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Involvement of MDR1 Function in Proliferation of Tumour Cells

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Mdr1 is a multi-drug-resistance protein, a member of the adenosine triphosphate-binding cassette family of drug transporters. Mdr1 is expressed in wide variety of cells and limits absorption of toxicants into the body or tissue; however, it is also expressed in many cancer cells and can render tumour cells resistant to many anti-cancer drugs. Mdr1 is well studied as a multi-drug resistance transporter, but little is known regarding its other role in tumour cells. In the present study, we investigated *mdr1* function in tumour cell proliferation. We silenced the *mdr1* gene in tumour cells by using an RNA interference method that employed short hairpin RNA. The result showed that knockdown of *mdr1* gene suppressed tumour cell proliferation *in vitro*, and induced the passage of the cell cycle into the G1/G0 phase. Furthermore, in a mice xenograft tumour formation assay, *mdr1* knockdown of tumour cells inhibited tumour expansion. These results suggest that Mdr1 plays a role in regulation of tumour cells proliferation.

Key words: Mdr1, tumour, proliferation, RNAi, drug resistance.

Abbreviations: ABC transporter, ATP-binding cassette transporter; CyA, cyclosporine A; DXR, doxorubicin; BrdU, bromodeoxyuridine; ERK1/2, extracellular signal-regulated kinase ½; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MDR, multi-drug resistance; MEFs, mouse embryonic fibroblasts; Mrp, multi-drug resistance associated protein; MTT, 3-[4,5-dimethylthiazol-2-yl]- 2,5-diphenyl-tetrazolium bromide; PI, propidium iodide; RNAi, RNA interference; Rs, reserpine; RT-PCR, reverse transcription-polymerase chain reaction; shRNA, short hairpin RNA.

The expression of multi-drug resistance (MDR) in tumour cells remains one of the most significant causes of failure in anti-tumour chemotherapy (*1*). It has been widely accepted that the mechanisms leading to chemoresistance of tumour cells is the increased activity of transporter proteins (*2*). MDR1/P-glycoprotein (p-gp) is a well-characterized transporter protein and various types of compounds and techniques for its inhibition have been developed (*3–5*). For this purpose, knowledge of the physiological function of MDR1 should provide a means for the suppression of MDR1 without side effects.

MDR1 belongs to a superfamily of ATP-binding cassette (ABC) transporters and is widely expressed in tissues and cells, including the blood–brain barrier, proximal tubular renal epithelium, gut, liver, adrenal glands, lymphocytes, haematopoietic stem cells and cancer stem cells (*6–8*). The physiological role of MDR1 is in cellular detoxification of bacterial products, hormone secretion and transport of some metabolites (*9–11*). This phenomenon is due to the ability of MDR1 to pump several types of molecules from the inside of the cell to the extracellular space (*5*).

Mice express two genes encoding Mdr1, *mdr1a* (also called *mdr3*) and *mdr1b* (also called *mdr1*) (*12*). In humans, a single MDR1 product displays both functions of murine Mdr1a and Mdr1b (*13*). Therefore, the physiological function of MDR1 in human has been deduced from the phenotype of mice in *mdr1a* and *mdr1b* gene knockout studies. Based on the analysis of mice with a genetic disruption of *mdr1a*, *mdr1b* or both *mdr1a* and *mdr1b*, it has been suggested that these genes are not essential for organ development but play important roles for drug transport (*14–16*).

In cancer, MDR1 activity appears to be the major reason for failure of chemotherapy; therefore, research has focused on the mechanism of drug resistance. However, recently, MDR1 has been suggested to directly associate with malignant proliferation of cells. One line of data showed involvement of Mdr1 in intestinal tumourigenesis through association with the tumour suppressor APC pathway (*17*). Therefore, MDR1 may regulate cell proliferation in tumours, although lack of MDR1 did not affect normal cell development in gene knockout studies (*14*, *15*). In this study, we report suppression of tumour cell proliferation by down-regulation of Mdr1 in cancer cell lines.

MATERIALS AND METHODS

Cells—Mouse rectum carcinoma cell line Colon 26 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma, St Louis, MO, USA). Mouse skin melanoma cell line B16 cells were cultured in DMEM medium supplemented with 10% heat-inactivated FBS. Fibroblast-like cell line OP9 derived from mouse calvaria was cultured in αMEM medium supplemented with 20% heat inactivated FBS. Mouse embryonic fibroblasts (MEFs) were prepared from

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embryonic day 14.5 Balb/c mouse embryos as described (*18*). MEFs were cultured in DMEM medium supplemented with non-essential amino acids plus 1 mM pyruvic acid (Invitrogen, Carlsbad, CA, USA) and 10% heat-inactivated FBS. Stably transformed Colon 26 cells were cultured in RPMI 1640 medium supplemented with 300 μg/ml geneticin (Invitrogen). All media were supplemented with 100 U/ml penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen).

Cell Proliferation Assay Using Multi-drug Resistance Transporter Inhibitor—Cell proliferation was determined by WST-8 reagent (Dojindo, Kumamoto, Japan) under the manufacturer's instruction. This assay is basically similar to the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded at a density of 3×10^3 cells per well in 96-well plates, and after overnight cultivation, Cyclosporine A (CyA; Wako, Osaka, Japan) of concentrations ranging from 1 to 10 μM or 20 M reserpine (Rs; Sigma) was added to wells. After 48 h of incubation with drug, WST-8 reagent was added to each well and cells were incubated for an additional 2 h. Absorbance at 405 nm was determined using a Biotrac II platereader (Amersham biosciences, Piscataway, NJ, USA).

Transient Knockdown of mdr1a/1b—Transient knockdown of *mdr1a/1b* gene of Colon 26 and B16 cells was performed using the short hairpin RNA (shRNA) method. shRNA coding for *mdr1a/1b* was inserted into the pSINsi-hU6 DNA vector (Takara, Shiga, Japan) at the BamH1 and Cla1 ligation sites according to the manufacturer's instruction. shRNA oligonucleotides were synthesized corresponding to the published sequence of *mdr1a* and *mdr1b* mRNAs (*mdr1a*: NM_011076, *mdr1b*: NM_011075). The following specific DNA inserts were used: *mdr1a/1b* shRNA (insert 1; forward strand: 5′-gatcc GTG TGT TCA TAG TTG TCT ATA ttc aag aga TGT AGG CAA CTA TGA GCA CAC tttttt at-3′, reverse strand: 5′-cgat aaaaaa GTG TGC TCA TAG TTG CCT ACA tct ctt gaa TAT AGA CAA CTA TGA ACA CAC g-3′), (insert 2; forward strand: 5′-gattc GAT CGT TGG TGT GGT GGG TCG ttc aag aga TGA CTC ACC ACA CCA ATG ATC tttttt at-3′, reverse strand: 5′-cgat aaaaaa GAT CAT TGG TGT GGT GAG TCA tct ctt gaa CGA CCC ACC ACA CCA ACG ATC g-3′) and a non-specific oligonucleotide insert for negative control (forward strand: 5′-gatcc GAT CGT TGG TGT GGT GGG TCG ttc aag aga ACT ACC ATG CTC CCA TGA ACA tttttt at-3′, reverse strand: 5′-cgat aaaaaa TGT TCA TGG GAG CAT GGT AGT tct ctt gaa CGA CCC ACC ACA CCA ACG ATC g-3′). Colon 26 and B16 cells were seeded at a density of 3×10^5 cells per well in 24-well plates. The following day, a plasmid containing one of the DNA inserts (noted above) was transfected into the cells using lipofectamine 2000 reagent (Invitrogen) according to the manufacture's instruction. For the evaluation of *mdr1a/1b* knockdown, *mdr1a/1b* expression was determined by real-time reverse transcription (RT) and polymerase chain reaction (PCR) (RT-PCR) and immunoblotting. Total RNA, for real-time RT-PCR, was collected from cells 48 h after transfection. Total cell lysate for immunoblotting was prepared from cells after 72 h of transfection. For growth assay, transfected cells were harvested with trypsin and re-seeded at a density of 3×10^4 cells in 6-well plates after 1 day of transfection. Four days later, cells were harvested and the total cell number was determined.

Generation of mdr1a/1b Knockdown Cells—Stable knockdown of *mdr1a/1b* gene of Colon 26 cells was achieved using the shRNA. shRNA vectors with mdr1a/1b-specific insert 1, non-specific insert or a mock vector was used. Colon 26 cells were seeded at a density of 1×10^4 cells per well in 24-well plates. The following day, a plasmid containing the DNA insert (noted above) was transfected by using lipofectamine 2000 reagent (Invitrogen) using the manufacture's instruction. Forty-eight hours after infection, the cells were harvested by trypsinization and re-seeded at a density of 2×10^4 cells in 10-cm culture dishes. The following day, geneticine was added to a final concentration of 300 μg/ml. Eight days after addition of geneticine, colonies were picked by using a cloning ring (Asahi techno glass corporation, Tokyo, Japan) and re-seeded in the culture plate. For the evaluation of *mdr1a/1b* knockdown, *mdr1a/1b* expression was determined by real-time RT-PCR.

RNA Isolation and RT-PCR Analysis—Total RNA was collected using Isogen reagent (Invitrogen) as previously reported (*19*). Total RNA was reverse transcribed using the MMLV Reverse Transcriptase (Invitrogen). The cDNA was amplified using *Taq* polymerase (Takara) in a GeneAmp PCR system model 9700 (Applied Biosystems, Foster City, CA, USA) using 32 cycles. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min. Primers used for RT-PCR were as follows; *mdr1a* (sense: GAA TTG GTG ACA AAA TCG GA, anti-sense: TGT CTA TAC TGG GCT TAT TA), *mdr1b* (sense: GGA ACT CTC GCT GCT ATT AT, anti-sense: GGT TAG CTT CCA ACC ACT TA), *mdr2* (sense: AAC ACA GCC AAC CTT GGA AC, anti-sense: CAG CCC TTC TCC ACT GTA GC), *mrp1* (sense: CCA CTC TGG GAC TGG AAT GT, anti-sense: GGG GTG AGC AGT CTG AGA AG), *beta-actin* (sense: CCT AAG GCC AAC CGT GAA AAG, anti-sense: TCT TCA TGG TGC TAG GAG CCA).

For real-time RT-PCR, reactions were performed by using platinum SYBR green qPCR supermix UDG reagent (Invitrogen). Cycling conditions were 2 min at 50°C, 10 min at 95°C, followed with 41 cycles of alternating 15 s at 95°C and 1 min at 60°C. The quantity of *mdr1a*, *mdr1b*, *mrp1* detected in each reaction tube was normalized to the level of the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) internal control. Primers used for real-time RT-PCR were as follows; *mdr1a* (sense: AGT GTC AGC ATC CCA CAT CA, anti-sense: TTG CAC ATT TCC TTC CAA CA), *mdr1b* (sense: GGA CAT CCT TGG TCC TCT CA, anti-sense: CTA TGT TTG GGG CCA AGT GT), *mrp1* (sense: TTG GTG TGA GCT GGT CTC TG, anti-sense: TTT GGG TTT TGT CTG GGA AG), *GAPDH* (sense: TGG CAA AGT GGA GAT TGT TGC C, anti-sense: AAG ATG GTG ATG GGC TTC CCG). Data were collected using an ABI prism 7900 HT RT-PCR System (Applied Biosystems).

Immunoblotting—Total cell lysates from each transfectant were prepared by lysis in buffer [10 mM Tris–HCl

(pH7.4), 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 1 mM EDTA-2Na] and the proteins were separated by electrophoresis on 10% polyacrylamide gels containing SDS. The proteins were transferred by electroblotting to polyvinylidene difluoride membranes and the membranes were subsequently incubated with the following antibodies: anti p-gp rabbit polyclonal antibody (H-241; Santa Cruz, DE, CA, USA), anti GAPDH mouse monoclonal antibody (Chemicon, Temecula, CA, USA), anti-extracellular signal-regulated kinase 1/2 (ERK1/2) rabbit polyclonal antibody or anti-phospho-ERK1/2 (Thr202/Thr204) rabbit polyclonal antibody (Cell Signaling Technology, Beverly, MA). Proteins were detected with horseradish peroxidase (HRP)-conjugated anti-rabbit Ig (Dako Cytomation, Glostrup, Denmark), anti-mouse Ig (Dako) or anti-rabbit IgG (Cell Signaling Technology, for detection of P-ERK1/2). After extensive washing, the antigen was visualized using the ECL plus reagents (Amersham) according to the manufacturer's instructions.

Cell Kinetics Assays Using Mdr1 Knockdown Cell Line—For cytotoxicity assay, cells were seeded at a density of 3×10^3 cells per well in 96-well plate, and next day 0.5 μg/ml doxorubicin (DXR; Biovision, Mountain View, CA, USA) was added. After 48 h of incubation with drug, WST-8 reagent was added to each well and cells were incubated for an additional 2 h. Absorbance at 405 nm was determined using a Biotrac II plate reader (Amersham Biosciences). For cell growth assay, each clone was seeded at a density of 5×10^4 cells per well in 6-well plates. The following day and 3 days later, cells were collected by trypsinization, counted by using a cell counting chamber and the doubling time for cell division was calculated. For the analysis of DNA synthesis, each clone was seeded at a density of 3×10^4 cells per well in 24-well plates. Two days later, bromodeoxyuridine (BrdU; SIGMA) was added at 10 μM, and incubated for 4–5 h. To examine BrdU positive cell, cells were incubated with anti-BrdU rat IgG (abcam, Cambridge, Cambridgeshire, UK), followed with alexa 488-conjugated goat anti rat IgG antibody (molecular probes, Eugene, OR, USA), and examined by fluorescence microscopy (IX70, Olympus, Tokyo, Japan). For nuclear counter staining, Hoechst 33342 (Nacalai, Kyoto, Japan) was used. Immunocytochemical methods were performed as described previously (*20*). For cell cycle analysis, each clone was seeded at a density of 5×10^5 cells per 10-cm culture dishes. Two days later, cells were collected and stained with propidium iodide (PI) using standard protocol. Stained cells were detected by flow cytometry, FACS calibur system (Becton Dickinson, Franclin Lakes, NJ, USA), and analysed by Modfit LT software (Verity Software House, Augusta, ME, USA).

Mouse Xenograft Assay—Six- to 8-week-old female Balb/c mice were injected subcutaneously with 5×10^5 cells as described previously (*19*). Mice injected with cells were sacrificed 2 weeks post-injection, the tumour was dissected and the tumour weight was determined. Mice were purchased from SLC (Shizuoka, Japan). These studies were approved by the animal care committee of Osaka University and Kanazawa University.

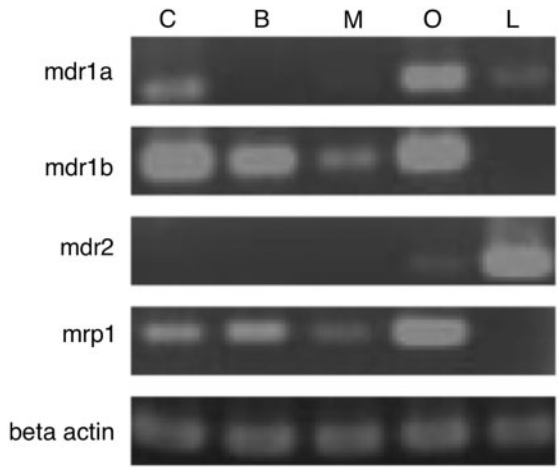

Fig. 1. **Expression of *mdr1a/1b* mRNA in tumour cells.** Total RNA from each cell type was subjected to RT-PCR analysis. C: Colon 26, B: B16 melanoma, M: MEFs, O: OP9, L: mouse whole liver. Liver was used as positive control for mdr2 detection; beta-actin was used as an internal control.

RESULTS

Suppression of Tumour Cell Proliferation by Inhibition of Mdr1 Function—Intestinal tumourigenesis usually observed in Min ($Apc^{Min/+}$) mice was reported to be suppressed in the *mdr1a/1b* double mutant background (*17*). This might suggest that malignant proliferation of tumour cells is dependent on Mdr1a/1b function. To test this property, we observed *mdr1a/1b* expression on cells from a mouse colon cancer cell line, Colon 26, and compared its expression to that on cells from a non-gastrointestinal cancer cell line, B16 (melanoma), osteoblastic cell line OP9 and primary normal cells, MEFs. As expected, Colon 26 cells expressed both *mdr1a* and *mdr1b* (Fig. 1). On the other hand, B16 melanoma cells and MEFs expressed *mdr1b* weakly but did not express *mdr1a*. Moreover, OP9 cells expressed both *mdr1a* and *mdr1b* as found in Colon26 cells. To test the role of Mdr1 in cell proliferation, firstly, we blocked the pump function of Mdr1 on these four different types of cells by using MDR modifier CyA or Rs. MEFs and OP9 cells could proliferate in the presence of increasing concentrations (up to 10 μM) of CyA but the growth of Colon 26 and B16 cells decreased by as much as 70–80% at the highest concentration (Fig. 2A). As with CyA, Rs suppressed the growth of Colon 26 and B16 cells but not that of MEFs. However, unlike CyA, Rs attenuated the proliferation of OP9 cells (Fig. 2B). These results suggested that MDR1 function is associated with proliferation of transformed cells that had acquired the property of permanent cell proliferation, like tumour cells. However, based on controversial result of CyA and Rs on the growth of OP9 cells, it appears that gene knockdown experiment of Mdr1 is required for the clarification of whether Mdr1 is actually involved in tumour cell growth or not. Moreover, there is another member of a multi-drug resistance transporter family of proteins Mrp1. Mrp1 is expressed on tumour cells and CyA also inhibit Mrp1 pump function (*5*). Indeed, both colon 26 cells and B16 melanoma cells expressed *mrp1* (Fig. 1). Therefore, to

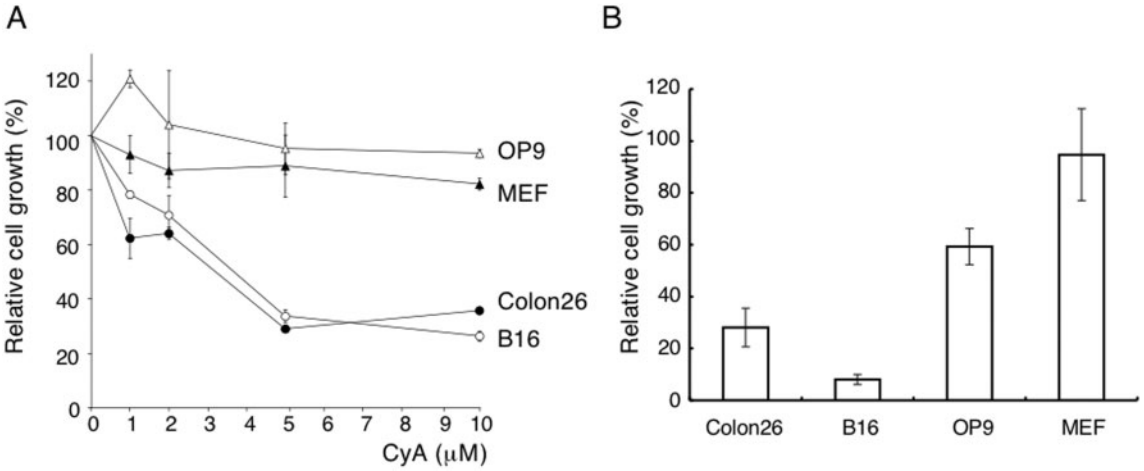

Fig. 2. **Effect of *mdr1a/1b* on cell growth.** (A) After 2 days of culturing with various concentration of CyA as indicated, cell growth was analysed with WST-8 assay. For each concentration of CyA, the percentage of O.D. measured in the presence of CyA relative to that in the absence of CyA is shown. Filled circle: Colon 26 cells, open circle: B16 cells, filled triangle: MEFs, open triangle: OP9. Each data point represents the mean and the bar shows the SD of three independent measurements. (B) After 2 days of culturing with 20 μM reserpin (Rs), cell growth was analysed using the WST-8 assay. The percentage of O.D. measured in the presence of Rs relative to that in the absence of Rs is shown.

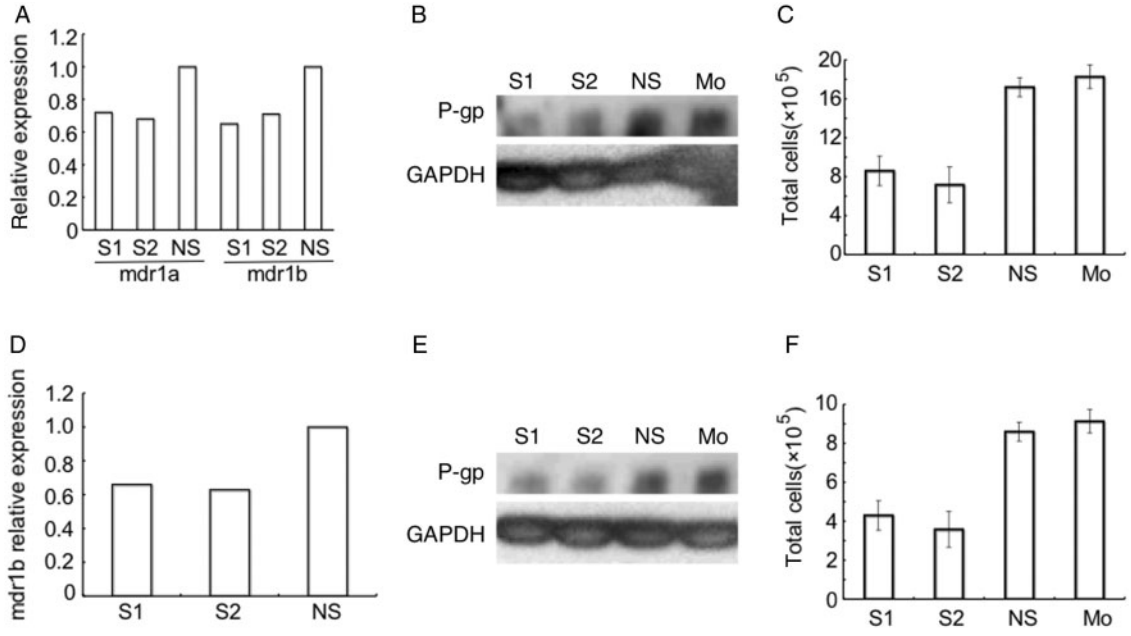

Fig. 3. **Effect of transient mdr1a/1b knockdown on tumour cell growth.** After transfection with S1 or S2 (two different vector containing shRNA for mdr1a/1b), NS (vector containing a non-specific insert) or Mo (mock vector containing no shRNA insert) into Colon 26 cells (A–C) or B16 cells (D–F), expression of mRNA for *mdr1a/1b* was determined (A, D) and protein level (B, E) and cell growth was measured in the cell cultures (C, F). (A and D) Total RNA from cells transfected with each vector was subjected to real-time RT-PCR analysis. Data show the relative expression level of *mdr1a* or *mdr1b* (A) or *mdr1b* (D) gene in S1 or S2 transfected cells compared with that in NS transfected cells. (B and E) Total protein from cells transfected with each vector was subjected to western blotting analysis. p-gp and GAPDH expression of the clones was analysed. GAPDH was used as an internal control. (C and F) Cells transfected with each vector were seeded in culture dishes; the total number of cells was determined after 4 days of culturing. Data show mean ± SD derived from representative result of three independent experiments.

test whether growth inhibition of tumour cells is actually regulated by *mdr1a/1b*, we ablated *mdr1a/1b* genes in Colon 26 and B16 cells by performing RNA interference (RNAi).

Transient Knockdown of mdr1a/1b Gene on Colon26 and B16 Cells Inhibits Cell Growth—To test whether attenuation of mdr1a/1b expression on Colon 26 or B16 cells inhibits cell growth or not, we performed gene silencing by using shRNA method (Fig. 3). Mice express two genes encoding *Mdr1*, *mdr1a* and *mdr1b* (*12*). In humans, a single MDR1 product displays both functions of murine Mdr1a and Mdr1b (*13*). Mouse *mdr1a* is

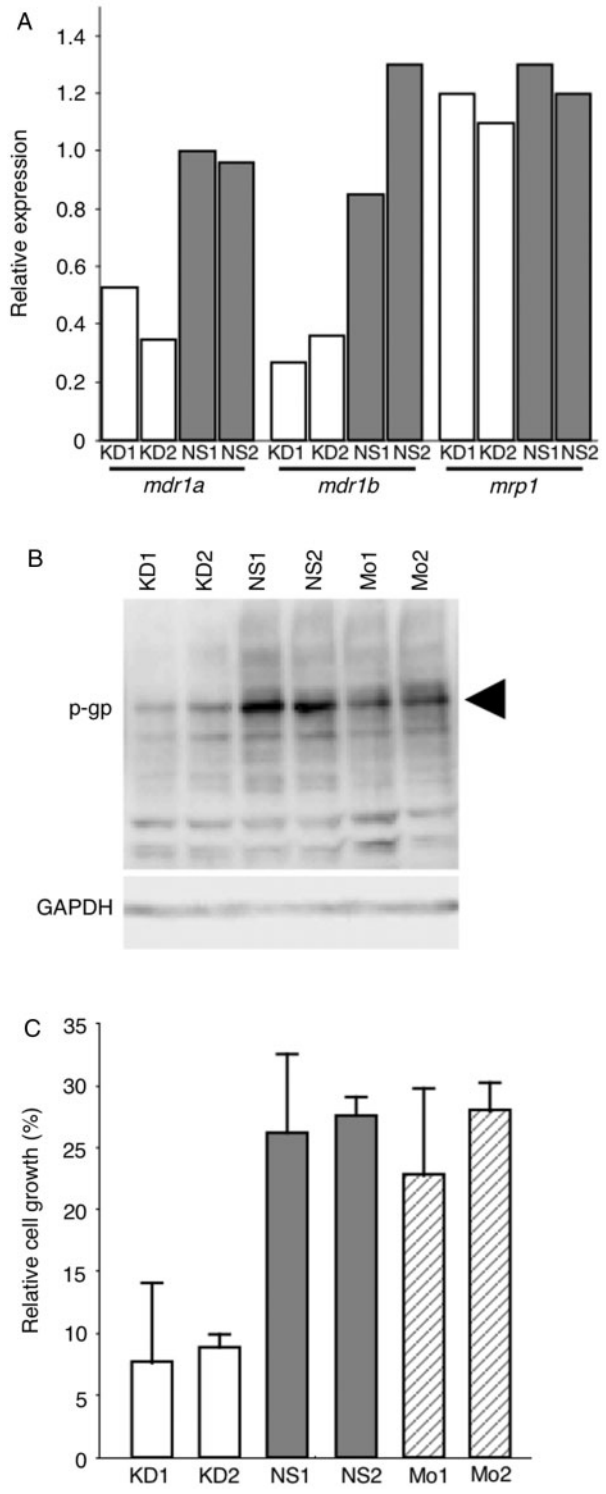

Fig. 4. **Generation of stable *mdr1a/1b* knockdown Colon26 cells.** (A) Total RNA from each clones were subjected to real-time RT-PCR analysis. Data show the relative expression level of RNA compared to Colon 26 clones transfected with a mock vector. In each clone, *mdr1a*, *mdr1b* and *mrp1* expression were analysed. (B)Total protein from each clone was subjected to western blotting analysis. p-gp and GAPDH expression of the clones were analysed. GAPDH was used as an internal control. (C) To evaluate the suppression of *mdr1a/1b*, each clone was incubated with 0.5 μg/ml DXR. Two days later after incubation with DXR, cell growth was analysed by WST-8 assay. Data represent relative ratio of O.D. from cells incubated with DXR compared with that incubated with control vehicle. Data show mean ± SD ($n = 3$) derived from representative result of three independent experiments. KD1, KD2: 2 independent clones of *mdr1a/1b* knockdown Colon 26 cells using *mdr1a/1b*-specific shRNA; NS1, NS2: 2 independent clones of control Colon 26 cells transduced with a vector containing a non-specific shRNA insert; Mo1, Mo2: 2 independent clones of control Colon 26 cells transduced with a mock vector containing no shRNA insert.

expressed in the intestinal epithelium and at the blood–brain and blood–testis barriers, whereas *mdr1b* is highly expressed in the adrenal gland, pregnant uterus and ovary. Although tissue distribution of *mdr1a* or *mdr1b* is different, functional discrimination between *mdr1a* and *mdr1b* has not been reported (*15*). Therefore, in order to know the function of Mdr1 in tumour cells, we used plasmids containing two different oligonucleotide inserts of shRNA (S1 or S2) that specifically recognize both of *mdr1a* and *mdr1b* sequences. With this method, we caused interference with the expression of *mdr1a* and *mdr1b* by using a common sequence shared by both *mdr1a* and *mdr1b* mRNAs. Upon transfection with plasmids (S1 and S2), both *mdr1a* and *mdr1b* expression of Colon 26 cells and *mdr1b* expression on B16 cells were reduced compared with the level of expression of control cells transfected with a plasmid containing a non-specific shRNA (NS) (Fig. 3A and D). Western blotting showed a reduction in mdr1a/1b (p-gp) protein expression in Colon 26 and B16 cells transfected with S1 or S2 containing vectors (Fig. 3B and E). Under these conditions of reduced mdr1a/1b expression, suppression of tumour cell growth was observed in both Colon 26 and B16 cells (Fig. 3C and F). Therefore, we confirmed that inhibition of pump function of tumour cells suppresses cell proliferation.

Stable Knockdown of mdr1a/1b Genes on Colon 26 Cells Inhibits Cell Growth—Finally, as it has been reported that intestinal tumourigenesis observed in Min ($Apc^{Min/+}$) mice was suppressed in the *mdr1a/1b* double mutant background (*17*), we tested how attenuation of mdr1a/1b expression affects *in vitro* and *in vivo* cell growth of Colon 26 intestinal cancer cells, when *mdr1a/1b* genes were stably knocked down in these cells.

Upon transfection with plasmids containing shRNA oligonucleotides (specific insert 1) that specifically recognized *mdr1a/1b* sequences, both *mdr1a* and *mdr1b* expression of Colon 26 cells (KD1 and KD2, two different subclones) was reduced compared to the level of expression when the cells were transfected with a plasmid containing non-specific shRNA (NS1 and NS2); however, expression of *mrp1* was not affected by this RNAi method (Fig. 4A). Western blotting showed a reduction in mdr1a/1b (p-gp) protein expression in clones (KD1 and KD2, two different subclones) (Fig. 4B), as found in the real-time PCR analyses for mRNA expression.

Using several independent knockdown transformants of Colon 26 cells (KD1 and KD2), we evaluated the sensitivity for anti-cancer drug, DXR, to confirm whether Mdr1a/1b function was reduced by the RNAi method. As showed in Fig. 4C, sensitivity for DXR was not affected in Colon 26 subclones transduced with non-specific shRNA (NS1 and NS2) and also with mock vector

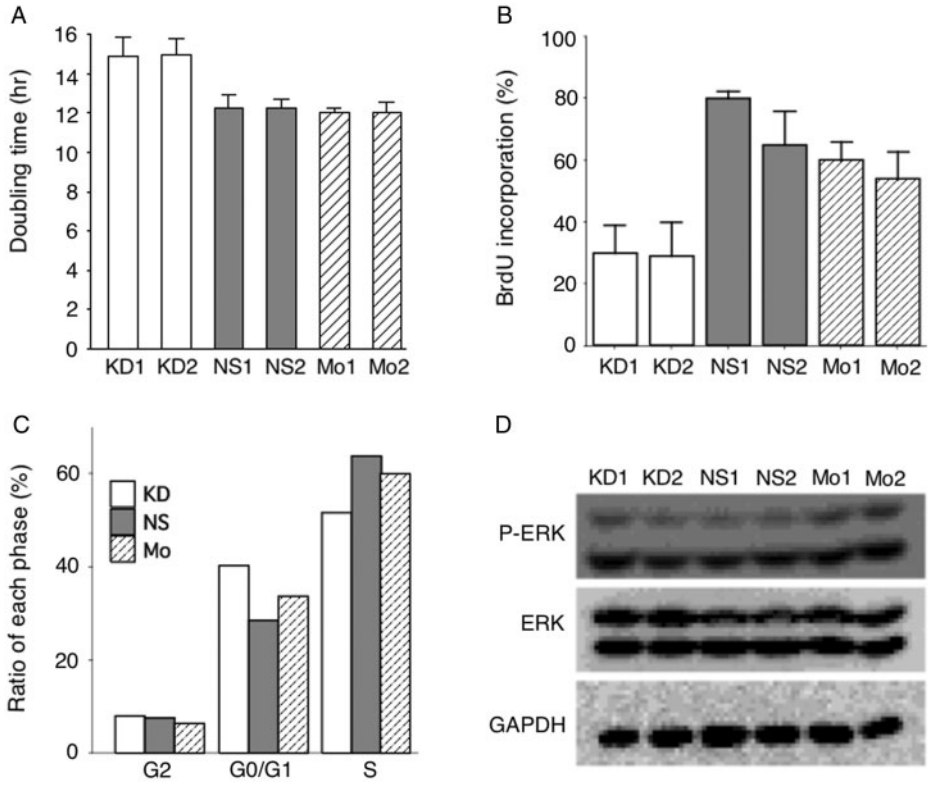

Fig. 5. **Knockdown of *mdr1a/1b* inhibits growth activity of tumour cells.** (A) Cell growth was affected by knockdown of *mdr1a/1b* on Colon 26 cells. Cells were seeded in culture dishes; the total number of cells was determined on Day 1 and Day 3 and the doubling time for cell division was calculated. Data shows doubling time; data represent the mean ± SD of three experiments ($n = 3$). (B) DNA synthesis affected by knockdown of *mdr1a/1b* on Colon 26 cells. Clones were seeded in culture dishes and 2 days later BrdU was added to the culture. Data show percentage of BrdU-positive cells/total cells. Bar graphs show the mean and the bar lines show the SD ($n = 3$). (C) Cell cycle was affected by knockdown of *mdr1a/1b* of Colon 26 cells. Cells were stained with PI and analysed by flowcytometry. The percentage of cells in G2, G0/1 and S phase are shown. Data are representative results from three independent experiments. (D) Total protein from each clone was subjected to western blotting analysis. P-ERK, ERK and GAPDH expression of the clones was analysed. GAPDH was used as an internal control. KD; *mdr1a/1b* knockdown Colon 26 cells using mdr1a/1b specific shRNA, NS; control Colon 26 cell transduced with a non-specific insert, Mo; control Colon 26 cells transduced with a mock vector. Numerals 1 and 2 for KD, NS and Mo in (A, B) indicate two independent clones as described in Fig. 4.

(Mo1 and Mo2, two different subclones). As expected, sensitivity for DXR was up-regulated by *mdr1a/1b*-specific RNAi in Colon 26 cells.

Next, we determined how attenuation of *mdr1a/1b* expression in Colon 26 cells affects cell growth. Doubling time for cell division suggested that rate of proliferation of *mdr1a/1b* knockdown Colon 26 cells (KD1 and KD2) was slower than that of control Colon 26 cells (NS1, NS2, Mo1 and Mo2) (Fig. 5A). BrdU staining showed that the number of positively stained cells was lower in case of *mdr1a/1b* knockdown Colon 26 cells compared to control cells (Fig. 5B). Cell cycle analysis showed that cells in Go phase were most abundantly observed in *mdr1a/1b* knockdown Colon 26 cells compared with control Colon 26 cells and cells in the S phase were fewest in case of *mdr1a/1b* knockdown Colon 26 cells compared with control Colon 26 cells (Fig. 5C). Taken together, these results indicate that the growth retardation observed in *mdr1a/1b* knockdown Colon 26 cells was caused by delay of cell cycle based on diminished DNA synthesis.

Suppression of In Vivo Tumourigenesis of Colon 26 Cells through Reduction of mdr1a/1b Function—In vitro analysis of cell growth demonstrated that *mdr1a/1b* is closely involved in cell proliferation. However, *in vitro* culture conditions may lack various molecular cues in tumour cell growth in cells with reduced *mdr1a/1b* mRNA. Therefore, finally, we investigated whether *in vivo* tumour development is also affected by reduction of *mdr1a/1b* in Colon 26 cells. Upon subcutaneous inoculation of Colon 26 cells transduced with *mdr1a/1b* specific shRNA or a non-specific control shRNA, tumour weight was significantly reduced in *mdr1a/1b* knockdown Colon 26 cells compared to that in control Colon 26 cells (Fig. 6). Therefore, we concluded that *mdr1a/1b* which has been thought as a drug-resistant molecule in intestinal tumour cells has a functional role in regulation of cell proliferation.

DISCUSSION

Recently, several reports indicated that MDR1 function in tumour cells is associated not only with drug resistance but also tumour cell kinetics. For example, a tumour suppressor protein p53 represses transcription

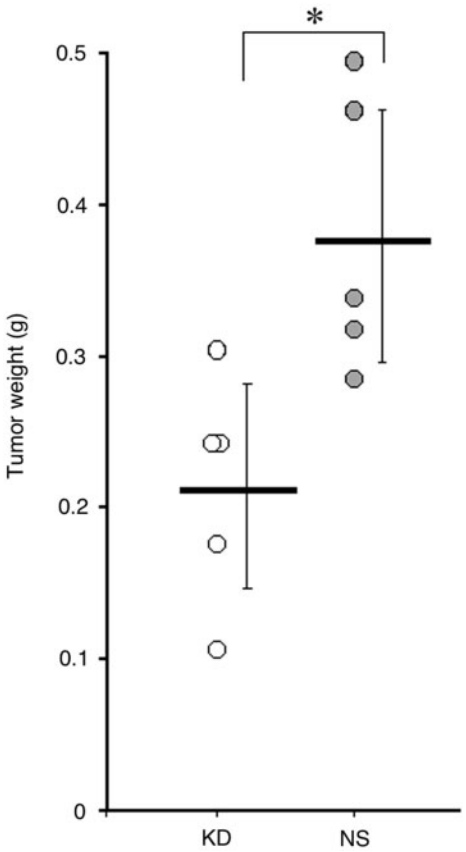

Fig. 6. **Knockdown of *mdr1a/1b* in Colon 26 cells inhibits tumour growth.** *mdr1a/1b* knockdown Colon 26 cells using *mdr1a/1b* specific shRNA (KD) or control Colon 26 cell transduced with a non-specific insert (NS) were inoculated into mice. Fourteen days later, the tumours were dissected and tumour weight was determined. Data shows representative result from three independent experiments; the two groups were statistically significantly different ($P < 0.05$).

of *MDR1* gene (*21–23*). MDR1 expression and up-regulation of its activity has been observed in various cells stimulated by growth factors such as EGF (*24*), IGF-I (*25*) and FGF6 (*26*). Moreover, early responsive genes, Fos/jun (AP-1) activated by such growth factors have been suggested to directly regulate *mdr1* expression (*27*). Taken together, these data strongly support the notion that Mdr1 is involved in the cell growth. However, at far as we could determine, attenuation of Mdr1 expression on Colon 26 cells did not affect MAPK activation in the culture condition we used (Fig. 5D). At present, although the mechanism whereby Mdr1 regulates cell proliferation is not clear, association with cell cycle check points, longevity of cells (failure of apoptotic mechanisms) and repair of damaged cellular targets are possible ways in which it can exert this effect. Further precise analysis of MDR1 involvement in the cell cycle of tumour cells is required.

In the present study, we evaluated the function of *mdr1a/1b* in tumour cell proliferation. Our strategy using shRNA that specifically recognized *mdr1a/1b*, knockdown of *mdr1a/1b* was not complete. If mdr1a/1b expression was completely attenuated, it may be possible to achieve more severe suppression of tumour growth. However, as shown in Fig. 1, another member of Mdr family, *mrp1* is expressed in tumour cells. MRP1 has been reported to be expressed in many cancers at high levels (*28*). Although MRP1 may contribute to the baseline drug resistance of cells including normal cells and tumour cells, the specific role of MRP1 in tumour cells remains to be demonstrated. However, if MRP1 has a potential association with cell proliferation as observed in *mdr1a/1b*, inhibition of both *mdr1a/1b* and *mrp1* may effectively inhibit tumour growth.

Taking into account the nature of MDR1 function for drug resistance in tumour cells, the design of non-toxic agents that would overcome MDR in tumour has been considered. However, because of their predicted toxicity, none of the drugs that inhibit MDR1 function are available in the clinic. To overcome this issue, less toxic agents should be developed. One such approach is gene therapy targeting the expression of MDR1 in tumours specifically. Our present data as well as other reports (*29*, *30*) showed that RNAi method could bring about a reduction in the expression and function of MDR1. If a suitable gene therapy system for delivery of RNAi into tumour site is developed, RNAi methods using *mdr1* gene as a target is a likely candidate to inhibit tumour growth.

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