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Iron, Copper, and Zinc Transport: Inhibition of Divalent Metal Transporter 1 (DMT1) and Human Copper Transporter 1 (hCTR1) by shRNA

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Abstract Iron (Fe), copper (Cu), and zinc (Zn) fulfill various essential biological functions and are vital for all living organisms. They play important roles in oxygen transport, cell growth and differentiation, neurotransmitter synthesis, myelination, and synaptic transmission. Because of their role in many critical functions, they are commonly used in food fortification and supplementation strategies globally. To determine the involvement of divalent metal transporter 1 (DMT1) and human copper transporter 1 (hCTR1) on Fe, Cu, and Zn uptake, Caco-2 cells were transfected with four different shRNA plasmids to selectively inhibit DMT1 or hCTR1 transporter expression. Fe and Cu uptake and total Zn content measurements were performed in shRNA-DMT1 and shRNA-hCTR1 cells. Both shRNA-DMT1 and shRNA-hCTR1 cells had lower apical Fe uptake (a decrease of 51% and 41%, respectively), Cu uptake (a decrease of 25.8% and 38.5%, respectively), and Zn content (a decrease of 23.1% and 22.7%, respectively) compared to control cells. These results confirm that DMT1 is involved in active transport of Fe, Cu, and Zn although Zn showed a different relative capacity. These results also show that hCTR1 is able to transport Fe and Zn.

Keywords Iron · Copper · Zinc · DMT1 · hCTR1 · shRNA

Iron (Fe), zinc (Zn), and copper (Cu) have important functions in the body. Iron is involved in transport and storage of oxygen, oxidative phosphorylation, metabolism of neurotransmitters, and DNA synthesis [1]. Zinc participates as a component of numerous enzymes, in maintenance of structural integrity of proteins and membranes, in binding of hormones to their receptors, and in regulation of gene expression [2–4]. Copper is a cofactor for several enzymes involved in electron transport, superoxide dismutation, melanin synthesis, cross-linking of collagen and elastin, neurotransmitter synthesis, and iron oxidation [5]. Divalent metal transporter 1 (DMT1) is involved in iron uptake at the enterocyte [6, 7] while copper uptake occurs via both DMT1 and human copper transporter 1 (hCTR1) [6–8]. The two zinc transporter families are the ZnT (solute-linked carrier SLC30 which are mainly exporters) and the Zip (solute-linked carrier SCL39 which are mainly importers). To date, 10 ZnT and 14 Zip transporters have been identified in mammals [9].

Deficiencies of trace elements such as iron and zinc are important nutritional and public health problems affecting the populations of developing countries as well as some developed countries. Iron deficiency is the most prevalent nutritional disorder in the world and the principal cause of anemia in infants, children, adolescents, and women of

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childbearing age [10]. In the developing world, iron deficiency coexists with other micronutrient deficiencies, such as zinc, copper, and vitamin A [11]. The prevalence of zinc deficiency is not well defined because, unlike iron, zinc does not have laboratory biomarkers with high sensitivity and reliability [12, 13] and is therefore more difficult to study. However, it is suggested that zinc deficiency is highly prevalent in developing countries and at a magnitude similar to iron deficiency [14, 15]. Copper deficiency has been identified mainly in malnourished children and children fed diets based on nonfortified milk, especially if they were premature or have recurrent episodes of diarrhea [5].

Dietary modifications and/or diversification, food fortification, and supplementation are the main strategies used to prevent micronutrient deficiencies globally [14, 16]. There is concern about the potential competition between iron, zinc, and copper when all three elements are used together. Current knowledge of the interaction between Zn to Fe and Cu to Fe at the cellular level and in the whole organism is incomplete. A negative interaction between Fe and Zn when taken together has been explained by an increase in the relative abundance of ions that compete for the same route of intestinal absorption. We recently provided evidence of this by demonstrating that combined administration of zinc and of iron at the molar ratios Zn to Fe ≥5:1 in aqueous solutions under fasting conditions lowers the bioavailability of iron in a dose-dependent way [17].

Additional studies have provided information about uptake of Fe, Zn, and Cu at the cellular level. Using Caco-2 cells (a human cell line of intestinal epithelium), it was shown that Zn promotes the transepithelial flux of Fe by increasing DMT1 gene expression and concentration [18]. Cu uptake by Caco-2 cells was reduced by ~50% when DMT1 expression was blocked with an antisense oligonucleotide [7]. In addition, we have provided evidence that Fe and Cu compete for DMT1 transport at the enterocyte [19] (though in human studies we did not observe a negative effect of copper on iron absorption [20]). While there is substantial information regarding the role of DMT1 in Fe and Cu transport, a role in Zn transport has been questioned [21–23]. Zn is preferentially taken up by the enterocyte by the Zip 4 transporter [24], however its divalent condition means it can be incorporated by DMT1. Zn could potentially affect tissue utilization of Fe because competition for DMT1 could occur not only in intestinal cells, but in other tissues and organs, regardless of the actual role of DMT1 in Zn transport. The aim of the current study was to provide evidence regarding the role of DMT1 and hCTR1 on Fe, Cu, and Zn transport using an intestinal epithelial cell line.

Materials and Methods

Transfection of Caco-2 Cells with shRNA

Caco-2 cells were seeded in six-well plates and transfected with vectors SureSilencing shRNA Plasmids (SABioscience, Valencia, Ca, USA): (a) shRNA-DMT1 (Cat. KH05760N), clon 1 to 4 and negative control and (b) shRNA-hCTR1 (Cat. KH06147N), clone 1 to 4 and negative control. Briefly, 2.5 μg of each vector was diluted with 500 μL DMEM media without fetal bovine serum (FBS) and incubated with 2.5 μL PLUS Reagent (Invitrogen, Cat. 11514-015), and 8 μL Lipofectamine LTX (Invitrogen, Cat. 15338-500) for 25 min. The mix was added to cells with 2 mL of complete media. After 48 h of incubation at 37°C, 5% CO_2, and 80% humidity, transfected cells (shRNA cells) were trypsinized and reseeded at a dilution of 1:10. After 24 h of culture in regular media, cells were incubated with selection media DMEM, FBS 10%, with Geneticin 800 μg/mL (G418, Gibco; Cat. 11811031).

DMT1 and hCTR1 Real-Time PCR After cell selection, total RNA was isolated from shRNA-DMT1 and shRNA-hCTR1 cells using TRIzol reagent (Invitrogen, Cat. 15596-026), according to manufacturer's instructions. RNA was digested with DNase TURBO DNA-free (Ambion, AM1907) and quantified using Quant-iTTM RiboGreen RNA Reagent (Invitrogen, Cat. R11491). RNA (1 μg) was used to synthesize cDNA using AffinityScript cDNA Synthesis Kit (Stratagene, Cat. 600559). The relative abundance of DMT1 and hCTR1 RNA were quantified in LightCyclerTM system (Roche) using the Brilliant II SYBR Green QPCR Master Mix (Stratagene, Cat. 929548) from a 1:10 dilution of synthesized cDNA. The primers used were: DMT1-s: TTTGGAGCTTTCCTTCCAGA and DMT1-a: AGACCATCCATCCAGTCTGC; hCTR1-s: AGCAAGACCCTGTCTCAGAA and hCTR1-a: ATCCTTTGGCAGCCAGTCTA; and Actin-s: TGGCACCCAGCACAATGAAGA and Actin-a: GAAGCATTTGCGGTGGACGAT. The following program was used for the amplification of three genes: 10 min at 95°C and then 50 cycles, 5 s at 95°C, 15 s at 60°C, and 15 s at 72°C. The products of the PCR reaction were confirmed by a melting curve of PCR product. The relative abundances of RNA were calculated according to Pfaffl et al. [25], using actin as an expression control.

DMT1 and hCTR1 Western Blot

Cell lysate from shRNA cells was prepared incubating the cells for 15 min in lysis buffer [in millimolars: Tris–HCl 50,

pH 7.5, NaCl 150, EDTA 1, DTT 1, glycerol 5%, Triton X-100 1%, protease inhibitor cocktail 1× (Invitrogen)]. Protein concentration was determined by the Lowry method [26]. For DMT1 and hCTR1 immunodetection, 40 μg of cell lysate were separated in a 8% and 12% SDS-PAGE gel, respectively. Then, proteins were transferred to a nitrocellulose membrane (PROTRAD, Perkin Elmer) and blocked for 2 h at room temperature with 5% low fat milk in 1× TBST buffer (Tris base 20 mM, NaCl 150 mM, Tween-20 0.05%). DMT1 and hCTR1 were detected with a polyclonal antibody (DMT1 and hCTR1 were kindly donated by Dr. MT Nuñez and Dr. Magdalena Araya, University of Chile) diluted 1:1,000 in 1× TBST at 4°C over night. The membrane was washed three times for 10 min with 1× TBST and incubated with an anti-rabbit IgG antibody coupled to horseradish peroxidase 1:10,000 (Pierce, Cat. 31490) for 3 h at room temperature. The signal was detected with a chemiluminescent kit (SuperSignal West Pico Chemiluminescent Substrate, Pierce, Cat. 34080) and autoradiography. Membrane was stripped and reprobed againts actin (monoclonal IgG antibody 1:20,000, Sigma) as a loading control and a secondary antibody (anti-mouse IgG 1:20,000, Sigma).

^{55}Fe and ^{64}Cu Uptake Experiments

Control Caco-2 (cells transfected with negative control), DMT1-shRNA3, and hCTR1-shRNA1 cells were seeded in bicameral inserts of 0.33 cm^2 (Transwell, Costar) and the confluence was monitored by measuring the transepithelial electrical resistance (TEER) of the inserts. Cells were used when TEER reached a value over 250 Ωcm^2. The day of the experiment, cells were washed with transport buffer (in millimolars: HEPES 10, NaCl 140, KCl 5, $CaCl_2$ 1, Na_2HPO_4 1, $MgCl_2$ 0.5, glucose 5, pH 7.5). The apical compartment contained uptake buffer (in millimolars: PIPES 10, NaCl 140, KCl 5, $CaCl_2$ 1, Na_2HPO_4 1, $MgCl_2$ 0.5, glucose 5, pH 6.2), ^{55}Fe uptake solution (0,5–50 μM Fe as $FeCl_2$ to NTA 1:2, 2,0 μCi/mL, ascorbic acid 1:5) or ^{64}Cu uptake solution (0,5–50 μM Cu as $CuSO_4$, 1,6 μCi/mL, ascorbic acid 1:5). Transport buffer was added in basolateral compartment. Cells were incubated for 60 min at 37°C, then washed twice with cold PBS, 1 mM EDTA to remove nonspecifically membrane Fe bound, and then washed again with 1× PBS. The membranes and the basolateral media were placed in scintillation vials, and Ultra Fluoride solution was added. Radioactivity was determined in a beta counter.

Quantification of Intracellular Zn Concentration

Control Caco-2 (cells transfected with negative control), DMT1-shRNA3, and hCTR1-shRNA1 cells were seeded in bicameral inserts (4.7 cm^2), and the same protocol described above was followed. In the apical compartment, uptake buffer with Zn was added (5 and 100 mM Zn as $ZnSO_4$, ascorbic acid 1:5). Transport buffer was added in the basolateral compartment. Cells were incubated for 90 min at 37°C, then inserts were washed twice with cold PBS, 1 mM EDTA and once with PBS. The membranes and the basolateral media were placed in 1.5-mL tubes and digested with concentrated 65% nitric acid, diluted 1:1 with H_2O. The concentration of Zn was determined by flame atomic absorption spectrometry

Results

DMT1 and hCTR1 shRNA Transfections

Transfection of Caco-2 cells with shRNA plasmids to inhibit DMT1 or hCTR1 transporters expression produced four different clones. For uptake experiments, we used clones that showed the higher inhibition of relative abundance of mRNA and protein expression for DMT1 and hCTR1. Relative abundance of mRNA for *dmt1* and *hctr1* (a), western blot for DMT1 and hCTR1 (b), and a densitometric analysis (c) of western blot are showed in Fig. 1. We decided to use clone 3 for DMT1 and clone 1 for hCTR1 for ^{55}Fe uptake and competition studies.

Fe Uptake in shRNA-DMT1 and shRNA-CTR1 Cells

Both shRNA-DMT1 and shRNA-hCTR1 cells had lower apical Fe uptake than control cells. Fe uptake in shRNA-DMT1 and shRNA-hCTR1 cells increased from 0.005 to 0.149 and from 0.007 to 0.174 nmol Fe/mg protein/h, respectively, compared with control cells (0.005 to 0.276 nmol Fe/mg protein/h; two-way ANOVA, $p<0.001$ for Fe treatment, cell type, and interaction). Fe uptake in shRNA-DMT1 and shRNA-hCTR1 cells decresed 51% and 41%, respectively (Fig. 2). Kinetic parameters showed that the V_{max} in shRNA-DMT1 and shRNA-hCTR1 cells was half that of control cells (0.245±0.029; 0.261±0.027, and 0.424±0.034 nmol Fe/mg protein/h, respectively; one-way ANOVA, $p<0.005$; Tukey's multiple comparison test compared shRNA-DMT1 and shRNA-CTR1 to control, $p<0.001$). The highest K_m was observed in shRNA-DMT1 cells compared to shRNA-hCTR1 and control cells (33.5±7.6, 26.7±5.4, and 27.4±4.5 μM, respectively; one-way ANOVA, p = NS).

Cu Uptake in shRNA-DMT1 and shRNA-CTR1 Cells

^{64}Cu uptake in DMT1-3 and hCTR1-1 shRNA clones showed that Cu uptake (Fig. 3) was lower than in control

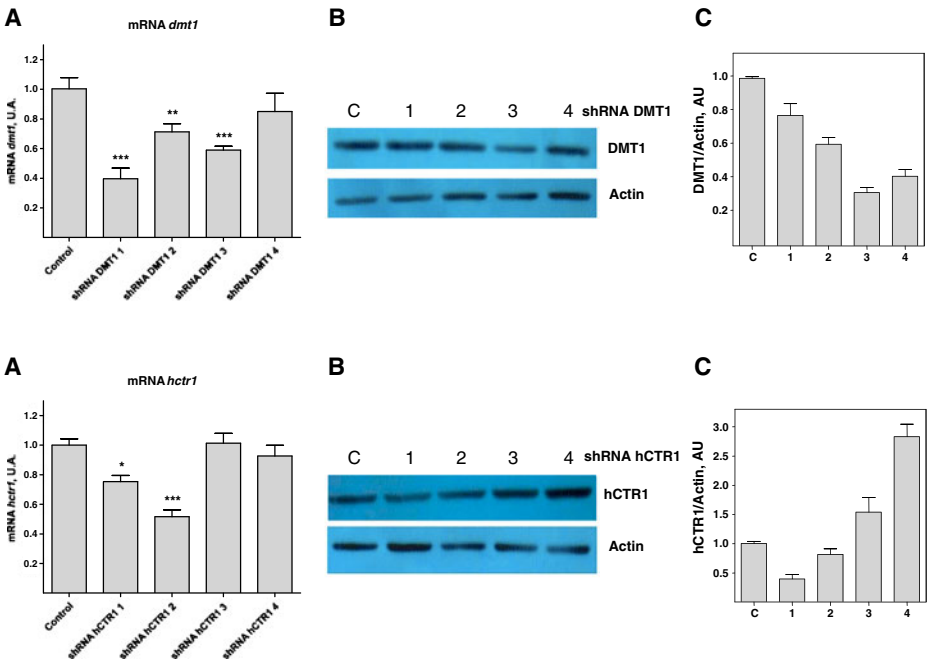

Fig. 1 DMT1 and hCTR1 shRNA transfections. Four clones of DMT1 and hCTR1 shRNA were transfected in Caco-2 cells to inhibit DMT1 or hCTR1 transporters expression. **(a)** Relative abundance of mRNA for DMT1 and hCTR1; **(b)** Western blot for DMT1 and hCTR1 (figure representative of three different experiments); **(c)** densitometric analysis of western blot **(b)** (n=3)

cells. In DMT1-3 and hCTR1-1 cells Cu uptake increased from 0.39 to 109.9 and 0.36 to 89.4 nmol/mg protein/h, respectively, compared to control cells (1.2 to 146.7 nmol/mg protein/h). These results indicate a decrease of 25.8% for DMT1-3 cells and 38.5% in hCTR1-1 cells (two-way ANOVA, $p<0.001$ for Cu concentration, cell type and interaction). Kinetic parameters showed a similar behavior in V_{max} (0.138±0.017, 0.161±0.025 and 0.148±0.004 nmol/mg protein/h in shRNA-DMT1, shRNA-hCTR1 and control cells, respectively; one-way ANOVA, p = NS). However, the K_m was higher in shRNA-DMT1 and shRNA-hCTR1 cells compared to control cells (27.1±6.8, 55.1±14.1 and 13.2±1.1 μM, respectively; one-way ANOVA, $p<0.009$, Tukey's multiple comparison test shRNA-hCTR1 vs. control cells, $p<0.01$).

Total Intracellular Zn in shRNA-DMT1 and shRNA-hCTR1 Cells

Intracellular Zn in DMT1-3 and hCTR1-1 shRNA clones showed that total Zn at baseline were similar to control cells (3.99±0.27, 4.82±0.26 and 4.49±0.62 nmol/mg protein, respectively; one-way ANOVA, NS; Fig. 4). After 90 min incubations with 100 μM of Zn, intracellular Zn in DMT1-

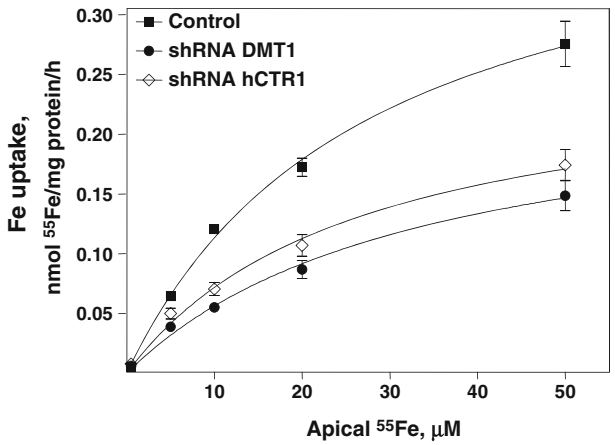

Fig. 2 Fe uptake by Caco-2 cells transfected with DMT1 or hCTR1 shRNA. shRNA-DMT1 (*filled circle*), shRNA-hCTR1 cells (*empty diamond*), and control cells (*filled square*) were incubated with different ^{55}Fe concentrations (0.5–50 μM). Fe uptake was expressed as a nanomoles ^{55}Fe/milligram protein/hour

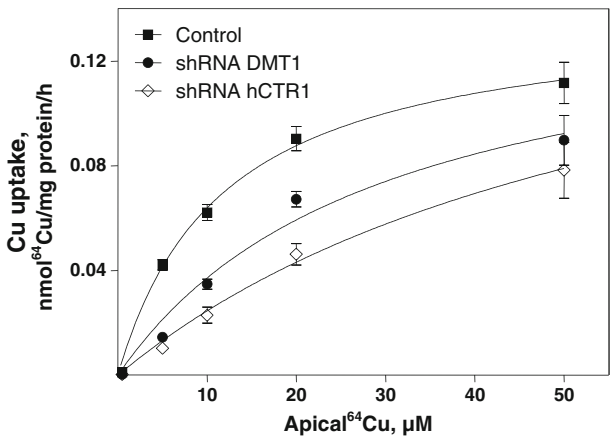

Fig. 3 Cu uptake by Caco-2 cells transfected with DMT1 or hCTR1 shRNA. shRNA-DMT1 (*filled circle*), shRNA-hCTR1 cells (*empty diamond*), and control cells (*filled square*) were incubated with differents ^{64}Cu concentrations (0.5–50 μM). Cu uptake was expressed as a nanomoles ^{64}Cu/milligram protein/hour

3 and hCTR1-1 shRNA cells was lower than in control cells (7.37±0.41 and 7.36±0.48 nmol/mg protein compared to 9.48±0.43 nmol/mg protein, respectively; two-way ANOVA: concentration $p<0.001$, treatment $p<0.01$ and interaction $p<0.02$; Fig. 4).

Discussion

Fe, Cu, and Zn are essential metals that interact and can exhibit competitive inhibition in transport. In this study we analyzed DMT1 and hCTR1-mediated uptake of Fe, Cu, and Zn in Caco-2 cells, inhibiting both transporters with shRNA. The V_{max} of Fe uptake decreased in both shRNA-DMT1 and shRNA-hCTR1cells, which confirms a decrease in the number of transporters in the cells.

As expected Fe uptake showed a greater decrease in shRNA-DMT1 cells. Cu uptake, on the other hand, was more greatly reduced in shRNA-hCTR1, confirming that hCTR1 is the main copper uptake protein [8, 27]. Finally, we showed that Fe and Cu uptake and total intracellular Zn concentration were decreased in both shRNA-DMT1 and shRNA-hCTR1 cells as compared to control cells. This demonstrates that under these experimental conditions, hCTR1 is able to transport Fe and Zn as well as Cu.

Previously, using antisense oligonucleotide againts DMT1, we showed that DMT1 is the main Fe transport (over 90% of the Fe) and transports Cu (around 50% of Cu) [7] in addition to hCTR1. In the current study, intracellular Zn content was lower in both shRNA-DMT1 and shRNA-hCTR1 cells compared to control cells. These results agree with those from Iyengar et al. [28], who reported similar V_{max} and K_m values and showed that cellular zinc status

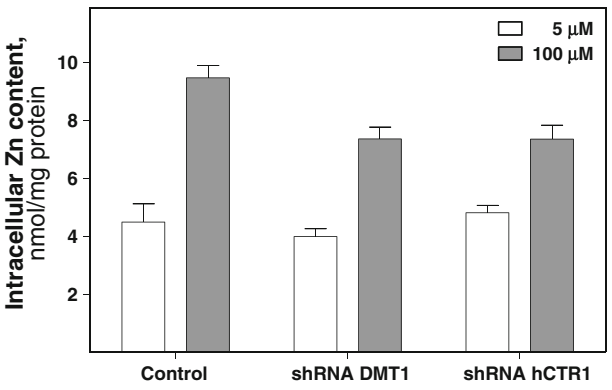

Fig. 4 Intracellular Zn concentration by Caco-2 cells transfected with DMT1 or hCTR1 shRNA. shRNA-DMT11 (*filled circle*), shRNA-hCTR1 cells (*empty diamond*), and control cells (*filled square*) were incubated with two differents Zn concentrations (5 or 100 µM). Intracellular Zn concentrations were expressed as a nanomoles Zn/milligram protein

influenced iron uptake and interaction with zinc during uptake. This implies that DMT1 might not simultaneously transport iron and zinc. Our data also agree with Gunshing et al. [6], who showed that DMT1 was able to transport Fe, Cu, and Zn.

The K_m values differed between cells only for Cu uptake. The higher value for the K_m was in shRNA-hCTR1 cells showing an effect of the RNA interference and also suggesting the possibility of a second transporter for Cu. For Fe and Zn there was no change in affinity for the metals, and hCTR1 was shown to be capable of transporting them both. Our results confirm that DMT1 has a lower affinity for zinc [29]. These results demonstrate that both DMT1 and hCTR1 can transport Fe, Cu, and Zn under experimental conditions.

Our results also support a recent finding from a human study, in which the coadministration of copper and iron, given in an aqueous solution, did not inhibit iron absorption even at Cu to Fe molar ratio of 8:1, suggesting that DMT1 is not a relevant transporter of Cu in humans [20]. It appears that in the absence of DMT1, both Fe and Zn would be able to stimulate hCTR1 expression, favoring Cu uptake. But when hCTR1 is inhibited, Cu uptake is not facilitated, which shows the importance of the hCTR1 transporter in Cu metabolism.

In regards to intracellular Zn concentrations, there are a wide range of reported values. Discrepancies in Zn measurements may reflect true variation in concentration in different cell types and/or cell state. Qin et al. [30] reported that Zn levels in HT29 cells change with cell state from 614 pM in resting to 1.25 nM in differentiated cells. In our study the incubation with 5 or 100 µM Zn produced a change in the intracellular Zn of 1.8-, 1.5-, and 2.1-folds in shRNA-DMT1, shRNA-hCTR1, and control cells, respectively. This suggests that both transporters may carry Zn. Currently 24 zinc transporters have been identified belonging to two gene families, ZnT and Zip. Both appear to have opposite roles in cellular zinc homeostasis: ZnT transporters promote zinc efflux from cells and into intracellular vesicles while Zip transporters increase intracellular cytoplasmic zinc [31]. The existence of numerous Zn transporters raises the question of whether DMT1 plays a physiological role in Zn metabolism [29]. Additionally, we have to consider the participation of other transporters not included in our experiment; Zip14, a member of the SLC39A zinc transporter family that is highly expressed in mouse duodenum, would be able to transport nontransferrin-bound iron into cells [32].

In the present study, we provide new evidence about the roles of DMT1 and hCTR1 in the metabolism of Fe, Cu, and Zn. Furthermore, our observations in cells exposed to Zn confirm the participation of both DMT1 and hCTR1 in Zn metabolism.

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