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MicroRNA-103a regulates sodium-dependent vitamin C transporter-1 expression in intestinal epithelial cells

Veedamali S. Subramanian^{1,2,3}, Subrata Sabui^{1,2,3}, Jonathan S. Marchant⁴, and Hamid M. Said^{1,2,3}

¹Department of Medicine, University of California, Irvine, CA 92697

²Department of Physiology/Biophysics, University of California, Irvine, CA 92697

³VA Medical Center, Long Beach, CA 90822

⁴Department of Cell Biology, Neurobiology & Anatomy, Medical College of Wisconsin, WI 53226

Abstract

Intestinal absorption of ascorbic acid (AA) occurs via a Na⁺-dependent carrier-mediated process facilitated through the human sodium-dependent vitamin C transporters-1 & -2 (hSVCT1 and hSVCT2). Many studies have shown that hSVCT1 (product of the *SLC23A1* gene) is expressed on the apical membrane of polarized enterocytes where it mediates AA absorption. hSVCT1 expression levels are therefore an important determinant of physiological vitamin C homeostasis. However, little is known about posttranscriptional mechanisms that regulate hSVCT1 expression in intestinal epithelia. In this study, we investigated regulation of hSVCT1 by microRNA (miRNA). A pmirGLO-*SLC23A1*-3'-UTR construct transfected into human intestinal cell lines (Caco-2 and NCM460 cells) showed markedly reduced luciferase activity. Bioinformatic analysis of the *SLC23A1*-3'-UTR predicted five miRNA binding sites (miR-103a, miR-107, miR-328, miR-384, and miR-499-5p) in the 3'-UTR. Expression of mature miR-103a was markedly higher compared to the other four putative miRNA regulators in both intestinal cell lines and mouse jejunal mucosa. Addition of a miR-103a mimic, but not a miR-103a mutant construct, markedly reduced the luminescence of the pmirGLO-*SLC23A1*-3'-UTR reporter. Reciprocally, addition of a miR-103a inhibitor significantly increased luciferase reporter activity. Addition of the miR-103a mimic led to a significant inhibition in AA uptake, associated with decreased hSVCT1 mRNA and protein expression in Caco-2 cells. In contrast, the miR-103a inhibitor increased AA uptake, associated with increased levels of hSVCT1 mRNA and protein. These findings provide the first evidence for posttranscriptional regulation of hSVCT1 by miRNA in intestinal epithelial cells.

Keywords

Vitamin C; *SLC23A1*; microRNA; transport; regulation; intestinal epithelia

Correspondence to: V. S. Subramanian, Med. Sci. I, C370, University of California, Irvine, CA 92697, Tel: 949-824-3084, vsubrama@uci.edu.

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1. Introduction

Vitamin C (ascorbic acid, AA) is an essential micronutrient needed for numerous cellular functions. This vitamin is a potent antioxidant and scavenger of free radicals and serves as a cofactor for several important enzymatic reactions [1]. Vitamin C deficiency can occur in settings where absorption and handling of this micronutrient is impaired: for example, within the elderly population, alcoholics and smokers [2–4]. Optimal vitamin C body homeostasis appears to protect against gallbladder diseases, cardiovascular disease, cancer, osteoporosis and cataract formation [5–8]. A growing body of evidence implicates vitamin C deficiency as a contributing factor in the pathogenesis of inflammatory bowel disease (IBD) [9, 10].

Humans have lost the ability to synthesis vitamin C endogenously due to the absence of the enzyme, gulonolactone oxidase (GLO), which catalyzes the last enzymatic step in ascorbate synthesis [11]. Humans therefore obtain vitamin C via intestinal absorption. Vitamin C exists in two forms as ascorbic acid (AA; reduced active form) and dehydroascorbic acid (DHA; oxidized form). Absorption of AA by intestinal epithelial cells occurs via a Na⁺-dependent carrier-mediated process that involves two human sodium-dependent vitamin C transporters –1 and –2 (hSVCT1 and hSVCT2, products of *SLC23A1* and *SLC23A2* genes, respectively) [12–14]. Both vitamin C transporters are expressed in the intestinal epithelial cells with hSVCT1 and hSVCT2 differentially targeted to the apical and basolateral membrane domains, respectively [15–17].

Recent studies have begun to decipher how the vitamin C uptake process is transcriptionally regulated, assisted by the cloning and characterization of the 5′-regulatory regions containing the promoters of the *SLC23A1* and *SLC23A2* genes [18, 19]. Much less is known about posttranscriptional regulation of *SLC23A1*, including the role of microRNAs (miRNA) in expression of this transport system in the gut. miRNAs are a class of small non-coding RNA molecules that regulate gene expression by binding to the 3′-untranslated region (3′-UTR) of their target mRNAs to suppress translation and/or regulate degradation [20–25]. Expression of miRNA is tissue-specific, and this localized expression is important for their tailored roles in development and regionalized function [26]. miRNAs play important roles in posttranscriptional regulation of many genes including several water-soluble vitamin transporter genes (*SLC23A2*, *SLC19A2*, *SLC52A3*, *SLC26A3* and *SLC15A1*) [20, 21, 22, 24, 25, 27]. In the intestine, non-coding miRNAs also play roles in differentiation, cell migration, and barrier function [28]. Intriguingly, the expression patterns of intestinal miRNAs are altered in various intestinal diseases, including small bowel syndrome and IBD [29–32].

Here, we have examined the role of miRNAs in posttranscriptional regulation of hSVCT1 and vitamin C uptake using intestinal epithelial cell lines. Results demonstrate a regulation of hSVCT1 by miR-103a which interacts with the 3′-UTR region of *SLC23A1* to decrease transcription and lower AA uptake.

2. Materials and methods

2.1. Materials

¹⁴C-AA (specific activity 2.8 mCi/mmol; radiochemical purity > 97%) was from Perkin Elmer (Boston MA). Caco-2 and NCM460 cells were from American Type Culture Collection (ATCC, Manassas, VA) and INCELL (San Antonio, TX), respectively. Primers for PCR amplifications, microRNA mimics and inhibitors were from Sigma (Sigma, St. Louis, MO/Thermo Fisher, Huntington Beach, CA). TaqMan probes and primers for mature microRNAs amplification were from Applied Biosystems (Foster City, CA). The monoclonal hSVCT1 antibody was from Santa Cruz Biotechnology Inc., (Santa Cruz, CA).

2.2. Generation of 3'-UTR reporter constructs for SLC23A1

A full length (453bp) and truncated fragments (322 and 240bp) of the *SLC23A1*-3'-UTR were amplified from human intestinal cDNA (Clontech, CA) by PCR. These sequences were cloned using primer sequences containing the *XhoI* and *SalI* sites into the multiple cloning site of the pmirGLO vector (Promega, Madison, WI; Table 1) as described previously [25]. The cloned full-length and truncated pmirGLO- *SLC23A1*-3'-UTR constructs were confirmed by DNA sequencing (Laragen, CA).

2.3. Bioinformatic analyses

The commonly used prediction algorithms TargetScan (<http://www.targetscan.org/>), MicroCosm Targets (<http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/>) and microRNA.org (<http://www.microRNA.org/microna/home.do>) were used to identify putative miRNAs targeting the *SLC23A1*-3'-UTR (hSVCT1) [20, 21, 25, 33]. The putative regulatory miRNAs were prioritized based on their target/free energy scores (Table 2) as well as their expression levels within the intestine [34–39].

2.4. Site-directed mutagenesis

Site-directed mutations were carried out in the miR-103a binding site of *SLC23A1*-3'-UTR using the Quick-change XL site-directed mutagenesis kit (Agilent, La Jolla, CA) and a mutant primer set (Table 1) as described previously [25]. The specific mutation in the *SLC23A1*-3'-UTR (103a binding site) were verified by DNA sequencing (Laragen, CA).

2.5. Cell culture, transient transfection, and luciferase assay

Human intestinal epithelial Caco-2 cells and human-derived colonic epithelial NCM460 cells were grown in Eagle's minimal essential medium (EMEM) and Ham's F-12 culture medium, respectively. Media was supplemented with fetal bovine serum (10% for Caco-2 cells and 20% for NCM460), penicillin, and streptomycin at 37°C in 5% CO₂-95% air environment in T-75 flasks. Caco-2 and NCM460 cells, grown in 12 well plates, were transiently transfected with 1.5µg of full-length or truncated pmirGLO-*SLC23A1*-3'-UTR or pmirGLO vector alone. The Caco-2 cells were transiently transfected with 1.5µg of pmirGLO-*SLC23A1*-3'-UTR or pmirGLO-*SLC23A1*-3'-UTR [103a mutant] in combination with 20 nM of different miRNA mimics (miR-103a, miR-107, miR-328, miR-384 and miR-499-5p), or a negative control miRNA mimic (Sigma /Thermo Fisher).

For inhibitor experiments, Caco-2 cells were transiently transfected with 1.5µg of pmirGLO-*SLC23A1-3'-UTR*, or pmirGLO-*SLC23A1-3'-UTR* [103a mutant], in combination with miR-103a inhibitor or negative control miRNA inhibitor (50 nM; Sigma, St. Louis, MO/ Thermo Fisher). Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used for transfecting miRNA mimics/inhibitor and/or the negative control mimic/inhibitor along with plasmids (*SLC23A1-3'-UTR* or pmirGLO vector). RNAiMax reagent (Invitrogen) was used for transfecting mimic/inhibitor or negative control mimic/inhibitor in Caco-2 cells. After 48h of post-transfection, cells were lysed in passive lysis buffer (Promega) and luciferase activity was measured using a dual luciferase assay kit (Promega). The 3'-UTR activity on the luciferase reporter gene was calculated as a ratio of firefly luciferase to renilla luciferase luminescence and expressed as percentage of relative luciferase units of control samples.

2.6. ¹⁴C-AA uptake

AA uptake by confluent Caco-2 monolayers treated with either miRNA mimic or inhibitor was performed as described previously [40, 41]. Briefly, Caco-2 cells were incubated in Krebs-Ringer (K-R) buffer (pH 7.4) at 37°C in the presence of ¹⁴C-AA (0.4µCi/ml) for 30 min. Monolayers were washed twice with ice-cold K-R buffer after this incubation period, before cells were lysed with NaOH followed by a neutralization with HCl. Radioactivity was measured using liquid scintillation counting (Beckman Coulter).

2.7. Real-time PCR (RT-qPCR)

Total RNA (2µg) isolated from Caco-2 cells was used to synthesize cDNA using i-Script reverse transcriptase kit (Bio-Rad, CA). These samples were used for quantitative real-time PCR (RT-qPCR) analyses with hSVCT1 or human β-actin (hβ-actin) specific primers (Table 1) as reported previously [40, 41]. hSVCT1 mRNA expression level was normalized relative to hβ-actin as a representative house-keeping gene and quantified by Ct method [42].

2.8. Quantification of mature miRNA expression

To quantify expression levels of mature miRNAs, total RNA was isolated from Caco-2, NCM460 cell lines and mouse jejunal mucosa (C57BL/6J wild-type, 4-6 weeks old male mice were euthanized by cervical dislocation and jejunum was removed, washed with cold PBS, then longitudinally opened to separate the mucosa) using the miRNeasy mini kit (Qiagen, Germantown, MD). Total RNA (10ng) was reverse transcribed using the TaqMan microRNA reverse transcription kit (Applied Biosystems, Foster city, CA) to synthesize cDNA. The reverse-transcription primers used for cDNA synthesis were provided within the TaqMan miRNA assay kit (Applied Biosystems). Mature miRNA levels [miR-103a (assay ID 000439), miR-107 (assay ID 000443), miR-328 (assay ID 000543), miR-384 (assay ID 000574) and miR-499-5p (assay ID 001352)] in Caco-2, NCM460 cell lines, and mouse jejunum were quantified utilizing TaqMan probes and Universal PCR master mix (Applied Biosystems). The constitutively expressed RNU6B miRNA (assay ID 001093) supplied by Applied Biosystems was used as a representative house-keeping gene. Animal use in this study was approved by the Institutional Animal Care and Use Committee (IACUC) at the Veterans Affairs Medical Center, Long Beach, CA.

2.9. Western blot analysis

Protein samples were isolated from '103a mimic' or '103a inhibitor' transfected Caco-2 cells as described previously [40, 41]. To determine the hSVCT1 expression level, 60 µg of total protein was loaded onto a NuPAGE 4-12% mini gel (Invitrogen). After separation, the gel was transferred to polyvinylidene difluoride (PVDF) membranes. To minimize non-specific binding, the blot was blocked with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) and membranes were probed with hSVCT1 (1:200 dilution) and hβ-actin (1:3000 dilution) antibodies. The membranes were then washed three times (7 min each) with a wash buffer containing PBS and 0.01% Tween-20. Subsequently, the PVDF membranes were probed with corresponding secondary antibodies (1:30000, anti-rabbit IRDye 800 and/or anti-mouse IRDye 680; LI-COR Biosciences) for 1 h at room temperature. Fluorescence of immunoreactive bands was visualized using Odyssey infrared imaging system and specific band intensity was quantified using Odyssey application software [LI-COR Biosciences, (version 3.0)].

2.10. Statistical analyses

AA uptake data represent mean ± SE of at least three independently transfected experiments. Uptake was expressed as percentage relative to levels achieved with simultaneously performed negative controls. The carrier-mediated AA uptake process was determined by subtracting uptake of ¹⁴C-AA in the presence of 1 mM of unlabeled AA from uptake in its absence. Statistical significance was determined using the student's *t*-test with the cutoff for statistical significance set at *P* < 0.05. Quantification of luciferase assays, western blotting and RT-qPCR analyses are expressed as mean ± SE of at least three independent experiments from different batches of cells.

3. Results

3.1. SLC23A1-3'-UTR regulates luciferase expression in intestinal cell lines

To determine whether the *SLC23A1*-3'-UTR is a target for miRNAs in intestinal epithelial cells, we generated a reporter construct in which the firefly luciferase gene (from the pmirGLO dual luciferase miRNA target expression vector) was ligated to full-length *SLC23A1*-3'-UTR (453bp). When the cloned construct (pmirGLO-*SLC23A1*-3'-UTR) was expressed in intestinal epithelial cells, results revealed a significant decrease in luciferase activity for the pmirGLO-*SLC23A1*-3'-UTR construct in both Caco-2 and NCM460 cells (*P* < 0.01 for both cell lines) compared to cells transfected with pmirGLO empty vector (Fig. 1). These results suggest that miRNAs may regulate the *SLC23A1*-3'-UTR.

3.2. MiRNAs that target the SLC23A1-3'-UTR and their expression in Caco-2, NCM460 cell lines and mouse jejunum mucosa

To identify miRNAs that may regulate *SLC23A1* (hSVCT1) expression, we utilized three bioinformatic algorithms (TargetScan, microcosm, miRbase [20, 21, 25, 33]) to predict miRNAs that may bind within the *SLC23A1*-3'-UTR. These prediction programs identified five potential miRNA interactors and three putative binding sites in the *SLC23A1*-3'-UTR (Fig. 2A). These miRNAs were miR-103a, miR-107, miR-328 (predicted by TargetScan

only), miR-384, and miR-499 (Fig. 2A). From these predictions, miR-103a and miR-107 were associated with a high context score and percentile predictor (Table 2). Next, the level of expression of each miRNA (miR-103a, miR-107, miR-328, miR-384, and miR-499-5p) was determined in both Caco-2, NCM460 cell lines as well as mouse jejunum mucosa (Fig. 2B). The level of expression of the mature miR-103a was significantly higher than that of other candidate miRNA regulators in each system (Fig. 2B).

3.3. Effect of truncation of *SLC23A1*-3'-UTR on firefly luciferase activity in Caco-2 cells

In order to determine which region(s) within the *SLC23A1*-3'-UTR are important for miRNA regulation, two truncation constructs (1-240bp and 1-322bp) were made in the *SLC23A1*-3'-UTR (Fig. 3A). As seen previously, transient transfection of Caco-2 cells with the full-length *SLC23A1*-3'-UTR construct caused a decrease in luciferase activity relative to the control construct (Fig. 3B). However, transfection of either truncated constructs did not cause any decrease in observed luminescence values in Caco-2 cells (Fig. 3B). These data implicate the region between 322-453bp as an important region for miRNA binding within the *SLC23A1*-3'-UTR. Both miR-103a and miR-107 are predicted to bind within this region (Fig. 3). Collectively both the mature miRNA expression data (Fig. 2B) and truncation analyses (Fig. 3) pointing towards miR-103a as an important interactor with *SLC23A1*-3'-UTR. Therefore, our subsequent studies were focused on determining the role of miR-103a in posttranscriptional regulation of *SLC23A1* gene.

3.4. MiR-103a targets *SLC23A1*-3'-UTR in Caco-2 cells

To examine whether miR-103a directly targets the *SLC23A1*-3'-UTR, the pmirGLO-*SLC23A1*-3'-UTR construct was transfected into Caco-2 cells together in the presence of either a miR-103a mimic, or a negative control miR mimic. Luciferase activity was not changed when pmirGLO vector with 103a mimic transfected compared to pmirGLO vector with 103a negative control mimic (Fig. 4). However, transfection of the Caco-2 cells with the pmirGLO-*SLC23A1*-3'-UTR in the presence of the 103a mimic led to a significant ($P < 0.05$) inhibition in luciferase activity compared to cells transfected with the pmirGLO-*SLC23A1*-3'-UTR with negative control mimic (Fig. 4). Further to determine the specific interaction of the miR-103a with *SLC23A1*-3'-UTR, we also examined the effect of other predicted miR-107, miR-328, miR-384 and miR-499-5p in Caco-2 cells. The results showed that there is no significant inhibition in luciferase activity between pmirGLO-*SLC23A1*-3'-UTR with miR-107 or miR-328 or miR-384 or miR-499-5p and pmirGLO-*SLC23A1*-3'-UTR with negative control mimic alone transfected Caco-2 cells (73.41 ± 4.21 and 63.13 ± 4.82 ; 71.69 ± 3.82 and 61.38 ± 4.13 ; 72.75 ± 4.82 and 80.30 ± 4.66 and 76.0 ± 3.37 and 78.44 ± 3.31 for miR-107, miR-328, miR-384 and miR-499-5p, respectively). These findings suggest that miR-103a has a role in modulating hSVCT1 expression in Caco-2 cells.

3.5. Effect of mutating the miR-103a putative site in the *SLC23A1*-3'-UTR on luciferase activity

To further assess the interaction between miR-103a and its predicted binding site in the *SLC23A1*-3'-UTR region, we mutated the miR-103a binding site (Fig. 5A). First, we transfected Caco-2 cells with wild-type and miR-103a binding mutant construct. After 48 h

of transfection, we determined the luciferase activity. The results showed a significant ($P < 0.05$) increase in luciferase activity in mutant transfected cells compared to wild-type (Fig. 5B). Next, the wild-type or miR-103a binding mutant construct were transfected into Caco-2 cells in the presence of the miR-103a mimic or a miR-103a inhibitor. The results showed that miR-103a mimic reduced significantly the luciferase activity in Caco-2 cells transfected with wild-type construct ($P < 0.01$, Fig. 5C) and this inhibition was lost when the miR-103a binding mutant construct was used (Fig. 5D). On the other hand, the miR-103a inhibitor increased significantly ($P < 0.01$) the luciferase activity in Caco-2 cells transfected with wild-type (Fig. 5E) but not with mutant construct (Fig. 5F). These findings suggest that the miR-103a putative site in the *SLC23A1*-3'-UTR plays a role in the interaction between the miR-103a and the *SLC23A1*-3'-UTR.

3.6. Effect of miR-103a mimic on carrier-mediated AA uptake in Caco-2 cells

To determine whether miR-103a regulation impacts the function of hSVCT1, we transfected a miR-103a mimic (20 nM) into Caco-2 cells. After 48 h of transfection, AA uptake was performed and results showed a significant ($P < 0.01$) inhibition in AA uptake in cells transfected with the miR-103a mimic compared with those transfected with a negative control mimic (Fig. 6A). Additionally, transfecting cells with a mimic for miR-499-5p showed no significant effect on AA uptake (Fig. 6). On the other hand, transfecting Caco-2 cells with miR-103a inhibitor (50 nM) showed a significant ($P < 0.01$) increase in AA uptake compared to negative control inhibitor or miR-499-5p inhibitor transfected cells (Fig. 6B). These results suggest that miR-103a interaction with the *SLC23A1*-3'-UTR has functional relevance for intestinal AA transport.

3.7. Effect of miR-103a on hSVCT1 mRNA and protein expression in Caco-2 cells

To determine whether miR-103a directly involved in regulating hSVCT1 mRNA and protein expression, we transfected miR-103a mimic and negative control mimic into Caco-2 cells. After 48h, we examined the hSVCT1 mRNA and protein expression levels. The results showed that hSVCT1 mRNA and protein expression were significantly ($P < 0.01$ for both) decreased compared to negative control mimic transfected cells (Fig. 7A & B). In another study, we also transfected miR-103a inhibitor and negative control inhibitor to further confirm the miR-103a role in regulating hSVCT1 mRNA and protein expression in Caco-2 cells. Our results showed that hSVCT1 mRNA and protein expression was significantly ($P < 0.01$ for both) increased compared to negative control inhibitor transfected cells (Fig. 7C & D). These finding shows that miR-103a directly involved in regulating hSVCT1 expression in Caco-2 cells.

4. Discussion

Vitamin C is an essential micronutrient for normal cellular function, growth and development. The apically localized intestinal vitamin C transporter-1 (hSVCT1) plays a key role in AA absorption in the intestine [15–17]. Studies on the transcriptional regulation of this transport system have been forthcoming in recent years, including demonstration of the effects of external/internal regulators such as lipopolysaccharides (LPS) and pro-inflammatory cytokines (e.g., TNF- α) [40, 41]. However, far less is known about

posttranscriptional regulation of this transport system including the role of microRNAs which play an important role in the posttranscriptional regulation of transporters involved in nutrient absorption [20–22, 24, 25, 27]. For these reasons, we investigated the role of miRNA in the posttranscriptional regulation of hSVCT1 in intestinal epithelial cells.

First, we subjected the *SLC23A1*-3'-UTR to different bioinformatic analyses and identified five putative potential miRNA binding sites (miR-103a, miR-107, miR-328, miR-384 and 499-5p). In order to establish whether the 3'-UTR of *SLC23A1* is a target for miRNA binding/interaction in intestinal epithelial cells, we transiently over expressed pmirGLO-*SLC23A1*-3'-UTR and pmirGLO vector alone in Caco-2 and NCM460 cells. Initial transient expression of this construct showed the relative luciferase activity was markedly decreased compared to pmirGLO vector alone establishing that the *SLC23A1*-3'-UTR harbors interacting sites for miRNAs binding in intestinal epithelial cells. Next, we determined the expression level of the five putative regulatory miRNAs in intestinal epithelial cells and mouse jejunum tissue. We found that the expression level of miR-103a was markedly higher than that of the other four miRNAs in Caco-2 and NCM460 cells as well as native mouse jejunum. It is worth mentioning here that the expression of miR-103a is also higher in carcinogenic human Caco-2 cells compared to either normal human colonic NCM460 cells or native normal mouse jejunum tissue (Fig. 2B). These findings are similar to results from Geng *et al.* demonstrating miR-103a expression is higher in cancerous samples compared to normal intestinal samples [35]. Interestingly, the genes for miR-103 and *SLC23A1* are located in the long arm of chromosome 5 (5q) [34, 43]. Further, this region of chromosome 5 (5q31-33) is associated with enhanced risk of developing gastrointestinal disorders such as IBD [44]. In addition, a genetic polymorphism in *SLC23A1* gene has been shown to be associated with IBD [43] and low levels of vitamin C have been found in IBD patients [9].

To investigate whether the interaction between miR-103a and *SLC23A1*-3'-UTR has a functional role, we examined the effect of treatment with a miR-103a mimic or inhibitor on AA uptake in Caco-2 cells. We found that the miR-103a mimic inhibited AA uptake, whereas the miR-103a inhibitor increased AA uptake. This effect may be specific for *SLC23A1* (hSVCT1) and not *SLC23A2* (hSVCT2) which expresses at the basolateral membrane domain of intestinal epithelial cells [15] as we did not find the binding/interacting site for miR-103a in *SLC23A2*-3'-UTR using bioinformatics analyses. This inhibitory effect of miR-103a mimic is associated with reduced expression of hSVCT1 mRNA and protein. In contrast, miR-103a inhibitor markedly increased AA uptake and expression of hSVCT1 mRNA and protein levels in intestinal epithelial cells.

Collectively, these data suggest that hSVCT1 is a target for posttranscriptional regulation by miR-103a in intestinal epithelial cells, and this regulation has functional significance on intestinal AA absorption.

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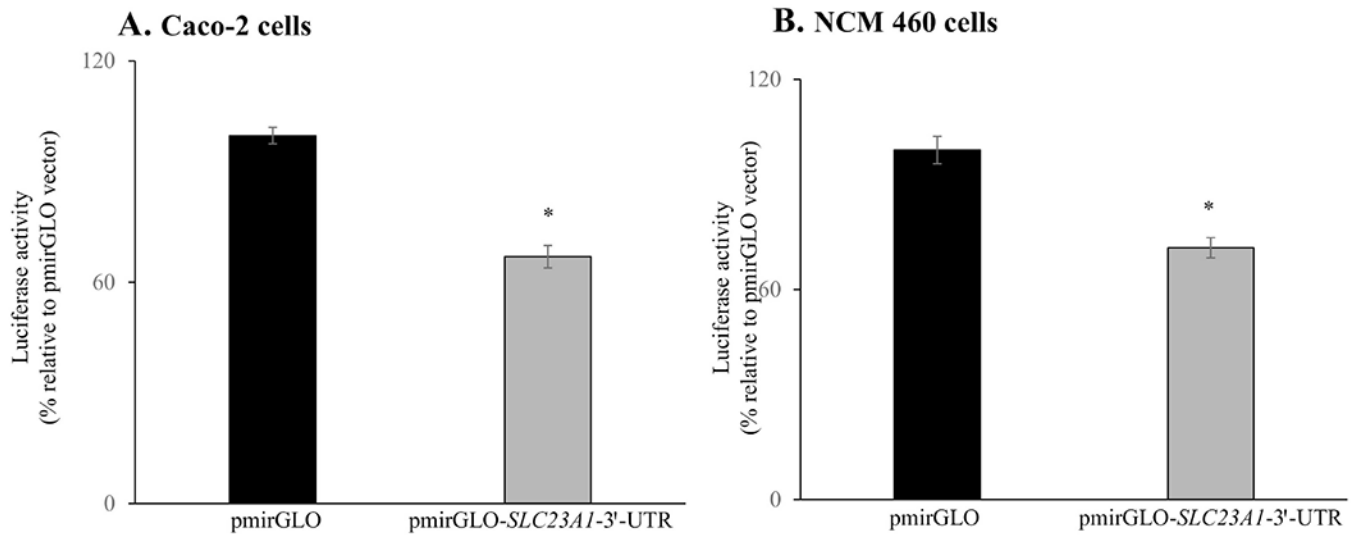


Fig. 1. Effect of cloned pmirGLO-SLC23A1-3'-UTR construct on firefly luciferase activity: (A) Caco-2 and (B) NCM460 cells were transiently transfected with pmirGLO vector or the pmirGLO-SLC23A1-3'-UTR construct. After 48 h of transfection, cells were used for determining the luciferase activities as described in the "Methods". Data are mean \pm SE of 3-5 independent experiments (*P < 0.01).

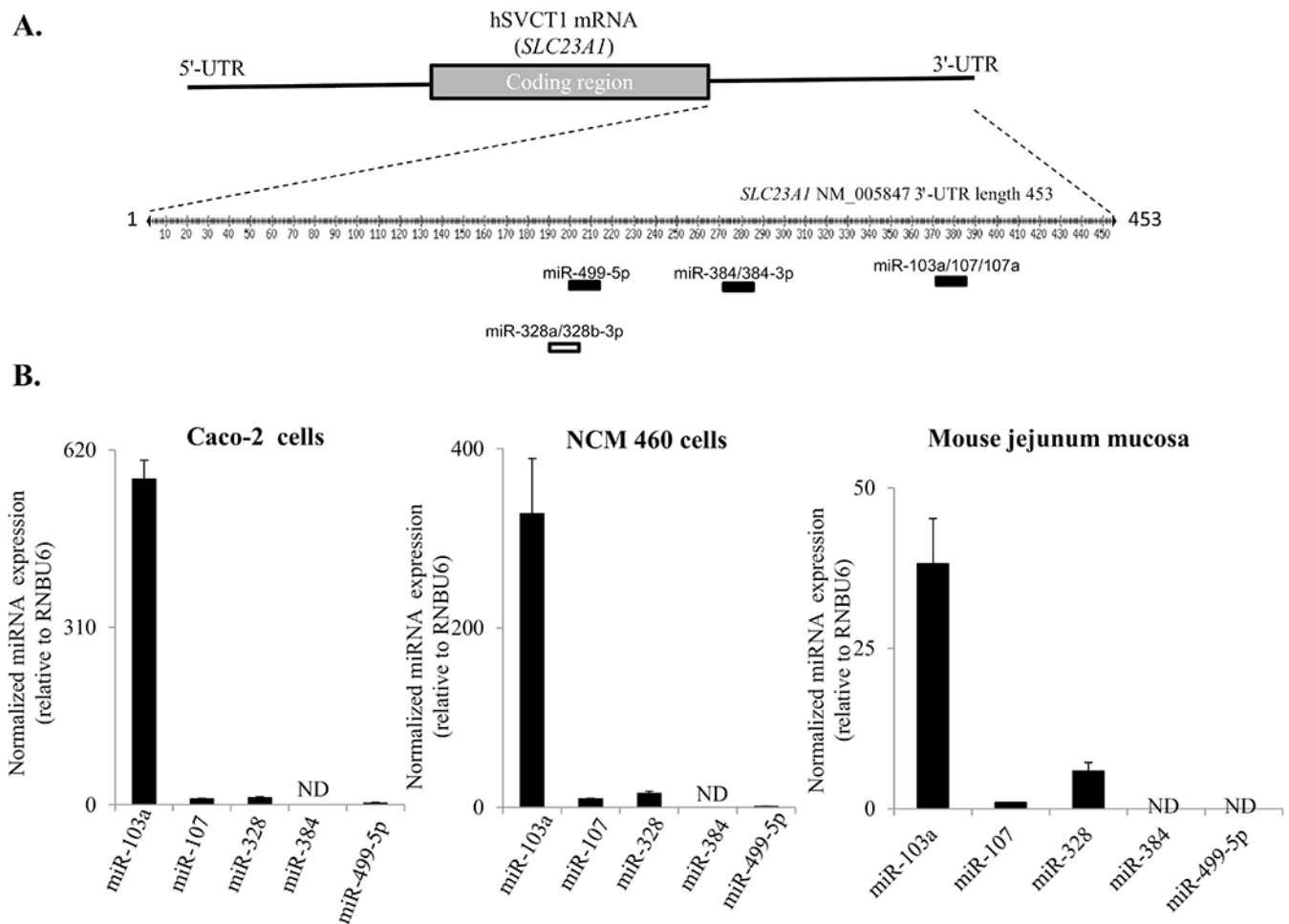


Fig. 2.

(A) *The SLC23A1-3' UTR and location of target scan predicted miRNA target sites.*

Depicted figure is based on website <http://www.targetscan.org>. (B) *Relative expression of*

mature miRNAs in Caco-2 and NCM460 cell lines and mouse jejunum mucosa: The mature miRNA expression levels were determined utilizing TaqMan microRNA assay system as described in "Methods". Data of miRNA expression levels were determined after normalizing constitutively expressed RNU6B (house-keeping gene) miRNA. Values are mean \pm SE of at least from 3 independent experiments with different batches of cells. Open rectangle: predicted by only targetScan and closed rectangle: predicted by all three programs as described in "Methods". ND-not detectable.

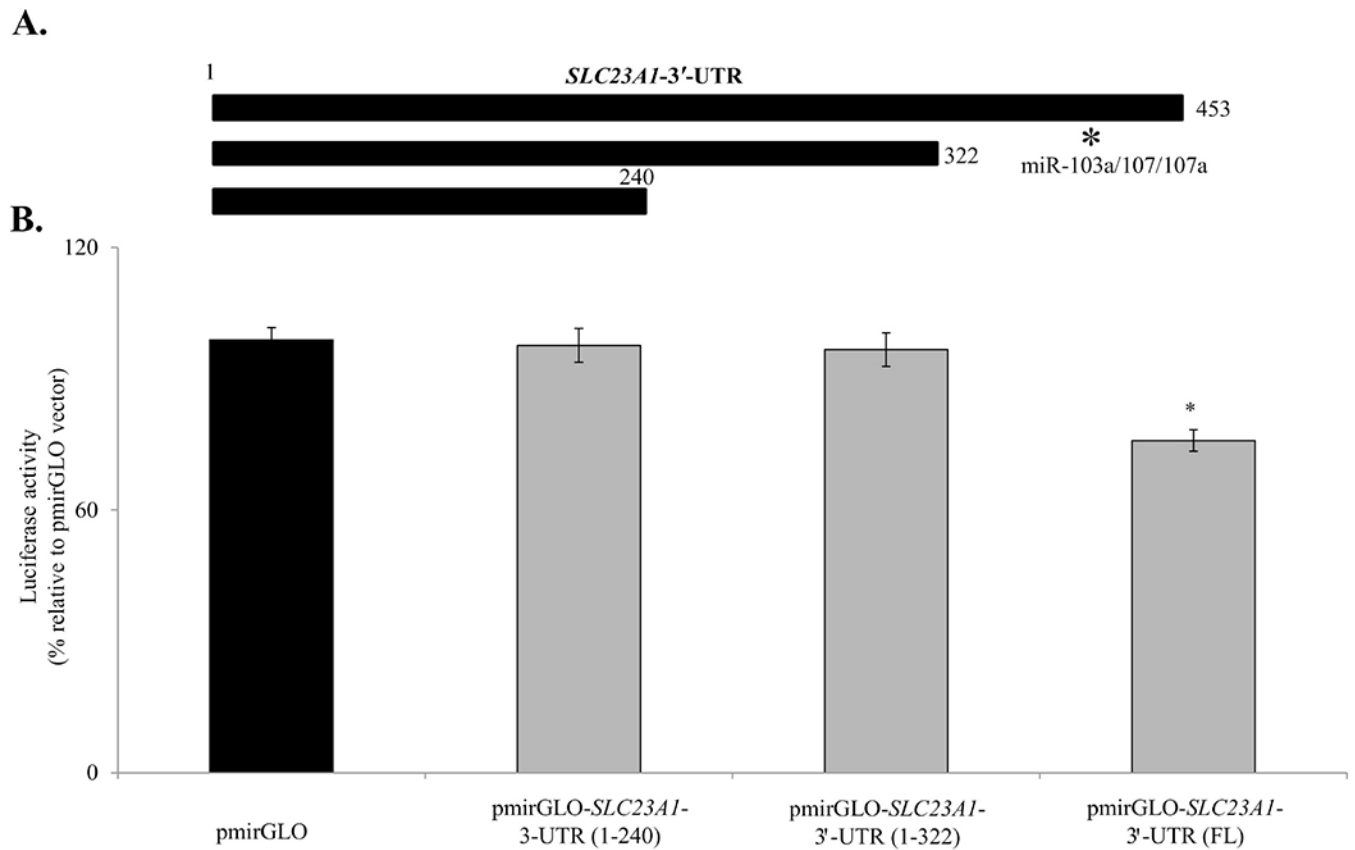


Fig. 3. Effect of truncation of *SLC23A1*-3'-UTR on firefly luciferase activity in Caco-2 cells: (A) Schematic representation of the regions of *SLC23A1*-3'-UTR used to generate truncated constructs. (B) Caco-2 cells were transiently transfected with full-length and truncated constructs and luciferase activity was determined after 48 h. Data are mean \pm SE of at least from 3 independent experiments with different batches of cells. (* $P < 0.01$). *indicates the approximate location of the miR-103a/107/107a in 3'-UTR of *SLC23A1*.

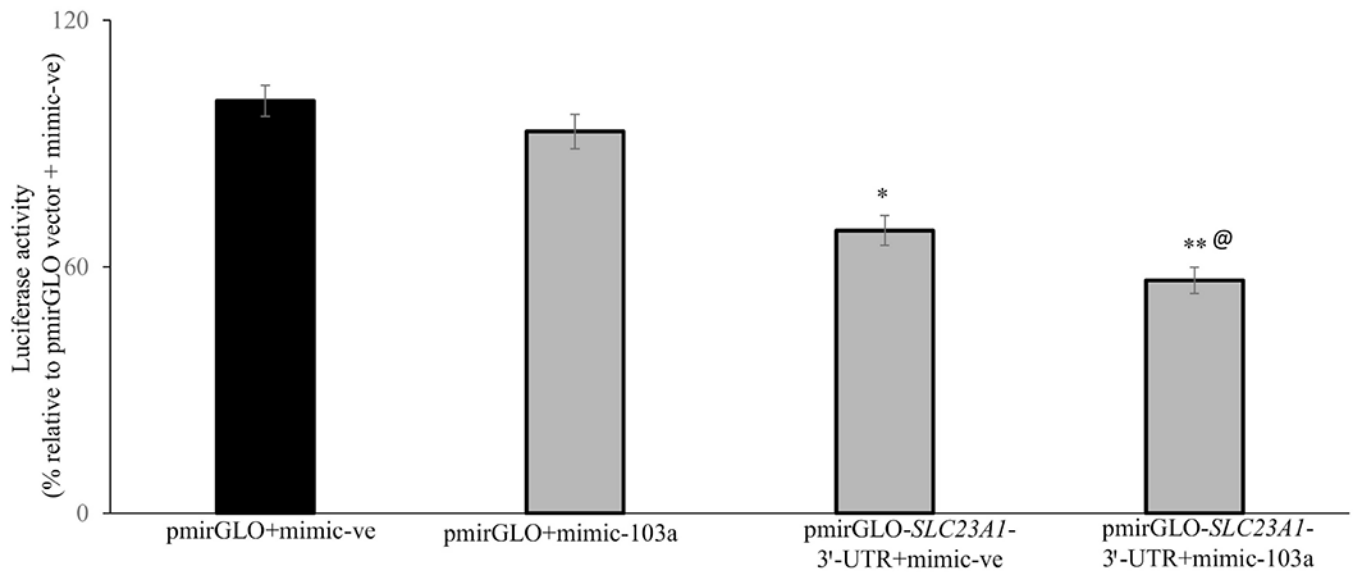


Fig. 4. Effect of transient co-transfection of mimic-103a with pmirGLO-*SLC23A1*-3'-UTR on luciferase activity in Caco-2 cells:

Caco-2 cells were transiently co-transfected with pmirGLO-vector alone or the pmirGLO-*SLC23A1*-3'-UTR plasmid along with miRNA mimic (either negative control mimic or mimic-103a, 20nM, 48h) and measured luciferase activity. Data are mean \pm SE of at least 3 independent experiments. @ indicates the pmirGLO-*SLC23A1*-3'-UTR with mimic-103a is significantly decreased in the luciferase activity compared to pmirGLO-*SLC23A1*-3'-UTR with mimic-ve (**P < 0.05, *P < 0.01).

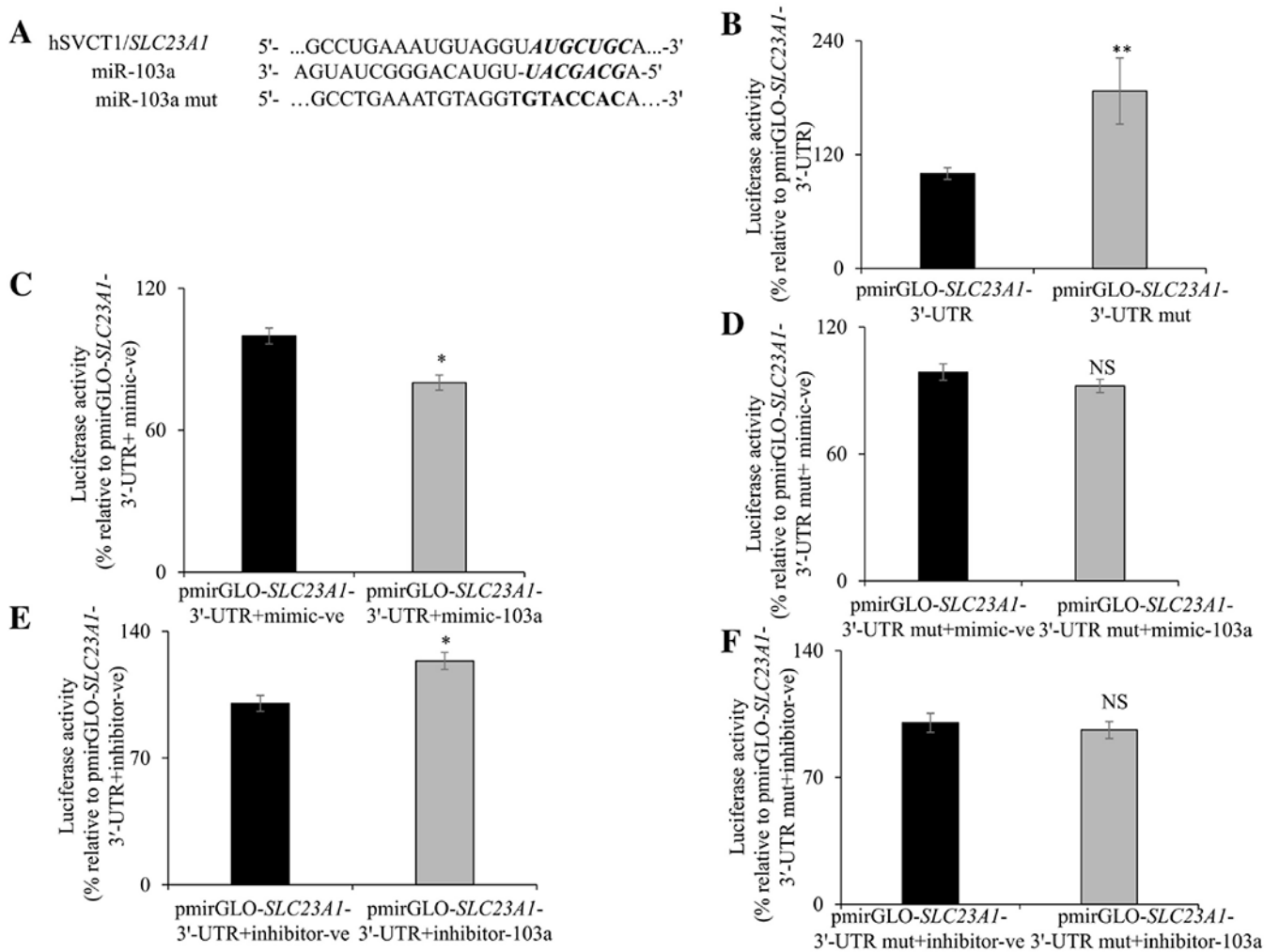


Fig. 5. Effect of mutating the miR-103a putative binding site in the *SLC23A1*-3'-UTR on luciferase activity in Caco-2 cells:

miR-103a binding mutant nucleotide sequence (A). pmirGLO-*SLC23A1*-3'-UTR miR-103a binding mutant construct transfected Caco-2 cells increases the firefly luciferase activity (B). MiR-103a mimic transfected Caco-2 cells reduced the luciferase activity of wild-type construct (C) but not the 103a mutant construct (D). MiR-103a inhibitor transfected Caco-2 cells increased the luciferase activity of wild-type construct (E) but not the miR-103a mutant construct (F). Firefly luciferase activities were measured and normalized with respective renilla luciferase activities. Data are mean \pm SE of at least 3 independent experiments (* $P < 0.01$, NS-not significant).

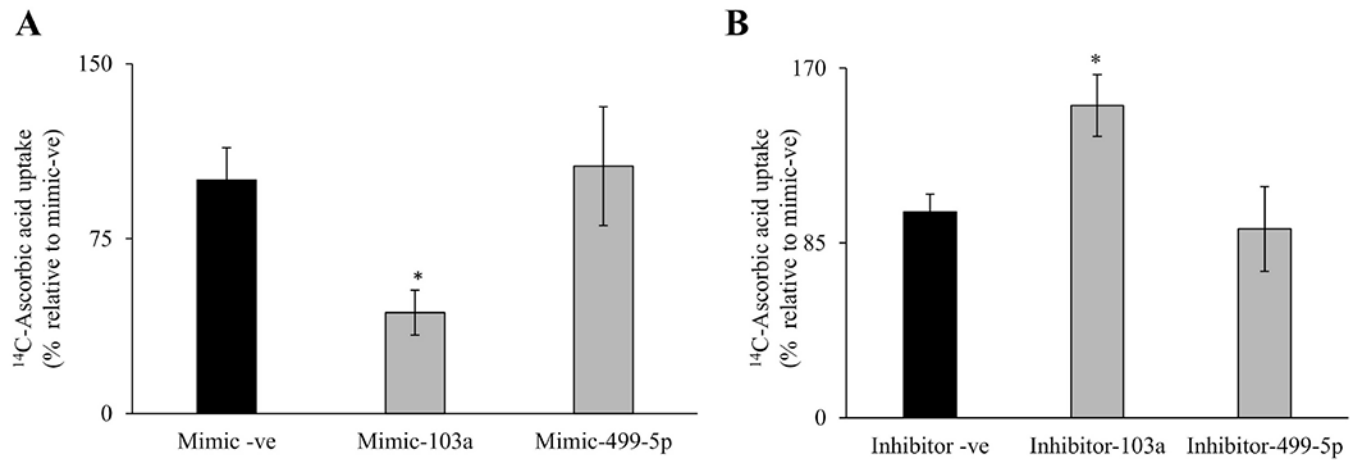


Fig. 6. Effect of transfection with miR-103a mimic or inhibitor on carrier-mediated intestinal AA uptake:

Carrier-mediated AA uptake was determined after transiently transfecting with (A) mimic [negative control mimic, or mimic-103a or mimic-499-5p (20 nM, 48h)] or (B) inhibitor [negative control inhibitor, or inhibitor-103a or inhibitor-499-5p (50 nM, 48h)]. Data are mean \pm SE of at least from 3 independent experiments (* $P < 0.01$).

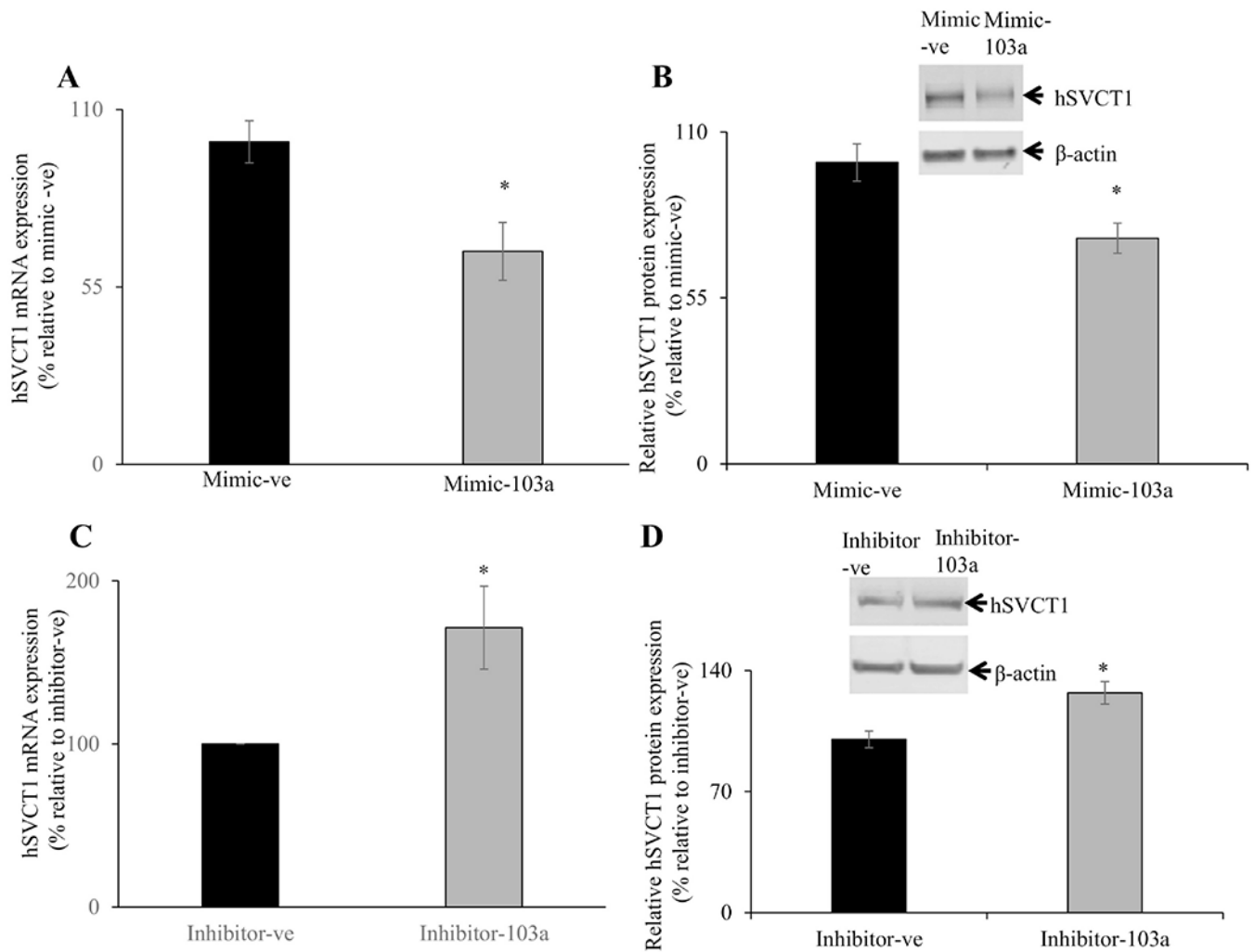


Fig. 7. Effect of miR-103a mimic or inhibitor transfection on level of hSVCT1 mRNA and protein expression: (A&C) total RNA was isolated from cells transfected with either negative control mimic/inhibitor or mimic/inhibitor-103a and hSVCT1 mRNA levels were determined by RT-qPCR. (B&D) membranous fractions isolated from cells transfected with either negative control mimic/inhibitor or mimic/inhibitor-103a and hSVCT1 protein expression levels were determined by western blot analysis. Data are mean \pm SE of at least from 3 independent experiments (* $P < 0.01$).

Table 1.

Combination of primers used in this study

Gene name	Forward and Reverse Primers (5'-3')
Primers used for cloning <i>SLC23A1</i>-3-UTR and truncations in pmirGLO vector	
<i>SLC23A1</i> (1-453)	CCG CTCGAG ATGACTTCCAGGAAAGGAA; GCG TCGAC ATTTCTCAACATGATGTTTA
<i>SLC23A1</i> (1-322)	CCG CTCGAG ATGACTTCCAGGAAAGGAA; GCG TCGAC TTTCAACTGCAGTCTAA
<i>SLC23A1</i> (1-240)	CCG CTCGAG ATGACTTCCAGGAAAGGAA; GCG TCGACA AGATTTAGAGACATAGC
Real-time PCR	
hSVCT1	TCATCCTCTTCTCCCAGTACCT; AGAGCAGCCACACGGTCAT
h β -actin	CATCCTGCGTCTGGACCT; TAATGTCACGCACGATTTC
Site-directed mutagenesis	
<i>miR-103a</i>	
	CCAGGGCCTGAAATGTAGGTG TACCACA AGGCTTTTACATTGAATTTG; CAAATTC AATG TAAAAGCCT TGTGGT ACACCTACATTT CAGGCCCTGG

Bold italics *XhoI*; Bold face *Sal I*

Table 2.

The context score and context score percentile based on TargetScan 7.1 program for predicted miRNAs to target 3'-UTR of *SLC23A1*.

miRNAs name	Context score	Context score Percentile	Conserved branch length	Binding position in <i>SLC23A1</i> -3'-UTR
<i>miR-103/107</i>	-0.40	98	0.32	372-379
<i>miR-328</i>	-0.28	94	2.02	186-192
<i>miR-384</i>	-0.15	90	0.22	271-277
<i>miR-499-5p</i>	-0.07	65	0.55	199-205

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