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**MESENCHYMAL TO EPITHELIAL REVERTING TRANSITION IN BREAST
CANCER METASTASES: A KEY ROLE FOR RE-EXPRESSION OF E-CADHERIN**

by

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MESENCHYMAL TO EPITHELIAL REVERTING TRANSITION IN BREAST CANCER METASTASES: A KEY ROLE FOR RE-EXPRESSION OF E-CADHERIN

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University of Pittsburgh, 2011

Metastasis is a major contributor to breast cancer mortality, as currently available therapies are unable to ensure progression or disease-free survival. Little is known about the molecular pathogenesis of metastasis, and the role of the surrounding microenvironment is only beginning to be understood. In vitro studies have repeatedly shown that epithelial to mesenchymal transition (EMT) and loss of E-cadherin expression are critical events in the initiation of metastasis and can be induced by the microenvironment. However, metastases are often well-differentiated and epithelial in phenotype, suggesting that EMT is reversible. The role of E-cadherin expression and mesenchymal to epithelial reverting transition (MErT) in metastatic colonization of the secondary site remains ill-defined. Evidence for E-cadherin re-expression and partial MErT was observed in metastases of breast and prostate cancer patients, and suggests that MErT is unstable and reversible. MDA-MB-231 breast cancer cells cultured with hepatocytes also resulted in E-cadherin re-expression and partial MErT, suggesting that such phenotypic plasticity can be induced by the microenvironment of the liver, a key site of breast cancer metastases. Re-expression of E-cadherin following hepatocyte coculture not only results in heterotypic ligation between cancer and liver parenchymal cells, but also activates Erk survival signaling and increases resistance to nutrient-deprivation and chemotherapy. Taken together, our results indicate that the distant organ microenvironment may induce E-cadherin re-expression and partial MErT to enhance the survival of metastatic cancer cells at the secondary organ.

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PREFACE

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1.0 INTRODUCTION

Breast cancer is a significant clinical problem that will afflict 1 in 8 women in her lifetime. Even with increased earlier detection and utilization of targeted therapies such as tamoxifen and Herceptin, the rates of patients diagnosed with metastatic breast cancer has not changed. Metastasis is still the major cause of breast cancer mortality, with no currently available therapies capable of ensuring disease-free, or even progression-free survival. Throughout metastasis, the cancer cell exhibits remarkable plasticity during its trek from primary site to distant organ, enabling it to survive through drastically different microenvironments. While previously this plasticity had been attributed to intrinsic genetic instability enabling in vivo selection of adaptability, current understanding is that the tumor behaves like a complex organ. Whether it is through interactions with immune and stromal cells or with the extracellular matrix, cancer cells are constantly engaging with the microenvironment, which greatly contributes to the plasticity of cancer cells. Also critical to the idea of phenotypic plasticity is that the metastatic cancer cell is a moving target, both literally and figuratively, and that therapies that work to prevent invasion in the primary tumor may have a different effect on already disseminated cells. Thus, elucidating the role of the microenvironment on cancer cell plasticity may significantly affect the way new therapeutics are developed, as targeting the microenvironment in tandem may lead to greater therapeutic success.

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1.1 BREAST CANCER

Approximately 200,000 women are diagnosed with breast cancer each year, making breast cancer the most common malignancy that strikes women in the United States and the second leading cause of cancer-related deaths. Women born today have a lifetime-risk of 12.5% of developing breast cancer later in life (Altekruse et al., 2010). Male breast cancer accounts for only 1% of all cases, with 2,000 new diagnoses each year. While increased screening has improved detection of breast cancer at earlier stages, mortality rates have not decreased because the number of women who develop advanced or metastatic disease has stayed the same.

1.1.1 Breast Anatomy

Breast carcinoma is a disease of the epithelia of the mammary gland, the presence of which defines the biological class of mammals. In humans, each breast comprises 5 to 9 mammary glands. Each mammary gland, also known as a ductal system, is composed of milk-secreting alveoli that cluster to form lobules that drain milk into ducts leading to the nipple (Love and Barsky, 2004). On a cellular level, alveoli are lined with cuboidal cells that produce and secrete milk. Ducts are composed of a bilayer of luminal epithelial cells and basal, or myoepithelial, cells that sit on a basement membrane. Mammary stroma consisting of extracellular matrix (ECM), fibroblasts, adipocytes, and inflammatory cells surrounds the ductal systems (Kuperwasser et al., 2004; Egeblad et al., 2010).

Starting in puberty, each simple mammary gland begins elaborate branching that results in a tree of multiple ducts and lobules. During menstruation and pregnancy there are additional cycles of cell proliferation and budding. Thus stem cells are necessary to replenish the ductal and

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lobular cells at these times. Based on expression of cell surface markers, studies have uncovered distinct cell populations in the breast: a mammary stem cell (MSC) population gives rise to basal and luminal progenitors and mature luminal and basal populations. Mammary stem cells are basally located in ducts and surrounded by proliferating progenitor cells. MSCs are believed to represent about 1:2000 of mammary epithelial cells (Stingl et al., 2001; Villadsen et al., 2007; Lim et al., 2009).

1.1.2 Classification

Breast cancer patients are clinically stratified according to stage, histopathology, and hormone receptor/marker expression to determine prognosis, risk of recurrence, and course of treatment. Tumors are staged according to the tumor node metastasis (TNM) system, which is based on tumor size and spread to adjacent lymph nodes or distant organs. Breast carcinomas can be categorized histopathologically into 18 subtypes with infiltrating ductal (IDC) and invasive lobular (ILC) being the most common at 75 and 15 percent of cases, respectively (Li et al., 2005; Harris et al., 2009). Breast cancers can also be classified according to expression of estrogen (ER) and progesterone receptors (PR) and amplification of the oncogene HER2/Neu.

While use of the criteria described above has been able to provide prognostic value, response to therapy within groups is still very heterogeneous. As a result, efforts are being made to discover molecular profiles or biomarkers to more accurately predict outcome. Recently, differences in gene expression patterns have resulted in the introduction of 6 molecular subtypes: Luminal A, Luminal B, HER2-enriched, Basal-like, Claudin-low and Normal Breast-like. Luminal A tumors are characterized by high ER/PR expression, low HER2 expression, and low expression of markers of proliferation. Luminal B tumors have weaker ER/PR expression

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compared to Luminal A tumors, variable HER2 expression, and high expression of proliferation markers. Tumors of the HER2-enriched subtype are marked by high HER2 amplification with absent expression of ER/PR while the Basal-like subtype typically consists of tumors negative for ER/PR and HER2 (commonly called triple negative). The Claudin-low subtype was previously considered a sub-population of the Basal-like subtype and includes triple negative tumors that exhibit low expression of cell adhesion molecules, high expression of mesenchymal and stem cell markers, and lower expression of proliferation markers (Perou et al., 2000; Sorlie et al., 2001; Herschkowitz et al., 2007; Prat et al., 2010). While these molecular subtypes are not currently used to affect treatment, they have been shown to correlate with prognosis, with Luminal A and B carrying the best and Basal-like carrying the worst prognosis (O'Brien et al., 2010).

It has been hypothesized that the molecular subtypes originate from different cell types (Prat and Perou). Women with BRCA-1 mutations are more likely to develop Basal-like tumors, and recent evidence suggests that they result from transformation of a luminal progenitor population. In addition, gene expression of the MSC population most closely aligns with the Claudin-low subtype while the mature luminal signature is closest to the Luminal A and B subtypes (Lim et al., 2009). Importantly, while the majority of cases can be segregated into the molecular subtypes according to expression of ER/PR/HER2, this is not always the case. For example, not all Basal-like tumors are triple negative and vice versa (Prat and Perou, 2010).

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1.1.3 Tumorigenesis

About 5-10% of breast cancer cases are hereditary, or the result of familial genetic mutations BRCA1 and BRCA2. The incidence of breast cancers that are attributed to genetic mutations increases in younger patient cohorts, with about 36% of patients diagnosed with breast cancer between the ages of 20 to 29 exhibiting mutations. Mutations in BRCA, a gene involved in the cell response to DNA damage, are autosomal dominant with varied penetrance. Thus reports of the lifetime risk of cancer of patients with BRCA mutations range from 36% to as high as 97% lifetime risk (Harris et al., 2009).

Besides the small percentage of cases that exhibit hereditary genetic mutations, breast cancer is for the most part a remarkably heterogeneous disease as there are few genetic insults that are common among all cases, unlike some other carcinomas. Tumorigenesis is a multi-step process that results from several genetic alterations that affect basic cell physiology resulting in malignant growth. Alterations that affect active growth signals, evasion of apoptosis, resistance to anti-growth signals, limitless replication, sustained angiogenesis, and tissue invasion can all contribute to tumorigenesis (Hanahan and Weinberg, 2000). Adding to the complexity, genetic insults do not even necessarily have to occur in the same cell. In *Drosophila*, tumor formation can result from different mutations in two adjacent epithelial cells (Wu et al., 2010). It has been proposed from pathologic observation that tumor transformation and progression is linear, with epithelial atypia, hyperplasia, and carcinoma in situ (DCIS or LCIS) as precursors to the invasive ductal and lobular carcinomas that are most common in breast cancer patients (Bombonati and Sgroi, 2011). However, genetic studies comparing chromosomal abnormalities in DCIS and IDC suggest that linear progression may not necessarily be the case (Buerger et al., 1999).

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There is much debate about the cancer cell of origin. The clonal model posits that any epithelial cell is capable of garnering the mutations needed to initiate a tumor. The cancer stem hypothesis argues that only a small population of cells is capable of initiating a tumor – either tissue stem or progenitor cells or cells that have acquired mutations leading to stem-like abilities. Because stem cells are long-lived and have the capacity for self-renewal and asymmetric division, they are more likely to acquire the genetic alterations for transformation, maintain the stem cell population and generate the heterogeneous tumor (Al-Hajj et al., 2003). Although transformed stem or progenitor cells are well-established in hematopoietic malignancies, the existence of a breast cancer stem cell is still controversial as tumor-initiating capacity depends widely on the experimental model used (Kelly et al., 2007; Quintana et al., 2008; Rosen and Jordan, 2009).

1.1.4 Primary tumor microenvironment

Despite the fact that a “carcinoma” indicates that the cancer is derived from an epithelial cell, tumors are like complex ecosystems in that they consist of many other cell types besides epithelial cells. The mammary gland derives from the ectoderm, but responds to underlying mesenchyme to form a breast bud (Anbazhagan et al., 1998). Even in adults the breast remains very responsive to signals from mesenchymal cells and embryonic mammary mesenchyme can induce differentiation in breast cancer explants (DeCosse et al., 1973). Thus, the mammary stroma is a significant part of the breast cancer tumorigenesis and participates in bi-directional interactions with epithelial cells. Interactions with recruited immune cells and endothelial cells can also affect tumorigenesis as failed immune surveillance of transformed cells results in frank tumor growth (Tlsty and Coussens, 2006). During the DCIS to IDC transition, there is significant

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upregulation of extracellular matrix components and metalloproteases in the stromal compartment, suggesting that ECM remodeling is an important part of the transformation process (Ma et al., 2009). In response to the primary tumor, the stroma often undergoes a desmoplastic response with increased deposition of ECM. The conversion of normal fibroblasts to carcinoma-associated fibroblasts is a major contributor of ECM deposition (Elenbaas and Weinberg, 2001). Collagen deposition results in increased stiffness of the breast during the transition from normal to premalignant to invasive cancer, which along with changes in other mechanical properties affects cell signaling and behavior. Inhibition of collagen cross-linking delays tumor onset and decrease tumor incidence (Levental et al., 2009; Yu et al., 2011).

Although gene expression changes are evident in all cell types comprising the tumor, genetic mutations are mainly found in the epithelial fraction, suggesting paracrine activation of stromal cells (Allinen et al., 2004; Hu et al., 2005). However, genetically abnormal stroma can initiate tumors or increase the mutation rate in adjacent epithelial cells (Radisky et al., 2005). Paracrine signals from cancer-associated fibroblasts (CAFs) promote tumor growth in non-tumorigenic and pre-malignant epithelial cells (Olumi et al., 1999; Shekhar et al., 2001; Cheng et al., 2005). In a study of gene expression profiles of all cell types in the breast microenvironment, DCIS-associated myoepithelial cells exhibited upregulation of genes involved in paracrine/autocrine signaling necessary for cell migration (Allinen et al., 2004). In pancreatic cancer, increased Hedgehog (Hh) expression in cancer cells results in Hh signaling in the CAFs. Therapeutic targeting of only CAFs is sufficient to reduce tumor progression (Yauch et al., 2008). These results suggest that targeting the more genetically stable microenvironment may be more effective than targeting the transformed epithelial cells themselves.

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1.2 METASTATIC BREAST CANCER

At most 5% of patients are diagnosed with metastatic breast cancer at presentation, yet around 30% of patients will be diagnosed with metastases at some point in the course of disease. The 5-year overall relative survival is a high 98% for those with localized disease to the breast, 83.6% for cancer that has spread to regional lymph nodes, and 23% for cancer that has metastasized at diagnosis (Altekruse et al., 2010). Recurrence is most common in the first five years after initial diagnosis, but relapses can occur as many as 30 years later. Only 2 to 5 percent of those diagnosed with advanced disease survive longer than 10 years as the therapies available today are mainly palliative, temporary, and not curative.

Like the pathogenesis of the primary tumor, metastasis results from a complex progression of cellular and molecular events. First, the tumor cells detach from the primary mass to invade into the surrounding stroma and intravasate into lymph or blood vessels. Secondly, following intravasation tumor cells must survive in the circulation and eventually embolically arrest in a capillary bed. Third, the cancer cell extravasates out of the vasculature into the secondary organ. Finally, these post-extravasation individual or clusters of cells undergo apoptosis, lie dormant for an indeterminate length of time, or proliferate to form a micro- and then macrometastasis. These steps must all occur in step-wise succession, and as a result, metastasis is a highly inefficient process. Failure to complete any one of these steps can prevent metastasis, but only a few are considered rate-limiting and contribute to metastatic inefficiency. In a chick embryo assay, all cancer cells injected into the circulation survive and extravasate into surrounding tissue by 24 hours (Koop et al., 1995), yet only 0.01% of cancer cells injected into the circulation form metastatic foci (Fidler, 1970). This was confirmed in rodent models of liver

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metastases, indicating that post-extravasation survival followed by initial and persistent growth of metastatic cancer cells are rate-limiting steps (Luzzi et al., 1998; Cameron et al., 2000).

1.2.1 Invasion

Invasion is the first step of the metastatic process. While invasion is necessary for metastasis, the finding of invasive or infiltrating cancer cells does not necessarily mean that the cancer has metastasized. The ability to invade involves the loss of tumor cell adhesion, processing of the ECM, changes in integrin expression, and induction of pro-migratory cell signaling (Wells et al., 2011). In general, cancer cells employ either single cell or collective cell migration. The mode of migration is dependent on differences in extracellular proteases, integrin-mediated cell-matrix adhesion, cadherin-mediated cell-cell adhesion, cell polarity, and cytoskeletal arrangement (Yilmaz and Christofori, 2010). Even within single cell motility, intravital imaging has revealed that there are very motile single cells and slower collectively moving cells, and that the mode of motility determines spread through the blood or lymphatic system (Giampieri et al., 2009).

For single motile cells, there are two forms of migration: mesenchymal-like or amoeboid. Mesenchymal-like migration follows employment of cancer-associated epithelial to mesenchymal transition (EMT), which will be discussed in detail in Section 1.4. Amoeboid migration is characterized by limited interaction with the ECM and independence from extracellular proteases. This type of movement is exhibited by immune cells and is often observed in hematopoietic malignancies. Cancer cells can switch quickly between these two modes, and specific GTPases, Rac or Rho, determine the mode of migration (Sanz-Moreno et al., 2008). Collectively motile cells, on the other hand, invade with cell-cell adhesions intact. Invasion can occur as strands, clusters, sheets or tubes and is usually led by a subset of cells at

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the front that generate enough force to pull the cells at the rear. This type of migration requires extensive degradation of the ECM (Wolf et al., 2007).

Chemokines and cytokines secreted by cells in the tumor microenvironment are also involved in the induction of cell motility during invasion by producing a chemoattractant gradient. One of the best-described examples is that of the tumor-associated macrophages (TAM) that chemotax breast cancer cells in reciprocal paracrine signaling of CSF-1 and EGF (Wyckoff et al., 2004; Patsialou et al., 2009). The onset of invasion also coincides with changes in ECM structure and composition. The ability of cancer cells to alter the stromal microenvironment correlates with the tumor invasive potential (Li et al., 2009; Soikkeli et al., 2010). Cancer cells induce gene expression changes in fibroblast and other stromal cells to produce ECM molecules that promote tumor migration and increase MMP production to loosen the stiffness of the matrix (Gallagher et al., 2005; Adam et al., 2006). Various ECM proteins are upregulated in invasive carcinomas, including collagens I and IV, laminins, tenascin C, fibronectin and vitronectin. Modulation of cell motility is achieved by varying the expression of adhesive and anti-adhesive ECM proteins as well as their ligands. The release of embedded growth factors following proteolytic cleavage of ECM components provides a positive feedback loop to augment motility (Friedl and Wolf, 2010).

1.2.2 Intravasation

Intravasation is the process of entering the circulation, and can occur into the blood or lymphatics. Increased tumor size correlates both with lymph node involvement and distant metastases (Carter et al., 1989). Intravital imaging has revealed that although both metastatic and non-metastatic cells may be motile, metastatic cells exhibit greater polarization toward blood

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vessels, which is generated by a chemoattractant EGF gradient released by macrophages lining the vasculature (Condeelis et al., 2000; Wyckoff et al., 2000). Intravasation into the lymphatic system may be more passive than hematogenous dissemination, as lymphatics lack tight junctions, layers of pericytes, and basement membranes observed in endothelial cells. The leakiness and low pressure of the lymphatic vessels seem to favor amoeboid-like and collective migration (Byers et al., 1995). Interestingly, inhibiting lymphatic spread has no effect on distant metastasis, suggesting different mechanisms (Wong and Hynes, 2006).

The mechanisms for intravasation and extravasation are similar. During the transendothelial migration of hematogenous dissemination, the cancer cell must break the bonds of intercellular adhesion formed by VE-cadherin between endothelial cells (Sandig et al., 1997). Expression of N-cadherin has been shown to be necessary for cancer cell adhesion to endothelial cells, which upregulates Src and causes the down-regulation of VE-cadherin on the attached endothelial cell (Qi et al., 2006). During prolonged heterotypic ligation of cancer cells and endothelial cells, activation of Src leads to phosphorylation of N-cadherin, facilitating the temporary dissociation of β -catenin and consequently migration across the endothelial barrier. The generation of fibrosarcoma cell variants that differ in their ability to intravasate, but not in ability to form primary tumors or metastases, has revealed additional cell-surface molecules involved in intravasation: cell adhesion molecules N-CAM and JAM-C, and tissue factor, a surface receptor active in the coagulation cascade (Conn et al., 2008). An in vitro model of intravasation using MDA-MB-231 cells and HUVEC cells separated by a transwell filter showed that transendothelial migration induced apoptosis in HUVEC cells (Peyri et al., 2009).

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1.2.3 Survival in circulation

Following intravasation, metastatic cells must survive in the circulation by evading anoikis, or detachment-induced cell death. Metastatic and non-metastatic melanoma cells are separated by their ability to evade apoptosis upon arrest in the vasculature (Kim et al., 2004). Normally, attachment to the ECM by integrins is necessary for survival of mammary epithelial cells (Streuli and Gilmore, 1999). Furthermore, not just any attachment is sufficient; mammary epithelial cells undergo apoptosis on fibronectin or collagen-coated surfaces but survive on laminin-rich surfaces reminiscent of the basement membrane (Boudreau et al., 1996). The EMT that enables dissemination results in disruption of polarity and unfettered growth factor signaling and loss of E-cadherin is sufficient to induce anoikis (Grossmann, 2002).. Excessive growth factor stimulation induces cell rounding and anoikis, applying selection pressure on the cancer cell to develop resistance to anoikis (Frisch and Screaton, 2001). Constitutively active FAK signaling upstream of integrin signal transduction can increase resistance to anoikis (Frisch and Ruoslahti, 1997). Other mechanisms of evasion include upregulation of the caspase inhibitors XIAP or FLIP or increased degradation of the pro-apoptotic protein Bim (Simpson et al., 2008). Inhibition of FLIP or XIAP does not prevent orthotopic growth but prevents metastases when cancer cells are injected into the circulation

Circulating tumor cells (CTCs) can be detected in the blood of breast cancer patients, even before the presentation of clinical metastases and even in some cases of DCIS (Ignatiadis et al., 2011). However, the presence of detectable CTCs in the bloodstream indicates that a large number of cancer cells are shed, with one study estimating this figure at 4×10^6 cells per gram of tumor released daily (Butler and Gullino, 1975). Whether or not detection of CTCs is prognostic for metastasis is still up for debate (Braun and Naume, 2005; Harris et al., 2007). Furthermore,

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once disseminated, CTCs do not remain in the circulation for long, as demonstrated by the chick chorioallantoic membrane assay for metastasis where almost 90% of cells had extravasated 3 days after injection (Luzzi et al., 1998).

1.2.4 Extravasation

In the circulation, tumor cells arrest in organ capillary beds due to size restriction or embolization following binding to coagulation factors. Although mechanical entrapment of circulating tumor cells can occur, tumor cells must also adhere to the vasculature and extravasate, or transmigrate through the endothelium into organ parenchyma. While the mechanisms behind intravasation and extravasation are similar, studies suggest that they are not identical. Cellular motility mediated by the tetraspannin CD151 was found to be necessary for both intravasation and for migration within the secondary site, but not for extravasation (Zijlstra et al., 2008).

The process of extravasation is similar to diapedesis exhibited by leukocytes in inflammation. During diapedesis, leukocytes adhere to and roll along the vasculature and then migrate between endothelial cells. The initial attachment of cells to the endothelium is mediated by a class of cell adhesion molecules called selectins, followed by stronger adhesions facilitated by immunoglobulin adhesion molecules, integrins and cadherins. Expression of many of these same cell adhesion molecules are necessary for extravasation of disseminated carcinoma cells (Glinskii et al., 2005). Cancer cell rolling mediated by selectins has been observed in vitro (Giavazzi et al., 1993; Hsu et al., 2011; Myung et al., 2011).

Selectins are a family of adhesion receptors that bind to carbohydrate ligands. Presentation of selectin ligands on cancer cells is believed to be critical to extravasation.

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Interactions of circulating cancer cells with platelets and leukocytes via P- and L-selectins may support tumor cell embolic arrest and immune evasion in the vasculature (Paschos et al., 2009). Cancer cell binding to E-selectin on endothelial cells is critical to the extravasation of colon cancer cells in metastatic colonization of the liver. Attachment of cancer cells to the endothelium and subsequent formation of metastases can be inhibited by addition of antibodies against E-selectin (Brodt et al., 1997). Furthermore, selectin-dependent adhesion to endothelial cells results in morphology changes, reorganization of the cytoskeleton, and tyrosine phosphorylation, suggesting that these interactions are not limited to adhesion and may have downstream signaling effects (Di Bella et al., 2003). Differential expression of selectin ligands can also influence the site of metastatic colonization and account for organotropism (Gout et al., 2008).

Following the attachment initiated by selectin binding, other adhesion molecules may further strengthen adhesion between cancer cells and endothelial cells. Expression of immunoglobulin cell adhesion molecule (IgCAM) family members ICAM and VCAM has been observed in distant metastases of colorectal cancer. The attachment of metastatic cells to endothelial cells and to extracellular matrix is necessary for metastasis (Schmidmaier and Baumann, 2008). The cadherin switch that sometimes occurs during EMT results in the down-regulation of E-cadherin and the upregulation of N-cadherin. N-cadherin expression on endothelial cells may facilitate the heterotypic binding of cancer cells to endothelial cells. Indeed, N-cadherin has been shown to mediate attachment of MCF-7 breast cancer cells to endothelial monolayers as well as the transendothelial migration of melanoma cells (Sandig et al., 1997; Hazan et al., 2000). Similarly, exogenous expression of gap junction molecule connexin 43 (Cx43) in MDA-MET, a breast cancer cell line variant that is highly metastatic to bone, results in increased adhesion to endothelial cells. Others have shown similar heterophilic

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binding between cancer cells and endothelial cells in melanoma and lung cancer (el-Sabban and Pauli, 1994; Ito et al., 2000). Finally, engagement of integrins expressed on cancer cells contributes to adhesion to the microvasculature, as antibodies against $\beta 1$, $\alpha 2$, and $\alpha 6$ integrins inhibit adhesion to and migration through sinusoids in colorectal metastases to the liver (Enns et al., 2004). Although cancer cells may arrest in capillaries due to size-restriction, these studies show that adhesion to endothelial cells is nonetheless a required step of extravasation.

1.2.5 Colonization

Once integrated into the parenchyma, the stimulation or inhibition of tumor growth can be affected by the specific growth factor milieu in the organ, which will be discussed further in Section 1.2.6.2. Once in the new receptive organ, metastatic cancer cells can undergo apoptosis, become dormant, or proliferate (Fidler, 2002). Dormancy occurs as single or clusters of cells in a state of quiescence –not slow growing, as studies suggest that rapid growth upon emergence from dormancy is more likely rather than continual slow growth (Demicheli et al., 2007). Interestingly, there is a bimodal distribution of recurrence, with a peak at 2 years post diagnosis and another at 5 years, which is across the board for all organ sites, suggesting that dormancy is the product of something intrinsic within the cancer cell, not the site of metastasis (Demicheli et al., 2008). Dormant, viable cells can be isolated from metastasis-free organs in vivo and then go on to generate tumors in other animals (Naumov et al., 2002; Suzuki et al., 2006). The factors that determine post-extravasation survival over dormancy or cell death are still unknown. Studies have shown Ras overexpression is one factor that is necessary to tip the proliferation:apoptosis ratio in favor of proliferation to make the transition from micro to macrometastases (Varghese et al., 2002).

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1.2.6 Seed and soil hypothesis

Stephen Paget observed as early as 1889 that cancers display a characteristic pattern of metastasis -- that some tumors have a proclivity for forming metastases in certain organs. He termed his explanation for this the “seed and soil” hypothesis (Paget, 1889). In breast cancer, the most common sites of distant metastases are bone, liver, lung, and brain. This pattern of metastases cannot be explained solely by mechanical arrest in the first capillary bed encountered after leaving the breast (Tarin and Price, 1981; Tarin et al., 1984; Glinskii et al., 2005). Rather, it has been proposed that the ability of the cancer cells, or “seeds”, to survive and to proliferate in the secondary organ is dependent on appropriate signals and supportive environment, or the “soil” (Fidler, 1970). The heterogeneous nature of tumors suggests that cells within a primary tumor have different metastatic properties in that some may be more targeted to certain organs while others have lower metastatic potential overall (Fidler and Kripke, 1977; Hart et al., 1981). The histopathology of the primary tumor has been shown to be associated with the specific site of metastasis: extent of fibrosis is associated with bone metastases, tumor necrosis and ER/PR negative status with lung, and 10 or more nodal metastases with liver metastases (Hasebe et al., 2008).

1.2.6.1 The Seed

The notion of the “seed” is that cancer cells exhibit intrinsic heterogeneity that predispose to sites of metastasis or may express molecules to home to specific organs. Examples of this are the overexpression of CXCR4, integrins, and other adhesion molecules that allow selective homing and migration to bone (Woodhouse et al., 1997; Roodman, 2004). Cancer cells often engage in molecular mimicry in which they secrete parathyroid hormone-related protein (PTHrP), which

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activates osteoclast-mediated degradation of bone matrix, resulting in the release of growth-stimulating factors that create a vicious cycle of cancer cell growth and the release of more PTHrP (Steeg, 2006). Chemokines CXCR3 and CCR7 are highly expressed in cancer cells and involved in homing to lung, bone, and lymph nodes, where the ligands CXCL12/SDF-1 and CCL21 are expressed. Similarly, CXCR4-expressing breast cancer cells respond to the high CXCL12 activity in lung tissue, helping to explain the lung specificity seen in breast cancer metastasis. Blocking presentation of the ligand reduces lung metastasis in vivo (Muller et al., 2001).

Within tumors a subset of cells are genetically programmed to metastasize to certain sites. For example, increased hypoxia in the primary tumor generates a hypoxia response signature that primes for metastasis to the lungs (Lu et al., 2010). Variants of the MDA-MB-231 cell line that exhibit tropism to metastasize to the bone or lung have been isolated (Minn et al., 2005). Microarray analysis of their gene expression profiles reveals a lung metastasis signature and a bone metastasis signature that are distinct from the poor prognosis signature (Van't Veer et al., 2002; Kang et al., 2003; Minn et al., 2005). Genes differentially expressed by lung metastasis variants include the cell adhesion molecules *SPARC* and *VCAM2*, the chemokine *CXCL1*, and metalloproteases *MMP1* and *MMP2*. Similarly, the bone metastasis variant demonstrates upregulation of CXCR4, activator of osteoclast differentiation interleukin-11, and osteopontin, which promotes osteoclast adhesion to bone matrix. These variants were also found to be pre-existing in the parental population and selected for in vivo. To demonstrate that the propensity to metastasize to one organ over another is intrinsic, fusion of the two cell variants generates clones that have dual organotropism (Lu and Kang, 2009).

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1.2.6.2 The Soil

The soil aspect of the seed and soil hypothesis posits that the specific organ microenvironment supports tropism. For example, the bone microenvironment is considered “fertile”, as it is rich with growth factors bound to the bone matrix that are released upon degradation by osteoclasts. These growth factors such as EGF and VEGF cause selective growth of cells that metastasize to bone (Woodhouse et al., 1997). In the brain, astrocytes abundantly secrete cytokines such as IL-1, IL-3, IL-6, TNF α , IGF-1, and PDGF-1, of which tumor cells take advantage to increase their survival and growth (Lu and Kang, 2007). The ECM and conditioned media of liver parenchymal cells has also been shown to preferentially stimulate the growth of cancers that metastasize there (Zvibel et al., 1998).

Changes in the microenvironment, through the infiltration of immune cells, matrix remodeling, and increases in reactive oxygen species have been observed in distant organs prior to metastasis, suggesting that the preparation of a premetastatic niche may contribute to organ-specific metastasis (Kaplan et al., 2006). Infiltration of bone marrow-derived hematopoietic progenitor cells (HPCs) are found in the premetastatic niche, prepping the “soil” for extravasating cancer cells. The HPCs are mobilized to the premetastatic niche by factors secreted by cancer cells, which is cell-type specific. Conditioned media from Lewis lung carcinoma (LCC) cells results in clustering of HPCs in lung and liver while conditioned media from B16 melanoma cells results in mobilization to lung, liver, testis, spleen and kidney, all common sites of melanoma metastasis. Interestingly, LLC cells can be reprogrammed to metastasize to the sites observed by B16 melanoma cells by preconditioning with B16 conditioned media. Furthermore, these HPCs express VEGFR and secrete MMP9 to degrade the basement membrane to allow for further infiltration and to alter the local microenvironment, making it

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more suitable for tumor cell attachment, survival, and growth. Clusters of VEGFR+ cells have been observed in human tissue in common sites of metastasis in the absence of tumor (Kaplan et al., 2005). However, the use of monoclonal antibodies to block VEGFR does not affect metastatic tumor growth *in vivo*, suggesting that there are alternate pathways (Duda and Jain, 2010).

1.2.7 Liver metastases

The liver is the most common site of metastases, so much that liver metastases are more common than primary hepatic tumors. The liver is not a common initial site of metastases but 40-75% of women diagnosed with metastatic breast cancer will present with liver metastases at some point during the course of disease (Viadana et al., 1973; Lermite et al., 2010). The median survival of breast cancer patients diagnosed with only liver metastases is less than 25 months and decreases to 15.5 months when metastases at other organ sites are also present (Atalay et al., 2003). Liver metastases present stealthily, with abdominal pain, hepatomegaly, or abnormal liver function values as the main clinical symptoms. Only a few patients will present with the more noticeable symptoms of ascites or jaundice.

Grossly, the liver consists of four lobes and is supplied by the hepatic artery and portal vein, which drains the spleen and gastrointestinal system. Blood flows through the sinusoids and empties into the central vein of each lobule. Hepatocytes are the epithelial cells that constitute around 70% of the liver, and are organized in plates a single-cell thick along sinusoids. Fenestrated endothelial cells that lack basement membranes, a unique feature of the liver, line sinusoids. The space of Disse, which drains lymph, provides a separation between hepatocytes

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and sinusoids. Kupffer cells are scattered between endothelial cells and phagocytose red blood cells. Stellate cells produce ECM and actively respond to liver injury by secreting collagen.

In general, liver metastases exhibit several patterns of growth. Desmoplastic lesions are separated from the parenchyma by a fibrotic ring and do not conserve the existing liver architecture. In the “pushing” pattern of growth, which is similar to desmoplasia but without fibrosis, tissue architecture is also not conserved. In the replacement pattern, cancer cells take the place of hepatocytes and co-opt the existing vasculature, conserving tissue architecture. Breast cancer liver metastases most commonly exhibit the replacement pattern. Hypoxia and angiogenesis thus occur much later compared to other modes of tumor growth (Stessels et al., 2004).

Not much is known about the signals from the liver that promote colonization but there are several features unique to the liver that may contribute to a ripe environment for metastases. First, fenestration of the sinusoid endothelium may allow for easier extravasation and access into the parenchyma. In addition, factors secreted by liver sinusoidal endothelium and not lung endothelium induce the motility of metastatic cancer cells (Sawada et al., 1996). Likewise, Kupffer cells of the monocyte lineage secrete soluble and matrix factors trophic for cancer cells (Wyckoff et al., 2004). The unique composition of the liver ECM also plays a role, as it has been shown that organ-specific metastasis correlates with the ability for cancer cells to survive on organ-derived matrix (Doerr et al., 1989). Heparin proteoglycans in the liver ECM regulate autocrine EGF activation in colon cancer cells and hepatocyte-derived growth factors strongly stimulate the growth of highly liver metastatic cells (Zvibel et al., 1998). In vivo selection for a liver-aggressive variant of 4T1 breast cancer cells revealed that claudin-2 is upregulated in liver metastases and improves adhesion of liver-aggressive cells to fibronectin and collagen IV, key

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components of the liver ECM, through increased recruitment of $\alpha 5\beta 1$ - and $\alpha 2\beta 1$ -integrin complexes (Tabaries et al., 2010).

Invasive breast carcinomas have been shown to express higher levels of hepatocyte growth factor (HGF) and its receptor MET (Jin et al., 1997) when compared to ductal carcinoma in situ. Comparative proteomic analysis of variants of the metastatic MDA-MB-435 breast cancer cell line selected in vivo for metastasis to the lung or to the liver revealed a panel of proteins that were differentially expressed in liver but not lung metastases that included pathways involving HGF, PDGF, VEGF, and EGF (Martin et al., 2008). Similar to the primary tumor microenvironment, metastasis-associated fibroblasts also express different gene expression profiles compared to normal liver fibroblasts or fibroblasts derived from other organs, which include the up-regulation of many adhesion molecules such as VCAM, ICAM, K-cadherin, and N-cadherin (Nakagawa et al., 2004).

1.2.8 Experimental models of metastasis

As ethical constraints prevent testing in humans, most in vivo studies of metastasis are performed in immunocompromised mouse or rat animal models. There are two well-established methods of studying metastasis in animal models: spontaneous and experimental metastasis. In spontaneous metastasis, breast cancer cells are injected at the orthotopic site, or the mammary fat pad, and allowed to metastasize. All the steps of the metastatic cascade must be completed to observe metastases using this method. For experimental metastasis, the steps of invasion and dissemination are bypassed as cells are injected directly into the circulation via tail vein, intracardiac, or intrasplenic injection.

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The use of immunocompromised animal models is necessary for human cell inoculation, but then the role of the immune system cannot be ascertained. Another issue with animal studies is that the specific pattern of metastasis observed in humans is either not always replicated in animal models or may be limited by other factors. For example, because the liver is not usually the site of initial metastases, most experiments are ended prior to observation of liver metastases due to excessive tumor burden. Finally, animal studies are end-stage in that the endpoints are whether metastases develop or do not develop, thus preventing observation of the actual process.

Intravital or in vivo imaging techniques seek to overcome this problem. Whole body imaging using bioluminescence or fluorescence enables the detection of developing metastases in the same animal over time. Imaging at the single cell level through an imaging window and imaging using multiphoton confocal microscopy have provided insights into cell motility and invasion during intravasation and extravasation, but is technically limited by depth of field and length of time of observation (Sahai, 2007; Kedrin et al., 2008). Imaging of biological processes in tumors can exploit reporter gene or GFP-tagged proteins to investigate molecules or pathways of interest in vivo. Probes to detect MMP activation, apoptosis, and hypoxia have been used (Bouvet et al., 2006; Sahai, 2007). Using a GFP transgenic mouse and color-coded tumor and stromal cells, the recruitment of fibroblasts to the metastatic tumor microenvironment was observed in the liver in real time (Suetsugu et al., 2010).

Given that liver metastases in animal models are not commonly observed, organotypic bioreactors are a viable option. 2D culture of isolated primary hepatocytes is limited because of the quick loss of liver-specific functions. Expression of important liver enzymes is lost within one day of in vitro adherent culture of primary hepatocytes. Liver tissue slices have been used, but without perfusion, areas of the slice become quickly necrotic (Cross and Bayliss, 2000). In

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3D culture, hepatocytes form spheroid aggregates that can include non-parenchymal cells and extracellular matrix components. Liver bioreactors are such an improvement over 2D cultures that their use has been considered for extracorporeal liver support for those awaiting transplant (Sauer et al., 2003; Gerlach, 2006). Primary hepatocytes cultured in a 3D perfused bioreactor maintain a phenotype much closer to hepatocytes in vivo in terms of both gene expression profiles and drug metabolism (Sivaraman et al., 2005). These bioreactors are designed to mimic physiologic fluid stress experienced in the liver. In many cases, imaging in real-time is possible (Powers et al., 2002). Non-parenchymal cells (NPCs) can also be introduced, as ECM and interactions with NPCs undoubtedly affect hepatocyte function. The ability to add NPCs enables layers of complexity to probe the metastatic process (Zeilinger et al., 2004; Hwa et al., 2007).

1.3 TREATMENT

Tumor stage, histology and ER/PR/HER2 marker expression are a constellation of factors that affect the course of treatment for the primary tumor. If the tumor has not spread to surrounding lymph nodes or distant organs, local resection with radiation is usually sufficient. Chemotherapy prior to surgical resection may be indicated for large tumors to shrink the tumor. Otherwise, adjuvant systemic therapy is clinically indicated following surgical removal for most node-negative and node-positive cancers. Systemic therapy can include any combination of chemotherapy, hormone therapy (such as tamoxifen or an aromatase inhibitor), or HER2-targeted monoclonal antibody (trastuzumab or Herceptin), depending on ER, PR, and HER2 status (Goldhirsch et al., 2009).

For breast cancer liver metastases, surgical resection is only indicated for isolated

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metastases. Isolated metastases are observed in 5% of patients with liver nodules, and post-resection there is a 50% recurrence rate. Thus the survival rate of patients presenting with metastatic nodules in the liver is low (Lubrano et al., 2008; Lermite et al., 2010). The high rate of post-hepatectomy tumor recurrence may be explained by growth factor release (HGF, EGF, and VEGF) and ECM remodeling that occurs during liver regeneration following hepatectomy, which may promote the growth of dormant metastases (Paschos and Bird, 2010). Because most patients present with multiple liver metastases, systemic chemotherapy is therefore indicated, which can be combined with transarterial chemoembolization (TACE) to more directly target the liver. Even still, systemic chemotherapy and endocrine therapy only have a 60% response rate. Methods of palliation of liver metastases include radiofrequency ablation, selective internal radiotherapy (SIRT) using resin microspheres labeled with Yttrium-90, or stereotactic body therapy (SBRT) (Lubrano et al., 2008; Lermite et al., 2010). These treatments are palliative but do not prevent progression (Diamond et al., 2009). Although initially promising, antiangiogenic drugs such as Avastin (VEGF-A inhibitor) have had little clinical success. For breast cancer liver metastases specifically, the replacement pattern of liver metastatic growth in which existing vessels are co-opted in place of angiogenesis, suggests that anti-angiogenic therapies may not be beneficial on micrometastases (Stessels et al., 2004).

1.3.1 Personalized medicine/ Targeted therapies

While ER/PR/HER2 status provides a guideline for treatment, within each group there is still a large amount of heterogeneity of response to therapy. Thus efforts are turning to more closely tailored therapy based on the molecular profile of each patient's tumor. While the classification of tumors into molecular subtypes has yet to directly influence clinical treatment, gene

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expression profiles or molecular signatures are being used to predict outcome and response to treatment in a subset of tumors. These gene signatures are used to predict response to additional adjuvant systemic therapy and are not used in place of traditional clinical factors. Oncotype DS and MammaPrint are the most well-validated of these molecular profiling prognostic assays. Oncotype DS uses a 21-gene signature to predict low, intermediate, or high risk of recurrence rate in node-negative, ER-positive tumors. The MammaPrint assay uses a 71-gene signature that requires fresh tissue and identifies low and high risk of relapse (Harris et al., 2007).

1.3.2 Chemoresistance

Tumors can be intrinsically resistant to chemotherapy or acquire resistance over the course of treatment. Even within ER+ and HER2+ tumors, not all tumors respond to endocrine therapy or Herceptin. Gene profiles delineating the responders from the non-responders are being developed to predict which patients will have therapeutic success. Little is known about the specific mechanisms behind both intrinsic and acquired chemoresistance, but because of genetic instability of cancer cells, selection pressure can lead to adaptations promoting resistance: drug efflux, drug inactivation, mutation of drug targets, DNA repair, activation of survival signaling, and evasion of apoptosis (Wilson et al., 2006). Overexpression of ATP-binding Cassette (ABC) transporters such as P-glycoprotein has been observed in cancer cells resistant to common chemotherapy drugs like camptothecin, vincristine, and 5-fluorouracil (5FU). Many drugs must be converted to active forms using endogenous enzymes and cancer cells can become resistant by decreasing expression of these enzymes or by increasing expression of enzymes that increase clearance or inactivation. Similarly, mutation or overamplification of the drug target also occurs. For example, cell lines resistance to 5FU exhibit overexpression of target thymidylate synthase.

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Increased expression of DNA damage repair proteins leads to resistance to drugs whose mechanism of action is DNA damage. Over-expression of anti-apoptotic proteins like Bcl-2 or decreased expression of apoptotic proteins like Fas or p53 are common methods of mediating drug resistance. Conversely, overexpression of protein tyrosine kinases that activate pro-survival pathways like Akt and Erk are alternative mechanisms of chemoresistance.

Under the cancer stem cell hypothesis, one of the prevailing explanations for the chemoresistance observed in cancer is that current therapies are cytotoxic to replicating cells but leave slow cycling tumor-initiating cells intact (Chuthapisith et al., 2010). Stem cells are also loaded with multiple drug transporters, enhancing drug efflux. If cancer stem cells are responsible for generating tumors and metastases, finding effective cancer therapies is a qualitative versus a quantitative problem, as killing the bulk of the tumor will have little effect. Recently, high-throughput screens have isolated several compounds that selectively target the small population of tumor-initiating cells (Gupta et al., 2009).

Given the role of the microenvironment in tumorigenesis and metastatic progression, it is not surprising that interactions with stromal cells facilitate chemoresistance. In metastases of melanoma and breast cancer to the brain, interactions with astrocytes increase chemoresistance (Lin et al., 2010). Stromal ECM protects colon cancer cells from cell death induced by camptothecin or etoposide while collagen I, fibronectin and fibroblast-derived ECM increase the chemosensitivity of cancer cells to 5-FU (Kouniavsky et al., 2002). Hypoxia of the tumor microenvironment also increases the radio- and chemoresistance of tumors (Brown and Wilson, 2004). With both intrinsic and extrinsic contributions to chemoresistance and cancers often exhibiting multidrug resistance, developing effective therapeutics is clearly a difficult task.

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1.4 EPITHELIAL TO MESENCHYMAL TRANSITION

One mechanism that is important to metastatic progression is the re-employment of epithelial-mesenchymal transition (EMT). During EMT, cells lose apico-basal polarity, gain motility, and lose cell-to-cell contacts through the loss of cadherins and other specialized structures for adhesion, allowing for the detachment and dissemination of cancer cells (Thiery, 2002; Tse and Kalluri, 2007). EMT is a program deployed during embryonic development that is required for gastrulation as well as morphogenesis of the neural crest, musculoskeletal system, craniofacial structures, and the peripheral nervous system (Thiery, 2002). EMT is also utilized in adult physiological processes such as wound healing, where keratinocytes undergo a process akin to EMT during re-epithelialization of the wound, as well as pathological processes such as kidney fibrosis, idiopathic pulmonary fibrosis, and cataract formation in the lens (Thiery and Sleeman, 2006). As there are subtle differences between the pathologic and physiologic phenotypic transitions, EMT is now classified as developmental EMT, fibrosis-associated EMT, and cancer-associated EMT (Kalluri and Weinberg, 2009). The cancer-associated EMT is more loosely defined as plasticity exhibited by an epithelial cell rather than as true differentiation into a mesenchymal cell.

There is increasing evidence for the role of EMT in tumor progression. Many studies have shown that tumor progression and poor prognosis correlate with expression of proteins characteristic to mesenchymal cells, such as vimentin, fibronectin, and matrix metalloproteinases, as well the down-regulation of proteins typical to epithelial cells such as E-cadherin (Thiery and Sleeman, 2006). In addition, comparison of large-scale transcriptional profiles of cells from metaplastic breast carcinoma, a highly invasive and metastatic subtype of breast cancer, with cells from ductal carcinoma, a less metastatic subtype, showed differential

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overexpression of several genes involved in EMT (Lien et al., 2007). This implies that genes responsible for the phenotypic switch can also promote invasion and metastasis. Expression of genes that induce EMT enhance the metastatic potential of tumor cells in vivo (Grunert et al., 2003). Cell fate mapping strategies and methods to differentially mark epithelial and stroma cells provide direct in vivo evidence for the occurrence of EMT (Trimboli et al., 2008). EMT is most discernable at the invasive front of primary carcinomas and has been visualized as individual or a group of cells migrating into the surrounding tissue (Wyckoff et al., 2000).

Several critical changes in cell properties and behaviors underline the ability for cancer-associated EMT to enable metastatic dissemination. These include the loss of cell-cell adhesions that physically tether cancer cells together, sequester molecules that would otherwise activate invasive signaling pathways, and prevent inappropriate growth factor signaling. Following loss of cell adhesion, the emancipated cancer cell then penetrates the basement membrane and actively migrates into the surrounding stroma, supported by proteolytic degradation of the matrix (Wells et al., 2011).

1.4.1 Loss of cell adhesion

One of the main distinguishing characteristics of epithelia is that epithelial cells are linked by cell adhesion molecules to form contiguous sheets. These intercellular physical interactions not only limit motility but also establish apico-basal polarity that regulates signaling between cells and the surrounding environment. In contrast, mesenchymal cells exhibit transient and changeable front-back polarity and present loose and readily tractable intercellular contacts. As an important mediator of the transformation between epithelial and mesenchymal phenotypes during EMT, loss of cell adhesion molecules has been repeatedly documented to be associated

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with invasion and poor prognosis in many carcinomas. Loss of cell-cell adhesions is a critical step during EMT that allows for physical detachment of individual or groups of cancer cells from the primary tumor and for autocrine activation of signaling pathways that enable migration.

There are four main types of cell-cell junctional molecules that connect epithelial cells. Tight junctions provide a barrier for solutes and small molecules along the apical surface of cells. Adherens junctions provide strong mechanical cohesion through connection to the actin cytoskeleton. Desmosomes also mediate intercellular contacts, but through anchorage to intermediate filaments. Gap junctions form intercellular junctions that allow the passage of ions and small molecules. In addition, integrins are cell-substratum adhesion molecules that are located on the basal surface of epithelial cells and facilitate interactions between the ECM and the cytoskeleton. Members of all these different families of cell adhesion molecules act in concert to contribute to a fully polarized epithelial phenotype.

Components of each one of these cell adhesion molecules have been shown to be dysregulated during invasion. Cadherins, the main component of adherens junction, will be discussed in depth in Section 1.5, particularly the epithelial cadherin, E-cadherin. Dissolution of tight junctions is an early event in EMT and several tight junction components are dysregulated in cancer progression. Expression of occludin in breast cancer cells decreases invasion and migration *in vitro* and *in vivo* (Osanai et al., 2006). Similarly, levels of claudins are downregulated in invasive carcinomas and exogenous introduction of claudins increases adhesion and prevents migration and invasion (Osanai et al., 2007; Martin et al., 2008). Desmosomal components are also commonly downregulated in carcinomas and associated with presentation of distant metastases, especially in cancers of the head and neck (Depondt et al., 1999). Loss of the desmosomal plaque proteins plakophilin-1 and -3 increases cell motility and

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metastasis of carcinoma cells (Sobolik-Delmaire et al., 2007; Kundu et al., 2008). Transfection of desmosomal components desmocollin, desmoglein, and plakoglobin into L929 fibroblasts results in intercellular adhesion and suppression of invasion into collagen gels, even in the absence of the assembly of full desmosome complexes with linkage to intermediate filaments (Tselepis et al., 1998). Lastly, inhibition of cell motility of prostate carcinoma and melanoma cells correlates with increased localization of gap junction molecule connexin 43 (Cx43) at cell-cell contacts (Daniel-Wojcik et al., 2008).

As cancer cells break free from the tumor mass and begin to interact with the extracellular matrix, integrins gain importance. Not only do they provide anchorage to the actin cytoskeleton and connect the cell to the ECM during migration, but they also transmit outside-in signaling. Integrins are composed of α and β subunits that form a heterodimeric complex to determine specificity to ligands. Some integrin heterodimers exhibit great promiscuity by binding to several different ECM components while others may recognize only unique ligands. Epithelial cells typically express the $\beta 1$ subunits, which recognize collagen and laminin, and the epithelial-specific $\alpha 6\beta 4$, $\alpha \nu \beta 3$ integrins (Matlin et al., 2003). Several studies have documented the differential expression, distribution, and ligand affinity of integrins in preneoplastic lesions and carcinomas. Expression of integrins and therefore adhesion to ECM is regulated by TGF- β , which is a potent inducer of EMT (Ignotz and Massague, 1987). TGF- β downstream targets Smads activate the expression of integrins and focal adhesion-associated proteins. Transformation of mammary epithelial cells with the Ras oncogene induces EMT and the upregulation of integrins $\alpha 2$, $\alpha 3$, $\alpha 5$, $\alpha 6$, and $\beta 1$ and consequently increases adhesion to matrix components collagen, fibronectin and laminin 1. EMT and integrin expression changes in these Ras transformed cells is maintained by an autocrine TGF $\beta 1$ loop (Maschler et al., 2005).

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1.4.2 Disruption of apico-basal polarity

Besides tethering cells together to prevent the detachment and migration of cells, tight and adherens junctions also establish tissue polarity. This is a critical regulatory mechanism, as most epithelial cells secrete growth factors from their apical surfaces but express the cognate receptors on their basolateral surfaces (Figure 1). When cell adhesion molecules are disrupted in carcinoma-associated EMT, this organization is lost, and the growth factor receptors that are located basolaterally can now come into unrestricted contact with their ligands. Furthermore, the growth factors now have access to the basement membrane and stromal compartment, and can affect changes in the tumor microenvironment to further promote motility. For example, induction of EMT through TGF β 1 expression normally leads to increased ECM production, and deposition and reconstruction of the basement membrane stops the autocrine loop. However, in tumors this feedback loop is disrupted and TGF β 1 is continuously produced and active.

1.4.3 Induction of cell motility

Following the loss of cellular adhesion that allows for detachment from the primary tumor mass, tumor cells must penetrate the basement membrane and migrate through the surrounding stroma to disseminate. As a result of EMT, gene expression changes both regulate the ability of the cell to migrate and the production of matrix metalloproteinases to degrade the ECM. Consequently, proteolytic degradation of the stroma causes the release of growth factors and other molecules that provide feedback signals for further active cell migration, helps to cause turnover of cell adhesion molecules, and paves a path through which the cancer cell migrates (Radisky and Radisky, 2010).

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Growth factors such as EGF, IGF, FGF, HGF and TGF- β that are present in the primary site can induce EMT and migration in cancer cells. Autocrine and paracrine activation of stromal cells and concurrent overexpression of growth factor and chemokine receptors such as EGFR, c-Met, and CXCR4 in cancer cells augments growth factor stimulation (Yilmaz and Christofori, 2010). TGF- β 1 secreted by cancer cells promotes a pro-migratory environment through paracrine signaling to myofibroblasts to induce ECM deposition. Cytokines and chemokines secreted by cells in the tumor microenvironment also contribute to cell motility. There is cross-talk between many of these growth factors and their receptors, adding overlapping and compensatory levels of motility signaling (Lorenzato et al., 2002).

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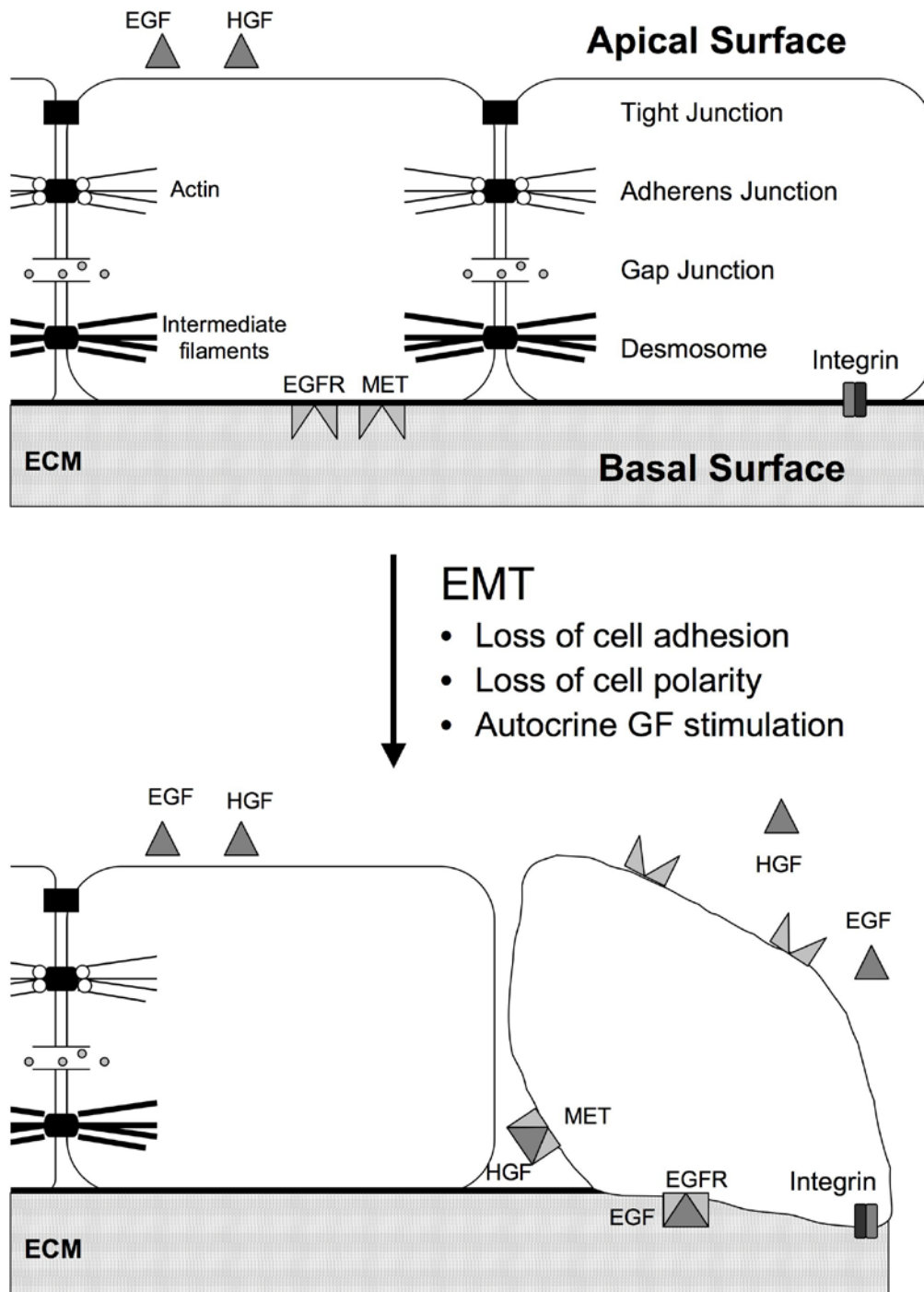


Figure 1. Epithelial to mesenchymal transition. Epithelial cells are characterized by 4 types of cell adhesion molecules (top). Cancer-associated EMT results in loss of cell adhesion and polarity and autocrine growth factor activation (bottom).

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1.5 E-CADHERIN

Loss of cell-cell adhesions is a critical step during both developmental and cancer-associated EMT that allows for physical detachment of individual or groups of cancer cells from the primary tumor. Loss of epithelial-cadherin, or E-cadherin, is widely recognized as the hallmark of EMT and is vital for the initiation of tumor invasion and dissemination. Required for epithelial morphology, E-cadherin is silenced during EMT as epithelial cells change to a mesenchymal phenotype (Takeichi, 1995).

1.5.1 Function and structure

E-cadherin is a member of the cadherin classical family of transmembrane glycoproteins that mediate calcium-dependent homophilic interactions and is the only cadherin expressed in epithelial cells. The other cadherin members most studied besides E-cadherin are N-cadherin (neural), R-cadherin (retinal), P-cadherin (placental), OB-cadherin (bone), and VE-cadherin (endothelial). Cadherins are the core components of adherens junctions, which are integral to tissue homeostasis and to creating polarized sheets of cells through homotypic cell-cell adhesion. During morphogenesis, the cell sorting that results in the formation of different layers and structures is largely facilitated by cadherins (Hirano et al., 1987).

The basic structure of a cadherin is an amino-terminal extracellular domain, a transmembrane domain, and a carboxy-terminal cytoplasmic domain (Figure 2). The extracellular domain consists of 5 repeating units of the EC domain with EC1 being the most distal ectodomain and EC5 the most proximal. Recently it has been shown that the EC1 domains form *trans* interactions with EC1 domains on the opposing cell (Zhang et al., 2009). *Cis*

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clustering of E-cadherin monomers in the same cell strengthen these *trans* adhesions. Calcium is required for the rigidity of the extracellular domains. The structures of cadherins family members exhibit 57% homology in the extracellular domain, which is responsible for the adhesive function (Hirohashi and Kanai, 2003). However, the cytoplasmic domain is highly conserved and binds to β -catenin and p120, which through binding to α -catenin link the cadherins to the actin cytoskeleton. E-cadherin and β -catenin associate in the endoplasmic reticulum and transport to the cell surface together. Impairment of β -catenin binding results in degradation of E-cadherin, as binding of β -catenin blocks the motif recognized by ubiquitin ligases (Huber et al., 2001). Importantly, β -catenin is a nuclear transcriptional co-activator for Wnt signaling and the mitogenic LEF/TCF family of transcription factors, so sequestration of this molecule by cadherins prevents activation of downstream signaling pathways (Heuberger and Birchmeier, 2010).

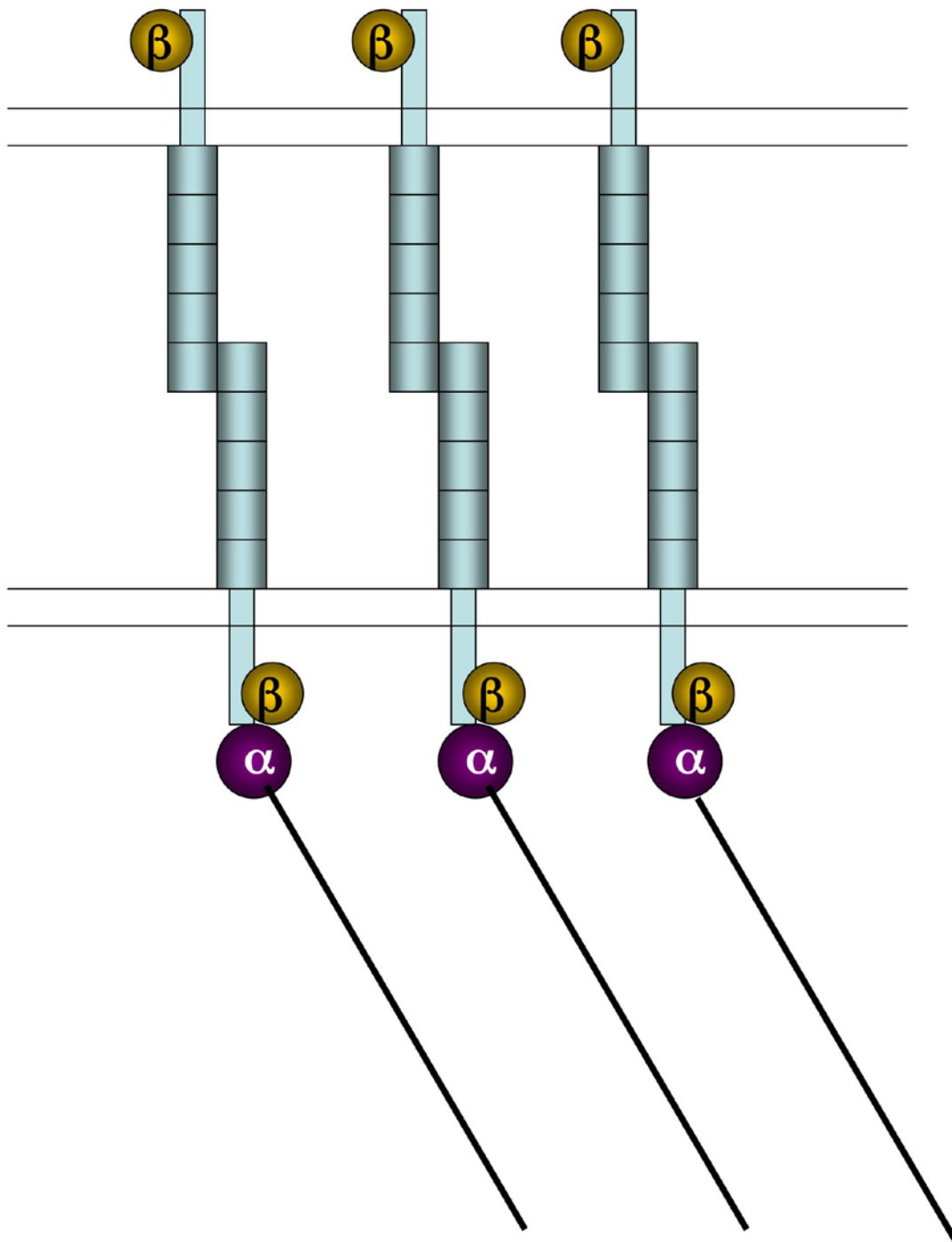


Figure 2. Structure of E-cadherin.

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1.5.2 Role as tumor-suppressor

As the only cadherin expressed by epithelial cells, E-cadherin has been described as the “caretaker” of the epithelial phenotype and thus loss of E-cadherin is central to cancer-associated EMT. Downregulation of E-cadherin expression correlates with the progression of most carcinomas including breast carcinomas (Oka et al., 1993; Takeichi, 1993; Peinado et al., 2004). Generally, E-cadherin expression is present in well-differentiated cancers and reduced in undifferentiated, highly invasive cancers. This relationship also correlates with patient survival (Umbas et al., 1994). E-cadherin is considered an invasion suppressor, as transfection of invasive E-cadherin-negative carcinoma cell lines with E-cadherin cDNA decreases invasiveness, which is reversed after treating transfected cells with an anti-E-cadherin function-blocking antibody (Frixen et al., 1991; Vleminckx et al., 1991). Loss of E-cadherin is sufficient to increase the metastatic behavior of noninvasive breast cancer cells and is a rate-limiting step of the transition from adenoma to invasive carcinoma (Perl et al., 1998).

Perturbation of E-cadherin expression promotes cell motility in several ways. Physical adhesion mediated by E-cadherin prevents the dissociation and migration of cells. In addition, E-cadherin down-regulation releases β -catenin from the membrane, where it can then translocate into the nucleus and act as a transcription co-activator in downstream signaling pathways. Studies using E-cadherin mutants suggest that the β -catenin binding function and not adhesion is responsible for invasion suppression (Wong and Gumbiner, 2003). In addition, loss of E-cadherin alone is not sufficient to drive β -catenin signaling, so it is likely that E-cadherin regulates the threshold of β -catenin signaling (Jeanes et al., 2008). Furthermore, use of a dominant-negative E-cadherin that results in subcellular localization and prevents intercellular

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contacts is sufficient to induce the invasive phenotype, but expression of a constitutively active β -catenin is not (Berx and Van Roy, 2001).

Although down-regulation of E-cadherin has been shown to be sufficient to induce the changes in cell behavior downstream of EMT, in some cases expression of the mesenchymal cadherins can be sufficient or dominant. Downregulation of E-cadherin is often, but not always, accompanied by an upregulation of N-cadherin suggesting a cadherin switch in EMT (Lehembre et al., 2008). However, colocalization of both E-cadherin and N-cadherin has been observed (Hazan et al., 2000). In addition, forced expression of N-cadherin in the absence of changes in E-cadherin has been shown to induce migration and invasiveness of cancer cells either through FGFR signaling or through interactions with N-cadherin expressed by the surrounding stromal cells. Similarly, expression of R-cadherin in BT-20 breast cancer cells leads to downregulation of E- and P-cadherins and induction of cell motility through sustained activation of Rho GTPases (Depondt et al., 1999). Although seemingly contradictory, these studies suggest that E-cadherin and the mesenchymal cadherins may induce motility via different mechanisms intrinsic to the disparate functions of the cadherins.

1.5.3 Regulation

The molecular mechanism of E-cadherin suppression in breast cancer is distinct from most other tumor suppressor molecules where irreversible genetic deletion or mutation is the rule. Except for invasive lobular breast carcinomas and hereditary gastric cancer, few somatic mutations in E-cadherin are found. Ablation of both E-cadherin and p53 in a mouse model is sufficient to cause metastatic infiltrating lobular carcinoma (Derksen et al., 2006). Instead, in most carcinomas E-cadherin is directly silenced by promoter hypermethylation, which can be reversed by the DNA

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methyltransferase inhibitor 5-azacytidine in human cancer lines (Yoshiura et al., 1995). Methylation of CpG islands in the promoter increases with malignant progression (Graff et al., 1995; Nass et al., 2000). Importantly, methylation of the E-cadherin promoter is dynamic, and can be modulated by the microenvironment or by changes in tissue architecture (Graff et al., 1997; Graff et al., 2000).

E-cadherin is silenced by transcriptional repressors Snail, Slug, and Twist. Upregulation of these repressors in invasive breast carcinomas correlates with EMT and loss of E-cadherin expression (Hajra et al., 2002; Yang et al., 2004; Olmeda et al., 2007). Multiple micro-RNAs are specific for these transcriptional repressors, thereby blocking protein translation and resulting in increased E-cadherin expression. Many of these micro-RNAs are aberrantly expressed in breast cancer (Burk et al., 2008; Tryndyak et al., 2010). Other mechanisms of perturbing E-cadherin include increasing endocytosis and degradation via phosphorylation of the cytoplasmic domain, which result from activation of proto-oncogenes EGFR, c-Met, and Src (Berx and van Roy, 2009). Destabilization of binding partners p120 or β -catenin also increases E-cadherin turnover. Matrix metalloproteinases and other proteases proteolytically cleave E-cadherin at the extracellular domain close to the plasma membrane, resulting in soluble form of E-cadherin that act as a dominant negative to antagonize normal E-cadherin ligation (Figure 3).

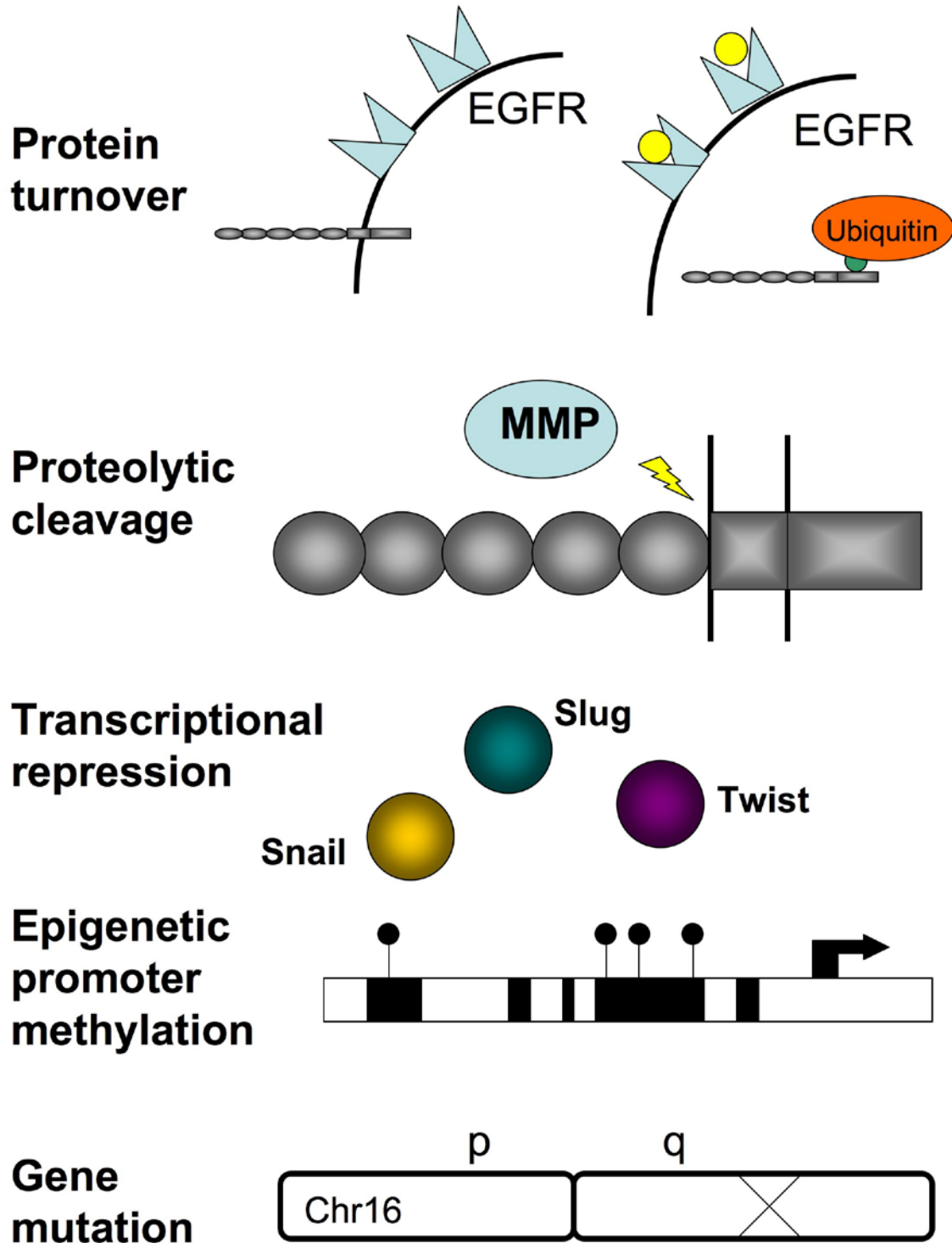


Figure 3. Regulation of E-cadherin in cancer

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1.6 MESENCHYMAL TO EPITHELIAL REVERTING TRANSITION

Despite the wealth of studies describing EMT in carcinoma cells in vitro, and the strong clinical association between loss of expression of adhesion molecules and invasion and poor prognosis, metastases often present a well-differentiated, epithelial phenotype, bringing into question whether EMT is reversible. It is well described that signals from the primary tumor microenvironment greatly contribute to induction of EMT at the primary tumor, so dissemination not only removes cancer cells from these signals but also exposes them to new ones at the secondary organ site. Whether the microenvironment at the secondary organ affects the plasticity of cancer cells to undergo a mesenchymal to epithelial reverting transition (MErT) is a question of great interest. As evidence of the phenotypic plasticity of cancer cells, PC3 prostate cancer cells cultured in 3D Matrigel form cell-cell contacts, tight junctions, and decrease in mesenchymal gene expression, suggesting that a change in tissue architecture is enough to induce such morphological changes (Lang et al., 2001).

While the mesenchymal phenotype that results from EMT may promote invasion and dissemination, there is evidence that metastatic colonization favors an epithelial phenotype. In bladder carcinoma, cell lines selected in vivo for increasing metastatic ability reacquire epithelial morphology and gene expression. When these cells are injected orthotopically, they show a decreased ability to colonize the lung when compared to the more mesenchymal parental cell line. However, when they are injected via intracardiac or intratibial inoculation, they exhibit an increased ability to colonize the lung compared to the parental cell line (Chaffer et al., 2006). Therefore, while induction of EMT through loss of E-cadherin promotes tumor invasion and spread, MErT through E-cadherin re-expression may allow the metastatic cancer cell to complete the last steps of the metastatic process and survive in the new organ.

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A few studies of tumor histopathology suggest that this cancer cell plasticity occurs in patients: a number of studies have shown that E-cadherin-positive metastases may derive from E-cadherin-negative primary carcinomas (Mayer et al., 1993; Bukholm et al., 2000; Rubin et al., 2001; Kowalski et al., 2003; Saha et al., 2007). Similarly, changes in β -catenin localization have been documented (Brabletz et al., 2001) and a study of breast cancer found increased expression of Cx26 and Cx46 in metastatic lymph nodes compared to the primary tumors, with even positive foci originating from connexin-negative primaries (Kanczuga-Koda et al., 2006; Kanczuga-Koda et al., 2007).

1.7 HYPOTHESIS

Even with the abundant evidence for E-cadherin's role in initiating metastasis through EMT, few have looked at E-cadherin expression during the last steps of the metastatic process, particularly in colonization of the secondary organ. Given that metastases can look histologically similar to the primary tumor, it is possible that the reciprocal process to EMT, or mesenchymal-to-epithelial reverting transition (MErT), is involved in the establishment of metastases at the secondary site. As E-cadherin down-regulation in invasive carcinomas is largely the result of promoter methylation and transcriptional repression, cancer cells can easily switch between epithelial and mesenchymal phenotypes. Promoter hypermethylation leading to E-cadherin suppression is dynamic and reversible and therefore re-expression in response to changes in the microenvironment is possible (Graff et al., 2000). The question remains whether the well-differentiated phenotype observed in metastases is the result of an expansion of epitheloid cells or from actual reversion of EMT and a transition back to an epithelial phenotype from a

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mesenchymal state. While studies suggest that both methods may be possible, few studies have directly shown that E-cadherin re-expression and MErT can be induced by the secondary organ microenvironment.

Previous work in our laboratory has shown that prostate cancer cells re-express E-cadherin when cultured with hepatocytes. While there are added levels of complexity to E-cadherin regulation in breast cancer cells compared to prostate cancer cells, we aim to show that the liver microenvironment is capable of inducing E-cadherin re-expression in breast cancer cells. Furthermore, we aim to discern whether mesenchymal to epithelial reverting transition follows E-cadherin re-expression and whether these phenotypic transitions occur in breast cancer patients. Finally, given that E-cadherin expression and cell adhesion have been shown to protect against chemotherapy- and detachment-induced cell death, we aim to show that such phenotypic plasticity has functional significance. **In short, we hypothesize that metastatic breast carcinoma cells, as a result of E-cadherin re-expression, undergo a mesenchymal to epithelial transition in the liver to confer a survival advantage.**

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2.0 PARTIAL MESENCHYMAL TO EPITHELIAL REVERTING TRANSITION IN BREAST AND PROSTATE CANCER METASTASES

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2.1 ABSTRACT

Epithelial to mesenchymal transition (EMT) is an oft-studied mechanism for the initiation of metastasis. Despite a wealth of *in vitro* and *in vivo* studies, evidence of MErT in human specimens is difficult to document because clinically detectable metastases are typically past stage at which this transition is most likely evident. We obtained primary and metastatic tumors from breast and prostate cancer patients and evaluated expression of 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 and found that while E-cadherin expression was increased compared to the primary lesion, the expression inversely correlated with size of the metastasis and distance from organ parenchyma. This suggests that a second EMT may occur in the ectopic site for tumor growth or to seed further metastases. 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.

2.2 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 (Wells et al., 2011). 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 (Wyckoff et al., 2000). However, the true extent of EMT in human cancer specimens is still open to debate, as is the role of EMT in metastatic seeding (Tarin et al., 2005; Wells et al., 2008; Wells et al., 2011).

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. Others and we have proposed that a reverse EMT, or mesenchymal to epithelial reverting transition (MErT), occurs to enable metastatic colonization (Thiery, 2002; Chaffer et al., 2006; Hugo et al., 2007; Wells et al., 2008). Therefore, while induction of EMT through loss of E-cadherin promotes 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.

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Opponents of cancer-associated EMT argue that there is a lack of convincing evidence in clinical samples that support the *in vitro* findings (Tarin et al., 2005). 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 phenotypes since 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 treat cancer.

We have reported that E-cadherin-negative DU-145 prostate cancer cells can be induced to re-express E-cadherin by *in vitro* coculture with liver parenchymal cells (Yates et al., 2007). 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 occurs. Thus, for the present study we evaluated the expression of mesenchymal and epithelial markers in a 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

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markers was increased in metastases while expression of mesenchymal markers fibroblast-specific protein (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 both increasing metastatic tumor size and distance from secondary organ parenchyma. This observation suggests that MErT is also reversible and helps to answer the question of whether metastases generate other metastases or if all metastases arise from the primary tumor.

2.3 RESULTS

2.3.1 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 (Bukholm et al., 2000; Kowalski et al., 2003). However, the cases analyzed in these studies included metastases to lymph nodes or uncommon sites of breast cancer metastasis. 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 (12 cases), liver (3), and brain (6). Besides bone, these comprise the most common sites of breast cancer metastases. Both primary tumor and metastases were immunostained for E-cadherin. E-cadherin positive cells were counted based on high intensity membrane staining. Percentage of E-cadherin positivity was calculated as the number of E-cadherin-positive cells over the total number of

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cancer cells in each field. We quantified 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. Overall, 17/20 (85%) cases showed increased membranous E-cadherin expression in the metastases compared to the primary tumors (Figure 4a), 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 is a critical suppressor of cancer cell migration and invasion as it forms part of a stable adherens junction (Brabletz et al., 2001; Wong and Gumbiner, 2003). We therefore evaluated primary and metastatic tumors for membranous β -catenin expression (Figure 4b). 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.

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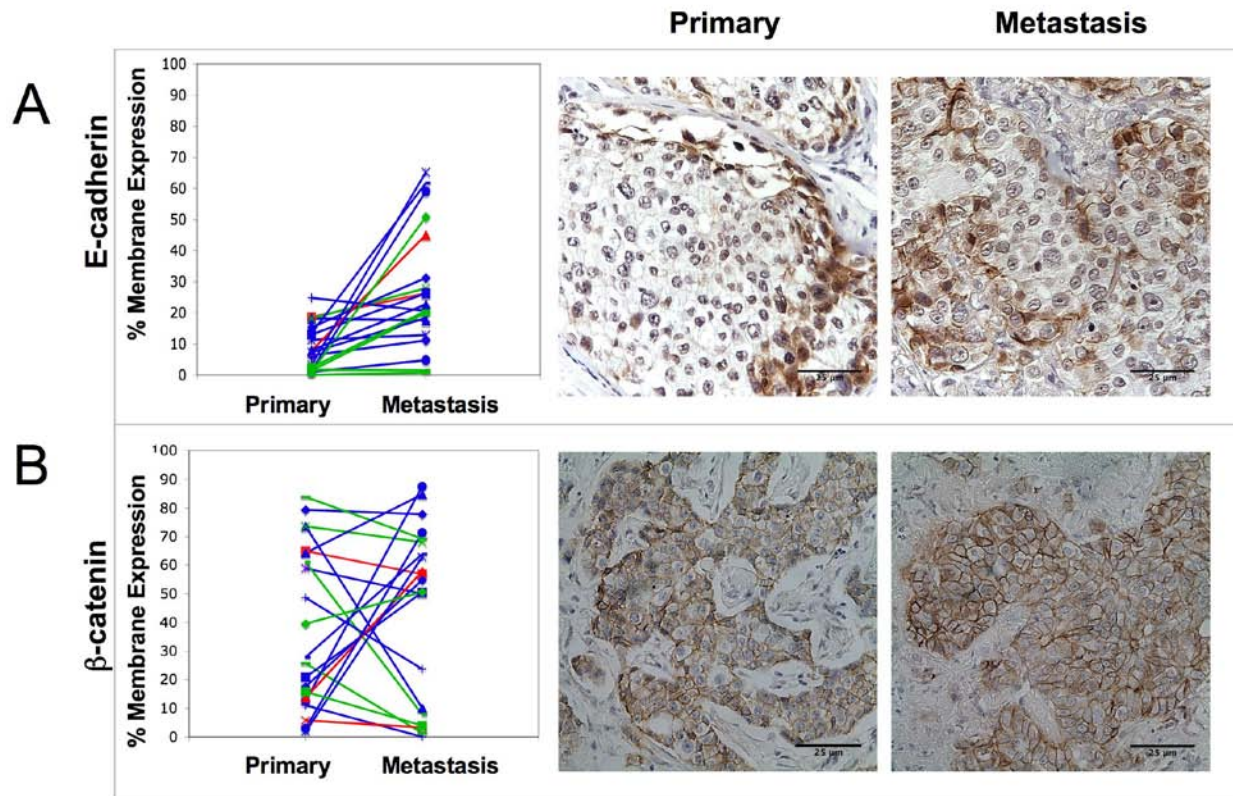


Figure 4. Breast cancer metastases exhibit increased localization of adherens junctions components to the membrane. A) Quantification of membrane-bound E-cadherin in breast cancer primary tumors and metastases. Representative images of a primary tumor exhibiting cytoplasmic or absent E-cadherin and the 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.

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2.3.2 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 (Monaghan et al., 1996). Loss of Cx26 and Cx43 correlates with tumor progression in breast and colorectal cancer and over-expression of Cx43 reduces breast cancer metastasis (Kanczuga-Koda et al., 2005; McLachlan et al., 2006; Li et al., 2008). 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 MERF (Kanczuga-Koda et al., 2006; Kanczuga-Koda et al., 2007). 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 5a). 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 5b). 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 were strikingly increased in metastases to the brain.

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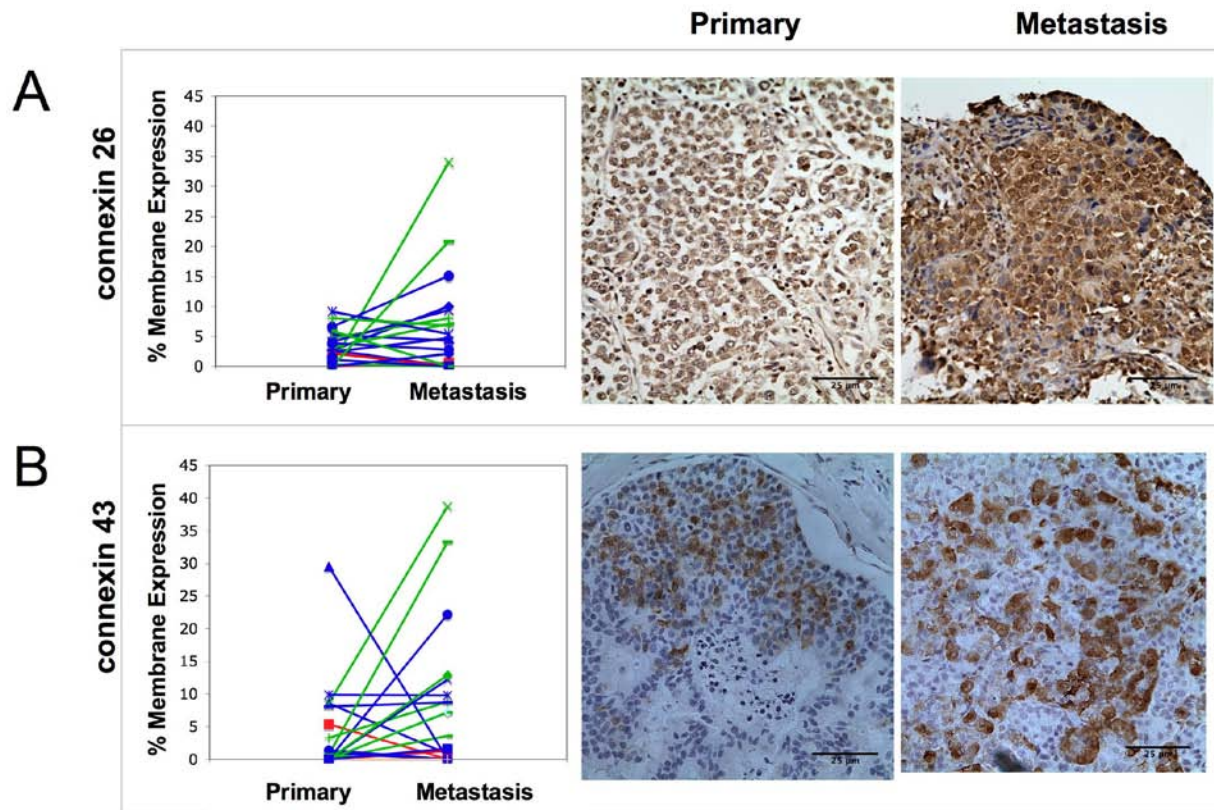


Figure 5. Expression of gap junctional proteins is increased in breast cancer metastases to the brain. Quantification of membranous Cx26 (A) and Cx43 (B) staining in primary and metastatic tumors. Shown are representative images of connexin staining in primary tumors and brain metastases. Organ sites of metastases are color-coded: lung (blue), red (liver), and brain (green). Size bar in the photomicrographs is 25 microns.

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2.3.3 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 (Okada et al., 1997; Trimboli et al., 2008). 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 (Mendez et al., 2010; Vuoriluoto et al., 2010). 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 6a). 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 6b). 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.

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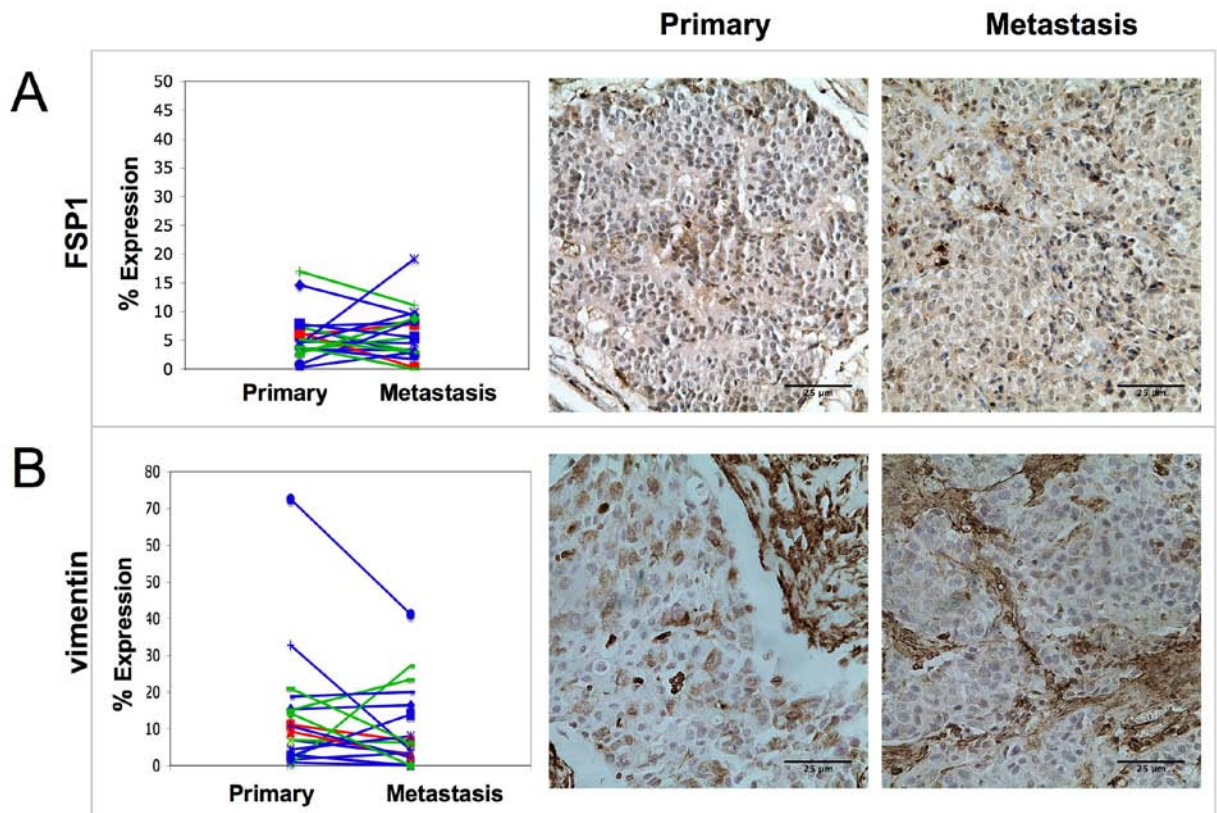


Figure 6. Mesenchymal markers persist in metastases, suggesting only a partial MERT. Quantification of immunostaining for mesenchymal markers FSP1 (A) and vimentin (B). Images of FSP1 and vimentin staining in primary tumors and metastases. Organ sites of metastases are color-coded: lung (blue), red (liver), and brain (green). Size bar in the photomicrographs is 25 microns.

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2.3.4 Size of metastasis and distance from organ parenchyma affects E-cadherin expression

To extend our findings beyond breast cancer, 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 7a). 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 7b). 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 7c), suggesting that the partial MERT that allows for metastatic colonization is transient and reversible like the EMT that enables metastatic dissemination.

Additionally, the distribution of E-cadherin expression within metastases did not appear to be random. In many cases, E-cadherin expression was increased at the edges of contact with organ parenchymal cells. For example, a liver metastasis demonstrating increased expression at the hepatocyte-cancer cell interface and decreased expression centrally is shown, suggesting that E-cadherin is directly regulated by hepatocyte interactions (Figure 8a). Quantification of staining

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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 8b).

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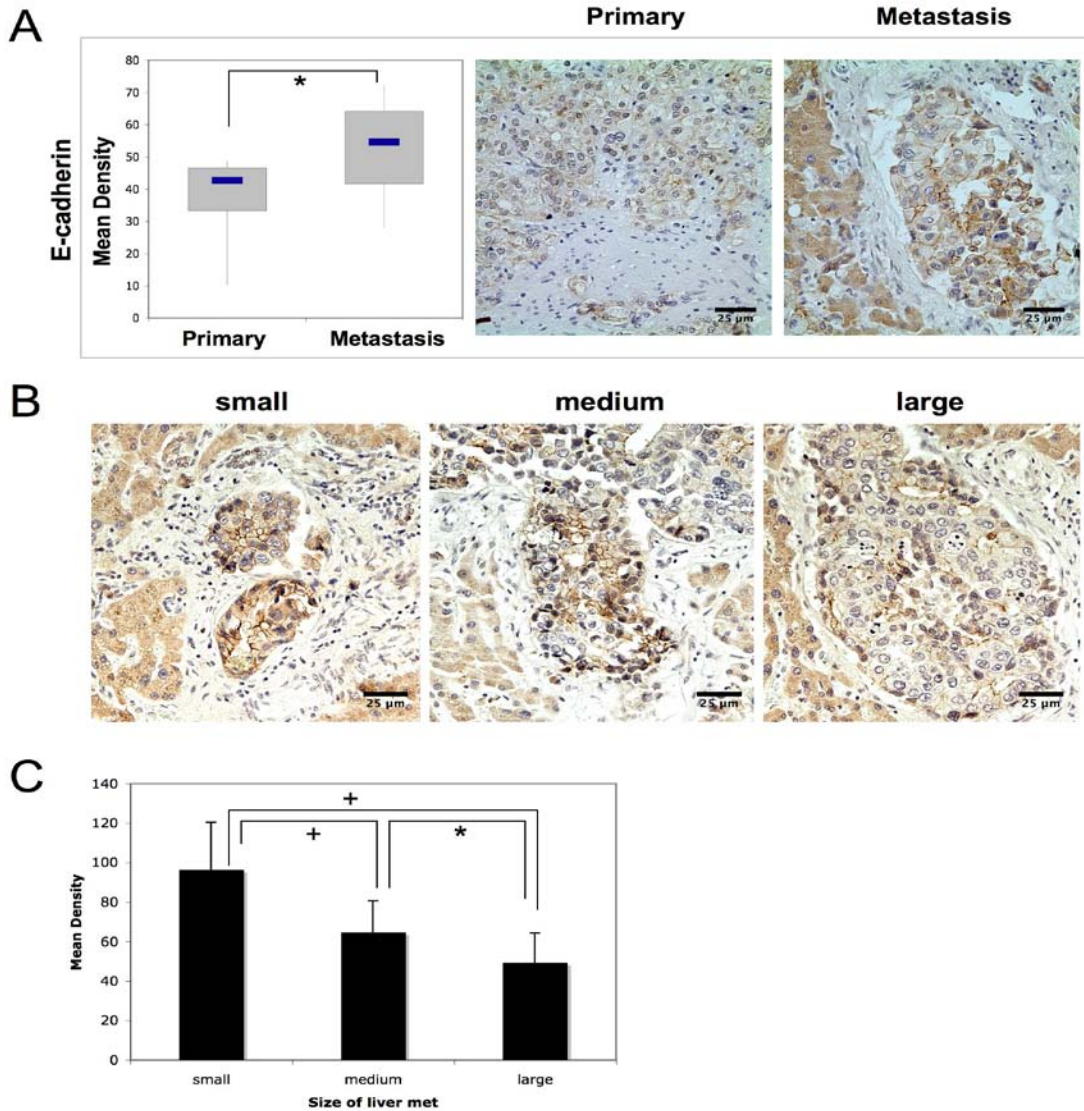


Figure 7. E-cadherin expression in prostate cancer metastases is inversely correlated with size of metastasis. A) Quantification and representative images of prostate cancer primary and metastatic tumors immunostained for E-cadherin. * $p < 0.05$ B) Images of metastatic tumors stained for E-cadherin as categorized by size: small (less than 50 μm in diameter), medium (between 50 μm and 100 μm) and large (bigger than 100 μm). 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.

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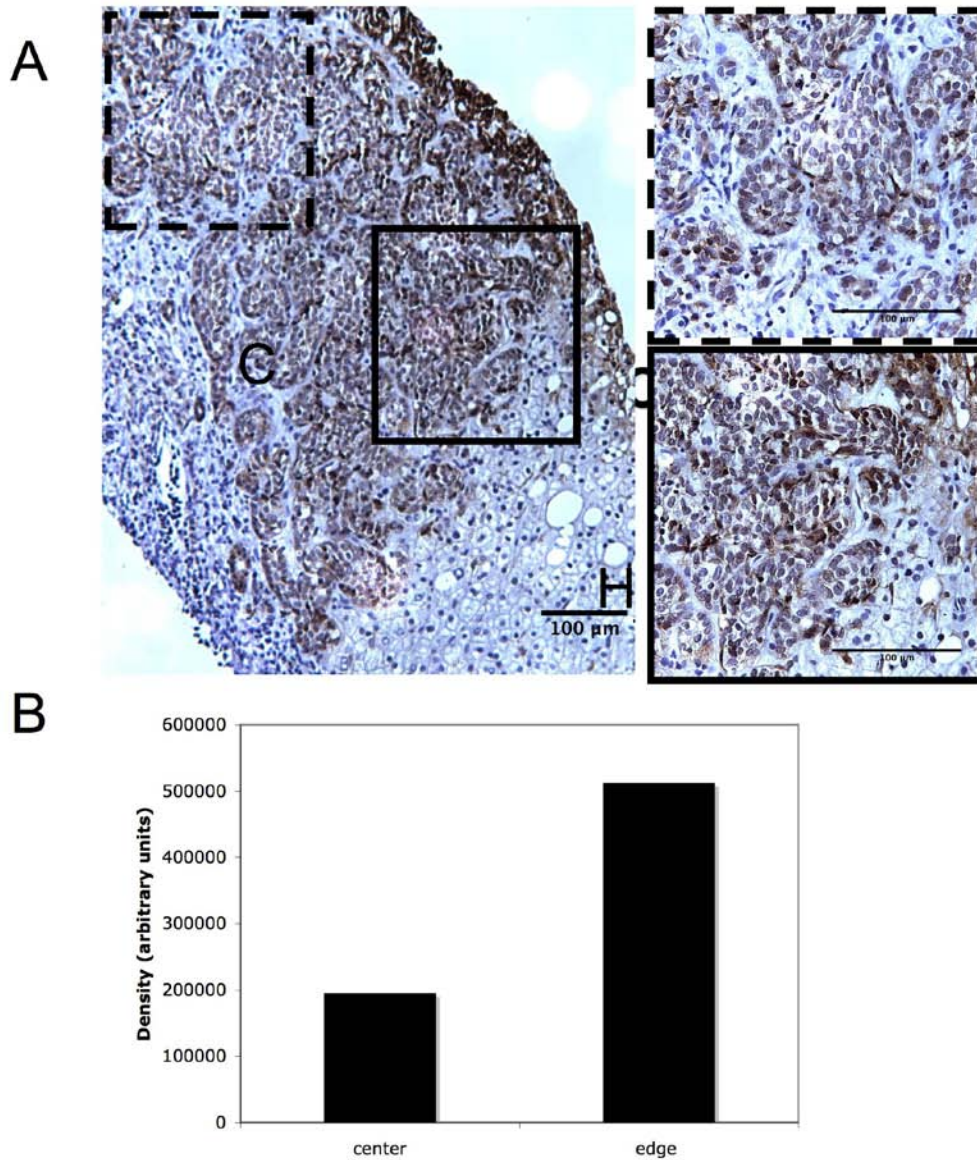


Figure 8. E-cadherin expression decreases with increased distance from organ parenchyma. A) Heterogeneous expression of E-cadherin in the center (dashed inset) versus edge (solid inset) of a liver metastasis. “C” denotes tumor and “H” denotes hepatocytes. B) Quantification of E-cadherin staining in the center and edge of the liver metastasis.

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Table 1. Summary of epithelial and mesenchymal marker expression data.

	Case	E-cad	b-cat	Cx26	Cx43	FSP1	Vimentin
LUNG	1	UP	DOWN	UP	NO CHANGE	UP	DOWN
	4	DOWN	DOWN	DOWN	DOWN	UP	DOWN
	8	UP	DOWN	DOWN	DOWN	DOWN	UP
	9	UP	UP	NO SAMPLE	NO SAMPLE	NO SAMPLE	NO SAMPLE
	10	NO SAMPLE	DOWN	UP	UP	DOWN	DOWN
	13	UP	UP	NO SAMPLE	UP	NO SAMPLE	DOWN
	14	UP	DOWN	DOWN	DOWN	UP	UP
	16	UP	NO SAMPLE	DOWN	DOWN	UP	DOWN
	18	UP	UP	UP	UP	UP	UP
	19	UP	UP	UP	DOWN	UP	DOWN
	20	DOWN	DOWN	DOWN	UP	UP	UP
	21	UP	UP	DOWN	NO CHANGE	DOWN	UP
	26	UP	UP	UP	UP	DOWN	DOWN
LIVER	3	UP	DOWN	UP	DOWN	UP	DOWN
	15	UP	UP	DOWN	UP	DOWN	DOWN
BRAIN	4	UP	DOWN	UP	UP	UP	DOWN
	11	DOWN	DOWN	UP	UP	DOWN	UP
	12	UP	DOWN	UP	UP	DOWN	UP
	22	UP	DOWN	DOWN	UP	DOWN	DOWN
	24	UP	UP	DOWN	UP	UP	DOWN
	25	UP	DOWN	UP	UP	UP	DOWN

■ UP
■ DOWN
■ NO CHANGE
■ NO SAMPLE

2.4 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 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 (Yates et al., 2007; Giampieri et al., 2010; Stoletov et al., 2010). 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. Loss of expression of E-cadherin is widely considered to be a hallmark of EMT. If metastases are the result of clonal expansion of 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.

Paget's "seed and soil" hypothesis posits that cancer cells 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 adapt to the foreign soil of ectopic organ microenvironments. Expression of adhesion molecules is necessary to complete the final steps of the metastatic cascade including intravasation and colonization (Glinkii et al., 2005). We also

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included analysis of 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 MER-T 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 (Li et al., 2008; Huang et al., 2010). 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

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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 (Monaghan et al., 1996). 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 (Wells et al., 2008). 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 (Langley et al., 2009; Fidler et al., 2010; Lin et al., 2010).

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, or desmoplasia, as well as normal stromal cells that stain positively for mesenchymal markers.

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Therefore, 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 of intermediate phenotypes 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 (Friedl and Gilmour, 2009). Similarly, during extravasation, cancer cells re-express molecules that permit adhesion to endothelial cells yet still maintain the ability for transendothelial migration (Gout et al., 2008; Schmidmaier and Baumann, 2008).

Finally, we 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 (Yates et al., 2007; Chao et al., 2010; Wells et al., 2011). However, pathological examination of large metastases removed for palliative or diagnostic needs often 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. Similar changes were observed in a study of claudin expression in primary tumors and liver metastases. Several of the larger liver lesions exhibited re-expression of claudin 4,

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compared to earlier, smaller tumors (Erin et al., 2009). In addition, re-expression of other adhesion molecules such as claudin 7 and γ -catenin have been demonstrated in lymph node metastases (Park et al., 2007). E-cadherin expression was also increased at the edge of metastases compared to the center. This suggests that soluble or ECM-derived factors or physical interaction with the secondary organ may be necessary to maintain MErT. 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 is lower when metastases to the lung, liver, or bone are not already present (Viadana et al., 1973). In a mouse model, systemic metastases arise in mice with large lung metastases in the absence of the primary tumor (Alterman et al., 1985; Stackpole, 1990). One explanation is dormant cells were already seeded in the lung prior to primary tumor removal, but parabiosis experiments reveal that the non-tumor bearing partner develop metastases (Hoover and Ketcham, 1975). 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.

2.5 MATERIALS AND METHODS

2.5.1 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.

Samples were deparaffinized for 1 hour at 55°C and hydrated. Sections underwent antigen retrieval in citrate buffer, pH 6.0 (Dako) followed by 10 minutes of peroxidase blocking in 0.3% hydrogen peroxide and 10 minutes of protein blocking in 5% goat serum. Samples were then incubated with primary antibodies for one hour: 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 incubation for one hour (Jackson Laboratories). After washing several times, slides were then incubated with Streptavidin-Peroxidase (Vectastain) followed by chromagen substrate (Vector Laboratories). The reaction was quenched once DAB staining was visualized. Lastly, samples were counterstained with Mayer's hematoxylin. Staining using only secondary antibody 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

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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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3.0 BREAST CARCINOMA CELLS RE-EXPRESS E-CADHERIN DURING MESENCHYMAL TO EPITHELIAL REVERTING TRANSITION

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3.1 ABSTRACT

Epithelial to mesenchymal transition (EMT), implicated as a mechanism for tumor dissemination, is marked by loss of E-cadherin and induction of cell motility. Epigenetic methylation of the promoter leads to dynamic regulation of E-cadherin expression that is open to modulation by the microenvironment. We have previously shown increased E-cadherin expression along with persistent expression of mesenchymal markers in metastases compared to the primary tumor, indicating a partial M_ErT at metastatic sites. These observations led to the question of whether the positive metastatic foci arose from expansion of E-cadherin-positive cells or from M_ErT of originally E-cadherin-negative disseminated cells. Thus, we aimed to determine if mesenchymal-like MDA-MB-231 breast cancer cells could undergo a M_ErT through the re-expression of E-cadherin. 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. Our clinical observations and experimental data indicate that the secondary organ microenvironment can induce the re-expression of E-cadherin and consequently M_ErT. This phenotypic change is reflected in altered cell behavior and thus may be a critical step in cell survival at metastatic sites.

3.2 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 (Punglia et al., 2007). 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 mesenchymal-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 epithelial to mesenchymal transition (EMT) (Lee et al., 2006). 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 (Tse and Kalluri, 2007; Trimboli et al., 2008). After undergoing EMT, thereby enabling access to hematogenous or lymphatic routes of dissemination, tumor cells 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 (Hugo et al., 2007). A few studies have charted switches between EMT and MET phenotypes throughout malignant progression such as in colorectal cancer (Brabletz et al., 2001), bladder cancer (Chaffer et al., 2006), and ovarian cancer (Hudson et al., 2008). 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.

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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 (Polyak and Weinberg, 2009). 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 (Hirano et al., 1987; Takeichi, 1991). 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. This duality of functionalities—intercellular cohesion and regulation of intracellular signaling cascades—suggests that E-cadherin impacts 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 MErT depending on the microenvironment (Graff et al., 2000). Interestingly, metastases often resemble the epithelial-like phenotype of the primary tumor rather than the mesenchymal phenotype observed at the invasive front. Several pathological studies, including one described previously in Section 2.0, 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 MErT at the metastatic site (Kowalski et al., 2003; Yates et al., 2007). However, one limitation of these pathological studies is that it is

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impossible to determine whether these E-cadherin-positive metastases result from the rare escape and expansion of epithelial 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.

Therefore, we aimed to experimentally 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 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.

3.3 RESULTS

3.3.1 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

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of E-cadherin-positive cells or from M_{Er}T 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 epithelial 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/M_{Er}T 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 (Vizirianakis et al., 2002; Fedor-Chaiken et al., 2003). We postulated that expressing only the cytosolic tail of E-cadherin would allow for a partial M_{Er}T 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 (Figures 9 and 10). 231-Ecad and 231-H2kd mutants display colocalization with the catenins at the membrane (Figure 10). 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 were 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 (Figure 9). As expected, 231-H2kd cells did not form cell-cell contacts. It is important to note that this culture was performed at low cell

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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 (Gotzmann et al., 2004; Kokkinos et al., 2007). 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 (Figure 11a). 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 11b). 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. 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. We therefore tested the two key properties related to tumor escape enabled by EMT: migration

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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 12a). 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 12b). 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 wild-type 231 cells in terms of mesenchymal and epithelial gene expression, β -catenin localization differed (Figure 10a). 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 (Wong and Gumbiner, 2003).

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

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key signaling proteins during the mesenchymal to epithelial transition can have profound effects in mitigating the mesenchymal nature of an invasive cell.

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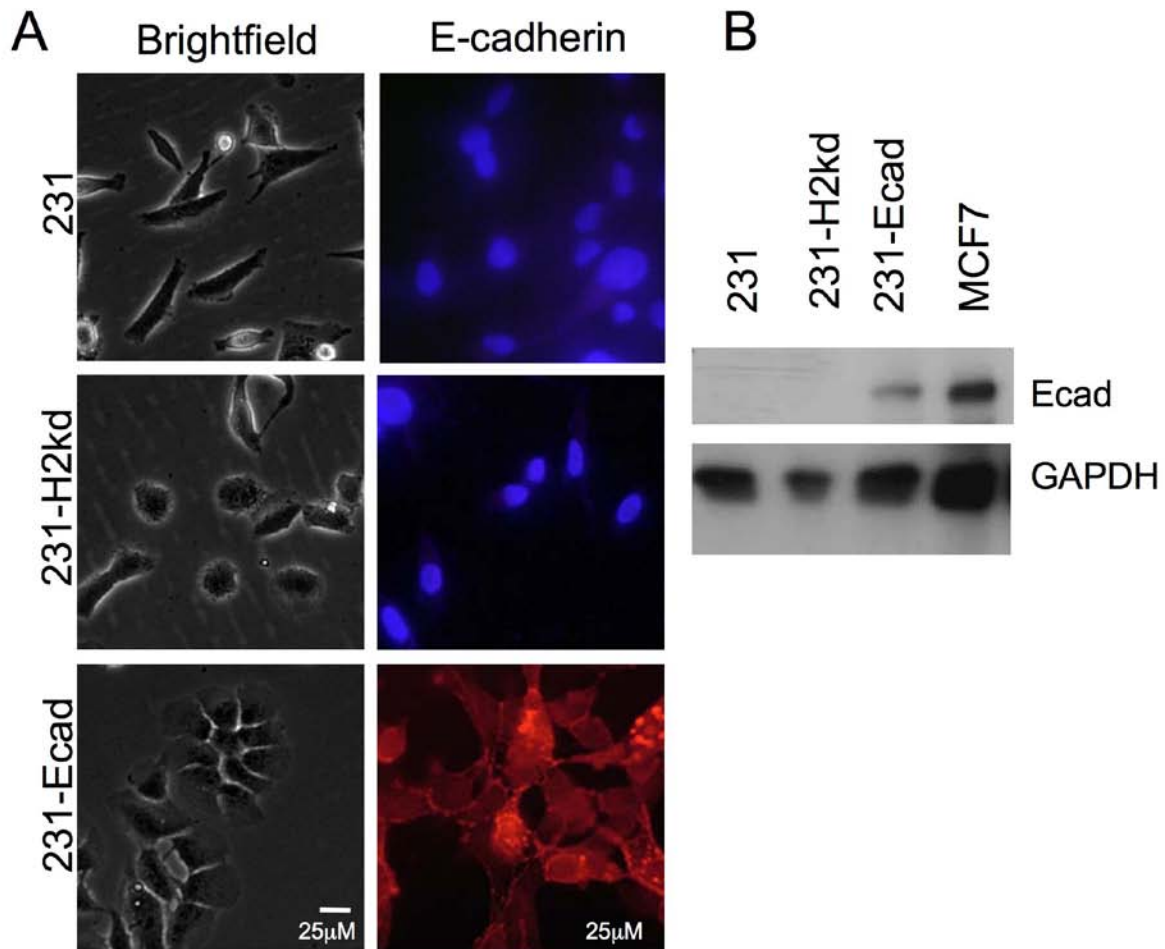


Figure 9. Expression of full-length and cytosolic E-cadherin in MDA-MB-231 cells alter morphology A) Cell morphology as examined by phase contrast microscopy (left column) and E-cadherin expression (red) as detected by immunofluorescence (right column) B) Immunoblot analysis illustrates ectopic expression of E-cadherin in 231-Ecad cells.

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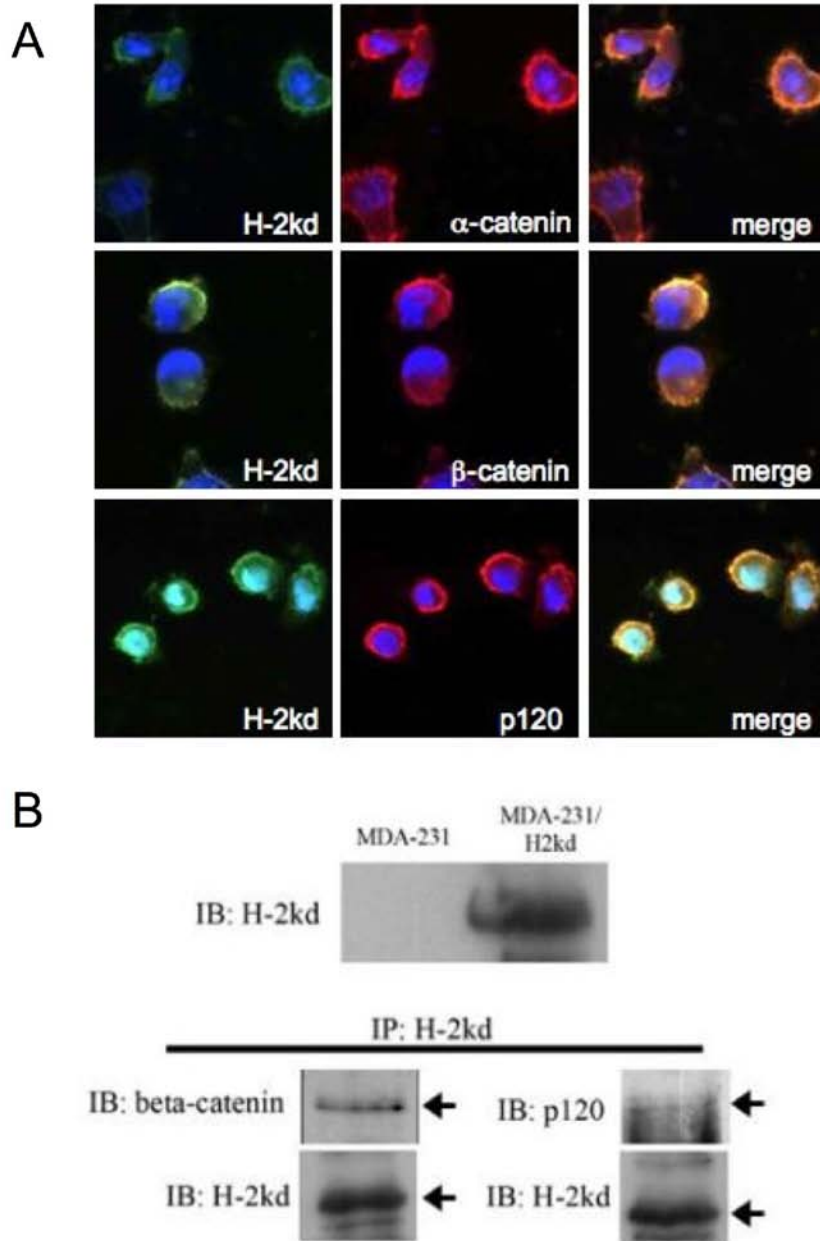


Figure 10. 231-H2kd mutants localize β -catenin to the membrane A) H2kd, left panel, green; α -, β - or p120-catenin, middle panel, red; merge, right panel, yellow. B) Transfected MDA-231 cells express the H2kd fragment. When 231-H2kd whole cell lysates are probed with an H2kd antibody and immunoprecipitated, both beta- and p120 catenins coimmunoprecipitate as determined by immunoblot.

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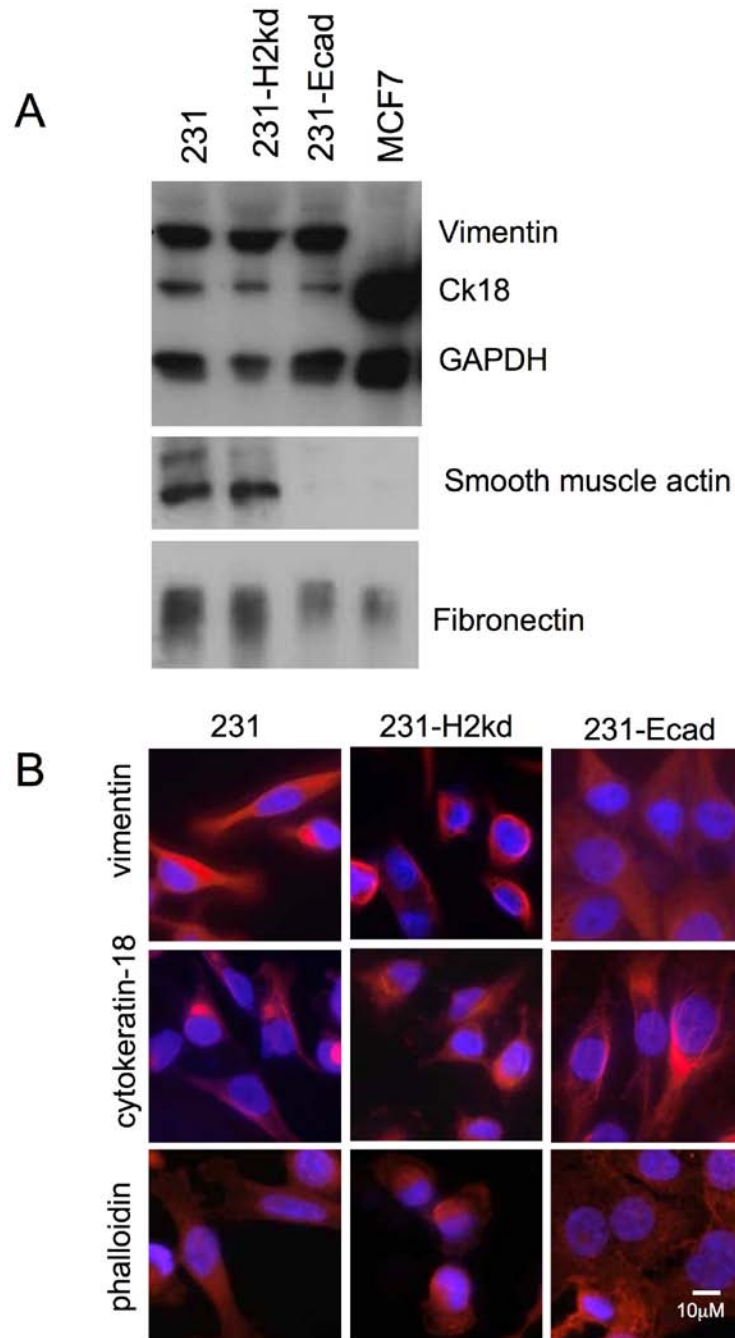


Figure 11. Expression of epithelial and mesenchymal markers in E-cadherin mutants A) Immunoblot of epithelial and mesenchymal marker expression in the E-cadherin mutants. B) Immunofluorescence of vimentin, cytokeratin-18 and actin cytoskeleton (rhodamine phalloidin). Shown are representative of at least three different assessments using one of two independent clones of each cell variant.

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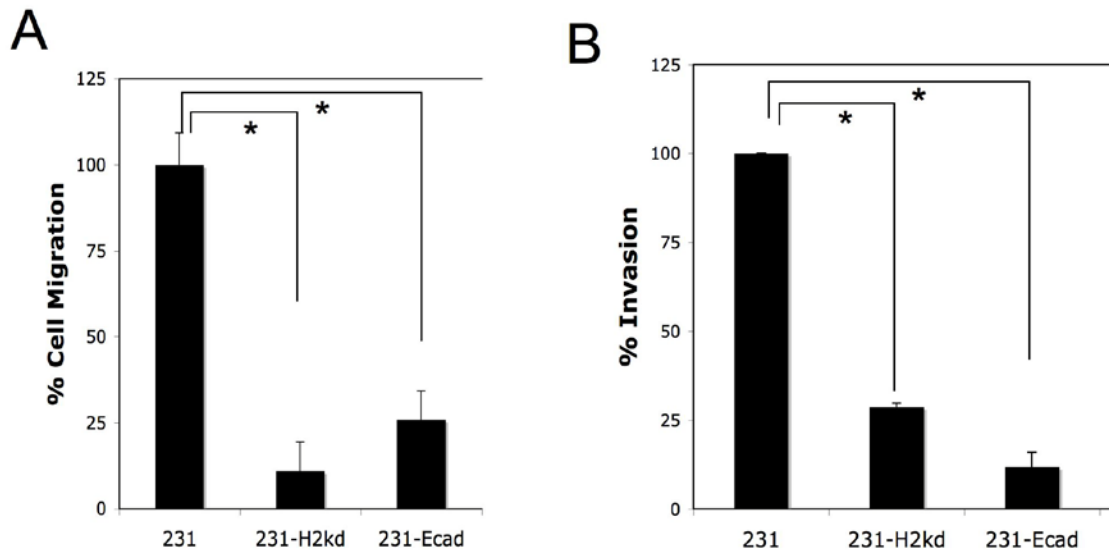


Figure 12. Expression of full-length and cytosolic E-cadherin suppresses migration and invasion A) Cell migration was analyzed using a scratch assay. Scratch closure was measured over a period of 24 hours and the fraction closure was quantified by Metamorph software (n=3). B) Invasion was measured using 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.

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3.3.2 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 is possible 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 13a). 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 13b). 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% 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 13c). Increased expression of E-cadherin mRNA was also detected by qRT-PCR (Figure 13d). 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.

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

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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 14). This reversion to an epithelial morphology was not observed when 231 cells were cocultured with primary fibroblasts for 6 days (Figure 15). Immunofluorescence confirmed that the shRNA construct prevented re-expression of E-cadherin (Figure 16, 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 16, 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.

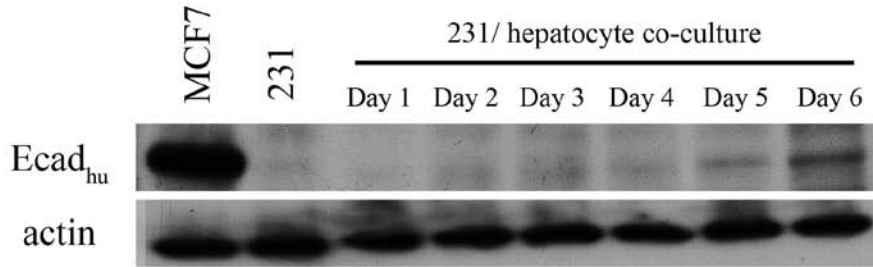
Immunohistochemistry revealed that besides E-cadherin, expression of gap junction molecules connexin 26 (Cx26) and connexin43 (Cx43) were also increased in metastases compared to primary tumor. As MDA-MB-231 cells do not normally express these connexins, we tested whether re-expression of connexins could also occur. MDA-MB-231 cells cultured with human hepatocytes for 6 days revealed punctate localization of Cx43 whereas breast cancer cells cultured in the absence of hepatocytes remained Cx43-negative (Figure 17). Lack of a human-specific Cx43 antibody prevented the detection of overall protein levels by immunoblot.

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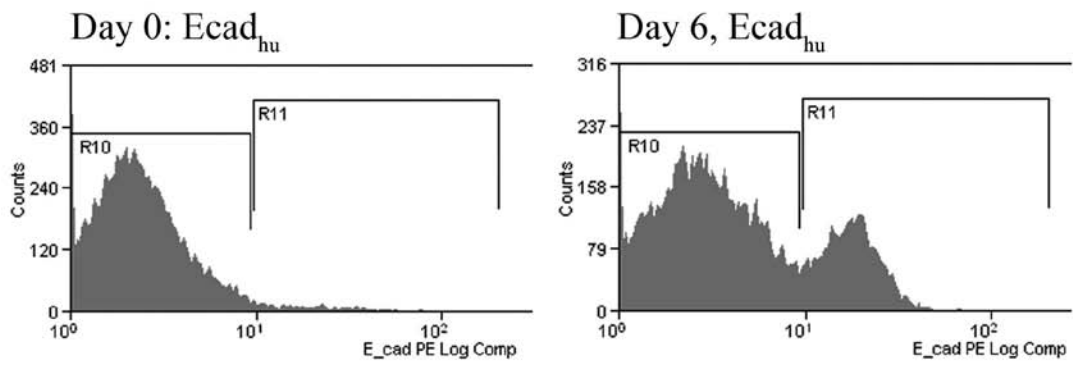
As we demonstrated that it was possible for mesenchymally-transitioned carcinoma cells to revert to a more epithelial phenotype, we next tested whether primary explants of human breast tumors could also re-express E-cadherin in hepatocyte coculture. Explants were obtained from breast tumors without current 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 18a). 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 18b). 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.

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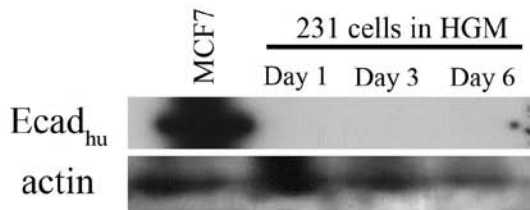
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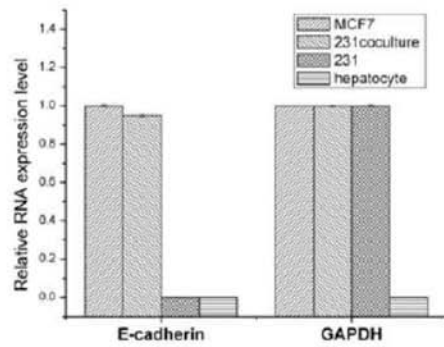
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Figure 13. MDA-MB-231 cells re-express E-cadherin in hepatocyte coculture A) Immunoblot of MDA-MB-231/hepatocyte co-cultures using a human-specific antibody. B) Flow cytometry shows a unimodal population on day 0 and a bimodal population on day 6. 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.

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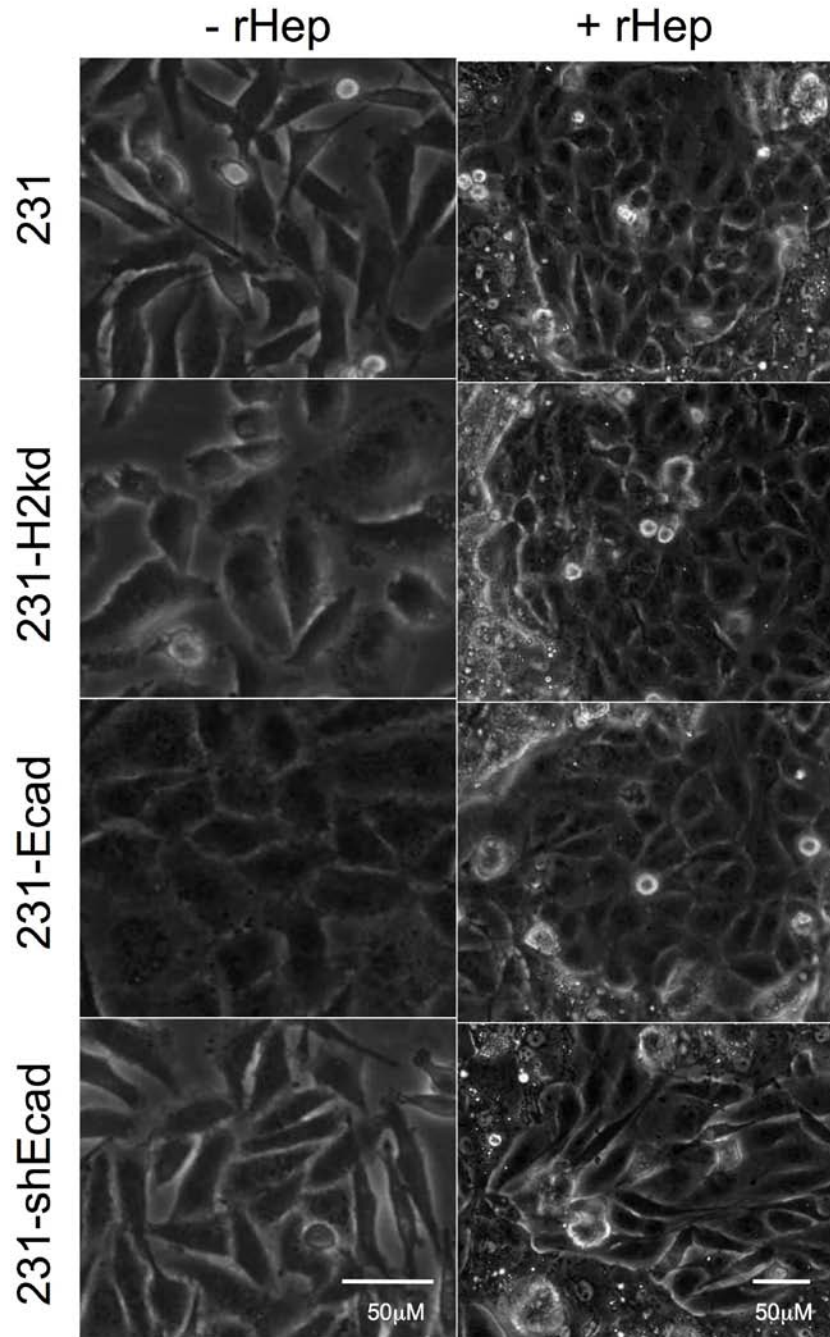


Figure 14. Breast cancer cells cultured with hepatocytes revert to an epithelial morphology.

Phase contrast images of 231, 231-H2kd, 231-Ecad, and 231-shEcad breast cancer cells cultured with rat hepatocytes for 6 days.

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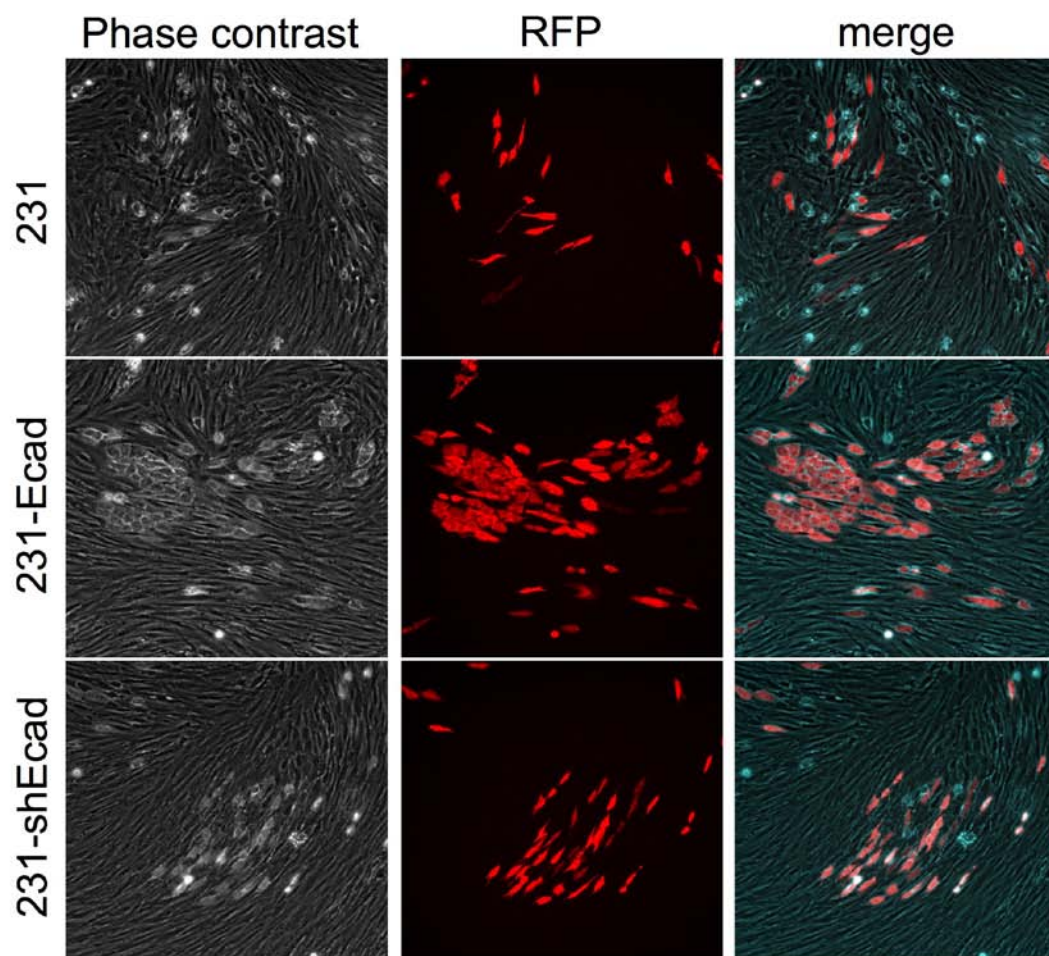


Figure 15. 231 cells do not revert to an epithelial phenotype in fibroblast coculture. RFP-labeled 231, 231-Ecad and 231-shEcad cells cultured for 6 days with primary human fibroblasts.

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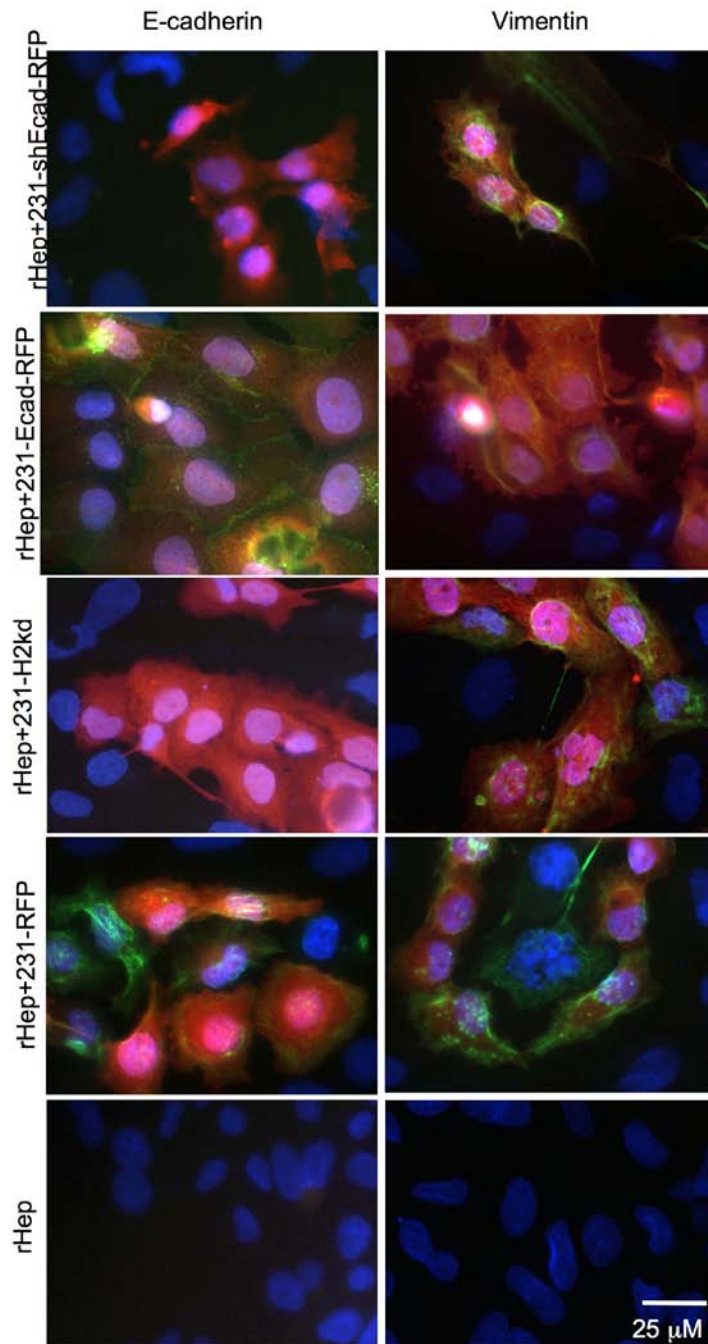


Figure 16. Breast cancer cells culture with hepatocytes re-express E-cadherin but maintain vimentin Immunostaining of RFP-labeled breast cancer cells in hepatocyte coculture; E-cadherin (green), RFP (red), DAPI (blue) and vimentin (green), RFP (red), DAPI (blue).

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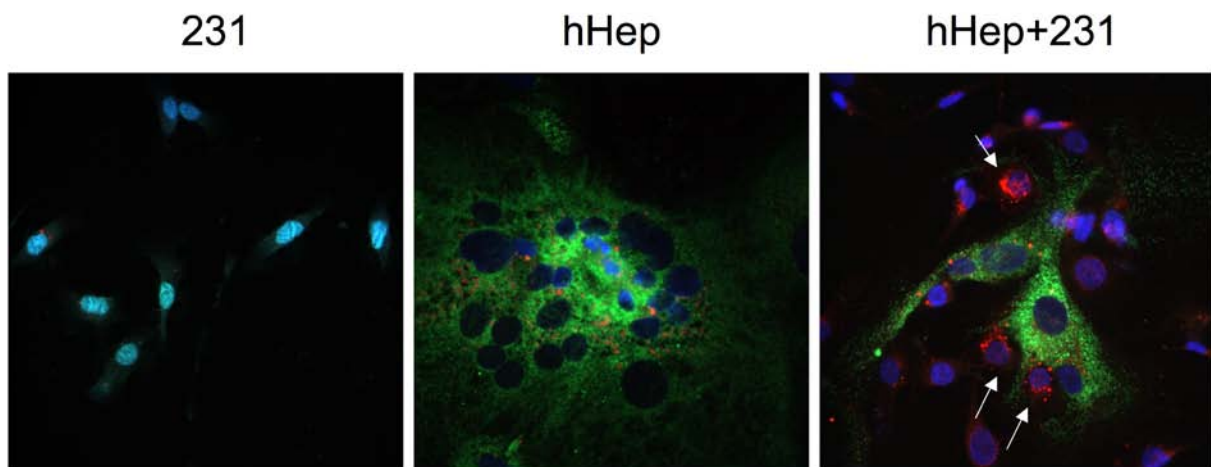


Figure 17. MDA-MB-231 cells re-express connexin 43 after coculture. Immunofluorescence of connexin 43 expression in MDA-MB-231 cells with and without hepatocyte coculture. Blue (DAPI), green (hepatocyte-specific Hep Par 1), red (connexin43).

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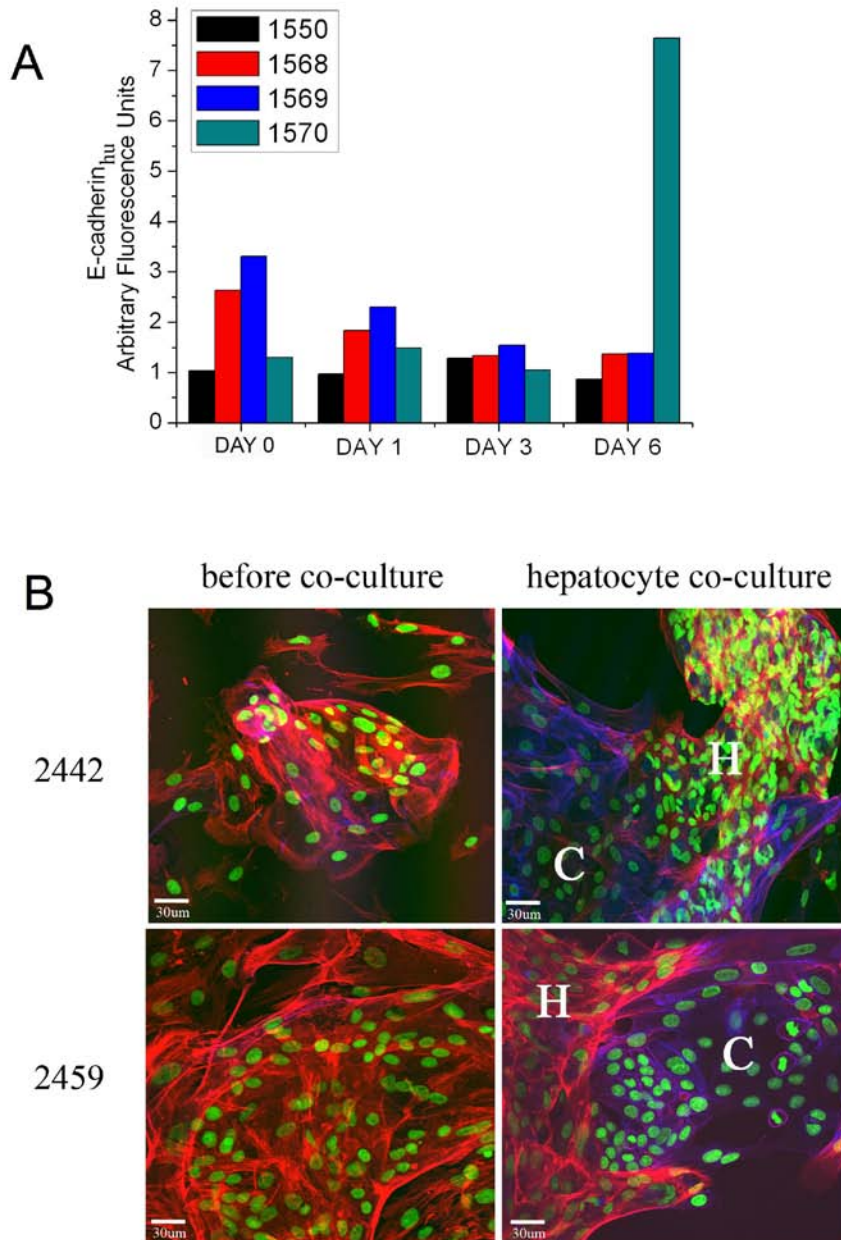


Figure 18. Primary breast carcinoma explants re-express E-cadherin when cocultured with hepatocytes. A) Flow cytometry analysis of primary explants using a human-specific E-cadherin antibody. B) Confocal microscopy of two positive explants. Explants (C), hepatocytes (H). Human-specific E-cadherin, blue; actin, red; nuclei, green.

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3.3.3 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 (Graff et al., 1995). 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 (Kallakury et al., 2001). Following coculture, total genomic DNA was isolated for methylation-specific PCR (MS-PCR) (Corn et al., 2000). 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 19a). However, in the absence of hepatocytes, MDA-MB-231 cells remained hypermethylated (Figure 19b).

Because cancer cells are 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 (Lucifero et al., 2002). 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

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unchanged at all time points indicating that global hypomethylation is not responsible for the changes observed at the E-cadherin promoter (Figure 19c).

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 18d). Furthermore, addition of mitomycin-C also prevented re-expression of E-cadherin at the protein level (Figure 19e). 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 19f). Taken together, these data point to passive loss of methylation as the mechanism by which E-cadherin is re-expressed.

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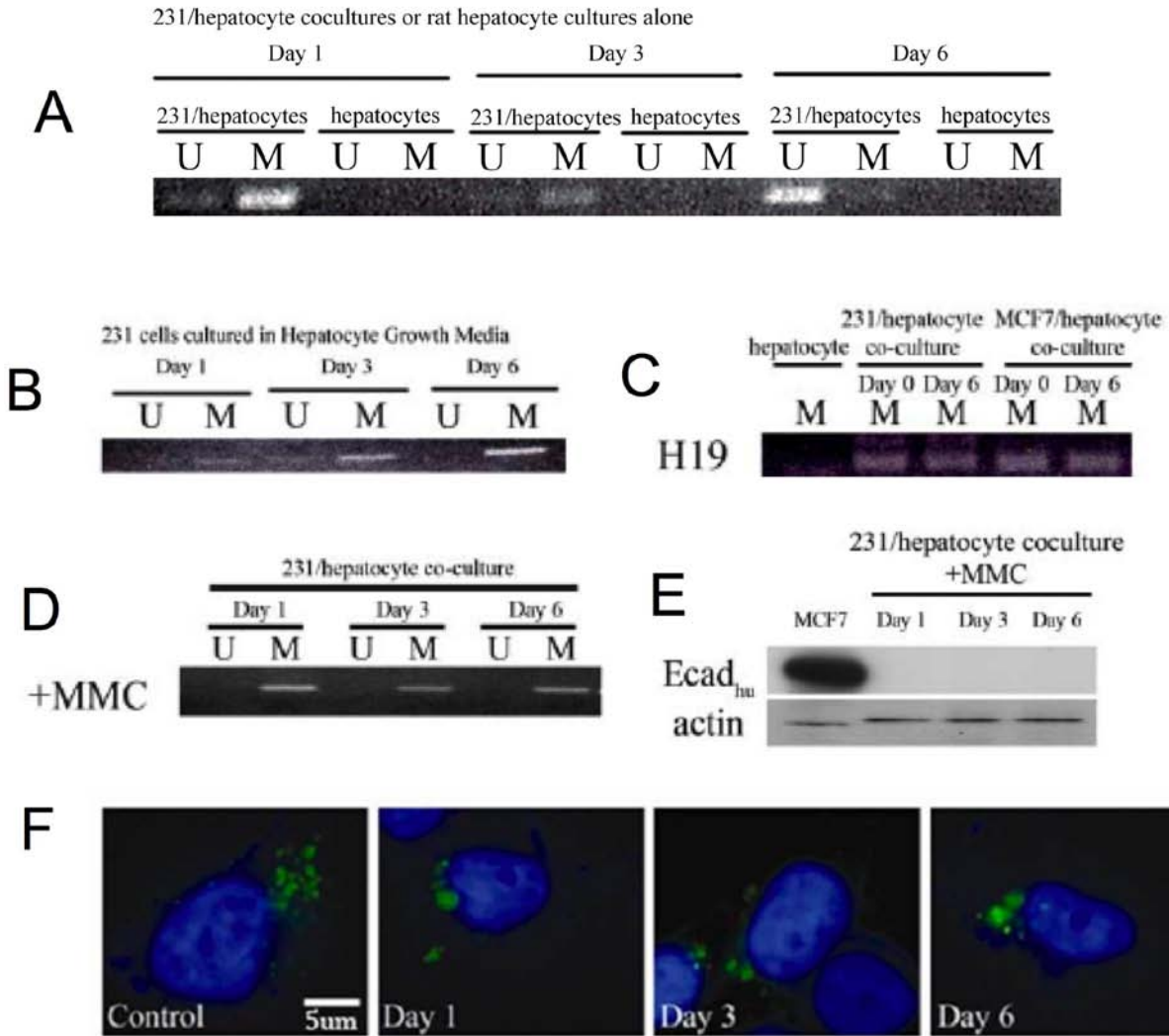


Figure 19. Re-expression of E-cadherin follows a proliferation-dependent demethylation of the E-cadherin promoter. A) MS-PCR of MDA-MB-231 cultured alone in hepatocyte growth media B) MS-PCR using human-specific primers that amplify the imprinted H19 gene. C) MS-PCR of E-cadherin promoter following addition 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.

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3.3.4 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. 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 20a, 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 20a, 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 20b). 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.

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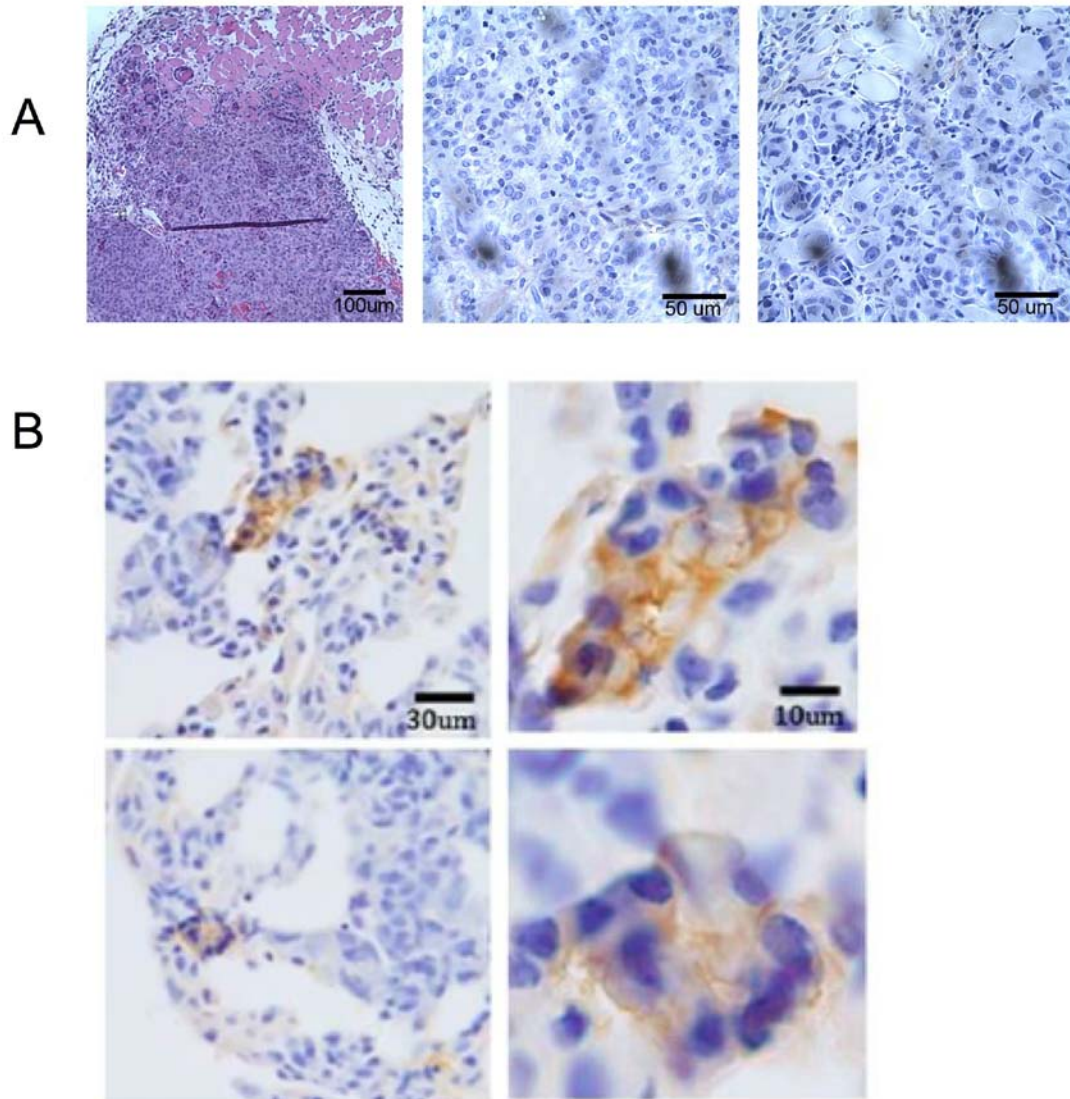


Figure 20. E-cadherin positive metastatic foci originate from E-cadherin negative primary tumors. A) Left, human MDA-MB-231 breast cancer cell xenograft in a mouse inguinal fat pad (H&E); middle, human-specific E-cadherin antibody indicates the absence of E-cadherin expression in the primary tumor B) 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.

3.4 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" (Fidler, 2003). 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 (Koop et al., 1995; Luzzi et al., 1998; Kienast et al., 2010). 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 (Lu and Kang, 2007). 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 (Bukholm et al., 2000; Kowalski et al., 2003). Re-expression of adhesion molecules could therefore be one way in which the secondary organ microenvironment promotes survival of metastatic cells as

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cadherin-cadherin ligandation promotes activation of cell survival signaling pathways (Pece and Gutkind, 2000).

Despite our previous findings of increased E-cadherin expression and partial MErT at the secondary site, it was possible that these E-cadherin-positive tumor cells disseminated from the primary tumor as epithelioid cells and formed secondary metastatic lesions. These results corroborate our immunohistochemistry results in breast cancer patient samples, where E-cadherin expression was observed in metastases but not accompanied by drastic decrease in expression of mesenchymal markers. 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 (Fedor-Chaiken et al., 2003). 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 (Wong and Gumbiner, 2003; Onder et al., 2008; Sarrío et al., 2009).

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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 lead to a weak re-expression of E-cadherin (data not shown). These results are in line with observations made in patient samples, where E-cadherin expression decreased with increasing distance from contact with organ parenchymal cells, suggesting that a gradient of soluble factors and contact with parenchymal cells or parenchyma-derived matrix is required to maintain E-cadherin expression. Re-expression secondary to loss of methylation of the E-cadherin promoter was also observed in the cell line MDA-MB-435 (data not shown), which is now considered to be a melanoma derivative, but is nonetheless useful as this neuroectodermal lineage expresses E-cadherin as melanocytes but loses expression during melanoma progression (Silye et al., 1998; Sanders et al., 1999; Rae et al., 2004). Furthermore, this reversion is not likely unique to hepatocytes, 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 (Li et al., 2010). Another study suggests that laminin-1 may be one component of the extracellular matrix that contributes to E-cadherin re-expression (Benton et al., 2009). 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

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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.

Furthermore, probing of mesenchymal marker vimentin reveals that similar to the *in vivo* data, expression of vimentin persists to suggest a partial MERT. Evaluation of epithelial marker Cx43 suggested similar re-expression of gap junction molecules, but it is unknown whether the gap junctions are functional. Dye transfer studies would need to be performed to determine if re-expressed Cx43 is functional. Heterotypic formation of gap junctions between breast cancer cells and parenchymal cells has been shown previously (el-Sabban and Pauli, 1994; Kanczuga-Koda et al., 2007). Not only that, but the ability to form heterotypic gap junctions over homotypic gap junctions is increased in metastatic cancer cells compared to non-metastatic cells (Woodward et al., 1998).

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 (Yates et al., 2007). 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 (Graff et al., 1995; Cano et al., 2000; Hajra

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et al., 2002; Onder et al., 2008). 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 (Nam et al., 2004). 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 (Holler et al., 1988; Brandeis et al., 1994; Silke et al., 1995; Matsuo et al., 1998). 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 (Goldstein, 2002), suggesting that the epigenetic reversion at distant secondary sites is also relevant. The xenograft model in which MDA-MB-

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231 cells formed E-cadherin-negative primary tumors in the mammary fat pads but E-cadherin-positive micrometastases and the finding that primary breast carcinoma explants 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 (Chambers et al., 1995; Kienast et al., 2010). While E-cadherin typically mediates homotypic cell-cell adhesions, heterophilic ligation between different cell types has been documented (Shiraishi et al., 2005). Cancer cell adhesion facilitates extravasation and colonization of distant organs (Glinskii et al., 2005; Chu et al., 2008; Gassmann and Haier, 2008). Epithelial phenotypic reversion in vivo may therefore enhance the integration and survival of cancer cells at the metastatic site by cloaking the cancer cell with epithelial-like characteristics, or may act to transmit mitogenic or motogenic signals. E-cadherin expression has been shown to suppress cell growth, which may account for the dormancy period between clinical presentation of metastases (St Croix et al., 1998; Perrais et al., 2007). 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 (Section 4.0). Cellular adhesion has long been implicated in intrinsic or acquired resistance of

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solid tumors to multiple anticancer therapeutics not restricted to chemotherapy (St Croix and Kerbel, 1997). 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 (Green et al., 2004; Wang et al., 2009). 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 MERF suggests that they may be appropriate therapeutic targets for preventing the establishment of metastases in breast cancer.

3.5 MATERIALS AND METHODS

3.5.1 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 (Vizirianakis et al., 2002). 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 (Origene) 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

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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.

3.5.2 Cell culture and hepatocyte coculture

MCF7, MDA-MB-231, and MDA-MB-435 cells were cultured in RPMI-1640 with 10% FBS as previously described (Yates et al., 2007). Primary rat hepatocytes were isolated by collagenase perfusion and cultured as described previously 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.

3.5.3 Invasion assay

Invasive potential was determined in vitro by migration through an artificial ECM. 2.5x10⁴ 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.

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3.5.4 Migration 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.

3.5.5 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 immunohistochemistry. 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).

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3.5.6 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 (Corn et al., 2000) 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).

3.5.7 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).

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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.

3.5.8 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).

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3.5.9 Primary explants

Polyclonal primary human tumor explants were obtained and cultured as previously reported (Ochs et al., 2003). Immunofluorescence labeling was performed as above.

3.5.10 Statistical analyses

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.

3.6 ACKNOWLEDGEMENTS

We thank Ioannis Vizirianakis for the H-2kd-Ecad construct and Stephen Strom, William Bowen, and Liang Kang for providing hepatocytes. These studies were supported by grants from the DOD CDMRP on Breast Carcinoma Cancer and the VA Merit Award Program.

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**4.0 RE-EXPRESSION OF E-CADHERIN INCREASES THE SURVIVAL AND
CHEMORESISTANCE OF BREAST AND PROSTATE CANCER CELLS**

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4.1 ABSTRACT

Metastatic colonization is a rate-limiting step of metastasis. Metastatic nodules are often refractory to current therapies, making necessary the elucidation of molecular changes that enable cancer cell survival and chemoresistance at the secondary site. Drug resistance exhibited by tumor spheroids is mediated by cell adhesion and can be abrogated with addition of E-cadherin blocking antibody. Indeed, exogenous expression of E-cadherin in MDA-MB-231 cells increased resistance to cell death induced by chemotherapy and nutrient deprivation. Similar results were obtained in DU-145 prostate cancer cells chemically induced to re-express E-cadherin by buserelin or PD153035. We have previously shown that MDA-MB-231 breast cancer cells can be induced to re-express E-cadherin in a partial mesenchymal to epithelial reverting transition (MErT) when co-cultured with hepatocytes. E-cadherin re-expression in breast cancer cells facilitated heterotypic adhesion to hepatocytes, activated Erk survival signaling, and decreased LC3 localization to autophagosomes, suggesting a functional pro-survival role for E-cadherin during metastatic colonization of the liver. In addition, MDA-MB-231 cells that re-expressed E-cadherin in hepatocyte coculture were more chemoresistant compared to cells stably transfected with E-cadherin shRNA. This effect was further amplified when MDA-MB-231 cells were cultured in 3D in a liver bioreactor, which more closely approximates the liver microenvironment. These results reveal that in the liver microenvironment, breast cancer cells undergo molecular changes not just limited to E-cadherin re-expression that confer a survival advantage and may help to elucidate why chemotherapy commonly fails to treat metastatic breast cancer.

4.2 INTRODUCTION

About 30% 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 (Viadana et al., 1973; Lermite et al., 2010). Breast cancer that metastasizes to the liver carries a very poor prognosis, with the median survival at around 24 months (Atalay et al., 2003). Only 5% of patients with liver metastases present with a singular nodule, and as a result 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 (Diamond et al., 2009). 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. Therefore elucidating the mechanisms behind chemoresistance in metastasis is 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 the colonization of breast cancer in the liver. Breast cancer exhibits organotropism, meaning it preferentially localizes to bone, liver, lung and brain, despite circulating through many other organs (Viadana et al., 1973). Lumen occlusion alone is insufficient for liver colonization (Gassmann et al., 2009). 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 (McGuire et al., 1984). Similarly, loss of claudins is associated with EMT while 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,

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key components of the liver ECM (Tabaries et al., 2010). 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 (Mannori et al., 1995; Paschos et al., 2009). Expression of cell adhesion molecule E-cadherin on breast cancer cells may be another mechanism to facilitate adhesion to hepatocytes, the E-cadherin expressing parenchymal cells that account for 70-80% of the liver.

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 can contribute 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 (Luebke-Wheeler et al., 2009). Similarly, endocytosis of E-cadherin induced by EGFR activation leads to anoikis of enterocytes (Lugo-Martinez et al., 2009). The assembly of adherens junctions coordinated by E-cadherin ligation leads to rapid activation of MAPK and Akt, signaling pathways critical for cell survival (Pece et al., 1999; Pece and Gutkind, 2000). VE-cadherin also controls endothelial cell survival through signaling through Akt and Bcl-2 (Carmeliet et al., 1999). Thus, breast cancer cells may activate survival signaling through heterotypic ligation with hepatocytes.

We have shown previously that E-cadherin expression and partial MERT are observed in human breast cancer metastases. We have also shown that E-cadherin is re-expressed in the liver microenvironment by culturing breast cancer cells with hepatocytes in vitro. Thus this study aims to determine whether there is a functional significance to E-cadherin re-expression. We show that E-cadherin can promote attachment to the secondary organ through heterotypic ligation, resulting in the activation of survival signaling. Furthermore, E-cadherin re-expression also

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confers a survival advantage by increasing the resistance of breast cancer cells to chemotherapy and nutrient deprivation-induced cell death in the liver microenvironment.

4.3 RESULTS

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

We have previously shown that E-cadherin-negative MDA-MB-231 cells re-express E-cadherin and revert to an epithelial morphology when cultured with hepatocytes (Chao et al., 2010). As mediating intercellular adhesion is a major function of E-cadherin, we hypothesized that post-extravasation survival of cancer cells at the secondary site could be facilitated by heterotypic adhesion between cancer cells and organ parenchymal cells. To test whether attachment to hepatocytes was dependent on E-cadherin expression we used previously characterized E-cadherin knock-in and knock-out mutants: E-cadherin-negative MDA-MB-231 cells, 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. Primary rat or human hepatocytes were plated on collagen-coated plates at 30% confluency and 2×10^4 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-

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negative cell lines (Figure 21a). 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 21b and 21c). While lack of E-cadherin expression initially impeded the ability of 231 cells to attach to hepatocytes, re-expression of E-cadherin following 6 days of hepatocyte coculture increased attachment, as measured by a centrifugal assay for fluorescence-based cell adhesion (data not shown).

E-cadherin ligation activates survival signaling pathways (Pece et al., 1999; Pece and Gutkind, 2000) so we queried whether heterotypic ligation between breast cancer cells and hepatocytes resulted in similar activation. To expose signaling only occurring in the breast cancer cells, hepatocyte membranes were isolated and adsorbed onto culture plates. Activation of the Erk pathway was probed after MDA-MB-231 cells isolated from cocultures were plated onto hepatocyte membranes. Maximal Erk activation was observed 30 minutes after addition of E-cadherin re-expressing 231 cells, which was similar to signaling observed following addition of E-cadherin expressing MCF7 cells (Figure 22). Erk activation was not detected when 231 cells cultured in the absence of hepatocytes, and therefore remained E-cadherin-negative, were plated onto hepatocyte membranes.

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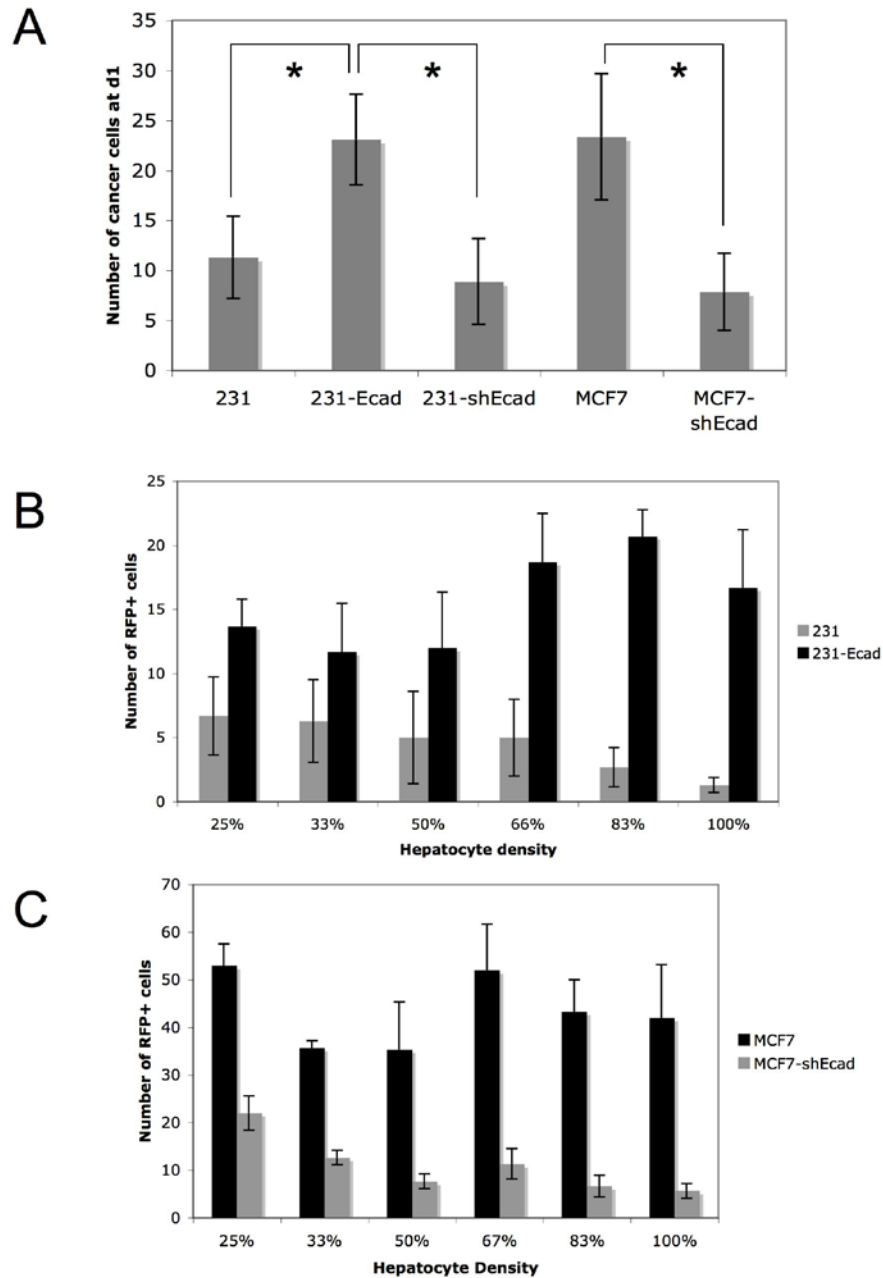


Figure 21. E-cadherin expression increases attachment to hepatocytes. A) Attachment of E-cadherin -negative and -positive breast cancer cells to hepatocyte plated at 30% confluency, 24 hours after plating B and C) Attachment of E-cadherin -negative and -positive breast cancer cells to hepatocytes plated at 25 to 100% confluency, 24 hours after plating.

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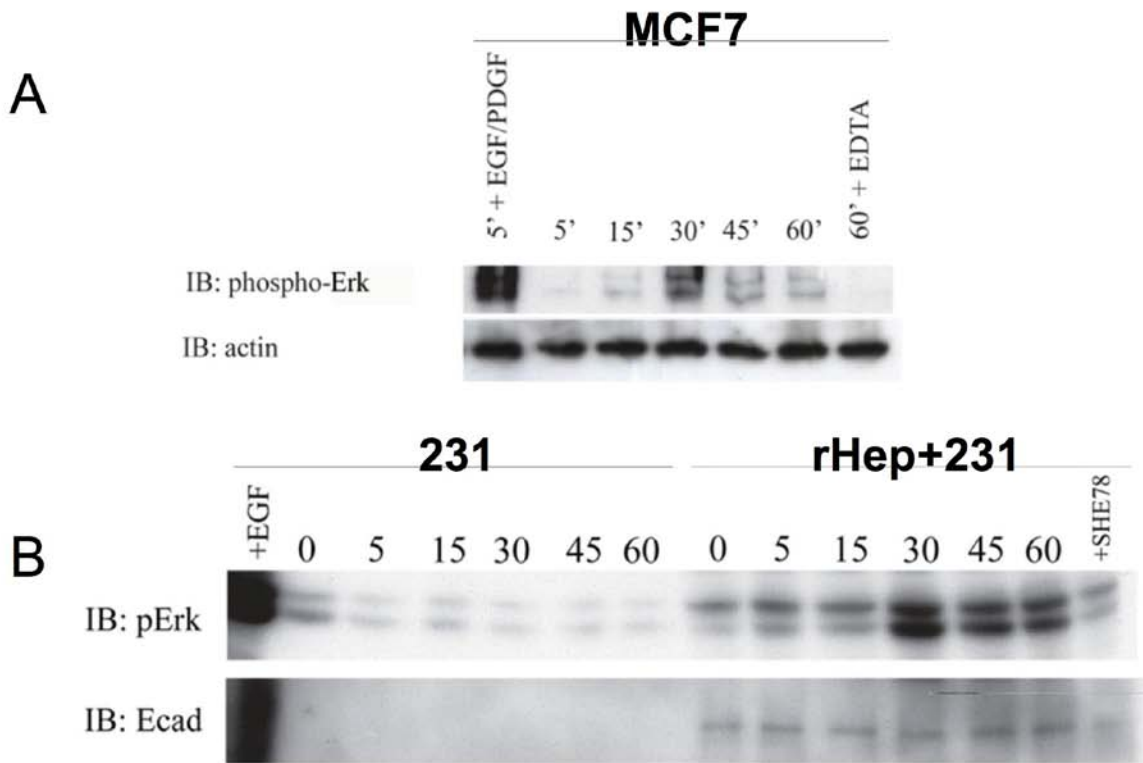


Figure 22. Hepatocyte coculture activates Erk survival pathways in breast cancer cells. Erk phosphorylation was measured at various time points following plating of breast cancer cells onto hepatocyte membranes. A) MCF7 cells B) 231 cells with and without hepatocyte coculture

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4.3.2 E-cadherin expression increases resistance to starvation

As the secondary organ microenvironment may be considerably different compared to the primary tumor microenvironment in terms of growth factor and nutrient milieu, we next tested whether E-cadherin expression affected the cancer cell response to starvation. To determine whether E-cadherin expression affected the ability of cancer cells to respond to starvation, we cultured our cell lines in growth media or serum-free media for 3 days and counted the number of surviving cells daily. By the end of the starvation period, there were more surviving cells in E-cadherin-positive cell lines (Figure 23a). There is increasing evidence that cancer cells exploit starvation by employing autophagy to catabolically degrade intracellular components for nutrients. We therefore stained nutrient deprived breast cancer cells and breast cancer cells cocultured with hepatocytes for autophagy marker LC3. When 231 cells were cultured in serum-free media, immunostaining revealed LC3 localized to autophagosomes. Autophagosomes were not observed in 231 cells cultured with hepatocytes, suggesting either a reduction in autophagy upon E-cadherin re-expression or an increase in nutrients produced by hepatocytes (Figure 23b).

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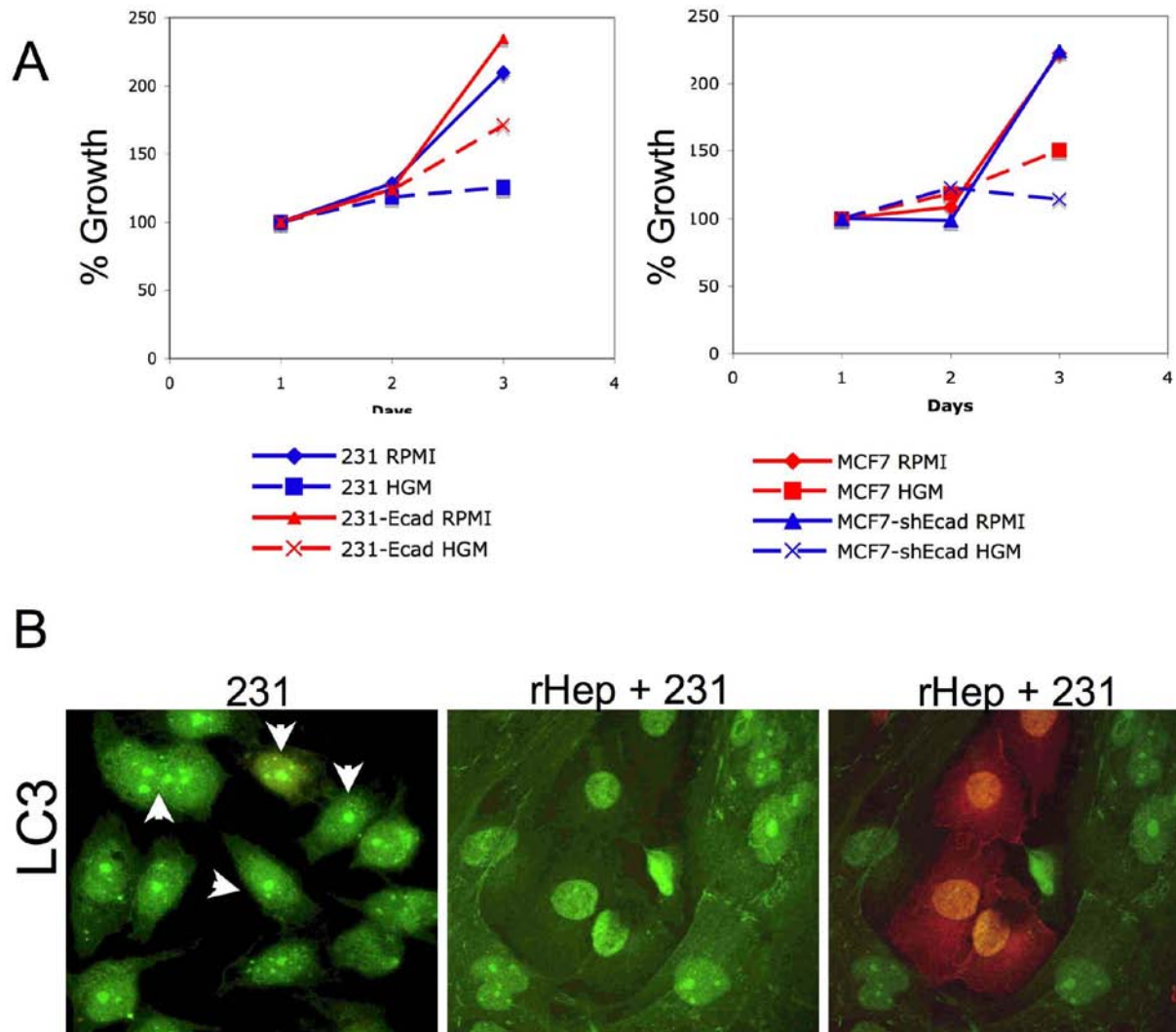


Figure 23. E-cadherin expression increases survival under starvation. A) E-cadherin – negative and –positive cells cultured in with (RPMI) and without serum (HGM) for 3 days. B) LC3 immunofluorescence of 231-RFP cells cultured with and without hepatocytes. Green (LC3), Red (RFP-labeled 231 cells)

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4.3.3 E-cadherin re-expression increases chemoresistance

Multiple studies have shown that E-cadherin protects against cell death and increases drug resistance (St Croix and Kerbel, 1997; Green et al., 2004; Wang et al., 2009). Treatment of the 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 24a and 24c). Addition of E-cadherin antibody abrogated the effect on 231-Ecad cells (data not shown). Furthermore, E-cadherin re-expression in hepatocyte coculture increased the chemoresistance of 231 cells to 231-Ecad levels, while 231-shEcad cells remained the most sensitive (Figures 24b and 24d). Interestingly, overall the breast cancer cells were less chemosensitive 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.

We corroborated these results in prostate cancer cells as we have previously shown that prostate cancer cells re-express E-cadherin upon repression of EGFR signaling (Yates et al., 2005; Yates et al., 2007). DU-145 prostate cancer cells were treated with 1 μ g/ml of buserelin, luteinising hormone-releasing hormone (LHRH) analog or 500nM EGFR kinase inhibitor PD153035 for 48hrs. Treatment with these agents resulted in re-expression of E-cadherin and an epithelial cluster morphology (Figures 25a and 25b). Following E-cadherin re-expression induced by these agents, DU-145 cells were more resistant to cell death induced by staurosporine and camptothecin (Figure 25c). The small degree of protection can be explained by the fact that not all of the prostate cancer cells re-express E-cadherin under the treatment.

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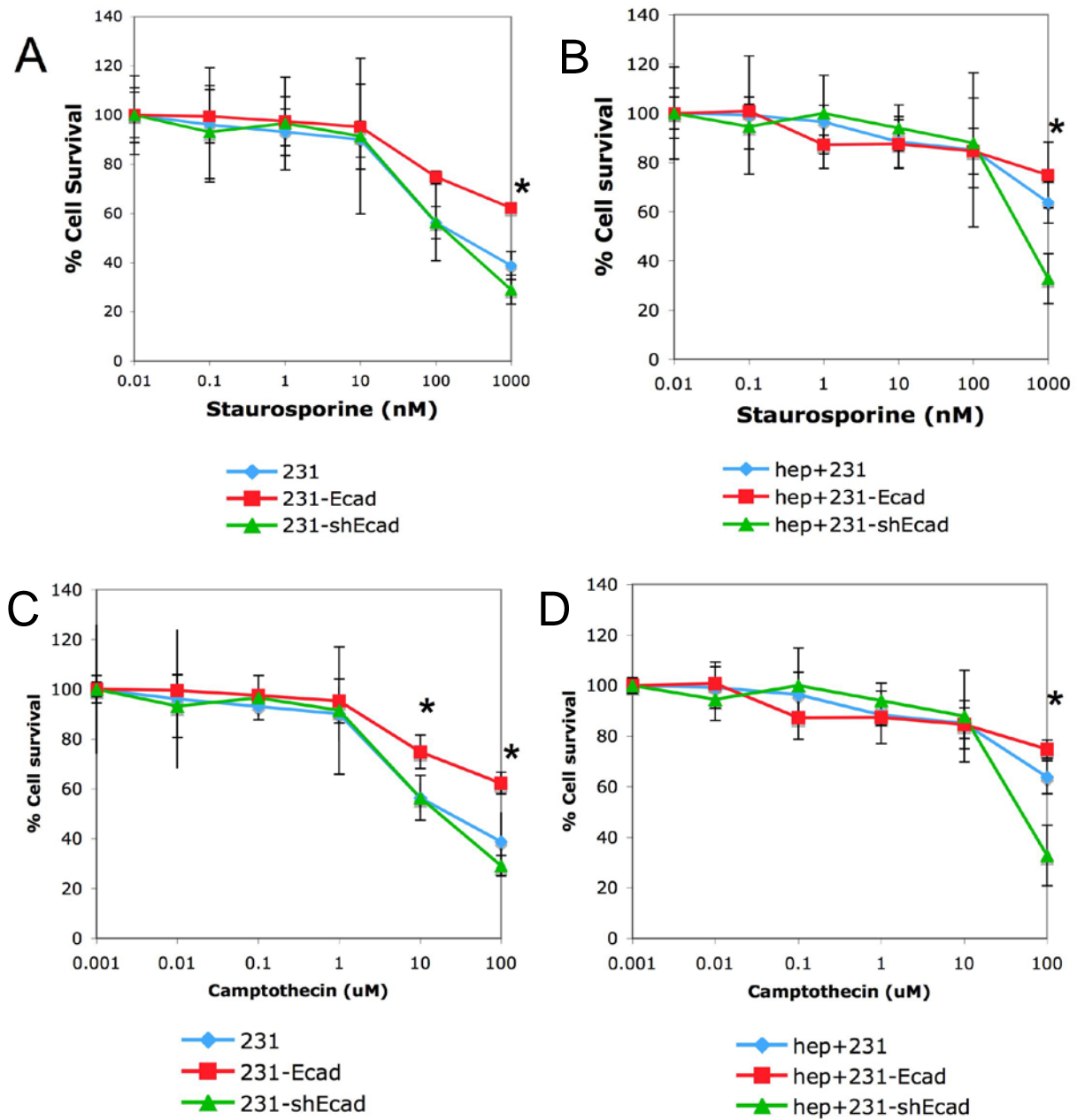


Figure 24. E-cadherin re-expression in hepatocyte coculture increases chemoresistance. A and C) Staurosporine- and camptothecin-induced cell death in breast cancer cells without hepatocytes B) Staurosporine- and camptothecin-induced cell death in breast cancer cells after 6 days of coculture with hepatocytes

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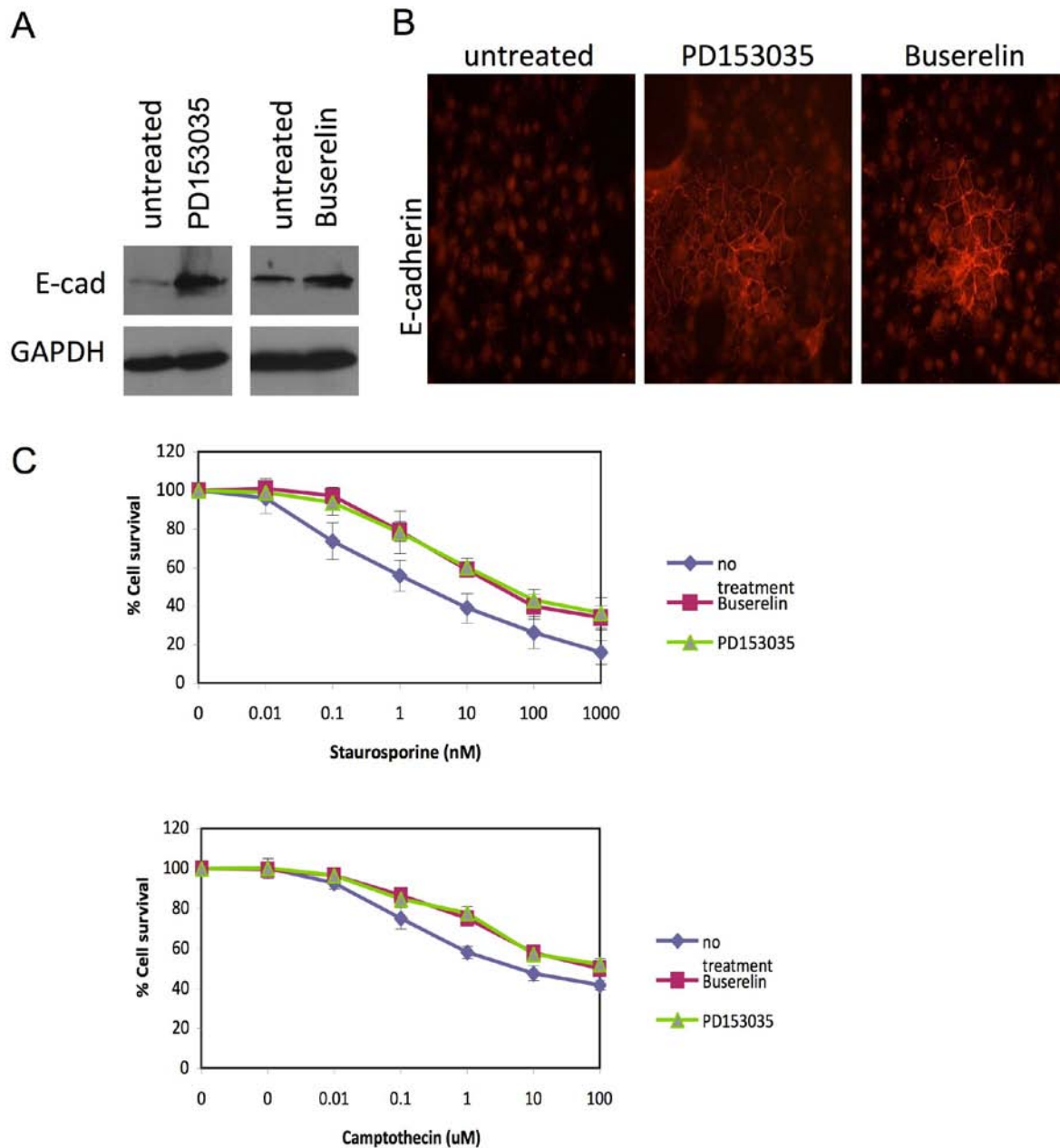


Figure 25. E-cadherin re-expression in prostate cancer cells increases chemoresistance. A) Immunoblot for E-cadherin following treatment with buserelin or PD153035. B) Immunofluorescence for E-cadherin following treatment C) DU-145 cells treated with camptothecin and staurosporine with or without re-expression of E-cadherin by buserelin and PD153035.

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4.3.4 E-cadherin re-expression in a organotypic liver bioreactor increases chemoresistance

The microenvironments of both the orthotopic and metastatic sites undoubtedly play a crucial role in tumor progression. In addition, significant experimental differences have been shown between 2D and 3D culture, with 3D culture able to recapitulate a more accurate microenvironment. We therefore used an organotypic bioreactor to more closely recapitulate the liver microenvironment, as fluid flow dynamics of circulation through hepatic sinusoids is captured in this system (Figure 27a). Liver toxicity studies performed in this particular bioreactor shows that hepatocytes cultured in this manner more closely resemble the gene, protein, and metabolic expression profiles of hepatocytes in vivo (Sivaraman et al., 2005). Hepatocytes were seeded in collagen-coated scaffolds in the bioreactor and allowed to organize into spheroids for 48 hours. RFP-labeled MDA-MB-231 cells were then seeded onto bioreactor scaffolds to incorporate within the hepatocyte spheroids. Following 10 days of culture, spheroids were gently detached from scaffolds and imaged. Both 231 and 231-Ecad cells exhibited a cluster morphology within spheroids, while 231-shEcad cells remained diffuse throughout spheroids, suggesting that the 231 cells had reverted to a morphology similar to the 231-Ecad cells. scaffolds were removed and fixed and stained for E-cadherin (Figure 26). Scaffolds were also fixed with spheroids still attached within the channels for immunostaining, which revealed E-cadherin-positive cells on RFP-labeled 231 cells within scaffold channels (Figure 27b). After 10 days of culture, different doses of staurosporine were added to the media and allowed to circulate for 24 hours. The next day scaffolds were removed intact from the bioreactor and placed into the wells of a 12-well plate. RFP fluorescence was measured using a microplate reader. While 231 and 231-shEcad cells exhibited similar drug response curves prior to

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hepatocyte coculture, preliminary results show that the response to staurosporine greatly diverged following E-cadherin re-expression in 231 cells in the liver bioreactor (Figure 27c).

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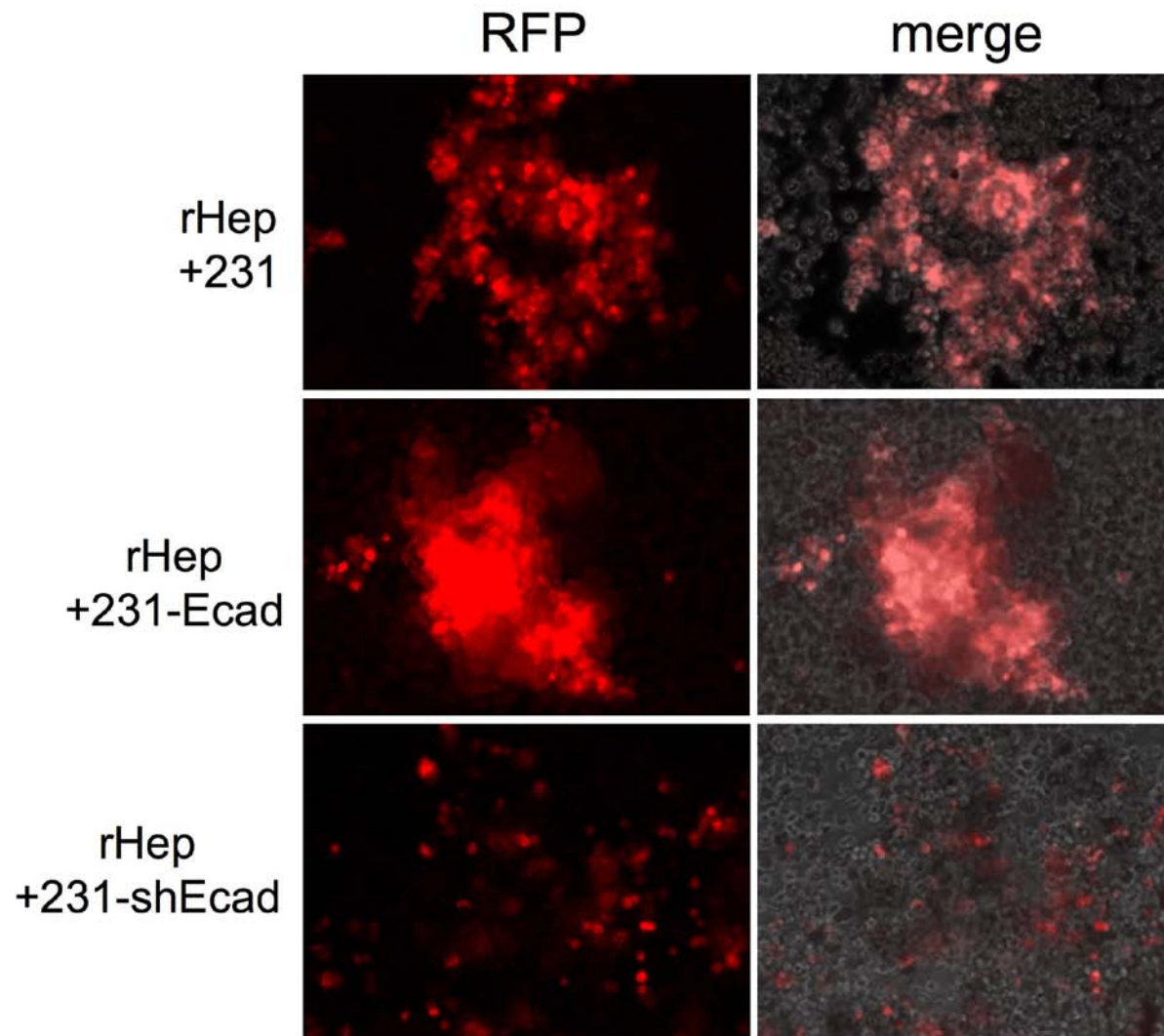


Figure 26. 231 cells revert to an epithelial morphology following 3D culture in a liver bioreactor.

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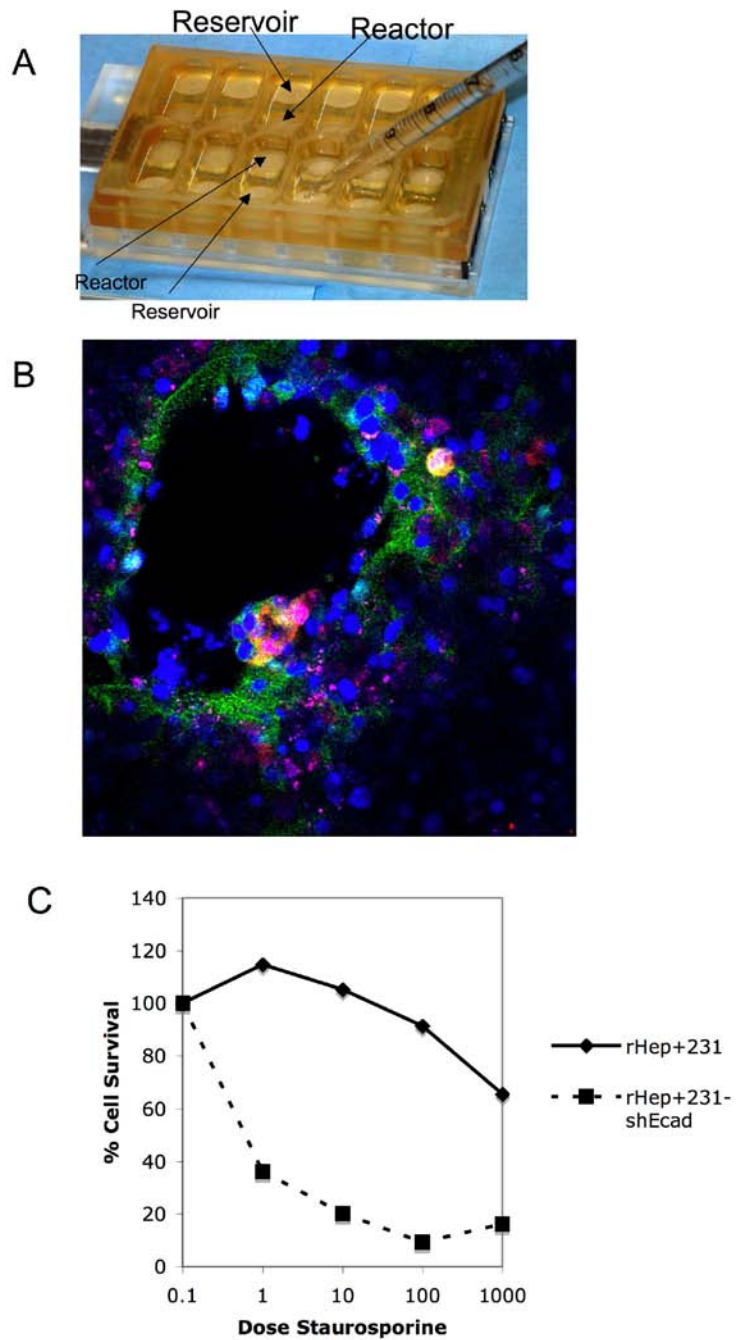


Figure 27. E-cadherin re-expression in 3D bioreactor culture increases chemoresistance A) Image of multi-well perfused bioreactor B) Immunofluorescence of E-cadherin expression after 10 days of culture in bioreactor, blue (DAPI), red (RFP), green (E-cadherin), yellow (RFP and E-cadherin merge).

4.4 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 (McGuire et al., 1984). We have shown previously that metastatic tumors from breast cancer patients express increased levels of E-cadherin compared to the primary tumor, which is accompanied by a partial mesenchymal to epithelial revert transition. 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 (Chao et al., 2010). 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 (Pece et al., 1999; Pece and Gutkind, 2000). E-cadherin binding of renal epithelial cells indeed promotes survival in a PI-3K–dependent fashion (Bergin et al., 2000). The finding that Erk is phosphorylated upon binding to hepatocytes by re-expressed E-cadherin on MDA-MB-231 cells implies relevant functional signaling occurs as a result of heterotypic ligation.

What remains is the question of whether E-cadherin expression is required for the establishment of metastases. E-cadherin re-expression could explain the propensity for breast cancer cells to metastasize to lung and liver, both lined with epithelia. 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

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(Chu et al., 2008; Li et al., 2008). Tsuji et al, approached this question by showing that EMT-induced cells transfected with E-cadherin failed to form metastases (Tsuji et al., 2009). However, it is possible that their method of EMT induction by overexpressing p12 interfered with reversion of the epithelial phenotype. An experimental model of metastasis through intrasplenic injection of 231, 231-Ecad, and 231-shEcad would answer the question of whether E-cadherin expression is necessary for metastatic colonization. It is likely that while not necessary, there is an advantage to expression of expression of adhesion molecules such as E-cadherin.

We have shown herein that one such advantage is the increased resistance to nutrient deprivation and chemotherapy-induced cell death. Previous studies have shown the protective role of E-cadherin in the face of chemotherapy and our studies of chemoresistance in breast and prostate cancer cells alone corroborate these results (St Croix and Kerbel, 1997). Another way in which E-cadherin expression can increase the survival of cancer cells is through autophagy. During tumorigenesis, mutations in the autophagic pathway can promote tumorigenesis; however once the cancer is established, activation of autophagy can help cancer cells to survive nutrient deprivation as they encounter different microenvironments (Karantza-Wadsworth and White, 2007; Kenific et al., 2009). However, autophagy only protects against short-term starvation; as prolonged nutrient deprivation still leads to cell death. E-cadherin expressing cells are more protected against starvation, and although we were unable to show a difference in LC3 protein expression we did observe a difference in localization to autophagosomes. The finding that there is decreased autophagy in hepatocyte cocultures suggests that the microenvironment is nutrient-sufficient. Treatment with autophagy inducer rapamycin in hepatocyte cocultures may reveal additional protective role of E-cadherin re-expression.

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Of particular interest is the finding that breast cancer cells in hepatocyte coculture, both in 2D and 3D, were more resistant to cell death-induced by staurosporine or camptothecin compared to cells cultured in the absence of hepatocytes. While E-cadherin re-expression increased the chemoresistance of 231 cells compared to 231-shEcad cells, even in hepatocyte coculture 231-shEcad cells were more resistant compared to 231-shEcad cells cultured alone. We have shown that in human samples and in hepatocyte coculture a partial mesenchymal to epithelial reversion occurs, suggesting that the liver microenvironment can induce other molecular changes besides E-cadherin expression during partial MErT (Chao et al., 2010). 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. Additionally, hepatocyte coculture induces re-expression of connexin43 in breast cancer cells. As we showed previously, brain metastases of breast cancer patients exhibit increased expression of E-cadherin, Cx43 and Cx 26. A recent study showed that astrocyte-cancer cell interactions mediated by gap junction expression protects cancer cells from chemotherapy-induced cell death (Langley et al., 2009; Lin et al., 2010). Thus adhesion, facilitated by gap junctions in this case, promotes the survival of cancer cells during metastatic colonization.

The mechanisms behind the increased chemoresistance in E-cadherin re-expressing cells in our model is still unknown. Possible mechanisms behind the chemoresistance include upregulation of anti-apoptotic proteins such as Bcl-2 or cell cycle inhibitors cyclin-dependent kinase inhibitor p27 (St Croix et al., 1996; Wang et al., 2009). Activation of Akt also contributes to chemoresistance (West et al., 2002). Another potential explanation for the increased chemoresistance is contact mediated growth inhibition governed by E-cadherin (St Croix et al., 1998). However, growth inhibition of MDA-MB-231 cells upon re-expression of E-cadherin was

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not observed in hepatocyte coculture (data not shown). Furthermore, there are several therapeutic implications raised by this study. Our previous results suggest that anti-adhesive therapy may not be the best method of overcoming E-cadherin-mediated chemoresistance, as disrupting E-cadherin cell adhesions would induce EMT in the metastatic tumor. 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.

4.5 MATERIAL AND METHODS

4.5.1 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 as previously described (Chao et al., 2010). For autophagy studies, cells were cultured in serum-free RPMI or HGM for 3 days.

4.5.2 Coculture

Primary rat and human hepatocytes were isolated and plated on 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). Media was replenished daily.

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4.5.3 Chemical re-expression of E-cadherin

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

4.5.4 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. 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.

4.5.5 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.

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4.5.6 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.

4.5.7 Bioreactor

A three-dimensional multiwell perfused liver bioreactor based on one previously described by Yates et al was used (Yates et al., 2007). 769-channeled polycarbonate scaffolds were coated with 30µg/ml collagen I and placed into reactor wells. 800,000 hepatocytes primary rat or human hepatocytes in single-cell suspension were seeded into scaffold channels and allowed to attach and reorganize for 48 hours. 20,000 RFP-labeled breast cancer cells were then seeded onto scaffolds and allowed to expand within the hepatocyte microtissue for 10 days. Media was changed every other day. For immunofluorescence to confirm E-cadherin expression, scaffolds were removed and fixed in 4% paraformaldehyde for 1 hour and permeabilized in 0.1% Triton-X for 30 minutes. Primary antibody incubation with E-cadherin (Cell Signaling) and DsRed2 (Santa Cruz) was performed overnight and 1 hour secondary antibody incubation (Alexafluor-488 and -594) at room temperature. Tissue structures within scaffold channels were visualized using an Olympus Fluoview 1000 confocal microscope (Olympus, Center Valley, PA). For the

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cell death assay, reactor wells were treated with 0 to 1000nM staurosporine on day 10. The next day scaffolds were removed and placed in PBS in the well of a 12-well plate. RFP fluorescence was analyzed using a Tecan SpectraFluor.

5.0 DISCUSSION

While the library of studies investigating cancer-associated epithelial to mesenchymal transition is rapidly expanding, the field of mesenchymal to epithelial transition in cancer is still nascent. We have provided *in vivo* evidence of differential expression of epithelial and mesenchymal markers in primary and metastatic tumors, suggesting that MERT is possible at the secondary site. We have shown increased expression of epithelial markers at the metastatic site compared to the primary tumor; however this change is accompanied by persistence of mesenchymal markers suggesting a partial MERT. Furthermore, we demonstrated organ-specific expression of adhesion molecules in the form of connexin expression in brain metastases, which may contribute to organotropism to the brain. Using an *in vitro* coculture of liver parenchymal cells with breast cancer cells, we also provide evidence that this increased epithelial expression observed in metastases can be due to reversion of the mesenchymal phenotype rather than expansion of epithelial cells from the primary tumor. Both the mechanism of E-cadherin regulation, methylation of the promoter, and persistence of mesenchymal markers facilitate the switching between different phenotypes. Finally, we have demonstrated that this E-cadherin expressed on different cell types, cancer cells and hepatocytes, can mediate heterotypic ligation that increases cancer cell attachment to the target organ. This ligation improves survival through activation of canonical signaling pathways and increased resistance to chemotherapy. In short, we have shown

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that the liver microenvironment is able to induce a part mesenchymal to epithelial reverting transition to confer a survival advantage to metastatic breast cancer cells in the liver.

5.1 FUTURE DIRECTIONS

5.1.1 Signals driving MErT

The signals from the microenvironment that cause E-cadherin re-expression are still unidentified. Previous work in the lab suggests that a combination of soluble factors secreted by hepatocytes and hepatocyte-derived matrix is required for E-cadherin re-expression (data not shown). These results are also supported by the finding that a gradient of E-cadherin expression can be observed in patient samples, with highest expression in areas in closest contact with organ parenchymal cells (Chao et al., 2010). One potential avenue of future research is thus the explication of these signals. Laminin-1 may be one such critical ECM component, as 3D culture of MDA-MB-231 cells in a laminin gel induces loss of methylation of the E-cadherin promoter and re-expression (Benton et al., 2009). 2D culture of MDA-MB-231 cells in hepatocyte-conditioned media on different ECM-coated surfaces has not uncovered other ECM components involved; however, 3D culture of ECM components may be more revealing. Similarly, a deductive method of applying blocking antibodies to conditioned media or the use of an antibody-array may illuminate the soluble factors involved. Whether or not physical contact between hepatocytes and E-cadherin is necessary for re-expression is undetermined.

Also unknown is whether an active signal to induce MErT is required, or if only a removal of EMT-inducing stimuli is necessary. Preliminary experiments suggest that there is no

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correlation between protein expression of EMT master regulators Snail, Slug, and Twist and E-cadherin expression in our system (data not shown). However, cellular localization of these proteins was not investigated. Mesenchymal to epithelial transition in development is well-studied in terms of kidney morphogenesis; therefore parallels in these processes may give some insight into signals that drive MErT. FGFs are necessary for epithelialization of nephric mesenchyme during kidney development (Urban et al., 2006). In a model of lung fibrosis, an example of fibrosis-associated EMT in which alveolar epithelial cells convert to fibroblasts/myofibroblasts, TGF- β induced EMT can be reverted by addition of FGF-1 and heparin in combination or HGF alone (Shukla et al., 2009; Ramos et al., 2010). Similarly, expression of hepatocyte nuclear factor 4 alpha (HNF α) in fibroblasts induces mesenchymal to epithelial transition (Parviz et al., 2003; Kanazawa et al., 2010). HNF4 α is a dominant driver of the epithelial phenotype in the liver as conditional knockout during hepatogenesis results in disorganized tissue architecture of hepatocytes that do not express E-cadherin nor form cell-cell contacts (Parviz et al., 2003). Further analysis reveals that HNF4a is responsible for expression of all cell junction genes, including adherens, tight, and gap junctions (Battle et al., 2006), suggesting that HNF4a is a key orchestrator of epithelial phenotype in the liver. As we have shown that E-cadherin re-expression increases the survival of cancer cells in the liver microenvironment, elucidating the signals that induce MErT may be critical to developing therapies to target metastatic colonization.

5.1.2 Organotypic bioreactor as an experimental tool for studying metastasis

The chemoresistance of breast cancer cells was seemingly amplified when cultured in 3D in the organotypic bioreactor compared to when cultured in 2D on collagen-coated plastic. This finding

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suggests that the bioreactor may thus be a more useful experimental tool that more closely approximates how cells behave *in vivo*. Similarly, studies in 3D are a more accurate reflection of how cells will respond to drugs and other therapeutics than in 2D cell culture. An added benefit of the bioreactor is that it allows for relatively high throughput testing of drugs. One of the major drawbacks to studies of EMT and MErT is that the transient dynamism of plasticity is difficult to capture using static techniques such as immunostaining or even gene expression profiling. Models like the bioreactor can combine 3D culture with real-time imaging of metastatic progression. The involvement of other cells in the liver microenvironment, such as endothelial, stellate, or Kupffer cells can also be elucidated by introduction into the bioreactor.

5.2 EPITHELIAL PLASTICITY IN CANCER

Epithelial to mesenchymal transition has captured great interest over the years as a developmental program that is re-articulated in pathological processes such as metastasis and fibrosis. However, there is a movement toward using terms such as “epithelial mesenchymal plasticity” instead of EMT to denote a difference between these reversible, and often partial or intermediate phenotypic switches, and the irreversible conversion to a mesenchymal phenotype observed in developmental EMT. Processes such as endothelial to mesenchymal transition (EndoMT) and epithelial to myofibroblast transition (EMyT) denoting similar phenotypic switches have also been defined (Kizu et al., 2009; Masszi et al., 2010). At the core of these phenotypic transitions is the idea that differentiated cells are much more plastic than originally thought, as also underlined by studies involving induced pluripotent stem cells and metaplasia. Most importantly, through epigenetic regulation that can be induced by the microenvironment,

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EMT and M_ErT allow cancer cells to easily switch between motile and immotile, adhesive and non-adhesive, proliferative and non-cycling states; these adaptations can all occur without the acquisition of genetic mutations.

5.2.1 The hunt for a master regulator

Many independent inducers of EMT have been discovered and hundreds of genes are differentially expressed between epithelial and mesenchymal phenotypes, initiating a search for a master regulator. It is becoming more evident that there is a hierarchy of regulation, with Snail and Twist at the top (Foubert et al., 2010). Besides these transcriptional regulators, microRNAs (miRNAs) are a family of non-coding RNAs that inhibit translation by binding target mRNAs. MicroRNAs are often dysregulated in cancer, which can consequently induce EMT (Valastyan et al., 2009; Tryndyak et al., 2010). Intriguingly, the miR-200 family of microRNAs is involved in a reciprocal repressive feedback loop with ZEB, a transcriptional repressor of E-cadherin and EMT inducer (Burk et al., 2008; Brabletz and Brabletz, 2010). In EMT, the balance shifts in favor of ZEB, which is also a transcriptional repressor of miR-200. In M_ErT, expression of miR-200 increases to inhibit translation of ZEB, which is then unable to repress transcription of E-cadherin, therefore pushing cells toward an epithelial phenotype. The signature of EMT-associated gene expression is also driven by master programs of chromatin organization, DNA methylation, and splicing (Han et al., 2008; Warzecha et al., 2010).

Recent evidence suggests that induction of EMT in cancer cells confers stem cell properties, providing a link between EMT and the cancer stem cell hypothesis. Ectopic expression of Snail or Twist not only results in induction of EMT as expected, but cells also acquire expression of stem cell markers CD44^{high}/CD24^{low} with increased ability to form

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mammospheres. Similarly, stem-like cells isolated *in vivo* express EMT markers (Mani et al., 2008; Morel et al., 2008). In breast cancer cell lines, there is a significant overlap in expression of genes in the EMT signature and mammary stem cell signature (Blick et al., 2010).

5.2.2 MErT in metastatic colonization

Many studies have shown that inducing EMT in the primary tumor can increase metastases, and in these studies EMT is usually irreversibly induced, suggesting that reversion at the secondary site is not necessary for establishment. However, use of the 4T1 cell line and its variants, which were derived from a single, spontaneously generated tumor, have provided some insight into the role of E-cadherin in colonization (Aslakson and Miller, 1992). The cell lines 67NR, 168FARN, 4T07, and 4T1 can all form primary tumor when injected orthotopically, but vary in the ability to form metastases, with 4T1 cells able to form macroscopic tumors in lung, liver, bone, and brain, and 67NR cells unable to intravasate and subsequently form metastases. 168FARN cells form only lymph node metastases and 4T07 cells disseminate to lung but do not form macroscopic lung metastases. Interestingly, not only is E-cadherin only expressed in 4T1 cells, but there is also dramatic up-regulation of the miR-200 family of miRNAs, which repress translation of E-cadherin transcription repressor ZEB2. Expression of vimentin is equal among all the cell lines, indicating some persistence of mesenchymal phenotype (Lou et al., 2008; Dykxhoorn et al., 2009). Repression of ZEB and increased expression of E-cadherin in 4T07 cells increases invasion and the ability to form macroscopic nodules. However, these studies only directly tested the roles of ZEB and miR-200, and not E-cadherin, in metastatic colonization, as both ZEB and miR-200 have many downstream targets besides E-cadherin.

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5.2.3 A model for EMT and MErT in cancer

Based on the literature and our findings we present a model for the role of EMT and MErT in metastatic progression (Figure 28). Although rarely captured in patient samples, in vivo imaging and in vitro studies have conclusively provided evidence that EMT contributes to metastatic dissemination. However, EMT is not the only mechanism of invasion nor is it absolutely required. EMT results in loss of E-cadherin expression and disruption of intercellular adhesion, disturbing the compartmentalized and polarized architecture of epithelia and resulting in autocrine growth factor activation and increased interactions with stroma cells and matrix. As a consequence of EMT, cancer cells slide toward a mesenchymal phenotype and gain motility and the ability to process and degrade the ECM en route to dissemination. Following survival in the circulation and extravasation, adhesion to the secondary organ is mediated by re-expression of E-cadherin and other adhesion molecules in a partial MErT. This model of a more differentiated phenotype at the metastatic site also supports the hypothesis that the cells that exhibit the EMT phenotype are stem-like. Post-extravasation survival, one of the rate-limiting steps of metastasis, is thus facilitated by heterotypic ligation between cancer cells and parenchymal cells. Following proliferation of cancer cells in the ectopic organ and angiogenesis that generates a macrometastasis, the exposure of cancer cells to microenvironmental signals decreases and new cycles of EMT can occur to generate metastases from metastases.

Where dormancy fits into this chronology is a question that should be explored further. Studies have shown that EMT involves a switch from a proliferative to a lower-cycling, migratory state, suggesting that after MErT cancer cells would become proliferative (Kent et al., 2009). However, the role of E-cadherin in contact-dependent growth inhibition is well-

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established, so it is unclear whether a period of dormancy would precede or succeed re-expression of E-cadherin.

Importantly, MErT is not a process that is limited to breast cancer and metastases to the liver. We have previously shown that prostate cancer cells cocultured with hepatocytes also re-express E-cadherin, although the mechanism of re-expression is through inhibition of EGFR signaling (Yates et al., 2007). Prostate cancer is the most common malignancy in men and exhibits a specific pattern of metastases similar to breast cancer: bone, lung, and liver (Bubendorf et al., 2000). Other similarities between breast and prostate cancer include dependency on hormones, roles of the stroma in tumor progression, and EMT as a mechanism for metastatic initiation (Henshall et al., 2001; Hugo et al., 2007; Risbridger et al., 2010). The prostate also relies on the underlying mesenchyme during development (Cunha, 2008). In our studies, immunohistochemistry of prostate cancer primary and metastatic tumors revealed increased E-cadherin expression as observed in breast cancer metastases. Cell death assays of prostate cancer cells chemically induced to re-express E-cadherin through EGFR inhibition suggest that the function of E-cadherin re-expression in prostate cancer may be similar.

Our results suggest that MErT is not limited to liver metastases but may be extrapolated to colonization of other organs as well. Tail vein injection of MDA-MB-231 cells in mice resulted in E-cadherin-positive foci in the lung. These results were supported by immunohistochemistry of human lung metastases as well as in vitro coculture of breast cancer cells and pneumocytes (Li et al., 2010). Breast cancer metastases to bone and brain also express E-cadherin, so it remains to be seen whether E-cadherin expression facilitates attachment to these organs where the parenchymal cells do not express E-cadherin. Although E-cadherin expression

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is observed in brain metastases, it may be other adhesions such as gap junctions that coordinate the survival and chemoresistance of cancer cells in the brain.

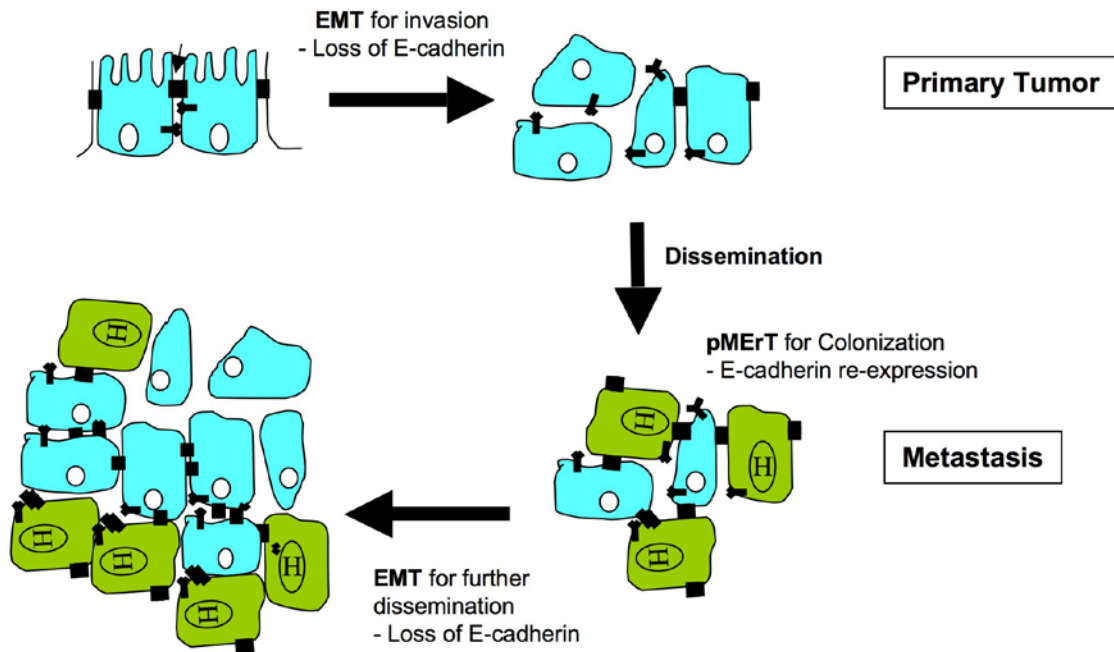


Figure 28. Model for EMT and MERt in metastatic progression

5.3 THERAPEUTIC IMPLICATIONS

Mortality of breast cancer is largely attributed to chemoresistance of metastases. The findings presented herein unfortunately do not reveal a clear therapeutic target; rather, the phenotypic plasticity of cancer complicates development of therapeutic interventions. When EMT was first described, induction of E-cadherin expression in the primary tumor was touted as a way to prevent invasion and metastasis. Given our results, such therapies may have the unwanted side effect of promoting secondary metastases. Conversely, anti-adhesive antibodies to prevent re-expression of E-cadherin and other adhesion molecules at the metastatic site are burdened with the possibility of inducing EMT and causing dissemination of metastases from metastases, a possibility supported by our *in vivo* analysis of E-cadherin expression correlated with metastatic tumor size. However, even if EMT is induced, anti-adhesive therapy may prevent attachment and colonization, resulting in circulating tumor cells without the formation of metastases, much like the 4TO7 cell line described earlier. An additional problem is that targeting one adhesive molecule may not be sufficient, as others can compensate. Strategies of targeting the so-called “master regulators” of EMT/MErT such as transcriptional repressors and microRNAs may be more effective at enacting multi-level molecular changes.

Similarly, our results that show increased chemoresistance in breast cancer cells following E-cadherin re-expression are complicated by the fact that many studies have also shown that cells that have undergone EMT are more resistant to chemotherapy and radiotherapy. One such example is in non-small cell lung cancer where the E-cadherin-positive cohort is more susceptible to treatment with EGFR inhibitor erlotinib compared to E-cadherin-negative tumors (Buck et al., 2007). Whether there are differences in the mechanisms behind resistance in EMT and the resistance in MErT is unknown. The key to overcoming the problem of cancer cell

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plasticity is probably multi-modal therapy to target multiple cell populations concurrently. Screens have identified several agents that selectively kill cancer stem cells/EMT cells (Gupta et al., 2009). Such agents may be more effective when used in combination with an agent to target cells that have already gone MERt, such as blocking the signals from the secondary microenvironment that induce E-cadherin re-expression.

Studies have independently evaluated the prognostic value of both EMT markers and EMT gene signatures; however, clinical implementation of evaluating EMT and MERt status is limited. The main constraints are temporal and spatial heterogeneity in expression and the lack of good phenotypic markers of EMT/MERt. The plasticity of cancer cells implies that the phenotype of the cell is constantly in flux, and as we have shown cells exist in intermediate states where both epithelial and mesenchymal markers are present. As a result, temporal and spatial heterogeneity means that phenotypic status can differ depending on the time course of cancer progression and even within the same tumor. Furthermore, as we have discussed in Section 2.4 there are no good markers to identify breast cancer cells that have undergone EMT and now exhibit a more mesenchymal phenotype. For example, circulating tumor cell assays isolate CTCs based on epithelial marker EpCAM, leaving out the population of circulating cancer cells that have gone through EMT and lost expression of EpCAM.

5.4 CONCLUSION

Our finding that E-cadherin re-expression and partial mesenchymal to epithelial reverting transition may have an important role in metastatic colonization has seemingly unleashed more questions than answers. However, the phenotypic plasticity exhibited by cancer cells throughout metastatic progression likely explains why singular therapies have limited success in the clinic. Hopefully this work will lead toward a combinatorial approach that is both targeted to tumor cells and the microenvironment, thus limiting these phenotypic transitions at multiple stages of progression.

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ABBREVIATIONS

5FU – 5-fluorouracil
ABC – ATP-binding cassette transporter
CAF – cancer-associated fibroblast
CAFCA – centrifugal assay for fluorescence-based cell adhesion
CK – cytokeratin
CMV - cytomegalovirus
CSF-1 – colony stimulating factor
CTC – circulating tumor cells
Cx26 – connexin 26
Cx43 – connexin 43
CXCR – C-X-C receptor
DCIS – ductal carcinoma in situ
DNMT – DNA methyltransferase
E-cadherin – Epithelial cadherin
ECM – extracellular matrix
EGF – epidermal growth factor
EGFR – epidermal growth factor receptor
EMT – epithelial to mesenchymal transition
ER – estrogen receptor
EpCAM – epithelial cell adhesion molecule
FACS – fluorescence-activated cell sorting
FAK – focal adhesion kinase
FGF – fibroblast growth factor
FSC – forward scatter
FSP1 – fibroblast specific protein
GFP – green fluorescent protein
HGF – hepatocyte growth factor
HGM – hepatocyte growth media
HNF – hepatocyte nuclear factor
HPC – hematopoietic progenitor cell
HUVEC – human vascular endothelial cell
ICAM – intercellular adhesion molecule
IDC – infiltrating ductal carcinoma
IL – interleukin
ILC – invasive lobular carcinoma
IGF – insulin growth factor
LHRH – luteinising hormone-releasing hormone
MErT – mesenchymal to epithelial reverting transition
MMC – mitomycin C
MMP – matrix metalloprotease
MSC – mammary stem cell

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MS-PCR – methylation specific polymerase chain reaction
N-cadherin – Neural cadherin
N-CAM – neural cell adhesion molecule
NPC – non-parenchymal cell
PDGF – platelet-derived growth factor
PR – progesterone receptor
PTHrP – parathyroid hormone-related protein
RFP – red fluorescent protein
SBRT – stereotactic body radiotherapy
SDF - stromal cell-derived factor
SIRT – selective internal radiotherapy
SSC – side scatter
TACE – transarterial chemoembolization
TAM – tumor associated macrophages
TGF – transforming growth factor
TNF – tumor necrosis factor
VCAM – vascular cell adhesion molecule
VE-cadherin – vascular endothelial cadherin
VEGF – vascular endothelial growth factor
XIAP - X-linked inhibitor of apoptosis protein

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