MicroRNA-145 Post-transcriptionally Regulates the Expression and Function of P-glycoprotein in Intestinal Epithelial Cells

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ABSTRACT

P-glycoprotein (P-gp/MDR1) is a multispecific efflux transporter regulating the pharmacokinetics of various drugs. Although P-gp expression in the small intestine is elevated after liver ischemia-reperfusion (I/R) injury, the regulatory mechanism remains to be clarified. MicroRNAs (miRNAs) play an important role in the post-transcriptional regulation of the expression of drug transporters. Here, we investigated the intestinal expression profile of miRNAs after liver I/R and the role of miRNAs in the post-transcriptional regulation of P-gp in intestinal epithelial cells. Microarray analysis showed that microRNA-145 (miR-145) level was decreased in the small intestine of I/R rats. This downregulation of miR-145 was further confirmed by real-time polymerase chain reaction. In silico analysis revealed that 3'-untranslated regions (UTRs) of rat Mdr1a, mouse Mdr1a, and human MDR1 mRNA retain binding sites for miR-145. Luciferase assays using MDR1 3'-UTR reporter plasmid in HEK293 cells showed that luciferase activity was decreased by the overexpression of miR-145, and the deletion of miR-145 binding site within MDR1 3'-UTR abolished this decreased luciferase activity. The downregulation of miR-145 in Caco-2 cells, an epithelial cell line derived from human colon, increased P-gp expression and efflux activity of rhodamine 123, but not MDR1 mRNA level. These findings demonstrated that miR-145 negatively regulates the expression and function of P-gp through the repression of miRNA by direct interaction on the 3'-UTR of MDR1 mRNA. In addition, the downregulation of miR-145 should significantly contribute to the elevated intestinal P-gp expression after liver I/R. Our results provide new insight into the post-transcriptional regulation of intestinal P-gp.

Introduction

P-glycoprotein (P-gp) encoded by multidrug resistance 1 (MDR1) is an ATP-binding cassette efflux transporter expressed in a broad range of tissues (Schwab et al., 2003); it plays a key role in the pharmacokinetics of P-gp substrates and the multidrug resistance of cancer cells (Cordon-Cardo et al., 1990; Gottesman et al., 2002). It is known that there are marked interindividual variations in the expression and function of P-gp (Masuda and Inui, 2006). In our previous study, we used rats after liver ischemia-reperfusion (I/R) injury as a model for the conditions within a graft liver immediately after liver transplantation, showing that the expression levels of P-gp in the small intestine were elevated after liver I/R (Ikemura et al., 2009). However, the mechanism for the elevated P-gp in the small intestine after liver I/R remained to be clarified.

MicroRNAs (miRNAs), which are small noncoding RNAs, have recently emerged as a new class of gene regulator (Eulalio et al., 2008). MiRNA post-transcriptionally regulates gene expression by binding to the 3'-untranslated region (UTR) of target mRNA to repress its translation or regulate its degradation (Kim, 2005; Filipowicz et al., 2008). Recent studies have demonstrated that the expression patterns of hepatic miRNAs are altered in various liver diseases, such as liver I/R injury (Xu et al., 2009), hepatitis C (Cermelli et al., 2011), hepatocellular carcinoma (Budhu et al., 2008), and toxic-induced liver injury (Yamaura et al., 2012). These studies also indicated that miRNAs play important roles in physiologic and pathologic processes in liver diseases. Moreover, the roles of miRNAs have received attention in the pharmacokinetic field (Nakajima and Yokoi, 2011), and some studies have demonstrated the importance of miRNAs in regulation of the expression of drug transporters, drug-metabolizing enzymes, and nuclear receptors (Takagi et al., 2008; Pan et al., 2009; Takagi et al., 2010; Dalmasso et al., 2011). Interestingly, it was reported that the stability of MDR1 mRNA and the rate of its translation are responsible for the individual variation in P-gp function (Wang and Sadee, 2006; Kimchi-Sarfaty et al., 2007), suggesting the importance

ABBREVIATIONS: CsA, cyclosporine A; CYP3A, cytochrome P450 3A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPLC, high-performance liquid chromatography; I/R, ischemia-reperfusion; MDR1, multidrug resistance 1; miRNA, microRNA; P-gp, P-glycoprotein; RT-PCR, real-time polymerase chain reaction; Rho123, rhodamine 123; UTR, untranslated region.
of further investigation into the mechanisms of post-transcriptional regulation of P-gp. Indeed, the recent studies suggested that miR-27a, miR-298, and miR-451 regulate P-gp expression in multidrug-resistant cancer cell lines (Zhu et al., 2008; Li et al., 2010; Bao et al., 2012). Therefore, the variation in the expression and function of P-gp should be explained in part by the alteration of expression patterns of miRNAs in pathologic conditions. However, the mechanisms regulating the expression and function of P-gp in the small intestine through miRNAs after liver I/R have not been fully understood.

In the present study, we investigated the profile of miRNA expression in the small intestine after liver I/R and the role of miRNAs in the post-transcriptional regulation of P-gp in intestinal epithelial cells.

**Materials and Methods**

**Materials.** MiR-145 precursor (Pre-miR miRNA precursor, AM17100, product ID PM11480), miR-145 inhibitor (Anti-miR inhibitor, AM17000, product ID AM11480), control for miR-145 precursor (Pre-miR negative control, AM17110), and control for miR-145 inhibitor (Anti-miR negative control, AM17010) were obtained from Ambion (Austin, TX). Rhodamine 123 (Rho123) was obtained from WAKO Pure Chemical (Osaka, Japan). All of the other chemicals used were of the highest purity available.

**Animals.** Nine-week-old male Wistar rats (SLC Japan Co., Shizuoka, Japan) were used for these experiments. The rats were fasted for 12 hours before the experiments, but allowed free access to water. The experiments were approved by the Mie University Review Board for animal investigation and were conducted according to the guidelines for animal experiments of the National Institutes of Health.

Liver I/R model rats were prepared according to our previous report (Ikemura et al., 2009). Briefly, after the rats were anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital, partial hepatic ischemia was achieved by clamping the blood vessels of the left portal vein and hepatic artery using a microvessel clip. The vascular clip was released after 60 minutes of hepatic ischemia. The sham-operated rats were treated in the same way as the liver I/R rats without occlusion of the left portal vein and hepatic artery. After 12 hours of reperfusion, the tissues of the small intestine (15-cm segments of the upper, middle, and lower intestine) were collected.

**Cell Culture.** The human colon carcinoma cell line Caco-2 and the human embryonic kidney cell line HEK293 were obtained from American Type Culture Collection (Rockville, MD). Caco-2 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% nonessential amino acids without antibiotics and were used between passage numbers 40 and 60. HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum without antibiotics and were used between passage numbers 60 and 70. These cells were maintained at 37°C under 5% CO2 in a humidified atmosphere.

**Transfection of miR-145 Inhibitor into Caco-2 Cells.** MiR-145 inhibitor (10, 30, and 50 nM), miR-control inhibitor (50 nM), or corresponding vehicle was transfected into Caco-2 cells cultured on plastic plates using SiPORT NeoFX transfection agent (Ambion) and Opti-MEMI reduced serum medium (Invitrogen, Carlsbad, CA) according to the manufacturers’ instructions. After 48 hours, total RNA and total protein were extracted from the cells.

RNA Extraction and Real-Time Polymerase Chain Reaction. Total RNA was isolated using the miVana miRNA isolation kit (Ambion). The cDNAs corresponding to miRNAs were reverse-transcribed from total RNA (10 ng) using the stem-loop hybridization based on TaqMan MicroRNA Reverse Transcription Kit and TaqMan MicroRNA Assay for miR-145 and U6 small nuclear RNA (Applied Biosystems, Foster City, CA). For mRNA expression, cDNA was synthesized from the total RNA (100 ng) using the PrimeScript RT Reagent Kit (Takara Bio Inc., Shiga, Japan) following the instructions provided by the manufacturer. Subsequently, cDNA was subjected to real-time polymerase chain reaction (RT-PCR) using SYBR Premix Ex Taq (Takara Bio Inc.). The primers used were as follows: human MDR1 sense 5’-TTTGCAAACTCTCTGCTCTGA-3’, rat Mdr1a sense 5’-GACGAAATGTAATAAGTGCGA-3’, rat Mdr1a antisense 5’-GTTAGCTCTAGGACGAGG-3’, and human and rat GAPDH antisense 5’-GGAAGGCATTGCAGCTGAG-3’. Amplification and detection of specific products were performed with ABI 7300 Sequence Detection System (Applied Biosystems). The miR-145 levels were normalized using the U6 small nuclear RNA level, and Mdr1a, Mdr1b, and MDR1 mRNA levels were normalized using the GAPDH level.

**RNA Labeling and Hybridization of miRXplore Microarray.** The fluorescence signals of the hybridized miRXplore Microarrays were detected using a laser scanner system (Agilent Technologies). The Imagenes software (Biodiscovery, El Segundo, CA) was used to determine mean signal and mean local background intensity for each spot on the miRXplore microarray images. Low-quality spots were flagged and excluded from data analysis, and unflagged spots were evaluated using the PIQOR Analyzer software (Milltenyi Biotec), which allows automated data processing of the raw data text files derived from the Imagenes software. After background subtraction, the net signal intensity was subjected to data normalization and calculation of the Hy5/Hy3 ratios for the species of interest. Only spots characterized by a signal that were equal to or higher than the 50% of the background signal intensity were taken into account for the calculation of the Hy5/Hy3 ratio. Two samples were compared using a cut-off value of 1.5-fold difference.

**Luciferase Assay.** The 683-bp cDNA fragment corresponding to the 3’-UTR of human MDR1 mRNA was inserted into the downstream region of the luciferase gene in the pLightSwitch reporter vector (Switchgear Genomics, Menlo Park, CA). Deletion mutant lacking the seed sequence (GACUGGA) of miR-145 binding site in 3’-UTR of human MDR1 mRNA was created using KOD Plus Mutagenesis kit (TOYOBO, Osaka, Japan) and primers 5’-GGTTAC-TTGTCCAAACTGCCTGTG-3’ (sense) and 5’-GATAGAAGTT-TAGTGTATATTATAGACTT-3’ (antisense) according to the manufacturers’ instructions. Deletion of miR-145 binding site in the insert was confirmed by direct DNA sequencing analyses. The reporter plasmid (50 ng) and precursor for miR-145 (1, 3, and 5 nM) or miR-control precursor (5 nM) were cotransfected into HEK293 cells cultured on 96-well plastic plates using Lipofectamine 2000 (Invitrogen). After 24 hours, the cells’ luciferase activities were measured with the LightSwitch Luciferase Assay System (Switchgear Genomics) and Applikisan luminometer (Thermo Scientific, Waltham, MA).

**Western Blotting Analyses of P-gp.** The protein (12.5 μg) isolated from Caco-2 cells was separated by 7.5% SDS-PAGE. Western blotting using C219 monoclonal antibody (Calbiochem, San Diego, CA) for P-gp was performed as described previously (Shimomura et al., 2002), and a polyclonal antibody for villin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used according to the manufacturer’s instructions. The relative densities of the bands in...
TABLE 1
The mRNA levels of Mdr1a and Mdr1b in the upper, middle, and lower small intestine of Sham and I/R rats.

The Mdr1a and Mdr1b mRNA levels were determined by RT-PCR and normalized using GAPDH level relative to the levels in the upper small intestine of Sham rats. Results are mean ± S.E. of five rats.

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<th>Mdr1a mRNA levels</th>
<th>Sham</th>
<th>I/R</th>
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<tr>
<td>Upper</td>
<td>1.00 ± 0.10</td>
<td>0.73 ± 0.08</td>
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<tr>
<td>Middle</td>
<td>2.30 ± 1.41</td>
<td>2.09 ± 0.56</td>
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<tr>
<td>Lower</td>
<td>4.33 ± 1.38</td>
<td>2.75 ± 0.69</td>
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<tr>
<th>Mdr1b mRNA levels</th>
<th>Sham</th>
<th>I/R</th>
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<tr>
<td>Upper</td>
<td>1.00 ± 0.31</td>
<td>0.69 ± 0.17</td>
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<tr>
<td>Middle</td>
<td>1.42 ± 0.64</td>
<td>1.42 ± 0.36</td>
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<tr>
<td>Lower</td>
<td>3.76 ± 0.77</td>
<td>1.97 ± 0.46</td>
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Statistical Analyses. All data are expressed as the mean ± S.E. Statistical analyses were carried out using one-way analysis of variance followed by Dunnett’s test or Tukey’s multiple comparison test and the differences between two groups were determined by the unpaired t-test with GraphPad Prism 5.02 (GraphPad Software Inc., San Diego, CA). The level of significance was P < 0.05.

Results
Effect of Liver I/R on the mRNA Levels of Mdr1a and Mdr1b in the Small Intestine. Our previous studies demonstrated that the expression levels of P-gp in the upper, middle, and lower small intestine were significantly increased after liver I/R (Ikemura et al., 2009, 2012). Therefore, we first evaluated the mRNA expression levels of the two distinct genes encoding the rat P-gp, Mdr1a (Abcb1a) and Mdr1b (Abcb1b), in the small intestine of Sham and I/R rats. Table 1 shows the mRNA levels of Mdr1a and Mdr1b in the upper, middle, and lower intestines, normalized by the corresponding GAPDH in Sham and I/R rats. The mRNA levels of GAPDH were not significantly different between the two groups. The rank order of Mdr1a and Mdr1b mRNA levels was upper < middle < lower small intestine of Sham and I/R rats, which is comparable to that in a previous report (Brady et al., 2002). The Mdr1a and Mdr1b mRNA levels in upper and lower intestine of I/R rats tended to decrease, but were not significantly different, compared with those of Sham rats.

Fig. 1. Predicted target site of miR-145 in the 3'-UTR of Mdr1a and MDR1 mRNA in human (A), rat (B), and mouse (C) by miRanda program.
Similarly, there were no significant differences in those in the middle intestine between the two groups.

**Expression Profiles of miRNAs in the Small Intestine after Liver I/R.** The RNA samples isolated from the upper intestine in Sham and I/R rats, which were quality-checked and showed sufficient quality for microarray experiments, were subjected first to screening using microarray, which contain 475 rat mature miRNAs according to miRbase version 16.0 (http://microrna.sanger.ac.uk/sequences/). The detected miRNAs upregulated or downregulated at least 1.5-fold are listed in Table 2. After liver I/R, the microarray analysis demonstrated that 10 miRNAs were downregulated and 4 miRNAs were upregulated. Among them, a search with the miRanda program (Betel et al., 2008) indicated that 3′-UTR of rat Mdr1a mRNA retains an miR-145 binding site (Fig. 1B). Similarly, the 3′-UTRs of human MDR1 mRNA and mouse Mdr1a mRNA retain an miR-145 binding site (Fig. 1, A and C). On the other hand, the 3′-UTR of rat and mouse Mdr1b mRNA retained no miR-145 binding site, because the Mdr1b mRNA has the sequence of 3′-UTR differed from Mdr1a mRNA. Moreover, other miRNAs in which changes were observed by microarray analysis did not retain the target-site accessibility in 3′-UTR of Mdr1a and Mdr1b mRNA. Next, the miR-145 levels in the small intestines were confirmed by RT-PCR using the total RNA isolated from Sham and I/R rats. Figure 2 shows the miR-145 levels normalized by the corresponding U6 small nuclear RNA of the small intestine in each rat group. The expression levels of U6 small nuclear RNA were not significantly different between the two groups. The rank order of miR-145 expression was upper, middle, lower small intestine, similarly to the results of Mdr1a and Mdr1b mRNA levels shown in Table 1. The expression levels of miR-145 in the upper, middle, and lower intestine after liver I/R were decreased to 33%, 47%, and 48% compared with the levels in corresponding Sham rats. Moreover, the expression levels of miR-143, which is located in close proximity to the miR-145 gene on the genome (Iio et al., 2010), showed similar decreases to those of miR-145 after liver I/R (Table 2).

**Effect of miR-145 for 3′-UTR of MDR1 mRNA in HEK293 Cells.** To examine whether miR-145 directly targets the 3′-UTR of MDR1 mRNA, the MDR1 3′-UTR was cloned into a luciferase reporter gene, and this construct was transfected into HEK293 cells in the presence of miR-145 precursor or miR-control precursor. The targeting of miRNA for the 3′-UTR of MDR1 was assessed by measuring the luciferase activity in HEK293 cells. As shown in Fig. 3A, the luciferase activity observed in the control was significantly decreased in a dose-dependent manner by cotransfection with the precursor of miR-145, whereas the miR-control precursor showed no significant effect on the luciferase activity. Moreover, the deletion of the miR-145 binding site in MDR1 3′-UTR abolished the downregulation of luciferase activity by cotransfection with the precursor of miR-145 (Fig. 3B).

**Effect of miR-145 Inhibitor on MDR1 mRNA and P-gp Levels in Caco-2 Cells.** To confirm the expressional regulation of P-gp through miR-145, miR-145 inhibitor, miR-control inhibitor, or vehicle (control) was transfected into Caco-2 cells. The transfection of miR-control inhibitor showed no significant effect on the miR-145 expression level. The miR-145 levels in
Caco-2 cells were significantly decreased in a dose-dependent manner by transfection with miR-145 inhibitor (Fig. 4A), suggesting the accomplishment of downregulation of miR-145 by this transfection. Figure 4B shows the mRNA levels of MDR1, normalized by the corresponding GAPDH. The expression levels of GAPDH mRNA were not significantly different among the three groups. The levels of MDR1 mRNA were not significantly altered by the transfection of miR-145 inhibitor or control inhibitor (Fig. 4B). Moreover, the expression levels of P-gp and villin protein were compared by Western blot analysis using the samples isolated from Caco-2 cells after 48 hours of transfection (Fig. 4C). Figure 4D shows the densitometric analyses of P-gp expression levels normalized by the corresponding villin signals. The expression levels of villin were not significantly different among the three groups. The expression levels of P-gp were increased to approximately 160% of the control by the miR-145 inhibitor, whereas miR-control inhibitor showed no significant effect on the expression levels of P-gp. However, there were no significant differences in P-gp expression in Caco-2 cells between with miR-145 inhibitor and those with miR-control inhibitor ($P = 0.07$).

**Effect of miR-145 Inhibitor on the Function of P-gp in Caco-2 Cells.** To assess the impact of the miR-145 inhibitor on the function of P-gp as an efflux pump, we examined the P-gp-mediated Rho123 transport in Caco-2 cells transfected with miR-145 inhibitor, miR-control inhibitor, or vehicle (control), using CsA as a P-gp inhibitor. As shown in Fig. 5A, the accumulation of Rho123 without CsA in Caco-2 cells transfected with miR-145 inhibitor tended to decrease, but was not significantly different compared with that in those transfected with vehicle. Moreover, the accumulation of Rho123 in Caco-2 cells was significantly increased by coincubation with CsA. Figure 5B shows the apparent efflux activity of Rho123 determined by subtracting the accumulation of Rho123 without CsA from that with CsA. The apparent efflux activity of Rho123 in Caco-2 cells transfected with miR-145 inhibitor was increased to approximately 290% of the control level. On the other hand, the transfection of miR-145 inhibitor control showed no significant effect on the efflux activity of Rho123.

**Discussion**

The mechanisms regulating the variation of P-gp expression in the small intestine have not been fully understood. To our knowledge, this is the first report on the effect of miRNA on the expression and function of P-gp in intestinal epithelial cells through the direct action of 3’-UTR of MDR1 mRNA.

The expression levels of P-gp, but not the levels of mRNA encoding Mdr1a and Mdr1b in upper, middle, and lower small intestine, were elevated after liver I/R, as shown in Table 1.

![Fig. 4. Effect of miR-145 inhibitor on the expression levels of miR-145 (A), MDR1 mRNA (B), and P-gp (C and D) in Caco-2 cells.](image-url)
and our previous report (Ikemura et al., 2012). These findings suggested that the same post-transcriptional regulation should contribute to the variation of P-gp expression after liver I/R. In the present study, we focused on the decreased miR-145 in the small intestine after liver I/R because the 3′-UTR of MDR1 as well as Mdr1a mRNA have a potential miR-145 target site as predicted by in silico analysis (Figs. 1 and 2, Table 2). The results from the luciferase assays using MDR1 3′-UTR reporter plasmid in HEK293 cells and the downregulation of miR-145 in Caco-2 cells demonstrated that miR-145 negatively regulates the expression and function of P-gp through the repression of mRNA by direct action on the 3′-UTR of MDR1 mRNA (Figs. 3, 4, and 5). Moreover, we demonstrated that the overexpression of miR-145 by the transfection of miR-145 precursor decreased P-gp expression with no significant change of MDR1 mRNA expression in Caco-2 cells (Supplemental Fig. 1). Therefore, it is strongly suggested that miR-145 regulates the expression and function of P-gp through the translational repression of MDR1 mRNA.

MiR-145 is ubiquitously expressed in normal human tissues, including the small intestine (7942 copies per cell), kidney (2130 copies per cell), brain (991 copies per cell), and liver (606 copies per cell) (Lee et al., 2008). Recently, miR-145 was shown to be highly enriched in smooth muscle cells (Cheng et al., 2009). Some studies have demonstrated that the expression level of miR-145 was elevated in the brain after cerebral I/R (Dharap et al., 2009) and in pulmonary artery smooth muscle cells after chronic hypoxia (Cheng et al., 2009). Meanwhile, miR-145 levels were downregulated in vascular smooth muscle cells after vascular disease (Cheng et al., 2009) and treatment with hydrogen peroxide as oxidative stress (Lin et al., 2009). Our previous study demonstrated that an antioxidant, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox; Hoffman-LaRoche, Basel, Switzerland), ameliorates the elevated P-gp in the small intestine after liver I/R injury by preventing oxidative stress (Ikemura et al., 2012). These findings suggest that the oxidative stress in blood may regulate the expression and function of P-gp through the repression of mRNA by direct action on the 3′-UTR of MDR1, Mdr1a, and Mdr1b mRNA (Fig. 1, Table 2). The expression of Mdr1b mRNA rather than Mdr1a mRNA has been indicated in intestinal epithelial cells in rat and human intestine, indicating higher levels of P-gp in the lower intestine than in the upper intestine, whereas the expression of cytochrome P450 3A (CYP3A) is less in the lower intestine than in the upper intestine (Thorn et al., 2005; Jin et al., 2006). Therefore, the site-dependent expressions of CYP3A and P-gp cooperatively function as a barrier against the invasion of various ingested toxic chemicals, including P-gp/CYP3A substrates (Tamura et al., 2003). In our study, the expression levels of mRNA and protein of P-gp were highest in the lower intestine and these regional differences in the intestinal P-gp expression were correlated with miR-145 expression levels, indicating that miR-145 may play an important role in the site-dependent expression of P-gp.

It is now known that CsA inhibits the transport mediated by P-gp (Loor et al., 2002), multidrug resistance protein 2 (Kamisako et al., 1999), and breast cancer resistance protein (Matsson et al., 2009). However, in silico analysis indicated that miR-145 retains no binding site in the 3′-UTR of multidrug resistance protein 2 and breast cancer resistance protein. Consequently, it is suggested that the increased expression of P-gp by the transfection of miR-145 inhibitor in Caco-2 cells is attributable to the elevated efflux activity of Rho123 (Fig. 5B). Moreover, 13 miRNAs excluding miR-145 observed by microarray analysis retain no binding site in the 3′-UTR of MDR1, Mdr1a, and Mdr1b mRNA (Fig. 1, Table 2). However, it is reported that miR-21 positively induced P-gp expression in breast tumor cells (Bourguignon et al., 2009). In our present study, the expression level of miR-21 was...
increased after liver I/R in the microarray results shown in Table 2. However, there were no significant differences in the expression of miR-21 in the small intestine between Sham and I/R rats by RT-PCR analysis (data not shown), indicating that the upregulation of P-gp in the small intestine should not be due to the regulation through miR-21 after liver I/R. Moreover, it is not likely that other miRNAs target the transcriptional factors of P-gp, leading to indirect transcriptional regulation of P-gp, because the expressions of Mdr1a and Mdr1b mRNA were not elevated after liver I/R injury (Table 1).

In conclusion, our study clearly demonstrated for the first time that miR-145 negatively regulates the expression and function of P-gp through the repression of mRNA by direct action on the 3′-UTR of MDR1 mRNA. Moreover, miR-145 expression should contribute to the regulation of intestinal P-gp expression after liver I/R. The present findings provide insight into the mechanism of P-gp expression/function as well as the etiology of liver I/R.

Authorship Contributions
Participated in research design: Ikemura, Iwamoto, Okuda. Conducted experiments: Ikemura, Yamamoto, Miyazaki, Mizutani. Performed data analysis: Ikemura, Mizutani, Iwamoto, Okuda. Wrote or contributed to the writing of the manuscript: Ikemura, Iwamoto, Okuda.

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Supplemental Figure 1  Effect of miR-145 precursor on the expression levels of miR-145 (A), MDR1 mRNA (B), and P-gp (C and D) in Caco-2 cells.  Cells were transfected with 50 nM miR-145 precursor or 50 nM miR-control precursor or vehicle (control) for 48 h.  (A) The miR-145 levels were determined by RT-PCR and normalized using the U6 small nuclear RNA levels relative to the control.  (B) The MDR1 mRNA levels were determined by RT-PCR and normalized using GAPDH mRNA levels relative to the control.  (C) The expression levels of P-gp and villin in Caco-2 cells were determined by Western blot analysis.  (D) Densitometric quantification of P-gp was performed by normalization of the result relative to the villin signals.  Each column represents mean ± S.E. of three separate experiments.  *: P<0.05, ***: P<0.001 compared with control.