Note

Riboflavin Transporters RFVT/SLC52A Mediate Translocation of Riboflavin, Rather than FMN or FAD, across Plasma Membrane

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Riboflavin (vitamin B2) plays a role in various biochemical oxidation-reduction reactions. Flavin mononucleotide (FMN) and FAD, the biologically active forms, are made from riboflavin. Riboflavin transporters (RFVTs), RFVT1-3/*Slc52a1-3*, have been identified. However, the roles of human (h)RFVTs in FMN and FAD homeostasis have not yet been fully clarified. In this study, we assessed the contribution of each hRFVT to riboflavin, FMN and FAD uptake and efflux using *in vitro* studies. The transfection of hRFVTs increased cellular riboflavin concentrations. The uptake of riboflavin by human embryonic kidney cells transfected with hRFVTs was significantly increased, and the efflux was accelerated in a time-dependent manner. However, the uptake and efflux of FMN and FAD hardly changed. These results strongly suggest that riboflavin, rather than FMN or FAD, passes through plasma membranes *via* hRFVTs. Our findings could suggest that hRFVTs are involved in riboflavin homeostasis in the cells, and that FMN and FAD concentrations are regulated by riboflavin kinase and FAD synthase.

Key words riboflavin; flavin mononucleotide; FAD; transporter; vitamin B2

The water-soluble vitamin riboflavin (vitamin B2) is essential for cellular growth and functions.[1)] Flavin mononucleotide (FMN) and FAD, which are biologically active forms, are made from riboflavin with riboflavin kinase (EC 2.7.1.26) and FAD synthase (EC 2.1.1.148). Chemical structures of riboflavin, FMN, and FAD are shown in Fig. 1. FMN and FAD participate in various biochemical oxidation-reduction reactions including the metabolism of carbohydrates, amino acids, and lipids. Humans are unable to synthesize riboflavin, and thus it has to be absorbed from the diet or, to a lesser extent, from production by the intestinal microflora. *In vivo*, *in situ*, and membrane vesicle experiments have suggested that riboflavin transporters are essential for the maintenance of riboflavin homeostasis to constantly keep the blood and tissue concentrations of riboflavin.[2–6)]

Human riboflavin transporters (hRFVTs), such as hRFVT1-3 encoded by *SLC52A1-3* genes, have been identified.[7–10)] We have revealed the functional properties of the RFVT family by comparing their functional and molecular characteristics with each other.[9–11)] hRFVT1, hRFVT2, and hRFVT3 show similar substrate specificities and affinities. hRFVT1 and hRFVT2 are widely expressed in several tissues, while hRFVT3 is expressed mainly in testis, small intestine, kidney, and placenta. In Rfvt3 knockout mice, the plasma and tissue riboflavin levels were dramatically lower than control mice.[11)] It was indicated that RFVTs should play an important role in riboflavin homeostasis. However, the roles of hRFVTs in FMN and FAD homeostasis remain unclear.

In the present study, we compared the effects of hRFVTs expression on the uptake and efflux of riboflavin and its analogs, and clearly indicated that hRFVTs mediated the transport of riboflavin, rather than FMN or FAD, across the plasma membrane.

MATERIALS AND METHODS

Cell Culture and Transfection Human embryonic kidney (HEK)-293 cells (American Type Culture Collection CRL-1573) were cultured in complete medium consisting of Dulbecco's Modified Eagle's medium (DMEM) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham,

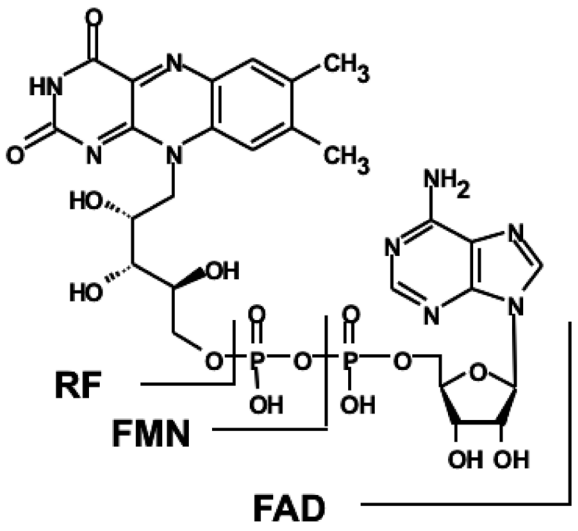

Fig. 1. Chemical Structures of Riboflavin (RF), Flavin Mononucleotide (FMN), and FAD

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MA, U.S.A.) in an atmosphere of 5% CO_2-95% air at 37°C. The concentrations of riboflavin, FMN, and FAD in the complete medium were 528.0, 3.2, and 22.4 nM, respectively. The plasmid DNAs of pcDNA3.1/Hygro(+) containing hRFVT1, hRFVT2, and hRFVT3 were previously constructed.[9] The plasmids were purified using the HispeedTM Plasmid Purification System (QIAGEN K.K., Tokyo, Japan). On the day before transfection, the cells were seeded onto poly-D-lysine-coated 12-well plates (Becton, Dickinson and Company, Franklin Lakes, NJ, U.S.A) at a density of 1.5×10^5 cells per well. The cells were transfected with 0.4 μg of plasmid DNA using 2 μL of Lipofectamine 2000 Reagent (Thermo Fisher Scientific) per well. Forty-eight hours after transfection, the cells were used for the subsequent experiments. The RNA interference (RNAi) system was performed according to a previous report with some modifications.[12] The target sequences of gene-specific duplexed Stealth RNAi small-interfering RNA (siRNA) (Life Technologies, U.S.A.) for SLC52A2 coding hRFVT2 were as follows: hRFVT2-siRNA I for sequence 5′-AGG CAC UGC GGG CUG AUA GAA GCU G-3′; hRFVT2-siRNA II for sequence 5′-ACC ACA GGU AGC UCC ACC CAG AUC C-3; and hRFVT2-siRNA III for sequence 5′-UAA GAA GCG AGG UGG CAG GUG GCU C-3′. Stealth RNAi Negative Control Duplexes (Life Technologies) were transfected as control. A dose of 60 pmol siRNA was transfected into HEK293 cells at a density of 1.5×10^5 cells per well of 12-well plates using 3 μL of Lipofectamine RNAiMAX (Life Technologies) per well according to the manufacturer's protocol.

Uptake and Efflux of Riboflavin and Its Active Forms HEK293 cells transfected with empty vector (as control) or hRFVT1-3 were incubated with incubation medium consisting of 145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM D-glucose, and 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.4). For the uptake study, the cells were pre-incubated with incubation buffer for 20 min at 37°C and incubated in incubation medium containing 10 μM riboflavin, FMN, or FAD for 10 min at 37°C. Additionally, the time course of uptake was measured at 0, 1, 2, 5, 10 min, respectively. HEK293 cells have endogenous riboflavin, FMN and FAD, and thus, the specific transport by hRFVTs was calculated by subtracting the amount of riboflavin, FMN, or FAD in the cells after the incubation with incubation buffer containing them from the amount in the cells after the pre-incubation with only incubation buffer. For the efflux study, the cells were incubated with incubation medium without riboflavin or its analogs for 10 or 20 min at 37°C. The cellular concentrations of endogenous riboflavin, FMN, and FAD were measured as described below.

Measurement of Riboflavin and Its Active Forms Cells were rinsed with cold incubation medium and recovered in 10 mM phosphate buffer (pH 5.5). The same volume of methanol was added, and the samples were mixed and centrifuged. The supernatants were filtered through a Cosmonice filter (0.45 μm; Merck Millipore, Billerica, MA, U.S.A). The concentrations of riboflavin, FMN, and FAD were determined by HPLC (LC-10ADVP equipped with an RF-10ASL variable wavelength fluorescence detector and LC solution data systems; Shimadzu, Kyoto, Japan) according to a previous report with some modifications.[12] The HPLC conditions were as follows: column, CHEMCOSORB 5-ODS-H (4.6×150 mm; Chemco Scientific Co., Ltd., Osaka, Japan); mobile phase, 10 mM phosphate buffer (pH 5.5) : methanol=65 : 35; flow rate, 1.0 mL/min; excitation wavelength, 445 nm; emission wavelength, 530 nm; injection volume, 20 μL; and column temperature, 40°C.

Real-Time PCR Total RNA was isolated from HEK293 cells without transfection using the RNeasy Mini Kit (QIAGEN K.K., Tokyo, Japan) and reverse-transcribed to cDNA. To determine the mRNA levels of SLC52A1-3, real-time PCR was performed as described previously.[9] TaqMan® Gene Expression Assays were purchased from Life Technologies: *i.e.*, SLC52A1 (ID: Hs01079030_g1), SLC52A2 (ID: Hs01859203_s1), and SLC52A3 (ID: Hs00364295_m1).

Statistical Analysis All values are expressed as the mean±standard error of the mean (S.E.M.), and the differences were analysed for significance using the unpaired Student's *t*-test. Multiple comparisons were performed by the Bonferroni's two-tailed test after a one-way ANOVA using GraphPad Prism (version 5.0b, GraphPad Software, Inc., La Jolla, CA, U.S.A). Differences where $p<0.05$ were considered significant.

RESULTS

Endogenous Riboflavin, FMN, and FAD Levels in hRFVTs-Expressing Cells At first, the endogenous expression of hRFVTs in HEK293 cells was examined. The mRNA levels of hRFVT2 was 0.54 amol/μg total RNA, while hRFVT1 and 3 were undetected. We examined the effect of hRFVT transfection on the intracellular amounts of endogenous riboflavin, FMN, and FAD in HEK293 cells. The cellular concentration of riboflavin was significantly higher by transfection with hRFVT3 as well as hRFVT2 and hRFVT1 compared with control cells (Fig. 2A). The amount of riboflavin was the highest in hRFVT3 transfectants. On the other hand, there was little difference of the cellular amounts of FMN or FAD between all hRFVTs-expressing cells and control one (Figs. 2B, C).

Involvement of hRFVTs in the Uptake of Riboflavin and Its Active Forms The uptake of riboflavin, FMN, or FAD was determined with HEK293 cells transfected with empty vector, hRFVT1, hRFVT2, and hRFVT3. The cells were incubated in incubation medium containing riboflavin, FMN, or FAD (10 μM) for 10 min after 20 min pre-incubation. Figure 3 shows the difference in the intracellular amounts before and after adding 10 μM riboflavin, FMN, or FAD. The time course of riboflavin uptake was shown in Fig. 3A. Riboflavin uptake by hRFVTs-expressing cells was significantly higher compared with control cells (Fig. 3B). The uptake of FMN by hRFVT1 and hRFVT3 was slightly increased (Fig. 3C). The amount of FAD remained unchanged in hRFVTs-expressing cells (Fig. 3D).

Involvement of hRFVTs in the Efflux of Riboflavin and Its Active Forms The efflux of endogenous riboflavin, FMN, and FAD was determined with HEK293 cells transfected with empty vector, hRFVT1, hRFVT2, or hRFVT3. The cells were incubated in the incubation medium without riboflavin, FMN, or FAD for 10 or 20 min. The intracellular riboflavin level in every cell were significantly decreased in a time-dependent manner (Fig. 4A). The plateau phase of riboflavin concentration in the cells transfected with hRFVT1 was significantly lower than control cells. Intracellular FAD levels in hRFVT1- and hRFVT3-expressing cells were significantly

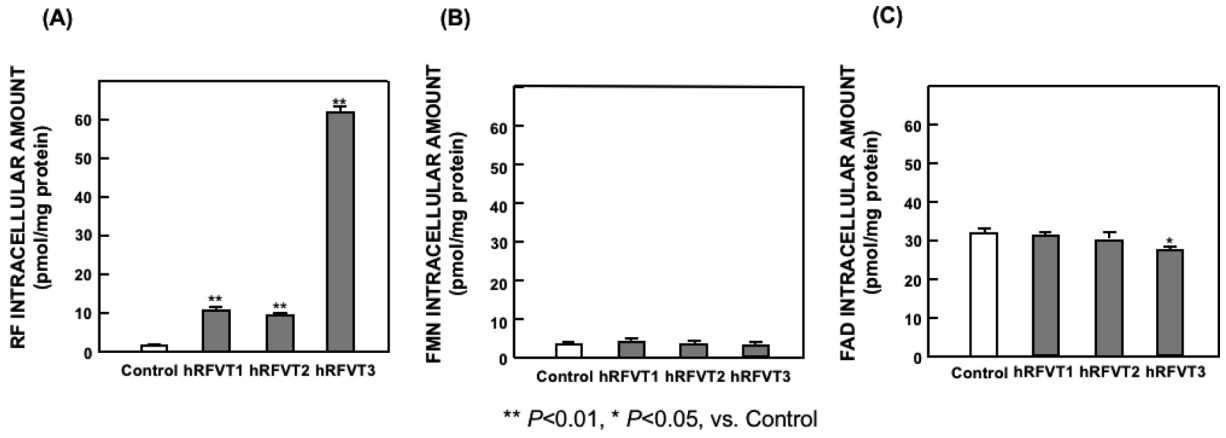

Fig. 2. Endogenous Amounts of Riboflavin (A), FMN (B), and FAD (C) in HEK293 Cells Transfected with Empty Vector, hRFVT1, hRFVT2, and hRFVT3

Forty-eight hours after transfection, concentrations were determined by HPLC. Each point represents the mean±S.E.M. of 3 wells. Differences where $p<0.05$ (*) or <0.01 (**) were considered significant when compared with control cells.

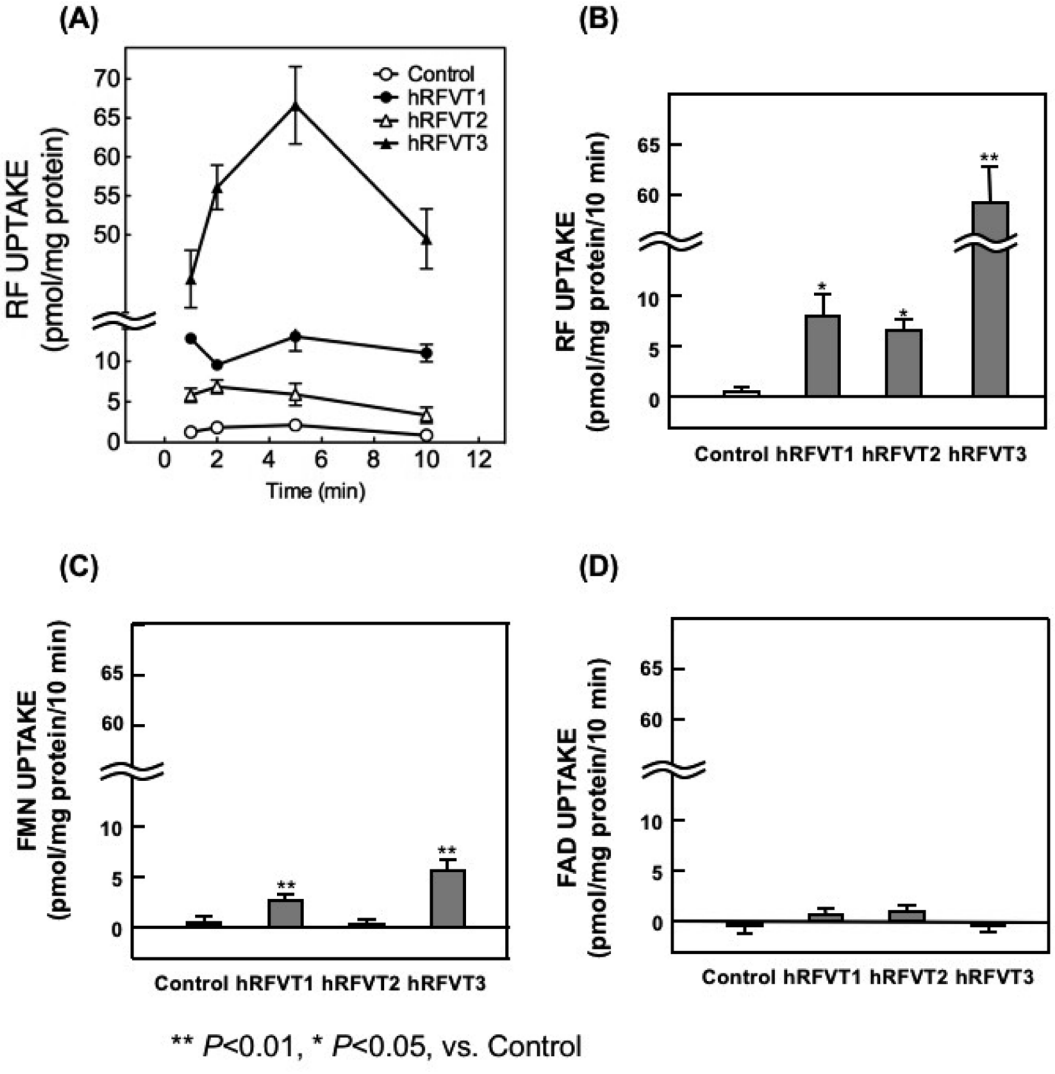

Fig. 3. The Time Course of Riboflavin Uptake, and the Points Were Collected at 0, 1, 2, 5, 10 min after 20 min Pre-incubation (A)

Uptake of riboflavin (B), FMN (C), and FAD (D) in HEK293 cells transfected with empty vector, hRFVT1, hRFVT2, and hRFVT3. The cells were incubated in incubation buffer (pH 7.4) containing riboflavin, FMN, or FAD for 10 min at 37°C. Concentrations at 0 and 10 min were determined by HPLC. The specific transport by hRFVTs was calculated by subtracting the amount of riboflavin, FMN, or FAD in the cells after the incubation with incubation buffer containing them from the amount in the cells after the pre-incubation with only incubation buffer. Each bar represents the mean±S.E.M. of 3 wells. Differences where $p<0.05$ (*) or <0.01 (**) were considered significant when compared with control cells.

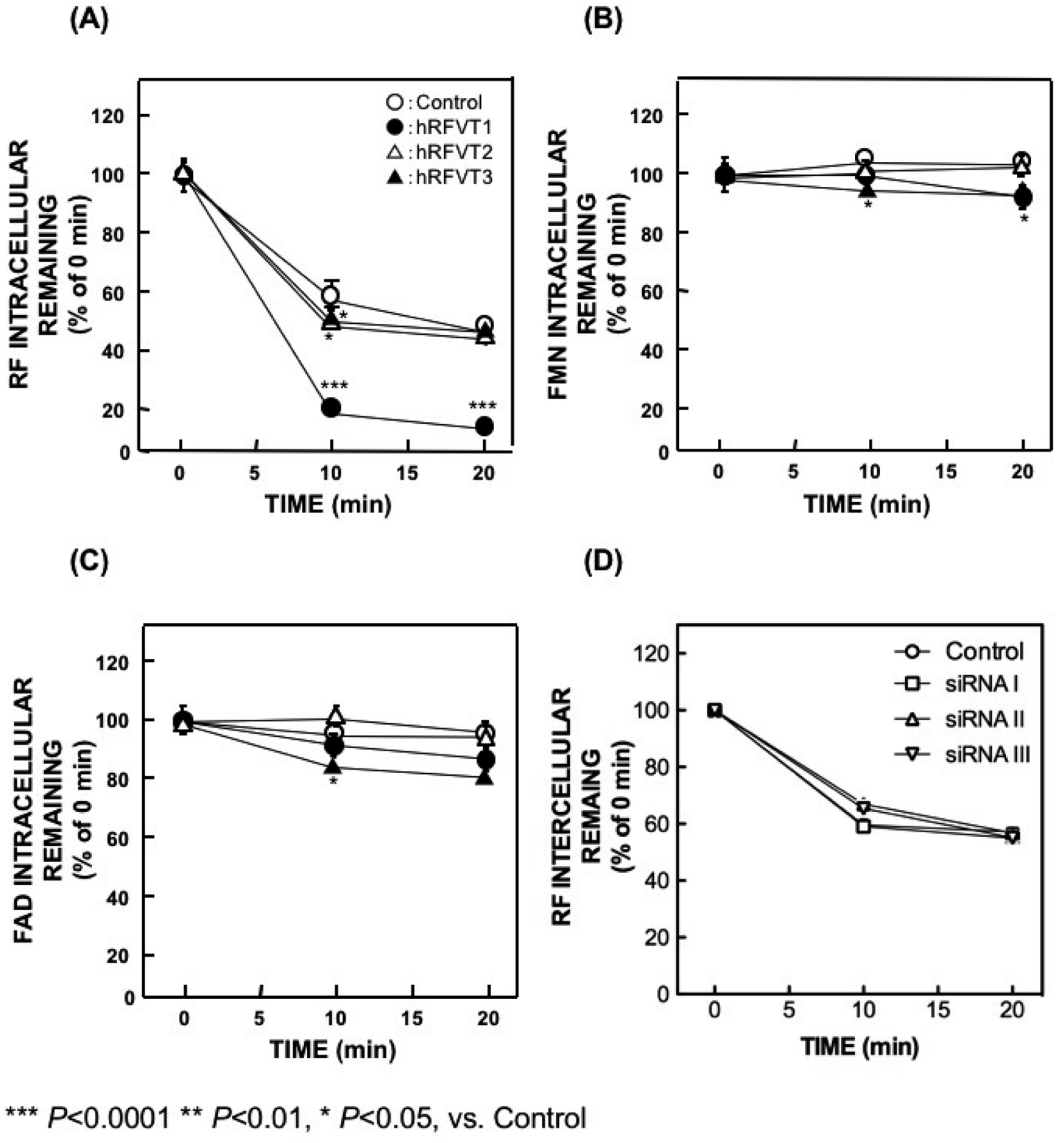

Fig. 4. Efflux of Riboflavin (A), FMN (B), and FAD (C) in HEK293 Cells Transfected with Empty Vector (Control), hRFVT1, hRFVT2, and hRFVT3

In addition, efflux of riboflavin in negative control (control), hRFVT2-siRNA (siRNA I, siRNA II, siRNA III) transfected HEK293 cells (D). The cells were incubated in incubation buffer (pH 7.4) without riboflavin or its active forms for 10 or 20 min at 37°C. Concentrations at 0, 10, and 20 min were determined by HPLC. Each point represents the mean±S.E.M. of 3 wells. Differences where $p<0.05$ (*), <0.01 (**) or <0.0001 (***) were considered significant when compared with control cells.

decreased, but slightly less effective (Fig. 4C). On the other hand, the efflux of FMN was not observed in all hRFVTs-expressing cells (Fig. 4B). As the endogenous expression of hRFVT2 was detected in HEK 293 cells, efflux of riboflavin in hRFVT2-siRNAs transfected cells was measured (Fig. 4D). Real-time PCR analysis confirmed that hRFVT2-siRNA significantly reduced the expression of hRFVT2 mRNA in HEK293 cells (data not shown). However, a knockdown of hRFVT2 by siRNA didn't reduce riboflavin efflux.

DISCUSSION

For the first time, we clearly demonstrated the contribution of hRFVTs to the transport of riboflavin, rather than that of FMN or FAD, across the plasma membrane. The uptake and efflux of riboflavin, but hardly FMN or FAD, by hRFVTs were observed in hRFVTs-expressing cells. Previously, the inhibition-based experiments indicated similar specificities as FMN and FAD were weakly recognized by hRFVTs.[9] In addition, it was also shown that riboflavin but hardly FMN or FAD was transported across T84 intestinal cells.[12] Therefore, it has been indicated that riboflavin, rather than FMN or FAD, passes through the plasma membranes *via* hRFVTs.

Cellular FMN and FAD levels were hardly changed in the present experiments. It was also reported that a reduction of FMN and FAD was smaller than that of riboflavin in the tissues of Rfvt3 knockout mice.[13] FMN and FAD are made from riboflavin by riboflavin kinase and FAD synthase. It was suggested that the homeostasis of FMN and FAD was not regulated by hRFVTs, but by riboflavin kinase and FAD synthase. Interestingly, the plateau phase of riboflavin concentration in the cells expressing hRFVT1 was lower than the others including mock-transfected cells in the efflux study. Although hRFVT2 was natively expressed on HEK293 cells, a knockdown of hRFVT2 did not affect the efflux of riboflavin. It was suggested that RFVT1 played a role in the efflux of riboflavin

from the cells.

Riboflavin consists of an isoalloxazine ring and ribose side chain. FMN and FAD are made from riboflavin with riboflavin kinase and FAD synthase. FMN has a phosphate group attached to the carbon of the ribityl side chain, and FAD has ADP attached at the ribose moiety. The chemical structures of these analogs are shown in Fig. 1. The uptake of FMN *via* hRFVTs was stronger than that of FAD, but weaker than that of riboflavin. Therefore, the attachment of phosphate group to riboflavin could disturb the binding to hRFVTs, and ADP could also obstruct it much more. It was previously reported that the riboflavin transport activity of hRFVTs was completely inhibited by lumiflavine, but not D-ribose, suggesting that the isoalloxazine ring of riboflavin, but not the ribose side chain, is necessary for the recognition of all hRFVTs.[9] The substrate specificities of hRFVTs were similar and relatively specific for riboflavin, as shown in this study and previous reports.[7–10] hRFVT might specifically recognize the isoalloxazine ring of riboflavin as substrates, and the binding of FMN or FAD to hRFVTs was disturbed by phosphate group and ADP.

Vitamin B2 exists in the forms of FMN and FAD in dietary sources.[14] In addition, many vitamin supplements contain FMN or FAD rather than riboflavin. However, the present study demonstrated that the transport of FMN and FAD was weaker than that of riboflavin in hRFVTs-expressing cells. The previous study showed that the apical-to-basal transports of FMN and FAD were lower than that of riboflavin in T84 cells.[12] In addition, it was suggested that RFVT3 play an important role in the absorption of riboflavin.[12] The present findings and previous reports suggest that the absorption of vitamin B2 would occur primarily in the form of riboflavin, attributable to substrate recognition by apical RFVT3. It was suggested that FMN and FAD were unavailable for absorption and, thus, might be hydrolyzed to riboflavin before absorption. Recently, mutations of hRFVTs were found in patients with Brown–Vialetto–Van Laere syndrome, and early treatment with high-dose riboflavin showed clinical improvement.[15–22] FMN and FAD are active forms of riboflavin, but are hardly transported by RFVTs. Further studies are needed to clarify which of riboflavin, FMN, or FAD is suitable for the treatment with this syndrome.

It was previously reported that riboflavin deficiency caused an increase in RFVT2 expression.[13] The present study indicated that hRFVT expression increased cellular contents of riboflavin. hRFVT mediates the uptake and efflux of riboflavin, depending on the concentration gradient. Therefore, it was speculated that hRFVT expression regulated cellular riboflavin concentrations, and maintained the homeostasis of riboflavin.

In conclusion, hRFVTs mainly mediate the transport of riboflavin and hRFVT expression increased the cellular riboflavin levels, suggesting that riboflavin, rather than FMN or FAD, passes through the plasma membranes *via* hRFVTs. Our findings could suggest that hRFVTs are involved in riboflavin homeostasis in the cells, and that FMN and FAD concentrations are regulated by riboflavin kinase and FAD synthase.

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Conflict of Interest The authors declare no conflict of interest.

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