Synergistic Therapeutic Effect of Cisplatin and Phosphatidylinositol 3-Kinase (PI3K) Inhibitors in Cancer Growth and Metastasis of Brca1 Mutant Tumors*

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Background: Metastasis is a serious problem that claims the lives of breast cancer patients. Results: Rapamycin and cisplatin synergistically inhibit CSC-mediated primary and metastatic cancer growth by blocking mTOR signaling and cytoskeletal remodeling. Conclusion: Cancer stem cells are involved in both primary and metastatic cancer growth of Brca1 tumors through distinct signaling pathways. Significance: Targeting cancer stem cell-specific pathways may reveal new therapeutic strategies.

Drug resistance and cancer metastasis are two major problems in cancer research. During a course of therapeutic treatment in Brca1-associated tumors, we found that breast cancer stem cells (CSCs) exhibit an intrinsic ability to metastasize and acquire drug resistance through distinct signaling pathways. Microarray analysis indicated that the cytoskeletal remodeling pathway was differentially regulated in CSCs, and this was further evidenced by the inhibitory role of reagents that impair this pathway in the motility of cancer cells. We showed that cisplatin treatment, although initially inhibiting cancer growth, preventing metastasis through blocking cytoskeletal remodeling, and retarding CSC motility, eventually led to drug resistance associated with a marked increase in the number of CSCs. This event was at least partially attributed to the activation of PI3K signaling, and it could be significantly inhibited by co-treatment with rapamycin. These results provide strong evidence that cytoskeletal rearrangement and PI3K/AKT signaling play distinct roles in mediating CSC mobility and viability, respectively, and blocking both pathways synergistically may inhibit primary and metastatic cancer growth.

Breast cancer is the most common malignancy among women as revealed by the Surveillance, Epidemiology and End Results Program, NCI, National Institutes of Health, which reports that 1 in 8 women born in the United States will be diagnosed with breast cancer at some time in their lives. Despite the progress in prevention, detection, and adjuvant therapy of breast cancer, metastasis remains the main cause of death, and metastatic breast cancer is typically incurable (1). Metastasis is a multistep process composed of invasion through the basement membrane barrier and extracellular matrix (ECM), entry of tumor cells into the circulation (intravasation), survival within the vasculature, extravasation into distant tissues, and finally the establishment and growth of secondary tumors (2, 3). These processes rely on the coordinated spatial-temporal expression of various genes and commensurate protein products, which allows tumor cells to successfully metastasize to distant tissues. Despite efforts to elucidate the mechanisms that control the metastatic process, the precise molecular circuitry that governs this process remains largely unknown, and this reflects the high rate of metastatic breast cancer reoccurrence over the years.

Triple-negative breast cancers (negative for estrogen, progesterone receptors, and epidermal growth factor receptor 2 (HER2)) account for about 15–20% of all breast cancers and represent a heterogeneous group of invasive breast cancers with a relatively poorer prognosis than the major breast cancer subtypes (4). The majority of familial breast cancers associated with mutations in breast cancer-associated gene 1 (BRCA1) belong to this category (5). Hereditary breast cancers account for 5–10% of all breast cancers, and of these, BRCA1 mutations are found in half of these cases. In contrast to estrogen-positive or HER2-positive breast tumors, currently there is no targeted therapy for triple-negative breast cancer. Thus, conditional Brca1 mouse models that develop mammary tumors with a strong resemblance to human BRCA1-mutated breast tumors can be very helpful in predicting response and allowing for the discovery of new therapeutic strategies for this highly aggressive subclass of breast tumors (6).

The failure to target the subpopulation of cells with the highest migration ability contributes to the ineffectiveness of current treatments for metastatic tumors. Growing evidence indicates that certain types of cancer are stem cell diseases (7–9).

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‡ The abbreviations used are: ECM, extracellular matrix; CSC, cancer stem cell; mTOR, mammalian target of rapamycin; ERM, ezrin, radixin, and moesin; S6RP, S6 ribosomal protein; BMI, Bio-Macrolide I.
The cancer stem cell theory predicts that not all cancer cells within a tumor exhibit the same tumor-growing ability, and it is only a rare population of cells with stem cell properties that drives tumor growth. This concept may have implications in the metastatic process as well, suggesting that not all cells possess the same metastatic potential, and cancer stem cells (CSCs) may share characteristics necessary to induce both tumorigenesis and metastasis. The first evidence of a cancer stem cell origin for breast cancer was reported in 2003 (7). Interestingly, in this study CSCs were initially derived mainly from metastases implying that both tumor growth and metastasis originate from CSCs. Furthermore, data from more recent studies also showed that CSCs from breast tumors possess an "invasiveness" gene signature associated with the active epithelial-mesenchymal transition program and exhibit higher metastatic potential than non-CSCs (10–13). Emerging evidence also indicates that CSCs are more resistant to irradiation and treatment with therapeutic drugs (11, 12, 14), although the underlying mechanism for both cancer metastasis and drug resistance mediated by CSCs is largely unknown.

During a course of therapeutic treatment in Brca1 mutant mammary tumors, we found that cisplatin is the most effective among the tested drugs in terms of slowing down tumor growth. However, our data also revealed that tumors gradually developed drug resistance accompanied by an increase in the population of CSCs and an unexpected decrease in primary tumor metastasis. We further demonstrated that this decrease is primarily due to the inhibitory role of cisplatin in cytoskeletal remodeling-mediated migration of CSCs, whereas treatment with both cisplatin and rapamycin effectively blocked cancer cell survival and metastasis.

**MATERIALS AND METHODS**

**Animals**—Mice carrying Cre-LoxP-mediated mammary-specific deletion of the full-length form of Brca1 and a heterozygous null mutation of p53 (Brca1<sup>C0/C0</sup>p53±;MMTV-Cre) were generated as described earlier (15) and maintained in a pathogen-free facility. The protocol for animal studies was approved by the Animal Care and Use Committee of the NIDDK.

**Drug Treatment**—Cells after trypsinization or single cells from primary tumors were resuspended in PBS, which then were mixed at a 1:1 ratio with Matrigel (BD Biosciences) on ice. For drug treatment experiments, 10<sup>6</sup> cells from primary mammary tumors were implanted into the fourth mammary fat pad of female immunocompromised mice. When the tumors became palpable, the recipient mice were treated with the following: ICI182,780 (Tocris, 5 mg/mouse was injected subcutaneously, once a week); U0126 (Cell Signaling Technology, 10 mg/kg, i.p. injection, every other day); mifepristone (RU-486) (made into a pellet, 35 mg/pellet, 60-day release, and 1 pellet was implanted underneath the skin at the back neck); rapamycin (Chempacific, 4 mg/kg, i.p. injection, every other day), or cisplatin (Sigma, 6 mg/kg, i.p. injection, twice a week). Tumor volumes were monitored daily during the first 60 days and then every other day. Tumor size was measured with a caliper when visible nodules were present. Tumor volume was calculated in mm<sup>3</sup> by using Equation 1,

\[ V = \frac{1}{2} r_x r_y^2 \]  

(Eq. 1)

where \( r \) is radius and \( x \) and \( y \) refer to each axis. For checking metastasis in vivo, at the end of the experiment animals were sacrificed, and multiple tissues were removed and examined for the presence of metastatic tumors with a Leica MZ10F stereomicroscope.

**Migration Assays**—Cells were grown to confluence either in 35-mm wells or 4-well chamber slides. Wounds were generated by scratching using a 200-μl pipette tip, and cells were washed three times with PBS followed by addition of fresh medium. After 24 h, new pictures were taken, and migration was analyzed by checking the maximum distance of the cell front into the wound edge. For experiments where different compounds were tested, wounds were scratched, and cells were allowed to migrate in the presence of the medium supplemented with the tested compound. Triplicate wells were used per condition. For transwell assays, cells were starved for 24 h and, after trypsinization, were plated in the top chambers of 8-μm pore transwells (BD Biosciences) in serum-free medium in 6-well plates (1.5 × 10<sup>5</sup> cells/well). Cells were allowed to migrate toward regular medium (DMEM, 10% FBS) over a period of 24 h. At the end of the assay, the cells at the top chamber were removed, and the cells at the bottom of the filter were fixed with 100% methanol for 20 min and stained with hematoxylin solution for 10 min. Then membranes were removed, placed in slides, coverslipped, and examined immediately using a Leica DMR microscope. Alternatively, after cell migration for 24 h, transwells were removed, and cells that migrated to the lower chamber were allowed to attach and form colonies. After 5–6 days, colonies were fixed with 100% methanol for 20 min and stained with hematoxylin solution for 10 min. Plates were checked for the presence of colonies with a Leica MZ10F stereomicroscope, and pictures were captured using an Olympus camera. When GFP-labeled cells were used for the migration assays, the presence of migrating cells was checked under a Leica DMR microscope. For real time monitoring of cellular migration, the xCELLigence Real Time Cell Analyzer (RTCA) DP instrument was used (Roche Applied Science) according to the manufacturer’s instructions. Cell migration was dynamically recorded and analyzed by using the RTCA software 1.2. Triplicate wells were used per condition in all experiments.

**Histology and Immunohistochemical Staining**—For histology, tissues were fixed in 10% formalin, blocked in paraffin, sectioned, stained with hematoxylin and eosin, and examined by light microscopy (Leica DMR microscope). For immunohistochemistry, a histostain staining kit (Zymed Laboratories Inc.) was used according to the manufacturer’s instructions.

**Western Blot**—For Western blotting, membranes were blocked with either 5% nonfat dried milk in PBS or 5% BSA in TBST and then incubated with primary antibodies against phosphorylated ERM, ERM, p-AKT(Ser-473), pan-AKT, p-S6RP(Ser-235/236), S6RP, p-4EBP1(T70), 4EBP1 (Cell Signaling), laminin, collagen IV (Abcam), β-actin, and α-tubulin (Sigma). Horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) were used, and protein was visualized using enhanced Immobilon Western Chemiluminescent HRP substrate (Millipore). Specifically for detection of phosphory-
lated and total levels of ERM, cells were lysed at 4 °C in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA) supplemented with complete protease inhibitor mixture (Roche Applied Science) and phosphatase inhibitor mixture (Pierce).

**Cell Labeling, Flow Cytometry, and Sorting**—For fluorescence-activated cell sorting (FACS) analysis, cells were stained at a concentration of 1 × 10^6 cells per 100 μl of buffer (PBS, pH 7.2, 0.5% BSA, 2 mM EDTA) with antibodies against CD24 (anti CD24-PE, Pharmingen), CD29 (anti CD29-FITC, Chemicon), CD49f (anti-CD49f-FITC, Pharmingen), and CD44 (anti CD44-APC, Pharmingen). After incubation at 4 °C for 25 min, analysis was done using the FACSCalibur flow cytometer (BD Biosciences). For sorting out different cell populations, the same procedure was followed, and sorting was carried out using a FACS Aria Cell Sorter (BD Biosciences).

**RNA Isolation and RT-PCR**—Total RNA was isolated from cells with STAT-60 following the manufacturer’s protocol (Tel-Test, Friendswood, TX). Complementary DNA was synthesized with Cells-to-cDNAII (Ambion, Austin, TX). For real time PCR, SYBR® Green PCR master mix was used (Applied Biosystems), and data were acquired with a 7500 real time PCR system (Applied Biosystems).

**Microarray Analysis**—Total RNA was isolated from the sorted subpopulations based upon CD24 and CD29 expression using the mirVana™ miRNA isolation kit (Ambion), followed by mRNA amplification using a T7 global amplification method (two-cycle target labeling kit; Affymetrix), DNA fragmentation, biotinylation, and hybridization onto Affymetrix Mouse Genome 430A 2.0 array chips. The microarray signals were referred by the Affymetrix Robust Multichip Average algorithm. Up- and down-regulated genes were selected based on p values (<0.05) and fold changes (> 2 or < −2) assessed by analysis of variance with pro software from Partek. The microarray analysis was performed with three independent biological sample sets. The microarray data have been submitted to the GEO database under the accession number GSE26621. The statistically significantly expressed genes were used for the gene ontology analysis by the commercial gene pathway analysis web tool.

**Immunofluorescence**—Cells were grown, treated, fixed, and stained directly either in chamber slides, or on coverslips. Growth medium was removed, and cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Specifically for staining of ERM proteins, cells were double-stained with primary antibodies in 3% BSA in PBS at 4 °C. Cells were rinsed three times with PBS for 5 min each, and fluorescence-conjugated secondary antibodies in 3% BSA were added for 1 h at room temperature avoiding light exposure. After three washes with PBS for 5 min each, slides were cover-slipped with Prolong® gold antifade reagents (Invitrogen), and cells were examined immediately using a Leica DMR microscope. For long term storage, slides were kept at 4 °C protected from light. Antibodies used were against F-actin (phalloidin-rhodamine, Invitrogen), phosphorylated ERM (Cell Signaling), CD24 (BD Biosciences), and CD29 (Millipore).

For paraffin-embedded tissues, sections were first deparaffinized and hydrated and then placed in a water bath at 95–100 °C for 15 min for antigen retrieval by using citrate buffer (Thermo Scientific®). Slides were allowed to cool for 20 min followed by three washes with PBS for 5 min each. For permeabilization and reduction of unspecific fluorescence, 0.5% Triton X-100 was used for 5 min at 37 °C, and 0.5 mg/ml sodium borohydride was used for 10 min at room temperature, respectively. Blocking as well as primary and secondary antibody incubations were performed as described above. For immunofluorescent detection of apoptotic cells, the ApopTag® fluororescin in situ apoptosis detection kit was used according to the manufacturer’s instructions (Millipore).

**RESULTS**

Cisplatin Induces Drug Resistance That Is Associated with an Increase in the Cancer Stem Cell Subpopulation—To study the drug response of Brca1 mutant mammary tumors, we transplanted cells isolated from primary tumors of Brca1 mutant mice into nude mice and treated the recipient mice with several drugs that are commonly used for various forms of cancers, including ICI1182,780, U1026, mifepristone (RU-486), rapamycin, and cisplatin (16–19). Our data revealed variable therapeutic effects of these drugs with cisplatin exhibiting the best anti-tumor effect, followed by rapamycin, whereas the other three drugs only partially inhibited tumor growth during the same period of the treatment (Fig. IA).

Because of this initial observation that cisplatin treatment significantly inhibited tumor growth, we further studied its therapeutic effect by starting the treatment when tumors reached about 200 mm³ in volume. We found that although cancer cells were initially very sensitive to cisplatin, they regressed quickly and then reached about 200 mm³ 14 days later, when another dose of drug was injected (Fig. IB). However, after the 4th dose of drug injection, tumors gradually became refractory to treatment. Of note, the drug resistance was accompanied by a significant increase in the number of cells double-positive for CD24 and CD29 (CD24⁺CD29⁺), which are markers for mammary cancer-initiating cells (or CSCs) in mice (13, 20–22). In particular, there was a 3-fold increase in the percentage of CD24⁺CD29⁺ cells in the cisplatin-treated tumors compared with mock-treated tumors at the end of the experiment, when tumor size was comparable between the two groups of tumors (Fig. 1, B and C) (75.3 ± 15.2% versus 26.4 ± 9.6%). Of note, when the presence of metastatic nodules in the lung was checked, we found that mice treated with cisplatin had a significantly lower incidence of metastasis. Although all (10/10) control mice developed lung metastasis (Fig. 1D, left panels), none of the 10 cisplatin-treated mice had visible metastatic tumors upon dissection, although three mice carried small focal areas of tumor cells in the lung when examined by H&E (Fig. 1D, right panels). The finding that cisplatin treatment markedly increased CSC population is consistent with the current view that drug resistance can be attributed to the failure of specifically targeting CSCs (14, 22, 23).
However, the significant decrease in tumor metastasis despite the enrichment of CSCs in primary tumors was, at least on the surface, contradictory to the widely accepted observation that CSCs mediate cancer metastasis (10, 13, 24, 25). Based on this information, we hypothesized that cisplatin treatment must have altered some features of CSCs, thereby decreasing their ability to metastasize.

**Microarray Analysis Reveals the Importance of Cytoskeletal Remodeling in CSCs**—In our effort to explain this paradoxical finding, we tried to better analyze CSCs by identifying pathways that may contribute to the enhanced metastatic ability. Therefore, microarray analysis was performed by using both FACS-sorted CSCs and non-CSCs from three different primary mammary tumors from Brca1<sup>CW/CW</sup>;MMTV-Cre mice based upon expression of CD24 and CD29 as described earlier (15, 20). RNA was isolated from each of the subpopulations, and gene expression was compared between the Lin<sup>−</sup>CD24<sup>+</sup>CD29<sup>+</sup> (CSCs) and the Lin<sup>−</sup>CD24<sup>−</sup>CD29<sup>−</sup> (non-CSCs) subpopulations. Genes differentially expressed with both p < 0.05 and fold change with absolute value of ≥2.0 using analysis of variance were identified in this comparison. Among the 1427 genes identified, 973 were up-regulated (ranging from 2- to 35-fold), and 454 genes were down-regulated (from 2- to 26-fold) (Fig. 2A and GEO database under submission number GSE26621). Analysis of these genes revealed that cytoskeletal remodeling and cell adhesion were among the 10 top pathways identified as differentially expressed in the cancer stem cell subpopulation (Fig. 2B). It is known that alteration of cytoskeletal remodeling and cell adhesion can have a significant effect on cell movement (27). Considering our previous observation that CSCs mediate cancer metastasis (13), we next compared four pathways that are involved in cytoskeletal remodeling, cell-matrix interaction, cell adhesion, and cell migration to identify the genes that are shared in these pathways (Fig. 2C). We identified 10 genes that are commonly up-regulated in the CSCs, including fibronectin, collagen IV (Col1a1, Col1a2, Col4a3, Col4a4, Col4a5, and Col4a6), and laminin (Lama1, -b1, and -c1) (Fig. 2D). In accordance with the microarray data, we confirmed increased expression of these genes by qRT-PCR in sorted out CSCs (Fig. 2E), and higher levels for both collagen and laminin were confirmed by Western blotting in cell lines positive for the presence of a CSC subpopulation (Fig. 2F).

**Blocking Cytoskeletal Rearrangement Inhibits Cancer Cell Migration in Vitro**—Collagen IV, laminins, and fibronectin are major proteins of the ECM. Collagen IV forms the basement membrane, in which laminins play important functions, including cell differentiation, growth, migration, and adhesion, whereas fibronectin is a glycoprotein that connects membrane-
Targeting Breast Cancer Stem Cells as a Therapeutic Strategy

A

B

C

D

Gene Symbol | Gene Name
---|---
Col4a1 | Collagen IV a1
Col4a2 | Collagen IV a2
Col4a3 | Collagen IV a3
Col4a4 | Collagen IV a4
Col4a5 | Collagen IV a5
Col4a6 | Collagen IV a6
Fn1 | Fibronectin
Lama1 | Laminin a1
Lamb1 | Laminin b1
Lamc1 | Laminin c1

E

F

Relative Levels

CD24-CD29- | CD4+CD29+
---|---
Col4a3 | ** | *
Col4a4 | 3.5 | **
Col4a5 | 2.5 |*
Col4a6 | 3 | **
Col4a1 | 4 | **
Col4a2 | 3 | **
Fn1 | 1 | *
Lama1 | 1 | *
Lamb1 | 1 | *
Lamc1 | 1 | *

Normalized levels

** | CSCs-negative
* | CSCs-positive

Collagen IV | Tubulin
Laminin | Tubulin
bound integrins and ECM (28). Integrins are also connected to actin stress fibers and mediate cytoskeletal remodeling, and in this way, they are involved in cell migration and cancer metastasis (29, 30).

Because cytoskeletal remodeling is directly involved in cell motility and we have previously shown that integrins are involved in CSC-mediated migration (13), we were very interested in exploring whether this process could be targeted to block the metastatic ability of CSCs. We first showed that formation of filopodia (cytoplasmic projections enriched for F-actin) could be detected in migrating cells under a microscope (Fig. 3A), as well as in sorted-out CSCs (CD24+/CD29− cells) after staining with phalloidin and antibodies to actin-binding proteins, such as ERM (Fig. 3B). ERM proteins provide a link between the actin cytoskeleton and the cellular membrane because their activation of the C-terminal threonine upon phosphorylation plays a prominent role in the determination of cell shape and motility (31–33). To disrupt cytoskeletal rearrangement, we targeted ERM phosphorylation by treating W0069 cells either with an ERM peptide, which corresponds to the highly conserved region containing the phosphorylation site and competes with the endogenous ERM proteins for phosphorylation, or a control peptide consisting of the same residues in the reverse order (34). In a wound healing assay, there was reduced cell migration (Fig. 3C) and less phosphorylated (active) ERMs in the wound areas (Fig. 3D). Transwell assay experiments detected a significantly decreased number of colonies formed by migrated cells (Fig. 4A) in the presence of ERM peptide compared with control peptide-treated cells. The impaired cell migration was associated with inhibition of cytoskeletal remodeling as shown by the decrease in stress fiber formation in cells adjacent to the wound areas (Fig. 4B).

To further confirm the role of cytoskeletal remodeling in these cells, we used a synthetic macrolide analog of migrastatin (BMI), which has been shown to inhibit cell migration by affecting formation of actin-rich structures at the leading edge of migrating cells (35). Using the W0069 cell line that was derived from a mammary tumor of Brca1 mutant mice (15, 36), this compound was found very effective in preventing migration as revealed by the decrease in the number of migrated cells (Fig. 4C). The same results were obtained after examination of the migrated cells at the bottom surface of the membrane in the Boyden chamber assay when GFP-labeled W0069 cells were treated with either BMI or pERM peptide (Fig. 4D). These results indicate that cytoskeletal remodeling is required for cancer stem cell-mediated migration.

Cisplatin Prevents CSC-mediated Migration by Affecting Cytoskeletal Remodeling—After the observation that cytoskeletal remodeling is a characteristic of CSCs, we checked whether the paradox regarding the decreased metastatic rate in cisplatin-treated tumors highly enriched for CSCs could be explained through a possible inhibitory role of cisplatin on cytoskeletal remodeling. After treating cells with cisplatin, we noticed significantly decreased migration as revealed by transwell assay. Strikingly, fewer migrated cells were found both at the lower surface of the filter (Fig. 5A) and at the lower chamber when cells were treated with two different concentrations of cisplatin (2.5 and 5 μg/ml) compared with mock-treated control cells (Fig. 5B), indicating that cisplatin is very effective in blocking migration. When cells were placed at lower density, cisplatin reduced long protrusions compared with the control-treated cells (Fig. 5C). In addition, when control cells were stained with phallolidin, stress fibers in the migrated cells could be seen at the edge of the wound area, although this was not observed in the cisplatin-treated cells (Fig. 5D).

These observations indicate that the effect of cisplatin on cell migration is mediated, at least in part, through inhibition of cytoskeletal rearrangement. Of note, the effect on migration was not attributed to cell killing as there was no significant decrease in cell survival when cells were treated with the same concentrations of cisplatin used in the cell migration experiments (Fig. 5, E and F).

Next, we investigated whether the finding that cisplatin may inhibit CSCs migration could also be observed in human breast cancer cells. For this, we used MDA-MB-231 cells, which are human breast cancer cells highly enriched for CSCs (37–39). Our data indicated that migration was significantly decreased in the presence of cisplatin as revealed by the number of colonies formed by the migrated cells (Fig. 5G). A similar effect was observed when real-time monitoring of cellular migration was performed (Fig. 5H). In particular, there was a dose-dependent inhibition of migration when MDA-MB-231 cells were treated with increasing doses of cisplatin.

Cisplatin and Rapamycin Synergistically Inhibit Cancer Formation in Vivo—Because cancer cells were initially very sensitive to cisplatin treatment and eventually became drug-resistant, primarily due to survival of the CSCs subpopulation, we hypothesized that there should be activation of a survival signaling pathway associated with this response. Indeed, after checking our microarray data, we found that the expression of many genes involved in the PI3K pathway was up-regulated in CSCs versus non-CSCs (Fig. 6A). In addition, Brca1 mutant tumors exhibited increased expression of mTOR downstream signaling compared with the normal mammary gland as revealed by Western blotting of total and phosphorylated S6RP (Fig. 6B). Thus, it is conceivable that although cisplatin treatment inhibits metastasis by preventing cytoskeletal rearrangement, cancer stem cells may survive after long-term treatment and maintain their proliferation status due to the activation of the PI3K/PTEN/AKT/mTOR signaling axis. Consistent with

FIGURE 2. Microarray analysis of cancer stem cells versus noncancer stem cells from mouse Brca1 mutant mammary tumors. A volcano plot of the analysis of variance results is presented. The vertical lines show the cutoff value of 2-fold, and the horizontal line shows the cutoff p value of 0.05. The top right square shows the total number of up-regulated probe set IDs, and the top left square shows the down-regulated probe set IDs after comparing CSCs to non-CSCs isolated from Brca1 mutant primary mammary tumors. B. GeneGo pathway analysis from microarray data after comparing CSCs versus non-CSCs. C. Venn diagram comparing genes in four pathways that are involved in cytoskeletal remodeling, cell-matrix interaction, cell adhesion, and cell migration. D. List of 10 genes, which are commonly up-regulated in all four pathways. E and F. Levels of gene expression as revealed by real-time PCR in sorted out CD24+/CD29− cells (E) and levels of collagen IV (upper) and laminin (middle) as revealed by Western blotting in cell lines positive for CSCs (W0069, CD24+/CD29−, 210, and 215) compared with cell lines negative for CSCs (CD24+/CD29−, 202, and 207). Lower panel shows quantification for collagen IV and laminin levels in CSC-positive cell lines relative to CSC-negative cell lines using the GelQuant.NET program. *, p < 0.05; **, p < 0.001.

AUGUST 29, 2014•VOLUME 289•NUMBER 35 JOURNAL OF BIOLOGICAL CHEMISTRY 24207
this, we observed that cisplatin-resistant tumors were in an active proliferating state after staining both mock- and cisplatin-treated tumors with an antibody against the cell proliferation marker Ki67 (Fig. 6C). These observations reinforced our initial drug test (Fig. 1A) that treatment with rapamycin, a potent inhibitor of mTOR signaling, could significantly inhibit tumor growth. In such a case, we could predict that rapamycin treatment might help overcome the cisplatin-induced resistance of CSCs. To test this, we treated Brca1 mutant tumors with both rapamycin and cisplatin as well as a single drug alone. Our data indicated that the combined rapamycin and cisplatin treatment was able to significantly inhibit growth of the cisplatin-resistant tumors compared with single drug alone (Fig. 6D). Similar results were obtained when we estimated the time...

**FIGURE 3.** Cytoskeletal remodeling is required for migration in vitro. Wound-healing assay shows fillopodia in the migrating W0069 cells as revealed by microscopy (higher magnification is shown on the right) (A), and staining of CD24^+ CD29^- sorted out CSCs with phalloidin and phospho-ERM antibody is shown (B). C, representative example of the wound healing assay in cells treated either with control or phospho-ERM peptide. Quantification of the results from three independent experiments is presented on the right (*, p < 0.05). D, impairment of cytoskeletal remodeling by using the phospho-ERM peptide. Phospho-ERM peptide reduces ERM activation as revealed by staining with a phospho-ERM antibody in migrating cells (right). pERM fluorescence intensity (arbitrary units) is measured by using ImageJ software. Ctr, control.
Targeting Breast Cancer Stem Cells as a Therapeutic Strategy

A

B

C

D

ctr

ctr peptide 2uM

pERM peptide 2uM

No colonies

ctr ctr pep pERM pep 2 uM pERM pep 4 uM

BMI 2 uM

BMI 1 uM

BMI (uM)

ctr 0.5 1 2 4

No colonies

ctr BMI 2 uM pERM peptide 2 uM
Cytoskeletal remodeling is required for migration in vitro. A, transwell migration assay in untreated (ctr) and treated cells with either control or phospho-ERM peptide. Quantification of the colonies from three different experiments is shown on the right. Numbers represent average ± S.D. (*, p < 0.05). B, wound was made in chambers with Brca1 mutant cells either in the presence (left) or absence of cisplatin (right). After 24 h, cells were fixed and stained with rhodamine/phalloidin. Arrows indicate filopodia, and arrowheads show submembranous cortical actin in control migrating cells that are absent in treated cells (higher magnifications are shown on the right). C, Boyden chamber assay when GFP-labeled W0069 cells were treated with either BMI or phospho-ERM peptide. D, cisplatin blocks migration by affecting cytoskeletal rearrangement in CSCs. A and B, transwell migration assay in control or cisplatin-treated cells. Cells that have migrated at the bottom of the filter were stained, and characteristic pictures are shown (A). ctr, control. Quantification of both migrated cells at the bottom of the filter and colonies formed from the migrated cells from three different experiments (lower) are shown (B). Numbers represent average ± S.D. (**, p < 0.01). C, morphology of cisplatin-treated (right panel) and untreated cells (left panel). Arrows point to cytoplasmic protrusions. D, wound was made in chambers with Brca1 mutant cells either in the presence of control (left) or phospho-ERM (right) peptide. After 24 h, cells were fixed and stained with rhodamine/phalloidin. Arrows indicate filopodia, and arrowheads show submembranous cortical actin in control migrating cells that are absent in treated cells (higher magnifications are shown on the right).
Targeting Breast Cancer Stem Cells as a Therapeutic Strategy

AUGUST 29, 2014 • VOLUME 289 • NUMBER 35
JOURNAL OF BIOLOGICAL CHEMISTRY

24211
required for tumor cells to grow back after drug treatment. We found that this time was significantly delayed in tumors treated with the combination of cisplatin and rapamycin compared with cisplatin alone (Fig. 6E).

Molecular analysis indicated that cisplatin-resistant tumors showed significant activation of the mTOR pathway, as revealed by detecting phosphorylation of mTOR, S6RP, and 4EBP1 (Fig. 6F). Because these tumors were significantly enriched for CSCs (Fig. 1, B and C), the data imply that the mTOR axis functions as a major survival signaling pathway for CSCs. In agreement with this, the inhibitory effect of rapamycin on tumor growth was associated with a significant decrease in mTOR signaling in tumors treated with a combination of cisplatin and rapamycin compared with cisplatin alone (Fig. 6F). This effect correlated with decreased proliferation (Fig. 6C, right panel), as well as increased apoptotic cell death (Fig. 6G) in tumors treated with both cisplatin and rapamycin. These findings suggest that rapamycin-mediated impairment of mTOR signaling inhibits the growth of CSC-enriched cisplatin-resistant tumors, indicating that this pathway plays a critical role in maintaining CSC survival and tumor growth.

**DISCUSSION**

Growing evidence indicates that CSCs play an important role in metastasis of many types of solid cancers (10, 13, 24, 25, 40). To identify key features of CSCs mediating metastasis, we have performed microarray analysis between CSCs and non-CSCs. We detected altered expression of genes in several pathways with the most significant expression changes in genes involved in cell adhesion and the cytoskeletal remodeling pathways. It is well known that normal cell movement, cell to cell interaction, and cell to extracellular matrix adhesion require dramatic remodeling of the actin cytoskeleton during which many molecular and cellular changes occur (41). The differential expression of the cytoskeletal remodeling pathway in CSCs versus non-CSCs indicates that CSCs use this common mechanism more effectively to migrate to distant organs. This is further evidenced by our results showing that inhibition of this pathway represses the CSC-mediated migration in Brca1 tumors. Once CSCs arrive at the target organs, they can differentiate to other cell populations and recapitulate the heterogeneity of the primary tumor formation, eventually forming metastatic secondary tumors.

Cisplatin is a commonly used potent drug for the therapeutic treatment of various types of solid cancers (42, 43). In addition to the established cytotoxic role of cisplatin, our data demonstrated that cisplatin treatment blocks CSC-mediated tumor metastasis. Previous studies showed that cisplatin treatment may affect the actin cytoskeleton and induces marked changes in cell morphology (44). Thus, we hypothesized that cisplatin might be used to specifically block CSC-mediated migration especially after the observation that cytoskeletal remodeling is required for the enhanced motility of CSCs. Interestingly, cisplatin treatment blocked migration in vivo both in mouse and human cell lines positive for the presence of CSCs. This effect was associated with changes in cell morphology and inhibition of filopodia formation in the migrated cells at doses that do not cause obvious cytotoxic effects. Of note, cisplatin proved to be very effective in blocking CSC-mediated metastasis in vivo as well, highlighting the importance of cytoskeletal remodeling in regulating the migration ability of this subpopulation of tumor initiating cells. Thus, it is possible that cisplatin may play a new inhibitory role in metastasis, which may have implications in the types of tumors originating from and/or depending on CSCs.

Notably, it seems that mono-treatment with cisplatin eventually results in drug resistance, which is accompanied by a significant increase in the number of CSCs. Cisplatin resistance can occur through multiple mechanisms, including but not limited to decreased drug accessibility to cancer cells, reduced drug uptake, increased drug efflux, reduced drug binding to targets, altered gene expression, drug sequestering, impaired DNA damage sensing, and defects in apoptotic pathways (45, 46). Here, we show that Brca1 mutant tumors have markedly increased levels of phosphorylated S6RP, a downstream target of mTOR, whereas cisplatin-resistant tumors actively proliferate and exhibit activation of this pathway as revealed by immuno blot analysis. Thus, the inhibitory role of rapamycin in tumor growth indicates that mTOR signaling is involved in CSC viability upon cisplatin treatment. The mTOR pathway is composed of many important signaling molecules, including upstream protein-tyrosine kinases, PI3K, AKT, TSC, and the downstream ribosomal S6 kinase (p70S6K), which activates S6K1 and S6K2, and the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) (47). Numerous studies have demonstrated that the PI3K/AKT/mTOR pathway is frequently activated in various human cancers and plays a critical role in protein synthesis, cell proliferation, survival, apoptosis, autophagy, and angiogenesis (47). Currently, many inhibitors for this pathway (PI3K, AKT, and mTOR) have been developed, and several are in clinical trials (48). Moreover, recent evidence shows that PI3K/mTOR signaling may be involved in orchestrating the biology of cancer stem-like cells (26, 49). These
results are in line with our finding that the mTOR axis is indispensable for proliferation and survival of cisplatin-resistant tumors. Given that these tumors are highly enriched for CSCs, mTOR inhibition may overcome cisplatin resistance by affecting the survival of CSCs. It is conceivable that finding drugs that target CSCs will be helpful in eradicating tumors more efficiently. Specifically in triple-negative tumors where there is no targeted therapy so far, many inhibitors can be tested in the future for their efficacy to inhibit growth in targeting tumors of cancer stem cell origin.

Here, we have investigated breast CSCs for their ability to mediate cancer metastasis and drug resistance in Brca1 mutant tumors. In summary, our results suggest the following: 1) cytoskeletal remodeling and cell adhesion pathways are differentially expressed in the cancer stem cell subpopulation, which may explain the functional role of integrins in the enhanced motility of CSCs; 2) tumor cell migration is associated with extensive remodeling of the cytoskeleton in CSCs; 3) cisplatin inhibits CSC-mediated metastasis by blocking cytoskeletal remodeling; however, the prolonged treatment may induce drug resistance due to the activation of cell survival signaling; and 4) treatment of tumors with both cisplatin and rapamycin significantly inhibits tumor progression than treatment with each alone. These findings could be a proof of concept for the development of effective therapeutic strategies to target pathways specifically activated in the CSC subpopulation.

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