
Hisakazu Komori, Daichi Fujita, Yuma Shirasaki, Qiunan Zhu, Yui Iwamoto, Takeo Nakanishi, Miki Nakajima, and Ikumi Tamai

Department of Membrane Transport and Biopharmaceutics (H.K., D.F., Y.S., Q.Z., Y.I., T.N., I.T.), Department of Drug Metabolism and Toxicology (M.N.), Faculty of Pharmaceutical Sciences, Institute of Medical, Pharmaceutical and Health Sciences, and WPI Nano Life Science Institute (M.N.), Kanazawa University, Kakuma-machi, Kanazawa, Japan

ABSTRACT

Plant-derived nanoparticles exert cytoprotective effects on intestinal cells by delivering their cargo to intestinal tissues. We previously reported that apple-derived nanoparticles (APNPs) downregulate the mRNA of the human intestinal transporter organic anion–transporting peptide 2B1 (OATP2B1)/SLCO2B1 and that the 3′-untranslated region (3′UTR) is required for the response to APNPs. Here, we investigated the involvement of microRNAs (miRNAs) in APNPs in suppressing OATP2B1 expression to demonstrate that APNP macromolecules directly interact with intestinal tissues. Using in silico analysis, seven apple miRNAs were predicted as candidate miRNAs that interact with the SLCO2B1-3′UTR. The APNP-mediated decrease in luciferase activity of pGL3/SLCO2B1-3′UTR containing an MRE for miR-7121d-h, was decreased by the miR-7121d-h mimic but decreased little by the other mimics. APNP also reduced the luciferase activity of truncated pGL3/SLCO2B1-3′UTR containing an MRE for miR-7121d-h. Thus, we demonstrated that mdm-miR-7121d-h contributes to the APNP-mediated downregulation of intestinal OATP2B1. Accordingly, plant macromolecules, such as miRNAs, may directly interact with intestinal tissues via nanoparticles.

SIGNIFICANCE STATEMENT

This study demonstrates that mdm-miR7121d-h contained in apple-derived nanoparticles downregulated the mRNA expression of SLCO2B1 by interacting with SLCO2B1-3′untranslated region directly and that SLCO2B1 mRNA might also be decreased by mdm-miR160a-e and -7121a-c indirectly. This finding that the specific apple-derived microRNAs influence human intestinal transporters provides a novel concept that macromolecules in foods directly interact with and affect the intestinal function of the host.

Introduction

Several foods, including grapes, grapefruits, and nuts, have been reported to contain nanoparticles (NPs) (Ju et al., 2013; Wang et al., 2014; Aquilano et al., 2019), which are vesicle-like particles composed of a lipid bilayer (Mu et al., 2014). Food-derived NPs have been characterized by an average particle size of approximately 100–400 nm and a negative z potential (−49.2 to 1.52 mV) (Zhang et al., 2016). They include macromolecules, such as microRNAs (miRNAs) and proteins, as well as low-molecular compounds (Soleti et al., 2018). NPs isolated from edible plants have been reported to exert beneficial effects (Xiao et al., 2018). For example, grape-derived NPs exert a protective effect by increasing intestinal stem cells in a dextran sulfate sodium–induced colitis mouse model (Ju et al., 2013). Moreover, NPs prepared from grapefruit suppress the exacerbation of dextran sulfate sodium–induced colitis by reducing the production of inflammatory cytokines from intestinal macrophages (Wang et al., 2015). Interestingly, miR-156c and miR-159a in nut nanovesicles decrease tumor necrosis factor z receptor expression and dampen the tumor necrosis factor z signaling pathway in mouse adipocytes (Aquilano et al., 2019). Thus, plant-derived NPs may interact with host tissues, and macromolecules such as miRNAs included in NPs can modulate mammalian genes, contributing to biological effects.

We previously reported that the intestinal transporter organic anion–transporting polypeptide 2B1 (OATP2B1) (SLCO2B1), which has a broad range of substrate specificity, is responsible for intestinal absorption of clinically used drugs, including antiallergic fexofenadine and lipid-lowering statins (Tamai, 2012; Hoshino et al., 2016). Furthermore, intestinal absorption of these drugs was reduced by the competitive

ABBREVIATIONS: Ago2, Argonaute 2; APNP, apple-derived NP; HEK293, human embryonic kidney 293; Mfe, minimum free energy; miRNA, microRNA; MRE, miRNA recognition element; NP, nanoparticle; OATP2B1, organic anion–transporting peptide 2B1; PCR, polymerase chain reaction; pGL3-p, pGL3-promoter vector; qRT-PCR, quantitative RT-PCR; RT-PCR, reverse-transcription PCR; 3′UTR, 3′-untranslated region.
inhibition of OATP2B1 by flavonoids in fruit juices, such as apples, grapefruits, and oranges (Shirasaka et al., 2013a). Also, apple and orange juice exhibited long-lasting effects, which is distinct from competitive inhibition (Shirasaka et al., 2013b). Accordingly, fruits have variable effects on the intestinal function when digested. More recently, we found that NPs obtained from apple [apple-derived nanoparticles (APNPs)] decreased mRNA expression of the intestinal transporters OATP2B1; apical sodium-dependent bile acid transporter (SLC10A2), which is essential for an enterohepatic circulation of bile acids; and carnitine transporter (SLC22A5) in intestinal epithelial cells, such as Caco-2 cells (Fujita et al., 2018). Concerning the downregulation of OATP2B1, the 3′-untranslated region (3′UTR) was required to respond to APNPs, suggesting that OATP2B1 might be downregulated by miRNAs contained in APNPs. Furthermore, low-molecular-weight compounds in apples, such as flavonoids, were not considered the underlying mechanism since their concentration in the APN fraction was very low (Fujita et al., 2018). The effect of food on the host has been mostly explained by small molecules in food and those formed by the digestion of food-derived macromolecules, such as proteins and carbohydrates in the intestinal lumen. If plant-derived miRNAs directly regulate intestinal transporters’ expression through NPs, they will be regarded as a novel food function.

Although miRNAs contribute to a wide range of physiologic functions, such as growth, regulation of differentiation, and response to nutrition in plants (Stepien et al., 2017), it is unclear whether orally administered miRNAs are absorbed in intestinal cells and transferred into circulating blood (Dickinson et al., 2013; Snow et al., 2013). Unlike animal miRNAs, plant miRNAs avoid degradation by exonucleases because of modifications on the 3′ end by 2′-O-methylation (Yu et al., 2005). Plant miRNAs are spliced out as double-stranded RNA from primary miRNAs in the cytoplasm. Then, miRNA is incorporated into the RNA-induced silencing complex, including Argonaute 2 (Ago2), Dicer, and HIV-1 transactivating-response-RNA-binding protein, and then degraded (Zhang et al., 2006; Matsui and Corey, 2017; Stepien et al., 2017). We previously reported that APNPs maintained particle configuration at acidic gastric juice pH (pH 1.0) with a particle size distribution of approximately 200 nm (Fujita et al., 2018). Li et al. (2019b) reported that plant miR-15b was detected in intestinal crypt cells in maize-fed mice and decreased intestinal cell proliferation by reducing Wnt/β-catenin signaling. Therefore, it is expected that miRNAs included in orally administered APNPs are incorporated and function in intestinal epithelial cells. Also, we recently found that miRNAs regulate OATP2B1 expression in the intestine (Liu et al., 2020).

Accordingly, in the present study, we investigated the involvement of miRNAs in APNPs to regulate OATP2B1 expression, thereby demonstrating a novel food function by direct interaction of food-derived macromolecules with host tissues.

Materials and Methods

Cell Culture. Caco-2 and human embryonic kidney 293 (HEK293) cells were purchased from RIKEN Cell Bank (Tsukuba, Japan) and Health Science Research Resource Bank (Osaka, Japan), respectively. Caco-2 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 0.1 mM nonessential amino acids, 100 U/ml benzylpenicillin, and 100 μg/ml streptomycin. HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 100 U/ml benzylpenicillin, and 100 μg/ml streptomycin. Cells were maintained in collagen-coated dishes at 37°C in an atmosphere consisting of 5% CO2 and 95% air.

Preparation of APNPs. The preparation of APNPs has been previously reported (Fujita et al., 2018). Briefly, whole apples (Malus pumila; Fuji, Iwate, Japan) were crushed without peeling after washing with running water for 10 minutes. The juice was collected after cheesecloth filtration. The supernatant collected after centrifugation at 2000 × g for 20 minutes at 4°C was then centrifuged at 13,000 × g for 70 minutes at 4°C. The resultant supernatant was centrifuged at 120,000 × g for 130 minutes at 4°C. The obtained pellets containing NPs were resuspended in PBS. The protein concentration of APNPs was determined using a Bio-Rad protein quantification assay kit (Bio-Rad Laboratories, Inc., Hercules, CA) with bovine serum albumin as the standard.

Plasmid Construction and Mutagenesis. Fragments of human SLC02B1 were PCR-amplified using Caco-2 cell genomic DNA as a template. The forward and reverse primers are shown in Supplementary Table 1. PCR products were digested with appropriate restriction enzymes and inserted into the pGL3-promoter vector (pGL3-p), which were predigested with the corresponding enzymes. Specific 3′UTR plasmids were prepared similarly using pGL3-p/SLCO2B1-3′UTR as a template, and primers are shown in Supplementary Table 1. The miRNA recognition element (MRE) of interest in the 3′UTR was mutated using PrimeSTAR MAX DNA polymerase (Takara Bio Inc., Shiga, Japan) and corresponding wild-type plasmids as templates. The primers used are shown in Supplementary Table 2. Plasmid DNA was prepared using NucleoSpin Plasmid QuickPure (Takara Bio Inc.).

Detection of miRNA. Total RNA from the APNP fraction or Caco-2 cells treated with APNPs was isolated with a mirCURY RNA Isolation kit (Exiqon, Vedbaek, Denmark) according to the manufacturer’s instructions and then reverse-transcribed to cDNA using the Mir-X miRNA qRT-PCR SYBR kit (Takara Bio Inc.; 638314). In the Mir-X cDNA synthesis reaction, miRNAs are poly(A)-tailed using poly(A) polymerase and then copied using a modified oligo(dT) primer (mRq 3′ primer) and SMART MMLV reverse transcriptase. Subsequently, first-strand cDNAs are amplified using a miRNA-specific forward primer and mRq 3′ primer by PCR. According to the manufacturer’s instructions, this system is designed to amplify the PCR product with a size of 80–90 bp. The forward primers for the predicted miRNAs are listed in Supplementary Table 3. Gel electrophoresis was carried out after RT-PCR to detect when the predicted RNAs were contained in APNPs or taken up by Caco-2 cells.

Immunoprecipitation Using Anti-Ago2 Antibody. Apple miRNAs interacting with Ago2 were isolated from Caco-2 cells treated with APNPs using the MagCapture microRNA isolation kit, Human Ago2 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the manufacturer’s instructions. After immunoprecipitation using magnetic beads conjugated with mouse anti-human Ago2 monoclonal antibody (clone 4G8), which is a component of the MagCapture microRNA isolation kit, miRNAs combined with Ago2 were eluted. Obtained miRNAs were reverse-transcribed to cDNA using the Mir-X miRNA qRT-PCR SYBR kit. The subsequent detection of miRNA was carried out in the same manner as described above.

Evaluation of the Effect of APNP on SLC02B1-3′UTR. HEK293 cells were plated in 48-well plates 24 hours before transfection. Plasmids of pGL3-p/SLCO2B1-3′UTR or its mutant plasmids were cotransfected with Renilla luciferase control reporter vector pRL-TK using Lipofectamine 2000. mirVana miRNA inhibitors, which are chemically modified, single-stranded oligonucleotides designed to specifically bind to and inhibit target miRNAs, were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). Each miRNA-specific inhibitor was cotransfected with pGL3-p/SLCO2B1-3′UTR and pRL-TK. Six hours after transfection, the cells were treated with APNPs at a final concentration of 40 μg/ml for 12 hours and collected for the dual-luciferase reporter assay. Alternatively, to study the effect of miRNAs, each microRNA mimic (mirVana miRNA mimics, Thermo Fisher Scientific Inc.) or negative control miRNA mimic (mirVana miRNA Mimic, Negative Control #1, Thermo Fisher Scientific Inc.) was cotransfected with pGL3-p/SLCO2B1-3′UTR or pRL-TK. Here, mirVana miRNA mimics are chemically modified double-stranded RNA molecules that are designed to mimic endogenous mature miRNAs. After 48 hours transfection, the cells were collected for the dual-luciferase reporter assay. The dual-luciferase reporter assay was performed using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI) following the manufacturer's instructions. Chemiluminescence of firefly and Renilla luciferase was measured using a Wallac 1420 ARVOx Multilabel plate reader (PerkinElmer, Inc, Waltham, MA). Firefly luciferase activity was normalized to Renilla luciferase activity.

Statistical Analysis. All data are expressed as mean ± S.D. for the indicated number of separate experiments, and statistical analysis was performed with Student’s t test and Dunnett’s test, with P < 0.05 as the criterion of significance.
Results

**Prediction of Putative Apple miRNA that Decreases OATP2B1.** According to miRBase (http://www.mirbase.org), a database of published miRNA sequences and annotations, 207 apple miRNAs were registered in 2018. Of these, 16 miRNAs were predicted as putative miRNAs that bind to the 3’UTR of OATP2B1 using RNAhybrid (Krüger and Rehmsmeier, 2006). It has been reported that the specific binding of miRNA to mRNA is likely to occur when the interaction site is located on the 3’-terminal side of the center in the 3’UTR and when the AU content (the proportion of adenine and uracil near the binding site) is high (Grimson et al., 2007). Based on this report, the prediction condition using RNAhybrid was set as follows: Relative position of the 3’UTR was less than 0.4 and greater than 0.6 from the 5’-end side, and local AU content in binding site was 0.3 or more. After narrowing down the candidate miRNAs by these conditions, seven miRNAs were selected (Table 1) and further tested for their relationship with OATP2B1 expression in Caco-2 cells.

The presence of the predicted miRNAs in the prepared APNPs was confirmed by RT-PCR using the Mir-X miRNA quantification system using isolated RNA from APNPs. In this assay, miRNAs were detected as amplified products with a size of 80–90 bp. Figure 1A shows the amplicons of all predicted miRNAs and human miR-1 and human U6, which were used as negative controls. Thus, the predicted miRNAs were confirmed to be present in APNPs. To further examine whether the candidate miRNAs are taken up into cells by APNP treatment, incorporating miRNAs into intracellular compartments was evaluated in APNP-treated Caco-2 cells by RT-PCR. As shown in Fig. 1B, only five of the seven PCR products, mdm-miR-160a-e, -395a-i, -477b, -7121a-c, and -7121d-h, were amplified with the expected size (approximately 90 bp). Furthermore, the band intensities of mdm-miR-160a-e, -395a-i, -471d, -7121a-c, and -7121d-h increased as the APNP concentration increased.

**Suppressive Effect of miRNA Inhibitors for Predicted microRNAs.** Our previous report showed that APNPs decreased the luciferase activity of the pG3/SLCO2B1-3’UTR (Fujita et al., 2018). Accordingly, apple miRNAs that cause such a reducing effect were investigated using an inhibitor for each miRNA. HEK293 cells were cotransfected with pG3/SLCO2B1-3’UTR and each inhibitor for candidate miRNAs and subsequently treated with APNPs. As shown in Fig. 2, APNPs significantly decreased luciferase activity in the absence and presence of miRNA inhibitors. However, the decrease in luciferase activity was partially but significantly reversed by the miRNA inhibitor for mdm-miR-160a-e, -7121a-c, and -7121d-h. Accordingly, we suggest that these three miRNA inhibitors disturb the effect of APNP on OATP2B1 expression. On the other hand, an incomplete reversal of luciferase activity by each specific miRNA inhibitor suggested that more than one miRNA is coordinately involved in the modulation of OATP2B1 expression. Based on the results obtained, three miRNAs, mdm-miR-160a-e, -7121a-c, and -7121d-h, were investigated in subsequent studies.

**Interaction of Apple miRNAs with Human Ago2.** To confirm the interaction of mdm-miR-160a-e, -7121a-c, and -7121d-h with SLCO2B1-3’UTR, their incorporation into human Ago2 was examined. When miRNAs act on target miRNAs, miRNAs bind to Ago2, which exerts RNase-like enzymatic activity and causes miRNA degradation complementary to miRNA. To verify that mdm-miR-160a-e, -7121a-c, and -7121d-h were incorporated into human Ago2, Ago2 was immunoprecipitated from Caco-2 cells treated with APNPs using human Ago2 antibody, miRNAs interacting with Ago2 were evaluated using the Mir-X miRNA RT-PCR system. All three miRNAs were detected in the immunoprecipitated Ago2 fraction (Fig. 3). Hence, these miRNAs can form a functional complex with Ago2.

**Downregulation of Apple miRNAs by Predicted miRNA Mimic.** Based on the simulation by RNAhybrid, the MRE for miR-160a-e was located at +2454 to +2461 (site 1a) and +2526 to +2533 (site 1b) of SLCO2B1, and those for both miR-7121a-c and -7121d-h were predicted at +2526 to +2832 (site 2) (Fig. 4A). A luciferase assay was performed using a plasmid mutated with MREs to investigate whether miRNAs in APNPs suppress their expression by recognizing these MREs. pGL3/SLCO2B1-3’UTR-Mut1a, -Mut1b, and -Mut2 were prepared by mutating at site1a, 1b, and 2 SLCO2B1-3’UTR, respectively. Although APNPs significantly decreased the luciferase activity of normal pGL3/SLCO2B1-3’UTR, as shown in Fig. 2, the decreased luciferase activity was not restored even when each MRE was mutated (Fig. 4B). This result seems that the miRNAs in APNPs did not interact with sites 1a, 1b, and 2. However, since APNPs contain several miRNAs, it is considered that even if MRE is mutated at only one site, it is suppressed by other miRNAs targeting different MREs. Thus, it was suggested that the suppression of OATP2B1 by APNP was regulated by multiple miRNAs rather than by a single miRNA.

Therefore, to verify whether mdm-miR-160a-e, -7121a-c, and -7121d-h downregulated OATP2B1 expression in Caco-2 cells, each miRNA mimic was transfected with Caco-2 cells. As shown in Fig. 4C, all three miRNA mimics significantly decreased the endogenous expression of OATP2B1 mRNA in Caco-2 cells.

### Table 1

<table>
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<th>MRE</th>
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<th>Relative Position</th>
<th>Local AU Content</th>
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<td>29.6 256 0.26 0.37</td>
<td>2526 to 2916</td>
<td>7121a-c</td>
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Fig. 1. Detection of predicted miRNAs. Total RNA from APNP fraction (A) or Caco-2 cells treated with APNP for 6 hours (B) was isolated. The presence of miR-160a-e, miR-390a-f, miR-391, miR-395a-i, miR-477b, miR-7121a-c, miR-7121d-h, human miR-1, and human U6 in APNP was probed by RT-PCR using Mir-X miRNA detection system.
Decreased Luciferase Activity of Truncated pGL3/SLC02B1-3’UTR by miRNA Mimics. Next, we examined whether these miRNA mimics directly interacted with the predicted MRE of the SLC02B1-3’UTR. Luciferase plasmid constructs containing approximately 200 bp sequences containing the potential MRE were prepared (Fig. 5A). pGL3/SLC02B1-3’UTR (+2343–+2562) includes site 1a and 1b, which are MREs for miR-160a-e. Meanwhile, pGL3/SLC02B1-3’UTR (+2715–+2913) includes site 2, that is, MRE for miR-7121a-c and -7121d-h. Luciferase activity of pGL3/SLC02B1-3’UTR (+2343–+2562) tended to be reduced by the miR-160a-e mimic, but the difference was not statistically significant (Fig. 5B). This reduction was canceled by mutating at site1a and site1b, which are MREs of mdm-miR160a-e (Fig. 5, C and D). The expression of pGL3/SLC02B1-3’UTR (+2715–+2913) was significantly suppressed by the miR-7121-dh mimic (Fig. 5F). The miR-7121a-c mimic showed a tendency to decrease luciferase activity, but this was not statistically significant (Fig. 5F). Since the latter plasmid responded well to the miR-7121-dh mimic, pGL3/SLC02B1-3’UTR (+2715–+2913)-Mut2, which is mutated at the MRE of miR-7121-dh, was prepared and used to confirm the specific interaction of the miR-7121-dh mimic with its predicted MRE. As expected, the miR-7121-dh mimic did not change the luciferase activity in mutated plasmid-transfected cells, similar to miR7121a-c (Fig. 5G). These results indicate that mdm-miR-7121-dh binds to the 3’UTR of SLC02B1, resulting in the suppression of SLC02B1 expression.

Interaction of miRNA Contained in APNP with SLC02B1-3’UTR. Since the 3’UTR (+2715–+2913) of SLC02B1 was suggested as the MRE of miRNA in APNs using miRNA mimic, the direct interaction of APNPs with the MRE was examined. A luciferase assay was performed using pGL3/SLC02B1-3’UTR (+2343–+2562) and -3’UTR (+2715–+2913) to exclude the effects of miRNAs other than a candidate miRNA. Consistent with the results obtained for miR-160a-e mimic shown in Fig. 5B, the luciferase activity of pGL3/SLC02B1-3’UTR (+2343–+2562) was slightly decreased by APNPs, but this reduction was not statistically significant (Fig. 6A). However, decreases of luciferase activities were attenuated when MREs for mdm-miR-160a-e were mutated (Fig. 6, B–D). On the other hand, the luciferase activity of pGL3/SLC02B1-3’UTR (+2715–+2913) was significantly decreased by APNPs (Fig. 6E). Furthermore, the APNP-mediated decrease in the luciferase activity of pGL3/SLC02B1-3’UTR (+2715–+2913) was abrogated by the disruption of MRE for miR7121-dh (Fig. 6F). These results show that mdm-miR-7121-dh contributes to the downregulation of OATP2B1 by APNPs.

Discussion

It has been reported that fruit juices alter oral drugs’ intestinal absorption through interactions with intestinal transporters (Imanaga et al., 2011; Shirasaka et al., 2013a; Tamai and Nakanishi, 2013). These effects on the host are accounted for by low-molecular-weight compounds in food and those formed by the digestion of food-derived macromolecules, such as proteins. We recently investigated the effect of
APNPs on intestinal transporters and suggested a novel mechanism by which macromolecules, such as miRNAs in APNPs, may downregulate OATP2B1 by binding to its 3' UTR (Fujita et al., 2018). In the present study, we revealed the mechanism by which apple miRNA downregulated the expression of OATP2B1 through its interaction with the SLCO2B1-3' UTR.

Candidate miRNAs that may bind to the SLCO2B1-3' UTR were predicted using RNAhybrid (Krüger and Rehmsmeier, 2006). Seven predicted mdm-miRNAs were detected in the RNA extracted from the APNPs (Fig. 1A). If APNPs contained these seven miRNAs, it was expected that all of them would be detectable in APNP-treated cells. However, five miRNAs were detected in the RNA extracted from Caco-2 cells treated with APNPs, except for mdm-miR390a-f and -391 (Fig. 1B). miRNAs are known to possess intrinsically distinct stabilities in cells (Marzi et al., 2016). Bail et al. (2010) compared the decay rates of miRNAs under transcriptional shutoff. As a result of miRNA microarray analysis, miR-382 was decreased by more than 50% after 8 hours of transcriptional inhibition with actinomycin D treatment. Its degradation was abrogated by knocking down the 5'–3' exoribonuclease Rrp41.

Moreover, the 3' terminus seven nucleotides of miR-382 are essential for its instability, suggesting that a specific sequence within a miRNA, especially the 3' terminus, influences its stability. In our experiment, miRNAs were harvested after 6 hours in Caco-2 cells treated with APNPs (Fig. 1B). Therefore, because of the difference in instability, mdm-miR-390a-f might not have been detected.

Next, the involvement of candidate miRNAs in the suppression of OATP2B1 expression by APNPs was examined using each candidate miRNA's inhibitors. The decrease in luciferase activity by APNPs was mildly abrogated by cotransfection of inhibitors for miR-160a-e, -7121a-c, or -7121d-h (Fig. 2). However, the sum of the three miRNA inhibitors' effects was not sufficient to completely reverse the APNP-induced decrease in OATP2B1 mRNA expression. Therefore, multiple miRNAs may exert their effects in a concerted manner, or they may indirectly affect other molecules' regulation. Moreover, even though the decreased luciferase activity by APNPs was not prevented by mutating each MREs in the full-length pGL3/SLCO2B1-3' UTR (Fig. 4), it was marginally diminished by cotransfection with miRNA inhibitors (Fig. 2). Considering that miRNA inhibitors specifically bind to
and inhibit target miRNAs, these results imply that the mdm-miR-160a-e, -7121a-c, and -7121d-h are bound to multiple MREs in SLC02B1-3'UTR. Furthermore, the mir-7121d-h mimic significantly decreased the luciferase activity of the fragmented 3'UTR (Fig. 5F), whereas this downregulation was canceled by mutating at site 2 (Fig. 5G). These results suggest that the mir-7121d-h mimic directly interacts with the SLC02B1-3'UTR at the predicted MRE. Alternatively, endogenous mRNA expression of SLC02B1 in Caco-2 cells was downregulated not only by mir-7121d-h but also by miR-160a-e and mir-7121a-c (Fig. 4C), although these three candidate miRNAs functionally existed in APNP-treated Caco-2 cells by being interacted with Ago2 (Fig. 3). As shown in Fig. 5, B and F, mimics for miR-160a-e and -7121a-c tended to decrease the luciferase activity of the fragmented 3'UTR, whereas they were not statistically significant. However, this suppression was restored by mutating at each MREs (Fig. 5, C-E and G). Accordingly, miR-160a-e and mir-7121a-c possibly interact with the SLC02B1-3'UTR. Otherwise, they can decrease SLC02B1 indirectly and at MREs different from the predicted ones.

In the study of miRNA mimics shown in Fig. 4C, cells were treated with the same concentration of miRNA mimics. Therefore, it is considered that the difference in the action of the candidate miRNA is due to the binding affinity. In mammals, miRNAs can recognize their target mRNAs by seed sequence at the second to eighth nucleotides from the 3' end of the miRNA, resulting in cleavage of the target mRNAs (Buck et al., 2008; Ha and Kim, 2014). In contrast, plant miRNAs usually have a near-perfect pairing with their mRNA targets, which causes cleavage of target mRNAs (Jones-Rhoade et al., 2006). In addition to the seed sequence, AU-rich nucleotide composition near the recognition site, proximity to Watson-Crick pairing to miRNA at nucleotides 13–16, and positioning away of MRE from the center of 3'UTRs also affects miRNA action (Grimson et al., 2007). The predicted binding pattern between candidate miRNAs and the target mRNA was analyzed using RNAhybrid (Fig. 7). Among the candidate miRNAs, the mir7121d-h mimic significantly decreased the luciferase activity of SLC02B1-3'UTR (+2715→−2913) (Fig. 5F). The sequences of mir7121a-c and mir7121d-h differed only in one base at the 3'end (Fig. 7). Thus, it seems that miRNA sequence recognition is strictly regulated following the principle of target recognition. Table 1 shows the minimum free energy (Mfe), relative position, and AU contents of each miRNA calculated using RNAhybrid, representing binding stability, MRE position within the 3'UTR, and AU content near the binding site, respectively (Grimson et al., 2007). Consistently, among 206 apple miRNAs registered in miRbase, the Mfe of miR160a-e, 7121a-c, and 7121d-h were the highest in the order of 1, 4, and 3, respectively. Additionally, comparing these parameters among these candidate miRNAs, mir7121d-h, in which a decrease in luciferase activity was observed, had the highest AU content. Thus, it is plausible that mdm-miR-7121d-h can decrease OATP2B1 by directly interacting with the predicted MRE on the 3'UTR.

Plant miRNAs are thought to cause degradation of mRNA because they have few mismatches with the target mRNAs (Jones-Rhoade et al., 2006). However, the detailed binding mechanism between plants and mammals, namely interspecies interactions, has not been clarified. Plant miR167e-5p was reported to suppress human β-catenin expression by decreasing mRNA expression, and 15 of the 21 miRNA bases (71.4%) were complementary to mRNA (Li et al., 2019a). Similar to this report, our study showed that the complementarity between mdm-mir7121d-h and the target site was 71.4%. This result suggests that plant miRNAs degrade mammalian mRNA by binding a near-perfect pairing with their targets. Furthermore, it has been argued whether plant miRNAs can reach target sites and suppress human mRNA. Several groups have shown that plant miRNAs are detected in body fluids, including serum, urine, and saliva (Arroyo et al., 2011; Turchinovich et al., 2011; Gallo et al., 2012), suggesting that circulating miRNAs may be broadly implicated in miRNA-mediated control of gene expression. In contrast, contradictory results have also reported a lack of detectable oral bioavailability of plant miRNAs after feeding in mice (Dickinson et al., 2013). On the other hand, when plant-derived NPs are orally administered, it is likely intestinal epithelial cells are directly exposed to miRNAs encapsulated in exosome-like NPs. Further studies are required to verify whether orally administered APNPs decrease the expression of OATP2B1 in intestinal epithelial cells in vivo.

In conclusion, we successfully demonstrated that mdm-miR7121d-h contained in APNPs downregulated the mRNA expression of SLC02B1 by interacting with SLC02B1-3'UTR directly and that SLC02B1 mRNA might also be decreased by mdm-miR160a-e and -7121a-c indirectly. Although low-molecular-weight compounds contained in apples have been reported to exert an inhibitory effect on OATP2B1, our finding that the specific apple-derived miRNAs influence human intestinal transporters provides a novel concept that macromolecules in foods directly interact with and affect the intestinal function of the host. To use food-derived miRNAs for the prevention and treatment of diseases, further studies are needed to clarify the physiologic actions of miRNAs as functional components in food.

**Authorship Contributions**

**Conducted experiments**: Komori, Fujita, Shirasaki, Zhu, Iwamoto.

**Performed data analysis**: Komori, Fujita, Shirasaki, Zhu, Iwamoto.

**Wrote or contributed to the writing of the manuscript**: Komori, Fujita, Nakajima, Tamai.

**References**


Address correspondence to: Ikumi Tamai, Ph.D., Department of Membrane Transport and Biopharmaceutics, Faculty of Pharmaceutical Sciences, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kakumamachi, Kanazawa 920–1192, Japan. E-mail: tamai@p.kanazawa-u.ac.jp