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14. ABSTRACT Metastasis contributes significantly to the mortality of breast cancer. The loss of E-cadherin expression is a critical event in the initiation of metastasis. However, these studies focus on the role of E-cadherin in dissemination but not colonization, or survival in a new organ environment, such as the liver, a main site of breast cancer metastasis. We hypothesize that signals from the liver cause breast cancer cells to undergo a mesenchymal to epithelial reverting transition (MERt) through the re-expression of E-cadherin, which consequently confers a survival advantage. Co-culture of E-cadherin-negative MDA-MB-231 breast cancer cells with hepatocytes results in the re-expression of E-cadherin as determined by immunoblot, flow cytometry, and immunofluorescence. To test whether E-cadherin expression protects breast cancer cells from chemotherapy-induced cell death, an E-cadherin knock-in and knock-out was generated. When cell death was induced by staurosporine, camptothecin, doxorubicin, or taxol, E-cadherin-positive cells were more resistant to cell death. Furthermore, MDA-MB-231 that have re-expressed E-cadherin following hepatocyte coculture are more chemoresistant compared to MDA-MB-231 cells cultured in the absence of hepatocytes. These results reveal that breast cancer cells cultured in the liver microenvironment undergo molecular changes that confer chemoresistance and may help to elucidate why chemotherapy commonly fails					
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E-Cadherin as a Chemotherapy Resistance Mechanism on Metastatic Breast Cancer

INTRODUCTION

Breast cancer is the most common malignancy in women in the United States. While the incidence and death rate of breast cancer is decreasing due to earlier detection and treatment, one third of women diagnosed will still develop metastases [1]. Because current therapies for metastatic breast cancer are generally unsuccessful due to chemotherapeutic resistance, distant metastases are the leading cause of mortality, with the five year survival rate around 20% [2]. Clearly, a better understanding of the molecular pathogenesis of metastasis is an important objective that may lead to more effective breast cancer therapy.

Many of the steps required for the initiation of metastasis are reminiscent of the epithelial-mesenchymal transition (EMT) that occurs during embryonic development. The loss of expression of E-cadherin, a cell adhesion molecule, has been shown to be a critical event in both EMT and metastasis. Numerous studies have shown that loss of E-cadherin contributes to tumor invasiveness and cell motility. However, these studies focus only on the role of E-cadherin in detachment and dissemination from the primary tumor. Few have examined E-cadherin expression during the last steps of the metastatic process, particularly the establishment of a metastatic niche in a secondary organ.

While loss of E-cadherin may promote tumor invasion and spread, E-cadherin re-expression may allow the metastatic cancer cell to survive in the new organ. In breast cancer, the most common sites of distant metastases are bone marrow, liver, lung, and brain, yet metastatic cancer cells may circulate through several other organs before reaching these target organs -- suggesting that metastases only form within organs that provide the appropriate signals and environment. Interestingly, when queried by pathology, a number of studies have observed E-cadherin-positive metastases arising from E-cadherin-negative primary tumors [3, 4]. Furthermore, in cancer, E-cadherin is mostly epigenetically regulated and responsive to changes in the microenvironment [5]. These studies suggest that E-cadherin may be re-expressed in a new organ environment such as the liver.

We hypothesize that signals from the liver cause breast cancer cells to undergo a mesenchymal to epithelial reverting transition (MErT) through the re-expression of E-cadherin, which consequently confers a survival advantage. Evasion of apoptosis is an important cellular adaptation that would account for the failure to treat metastatic breast cancer with current chemotherapies. This proposal aims to fill a gap in our understanding of the pathogenesis of breast cancer metastasis. The molecular basis of metastatic progression is still poorly understood and not much is known about the signals stemming from the liver that provide a hospitable environment for metastatic colonization. The identification of a molecule responsible for protecting metastatic cancer cells from cell death may therefore lead to novel therapeutic approaches for women and men diagnosed with metastatic breast cancer.

BODY

The Statement of Work outlined below is divided into training and research objectives of this award. A description of progress under the training plan will be addressed first, followed by a description of progress on the research plan.

Training Plan:

As an MD/PhD student with goals to become an academic physician scientist, the training plan contains elements to develop both research and clinical skills.

Task 1. Maintain clinical skills

- A. Participate in the Longitudinal Clerkship in the breast cancer clinic. I will spend one half day a week for 20 weeks in the clinic under the guidance of a physician. *Months 1-6*

Task 2. Develop oral presentation skills

- A. Attend Pathology Research Seminar weekly, where I will learn how to critically evaluate publications and present them to peers. *Months 1-36*
 - B. Present data at quarterly committee meetings. *Months 1-36*
 - C. Present data and articles at weekly lab meetings and journal clubs. *Months 1-36*
 - D. Present data at yearly MSTP and Department of Pathology retreats. *Months 1-36*
- Task 3. Complete graduate course work
- A. Complete courses in Angiogenesis and Mechanisms of Tissue Growth and Differentiation.
- Task 4. Broaden knowledge of the field of breast cancer research
- A. Attend at least one national/international meeting yearly (such as AACR, SABC). *Months 1-36*

Task 1. Maintain clinical skills. This task is completed. For 20 weeks, I spent half of a day per week in clinic with Dr. Adam Brufsky, director of the Breast Cancer Clinic at Magee Womens Hospital. I participated in the management and care of breast cancer patients of all types, from basic DCIS to advanced metastatic disease. This Longitudinal Clerkship imparted clinical relevance to my research and taught me skills on balancing clinical and research responsibilities, which will be invaluable for the future.

Task 2. Develop oral presentation skills. This task is completed. In the past year, I have given oral or poster presentations on my research at least 5 times at various symposiums and seminars at the University of Pittsburgh. For these efforts I was awarded the Scholar-in-Training at the annual AACR meetings in 2009 and 2010.

Task 3. Complete graduate course work. This task is completed. I have completed all the necessary graduate course work for the Cellular and Molecular Pathology graduate program, as well as the required course work for the Cellular Approaches to Tissue Engineering and Regeneration training program.

Task 4. Broaden knowledge of the field of breast cancer research. This task is completed. In April 2011 I presented a poster at the national meeting of the American Association for Cancer Research. At this meeting I attended talks by eminent researchers in many different subfields of breast cancer research. This experience has greatly contributed to my training. I look forward to attending the Era of Hope meeting in August to learn more about different fields of breast cancer research as well as learn patient and advocate perspectives.

Research Plan:

Task 1 (Specific Aim 1). To determine whether breast cancer cells upregulate E-cadherin expression within a metastatic niche. *Months 1-30*

- A. Co-culture of metastatic breast cancer cells with rat hepatocytes. E-cadherin expression will be evaluated. *Months 1-12*
- B. Inoculate metastatic breast cancer cells into rat spheroid cultures and evaluate E-cadherin expression. *Months 6-18*
- C. Seed an organotypic liver bioreactor, established with rat hepatocytes, with breast cancer cells and evaluate tumor cell distribution and E-cadherin expression and localization. *Months 12-30*

Task 2 (Specific Aim 2). To determine whether E-cadherin re-expression endows resistance to chemotherapy. *Months 12-36*

- A. E-cadherin expression will be blocked in co-cultures by using siRNA and E-cadherin blocking antibody. *Months 12-18*
- B. Induce tumor cell death using 2 different agents: TNF α and camptothecin. After sorting hepatocytes from breast cancer cells in 2D, we will measure cell death. *Months 12-24*
- C. Induce tumor cell death in hepatocytes spheroid co-cultures and evaluate apoptosis. *Months 18-30*
- D. Induce tumor cell death in liver bioreactor co-culture and evaluate tumor cell apoptosis and effects on the liver tissue. *Months 18-36*

Task 1A. Evaluation of E-cadherin re-expression in hepatocyte cocultures. This task is completed. We have successfully shown using several methods that breast cancer cells can re-express E-cadherin when cocultured with hepatocytes. Normally, MDA-MB-231 breast cancer cells do not express E-cadherin due to methylation of the E-cadherin promoter. However, following coculture of 231 cells with rat hepatocytes for 5 days, E-cadherin expression is detected by immunofluorescence. In addition, we transfected MDA-MB-231 cells with an E-cadherin shRNA construct, and as expected expression of this construct prevents re-expression of E-cadherin in the liver microenvironment (Figure 1). To show that this phenomenon is not rodent specific, 231 cells were also cocultured with human hepatocytes obtained from resected or donor livers. E-cadherin expression was detected following human hepatocyte coculture by immunoblot, immunofluorescence, and flow cytometry (Figure 2).

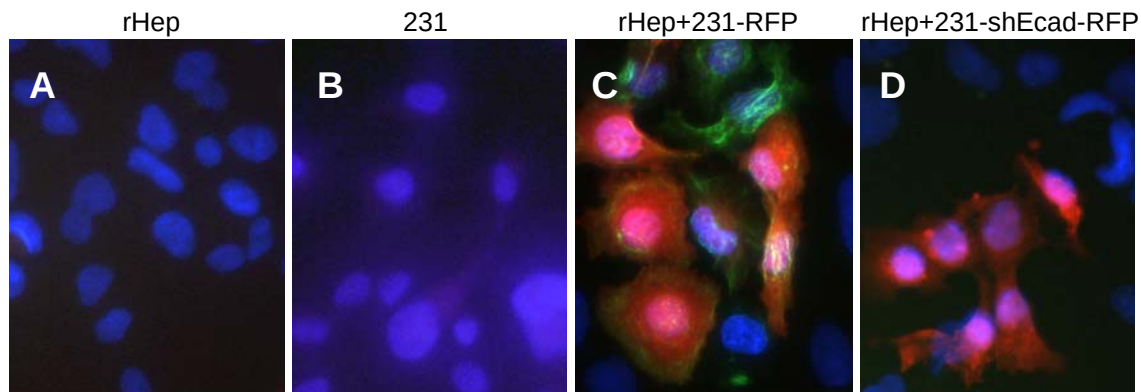


Figure 1. A) Rat hepatocytes cultured alone B) 231 cells cultured alone C) Rat hepatocytes cocultured with 231-RFP cells for 5 days D) Rat hepatocytes cocultured with 231-shEcad-RFP cells for 5 days. All samples were fixed, permeabilized, and immunostained for E-cadherin (green), RFP (red), and DAPI (blue).

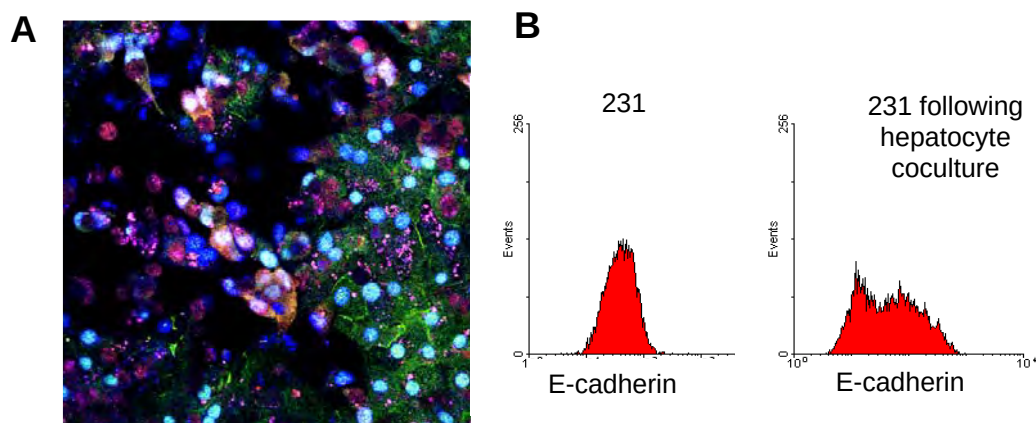


Figure 2. A) Human hepatocytes cocultured with 231-RFP cells for 10 days were immunostained for E-cadherin (green), RFP (red) and DAPI (blue). 231-RFP cells re-express E-cadherin (yellow). B) Human hepatocyte and 231-RFP cocultures were analyzed by flow cytometry. 231-RFP cells were gated from hepatocytes using FSC and SSC and stained for E-cadherin.

Task 1B. Evaluation of E-cadherin re-expression in hepatocyte spheroid cultures. This task is completed. Because hepatocytes form spheroids in the liver bioreactor, we have combined this task with Task 1C.

Task 1C. Evaluation of E-cadherin expression in an organotypic liver bioreactor. This task is completed. Analysis of breast cancer cell and hepatocyte interactions in a liver bioreactor have many

advantages over a 2D culture system. 3D bioreactors more accurately recapitulate many aspects of the liver microenvironment, including fluid dynamics, gene and protein expression, and metabolism. We therefore used this model to determine whether breast cancer cells form cohesive interactions with hepatocytes *ex vivo*. First, rat or human hepatocytes were seeded into the liver bioreactor and allowed to reorganize for 48-72 hours. In the bioreactor, hepatocytes reorganize into 3D spheroids situated inside collagen-coated channels of a polystyrene scaffold. RFP-labeled MDA-MB-231 cells were seeded into the liver bioreactor and cultured for 10 to 15 days. Scaffolds were then removed from the bioreactor and immunostained for E-cadherin and imaged by confocal microscopy. Similar to what was observed in Figure 2, MDA-MB-231-RFP cells stained positive for E-cadherin following culture in the liver bioreactor (Figure 3). These results indicate that breast cancer cells are able to cohere to hepatocytes in both 2D and 3D environments. However, we were unable to determine the localization of E-cadherin.

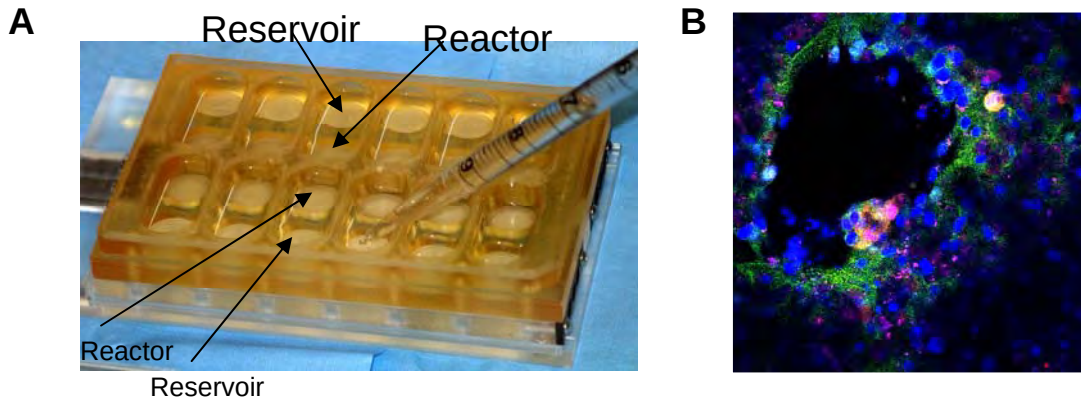


Figure 3. A) Image of the bioreactor. Hepatocytes and cancer cells are seeded into the reactor wells and media is circulated between the reservoir and reactor wells by a pneumatic pump. B) Confocal image of 1 of 769 channels in a single reactor scaffold. Scaffolds were immunostained with RFP (red), E-cadherin (green), and DAPI (blue).

Task 2A. Evaluate the affect of E-cadherin inhibition on chemoresistance. This task is completed. MCF7 is another commonly used breast cancer cell line; however, these cells express E-cadherin. We used an E-cadherin function blocking antibody to prevent the formation of extracellular contacts as well as siRNA to knockdown E-cadherin expression in MCF7 cells. When treated with $\text{TNF}\alpha$ and camptothecin, MCF7 cells that had been treated with E-cadherin siRNA were more sensitive to induction of cell death. We also created stable knockouts using an E-cadherin shRNA plasmid (MCF7-shEcad) and created an E-cadherin knock-in mutant by over-expressing full-length E-cadherin in MDA-MB-231 cells (231-Ecad) (Figure 4a). In the absence of hepatocytes, these four cell lines (231, 231-Ecad, MCF7 and MCF7-shEcad) were treated with staurosporine, an inducer of apoptosis to replicate the results reported by Wang *et al* [6] (Figure 4b and 4c). To make this experiment more clinically relevant, cells were also treated with taxol, doxorubicin, cyclophosphamide, and 5-fluorouracil, which are all chemotherapy drugs commonly used to treat breast cancer. All of the cell lines (both wild-type and mutant and regardless of E-cadherin expression) were resistant to both cyclophosphamide and 5-fluorouracil, which may be explained by the fact that the cell lines were derived from patients that had been treated with systemic chemotherapy. However, when cells were treated with camptothecin, doxorubicin, or taxol, 231-Ecad and MCF7 cells were more resistant to chemotherapy-induced cell death when compared to their E-cadherin-negative counterparts (Figure 5).

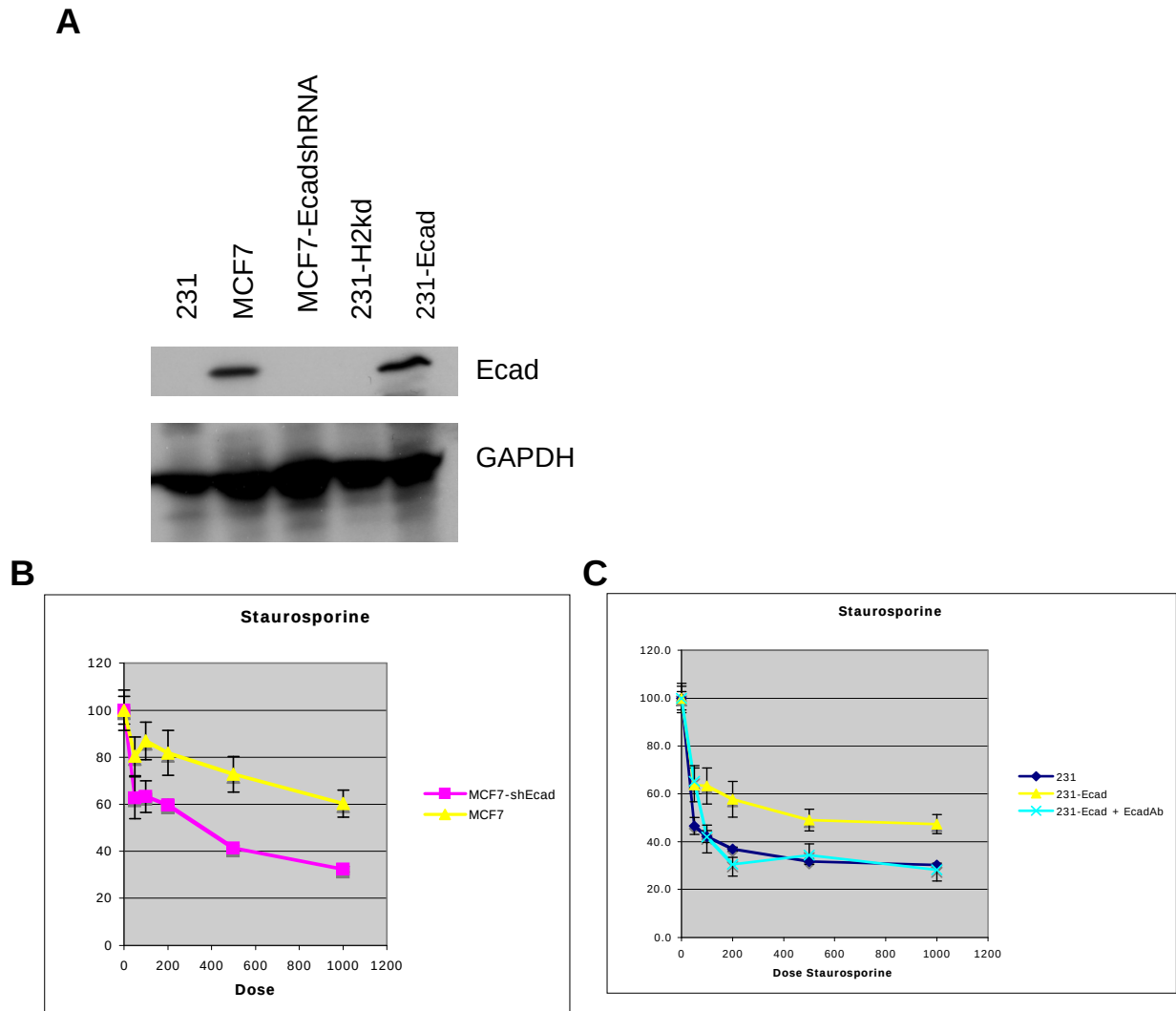


Figure 4. A) Immunoblot evaluating the E-cadherin expression of various E-cadherin knock-in and knock-out mutants. B) MCF7 cells are more resistant to staurosporine-induced cell death compared to E-cadherin-negative MCF7-shEcad cells. B) 231-Ecad cells are more resistant than 231 or 231-Ecad cells treated with E-cadherin blocking antibody. To evaluate survival, cells were stained with calceinAM following staurosporine treatment and fluorescence intensity was analyzed using a Tecan microplate reader.

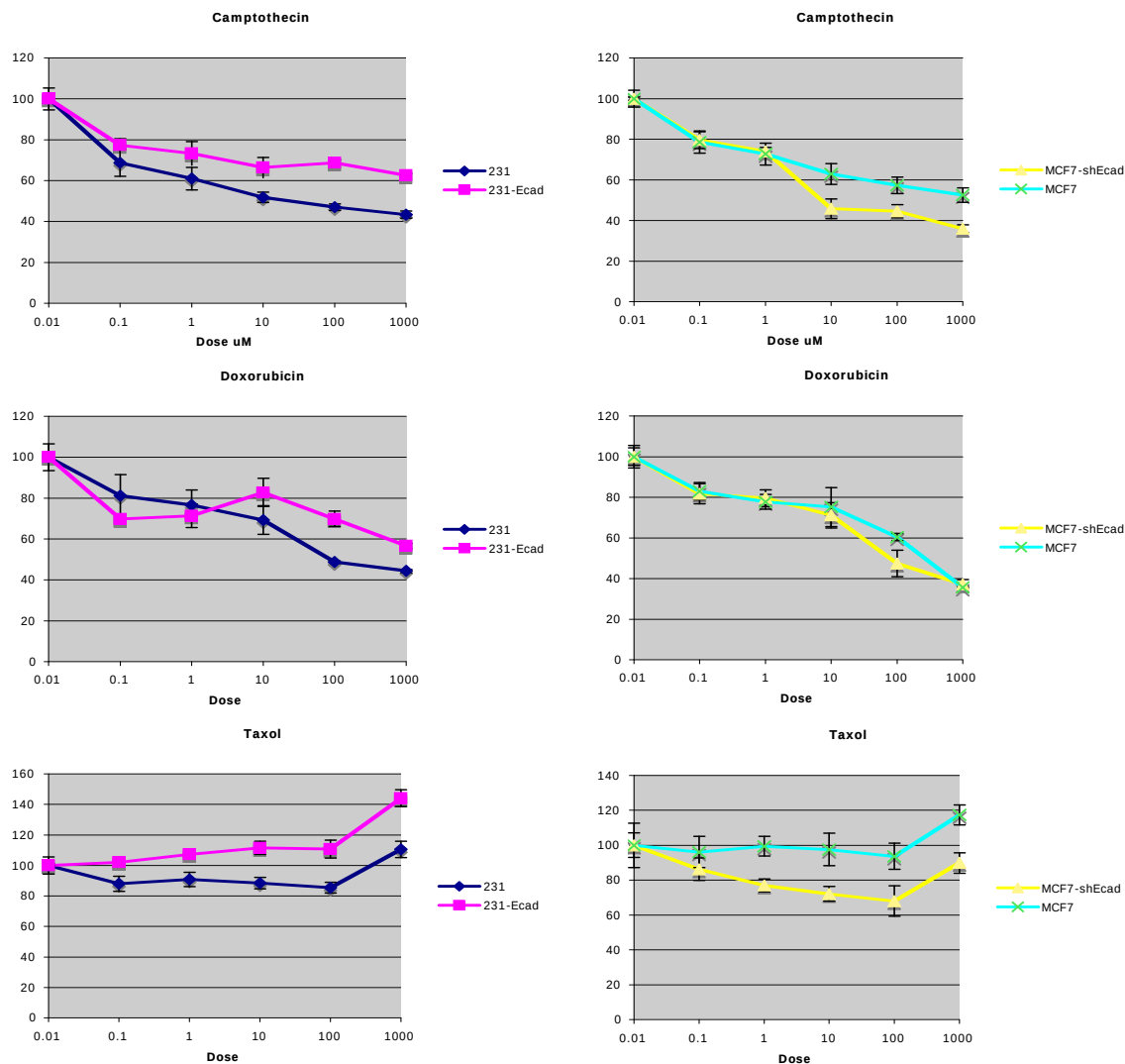


Figure 5. 231, 231-Ecad, MCF7, MCF7-shEcad were treated with camptothecin, doxorubicin, and taxol for 72 hours to determine whether cells expressing E-cadherin were more resistant to cell death. Following treatment with chemotherapy, cells were stained with calceinAM and the fluorescence intensity was detected using a microplate reader.

Task 2B. Evaluate the affect of E-cadherin re-expression on chemoresistance of breast cancer cells in 2D hepatocyte coculture. This task is completed. Given that expression of E-cadherin in breast cancer cells in the absence of hepatocytes protects against chemotherapy-induced cell death, the next step is to test whether re-expression of E-cadherin in the liver microenvironment also confers this survival advantage. MDA-MB-231 cells that have re-expressed E-cadherin following coculture with hepatocytes are more resistant to staurosporine and camptothecin-induced cell death compared to breast cancer cells cultured alone. 231-shEcad cells that are unable to re-express E-cadherin do not exhibit this increase in chemoresistance (Figure 6). Interestingly, the degree of protection due to E-cadherin expression seems to be greater following hepatocyte coculture. We hypothesize that during hepatocyte coculture, other molecular changes occur besides E-cadherin re-expression that provide for a more complete epithelial reversion than just E-cadherin expression alone.

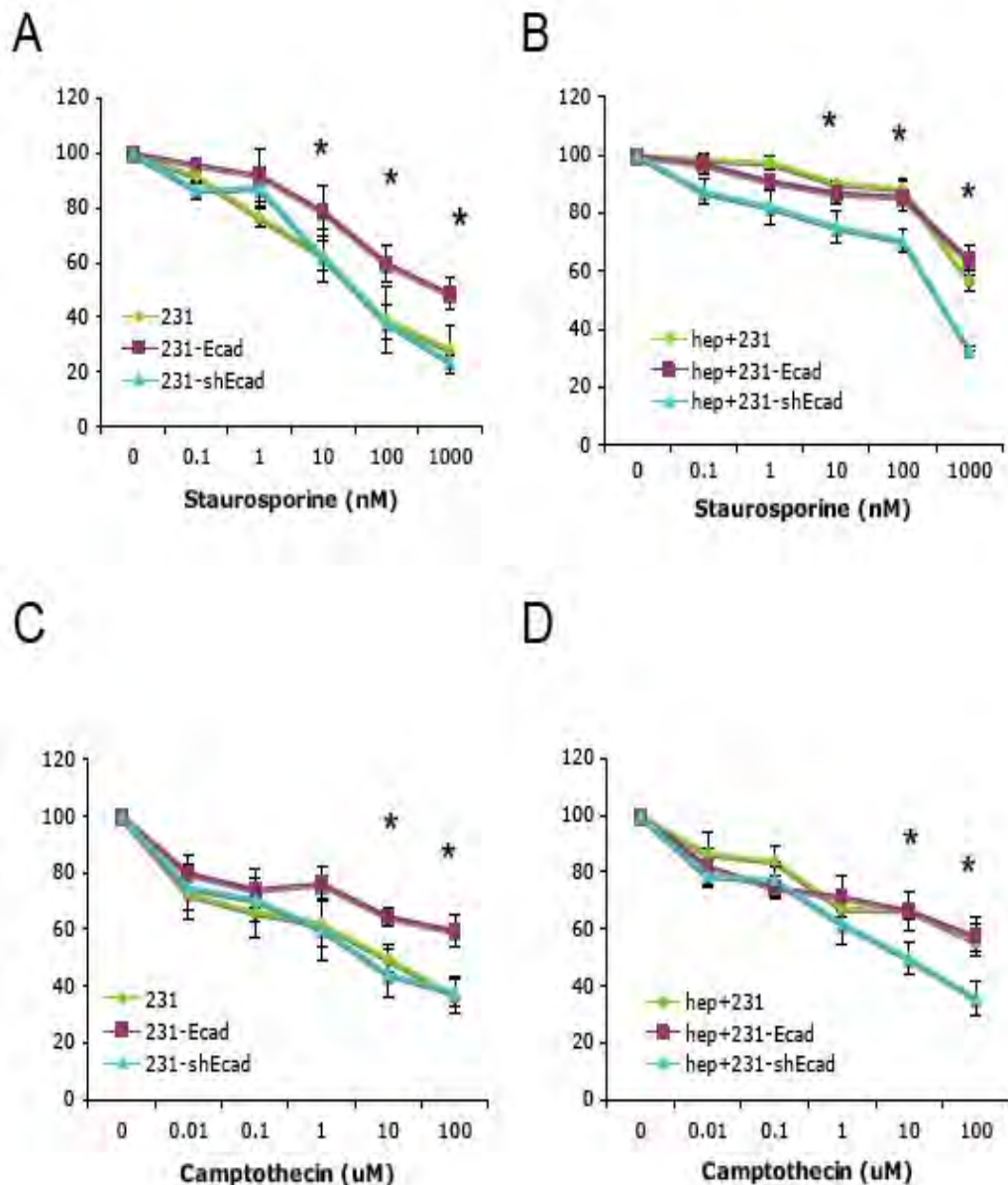


Figure 6. Exogenous and microenvironment-induced expression of E-cadherin in breast cancer cells increases the chemoresistance to staurosporine (A and B) and camptothecin (C and D).

Task 2C. Evaluate the affect of E-cadherin re-expression on chemoresistance of breast cancer cells in 3D hepatocyte spheroid culture. This task is completed. Similar to the 2D coculture experiments, we are still optimizing the best way to measure cancer cell survival in the cocultures without dissociating the two cell populations. Results from one experiment in which 231 and 231-shEcad cells were cocultured in the bioreactor for 6 days and then treated with staurosporine suggest that there is protection due to E-cadherin re-expression (Figure 7). The chemoprotection is greater than the protection observed in 2D coculture, suggesting that the 3D microenvironment of the bioreactor may result in a more complete reversion of phenotype.

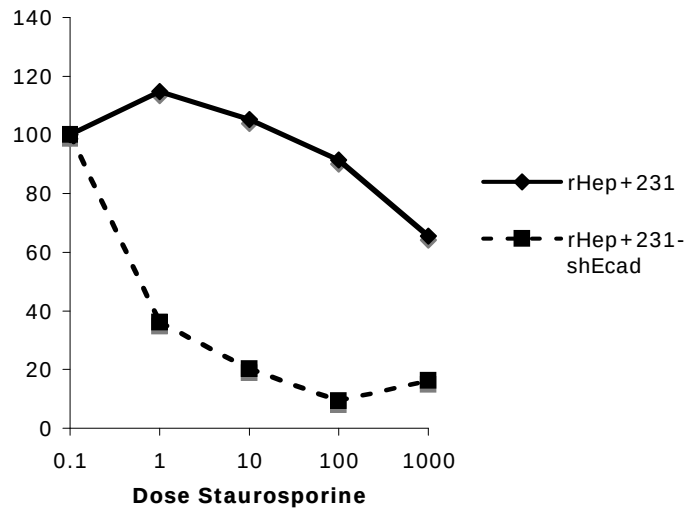


Figure 7. Bioreactor was seeded with rat hepatocytes and then seeded with 231-RFP or 231-shEcad-RFP cells 48 hours later. Following 6 days of coculture in the bioreactor, wells were treated with various doses of staurosporine for 24 hours. Bioreactor scaffolds were then removed with hepatocyte-cancer cells spheroids intact and RFP fluorescence was analyzed by microplate reader.

KEY RESEARCH ACCOMPLISHMENTS

1. When cultured in a liver microenvironment (both 2D and 3D), MDA-MB-231 cell can re-express E-cadherin. To initiate metastasis, breast cancer cells often epigenetically repress expression of E-cadherin; however, our results suggest that once the cells reach the liver, they may continue to undergo molecular changes that result in E-cadherin re-expression.
2. Cancer cells that express E-cadherin are more resistant to cell death induced by chemotherapies that are currently used to treat breast cancer. Our 2D and 3D coculture experiments suggest that the re-expression of E-cadherin in the liver protects against chemotherapeutic insult, providing an explanation for why metastases are commonly resistant to chemotherapy.

REPORTABLE OUTCOMES

Publications

1. **Chao Y**, Wu Q, Shepard C, and Wells A. "Hepatocyte induced re-expression of E-cadherin in breast and prostate cancer cells increases survival and chemoresistance." Submitted to Clinical and Experimental Metastasis (Appendix 1)
2. **Chao Y**, Wu Q, Acquafondata M, Dhir R, and Wells A. "Partial Mesenchymal to Epithelial Reverting Transition in Breast and Prostate Cancer." Submitted to Cancer Microenvironment (Appendix 2)
3. **Chao Y***, Shepard CR*, Wells A (2010). Breast carcinoma cells re-express E-cadherin during mesenchymal to epithelial reverting transition. Mol Cancer. Jul;9(1):179. (Appendix 3)

Reviews

1. Wells A, **Chao Y**, Grahovac J, Wu Q, Lauffenburger DA (2010). Cell motility in carcinoma metastasis as modulated by switching between epithelial and mesenchymal phenotypes. Frontiers in Biosciences.

Book Chapters

1. Wells A, **Chao Y**, Wu Q (2009). Biology of Cancer Metastases to the Liver. In Molecular Pathology of Liver Diseases (Ed: PS Monga, Springer Press).

Oral Presentations

1. **Chao Y** and Wells A "Evaluation of Mesenchymal to Epithelial Reverting Transition Markers in Human Primary Breast Carcinomas and Metastases." Academy of Clinical Laboratory Physicians and Scientists. Redondo Beach, PA. June 2009.
2. **Chao Y**, Shepard CR, Wells, A. "E-cadherin expression as a survival mechanism for breast cancer metastasis." ACLPS. Philadelphia, PA. June 2008.

Abstracts

1. **Chao Y** and Wells A. "E-cadherin expression as a chemotherapy resistance mechanism in metastatic breast cancer." American Society for Clinical Investigation. Chicago, IL. April 2010.
2. **Chao Y** and Wells A. "E-cadherin re-expression affects the growth and survival of breast cancer cells in metastatic colonization of the liver." American Association for Cancer Research. Washington, DC. April 2010.
3. **Chao Y** and Wells, A. "Re-expression of E-cadherin on metastatic breast cancer cells in the liver microenvironment." National MD/PhD Meeting. Keystone, CO. July 2009.
4. **Chao Y** and Wells A. "Re-expression of E-cadherin on metastatic breast cancer cells in the liver microenvironment." American Association for Cancer Research. Denver, CO. April 2009.

Awards

American Physician Scientists Association Travel Award, 2011
American Society for Clinical Pathology Award for Academic Excellence and Achievement, 2010
McGowan Trainee Career Advancement Program Travel Scholarship, 2010
Scholar-in-training Award, America Association of Cancer Research, 2010
Young Investigator Award, Academy of Clinical Laboratory Physicians and Scientists, 2009
Scholar-in-training Award, America Association of Cancer Research, 2009
Science Symposium Best Graduate Student Poster Award, University of Pittsburgh, 2008
Young Investigator Award, Academy of Clinical Laboratory Physicians and Scientists, 2008

CONCLUSIONS

With the end of the second year of the award, significant progress has been made toward completion of the aims outlined in the proposal. The first aim has been completed, with multiple lines of evidence showing that when breast cancer cells are cultured in a liver microenvironment, they can re-express E-cadherin. In addition, progress into the second aim suggests that the re-expression of E-cadherin in hepatocyte coculture may have functional significance in protecting the metastatic cancer cells from cell death. These results are important because not only do they further what is currently known about cancer pathogenesis but they also have clinical implications. A plethora of studies have implicated loss of E-cadherin and EMT as an initiator of metastasis but re-expression of E-cadherin and EMT may be necessary for colonization in the liver. As for the clinical implications, liver metastases are rarely surgically resected – instead they are treated with systemic chemotherapeutic drugs, many of which were tested in our studies. Our findings that breast cancer cells express E-cadherin in the liver and that E-cadherin expressing cells are more resistant to chemotherapy may therefore explain the chemoresistance of metastases.

REFERENCES

1. Jemal, A, R Siegel, E Ward et al (2007) Cancer statistics. CA Cancer J Clin 57(1): 43-66.
2. Gonzalez-Angulo, A, F Morales-Vasquez, and G Hortobagyi (2007) Overview of resistance to systemic therapy in patients with breast cancer. Adv Exp Med Biol (608): 1-22.
3. Kowalski, PJ, MA Rubin, and CG Kleer (2003) E-cadherin expression in primary carcinomas of the breast and its distant metastases. Breast Cancer Res 5(6): R217-22.
4. Bukholm, IK, JM Nesland, and AL Borresen-Dale (2000) Re-expression of E-cadherin, alpha-catenin and beta-catenin, but not of gamma-catenin, in metastatic tissue from breast cancer patients [see comments]. J Pathol 190(1): 15-9.
5. Graff, JR, E Gabrielson, H Fujii et al (2000) Methylation patterns of the E-cadherin 5' CpG island are unstable and reflect the dynamic, heterogeneous loss of E-cadherin expression during metastatic progression. J Biol Chem 275(4): 2727-32.
6. Wang, L, Z Li, C Wang et al (2009) E-cadherin decreased human breast cancer cells sensitivity to staurosporine by up-regulating Bcl-2 expression. Arch Biochem Biophys 481(1): 116-22.

Appendix 1: Manuscript submitted to *Clinical and Experimental Metastasis*

Hepatocyte induced re-expression of E-cadherin in breast and prostate cancer cells increases survival and chemoresistance

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Running title: Mesenchymal to Epithelial reverting Transition Protects Carcinoma Cells

Key words: Epithelial-to-Mesenchymal Transition, Mesenchymal-to-Epithelial reverting Transition

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ABSTRACT

Post-extravasation survival is a key rate-limiting step of metastasis; however, not much is known about the factors that enable survival of the metastatic cancer cell at the secondary site. Furthermore, metastatic nodules are often refractory to current therapies, necessitating the elucidation of molecular changes that affect the chemosensitivity of metastases. Drug resistance exhibited by tumor spheroids has been shown to be mediated by cell adhesion and can be abrogated by addition of E-cadherin blocking antibody. We have previously shown that hepatocyte coculture induces the re-expression of E-cadherin in breast and prostate cancer cells. In this study, we show that this E-cadherin re-expression confers a survival advantage, particularly in the liver microenvironment. E-cadherin re-expression in MDA-MB-231 breast cancer cells resulted in increased attachment to hepatocytes. This heterotypic adhesion between cancer cells and secondary organ parenchymal cells activated ERK MAP kinase, suggesting a functional pro-survival role for E-cadherin during metastatic colonization of the liver. In addition, breast and prostate cancer cells that re-expressed E-cadherin in hepatocyte coculture were more chemoresistant compared to 231-shEcad cells unable to re-express E-cadherin. Similar results were obtained in DU-145 prostate cancer cells induced to re-express E-cadherin in hepatocyte coculture or following chemical induction by EGFR inhibitors buserelin and PD153035. These results suggest that E-cadherin re-expression and other molecular changes imparted by a partial mesenchymal to epithelial reverting transition at the secondary site increase post-extravasation survival of the metastatic cancer cell and may help to elucidate why chemotherapy commonly fails to treat metastatic breast cancer.

INTRODUCTION

Approximately one-third of breast cancer patients will present with distant, non-nodal metastases, and as high as 60-70% of those patients will develop metastases in the liver [1, 2]. Breast cancer that metastasizes to the liver carries a very poor prognosis, with the median survival around 24 months [3]. Only 5% of patients with liver metastases present with a singular nodule; thus, surgical resection is not an option for most. Current treatment for liver metastases relies on a multi-modal approach of systemic chemotherapy, endocrine- or HER2-targeted therapy if dictated by ER/PR/HER2 status, and palliative therapy such as radiation [4]. Poor response to chemotherapy is a major reason for the high mortality for breast cancer patients with liver metastases, and for all metastatic cancer patients in general. Elucidating the mechanisms behind chemoresistance in metastasis is therefore valuable for developing more effective therapies.

Just as not much is known about why metastases are refractory to chemotherapy, little is known about the molecular mechanisms controlling metastatic colonization of the liver. The liver is a major organ site for cancer metastases, so much so that liver metastases are more common than primary hepatic tumors [5]. A few of the cancers that exhibit organotropism to the liver include breast, prostate, and colorectal carcinomas[6]. Lumen occlusion or mechanical arrest in the first capillary bed encountered is insufficient for liver colonization [7, 8]. Selective cellular adhesion accounts for some of the organotropism exhibited by cancers, as cancer cell line variants that exhibit increased liver metastasis potential show increased adhesion to embryonic mouse liver cells [9]. Similarly, loss of claudins is associated with EMT whereas the upregulation of other tight junction components occurs in liver metastases. In vivo selection for a liver-aggressive variant of 4T1 breast cancer cells reveals that claudin-2 is upregulated in liver metastases and improves adhesion of the liver-aggressive cells to fibronectin and collagen IV, key components of the liver extracellular matrix (ECM) [10]. Selectins are a family of cell adhesion molecules that are differentially expressed on the vascular endothelial cells of various organs; colon cancer cells express different selectin ligands to adhere to particular organs [11, 12]. Expression of the epithelial-marker and cell adhesion molecule E-cadherin on breast

cancer cells may be another mechanism to facilitate adhesion to hepatocytes, E-cadherin expressing parenchymal cells that account for 70-80% of the liver. Importantly, of the 4T1-derived cell lines with varying metastatic ability, only the 4T1 cells that express E-cadherin are able to form liver, lung, bone, and brain metastases while the E-cadherin-negative cell lines form only primary tumors [13, 14].

Besides mediating physical adhesion to organ parenchymal cells to facilitate colonization, expression of E-cadherin is also associated with cell survival. Although lacking intrinsic kinase activity, E-cadherin contributes to cell signaling through transactivation of EGFR. Expression of E-cadherin on hepatocyte spheroids in culture protects against detachment-induced cell death, or anoikis, in a caspase-independent manner [15]. Similarly, endocytosis of E-cadherin induced by EGFR activation leads to anoikis of enterocytes [16]. The assembly of adherens junctions coordinated by E-cadherin ligation quickly leads to sustained activation of MAPK and Akt, signaling pathways critical for cell survival [17, 18]. The related cadherin family member VE-cadherin likewise controls endothelial cell survival through signaling through Akt and Bcl-2 [19]. Thus, breast cancer cells may activate survival signaling through heterotypic ligation with hepatocytes.

We have shown previously that the liver microenvironment induces the re-expression of E-cadherin in breast and prostate cancer cells [20, 21]. Thus the aim of this study was to determine whether there is a functional significance to E-cadherin re-expression. We show that E-cadherin promotes attachment to the secondary organ through heterotypic ligation, resulting in the activation of survival signaling. Furthermore, E-cadherin re-expression also confers a survival advantage by increasing the resistance of breast and prostate cancer cells to chemotherapy-induced cell death in the liver microenvironment.

RESULTS

E-cadherin expression affects survival through heterotypic adhesion of breast cancer cells to hepatocytes

E-cadherin-negative MDA-MB-231 breast cancer and DU-145 prostate cancer cells re-express E-cadherin and revert to an epithelial morphology when cocultured with rat hepatocytes, a cell culture model for the liver microenvironment [20, 21]. This also happens upon coculture with lung parenchymal cells [22]. As mediating intercellular adhesion is a major function of E-cadherin, we hypothesized that post-extravasation survival of cancer cells at the secondary site is facilitated by heterotypic adhesion between cancer cells and organ parenchymal cells. To probe this role we used previously characterized E-cadherin knock-in and knock-down lines and derivatives: E-cadherin-negative MDA-MB-231 cells (231), MDA-MB-231 cells that exogenously express E-cadherin (231-Ecad), MDA-MB-231 cells stably expressing E-cadherin shRNA (231-shEcad), E-cadherin-positive MCF7 cells, and MCF7 cells stably expressing E-cadherin shRNA (MCF7-shEcad). All cell lines were RFP-labeled to facilitate detection of cancer cells in hepatocyte coculture. When cocultured with human hepatocytes for 6 days, 231 cells reverted to an epithelial morphology and re-expressed E-cadherin (Figure 1) (similar reversion is noted with rat hepatocytes, data not shown). In contrast, an analogous phenotypic change was not observed in cocultured 231-shEcad cells. The phenotypic effect of this change is mirrored in the cell distribution pattern in which the E-cadherin-expressing cells (231-Ecad, and 231 after coculture) cluster, suggesting cell-cell contacts, whereas the E-cadherin-negative cells (231-shEcad) remain as single cells interspersed among the hepatocytes. The three cell lines were also cocultured with primary human fibroblasts. Following 6 days of fibroblast coculture, 231 cells remained mesenchymal in phenotype and singularly interspersed (Figure 2). These cells remained E-cadherin negative, demonstrating that the re-expression is dependent on the hepatocytes (Figure 2).

To test whether attachment to hepatocytes is dependent on E-cadherin expression, hepatocytes were plated on collagen-coated plates at 30% confluency and cancer cells were seeded onto the monolayer the following day. 24 hours later, the number of RFP-positive cells in the monolayer was counted as a measure of attachment. The E-cadherin-positive 231-Ecad and MCF7 cell lines exhibited twice the number of adherent cancer cells compared to E-cadherin-negative cell lines (Figure 3a). However, it was possible that the differences in attachment were not entirely E-cadherin dependent, as the plating of hepatocytes at 30% confluency left portions of the collagen-coated plastic exposed. As a result, the cell lines were plated on differing hepatocyte densities ranging from 25 to 100% confluency. Thus, at higher hepatocyte densities attachment could only be generated by cancer cell adhesion to the hepatocyte monolayer. As expected, the ability of E-cadherin-positive 231-Ecad and MCF7 cells to attach was not affected by hepatocyte density while attachment of E-cadherin-negative 231 and MCF7-shEcad cells decreased with increasing hepatocyte density (Figures 3b and 3c). Re-expression of E-cadherin after hepatocyte coculture also increased the attachment of 231 cells to hepatocytes. While lack of E-cadherin expression initially impeded the ability of 231 cells to attach to hepatocytes, re-expression of E-cadherin in 231 cells following 6 days of hepatocyte coculture increased attachment, as measured by a centrifugal assay for fluorescence-based cell adhesion (CAFCA) (Figure 3d). Thus, the re-expressed E-cadherin was capable of establishing cell-heterotypic cell-cell adhesions. Control experiments using MCF7 cells revealed that the heterotypic attachment between breast cancer cells and hepatocytes is E-cadherin dependent, as addition of blocking antibody SHE78, calcium chelator EDTA, and E-cadherin siRNA all limited cell binding to hepatocytes as assessed by CAFCA (Supplemental Figure 1).

E-cadherin homotypic ligation activates survival signaling pathways [17, 18], so next we queried whether heterotypic ligation between breast cancer cells and hepatocytes resulted in similar activation. To isolate signaling only occurring in the breast cancer cells (apart from the cognate hepatocyte partner), hepatocyte membranes were isolated and adsorbed onto culture plates and labeled with Dil (Supplemental Figure 2a). Activation of the Erk MAP kinase pathway was probed

after MCF7 cells and MDA-MB-231 cells cultured with and without hepatocytes for 6 days were plated onto hepatocyte membranes. Maximal phospho-Erk expression was detected 30 minutes after plating E-cadherin-positive MCF7 cells onto hepatocyte membranes (Figure 4a). Erk activation was not observed in E-cadherin-negative 231 cells cultured in the absence of hepatocytes, but was observed 30 minutes after addition of E-cadherin re-expressing 231 cells (Figure 4b). Activation of Erk signaling was dependent on E-cadherin ligation as addition of E-cadherin blocking antibody SHE78 blocked the increase in pErk (Figure 4b). Heterotypic ligation of MCF7 cells and hepatocytes also activated Akt (Supplemental Figures 2c and 2d), suggesting that survival pathways in addition to Erk MAP kinase may be involved.

E-cadherin expression increases chemoresistance of breast and prostate cancer cells

Multiple studies have shown that E-cadherin protects against cell death and increases drug resistance of tumors [23-25]. Treatment of breast cancer cells with the protein kinase inhibitor staurosporine and chemotherapeutic drug camptothecin showed that 231-Ecad cells were less sensitive to cell death induced by these agents compared to E-cadherin negative 231 and 231-shEcad cells (Figures 5a and 5c). Addition of E-cadherin antibody abrogated the effect on 231-Ecad cells (Supplemental Figure 3). Similar results were observed in breast cancer cells treated with other chemotherapeutic drugs taxol and doxorubicin (data not shown).

To determine whether this chemoprotection is unique to breast cancer cells, we corroborated these results in prostate cancer cells chemically induced to express E-cadherin. We have previously shown that prostate cancer cells also re-express E-cadherin upon coculture [20, 22], or even just repression of EGFR signaling [20, 26]. DU-145 prostate cancer cells were treated with 1 μ g/ml of the luteinising hormone-releasing hormone (LHRH) analog buserelin or 500nM EGFR kinase inhibitor PD153035 for 48 hours. Treatment with these agents resulted in re-expression of E-cadherin and an epithelial cluster morphology (Figures 6a and 6b). Following E-cadherin re-expression induced by these agents, DU-145 cells were more resistant to cell death induced by staurosporine and

camptothecin (Figures 6c and 6d). The small degree of protection is explained by the fact that not all of the prostate cancer cells re-express E-cadherin under the treatment.

E-cadherin re-expression in the liver microenvironment increases the chemoresistance of breast and prostate cancer cells

The above provides a proof of concept of chemoprotection by E-cadherin, one that is consistent with literature reports [25, 27]. However, the extent of chemoprotection is modest, but this could simply be due to the artificial and limited extent of epithelial reversion based solely on E-cadherin re-expression. Thus, we tested whether similar chemoprotection could be effected in the liver microenvironment. On day 6 of hepatocyte coculture, breast and prostate cancer cells were treated with staurosporine and camptothecin and the number of surviving RFP-positive cells were counted after a further 24 (staurosporine) or 48 hours (camptothecin). E-cadherin re-expression in hepatocyte coculture increased the chemoresistance of 231 cells to 231-Ecad levels, while 231-shEcad cells unable to re-express E-cadherin remained the most sensitive (Figure 5b and 5d). Interestingly, overall the breast cancer cells were less sensitive to staurosporine treatment in hepatocyte coculture as the IC₅₀ was 10 fold higher in coculture, which may be explained by molecular changes besides E-cadherin re-expression that allow for a more complete reversion to the epithelial phenotype not observed when only E-cadherin is exogenously expressed.

DU-145 prostate cancer cells induced to re-express E-cadherin in the liver microenvironment also exhibited increased resistance to cell death (Figures 7a and 7b). This increase in chemoresistance was abrogated when DU-145 cells were transiently transfected with E-cadherin siRNA prior to coculture. Because primary isolation of hepatocytes often includes fibroblasts and other non-parenchymal cells, to show that this protective effect was mediated by E-cadherin re-expression induced by the hepatocytes, the chemosensitivity of prostate cancer cells following coculture with fibroblasts was also tested. Following staurosporine and camptothecin treatment, the

level of chemosensitivity of DU-145 cells cocultured with fibroblasts was similar to DU-145 cells cultured in the absence of hepatocytes.

There remains the question of whether the chemoprotection noted in the presence of the liver microenvironment is due to metabolism of the agents by the hepatocytes. It should be noted that hepatocytes in two-dimensional culture, as performed here in the cocultures, lose metabolic capacity over time with little remaining after 6 days [28-30] and therefore would not likely be active metabolizers. Still, this needed to be addressed experimentally. The prostate carcinoma cells were cocultured with hepatocytes isolated in a transwell system, which does not allow for epithelial reversion. In this situation, there was no evidence of chemoprotection (Figures 7c and 7d).

DISCUSSION

Alterations in adhesion have been shown to be necessary for many steps of metastasis, from down-regulation of E-cadherin in EMT during invasion to expression of selectin ligands or gap junction molecules for adherence to endothelial cells during extravasation [7, 9, 12, 31]. We have shown previously that metastatic tumors from breast and prostate cancer patients express increased levels of E-cadherin compared to the primary tumor, which is accompanied by a partial mesenchymal to epithelial reverting transition [20, 21, 32]. Furthermore, E-cadherin re-expression is also observed when cultured in a liver microenvironment in vitro and in lung metastases in an in vivo animal model [21]. Our findings herein show that the functional significance of E-cadherin expression in metastases may be to increase attachment and integration within organ parenchyma, and to subsequently increase post-extravasation survival through E-cadherin-mediated survival signaling. Besides physical intercellular adhesion, E-cadherin engagement also activates internal signaling pathways that promote survival through suppression of anoikis and canonical Erk and Akt pathways [17, 18]. E-cadherin binding of epithelial cells has also been shown to promote survival in a PI-3K –dependent fashion [33]. The finding that Erk is phosphorylated upon binding to hepatocytes by re-expressed E-cadherin on MDA-MB-231 cells implies that relevant functional signaling occurs as a result of heterotypic ligation between cancer cells and organ parenchymal cells.

A critical result of this reversion to a more epithelial phenotype is the resistance to induced cell death. Previous studies have shown the protective role of E-cadherin in the face of chemotherapy and our studies corroborate these results [23]. Of particular interest is the finding that breast and prostate carcinoma cells in hepatocyte coculture were more resistant to cell death-induced by staurosporine or camptothecin compared to cells cultured in the absence of hepatocytes. This is not due to hepatocyte metabolism of agents independent of the phenotypic reversion as shRNA to E-cadherin blunts this coculture protection, and coculture without physical juxtaposition, which does not alter the carcinoma cell phenotype, did not confer chemoprotection. While it remains to be experimentally dissected, we propose that the normal parenchymal cells induce a more complete

phenotypic shift. We have shown evidence that a partial mesenchymal to epithelial reversion occurs in human breast and prostate cancer metastases, suggesting that the liver microenvironment can induce other molecular changes besides E-cadherin expression during partial MErT [21, 32]. One such change can be re-expression of the gap junction protein connexins, which are frequently downregulated in EMT and have been shown to be upregulated in lymph node metastases; hepatocyte coculture induces re-expression of connexin43 in breast cancer cells (data not shown). Brain metastases of breast cancer patients exhibit increased expression of E-cadherin, Cx43 and Cx 26 [32]. A recent study showed that astrocyte-cancer cell interactions mediated by gap junction expression protects cancer cells from chemotherapy-induced cell death [34, 35]. Thus adhesion, facilitated by gap junctions in this case, promotes the survival of cancer cells during metastatic colonization.

The functional mechanisms behind the increased chemoresistance in E-cadherin re-expressing cells in our model are still unknown. Pro-survival pathways such as Erk MAP kinase and Akt are noted as activated upon E-cadherin re-expression. Akt signaling also contributes to chemoresistance [36]. Other possible molecular mechanisms behind the chemoresistance include upregulation of anti-apoptotic proteins such as Bcl-2 or cell cycle inhibitors cyclin-dependent kinase inhibitor p27 [25, 37]. Another potential explanation for the increased chemoresistance is contact mediated growth inhibition governed by E-cadherin [38]; however, growth inhibition of MDA-MB-231 cells upon re-expression of E-cadherin was not observed in hepatocyte coculture (data not shown). Molecular dissection of the operative pathways underlying this chemoprotection lies beyond the scope of the present manuscript, but remains a key area for further investigation.

Also remaining is the question of whether E-cadherin expression is required for the initial establishment of metastases. E-cadherin re-expression could explain the propensity for breast cancer cells to metastasize to lung and liver, both lined with epithelial cells expressing this cell recognition molecule. In support of a proposed cell-cell recognition moiety is that fact that aberrant expression of osteoblast cadherin, also known as OB-cadherin and cadherin-11, on breast and prostate cancer

cells, increases metastases to the bone by increasing migration and intercalation with osteoblasts [39, 40]. It is also possible that the chemoprotection conferred by E-cadherin re-expression and ligandation also promotes the survival of disseminated carcinoma cells in the face of a challenging ectopic environment or any intrinsic inflammatory response upon metastatic seeding.

This transitional step opens the role of phenotypic plasticity in tumor progression and the metastatic cascade. It is well-established that E-cadherin functions as a 'tumor suppressor' and its forced expression limits metastatic dissemination. Thus, the ability of E-cadherin to support metastasis has been brought into question [41]. Of interest, the phenotypic reversion to a more-epithelial phenotype is driven by the receptive microenvironment. Coculture of cancer cells with normal fibroblasts failed to produce the epithelial reversion and concomitant re-expression of E-cadherin, further suggesting that the phenotypic changes of the cancer cell reflect the microenvironment. Thus, it is likely that premature expression of E-cadherin interferes with steps in the metastatic cascade and would only promote metastatic competency at a somewhat later stage of carcinoma cell survival in the face of hostile ectopic site. An inverse correlation of E-cadherin with size of metastases suggests that this phenotypic reversion is not stable, and would only be advantageous for small micrometastases [32].

There are several therapeutic implications raised by this study, even with a number of open questions as noted above. Expressing E-cadherin or attempting to revert carcinoma phenotype towards a more epithelial state, while limiting escape from the primary tumor site, may perversely improve metastatic competency of the multitude of shed cells. On the other hand, downregulating E-cadherin would likely make the carcinomas more invasive and aggressive. As metastases constitute the major part of carcinoma mortality, new approaches should target the micrometastases to kill them prior to frank metastatic disease. Thus, the survival signals activated upon heterotypic E-cadherin ligation or the as yet unknown microenvironmental cues that initially induce expression of E-cadherin in the secondary organ may thus be the more effective therapeutic targets.

MATERIALS AND METHODS

Cell lines and cell culture

231-RFP, 231-Ecad-RFP, and 231-shEcad-RFP breast cancer cells and DU-145 prostate cancer cells were cultured in RPMI as previously described [21]. Human fibroblasts 10-1169F were cultured in DMEM.

Coculture

Primary rat and human hepatocytes were isolated and plated at 4×10^5 cells per well in 6-well plates coated with 10% rat tail collagen in dH₂O (BD Biosciences) at 30% confluency and allowed to attach overnight. The next day, 2×10^4 RFP-labeled cancer cells were seeded onto hepatocyte monolayers. Rat cocultures were maintained in Hepatocyte Growth Media (HGM) and human hepatocytes were maintained with Hepatocyte Maintenance Media (Lonza). For fibroblast cocultures, the fibroblast monolayer was initially plated at 1×10^5 cells per well in 6-well plates and seeded with 2×10^4 the following day. Media was replenished daily. For transwell coculture, inserts (Millipore) was coated with 10% rat tail collagen and plated with hepatocytes at 4×10^5 cells per insert. Cancer cells were seeded with 2×10^4 in the bottom chamber the following day. Cells were treated or collected for analysis after 5-day transwell coculture.

Chemical re-expression of E-cadherin

DU-145 cells were seeded in 96-well plates and treated with 1 μ g/ml buserelin or 500nM PD153035 for 48hrs. Immunoblot and immunofluorescence to confirm E-cadherin expression was performed using E-cadherin antibody (Cell Signaling).

Attachment assay

Primary hepatocytes were plated at densities ranging from 25-100% confluency on collagen-coated 6-well plates and allowed to attach overnight. The next day, 2×10^4 RFP-labeled cancer cells were

seeded in each well. 24 hours later, wells were washed once with PBS to remove any unattached cells and the number of RFP+ cells in each well was quantified.

Centrifugal assay for fluorescent cell adhesion (CAFCA)

This assay is a modification of the McClay and Giaccolmello assays (McClay, Wessel et al. 1981). Cancer cells were non-enzymatically dissociated and labeled with 5 M Calcein AM (Molecular Probes, Carlsbad, CA, USA). Labeled cancer cells were seeded at a density of 42 000 cells well in 96-well plates containing a densely confluent hepatocyte monolayer. The plates were centrifuged for <60s at 50g to pellet the cancer cells onto the hepatic monolayer, then incubated at 37°C. At defined times, the plates were inverted and centrifuged at 600g for 5 min and then gently washed to remove unbound cells from the hepatocyte monolayer. Fluorescence was measured with a 494/517 bandpass filter set-up from the bottom of the plate by a TECAN Spectra-Fluor plate fluorometer. Absolute emission measurements were background subtracted.

Chemoprotection assay

For cell death assays in the absence of hepatocytes, breast and prostate cancer cells were seeded in 96-well plates and treated with 0 to 1000nM of staurosporine for 24 hours or 0 to 100μM of camptothecin for 48 hours. Wells were then stained with 1uM calcein AM for 30 minutes and fluorescence was quantified with Tecan Spectrafluor. In the presence of hepatocytes, following induction of cell death with staurosporine or camptothecin, the number of RFP+ cells in each well was counted.

Hepatocyte membrane assay

Culture plates were coated with poly-L-lysine (Sigma) and hepatocyte membranes (2 mg protein/cm²) were allowed to adsorb onto poly-L-lysine-coated 6-well plates for 10 minutes. Hepatocyte membranes were labeled with Dil (Molecular Probes) for visualization. MDA-MB-231 cells were

sorted from hepatocyte cocultures and quiesced in serum-free media for 3 hours, then seeded 2E4 cells onto the membrane coated plates and centrifuged at 50g for 1 minute. RIPA lysates were taken at each time point and pErk (Santa Cruz Biotech) was detected by immunoblot.

REFERENCES

1. Viadana E, Bross ID, Pickren JW. An autopsy study of some routes of dissemination of cancer of the breast. *Br J Cancer* 1973; 27 (4): 336-40.
2. Lermite E, Marzano E, Chereau E, et al. Surgical resection of liver metastases from breast cancer. *Surg Oncol* 2010; 19 (4): e79-84.
3. Atalay G, Biganzoli L, Renard F, et al. Clinical outcome of breast cancer patients with liver metastases alone in the anthracycline-taxane era: a retrospective analysis of two prospective, randomised metastatic breast cancer trials. *Eur J Cancer* 2003; 39 (17): 2439-49.
4. Diamond JR, Finlayson CA, Borges VF. Hepatic complications of breast cancer. *Lancet Oncol* 2009; 10 (6): 615-21.
5. Wells A, Chao Y, Wu Q. Biology of Metastatic Liver Tumors. In Monga SPS (ed): *Molecular Pathology of Liver Diseases*: Springer US 2011; 859-66.
6. Viadana E, Cotter R, Pickren JW, et al. An autopsy study of metastatic sites of breast cancer. *Cancer Res* 1973; 33 (1): 179-81.
7. Gassmann P, Hempling-Bovenkerk A, Mees ST, et al. Metastatic tumor cell arrest in the liver-lumen occlusion and specific adhesion are not exclusive. *Int J Colorectal Dis* 2009; 24 (7): 851-8.
8. Glinskii OV, Huxley VH, Glinsky GV, et al. Mechanical entrapment is insufficient and intercellular adhesion is essential for metastatic cell arrest in distant organs. *Neoplasia* 2005; 7 (5): 522-7.
9. McGuire EJ, Mascali JJ, Grady SR, et al. Involvement of cell-cell adhesion molecules in liver colonization by metastatic murine lymphoma/lymphosarcoma variants. *Clinical and Experimental Metastasis* 1984; 2 (3): 213-22.
10. Tabaries S, Dong Z, Annis MG, et al. Claudin-2 is selectively enriched in and promotes the formation of breast cancer liver metastases through engagement of integrin complexes. *Oncogene* 2010.
11. Mannori G, Crottet P, Cecconi O, et al. Differential colon cancer cell adhesion to E-, P-, and L-selectin: role of mucin-type glycoproteins. *Cancer Res* 1995; 55 (19): 4425-31.
12. Paschos KA, Canovas D, Bird NC. The role of cell adhesion molecules in the progression of colorectal cancer and the development of liver metastasis. *Cell Signal* 2009; 21 (5): 665-74.
13. Lou Y, Preobrazhenska O, auf dem Keller U, et al. Epithelial-mesenchymal transition (EMT) is not sufficient for spontaneous murine breast cancer metastasis. *Dev Dyn* 2008; 237 (10): 2755-68.
14. Aslakson CJ, Miller FR. Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. *Cancer Res* 1992; 52 (6): 1399-405.
15. Luebke-Wheeler JL, Nedredal G, Yee L, et al. E-cadherin protects primary hepatocyte spheroids from cell death by a caspase-independent mechanism. *Cell Transplant* 2009; 18 (12): 1281-7.
16. Lugo-Martinez VH, Petit CS, Fouquet S, et al. Epidermal growth factor receptor is involved in enterocyte anoikis through the dismantling of E-cadherin-mediated junctions. *Am J Physiol Gastrointest Liver Physiol* 2009; 296 (2): G235-44.
17. Pece S, Chiariello M, Murga C, et al. Activation of the protein kinase Akt/PKB by the formation of E-cadherin-mediated cell-cell junctions. Evidence for the association of phosphatidylinositol 3-kinase with the E-cadherin adhesion complex. *J Biol Chem* 1999; 274 (27): 19347-51.
18. Pece S, Gutkind JS. Signaling from E-cadherins to the MAPK pathway by the recruitment and activation of epidermal growth factor receptors upon cell-cell contact formation. *J Biol Chem* 2000; 275 (52): 41227-33.

19. Carmeliet P, Lampugnani MG, Moons L, et al. Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. *Cell* 1999; 98 (2): 147-57.
20. Yates CC, Shepard CR, Stolz DB, et al. Co-culturing human prostate carcinoma cells with hepatocytes leads to increased expression of E-cadherin. *Br J Cancer* 2007; 96 (8): 1246-52.
21. Chao YL, Shepard CR, Wells A. Breast carcinoma cells re-express E-cadherin during mesenchymal to epithelial reverting transition. *Mol Cancer* 2010; 9: 179.
22. Li M, Aliotta JM, Asara JM, et al. Intercellular transfer of proteins as identified by stable isotope labeling of amino acids in cell culture. *J Biol Chem* 2010; 285 (9): 6285-97.
23. St Croix B, Kerbel RS. Cell adhesion and drug resistance in cancer. *Curr Opin Oncol* 1997; 9 (6): 549-56.
24. Green SK, Francia G, Isidoro C, et al. Antiadhesive antibodies targeting E-cadherin sensitize multicellular tumor spheroids to chemotherapy in vitro. *Mol Cancer Ther* 2004; 3 (2): 149-59.
25. Wang L, Li Z, Wang C, et al. E-cadherin decreased human breast cancer cells sensitivity to staurosporine by up-regulating Bcl-2 expression. *Arch Biochem Biophys* 2009; 481 (1): 116-22.
26. Yates C, Wells A, Turner T. Luteinising hormone-releasing hormone analogue reverses the cell adhesion profile of EGFR overexpressing DU-145 human prostate carcinoma subline. *Br J Cancer* 2005; 92 (2): 366-75.
27. Guriec N, Marcellin L, Gairard B, et al. E-cadherin mRNA expression in breast carcinomas correlates with overall and disease-free survival. *Invasion Metastasis* 1996; 16 (1): 19-26.
28. Brown LA, Arterburn LM, Miller AP, et al. Maintenance of liver functions in rat hepatocytes cultured as spheroids in a rotating wall vessel. *In Vitro Cell Dev Biol Anim* 2003; 39 (1-2): 13-20.
29. Dash A, Inman W, Hoffmaster K, et al. Liver tissue engineering in the evaluation of drug safety. *Expert Opin Drug Metab Toxicol* 2009; 5 (10): 1159-74.
30. Powers MJ, Domansky K, Kaazempur-Mofrad MR, et al. A microfabricated array bioreactor for perfused 3D liver culture. *Biotechnol Bioeng* 2002; 78 (3): 257-69.
31. Gassmann P, Haier J. The tumor cell-host organ interface in the early onset of metastatic organ colonisation. *Clin Exp Metastasis* 2008; 25 (2): 171-81.
32. Chao YL, Wu Q, Acquafondata M, et al. Partial mesenchymal to epithelial reverting transition in breast and prostate cancer metastases [abstract nr. 3357]. In: *Proceedings of the 102nd Annual Meeting of the American Association for Cancer Research*; Orlando, FL: Philadelphia (PA): AACR 2011.
33. Bergin E, Levine JS, Koh JS, et al. Mouse proximal tubular cell-cell adhesion inhibits apoptosis by a cadherin-dependent mechanism. *Am J Physiol Renal Physiol* 2000; 278 (5): F758-68.
34. Langley RR, Fan D, Guo L, et al. Generation of an immortalized astrocyte cell line from H-2Kb-tsA58 mice to study the role of astrocytes in brain metastasis. *Int J Oncol* 2009; 35 (4): 665-72.
35. Lin Q, Balasubramanian K, Fan D, et al. Reactive astrocytes protect melanoma cells from chemotherapy by sequestering intracellular calcium through gap junction communication channels. *Neoplasia* 2010; 12 (9): 748-54.
36. West KA, Castillo SS, Dennis PA. Activation of the PI3K/Akt pathway and chemotherapeutic resistance. *Drug Resist Updat* 2002; 5 (6): 234-48.
37. St Croix B, Florenes VA, Rak JW, et al. Impact of the cyclin-dependent kinase inhibitor p27Kip1 on resistance of tumor cells to anticancer agents. *Nat Med* 1996; 2 (11): 1204-10.
38. St Croix B, Sheehan C, Rak JW, et al. E-Cadherin-dependent growth suppression is mediated by the cyclin-dependent kinase inhibitor p27(KIP1). *J Cell Biol* 1998; 142 (2): 557-71.
39. Li Z, Zhou Z, Donahue HJ. Alterations in Cx43 and OB-cadherin affect breast cancer cell metastatic potential. *Clin Exp Metastasis* 2008; 25 (3): 265-72.
40. Chu K, Cheng CJ, Ye X, et al. Cadherin-11 promotes the metastasis of prostate cancer cells to bone. *Mol Cancer Res* 2008; 6 (8): 1259-67.
41. Tsuji T, Ibaragi S, Hu GF. Epithelial-mesenchymal transition and cell cooperativity in metastasis. *Cancer Res* 2009; 69 (18): 7135-9.

FIGURE LEGENDS

Figure 1. Breast cancer cells cultured with hepatocytes revert to epithelial cluster morphology and re-express E-cadherin (A) Phase contrast and fluorescent images of breast cancer cells cocultured with hepatocytes for 6 days. (B) Immunoblot of E-cadherin expression in breast cancer cells cultured with and without human hepatocytes.

Figure 2. Breast cancer cells cultured with fibroblasts fail to re-express E-cadherin. (A) Phase contrast and fluorescent images of breast cancer cells cocultured with fibroblasts for 6 days. (B) Immunoblot of E-cadherin expression in breast cancer cells cultured with human fibroblasts.

Figure 3. E-cadherin expression increases attachment to hepatocytes. (A) Attachment of E-cadherin -negative and -positive breast cancer cells to hepatocyte plated at 30% confluency, 24 hours after plating (B and C) Attachment of E-cadherin -negative and -positive breast cancer cells to hepatocytes plated at 25 to 100% confluency, 24 hours after plating. (D) Binding of breast cancer cells to hepatocytes by centrifugal assay for fluorescence based cell adhesion.

Figure 4. Heterotypic ligation between breast and prostate cancer cells activates Erk signaling (A) E-cadherin-positive MCF7 cells plated onto hepatocyte membranes; addition of EDTA prevents Erk activation (B) 231 cells with and without E-cadherin re-expression plated onto hepatocyte membranes; addition of E-cadherin blocking antibody SHE78 blocks Erk signaling in 231 cells that re-express E-cadherin

Figure 5. Exogenous and microenvironment-induced expression of E-cadherin in breast cancer cells increases the chemoresistance to staurosporine (A and B) and camptothecin (C and D).

Figure 6. E-cadherin re-expression in prostate cancer cells increases chemoresistance. (A) Immunoblot for E-cadherin following treatment with buserelin or PD153035. (B) Immunofluorescence for E-cadherin following treatment with buserelin or PD153035. (C) DU-145 cells treated with camptothecin (C) and staurosporine (D) with or without re-expression of E-cadherin by buserelin and PD153035.

Figure 7. Prostate cancer cells that re-express E-cadherin in hepatocyte coculture are more chemoresistant to staurosporine (A) and camptothecin (B). This effect is abrogated in fibroblast coculture and when cells are transiently transfected with E-cadherin siRNA prior to coculture.

Supplemental Figures

Figure 1. Heterotypic adhesion between cancer cells and hepatocytes is E-cadherin-dependent. A) Homotypic cohesion between MCF7-MCF7 cells develops in a single logarithmic step (triangles); heterotypic cohesion between MCF7-hepatocytes also develops in a single logarithmic step, though the half-maximal number of cells bound is significantly less (squares); heterotypic cohesion between 231-hepatocytes is indistinguishable from background levels (circles). B) Heterotypic MCF7-hepatocyte cohesion is E-cadherin dependent and can be abolished by either calcium chelation (triangles) or the E-cadherin function blocking antibody, SHE78 (circles). C) siRNA knock-down of E-cadherin in MCF7 cells. D) Heterotypic adhesion between MCF7-hepatocytes can be abolished with an E-cadherin-directed siRNA, but adhesion remains unaffected with a non-targeted siRNA. Shown are mean ($n=5$) $\pm$ s.d.

Figure 2. The Erk-MAPK and Akt pathways are activated in E-cadherin positive MCF7 cells upon ligation with hepatocyte E-cadherin. A) Hepatocyte membranes were isolated by differential centrifugation and labeled with Dil. Poly-L-lysine was used to passively adsorb membranes onto tissue culture slides, and the result was imaged using confocal microscopy, and compiled as a z-stack. B) E-cadherin positive MCF7 breast cancer cells were seeded onto culture plates decorated with hepatocyte membranes. Erk activation peaks at 30' after ligation and Akt activation peaks at 60' after contact; activation of Erk and Akt can be attenuated with calcium chelation or the function blocking antibody, SHE78. C) 60' time-course of Erk and Akt activation. D) *In vitro* kinase assay of Erk and Akt activation. Results are shown as fractions of maximal activation by 5' EGF or PDGF treatment, mean ($n=3$) $\pm$ s.d.

Figure 3. Exogenous expression of E-cadherin in MDA-MB-231 cells increases resistance to staurosporine induced cell death. This effect is abrogated when 231-Ecad cells are pretreated with the E-cadherin blocking antibody SHE78.

Appendix 2: Manuscript published in *Cancer Microenvironment*

Partial Mesenchymal to Epithelial Reverting Transition in Breast and
Prostate Cancer Metastases

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ABSTRACT

Epithelial to mesenchymal transition (EMT) is an oft-studied mechanism for the initiation of metastasis. We have recently shown that once cancer cells disseminate to a secondary organ, a mesenchymal to epithelial reverting transition (MErT) may occur, which we postulate is to enable metastatic colonization. Despite a wealth of *in vitro* and *in vivo* studies, evidence of MErT in human specimens is rare and difficult to document because clinically detectable metastases are typically past the micrometastatic stage at which this transition is most likely evident. We obtained paired primary and metastatic tumors from breast and prostate cancer patients and evaluated expression of various epithelial and mesenchymal markers by immunohistochemistry. The metastases exhibited increased expression of membranous E-cadherin compared to primary tumors, consistent with EMT at the primary site and MErT at the metastatic site. However, the re-emergence of the epithelial phenotype was only partial or incomplete. Expression of epithelial markers connexins 26 and/or 43 was also increased on the majority of metastases, particularly those to the brain. Despite the upregulation of epithelial markers in metastases, expression of mesenchymal markers vimentin and FSP1 was mostly unchanged. We also examined prostate metastases of varied sizes and found that while E-cadherin expression was increased compared to the primary lesion, the expression inversely correlated with size of the metastasis. This not only suggests that a second EMT may occur in the ectopic site for tumor growth or to seed further metastases, but also provides a basis for the failure to discern epithelial phenotypes in clinically examined macrometastases. In summary, we report increased expression of epithelial markers and persistence of mesenchymal markers consistent with a partial MErT that readily allows for a second EMT at the metastatic site. Our results suggest that cancer cells continue to display phenotypic plasticity beyond the EMT that initiates metastasis.

INTRODUCTION

Recapitulation of the developmental process of epithelial to mesenchymal transition (EMT) has been proposed as a mechanism for enabling cancer cell invasion and dissemination. During cancer-associated EMT, loss of cell-cell adhesions via downregulation of E-cadherin allows for both physical detachment from the tumor mass and for external autocrine growth factor and internal signaling that activates cell migration [1]. EMT in cancer progression and metastasis has been widely studied through *in vitro* cell culture and *in vivo* animal models of cancer progression. In addition, EMT has been visualized at the invasive front of primary carcinomas as individual cells or a group of cells migrating into the surrounding tissue [2]. However, the true extent of EMT in human cancer specimens is still open to debate as is the role of EMT in metastatic seeding [1, 3, 4].

Despite the strong clinical association between decreased expression of adhesion molecules and invasion and poor prognosis, metastases can present a well-differentiated, epithelial phenotype, bringing into question whether EMT is reversible. We and others have proposed that a reverse EMT, or mesenchymal to epithelial reverting transition (MErT), occurs to enable metastatic colonization [4-7]. Therefore, while induction of EMT through loss of E-cadherin may promote tumor invasion and dissemination, MErT through re-expression of epithelial genes and downregulation of mesenchymal genes may allow the metastatic cancer cell to complete the last steps of the metastatic process and to survive in the secondary organ. However, just as it has been difficult to capture EMT *in vivo*, there is also a dearth of histological evidence for MErT.

Opponents of cancer-associated EMT argue that there is a lack of convincing evidence in clinical samples that support the *in vitro* findings [3]. However, lack of evidence in clinical samples does not mean that an EMT or MErT has not occurred at some point in time, as pathological specimens are often end-stage observations. Unless clinically indicated, only a small percentage of metastases undergo surgical resection or biopsy, as systemic adjuvant endocrine, chemotherapy, or palliative radiation is more commonly used as therapy. Furthermore, specimens of metastases that are resected or that undergo biopsy originate from tumors of various stage and size (and ER/PR/HER2/neu status for breast cancer), making direct comparisons between patients difficult. Tumors often exhibit areas of poor differentiation with morphological changes, such as cell scattering and spindle-shaped cells that are distinct from the bulk of the tumor; however, pathologists do not routinely stain for markers of epithelial or mesenchymal phenotype as diagnostic and prognostic value is absent. Despite these shortcomings, histological examination of epithelial and mesenchymal markers in primary tumors and their corresponding metastases is important to determine whether EMT and MErT occurs clinically, with implications for the development of new approaches to cancer.

Recently, we have reported that breast and prostate cancer metastases express increased levels of E-cadherin when compared to the matched primary tumor [8, 9]. In addition, E-cadherin-negative MDA-MB-231 breast cancer cells were induced to re-express E-cadherin by *in vitro* coculture with liver parenchymal cells or following spontaneous metastasis to the lung in a mouse

model [8]. However, despite the findings of E-cadherin re-expression and an accompanying morphological change, it remained to be seen whether a full or partial mesenchymal to epithelial transition had occurred. Thus, for the present study we evaluated the expression of mesenchymal and epithelial markers in a larger set of matched primary and metastatic tumor samples from breast cancer patients. We also focused on membranous expression of epithelial markers E-cadherin, β -catenin, connexin 26, and connexin43 as an indicator of normal function. Expression of epithelial markers was increased in metastases while expression of mesenchymal markers FSP1 and vimentin was variably changed, suggesting a partial MERT. In addition, we corroborated our results in a set of unmatched primary and metastatic prostate cancer samples and found that E-cadherin expression decreased with increasing metastatic tumor size, an observation that suggests that MERT is also reversible and helps to answer the question of whether metastases likely generate other metastases or if all metastases arise from the primary tumor.

RESULTS

Breast cancer metastases exhibit increased levels of localization of adherens junction components to the membrane

A few studies have compared E-cadherin expression in the primary tumor and distant metastases [3, 9-11]. We recently reported on a small set of matched primary breast carcinomas and their metastases to the lung, liver, and brain [8]; besides bone, these comprise the most common sites of breast cancer metastases. In that study, we quantified both cytosolic and membranous staining as positive E-cadherin expression because E-cadherin expression was not always localized to the membrane. We have re-analyzed the data to include only positive membranous staining, as functional E-cadherin that both participates in intercellular adhesion and sequestration of the catenins is only localized at the membrane. In addition, we expanded the sample set to include additional pairs of matched specimens. Percentage of membrane expression was calculated as the number of cells positive for E-cadherin expression localized to the membrane over the total number of cancer cells in each field. Overall, 17/20 (85%) cases showed increased membranous E-cadherin expression in the metastases compared to the primary tumors (Figure 1a), with this being consistent across the various sites; 2/2 (100%) of liver metastases, 5/6 (83%) of brain metastases, and 10/12 (83%) of lung metastases exhibited increased E-cadherin expression.

Localization of β -catenin at the cell membrane has been shown to be a critical suppressor of cancer cell migration and invasion as it forms part of a stable adherens junction [12, 13]. We therefore evaluated primary and metastatic tumors for membranous β -catenin expression (Figure 1b). Overall, 9/20 (45%) of metastases exhibited increased expression of membranous β -catenin; 7/12 (58%) of lung metastases, 1/2 (50%) of liver metastases, and 1/6 (17%) in brain metastases. When positive β -

catenin expression was quantified as including both membranous and cytoplasmic expression, increased β -catenin was evident in metastases compared to primary tumors, in 9/12 (75%) of lung metastases, 2/2 (100%) of liver metastases, and 1/6 (17%) of brain metastases (data not shown). Due to the activation of the downstream Wnt pathway, nuclear localization of β -catenin is most commonly associated with the invasive phenotype; therefore β -catenin involvement in an epithelial phenotype may be best quantified by membranous and cytoplasmic localization.

Expression of gap junction proteins is increased in breast cancer metastases to the brain

While adherens junctions facilitate intercellular adhesion, gap junctions mediate intercellular communication by the exchange of small molecules and ions through a membrane-spanning pore composed of connexins. In the breast, connexin 26 (Cx26) is expressed by luminal cells while connexin 43 (Cx43) is expressed by myoepithelial cells [14]. Loss of Cx26 and Cx43 has been shown to correlate with tumor progression in breast and colorectal cancer and over-expression of Cx43 reduces breast cancer metastasis [15-17]. Furthermore, just as re-expression of E-cadherin has been observed in metastases, increased expression of Cx26, Cx43, and Cx32 has been found in breast cancer lymph node metastases, suggesting that re-expression of gap junctions could also contribute to a MErT [18, 19]. We therefore surveyed the expression of membranous Cx26 and Cx43 in primary and metastatic tumors. For Cx26, 10/19 (53%) metastases showed increased membranous expression: 5/11 (45%) of lung metastases, 1/2 (50%) of liver metastases, and 4/6 (66%) of brain metastases (Figure 2a). Increased expression of membranous Cx43 expression was observed in 55% (11/20) of all metastases, specifically in 4/12 (33%) of lung metastases, 1/2 (50%) of liver metastases, and 6/6 (100%) of brain metastases (Figure 2b). For the most part, the two connexins changed, or stayed similar in parallel fashion within each metastasis. While there was no correlation in metastases to lung or liver, both Cx26 and Cx43 expression was strikingly increased in metastases to the brain.

Persistence of mesenchymal markers in metastases suggests a partial mesenchymal to epithelial reverting transition

To determine if the increase in epithelial markers signified the occurrence of a full MErT, which includes a loss or decrease in expression of mesenchymal markers in metastases, we next evaluated the expression of FSP1 and vimentin. FSP1 is considered one of the few truly fibroblast-specific markers and is commonly used as an early marker of EMT [20, 21]. Vimentin is also a widely accepted marker of the mesenchymal phenotype in EMT. During EMT, cells undergo a shift from using cytokeratin intermediate filaments to vimentin intermediate filaments, which are involved in the changes in adhesion and motility [22, 23]. Immunohistochemistry revealed that overall only 9/19 (47%) of metastases showed decreased expression of FSP1: 4/11 (36%) of lung metastases, 1/2 (50%) of liver metastases, and 4/6 (66%) of brain metastases (Figure 3a). Similarly, 13/20 (65%) of

metastases exhibited decreased expression of vimentin: 7/12 (64%) of lung metastases, 2/2 (100%) of liver metastases and 4/6 (66%) of brain metastases (Figure 3b). For metastases that did display a decrease in expression of FSP1 or vimentin, the degree of change was small relative to the change observed in E-cadherin. The lack of a dramatic downregulation of mesenchymal markers suggests that only a partial MErT occurs during metastatic colonization.

E-cadherin expression is inversely correlated with size of metastasis

To extend our findings beyond breast cancer metastases, we obtained a number of unmatched prostate carcinoma primary tumors and metastases. Organ sites of metastases included liver, lung, kidney, and thyroid. Primary and metastatic tumors were immunostained for E-cadherin and staining intensity was quantified with ImageJ. Metastases exhibited increased staining of E-cadherin compared to primary tumors ($p < .05$), suggesting that E-cadherin re-expression can occur in other cancers besides breast carcinoma (Figure 4a). Due to a shortage of specimens, staining for other epithelial and mesenchymal markers was not performed.

Several of the metastatic specimens from individual patients contained multiple foci of different sizes. The metastatic foci within one patient sample were divided into three categories based on size: less than 50 μ m in diameter (small), between 50 μ m and 100 μ m in diameter (medium), and larger than 100 μ m in diameter (large) (Figure 4b). The staining intensity of E-cadherin was quantified for each individual focus. Interestingly, E-cadherin expression inversely correlated with tumor size, with increased E-cadherin expression in small metastases compared to large ($p < .001$) (Figure 4c), suggesting that the partial MErT that allows for metastatic colonization is transient and reversible like the EMT that enables metastatic dissemination.

DISCUSSION

One of the major limitations of studying metastasis *in vivo* is that studies involving animal models and clinical samples are end-stage time points that can only provide a snapshot of the metastatic cascade at the point of tissue harvest. Although intravital imaging and use of organotypic bioreactors has improved the ability to visualize metastasis at various stages, the phenotypic plasticity exhibited during EMT and MErT is nonetheless difficult to capture [24-26]. Evidence of EMT and MErT in clinical specimens is rare and has been used as an argument that cancer-associated EMT does not occur during the course of disease. Using matched primary and metastatic tumors, we have examined expression of epithelial and mesenchymal markers in specimens obtained from human breast cancer patients. Our results show that the occurrence of cancer-associated EMT and MErT is possible.

Paget's "seed and soil" hypothesis posits that cancer cells can only survive and grow in appropriate environments; the reversible phenotypic plasticity of cancer cells during EMT and MErT is therefore one way in which cancer cells can adapt to the foreign soil of ectopic organ

microenvironments. Expression of adhesion molecules has been shown to be necessary to complete the final steps of the metastatic cascade including intravasation and colonization [27]. Based on previous observations of increased E-cadherin expression in metastases compared to primary tumors, we expanded our analysis to include E-cadherin binding partner β -catenin, gap junction molecules Cx26 and Cx43 and mesenchymal markers FSP1 and vimentin to discern whether a full or partial MErT occurs (summarized in Table 1). We limited our quantification of E-cadherin, β -catenin, Cx26, and Cx43 to expression localized to the membrane to account for proteins functioning in the epithelial phenotype, as dysfunctional proteins are commonly dislocated in the cytoplasm or nucleus during tumor progression. Increased expression of membranous E-cadherin was observed in metastases compared to primaries, across all organ sites of metastases. While we expected these results in metastases to lung and to liver where E-cadherin is expressed by pneumocytes and hepatocytes, it was surprising that 83% of metastases to the brain also exhibited increased E-cadherin expression. Breast cancer cells that metastasize to bone have been shown to express OB-cadherin, the cadherin expressed by osteocytes, so it was expected that metastases would exhibit increased expression of the adhesion molecule native to the ectopic organ [28, 29]. Thus, increased E-cadherin expression was not expected in metastases to the brain, which primarily expresses N-cadherin. When we queried N-cadherin expression in primary and metastatic tumors, only 2/5 brain metastases exhibited increased N-cadherin expression (data not shown).

It is not surprising that an overall corresponding increase in membranous β -catenin was not observed in metastases, as in all specimens the percentage of cells expressing β -catenin was higher than the E-cadherin-expressing cells. Thus, there was limited amount of increase that could be noted with β -catenin. This high level could be due to β -catenin binding to other cadherins. E-cadherin is not the only molecule that sequesters β -catenin, as the cytoplasmic domains are conserved among the type I classical cadherins. To test this, samples were also stained for N-cadherin (data not shown). While there was no consistent pattern of N-cadherin expression between primary tumors and metastases, high N-cadherin expression in the primary tumor was observed in many cases that exhibited no change or decreased localized β -catenin expression in metastases.

We also evaluated expression of gap junction molecules as another measure of epithelial gene expression in MErT. Cx26 and Cx43 are disparately expressed in the breast – luminal cells express Cx26 while myoepithelial cells express Cx43 [14]. Although the luminal and basal breast cancer subtypes arise from these two different cell types, there was no association between connexin expression and ER/PR/Her2 status, and therefore breast cancer subtype. Overall, metastases exhibited increased expression of Cx26 and Cx43 compared to the primary tumors. This was most striking in brain metastases, where 66% of brain metastases demonstrated increased Cx26 expression and 100% showed increased Cx43 expression. In the brain, Cx26 and Cx43 are expressed by astrocytes, which suggests that gap junctions and not adherens junctions may be the driving force behind brain metastases. We have hypothesized that MErT in metastatic colonization

serves to protect the metastatic cancer cell from inflammatory or chemotherapeutic insult [4]. Recent *in vitro* work by the Fidler group supports both our findings of increased connexin expression in brain metastases and also the theory that this re-expression confers a survival advantage. Melanoma or breast cancer cells cultured with astrocytes demonstrated reduced chemosensitivity, which was mediated by expression of connexins [30-32].

When immunostaining was performed for the mesenchymal markers FSP1 and vimentin, expression of these markers in metastases was either unchanged or slightly decreased, suggesting only a partial MErT. In addition, tumors are typically surrounded by reactive fibrosis and normal stromal cells that stain positive for mesenchymal markers so the possibility of false positives is high. Ideally, dual staining for breast cancer-specific and mesenchymal markers would overcome this problem; however, a reliable breast cancer-specific marker does not exist. Cell-cell adhesion and cell motility are usually viewed as attributes of opposing sides of the epithelial and mesenchymal phenotypic spectrum. However, partial EMT and MErT in which cells maintain some level of both is not an unusual phenomenon, as many examples can be found throughout cancer progression. During invasion, tumors have been shown to invade the ECM collectively as strands of cancer cells that maintain expression of adhesion molecules [33]. Similarly, during extravasation cancer cells re-express molecules that permit adhesion to endothelial cells yet still maintain the ability for transendothelial migration [34, 35].

Finally, we also found that E-cadherin expression decreases with increasing metastatic tumor size, suggesting that just as EMT is reversible, so is MErT. These data support earlier experimental evidence that the EMT that allows for escape from the primary lesion is not fixed but can be reverted during metastatic seeding [1, 8, 9]. However, often pathological examination of large metastases removed for palliative or diagnostic needs present de-differentiated cells reminiscent of the original EMT, which superficially appears at odds with our model of MErT. These data can be reconciled by our analysis of the prostate carcinoma micrometastases. In evaluating expression of E-cadherin based on metastasis size, we found the larger metastases (all still microscopic clinically) were less likely to express E-cadherin at the membrane, implying a re-emergence of EMT as with tumor growth. Thus, the phenotypic plasticity of carcinomas allows for continual repositioning of the tumor cell to provide a survival or dissemination advantage.

The reversibility of MErT at the secondary site alludes to the question of whether all metastases necessarily arise from the primary tumor or whether metastases can give rise to metastases. An autopsy study of breast cancer patients found that the frequency of metastases to non-common sites was lower when metastases to the lung, liver, or bone were not already present [36]. It has been shown in a mouse model that systemic metastases arise in mice with large lung metastases in the absence of the primary tumor [37, 38]. One explanation is dormant cells were already seeded in the lung prior to primary tumor removal, but parabiosis experiments revealed that the non-tumor bearing partner could develop metastases [39]. Despite these observations, the

mechanism by which these secondary metastases occur is still unknown. Here we suggest that EMT may occur following MErT in the metastatic site to engender these secondary metastases. Ultimately, the persistence of mesenchymal characteristics in MErT, despite the re-expression of epithelial genes and adhesion molecules, enables metastatic cancer cells to adeptly adapt to changing environments – from primary tumor to secondary organ and beyond.

MATERIALS AND METHODS

Immunohistochemistry

All studies were performed on de-identified specimens obtained during clinically-indicated procedures; these were deemed to be exempted (4e) from human studies by the University of Pittsburgh Institutional Review Board.

Paraffin-embedded patient samples, excess to clinical need, were obtained from the University of Pittsburgh Tissue Banks, primarily coming from Magee Womens Hospital of UPMC and UPMC Shadyside Hospital, under informed consent of patients undergoing diagnostic and therapeutic procedures. Sections underwent antigen retrieval in citrate solution (Dako) and were incubated with primary antibodies: E-cadherin (Cell Signaling), β -catenin (abcam), connexin 26 (abcam), connexin 43 (abcam), FSP1/S100A4 (abcam), and vimentin (abcam) followed by biotin-conjugated secondary antibody (Jackson Laboratories). Antigen staining was performed using DAB (Vector Laboratories) then counterstained with Mayer's hematoxylin. Secondary antibody alone served as a negative control and adjacent normal tissue served as an internal positive control. Images of three randomly-selected microscope fields of each sample were taken and the percentage of cancer cells with positive staining was quantified as the number of positive cells over the total number of cancer cells in that image. Microscope fields shown were selected to account for the heterogeneity of each sample. For the unmatched prostate cancer samples, mean density of E-cadherin staining was quantified using the Color Deconvolution plug-in for ImageJ software.

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REFERENCES

1. Wells, A, YL Chao, J Grahovac et al (2011) Epithelial and mesenchymal phenotypic switchings modulate cell motility in metastasis. *Frontiers in Bioscience* 16: 815-837.
2. Condeelis, JS, J Wyckoff, and JE Segall (2000) Imaging of cancer invasion and metastasis using green fluorescent protein. *European Journal of Cancer* 36: 1671-1680.
3. Tarin, D, EW Thompson, and DF Newgreen (2005) The fallacy of epithelial mesenchymal transition in neoplasia. *Cancer Res* 65(14): 5996-6000; discussion 6000-1.
4. Wells, A, C Yates, and CR Shepard (2008) E-cadherin as an indicator of mesenchymal to epithelial reverting transitions during the metastatic seeding of disseminated carcinomas. *Clin Exp Metastasis* 25(6): 621-628.
5. Thiery, JP (2002) Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* 2(6): 442-54.
6. Hugo, H, ML Ackland, T Blick et al (2007) Epithelial--mesenchymal and mesenchymal--epithelial transitions in carcinoma progression. *J Cell Physiol* 213(2): 374-83.
7. Chaffer, CL, JP Brennan, JL Slavin et al (2006) Mesenchymal-to-epithelial transition facilitates bladder cancer metastasis: role of fibroblast growth factor receptor-2. *Cancer Res* 66(23): 11271-8.
8. Chao, YL, CR Shepard, and A Wells (2010) Breast carcinoma cells re-express E-cadherin during mesenchymal to epithelial reverting transition. *Mol Cancer* 9: 179.
9. Yates, CC, CR Shepard, DB Stolz et al (2007) Co-culturing human prostate carcinoma cells with hepatocytes leads to increased expression of E-cadherin. *Br J Cancer* 96(8): 1246-52.
10. Kowalski, PJ, MA Rubin, and CG Kleer (2003) E-cadherin expression in primary carcinomas of the breast and its distant metastases. *Breast Cancer Res* 5(6): R217-22.
11. Bukholm, IK, JM Nesland, and AL Borresen-Dale (2000) Re-expression of E-cadherin, alpha-catenin and beta-catenin, but not of gamma-catenin, in metastatic tissue from breast cancer patients [see comments]. *J Pathol* 190(1): 15-9.
12. Wong, AS and BM Gumbiner (2003) Adhesion-independent mechanism for suppression of tumor cell invasion by E-cadherin. *J Cell Biol* 161(6): 1191-203.
13. Brabletz, T, A Jung, S Reu et al (2001) Variable beta-catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. *Proc Natl Acad Sci U S A* 98(18): 10356-61.
14. Monaghan, P, C Clarke, NP Perusinghe et al (1996) Gap junction distribution and connexin expression in human breast. *Exp Cell Res* 223(1): 29-38.
15. Li, Z, Z Zhou, DR Welch et al (2008) Expressing connexin 43 in breast cancer cells reduces their metastasis to lungs. *Clin Exp Metastasis* 25(8): 893-901.
16. McLachlan, E, Q Shao, HL Wang et al (2006) Connexins act as tumor suppressors in three-dimensional mammary cell organoids by regulating differentiation and angiogenesis. *Cancer Res* 66(20): 9886-94.
17. Kanczuga-Koda, L, S Sulkowski, M Koda et al (2005) Alterations in connexin26 expression during colorectal carcinogenesis. *Oncology* 68(2-3): 217-22.
18. Kanczuga-Koda, L, S Sulkowski, A Lenczewski et al (2006) Increased expression of connexins 26 and 43 in lymph node metastases of breast cancer. *J Clin Pathol* 59(4): 429-33.
19. Kanczuga-Koda, L, M Sulkowska, M Koda et al (2007) Increased expression of gap junction protein--connexin 32 in lymph node metastases of human ductal breast cancer. *Folia Histochem Cytobiol* 45 Suppl 1: S175-80.
20. Trimboli, AJ, K Fukino, A de Bruin et al (2008) Direct evidence for epithelial-mesenchymal transitions in breast cancer. *Cancer Res* 68(3): 937-45.
21. Okada, H, TM Danoff, R Kalluri et al (1997) Early role of Fsp1 in epithelial-mesenchymal transformation. *Am J Physiol* 273(4 Pt 2): F563-74.
22. Mendez, MG, S Kojima, and RD Goldman Vimentin induces changes in cell shape, motility, and adhesion during the epithelial to mesenchymal transition. *Faseb J* 24(6): 1838-51.
23. Vuoriluoto, K, H Haugen, S Kiviluoto et al Vimentin regulates EMT induction by Slug and oncogenic H-Ras and migration by governing Axl expression in breast cancer. *Oncogene*.
24. Stoletov, K, H Kato, E Zardouzan et al (2010) Visualizing extravasation dynamics of metastatic tumor cells. *J Cell Sci* 123(Pt 13): 2332-41.
25. Yates, C, CR Shepard, G Papworth et al (2007) Novel three-dimensional organotypic liver bioreactor to directly visualize early events in metastatic progression. *Adv Cancer Res* 97: 225-46.

26. Giampieri, S, S Pinner, and E Sahai (2010) Intravital imaging illuminates transforming growth factor beta signaling switches during metastasis. *Cancer Res* 70(9): 3435-9.
27. Glinskii, OV, VH Huxley, GV Glinsky et al (2005) Mechanical entrapment is insufficient and intercellular adhesion is essential for metastatic cell arrest in distant organs. *Neoplasia* 7(5): 522-7.
28. Huang, CF, C Lira, K Chu et al (2010) Cadherin-11 increases migration and invasion of prostate cancer cells and enhances their interaction with osteoblasts. *Cancer Res* 70(11): 4580-9.
29. Li, Z, Z Zhou, and HJ Donahue (2008) Alterations in Cx43 and OB-cadherin affect breast cancer cell metastatic potential. *Clin Exp Metastasis* 25(3): 265-72.
30. Fidler, IJ, K Balasubramanian, Q Lin et al (2010) The brain microenvironment and cancer metastasis. *Mol Cells* 30(2): 93-8.
31. Langley, RR, D Fan, L Guo et al (2009) Generation of an immortalized astrocyte cell line from H-2Kb-tsA58 mice to study the role of astrocytes in brain metastasis. *Int J Oncol* 35(4): 665-72.
32. Lin, Q, K Balasubramanian, D Fan et al (2010) Reactive astrocytes protect melanoma cells from chemotherapy by sequestering intracellular calcium through gap junction communication channels. *Neoplasia* 12(9): 748-54.
33. Friedl, P and D Gilmour (2009) Collective cell migration in morphogenesis, regeneration and cancer. *Nat Rev Mol Cell Biol* 10(7): 445-57.
34. Schmidmaier, R and P Baumann (2008) ANTI-ADHESION evolves to a promising therapeutic concept in oncology. *Curr Med Chem* 15(10): 978-90.
35. Gout, S, PL Tremblay, and J Huot (2008) Selectins and selectin ligands in extravasation of cancer cells and organ selectivity of metastasis. *Clin Exp Metastasis* 25(4): 335-44.
36. Viadana, E, ID Bross, and JW Pickren (1973) An autopsy study of some routes of dissemination of cancer of the breast. *Br J Cancer* 27(4): 336-40.
37. Alterman, AL, DM Fornabaio, and CW Stackpole (1985) Metastatic dissemination of B16 melanoma: pattern and sequence of metastasis. *J Natl Cancer Inst* 75(4): 691-702.
38. Stackpole, CW (1990) Intrapulmonary spread of established B16 melanoma lung metastases and lung colonies. *Invasion Metastasis* 10(5): 267-80.
39. Hoover, HC, Jr. and AS Ketcham (1975) Metastasis of metastases. *Am J Surg* 130(4): 405-11.

FIGURE LEGENDS

Figure 1: Breast cancer metastases exhibit increased localization of adherens junctions components to the membrane. A) Quantification of membrane-bound E-cadherin in breast cancer primary tumors and metastases. Representative images of a primary tumor exhibiting cytoplasmic or absent E-cadherin and the paired lung metastasis with membranous E-cadherin expression. B) Quantification of membranous β -catenin in primary and metastatic tumors. Images from a case that exhibited increased membranous β -catenin staining in a metastasis to the lung. Organ sites of metastases are color-coded: lung (blue), red (liver), and brain (green). Size bar in the photomicrographs is 25 microns.

Figure 2: Expression of gap junctional proteins is increased in breast cancer metastases to the brain. Quantification of membranous Cx26 (A) and Cx43 (B) staining in primary and metastatic breast cancer tumors. Shown are representative images of connexin staining in primary tumors and brain metastases. Organ sites of metastases are color-coded: lung (blue), red (liver), and brain (green). Size bar in the photomicrographs is 25 microns.

Figure 3: Mesenchymal markers persist in metastases, suggesting only a partial MErT. Quantification of immunostaining for mesenchymal markers FSP1 (A) and vimentin (B). Images of FSP1 and vimentin staining in primary tumors and metastases. Organ sites of metastases are color-coded: lung (blue), red (liver), and brain (green). Size bar in the photomicrographs is 25 microns.

Figure 4: E-cadherin expression in prostate cancer metastases is inversely correlated with size of metastasis. A) Quantification and representative images of prostate cancer primary and metastatic tumors immunostained for E-cadherin. $*p < 0.05$ Images of three random fields were quantified with ImageJ. B) Images of metastatic tumors stained for E-cadherin as categorized by size: small (less than $50\mu\text{m}$ in diameter), medium (between $50\mu\text{m}$ and $100\mu\text{m}$) and large (bigger than $100\mu\text{m}$). C) Quantification of E-cadherin expression in different sized prostate cancer metastases. $+p < 0.001$ $*p < 0.05$. Size bar in the photomicrographs is 25 microns.

Table 1. Summary of epithelial and mesenchymal marker expression data. Green, cases that exhibited an increased expression in metastases; red, decreased expression in metastases compared to primary tumors; yellow, absent or no change in expression; white, unable to quantify sample.

Figure 5: Model of reversible phenotypic transitions during metastasis. EMT and loss of E-cadherin enables dissemination, followed by E-cadherin re-expression and a partial MErT that facilitates

metastatic colonization at a secondary site. MErT is reversible, and with tumor growth may undergo an additional EMT.

Appendix 3: Manuscript published in *Molecular Cancer*, July 2010

Breast carcinoma cells re-express E-cadherin during mesenchymal to epithelial reverting transition

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Abstract

Background

Epithelial to mesenchymal transition (EMT), implicated as a mechanism for tumor dissemination, is marked by loss of E-cadherin, disruption of cell adhesion, and induction of cell motility and invasion. In most intraductal breast carcinomas E-cadherin is regulated epigenetically via methylation of the promoter. E-cadherin expression is therefore dynamic and open to modulation by the microenvironment. In addition, it has been observed that metastatic foci commonly appear more differentiated than the primary tumor, suggesting that cancer cells may further undergo a mesenchymal to epithelial reverting transition (MErT) in the secondary organ environment following the EMT that allows for escape.

Results

We first examined E-cadherin expression in primary breast tumors and their corresponding metastases to liver, lung and brain and discovered that 62% (10/16) of cases showed increased E-cadherin expression in the metastases compared to the primaries. These observations led to the question of whether the positive metastatic foci arose from expansion of E-cadherin-positive cells or from MErT of originally E-cadherin-negative disseminated cells. Thus, we aimed to determine whether it was possible for the mesenchymal-like MDA-MB-231 breast cancer cells to undergo an MErT through the re-expression of E-cadherin, either through exogenous introduction or induction by the microenvironment. Ectopic expression of full-length E-cadherin in MDA-MB-231 cells resulted in a morphological and functional reversion of the epithelial phenotype, with even just the cytosolic domain of E-cadherin yielding a partial phenotype. Introduction of MDA-MB-231 cells or primary explants into a secondary organ environment simulated by a hepatocyte coculture system induced E-cadherin re-expression through passive loss of methylation of the promoter. Furthermore, detection of E-cadherin-positive metastatic foci following the spontaneous metastasis of MDA-MB-231 cells injected into the mammary fat pad of mice suggests that this re-expression is functional.

Conclusions

Our clinical observations and experimental data indicate that the secondary organ microenvironment can induce the re-expression of E-cadherin and consequently MErT. This phenotypic change is reflected in altered cell behavior and thus may be a critical step in cell survival at metastatic sites.

Introduction

Breast cancer is the most frequently diagnosed cancer in women, and it is the second leading cause of cancer death in women of all ages [7]. Intraductal carcinoma, which originates from the epithelial cells lining the mammary ducts, is the most common type of breast cancer. Metastasis

occurs via a series of sequential steps, during which the cells acquire an amoeboid-like phenotype, become motile, disseminate, and colonize distant sites of the body, which in breast cancer are most commonly liver, lung, bone, and brain. The stages of this transformation are similar to the stages of the developmental process known as epithelial to mesenchymal transition (EMT) [8]. Much of the current literature supports the idea that EMT is the key mechanism by which tumor cells gain invasive and metastatic ability, as EMT enables separation of individual cells from the primary tumor mass as well as promotes migration [9, 10]. After undergoing EMT, thereby enabling access to hematogenous or lymphatic routes of dissemination, tumor cells can extravasate into secondary organs and establish micrometastases. We have hypothesized that EMT is reversible and that a reversion back towards the epithelial phenotype may occur at the secondary metastatic site (MErT). A similar reversion occurs in development when neural crest cells undergo a transient EMT followed by a permanent MET to generate tissues such as kidney epithelia [11]. A few studies have charted switches between EMT and MET phenotypes throughout malignant progression such as in colorectal cancer [12], bladder cancer [13], and ovarian cancer [14]. The phenotypic plasticity observed in these cases is unlikely to be generated by the acquisition of permanent genetic insults, suggesting that the microenvironment is capable of inducing epigenetic changes.

Numerous extracellular signals such as growth factors and stromal signals, and stressors such as hypoxia and ROS have been implicated in the induction of EMT [15]. However, at the core of the transition between an epithelial and a mesenchymal phenotype is the loss of E-cadherin expression. E-cadherin is a classical member of the cadherin family, whose extracellular domain facilitates homotypic intercellular adhesions while the cytosolic tail assembles catenins and other signaling and scaffolding molecules at the membrane to link to the actin cytoskeleton [16, 17]. E-cadherin-mediated cell-cell adhesions limit cell motility and establish apical-basal polarity. The loss of E-cadherin expression and disassembly of E-cadherin adhesion plaques on the cell surface enables tumor cells to disengage from the primary mass and move to conduits of dissemination [18]. This duality of

functionalities—intercellular cohesion and regulation of intracellular signaling cascades—suggests that E-cadherin may impact multiple aspects of epithelial homeostasis.

Thus, E-cadherin expression is intimately connected to a cell's degree of epitheliality – in both morphology and migratory and invasive abilities. In cancer pathogenesis, E-cadherin expression is dynamically regulated via epigenetic mechanisms, specifically methylation of the promoter, providing tumor cells the plasticity to switch between EMT and ME_{RT} depending on the microenvironment [5]. Interestingly, it has been observed that metastases often resemble the epithelial-like phenotype of the primary tumor rather than the mesenchymal phenotype observed at the invasive front. In addition, several pathological studies, including the one conducted herein, have observed increased E-cadherin expression in metastases compared to aberrant or loss of expression in the primary tumors, further challenging the notion that EMT is irreversible and suggesting that E-cadherin may be involved in ME_{RT} at the metastatic site [3, 19]. However, one limitation of these pathological studies is that it is impossible to determine whether these E-cadherin-positive metastases result from the rare escape and expansion of epitheloid carcinoma cells, such as in the cell cooperativity model, or whether they arise from a mesenchymal-like cell that has undergone a phenotypic reversion back to a more differentiated phenotype, as we hypothesize [20, 21].

Therefore, we aimed to experimentally determine whether it was possible for the mesenchymal-like MDA-MB-231 breast cancer cells to undergo an ME_{RT} through the re-expression of E-cadherin, either through exogenous introduction or through induction by the microenvironment. Ectopic expression of E-cadherin in MDA-MB-231 cells resulted in a reversion back to some degree of the epithelial phenotype, particularly with respect to morphology and functional suppression of migration and invasion. Furthermore, introduction of breast cancer cells and primary explants into a secondary organ environment led to the passive loss of methylation of the E-cadherin promoter and re-expression of this cell-cell adhesion molecule, demonstrating a mechanism for this reversion of EMT. *In vivo* experiments in mice revealed similar results in lung metastases, suggesting that re-

expression of E-cadherin may be a critical step in metastatic colonization of not only the liver but lung as well.

Results

E-cadherin is expressed in distant metastases of E-cadherin-negative primary tumors

Loss of E-cadherin expression in the primary tumor is correlated with poor prognosis and survival [3, 4]. A few studies have examined E-cadherin expression in the primary tumor and distant metastases, but the cases analyzed in these studies included metastases to lymph nodes or uncommon sites of breast cancer metastasis [19]. To conduct our own survey focusing on metastases to the most common sites, we obtained specimens of primary tumors and the corresponding metastases from 16 patients with infiltrating ductal carcinoma. Metastatic sites from which tissue was obtained included the lung (10 cases), liver (3), and brain (3). Both primary tumor and metastases were immunostained for E-cadherin. E-cadherin positive cells were counted based on high intensity membrane or cytoplasmic staining. Percentage of E-cadherin positivity was calculated as the number of E-cadherin-positive cells over the total number of cancer cells in each field (Additional file 1). Overall, 62% (10 of 16) cases showed increased E-cadherin expression in the metastases compared to the primary tumors (Figure 1a), with this being consistent across the various sites; 66% (2/3) of liver metastases, 66% (2/3) of brain metastases, and 60% (6/10) of lung metastases exhibited increased E-cadherin expression. There was no correlation between hormone receptor or Her2/neu status and E-cadherin expression. In some cases, closer examination of the specimens revealed striking differences of E-cadherin expression between the primary tumor and the metastasis, with the primary tumor wholly negative and the metastasis mostly positive for E-cadherin expression; one such liver metastasis is shown (Figure 1b). E-cadherin expression within both the primary tumors and the metastases was often heterogeneous, which was accounted for by quantifying areas of the tumor that best approximated the heterogeneity observed in the sample. However, even with this heterogeneity the levels of E-cadherin positivity were increased in the

metastases (Additional file 1). In addition, the sizes of metastases ranged greatly, from micrometastases less than 1mm to macrometastases greater than 2cm in diameter. The trend appeared likely that heterogeneity of E-cadherin expression was positively correlated with tumor size; however, due to our small sample size we were unable to statistically assess such a correlation.

Of interest, E-cadherin expression in the metastases did not appear to be random. Shown is a liver metastasis demonstrating increased expression at the hepatocyte-cancer cell interface and decreased expression centrally, suggesting that E-cadherin is directly regulated by hepatocyte interactions (Figure 1c). Quantification of staining intensity confirmed an increase in E-cadherin expression in the area outlined by the solid inset compared to the area outlined by the dashed inset located further away from hepatocytes (Figure 1d). E-cadherin staining in the tissue samples is observed both at the membrane and in the cytoplasm, as autocrine EGFR signaling generally present in breast cancer drives E-cadherin internalization [22, 23]. This overview of a small number of paired specimens provides insights into whether MErT is possible. If metastases are the result of expansion of a clonal population of cells originating from a primary tumor cell that has undergone EMT, then one would expect metastases to be E-cadherin-negative unless this phenotype is plastic. The finding of E-cadherin-positive metastases suggests that non-EMT cells can establish metastases or that MErT at the metastatic site can occur.

Ectopic expression of E-cadherin partly reverts breast cancer cells towards an epithelial phenotype

The finding of more prevalent E-cadherin expression in metastases compared to the paired primary tumors led to the question of whether the positive metastatic foci arose from expansion of E-cadherin-positive cells or from MErT of originally E-cadherin-negative cells. Thus, we aimed to determine whether it was possible for the mesenchymal-like MDA-MB-231 breast cancer cells to become more epithelioid following expression of E-cadherin. In MDA-MB-231 cells, E-cadherin expression is suppressed by methylation of the promoter. We stably transfected full-length E-cadherin

driven by a CMV promoter and generated single cell clones (231-Ecad). In addition, because the possibility of intermediate EMT/MErT phenotypes has been proposed, we also stably transfected MDA-MB-231 cells with a construct composed of the intracellular and transmembrane domains of E-cadherin coupled to the class I major histocompatibility complex antigen (H-2kd) extracellular domain (231-H2kd). Such a construct was originally used to examine the contribution of internal E-cadherin signaling in the absence of E-cadherin-mediated intercellular interactions [24, 25]. We postulated that expressing only the cytosolic tail of E-cadherin would allow for a partial MErT through the intracellular sequestration of adherens junction components and other effector proteins that is observed in epithelial cells but absent in mesenchymal cells. Immunoblot and immunofluorescence confirmed the exogenous expression of E-cadherin and E-cadherin-H2kd in MDA-MB-231 cells (Figure 2 and Additional file 2). 231-Ecad and 231-H2kd mutants display colocalization with the catenins at the membrane (Additional file 2b). E-cadherin expressing MCF7 breast cancer cells were used as a positive control. 231-Ecad cells exhibited cobblestone or cell-cell clustered morphology and formed cell contacts, which was not observed in control transfected MDA-MB-231 cells. 231-H2kd cells demonstrated a more flattened morphology that did not fully resemble either epithelial or mesenchymal phenotypes (Figures 2a and 5). As expected, 231-H2kd cells did not form cell-cell contacts. It is important to note that this culture was performed at low cell density, so that cells were limited in establishing cell-cell connections. Thus, outside-in signaling mediated by E-cadherin was not necessary for the morphology change.

We next analyzed the expression of epithelial and mesenchymal markers in the various cell lines to monitor the penetrance of the epithelial/mesenchymal phenotypes. We evaluated the expression of a spectrum of cytokeratins including cytokeratin-18 (CK-18), the primary intermediate filament present in epithelial cells. Expression of vimentin, smooth muscle actin, and fibronectin were used as markers of the mesenchymal phenotype. Loss of cytokeratins and increased expression of vimentin, smooth muscle actin, or fibronectin have been shown to occur concurrently with EMT in adenocarcinomas [26]. The survey of these epithelial and mesenchymal markers revealed that 231-

Ecad cells demonstrated decreased expression of smooth muscle actin, fibronectin, and vimentin and increased expression of cytokeratins (Figures 2b and 2c). Upregulation of N-cadherin has been observed in EMT, but because N-cadherin is not expressed in MDA-MB-231 cells this mesenchymal marker was not tested. 231-Ecad cells displayed increased cytokeratin-18 and decreased vimentin expression as assayed by immunofluorescence (Figure 2c). As epithelial and mesenchymal cells also differ in their cytoskeletal architecture, phalloidin was used to visualize the actin cytoskeleton. Expression of the entire E-cadherin molecule (231-Ecad) provided a more epithelial-like reticular actin filament meshwork (Figure 2c). The persistence of mesenchymal markers and failure to fully express epithelial markers in 231-Ecad cells compared to the epithelial MCF7 cells suggests that MDA-MB-231 cells transfected with E-cadherin (either wild-type or cytosolic tail) still maintain some aspects of mesenchymal phenotype.

Mesenchymal and epithelial phenotypes also confer functional behaviors on tumor cells. As such we tested the two key properties related to tumor escape enabled by EMT: migration and invasion. After an *in vitro* scratch assay, which measures migration, we observed that expression of full-length or the cytosolic region of E-cadherin resulted in suppressed migration almost down to low levels noted for the epithelial MCF7 cancer line (Figure 3a). Similar trends were observed in the Matrigel invasion assay, which integrates motility with other properties such as matrix remodeling to better recreate the movement through bioactive matrices that defines tumor invasion. The invasive ability of both 231-Ecad and 231-H2kd cells was suppressed compared to MDA-MB-231 cells (Figure 3b). That suppression of migration and invasiveness were observed in 231-H2kd cells in the absence of changes in expression in the marker genes suggests that these functional behaviors may be independent of a mesenchymal to epithelial transition. While 231-H2kd cells may be similar to wildtype 231 in terms of mesenchymal and epithelial gene expression, β -catenin localization differed (Additional file 2); while 231 cells exhibit cytoplasmic distribution of β -catenin, 231-H2kd cells localize α -catenin, β -catenin, and p120 to the cell membrane as do the epithelial counterparts 231-Ecad and

MCF7 cells. As reported by other groups, this alteration alone is sufficient to account for the invasion suppressor phenotype [27].

In summary, these results indicate that expression of exogenous E-cadherin (wild-type or cytosolic tail) in MDA-MB-231 cells results in a morphological shift toward the epithelial end of the spectrum. The expression of both epithelial and mesenchymal markers in 231-Ecad and 231-H2kd cells demonstrate that these cells may not have undergone a complete MErT, but the migration and invasion assay data suggest that expression of the full-length and cytosolic domains of E-cadherin are sufficient to induce a more epithelial-like phenotype in terms of cell motility and invasiveness. Furthermore, suppression of invasion and migration in 231-H2kd was comparable to the suppression in 231-Ecad cells, indicating that changes to the localization of key signaling proteins during the mesenchymal to epithelial transition can have profound effects in mitigating the mesenchymal nature of an invasive cell.

E-cadherin expression is induced by a secondary organ microenvironment

Our previous results demonstrating E-cadherin expression in metastases suggested that a reversion to a more epithelial phenotype could occur at the metastatic site. We therefore hypothesized that a secondary organ microenvironment could induce re-expression of E-cadherin. To test this hypothesis, we cultured MDA-MB-231 cells with rat hepatocytes, as the liver is one of the main organs to which breast cancer cells metastasize. After 6 days of culture, expression of E-cadherin was detected using a human specific E-cadherin antibody (Figure 4a). Control experiments confirmed that the human-specific antibody did not cross-react with E-cadherin of rat origin, indicating that the E-cadherin was re-expressed by MDA-MB-231 cells (data not shown). Expression was also detected by flow cytometry (Figure 4b). Side and forward scatter as well as hepatocyte-specific autofluorescence gating were used to exclude the hepatocyte population. Flow cytometry analysis of MDA-MB-231 cells after 6 days of co-culture with hepatocytes formed a bimodal distribution, with 22.32% of cells forming a distinct population of E-cadherin positive cells. Culture of MDA-MB-231

cells in hepatocyte growth media alone did not result in re-expression, indicating that the re-expression is driven by hepatocytes (Figure 4c). Increased expression of E-cadherin mRNA was also detected by qRT-PCR (Figure 4d). After 6 days of culture with hepatocytes, MDA-MB-231 exhibited levels of E-cadherin transcript comparable to E-cadherin-positive MCF7 cells, while MDA-MB-231 cells cultured in the absence of hepatocytes presented undetectable mRNA levels. The fact that the E-cadherin mRNA level appears to be similar to that in MCF-7 cells despite lower protein levels is likely due to autocrine EGFR signaling driving E-cadherin internalization and degradation [15].

To prevent re-expression of E-cadherin in coculture and to validate that the changes noted were from E-cadherin and not another undefined co-expressed protein, we stably transfected MDA-MB-231 cells with an E-cadherin shRNA plasmid construct and generated single cell clones (231-shEcad). In addition, breast carcinoma cells were RFP-labeled to more easily discriminate cancer cells from hepatocytes in coculture. While MDA-MB-231, 231-H2kd, and 231-Ecad cells reverted to an epithelial clustered morphology following hepatocyte coculture, 231-shEcad cells remained fibroblastic (Figure 5). Immunofluorescence confirmed that the shRNA construct prevented re-expression of E-cadherin (Figure 6, left column). To evaluate whether MERT occurs following E-cadherin re-expression, cocultures were immunostained for the mesenchymal marker vimentin. Just as expression of mesenchymal markers persisted in 231-Ecad cells, E-cadherin re-expression in coculture did not completely suppress expression of vimentin (Figure 6, right column). However, vimentin expression appeared more heterogeneous, with some cells expressing more than others. It is important to note that compared to 231-Ecad cells where E-cadherin was exogenously expressed, there may be other unexplored molecular changes in MDA-MB-231 cells following hepatocyte coculture besides E-cadherin re-expression.

As we demonstrated that it was possible for mesenchymally-transitioned carcinoma cells to revert to a more epithelioid phenotype, we next tested whether primary explants of human breast tumors could also re-express E-cadherin in hepatocyte coculture. Explants were obtained from breast tumors without current evidence of dissemination and cultured for at most 3 passages prior to

experimentation. In total, four cocultured primary explants were assayed by flow cytometry and seven primary explants were analyzed by immunofluorescence following hepatocyte coculture. Analysis by flow cytometry indicated that although initially E-cadherin negative, one of the four explants tested expressed E-cadherin after coculture (Figure 7a). Similarly, tumor cells in two of seven explants that were originally E-cadherin negative, expressed robust and well-localized E-cadherin after 6 days of co-culture with the hepatocytes (Figure 7b). We were unable to ascertain the promoter methylation status in these cells due to the limited number and passage integrity of the primary cells; nonetheless, this line of evidence strongly suggests that primary human breast cancer cells may undergo similar molecular changes as MDA-MB-231 cells when cultured in a hepatic microenvironment.

E-cadherin re-expression in the liver microenvironment is due to loss of promoter methylation

In the absence of hepatocytes, E-cadherin expression in MDA-MB-231 cells is transcriptionally repressed by methylation of the E-cadherin promoter. Most intraductal breast carcinomas in which E-cadherin is downregulated also exhibit similar promoter hypermethylation [28]. Therefore loss of promoter methylation was examined as a possible mechanism for the re-expression of E-cadherin. We assayed a CpG island that was proximal to the E-cadherin transcription start site, whose methylation correlates inversely with E-cadherin expression [29]. Following coculture, total genomic DNA was isolated for methylation-specific PCR (MS-PCR) [30]. Species-specific primers were used to guarantee measurement of CpG methylation in only the human cancer cells and not rat hepatocytes. When human MDA-MB-231 cells were co-cultured with rat hepatocytes over a period of 6 days, the methylation status of the E-cadherin promoter region changed from a hypermethylated state to a hypomethylated state (Figure 8a). However, in the absence of hepatocytes, MDA-MB-231 cells remained hypermethylated (Figure 9a). To capture the dynamic loss of methylation of the CpG sites along the length of the E-cadherin promoter region, bisulfite sequencing was performed on MDA-MB-231 cells. MCF7 cells were used as an unmethylated control for E-cadherin promoter analysis. As expected, the promoter regions of the MDA-MB-231 cells were highly methylated before

co-culture with hepatocytes, as denoted by the filled in circles of the control row. After coculture, much of the methylation was lost from these specific CpG islands (Figure 8b). Thus, the bisulfite sequencing validates our MS-PCR results and shows that E-cadherin promoter methylation decreases upon co-culture with hepatocytes, resulting in re-expression.

Because cancer cells are often globally hypomethylated, we evaluated whether the loss of methylation was specific to the E-cadherin promoter or the result of global hypomethylation. The *H19* gene is a paternally imprinted gene whose methylation is modulated during gametogenesis and does not change after terminal differentiation of a cell line [31]. We performed bisulfite MS-PCR analysis on MDA-MB-231 cells before coculture and following 1,3, and 6 days of coculture with hepatocytes, examining a previously reported CpG site of *H19*. Evaluation of the data revealed that the average methylation of *H19* remained unchanged at all time points indicating that global hypomethylation is not responsible for the changes observed at the E-cadherin promoter (Figure 9b).

Loss of promoter methylation can result from either a passive mechanism (lack of maintenance methylation subsequent to mitosis) or an active mechanism (enzyme-mediated excision), though there are currently no well-defined demethylases. The presence of intermediate stages of promoter methylation on day 3 and extended time period to unmethylated status (6 days) suggested a passive mechanism. To test whether the loss of methylation was dependent on proliferation of the cancer cells, we inhibited proliferation of the cancer cells with mitomycin-C. This treatment completely prevented loss of methylation of the promoter as demonstrated by MS-PCR (Figure 9c). Furthermore, addition of mitomycin-C also prevented re-expression of E-cadherin at the protein level (Figure 9d). Inhibition of DNA methyltransferases, which mediate CpG island methylation, could also account for loss of methylation. However, immunostaining for DNA methyltransferase DNMT1 showed neither decrease in expression nor change in nuclear localization (Figure 9e). Taken together, these data point to passive loss of methylation as the mechanism by which E-cadherin is re-expressed.

E-cadherin re-expression occurs in vivo

To determine whether reversion of E-cadherin repression could be induced *in vivo*, we injected MDA-MB-231 cells into the mammary fat pads of mice. Mice were sacrificed after four weeks, to allow for dissemination from the primary tumor. Because MDA-MB-231 cells inoculated into the mouse mammary fat pad mainly metastasize to lung and not to liver when allowed to spontaneously metastasize, mice were examined for lung metastases by histopathological examination of the tissues. Our use of human breast cancer cells in a mouse host allowed for a human-specific E-cadherin antibody to discern the source of E-cadherin expression between the cancer cells and the epithelial mouse parenchyma. We first confirmed that the primary xenograft transplants in the inguinal mammary fat pads did not express E-cadherin (Figure 10a, left panel). There was no change in E-cadherin status of the invading cells in the primary xenograft, as we observed both the central and peripheral areas of the tumor to be devoid of E-cadherin as detected by immunoperoxidase staining (Figure 10a, middle and right panels). Two representative images of lung micrometastases less than 2mm in diameter showed a markedly different pattern of E-cadherin expression. When immunoperoxidase labeling was performed on these sections, isolated islands expressing E-cadherin localized to the cell membrane were detected (Figure 10b). The human-specific antibody identified the disseminated MDA-MB-231 cells with robust E-cadherin expression, while not labeling the surrounding mouse lung tissue. Other fields of the same lung, unaffected and clear of metastatic lesions, did not display positive staining. Although we were unable to obtain metastases to the liver in the animal model, E-cadherin re-expression was observed in lung metastases in both the animal model and in clinical samples, suggesting that re-expression of E-cadherin may not be limited to the liver microenvironment.

Discussion

Paget's seed and soil hypothesis has long postulated that cancer cells, or the "seeds", will only grow in a specific microenvironment, or "soil" [27, 32-34]. Indeed, despite the fact that tumors are continually shedding cells, very few circulating tumor cells actually establish metastases, suggesting

that post-extravasation survival is a crucial rate-limiting step [35]. The clinical observations that breast cancer displays a characteristic pattern of metastasis, specifically to the lung, liver, bone, and brain, indicate that these organs provide the most conducive microenvironment for metastatic growth. In addition, cancer cells themselves may exhibit an inherent gene signature predisposing them to homing to a particular organ site [3, 4]. The precise environmental factors that enable the organotropism of metastases are yet to be fully discovered, but even less well known is why only a tiny fraction of circulating carcinoma cells form metastases.

Prior to extravasation, cancer cells must survive through invasion and emigration, anchorage-independent dissemination, and extravasation into the ectopic organ. These behaviors are thought to be conferred by molecular changes as a result of EMT. However, post-extravasation, cancer cells encounter a new set of challenges, notably integration within organ parenchyma and establishment of blood supply, which mesenchymal-like cells appear poorly equipped to handle. Despite the importance of EMT in promoting metastatic progression, there is mounting evidence that EMT is not an irreversible switch in cancer cell phenotype. Analysis of primary tumors and their corresponding metastases reveal that even though an EMT may have occurred to engender metastases, the phenotypes of the two can be strikingly similar. E-cadherin expression has been detected in lymph node and non-nodal metastases in carcinomas not limited to breast [36]. Re-expression of adhesion molecules could therefore be one way in which the secondary organ microenvironment promotes survival of metastatic cells as cadherin-cadherin engagement promotes activation of cell survival signaling pathways [22].

To ascertain whether these earlier reports of E-cadherin-expressing metastases held for intraductal breast carcinomas, we surveyed a small set of matched primary and metastatic tumors. Some 2/3 of metastases to the lung, liver, or brain expressed increased E-cadherin compared to the primary tumors, which largely exhibited aberrantly low to negative E-cadherin expression. Not all metastases exhibited high levels of E-cadherin expression, which is not surprising as metastases are

known to evolve and give rise to further disseminations, suggesting that a second EMT may occur within more aggressive nodules.

Interestingly, E-cadherin expression even within metastases was heterogeneous, with increased E-cadherin expression seemingly correlated with proximity to normal parenchymal cells. This heterogeneity suggests that constant interaction with hepatocytes in liver may be necessary. Still, despite these observations, it was possible that these E-cadherin-positive tumor cells disseminated from the primary tumor as epithelioid cells and formed secondary metastatic lesions. Thus, we sought to provide proof-of-principle that cancer cells could be engineered to approach a mesenchymal-to-epithelial reverting transition by altering E-cadherin expression, either exogenously or via the microenvironment. We first hypothesized that we could engineer a MErT in MDA-MB-231 cells by expressing wild-type E-cadherin or by sequestering the E-cadherin-associated catenins with a non-binding E-cadherin construct. After transfecting the MDA-MB-231 cells with the cytosolic domain of E-cadherin linked to the MHC external domain, we saw that the dominant negative protein sequestered α -, β - and p120- catenins. The advantage of using this dominant negative is that the catenin signaling could be parsed from other activities of the extracellular domain of E-cadherin including cell adhesion through *trans*-ligation and EGFR *cis*-modulation [26, 37, 38]. While neither construct could completely revert MDA-MB-231 cells to an epithelial phenotype, expression of either construct resulted in morphological transformations and behavioral changes noted as suppression of migration and invasion. Our results also corroborate the findings of other studies focusing on the role of E-cadherin as a tumor or invasion suppressor [39-41].

When cultured in a hepatic microenvironment, MDA-MB-231 exhibited a similar reversion to an epithelial phenotype, both in morphology and E-cadherin re-expression. The nature of the signals that drive the reversion back to an epithelioid phenotype are not known and likely to be complex. Initial studies found that neither conditioned media nor hepatocyte-derived matrix could trigger E-cadherin re-expression in this breast carcinoma line, though the combination of the two was noted to lead to a weak re-expression of E-cadherin (data not shown). Re-expression secondary to loss of

methylation of the E-cadherin promoter was also observed in the cell line MDA-MB-435 (Additional file 3), which is now considered to be a melanoma derivative, but is nonetheless useful as this neurectodermal lineage expresses E-cadherin as melanocytes but loses expression during melanoma progression [42]. Furthermore, this reversion is not likely unique to the liver microenvironment, based on the findings in human metastases and in our in vivo mouse model. Recently, we have found that lung parenchymal cells can drive E-cadherin expression in prostate tumor cells [43]. A recent study suggests that laminin-1 may be one component of the extracellular matrix that contributes to E-cadherin re-expression [19]. One key difference between our studies is the microenvironment used to induce E-cadherin re-expression in MDA-MB-231 cells. While Benton *et al* used a three-dimensional laminin-1 hydrogel, we chose to simulate a secondary organ microenvironment by culturing breast cancer cells with hepatocytes, thereby exposing them to hepatocyte-derived soluble factors and extracellular matrix. Their finding of DNMT1 downregulation as the mechanism for E-cadherin expression was not observed in our system (data not shown), suggesting that tissue architecture may induce MErT by alternative mechanisms. Thus, the search for this signaling 'cocktail' is likely to be complex and lies beyond the scope of the present communication.

That E-cadherin re-expression is caused by loss of methylation suggests a functional mechanism by which the microenvironment modulates the mesenchymal to epithelial phenotypic switch. E-cadherin is predominantly downregulated in carcinomas at the post-translational and/or transcriptional levels. Regulation of E-cadherin is therefore unique among tumor suppressors in which loss or mutation appears to be the rule, but this epigenetic regulation of E-cadherin allows for increased phenotypic plasticity. We have previously reported that prostate cancer cells cultured with hepatocytes also re-express E-cadherin, but as a result of inhibition of the EGF receptor signaling [27, 38, 44, 45]. However, in breast cancers E-cadherin is silenced directly at the transcriptional level by promoter hypermethylation or indirectly through its transcriptional suppressors Snail, Slug, and Twist [46]. No differences in expression of these transcriptional suppressors were observed following

hepatocyte coculture (data not shown). In MDA-MB-231 cells, representative of the basal subtype of infiltrating ductal carcinomas, the CpG islands in the promoter region most proximal to the E-cadherin initiation site are fully methylated, which exerts a profound effect on mesenchymal nature. Demethylation of these islands by the chemical agent 5-aza-deoxycytidine causes re-expression of E-cadherin and loss of invasive ability [47-50]. Coculturing of MDA-MB-231 cells with primary hepatocytes resulted in loss of methylation of the E-cadherin promoter and expression of E-cadherin mRNA and protein. We observed that the loss of methylation was dependent on the proliferation of the cancer cells. This finding was not unique to the breast carcinoma cells, as the MDA-MB-435 line also demonstrated loss of promoter hypermethylation upon coculturing with hepatocytes. Importantly, this loss of methylation was at least semi-specific and not global as the imprinted H19 gene remained methylated. The ubiquitous transcription factor Sp1 has been implicated in the regulation of methylation status by binding loci of hemimethylated DNA, protecting sequences from *de novo* methylation, preferential demethylation, or passive demethylation mechanisms [51]. Sp1 was necessary for loss of methylation in coculture (data not shown), strongly suggesting active signaling from the microenvironment.

The foundation of our findings rest on the epigenetic reversion observed when breast cancer cells are cocultured with primary hepatocytes. The epigenetic status of the primary tumor and disseminated metastases is most likely important, since primary tumors that have high E-cadherin levels have very little systemic disease [34, 52], suggesting that the epigenetic reversion at distant secondary sites is also relevant. The xenograft model in which E-cadherin negative MDA-MB-231 cells formed E-cadherin-negative primary tumors in the mammary fat pads but E-cadherin-positive micrometastases and the finding that at least some E-cadherin-negative primary breast carcinoma cells can re-express this molecule support the idea that this reversion is possible. Furthermore, the xenograft experiment demonstrates that the molecular changes can occur in the secondary site. However, these experiments do not mean that all E-cadherin-positive metastases necessarily arise from the reversion of E-cadherin-negative cancer cells. Further molecular dissections and a much

larger breast tumor survey, challenging due to the paucity of matched primary and non-nodal metastases, are needed to determine the extent of this MErT in early metastatic seeding.

The potential implications of E-cadherin re-expression and MErT are many. There are several possible outcomes or combinations of outcomes after a cell extravasates into a metastatic target tissue: apoptosis, dormancy, or sustained proliferation, with the latter appearing the rarest [53]. While E-cadherin typically mediates homotypic cell-cell adhesions, heterophilic ligation between different cell types has been documented [54-56]. Cancer cell adhesion has been shown to facilitate extravasation and colonization of distant organs [57, 58]. Phenotypic reversion to epitheliality *in vivo* may therefore enhance the integration and survival of cancer cells at the metastatic site by cloaking the cancer cell with epithelioid-like characteristics, or may act to transmit mitogenic signals. E-cadherin expression has also been shown to suppress cell growth, which may account for the dormancy period between clinical presentation of metastases [59]. However, preliminary results in a parallel study reveal that one important survival advantage conferred by E-cadherin expression is increased resistance to cell death induced by chemotherapeutic agents such as camptothecin, doxorubicin, and taxol (data not shown). Cellular adhesion has long been implicated in intrinsic or acquired resistance of solid tumors to multiple anticancer therapeutics not restricted to chemotherapy [6, 60]. The addition of E-cadherin function blocking antibodies sensitizes multicellular spheroids to treatment with various chemotherapeutic agents and E-cadherin-positive cells are more resistant to staurosporine-induced cell death than E-cadherin-negative breast cancer cells [23]. A similar survival advantage may be conferred when disseminated cells face apoptotic cytokines, thus providing a selective pressure that then confounds adjuvant therapies. The finding that E-cadherin re-expression and catenin sequestration can contribute to a MErT suggests that they may be appropriate therapeutic targets for preventing the establishment of metastases in breast cancer.

Materials and Methods

Generation of cell lines

231-H2kd cells were generated using the *Myc/His* encoding H-2kd-E-cad dominant negative E-cadherin construct, a kind gift from Vizirianakis *et al* [19]. 231-H2kd cells were selected by FACS using the H-2kd (SF1-1.1) antibody (BD Pharmingen; San Jose, CA) and were maintained in 900µg/ml G418 until used for experimentation. 231-Ecad cells were made by co-transfecting a plasmid encoding the E-cadherin full-length cDNA sequence (Open Biosystems) with the pcDNA 3.1 plasmid (Invitrogen) and cultured in 900µg/ml G418 to select for stable transfectants. 231-shEcad cells were generated using an E-cadherin shRNA plasmid (Santa Cruz Biotechnology) and stable transfectants were selected using 5ug/ml of puromycin and confirmed by RT-PCR. At least two single cell clones of each mutant were generated by selecting for resistance to G418 (231-H2kd and 231-Ecad) or puromycin (231-shEcad). Control clones transfected with pcDNA 3.1, DsRed2, and control shRNA were also generated and tested. Single cell clones of each mutant line were subsequently transfected with the DsRed2 plasmid vector and FACS sorted for RFP fluorescence for use in hepatocyte cocultures. In all cases the experiments were performed at least once with the different clones, rendering similar results.

Cell culture and co-culture

MCF7, MDA-MB-231, and MDA-MB-435 cells were cultured in RPMI-1640 with 10% FBS as previously described [19]. Primary rat hepatocytes were isolated by collagenase perfusion and cultured as described previously [61] and plated onto collagen-coated 6-well plates at 60,000 cells/cm². The following day, cancer cells were seeded onto the hepatocyte monolayer at 3,000 cells/cm² and cocultured for 6 days.

Immunohistochemistry

Paraffin-embedded patient samples were obtained from Magee Womens Hospital. Sections underwent antigen retrieval in citrate solution and were incubated with E-cadherin primary antibody (Cell Signaling). Antigen staining was performed using DAB (Vector Laboratories) then counterstained with Mayer's hematoxylin. Secondary antibody alone served as a negative control and adjacent normal tissue served as an internal positive control. Images of three randomly-selected microscope fields of each sample were taken and the percentage of E-cadherin positive cancer cells was quantified as the number of E-cadherin positive cells over the total number of cancer cells in that image. Microscope fields shown were selected to account for the heterogeneity of each sample. Relative staining intensity of the liver metastasis was quantified using ImageJ software.

Invasion assay

Invasive potential was determined *in vitro* by migration through an artificial ECM [29]. 2.5×10^4 cells were challenged in growth-factor reduced matrigel invasion chambers (BD Biosciences). Cells were seeded into the top chamber with serum-free media and media containing 10% serum was added to the lower chamber for the remainder of the assay. After 24 hours, the remaining cells and ECM in the top chamber were removed by cotton swab. Cells that invaded through the matrix to the bottom of the filter were then fixed and stained with DAPI and counted. Individual experiments were performed in triplicate.

Scratch Assay

A monolayer of cells was grown to confluence in a 6-well plate and at experimental time zero a scratch was made in each well using a pipette tip. The well was imaged at time zero and again 24 hours later. Using Metamorph, a measurement was taken for how much the denuded area had filled in the 24-hour period.

Xenografts

The Institutional Animal Care and Use Committee at the Veterans Affairs Hospital in Pittsburgh approved all animal procedures. Experiments were performed in 8 week old female athymic nude mice. One million MDA-MB-231 cells were injected into the right mammary fat pad; injection vehicle was the culture medium (0.2 mL/site). Mice were sacrificed 4-5 weeks after tumor cell implantation and the primary xenograft and lungs removed.

Xenograft and other harvested tissues were fixed in 4% buffered formalin and 4 μ m thick paraffin sections underwent antigen retrieval for 5 min in 95°C 10mM citrate solution in preparation for H&E and immunochemistry. With the use of the Mouse on Mouse Kit (Vector Labs, Berlingame, CA), positive labeling was confirmed by comparing serial sections incubated with the primary human-specific E-cadherin antibody (67A4 1:100; Santa Cruz Biotechnology, Santa Cruz, CA) or the biotinylated secondary antibody alone. Labeling was visualized with the Vectastain Elite kit (Vector Labs).

Methylation Specific PCR and bisulfite sequencing

DNA was isolated from co-culture using the DNeasy Blood and Tissue Kit (Qiagen, Velencia, CA). 2000ng of isolated DNA was subjected to bisulfite treatment using the EZ DNA Methylation Gold Kit (Zymo, San Diego, CA) per the manufacturer's specifications. MSP was performed in the way of Corn et al [62] or using the CpG WIZ E-cadherin Amplification Kit per the manufacturer's instructions (Millipore, Temecula, CA). Briefly, in the method of Corn, a nested PCR method was used, in which the first primer set generated a 270bp fragment that was subsequently sequenced. The second round of PCR used either nested primers that were specific to either the unmethylated or methylated allele, which amplified the first CpG island after the transcription start site. The product size of the methylated reaction was 112bp and 120bp for the unmethylated.

MSP of H19 after bisulfite conversion was performed using the following primers: F 5'-TTA TAA AAT CGA AAA TTA CGC GCG A-3' R 5'-TTT TAG ATG ATT TTT GTG AAT TTT-3'. Cycling conditions were 95 °C for 15 min, 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min with

a final extension of 5 min at 72 °C. All reactions were performed using Platinum Taq SuperMix (Invitrogen).

Real-time quantitative PCR

RNA was isolated from hepatocyte-cancer cell co-cultures with the PureYield RNA Midiprep System (Promega, Madison, WI). cDNA was obtained with High Capacity cDNA RT Kit (Applied Biosystems, Foster City, CA). The human-specific TaqMan Gene Expression Assay Hs00170423_A1 CDHI probe was obtained from Applied Biosystems (Foster City, CA). Amplification and analysis in quadruplicate was run in an Applied Biosystems 7500 Real-Time PCR System. Relative values were normalized by using GAPDH levels as a reference using TaqMan Pre-Developed Human GAPDH Assay Reagent by Applied Biosystems.

Immunoblotting, Immunofluorescence, and Flow Cytometry

Cell lysate proteins were resolved on 7.5% SDS-PAGE and transferred to PVDF membranes. After blocking, membranes were incubated with primary antibodies against E-cadherin (Santa Cruz), pan cytokeratin (abcam), smooth muscle actin (Cal Biochem), fibronectin (Rockland Inc), GAPDH (Sigma) and actin (Sigma), followed by incubation with peroxidase-conjugated secondary antibodies and chemiluminescence detection.

For flow cytometry, co-cultures were non-enzymatically dissociated from the culture plates and vortexed into a single-cell suspension. The cells were fixed in 2% Paraformaldehyde for 30 minutes, permeabilized with 1% Triton for 3 minutes, and incubated with a PE-conjugated E-cadherin antibody (67A4) for 30 minutes. The mixed hepatocyte-cancer cell suspension was gated as to exclude hepatocytes using the appropriate SSC/FSC parameters. Data were collected on at least 10^6 cells in the appropriate SSC/FSC region.

Immunofluorescence was performed by overnight primary antibody incubation with E-cadherin (Santa Cruz), DNMT1 (Santa Cruz), DsRed (Santa Cruz), Alexa 488-phalloidin (Molecular Probes),

cytokeratin-18 (abcam) or vimentin (abcam) followed by incubation with the appropriate fluorophore-labeled secondary antibody. Visualization was performed on an Olympus Fluoview 1000 confocal microscope (Olympus, Center Valley, PA).

Primary explants

Polyclonal primary human tumor explants were obtained and cultured as previously reported [29]. Immunofluorescence labeling was performed as above.

Statistical Analysis

All quantitative data are presented as mean $\pm$ sd obtained from independent experiments. p-value significance was determined using a two-tailed unpaired Student t-test, and set at 0.05 as a minimum. All images were representative of at least three independent observations.

Competing Interests

The authors declare that they have no competing interests

Authors' Contributions

YC and CS performed experiments, analyzed data, and drafted the manuscript. AW participated in the design of the study, interpretation of data, and edited the manuscript. All authors read and approved the final manuscript.

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References

1. Jemal, A, R Siegel, E Ward et al (2007) Cancer statistics. *CA Cancer J Clin* 57(1): 43-66.
2. Gonzalez-Angulo, A, F Morales-Vasquez, and G Hortobagyi (2007) Overview of resistance to systemic therapy in patients with breast cancer. *Adv Exp Med Biol* (608): 1-22.
3. Kowalski, PJ, MA Rubin, and CG Kleer (2003) E-cadherin expression in primary carcinomas of the breast and its distant metastases. *Breast Cancer Res* 5(6): R217-22.
4. Bukholm, IK, JM Nesland, and AL Borresen-Dale (2000) Re-expression of E-cadherin, alpha-catenin and beta-catenin, but not of gamma-catenin, in metastatic tissue from breast cancer patients [seecomments]. *J Pathol* 190(1): 15-9.
5. Graff, JR, E Gabrielson, H Fujii et al (2000) Methylation patterns of the E-cadherin 5' CpG island are unstable and reflect the dynamic, heterogeneous loss of E-cadherin expression during metastatic progression. *J Biol Chem* 275(4): 2727-32.
6. Wang, L, Z Li, C Wang et al (2009) E-cadherin decreased human breast cancer cells sensitivity to staurosporine by up-regulating Bcl-2 expression. *Arch Biochem Biophys* 481(1): 116-22.
7. Punglia, RS, M Morrow, EP Winer et al (2007) Local therapy and survival in breast cancer. *N Engl J Med* 356(23): 2399-405.
8. Lee, JM, S Dedhar, R Kalluri et al (2006) The epithelial-mesenchymal transition: new insights in signaling, development, and disease. *J Cell Biol* 172(7): 973-81.
9. Trimboli, AJ, K Fukino, A de Bruin et al (2008) Direct evidence for epithelial-mesenchymal transitions in breast cancer. *Cancer Res* 68(3): 937-45.
10. Tse, JC and R Kalluri (2007) Mechanisms of metastasis: epithelial-to-mesenchymal transition and contribution of tumor microenvironment. *J Cell Biochem* 101(4): 816-29.
11. Hugo, H, ML Ackland, T Blick et al (2007) Epithelial--mesenchymal and mesenchymal--epithelial transitions in carcinoma progression. *J Cell Physiol* 213(2): 374-83.
12. Brabletz, T, A Jung, S Reu et al (2001) Variable beta-catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. *Proc Natl Acad Sci U S A* 98(18): 10356-61.
13. Chaffer, CL, JP Brennan, JL Slavin et al (2006) Mesenchymal-to-epithelial transition facilitates bladder cancer metastasis: role of fibroblast growth factor receptor-2. *Cancer Res* 66(23): 11271-8.
14. Hudson, LG, R Zeineldin, and MS Stack (2008) Phenotypic plasticity of neoplastic ovarian epithelium: unique cadherin profiles in tumor progression. *Clin Exp Metastasis* 25(6): 643-55.
15. Polyak, K and RA Weinberg (2009) Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. *Nat Rev Cancer* 9(4): 265-273.
16. Hirano, S, A Nose, K Hatta et al (1987) Calcium-dependent cell-cell adhesion molecules (cadherins): subclass specificities and possible involvement of actin bundles. *J Cell Biol* 105(6 Pt 1): 2501-10.
17. Takeichi, M (1991) Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* 251(5000): 1451-5.
18. Wells, A, C Yates, and CR Shepard (2008) E-cadherin as an indicator of mesenchymal to epithelial reverting transitions during the metastatic seeding of disseminated carcinomas. *Clin Exp Metastasis* 25(6): 621-628.
19. Yates, CC, CR Shepard, DB Stolz et al (2007) Co-culturing human prostate carcinoma cells with hepatocytes leads to increased expression of E-cadherin. *Br J Cancer* 96(8): 1246-52.
20. Takeichi, M (1993) Cadherins in cancer: implications for invasion and metastasis. *Curr Opin Cell Biol* 5(5): 806-11.
21. Peinado, H, F Portillo, and A Cano (2004) Transcriptional regulation of cadherins during development and carcinogenesis. *Int J Dev Biol* 48(5-6): 365-75.

22. Fedor-Chaiken, M, TE Meigs, DD Kaplan et al (2003) Two regions of cadherin cytoplasmic domains are involved in suppressing motility of a mammary carcinoma cell line. *J Biol Chem* 278(52): 52371-8.
23. Vizirianakis, IS, YQ Chen, SS Kantak et al (2002) Dominant-negative E-cadherin alters adhesion and reverses contact inhibition of growth in breast carcinoma cells. *Int J Oncol* 21(1): 135-44.
24. Gotzmann, J, M Mikula, A Eger et al (2004) Molecular aspects of epithelial cell plasticity: implications for local tumor invasion and metastasis. *Mutat Res* 566(1): 9-20.
25. Kokkinos, MI, R Wafai, MK Wong et al (2007) Vimentin and epithelial-mesenchymal transition in human breast cancer--observations in vitro and in vivo. *Cells Tissues Organs* 185(1-3): 191-203.
26. Wong, AS and BM Gumbiner (2003) Adhesion-independent mechanism for suppression of tumor cell invasion by E-cadherin. *J Cell Biol* 161(6): 1191-203.
27. Graff, JR, JG Herman, RG Lapidus et al (1995) E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. *Cancer Res* 55(22): 5195-9.
28. Kallakury, BV, CE Sheehan, E Winn-Deen et al (2001) Decreased expression of catenins (alpha and beta), p120 CTN, and E-cadherin cell adhesion proteins and E-cadherin gene promoter methylation in prostatic adenocarcinomas. *Cancer* 92(11): 2786-95.
29. Corn, PG, BD Smith, ES Ruckdeschel et al (2000) E-cadherin expression is silenced by 5' CpG island methylation in acute leukemia. *Clin Cancer Res* 6(11): 4243-8.
30. Lucifero, D, C Mertineit, HJ Clarke et al (2002) Methylation dynamics of imprinted genes in mouse germ cells. *Genomics* 79(4): 530-8.
31. Fidler, IJ (2003) The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nat Rev Cancer* 3(6): 453-8.
32. Luzzi, KJ, IC MacDonald, EE Schmidt et al (1998) Multistep nature of metastatic inefficiency: dormancy of solitary cells after successful extravasation and limited survival of early micrometastases. *Am J Pathol* 153(3): 865-73.
33. Koop, S, IC MacDonald, K Luzzi et al (1995) Fate of melanoma cells entering the microcirculation: over 80% survive and extravasate. *Cancer Res* 55(12): 2520-3.
34. Kienast, Y, L von Baumgarten, M Fuhrmann et al Real-time imaging reveals the single steps of brain metastasis formation. *Nat Med* 16(1): 116-22.
35. Lu, X and Y Kang (2007) Organotropism of breast cancer metastasis. *J Mammary Gland Biol Neoplasia* 12(2-3): 153-62.
36. Pece, S and JS Gutkind (2000) Signaling from E-cadherins to the MAPK pathway by the recruitment and activation of epidermal growth factor receptors upon cell-cell contact formation. *J Biol Chem* 275(52): 41227-33.
37. Sarrio, D, J Palacios, M Hergueta-Redondo et al (2009) Functional characterization of E- and P-cadherin in invasive breast cancer cells. *BMC Cancer* 9: 74.
38. Onder, TT, PB Gupta, SA Mani et al (2008) Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. *Cancer Res* 68(10): 3645-54.
39. Rae, JM, SJ Ramus, M Waltham et al (2004) Common origins of MDA-MB-435 cells from various sources with those shown to have melanoma properties. *Clin Exp Metastasis* 21(6): 543-52.
40. Sanders, DS, K Blessing, GA Hassan et al (1999) Alterations in cadherin and catenin expression during the biological progression of melanocytic tumours. *Mol Pathol* 52(3): 151-7.
41. Silye, R, AJ Karayiannakis, KN Syrigos et al (1998) E-cadherin/catenin complex in benign and malignant melanocytic lesions. *J Pathol* 186(4): 350-5.
42. Li, M, JM Aliotta, JM Asara et al Intercellular transfer of proteins as identified by stable isotope labeling of amino acids in cell culture. *J Biol Chem* 285(9): 6285-97.

43. Benton, G, E Crooke, and J George (2009) Laminin-1 induces E-cadherin expression in 3-dimensional cultured breast cancer cells by inhibiting DNA methyltransferase 1 and reversing promoter methylation status. *Faseb J* 23(11): 3884-95.
44. Cano, A, MA Perez-Moreno, I Rodrigo et al (2000) The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol* 2(2): 76-83.
45. Hajra, KM, DY Chen, and ER Fearon (2002) The SLUG zinc-finger protein represses E-cadherin in breast cancer. *Cancer Res* 62(6): 1613-8.
46. Nam, JS, Y Ino, Y Kanai et al (2004) 5-aza-2'-deoxycytidine restores the E-cadherin system in E-cadherin-silenced cancer cells and reduces cancer metastasis. *Clin Exp Metastasis* 21(1): 49-56.
47. Holler, M, G Westin, J Jiricny et al (1988) Sp1 transcription factor binds DNA and activates transcription even when the binding site is CpG methylated. *Genes Dev* 2(9): 1127-35.
48. Silke, J, KI Rother, O Georgiev et al (1995) Complex demethylation patterns at Sp1 binding sites in F9 embryonal carcinoma cells. *FEBS Lett* 370(3): 170-4.
49. Brandeis, M, D Frank, I Keshet et al (1994) Sp1 elements protect a CpG island from de novo methylation. *Nature* 371(6496): 435-8.
50. Matsuo, K, J Silke, O Georgiev et al (1998) An embryonic demethylation mechanism involving binding of transcription factors to replicating DNA. *Embo J* 17(5): 1446-53.
51. Goldstein, NS (2002) Does the level of E-cadherin expression correlate with the primary breast carcinoma infiltration pattern and type of systemic metastases? *Am J Clin Pathol* 118(3): 425-34.
52. Chambers, AF, IC MacDonald, EE Schmidt et al (1995) Steps in tumor metastasis: new concepts from intravital videomicroscopy. *Cancer Metastasis Rev* 14(4): 279-301.
53. Shiraishi, K, K Tsuzaka, K Yoshimoto et al (2005) Critical role of the fifth domain of E-cadherin for heterophilic adhesion with alpha E beta 7, but not for homophilic adhesion. *J Immunol* 175(2): 1014-21.
54. Chu, K, CJ Cheng, X Ye et al (2008) Cadherin-11 promotes the metastasis of prostate cancer cells to bone. *Mol Cancer Res* 6(8): 1259-67.
55. Gassmann, P and J Haier (2008) The tumor cell-host organ interface in the early onset of metastatic organ colonisation. *Clin Exp Metastasis* 25(2): 171-81.
56. Glinskii, OV, VH Huxley, GV Glinsky et al (2005) Mechanical entrapment is insufficient and intercellular adhesion is essential for metastatic cell arrest in distant organs. *Neoplasia* 7(5): 522-7.
57. Perrais, M, X Chen, M Perez-Moreno et al (2007) E-cadherin homophilic ligation inhibits cell growth and epidermal growth factor receptor signaling independently of other cell interactions. *Mol Biol Cell* 18(6): 2013-25.
58. St Croix, B, C Sheehan, JW Rak et al (1998) E-Cadherin-dependent growth suppression is mediated by the cyclin-dependent kinase inhibitor p27(KIP1). *J Cell Biol* 142(2): 557-71.
59. St Croix, B and RS Kerbel (1997) Cell adhesion and drug resistance in cancer. *Curr Opin Oncol* 9(6): 549-56.
60. Green, SK, G Francia, C Isidoro et al (2004) Antiadhesive antibodies targeting E-cadherin sensitize multicellular tumor spheroids to chemotherapy in vitro. *Mol Cancer Ther* 3(2): 149-59.
61. Zhou, W, JR Grandis, and A Wells (2006) STAT3 is required but not sufficient for EGF receptor-mediated migration and invasion of human prostate carcinoma cell lines. *Br J Cancer* 95(2): 164-71.
62. Ochs, RL, J Fensterer, NP Ohori et al (2003) Evidence for the isolation, growth, and characterization of malignant cells in primary cultures of human tumors. *In Vitro Cell Dev Biol Anim* 39(1-2): 63-70.

Figure Legends

Figure 1. E-cadherin expression is increased in metastases compared to primary tumors. A) Percentage of E-cadherin-positive cells is increased in metastases compared to the primary tumors. Organ sites of metastases are organized by color: liver (red), lung (blue), and brain (yellow) B) Example of a case showing strong expression of E-cadherin in the metastasis (right) compared to negative expression in primary (left). C) Heterogeneous expression of E-cadherin in the center (dashed inset) versus edge (solid inset) of a liver metastasis. "C" denotes tumor and "H" denotes hepatocytes. D) Quantification of E-cadherin staining in the center and edge of the liver metastasis.

Figure 2. E-cadherin expression alters cell morphology. A) Cell morphology as examined by phase contrast microscopy (left column) and E-cadherin expression (red) as detected by immunofluorescence (right column) B) Immunoblot analysis illustrates ectopic expression of E-cadherin in 231-Ecad cells as well as expression of various epithelial and mesenchymal markers in the E-cadherin mutants. C) Immunofluorescence of vimentin, cytokeratin-18 and actin cytoskeleton (rhodamine phalloidin). Shown are representative of at least three different assessments using one of two independent clones of each cell variant. D) Quantification of fluorescence using ImageJ, n=20 cells, $p < 0.05$.

Figure 3. E-cadherin expression suppresses migration (A) and invasion (B). Cell migration was analyzed using a scratch assay. Scratch closure was measured over a period of 24 hours and the fraction closure was quantified by Metamorph software (n=3). Invasion was measured in using a Matrigel invasion assay in which cells were allowed to migrate through a Matrigel-coated transwell insert for a period of 24 hours. N = 3 in triplicate; mean $\pm$ s.e.m. Results shown are representative of one of two independent clones of each mutant.

Figure 4. Hepatocytes drive the re-expression of E-cadherin in MDA-MB-231 breast cancer cells. A) Immunoblot of proteins lysates from MDA-MB-231/hepatocyte co-cultures using a human-specific antibody. B) Flow cytometry of the MDA-MB-231 population using a human-specific antibody shows a unimodal population on day 0 and a bimodal population on day 6. C) MDA-MB-231 cells do not express E-cadherin without hepatocytes. D) RT-PCR using human-specific primers of MDA-MB-231 cells after 6 days of co-culture with hepatocytes. Means ($n=4$) $\pm$ s.d. Note that species-specific primers do not amplify E-cadherin or GAPDH from hepatocytes.

Figure 5. Breast cancer cells cultured with hepatocytes revert to an epithelial morphology. Phase contrast images of 231, 231-H2kd, 231-Ecad, and 231-shEcad breast cancer cells cultured with rat hepatocytes for 6 days.

Figure 6. Breast cancer cells culture with hepatocytes re-express E-cadherin but maintain vimentin A) Immunostaining of RFP-labeled breast cancer cells in hepatocyte coculture; E-cadherin (green), RFP (red), DAPI (blue) B) Immunostaining for vimentin (green), RFP (red), DAPI (blue). Shown are representative of at least three different assessments using at least two independent clones of each cell variant.

Figure 7. A subset of primary breast carcinoma explants re-express E-cadherin when cocultured with primary hepatocytes. A) Flow cytometry analysis of primary explants using a human-specific E-cadherin antibody. A fluorescence unit of 1 indicates that the fluorescence intensity was equal to the same gate performed without addition of antibody. B) Confocal microscopy of two positive explants. Explants (C), hepatocytes (H). Human-specific E-cadherin, blue; actin, red; nuclei, green.

Figure 8. Breast cancer cells lose methylation of E-cadherin promoter methylation following hepatocyte coculture. A) Nested PCR method to detect methylation status of the E-cadherin

promoter in a six day time course of hepatocyte coculture. Above, bisulfite-treated DNA is amplified with primers that exclude CpG islands to amplify a 270bp region independent of methylation status. Below, nested primers anneal to the 270bp target to amplify a methylated (112bp) or unmethylated (120bp) fragment in the six day time course. MCF7 is used as an unmethylated control. B) Bisulfite sequencing of CpG islands in the E-cadherin promoter. Figure adapted from Corn *et al* . CpG islands are indicated as vertical lines on map; each CpG island is represented a circle. MCF7, MDA-MB-231, and MDA-MB-435 were sequenced on days 1,3, and 5 coculture. Open circle, unmethylated CpG; closed circle, methylated CpG; filled circle, mixed quality values.

Figure 9. Re-expression of E-cadherin follows a proliferation-dependent demethylation of the E-cadherin promoter. A) MS-PCR of MDA-MB-231 cultured alone in hepatocyte growth media B) MS-PCR using human-specific primers that amplify the imprinted H19 gene. C) MS-PCR of E-cadherin promoter following addition of MMC D) Addition of MMC prevents E-cadherin re-expression at the protein level. E) The maintenance demethylase DNMT1 does not change in localization or intensity in MDA-MB-231 cancer cells when cocultured with hepatocytes. DNMT1, red; DAPI, blue.

Figure 10. E-cadherin positive metastatic foci originate from E-cadherin negative primary tumors. A) Left, human MDA-MB-231 breast cancer cell xenograft in a mouse inguinal fat pad (H&E); middle, human-specific E-cadherin antibody indicates the absence of E-cadherin expression in the center of the primary tumor; right, absence of human-specific E-cadherin labeling at the periphery of the tumor. B) Micrometastases in the lung originating from the primary xenograft in A. Immunoperoxidase labeling of diseased portions of the mouse lung indicate the presence of human E-cadherin-positive MDA-MB-231 cancer cells; bottom adjacent.

Additional Files

Additional file 1. Tables of quantification of E-cadherin staining in primary and metastatic tumors of breast cancer patients. Metastases are color-coded to mirror Figure 1A. Three microscope fields of each specimen were selected and quantified except when limited by the size of the sample.

Additional file 2. β - and p120-catenin are sequestered by the Ecad/H2kd fragment. A) β - or p120-catenin, left panel, green; H2kd, middle panel, red; merge, right panel, yellow. In the merged images, the catenins colocalize with the H2kd molecules. B) β -catenin staining of 231, 231-Ecad and MCF7 cells. β -catenin is localized at the membrane in 231-Ecad and MCF7 cells but in the cytoplasm in 231 cells. C) Transfected MDA-231 cells express the H2kd fragment. When 231-H2kd whole cell lysates are probed with an H2kd antibody and immunoprecipitated, both beta- and p120 catenins coimmunoprecipitate as determined by western blot.

Additional file 3. MDA-MB-435 cells re-express E-cadherin. A) Immunoblot of MDA-MB-435 cells cultured with hepatocytes for 6 days and probed with an E-cadherin antibody. B) Methylation-specific PCR of MDA-MB-435/hepatocyte samples reveals loss of methylation of the E-cadherin promoter.