MicroRNAs in apple-derived nanoparticles modulate intestinal expression of

OATP2B1/SLCO2B1 in Caco-2 cells

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Running title: Regulation of OATP2B1 by apple miRNA in nanoparticles

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Abbreviations
APNP, apple-derived nanoparticle; miRNA, microRNA; MRE, microRNA recognition element, NP, nanoparticle; OATP2B1, organic anion transporting peptide 2B1: 3'UTR, 3'-untranslated region
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 OATP2B1/SLC02B1 and that the 3’UTR region 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 SLC02B1-3’UTR. The APNP-mediated decrease in luciferase activity of pGL3/SLCO2B1-3’UTR was abrogated by inhibitors of mdm-miR-160a-e, -7121a-c, or -7121d-h. Each miRNA mimic reduced the endogenous expression of SLC02B1 mRNA in Caco-2 cells. The luciferase activity of the truncated pGL3/SLCO2B1-3’UTR, which contains approximately 200 bp around each miRNA recognition element (MRE), was decreased by the miR-7121d-h mimic but 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

We demonstrated that mdm-miR7121d-h contained in APNPs downregulated the mRNA expression of $SLCO2B1$ by interacting with $SLCO2B1$-3’UTR directly and that $SLCO2B1$ mRNA might also be decreased by mdm-miR160a-e and -7121a-c indirectly. This finding that the specific apple-derived miRNAs influence human intestinal transporters provides a novel concept that macromolecules in foods directly interact and affect the intestinal function of the host.
1. Introduction

Several foods, including grape, grapefruit, 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 to 400 nm and a negative zeta 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 TNFα receptor expression and dampen the TNFα 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
(OATP) 2B1 (SLCO2B1), which has a broad range of substrate specificity, is responsible for intestinal absorption of clinically used drugs, including anti-allergic fexofenadine and lipid-lowering statins (Tamai, 2012; Hoshino et al., 2016). Furthermore, intestinal absorption of these drugs was reduced by the competitive 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 (APNPs) decreased mRNA expression of the intestinal transporters OATP2B1; apical sodium-dependent bile acid transporter (ASBT, SLC10A2), which is essential for an enterohepatic circulation of bile acids; and carnitine transporter (OCTN2, SLC22A5) in intestinal epithelial cells such as Caco-2 cells (Fujita et al., 2018). Concerning the downregulation of OATP2B1, the 3’-untranslated region (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 APNP 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 physiological 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 pri-miRNAs in the cytoplasm. Then, miRNA is incorporated into the RNA-induced silencing complex (RISC), including Argonaute 2 (Ago2), Dicer, and tar-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. reported that plant miR-156 was detected in intestinal crypt cells in maize-diet-fed mice and decreased intestinal cell proliferation by reducing Wnt/β-catenin signaling (Li et al., 2019b). 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 HEK293 cells were purchased from RIKEN Cell Bank (Tsukuba, Japan) and Health Science Research Resource Bank (Osaka, Japan), respectively. Caco-2 cells were cultivated in DMEM supplemented with 10% FBS, 0.1 mM nonessential amino acids, 100 U/mL benzylpenicillin, and 100 μg/mL streptomycin. HEK293 cells were cultured in DMEM 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 min. The juice was collected after cheesecloth filtration. The supernatant collected after centrifugation at 2,000 × g for 20 min at 4 °C was then centrifuged at 13,000 × g for 70 min at 4 °C. The resultant supernatant was centrifuged at 120,000 × g for 130 min 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, USA) with BSA as the standard.

**Plasmid construction and mutagenesis**

Fragments of human \( SLCO2B1 \) were PCR-amplified using Caco-2 cell genomic DNA as a template. The forward and reverse primers are shown in Supplemental Table S1. 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 Supplemental Table S1.

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 Supplemental Table S2. 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., Catalog # 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 manufacturer’s information, 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 Supplemental Table S3. 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 No. 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 SLC2B1-3’UTR**

HEK293 cells were plated in 48-well plates 24 h before transfection. Plasmids of pGL3-p/SLCO2B1-3’UTR or its mutant plasmids were co-transfected with pRL-TK using Lipofectamine® 3000. mirVana™ miRNA inhibitor, 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 co-transfected with pGL3-p/SLCO2B1-3’UTR and pRL-TK. 6 h after transfection, the cells were treated with APNPs at a final concentration of 40 μg/mL for 12 h 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 co-transfected 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 h 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 ARVOsx Multilabel plate reader (PerkinElmer, Inc. Waltham, MA). Firefly luciferase activity was normalized to Renilla luciferase activity.

**Statistical analysis**

All data are expressed as means ± 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 near the binding site is high (Grimson et al., 2007). Based on this report, the predict 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, 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. Fig. 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, -477b -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 pGL3/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 co-transfected with pGL3/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 mRNAs, miRNAs bind to Ago2, which exerts RNase-like enzymatic activity and causes mRNA 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 OATP2B1 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 that 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 site 1a, 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.
Decreased luciferase activity of truncated pGL3/SLCO2B1-3’UTR by miRNA mimics

Next, we examined whether these miRNA mimics directly interacted with the predicted MRE of the SLCO2B1-3’UTR. Luciferase plasmid constructs containing approximately 200 bp sequences containing the potential MRE were prepared (Fig. 5A). pGL3/SLCO2B1-3’UTR (+2343→2562) includes site 1a and 1b, which are MREs for miR-160a-e. Meanwhile, pGL3/SLCO2B1-3’UTR (+2715→2913) includes site 2, that is, MRE for miR-7121a-c and -7121d-h. Luciferase activity of pGL3/SLCO2B1-3’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 site 1a and/or site 1b, those are MREs of mdm-miR160a-e (Fig. 5C-E). The expression of pGL3/SLCO2B1-3’UTR (+2715→2913) was significantly suppressed by the miR-7121d-h 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-7121d-h mimic, pGL3/SLCO2B1-3’UTR (+2715→2913)-Mut2, which is mutated at the MRE of miR-7121d-h, was prepared and used to confirm the specific interaction of the miR-7121d-h mimic with its predicted MRE. As expected, the miR-7121d-h mimic did not change the luciferase activity in mutated
plasmid-transfected cells, similar to miR-7121a-c (Fig. 5G). These results indicate that mdm-miR-7121d-h binds to the 3’UTR of SLCO2B1, resulting in the suppression of SLCO2B1 expression.

**Interaction of miRNA contained in APNP with SLCO2B1-3’UTR**

Since the 3’UTR (+2715–2913) of SLCO2B1 was suggested as the MRE of miRNA in APNPs using miRNA mimic, the direct interaction of APNPs with the MRE was examined. A luciferase assay was performed using pGL3/SLCO2B1-3’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/SLCO2B1-3’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. 6B-D). On the other hand, the luciferase activity of pGL3/SLCO2B1-3’UTR (+2715–2913) was significantly decreased by APNPs (Fig. 6E). Furthermore, the APNP-mediated decrease in the luciferase activity of pGL3/SLCO2B1-3’UTR (+2715–2913) was abrogated by the disruption of MRE for miR-7121d-h (Fig. 6F). These results show that mdm-miR-7121d-h 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. compared the decay rates of miRNAs under transcriptional shutoff (Bail et al., 2010). As a result of miRNA microarray
analysis, miR-382 was decreased by more than 50% after 8 h of transcriptional inhibition with actinomycin D treatment. Its degradation was abrogated by knocking down the 5′-3′ exoribonuclease Rrp41. Moreover, the 3′ terminus 7 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 h 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 co-transfection 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 were not prevented by mutating each MREs in the full-length pGL3/SLOC02B1-3’UTR (Fig. 4), it was marginally diminished by co-transfection with miRNA inhibitors (Fig. 2). Considering that miRNA inhibitors are 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. 5B and F, mimics for miR-160a-e and -7121a-e tended to decrease the luciferase activity of the fragmented 3’UTR, while they were not statistically significantly. However, this suppression was restored by mutating at each MREs (Fig. 5C-E, G). Accordingly, miR-160a-e and miR-7121a-c possibly interact with the \( SLC02B1 \)-3’UTR. Otherwise, they can decrease \( SLC02B1 \) indirectly and/or 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 2–8th nucleotides from the 5’ end of the miRNA, resulting in cleavage of the
target mRNAs (Baek 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-Rhoades 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 \( SLC22A1 \)-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-Rhoades 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 supports 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, 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 *SLCO2B1* by interacting with *SLCO2B1*-3’UTR directly and that *SLCO2B1* 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 and affect the intestinal function of the host. In order to use food-derived miRNAs for the prevention and treatment of diseases, further studies are needed to clarify the physiological actions of miRNAs as functional components in food.
Author contribution

H.K. and D.F. designed the study, performed experiments, analyzed data, and wrote the paper; Y.S., Q.Z., and Y.I. performed the experiments and analyzed the data; M.N., T.N., and I.T. designed the study and wrote the paper.
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Footnotes

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The authors declare no conflicts of financial interest.
### Tables

Table 1 Predicted miRNAs which bind to *SLCO2B1*-3′UTR

<table>
<thead>
<tr>
<th>Mfe (kcal/mol)</th>
<th>Position</th>
<th>Relative position</th>
<th>Local AU content</th>
</tr>
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<tbody>
<tr>
<td>miR-160a-e</td>
<td>-29.6</td>
<td>256</td>
<td>0.139</td>
</tr>
<tr>
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<td>miR-7121a-c</td>
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<td>628</td>
<td>0.342</td>
</tr>
<tr>
<td>miR-7121d-h</td>
<td>-29.4</td>
<td>628</td>
<td>0.342</td>
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Figure Legends

Fig. 1 Detection of predicted miRNAs

Total RNA from APNP fraction (A) or Caco-2 cells treated with APNP for 6 hr (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 were probed by RT-PCR using Mir-X™ miRNA detection system.

Fig. 2 Inhibition of APNP-induced decrease of \textit{SLCO2B1}-3′UTR luciferase activity by miRNA inhibitor

HEK293 cells were co-transfected miRNA inhibitors with pGL3/SLCO2B1-3′UTR. After 6 hr, these cells were treated with 40 µg/mL APNP for 6 hr. Firefly luciferase activity was normalized to the \textit{renilla} luciferase activity. * and # indicate significant difference from the APNP-untreated group and the negative control group, respectively (p < 0.05). Data were shown as means ± S.D. of six separate experiments.

Fig. 3 Incorporated apple miRNAs in human Ago2 in Caco-2 cells

Caco-2 cells were treated with 40 µg/mL APNP fraction for 24 h. Cells were lysed and were immunoprecipitated using anti-human Ago2 antibody. Expressions of miR-160a-e, -7121a-c and -7121d-h
incorporated in Ago2 were evaluated by Mir-X™ miRNA detection system.

Fig. 4 Influence of APNP on SLCO2B1-3’UTR mutated on MRE for predicted miRNAs

(A) The model for MRE of predicted miRNAs in pGL3/SLCO2B1-3’UTR. Numbers indicates locations of 3’UTR of SLCO2B1 (NM_001145211.3) and potential MREs. (B) HEK293 cells were transfected with pGL3-p/SLCO2B1-3’UTR or pGL3-p/SLCO2B1-mutated 3’UTR. After 6 hr from transfection, HEK293 cells were treated with 40 μg/mL APNP for 6 hr. Firefly luciferase activity was normalized to the renilla luciferase activity. (C) Caco-2 cells were transfected with 10 nM miRNA mimics. After 24 h from transfection, OATP2B1 mRNA was measured by qRT-PCR. Each bar represents the mean ± S.D. of three separate experiments. * indicates a significant difference from the APNP-untreated group (p < 0.05).

Fig. 5 Effect of miRNA mimics on truncated SLCO2B1-3’UTR

(A) The model for MRE of predicted miRNAs in fragmented pGL3/SLCO2B1-3’UTR. (B-D) HEK293 cells were co-transfected 10 nM miRNA mimics with pGL3/SLCO2B1-3’UTR (+2343 - +2562), -3’UTR (+2715 - +2913), and -3’UTR (+2715 - +2913)-Mut2, respectively. After 48 hr, Firefly luciferase activity was normalized to the renilla luciferase activity. Each bar represents the mean ± S.D. of three separate experiments.
experiments. * indicates a significant difference from the negative control (p < 0.05).

Fig. 6 Suppression of luciferase activity by APNP on truncated SLCO2B1-3′UTR

HEK293 cells were transfected with pGL3/SLCO2B1-3′UTR(+2343 – +2562), -3′UTR(+2715 – +2913), and -3′UTR(+2715 – +2913)-Mut2, respectively. After 6 hr, cells were treated with 40 µg/mL APNP for 48 hr, and firefly luciferase activity was normalized to the renilla luciferase activity. Each bar represents the mean ± S.D. of three separate experiments. * indicates a significant difference from the APNP-untreated group (p < 0.05).

Fig. 7 Schematic representation of predicted OATP2B1 site targeted by candidate miRNA
Figure 2

miRNA inhibitor for

Relative Luciferase Activity (Firefly / Renilla)

- +

APNP

Negative control

miR-160a-e

miR-390a-f

miR-391

miR-395a-i

miR-477b

miR-7121a-c

miR-7121d-h

* # * *

* # * *

0.2 0.4 0.6 0.8 1.0 1.2
A pGL3/SLCO2B1-3'UTR

Site1a: +2454~+2461
Site1b: +2526~+2533
Site2: +2825~+2832

Mut1a: GGCCAGGC→CCGGTCCG
Mut1b: GGCCAGGC→CCGGTCCG
Mut2: TCAAGAGG→AGTTCTCC

B

Relative Luciferase Activity (Firefly / Renilla)

APNP:
- + 3'UTR 3'UTR-Mut1a 3'UTR-Mut1b 3'UTR-Mut2

C

SLCO2B1 mRNA Expression (Relative to negative control)

Mimic: Negative miR-160a-e miR-7121a-c miR-7121d-h Control
Figure 5

A. pGL3/SLCO2B1-3'UTR(+2343 – +2562)

B. 3'UTR(+2343 – +2562)

C. 3'UTR(+2343 – +2562)-Mut1a

D. 3'UTR(+2343 – +2562)-Mut1b

E. 3'UTR(+2343 – +2562)-Mut1a/1b
Figure 6

A  
B  
C  
D  
E  
F

Relative Luciferase Activity (Firefly / Renilla)

- APNP

3'UTR(+2343 – +2562)  
3'UTR(+2343 – +2562)-Mut1a  
3'UTR(+2343 – +2562)-Mut1b  
3'UTR(+2343 – +2562)-Mut1a/1b  
3'UTR(+2715 – +2913)  
3'UTR(+2715 – +2913)-Mut2
miR160a-e to Site1a

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miR160a-e to Site1b

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miR7121a-c to Site2

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miR7121d-h to Site2

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<tbody>
<tr>
<td>miRNA</td>
<td>3’-CGUCCCGCUAGGGUCUCC-5’</td>
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