplification (Ross et al., 1999; Knutsen et al., 2000; Volk et al., 2002) has been shown to be an important mechanism for elevated ABCG2 expression in drug-resistant cancer cells. Recent studies have demonstrated that transcriptional factors [i.e., nuclear receptors (Ee et al., 2004b; Ebert et al., 2005; Szatmari et al., 2006; Honorat et al., 2008; Narang et al., 2008; Vore and Leggas, 2008; Wang et al., 2008b) and epigenetic factors (To et al., 2006; Turner et al., 2006; Calcagno et al., 2008; Nakano et al., 2008; To et al., 2008a)] play important roles in the regulation of ABCG2 in different model systems. Potential regulation of ABCG2 at its 3'-untranslated region (3'-UTR) is just beginning to be explored (To et al., 2008b).

MicroRNAs (miRNAs) represent a newly recognized family of short, noncoding RNAs that govern post-transcriptional

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ABBREVIATIONS: 3'-UTR, 3'-untranslated region; miRNA, microRNA; miR-328, microRNA-328; ABCG2, ATP-binding cassette, subfamily G (white), member 2; BCRP, breast cancer resistance protein; FTC, fumitremorgin C; PBS, fetal bovine serum; bp, base pair(s); MRE, microRNA response element; qPCR, quantitative real-time polymerase chain reaction; PCR, polymerase chain reaction; CDS, coding sequence; hsa-miR-328, human miR-328.

expression of target genes (Ambros, 2004; Bartel, 2004; He and Hannon, 2004). These miRNAs exhibit unique expression patterns in specific tissues and/or cells, at certain developmental stages, or in response to distinct stressors. They usually act through base pairing to partially complementary segments within the 3'-UTR transcript of target gene, leading to translation inhibition and/or mRNA cleavage and, consequently, negative regulation of the target gene. More and more studies have demonstrated the importance of miRNAs in drug metabolism and disposition via the regulation of drug-metabolizing enzymes, drug transporters or nuclear receptors (Tsuchiya et al., 2006; Yu, 2007; Kovalchuk et al., 2008; Liao et al., 2008; Takagi et al., 2008; To et al., 2008b; Zhu et al., 2008), which may not only provide insight into miRNA biological functions but also advance the understanding of integrated response of cells to xenobiotics. Indeed, the two most recent studies suggest that miR-519c and 520h may regulate ABCG2, in which miR-520h was nicely assessed by luciferase reporter assay (Liao et al., 2008) and miR-519c inhibitor or mimic were clearly shown to alter ABCG2 protein expression in A549 cells (To et al., 2008b). In the present study, however, we show that human miR-328 (hsa-miR-328) would readily target ABCG2, concerning the target-site accessibility (Kertesz et al., 2007), and given the finding that levels of ABCG2 protein and mature miR-328 in drug-resistant and parental MCF-7 cells are inversely related. Furthermore, we present data suggesting that miR-328 negatively regulates ABCG2 protein expression by acting on the 3'-UTR segment and that repression of ABCG2 expression via miR-328-mediated pathway is translated into significantly increased drug sensitivity in cancer cells.

Materials and Methods

Materials. Mitoxantrone was purchased from Sigma-Aldrich (St. Louis, MO). Fumitremorgin C (FTC) was bought from Alexis (San Diego, CA). RPMI 1640 medium, fetal bovine serum, and phosphate-buffered saline (PBS) were purchased from Invitrogen (Carlsbad, CA). The MCF-7/MX100 cells were kindly provided by Dr. Susan E. Bates (National Cancer Institute, Bethesda, MD).

Bioinformatics. The 3'-UTR sequence of human *ABCG2* (GenBank sequence NM_004827) was retrieved using Entrez (<http://www.ncbi.nlm.nih.gov/Entrez/>), and we searched for antisense matches to individual miRNAs with the use of PITA (Kertesz et al., 2007), TargetScan (Lewis et al., 2005), and miRBase Targets (Griffiths-Jones et al., 2006).

Cell Culture. Human breast cancer MCF-7 (parental) and MCF-7/MX100 (mitoxantrone-selected) cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO₂/95% air. Cell culture media consisted of 100 units/ml penicillin and 100 µg/ml streptomycin. Mitoxantrone (100 nM) was also added for the culture of MCF-7/MX100 cells.

Plasmids. Human miR-328 precursor (392 bp) was cloned into pSilencer 4.1 (Ambion, Austin, TX) using the primers 5'-CGCGGATCCCCAGCTCCACCCACCCACCTTGTGCGAA-3' (sense) and 5'-CCCAAGCTTGTGGAGAACAGCAGCCGCGAGGAC-3' (antisense). The correct plasmid was named pS-miR-328, compared with control plasmid (pS-Neg), consisting of a scrambled sequence (Ambion). Human ABCG2 3'-UTR (0–1296 bp) containing the predicted miR-328 response element (MRE) was cloned into pGEM-T Easy vector (Promega, Madison, WI) and then subcloned into downstream of the *Renilla reniformis* luciferase gene within psiCHECK-II (Promega) using primers 5'-CCGCTCGAGATGTACTGGCGAAGAATATTTGGTAAAGCAGGG-3' (sense) and 5'-TTGCGGCCGGGTAAGGAAGGAAGTAGTGACTGGGAGAATGG-3' (antisense). The miR-328 MRE mutant

containing four nucleotide transitions (G618C, G620C, G621C, and A625C) was created using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) and primers 5'-TGTGGGTAGAGTACACCGCCCGGAGTCCAGTAATAG-3' (forward) and 5'-CTATTACTGGACTCCGGGCGGTGTACTCTACCCACA-3' (reverse) (mutated nucleotides are underlined). In addition, Let-7a precursor (319 bp) and its targeted Lin-41 3'-UTR segment (124 bp) were cloned into pSilencer 4.1 and psiCHECK-II, respectively, which were used as positive controls in luciferase experiments. Cloning primers were 5'-CGCGGATCCTTATCACTCACTCACACAGAAACCAGGATTACC-3' (sense) and 5'-CCCAAGCTTGGCCATAACAAATGCTGCACTACATC-3' (antisense) for Let-7a, 5'-GCTCTAGATTTCTCAAATTGCA-CCAATCAAGTATACCT-3' (sense) and 5'-AAGGCCGCGCCCAATGGTTCCAGAGCAGAATGGTTGTATAAAA-3' (antisense) for Lin-41, respectively. All inserts were confirmed by direct DNA sequencing analyses.

Quantitative Real-Time Reverse Transcription-PCR Analysis. Total RNA was prepared with the SV Total RNA Isolation System (Promega) and reverse-transcribed to cDNA with the Superscript II Reverse Transcription-PCR kit (Invitrogen, Carlsbad, CA). The primers for analysis of ABCG2 coding sequence (CDS) transcript were 5'-CAGGTGGAGGCAAATCTTCGT-3' (forward; exon 3) and 5'-ACACACCACGGATAAACTGA-3' (reverse; exon 6), and primers for ABCG2 3'-UTR mRNA consisting of the miR-328 MRE segment were 5'-TCTGGCATTTGTTTCCTC-3' (forward) and 5'-CTCCTGGCCCTCTACTCT-3' (reverse). Amplification of glyceraldehyde-3-phosphate dehydrogenase (internal control) was carried out with the primers 5'-ATCACCATCTTCCAGGAGCGA-3' (forward) and 5'-GCTTCACCACCTTCTTGATGT-3' (reverse).

Small RNAs were isolated with the mirPremier microRNA isolation kit (Sigma-Aldrich, St. Louis, MO) and stem-loop reverse transcription of mature hsa-miR-328 was conducted as described previously (Chen et al., 2005) using the primer 5'-TGTCAGGCAACCGTATTCACCGAGTGGTGGGAAG-3'. U6 small nuclear RNA was used as internal control. qPCR reactions were performed with the following primers: for hsa-miR-328, 5'-GCTGGCCCTCTCTGCCC-3' (forward) and 5'-CGTCAGATGTCCGAGTAGAGG-3' (reverse); and for U6, 5'-CTCGCTTCGGCAGCACACA-3' (forward) and 5'-AACGCTTCACGAATTTGCGT-3' (reverse).

SYBR Green qPCR was performed on Stratagene 3005P real-time PCR system. All reactions were carried out using cells cultured in triplicate or quadruplicate, and experiments were repeated once with separate cultures. The cycle number (C_T) at which the amplicon concentration crossed a defined threshold was determined for each individual miRNA. The relative level of each analyte over internal standard (glyceraldehyde-3-phosphate dehydrogenase or U6) was calculated by using the equation $2^{-\Delta C_T}$, where $\Delta C_T = C_T$ (analyte) - C_T (internal standard), and then compared between different groups or treatments.

Luciferase Assay. All transfections were conducted with Lipofectamine 2000 (Invitrogen). MCF-7/MX100 cells were cotransfected with ABCG2 3'-UTR-luciferase reporter construct (0.1 µg) and pS-miR-328 or pS-Neg plasmid (0.4 or 1.0 µg). As positive control, cells were transfected with Let-7 and Lin-41 constructs. Likewise, MCF-7 cells were cotransfected with the luciferase constructs (0.1 µg) and miR-328 antagomir or a scrambled control (5 or 10 nM) (Dharmacon, Chicago, IL). Luciferase activities were assayed using Dual-Luciferase Reporter Assay System (Promega, Madison, WI) 48 h after transfection. Triplicate transfections were tested. *R. reniformis* luciferase activity was normalized to firefly luciferase activity and then compared between different treatments.

Immunoblot Analysis. Cells were harvested after being washed with PBS. Cell lysates were prepared with a lysis buffer (20 mM Tris, pH 7.5, 120 mM sodium chloride, 100 mM sodium fluoride, 1% nonidet P-40, 200 µM sodium orthovanadate, 50 mM β-glycerolphosphate, 10 mM sodium pyrophosphate) and the complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentrations were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL).

Whole-cell proteins (50 μ g) were separated on 7.5% SDS-polyacrylamide gels and electrotransferred onto nitrocellulose membranes (Invitrogen). Membranes were then incubated overnight in Tris-buffered saline containing 0.2% (v/v) Tween 20 and 5% (w/v) fat-free dry milk at 4°C. Analysis of BCRP/ABCG2 protein was achieved after incubation with the *BCRP-21* monoclonal antibody (Kamiya Biomedical, Thousand Oaks, CA) at room temperature for 2 h and with a horseradish peroxidase sheep anti-mouse IgG (GE Healthcare, Little Chalfont, Buckinghamshire, UK) for 1.5 h, and visualization with an enhanced chemiluminescence detection system (GE Healthcare). Densitometric analysis was conducted using the Kodak Image Station (New Haven, CT).

Mitoxantrone Cytotoxicity. Cytotoxicity studies were performed as described previously (Ee et al., 2004a; Zhang et al., 2004). In brief, 48 h after transfection with pS-Neg or pS-miR-328 plasmids (0.4 μ g), MCF-7-MX100 cells were cultured with fresh medium containing 0 to 1 mM mitoxantrone. Additional controls were untransfected cells treated with 0 to 1 mM mitoxantrone supplemented with FTC (10 μ M) or vehicle (0.1% DMSO). After 24-h incubation, drug-containing medium was removed. Cells were washed twice with PBS buffer, and cultured in drug-free medium for another 24 h. Cell growth was determined by sulforhodamine B assay. Inhibition (IC_{50} value) of cell growth by mitoxantrone was estimated by fitting the percentage of cell growth (vehicle control plus 0 mM mitoxantrone treatment as 100%) to the Hill equation with Prism software (Graph-Pad Software, San Diego, CA). All experiments were carried out in triplicate and repeated once with separate cultures.

Statistical analysis. All values were expressed as mean \pm S.D. Different treatments (qPCR and luciferase data) were compared by unpaired Student's *t* test, and multiple variances (cytotoxicity) were analyzed by two-way analysis of variance (Prism). Difference was considered as significant if the probability was less than 0.05 ($P < 0.05$).

Results and Discussion

Bioinformatic Analyses Reveal That ABCG2 3'-UTR May Be Readily Targeted by hsa-miR-328. To investigate miRNA regulation of ABCG2, we first employed multiple algorithms, PITA (Kertesz et al., 2007), TargetScan (Lewis et al., 2005), and miRBase Targets (Griffiths-Jones et al., 2006) to screen antisense matches of ABCG2 3'-UTR against human miRNAs. Each algorithm identified more than 10 candidate MRE sites, and all included the miR-520 MRE being validated recently by luciferase assay (Liao et al., 2008). However, concerning miRNA target-site accessibility, PITA analysis showed that miR-328 would be the top candidate, with the lowest interaction free energy (ΔG , -13.8 kcal/mol; difference between free binding energy of a miRNA to the target, ΔG_{duplex} , and free energy lost by opening the target site, ΔG_{open}) and the lowest free binding energy (ΔG_{duplex} , -31.3 kcal/mol) (Table 1). The same miR-328 MRE (Fig. 1A) was identified by TargetScan, whereas this site was not predicted by miRBase Targets. In contrast, the miR-519c MRE site was not predicted by PITA but by miRBase Targets, which has been shown to affect ABCG2 expression by miR-519c precursor and inhibitor in A549 cells (To et al., 2008b). The discrepancy in prediction of MRE sites is presumably due to the difference in method and database used by individual programs, which also indicates the need for validation by well-controlled biological experiments.

Levels of miR-328 and ABCG2 Are Inversely Related in Cancer Cells, and Deficient Expression of miR-328 Can Be Rescued by Transfection of MCF-7/MX100 Cells with pS-miR-328 Plasmid. To assess potential interaction between miR-328 and ABCG2 3'-UTR, we first investigated

the expression of miR-328 and ABCG2 3'-UTR mRNA in parental MCF-7 and mitoxantrone-resistant MCF-7/MX100 cells, the latter of which is characterized by ABCG2 overexpression. Consistent with a 6-fold higher level of ABCG2

TABLE 1

Human miRNAs that might target ABCG2 3'-UTR

Listed are the top 10 candidates predicted by PITA algorithm concerning target-site accessibility. Note that hsa-miR-328 exhibits the lowest ΔG_{duplex} value, as well as the lowest ΔG .

microRNA	Position	ΔG_{duplex}	ΔG_{open}	ΔG
kcal/mol				
hsa-miR-328	618	-31.3	-17.5	-13.8
hsa-miR-134	1106	-19.0	-7.26	-11.7
hsa-miR-520d-3p	43	-21.7	-12.4	-9.29
hsa-miR-146b-5p	1832	-16.6	-8.08	-8.51
hsa-miR-146a	1832	-16.4	-8.08	-8.31
hsa-miR-520e	43	-20.2	-12.4	-7.79
hsa-miR-520f	42	-18.7	-11.6	-7.10
hsa-miR-520c-3p	43	-19.5	-12.4	-7.09
hsa-miR-373	43	-19.1	-12.4	-6.71
hsa-miR-520g	45	-19.9	-13.7	-6.23

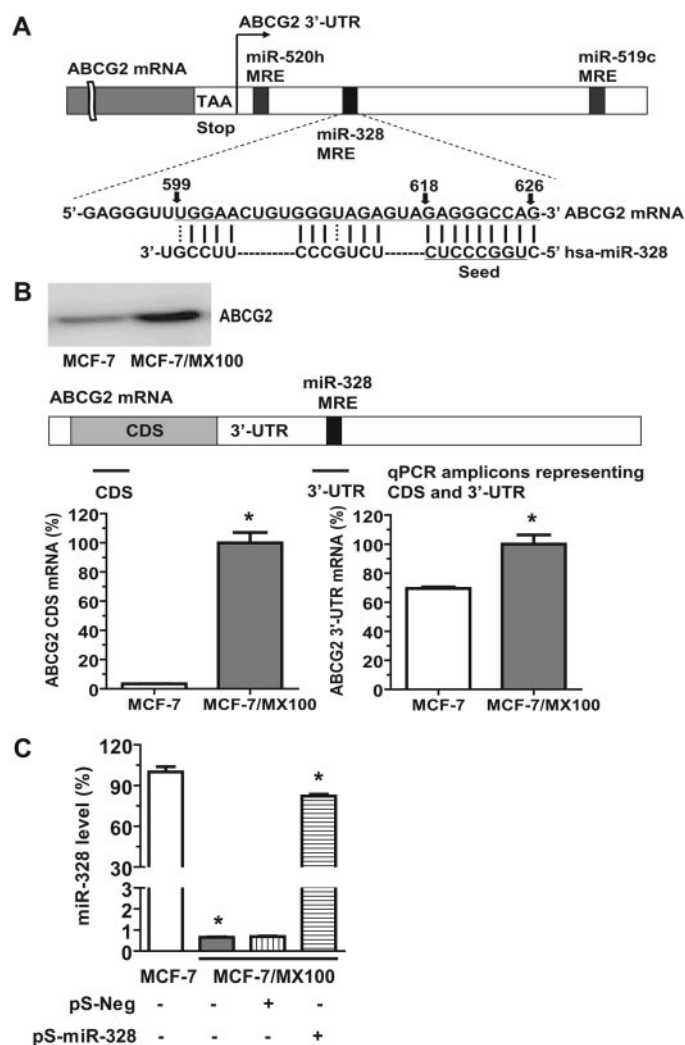


Fig. 1. Potential interaction between human miR-328 and ABCG2. The miR-328 MRE site (A), located on 599 to 626 within 3'-UTR of ABCG2, was identified by PITA and TargetScan algorithms. Overexpression of ABCG2 protein and CDS mRNA in MCF-7/MX100 cells (B) was associated with sharply reduced miR-328 expression, and deficient miR-328 expression could be rescued by transfection with pS-miR-328 plasmid (C). *, $P < 0.05$, compared with the control ($n = 4$ in each group).

protein estimated from immunoblot, the level of ABCG2 CDS mRNA, determined by qPCR analysis of a region from exon 3 to 6, was 30-fold higher in MCF-7/MX100 cells than MCF-7 cells (Fig. 1B). The level of ABCG2 3'-UTR mRNA containing miR-328 MRE was also significantly higher in MCF-7/MX100 cells. The more dramatic difference in ABCG2 CDS amplicons observed in the two cell lines is probably caused by the difference in transcription of *ABCG2*, which may be due to *ABCG2* gene amplification in drug-resistant cells (Ross et al., 1999; Knutsen et al., 2000; Volk et al., 2002). It is noteworthy that hsa-miR-328 level in MCF-7/MX100 cells was less than 1% of that in MCF-7 cells (Fig. 1C). The results suggest that miR-328 levels are inversely related to ABCG2 levels in the two cancer cell lines. In addition, hsa-miR-328 expression could be restored in MCF-7/MX100 cells after transfection with pS-miR-328, which was comparable with the levels in MCF-7 cells without any treatment (Fig. 1C). In contrast,

miR-328 levels in MCF-7/MX100 cells remained unchanged after transfection with pS-Neg control plasmid (Fig. 1C). Based on these findings, miRNA gene overexpression and silencing approach (Krutzfeldt et al., 2006) may be employed to delineate potential action of miR-328 on ABCG2 in MCF-7/MX100 and MCF-7 cells, respectively.

Luciferase Reporter Assays Suggest That miR-328 Acts on the 3'-UTR of ABCG2. To validate miR-328 MRE site, we first constructed a ABCG2 3'-UTR-luciferase reporter plasmid. Utilization of native 3'-UTR sequence containing the predicted MRE site would be more relevant than the use of an artificial sequence containing multiple MRE sites. As positive control, Lin-41-luciferase activities were reduced by more than 50% when cells were transfected with plasmid expressing Let-7a. As expected, ABCG2 3'-UTR-luciferase activity was decreased more than 50% in MCF-7/MX100 cells transfected with pS-miR-328 plasmid, compared with control pS-Neg plasmid (Fig. 2A). On the other hand, silencing miR-328 with selective antagomir led to a 100% increase in luciferase activity in MCF-7 cells (Fig. 2B). Fi-

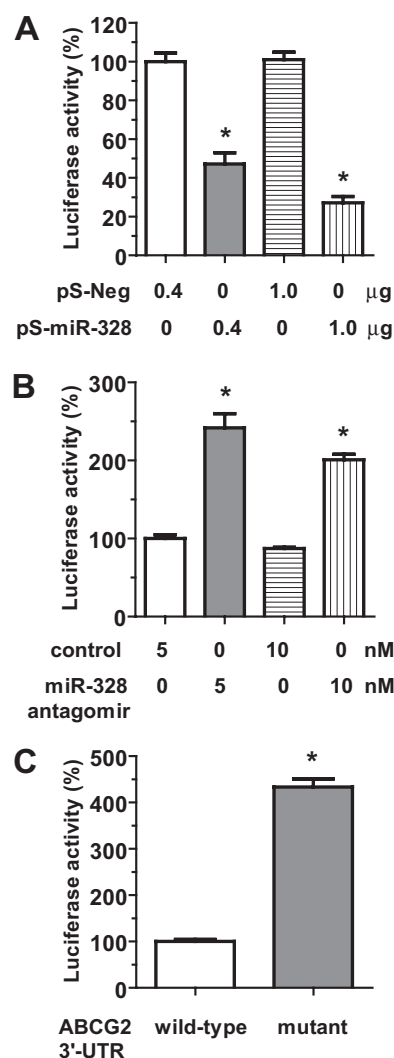


Fig. 2. Luciferase reporter assays suggest that human miR-328 directly targets the 3'-UTR of ABCG2. Overexpression of miR-328 led to more than 50% decrease in ABCG2 3'-UTR-luciferase activity in MCF-7/MX100 cells (A). Inhibition of miR-328 by selective antagomir resulted in 100% increase in luciferase activity in MCF-7 cells (B). ABCG2 3'-UTR mutant showed 300% higher luciferase activity than the wild-type 3'-UTR in MCF-7 cells (C). *, $P < 0.05$, compared with corresponding control ($n = 3$ in each group).

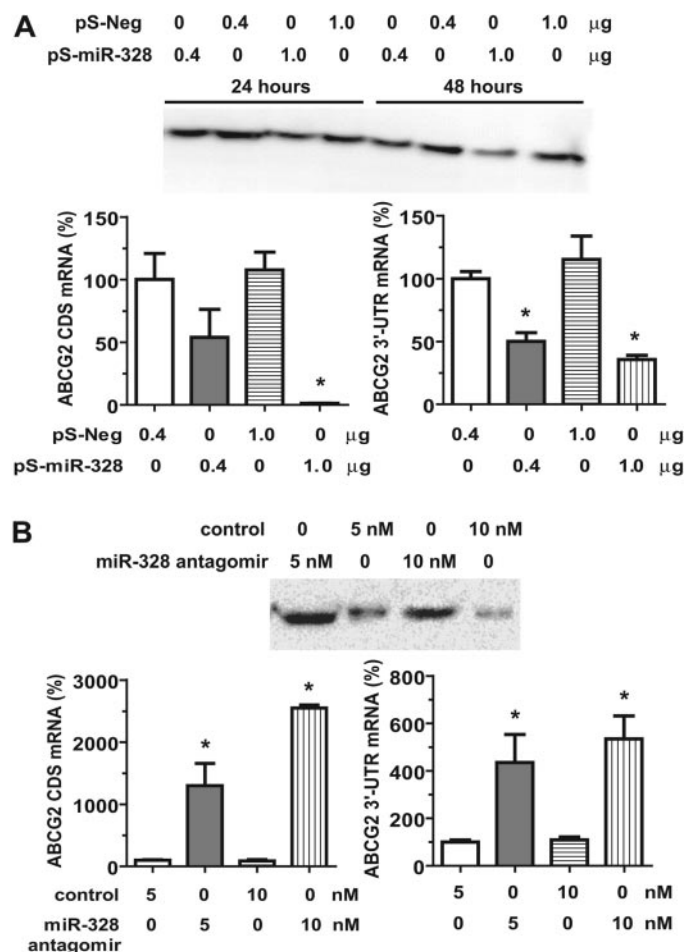


Fig. 3. Human miR-328 negatively regulates ABCG2 expression in breast cancer cells, and the mechanisms might include ABCG2 mRNA cleavage. MCF-7/MX100 cells transfected with pS-miR-328 plasmid had much lower levels of ABCG2 protein, CDS transcript, and 3'-UTR mRNA compared with cells transfected with pS-Neg control plasmid (A). Transfection with selective miR-328 antagomir consistently resulted in much higher expression of ABCG2 protein, CDS mRNA, and 3'-UTR transcript in MCF-7 cells (B). *, $P < 0.05$, compared with corresponding control ($n = 3$ in each group).

nally, disruption of miR-328 MRE within ABCG2 3'-UTR, particularly the segment complementary to 8-bp seed sequence (Fig. 1A), resulted in a 300% increase in luciferase activity in MCF-7 cells (Fig. 2C). These results suggest that miR-328 targets the 3'-UTR of ABCG2, namely the predicted miR-328 MRE.

ABCG2 Expression Is Negatively Regulated by miR-328, Which May Involve the Mechanism of mRNA Cleavage. Likewise, we employed a gene overexpression and knockdown approach to examine the effects of miR-328 on ABCG2 protein and mRNA expression in MCF-7/MX100 and MCF-7 cells, respectively. In MCF-7/MX100 cells, transfection with pS-miR-328 plasmid led to a dose- and time-dependent down-regulation of ABCG2 protein (Fig. 3A). It is noteworthy that ABCG2 protein expression was suppressed more than 70% at 48 h after transfection with a higher dose (1.0 μg) of pS-miR-328 plasmid. Furthermore, we determined the ABCG2 3'-UTR and CDS mRNA levels using qPCR, which were both sharply reduced in MCF-7/MX100 cells transfected with miR-328 plasmid (Fig. 3A). In contrast to effective ABCG2 regulation by high dose (≥ 40 nM) of miR-519c inhibitor in A549 cells (To et al., 2008b), a lower dose (5 nM) of miR-328 inhibitor resulted in a 2-fold increase in ABCG2 protein expression in MCF-7 cells, which was associated with a 12-fold increase in ABCG2 CDS mRNA and a 4-fold increase in 3'-UTR transcript (Fig. 3B). These findings suggest that miR-328 effectively regulates ABCG2, and the regulatory mechanisms are likely to include mRNA cleavage or other indirect effects. Meanwhile, one cannot exclude the possibility that miR-328 also targets the transcriptional factors of ABCG2, leading to an "indirect" transcriptional regulation of ABCG2 (Figs. 1B and 3B).

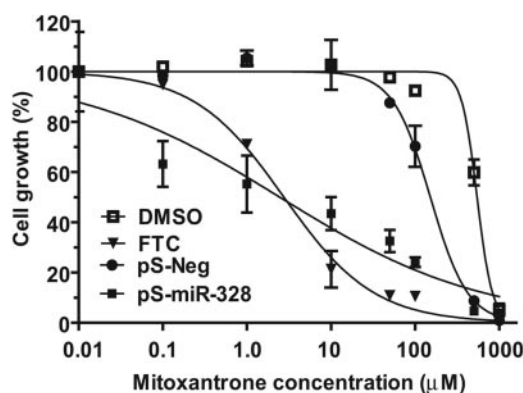


Fig. 4. ABCG2-mediated drug sensitivity can be altered via miR-328 pathway. MCF-7/MX100 cells transfected with pS-miR-328 plasmid were much more sensitive to mitoxantrone, compared with cells transfected with pS-Neg plasmid. As controls, inhibition of ABCG2 activity by FTC led to much higher sensitivity to mitoxantrone. Note that drug sensitivities are significantly ($P < 0.001$; two-way analysis of variance) different, not only between FTC and vehicle (DMSO) controls but also between pS-miR-328 and pS-Neg treatments ($n = 3$ at each concentration).

TABLE 2

Estimated IC_{50} and Hill slope values for mitoxantrone cytotoxicity in MCF-7/MX100 cells with different treatments

	DMSO	FTC	pS-Neg	ps-miR-328
IC_{50} (μM)	544 ± 30	$2.75 \pm 1.13^*$	151 ± 32	$2.46 \pm 1.64^*$
Hill slope	-4.75 ± 0.82	$-0.81 \pm 0.06^*$	-1.95 ± 0.28	$-0.36 \pm 0.06^*$

* Significantly ($P < 0.01$) different from corresponding control.

MicroRNA-328-Directed Down-Regulation of ABCG2 Is Translated into an Increased Drug Sensitivity of Cancer Cells.

To assess the impact of the miRNA pathway on drug disposition, we assayed mitoxantrone cytotoxicity in miR-328-transfected MCF-7/MX100 cells. As expected, coadministration of the ABCG2-selective inhibitor FTC significantly increased the sensitivity to mitoxantrone compared with vehicle control (Fig. 4). The corresponding IC_{50} values were 2.75 ± 1.13 and 544 ± 30 μM , respectively (Table 2), which are consistent with those reported (Zhang et al., 2004). Compared with cells transfected with pS-Neg plasmid, cells transfected with pS-miR-328 became much more sensitive to mitoxantrone (Fig. 4). This is indicated by a significantly lower IC_{50} value (2.46 ± 1.64 μM) in cells transfected with pS-miR-328 plasmid, compared with that (151 ± 32 μM) in cells transfected with pS-Neg. These results indicate that intervention of miRNA pathway can alter drug disposition and sensitivity of cancer cells.

Of particular note, mature miR-328 was shown to be extensively expressed in normal human tissues, including small intestine (~ 1600 copies per cell) and liver (~ 1100 copies per cell) (Lee et al., 2008). A comparison of miRNA expression is the liver-specific miR-122a, showing a level of ~ 4100 copies per cell in human liver. Furthermore, miR-328 expression seems to be much lower in all examined human colorectal cancer cell lines (less than 800 copies per cell; Lee et al., 2008). Very recent studies have also shown that miR-328 regulates zonation morphogenesis by targeting CD44 expression (Wang et al., 2008a), and mouse miR-328 regulates the expression of β -amyloid precursor protein-converting enzyme in neuronal cells (Boissonneault et al., 2009). Taken together, one cannot underestimate the physiological role of miR-328 nor exclude the importance to interindividual difference in ABCG2/BCRP expression, drug disposition, and multidrug resistance.

In summary, our results show that miR-328 targets the 3'-UTR of ABCG2 and negatively regulates ABCG2 protein expression, and suggest that suppressed miR-328 expression may be another underlying mechanism for ABCG2 overexpression in drug-resistant breast cancer cells (MCF-7/MX100). The findings also indicate that ABCG2-mediated drug resistance can be modified via interference of miRNA pathway in cancer cells. Furthermore, in light of our observations and those reported by others (Kertesz et al., 2007), the miRNA target-site accessibility should be considered an important factor in understanding gene regulation by miRNAs. Nevertheless, the above hypotheses await further examination in more complex model systems, and contribution of miR-328 and other evaluated miRNAs (Liao et al., 2008; To et al., 2008b) to ABCG2 expression and interindividual variability deserves investigation.

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References

- Ambros V (2004) The functions of animal microRNAs. *Nature* **431**:350–355.
- Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**:281–297.
- Boissonneault V, Plante I, Rivest S, and Provost P (2009) MicroRNA-298 and MicroRNA-328 regulate expression of mouse β -amyloid precursor protein-converting enzyme 1. *J Biol Chem* **284**:1971–1981.
- Calcagno AM, Fostel JM, To KK, Salcido CD, Martin SE, Cheung KJ, Wu CP,

- Varticovski L, Bates SE, Caplen NJ, et al. (2008) Single-step doxorubicin-selected cancer cells overexpress the ABCG2 drug transporter through epigenetic changes. *Br J Cancer* **98**:1515–1524.
- Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Andersen MR, et al. (2005) Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res* **33**:e179.
- Dean M, Fojo T, and Bates S (2005) Tumour stem cells and drug resistance. *Nat Rev Cancer* **5**:275–284.
- Ebert B, Seidel A, and Lampen A (2005) Identification of BCRP as transporter of benzo[a]pyrene conjugates metabolically formed in Caco-2 cells and its induction by Ah-receptor agonists. *Carcinogenesis* **26**:1754–1763.
- Ee PL, He X, Ross DD, and Beck WT (2004a) Modulation of breast cancer resistance protein (BCRP/ABCG2) gene expression using RNA interference. *Mol Cancer Ther* **3**:1577–1583.
- Ee PL, Kamalakaran S, Tonetti D, He X, Ross DD, and Beck WT (2004b) Identification of a novel estrogen response element in the breast cancer resistance protein (ABCG2) gene. *Cancer Res* **64**:1247–1251.
- Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, and Enright AJ (2006) miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res* **34**:D140–D144.
- He L and Hannon GJ (2004) MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet* **5**:522–531.
- Honorat M, Mesnier A, Di Pietro A, Lin V, Cohen P, Dumontet C, and Payen L (2008) Dexamethasone down-regulates ABCG2 expression levels in breast cancer cells. *Biochem Biophys Res Commun* **375**:308–314.
- Kertesz M, Iovino N, Unnerstall U, Gaul U, and Segal E (2007) The role of site accessibility in microRNA target recognition. *Nat Genet* **39**:1278–1284.
- Knutsen T, Rao VK, Ried T, Mickley L, Schneider E, Miyake K, Ghadimi BM, Padilla-Nash H, Pack S, Greenberger L, et al. (2000) Amplification of 4q21–q22 and the MXR gene in independently derived mitoxantrone-resistant cell lines. *Genes Chromosomes Cancer* **27**:110–116.
- Kovalchuk O, Filkowski J, Meservy J, Ilnytsky Y, Tryndyak VP, Chekhun VF, and Pogribny IP (2008) Involvement of microRNA-451 in resistance of the MCF-7 breast cancer cells to chemotherapeutic drug doxorubicin. *Mol Cancer Ther* **7**:2152–2159.
- Krützfeldt J, Poy MN, and Stoffel M (2006) Strategies to determine the biological function of microRNAs. *Nat Genet* **38** (Suppl):S14–S19.
- Lee EJ, Baek M, Gusev Y, Brackett DJ, Nuovo GJ, and Schmittgen TD (2008) Systematic evaluation of microRNA processing patterns in tissues, cell lines, and tumors. *RNA* **14**:35–42.
- Lewis BP, Burge CB, and Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* **120**:15–20.
- Liao R, Sun J, Zhang L, Lou G, Chen M, Zhou D, Chen Z, and Zhang S (2008) MicroRNAs play a role in the development of human hematopoietic stem cells. *J Cell Biochem* **104**:805–817.
- Mao Q and Unadkat JD (2005) Role of the breast cancer resistance protein (ABCG2) in drug transport. *AAPS J* **7**:E118–E133.
- Nakano H, Nakamura Y, Soda H, Kamikatahira M, Uchida K, Takasu M, Kitazaki T, Yamaguchi H, Nakatomi K, Yanagihara K, et al. (2008) Methylation status of breast cancer resistance protein detected by methylation-specific polymerase chain reaction analysis is correlated inversely with its expression in drug-resistant lung cancer cells. *Cancer* **112**:1122–1130.
- Narang VS, Fraga C, Kumar N, Shen J, Throm S, Stewart CF, and Waters CM (2008) Dexamethasone increases expression and activity of multidrug resistance transporters at the rat blood-brain barrier. *Am J Physiol Cell Physiol* **295**:C440–C450.
- Ross DD, Yang W, Abruzzo LV, Dalton WS, Schneider E, Lage H, Dietel M, Greenberger L, Cole SP, and Doyle LA (1999) Atypical multidrug resistance: breast cancer resistance protein messenger RNA expression in mitoxantrone-selected cell lines. *J Natl Cancer Inst* **91**:429–433.
- Szatmari I, Vámosi G, Brazda P, Balint BL, Benko S, Széles L, Jeney V, Ozvegy-Laczka C, Szántó A, Barta E, et al. (2006) Peroxisome proliferator-activated receptor γ -regulated ABCG2 expression confers cytoprotection to human dendritic cells. *J Biol Chem* **281**:23812–23823.
- Takagi S, Nakajima M, Mohri T, and Yokoi T (2008) Post-transcriptional regulation of human pregnane X receptor by micro-RNA affects the expression of cytochrome P450 3A4. *J Biol Chem* **283**:9674–9680.
- To KK, Polgar O, Huff LM, Morisaki K, and Bates SE (2008a) Histone modifications at the ABCG2 promoter following treatment with histone deacetylase inhibitor mirror those in multidrug-resistant cells. *Mol Cancer Res* **6**:151–164.
- To KK, Zhan Z, and Bates SE (2006) Aberrant promoter methylation of the ABCG2 gene in renal carcinoma. *Mol Cell Biol* **26**:8572–8585.
- To KK, Zhan Z, Litman T, and Bates SE (2008b) Regulation of ABCG2 expression at the 3' untranslated region of its mRNA through modulation of transcript stability and protein translation by a putative microRNA in the S1 colon cancer cell line. *Mol Cell Biol* **28**:5147–5161.
- Tsuchiya Y, Nakajima M, Takagi S, Taniya T, and Yokoi T (2006) MicroRNA regulates the expression of human cytochrome P450 1B1. *Cancer Res* **66**:9090–9098.
- Turner JG, Gump JL, Zhang C, Cook JM, Marchion D, Hazlehurst L, Munster P, Schell MJ, Dalton WS, and Sullivan DM (2006) ABCG2 expression, function, and promoter methylation in human multiple myeloma. *Blood* **108**:3881–3889.
- van Herwaarden AE and Schinkel AH (2006) The function of breast cancer resistance protein in epithelial barriers, stem cells and milk secretion of drugs and xenotoxins. *Trends Pharmacol Sci* **27**:10–16.
- Volk EL, Farley KM, Wu Y, Li F, Robey RW, and Schneider E (2002) Overexpression of wild-type breast cancer resistance protein mediates methotrexate resistance. *Cancer Res* **62**:5035–5040.
- Vore M and Leggas M (2008) Progesterone acts via progesterone receptors A and B to regulate breast cancer resistance protein expression. *Mol Pharmacol* **73**:613–615.
- Wang CH, Lee DY, Deng Z, Jeyapalan Z, Lee SC, Kahai S, Lu WY, Zhang Y, and Yang BB (2008a) MicroRNA miR-328 regulates zonation morphogenesis by targeting CD44 expression. *PLoS ONE* **3**:e2420.
- Wang H, Lee EW, Zhou L, Leung PC, Ross DD, Unadkat JD, and Mao Q (2008b) Progesterone receptor (PR) isoforms PRA and PRB differentially regulate expression of the breast cancer resistance protein in human placental choriocarcinoma BeWo cells. *Mol Pharmacol* **73**:845–854.
- Yu AM (2007) Small interfering RNA in drug metabolism and transport. *Curr Drug Metab* **8**:700–708.
- Zhang S, Yang X, and Morris ME (2004) Flavonoids are inhibitors of breast cancer resistance protein (ABCG2)-mediated transport. *Mol Pharmacol* **65**:1208–1216.
- Zhu H, Wu H, Liu X, Evans BR, Medina DJ, Liu CG, and Yang JM (2008) Role of MicroRNA miR-27a and miR-451 in the regulation of MDR1/P-glycoprotein expression in human cancer cells. *Biochem Pharmacol* **76**:582–588.

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