Candidate Antimetastasis Drugs Suppress the Metastatic Capacity of Breast Cancer Cells by Reducing Membrane Fluidity

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Abstract

Despite the high mortality from metastatic cancer, therapeutic targets to prevent metastasis are limited. Efforts to identify genetic aberrations that predispose tumors to metastasis have been mostly unsuccessful. To understand the nature of candidate targets for metastatic disease, we performed an in silico screen to identify drugs that can inhibit a gene expression signature associated with epithelial–mesenchymal transition (EMT). Compounds discovered through this method, including those previously identified, appeared to restrict metastatic capacity through a common mechanism, the ability to modulate the fluidity of cell membranes. Treatment of breast cancer cell lines with the putative antimetastasis agents reduced membrane fluidity, resulting in decreased cell motility, stem cell–like properties, and EMT in vitro, and the drugs also inhibited spontaneous metastasis in vivo. When fluidity was unchanged, the antimetastasis compounds could no longer restrict metastasis, indicating a causal association between fluidity and metastasis. We further demonstrate that fluidity can be regulated by cellular cholesterol flux, as the cholesterol efflux channel ABCA1 potentiated metastatic behaviors in vitro and in vivo. The requirement for fluidity was further supported by the finding in breast cancer patients that ABCA1 was overexpressed in 41% of metastatic tumors, reducing time to metastasis by 9 years. Collectively, our findings reveal increased membrane fluidity as a necessary cellular feature of metastatic potential that can be controlled by many currently available drugs, offering a viable therapeutic opportunity to prevent cancer metastasis. Cancer Res; 76(7); 2037–49. ©2016 AACR.

Introduction

To metastasize, a cancer cell must acquire a set of capabilities that are distinct from and potentially incompatible with its ability to flourish in the primary tumor. One explanation for how cells can acquire metastatic capacity is that they activate a latent epithelial–mesenchymal transition (EMT) program, which provides cancer cells with increased motility and stem cell properties. Regardless of mechanism, the diversity of signals that can activate the metastatic cascade poses a challenge in developing therapeutic strategies. A model that explains the acquisition of metastatic traits using a more limited set of targetable cellular events would help in the development of broadly applicable treatments. Despite the wealth of signals known to promote metastasis, the search for small molecules to target this fatal process has been hampered by a lack of knowledge of a targetable addiction of metastatic cells. Efforts to identify genetic alterations that predispose tumors to metastasis have thus far been unsuccessful. To address this deficiency, unbiased in vitro screens for drugs have focused on the stem cell phenotype because metastasis requires the ability to recapitulate a primary tumor in a distant site with the same hierarchy of cell types. Recent screens against stem cells have identified two leading candidates, salinomycin, a potassium channel ionophore; and thioridazine, a dopamine receptor antagonist. Both in both cases, their primary targets have little known bearing on the known biology of metastasis. However, salinomycin has later been shown to inhibit Wnt signaling through an unknown mechanism dependent upon a transmembrane receptor. For thioridazine, an association between dopamine receptor activation and stem cells was originally suggested, but an independent screen recovered both dopamine receptor agonists and antagonists, suggesting that the compound likely works through an alternative mechanism. Given these results, the only apparent similarity in targets is the dependence upon membrane associated receptors. The lack of a deeper explanation for how these compounds work reveals a limitation in our understanding of the important determinants of metastatic capacity.

To better understand the targets and dependencies of metastasis, our study demonstrates that the fluidity of cell membranes, whether changed by pharmacologic or genetic inducers, controls...
metastatic capacity. Decreasing fluidity prevents and reverses an EMT and inhibits metastasis in mouse models. In human breast cancers, overexpression of ABCA1, a regulator of membrane cholesterol and fluidity, is associated with increased metastasis. Taken together, these disparate lines of evidence indicate that membrane fluidity is a critical determinant of metastatic capacity and suggest that it may be a viable therapeutic target.

Materials and Methods

Cell culture

T47D, MCF7, AU565, MDA-MB-231, MDA-MB-361, and ZR-75-1 cell lines were purchased from ATCC in 2011. 4T1 cells were purchased from ATCC in 2013. SUM-159 were obtained from Dr. Jeffrey A. Frost (University of Texas Health Science Center at Houston, Houston, TX); human mammary epithelial (HMLE) and HMLE ER-Twist cells were kindly provided by Dr. Sendurai A. Mani (University of Texas MD Anderson Cancer Center, Houston, TX) in 2013. All cell lines used in this study were used at low passage and shown to be free of mycoplasma (Mycoplasma Detection Kit, Lonza #LT37-618). Except for SUM-159, all cell lines were obtained either from ATCC or the original source (HMLE). We did no further authentication on the SUM-159 cell line.

Additional procedures are described in Supplementary Materials and Methods.

Results

An in silico phenotype screen predicts small molecules that repress metastatic capacity

We searched for compounds that can repress a metastatic phenotype (i.e., the set of cellular characteristics associated with metastasis), even if they do not lead to death as in prior screens (8, 9), using an EMT gene expression signature as a model (Fig. 1A). We created a signature based on embryonic development using an approach we previously described (12), although other signatures have been developed (13, 14). Noticing that EMT inducers TWIST1 and ZEB1 (6, 15) are upregulated at day 9.5 (Supplementary Fig. S1A), we selected for our signature the 219 genes that are either induced or repressed at this time (Fig. 1B; Supplementary Table S1). We found that the scores from this signature are significantly higher in cells after undergoing an EMT (across 6-gene expression data sets; Fig. 1C). To further characterize the signature, we performed pathway analysis using GATHER (16) and found 67 significant processes (FDR < 0.05), including many associated with differentiation, development, and motility (Supplementary Fig. S1B). We also generated a network of the altered pathways (17–19) and saw that the signature is associated with upregulation of Wnt signaling, which is an inducer of Snail and EMT (Supplementary Fig. S1C, ref. 20). These results indicate that our EMT signature captures the distinction between the epithelial and mesenchymal phenotypes in cancer.

Figure 1.
An in silico screen identifies small-molecule compounds that inhibit metastatic phenotypes and EMT in vitro. A, we generated an EMT gene expression signature to represent a metastatic phenotype. Then, we used the Connectivity Map to predict drugs that can repress this signature and metastatic capacity. B, heatmaps showing the expression profiles of genes in the EMT signature across day e6.5 to e10.5 (left) and adult tissues (right). C, plots showing (y-axis) the gene expression of GAPDH (top) or the scores of the EMT signature (bottom) in cells undergoing an EMT. Each dot represents a cell that is either epithelial (EPI) or mesenchymal (MES). D, diagram showing an example of the gene expression profiles of the antimetastasis drugs. E, Connectivity Map analyzes a library of compounds and generates a connectivity score (x-axis). In this volcano plot, each point is a compound. The ones with the negative connectivity scores (left) are predicted to repress metastatic phenotypes. Compounds studied in this manuscript are highlighted, where blue are controls and green are predicted as antimetastasis drugs.
Membrane Fluidity Controls Metastasis

With the validated EMT signature, we identified drugs that can perturb it using the Connectivity Map, a database of gene expression changes triggered by pharmacologic compounds (Fig. 1D; ref. 21). Analysis of 1,309 compounds, including FDA-approved drugs and other bioactive molecules, yielded predictions of drugs that repress an EMT gene expression signature (antimetastasis drugs) (Fig. 1E; Supplementary Table S2). The top scoring anti-metastasis drugs are inhibitors of known EMT pathways, such as vimentin expression (withaferin A; ref. 22). HDAC dependency (vorinostat, trichostatin A; ref. 23), HSP90 response (tanespimycin, geldanamycin; ref. 24), or PI3K activation (LY294002; ref. 25). Furthermore, one of the compounds identified in a prior screen, thioridazine, is also predicted to be antimetastasis. The other, salinomycin, was not profiled in this database. To validate the robustness of the results, we found a statistically significant overlap ($P = 0.0004$) with the connectivity scores obtained using a previously published EMT signature (13). All but thioridazine were confirmed by the core EMT signature to be antimetastatic ($P = 0.007$; Supplementary Fig. S1D).

Predicted antimetastasis drugs inhibit cell motility, mammosphere formation, and the EMT

To test whether the drugs predicted in silico can alter metastatic phenotypes in vitro, we focused on four with a range of mechanisms: alprostadil (prostaglandin agonist), amitriptyline (tricyclic antidepressant), haloperidol (dopamine receptor antagonist), and maprotiline (tetracyclic antidepressant); as well as two previously identified drugs, salinomycin and thioridazine.

To validate whether the predicted drugs can affect metastatic capacity, we tested whether they impact cell motility or mammosphere formation, cellular characteristics that are necessary for metastasis. To measure motility, we performed transwell migration and wound healing assays and found that the drugs significantly decreased the motility of cells (Fig. 2A–C and Supplementary Fig. S2A–S2C). In addition, the drugs could also repress the ability of the cells to form mammospheres, an in vitro assay for early progenitor stem cell characteristics in breast cancer cells (Fig. 2D and Supplementary Fig. S2D; ref. 26). The drug concentrations used varied from 1 to 20 μmol/L (see Supplementary Materials and Methods).

To evaluate whether the assays were confounded by an impact on cell proliferation or death, we measured the impact of the drugs on cell number. At seven days, the maximum duration of the prior assays, drug treatment minimally impacted the cells, leading to a 17% to 18% decrease that cannot explain the magnitude of the change is a correlated byproduct of, or a requirement for, EMT and metastasis.

The prior experiments demonstrated that antimetastasis drugs could decrease metastatic phenotypes. However, in tumors, a metastatic phenotype may be maintained by signaling sustained via genetic or epigenetic changes or from extracellular sources. To see whether the target of the drugs is dominant over the presence of a continuous metastasis-inducing signal, we next tested whether antimetastasis drugs can reverse an EMT in cells that have undergone the transition or prevent cells from entering one. For the first experiment, we used HMLE cells (nontumorigenic immortalized human mammary epithelial cells; ref. 27) that stably express Twist, which forced the cells to undergo an EMT as seen by loss of the E-cadherin epithelial marker and gain of mesenchymal ones (Fig. 2E and I). In these cells, five days of antimetastasis drug treatment resulted in reassertion of E-cadherin and loss of mesenchymal markers, even under continued Twist expression, showing that the cells reverted to an epithelial state despite continued expression of a potent EMT inducer.

To test whether the drugs can prevent an EMT, we used an inducible EMT system comprised of HMLE cells carrying tamoxifen-inducible Twist (2). We treated cells with antimetastasis drugs for seven days and then activated Twist with tamoxifen. While untreated cells underwent an EMT, cells treated with antimetastasis drugs did not undergo the same change (Fig. 2G). This shows that antimetastasis drugs can prevent the EMT despite the presence of an EMT-inducing signal. We confirmed that a stem cell phenotype was also reverted in a mammosphere assay (Fig. 2H and I). Similar results could also be seen in T47D cells using the EMT inducer Snail (Supplementary Fig. S2F and S2G).

Taken together, these experiments demonstrate that drugs with a broad range of established targets can inhibit both metastatic phenotypes and EMT across a range of breast cancer cell lines (T47D, nonmetastatic, luminal; MCF7, nonmetastatic, luminal; MDA-MB-231, metastatic, claudin low; and SUM-159, metastatic, claudin low; ref. 28). Combined with the observation that they can inhibit an EMT, it suggests that they affect a target that is necessary for an EMT but also has a general capacity to tune metastatic phenotypes across cell states.

Antimetastasis drugs reduce plasma membrane fluidity

Due to the large number of pathways and the breadth of drugs that can reduce metastatic phenotypes, we surmised that they may be controlled by a broad cellular characteristic. To identify it, we examined the drugs found in our in silico screen and noticed a strong correlation between the induction of an EMT signature and a reported ability to alter plasma membrane fluidity (defined as the rate that a molecule can diffuse laterally across the membrane; Fig. 3A). This mechanism is supported by prior studies showing that cell lines from lung (29), sarcoma (30), and breast tumors (31) that metastasize have higher fluidity. On the basis of these results, we asked whether the observed fluidity change is a correlated byproduct of, or a requirement for, EMT and metastasis.

To test whether our predicted antimetastasis drugs can alter plasma membrane fluidity, we used a FRAP assay (Fig. 3B). After treating cells, we found that haloperidol decreases the fluidity of the plasma membrane (Fig. 3C), showing a lower number of mobile molecules as well as a decreased rate of diffusion (Fig. 3D).

To test a broader range of molecules, we switched to a more tractable pyrene fluidity assay. As a control, we included oleic acid, an 18-chain monounsaturated fatty acid that fluidizes membranes by disrupting lipid packing (32). Using this approach, we confirmed that EMT inducers and oleic acid increase fluidity, whereas antimetastasis drugs decrease it across a range of cell lines (Fig. 3E; Supplementary Fig. S3A).

The prior experiments were performed in a monolayer culture that lacks the 3D organization of the in vivo state, which has been seen to alter the response of cells to drugs such as chemotherapies (33). Therefore, to determine whether 3D organization impacts the ability of drugs to alter fluidity, we cultured MDA-MB-231 and T47D cells in 3D, where they assumed stellate (characterized by invasive projections) and mass (with disorganized nuclei and...
robust cell–cell adhesions) morphologies, respectively (Supplementary Fig. S3B; ref. 34). Although cells in 3D culture exhibit higher fluidity than in 2D, the drugs could nevertheless reduce fluidity similar to that seen in 2D (Fig. 3F and G and Supplementary Fig. S3C and S3D).

Next, to distinguish whether fluidity is correlated with, or causally represses, metastatic phenotypes, we isolated the molecular targets of the drugs from fluidity by treating cells concurrently with both an antimetastasis drug (that decreases fluidity) and oleic acid (that increases fluidity), resulting in no net change (Fig. 3H and Supplementary Fig. S3E). When fluidity was unchanged, antimetastasis drugs could no longer decrease either cell migration or mammosphere formation in MDA-MB-231 or T47D cells (Fig. 3I and J and Supplementary Fig. S3F and S3G). This demonstrates that antimetastasis drugs decrease metastatic phenotypes through a fluidity-dependent mechanism.

Antimetastasis drugs decrease membrane fluidity and prevent metastasis in vitro

Metastasis is comprised of complex physiologic events that are not present in vitro, including influences from the microenvironment and immune system. To model these factors, we tested our antimetastasis drugs in a syngeneic immunocompetent mouse model of metastasis. We injected 4T1 cells orthotopically into the mammary fat pads, a model of the stroma of normal mammary glands, of BALB/c mice. Once a day, we injected mice intraperitoneally with saline, alprostadil, or haloperidol at doses analogous to those given to humans clinically. To isolate fluidity from other targets of the drugs, we also treated with a combination of all the drugs and oleic acid. During the course of the experiment, all mice developed primary tumors in the mammary gland and were sacrificed after 31 days. The haloperidol-treated mice exhibited lethargy, a well-known side effect of the drug, immediately after injections, which diminished over the next hours, while the alprostadil-treated mice showed no obvious changes in behavior or weight. The sizes of the primary tumors were similar across all groups, indicating that the drugs had little or no impact on tumor growth (Fig. 4A). However, the fluidity of the tumors treated with drugs decreased, showing that the drugs affected the tumors, except when balanced by oleic acid injections [Fig. 4B]. Furthermore, drug treatment led to significantly fewer metastatic nodules on the surface of the lung, which was reversed when fluidity was restored with oleic acid (Fig. 4C and D). The tumors treated with oleic acid showed an increased number of nodules that did not achieve statistical significance. However, half these lungs were permeated with metastatic growth such that nodules could not be counted accurately, exceeding the limits of the assay. To more sensitively measure tumor burden, we sectioned the lungs, stained with hematoxylin and eosin, and calculated the metastatic burden as the fraction of cells that were cancerous. This revealed that oleic acid does significantly increase metastatic burden (Fig. 4E and F).

Finally, to check if the antimetastasis drugs can affect the mesenchymal status of the tumors, we stained for the expression of vimentin, revealing that the antimetastasis drugs resulted in tumors with decreased vimentin expression, while oleic acid had the opposite effect (Fig. 4G and H). Taken together, these results demonstrate that antimetastasis drugs significantly reduced breast cancer metastasis, dependent upon a decrease in the membrane fluidity of the primary tumor.

Cellular cholesterol alters membrane fluidity and metastatic phenotypes

As a more fluid membrane is required for metastasis, we reasoned that metastatic tumor cells must somehow increase their fluidity. One possible mechanism is an alteration in cellular cholesterol, a determinant of membrane fluidity (35), which we confirmed by titrating cholesterol in MDA-MB-231 cells (Fig. 5A and B). We treated MDA-MB-231 cells with our antimetastasis drugs and found that they increased cholesterol levels (Fig. 5C), consistent with the decrease in fluidity. Furthermore, cholesterol also leads to a loss of metastatic phenotypes, as measured by migration and mammosphere formation (Fig. 5D–G). Indeed, the total cellular cholesterol levels are inversely correlated with membrane fluidity and metastatic phenotypes.

The cholesterol efflux channel ABCA1 drives metastasis in human breast cancer

As cholesterol is a potent inhibitor of metastatic phenotypes, we predicted that the EMT program may increase metastatic capacity by reducing cholesterol. To identify this mechanism, we searched for changes in the expression of genes that control cellular cholesterol levels across an EMT (36). This bioinformatic analysis revealed that an ATP-binding cassette transporter ABCA1, which functions as a cholesterol reverse transporter (37), is significantly upregulated in an EMT (Fig. 6A). To confirm this, we expressed Snail or Twist in HMLE cells and a range of luminal breast cancer cell lines and measured ABCA1 gene expression. In these conditions, the expression of ABCA1 was increased 4- to 12-fold (Fig. 6B).

As a potential mechanism to explain their activity, we tested whether the antimetastasis drugs reduce ABCA1 expression. We treated T47D cells induced to undergo an EMT and MDA-MB-231 cells with a range of antimetastasis drugs and found that ABCA1 gene and protein expression was significantly decreased (Fig. 6C and D). Further, in sections from lung metastases, the tumors from mice treated with antimetastasis drugs had significantly fewer cells with high ABCA1 expression (Fig. 6E and F). Thus,
Figure 3.
Predicted drugs induce changes in membrane fluidity. A, connectivity score (x-axis) of each drug (dots) separated into those that increase (+) or decrease (−) membrane fluidity based on prior reports in the literature. B, confocal images from a FRAP assay showing plasma membrane fluidity changes in cells treated with either vehicle (left) or haloperidol (right). Top, we focused on 1 μm² areas of the plasma membrane stained with a lipophilic dye. The subsequent rows, at higher magnification, show the remaining phases of the assay: Acquiring (A) the baseline intensity level for 30 seconds, photobleaching the area for 30 seconds, and then measuring the recovery (R) of the fluorescence for two minutes. C, plot showing the fluorescence recovery for MDA-MB-231 cells in a FRAP assay after treatment with the antimetastasis drug haloperidol for three days. The x-axis shows time since the beginning of the recovery period and y-axis the relative fluorescence intensity. D, for the recovery plot, we calculated the mobile fraction (the fraction of fluorescent molecules that can diffuse back into the section) and the diffusion constant (the rate at which molecules diffuse back into the section). E, pyrene assay shows the relative fluidity of MDA-MB-231 cells grown in monolayer culture either expressing EMT inducers (red bars), treated with antimetastasis drugs (green bars), or treated with a membrane fluidizer oleic acid (blue bars). The fluidity is the ratio of the fluorescence intensity of excimers to monomers (IE/IM) and then normalized to the appropriate control. F, we cultured MDA-MB-231 cells in monolayer and 3D culture (2% Matrigel). This compared the membrane fluidity (y-axis) of MDA-MB-231 cells in the two conditions. G, plot showing the relative fluidity of MDA-MB-231 cells grown in 3D culture after treatment with two drugs. H, membrane fluidity (y-axis) of MDA-MB-231 cells after treatment with drugs with and without oleic acid. Blue bars, treated with oleic acid; green, treated with drugs; and yellow, treated with a combination of drugs and oleic acid. I, number of migrating cells from a transwell assay, normalized to vehicle, is shown (y-axis). J, number of mammospheres formed is shown on the y-axis. *, P < 0.05; **, P < 0.01; *** P < 0.001; NS, nonsignificant, P > 0.05.
Antimetastasis drugs decrease fluidity and prevent metastasis in vivo. A, we injected mouse breast cancer cells orthotopically and measured the volume of the primary tumors (y-axis) after sacrifice. Treatments (saline or drugs, with and without oleic acid) are shown on the x-axis (beneath E), and each point represents the primary tumor from a mouse in the study. Each condition is compared with the saline group with no oleic acid. B, y-axis shows the membrane fluidity of the primary tumor from each mouse. C, y-axis shows the number of metastatic nodules seen in the lungs from each mouse. D, representative lungs (the one with the median number of nodules) from each treatment group is shown. The top row shows the anterior, and the bottom row shows the posterior surface. E, metastatic burden, the percent of lung sections that are comprised of tumor tissue, is shown on the y-axis. F, H&E staining for a representative lung from each treatment group is shown. Arrows, colonies of metastatic cells. G, percentage of tumor cells in the lung metastasis that are vimentin positive is shown on the y-axis. H, representative fields of lung metastases stained for the EMT marker vimentin and counterstained with hematoxylin. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; NS, nonsignificant, P ≥ 0.05.
ABCA1 is an EMT-responsive gene that can be inhibited by the treatment of antimetastasis drugs. Although ABCA1 was previously found to decrease cell proliferation, its relationship with metastasis is still unknown (38). To determine whether ABCA1-regulated cholesterol levels control metastatic capacity, we used an RNAi strategy and knocked down ABCA1 in MDA-MB-231 cells (Supplementary Fig. S4A). This led to an increase in cellular free cholesterol, consistent with a diminished ability of the cells to efflux cholesterol (Fig. 7A). The cholesterol levels were also inversely correlated with changes in membrane fluidity (Fig. 7B). We measured cell proliferation for one week and observed no change (Supplementary Fig. S4B). As our model would predict, we also observed in these cells a decreased cell motility (Fig. 7C and D and Supplementary Fig. S4C). In each of these experiments, we could rescue the phenotype by restoring cellular cholesterol with MβCD, a compound that extracts cholesterol from cell membranes (39). This demonstrates that the changes in phenotype were dependent upon its impact on cholesterol and not another function of ABCA1. Taken together, these results show that ABCA1 determines cell migration through the regulation of cholesterol levels.

Seeing that knockdown of ABCA1 can alter metastatic phenotypes, we next tested whether cyclosporin A, an ABCA1 inhibitor, has similar effects (40). Treatment of MDA-MB-231 cells with this compound led to a significant increase in cellular cholesterol concomitant with a reduction in fluidity (Supplementary Fig. S4D and S4E). Consistent with this result, we also observed a decrease in migration and mammosphere formation (Supplementary Fig. S4F–S4H). Finally, we tested whether cyclosporin A can inhibit an EMT in a T47D model. We induced an EMT by ectopic expression of Snail, and found that it can restore epithelial transcriptional...
Figure 6.

**ABCA1** is increased in an EMT and decreased upon treatment with antimetastasis drugs. A, plots showing the expression of cholesterol regulatory genes in cells before and after an EMT, as in Fig. 1C. Four groups of genes are shown: EMT markers (controls); synthesis, cholesterol synthesis; import, cholesterol import; and export, cholesterol export. B, relative gene expression of **ABCA1** (y-axis), measured by qRT-PCR, before and after induction of EMT in HMLE and a range of luminal breast cancer cells by ectopic expression of Snail or Twist. C, gene expression (y-axis) of **ABCA1** in T47D cells via ectopic expression of Snail or Twist is shown after treatment with a range of antimetastasis drugs or vehicle controls (x-axis). D, gene expression (y-axis; bar plot) and protein expression (immunoblots) of **ABCA1** in MDA-MB-231 cells are shown after treatment with a range of antimetastasis drugs or vehicle controls (x-axis). E, we stained the sections from the lung metastases of mice treated with antimetastatic drugs for the presence of **ABCA1**. The percentage of tumor cells positive for **ABCA1** is shown (y-axis). F, representative sections from lung metastases stained for **ABCA1** and counterstained with hematoxylin. Red arrows, positively stained cells. *, P < 0.05; **, P < 0.01; NS, nonsignificant, P ≥ 0.05.
Figure 7.
ABCA1-driven reduction of cholesterol and metastasis in human breast cancer. A, free cholesterol level (y-axis) in MDA-MB-231 cells. The cells with a gray bar express a control shRNA, green express shRNAs targeting ABCA1, and yellow express both ABCA1-targeting shRNAs and MβCD (2 mmol/L). B and C, fluidity and migration efficiency (y-axis) are shown under the same conditions. D, representative images show the impact of control shRNA, shABCA1, or shABCA1+MβCD treatment on cell migration. Scale bar, 300 µm. E, each point represents the number of metastatic nodules found on the lung (y-axis) after injections of MDA-MB-231 cells transfected with control shRNA and shRNAs targeting ABCA1 (x-axis) in a mouse lung colonization assay. F, representative lungs (the one with the median number of nodules) from each group are shown. The top row shows the anterior, and the bottom row shows the posterior surface. G, images showing the representative lung from each treatment group. The lungs were stained with H&E, and colonies of metastatic cells are indicated with arrows. Right, the area shown at higher magnification. H, Kaplan–Meier plots showing the percentage of human breast cancer patients (y-axis) free of distant metastasis over time (x-axis) across four independent datasets. The patients are discretized into normal or high ABCA1 groups. Hash marks, censored patients. I, model; metastasis is driven by increased membrane fluidity due to the aberrant regulation of cholesterol levels. *, P < 0.05; **, P < 0.01; ***, P < 0.001; NS, nonsignificant, P ≥ 0.05.
markers, along with a reduction in mammosphere-forming ability (Supplementary Fig. S4I and S4J). These results confirm that the inhibition of ABCA1 function decreases metastatic phenotypes.

Having seen the importance of ABCA1 in vitro, we tested whether it drives metastasis in vivo. We injected the control and MDA-MB-231/shABCA1 cells (with two different shRNAs) into the tail veins of immunocompromised NOD/SCID mice and sacrificed them after 49 days. We stained the lungs for expression of ABCA1 and confirmed that the tumors from ABCA1 knockdown had lower ABCA1 expression (Supplementary Fig. S5A and S5B). At that time, they had significantly more metastatic nodules both on the surface (Fig. 7E and F) and in the interior of the lungs (Fig. 7G). To check the growth rate of the cells in vivo, we injected MDA-MB-231/control and MDA-MB-231/shABCA1-3 cells orthotopically into the mammary fat pads of NOD/SCID mice and saw no difference in the size of the resultant tumors, in contrast to a prior report, potentially because in the prior study, ABCA1 was ectopically overexpressed in a genetic background, where it promotes apoptosis (Supplementary Fig. S3C; ref. 38). In any case, our results demonstrate that ABCA1 significantly enhances metastatic colonization in vivo.

Given that ABCA1 is expressed during EMT and can drive metastatic properties in vitro and metastasis in vivo, we predicted that it may be a mechanism used by human tumors to increase metastatic capacity. To test this, we examined ABCA1 expression across four publicly available datasets of breast cancer patients that were monitored for distant metastases (Supplementary Fig. S5D). Among tumors that metastasized within five years, 41%±28% had high ABCA1 expression (Fig. 7H). Those patients acquired metastases significantly earlier than those with normal ABCA1 expression. The time required for 25% of the tumors to metastasize was 9±4 years shorter when ABCA1 was high. Hundred percent of patients with high ABCA1 had metastases by 12.5 years, compared with only 28% of patients with normal ABCA1. The strong association between ABCA1 and distant metastases in human breast cancers, coupled with the preclinical evidence revealing the mechanism, shows that ABCA1 is a potent inducer of metastatic capacity in human breast cancers. This demonstrates a novel strategy for human tumors to acquire metastatic capacity, namely the regulation of fluidity through alteration in cellular cholesterol homeostasis.

Discussion

Our investigations revealed that membrane fluidity is causally associated with metastasis, provides a mechanism that explains the efficacy of previously and newly identified antimetastasis drugs, and shows that breast tumors have altered cholesterol efflux associated with metastasis (Fig. 7I). The fact that the efficacy of antimetastasis drugs depends on their ability to reduce fluidity enables the search for more potent therapeutics.

The unexpected alterations of cholesterol efflux in breast cancer raise the possibility that systemic cholesterol levels may affect metastasis. This question has been addressed with epidemiologic studies starting 85 years ago with the observation that cancer patients have less heart diseases, initiating investigations that continue almost a century later (41). A range of studies found a correlation between low levels of plasma cholesterol and increased mortality from cancer, although a minority saw no significant effect (Supplementary Table S3).

One explanation for the correlation is that some patients may harbor undiagnosed tumors that are metabolizing cholesterol, leading to lower plasma cholesterol levels (42). This is supported by data showing that cholesterol and its metabolites can promote tumor initiation (43). However, even after controlling for this by lag-time analyses, the association remains.

The conflicting interpretations of the association between cholesterol and cancer suggest a complex relationship between cholesterol and disease that is not yet fully understood. As cholesterol affects tumor initiation, proliferation, and metastasis through a range of mechanisms, it is likely that cholesterol plays both promoting and inhibiting roles throughout tumor development. To better understand it, studies must be carried out that carefully isolate the contribution of cholesterol to different hallmarks of the cancer phenotype. This study reveals the impact of fluidity and cholesterol specifically on metastasis. While cells may utilize ABCA1 to promote metastasis, the same gene may also inhibit the development of tumors by inhibiting cellular proliferation (38). Similarly, as inhibitors of cholesterol synthesis, one would predict that statins would promote metastasis. However, the literature on statins is ambiguous, showing both tumorigenic and antitumorigenic effects, potentially due to pleiotropic effects of the drug.

Finally, the ability of fluidity to inhibit metastasis has implications for therapy. Nearly all the antimetastasis drugs we identified are FDA approved. These drugs were able to reduce the fluidity of primary tumors and decrease metastatic capacity in vivo at dosages analogous to those given to humans with no obvious additional systemic toxicity. However, this raises questions regarding the impact of long-term nonspecific decrease of fluidity across an organism. Suggesting that the treatment is not evidently toxic is the fact that one of the drugs, haloperidol, is used to treat chronic psychiatric disorders. Thus, the apparent wealth in available drugs that can affect membrane fluidity, observed by others and ourselves, suggests that a viable antimetastatic therapy may already exist.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: W. Zhao, J.T. Chang
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