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Research Paper

Construction of a Functional Transporter Analysis System Using *MDR1* Knockdown Caco-2 Cells

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Purpose. The efflux transporter, P-glycoprotein (P-gp), located in the brush-border membrane of intestinal absorptive cells, reduces the bioavailability of a wide range of orally administered drugs. Using P-gp inhibitors in transport experiments in Caco-2 cell monolayers is widely accepted as an efficient way to estimate the contribution of P-gp to the intestinal absorption of drugs. However, there still remain some arguments that the inhibitors might affect the function of other proteins. Multidrug resistance 1 gene (*MDR1*) specifically inhibited Caco-2 cells were constructed, therefore, as a better *in vitro* evaluation system of intestinal drug absorption.
Methods. The effective sites of RNAi were selected using siRNA libraries and single siRNAs and *MDR1* stable knockdown Caco-2 cells were constructed using a $tRNA^{val}$-shRNA expression vector.
Results. In siRNA stably expressed Caco-2 cells, the expression level of *MDR1* was reduced at mRNA and protein levels. Transcellular transport studies using digoxin revealed that the P-gp function was suppressed completely, similar to that in verapamil-treated cells.
Conclusions. *MDR1* stable knockdown Caco-2 cells were successfully constructed by RNAi technology. This will consequently allow the development of a selection system for candidate drugs with improved absorption properties.

KEY WORDS: Caco-2 cells; *MDR1*; RNAi.

INTRODUCTION

P-glycoprotein (P-gp), a product of the multidrug resistance 1 (*MDR1*) gene, acts as an ATP-dependent efflux pump that exports its substrates from cells. It was first discovered in tumor cells and contributes to the phenomenon of multidrug resistance to anti-cancer agents (1). P-gp is expressed not only in tumor cells, but also in the cells of several normal tissues that exhibit excretory functions such as the intestine, liver, kidneys, and in capillary endothelial cells of the brain, placenta and testis (2–4). P-gp has a very broad substrate specificity with a relatively hydrophobic and amphophilic nature (5). P-gp is considered to be a protective barrier against xenobiotics. In the small intestine, P-gp is localized in the brush-border membranes (6) and mediates excretion of its substrates into the gut lumen. Because the absorption from the intestine is one of the key factors in determining the bioavailability of orally administered drugs, intestinal P-gp plays an important role in their pharmacokinetics.

Caco-2 cells, derived from human colorectal adenocarcinoma, have been reported to express P-gp (6) and other transporters (7,8), the expression levels of which are in good agreement with those of the normal human jejunum, except for breast cancer resistance protein (*BCRP*). Monolayers of Caco-2 cell have been widely used as a potent *in vitro* model to predict drug absorption in humans (9–11). At present, although inhibitors of P-gp, such as cyclosporine A (12), PSC833, verapamil, and ketoconazol (13), are used to examine the contribution of P-gp to intestinal drug absorption, the possibility cannot be excluded that these inhibitors block the functions of other proteins in such experiments. Consequently, it would be very useful to develop alternative and more efficient strategies to estimate the contribution of P-gp.

RNA interference (RNAi) was initially identified as a phenomenon of sequence-specific post-transcriptional gene silencing in worms and plants (14,15). It was discovered that synthetic 21 bp siRNAs specifically suppressed the expression of endogenous genes without activating protein kinase R (PKR) and 2′, 5′-oligoadenylate synthetase in several mammalian cell lines (16) and now synthetic siRNA has become a powerful tool for investigating gene function in cell cultures. The efficacy of siRNA depends directly on the target

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ABBREVIATIONS: *MDR1*, multidrug resistance 1; P-gp, p-glycoprotein; RNAi, RNA interference; ShRNA, short hairpin RNA; SiRNA, small interference RNA.

sequence. Although rational rules for effective siRNA target sequencing have recently been reported (17–19), it remains difficult to predict the most effective target site from long genes, such as *MDR1*. In this study, therefore, siRNA libraries (20), which are not necessary to select a siRNA sequence within a target gene, were constructed against two approximately 500-bp fragments of the human *MDR1* gene. After evaluation of the siRNA libraries, single siRNAs were selected within the target sequences of the effective siRNA libraries and, finally, *MDR1* stable knockdown Caco-2 cells were constructed using a vector system. Transcellular transport studies using these cell lines demonstrated that the P-gp function was completely inhibited by RNAi. This result strongly suggests that these cell lines will be very useful tools to evaluate intestinal drug absorption *in vitro*.

MATERIALS AND METHODS

Materials and Antibodies

[^{3}H]digoxin (23.4 Ci/mmol) and [^{3}H]daunomycin (16 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA) and [^{3}H]vincristine sulfate (4.6 Ci/mmol) and [^{14}C]mannitol (59 mCi/mmol) were purchased from Amersham Biosciences (Piscataway, NJ). Unlabeled verapamil and rhodamine 123 was purchased from Sigma-Aldrich (St. Louis, MO). Human P-gp antibody (C219) and actin antibody (No. MAB 150R) were purchased from Alexis (San Diego, CA) and CHEMICON (Temecula, CA), respectively.

Cell Culture

Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD) at passage 17 and were used between passage numbers 40 and 70. Cells were maintained in a culture medium (CM) consisting of Dulbecco's modified Eagle's medium with 4500 mg/L of glucose (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), nonessential amino acids (NEAA; Invitrogen), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Invitrogen) at 37°C with 5% CO_2 and 95% humidity.

siRNA Library Construction

Two sets of libraries (siRNA library A site; 1859–2405, siRNA library B site; 2709–3093; Fig. 1) against *MDR1* mRNA specific sequence (GeneBank accession number NM 000927) were designed. Two sets of template DNA for *in vitro* transcription of dsRNAs were amplified from cDNA of Caco-2 cells by polymerase chain reaction (PCR) with specific primers that contained the T7 promoter. Using the PCR products, two pools of 22 bp siRNAs, named siRNA libraries, were generated according to the manufacturer's instructions (Dicer siRNA Generation Kit; Gene Therapy Systems, San Diego, CA).

siRNA Synthesis

Three-target sequences of 21–25 bp siRNAs were selected in each fragment of the siRNA library [A-1(1971–1994): 5′-TTCAAGATCCAGTCTAATAAGAAA-3′, A-2(2276–2297): 5′-TTCTAGCCCTTGGAATTATTTC-3′, A-3(2311–2332): 5′-TTCCTTCAGGGTTTCACATTTG-3′, B-1(2730–2752): 5′-GACTCAGGAGCAGAAGTTTGAAC-3′, B-2(3009–3033): 5′-ATCAGCAGCCCACATCATCATGATC-3′, B-3(3051–3073): 5′-GATTGACAGCTACAGCACGGAAG-3′]. The sequence derived from *Euglena*

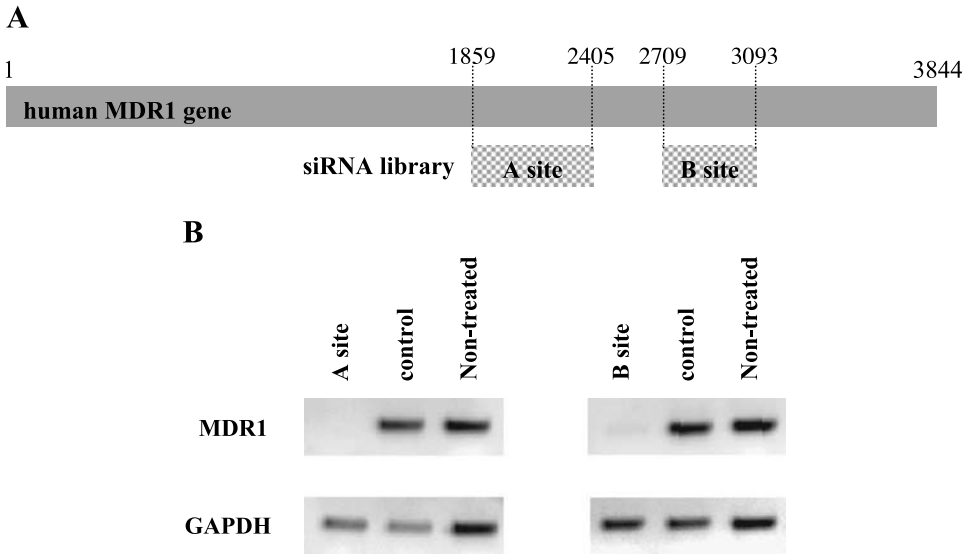

Fig. 1. Effects of *MDR1* siRNA libraries in Caco-2 cells. (A) Schematic representation of the sequences of siRNA libraries. The lower bars represent the sites of the siRNA libraries in the human *MDR1* gene. (B) The level of *MDR1* mRNA in the siRNA library transfected Caco-2 cells. The siRNA libraries of A site and B site and the control siRNA were transfected into Caco-2 cells. After transfection for 48 hr, total RNAs were isolated from the cells and *MDR1* mRNA was amplified by RT-PCR. GAPDH mRNA served as an internal control. A site, siRNA library A site; B site, siRNA library B site; control, control siRNA; non-treated, medium only.

gracilis, which has no homology with the human genome, was used as a control sequence (5′-TTGCGCGCTTTGTAG GATTCGTT-3′). The siRNAs were synthesized using a CUGA®7 *in vitro* siRNA Synthesis Kit (NIPPON GENE, Tokyo, Japan). The length of the purified siRNAs was confirmed by electrophoresis on 8% nondenaturing polyacrylamide gel.

Vector Construction and Transfection

Chemically synthesized oligonucleotide encoding an siRNA sense strand, loop (5′-cttcctgtca-3′) (21) and an siRNA antisense strand was annealed with the corresponding complementary single-stranded DNA oligonucleotide. The resulting double-stranded DNA (dsDNA) was inserted into *Sac*I and *Kpn*I sites of the piGENE™ tRNA Pur (iGENE, Ibaraki, Japan), *MDR1* siRNA expression vector.

Caco-2 cells were seeded in 12-well plates at 1.8×10^5 cells per well, grown for 12 h, then transfected with the siRNA library (100 nM) and the synthetic siRNA (200 nM) using a GeneSilencer™ siRNA Transfection Reagent Kit (Gene Therapy Systems) and Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocols, respectively. To construct stable cell lines, cells were transfected with constructed *MDR1* siRNA expression vector using Lipofectamine™ 2000 (Invitrogen) and selected using a culture medium containing puromycin (5.0 μg/ml) for 3 weeks.

RT-PCR Analysis

Total RNA was isolated from cells with Isogen™ (NIPPON GENE) according to the manufacturer's protocol. The reverse transcription PCR (RT-PCR) reaction was performed using TaKaRa RT-PCR Kit (AMV) Ver.3.0 (Takara, Shiga, Japan) according to the manufacturer's protocol. The level of *MDR1* mRNA was confirmed by PCR with *MDR1* specific primers (for A site: 5′-GCCTCATAAAT TTGACACCC-3′ and 5′-TTCAGTGCTTGTCCAGACAA-3′, for B site: 5′- TGAGTTGGTTTGATGACCCT-3′ and 5′-TGCCCTCACAATCTCTTCCT-3′). GAPDH specific primer (5′-ATGGTGAAGGTCGG-3′ and 5′-TTACTCCTTGGA GGCCATGT-3′) was used as an internal control. PCR products were electrophoresed on 0.8% agarose gels.

Western Blot Analysis

Crude membrane fractions were prepared from cells as described previously (22). The fractions were diluted with phosphate-buffered saline (PBS) and samples (50 μg of proteins for *MDR1* and 12.5 μg of proteins for actin) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Pall, East Hills, NY). The membrane was probed using various antibodies, as mentioned earlier, and then with horseradish peroxidase-conjugated anti-mouse IgG antibody (Amersham Bioscience). Immunoreactions were detected using an ECL Advance Western Blotting Detection Kit (Amersham Bioscience).

Accumulation Study

Transport studies were carried out as described previously (23). Caco-2 cells were seeded in 12-well plates at 1.8×10^5 cells per well, grown for 12 h, then transfected with the synthetic siRNAs (200 nM) using Lipofectamine™ 2000 (Invitrogen). Medium was replaced every 2 days, and after 4 days transport studies were performed. Hank's balanced salt solution (HBSS) supplemented with 25 mM glucose was used as the medium for drug transport (transport medium) in all studies after adjusting the pH to 7.4 with *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethansesulfonic acid (HEPES) or 2-(*N*-morpholino)ethanesulfonic acid (MES). The cells were washed twice before they were incubated with transport medium at 37°C for 15 min, and uptake was initiated by adding radiolabeled digoxin (0.1 μCi/ml, 4 nM) with or without verapamil (100 μM) to the transport medium (13). The incubation was terminated after 10 min by adding ice-cold transport medium. After the cells were washed twice with 1 ml of ice-cold transport medium, they were dissolved and the radioactivity and protein concentration were measured, as described previously (23).

Transcellular Transport Study

siRNA stably expressed Caco-2 cells were differentiated using our recently established method (24). Briefly stated, cells were seeded on 24-well fibrillar collagen precoated inserts (BIOCOAT® cell culture inserts Fibrillar Collagen, Type I Rat Tail, 1.0-μm pores; Becton Dickinson Bioscience, Bedford, MA) at a density of 2.0×10^5 cells per well. After 2 days of seeding, CM was changed to a differentiation medium (DM, ENTERO-STIM Differentiation Medium containing MITO+™ Serum Extender, Becton Dickinson Bioscience) and, after 4 days of seeding, the medium was replaced with fresh DM.

Transport studies were carried out as described previously (22,25,26). For transcellular transport studies, cells were washed and incubated with a transport medium for 15 min at 37°C. The experiment was initiated by adding [^{3}H]digoxin (1 μCi/ml, 43 nM) (27,28) with or without verapamil (100 μM) (13), [^{3}H]vinbcristine (2 μCi/ml, 434 nM) (28), rhodamine 123 (10 μM) (24), [^{3}H]daunomycin (1 μCi/ml, 62.5 nM) (27–29) or [^{14}C]mannitol (0.5 μCi/ml 8.5 μM) to the transport medium on either the apical or the basal side of the cell layer. The efflux by P-gp is not saturable under these experimental conditions. For the previously described isotopically labeled compounds, the radioactivity associated with incubation buffer was measured in liquid scintillation counter (LS6000SE; Beckman Instrument, Fullerton, CA). For Rhodamine 123, the fluorescence (excitation = 501 nm, emission = 524 nm) was measured in a spectrofluorometer. The cells were dissolved and the protein concentration were measured, as described previously (22,25,26).

RESULTS AND DISCUSSION

Suppression of *MDR1* Gene Expression by siRNA Libraries

Two sets of different *MDR1*-specific siRNA libraries that have no conserved sequences with other gene were

designed (Fig. 1A) and constructed. After transfection with these siRNA libraries, the expression level of *MDR1* mRNA was confirmed by RT-PCR. *MDR1* mRNA was not detected in either of the siRNA libraries transfected Caco-2 cells at all, while it was clearly detected in the control siRNA transfected Caco-2 cells (Fig. 1B). This clear reduction in *MDR1* gene expression indicates that both siRNA libraries contain effective siRNAs and, therefore, we identified the effective siRNA sequences in the approximately 500-bp fragments of the effective siRNA libraries.

Suppression of *MDR1* Gene Expression by Synthetic siRNAs

In the approximately 500-bp fragment of the effective siRNA libraries, five sequences (A1, A2, A3, B1, and B2) of 21–25 bp siRNAs were designed based on the guideline for the selection of highly effective sequences and one sequence (B3) was designed against the guideline (17) (Fig. 2A). These synthetic siRNAs were transfected into Caco-2 cells. RT-PCR analysis demonstrates that all five designed siRNAs

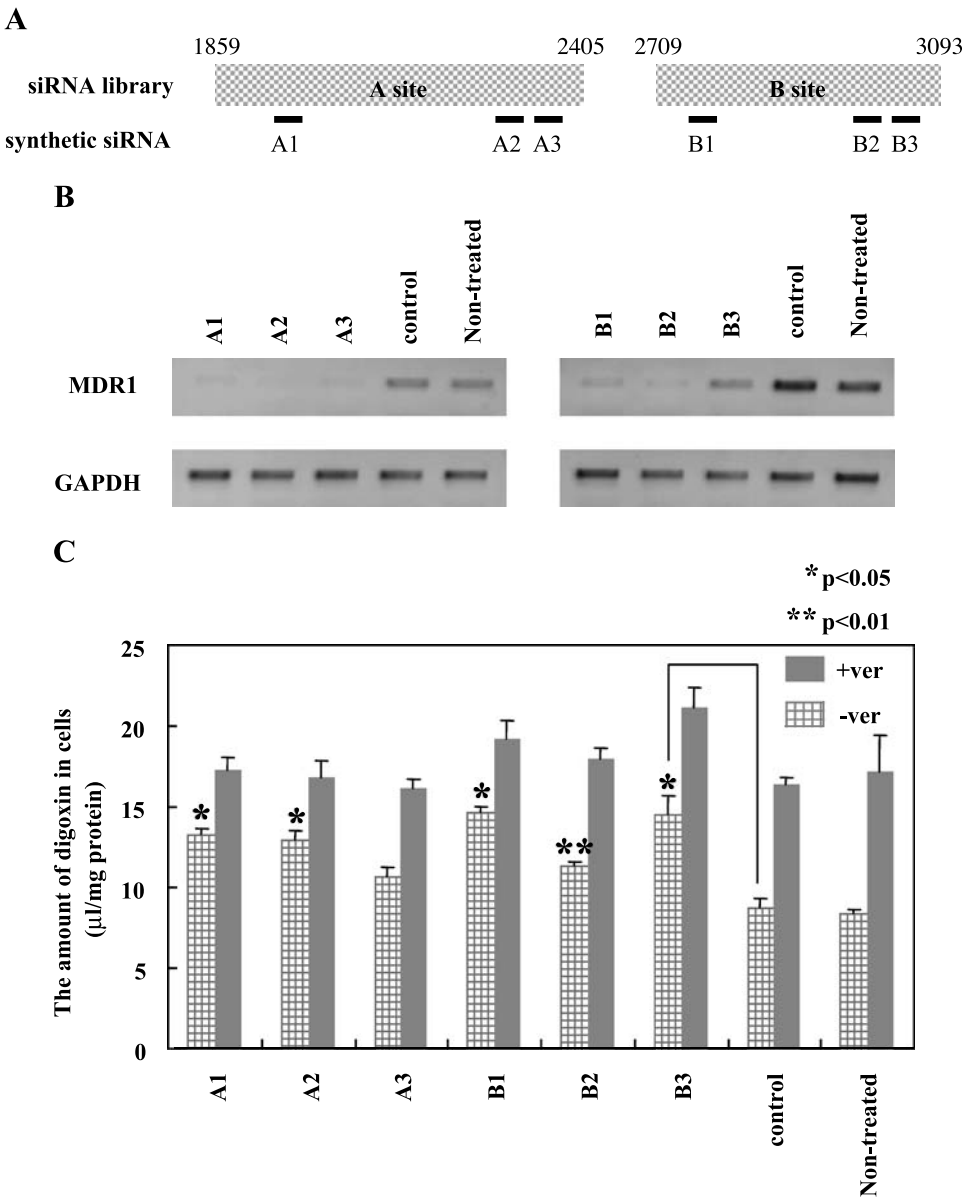

Fig. 2. Effects of synthetic *MDR1* siRNAs in Caco-2 cells. (A) Schematic representation of the sequences of the synthetic siRNAs within the sites of siRNA libraries. The black lines with the siRNA numbers represent the target sites of the designed siRNAs in the siRNA libraries. (B) The level of *MDR1* mRNA in the synthetic siRNA transfected Caco-2 cells. The synthetic siRNAs were transfected into Caco-2 cells using lipofectamine 2000. After transfection for 48 h, total RNAs were extracted from the cells and *MDR1* mRNA was amplified by RT-PCR. GAPDH mRNA served as an internal control. (C) Accumulation study of the synthetic siRNA transfected Caco-2 cells. After transfection for 4 days, the amount of [^{3}H]digoxin (4 nM), with or without verapamil (100 μM), in the cells was examined. Each bar represents the mean ± SEM (n = 3). control, control siRNA; non-treated, medium only.

based on Dr. Saigo's guideline (17) specifically inhibited the expression of *MDR1* at mRNA level but one siRNA against the guideline (B3) failed to exhibit strong inhibition (Fig. 2B). Also, the designed siRNAs based on this guideline for another transporter gene, mouse multidrug resistance associated protein 2 (*Mrp2*), significantly suppressed mRNA expression (unpublished data). These results indicate that this guideline for the selection of effective siRNA sequences is very useful in practical terms. In addition, to evaluate the function of the cells transfected with the synthetic siRNAs, accumulation studies were performed using digoxin and verapamil as a typical substrate and inhibitor of *MDR1*, respectively (Fig. 2C). The accumulated amount of digoxin significantly increased in the siRNAs-transfected Caco-2 cells in comparison with the control siRNA-transfected cells. The results of the accumulation studies indicate that the P-gp function is inhibited by RNAi.

Construction of siRNA Stably Expressed Caco-2 Cells

The siRNA libraries and the synthetic siRNAs significantly suppressed the expression of the *MDR* gene but their effects are only transient and there is heterolytic expression. Therefore, siRNA stably expressed Caco-2 cells were constructed using the piGENE™ tRNA Pur vector, which expresses $tRNA^{val}$ fused shRNA (tRNA-shRNA) (Fig. 3A).

Caco-2 cells were transfected with *MDR1* siRNA expression vectors (A1–A3, B1–B3, and the control), respectively. After selection by puromycin, three clones per type of siRNA expressed cells were established and evaluated by RT-PCR and accumulation study. In the two clones, A2-2 and B2-2, which express A2 and B2 siRNA sequences, respectively, high RNAi efficacy was observed. This activity was retained for at least seven passages after reculture from frozen stock when the cells were divided once a week. RT-PCR analysis demonstrated that the level of *MDR1* mRNA in the A2-2 and the B2-2 was reduced to 75% and 90%, respectively, of that found in the control siRNA expressed cells (Fig. 3B). In addition, no P-gp protein was detected in the two clones, although this protein was clearly detected in the control cells (Fig. 3C). It was confirmed that the expression of P-gp protein was reduced by the effect of RNAi in both clones. Next, the P-gp function of those constructed cell lines was examined by transcellular transport

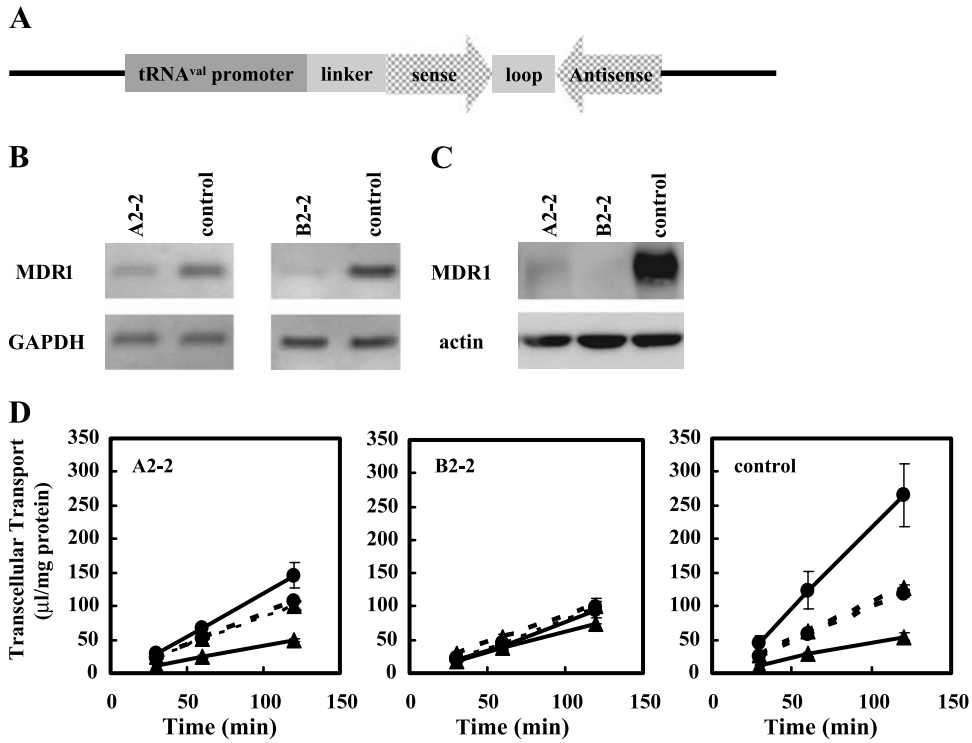

Fig. 3. Effects of tRNA-shRNA expression vectors in Caco-2 cells. (A) Schematic representation of the tRNA-shRNA expression vector. The vectors include the human $tRNA^{val}$ promoter linker, sense-strand of siRNA, loop, and antisense-strand of siRNA, sequentially. (B) RT-PCR of siRNA stably expressed Caco-2 cells. Total RNAs were extracted from the stable cell lines and *MDR1* mRNA was detected by RT-PCR. GAPDH mRNA served as an internal control. (C) Western blot of siRNA stably expressed Caco-2 cells. Preparation of crude membrane is described in Materials and Methods. 50 μg of protein for *MDR1* and 12.5 μg of protein for actin were examine for each lane. (D) Time profiles of transcellular transport across siRNA stably expressed Caco-2 cells. The transcellular transport of [^{3}H]digoxin (43 nM), with or without verapamil (100 μM), across siRNA stably expressed Caco-2 cells was examined at 37°C. The circles and the triangle with the solid lines represent the transcellular transport in the apical-to-basal and the basal-to-apical directions without verapamil, respectively, and the circles and the triangle with the dotted lines represent the transcellular transport in the basal-to-apical direction with verapamil. In the B2-2, the four points almost overlap. Each point represents the mean ± SEM (n = 3).

study of a typical substrate, digoxin, using a short-term Caco-2 cell culture system (24). In the B2-2 clone, the basal-to-apical flux of digoxin (Fig. 3C, solid line) was reduced to the level of inhibition of P-gp function by verapamil (Fig. 3C, dotted line). At the same time, the apical-to-basal flux increased to the same level as that obtained in the opposite direction, indicating that the P-gp function in the B2-2 clone was almost completely inhibited by RNAi. Also, in the A2-2 clone, the P-gp function was moderately inhibited. The results from the transcellular transport study were consistent with the degrees of mRNA inhibition obtained by RT-PCR and the protein levels found on Western blot analysis. In addition, RT-PCR analysis demonstrates that the expression levels of *MRP1-6* and *BCRP*, which express in Caco-2 cells, were not significantly different between the B2-2 clone and the control cells (data not shown). The integrity of monolayers of the modified Caco-2 cells, estimated by both transepithelial electrical resistance (TEER) and [^{14}C]mannitol permeability, was similar to that of normal Caco-2 cells (24). These results indicate that there was specific inhibition of *MDR1* expression in the B2-2 clone and this cell line will be a very useful *in vitro* system. To further characterize, the B2-2 clone, the transcellular transport of vincristine, rhodamine 123 and daunomycin was examined (Fig. 4A–C). It has been reported that vincristine and rhodamine 123 are typical substrates of P-gp (24,27,28) and daunomycin is accepted by P-gp and BCRP (27–29). Using vincristine and rhodamine 123, the basal-to-apical flux was almost the same as in the opposite direction but the basal-to-apical flux was slightly higher than the apical-to-basal flux. It is possible that the suppression of *MDR1* gene expression was not complete and that the other transporters endogenously expressing in Caco-2 cells were able to transport these drugs. In the case of daunomycin, the basal-to-apical flux was the same as in the opposite direction. Daunomycin is a substrate of P-gp and BCRP (27,28) but no vectorial transport was observed. These results suggested that BCRP protein is not expressed or its expression level is very low in Caco-2 cells. In the transcellular transport study of diazepam (27), used as a negative substrate, there was no difference between the B2-2 clone and the control cells and no vectorial transport could be observed (data not shown). It was confirmed that this *MDR1* specific inhibited cell line will provide more important information to investigate the contribution of P-gp to intestinal absorption in place of the P-gp inhibitors that have been used to date.

In some instances intestinal P-gp may be involved in human drug–drug interactions associated with absorption (30–32). In addition, genetic polymorphisms in human P-gp may also contribute to inter-individual differences in the response to drugs (33,34). The use of P-gp inhibitors in transport experiments in Caco-2 cell monolayers is widely accepted as an efficient way of estimating the contribution of P-gp to the intestinal absorption of drugs. However, there still remain some arguments that the inhibitors might affect the function of other proteins. Therefore, the *MDR1* stable knockdown Caco-2 cells developed here should be a better way of evaluating the importance of the role of P-gp in drug absorption from the small intestine. It is also difficult to identify the specific inhibitors of other transporters, such as MRPs and BCRP. The RNAi technique can be applied to other transporters and we can now predict the importance of intestinal transporters using transporter knockdown Caco-2 libraries. This will allow the more efficient selection of candidate drugs with improved properties.

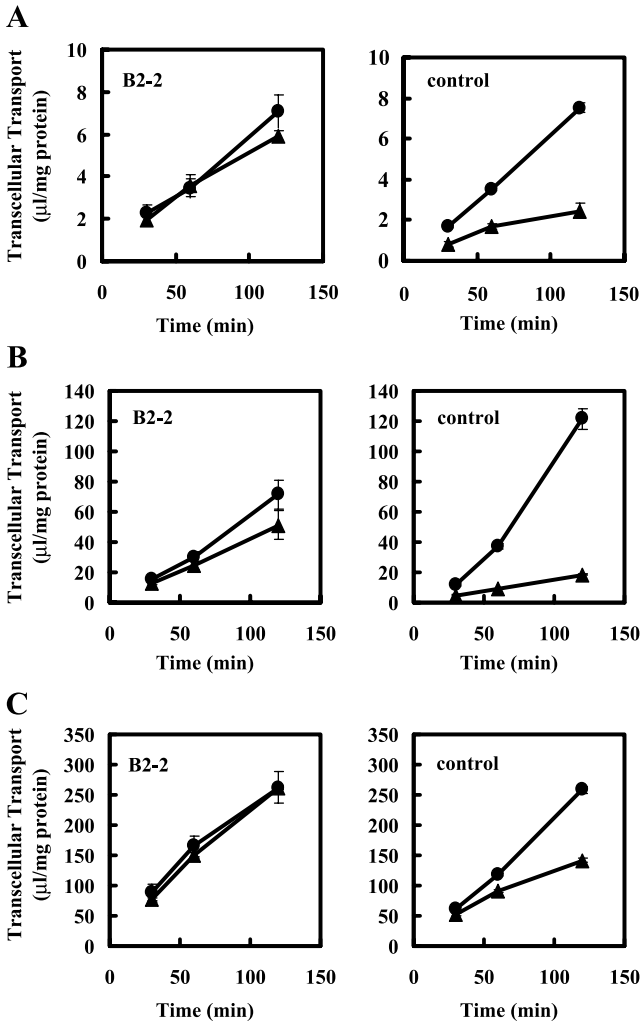

Fig. 4. Transcellular transport study of *MDR1* substrates. Time profiles for the transcellular transport across the B2-2 clone and the control cells. (A) Vincristine (435 nM). (B) Rhodamine 123 (10 μM). (C) Daunomycin (62.5 nM). The circles and the triangle with the solid lines represent the transcellular transport in the apical-to-basal and the basal-to-apical directions, respectively. Each point represents the mean ± SEM (n = 3).

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