Major Vault Protein Does Not Play a Role in Chemoresistance or Drug Localization in a Non-Small Cell Lung Cancer Cell Line†

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ABSTRACT: The human major vault protein (MVP) is the primary component of the 13 MDa vault complex. MVP has been implicated in the development of non-P-glycoprotein-mediated drug resistance in cancer cells. Here we present several lines of evidence that dispute this assertion. siRNAs capable of specifically and efficiently knocking down expression of MVP do not alter the ability of resistant cells to remove doxorubicin from the nucleus and do not increase sensitivity to the drug. Conversely, upregulation of MVP in chemosensitive cells does not confer increased drug resistance. In multi-drug resistant (MDR) lung carcinoma cells, fluorescence microscopy reveals that doxorubicin enters the nucleus and is then removed, inconsistent with suggestions that vaults either act to prevent the drug from entering the nucleus or are involved as a nuclear efflux pump. These data suggest that vaults play no direct role in the MDR phenotype in non-small cell lung carcinoma cells and that their cellular function remains unknown. These results also have important implications concerning the value of MVP as a drug target and as a prognostic marker for chemotherapy failure. Our results suggest the need for further investigation into the link between upregulation of vaults and malignancy, the mechanism behind non-P-gp-mediated drug resistance, and the role of vaults in human cells.

The human vault particle is a 13 MDa ribonucleoprotein complex comprised of the major vault protein (MVP),[1] vault-associated poly(ADP-ribose) polymerase (vPARP), telomerase-associated protein, and three small untranslated RNAs (*1*−*5*). It is the largest ribonucleoprotein particle described to date. Vaults nearly identical in size and morphology have been identified in many phylogenetically diverse species, including vertebrates, echinoderms, and protozoa (*6*, *7*). The remarkable conservation of vault structure and their ubiquitous presence suggest a universal and essential role in cellular activities. Although no specific function has been assigned to this complex, it has been widely suggested that vaults may be involved in the development of drug resistance in human cancers (*8*, *9*).

Vaults were first discovered in preparations of clathrin-coated vesicles from rat liver and were so named because their morphology is reminiscent of vaulted ceilings in cathedrals (*1*). They are highly regular, hollow, barrel-shaped particles with an invaginated region encircling the center of the complex and two protruding end caps (*10*, *11*). The majority of vault complexes are localized to the cytoplasm, although some evidence suggests a small percentage of vaults associate with the nuclear membrane or possibly the nuclear pore complex (*12*, *13*).

Seventy percent of the particle mass can be attributed to MVP which comprises the body of the complex. In the absence of TEP 1, vPARP, and vRNA, expression of MVP in insect cells does result in the presence of vault-like particles (*14*). Conversely, liver lysates from MVP-deficient mice show no vault particles, underscoring the importance of MVP in the formation of vaults (*15*).

The role of vaults in cellular function remains unknown. Characterizations of vault structure, localization, and protein associations have led some to suggest that they are involved in intracellular and possibly nucleocytoplasmic transport (*12*, *13*, *16*, *17*). Studies in humans have shown that the expression of MVP (also known as LRP, lung resistance-related protein) is tissue-dependent, with higher levels of MVP found in cells more frequently exposed to xenobiotics, such as epithelial, gastrointestinal, and lung cells (*18*, *19*). Recent evidence suggests that vault may serve as a scaffold protein promoting epidermal growth factor signaling (*20*).

Several lines of evidence indicate that vaults make an important contribution to drug resistance in some human cancer cells. Expression of MVP and other vault components is enhanced in cells displaying a non-P-glycoprotein-dependent MDR phenotype (*21*−*23*). In clinical samples, MVP expression levels closely reflect the chemoresistance profile of several histogenetically unrelated tumor lines (*24*, *25*). A number of studies have suggested that an increased level of MVP expression is indicative of a poor response to chemotherapy (*26*−*28*), although this correlation has not been consistent (reviewed in ref *25*).

The Akiyama group was the first to report a direct linkage between vaults and chemoresistance (*29*, *30*). They observed

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[1] Abbreviations: MVP, major vault protein; MDR, multidrug resistance.

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that MVP overexpression in SW620 colon carcinoma cells led to an increase in resistance to various chemotherapeutic drugs. Conversely, knockdown of MVP expression by MVP specific ribozymes caused a decrease in resistance to these drugs. They also demonstrated that doxorubicin is more rapidly removed from nuclei isolated from cells that overexpress vaults and that treating isolated nuclei with polyclonal MVP antibodies appears to hamper drug removal. This work suggests that vaults may be involved in nuclear drug efflux, possibly acting in a fashion similar to that of the ATP-binding cassette (ABC) drug transporters.

Balanced against this evidence for a direct linkage between vaults and drug resistance is the observation that overexpression of MVP in ovarian and lung carcinoma lines does not result in an increase in drug resistance (*23*, *31*). Recent studies demonstrate that embryonic cells extracted from MVP-deficient mice show no more susceptibility to cytotoxic drugs than their wild-type counterparts (*15*).

In this study, we directly assess the contribution of vaults to drug resistance by knocking down MVP expression in a human non-small cell lung carcinoma line with siRNA. Using both chemosensitive and drug resistant cell lines, we examined the effects of a reduced level of MVP expression on cellular localization and sensitivity to doxorubicin. In addition, we used sodium butyrate to upregulate endogenous MVP expression in chemosensitive lines to examine any potential effects on drug sensitivity. We find that manipulating the expression of endogenous MVP does not alter the cellular localization of or resistance to doxorubicin.

MATERIALS AND METHODS

Cell Culture. The human cervical cancer HeLa cell line was obtained from American Type Cell Culture Collection (ATCC, CCL-2). The multiple-drug resistant (MDR) tumor cell line SW1573-2R120 (non-small cell lung carcinoma) and its parental chemosensitive line, SW1573, were the kind gifts of G. Scheffer from the Free University Hospital (Amsterdam, The Netherlands). All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 2 mM L-glutamine, 500 units/mL penicillin, and 0.1 mg/mL streptomycin. Cells were cultured at 37 °C with a 5.0% CO_2 atmosphere.

Assessment of Cell Proliferation. The cells were seeded at a density of 5000−8000 cells/well (0.1 mL) in 96-well plates and incubated overnight at 37 °C. The next day, the cells were exposed to varying concentrations of doxorubicin (all agents from Sigma-Aldrich) for 48 or 72 h. At the end of the experiments, 20 μL of CellTiter 96 AQ_{ueous} One solution reagent MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt] (Promega, Madison, WI) in 100 μL of Opti-MEM was added to each well, and cells were incubated for 15−30 min, based on the rate of color change. Cell viability was estimated by monitoring the absorbance at 490 nm using a MR5000 microtiter plate reader (Dynatech).

siRNA Preparation. The following siRNA sequences were synthesized: MVP-1 (plus strand, 5′-uaggagucaccauggcaacTT-3′; minus strand, 5′-guugccauggugacuccuaTT-3′), MVP-UTR (plus strand, 5′-uuccccaucugaggcguuuTT-3′; minus strand, 5′-aaacgccucagaugggggaaTT-3′), MVP-ORF (plus strand, 5′-gagaggguacuguuugcccTT-3′; minus strand, 5′-gggcaaacaguaccucucTT-3′), and MVP-Scr (plus strand, 5′-guugccauguccuagugacTT-3′; minus strand, 5′-gucacuaggacauggcaacTT-3′). Deoxyribonucleotides are represented by capital letters and are located at the 3′-end of the siRNA sequences listed above. All siRNA oligomers were synthesized on the 0.2 μM scale by the RNA Synthesis Core Laboratory at the Center for Biomedical Inventions (The University of Texas Southwestern Medical School). All RNAs were provided as lyophilized single strands.

RNA duplexes are made by first hydrating oligomers in DEPC and water and then annealing them in a solution containing 2.5× PBS [10× phosphate-buffered saline (pH 7.4) without calcium or magnesium chloride, 10 mM KH_2PO_4, 1.55 M NaCl, and 30 mM $Na_2HPO_4 \cdot 7H_2O$; Invitrogen]. Annealing of RNA duplexes was performed in a thermal cycler according to the following temperature profile. Reductions in temperatures occurred in 1 min with the following hold times (, min): 95 °C for 5 min, 85 °C for 1 min, 75 °C for 1min, 65 °C for 5 min, 55 °C for 1 min, 45 °C for 1 min, 35 °C for 5 min, 25 °C for 1 min, and 15 °C for 1 min, held at 15 °C. After annealing, the RNA duplexes were stored at −20 °C.

siRNA Transfection. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4 g/L glucose (Sigma) supplemented with 10% fetal bovine serum (Atlanta Biologicals), 20 mM HEPES buffer (final concentration, pH 7.4), 4 mM L-glutamine, 500 units/mL penicillin, 0.1 mg/mL streptomycin, and 0.01 mg/mL tylosin (anti-mycoplasma reagent) (Sigma). Cells were cultured at 37 °C with a 5.0% CO_2 atmosphere. Cells were seeded in 6-, 12-, or 24-well plates at an appropriate density and allowed to attach to the culture vessel for 8−12 h prior to transfection. Duplex siRNAs were prepared as described above at stock concentrations of 100 μM. Lipid−siRNA complexes were prepared at 200 nM, and serial dilutions were performed after formation of siRNA−lipid complexes. OligofectAMINE (Invitrogen, Carlsbad, CA) was used to deliver siRNAs. Transfection solutions were prepared according to manufacturer protocols with Opti-MEM reduced-serum antibiotic free medium (Invitrogen), and 400 μL of the transfection solution was dispensed per well. Transfections were conducted overnight (∼10−14 h), after which the solution was removed and replaced with growth medium containing antibiotics. Cells were harvested for Western blot analysis 72 h post-transfection (the time required for cells to reach confluence within the wells).

Western Analysis. Cells were harvested by washing the cells once with 1× PBS, aspirating, and treating them with 50 μL of a trypsin solution (0.05% trypsin and 0.53 mM EDTA·4Na; Invitrogen) at 37 °C for 2 min. Trypsin was inactivated by the addition of culture medium containing 20% FBS. The contents of each well were transferred separately into 1.5 mL microfuge tubes and centrifuged at 1000*g* for 5 min at 4 °C. The supernatant fluid was discarded, and the cell pellet was suspended in 400 μL of 1× PBS. The contents were again centrifuged at 1000*g* for 5 min at 4 °C. Cells were then lysed with 40−50 μL of ice-cold lysis buffer [120 mM Tris base (pH 7.4), 120 mM NaCl, 1 mM Na_2-EDTA, 1 mM DTT, 10 mM β-glycerophosphate, 0.1 mM sodium fluoride, 0.1 mM sodium vanadate, and 0.5% (v/v) Nonidet

P-40] containing Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN). Tubes were vortexed for 10−20 s and then allowed to incubate for 5−10 min at room temperature.

Samples that were used immediately were centrifuged at 12000*g* for 5 min at 4 °C to pellet debris. Samples not used for immediate analysis were stored at −20 °C. The protein content was determined for each sample in a 96-well plate by the BCA method (Pierce, Rockford, IL).

Western analysis by SDS−PAGE was performed using standard methods. The primary antibody for MVP was mouse monoclonal anti-human LRP (BD Transduction Laboratories, San Jose, CA). Anti-LRP antibodies were diluted 1:2000 in Tween Tris-buffered saline (TTBS) and placed on a rocker platform for 1 h at room temperature. The membranes were washed twice for 5 min each in TTBS. Secondary antibody conjugates (HRP-conjugated goat anti-rabbit or goat anti-mouse) were diluted 1:5000 in TTBS and placed on a rocker platform for 1 h at room temperature. Membranes were then washed four times for 5 min each in TTBS. Each membrane was incubated for 5 min in 4 mL of SuperSignal West Pico Chemiluminescent substrate (Pierce), then drained, placed in a transparent sheet protector, and exposed to BioMax Light film (Eastman Kodak Co., Rochester, NY) for 1−60 s, and the film was developed according to the manufacturer's recommendations.

Films were quantified from the scanned images using Sigma Gel analysis software (SPSS Science, Chicago, IL). The housekeeping gene α-tubulin was included as a control in all Western blots to ensure consistent protein loading. All α-tubulin controls were probed with monoclonal mouse anti-α-tubulin (clone B-5-1-2) (Sigma).

Long-Term Transfections. HeLa, SW1573, and 2R120 cells were initially seeded in 12-well dishes at a density of 40 000 cells per well. Cells were transfected with siRNAs (200 nM) the next day as described above. Three days after transfection, cells were trypsinized and cell numbers of each well were determined using a Coulter Z Series cell counter (Beckman Coulter, Fullerton, CA). Cells from each well were counted three times, and the average numbers were used for the comparison among different treatment groups; cell numbers were expressed as percentages of lipid controls. Cells were transferred and reseeded at a density of 40 000 cells per well in a new dish and were transfected again with the corresponding oligonucleotides the following day. The cycle of transfections and counting were carried out three times. All assays were performed in triplicate, and the reported values for inhibition are averages of these triplicate determinations.

Microscopy. To assess the steady-state distribution of doxorubicin inside drug sensitive and drug resistant cells, fluorescence microscopy was performed. Cells were seeded in Lab-Tek four-well chambered coverglass slides (Nalgene Nunc International, Naperville, IL), allowed to adhere overnight, and then treated with 10 μM doxorubicin for 15 min at 37 °C. Doxorubicin was then aspirated, and the cells are washed five times with OPTI-MEM serum reduced media without phenol red (Invitrogen). After the last wash, slides were analyzed using a Zeiss Axiovert 200 M inverted transmitted light microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) equipped with a digital imaging system and Slidebook imaging software (Intelligent Imaging Innovations, Inc., Denver, CO). Cells were imaged at various times after doxorubicin treatment using the Cy3 filter. Nuclear staining was visualized using a 50% (v/v) solution of Opti-MEM and PBS containing 0.05 mg/mL Hoechst 33258 stain (Sigma) and then washed five times with 500 μL of Opti-MEM. Hoechst-stained cells were imaged using the UV filter. All images shown were taken at a magnification of 63×.

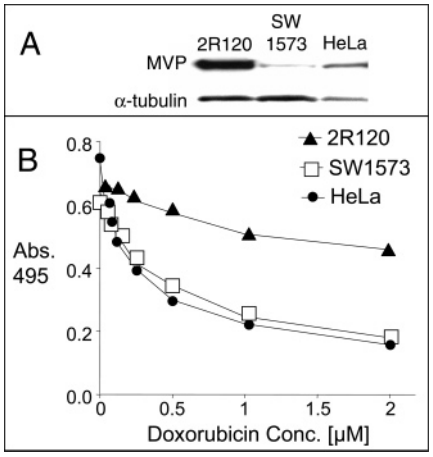

FIGURE 1: MVP is upregulated in the doxorubicin resistant SW1573/2R120 cell line relative to HeLa or parental SW1573 cells. (A) Western analysis of MVP expression in SW1573-2R120, SW1573, and HeLa cells. Below MVP bands, α-tubulin is included as a protein loading control. (B) Sensitivity of SW1573-2R120, SW1573, and HeLa cells to doxorubicin determined by the MTS assay performed 48 h after addition of the drug.

Heat Shock. HeLa cells are plated in six-well plates at a density of 100 000 cells per well. Cells are transfected with siRNA as described above. After transfection, the plates are floated on top of the water in a bath heated to 45 °C for 1 h. This method ensures direct contact with the water, resulting in quick, consistent, and even heating of the cells. To determine whether cells have been appropriately heat shocked, cells are trypsinized and lysed for either 16 or 43 h after heating. Lysates are probed for upregulation of Hsp70 and knockdown of MVP by Western blot analysis (Hsp70 antibody, SPA-812C, Stressgen). Duplicate experiments are conducted for the purpose of examining cell survival. Cells are trypsinized and counted using a Coulter cell counter. Cells are also analyzed by Trypan Blue exclusion to determine the percentage of cell death. All experiments were performed in triplicate.

RESULTS

Overexpression of MVP and Localization of Doxorubicin. To study the role of MVP in drug resistance, we obtained two non-small cell lung carcinoma lines, the parental chemosensitive SW1573 cell line and the related SW1573/2R120 cell line that had been selected for resistance to doxorubicin (*32*). For comparison, we also used a second doxorubicin sensitive cell line, HeLa-CCL2.

Previous studies have shown that SW1573/2R120 cells are resistant to higher concentrations of doxorubicin and express elevated levels of MVP relative to the parental SW1573 line (*21*). We confirmed that both SW1573 and HeLa cells express MVP at much lower levels than SW1573/2R120 cells (Figure 1A). MTS assays confirmed that SW1573/2R120

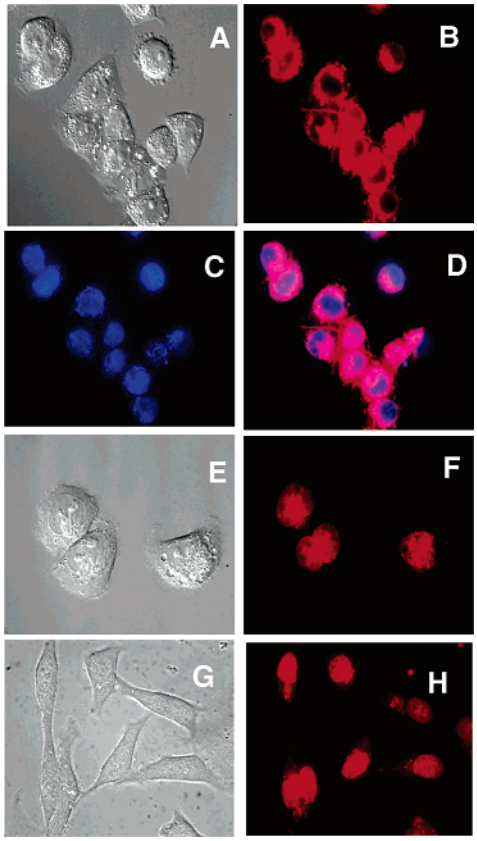

FIGURE 2: Doxorubicin is excluded from nuclei of doxorubicin resistant SW1573/2R120 cells but enters the nuclei of doxorubicin sensitive SW1573 and HeLa cells. All images were taken 16 h after doxorubicin treatment. (A) SW1573-2R120 cells shown in a normal contrast (DIC) image. (B) Cells shown in panel A imaged with the Cy3 filter to visualize doxorubicin. (C) Cells shown in panel A using the UV filter to visualize Hoechst staining of the nucleus. (D) Images of the cells shown in panels B and C overlaid to show doxorubicin has been excluded from the nucleus. (E) DIC image of SW1573 cells. (F) Cells shown in panel E imaged using the Cy3 filter to visualize doxorubicin. (G) DIC image of HeLa cells. (H) Cells shown in panel G imaged using the Cy3 filter to visualize doxorubicin.

cells are more resistant to doxorubicin than either HeLa or SW1573 cells (Figure 1B).

Doxorubicin is a naturally fluorescent molecule that exerts its cytotoxic effects in the nucleus. We took advantage of this fluorescence to monitor doxorubicin uptake and localization using fluorescence microscopy. We imaged live SW1573/2R120 cells 16 h after treatment with doxorubicin and observed no drug accumulation in the nucleus (Figure 2A−D). By contrast, doxorubicin accumulated in the nuclei of chemosensitive HeLa and SW1573 cells (Figure 2E−H). The drug remains visible in the nuclei of chemosensitive cells for more than 24 h, after which the cells begin to undergo apoptosis (data not shown).

Time Course of Doxorubicin Efflux. There are two possible explanations for the absence of doxorubicin from the nucleus of SW1573/2R120 cells. (1) Doxorubicin might somehow be blocked from entering the nucleus, or (2) doxorubicin might be removed after initially achieving entry. To examine these hypotheses, SW1573/2R120 cells were cultured in chambered coverglass slides and treated with 10 μM doxorubicin for 15 min. The cells were then washed to remove excess doxorubicin and imaged after 15 min, 1 h, or 16 h.

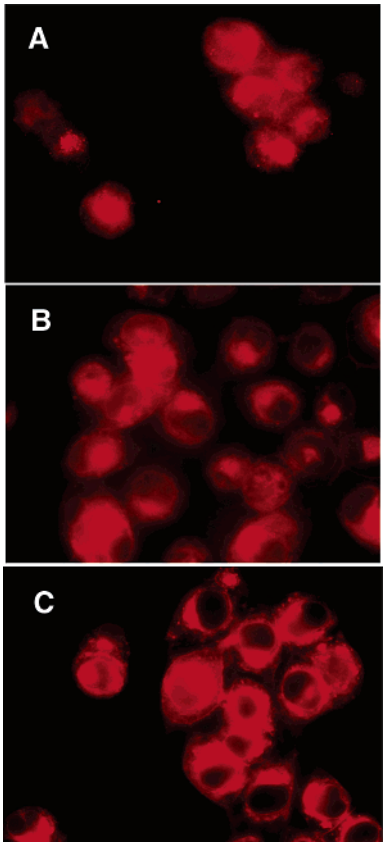

FIGURE 3: Doxorubicin enters the nucleus of drug resistant cells and is then removed. Fluorescence microscopy images of SW1573/2R120 cells treated with doxorubicin and imaged various times after washing: (A) 15 min after treatment with doxorubicin, (B) 1 h after treatment with doxorubicin, and (C) 16 h after treatment with doxorubicin.

Fifteen minutes after the cells had been washed, we observed that the nuclei of the SW1573/2R120 cells contained doxorubicin (Figure 3A) and appeared similar to the nuclei of the chemosensitive HeLa and SW1573 lines (Figure 2F,H). The ability of doxorubicin to enter the nuclei of resistant cells suggests that vaults do not act as barriers to prevent the drug from reaching the nucleus, nor do they act as containers for sequestering a drug.

One hour after washing the cells, we observed a reduction in the nuclear doxorubicin fluorescence with an increase in the fluorescence surrounding the nucleus (Figure 3B). After 16 h, the drug had been mostly cleared from the nuclei and accumulated in an area surrounding the nuclear membrane (Figure 3C) (*31*, *33*). Fluorescence microscopy of doxorubicin-treated SW1573 and HeLa cells did not show any observable nuclear efflux of doxorubicin after 24 h (data not shown).

Knockdown of MVP Does Not Affect Doxorubicin Localization. To investigate the role of vaults in the doxorubicin resistance phenotype of SW1573/2R120 cells, we targeted the MVP mRNA using siRNA to reduce the level of expression of the protein. Previous studies have shown that removal of MVP results in loss of vault particles (*15*). Furthermore, MVP has been shown to be the limiting component in the formation of vaults *in vivo* (*22*, *23*).

Three siRNAs, MVP-1, MVP-ORF, and MVP-UTR, were designed to target the translational start site, the open reading frame, and the 5′-untranslated region, respectively. MVP-

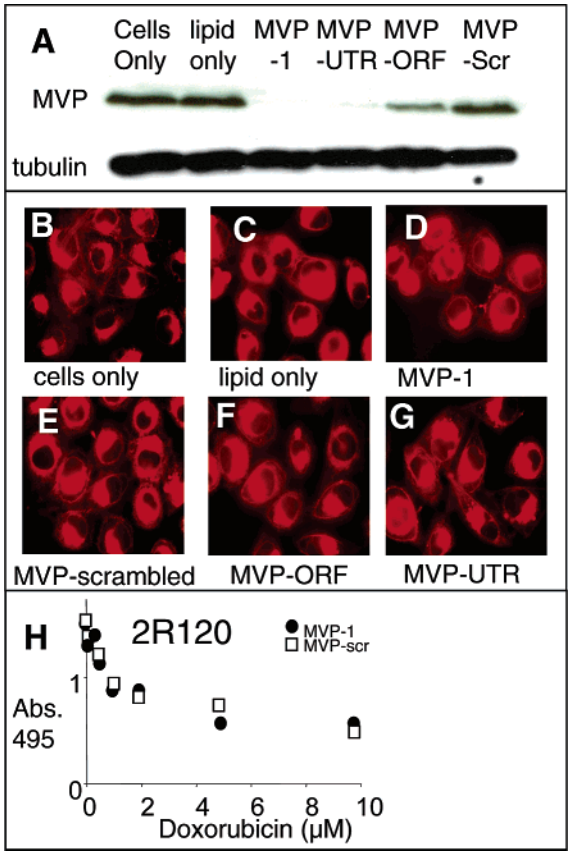

FIGURE 4: Reduction of the level of MVP expression does not alter doxorubicin localization or affect resistance to doxorubicin. (A) Western analysis of MVP expression after treatment of SW1573/2R120 cells with various siRNAs. Tubulin is included as a loading control. (B−G) Microscopy showing uptake of doxorubicin by SW1573/2R120 cells treated with the indicated duplex RNAs. (H) MTS assay of SW1573/2R120 cells transfected with MVP-1 and treated with doxorubicin.

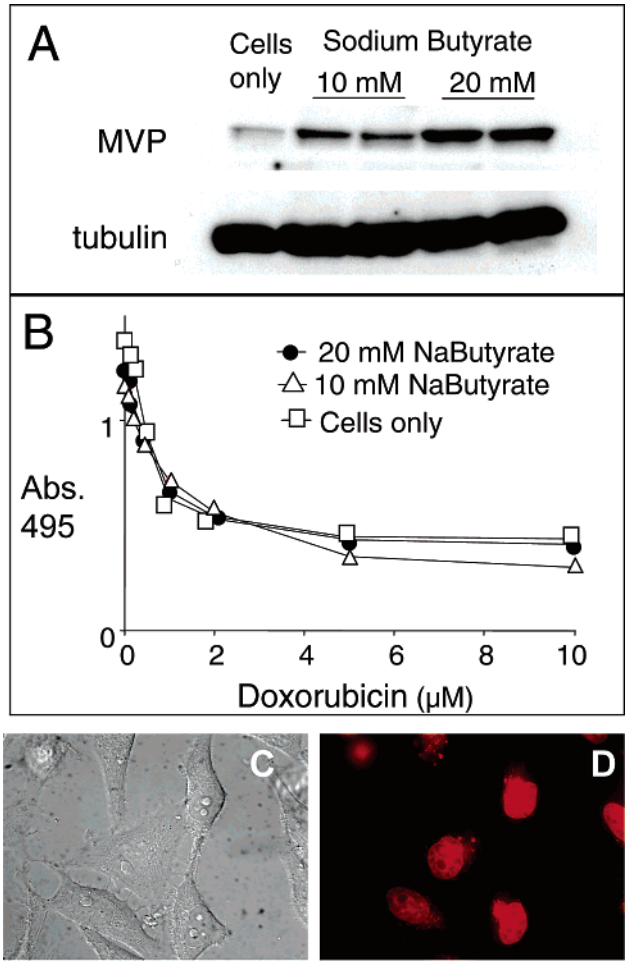

FIGURE 5: Treatment with sodium butyrate increases levels of MVP but does not increase resistance to doxorubicin in HeLa cells. HeLa cells were treated with sodium butyrate for 24 h and then subjected to analysis. (A) Western analysis of cells treated with sodium butyrate at 10 or 20 mM. Tubulin is included as a loading control. (B) MTS assay of untreated cells and cells treated with sodium butyrate at 10 or 20 mM. (C) DIC image of HeLa cells treated with 20 mM sodium butyrate and doxorubicin. (D) Cy3 filter image of HeLa cells treated with sodium butyrate and doxorubicin.

Scr was designed as a scrambled control of MVP-1. An unrelated siRNA, hCAV1, which targets the transcript for human caveolin-1, was also used as a control during early experiments and did not affect vault expression (data not shown). Cells were transfected with siRNAs using a cationic lipid. The transfected cells were then analyzed by Western blot to measure protein levels.

Two siRNAs, MVP-1 and MVP-UTR, were more than 90% effective in knocking down MVP expression (Figure 4A). There was some residual expression of MVP, but the reduced levels of MVP expression were lower than those observed in the chemosensitive cell lines HeLa and SW1573. The MVP-ORF siRNA was also able to knock down MVP expression by more than 50%. MVP expression levels in the untreated, lipid-treated, and MVP-Scr experiments were almost equivalent. These data demonstrate that siRNA can be an efficient tool for the selective and efficient reduction of the level of MVP expression.

We transfected SW1573-2R120 cells with varied siRNAs, treated them with doxorubicin, and then examined the living cells by fluorescence microscopy (Figure 4B−G). We observed that doxorubicin was excluded from the nucleus of drug resistant cells regardless of whether MVP expression was minimal or at high levels, suggesting that MVP does not play a direct role in nuclear efflux (Figure 4B−G). We also observe that knockdown of MVP does not alter the intracellular distribution of doxorubicin, suggesting that the drug is not sequestered by vaults.

Knockdown of MVP Does Not Affect Cell Survival. We also investigated the effect of MVP knockdown on the sensitivity of SW1573-2R120 cells to the toxic effects of doxorubicin using MTS assays. If MVP were involved in chemoresistance, inhibition of MVP expression would be expected to enhance sensitivity to doxorubicin. We observed, however, that knockdown of MVP expression has no effect on the doxorubicin sensitivity of SW1573/2R120 cells (Figure 4H). Similarly, we find that a reduced level of expression of MVP in drug sensitive SW1573 and HeLa cells did not further increase doxorubicin cytotoxicity (data not shown). These data further support the conclusion that MVP is not directly involved in drug resistance.

Upregulation of MVP Does Not Increase Nuclear Efflux or Resistance to Doxorubicin. We investigated the possibility that elevated MVP levels may result in increased resistance to doxorubicin. It has been established that treating cells with sodium butyrate, a histone deacetylase (HDAC) inhibitor, causes an increase in the level of MVP expression (*29*). We

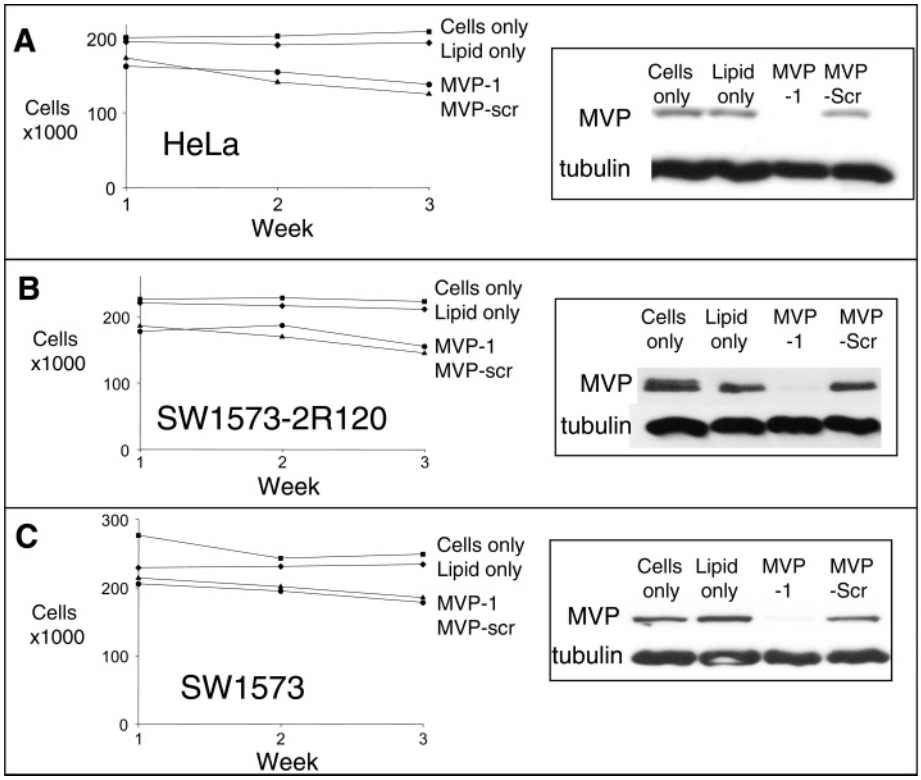

FIGURE 6: Prolonged inhibition of MVP expression does not cause a decreased rate of cell proliferation. Proliferation of HeLa (A), SW1573/2R120 (B), and SW1573 cells (C). Cells were treated individually with either MVP-1, MVP-Scr, or lipid or left untreated over a 3 week period. Cell proliferation was evaluated by the MTS assay, with accompanying Western blot analysis showing the diminished level of MVP expression.

treated drug sensitive HeLa cells with sodium butyrate to induce endogenous MVP expression (Figure 5A). After induction by sodium butyrate, we observed no increase in resistance to doxorubicin and no increase in the rate of drug efflux from the nucleus (Figure 5B,C). These data further support the conclusion that vaults have no direct role in developing resistance to doxorubicin.

Prolonged Inhibition of MVP Expression Does Not Affect Cell Growth. MVP is expressed at varying levels in many different types of human cells, suggesting that it has an important, yet unknown, biological function. To begin investigating potential functions of MVP, we used our siRNAs to examine long-term effects of MVP knockdown in human cancer cells.

SW1573, SW1573/2R120, and HeLa cells were transfected once every 5 days during a 3 week period to investigate any abnormalities that may develop as a result of a loss of MVP. We observed no decrease in the rate of proliferation of cells treated with siRNA MVP-1 relative to treatment with a scrambled siRNA control, MVP-Scr (Figure 6A−C). We also do not observe any general morphological changes in the cultured cells during this experiment. These data suggest that MVP does not play a role in the normal proliferation of cultured human cancer cells. Our findings are consistent with data published by Wiemer and colleagues that show no observable defects in MVP-deficient mice (*15*).

Effect of Inhibition of MVP on the Tolerance to Heat Shock. Our data suggest that MVP does not play a direct role in chemoresistance and is not necessary for the normal growth of cultured cells. The hypothesis that vaults are involved in stress response has been suggested by Wiemer and colleagues (*31*). To begin examining the potential linkage between vaults and stress response, we evaluated the effect of decreased MVP levels on heat tolerance of cancer cells.

After transfection with siRNA, cells were shocked for 1 h at 45 °C in a water bath and allowed to recover for 16 or 43 h prior to being harvested. We noted that cell counts were reduced by 30−50% after heat shock and that Hsp70 expression was upregulated (data not shown), indicating that valid heat shock conditions were achieved. We observed that MVP-1 was more than 90% efficient in knocking down MVP expression in heat-shocked cells (Figure 7A).

We observed that cell numbers remained the same regardless of whether cells were treated with MVP-1 siRNA or the scramble control siRNA (Figure 7B). Similarly, quantitation of cell death using Trypan Blue also revealed no significant difference between treatments with MVP-1 and the scramble control (Figure 7C). These observations suggest that a reduced level of vault expression does not affect the survival of heated cells in culture.

DISCUSSION

Linkage between Vaults and Drug Resistance. The development of resistance to chemotherapeutic agents is the major cause of poor outcomes for cancer patients. Obtaining a detailed understanding of the molecular mechanisms underlying the evolution and maintenance of drug resistance is important for developing better treatment strategies. Multidrug resistance (MDR) is characteristically associated with members of the ABC transporter family, particularly P-gp, MRP1, and BRCP (*34*).

A potentially novel drug resistance mechanism was suggested when the Broxterman group was able to select

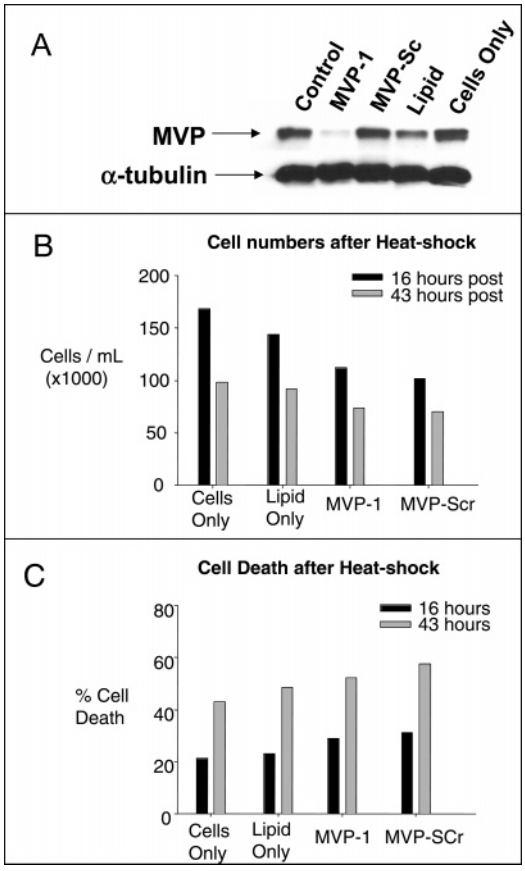

FIGURE 7: Reduced levels of MVP expression do not affect cell survival after heat shock. (A) siRNA MVP-1 reduces the level of MVP expression in heat-shocked HeLa cells. (B) Cell counts performed after the heat shock protocol. Cells were untreated, treated with lipid only, or treated with lipid in combination with MVP-1 or MVP-Scr. (C) Trypan Blue assay performed after the heat shock protocol. Cells were untreated, treated with lipid only, or treated with lipid in combination with MVP-1 or MVP-Scr.

non-small cell lung carcinoma lines exhibiting a form of drug resistance not associated with the P-glycoprotein (*32*, *35*). They discovered an overexpressed protein in these lines that they termed the lung resistance-related protein (LRP) (*21*). Subsequent studies identified LRP as MVP, providing a suggestive link between vaults and drug resistance (*2*).

A number of studies have demonstrated a correlation between an increased level of MVP expression and drug resistance in various cancer cell lines and primary tumors (*18*, *19*, *22*). In addition, studies of clinical specimens from patients with various unrelated cancers, including myelomas, leukemias, and colorectal cancers, have shown some correlation between MVP expression and poor response to chemotherapy (*9*, *26*−*28*). Clinical studies have sought to use MVP expression as a prognostic marker for chemotherapeutic failure.

The strongest evidence supporting a link between MVP and drug resistance was provided by reports from the Akiyama group indicating that manipulation of MVP expression can alter the sensitivity of SW620 cells to various antiproliferative agents (*29*, *30*). Their initial study indicated that treatment of SW620 cells with sodium butyrate increased the resistance to doxorubicin as well as other cytotoxic agents, and caused the upregulation of MVP expression (*29*). They were able to overcome the resistant phenotype by transfecting the cells with LRP specific ribozymes or treating the isolated nuclei with LRP antibodies. In a second study, they also identified a pyridine analogue, PAK-104P, as an inhibitor of sodium butyrate-induced drug resistance (*30*).

MVP and Vaults Are Not Directly Involved in Chemoresistance. Our results do not support a direct link between MVP and chemoresistance for several reasons.

(1) Reduction of the level of MVP expression by siRNAs does not increase doxorubicin sensitivity in the resistant SW1573-2R120 cell line.

(2) Drug sensitive HeLa and SW1573 cell lines do not become hypersensitive to doxorubicin in the absence of MVP.

(3) Reduction of the level of MVP expression does not decrease the rate of nuclear efflux in the resistant cell line, SW1573-2R120.

(4) The cytosolic distribution of doxorubicin does not change after MVP knockdown in drug resistant cells. If vaults were able to sequester the drug, then the expectation would be that doxorubicin would more freely diffuse and not remain concentrated in structures surrounding the nucleus.

(5) Induction of endogenous MVP expression using sodium butyrate does not alter doxorubicin localization in chemosensitive HeLa or SW1573 cells, nor does it decrease the sensitivity of these cell lines to doxorubicin.

(6) Fluorescence microscopy of SW1573/2R120 cells treated with doxorubicin reveals that the drug initially enters the nuclei of the resistant cells and is then removed. This result suggests that vaults do not act to prevent the drug from entering the nucleus.

Our findings are in agreement with recently published data from Wiemer and colleagues (*31*). In SW1573 cells stably transfected with a plasmid that overexpresses an MVP−green fluorescent protein (MVP−GFP) conjugate, they find no evidence that GFP-tagged vaults respond to daunorubicin treatment by either mass movement toward or accumulation at the nuclear membrane. In addition, they note no accumulation of GFP-tagged vaults at or near drug-filled vesicles. These results, considered together with data from our experiments, strongly argue against vaults participating as nuclear drug shuttles.

Our results in human cancer cells also support the conclusions of studies using embryonic cells removed from MVP knockout mice (*15*). These studies demonstrate no increased chemosensitivity to anthracyclines in the absence of MVP. Studies of MVP-deficient mice also indicate that vaults are dispensable for normal growth and development. Similarly, we have found that long-term reduction of MVP levels does not slow proliferation of human cancer cells under normal culture conditions. These data suggest that vault particles, although evolutionarily conserved, have no apparent function during growth under permissive conditions.

MVP, a Stress Response Protein? There is circumstantial evidence that vaults may be involved in cellular responses to stress. Analysis of the MVP promoter has uncovered GC elements, which are hallmarks of some of the HSP genes in human cells (*36*). Recent studies have shown that vaults are responsive to temperature (*37*). When cells are exposed to reduced temperature (21 °C), vaults congregate in structures termed "vault tubes", which disappear when the temperature is returned to 37 °C. At present, the purpose of these vault tubes is unknown.

It is reasonable to hypothesize that large hollow vault particles might assist in protein folding or protein complex assembly and that this assistance becomes significant when cells are challenged by adverse changes in their environment. This type of role for vaults would explain why we failed to observe a phenotype when MVP expression was inhibited in cells growing under permissive culture conditions. The size, morphology, and complexity of the vault particle would allow for any number of possible scenarios in which vault components could interact with or enhance the activities of other proteins. An increased level of expression of certain heat shock protein genes has been associated with drug resistance, although the extent of their involvement in the MDR mechanism is unknown (*38*, *39*).

In an effort to begin investigations into this possibility, we examined the most universal stress test, heat shock response. However, under heating conditions in which Hsp70 is upregulated, we do not observe an increase in the rate of cell death when MVP is knocked down, suggesting that MVP is not involved in response to heat stress. Furthermore, we do not see upregulation of MVP in Western blots using lysates from SW1573 and HeLa cells subjected to heat shock (data not shown). We also do not observe MVP upregulation in murine tissue samples recovered from heat-shocked animals (data not shown).

Although we find no increase in the rate of cell death when the level of MVP expression is reduced in heat shock experiments, our negative findings are not conclusive and the stress response hypothesis is worthy of further investigation.

Conclusion. We have used siRNAs and sodium butyrate-mediated overexpression to manipulate expression of MVP in cultured cancer cells and have found that MVP does not play a direct role in chemoresistance. Investigating the role of vaults in more general cell functions reveals that knockdown of MVP expression does not affect long-term cell proliferation and has no effect on cell survival after heat shock. The cellular function of vaults remains unknown, and our inability to detect a phenotype in MVP-deficient cells suggests that vaults may only become important for cell survival under a limited set of growth conditions.

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