Published in final edited form as:

Clin Exp Metastasis. 2012 January ; 29(1): 39–50. doi:10.1007/s10585-011-9427-3.

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

Yvonne Chao*, Qian Wu*, Christopher Shepard, and Alan Wells

Department of Pathology, Pittsburgh VAMC and University of Pittsburgh, Pittsburgh, PA, 15213, USA

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 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 the GnRH agonist buserelin or the EGFR inhibitor 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.

Keywords

Epithelial-to-Mesenchymal Transition; Mesenchymal-to-Epithelial reverting Transition; Chemoresistance; Cell death

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,

Address correspondences to: Alan Wells, 3550 Terrace Street, S713 Scaife Hall, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261, tel (412) 647-8409, fax (412)-624-8946, wells@upmc.edu.

*These authors contributed equivalently to this work

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. Expression of E-cadherin on hepatocyte spheroids in culture protects against detachment-induced cell death, or anoikis, in a caspase-independent manner [15]. This is consistent with a report that 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, in a mode of signaling for these pathways that leads to cell survival rather than proliferation [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 parenchymal cells through heterotypic ligation, with this resulting in the sustained activation of ERK MAP kinase. Furthermore, E-cadherin re-expression also confers a functional 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]. These results were also observed 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: 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 was 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 was mirrored in the cell distribution pattern in which the E-cadherin-expressing cells (231-Ecad, and 231 after coculture) clustered, suggesting cell-cell contacts, whereas the E-cadherin-negative cells (231-shEcad) remained 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 was mediated by E-cadherin expression, hepatocytes (which express E-cadherin) or fibroblasts (which lack E-cadherin) were plated on collagen-coated plates at 30% confluency and cancer cells were seeded onto the monolayer the following day. Four hours later, the number of RFP-positive cells in the monolayer was counted as a measure of attachment. On the hepatocyte monolayer, E-cadherin-positive cells 231-Ecad and MCF7 exhibited significantly increased attachment compared to E-cadherin-negative cells ($p = 0.05$). (Figure 3a). However, when cultured on the fibroblast monolayer to account for nonspecific adhesion and adhesion to exposed collagen matrix there was no statistically significant difference in attachment between the various cell lines. However, it was possible that the differences in attachment were not

entirely E-cadherin dependent, as the plating of hepatocytes and fibroblasts at 30% confluency left portions of the collagen-coated plastic exposed. As a result, the cell lines were plated on densities ranging from 25 to 100% confluency. Thus, at higher hepatocyte densities attachment would 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 diminished by hepatocyte density while attachment of E-cadherin-negative 231 and MCF7-shEcad cells decreased with increasing hepatocyte density ($p < 0.05$ between 25% and 100%) (Figures 3b and 3d). In contrast, attachment of all cell lines decreased with increasing density of fibroblasts, further confirming that cancer cell-hepatocyte attachment is mediated by E-cadherin (Figure 3c and 3e). 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). This adhesion was not observed when 231 cells were cocultured with fibroblasts (Figure 3f). Thus, the re-expressed E-cadherin was capable of establishing 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 the E-cadherin 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 DiI (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 Figure 2), 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 suggested that E-cadherin ligation protects against cell death and increases drug resistance of tumors [23–25]. To evaluate chemoresistance, we tested the cytotoxic effect of various chemotherapeutic agents commonly used to treat breast cancer on our cell lines. Likely because 231 cells were derived from a patient who was treated with many of these agents, these cells were only slightly sensitive at best to many of the drugs tested: 5-fluorouracil, cyclophosphamide, doxorubicin, and taxol (Supplemental Figure 3). We therefore selected the protein kinase inhibitor and apoptosis-inducer staurosporine and DNA topoisomerase inhibitor camptothecin to induce cancer cell death in the following

studies; these agents are representatives of two categories of chemotherapy drugs. The TC50 was 54.18nM for staurosporine and 6.35μM for camptothecin (Supplemental Figure 4a and 4b); therefore, a range of 0.01 to 1000nM staurosporine and a range of 0.001 to 100μM camptothecin was used for cell survival analysis. Treatment of breast cancer cells with staurosporine and 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 5). The TC50s for staurosporine and camptothecin treatment of 231-Ecad was higher than for 231 and 231-shEcad cells, further demonstrating the chemoprotection effects from E-cadherin expression in breast cancer cells (Supplemental Figure 4). Similar results were observed in breast cancer cells treated with other chemotherapeutic drugs taxol and doxorubicin (Supplemental Figure 3c and 3d).

As noncycling cells are more difficult to kill than cycling cells, the findings of limited chemoprotection may simply reside from differences in mitogenesis between the lines. This was not the case, as all lines proliferated and expanded indistinguishably (Supplemental Figure 6).

To determine whether this chemoprotection was 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 with parenchymal cells of target organs [20, 22], or even just repression of EGFR signaling by EGFR kinase inhibition by a direct agent (PD153035) or indirectly by a gonadotropin-releasing hormone (GnRH) agonist [20, 26]. DU-145 prostate cancer cells were treated with 1 g/ml of the GnRH agonist 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). Addition of E-cadherin blocking antibody abrogated the effect on DU-145 cells (Supplemental Figure 7), indicating the chemoprotection was a result of E-cadherin re-expression. At least part of the limited degree of noted protection can be explained by the fact that not all of the prostate cancer cells re-express E-cadherin under the treatment (Figure 6b).

To understand the mechanism behind the chemoprotection exhibited by E-cadherin-positive cells, caspase 3 activity was assessed in 231, 231-Ecad and 231-shEcad cells following staurosporine or camptothecin treatment (Supplemental Figure 8). Mild reduction of caspase3 activity was observed in 231-Ecad cells compared to 231 and 231-shEcad cells after drug treatment, suggesting E-cadherin re-expression diminishes activation of apoptotic signals in breast cancer cells.

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 exogenous induction of E-cadherin 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 were also induced to heterogeneously re-express E-cadherin in the liver microenvironment (Figure 7a and Supplemental Figure 9a). A notable increase of E-cadherin expression localized to the membrane of DU-145 cells was observed after coculture with human hepatocytes (Figure 7b and 7c). This heterogeneous E-cadherin re-expression also exhibited increased resistance to cell death (Figures 7d and 7e). The increased chemoresistance was abrogated when DU-145 cells were transiently transfected with E-cadherin siRNA prior to coculture (Figure 7d, 7e and Supplemental Figure 9d). 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. No E-cadherin re-expression was observed in DU-145 and fibroblast coculture (Supplemental Figure 9b). 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 (Figure 7d and 7e).

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 (Supplemental Figure 9c) though hepatocyte metabolism of agent would still occur; in this situation, there was no evidence of chemoprotection (Figures 7d and 7e). Similar protection was obtained in DU-145 cells treated with cisplatin, a chemotherapy drug, after E-cadherin re-expression induced by hepatocyte coculture (Supplementary Figure 10).

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.

Some of the functional mechanisms behind the increased chemoresistance in E-cadherin re-expressing cells in our model have been revealed. 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]. Furthermore, we showed decreased activity of apoptosis effector caspase 3 in E-cadherin-positive cells, providing a second possible mechanism for the chemoprotection. Other studies suggest that anti-apoptotic proteins such as Bcl-2 or cell cycle inhibitors cyclin-dependent kinase inhibitor p27 may also be involved [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 either 231-Ecad cell lines or hepatocyte coculture (data not shown). Deeper 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 and prostate 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. 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.

MATERIAL 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]. Selected cells were isolated on more than one occasion with little difference between the selections; transient transfections also provided similar cell responses but were not used due to the cell-cell heterogeneity making cell quantitation difficult (data not shown). 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). E-cadherin blocking antibody was used at 5 μ g/ml (Invitrogen).

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, 2E4 RFP-labeled cancer cells were seeded in each well. Four hours later, wells were washed once with PBS to remove any unattached cells and the number of RFP positive cells in each well was quantified.

Centrifugal assay for fluorescent cell adhesion (CAFCA)

This assay is a modification of the McClay and Giacomello 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 100 M of camptothecin for 48 hours. Wells were then stained with 1 μ M 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-positive 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 DiI (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.

TC50 analysis

Breast and prostate cancer cells were plated in 96-well plates and treated with 0 to 1μM of staurosporine for 24 hours or 0 to 1000 M of camptothecin for 48 hours. Vybrant MTT cell proliferation assay was performed according to the manufacturer's protocol (Invitrogen).

Caspase 3 activity assay

Breast cancer cells were seeded in 8-well chamber slides and treated with 10nM and 100nM of staurosporine for 8 hours or 10μM and 100μM of camptothecin for 16 hours. Caspase 3 activity was measured by CaspaTag Caspase 3 in-situ assay kit according to the manufacture's protocol (Chemicon).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

These studies were supported by a Merit Award from the Veterans Administration and an IDEA Award from the Department of Defense (USA) CDMRP in Breast Cancer. We thank members of the Wells laboratory for discussions and suggestions.

Abbreviations

EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial to Mesenchymal Transition
GnRH agonist	Gonadotropin-releasing hormone agonist
MErT	Mesenchymal to Epithelial reverting Transition
PI-3k	Phospho-Inositide – 3OH kinase
shRNA	Short Hairpin RNA

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. [PubMed: 4701705]
2. Lermite E, Marzano E, Chereau E, et al. Surgical resection of liver metastases from breast cancer. *Surg Oncol*. 2010; 19 (4):e79–84. [PubMed: 19592234]
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. [PubMed: 14602130]
4. Diamond JR, Finlayson CA, Borges VF. Hepatic complications of breast cancer. *Lancet Oncol*. 2009; 10 (6):615–21. [PubMed: 19482250]
5. Wells, A.; Chao, Y.; Wu, Q. Biology of Metastatic Liver Tumors. In: Monga, SPS., editor. *Molecular Pathology of Liver Diseases*. Springer US; 2011. p. 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. [PubMed: 4682319]
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. [PubMed: 19319542]
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. [PubMed: 15967104]
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. [PubMed: 6549550]
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. [PubMed: 7545541]
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. [PubMed: 19167485]
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. [PubMed: 18773493]
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. [PubMed: 1540948]
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. [PubMed: 20003757]
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. [PubMed: 19056766]
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. [PubMed: 10383446]
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. [PubMed: 10969083]

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. [PubMed: 10428027]
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. [PubMed: 17406365]
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. [PubMed: 20609236]
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. [PubMed: 20026604]
23. St Croix B, Kerbel RS. Cell adhesion and drug resistance in cancer. *Curr Opin Oncol*. 1997; 9 (6): 549–56. [PubMed: 9370076]
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. [PubMed: 14985455]
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. [PubMed: 18983973]
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. [PubMed: 15655536]
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. [PubMed: 8830762]
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. [PubMed: 12892522]
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. [PubMed: 19637986]
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. [PubMed: 11920442]
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. [PubMed: 18058027]
32. Chao, YL.; Wu, Q.; Acquafondata, M., et al. Partial mesenchymal to epithelial reverting transition in breast and prostate cancer metastases [abstract nr. 3357]. 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. [PubMed: 10807587]
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. [PubMed: 19724901]
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. [PubMed: 20824051]
36. West KA, Castillo SS, Dennis PA. Activation of the PI3K/Akt pathway and chemotherapeutic resistance. *Drug Resist Updat*. 2002; 5 (6):234–48. [PubMed: 12531180]
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. [PubMed: 8898746]
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. [PubMed: 9679152]

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. [PubMed: 18193170]
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. [PubMed: 18708358]
41. Tsuji T, Ibaragi S, Hu GF. Epithelial-mesenchymal transition and cell cooperativity in metastasis. *Cancer Res*. 2009; 69 (18):7135–9. [PubMed: 19738043]

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. Shown are representative of three experiments.

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. Shown are representative of two experiments.

Figure 3.

E-cadherin expression increases attachment to hepatocytes. (A) Attachment of E-cadherin - negative and -positive breast cancer cells to hepatocytes or fibroblasts plated at 30% confluency. (B and C) Attachment of 231 and 231-Ecad breast cancer cells to hepatocytes or fibroblasts plated at 25 to 100% confluency. (D and E) Attachment of MCF7 and MCF7-shEcad breast cancer cells to hepatocytes or fibroblasts plated at 25 to 100% confluency. (F) Binding of breast cancer cells to hepatocytes or fibroblasts by centrifugal assay for fluorescence based cell adhesion. Shown are mean $\pm$ s.e.m. (n=3, in triplicate) ($P < 0.05$).

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. Shown are representative of at least three experiments.

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). Shown are mean $\pm$ s.e.m. (n=3, in triplicate) ($P < 0.05$).

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. In (A) and (B), shown are representative of three experiments. In (C) and (D), shown are mean $\pm$ s.e.m. (n=3, in triplicate) ($P < 0.05$).

Figure 7.

Prostate cancer cells that re-express E-cadherin in hepatocyte coculture are more chemoresistant to staurosporine and camptothecin. (A) E-cadherin re-expression in DU-145 cells after hepatocyte coculture. DU-145 cells are labeled by RFP. E-cadherin is shown in green. Left column, DU-145 only; Middle column, an example of E-cadherin re-expression in DU-145 cells with coculture; Right column, an example of negative E-cadherin expression in DU-145 cells with coculture. (B) Quantification of percentage of DU-145 cells with none, low, mid and high levels of Ecadherin. (C) Quantification of E-cadherin localization in DU-145 cells with and without hepatocytes coculture. (D and E) DU-145 cells are more chemoresistant to (D) staurosporine and (E) camptothecin. This effect is abrogated in fibroblast coculture or when cells are transiently transfected with E-cadherin siRNA prior to coculture, or transwell coculture with hepato. Shown are mean $\pm$ s.e.m. (n=3, in triplicate) ($P < 0.05$).