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The Fowler Syndrome-Associated Protein FLVCR2 Is an Importer of Heme▽

Simon P. Duffy,[1,2] Jennifer Shing,[1] Punit Saraon,[1] Lloyd C. Berger,[2] Maribeth V. Eiden,[3] Andrew Wilde,[2] and Chetankumar S. Tailor[1,2]*

Program in Cell Biology, Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada[1]; Department of Molecular Genetics, University of Toronto, Toronto, Ontario M5S1A8, Canada[2]; and National Institute of Mental Health, Laboratory of Cellular and Molecular Regulation, Bethesda, Maryland 20892[3]

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Mutations in FLVCR2, a cell surface protein related by homology and membrane topology to the heme exporter/retroviral receptor FLVCR1, have recently been associated with Fowler syndrome, a vascular disorder of the brain. We previously identified FLVCR2 to function as a receptor for FY981 feline leukemia virus (FeLV). However, the cellular function of FLVCR2 remains unresolved. Here, we report the cellular function of FLVCR2 as an importer of heme, based on the following observations. First, FLVCR2 binds to hemin-conjugated agarose, and binding is competed by free hemin. Second, mammalian cells and *Xenopus laevis* oocytes expressing FLVCR2 display enhanced heme uptake. Third, heme import is reduced after the expression of FLVCR2-specific small interfering RNA (siRNA) or after the binding of the FY981 FeLV envelope protein to the FLVCR2 receptor. Finally, cells overexpressing FLVCR2 are more sensitive to heme toxicity, a finding most likely attributable to enhanced heme uptake. Tissue expression analysis indicates that FLVCR2 is expressed in a broad range of human tissues, including liver, placenta, brain, and kidney. The identification of a cellular function for FLVCR2 will have important implications in elucidating the pathogenic mechanisms of Fowler syndrome and of phenotypically associated disorders.

Membrane transporters play essential roles in cellular homeostasis by importing substrates critical for cell growth and differentiation or by exporting substrates that cause toxicity. There are five major categories of membrane transporters consisting of over 550 transporter superfamilies (41). The major facilitator superfamily (MFS) is the largest and most diverse superfamily, consisting of over 10,000 members (31, 41). Transporters in this superfamily consist of 12 to 14 transmembrane (TM)-spanning segments and transport substrates as diverse as sugars, polyols, drugs, neurotransmitters, amino acids, organic/inorganic ions, and peptides (31). Recently, a disruption of MFS transporters that is associated with human diseases has been described, further confirming their role in the maintenance of normal cell homeostasis. The DIRC2 MFS transporter (substrate transported unknown) is disrupted in renal cell carcinoma cosegregating with a t(2;3)(q35;q21) chromosomal translocation (4). Mutations in the thiamine transporter THTR1 have been shown to be responsible for Rogers syndrome (14, 21), a thiamine-responsive megaloblastic anemia. We have recently reported that a disruption in the heme exporter FLVCR1 (MFSD7B) plays a role in Diamond Blackfan anemia (DBA) (40), a fatal infant anemia characterized by a block in erythroid progenitor cell development (3, 12, 13). The abrogation of FLVCR1 function in primary human hematopoietic stem cells (40) or in a human erythroid cell line (37) specifically disrupts erythropoiesis, mimicking the hematological features observed for patients with DBA. We have reported previously that FLVCR1 is disrupted not as a consequence of mutations in the FLVCR1 coding region but due to the aberrant splicing of specific FLVCR1 exons that reduces the expression and cell surface localization of the encoded FLVCR1 protein (40). Interestingly, the THTR1 and FLVCR1 proteins were shown previously to function as receptors for entry by feline leukemia retrovirus (FeLV) subgroup A (FeLV-A) (25) and FeLV-C (36, 46), respectively. These viruses disrupt the cellular function of these proteins in infected cats and can induce diseases that correlate with Rogers syndrome (17) and DBA (1, 28).

Recently, mutations in the cell surface protein FLVCR2 (MFSD7C), an MFS transporter member, have been shown to be associated with Fowler syndrome (22, 26), a proliferative vascular disorder of the brain (16). A previous study (6) suggested that FLVCR2 functions as a calcium-chelate transporter based on its expression in murine and human tissues involved in calcium homeostasis. We have shown previously that FLVCR2 is highly related to the heme exporter/retroviral receptor FLVCR1 (7), and we have recently shown it to function as a receptor for the subgroup C FeLV variant FY981 (42). Based on its close sequence relationship to the heme exporter/retroviral receptor FLVCR1 and based on previous reports showing that retroviruses often adapt to use closely related cell surface proteins as receptors for infection (27, 30, 44), we investigated the heme transport function of FLVCR2. Here, we show the physiological function of FLVCR2 as an importer of heme.

MATERIALS AND METHODS

Cells. Chinese hamster ovary (CHO) cells were cultured in minimum essential alpha medium, human K562 cells were cultured in Iscove's modified Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Canada), and TELCeB6 (T6) (11) and human TE671 cells were cultured in DMEM with low glucose. Human

* Corresponding author. Mailing address: Hospital for Sick Children, Program in Cell Biology, Room 7129 Elm Wing, 555 University Avenue, Toronto, ON M5G 1X8, Canada. Phone: (416) 813-6429. Fax: (416) 813-5993. E-mail: chetankumar.tailor@sickkids.ca.

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embryonic kidney 293T (HEK293T) cells were maintained in DMEM with high glucose. All media were supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Burlington, ON, Canada).

Expression of FLVCR1, FLVCR2, and THTR1 in CHO cells. CHO cells expressing hemagglutinin (HA)-tagged human FLVCR1 (huFLVCR1), human FLVCR2 (huFLVCR2), or feline THTR1 (feTHTR1) were generated as described previously (42). The constructs were cloned into the pFBneo retroviral vector (Agilent Technologies, Mississauga, ON, Canada). Expression is driven by the retroviral long terminal repeat (LTR). HEK293T cells were transfected with a murine leukemia virus (MLV) *gag-pol* expression vector, a phCMV-VSVG expression vector (expression vector provided by Eyal Grunebaum, Hospital for Sick Children, Toronto, Canada), and a pFBneo retroviral vector containing HA constructs (see above) to generate vesicular stomatitis virus (VSV) pseudotype viruses carrying the FeLV receptor sequences. The virus supernatant from transfected cells was then used to infect CHO cells, and transduced cells were selected by using G418 Geneticin sulfate (1.5 mg/ml) (Invitrogen). Pooled resistant cells were then used for heme uptake experiments.

Hemin-agarose precipitation assay. The hemin-agarose precipitation assay was modified from that reported previously by Krishnamurthy and colleagues (20). Cells were harvested and suspended in buffer (210 mM mannitol, 70 mM sucrose, 5 mM Tris-HCl [pH 7.5], and 1 mM EDTA). Approximately 100 μg of protein suspension was incubated with 167.5 nM hemin-agarose (Sigma-Aldrich, Canada) for 15 min at room temperature. The reaction mixture was centrifuged at 10,000 × *g* for 1 min at 4°C, and the pellet was washed with 1 ml of lysis buffer (150 mM NaCl, 1% Nonidet P-40, and 50 mM Tris [pH 8]). The pellet was resuspended in 20 μl of 2× SDS sample-loading buffer, briefly mixed by vortexing, and centrifuged at 10,000 × *g* for 2 min at 4°C. The supernatant was resolved on a 10% SDS-PAGE gel and probed with peroxidase-conjugated anti-HA antibody (Sigma-Aldrich).

ZnMP uptake assay. Parental CHO cells and CHO cells expressing huFLVCR1 or huFLVCR2 were assayed for zinc mesoporphyrin (ZnMP) uptake. Target cells were seeded at 5×10^5 cells/well in a six-well plate 18 to 24 h prior to the uptake assay. The cells were then incubated with 5 μM ZnMP (dissolved in dimethyl sulfoxide [DMSO]) in uptake buffer (0.1% bovine serum albumin [BSA], 25 mM HEPES [pH 7.4], 130 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$) for 20 min at 37°C. Cells were then washed three times with ice-cold uptake buffer, dislodged by using a cell dissociation buffer (Invitrogen), and fixed with 1% paraformaldehyde, and the mean fluorescence intensity (MFI) values were determined by flow cytometry.

[^{55}Fe]hemin uptake in *Xenopus* oocytes. [^{55}Fe]hemin uptake in *Xenopus laevis* oocytes was carried out as described previously by Shayeghi and colleagues (43). *Xenopus* oocytes were injected with approximately 30 ng of huFLVCR2 cRNA, and control oocytes were injected with water. At 2 days postinjection, oocytes were incubated for 2 h at room temperature in Barth's solution (80 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES [pH 7.4]) containing 27 μM [^{55}Fe]hemin (0.66 mg; specific activity, 153 μCi/mg) (RI Consultant LLC, Hudson, NH) initially diluted in 200 mM arginine buffer (pH 8.0). Oocytes were rinsed twice in Barth's solution and then lysed with 100 μl of 0.1 M NaOH. Lysed oocytes were transferred into a scintillation vial, scintillation fluid was added, and presence of the radioisotope was determined by using a Beckman scintillation counter.

Heme toxicity assay. Parental CHO cells and CHO cells expressing huFLVCR2 or huFLVCR1 were plated in a 24-well plate 1 day prior to exposure with hemin. The following day, cells were incubated with medium containing increasing concentrations of hemin (50 μM to 250 μM) for 24 h. Cells were then stained with trypan blue, and the percentage of unstained viable cells compared to blue-stained (dead) cells was calculated to assess percent cell survival.

FLVCR2 mRNA expression analysis. cDNAs from multiple human tissues (MTC human panel I and human immune system panel; Clontech Laboratories, Mountain View, CA) were analyzed for FLVCR2 gene expression by quantitative PCR (QPCR) using FLVCR2 primers (5′-TTGTCCTGGTGTT TAGCTGCTACT-3′ and 5′-AGTCAATGGCAAAGGCACTGACAC-3′) and the MX3000 real-time thermocycler with PerfeCTa SYBR green supermix (Quanta Biosciences, Gaithersburg, MD). FLVCR2 transcript expression was normalized against actin transcript expression (actin-specific primers ActB-F [5′-TGCGTGACATTAAGGAGAAG-3′] and ActB-R [5′-AGGAA GGAAGGCTGGAAGAG-3′]).

Knockdown of FLVCR2. Two 25-base-length small interfering RNA (siRNA) oligonucleotides, S1 (FLVCR2HSS124723) and S2 (FLVCR2HSS124724), specific to human FLVCR2 (Invitrogen), were used to knock down FLVCR2 expression. The specificities of FLVCR2 siRNAs were validated as previously described (42). For the knockdown of FLVCR2, target cells were transfected with S1, S2, or scrambled (Scr) siRNA (Invitrogen) by using procedures described previously (42). At 2 days posttransfection, cells were assayed for ZnMP uptake.

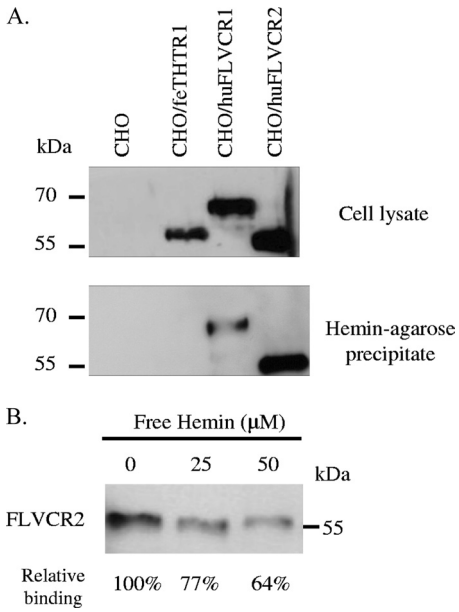


FIG. 1. Hemin binding by FLVCR1 and FLVCR2. (A) Immunoblots of cell lysate samples from CHO cells and CHO cells expressing the HA-tagged huFLVCR1, huFLVCR2, and feTHTR1 proteins incubated with and without hemin-agarose. Proteins were detected by using anti-HA-horseradish peroxidase (HRP) antibody. (B) Hemin-agarose binding by HA-tagged huFLVCR2 after preincubation with 25 μM and 50 μM free hemin. The percent bound huFLVCR2 relative to the control (0 μM) is shown below the blot.

RESULTS

FLVCR2 binds heme. Based on previous findings that retroviruses can use functionally related proteins as receptors for infection (5, 24, 45), we hypothesized that FLVCR2 transports heme in a manner analogous to that of FLVCR1. To investigate this, we first tested the ability of FLVCR2 to bind to heme. This was determined by a "pulldown" assay using hemin-agarose and cell lysates from Chinese hamster ovary (CHO) cells expressing hemagglutinin (HA)-tagged human FLVCR2 (CHO/huFLVCR2) (42). We also used cell lysates prepared from control CHO cells, CHO cells expressing HA-tagged human FLVCR1 (CHO/huFLVCR1), and CHO cells expressing the feline leukemia virus subgroup A (FeLV-A) receptor feTHTR1 (CHO/feTHTR1) (25), identified previously as a transporter of thiamine (15). As shown in Fig. 1A, the huFLVCR2, huFLVCR1, and feTHTR1 proteins were efficiently expressed in CHO cells. As expected, we found that HA-tagged huFLVCR1 coprecipitated with hemin-agarose, consistent with FLVCR1 being a heme transporter, whereas the thiamine transporter/receptor feTHTR1 failed to coprecipitate with hemin-agarose (Fig. 1A). Interestingly, huFLVCR2 also coprecipitated with hemin-agarose (Fig. 1A), and the preincubation of huFLVCR2 cell lysate with 25 μM or 50 μM free hemin reduced the binding of huFLVCR2 to hemin-agarose by 23% and 36%, respectively (Fig. 1B). These results indicate that huFLVCR2 binds heme.

FLVCR2 is an importer of heme. Because both FLVCR1 and FLVCR2 bind heme (Fig. 1A) and function as receptors for the FeLV-C variant FY981 (42), we initially speculated that FLVCR2, like FLVCR1, is a heme exporter. However, Quigley

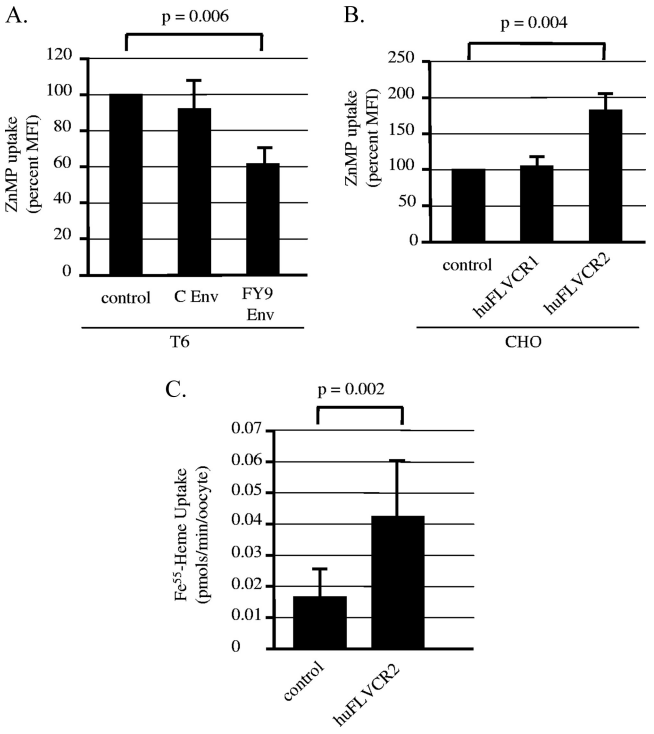


FIG. 2. Heme import by human FLVCR2. (A) Inhibition of ZnMP uptake by expression of the FY981 FeLV envelope (Env) protein in TELCeB6 (T6) cells. T6 cells (control) and T6 cells expressing the FeLV-C (T6/C Env) or FY981 (T6/FY Env) Env protein were incubated with ZnMP and analyzed by flow cytometry to determine the mean fluorescence intensity (MFI). The percent MFI value of ZnMP uptake was determined relative to the uptake by control T6 cells. MFI values are averages from three uptake experiments. Standard deviation (SD) bars are shown. (B) CHO cells (control) and CHO cells expressing huFLVCR1 or huFLVCR2 were incubated with ZnMP, and the MFI was determined by flow cytometry. ZnMP uptake is relative to that of control CHO cells and is the average of data from three uptake experiments. (C) [^{55}Fe]hemin uptake in *Xenopus* oocytes. Oocytes injected with huFLVCR2 cRNA or water were incubated with [^{55}Fe]hemin at room temperature. [^{55}Fe]hemin uptake is an average of 10 oocytes used per group for each experiment. Results were repeated in three separate uptake experiments. The *P* value was calculated by using a Student's *t* test.

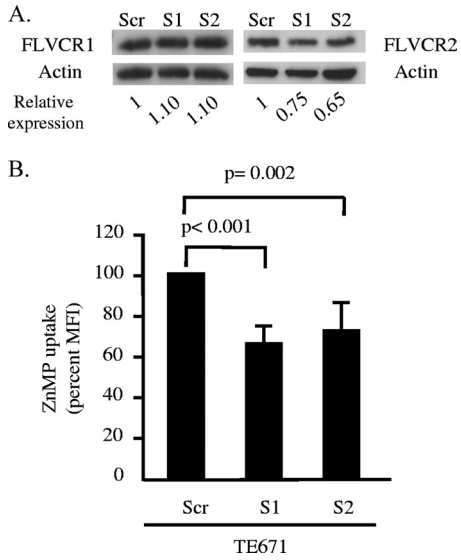
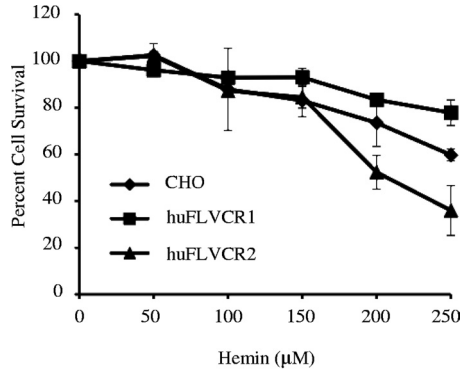


FIG. 3. Downregulation of human FLVCR2 disrupts heme uptake. (A) Validation of FLVCR2 knockdown by FLVCR2 siRNA. CHO/huFLVCR2 or CHO/huFLVCR1 cells were transiently transfected with huFLVCR2-specific siRNA oligonucleotide S1 or S2 or with scrambled (Scr) siRNA, and the expression of the HA-tagged huFLVCR2 or huFLVCR1 (control) protein was analyzed by Western blotting. (B) ZnMP uptake in human TE671 cells transiently transfected with FLVCR2-specific siRNA oligonucleotide S1 or S2 or scrambled (Scr) siRNA. The MFI value of ZnMP uptake was determined by flow cytometry, and the percent MFI was calculated relative to ZnMP uptake by TE671 cells expressing the Scr siRNA oligonucleotide. The MFI is the average of data from three uptake experiments. SD bars are shown. The *P* value was calculated by using a Student's *t* test.

and colleagues (37) previously reported that huFLVCR2 (hFLVCR14q in their study) does not export of heme. Therefore, we assessed the ability of huFLVCR2 to import heme. We first analyzed the ability of human cells expressing the envelope (Env) protein from FeLV-C (C-Env) or the Env protein from the FeLV-C variant FY981 (FY9-Env) to import zinc mesoporphyrin (ZnMP), a fluorescent heme analog. Because the binding of the retroviral envelope protein to the virus receptor in infected cells disrupts the normal function of the receptor (18, 37, 39), we hypothesized that if huFLVCR2 is an importer of heme, then the expression of the FY981 FeLV Env protein should disrupt the uptake of ZnMP in human cells. We therefore tested ZnMP uptake in human TELCeB6 (T6) cells expressing C-Env or FY9-Env (42). T6 cells are retroviral packaging cells derived from human TE671 (11), which express both huFLVCR1 and huFLVCR2 (7). T6/C-Env and T6/FY9-Env cells were previously used by our group to produce infectious *lacZ*-harboring pseudotype FeLVs (42). Control T6, T6/C-Env, and T6/FY9-Env cells were incubated with ZnMP, and the mean fluorescence intensity (MFI) of ZnMP uptake was determined by flow cytometry. Interestingly, T6/FY9-Env cells showed an approximately 40% reduced uptake of ZnMP compared to ZnMP uptake by control T6 cells (Fig. 2A). There was no significant change in ZnMP uptake by T6/C-Env cells compared to the control. This finding shows that the expression of FY9-Env downmodulates ZnMP uptake and indicates a heme import function for FLVCR2. To further validate FLVCR2 function as an importer of heme, we tested ZnMP uptake in CHO (control), CHO/huFLVCR1, and CHO/huFLVCR2 cells. We observed an approximately 1.8-fold increase in ZnMP uptake by CHO/huFLVCR2 cells compared to CHO cells, whereas there was no significant change in ZnMP uptake by CHO/huFLVCR1 cells (Fig. 2B). We also tested heme transport by FLVCR2 in *Xenopus* oocytes using [^{55}Fe]hemin. We observed a 2.3-fold increase in [^{55}Fe]hemin uptake in oocytes injected with human FLVCR2 cRNA compared to oocytes injected with water (Fig. 2C). These results collectively indicate that huFLVCR2 is an importer of heme.

Expression of FLVCR2-specific small interfering RNA inhibits heme import. To further confirm the cellular function of huFLVCR2 as a heme importer, we tested ZnMP uptake by human TE671 cells expressing either of two FLVCR2-specific small interfering RNA (siRNA) oligonucleotides (S1 and S2). We have previously reported that the expression of S1 and S2 in CHO/huFLVCR2 cells reduces the expression of huFLVCR2, whereas S1 and S2 siRNAs do not downregulate huFLVCR1 protein expression (Fig. 3A) (42). The transient



	p-value	
	200μM	250μM
huFLVCR2	0.013	0.040

FIG. 4. FLVCR2-expressing CHO cells are more sensitive to heme toxicity. CHO cells (control) and CHO/huFLVCR1 and CHO/huFLVCR2 cells were plated in a 24-well plate and exposed to increasing doses of hemin for 24 hours. Cell survival was analyzed by staining with trypan blue (which stains dead cells), and the percentage of unstained viable cells compared to stained dead cells was calculated. Results are averages of data from three experiments. SD bars and *P* values are shown. The *P* value was calculated by using a Student's *t* test.

expression of S1 and S2 in human TE671 cells caused 34% and 25% decreases in ZnMP uptake, respectively, relative to ZnMP uptake by TE671 cells expressing scrambled siRNA (Fig. 3B), thus confirming an import function for FLVCR2.

FLVCR2-expressing cells are more sensitive to heme toxicity. We hypothesized that CHO/huFLVCR2 cells would be more sensitive to heme toxicity due to enhanced heme uptake. Using a previously described heme toxicity assay (35), we exposed CHO, CHO/huFLVCR1, and CHO/huFLVCR2 cells to enhanced doses of hemin, and the percent cell survival was determined by using trypan blue staining. Hemin concentrations of 200 μM and 250 μM resulted in 52% and 36% survivals of CHO/huFLVCR2 cells, respectively, compared to 74% (200 μM) and 60% (250 μM) survivals of control CHO cells (Fig. 4). CHO/huFLVCR1 cells were more resistant to heme toxicity, with approximately 83% and 78% survival rates at concentrations of 200 μM and 250 μM hemin, respectively. These findings show that CHO/huFLVCR2 cells are more sensitive to heme toxicity.

Expression of FLVCR2 in human tissues. We next analyzed the tissue distribution of FLVCR2 mRNA by quantitative real-time PCR using cDNA isolated from various human tissues and primers specific for the huFLVCR2 sequence. As depicted in Fig. 5, relative to β-actin expression, we found that huFLVCR2 mRNA was expressed in nonhematopoietic tissues, with relatively abundant expression in brain, placenta, lung, liver, and kidney. FLVCR2 mRNA was also expressed in hematopoietic tissues (fetal liver, spleen, lymph node, thymus, leukocytes, and bone marrow). Our finding suggests that FLVCR2 mRNA is expressed in a broad range of human tissues.

DISCUSSION

Mutations in the MFS transporter member FLVCR2 were recently shown to be associated with Fowler syndrome (22, 26). In this study, we provide data that collectively indicate that the cellular function of FLVCR2, originally identified by our group as a receptor for the FeLV-C variant FY981 (42), is as an importer of heme and is distinct from that of the sequence-related protein FLVCR1, which was identified to function as an exporter of heme (37). Our findings provide a physiological function for FLVCR2 that could have important implications in our understanding of the pathology of Fowler syndrome.

Previous characterization of FLVCR2 implicated this protein to be a calcium-chelate transporter (6). This was based on FLVCR2 expression in cellular structures associated with rapid calcium exchange. Although we cannot exclude the pos-

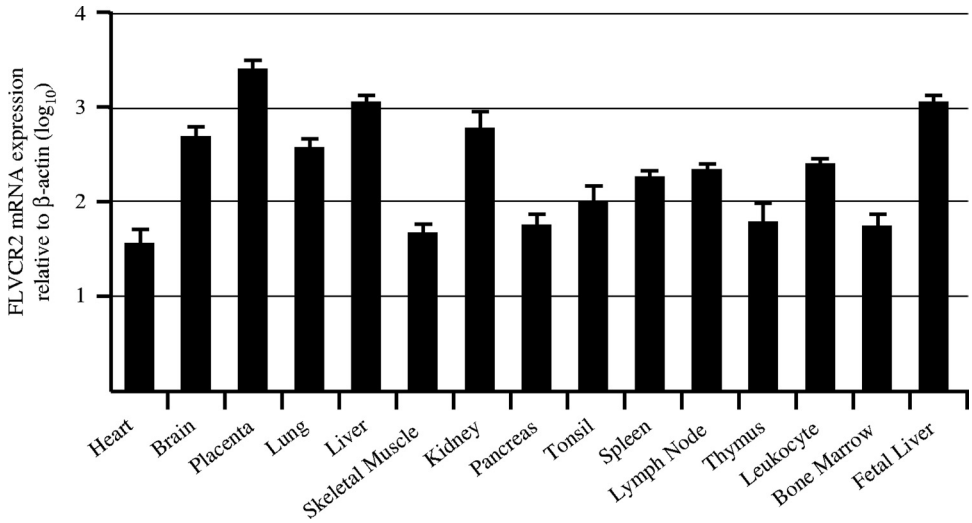


FIG. 5. FLVCR2 transcript expression in human tissues. The tissue distribution of FLVCR2 mRNA was determined by quantitative real-time PCR using cDNA from various hematopoietic and nonhematopoietic human tissues and using FLVCR2-specific primers. The FLVCR2 mRNA expression level is relative to that of β-actin mRNA expression. Data presented are averages of data from three independent real-time PCR experiments. SD are shown.

sibility that FLVCR2 transports calcium, our results clearly indicate a heme import function for FLVCR2. We show that FLVCR2 binds heme (Fig. 1A), which is consistent with the heme binding property of the heme exporter FLVCR1 (Fig. 1A) and of the previously described heme/porphyrin transporter ABCG2 (20). FLVCR2 but not the thiamine transporter THTR1 coprecipitated with hemin-agarose (Fig. 1A). Furthermore, the binding of FLVCR2 to hemin-agarose was competed by free hemin (Fig. 1B). The sequence homology of FLVCR2 and FLVCR1 (7, 36, 42, 46) initially implicated the cellular function of FLVCR2 as a heme exporter. However, a report by Quigley and colleagues (37) clearly shows that FLVCR2 does not export heme. In this study, we carried out several distinct experiments that collectively implicate FLVCR2 as an importer of heme. First, we consistently observed a 1.8-fold increase in the uptake of ZnMP, a heme analog, by FLVCR2-expressing CHO (CHO/huFLVCR2) cells compared to uptake by control CHO and CHO/huFLVCR1 cells (Fig. 2B). Furthermore, *Xenopus* oocytes injected with FLVCR2 cRNA showed a 2.3-fold-enhanced uptake of [^{55}Fe] hemin compared to oocytes injected with water (Fig. 2C). The approximately 2-fold change in heme transport by FLVCR2 is consistent with the heme transport reported previously for the heme/folate transporter HCP-1/PCFT (43), the heme transporter HRG-1 (38), and the heme exporter FLVCR1 (37). The fold change in transport is also similar to that reported for other retroviral receptors that have been identified as transporter proteins. These include transport by the neutral amino acid transporter ASCT2, used as a receptor for entry by the feline RD114 endogenous retrovirus, and the P_iT1 (SLC20A1) and P_iT2 (SLC20A2) inorganic phosphate symporters, used as receptors by FeLV-B, gibbon ape leukemia virus, and the 10A1 and amphotropic murine leukemia viruses (30). It is interesting that we did not observe a reduced uptake of ZnMP in CHO cells expressing FLVCR1, which is an exporter of heme. This can be explained by the protocol that we used in this experiment, which did not contain a washout stage after the uptake of ZnMP, thus preventing the measurement of heme export. Second, based on receptor interference experiments, the expression of the FeLV FY981 Env protein downregulated ZnMP uptake in human cells, whereas ZnMP uptake was not disrupted in cells expressing the FeLV-C Sarma isolate Env protein (Fig. 2A), which uses FLVCR1 as a receptor. Third, the expression of siRNA oligonucleotides S1 and S2, which specifically inhibit FLVCR2 expression (Fig. 3A), disrupted ZnMP uptake in human TE671 cells (Fig. 3B), the cells that served as the source of the original sequenced FLVCR2 clone (7). Finally, we found that FLVCR2-expressing CHO cells were more sensitive to heme toxicity than CHO and CHO/huFLVCR1 cells (Fig. 4). We infer from this result that the overexpression of FLVCR2 in CHO cells enhances the uptake of heme, leading to increased sensitivity to heme toxicity. We also found that FLVCR1-expressing CHO cells were less sensitive to heme toxicity, consistent with the heme export function of FLVCR1 (37).

Our results indicate that FLVCR2 mRNA is present in a variety of human tissues with strong expression in the brain (Fig. 5). This is in agreement with the expression profile reported in the database (http://www.genecards.org/cgi-bin/carddisp.pl?gene=FLVCR2) and with the *in situ* hybridization patterns observed with murine FLVCR2 probes in mouse tissue, where expression was robustly observed in brain and the spinal cord (23, 26, 48). We must emphasize that the FLVCR2 mRNA expression level in human tissues may not necessarily correlate with the protein expression level. Because an FLVCR2-specific antibody is not available, we were unable to determine FLVCR2 protein expression in human tissues.

It is interesting that FLVCR2 and FLVCR1 function as receptors for the FeLV-C variant FY981 (42) yet have opposing heme transport functions. Although retroviruses have been shown to use receptor proteins that are structurally and functionally related (30, 44), it is clear that retroviruses adapt to use related receptor proteins because of sequence and structural similarities and not because of functional similarities (49). We have previously reported (7) that an N463D mutation in extracellular loop 6 of the FLVCR2 heme importer is sufficient to allow FLVCR2 to mediate infection by FeLV-C Sarma, whose nascent receptor is FLVCR1 (36, 46). The distinct functions of FLVCR2 and FLVCR1 are consistent with the association of these proteins with different diseases. Whereas mutations in FLVCR2 have been associated with Fowler syndrome (22, 26), FLVCR1 disruption is suggested to play a major role in the erythropoietic defect observed for Diamond Blackfan anemia (19, 37, 40). Interestingly, the disruption of FLVCR1 in DBA is not caused by mutations in the FLVCR1 coding region but by enhanced aberrant splicing of the FLVCR1 transcript that disrupts FLVCR1 protein expression, cell surface localization, and heme export functions (19, 40). It is not clear how mutations in FLVCR2 are associated with Fowler syndrome. Fowler syndrome is a recessively inherited lethal disorder characterized by proliferative vasculopathy; brain stem, basal ganglia, and spinal cord ischemic lesions with calcification; and hydranencephaly-hydrocephaly (16). Five affected fetuses from three consanguineous families were found to have a common Thr430Arg mutation in FLVCR2. The Thr430 residue is located in an intracellular loop region of FLVCR2 and is highly evolutionarily conserved not only among different FLVCR2 orthologs (26) but also between FLVCR1 and FLVCR2 (7). In two additional consanguineous families, FLVCR2 mutations were identified at residues 110/112, 158, 280, and 398, which are also evolutionarily conserved among FLVCR2 orthologs (26) and between FLVCR2 and FLVCR1 (7). The mutation at residue 158 introduces a stop codon, causing the premature termination of the transporter protein. The other mutations are substitutions, except for the deletion and insertion present at residue 110/112. It is probable that mutations at these conserved residues result in a disruption of the function of FLVCR2, thereby affecting heme homeostasis. Heme (iron and protoporphyrin IX) not only is an important source of iron (8) but also is found as a major component of hemoproteins, such as cytochromes, nitric oxide synthase, and hemoglobin, that play pivotal roles in many cellular processes (32, 34). It is interesting that three cases of Fowler syndrome from one nonconsanguineous family were suggested to also have a deficiency in complexes III and IV of the mitochondrial respiratory chain/electron transport chain (ETC) (9, 10). The mitochondrial ETC consists of four complexes (complexes I to IV) that are essential for the production of ATP by oxidative phosphorylation (29). Complexes III and IV (cytochrome *c* reductase and oxidase, respectively) are mul-

tisubunit complexes consisting of distinct cytochromes. Interestingly, heme deficiency was shown to selectively interrupt the assembly of complex IV in human fibroblasts (2). It is tempting to speculate that a disruption in FLVCR2 could lead to a disruption of complex IV assembly and the subsequent dysfunction of the ETC. Since young neurons that migrate to the neocortex become aerobic and dependent on oxidative phosphorylation, a severe dysfunction of the ETC caused by FLVCR2 mutations may cause neurodegeneration and developmental abnormalities, leading to the hydranencephaly-hydrocephaly found for Fowler syndrome (9). It is unclear whether the proliferative vasculopathy that is also observed for Fowler syndrome is primary or secondary to the neurodegeneration. Additional investigations into the role of FLVCR2 in the function of the ETC may provide further insight into the mechanism of Fowler syndrome.

The mutations in the heme importer FLVCR2 observed for Fowler syndrome could also result in an inappropriate regulation of heme import resulting in iron accumulation. Iron accumulation is relevant not only to Fowler syndrome but also to other neurodegenerative diseases such as Friedreich ataxia and Halleryorden-Spatz syndrome (33). Iron overload could also account for the severe bone abnormalities observed upon fetal autopsy in individuals with Fowler syndrome and represents a hallmark of a hypervasculated, poorly developed brain. Furthermore, it was recently suggested by reports of Tsay et al. (47) that iron overload results in bone abnormalities in mice which are directly attributable to bone readsorption and oxidative stress. Thus, a dysregulation of a heme importer resulting in heme iron overload could play a causal role in Fowler syndrome.

In conclusion, the results of this study identify FLVCR2 as an importer of heme. The identification of the cellular function of FLVCR2 could have major implications for our understanding of the disease, particularly in light of the recent identification of a close association of mutations in FLVCR2 to Fowler syndrome (22, 26).

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ADDENDUM IN PROOF

Since the acceptance of this paper, a new paper describing the association of FLVCR2 mutations with Fowler syndrome was published (S. Thomas, F. Encha-Razavi, L. Devisme, H. Etchevers, B. Bessieres-Grattagliano, G. Goudefroye, N. Elkhartoufi, E. Pateau, A. Ichkou, M. Bonnière, P. Marcorelle, P. Parent, S. Manouvrier, M. Holder, A. Laquerrière, L. Loeuillet, J. Roume, J. Martinovic, S. Mougou-Zerelli, M. Gonzales, V. Meyer, M. Wessner, C. B. Feysot, P. Nitschke, N. Leticee, A. Munnich, S. Lyonnet, P. Wookey, G. Gyapay, B. Foliguet, M. Vekemans, and T. Attié-Bitach, Hum. Mutat. **31:**1134–1141, 2010).

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