The CD44 Receptor Interacts with P-Glycoprotein to Promote Cell Migration and Invasion in Cancer

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Abstract

Invasion and metastases of cancer cells and the development of resistance to anticancer therapies are the main causes of morbidity and mortality from cancer. For more than two decades, these two important but not clearly related aspects in the biology of cancer have been extensively studied. Specifically, P-glycoprotein and CD44 have been characterized and are known to be determinants of multidrug resistance (MDR) and metastases. Despite this body of knowledge, few reports have linked the two phenotypes and only recently have there been reasons to suspect a direct connection. In this report, we show that a novel physical and genetic interaction between CD44s and P-glycoprotein is in part responsible for the correlation between MDR and invasive potential in cancer cells. P-glycoprotein–specific substrates that interfere with its function reduced *in vitro* invasion, migration, and the physical colocalization of CD44s and P-glycoprotein. *CD44* expression in sensitive cells promoted the expression of P-glycoprotein and the MDR phenotype. RNA interference of *MDR1* inhibited the rate of cell migration. These data indicate that there is a close interaction between CD44 and P-glycoprotein that results in the concurrent expression and modulation of two malignant phenotypes, invasion and MDR. (Cancer Res 2005; 65(15): 6660-7)

Introduction

The major causes of treatment failure in cancer are the development of metastases and drug resistance. There is indirect evidence that these two phenotypes may be functionally linked. However, the relationship between metastasis and drug resistance is still unclear. The original work by Meyers and Biedler (1) in highly resistant multidrug-resistant (MDR) cell lines suggested that P-glycoprotein overexpression led to decreased tumorigenicity. Two recent reports make the connection between these two phenotypes more apparent. Yang et al. (2) reported that the extracellular matrix metalloproteinase inducer (EMMPRIN), a cell membrane glycoprotein involved in invasion and metastases (3, 4), is overexpressed in MDR cells and not in drug-sensitive parental cell lines. Misra et al. (5) reported that MDR in cancer cells could be regulated by a ubiquitous extracellular matrix component, hyaluronan, a major ligand for the metastases-related CD44 receptor (6, 7). We now present evidence for a direct link between drug resistance, cell motility, and invasion by uncovering a physical and functional interaction between P-glycoprotein (MDR) and CD44 (motility, invasion, and metastases).

CD44 is a membrane receptor implicated in cell adhesion, motility, and metastases (7). The gene that encodes CD44 contains 19 exons and it is alternatively spliced giving rise to many CD44 isoforms. When the central 10 exons are spliced out the CD44 "standard isoform" (CD44s) is expressed. CD44s is a M_r ~85 000 glycoprotein and is a major receptor for hyaluronan (8, 9). Hyaluronan, is the principal glycosaminoglycan found in all types of mammalian extracellular matrix.

P-glycoprotein, the product of the *MDR1* (*ABCB1*) gene, is a transmembrane ATP-dependent transporter, associated with poor outcomes in cancer patients, presumably because it imparts resistance to cancer treatment (10–13). Alternatively, P-glycoprotein may have other activities that lead to a poor prognosis. One possibility is that P-glycoprotein (+) cells may acquire a propensity towards invasion and metastasis, as suggested in colon cancer cells (14, 15).

The purpose of the present study was to investigate whether or not a linkage exists between MDR and metastatic potential. We found a correlation between the expression of P-glycoprotein and CD44 and showed that the two proteins coimmunoprecipitate and colocalize within the cell membrane. Furthermore, we show that one protein directly influences the expression of the other and that disruption of this interaction has profound effects on drug resistance, cell migration, and *in vitro* invasion.

Materials and Methods

Cell culture. The human breast cancer cell line MCF-7, the MDR derivative MCF-7/AdrR (16) and MCF-7/BC-19 (a human *MDR1* gene stable transfectant clone) which were kindly supplied by Dr. Kenneth Cowan of the Eppley Institute for Research in Cancer (Omaha, NE), and the *CD44s* stable transfectant clones (MCF-7/CD44s and MCF-7/BC19/CD44s) were maintained in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS). The human ovarian cancer cell lines A2780 (sensitive) and A2780Dx (resistant; ref. 17) were provided by Dr. Youcef Rustum (Roswell Park Cancer Institute, Buffalo, NY) and were maintained in DMEM containing 10% FBS. The cell lines KB3-1 (sensitive) and KBV-1 (resistant to vinblastine; ref. 18) were also maintained in FBS-complemented DMEM. All cells were cultured at 37°C in a humidified atmosphere (95% air, 5% CO_2).

Total RNA extraction and reverse transcription-PCR. Trizol reagent (Invitrogen, Carlsbad, CA) or the PARIS kit (Ambion, Austin, TX) was used

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to extract total RNA from culture cells as suggested by the manufacturer. Semiquantitative reverse transcription-PCR (RT-PCR) of each RNA template was carried out using the One-step RT-PCR kit (Qiagen, Valencia, CA) as suggested by the manufacturer with the modification that 1 μL of enzyme mixture instead of 2 μL was used. Briefly, reverse transcription of each mRNA transcript was done by 30-minute incubation at 50°C followed by 15-minute incubation at 95°C. In the same tube, PCR amplification for *CD44s* (sense primer, 5′-CAGCAACCCTACTGATGATGACG-3′; antisense primer, 5′-GCCAAGAGGGATGCCAAGATGA-3′) or *MDR1* (sense primer, 5′-TTCCTCTCATGATGCTGGTG-3′; antisense primer, 5′-ACCTCTTCAGC-TACTGCTCC-3′) was as follows: two cycles of 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds, followed by 10 cycles of 94°C for 30 seconds, 57°C for 30 seconds with a 1°C decrease per cycle, and 72°C for 30 seconds, followed by 18 cycles of 94°C for 30 seconds, 4°C for 30 seconds, and 72°C for 30 seconds, followed by a 5-minute incubation at 72°C. The ribosomal protein *L19* gene (sense primer, 5′-AGTATGCT-CAGGCTTCAGAAG-3′; antisense primer, 5′-TTCCTTGGTCTTAGACCTGCG-3′), used as a loading control, was reverse transcribed as above and PCR amplified as follows: five cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds, followed by 25 cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 45 seconds, followed by a 5-minute incubation at 72°C.

Immunoblotting analysis and immunoprecipitation assays. Confluent cell cultures in 150-mm culture plates were washed twice with ice-cold PBS buffer containing 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), scraped off the plates, transferred to microcentrifuge tubes, and centrifuged at 500 × *g* for 10 minutes. After the supernatant was removed, the cells were lysed by resuspending the cell pellet in 500 μL of cold TNT buffer [20 mmol/L Tris-HCl (pH 7.4), 200 mmol/L NaCl, 1% Triton X-100, 1 mmol/L PMSF, 1% aprotinin] for 45 minutes with occasional tapping of the microtubes. The cell lysate was clarified by centrifugation at 12,000 × *g* for 40 minutes at 4°C. Total protein concentration was determined by a modified Bradford assay (Bio-Rad, Hercules, CA) as suggested by the manufacturer. About 25 to 100 μg of protein were resuspended in Laemmli sample buffer and resolved by SDS-PAGE in a discontinuous (4-7%) gel under reducing conditions. The gel was electrotransferred using an electroblotter system (Bio-Rad) onto nitrocellulose membrane (Amershan Pharmacia Biotech, Piscataway, NJ). The membrane was incubated in blocking solution (4% nonfat milk in TBS) and immunoblotted with an anti-P-glycoprotein (Calbiochem-Novabiochem Corp., La Jolla, CA) or an anti-human CD44H (R&D Systems, Minneapolis, MN) monoclonal antibody. Signal detection was done using an enzyme-linked chemiluminescence reagent kit (Pierce Biotechnology, Rockford, IL). Anti-β-actin (Sigma, St. Louis, MO) or anti-tubulin (Sigma) was used as protein loading control. For the immunoprecipitation assays, protein preparations were similar as for immunoblottings with the exception that before resolving the cell lysate by SDS-PAGE, 1.5 mg of total protein was immunoprecipitated with 2 μg of mdr (ab-1) antibody (Oncogene, San Diego, CA) or with 20 μg of CD44s or CD44v3-10 polyclonal antibody (Biosource, Camarillo, CA) by overnight incubation at 4°C after adjusting the volume to 0.5 mL with ice-cold NET buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.1% NP40, 1 mmol/L EDTA, 0.25% gelatin, 0.02% sodium azide, 1 mmol/L PMSF, 1% aprotinin]. The immune complex was precipitated with protein G-Sepharose and washed thrice with NET buffer and once with PBS. The immunoprecipitated proteins were resuspended in Laemmli sample buffer and resolved by SDS-PAGE and electrotransferred onto nitrocellulose membrane as previously mentioned. The membrane was incubated and immunoblotted with anti-P-glycoprotein or anti-human CD44H and the signal detected as mentioned above. Densitometry analysis was done and quantification was done relative to β-actin levels. Confirmatory immunoprecipitation assays were done using a high-stringency lysis buffer as described (19) but with octylglucoside at 2% or 4%. Briefly, 90% cell confluent cultures were washed thrice with 5 mL PBS, scraped, and counted. Cell pellets corresponding to 5×10^6 cells were lysed with 2% or 4% octylglucoside-containing lysis buffers. One milligram of precleared samples were incubated with either isotype-match nonspecific IgG control (unlabeled clone 20102, R&D Systems) or with anti-P-glycoprotein monoclonal antibody (clone C219, Calbiochem) for 1 hour. Protein G-Sepharose beads were added and incubated for 90 minutes to precipitate the immune complexes. After washing in their respective lysis buffers, bead pellets were resuspended in 2× SDS sample buffer plus 100 mmol/L DTT, resolved by SDS-PAGE gels, and transferred as above. Anti-human CD44s antibody (H-300, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used for immunoblotting detection.

Tumor cell migration and invasion (Matrigel) assays. *In vitro* cell migration assays were conducted using Falcon cell culture insert with 8-μm-pore polyethylene terephthalate membranes (Becton Dickinson Labware, Franklin Lakes, NJ). Similar inserts covered with Matrigel were used to determine *in vitro* invasive potential. After growing to 90% to 100% confluent cultures, MCF-7 parental and derived cell lines were tripsinized and resuspended in RPMI 1640 plus 0.5% FBS. From this single-cell suspension, the cells were seeded at 5×10^4 in the migration assay insert and at 1×10^5 in the Matrigel invasion assay insert. The lower companion plate well contained RPMI 1640 plus 10% FBS. After a 48-hour incubation, the cells on the upper side of the membrane were removed by wiping with a cotton swab. The cells that migrated to the lower side of the membrane were fixed and stained using the Diff-quik stain set (Dade Behring AG, Düdingen, Switzerland). The number of cells in five randomly selected microscopic fields (200×) were counted. Similar experiments were done in the presence of *trans*-flupenthixol (tFPT), vinblastine (Sigma), or anti-human CD44H antibody (R&D Systems), both for the migration and invasion assays. Each assay was done in triplicate and repeated at least thrice. The data were analyzed using Student's *t* test and the statistical significance was set at $P < 0.01$.

Wound-healing assay. Cells were grown to 100% confluency in RPMI 1640 plus 10% FBS and serum deprived for 18 hours. A sterile pipette tip was used to make a straight line wound on the confluent cell culture. After washing the plate to remove the mechanically detached cells and to expose the "wound," the remaining cells were incubated for 48 hours in RPMI 1640 plus 0.5% FBS in the presence or absence of tFPT. At the indicated time points, the cell culture was photographed using a phase-contrast microscope (Eclipse TE 200, Nikon Inc., Melville, NY).

Immunofluorescence confocal microscopy analysis. To determine the cellular localization of CD44s and P-glycoprotein in MCF-7/AdrR cells, immunofluorescence analysis was done. Cells were seeded, incubated overnight on sterile glass coverslips, and after a brief washing with PBS, were fixed by adding 3.7% formaldehyde in PBS for 5 to 15 minutes. The fixed cells were washed thrice with PBS and then incubated with 10% normal goat serum in PBS for 20 minutes to suppress nonspecific binding of IgG. After another washing with PBS, the cells were incubated with anti-CD44s (2 μg/mL) and anti-P-glycoprotein (5 μg/mL) antibody for 1 hour followed by a-45 minute incubation with FITC- and rhodamine-conjugated secondary antibody (5 μg/mL) in PBS with 1.5% goat serum. The labeled cells were analyzed by laser scanning confocal microscopy. To detect nonspecific antibody binding, fluorescein-conjugated cells were incubated with rhodamine-conjugated preimmune rabbit IgG. The control samples did not show any significant cell labeling (data not shown).

Plasmid construction and *CD44s* transduction of MCF-7. The retroviral vector pLXIN (Clontech, Palo Alto, CA) was used to transduce the human *CD44s* cDNA sequence into MCF-7 and MCF-7/BC19 cells. The *CD44s* cDNA, cloned in the plasmid pAZ was a generous gift of U. Gunthert (Basel Institute, Switzerland). The *Eco*RI digestion fragment, which includes the entire *CD44s* cDNA sequence (Genbank accession no. AJ251595) was treated with T4 DNA polymerase (Invitrogen) to produce a DNA fragment with blunt ends. The same enzyme was used to fill-in the ends of pLXIN linearized with *Hpa*I, which was then ligated to the *CD44s* cDNA insert using T4 DNA ligase (Invitrogen). This recombinant plasmid (named pLXIN/CD44s) sequence orientation was confirmed by restriction digestion with *Bam*HI. pLXIN/CD44s was then transfected into the packaging cell line RetroPack PT67 (Clontech) as suggested by the manufacturer. Supernatant from transfected PT67 culture plates was collected from 24 to 72 hours after transfection. About 5×10^5 cells were seeded in 100-mm plates, grown overnight, and infected with the virus-containing supernatant from PT67 plates. Single colonies of stable transductants were selected using G-418 for

1 to 2 weeks, and transferred to 24-well plates for another 1 to 2 weeks of incubation in the presence of G-418. Cell clones that grew to confluency were transferred to 6-well plates and then to 100-mm plates for the intended experimental use.

Taxol accumulation assay. This assay was done as described (20) with modifications. ^{3}H-labeled paclitaxel (Taxol; Movarik) was used instead of ^{3}H-labeled vinblastine and verapamil at different concentrations instead of epidermal growth factor. Four wells were analyzed per experimental group: cell counting, control cells (^{3}H alone, 2 μl/mL, 6.7 Ci/mmol), and Taxol (250 nmol/L, 3.2 mCi/mmol) plus verapamil-treated cells. Briefly, MCF-7 and MCF-7/CD44s clones were seeded in 24-well tissue culture plates (5×10^5 cells per well) and incubated for 36 hours (~ 70% cell confluency) in RPMI 1640 plus 10% FBS (1 mL). The medium was aspirated and the cells were washed twice with sterile PBS (cell-counting wells) or serum-free medium (experimental and control wells). Serum-free medium containing Taxol alone, Taxol plus verapamil, or tritium only was added to the wells and the plates were incubated at 37°C for 3 hours. The plates were washed twice with cold PBS and the cells lysed with 1% SDS for 40 minutes. The cell lysates were transferred into scintillation vials containing 7 mL of scintillation fluid (Ecosint H, National Diagnostic, Atlanta, GA) and the radioactivity measured in a scintillation counter (LS6500, Beckman, Fullerton, CA).

MDR1 and nonspecific control small interference RNAs. The following small interference RNAs (siRNA) were used to target the MDR1 mRNA sequence. Two sequences previously published by others were used: MDR1-ORF (21), 5′-r(GGAAAAGAAACCAACUGUC)d(TT)-3′ and MDR1-3′ UTR (22), 5′-r(CAUCAUCAAGUGGAGAGAA)d(TT)-3′. A nonspecific control siRNA was designed: 5′-r(GGAUUACUGACGAACUUGU)d(TT)-3′. A sequence analysis of the nonspecific control siRNA by BLAST search (National Center for Biotechnology Information database) showed no homology to any human transcripts in records.

Inhibition of *MDR1* expression by RNA interference. To generate *MDR1*-negative MCF-7/CD44s cells, an *MDR1*-targeted RNA interference (RNAi) experiment was done. Two MDR1 sequences were siRNA targeted; the open reading frame (ORF; ref. 21) and the 3′ untranslated region (UTR; ref. 22). The nonspecific control siRNA was used as a negative control. Briefly, 5×10^4 cells were seeded in 24-well plates in triplicates and after an overnight incubation the cells were transfected with 10 or 100 nmol/L siRNA in serum-free Opti-MEM medium (Life Technologies, Gaithersburg, MD) using 1 μL of siPORT transfection reagent (Ambion) as suggested by the manufacturer. Forty-eight hours posttransfection, total RNA was extracted (PARIS kit, Ambion) and pooled together. Gene expression was determined by semiquantitative RT-PCR. The housekeeping gene *rpL19* was used as a loading control.

MDR1 small interference RNA–transfected cells migration assay. To detect phenotypic changes in migration in *MDR1* siRNA-transfected cells, a modified cell migration assay was done using similar Falcon cell culture inserts as described above. MCF-7/CD44s cells grown to 90% to 100% confluency were tripsinized and resuspended in serum-free Opti-MEM (Life Technologies). From this single-cell suspension, 5×10^4 cells were seeded in the inserts that were then placed in the lower companion 24-well plate without cell culture medium. After 24 hours of incubation, the cells in the inserts were transfected as mentioned above and the plate wells were filled with RPMI 1640 plus 10% FBS without removing the inserts. After another 24 hours of incubation, the cells on the upper side of the membrane were removed by wiping with a cotton swab. The cells that migrated to the lower side of the membrane were fixed and stained using the diff-quik stain set (Dade Behring). The cells in five randomly selected microscopic fields (magnification, 200×) were counted. Each assay was done in triplicate. The data were analyzed using Student's t test and the statistical significance was set at $P < 0.05$.

Results

P-glycoprotein and CD44 are expressed in multidrug resistant but not in parental, sensitive cell lines. To determine whether or not there is a relationship between P-glycoprotein and CD44 expression, three MDR cell lines that overexpress P-glycoprotein and their respective P-glycoprotein-negative, drug-sensitive, parental cell lines were analyzed by immunoblotting and RT-PCR. As shown in Fig. 1*A, a* and *b*, sensitive MCF-7 cells did not contain *MDR1* or *CD44s* mRNA transcripts nor P-glycoprotein or CD44 protein. In contrast, the resistant MCF-7/AdrR cell line expressed *MDR1* mRNA and P-glycoprotein as well as *CD44s* mRNA and protein. In the squamous cell cancer cell lines KB3-1 (sensitive) and KBV-1 (resistant), both proteins, P-glycoprotein and CD44s were also overexpressed in the resistant cell line but completely absent or negligibly expressed in the sensitive parental cell line (Fig. 1*A, a*). Interestingly, *CD44s* mRNA was present at relatively similar amounts in KB3-1 and KBV-1 cells (Fig. 1*A, b*) suggesting that posttranscriptional events regulate the decreased protein expression of CD44s in KB3-1 cells. Cannistra et al. (23) has shown that deregulation of *CD44* expression at the RNA level does not always correlate with the protein expression.

The relationship between CD44 and P-glycoprotein protein expression was also observed in the ovarian cancer cell line, A2780, and its resistant counterpart, A2780Dx. However, in this example, the variant isoform CD44v10 correlated with the expression of P-glycoprotein because these cell lines did not express CD44s (Fig. 1*B, a*). Similar to the *CD44s* mRNA expression in KB3-1 and KBV-1, the *CD44v10* mRNA transcript does not seem to change significantly in A2780 compared with A2780Dx (Fig. 1*B, b*). These results also suggest the involvement of posttranscriptional mechanism(s) in the down-regulation of CD44v10 protein expression in A2780 cells. The results are summarized on Table 1. The simultaneous expression of CD44 and P-glycoprotein proteins in the MDR cell lines suggested a possible link between the regulation of these two molecules.

P-glycoprotein and CD44s coimmunoprecipitate in multidrug resistant cancer cell lines. To explore the possibility of a physical interaction between P-glycoprotein and CD44, we did coimmunoprecipitation assays using cell lysates from sensitive and resistant cell lines. As shown in Fig. 1*C, a* and *b*, immunoprecipitation with anti-CD44s coprecipitated P-glycoprotein in cell lysates from the resistant cell lines MCF-7/AdrR and KBV-1. Immunoprecipitation with anti-P-glycoprotein also coprecipitated CD44s in MCF-7/AdrR cell lysates (Fig. 1*C, d*). P-glycoprotein also coprecipitated with the CD44v3-10 isoform in the resistant A2780Dx cell line (Fig. 1*C, c*). Lysates from the parental sensitive cell lines that do not express CD44 or P-glycoprotein were used as negative controls. Another control was the MCF-7/BC19 cells, a MCF-7 cell line infected with a *MDR1* retroviral vector. As expected in this CD44 (−) control anti-P-glycoprotein antibodies did not immunoprecipitate CD44s (data not shown). We confirmed these findings using high-stringency coimmunoprecipitation with 2% and 4% octylglucoside. As shown in Fig. 1*C, e*, CD44 and P-glycoprotein coimmunoprecipitation after solubilization by octylglucoside was clearly detected at both concentrations. A nonspecific antibody control did not immunoprecipitate CD44s. This result supports the hypothesis of a strong and specific interaction between CD44s and P-glycoprotein.

P-glycoprotein colocalizes with CD44s on the cell membrane of multidrug resistant cells. To analyze whether the immunoprecipitation experiments using cell lysates represent a plausible interaction at the cell membrane between P-glycoprotein and CD44s, we analyzed the cellular localization of each protein by confocal microscopy. As shown in Fig. 1*D*, both P-glycoprotein and

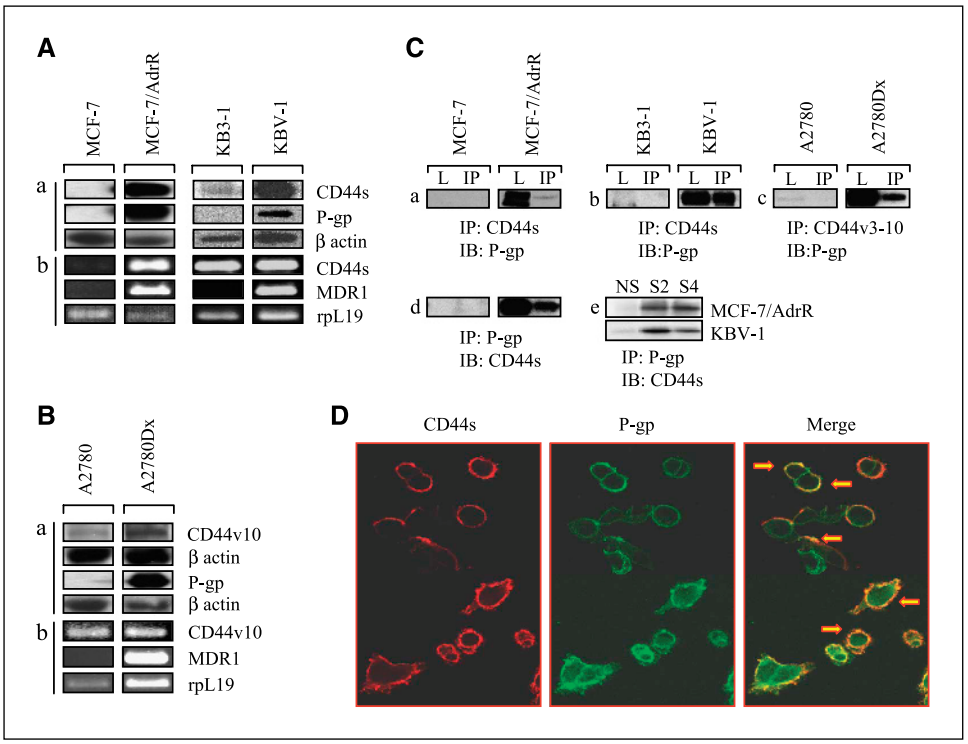

Figure 1. Sensitive and MDR cancer cells differentially express CD44 and P-glycoprotein (*P-gp*). *A,* immunoblotting and RT-PCR. Cell lysates from sensitive (MCF-7 and KB3-1) and drug resistance (MCF-7/AdrR and KBV-1) cell lines were immunoblotted with an anti-P-glycoprotein or an anti-CD44s antibody (*a*). Anti-β-actin antibody immunoblotting was used as a loading control. Total RNA from sensitive and drug-resistant cells was analyzed by a *CD44s* and *MDR1* semiquantitative RT-PCR protocol (*b*). The ribosomal protein *L19* mRNA was amplified by a semiquantitative RT-PCR and used as a loading control. *B*, cell lysates and total RNA from A2780 and A2780Dx were analyzed as in (*A*) but using an anti-CD44v10 (*a*) antibody and oligoprimers specific for *CD44v10* mRNA (*b*). *C,* immunoprecipitation (*IP*) and immunoblotting (*IB*). Cell lysates were immunoprecipitated with anti-human CD44s (*a* and *b*) or CD44v3-v10 (*c*) antibodies and immunoblotted with an anti-P-glycoprotein antibody. The MCF-7 and MCF-7/AdrR cell lysates were also immunoprecipitated with anti-P-glycoprotein (*d*) antibody and immunoblotted with anti-CD44s antibody. Preimmunoprecipitation lysate (*L*) was used a control. Coimmunoprecipation of P-glycoprotein and CD44s was also determined using cell lysates solubilized with 2% (*S2*) or 4% (*S4*) octylglucoside in KBV-1 and MCF-7/AdrR cells (*e*). A nonspecific antibody (*NS*) was used as a negative control. *D,* cells were incubated with anti-human CD44s and anti-P-glycoprotein antibodies followed by FITC- and rhodamine-conjugated secondary antibodies and immunofluorescence confocal microscopy analysis was done.

CD44s are expressed at the cell membrane and the location of each protein within the membrane overlaps except in regions of cell-to-cell contact.

Effect of P-glycoprotein substrates on cell migration and invasion. To investigate whether CD44s had a role in cell migration and *in vitro* invasion, we measured the cell migration in MCF-7/AdrR cells treated with anti-CD44s antibody. As shown in Fig. 2*A, graph a*, anti-CD44s antibody inhibited both cell migration and *in vitro* invasion of MCF-7/AdrR cells compared with controls. To determine whether inhibitors of P-glycoprotein function affected migration and *in vitro* invasion, we treated MCF-7/AdrR cells with tFPT (24). Figure 2*A, graph b*, shows that at noncytotoxic concentrations, tFPT decreased *in vitro* cell invasion and migration of MCF-7/AdrR in a dose-dependent manner. These results suggest that tFPT may interrupt the interaction between CD44s and P-glycoprotein by either affecting the P-glycoprotein pump function or alternatively interfering either physically or biochemically with the interaction between P-glycoprotein and CD44s. We examined these possibilities by analyzing the *in vitro* migration and invasion of cells treated with vinblastine, a cytotoxic drug, and a P-glycoprotein substrate (Fig. 2*A, graph c*). We found that vinblastine also decreased *in vitro* invasion in a dose-dependent manner, indicating that these drugs are likely to be destabilizing the P-glycoprotein/CD44s interaction or affecting an unknown function of P-glycoprotein. Alternatively, the observed effect could be caused by drug-directed modulation of cytoskeletal processes. Analysis of MCF-7/AdrR cells treated with tFPT or vinblastine showed no change in the relative expression of CD44s or P-glycoprotein (Fig. 2*B*). Therefore, changes in migration and invasion induced by these drugs do not seem to be due to a change in protein content.

Cell migration was also tested using a "wound-healing" assay. In these experiments, cells are grown to confluency and an incision

Table 1. Carcinoma cell lines and their respective P-glycoprotein and CD44 expression

Cell type	Cell line	P-glycoprotein	CD44
Breast carcinoma	MCF-7	Negative	Negative
	MCF-7/AdrR	Positive (24)	Positive (21)
Oral carcinoma	KB3-1	Negative	Negative
	KBV-1	Positive (5)	Positive (2)
Ovarian carcinoma	A2780	Negative	Negative
	A2780Dx	Positive (20)	Positive (2)

NOTE: Fold increase compared with sensitive parental cell line are in parentheses.

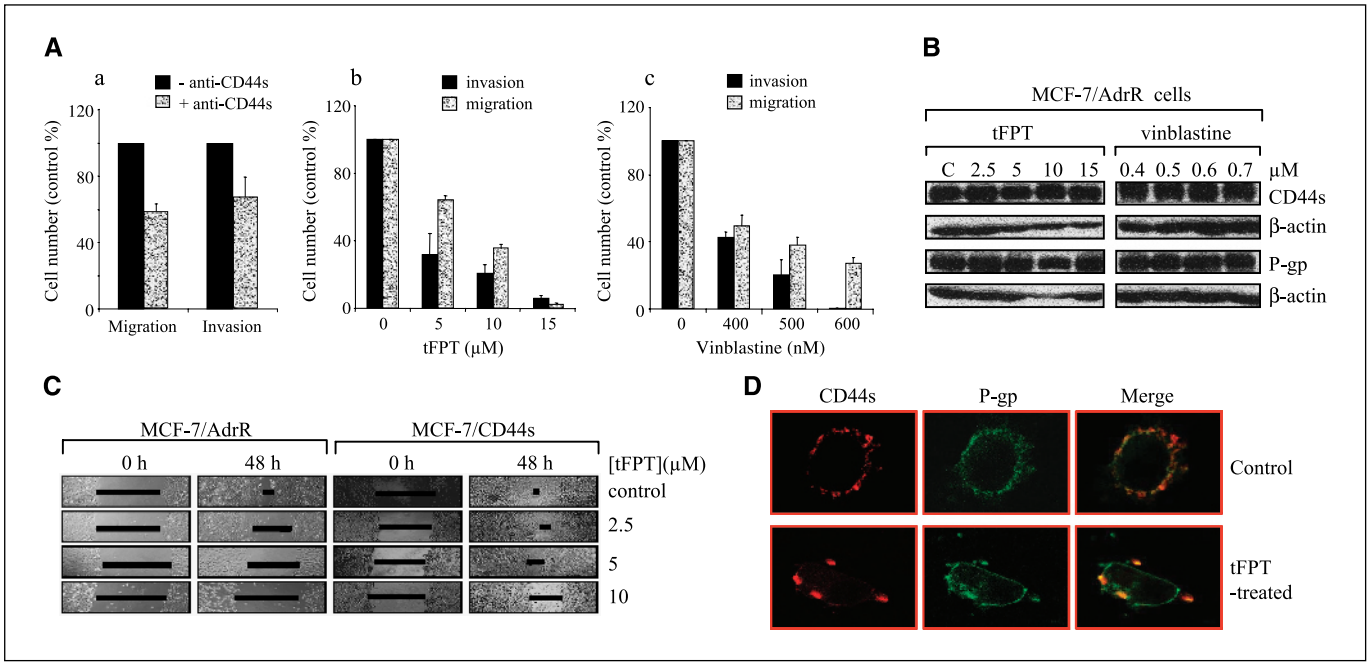

Figure 2. P-glycoprotein (*P-gp*) substrates and anti-CD44s antibodies mediate MCF-7/AdrR cell invasion and migration inhibition. *A, in vitro* cell migration and invasion in the presence of anti-CD44s (*a*) antibody (25 μg/mL for migration and 50 μg/mL for invasion), tFPT (*b*), or vinblastine (*c*). *B,* cell lysates of tFPT- or VBL-treated cells were immunoblotted with anti-P-glycoprotein or anti-CD44s antibodies. Anti-β-actin antibody immunoblotting was used as a loading control. *C,* wound-healing assay. MCF-7/AdrR or MCF-7/CD44s cells were grown to confluency and a linear "wound" was made with a pipette tip. After 48 hours of incubation in the absence (*control*) or presence of different concentrations of tFPT a microscopic photograph was taken (original magnification, 100×). *D,* MCF-7/AdrR cells were grown for 48 hours in the absence (*control*) or presence of tFPT and incubated with anti-human CD44s and anti-P-glycoprotein antibodies followed by FITC- and rhodamine-conjugated secondary antibodies. Immunofluorescence confocal microscopy analysis was done.

was made in the center of the plate. The relative movement of cells to cover the "wound" was measured after 48 hours. Figure 2*C* shows that when cells are treated with tFPT, cell migration is decreased in a dose-dependent manner.

***trans*-Flupenthixol promotes capping of the CD44 receptor.** To determine whether tFPT influenced cell migration and *in vitro* invasion by affecting the colocalization of P-glycoprotein and CD44s on the cell membrane, MCF-7/AdrR cells were treated with tFPT, labeled with fluorescent anti-CD44s and anti-P-glycoprotein antibodies, and analyzed by confocal microscopy. Figure 2*D* shows that tFPT promoted the "capping" of CD44s on the cell membrane, as shown by aggregated CD44s in a few well-defined regions. This physical "capping" effect of CD44s correlated with the decrease in cell migration caused by tFPT, suggesting that capped CD44s is less functional. Vehicle-treated MCF-7/AdrR cells did not show changes in CD44s localization or aggregation.

***trans*-Flupenthixol inhibits migration of MCF-7/CD44s and MCF-7/BC19/CD44s stable transfectants.** To determine whether each protein individually increased migration, we tested three different cell lines: MCF-7 (P-glycoprotein and CD44s negative), MCF-7/AdrR (P-glycoprotein and CD44s positive), and MCF-7/BC19 (P-glycoprotein positive, CD44s negative). We found that MCF-7/AdrR cells had the greatest rate of migration when compared with MCF-7 or MCF-7/BC19 cells (Table 2). These results indicate that expression of P-glycoprotein alone does not increase migration.

To test whether cell migration and invasion were dependent on CD44s or on the interaction between CD44s and P-glycoprotein, we searched for a cell line that expressed CD44s but not P-glycoprotein. In fact, in a screen of 6 ovarian cancer cell lines, we could not identify a CD44 (+), P-glycoprotein (−) cell line (data not shown). Therefore, we generated *CD44s* stable clones in MCF-7 parental cells. We also transfected MCF-7/BC19 cells that are P-glycoprotein (+) as a control. After selecting several stable clones in G-418-containing medium, CD44s expression was confirmed by immunoblotting. Unexpectedly, all five MCF-7/CD44s transfected clones also expressed P-glycoprotein (Fig. 3*A, a*).

Figure 3*A, b* and *B* shows CD44s expression in MCF-7/BC19/CD44s transfectants and coimmunoprecipitations of CD44s and P-glycoprotein from one of the MCF-7/CD44s clones. Cell migration of MCF-7/BC19/CD44s clones and MCF-7/CD44s clones (both CD44s and P-glycoprotein positive) paralleled that of MCF-7/AdrR cells (Table 2). Furthermore, tFPT or vinblastine inhibited cell migration in MCF-7/BC19/CD44s transfectants (Fig. 3*C*). These results were confirmed by testing migration in wound-healing assays of MCF-7/CD44s cells (Fig. 2*C*).

***CD44s* transfectant shows the multidrug-resistant phenotype.** The intracellular accumulation of paclitaxel (Taxol), a P-glycoprotein substrate, was measured to characterize the functionality of *CD44s*-induced expression of P-glycoprotein in

Table 2. Cell migration relative to MCF-7/AdrR

Cell line	P-glycoprotein	CD44	Migration (%)
MCF-7/AdrR	+	+	100
MCF-7	−	−	39.5 ± 3.8
MCF-7/BC19	+	−	17.8 ± 1.3
MCF-7/CD44s	+	+	100.5 ± 16.8
MCF-7/BC19/CD44s	+	+	76.5 ± 20.1

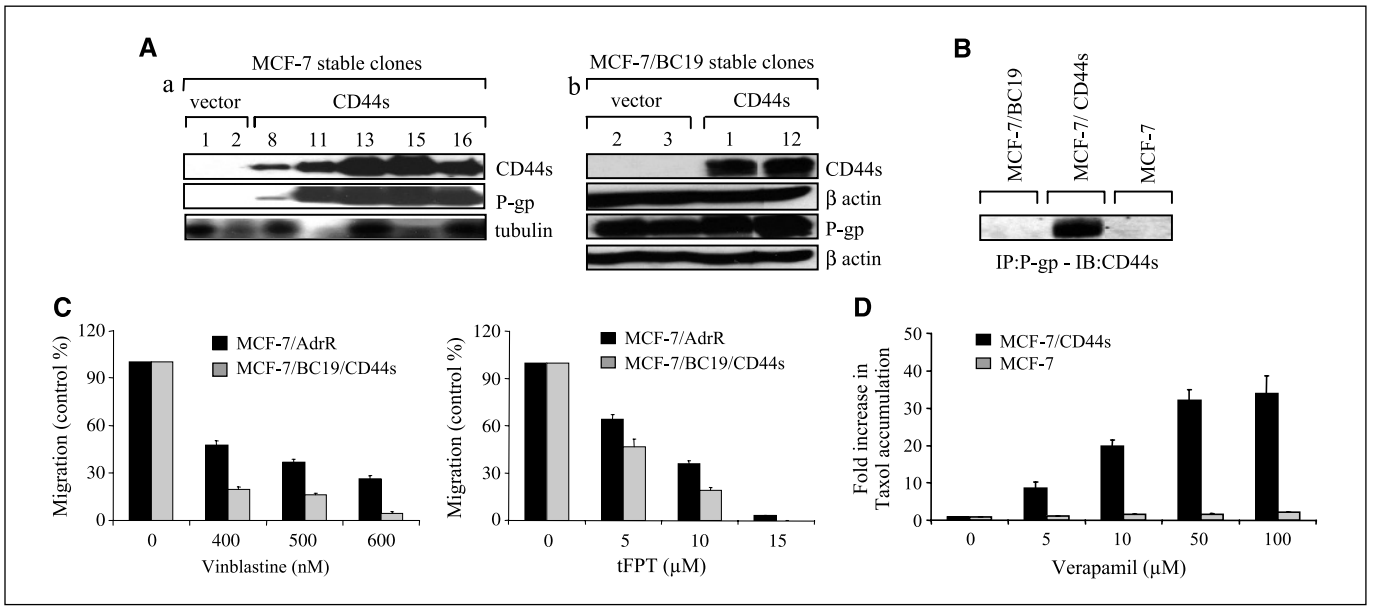

Figure 3. Stable expression of *CD44s* in MCF-7 cells promotes the MDR phenotype by inducing the expression of *MDR1*. *A,* immunoblotting. Cell lysates from MCF-7/vector (negative control) and MCF-7/CD44s stable transfectants were immunoblotted with anti-P-glycoprotein (*P-gp*) or anti-CD44s antibody (*a*). An anti-tubulin antibody immunoblotting was used as a loading control. Cell lysates from MCF-7/BC19/vector (negative control) and MCF-7/BC19/CD44s stable transfectants were analyzed as in *a* but using β-actin immunoblotting as loading control (*b*). *B,* immunoprecipitation. MCF-7/CD44s cell lysate was immunoprecipitated with anti-P-glycoprotein antibody and immunoblotted with anti-CD44s antibody. Cell lysates from MCF-7/BC19 (P-glycoprotein positive, CD44s negative) and MCF-7 (P-glycoprotein and CD44s negative) were used as experimental negative controls. *C,* cell migration analysis. MCF-7/AdrR and MCF-7/BC19/CD44s clone 12 were seeded in *in vitro* migration inserts in the presence or absence of vinblastine or tFPT. After 48 hours, the cells that migrated to the other side of the membrane were fixed, stained, and counted. Percentage of migration is based on the number of migrating MCF-7/Adr cells. Three wells per cell line were analyzed. *D,* drug accumulation assay. MCF-7/CD44s and MCF-7 cells were incubated in the presence or absence of verapamil and treated with tritium-labeled Taxol for 3 hours and the Taxol uptake was quantified in a scintillation counter.

the MCF-7/CD44s transfectant and in the P-glycoprotein-negative MCF-7 parental cell line. The *CD44s*-transfected MCF-7 clone (MCF-7/CD44s) accumulated 100-fold less Taxol than the parental cells (data not shown). Verapamil, a P-glycoprotein modulator, sensitized MCF-7/CD44s cells to Taxol in a dose-dependent manner but had no effect on MCF-7 parental cells (Fig. 3*D*).

Inhibition of *MDR1* expression by RNA interference decreases cell migration. The effect of inhibiting the expression of *MDR1* by RNAi on cell migration was tested (Fig. 4). The expression of *MDR1* was decreased by two independent sequences of *MDR1*-targeted siRNAs but not by the nonspecific control siRNA or the mock transfection control (Fig. 4*A-C*). P-glycoprotein expression also decreased by a modest amount after siRNA treatment. Furthermore, *MDR1*-targeted siRNA decreased the rate of migration of MCF-7/CD44s significantly, whereas negative controls had no effect on migration (Fig. 4*D*).

Discussion

This study shows that the expression of *CD44* and *MDR1* are coregulated and may begin to explain the association of advanced malignancy with drug resistance. Several types of experiments support this conclusion. First, as shown in Fig. 1 and summarized in Table 1, *CD44* expression was increased in cells that also overexpressed P-glycoprotein. Several publications suggest that this is the case in many cancers. For example, in an immunohistochemical analysis of breast cancer tissue from untreated female patients, de la Torre et al. (25) found a statistical correlation between CD44 and P-glycoprotein expression. There was no correlation between CD44 and age, tumor size, tumor grade, DNA ploidy, S-phase fraction, or hormone receptor content. Using a group of breast cancer cell lines including MCF-7 and MCF-7/AdrR, Fichtner et al. (26) showed that with the development of MDR, there is a change in the expression of metastasis-related transmembrane proteins including CD44. Staroselsky et al. (27) showed a direct correlation between the metastatic potential of B16 melanoma cells and the MDR phenotype in a mouse model. B16/Col/R MDR cells increased metastasis to the lung and also showed higher aggregability, cell motility, and adhesion to endothelial cells. Furthermore, the activation of the *MDR1* upstream promoter, the major *MDR1* promoter associated with P-glycoprotein overexpression, has been found to correlate with metastases to the lymph nodes in breast carcinoma cells (28).

Second, antibodies against P-glycoprotein coprecipitate P-glycoprotein and CD44 (Fig. 1*C, d* and *e*). Consistent with this result, anti-CD44 antibodies coimmunoprecipitate CD44 and P-glycoprotein (Fig. 1*C*). The possibility that this represents a true cellular interaction is strengthen by confocal microscopy that shows colocalization of both proteins within the plasma membrane (Fig. 1*D*).

Third, drugs that interfere with the function of P-glycoprotein also interfere with the cell's motility and invasion (Fig. 2). Nokihara et al. (29) confirmed the importance of P-glycoprotein in metastatic changes when they showed that the rate at which metastatic sites were formed by MDR human small cell lung cancer cells (SBC-3/ADM, P-glycoprotein positive) was higher than that produced by the sensitive parental cells (SBC-3, P-glycoprotein negative) in SCID mice. Bjornland et al. (30) observed that MDR hepatoma cells displayed an elevated capacity to migrate when compared with that of P-glycoprotein (−) parental cells. Treatment of the MDR hepatoma cells with the P-glycoprotein inhibitor PSC833 decreased their migration rate. Our results reflect the rate of cell migration after 48 hours of treatment. P-glycoprotein may have a prolonged

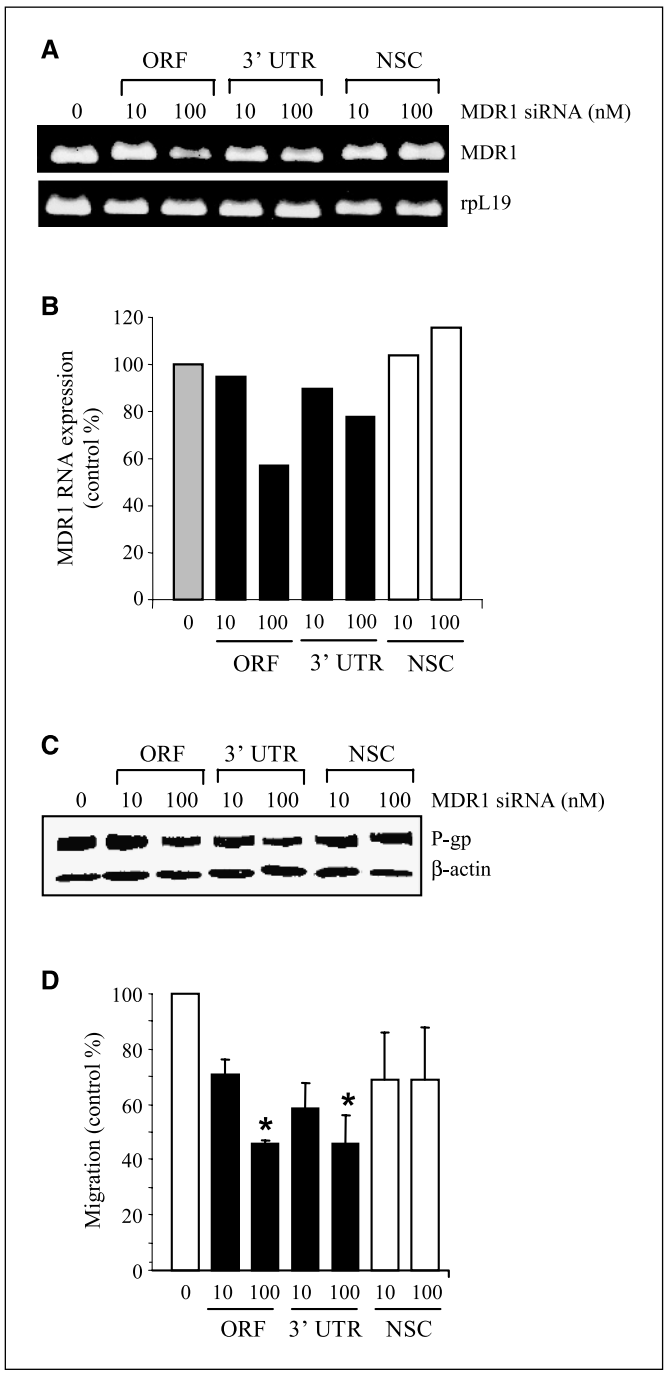

Figure 4. RNA interference of *MDR1* decreases cell migration. *A,* MCF-7/CD44s cells were transfected with *MDR1*-targeted siRNAs (e.g., ORF 3′ UTR) and a nonspecific control (*NSC*) siRNA. Forty-eight hours posttransfection, the total RNA was extracted. A semiquantitative RT-PCR with *MDR1* oligo-primers was done. A semiquantitative RT-PCR with *rpL19* oligo-primers was done as a loading control. *B,* the MCF-7/CD44s *MDR1* and *rpL19* PCR product bands were scanned and their intensities calculated. The *rpL19* intensity values were used to normalize the ones for *MDR1*. *C,* from MCF-7/CD44s cells treated as above, protein lysates were analyzed by immunoblotting using antibodies against P-glycoprotein (*P-gp*) and β-actin, which was used to normalized the P-glycoprotein bands. *D,* cells in serum-free Opti-MEM were seeded in inserts, incubated overnight and transfected with *MDR1* and nonspecific control siRNAs. After 24 hours, 10% FBS RPMI 1640 was added to the companion wells and the cells were allowed to migrate for 24 hours. The cells that migrated to the lower side of the membrane were fixed, stained, and counted. Percentage of migration was calculated based on the number of untransfected migrating cells (mock control). Three inserts per *MDR1* siRNA concentration were analyzed. *, $P < 0.05$.

half-life that dramatically increases up to 72 hours in conditions of serum deprivation (31). Therefore, it is difficult to observe phenotypic differences in a shorter period of time. However, it is important to point that changes in migration after 48 hours could be secondary to differences in cell proliferation.

We found that drugs that interfere with P-glycoprotein also affect the localization of CD44 on the cell membrane and promote CD44 capping (Fig. 2*D*). This capping of CD44 in the presence of drugs that affect P-glycoprotein function correlates with the decrease in migration caused by tFPT, suggesting that capped CD44s is less functional. It is possible that vinblastine or tFPT effect on capping is secondary to these drugs effect on actin polymerization. Yu and Stamenkovic (32) observed in TA3 mammary carcinoma cells that CD44 colocalized on the cell membrane with yet another protein, MMP-9. They observed that CD44 formed caps spontaneously. MMP-9 colocalized in the caps, however, surface CD44 expression was not limited to the caps, whereas MMP-9 was primarily detected in the caps. However, drug-induced capping of CD44 could be secondary to the direct effect of drug on cytoskeletal function.

Fourth, our studies also indicate that the expression of *CD44s* and *MDR1* are coregulated. For example, transfection of *CD44s* into *CD44s* (−) cell lines induces the expression of *MDR1* (Fig. 3*A, a*) and produces a functional P-glycoprotein (Fig. 3*D*). Even in transfectants that already express P-glycoprotein, the introduction of *CD44s* increases expression of P-glycoprotein when compared with vector only transfectants (Fig. 3*A, b*). Furthermore, *MDR1*-targeted siRNA decreases both *MDR1* expression and the rate of cell migration. Taken together with the experiments summarized on Table 2, which show that the expression of P-glycoprotein alone does not increase migration rate, we infer that it is the interaction of P-glycoprotein with CD44 that affects cell migration.

Similar results were reported by Honig et al. (33) in normal T lymphocytes. They showed that P-glycoprotein needed to be active in T lymphocytes for their proper migration to lymph nodes. Migration was also dependent on VLA-4α and CD44. They were able to inhibit T-lymphocyte migration with PSC833, a P-glycoprotein antagonist. Our findings indicate that cancer cells use the P-glycoprotein transporter in conjunction with CD44 in a similar fashion as that reported for normal T lymphocytes during activation and lymph node homing.

Finally, it is important to note that this phenotypic correlation seems cell type and/or drug related, which has led to the publication of apparent conflicting reports (34–37). For example, Liang et al. (38) exposed a drug-sensitive human nasal carcinoma cell line to melphalan or Taxol. The melphalan-exposed cells had increased *in vitro* invasiveness, whereas the Taxol-exposed cells did not. This indicates that other steps in the metastatic process, probably downstream of the CD44/P-glycoprotein interaction have to be optimized in order for the cancer cell to migrate, invade, and metastasize. For example, the correct localization of MMPs by CD44 at the cell surface, such as MMP-9 and MMP-7, and the presence or absence of tissue inhibitors of metalloproteinases are contributing steps (39). Furthermore, other membrane transporters downstream from P-glycoprotein may be necessary for the cells to migrate. Honig et al. (33) showed that T cells not only need P-glycoprotein and CD44, but downstream of this interaction another cell transporter ABCC1 (MRP1) is also required for T-cell migration. Studies to elucidate the possible role of other membrane transporters in MDR-related cancer cell migration are in progress.

Because vinblastine and taxol can inhibit the microtubule system, it is possible that the decrease in cell migration and *in vitro* invasion is in part due to a direct effect of these drugs on the cytoskeleton.

In summary, our data begin to clarify the observed relationships between MDR and metastatic behavior. The data make evident the physical (i.e., protein-protein) and genetic (i.e., gene "cross-talk") functional interactions between CD44 and P-glycoprotein. It seems that the functional interaction between CD44s and P-glycoprotein is one step in a complex molecular organization that results in the concomitant phenotype of MDR, increased cell migration, *in vitro* invasion, and metastasis.

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The CD44 Receptor Interacts with P-Glycoprotein to Promote Cell Migration and Invasion in Cancer

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