Monocarboxylate Transporter 4 Regulates Maturation and Trafficking of CD147 to the Plasma Membrane in the Metastatic Breast Cancer Cell Line MDA-MB-231

Shannon M. Gallagher,[1] John J. Castorino,[2] Dian Wang,[1] and Nancy J. Philp[1]

Departments of [1]Pathology, Anatomy and Cell Biology and [2]Dermatology, Thomas Jefferson University, Philadelphia, Pennsylvania

Abstract

Metastatic cancer cells increase glucose consumption and metabolism via glycolysis, producing large quantities of lactate. Recent work has shown that lactate efflux is mediated by monocarboxylate transporters (MCT), which are composed of a catalytic unit (MCT) and an accessory subunit (CD147), comprising the functional lactate transporter. CD147, an extracellular matrix metalloproteinase (MMP) inducer, is highly expressed in metastatic cancer cells. Because aerobic glycolysis is a hallmark of metastatic cancer, we examined whether increases in CD147 expression were linked to MCT expression in MDA-MB-231, a highly metastatic breast cancer cell line. MCT4 mRNA and protein expression were increased in MDA-MB-231 cells compared with cells derived from normal mammary tissue. MCT4 colocalized with CD147 in the plasma membrane and in membrane blebs shed from the cell surface. Small interfering RNA–mediated silencing of MCT4 impaired the maturation and trafficking of CD147 to the cell surface, resulting in accumulation of CD147 in the endoplasmic reticulum. Silencing MCT4 also resulted in fewer membrane blebs and decreased migration of MDA-MB-231 cells *in vitro*. Knockdown of CD147 resulted in loss of MCT4 in the plasma membrane and accumulation of the transporter in endolysosomes. These studies establish for the first time that increased expression of CD147 in metastatic cancer cells is coupled to the up-regulation of MCT4. The synergistic activities of the MCT/CD147 complex could facilitate migration of tumor cells by CD147-mediated MMP induction and lactate-stimulated angiogenesis and hyaluronan production. These data provide a molecular link between two hallmarks of metastatic cancer: the glycolytic switch and increased expression of CD147. [Cancer Res 2007;67(9):4182–9]

Introduction

A hallmark of invasive cancer is the up-regulation of glycolytic genes and an increased dependence on glycolysis for the production of ATP (1, 2). The switch from oxidative to glycolytic metabolism of glucose is accompanied by increased expression of the glucose transporter GLUT1 and glucose uptake. This feature of cancer cells, first described by Warburg more than 50 years ago, has been confirmed in animal models and humans through the use of 18-fluoro-deoxyglucose positron emission tomography (3, 4).

Requests for reprints: Nancy J. Philp, Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, 1020 Locust Street, Room 534, Philadelphia, PA 19107. Phone: 215-503-7854; Fax: 215-923-3808; E-mail: nancy.philp@jefferson.edu.

doi:10.1158/0008-5472.CAN-06-3184

Metabolism of glucose via glycolysis results in the production of large quantities of lactate that must be transported out of cells. Transport of lactate across the plasma membrane is mediated by a family of proton-coupled monocarboxylate transporters (MCT). At least 14 members of this family have been cloned and are distinguished by their kinetic properties and tissue distribution (5, 6). MCT1 is the most widely expressed member of this family and has recently been shown to be elevated in a variety of cancers, including neuroblastoma (7) and colorectal carcinomas (8). MCT4 is expressed preferentially in tissues dependent on glycolysis for the metabolism of glucose, including fast twitch muscle, leukocytes, and the neural retina (5, 6, 9–12). Expression of MCT4 protein in metastatic cancer cells has not been examined; however, *MCT4* recently appeared on a list of genes that are up-regulated by the transcription factor hypoxia-inducible factor (HIF)-1, which can regulate the metabolic switch in metastatic cancer cells (2, 13).

Recent studies have shown that MCT1, MCT3, and MCT4 are heteromeric transporters composed of a catalytic α-subunit (MCT) and an accessory β-subunit (CD147; refs. 9, 11). CD147 was characterized independently by several different laboratories as a type I transmembrane glycoprotein and a member of the immunoglobulin superfamily (14). In cancer cells, CD147 was identified as an extracellular matrix metalloproteinase (MMP) inducer because the purified protein was found to induce the expression of MMPs in fibroblasts and cancer cells (14, 15). It was subsequently shown that CD147 is expressed at high levels in metastatic tumor cells both *in vivo* and *in vitro* (15, 16). MCT1, MCT3, and MCT4 require association with CD147 in the endoplasmic reticulum (ER) for trafficking to the plasma membrane (9, 11, 17–19). In the absence of CD147, MCTs are targeted for degradation. A role for CD147 in inducing the expression of MMP activity has been extensively studied in cancer cells, but its association and dependence on MCTs for cell surface expression has not yet been addressed. The purpose of this study was to evaluate whether the increase in expression of CD147 observed in the metastatic breast cancer cell line MDA-MB-231 is linked to an increase in MCT expression.

Materials and Methods

Cell culture and reagents. Cell culture reagents were purchased from Mediatech, Inc. MDA-MB-231, HEK293, HL-60, K562, and HTB-125 cells were obtained from the American Type Culture Collection. HeLa and MCF-7 cells were kindly provided by Dr. David Herrick and Dr. Renato Iozzo, respectively (Thomas Jefferson University, Philadelphia, PA). MDA-MB-231 and HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). HTB-125 cells were maintained in DMEM supplemented with 30 ng/mL epidermal growth factor (Sigma) and 10% FBS. HEK293 cells were maintained in DMEM supplemented with 5% FBS. HL-60 cells were cultured in RPMI 1640 supplemented with MEM vitamins,

MEM nonessential amino acids, and 10% heat-inactivated FBS. K562 cells were cultured in RPMI 1640 supplemented with 10% FBS. ARPE-19, a human retinal pigment epithelium (RPE) cell line, was cultured as described previously (20). MCF-7 cells were maintained in MEM (Eagle) and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate and supplemented 0.01 mg/ml bovine insulin and 10% FBS. MCF-7 cells did not receive penicillin/streptomycin antibiotic. Culture media were supplemented with 2 mmol/L L-glutamine and 100 μg/mL penicillin/streptomycin antibiotic, with the exception of the HeLa cells. Cells were cultured at 37°C in a 5% CO_2 atmosphere.

Reverse transcription-PCR analysis. Expression of MCTs and CD147 in MDA-MB-231 and HTB-125 cells was analyzed by reverse transcription-PCR (RT-PCR) using isoform-specific primers. First-strand cDNAs were prepared using 1.5 μg of total RNA and 3′-rapid amplification of cDNA ends adaptor primer (5′-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT-3′; Life Technologies, Inc.). The following primer sets were used: MCT1, 5′-TTTC-TTTGCGGCTTCCGTTGTTG-3′ (forward) and 5′-TCAATTTACCCTT-CAGCCCCATGG-3′ (reverse); MCT4, 5′-TTTTGCTGCTGGGCAACTTCTT-CTG-3′ (forward) and 5′-TCACGTTGTCTCGAAGCATGGGTTT-3′ (reverse); CD147, 5′-AGGACCGGCGAGGAATAGGA-3′ (forward) and 5′-TGCAAG-CACTGGGAGTGGAC-3′ (reverse); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-GCAATGCCTCCTGCACCACCA-3′ (forward) and 5′-CCCCAGCGTCAAAGGTGGAGG-3′ (reverse). The following cycling variables were used: 94°C, 30 s; 54°C, 30 s; 72°C, 60 s; 30 cycles. PCR conditions for the small interfering RNA (siRNA) experiments were as follows: 94°C, 30 s; 54°C, 30 s; 72°C, 60 s; 23 cycles. PCR products were separated on 1.0% agarose gels.

Antibodies. MCT1 and MCT4 antibodies were generated and characterized previously (9). Anti-human CD147 antibodies were purchased from Research Diagnostics, Inc. for immunostaining and immunoprecipitation, and from Santa Cruz Biotechnology, Inc. for immunoblotting. Human GLUT1 antibodies were a generous gift from Dr. Ian Simpson (Pennsylvania State University, Hershey, PA). Additional antibodies used in these studies were anti-human cyclophilin A (Biomol Research Laboratories, Inc.), anti-β-actin (Sigma), and anti-GRP78 (Sigma). Anti-LAMP-1 was a generous gift from Dr. Enrique Rodriguez-Boulan (Weill Medical College, Cornell University, New York, NY). TO-PRO-3 iodide and Alexa Fluor–tagged secondary antibodies were purchased from Molecular Probes. Horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Bio-Rad Laboratories and Molecular Probes.

Immunoblot analysis. Cells were washed with PBS and lysed with ice-cold lysis buffer [25 mmol/L HEPES buffer (pH 7.4), 150 mmol/L NaCl, 5 mmol/L $MgCl_2$, 1% Triton X-100] containing protease inhibitors (Complete Mini, Roche) for 30 min, then centrifuged at 14,000 × *g* at 4°C for 30 min. The protein concentration of the cleared lysates was determined using bicinchoninic acid reagent (Pierce). The lysates were diluted in 2× LDS sample buffer (Invitrogen), and equal amounts of protein were loaded onto 4% to 12% NuPAGE Bis-Tris gels (Invitrogen). Separated proteins were transferred electrophoretically from gels to Immobilon-P membranes (Millipore). Membranes were incubated for 1 h at room temperature in blocking buffer [20 mmol/L Tris, 137 mmol/L NaCl (pH 7.5), 5% dry skim milk] followed by 1-h incubation with primary antibodies and 30-min incubation with HRP-conjugated secondary antibodies diluted 1:5,000. Blots were probed with the following antibodies: CD147 (goat; 1:500), MCT1 (rabbit; 1:1,000), MCT4 (rabbit; 1:10,000), and GLUT1 (rabbit; 1:5,000). Cyclophilin A (rabbit; 1:20,000) and β-actin (mouse; 1:20,000) were used as internal controls. Reactive bands were visualized with electrochemiluminescent Western blotting detection reagents (GE Healthcare Bio-Sciences Corp.). Total protein (5 μg) was loaded in all lanes unless otherwise indicated.

Vesicle collection. Plasma membrane vesicles shed from MDA-MB-231 cells were harvested as described previously (21, 22). Briefly, cells were cultured in DMEM containing 5% FBS for 17 h. The conditioned medium was removed from cells and centrifuged for 15 min at 1,000 × *g* to remove any large cellular debris. The supernatant was then centrifuged at 150,000 × *g* for 75 min at 4°C. Pelleted vesicles were washed with PBS and centrifuged again at 150,000 × *g* for 75 min at 4°C. Vesicles were resuspended in 2× LDS sample buffer. Presence of MCT4 and CD147 in purified vesicles was determined by immunoblot analysis.

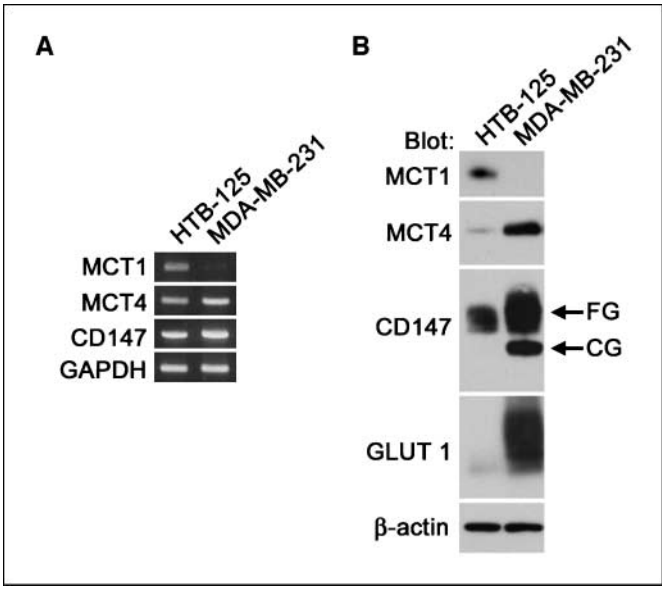

Figure 1. CD147 and MCT4 are up-regulated in breast cancer cells. *A,* CD147 and MCT4 mRNA levels are increased in MDA-MB-231 cells. GAPDH mRNA was used as an internal control. *B,* MDA-MB-231 cells express high levels of MCT4, CD147, and GLUT1. Detergent-soluble lysates prepared from MDA-MB-231 and HTB-125 cells were analyzed by immunoblot using antibodies against MCT1, MCT4, CD147, GLUT1, and β-actin (as an internal control). Total protein (5 μg) was loaded in each lane. *FG,* fully glycosylated form; *CG,* core-glycosylated form.

Immunoprecipitation. MDA-MB-231 cells were washed with PBS and lysed with ice-cold lysis buffer [25 mmol/L HEPES buffer (pH 7.4), 150 mmol/L NaCl, 5 mmol/L $MgCl_2$, 1% CHAPS detergent] containing protease inhibitors (Complete Mini) for 30 min. The lysate was clarified by centrifugation at 14,000 × *g* for 30 min. An aliquot of the supernatant was removed for protein determination, and the remaining supernatant was incubated with 5 μL of anti-CD147 antibody or anti-MCT4 antibody overnight with end-over rotation at 4°C. The following day, 25 μL of EZview Red protein G affinity gel beads (Sigma) were added to the samples for 1 h with end-over rotation at 4°C. The samples were centrifuged at low speed, and the supernatant was saved for analysis by SDS-PAGE immunoblotting. Agarose pellets were washed with lysis buffer, precipitated proteins were eluted with 2× LDS sample buffer and analyzed by immunoblotting.

Biotinylation of cell surface proteins. Cells were washed with PBS and incubated in PBS containing 0.5 mg/mL EZ-Link Sulfo-NHS-LC-Biotin (Pierce) for 30 min at room temperature. The reaction was quenched with DMEM. Protein was extracted from cells as described above in 1% Triton X-100 lysis buffer, and biotinylated proteins were precipitated from the detergent-soluble lysates by incubation overnight with 20 μL of avidin-agarose beads (Sigma). Beads were washed with lysis buffer, and precipitated proteins were eluted with 2× LDS sample buffer. Avidin-precipitated proteins were analyzed with the control lysates by immunoblotting.

Immunofluorescence. MDA-MB-231 cells were cultured on two-well glass slides or 35-mm dishes and allowed to reach 40% to 50% (for colocalization studies) or 70% to 90% (for siRNA experiments) confluence. Cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) in PBS for 5 min at room temperature and then for 25 min on ice. Cells were washed in PBS and permeabilized with methanol for 3 min at −20°C. Cells were blocked using 5% bovine serum albumin (BSA) in PBS with 0.1% Tween 20 (PBST) and incubated with primary antibody overnight at 4°C. The next day, cells were washed with PBST and incubated in secondary antibody for 30 min, washed, and mounted with gelvatol. Antibodies were used at the following dilutions: CD147 (mouse; 1:500), MCT4 (rabbit; 1:500), GRP78

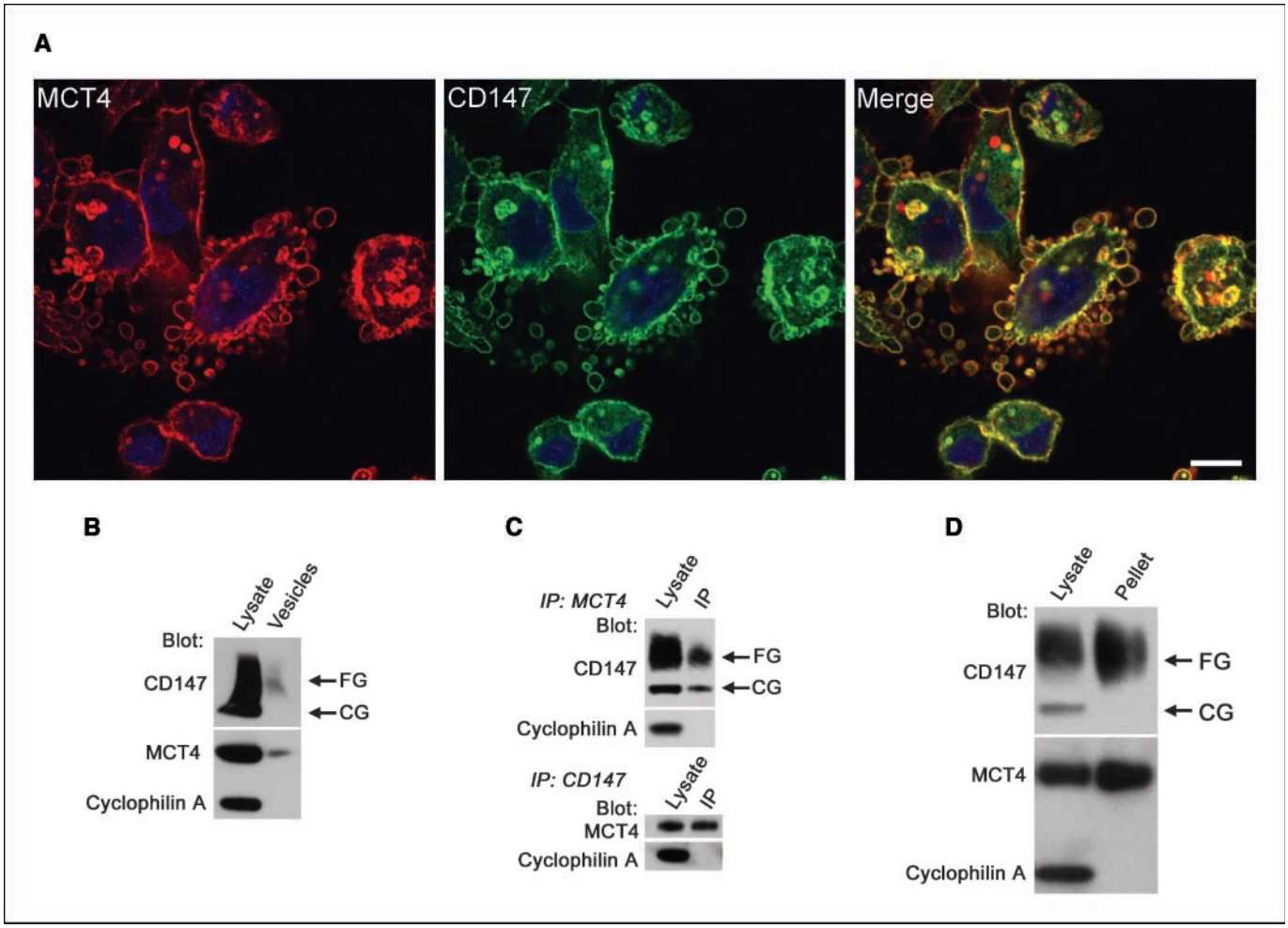

Figure 2. MCT4 and CD147 colocalize to the cell surface of MDA-MB-231 cells where they form MCT4/CD147 complexes. *A,* CD147 and MCT4 colocalize in the plasma membrane and in membrane blebs. MDA-MB-231 cells were immunolabeled with anti-MCT4 (*red*) and anti-CD147 (*green*) antibodies and imaged by confocal laser scanning microscopy. TO-PRO-3 iodide (*blue*) was used to label nuclei. Note the extensive blebbing at the surface of these cells and the colocalization of these proteins (*Merge, yellow*). *Bar*, 20 μm. *B,* MCT4 and fully glycosylated CD147 are expressed in membrane vesicles. Immunoblot analysis of membrane vesicles isolated from conditioned medium of MDA-MB-231 cells. Vesicles were analyzed by immunoblot using antibodies against CD147, MCT4, and cyclophilin A (as an internal control). Total protein (5 μg) was loaded in Lysate lane. Pellet fraction (25 μL) was loaded into the Vesicles lane. *C,* CD147 and MCT4 form a complex in MDA-MB-231 cells. Detergent-soluble lysates were immunoprecipitated with anti-CD147 or anti-MCT4. Soluble and pellet fractions were analyzed by immunoblot using antibodies against CD147, MCT4, and cyclophilin A (as an internal control). *D,* both MCT4 and fully glycosylated CD147 are expressed at the cell surface of MDA-MB-231 cells. After streptavidin-agarose precipitation, samples were analyzed by immunoblot using antibodies against CD147, MCT4, and cyclophilin A (as an internal control). Total protein (5 μg) was loaded in Lysate lanes in (*C*) and (*D*); pellet fraction (25 μL) was loaded in the IP lane (*C*) and Pellet lane (*D*). *IP,* immunoprecipitate.

(rabbit; 1:500), LAMP-1 (mouse; 1:250), TO-PRO-3 iodide (1:1,000), Alexa Fluor 488 (mouse; 1:500), and Alexa Fluor 555 (rabbit; 1:500). Images were collected on a laser scanning confocal microscope (Zeiss LSM510) with a 63× oil objective and processed with LSM510 software and Adobe Photoshop 7.0.

Stable transfection of HEK293 cells. HEK293 cells were stably transfected with hMCT4 and hCD147 constructs using Fugene 6 according to the manufacturer's instructions. Human CD147 was amplified from cDNA prepared from RPE. The 5′ primer (CCGCTCGAGCCACCATGGCGGCT-GCGCTGTTC) included the *Xho*I restriction site and an improved Kozak consensus sequence. 3′ Primers for the complete coding region (GCCAGAG-GAACTCTTCCTGGGTACCCCG) included *Kpn*I sites. PCR products were ligated into the pCRII vector (Invitrogen), excised with *Xho*I and *Kpn*I, and ligated into the *Xho*I and *Kpn*I sites of the pEGFP-N1 vector (Clontech), resulting in fusions CD147-green flourescent protein (GFP). The integrity of the constructed plasmids was confirmed by sequencing. All plasmids were purified using the Qiagen Maxiplasmid kit. hMCT4 constructs were a generous gift from Dr. Ami Deora (Weill Medical College, Cornell University) and generated as described in ref. 17.

siRNA-mediated silencing of MCT4 and CD147. MCT4 and CD147 expression was silenced with siRNA using manufacturer's protocols. Cells were plated in six-well plates and allowed to grow to 60% to 80% confluence. siRNAs were purchased from Dharmacon and Santa Cruz Biotechnology. MDA-MB-231 and HeLa cells were transfected with siRNAs specific for hCD147 (accession no. NM_001728), MCT1 (accession no. NM_003051), or MCT4 (accession no. NM_004207) using the DharmaFECT 2 transfection reagent (Dharmacon). MCT5 (accession no. NM_004696) siRNA was used as a negative control. Similar results were obtained using siRNAs from Dharmacon and Santa Cruz. As an additional control, cells were mock transfected using transfection reagent alone. HL-60 and K562 cells were electroporated with 10 μL of a 10 μmol/L solution of MCT1, MCT4, or MCT5 (negative control) siRNA in a BTX ECM 630 electroporator using 4-mm gap cuvettes with 500 μL of a 2×10^7 cells/mL suspension in serum-free RPMI 1640. K-562 cells were electroporated at 215 V, 850 μF, and no resistance. HL-60 cells were electroporated at 360 V, 450 μF, and no resistance. All cells were harvested or fixed 72 h after transfection for protein isolation and immunostaining and 48 h after transfection for RNA.

All siRNA experiments were done in triplicate for each condition. Similar results were obtained in all experiments.

Transwell migration assay. Migration assays were done using Corning Costar chambers. Filters were coated with 20 μg/mL fibronectin (Sigma) for 1 h at 37°C. MDA-MB-231 cells were transfected with MCT5 siRNA (control) or MCT4 siRNA. Seventy-two hours after transfection, cells were dissociated from the tissue culture plate with 1× nonenzymatic cell dissociation solution (Sigma) and resuspended in DMEM containing 1% BSA. An equal number of cells were added to the upper chamber of all wells. DMEM containing 10% FBS was added to the lower chamber. Cells were incubated in the chambers for 6 h at 37°C in a 5% CO_2 atmosphere. Following incubation, cells were removed from the upper chamber by gently scrubbing the filter surface with a cotton swab. The filters were washed in PBS and fixed in 4% paraformaldehyde. Migrated cells found on the bottom surface of the filter were visualized by crystal violet staining (0.5 g crystal violet, 20 mL methanol, 80 mL dH_2O) followed by light microscopy. A total of 15 fields was quantified for each condition (three fields per filter, five filters per condition) by manual cell counting. Experiments were done twice in duplicate (experiment 1) or triplicate (experiment 2). Data were analyzed using Microsoft Excel. Data were determined to be significant at $P < 0.05$ using a Student's t test.

Results

MDA-MB-231 cells express high levels of MCT4 and its accessory protein CD147. Because many cancer cells exhibit an increase in glucose consumption and metabolism via glycolysis, we compared MCT, CD147, and GLUT1 expression in the highly invasive breast cancer cell line MDA-MB-231 with a breast cell line derived from a normal mammary gland (HTB-125). Using RT-PCR analysis, we found that MCT4 and CD147 mRNAs were expressed at higher levels in MDA-MB-231 cells than in HTB-125 cells. MCT1 mRNA was expressed in normal breast cell lines but was not expressed at detectable levels in MDA-MB-231 cells (Fig. 1*A*). Immunoblot analysis revealed that HTB-125 cells expressed the mature, fully glycosylated form (45–65 kDa) of CD147 and MCT1 and low levels of MCT4 and GLUT1. In contrast, in MDA-MB-231 cells, levels of expression of MCT4 and CD147 were increased compared with the normal cells (Fig. 1*B*). Both the core-glycosylated (lower CD147 band, 30 kDa) and fully glycosylated forms of CD147 were detected in MDA-MB-231 cell lysates, suggesting an increase in synthesis of CD147. GLUT1 expression was greatly increased in MDA-MB-231 cells consistent with a shift to glycolytic metabolism (Fig. 1*B*; ref. 23).

MCT4 and CD147 colocalize in the plasma membrane and in membrane blebs. Metastatic cancer cells are distinguished morphologically from normal cells by extensive membrane blebbing (21, 22, 24, 25). Because MCTs form a heteromeric complex with CD147, we used immunofluorescence localization and confocal microscopy to determine whether MCT4 and CD147 were colocalized in membrane blebs of MDA-MB-231 cells. In cells colabeled with anti-MCT4 and anti-CD147 antibodies, MCT4 was detected in the plasma membrane as well as in the membrane blebs. CD147 exhibited a similar distribution as shown by the extensive colocalization observed (Fig. 2*A, Merge*). In addition to MCT4 and CD147, other plasma membrane proteins, such as GLUT1 and the amino acid transporter CD98, were detected in the blebs (data not shown). Expression of MCT4 and CD147 was detected in isolated vesicles by immunoblot analysis (Fig. 2*B*).

Because both the fully glycosylated and core-glycosylated forms of CD147 coimmunoprecipitated with CD147 (Fig. 2*C*), we next wanted to determine whether both complexes were expressed at the cell surface (Fig. 2*C*). Biotinylation revealed that both fully glycosylated CD147 and MCT4 were expressed at the plasma membrane (Fig. 2*D*). However, we found that core-glycosylated CD147 was not biotinylated, providing further evidence that assembly of the complex occurs in the ER (Fig. 2*D*).

Increased cell surface expression of CD147 by ectopically expressed MCT4. To determine whether the catalytic subunit (MCT) or the accessory subunit (CD147) regulates the abundance of the mature transporter in the plasma membrane, MCT4 and CD147 were overexpressed in HEK293 cells. Stable expression of MCT4 increased the level of expression of CD147 at the plasma membrane (Fig. 3*A*). In contrast, stable expression of CD147 did not increase the level of expression of the endogenous transporter MCT1 (Fig. 3*B*). These data show that the level of the MCT/CD147 transporter complex expressed in these cells is regulated by the MCT rather than CD147.

MCT4 is required for maturation and cell surface expression of CD147. To determine whether cell surface expression of CD147 requires association with MCT4, siRNA was used to silence MCT4 expression in MDA-MB-231 cells. PCR and immunoblot analysis were used to evaluate changes in MCT4 and CD147 mRNA and protein in treated cells. MCT4 siRNA reduced the level of MCT4 but not CD147 mRNA (Fig. 4*A*). Immunoblot analysis confirmed a loss of MCT4 expression in cells treated with MCT4 siRNA.

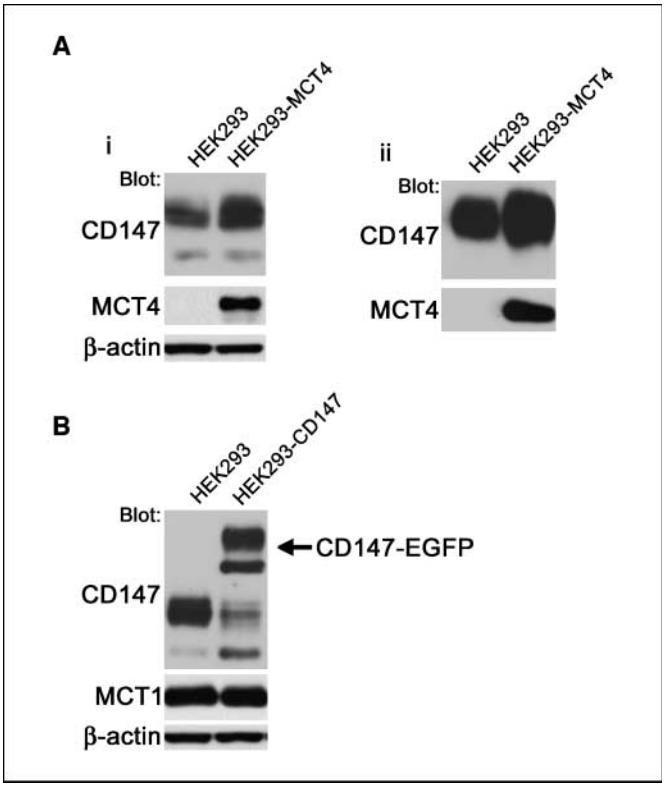

Figure 3. MCT4 increases CD147 expression in HEK293 cells. *A, i,* immunoblot of soluble lysates from HEK293 cells and HEK293 cells stably expressing MCT4 (*HEK293-MCT4*). Stable expression of MCT4 in HEK293 cells results in increased expression of both the core-glycosylated and fully glycosylated forms of CD147. *ii,* HEK293 and HEK293-MCT4 cells were biotinylated and avidin bound precipitates were analyzed by immunoblot. Precipitates (25 μL) were loaded in each lane. *B,* stable expression of CD147 in HEK293 cells decreased endogenous CD147 expression, but did not alter MCT1 expression in HEK293 cells. *CD147-EGFP,* exogenously expressed CD147 tagged with enhanced GFP. β-Actin was used as an internal control. Protein (10 μg) was loaded in each lane in (*A, i*) and (*B*).

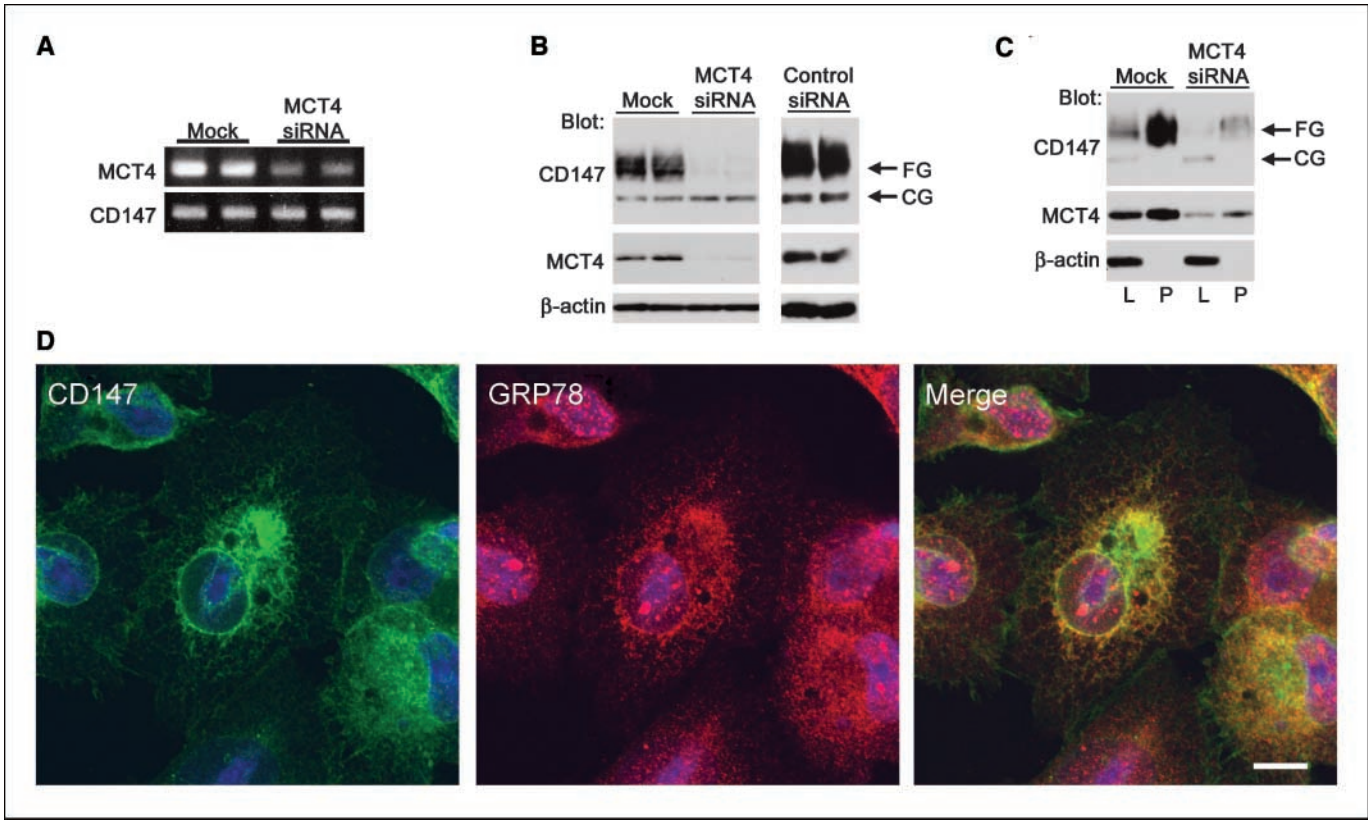

Figure 4. MCT4 silencing in MDA-MB-231 cells leads to a reduction of the fully glycosylated form of CD147 and accumulation of CD147 in the ER. *A,* MCT4 siRNA effectively reduces expression of MCT4 mRNA and has no effect on CD147 mRNA levels in MDA-MB-231 cells. Duplicate samples of each condition. *B,* silencing of MCT4 leads to reduction in the expression of fully glycosylated but not the core-glycosylated form of CD147. Lysates were analyzed by immunoblot using antibodies against MCT4, CD147, and β-actin (as an internal control). Cells were transfected with MCT5 siRNA as a control (*Control siRNA*) because MDA-MB-231 cells do not express this MCT isoform. Total protein (5 μg) was loaded in each lane. Duplicate samples of each condition. *C,* the core-glycosylated form of CD147 is not expressed at the cell surface following silencing of MCT4. Unbound lysates and streptavidin-agarose precipitates were analyzed by immunoblot using antibodies against CD147, MCT4, and β-actin (as an internal control). Unbound lysate (5 μg) was loaded in L lanes. Pellet fraction (20 μL) was loaded in the P lanes. *L,* lysate; *P,* pellet. *D,* CD147 accumulates intracellularly in the ER when cells are treated with MCT4 siRNA. Cells were immunolabeled with CD147 (*green*) or GRP78 (*red*) antibodies. TO-PRO-3 iodide (*blue*) was used to label nuclei. *Merge,* colocalization of CD147 with the ER marker GRP78, suggesting accumulation of CD147 in this organelle. *Bar,* 20 μm.

In cells treated with MCT4 siRNA, the maturation of CD147 was blocked as evidenced by the loss of fully glycosylated CD147 and increased core-glycosylated CD147 protein (Fig. 4*B*). Transfection of cells with MCT5 siRNA, a gene that is not expressed in these cells, resulted in no observable differences in protein expression relative to mock-transfected cells (Fig. 4*B, Control siRNA*). Because core-glycosylated CD147 persisted in cells transfected with MCT4 siRNA, we used cell surface biotinylation to evaluate whether this form of CD147 was trafficked to the plasma membrane. The core-glycosylated form of CD147 was not biotinylated, indicating that it is not expressed at the cell surface (Fig. 4*C*).

Next, we examined the subcellular distribution of CD147 in MDA-MB-231 cells treated with MCT4 siRNA by immunofluorescence confocal microscopy. CD147 was detected in the ER, as determined by colocalization with the ER marker GRP78, but was not detected in the plasma membrane (Fig. 4*D*). Interestingly, we also noted a decrease in the number of blebs present on the surface of cells transfected with MCT4 siRNA (Fig. 4*D*) compared with untreated cells (Fig. 2*A*).

Silencing CD147 results in degradation of MCT4. siRNA was used to evaluate the effect of CD147 silencing on the subcellular distribution of MCT4 in MDA-MB-231 cells. CD147 siRNA efficiently silenced CD147 mRNA, whereas no change was observed in MCT4 mRNA levels compared with mock-transfected cells (Fig. 5*A*) or cells transfected with the control siRNA (data not shown). Immunoblot analysis revealed a decreased level of expression of both core-glycosylated and fully glycosylated CD147 as well as MCT4 in cells treated with CD147 siRNA compared with controls (Fig. 5*B*). There was no significant reduction in expression of the amino acid transporter CD98, caveolin-1, or β_1-integrin, all of which have been shown to interact with CD147 at the plasma membrane (data not shown; refs. 26, 27). Using immunofluorescence confocal microscopy, we observed a loss of expression of MCT4 and CD147 in the plasma membrane in cells treated with CD147 siRNA (Fig. 5*C*; data not shown). MCT4 colocalized with the endosomal marker LAMP-1, suggesting that absence of CD147 resulted in degradation of MCT4 (Fig. 5*C, bottom, Merge*).

To determine if the increased expression of MCTs and their role in regulating CD147 expression was unique to MDA-MB-231 cells, we evaluated the expression of these proteins in four additional cancer cell lines, MCF-7 (nonmetastatic breast cancer), HeLa (cervical cancer), and HL-60 and K562 (leukemias). We found that the nonmetastatic MCF-7 cell line expressed low levels of CD147 and MCTs (Supplementary Fig. S1*A*). The ARPE-19 cell line, derived from human RPE, was used as a positive control because

the RPE expresses CD147 at a higher level than all other cell types (Supplementary Fig. S1*A*). Investigation of CD147 and MCT expression in the additional cell lines revealed patterns similar to that seen in MDA-MB-231 cells. HeLa cells exhibited high levels of expression of CD147 and MCT4 as well as some MCT1. Furthermore, when MCT1 or MCT4 were silenced in these cells using siRNA, a marked decrease in CD147 expression was also observed (Supplementary Fig. S1*B*). Similar results were obtained for the HL-60 and K562 cell lines, which express MCT4 and MCT1, respectively (Supplementary Fig. S1*C* and *D*). These results are consistent with the present findings with MCT4 as well as previous work with CD147 (14, 15, 28–30), showing that MCTs regulate the maturation of CD147.

Silencing MCT4 leads to decreased cell migration. A Transwell migration assay was used to evaluate the effect of silencing MCT4 on chemotaxis of MDA-MB-231 cells. Cells transfected with control (MCT5) siRNA or MCT4 siRNA were seeded onto Transwell inserts as described in Materials and Methods. Silencing of MCT4 resulted in a significant decrease (~85%) in cell migration (Fig. 6).

Discussion

In the present studies, we examined whether the high rate of glucose uptake and metabolism to lactate in metastatic breast cancer cells was accompanied by an increase in expression of lactate transporters. We found that MCT1 was the predominant isoform expressed in cells derived from normal mammary tissue, whereas MCT4 was more abundant in MDA-MB-231 cells (Fig. 1). MCT1 is widely expressed, whereas MCT4 is primarily found in cells or tissues reliant on glycolysis for the generation of ATP. The increased expression of MCT4 in MDA-MB-231 cells suggests that it is up-regulated with other genes encoding glycolytic transporters, such as *GLUT1*, and provides a mechanism for transporting excess lactate out of cells.

It has been shown both *in vitro* and *in vivo* that MCTs 1, 3, and 4 require an accessory protein, CD147, for trafficking to the cell surface (9, 11, 17, 19). Our results show that this is also true in cancer cells since knocking down CD147 expression in MDA-MB-231 cells resulted in a loss of cell surface expression of MCT4. In the absence of CD147, MCT4 is targeted to the endolysosomal compartment. CD147 has been reported to interact with several transmembrane and cytoplasmic proteins at the cell surface, including the chaperone proteins caveolin-1 and cyclophilin-60, as well as transport and adhesion proteins, such as CD98 and β_1-integrin (26, 27, 31, 32). Nonetheless, we found that the level of expression of these proteins was not changed when CD147 was silenced, indicating that CD147 does not directly regulate their expression and subcellular distribution (data not shown).

Although CD147 is constitutively expressed in most cell types, the mechanism regulating its enhanced cell surface expression has not

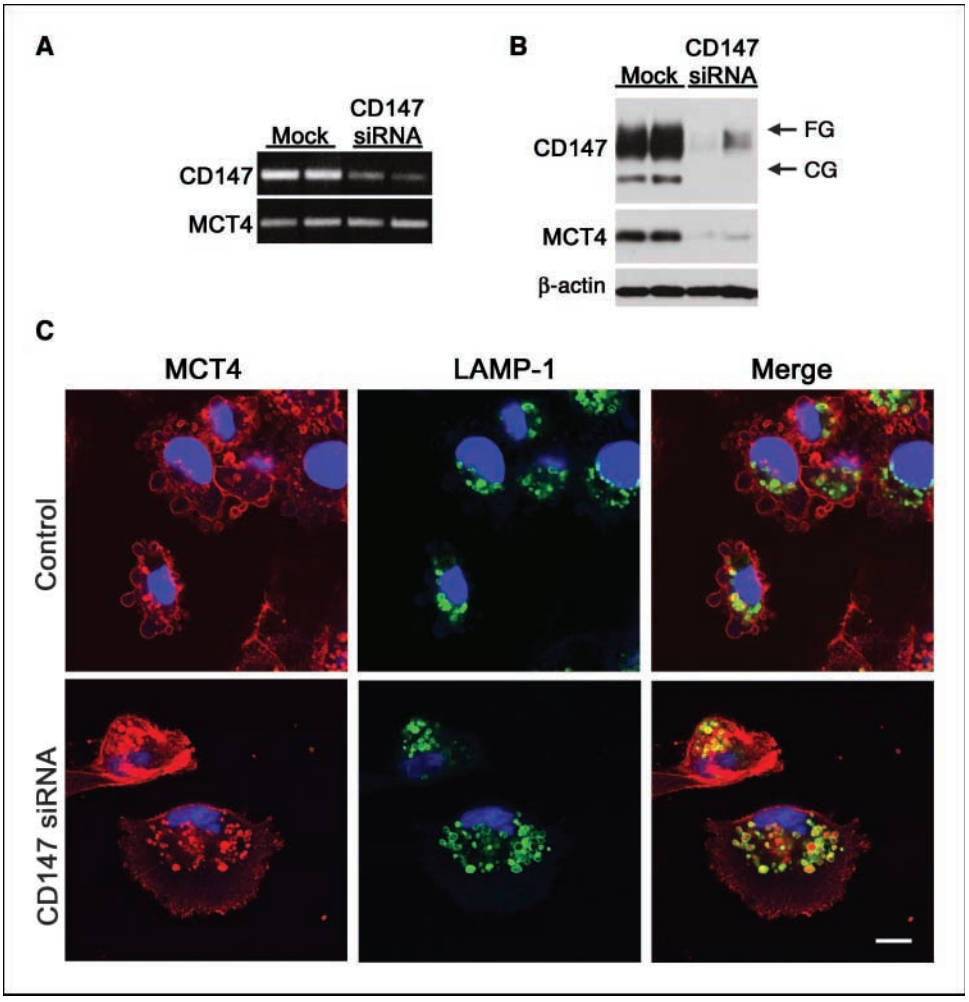

Figure 5. Silencing of CD147 in MDA-MB-231 cells leads to intracellular accumulation and decreased expression of MCT4. *A,* CD147 siRNA effectively decreases levels of CD147 mRNA without affecting MCT4 mRNA levels. Duplicate samples for each condition. *B,* CD147 siRNA leads to reduction of both MCT4 and CD147 protein expression levels in MDA-MB-231 cells. Immunoblots of cell lysates were probed with antibodies against CD147, MCT4, and β-actin (as an internal control). Total protein (5 μg) was loaded in each lane. Duplicate samples of each condition. *C,* treatment of MDA-MB-231 cells with CD147 siRNA results in the accumulation of MCT4 in the endolysosomal compartment. Cells were immunolabeled with MCT4 (*red*) and LAMP-1 (*green*) antibodies and imaged by confocal laser scanning microscopy. TO-PRO-3 iodide (*blue*) was used to label nuclei. *Top,* localization of MCT4 and LAMP-1 in cells transfected with control siRNA; *bottom,* localization of these proteins in cells transfected with CD147 siRNA. *Bar*, 20 μm.

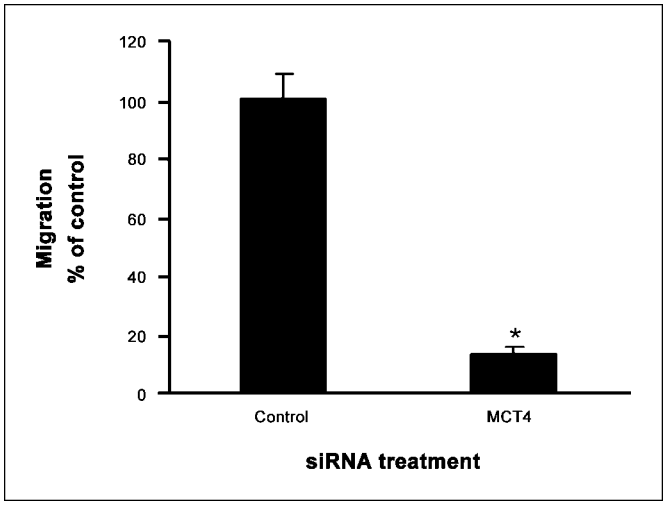

Figure 6. Silencing of MCT4 results in decreased migration of MDA-MB-231 cells *in vitro*. MDA-MB-231 cells were seeded onto Transwell filters following transfection with either control (MCT5) siRNA or MCT4 siRNA. Data are representative of two independent experiments. *, $P < 0.05$, Student's *t* test.

been elucidated. Therefore, we investigated if the abundance of CD147 in the plasma membrane could be controlled by silencing or overexpressing MCT4. We found that silencing MCTs in MDA-MB-231, as well as HeLa, HL-60, and K562 cells resulted in a loss of expression of the fully glycosylated CD147; however, the level of expression of the core-glycosylated protein and mRNA was unchanged (Fig. 3; Supplementary Fig. S1*B–D*). In addition, we found by confocal immunomicroscopy of MDA-MB-231 cells that, in the absence of MCT4, CD147 remained in the ER. These data therefore suggest that, in the absence of MCTs, CD147 is synthesized but not trafficked out of the ER.

To determine whether up-regulation of MCT expression could increase the abundance of the mature MCT/CD147 transporter complex in the plasma membrane, we stably expressed MCT4 in HEK293 cells. Cell surface biotinylation showed that overexpression of MCT4 increased the abundance of the fully glycosylated protein in the plasma membrane. The results of these studies show that up-regulation of MCT4 with other glycolytic genes would result in increased CD147 trafficking to the plasma membrane. Overexpression of MCT4 did not affect cellular levels of MCT1, suggesting that CD147 is constitutively expressed. Consistent with previous work from our laboratory (33), these studies show that it is expression of MCTs that regulates the abundance of the mature transporter complex. This interpretation is further supported by our findings showing that stable expression of CD147 in HEK293 cells did not increase the level of MCT1. Similar results have been reported for regulation of expression of the heteromeric neutral amino acid transporter CD98/LAT (34). Taken together, these results show that overexpression of the transporter, but not the accessory protein, can increase expression of the functional MCT/CD147 transporter complex.

In tumor cells, glycolysis provides the energy required for cell migration (35). Glycolytic enzymes are known to bind to the actin cytoskeleton and are found in pseudopodia purified from cancer cells. Inhibition of glycolysis can block cell migration by preventing phosphorylation of proteins which are required for the formation and protrusion of lamellipodia (36, 37). In the present study, we found that silencing of MCT4 in MDA-MB-231 cells impaired their migration. Because lactate is cotransported out of the cell with a proton, silencing MCT4 would be expected to decrease intracellular pH and increase extracellular pH. Studies have shown that integrin-mediated cell migration is pH sensitive and is inhibited by alkanization of the extracellular environment (37, 38). Therefore, functional MCTs are absolutely crucial to cancers because lactate efflux at the leading edge of tumor cells not only ensures continued ATP production required for survival but also serves to acidify the surrounding microenvironment, enhancing cell migration (39, 40).

Two transcription factors, HIF-1 and c-*MYC*, have been shown to increase the transcription of glycolytic genes in cancer cells (41–44). Recent work has shown that MCT4 mRNA and protein are increased in response to hypoxia and hormonal stimulation via HIF-1α–induced gene expression (42, 45, 46). *CD147* was not among the genes reported to be induced by HIF-1, supporting our hypothesis that enhanced MCT expression regulates the increase in CD147.

In sum, these studies provide a molecular explanation for the increased expression of CD147 in the metastatic cancer cell line MDA-MB-231. Our results show that surface expression and trafficking of CD147 is dependent on its association with MCTs. In addition, we have found that, in the absence of MCT4, cell migration is considerably impaired *in vitro*. These data suggest that MCTs and CD147 function synergistically to enhance metastatic potential through acidification of the tumor microenvironment and degradation of extracellular matrix via lactate efflux and induction of MMPs. Elucidating this link between two hallmarks of metastatic cancer, the glycolytic switch and elevated CD147 expression, has implications for designing new anticancer therapeutics.

Acknowledgments

Received 8/28/2006; revised 2/14/2007; accepted 2/21/2007.

Grant support: NIH grant EY012042 (N.J. Philp) and National Institute on Alcohol Abuse and Alcoholism training grant AA07463 (S.M. Gallagher).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Diane Durante for her technical assistance and Dr. Ami Deora for the MCT4-myc construct used to generate the overexpressing HEK293 cells.

References

1. Plas DR, Thompson CB. Akt-dependent transformation: there is more to growth than just surviving. Oncogene 2005;24:7435–42.
2. Semenza GL, Artemov D, Bedi A, et al. 'The metabolism of tumours': 70 years later. Novartis Found Symp 2001;240:251–60; discussion 260–4.
3. Warburg O. On respiratory impairment in cancer cells. Science 1956;124:269–70.
4. Czernin J, Phelps ME. Positron emission tomography scanning: current and future applications. Annu Rev Med 2002;53:89–112.
5. Halestrap AP, Meredith D. The SLC16 gene family—from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. Pflugers Arch 2004;447:619–28.
6. Enerson BE, Drewes LR. Molecular features, regulation, and function of monocarboxylate transporters: implications for drug delivery. J Pharm Sci 2003;92:1531–44.
7. Fang J, Quinones QJ, Holman TL, et al. The H^+-linked monocarboxylate transporter (MCT1/SLC16A1): a potential therapeutic target for high-risk neuroblastoma. Mol Pharmacol 2006;70:2108–15.
8. Koukourakis MI, Giatromanolaki A, Harris AL, Sivridis E. Comparison of metabolic pathways between cancer cells and stromal cells in colorectal carcinomas: a metabolic survival role for tumor-associated stroma. Cancer Res 2006;66:632–7.
9. Philp NJ, Ochrietor JD, Rudoy C, Muramatsu T, Linser

PJ. Loss of MCT1, MCT3, and MCT4 expression in the retinal pigment epithelium and neural retina of the 5A11/basigin-null mouse. Invest Ophthalmol Vis Sci 2003;44:1305–11.
10. Dimmer KS, Friedrich B, Lang F, Deitmer JW, Broer S. The low-affinity monocarboxylate transporter MCT4 is adapted to the export of lactate in highly glycolytic cells. Biochem J 2000;350 Pt 1:219–27.
11. Kirk P, Wilson MC, Heddle C, Brown MH, Barclay AN, Halestrap AP. CD147 is tightly associated with lactate transporters MCT1 and MCT4 and facilitates their cell surface expression. EMBO J 2000;19:3896–904.
12. Halestrap AP, Price NT. The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. Biochem J 1999;343 Pt 2:281–99.
13. Kim JW, Dang CV. Multifaceted roles of glycolytic enzymes. Trends Biochem Sci 2005;30:142–50.
14. Biswas C, Zhang Y, DeCastro R, et al. The human tumor cell-derived collagenase stimulatory factor (renamed EMMPRIN) is a member of the immunoglobulin superfamily. Cancer Res 1995;55:434–9.
15. Yan L, Zucker S, Toole BP. Roles of the multifunctional glycoprotein, emmprin (basigin; CD147), in tumour progression. Thromb Haemost 2005;93:199–204.
16. Tang Y, Kesavan P, Nakada MT, Yan L. Tumor-stroma interaction: positive feedback regulation of extracellular matrix metalloproteinase inducer (EMMPRIN) expression and matrix metalloproteinase-dependent generation of soluble EMMPRIN. Mol Cancer Res 2004;2:73–80.
17. Deora AA, Philp N, Hu J, Bok D, Rodriguez-Boulan E. Mechanisms regulating tissue-specific polarity of monocarboxylate transporters and their chaperone CD147 in kidney and retinal epithelia. Proc Natl Acad Sci U S A 2005;102:16245–50.
18. Wilson MC, Meredith D, Fox JE, Manoharan C, Davies AJ, Halestrap AP. Basigin (CD147) is the target for organomercurial inhibition of monocarboxylate transporter isoforms 1 and 4: the ancillary protein for the insensitive MCT2 is EMBIGIN (gp70). J Biol Chem 2005; 280:27213–21.
19. Wilson MC, Meredith D, Halestrap AP. Fluorescence resonance energy transfer studies on the interaction between the lactate transporter MCT1 and CD147 provide information on the topology and stoichiometry of the complex *in situ*. J Biol Chem 2002;277:3666–72.
20. Philp NJ, Wang D, Yoon H, Hjelmeland LM. Polarized expression of monocarboxylate transporters in human retinal pigment epithelium and ARPE-19 cells. Invest Ophthalmol Vis Sci 2003;44:1716–21.
21. Sidhu SS, Mengistab AT, Tauscher AN, LaVail J, Basbaum C. The microvesicle as a vehicle for EMMPRIN in tumor-stromal interactions. Oncogene 2004;23:956–63.
22. Dolo V, Ginestra A, Ghersi G, Nagase H, Vittorelli MI. Human breast carcinoma cells cultured in the presence of serum shed membrane vesicles rich in gelatinolytic activities. J Submicrosc Cytol Pathol 1994;26:173–80.
23. Gatenby RA, Gillies RJ. Why do cancers have high aerobic glycolysis? Nat Rev Cancer 2004;4:891–9.
24. Dolo V, D'Ascenzo S, Violini S, et al. Matrix-degrading proteinases are shed in membrane vesicles by ovarian cancer cells *in vivo* and *in vitro*. Clin Exp Metastasis 1999;17:131–40.
25. Ginestra A, La Placa MD, Saladino F, Cassara D, Nagase H, Vittorelli ML. The amount and proteolytic content of vesicles shed by human cancer cell lines correlates with their *in vitro* invasiveness. Anticancer Res 1998;18:3433–7.
26. Xu D, Hemler ME. Metabolic activation-related CD147-CD98 complex. Mol Cell Proteomics 2005;4: 1061–71.
27. Cho JY, Fox DA, Horejsi V, et al. The functional interactions between CD98, β_1-integrins, and CD147 in the induction of U937 homotypic aggregation. Blood 2001;98:374–82.
28. Riethdorf S, Reimers N, Assmann V, et al. High incidence of EMMPRIN expression in human tumors. Int J Cancer 2006;119:1800–10.
29. Nabeshima K, Iwasaki H, Koga K, Hojo H, Suzumiya J, Kikuchi M. Emmprin (basigin/CD147): matrix metalloproteinase modulator and multifunctional cell recognition molecule that plays a critical role in cancer progression. Pathol Int 2006;56:359–67.
30. Zucker S, Hymowitz M, Rollo EE, et al. Tumorigenic potential of extracellular matrix metalloproteinase inducer. Am J Pathol 2001;158:1921–8.
31. Pushkarsky T, Yurchenko V, Vanpouille C, et al. Cell surface expression of CD147/EMMPRIN is regulated by cyclophilin 60. J Biol Chem 2005;280:27866–71.
32. Tang W, Chang SB, Hemler ME. Links between CD147 function, glycosylation, and caveolin-1. Mol Biol Cell 2004;15:4043–50.
33. Fanelli A, Grollman EF, Wang D, Philp NJ. MCT1 and its accessory protein CD147 are differentially regulated by TSH in rat thyroid cells. Am J Physiol Endocrinol Metab 2003;285:E1223–9.
34. Padbury JF, Diah SK, McGonnigal B, et al. Transcriptional regulation of the LAT-1/CD98 light chain. Biochem Biophys Res Commun 2004;318:529–34.
35. Beckner ME, Stracke ML, Liotta LA, Schiffmann E. Glycolysis as primary energy source in tumor cell chemotaxis. J Natl Cancer Inst 1990;82:1836–40.
36. Nguyen TN, Wang HJ, Zalzal S, Nanci A, Nabi IR. Purification and characterization of β-actin-rich tumor cell pseudopodia: role of glycolysis. Exp Cell Res 2000; 258:171–83.
37. Betapudi V, Licate LS, Egelhoff TT. Distinct roles of nonmuscle myosin II isoforms in the regulation of MDA-MB-231 breast cancer cell spreading and migration. Cancer Res 2006;66:4725–33.
38. Stock C, Gassner B, Hauck CR, et al. Migration of human melanoma cells depends on extracellular pH and Na+/H+ exchange. J Physiol 2005;567:225–38.
39. Fantin VR, St-Pierre J, Leder P. Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. Cancer Cell 2006;9:425–34.
40. Udabage L, Brownlee GR, Waltham M, et al. Antisense-mediated suppression of hyaluronan synthase 2 inhibits the tumorigenesis and progression of breast cancer. Cancer Res 2005;65:6139–50.
41. Hochachka PW, Rupert JL, Goldenberg L, Gleave M, Kozlowski P. Going malignant: the hypoxia-cancer connection in the prostate. Bioessays 2002;24:749–57.
42. Ullah MS, Davies AJ, Halestrap AP. The plasma membrane lactate transporter MCT4, but not MCT1, is up-regulated by hypoxia through a HIF-1α-dependent mechanism. J Biol Chem 2006;281:9030–7.
43. Jamerson MH, Johnson MD, Dickson RB. Of mice and myc: c-Myc and mammary tumorigenesis. J Mammary Gland Biol Neoplasia 2004;9:27–37.
44. Liao DJ, Dickson RB. c-myc in breast cancer. Endocr Relat Cancer 2000;7:143–64.
45. Moeller LC, Dumitrescu AM, Refetoff S. Cytosolic action of thyroid hormone leads to induction of hypoxia-inducible factor-1α and glycolytic genes. Mol Endocrinol 2005;19:2955–63.
46. Hausmann S, Claus R, Walzel H. Short-term culture of surface-biotinylated cells: application in non-radioactive analysis of surface protein shedding. Immunol Lett 1995; 48:175–80.

Monocarboxylate Transporter 4 Regulates Maturation and Trafficking of CD147 to the Plasma Membrane in the Metastatic Breast Cancer Cell Line MDA-MB-231

Shannon M. Gallagher, John J. Castorino, Dian Wang, et al.

Cancer Res 2007;67:4182-4189.