EPITHELIAL CADHERIN RE-EXPRESSION IN METASTATIC BREAST CANCER AS A STRATEGY FOR METASTATIC COLONIZATION OF THE LIVER

by

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ABSTRACT

Epithelial-cadherin downregulation enables cancer cells to escape from the primary mass; however, E-cadherin has been found to be expressed on metastatic foci, bringing into question the role of this molecule in tumor progression. We define a novel role for the cellular adhesion molecule E-cadherin, in which the protein's re-emergence promotes carcinoma-parenchymal interactions in ectopic sites. Non-metastatic E-cadherin positive MCF7 breast cancer cells form heterotypic cohesions mediated by E-cadherin, and in invasive and metastatic MDA-MB-231 cells, the E-cadherin promoter hypermethylation that prevents endogenous E-cadherin expression is reversed when these cells are cultured with hepatocytes. The function of this re-expression is suggested by the E-cadherin-dependent sustained activation of Erk-MAP kinase and Akt in these breast carcinoma cells. Thus, we propose that E-cadherin expression and subsequent heterocellular interactions direct cell fate decisions that may ultimately enable colonization of a secondary site by an invasive cancer cell.

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binding domains. In an untransformed cell, p120 is thought to stabilize E-cadherin at the surface, β-catenin is sequestered from forming a complex with axin and, in this location also functions as an adaptor protein for α-catenin, which in turn anchors E-cadherin to the cytoskeleton. B) In many carcinomas, E-cadherin is silenced by promoter methylation allowing β-catenin to translocate to the nucleus and p120 to promote an epithelial phenotype. However the mechanisms of how these catenins act and whether they act individually or in concert are not settled. C) E-cadherin dependent adhesion in itself is not a dominant stop mechanism to inhibit invasion. In studies where the β-catenin binding domain was deleted from the E-cadherin intracellular domain, but E-cadherin was still able to mediate adhesion through direct crosslinking with α-catenin and therefore interaction with the actin cytoskeleton, β-catenin was free to signal in the cell cytoplasm and led to an invasive phenotype (though this phenotype was independent of its TCF-mediated transcriptional activity). Therefore, cytoplasmic localization of β-catenin is thought to contribute to the mesenchymal nature of cells. D) In studies of cell that had low levels of E-cadherin and cytoplasmic localization of p120, tyrosine phosphorylation on p120's amino-terminal by the pro-oncogene Src was thought to contribute to modulate its contribution to cell migration. When p120 is knocked down, the equilibrium shift to Rho-GDP promotes actin polymerization, stress fiber formation, a flattened morphology and less invasive phenotype. Therefore, cytoplasmic localization of p120 is thought to contribute to the mesenchymal nature of cells. These four scenarios provide data that show the critical role of E-cadherin as a signal modulation molecule by sequestering catenins, primarily p120 and α-catenin. In the absence of E-cadherin homotypic binding, this plaque is unstable and the catenins are now free to relocalize. 12

PREFACE

This thesis could not have been written without the help from many people.

Firstly, I would like to thank my PhD mentor, Alan Wells. I could not have imagined a better advisor and mentor for my PhD, and I hope to work with him for a very long time. He gave me the independence I needed, but at the same time, I would have been lost without him.

My thesis committee has also been particularly supportive. I have been inspired by their intelligence, direction, and high expectations. I would be honored to work with them in the future.

Next, I would like to thank all the Members of the Wells Lab throughout the years. They have always been there when I needed their help, even though I can be sometimes difficult to get along with while in pursuit of good data.

Lastly, I would like to thank my family. Even though my parents thought I was working towards an M.D. for the first two years of my graduate work, I could not have succeeded without their love and support. Also to Pat, through whom I have lived vicariously since graduating from college.

1.0 INTRODUCTION

As is the case with scientific investigations, a macroscopic perspective is required to understand the significance of the individual steps taken to arrive at the conclusions of the study. Cancer pathology, a disease of vastly heterogeneous etiology, is especially suited to such perspective, as dissection of sequential steps of the metastatic process without context results in meaningless segmentation of the disease. The following introduction describes the events in the transformation of normal breast cells to their invasive counterparts and metastatic disease of the liver, all elements essential to understanding the significance of the thesis as a whole.

1.1 BREAST CANCER

The normal mammary gland has the structure of a modified sweat gland. The breasts' anatomy consists of the skin, the subcutaneous adipose tissue, and the glandular tissue, comprising the stromal and parenchymal architectures. The breasts are in systemic linkage to the rest of the body by way of the arterial (thoracic, thoracoacromial, posterior intercostal) and venous (axillary) blood supply and drainage, respectively. The breasts also have rich lymphatic network, most of which flows to the axillary lymph nodes. The lumen of the lymphatic structures are open-ended and in continuity with the extracellular tissue spaces, which allows the

free flow of lymph and cellular content. The relationship of structure to function of the human female breast has been covered in much greater depth elsewhere (Hovey and Trott 2004); the most relevant part of the breast microanatomy to the thesis herein is the epithelial parenchymal component.

The breast is divided into six to 10 duct systems, each of which is subdivided into lobules, the organizational unit of the mammary parenchyma. Iterative branching of the large ducts results in the eventual terminal duct. The distal proliferation of these terminal ducts at the beginning of female menarche results in lobules composed of ductules lined with epithelium. This architectural unit is known as the terminal duct lobular unit. Carcinoma *in situ* is thought to develop when genetic or epigenetic changes allow the epithelial cells in the terminal duct lobular units to take on a transformed phenotype, which is antecedent to a more motile cell type. Subsequent dissemination and colonization of distant organs throughout the body is mostly responsible for the pathologies of invasive cancers.

The symbolic and esthetic value of the female breast has increased public awareness of the breast cancer for the past half-century. Previous theories on breast cancer pathology motivated the radical mastectomy procedure, which was overwhelmingly favored by physicians for the first half of the century. This radical procedure was unsuccessful though, as most patients relapsed in systemic disease (Fisher 1999). More modern locoregional procedures to control the spread of breast cancer also proved deficient. The modern treatment regimen combines both local and systemic treatment, taking advantage of new tools that allow clinicians to personalize therapy based on the molecular variants of the disease in the individual person.

The etiology of breast cancer is heterogeneous. The greatest risk factors include advanced age, being born in North America or Northern Europe, high premenopausal insulin-like

growth factor (IGF)-1 level, high postmenopausal blood estrogen level, and a familial history of breast cancer. Genetic predisposition to the disease is responsible for 5-10% of diagnosed cases (Oldenburg, Meijers-Heijboer et al. 2007). *BRCA1* and *BRCA2* account for the majority of autosomal predisposition. Though there are other germ line mutations (*p53*, Cowden syndrome 10q locus, *ATM*) that can act as predictive criteria for the development of metastatic disease, the precise determination of who is at risk has been impossible and therefore proactive management has also been unachievable.

The preponderance of evidence supports that there is no single reason for the cellular transformation to neoplastic phenotypes. For that reason, targeted molecular therapies have had the most success for cytotoxic and cytostatic cancer treatment. This thesis investigates two targetable molecules for potential therapeutic intervention: Phospholipase-C-γ (PLCγ), a molecule important to cellular motility, and Epithelial-cadherin, a cell-adhesion molecule integral to the maintenance of epithelial architecture.

1.2 EPITHELIAL TO MESENCHYMAL TRANSITION IN CANCER

The epithelial to mesenchymal transition (EMT) was recognized as early as 1908 (Lillie 1908). It was characterized as being a critical part of embryonic development in many animal species. It was not until 1982, when scientists found that if Madaline-Darby canine kidney cells were treated with conditioned fibroblast media, they could be converted to a nonpolarized migratory cell type (Greenburg and Hay 1982). The connection between EMT and cancer progression came even later primarily because it is impossible to recognize EMT *in vivo* during carcinoma progression because of the spatial and temporal complexity of metastasis (Thiery 2002).

Epithelial cell plasticity has been conserved and is integral to development of all metazoans. The remodeling of epithelial sheets in higher organisms is conserved from the basic remodeling actions of delamination, invagination, and cavitation in the most primitive organisms. It has been reported that in most metazoans, epithelial sheets can undergo both EMT and the mesenchymal-epithelial transition (MET) (Gilbert 1997). In humans, the MET is particularly important in the normal developmental process of kidney ontogenesis (Stark, Vainio et al. 1994).

The loss of the E-cadherin cell adhesion molecule is of particular importance to the EMT, and it has even been given the name "caretaker of the epithelial phenotype" (Thiery 2002). In normal development, E-cadherin is responsible for organizing cells into their constituent organs, because the cell adhesion molecule allows cells of the same type to organize together. In turn, E-cadherin foci form adherence junctions and in some instances promote the formation of desmosomes (Kowalczyk, Bornslaeger et al. 1999).

The stability of these cohesive junctions depends on the stability of the E-cadherin molecule, including E-cadherin's transcription, translation, trafficking to and from the cell surface, and effector control over the cell's presentation on the cell surface. Function-blocking antibodies or siRNA directed to E-cadherin will cause loss of integrity of epithelial sheets *in vivo* or loss of cobblestone morphology *in vitro* (Imhof, Vollmers et al. 1983). In the context of EMT and MET, E-cadherin is lost during the gastrulation of *Drosophila*, chick, and mouse (Edelman, Gallin et al. 1983; Burdsal, Damsky et al. 1993; Tepass, Gruszynski-DeFeo et al. 1996); and, importantly, E-cadherin is gained in the reciprocal transition of kidney ontogenesis mentioned above (Kuure, Vuolteenaho et al. 2000).

In modeling cellular behavior in three-dimensional collagen gels, it has become well known that if E-cadherin is abrogated, the epithelial nature of polarized cells is also compromised, and they will invade and degrade the collagen substratum (Chen and Obrink 1991). In studies where E-cadherin expression was rescued in mesenchymal cells, the previously motile and invasive cells will undergo partial or complete reversion to an epithelial type (Behrens, Mareel et al. 1989; Navarro, Gomez et al. 1991; Vleminckx, Vakaet et al. 1991). *In vivo*, there is a strong correlation between loss of E-cadherin in invasive cancers, number of metastases, and short survivor rate of cancer patients (Birchmeier and Behrens 1994; Hirohashi 1998). Notably, E-cadherin deactivation is usually by epigenetic means, which are reversible, opposed to the small minority of cancers that deactivate E-cadherin through mutation (Risinger, Berchuck et al. 1994; Yoshiura, Kanai et al. 1995). In Chapter 4, it is reported for the first time that cancer cells will re-expression E-cadherin in the appropriate physiological context, namely the ectopic hepatic parenchymal environment.

1.3 CANCER CELL DISSEMINATION

As the EMT is a precursor to a more motile and invasive phenotype, the view that invasive cancer is a disease of dysregulated cell motility has become more accepted (Kassis, Lauffenburger et al. 2001). The important reason to focus on motility is the ability to parse motility from proliferation, both caused by receptor tyrosine kinase activation (Turner, Chen et al. 1996; Turner, Epps-Fung et al. 1997). Motility is a key step in the metastatic cascade because migration and invasion into adjacent tissues is dependent on productive motility through the basement membrane (Figure 1).

EGF and TGFα, both EGFR ligands, as well as HGF/Scatter factor have all been linked to tumor cell invasiveness (Wells 2000). The amplification of EGFR and HER2 has been documented repeatedly in cancers associated with poor prognosis, and EGFR-family member signaling has been a particularly successful target in the clinic with the development of Trastuzumab, Cetuximab, and other function blocking antibodies to EGFR-family members. Downstream of receptor tyrosine kinase activation, signaling pathways must diverge into either pro-migratory or pro-proliferatory roles. Phospholipase-C-γ (PLCγ), in conjunction with actin modifying proteins, was initially described as being necessary for growth-factor induced cell motility (Chen, Murphy-Ullrich et al. 1996; Gilmore and Burridge 1996). Upon receptor tyrosine kinase activation, which activates PLCγ, phosphatidylinostitol biphosphate (PIP2) is cleaved at the membrane causing the release of gelsolin, profiling, and cofilin that direct changes in cytoskeletal architecture (Chen, Murphy-Ullrich et al. 1996). The cytoskeletal remodeling results in cellular polarization, which is necessary for motility (Wells, Ware et al. 1999). Abrogation of PLCγ activation does not block proliferation, an observation that confirms the independence of the growth factor induced responses (Bornfeldt, Raines et al. 1994; Chen, Xie et al. 1994).

Initial studies to determine this involved expressing an EGFR that could transmodulate PLCγ, the wild-type EGFR, and a construct that lacked the ability to transactivate PLCγ (Xie, Turner et al. 1995). Though both receptors were able to activate the pro-proliferatory pathway, the cells expressing the wild-type EGFR were significantly more invasive in invasion assays and

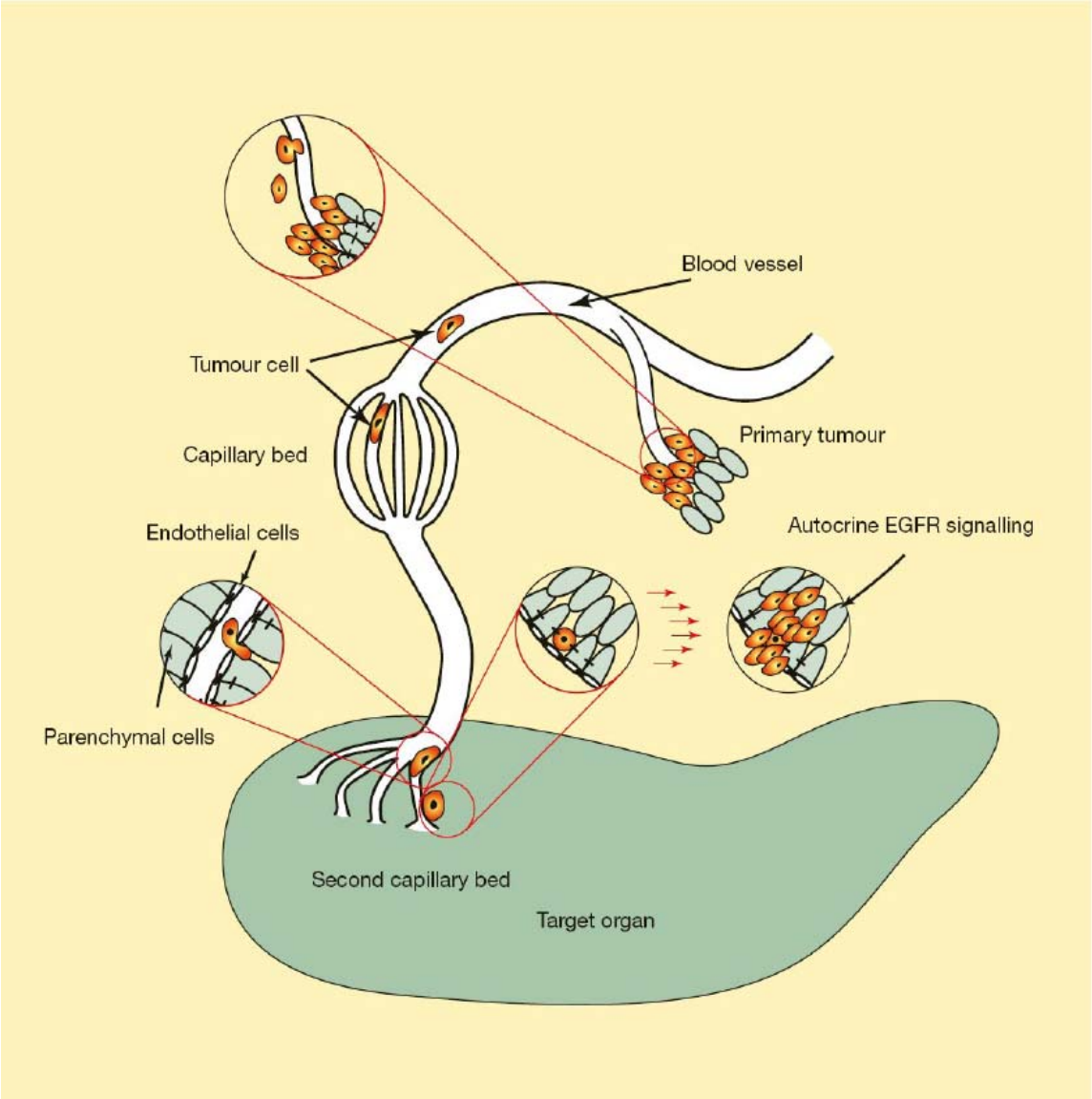

Figure 1. Motility, autocrine signaling, and loss of epithelial architecture are all key to transformative and metastatic progression. Epithelial tissues consist of sheets of normal cells (green) linked together by E-cadherin (thick black bars). This establishes a polarity that segregates apically secreted factors, such as EGF, from their basolaterally-presented receptors that normally are utilized by stromally derived factors, such as TGFβ. Due to genetic and epigenetic events, E-cadherin is lost during neoplastic transition (red/orange cells), allowing for autocrine signaling. This 'dedifferentiation' is the carcinoma-associated EMT. However, we propose that during metastatic seeding to other epithelial organs E-cadherin is re-expressed enabling linkages to normal parenchymal cells. This characterizes the mesenchymal to-epithelial reverting transition (MErT).

xenograft models (Xie, Turner et al. 1995; Turner, Chen et al. 1996). In studies that directly targeted PLCγ with the pharmacological inhibitor U73122, tumor invasiveness was inhibited while growth remained unchanged (Turner, Chen et al. 1996; Turner, Epps-Fung et al. 1997).

The evidence above in combination with other studies on autocrine-induced PLCγ activation and observations of PLCγ in other cancer cell lines (Kassis, Moellinger et al. 1999; Khoshyomn, Penar et al. 1999) underlines the important of PLCγ to tumor cell invasion. As PLCγ is also activated by the PDGF and IGF-1 receptors (Bornfeldt, Raines et al. 1994; Kundra, Escobedo et al. 1994), PLCγ may be a convergence point to negotiate the cross-talk between a number of pro-migratory and pro-proliferatory pathways (Kassis, Radinsky et al. 2002). In total, there is a well-supported proof-of-concept that PLCγ signaling is fundamental to neoplastic cell dissemination that causes the majority of cancer morbidity and mortality. In Chapter 2, the critical *in situ* data validating the proof-of-concept is reported, thus substantiating the *in vitro* data with its clinical presentation.

1.4 E-CADHERIN IN NORMAL AND TRANSFORMED CELLS

Epithelial-cadherin (E-cadherin) is central to the dynamic cellular and morphological changes that occur during both normal development and the EMT. Many biological processes, such as hemostasis, immunological response, inflammation, embryogenesis, and development of neural tissue depend on the precise selective molecular interactions that E-cadherin orchestrates (Petruzzelli, Takami et al. 1999). In addition, the spatiotemporal expression of E-cadherin is fundamental to the processes of normal development and progression of EMT / MET. The importance of such concepts to this thesis includes 1) E-cadherin has historically taken the role

of directing interactions between cells of the same type (homotypic interactions; and 2) the disappearance and reappearance of E-cadherin is a continual theme throughout development and EMT. Chapter Three of this thesis provides compelling information that the role of E-cadherin may be greatly expanded in the process of MET-related metastatic progression (Figure 1).

1.4.1 E-cadherin structure and function

Though many cadherins have been characterized, the classic (Type I) cadherins, the group to which E-cadherin belongs, have been best described and offer the best insight into the structure-function relationship of these proteins. The classical cadherins are a family of transmembrane proteins, whose extracellular domains control the adhesive function of the proteins and whose intracellular domains allow docking of cadherin substrates to stabilize the protein at the cell membrane and connection to the actin cytoskeleton. While the calcium dependent ligation of the extracellular domains without involvement of the actin cytoskeleton is sufficient for adhesion, the anchoring of the intracellular cadherin tail to cytoskeletal components significantly strengthens the adhesion (Yap, Brieher et al. 1997). Cadherin substrate molecules including β-catenin, α-catenin, and vinculin anchor cadherins to the actin cytoskeleton.

1.4.1.1 E-cadherin structure

The extracellular domain of E-cadherin is made up of 5 domains, labeled EC-1 through EC-5. EC-1, the domain most distal to the cell membrane was resolved by NMR spectroscopy and X-ray crystallography in 1995 (Overduin, Harvey et al. 1995; Shapiro, Fannon et al. 1995). The resolved structures bared highly conserved structure to the immunoglobulin variable-like domains. Within these structures, coordinated Ca2+ was determined to be necessary for

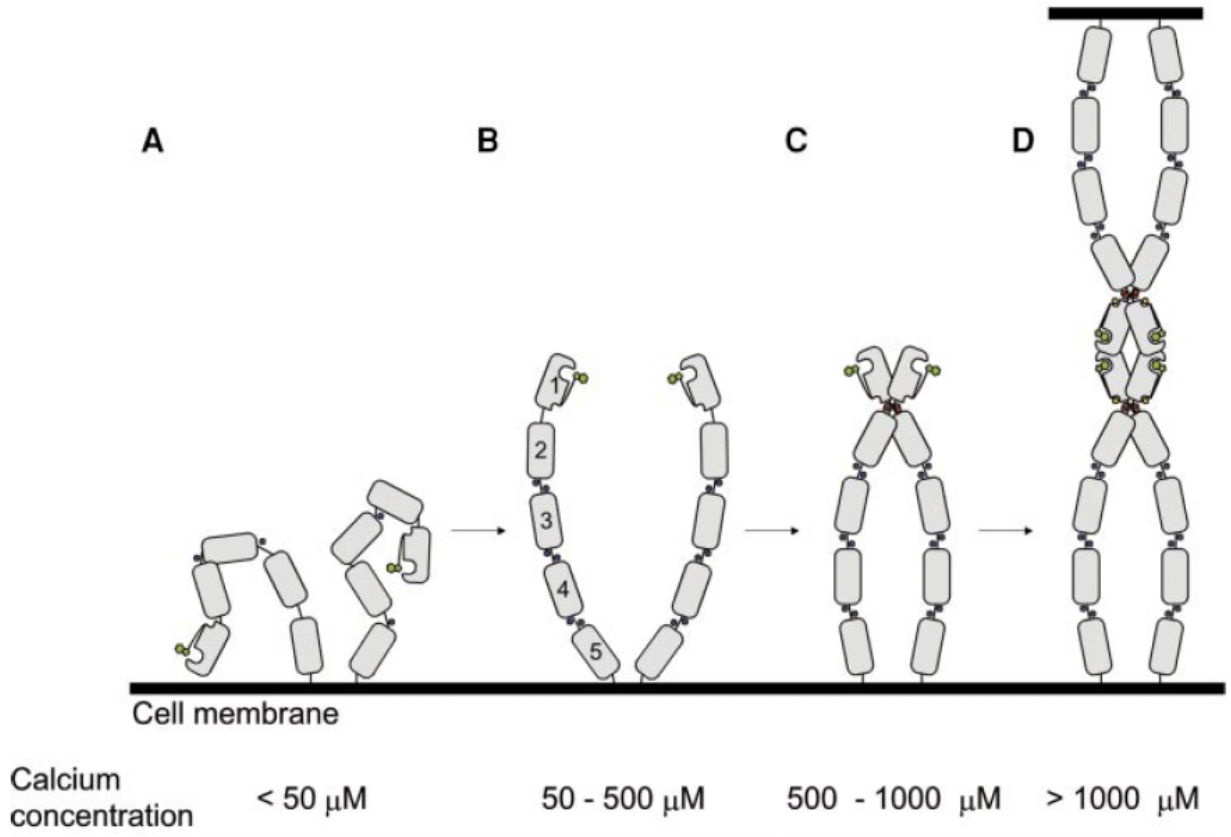

Figure 2. E-cadherin cis- and trans- ligation is calcium dependent. Calcium coordination rigidifies the 5 extracellular domains of the E-cadherin molecule (B). The unorganized molecular lattice of (A) proceeds to the ordered cis-interaction in (C), and then to trans-ligation of adjacent molecules in (D). Figure used with Open Access permission: Pertz et al. (1999). "A new crystal structure, Ca2+ dependence and mutational analysis reveal molecular details of E-cadherin homoassociation." EMBO **18**(7):1738-1747.

productive ligation by rigidifying domain structure and hence orientation (Nagar, Overduin et al. 1996).

Upon the appropriate engagement of adjacent cell membranes, E-cadherin undergoes rearrangement in the membrane form a disordered structure to an ordered lattice. The formation of the ordered lattice is also supported by Ca2+ coordination. Lattice coordination is thought to occur in two steps: cis-dimerization of E-cadherin proteins on the cell surface and then trans-dimerization of E-cadherin proteins on adjacent cell membranes (Shapiro, Fannon et al. 1995). The Ca2+ fortified lattice provides substantial protection again exogenous proteases (Yoshida and Takeichi 1982). Although dimer formation takes on a zipper appearance with interdigitated residue adducts, the attempt to find conserved amino acid recognition sequences for the dimerization structures that have held up to subsequent experimentation have eluded researchers to this point (Shapiro, Fannon et al. 1995).

1.4.1.2 E-cadherin substrates determining binding efficiency

The cytoplasmic domain of E-cadherin is crucially important to stable cell-cell adhesion (Figure 3). The intercellular domain is highly conserved across the classical cadherins and contains a membrane-proximal domain and a series of catenin-binding domains. The C-terminal residues of the E-cadherin cytoplasmic domain are very important for β-catenin-E-cadherin complex formation, and these residues are highly conserved in the classical cadherin family (Stappert and Kemler 1994). It has also been shown that phosphorylation of the E-cadherin cytoplasmic domain strengthens the interaction between the C-terminal residues and β-catenin (Stappert and Kemler 1994; Kinch, Clark et al. 1995). There is well documented role for β-catenin in the Wnt growth factor signaling pathway (Brennan and Brown 2004). β-catenin in its unactivated state and while not being sequestered on the E-cadherin binding domain, is targeted

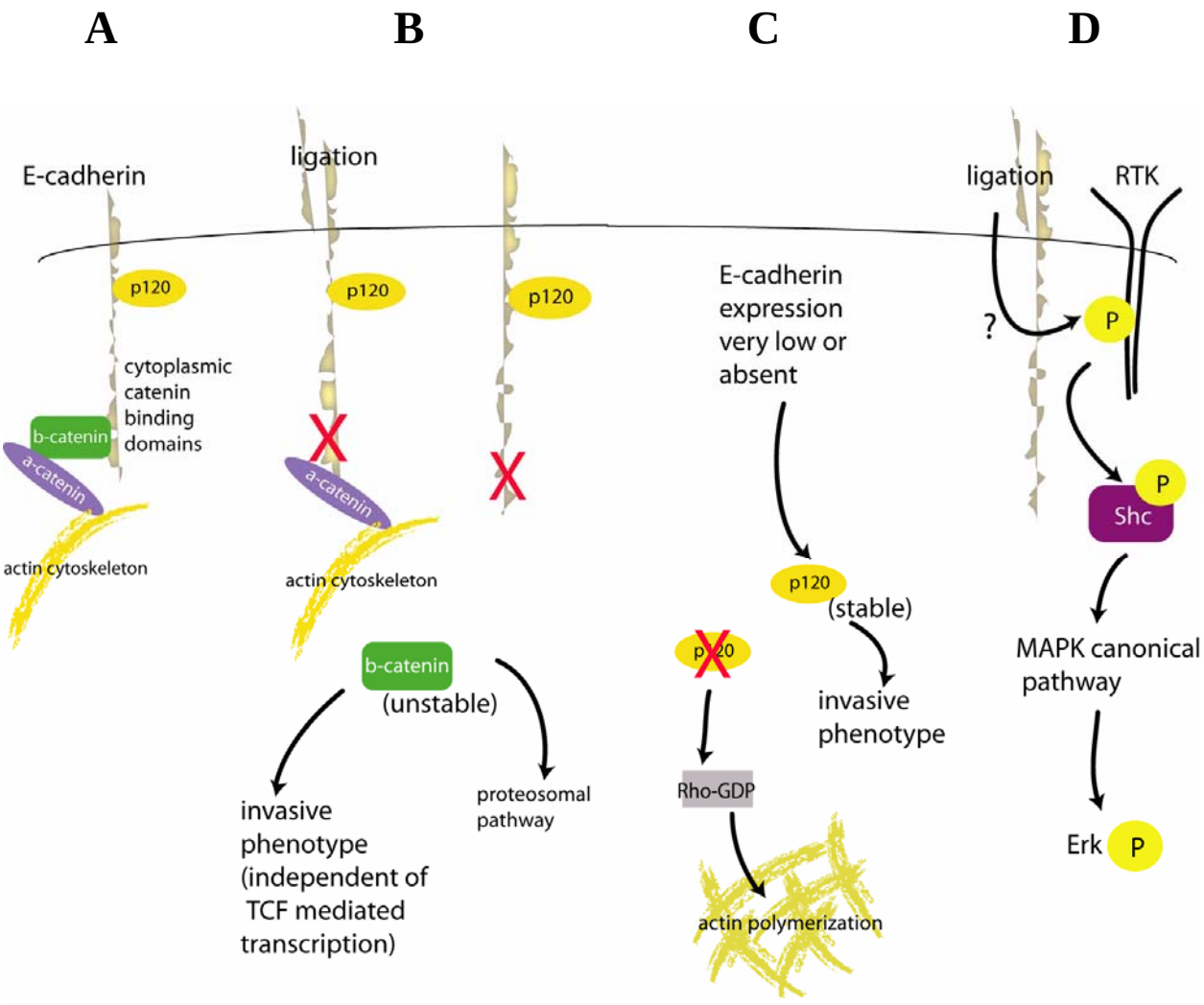

Figure 3. E-cadherin sequesters catenins and controls their signaling in addition to forming cell-cell adhesions. A) E-cadherin sequesters β- and p120- catenins on its intracellular catenin binding domains. In an untransformed cell, p120 is thought to stabilize E-cadherin at the surface, β-catenin is sequestered from forming a complex with axin and, in this location also functions as an adaptor protein for α-catenin, which in turn anchors E-cadherin to the cytoskeleton. B) In many carcinomas, E-cadherin is silenced by promoter methylation allowing β-catenin to translocate to the nucleus and p120 to promote an epithelial phenotype. However the mechanisms of how these catenins act and whether they act individually or in concert are not settled. C) E-cadherin dependent adhesion in itself is not a dominant stop mechanism to inhibit invasion. In studies where the β-catenin binding domain was deleted from the E-cadherin intracellular domain, but E-cadherin was still able to mediate adhesion through direct crosslinking with α-catenin and therefore interaction with the actin cytoskeleton, β-catenin was free to signal in the cell cytoplasm and led to an invasive phenotype (though this phenotype was independent of its TCF-mediated transcriptional activity). Therefore, cytoplasmic localization of β-catenin is thought to contribute to the mesenchymal nature of cells. D) In studies of cell that had low levels of E-cadherin and cytoplasmic localization of p120, tyrosine phosphorylation on p120's amino-terminal by the pro-oncogene Src was thought to contribute to modulate its

contribution to cell migration. When p120 is knocked down, the equilibrium shift to Rho-GDP promotes actin polymerization, stress fiber formation, a flattened morphology and less invasive phenotype. Therefore, cytoplasmic localization of p120 is thought to contribute to the mesenchymal nature of cells. These four scenarios provide data that show the critical role of E-cadherin as a signal modulation molecule by sequestering catenins, primarily p120 and α-catenin. In the absence of E-cadherin homotypic binding, this plaque is unstable and the catenins are now free to relocalize.

for degradation by the GSK3-APC-axin complex. In the presence of a Wnt activation signal, β-catenin acts as a transcriptional coactivator in complex with members of the Lef/Tcf family (Behrens, von Kries et al. 1996). This equilibrium is essential for stable cell-cell adhesion. In a transformed state, there is a large pool of free β-catenin that results in accumulation of the β-catenin-Tcf complex, which in turn activates transcription of a number of pro-mitogenic genes. In balanced equilibrium, most of the β-catenin is sequestered by E-cadherin and involved in stabilizing cell-cell adhesion and only a small fraction of free β-catenin exists in a soluble cytoplasmic pool.

Another catenin-protein capable of binding the E-cadherin cytoplasmic domain is p120ctn, although the binding of this catenin occurs at the membrane proximal domain (Gooding, Yap et al. 2004). Though the exact role for p120ctn has yet to be determined, p120ctn can affect E-cadherin's adhesiveness both negatively and positively (Ireton, Davis et al. 2002; Gumbiner 2005). p120ctn can also modulate actin modifying proteins such as RhoA, Rac, and Cdc42, which regulate actin clustering (Ireton, Davis et al. 2002), though there is no evidence that p120 links directly to the actin cytoskeleton. Modulating level of p120ctn have been found to influence levels of E-cadherin expression, though mechanisms of p120ctn adhesion modulation independent of E-cadherin protein levels have been found (Aono, Nakagawa et al. 1999; Thoreson, Anastasiadis et al. 2000; Davis, Ireton et al. 2003). Therefore, it is unclear as the most important role for p120ctn in the regulation of cadherin adhesive function.

1.4.1.3 E-cadherin presentation at the cell surface

Catenins control the regulation of adhesion by controlling the amount of E-cadherin available at the cell surface. To fully appreciate E-cadherin translocation on and off the membrane, the exocytic path of E-cadherin will be explored from the beginning. The trans-

Golgi network is the first place where delivery of E-cadherin is fated. The highly conserved dileucine sorting motif in the cytoplasmic tail of E-cadherin dictates its sorting to the basolateral surface (Miranda, Khromykh et al. 2001). β-catenin is associated with E-cadherin very early in the exocytic pathway, and is delivered to the membrane surface in a single complex (Chen, Stewart et al. 1999). In preconfluent or nonadherent cells, E-cadherin is trafficked constantly to and from the cell membrane (Bryant and Stow 2004). In cells making productive contacts, E-cadherin trafficking is minimized, and the protein is stably incorporated into adherens junctions. Only at the cell surface does p120ctn become associated with E-cadherin, and the turnover of E-cadherin is thought to be controlled by speed of p120ctn turnover from E-cadherin's membrane proximal domain (Davis, Ireton et al. 2003). Confluent cell populations have very small intracellular pools of E-cadherin (Bryant and Stow 2004). Further studies have revealed how contacts between cells are initiated, strengthened, compacted, and condensed (Adams, Chen et al. 1998). At early points of contact, E-cadherin assemble in puncta and eventually form plaques. These plaques colocalize with actin nucleating complexes, such as Arp2/3 (Kovacs, Goodwin et al. 2002; Verma, Shewan et al. 2004). Hence, the coordinated reorganization of E-cadherin and the actin cytoskeleton results in mature cell-cell contacts.

When cells become motile and break mature cell-cell contacts, E-cadherin is rapidly removed from the cell surface. The rapid turnover of E-cadherin at the cell surface is also linked to neoplastic events. In the context of most cancers, EGFR is intimately involved in the transformation of normal cells to their neoplastic counterparts. It has been repeatedly reported that increased receptor tyrosine kinase activity and loss of E-cadherin function are interconnected events associated with tumor progression. EGFR activation causes subsequent tyrosine phosphorylation of E-cadherin substrates, such as β-catenin, causing a loss of anchorage to the

cytoskeleton. E-cadherin is then internalized and either recycled or degraded (Pece and Gutkind 2002). Internalization occurs through clathrin mediated endocytosis, and ubiquitinated E-cadherin is directed to the early endosome, where it can either be trafficked to the late endosome or be recycled to the cell surface (Fujita, Krause et al. 2002).

1.4.1.4 Cadherin binding

Observations from early experiments found that different types of animal cells sort themselves from each other when artificially mixed (Moscona and Moscona 1952; Steinberg 1963; Steinberg and Gilbert 2004). This phenomenon was presumed to play a very large role during early ontogenesis. These results combined with previous experiments demonstrating that cells with different cadherins would segregate from one another *in vitro* (Takeichi, Atsumi et al. 1981; Nose, Nagafuchi et al. 1988) had yet to be reconciled. The first direct evidence that showed the presumable unique binding specificity of the cadherin subclasses was demonstrated by transfection of cells lacking endogenous cadherins with the different cadherin subclasses (Nose, Nagafuchi et al. 1988). The study was very straightforward: L-cells were transfected with cDNAs encoding two different subclasses of cadherin. When in spheroid culture, aggregates would form of cells only expressing similar cadherin subclass. They also found that cells without cadherin expression would not associate with those cells that expressed cadherins. They concluded that the homotypic aggregates overcame the affinities of the heterotypic aggregates, thus resulting in cell sorting.

Next came a study that built on the previous report to conclude that the amount of cadherin molecule presented on the cell surface was important for binding specificity (Friedlander, Mege et al. 1989). This study verified that cadherins allow cells to sort from one another, but it is a more complex process than merely subclass presentation on particular cell

type. The more complex view of cell sorting was verified many years in later in a modern study using six different cadherin subclasses. By this time, the Differential Adhesion Hypothesis had been well documented. The Differential Adhesion Hypothesis states that cell sorting rearrangements "result from the repeated exchange of weaker for stronger adhesions by intrinsically motile cells. The final configuration, approaching that of minimal interfacial free energy, is achieved when total cell-cell binding strength is maximized" (Steinberg and Roth 1964). The ultimate test of the Differential Adhesion Hypothesis was to determine the extent to which cadherin of different subclass could participate in heterocadherin bonding. The study determined that coaggregation of similar cadherin subclasses occurred when shear forces were low or absent, but at higher shear forces, subclasses intermixed (Duguay, Foty et al. 2003). The conclusion was that adhesions of the classic classical cadherin subclass must be of similar strength. The most recent study that verified the finding that the ability to sort cells that express different cadherin subclasses is determined by other mechanisms than simply the differential affinity of one subclass over the other (Niessen and Gumbiner 2002).

In total, these studies are important because they note the relative promiscuity of cadherin binding. Despite the many years of study, no one has shown a physiologically relevant context for the binding of cadherins on different types of cells. In Chapter 4, the notion is developed that transformed breast cells may be taking advantage of the cadherin ligation to establish functional contacts with hepatocytes.

1.4.2 E-cadherin in cancer progression

The invasive phenotype is gained upon disruption of tight epithelial cell-cell contacts, which results in release of invasive tumor cell from the primary site (Figure 1). E-cadherin has been

known as a tumor suppressor since 1991 (Frixen, Behrens et al. 1991; Sommers, Thompson et al. 1991), because the molecule's downregulation is a turning point from carcinoma *in situ* to a metastatic phenotype. It is also thought that the loss of E-cadherin protein expression, in addition to reducing coupling of epithelial cells, also solublizes β-catenin from its previously sequestered status. β-catenin can then travel to the nucleus to activate TCF-induced genes, such as c-myc, cyclin-D1, and matrilysin (Brennan and Brown 2004). Though mutation of the E-cadherin gene has been reported as the cause for E-cadherin silencing, the mutation rate of E-cadherin seems to be very low in a cancers (Kanai, Oda et al. 1994; Risinger, Berchuck et al. 1994). Epigenetic silencing due to methylation of the promoter region seems to be the most frequent cause of E-cadherin silencing in human cancers (Graff, Herman et al. 1995; Yoshiura, Kanai et al. 1995).

The first studies dealing with the transcriptional regulation of E-cadherin alluded to reduced activity of the promoter using a chloramphenicol acetyltransferase assay, which suggested negative regulation in a cis-regulatory manner (Behrens, Lowrick et al. 1991). The promoter region was also found not to be active in the TSU-pr1 prostate cancer cell line, but active in the PC-3 cell line (Bussemakers, Giroldi et al. 1994). The most likely cause of transcription repression from promoter silencing is the methylation of CpG islands proximal to the 5' regulatory regions of genes. Altered methylation profiles of cancer cells is widely reported (Liteplo, Frost et al. 1985; Das and Singal 2004; Issa 2004; Esteller 2005; Esteller 2005; Jair, Bachman et al. 2006). In fact, E-cadherin promoter methylation is common to a wide variety of cancers (Yoshiura, Kanai et al. 1995). It should be noted, however, that a methylation-free promoter does not guarantee protein expression on the cell surface. A variety of mechanisms including transcriptional repression (SLUG/SNAIL) and effector tyrosine kinase

activation (reviewed above) can prevent the positive functioning of E-cadherin (Maeda, Johnson et al. 2005) (Figure 3). It should further be noted that methylation is a reversible epigenetic mode of silencing, whether active or passive (Ramchandani, Bhattacharya et al. 1999; Lucifero, Mertineit et al. 2002; Das and Singal 2004; Turek-Plewa and Jagodzinski 2005). The demethylation of genes is a common event in the development-associated mesenchymal-epithelial transition. Chapter Four queries whether a MET-like demethylation is occurring in breast cancer cells in a hepatocyte microenvironment.

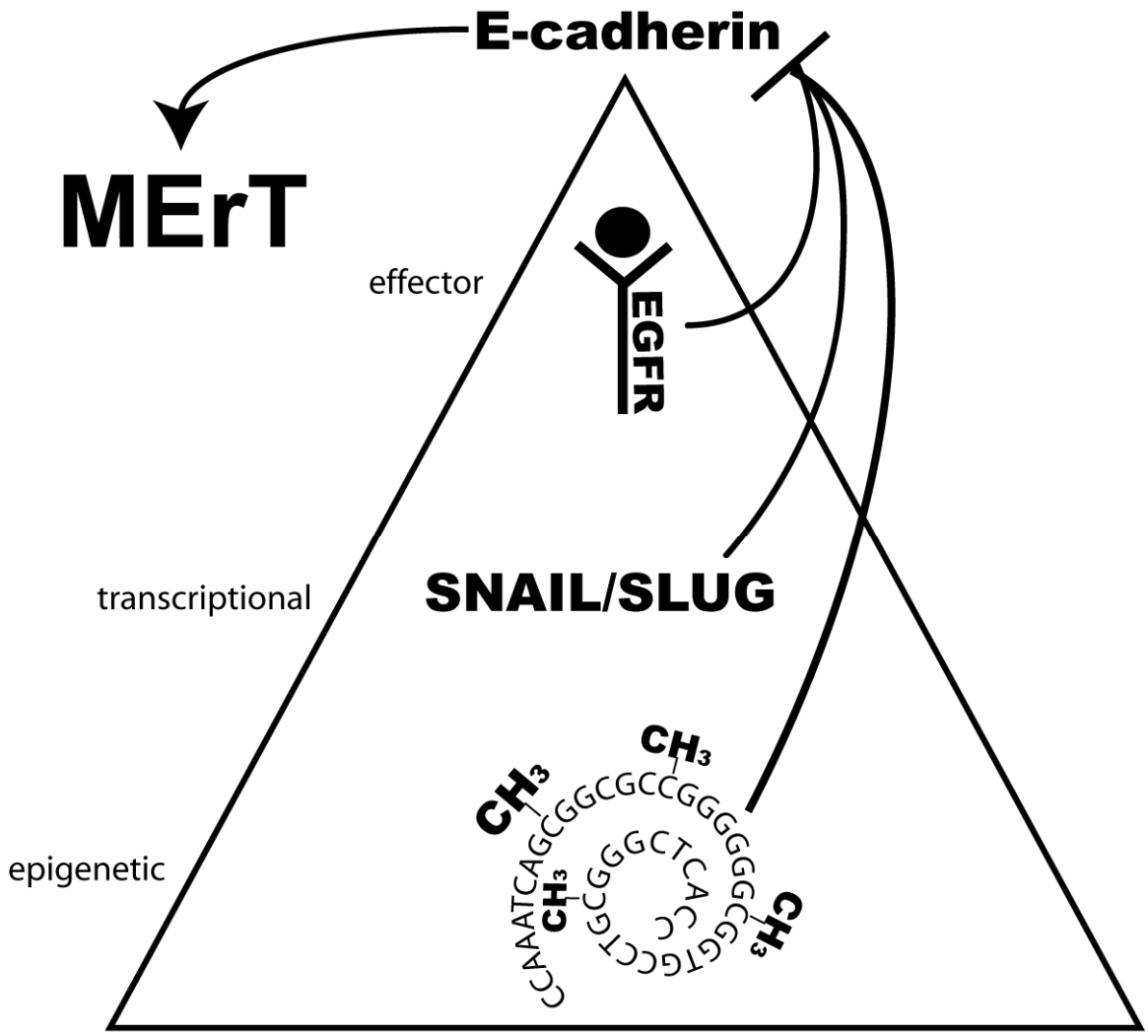

Figure 4. E-cadherin is regulated on multiple levels. E-cadherin is regulated on the epigenetic level by methylation of the promoter region, on the transcriptional level by the Snail/Slug transcription factors, and on the effector level by the receptor tyrosine kinase EGFR. Overcoming all of these repression mechanisms can result in a reversion of the EMT, or the so-called mesenchymal-epithelial reverting transition (MErT).

1.5 BREAST CANCER METASTATIC TO THE LIVER

The transition from *in situ* neoplastic growth in breast to metastatic disease is characterized by the ability of cells to invade the basement membrane into adjacent local tissue (Figure 1). Metastasis follows a series of sequential steps that allows for extravasation from the primary site, dissemination throughout the body, intravasation into ectopic tissues, and metastatic colonization of those tissues (Kassis, Lauffenburger et al. 2001). To successfully colonize an ectopic site, the cancer cells must strategically arrest at the appropriate site by either hematogenous or lymphatic routes of dissemination, elude immune surveillance, cope with hemostatic shear stress, reorient metabolic needs to the ectopic environment, and overcome other metastatic inefficiencies.

The distribution of metastases is strongly correlated to those soft tissue organs that circulate a large volume of lymph or blood. Appropriately, the liver is the second most common organ involved in metastatic disease after the lymph nodes. The dual blood supply and microvasculature significantly contribute to the formation of metastases. In fact, a focal liver lesion is more likely to represent a metastatic deposit than a primary malignancy (Bail, Foultier et al. 1994). Tumor emboli flowing through the blood stream are trapped by physical obstruction due to the narrowness of the venous branches or by other cells in the lumen of the hepatic environment, such as Kupffer cells. For extravasation, the normally fenestrated endothelium of the liver allows access to the underlying basement membrane-like layer. Implantation into the Space of Disse further allows access to diffusible nutrients from the hepatic capillaries. If a cancer cell progresses beyond arresting in the endothelium and begins to colonize the ectopic

site, the liver parenchymal hepatocytes are compressed, causing atrophy and clinically presenting as hepatolomegaly or acites. The process of colonization involves more than simply proliferation, but is a combination of dormancy, apoptosis, novel sets of cell-cell cell-tissue interactions, and altered responsiveness to paracrine factors (Steeg 2000).

Breast cancer metastasizes to the liver in 60.6% of all cases (O'Reilly, Richards et al. 1990); for non-nodal metastases, that frequency is second only to that of the lung. Patients presenting with breast cancer metastatic to the liver represent a poor median survival group with median survival rates of less than 6 months (Pentheroudakis, Fountzilas et al. 2005). Indeed, studies have suggested that colonization of the secondary-site is rate-limiting to the pathological nature of metastatic cancer (Steeg 2000). The most germane question remains whether metastatic colonization of the liver is a translational target.

2.0 PLCγ CONTRIBUTES TO METASTASIS OF *IN SITU*-OCCURING MAMMARY AND PROSTATE TUMORS

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2.1 ABSTRACT

PLCγ (phospholipase C-γ) has been implicated in tumor cell motility required for invasiveness and metastasis. Diminished tumor dissemination has been demonstrated in xenograft models, but studies in naturally-occurring tumors are lacking having been limited by the timing of the interventions. Therefore, we generated mice which express a doxycycline (DOX)-inducible dominant-negative fragment of PLCγ, PLCz; this approach avoids the *in utero* lethality caused by the absence of PLCγ. As we targeted two *de novo*-occurring carcinomas of the mammary (MMTV-driven polyoma middle T antigen model, PyVmT) and prostate (TRAMP model) glands, we limited expression to these epithelial cells by driving doxycycline transactivator from the prostatein C3 promoter. This avoids the confounding variable of potentially abrogating motility in stromal and endothelial cells. These mice developed normally in the presence of doxycycline, except for limited mammary development if treated before 6 weeks and immaturity of the prostate gland if treated before 2 weeks of age. DOX-mediated induction of PLCz from age 8 to 16 weeks in PyVmT mice decreased the number of lung metastases by >10 fold ($p < 0.06$) without a detectable effect on *in situ* tumor cell proliferation or tumor size. Lung metastases were also significantly decreased in the TRAMP model in which the mice expressed the PLCz fragment ($p < 0.05$). Doxycycline treatment itself had no effect on tumor size or metastasis in control mice, nor did it affect tumor dissemination in nontransgenic littermates. In conclusion, abrogation of the PLCγ signaling pathway can limit the metastatic potential of carcinomas.

2.2 INTRODUCTION

Cancer morbidity and mortality results mainly from invasion and dissemination of the primary tumor. This spread of the tumor requires cell proliferation, motility, and survival in an ectopic environment (Wells, Kassis et al. 2002; Fidler 2003; Wang, Goswami et al. 2005). Inhibition of any one of these steps would stop tumor dissemination; however, therapies aimed at cell proliferation and survival have been limited by toxicity as these properties are common to normal homeostatic mechanisms. On the other hand, the induced motility noted during tumor invasion and metastasis appears to be a re-iteration of that noted during organogenesis and regenerative wound repair (Wells 2000), and not molecularly analogous to the motility extant during homeostasis. This offers a novel avenue for intervention against tumor spread that could have minimal toxicity.

Key molecular switches have been identified during induced cell migration of tumor cells (Wells 2000; Ridley, Schwartz et al. 2003). These are downstream of growth factor receptors activated by autocrine and paracrine signals. These switches include phospholipase C-γ (PLCγ), activation of which lies upstream of cytoskeletal reorganization. PLCγ appears to be at a convergence point of various signaling pathways, and as such offers an opportunity for differential abrogation of multiple yet distinct signaling events (Kassis, Moellinger et al. 1999; Jones, Peak et al. 2005). In previous studies, we tested whether PLCγ signaling contributes to tumor invasion indirectly through an examination of EGF receptor-mediated invasiveness. We engineered EGFR constructs that were fully mitogenic, but either activated PLCγ (WT) or did not (c'973) in DU-145 androgen-independent human prostate cancer cells. The cells expressing the WT EGFR were significantly more invasive through Matrigel and xenograft models (Xie,

Turner et al. 1995; Turner, Epps-Fung et al. 1997). When this cascade was intervened at the level of PLCγ using pharmacological inhibitors (U73122) or a dominant negative fragment (PLCz), tumor invasiveness and dissemination was inhibited in xenograft models, but tumor growth was unaffected. Subsequent studies confirmed the applicability of PLCγ signaling of tumor cell invasiveness in other prostate tumor cells as well as those derived from breast, bladder and oral mucosa (Kassis, Moellinger et al. 1999; Price, Tiganis et al. 1999; Dittman, Husemann et al. 2002; Thomas, Coppelli et al. 2002).

Although these published reports provide a 'proof-of-concept' that PLCγ signaling can be targeted to limit carcinoma invasion (Mouneimne, Soon et al. 2004), *in vivo* experiments have been lacking to support this clinically relevant hypothesis that such inhibition limits tumor metastasis. Therefore, we have developed a double-transgenic model of oncogene-induced mammary or prostate carcinoma in which PLCγ can be conditionally regulated, thus avoiding tumor suppressive effects during early phases of transformation. By limiting the expression of a dominant-negative PLCγ fragment (PLCz) to the epithelial cells of the mammary and prostate glands (Guy, Webster et al. 1992; Tehranian, Morris et al. 1996), we also avoid any questions as to limiting stromal responsiveness or angiogenesis needed for metastases to become macroscopic. We tested the hypothesis in spontaneous tumor models of mammary and prostate carcinomas. In these models, induction of the dominant negative fragment PLCz clearly decreased the number of metastases to the lung. These results not only support tumor cell motility as a rate-limiting step in metastasis, but also are the first to limit dissemination of in situ de novo-occurring tumors by inhibiting PLCγ mediated motility.

2.3 RESULTS

2.3.1 Expression of the DOX-inducible PLCz transgene is restricted to mammary and prostate epithelia

A constitutively active PLCz transgene under the control of the C3(1) prostatein promoter (Guy, Webster et al. 1992; Tehranian, Morris et al. 1996) was constructed. The rat C3(1) prostatein promoter drives the expression of the reverse tetracycline transactivator (rTetR-VP16) which binds to TRE (tetracycline response element) and activates the transcription of PLCz in the presence of DOX (Figure 5A). We chose to utilize expression of a dominant-negative rather than an inducible excision/deletion situation for two reasons, one being that low level leakage of expression would lead to inadvertent deletion but not competition, and the second being the ability to revert to a PLCγ-positive state by withdrawal of DOX. The predicted 800bp PCR product was amplified from positive PLCz transgene founders (Figure 5B).

The expression of the transgene was confirmed by immunoblot analysis of protein from tissues of mice using a polyclonal anti-PLCγ antiserum. We treated mice between 4 and 8 weeks of age with DOX to determine if PLCz expression was inducible, and in which tissues it would be expressed. After one week of DOX treatment, PLCz was found only in the prostate and mammary glands of transgenic animals and not in the wild-type littermates. The 51kDa PLCz band was not detected in mice in the absence of DOX treatment, nor was it found in nontransgenic mice in the presence of DOX (Figure 6A). We did not detect PLCz in the ventral prostate and seminal vesicles, consistent with reports of C3(1) being expressed only in the dorsal prostate (Figure 6B) (Claessens, Celis et al. 1989; Maroulakou, Anver et al. 1994; Tehranian, Morris et al. 1996).

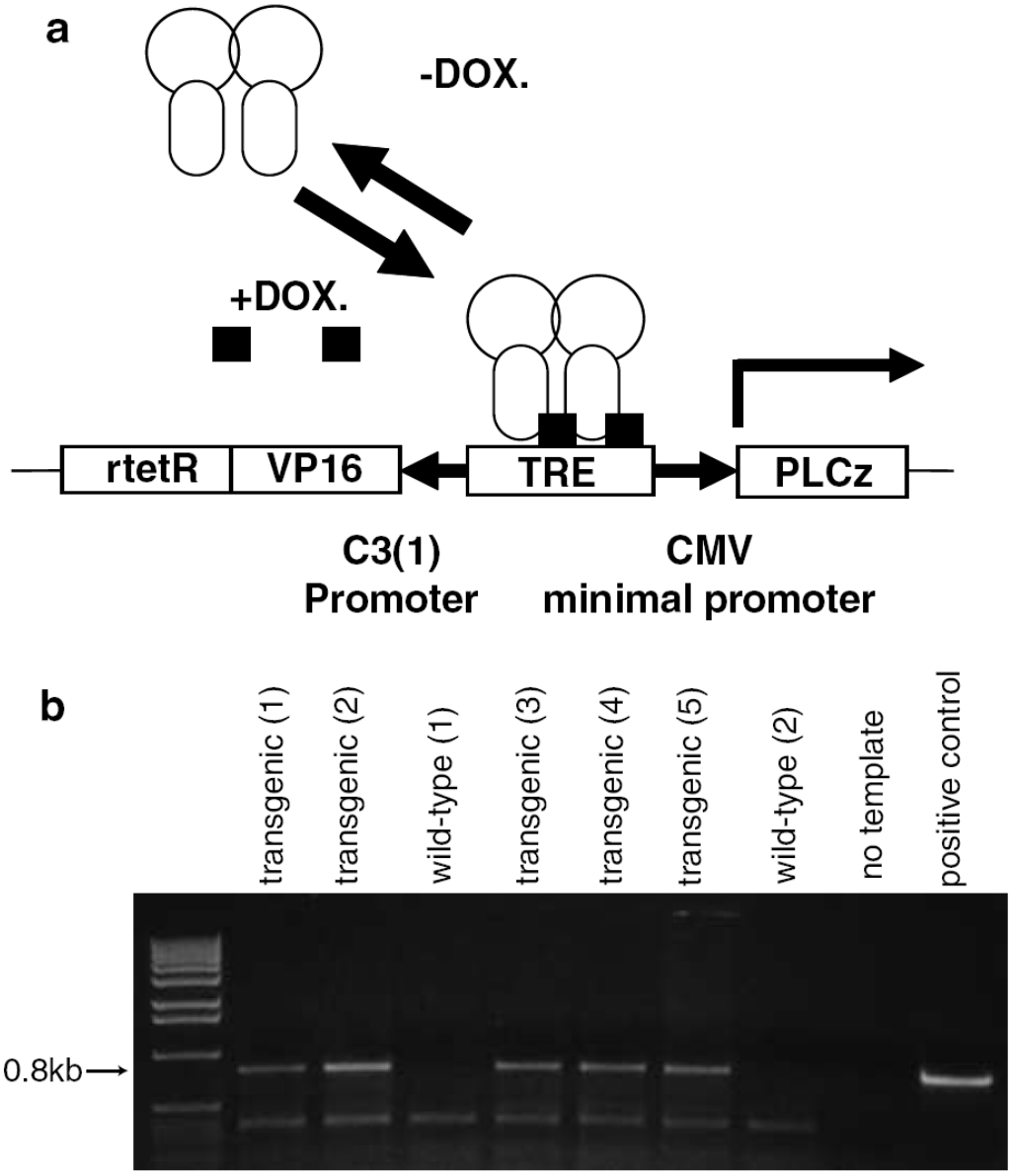

Figure 5. Generation of PLCz-expressing mice. The pC3(1)tPLCz bicistronic construct (a) contains the reverse tet repressor driven by the breast and prostate specific promoter, C3(1), that binds to the TRE (tetracycline response element) in the presence of doxycycline (DOX). VP-16 transcriptional activation domain of herpes simplex virus enhances expression of the downstream PLCz. Thus, PLCz expression is expressed only on DOX induction in a tissue-specific manner. To detect transmission of the pC3(1)tPLCz construct, 1 mg of genomic DNA from a tail of each transgenic or wild-type mouse was used to perform the PCR analysis. 10 pg of transgene fragment from pTGC3(1) was used as template for the positive control and there is no template with the negative control. (b) The arrow head indicates the 0.8 kb PCR products from the transgene.

The persistent expression of PLCz depended on continued treatment with DOX, as removal of the DOX-laced drinking water resulted in dramatically reduced PLCz expression after two weeks (Figure 6C). For both the induction and the reversion experiments, we probed the protein rather than mRNA, as protein processing and stability might be extended and thus alter the kinetics of biological response. As abrogation of signaling by dominant-negative constructs requires excess expression, we confirmed that PLCz expression was significantly higher than that of endogenous PLCγ, seen at low levels at 130kDa (Figure 6A). These results strongly indicate that the C3(1) promoter is able to induce the transgene expression in a tissue specific and regulatable manner in the mammary and prostate gland of transgenic mice.

2.3.2 Induction of PLCz inhibits invasiveness of the primary tumor and metastatic tumor formation

To determine whether a PLCγ-mediated motility-associated signaling pathway is associated with tumor cell dissemination, the pC3(1)tPLCz transgene founders were crossed with MMTV-PyVmT and TRAMP mice to generate double transgenic MMTV-PyVmT-PLCz (PyVmT-PLCz) mice and TRAMP-PLCz mice. MMTV-PymT transgenic mice develop mammary tumors with an average latency of 53 days and form lung metastases with 100% penetrance by 100 days of age (Muraoka, Dumont et al. 2002). TRAMP transgenic mice develop prostate tumors with an average latency of 84 days and form lung metastases with 95% penetrance by 24 weeks of age (Gingrich, Barrios et al. 1997).

In the absence of DOX, tumors arose with similar latencies to MMTV-PyVmT and TRAMP mice. Because the average tumor latency for the PyV-PLCz mice was ~8 weeks,

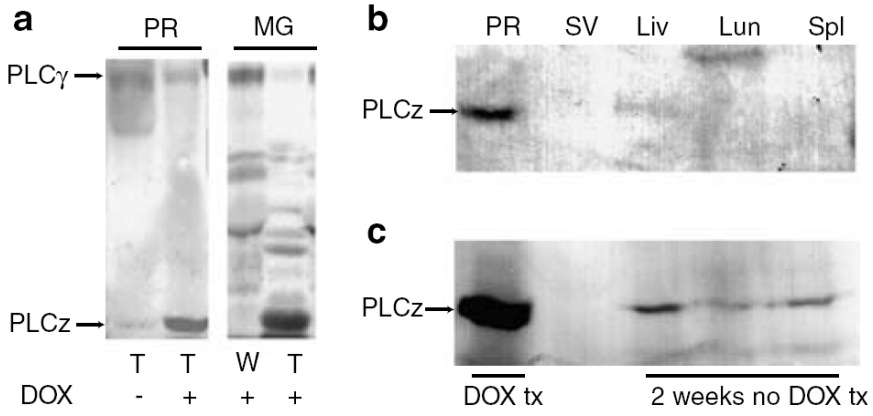

Figure 6. Inducible expression of PLCz in tissues from male and female mice. Proteins were analysed on 10% SDS–PAGE and immunoblotted with a rabbit polyclonal antibody that recognizes the Z region of PLCg. DOX was added to the drinking water to induce PLCz expression. One week after DOX addition, mice were killing and tissues were processed to get protein lysates. A selection of tissues and mice are shown to present evidence that PLCz expression is dependent on (a) transgene presence, (a) DOX induction (b) steroid-responsive tissues and (c) continued presence of DOX. (a) PLCz is induced by DOX in the prostate (PR) and mammary glands (MG) of transgenic (T) mice and is not expressed in PLCz- (W) mice. (b) PLCz is expressed in the PG of male T mouse in a tissue-specific fashion and is not expressed in the seminal vesicles (SV), liver (Liv), lungs (lun) or spleen (Spl). Nontransgenic mice were negative in all tissues. (c) Three different PLCz-transgenic female mice were treated with DOX for 1 week and then switched to normal drinking water and sacrificed 2 weeks later. The positive control was killed after the DOX treatment. The inguinal MG was dissected and protein lysates analysed. (c) is overexposed compared to (a) and (b) to detect lowlevel persistence of PLCz.

A	TRAMP/PLCz mice invasiveness of primary tumors	PLCz−/DOX tx	PLCz+/no DOX	PLCz+/DOX tx
	PLCz−/no DOX	0.48	0.44	0.02
B	TRAMP/PLCz mice extent of lung metastases	PLCz−/DOX tx	PLCz+/no DOX	PLCz+/DOX tx
	PLCz−/no DOX	0.12	0.14	0.03
C	PyVmT/PLCz mice extent of lung metastases	PLCz−/DOX tx	PLCz+/no DOX	PLCz+/DOX tx
	PLCz−/no DOX	0.48	0.47	0.06

Figure 7 Fisher's exact test was used to generate P-values to determine whether induction of PLCz had a significant influence on the extent of lung metastases. A, TRAMP and TRAMP/PLCz mice were scored for invasiveness of the primary prostate tumors. The TRAMP mice expressing the PLCz trangene had a significant reduction in the invasiveness of the primary tumor ($P<0.02$). B, TRAMP and TRAMP/PLCz mice were scored for the extent of the lung metastases. The TRAMP mice expressing the PLCz transgene had a significant decrease in the extent of lung metastasis ($P<0.06$). C, PyVmT and PyVmT/PLCz mice were scored as in (B). The PyVmT mice expressing the PLCz transgene had a significant decrease in the extent of lung metastasis ($P<0.03$).

DOX was administered no earlier than 9 weeks to allow both the mammary gland to form and primary tumor formation to occur. In TRAMP mice, tumor formation began soon after birth, so DOX was administered beginning immediately after weaning. The majority of DOX treated PyVmT-PLCz and TRAMP-PLCz mice exhibited signs of high carcinoma load-related distress at 16 weeks and 24 weeks, respectively, and were sacrificed.

The primary tumors in the prostate of the TRAMP-PLCz mice were scored for invasiveness after 24 weeks of DOX treatment. The TRAMP-PLCz mice displayed a significantly decreased invasiveness score of the tumor into the prostate parenchyma when compared with the untreated mice, while the no-insert controls and untreated controls remained indistinguishable (Table 1A). When comparing the invasiveness score of the DOX-treated PLCz-positive mice with the DOX-treated transgene-absent control mice, the DOX-treated PLCz positive mice displayed a significantly decreased invasvieness score of the tumor into the prostate parenchyma that was significantly lower than the DOX-treated transgene-absent mice ($p < 0.02$).

Lung metastases were counted at 24 weeks of age in the TRAMP mice and 16 weeks of age in the PyVmT-PLCz mice, based on literature-reported development and pilot experiments. In the TRAMP-PLCz mice, after less than 24 weeks of DOX treatment, the TRAMP-PLCz mice displayed a significantly decreased number of lung metastases when compared with TRAMP-PLCz mice without DOX treatment, while the controls, as above, remained indistinguishable (Table 1B).

To determine if this reduced metastasis also held in other tumors, we checked the mammary tumor spontaneously arising in the PyVmT mice. After 8 weeks of DOX treatment, the PyVmT-PLCz mice displayed a significantly decreased number of lung metastases when

compared with PyVmT-PLCz mice without DOX treatment ($p < 0.02$), while the "no insert" controls and positive insert/no treatment mice remained statistically indistinguishable (Table 1C; Figure 7, Figure 8A). Primary mammary tumors from DOX-treated PyVmT and PyVmT-PLCz mice had no differences in tumor volume due to DOX induction (Figure 8B). The number of lung metastases in PyVmT-PLCz mice not induced to express PLCz by DOX was not different from the number observed in MMTV-PyVmT mice.

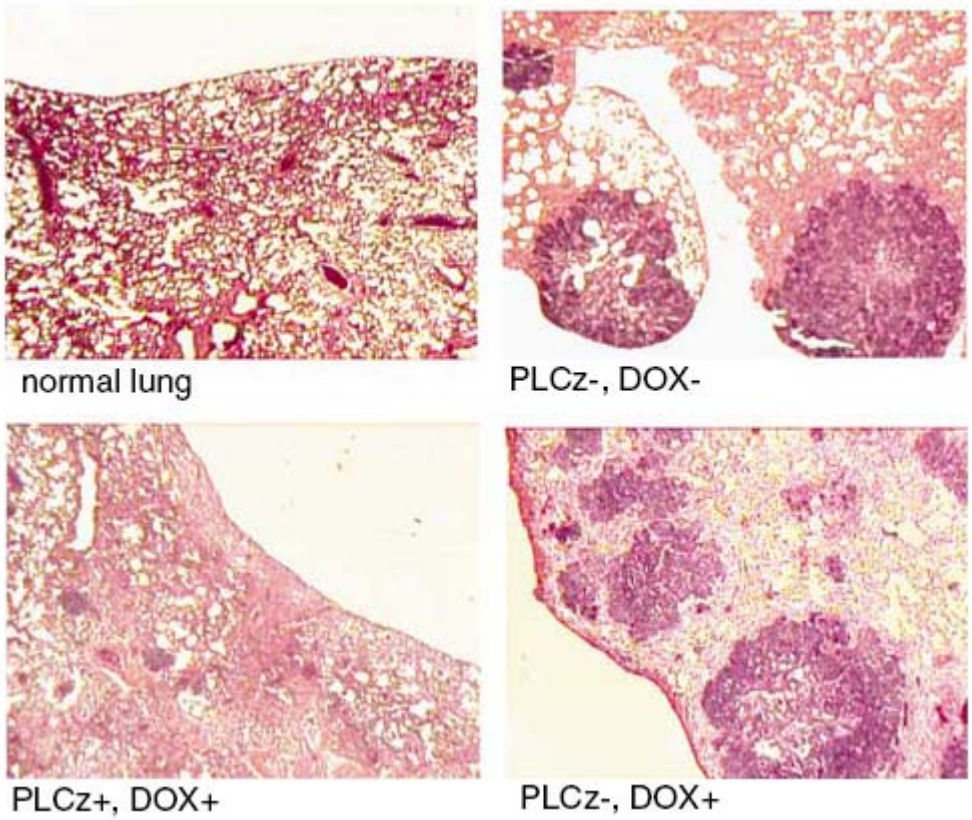

Figure 8. Metastatic tumors in the lungs of MMTV-PyV mice expressing PLCz. H&E sections of dissected lung tissue from representative mice. Normal mouse lung tissue (top left). Lung sections of transgenic PyVmT without the PLCz transgene show large metastatic tumors throughout the lung parenchyma that crowd alveolar septa (top right). PyVmT-PLCz mice whose PLCz expression is induced with DOX treatment markedly diminishes the number of tumors in the lung parenchyma (bottom left). DOXtreatment has no effect on metastatic lung deposits in PyVmT mice (bottom right).

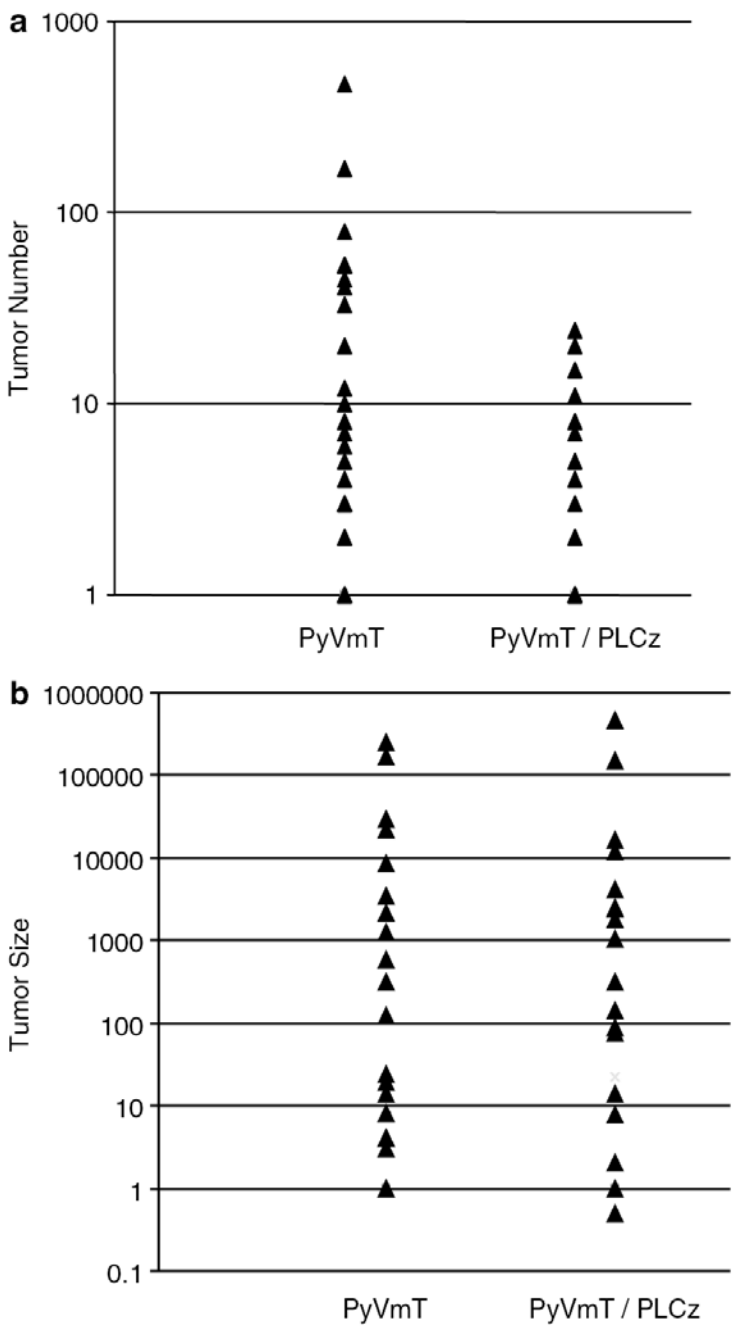

Figure 9. The number of metastatic lung tumors is drastically reduced in PLCz+ mice, while the tumor size remains unchanged. The number of tumors were counted and sized according to the Materials and methods section. (a) The number of metastatic lung tumors in PyVmT/PLCz+ mice is fewer compared to the number of metastatic tumors in PyVmT not expressing the PLCz transgene. (b) The PLCz transgene does not affect the size of metastatic lung tumors in PyVmT/PLCz mice when compared to the PyVmT not expressing the transgene.

2.4 DISCUSSION

Tumor invasion, both into adnexia and as the first step in dissemination, has been proposed to be a consequence of dysregulated cell motility (Kassis, Lauffenburger et al. 2001). The dissemination of cells from the primary tumor to metastatic sites is limiting for the tumor's progression. Although many of the molecules that regulate or effect cell motility have been investigated (Ridley, Schwartz et al. 2003), the dysregulation of those same molecular events has yet to be properly characterized in the context of the dissemination and metastatic seeding of de-novo arising cancers. The models examined herein, are of spontaneous metastasis of in situ carcinomas that arise secondary to oncogenic activation. We queried whether the dissemination of such tumors can be limited by blocking a key cell motility pathway, that of PLCγ.

PLCγ signaling is an enticing target to dissect the contributions of induced cell motility to tumor progression, as it distinguishes the cell motility response from that of proliferation (Chen, Xie et al. 1996). Obviously, any intervention that also prevents proliferation would be falsely scored as metastasis suppressing as metastasis requires subsequent proliferation. Despite the extensive investigations on motility in culture systems and confirmation in xenograft models, experimental evidence is lacking as to the relative or specific contributions of motility on the metastatic dissemination of *de novo*-occuring cancer cells. This is due to the pleiotropic nature of the signals during tumor progression, such as the autocrine stimulatory loops activating growth factor receptors that drive both motility and proliferation. For instance, current molecularly-targeted therapies inhibit signaling from the EGFR family members HER2 (erbB2) and HER1 (EGFR, erbB1). Both of these receptors initiate and sustain proliferation and migration in breast and prostate carcinoma cells. Thus, while these inhibitors limit dissemination, whether growth factor-enhanced motility underlies metastasis remains unproven. Abrogation of

PLCγ signaling by pharmacological or genetic means eliminates motility but not mitogenesis induced by EGFR, HER2 (Chen, Gupta et al. 1994; Chen, Xie et al. 1994) and other growth factor receptors (Reddy, Wells et al. 1996). By targeting this convergent downstream effector molecule, we defined a role for growth factor-induced cell motility in the dissemination of primary breast and prostate cancer *in vivo*.

In this study, we conditionally expressed a dominant-negative fragment of PLCγ in the mammary and prostate epithelium of TRAMP and MMTV-PyVmT. This conditional, post-natal expression was required as constitutive ablation of PLCγ is embryonic lethal (Ji, Ermini et al. 1998). Furthermore, by limiting expression to the target epithelial cells, we avoid issues of altering the microenvironment of the primary and metastatic tumor sites, as well as affecting tumor-associated angiogenesis (Bodnar, Yates et al. 2006). We chose a reversible, inducible system rather than conditional gene deletion (i.e. Cre-Lox) for the flexibility provided by the ability to revert to a functional phenotype (see Supplemental data).

Expression of the dominant-negative PLCz construct during prenatal and immediate post-natal periods retarded organogenesis specific to the prostate and mammary epithelium. This was reversible upon DOX withdrawal, validating our strategy and demonstrating that low level leakiness of the tetracycline-on system was not consequential as a dominant-negative is only effective when in stoichiometric excess.

To study the role of PLCγ signaling in metastasis, we allowed organogenesis and maturation to proceed, and spontaneous tumorigenesis to be initiated by the transgenic oncogenes. Forcing expression of PLCz limited the metastases from both the breast and prostate carcinomas; the lungs were chosen for ease and reproducibility of quantitation, though similarly decreased metastasis was noted in other internal organs (data not shown). This decrease in

number and size of metastases was not due to slower tumor growth as the primary tumor sizes were unaffected by PLCγ expression. These data confirm that cancer cell invasiveness is a determining factor of tumor progression *in vivo*.

These studies confirm that PLCγ function is necessary for tumor cell dissemination in both breast and prostate cancers. That tumorigenesis was incompletely abrogated is not unexpected, but may be due to several reasons. First, the expression of PLCz does not completely eliminate EGF-induced PIP_2 hydrolysis *in vitro* (Chen, Gupta et al. 1994) and may be similarly incomplete in *in vivo* systems. Second, some low level EGF-induced motility is noted in PLCγ-1 devoid cells (Ji, Ermini et al. 1998) this is due to other pathways that regulate motility-associated processes other than protrusion, such as ERK MAP kinase/m-calpain (Chen, Xie et al. 1996; Glading, Chang et al. 2000) and PKCd/MLC (Iwabu, Smith et al. 2004), as well some upregulation of PLCγ-2 expression. It should also be noted that while the targeted inhibition of PLCγ is being discussed as a downstream effector required for EGFR-mediated motility, we cannot be certain that the extracellular trigger for breast and prostate cancer dissemination are the EGFR ligands. EGFR signaling is likely to be the primary trigger as it has been implicated in the transformation, mitogenic and motogenic properties of tumor cells (Carpenter 2000); however, it is possible that other growth factors are signaling via PLCγ (Carpenter and Ji 1999; Jones, Peak et al. 2005). As PLCγ seems to serve as a point of convergence for PDGF, HGF, and IGF-1 in addition to EGF in signaling motility (Bornfeldt, Raines et al. 1994; Kundra, Escobedo et al. 1994), expression of PLCz would be expected to diminish cell motility signaled by all of these factors. Furthermore, PLCγ has been linked to integrin-related changes that result in cell motility in the context of invading cancer cells (Jones, Peak et al. 2005) The progression of invasive cancers to the morbidity and mortality of

metastasis encompasses a series of highly coordinated events, though the literature offers a compelling line of evidence for the general role of PLCγ throughout the metastatic process. We have demonstrated here that signaling through PLCγ is a necessary and potentially limiting step that may be targeted in a rational intervention.

2.5 MATERIALS AND METHODS

All animal experimentations were approved by the Institutional Animal Care and Use Committees respectful to where the experiments occurred, including the University of Pittsburgh, Pittsburgh VAMC, and the University of Alabama at Birmingham.

2.5.1 Generation of double transgenic TRAMP and MMTV-PyVmT with a DOX-inducible PLCz transgene

The reverse tetracycline plasmids (tet-on) PUHD13-6 and PUHD172-1neo were kindly provided by Dr. Hermann Bujard (Heidelberg) (Gossen, Freundlieb et al. 1995). The CMV promoter driving the reverse tet repressor (rTetR) of PHUD172-1neo was replaced with rat C3(1) prostatein promoter (kindly provided by Dr. Peter Barry, UC Davis (Tehranian, Morris et al. 1996)). The dominant-negative PLCγ fragment, PLCz, ((Homma and Takenawa 1992; Chen, Gupta et al. 1994)) was placed downstream of the TRE (tetracycline response element) with the TATA box from CMV minimal promoter of PUHD13-6. These two DNA fragments containing C3(1)rtetR and TRE-TATA-PLCz were ligated into the pBluescript to create a single bicistronic

construct; this was done to increase expression levels (Hofmann, Nolan et al. 1996) and simplify breeding schemes by physically linking the two halves of the system. The resulting plasmid, pC3(1)tPLCz, was used in microinjection (Fig.1A)

pC3(1)tPLCz was digested with NotI and PstI and electrophoresed through a 0.7% low melting point agarose gel and purified using Quiax DNA binding beads. Purified DNA was injected into one cell FBV zygotes by the University of Alabama at Birmingham transgenic polyomafacility (Carl Pinkert, Director). Following injection, viable eggs were transferred to the oviducts of pseudopregenant female mice. To identify positives, DNA was extracted from 1cm section of tails using Quiamp DNA extraction kit (Quiagen). DNA was resuspended with 200 μl of TE (10 mM Tris, 1 mM EDTA pH 8.0). Screening of transgenic mice was by polymerase chain reactions with one pair of oligonucleotides annealed to the PLCz. The PCR was performed on a DNA thermocycler (Ericomp) using following program: 94^{o}C for 1 minutes, 56^{o}C for 1 minute, and 72^{o}C for 1 minute. PCR product was analyzed by electrophoresis on a 1% agarose gel (Fig. 1B).

The PLCz mice were mated with TRAMP mice, kindly provided by Norman Greenburg (Gingrich, Barrios et al. 1996), or MMTV-PyV mice, the gift of Dr. WJ Muller (Webster, Martin-Soudant et al. 1998). Male PLCz mice were mated with female TRAMP mice or female PLCz were mated with male MMTV-PyV mice. The F1 crosses were used for all experiments. The mice lacking the PLCz transgene presented tumors at the expected rates and times.

2.5.2 PLCz expression

To induce expression of the PLCz transgene, doxycycline was also administered orally in drinking water (2 mg/ml) for varying times before sacrificing mice. Various tissues from wild type and transgenic mice were sonicated in PBS with protease inhibitors (10 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM phenylmethylsulfhnyl fluoride). The concentration of each sample was determined by measuring the absorbance at 595 nm using Biorad Bradford assay. 50 μg of lysates were subjected to 10% SDS-PAGE, and the resolved proteins were transferred electrophoretically to PVDF membrane. After shaking the blot in TBST with 5% non-fat dry milk (W/V) for 1 hour and blot was incubated with the anti-rabbit PLCγ antibody (Santa Cruz) at 4^{O}C for overnight. After incubation with primary antibody, the blot was washed in TBST for 15 minutes twice and incubated with secondary antibody (anti rabbit IgG, 2500:1 dilution, Promega). Protein bands were visualized with NBT and BCIP (Promega).

2.5.3 Histologic and immunohistochemical assessments of mammary and prostate glands

For histology, part of mammary gland and dorsal prostate gland were fixed in 10% neutral buffered formalin, processed for paraffin sectioning and stained with hematoxylin and eosin. Paraffin sections were deparaffinized, hydrated, pretreated with 2 N HCl for 20 minutes at 37^{O}C and exposed to 0.01% trypsin at 37^{O}C for 3 minutes, followed by staining using a mouse monoclonal anti-PCNA antibody and biotinylated secondary antibody. 3,3' Diaminobenzidine tetrahydrochloride was used as chromogen. Sections were also tested for apoptosis using the Apotag kit (Oncor). After hydration, sections were treated with proteinase K (20 μg/ml) and the

endogenous hydrogen peroxide was quenched by incubating with 2% hydrogen peroxide in PBS. Terminal deoxytransferase was added to sections followed by the incubation with anti-digioxigenin antibody conjugated with HRP. Color detection was accomplished with DAB as Chromogen.

The primary and metastatic tumors were scored for invasion of the parenchyma and were evaluated using a method modified from Eneroth (Jakobsson, Eneroth et al. 1973). The tumor invasion modes were scored into four histopathologic malignancy grade, given as (score), gross description: (0.5) micrometastasis; (1) well defined border; (2) cords/Indian files, less well defined border; (3) groups of cells, no distinct border; (4) diffuse growth.

2.5.4 Statistical analyses

Data were categorized into PLC-/No DOX, PLC+/No DOX, PLC-/DOX and PLCz+/DOX groups. Differences in continuous parameters (size, number) were evaluated by Wilcoxon-Rank Sum Test using the SAS statistical software package (Cary, NC). Fisher's exact test was used to evaluate the significance of differences in the parameters. All Ps are represented by one-tailed tests (Kassis, Moellinger et al. 1999) and were deemed statistically significant at $p < 0.10$; though levels of statistical differences are provided.

2.6 ACKNOWLEDGEMENTS

We thank Norman Greenberg for the gift of the TRAMP mice and Dr. William Muller for the gift of the MMTV-PyV mice. Dr. Peter Barry kindly provided the prostatein promoter construct.

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3.0 EXPRESSION OF THE CYTOSOLIC TAIL OF E-CADHERIN PARTIALLY REVERTS THE EPITHELIAL TO MESENCHYMAL TRANSITION IN AGGRESSIVE BREAST CARCINOMA CELLS

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3.1 ABSTRACT

Coculturing of the aggressive MDA-231 breast carcinoma cell and primary breast carcinoma cells with normal parenchymal cells of a metastatic target organ, the liver, resulted in renewed transcription of E-cadherin secondary to specific loss of promoter methylation. Despite the re-expression of E-cadherin, the MDA-231 cells do not fully revert to an epithelial cohesive-cluster morphology. We speculated that the MDA-231 cells that re-expressed E-cadherin may have an intermediate phenotype, wherein the cells benefit from E-cadherin-dependent canonical pathway activation, but also retain characteristics of invasive cancer cells. We tested this hypothesis by using a dominant negative E-cadherin, in which an intact intracellular domain is coupled to an extracellular domain from the class I major histocompatibility complex antigen (H-2kd). This dominant negative construct sequesters the E-cadherin plaque-associated molecules beta-catenin and p120, which, in the absence of E-cadherin, are conventionally thought to propagate oncogenic signals. The cells demonstrated a level of invasiveness *in vitro* intermediate between MDA-231 cells and non-aggressive MCF7 cells, and similar to that of partially-reverted MDA-231 cells cocultured with hepatocytes. These data suggest a model in which breast cancer cells in the metastatic environment partially downregulate catenin-driven oncogenic signaling by a phenotypic shift in the E-cadherin expression equilibrium. In this manner, the cells remain in a partial EMT or reversion thereof, whereby they are able to readily shift between epithelial-like dormancy and aggressive metastatic growth as determine by other micro-environmental cues.

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, Morrow et al. 2007). The majority of these cancers originate from the epithelial cells lining the mammary ducts. Transformation of normal ductal epithelial cells into their metastatic counterparts occurs in a series of sequential steps, during which the cells acquire a more amoeboidal phenotype, become motile, disseminate, and colonize distant sites of the body. The initial stages of this transformation are described as an epithelial-to-mesenchymal transition (EMT) (Lee, Dedhar et al. 2006). The EMT, which includes a loss of E-cadherin as a precursor to a more motile and invasive phenotype, is necessary to break away from the primary mass and either disseminate by hematagenous or lymphatic routes. Upon colonization of a distant ectopic organ, it is hypothesized that cancer cells undergo a reprogramming to a more epithelial phenotype, described as a mesenchymal-to-epithelelial reverting transition (MErT) (Yates, Shepard et al. 2007). MErT is hypothesized to play a role in the ability of cancer cells to colonize an ectopic locale and counter many of the metastatic inefficiencies and pharmaceutical challenges that affront the cancer cells.

A hallmark measure of malignant tumor cells is invasiveness. Invasiveness is the result of disconnect of E-cadherin-mediated cell-cell adhesions that limit cell dispersion and establish apical-basal polarity with segregation of signals. This phenomenon has been shown repeatedly in simulations of the cadherin switch phenomena in which E-cadherin presence or absence dictates the degree of epithelial-like phenotype in a cell. This has been demonstrated by expression of a dominant negative Epithelial-cadherin (H-2kd-Ecad), formed by fusion of an intact intracellular domain to the extracellular domain from the class I major histocompatibility complex antigen (H-2kd), in non-metastatic epithelial-like MCF-7 breast cancer cells causing

them to become more invasive (Vizirianakis, Chen et al. 2002). Thus, invasiveness can be considered an inverse measure of epithelial quality of a carcinoma cell (Heimann and Hellman 2000), and can be used to determine how restoration of the appropriate equilibriums restores the epithelial phenotype.

E-cadherin not only established cell-cell connections but alters the localization and signaling capacity of multifunctional signaling molecules, the catenins in particular. One molecular equilibrium necessary for the epithlelial phenotype is the appropriate stoichiometry between E-cadherin, β-catenin, and p120 (Aberle, Schwartz et al. 1996; Hirohashi 1998). Cell-cell binding of E-cadherin inhibits β-catenin and p120 signaling by sequestering the proteins at the cell surface; active β-catenin and p120 signaling have both been implicated in invasive phenotypes (Gottardi, Wong et al. 2001; Reynolds and Roczniak-Ferguson 2004). Noting that many cancer cell lines retain epithelial elements, we hypothesized whether epitheliality could be incrementally gained by simple sequestration of the effector catenins. Herein, we report that expression of the intracellular domain of E-cadherin binds the catenins, and partially reverts the EMT, limiting tumor cell invasiveness similar. This suggests that a major role of E-cadherin expression in breast carcinoma cells is to modulate catenin signaling.

3.3 RESULTS

3.3.1 Breast cancer cell lines retain epithelial elements

Two well-characterized model breast cancer cell lines, MCF7 and MDA-231, though both originating as exudative pleural effusions from ductal carcinoma *in situ*, possess seemingly

polar characteristics on the EMT spectrum (Soule, Vazguez et al. 1973; Cailleau, Young et al. 1974; Cailleau, Olive et al. 1978). MCF7 cells retain many features of their epithelial counterparts and are not invasive *in vivo*. MCF7 cells present cobblestone morphology when grown in monolayer and epithelial levels of E-cadherin expression, and have even been considered in some studies as immortalized mammary epithelial cells. MDA-231 cells, in contrast, are highly invasive and have a mesenchymal phenotype. MDA-231 cells grow individually and unconnected in monolayer cultures, do not express E-cadherin due to promoter methylation (which is characteristic of E-cadherin silencing in invasive carcinoma cells), and are highly invasive *in vivo*. Since the preponderance of literature suggests that carcinoma cells, in order to metastasize, must lose their epitheliality, specifically the cell adhesion molecule E-cadherin to decouple from the primary epithelia, we were curious as to what epithelial characteristics each cell line endogenously retained or reacquired before being isolated from the pleural effusion with the intention of engineering the equilibrium of these features further into the study. In order to determine the extent to which breast cancer cells retained epithelial elements we assayed two hallmark features: their ability to assume a mammary-specific epithelial morphology and their responsiveness to TGF-β.

To determine how E-cadherin expression effected the morphological clustering of MCF7 cells, we performed FACS analysis and gated the top 5% of E-cadherin expressors in a screen of 5e6 MCF7 cells. When these cells were cultured, they grew in dome-shaped, tightly adherent clusters (Figure 10A), in contrast to their more spread morphology prior to the sorting. This morphology has been seen in both primary isolates and clonally derived cell lines of mammary adenocarcinoma (Pickett, Pitelka et al. 1975; Zucchi, Bini et al. 2002), though this morphology does not occur spontaneously in MCF7 cells (Figure 10A). After four weeks of

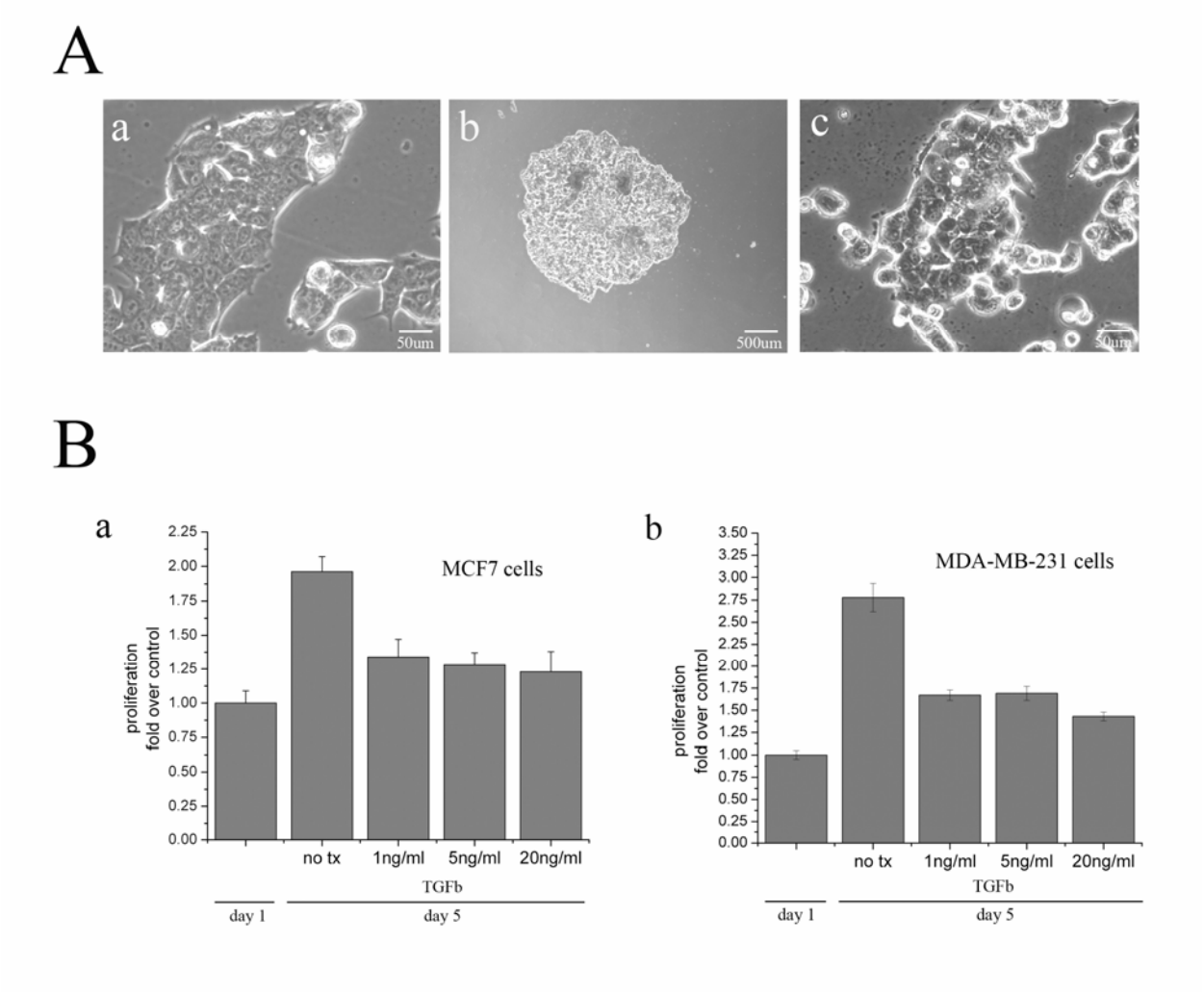

Figure 10. Invasive cancer cells retain epithelial characteristics. A) *a*, MCF7 cells grow in clustered epithelial sheets. *b*, when high-E-cadherin expressing MCF7 are plated, they grow in a very tightly adherent domes. *c*, four weeks after sorting out the high E-cadherin MCF7 cells, they have returned to a flattened morphology and a more similar morphology as seen in panel *a*. B) MDA-231 cells (*a*) and MCF7 cells (*b*) were kept in culture with and without varying concentrations of TGF-β. Both cells lines, which were isolated from exudative pleural effusions indicating that they had already metastasized, were responsive to the growth suppressive effects of TGF-β.

normal culture without any selective mechanism, the MCF7 cells return to their original morphology (Figure 10A). This qualitative data suggests that MCF7 cells retain their ability to undergo reversible shifts in differentiation. Contrary to other mammary adenocarcinoma cell lines that require *de novo* protein expression for dome formation (Zucchi, Bini et al. 2002), high E-cadherin expression is the primary phenotypic element that promotes dome-forming ability in MCF7 cells. The return of MCF7 cells to their original phenotype after sorting suggests that MCF7 have the plasticity to move along the spectrum of the EMT.

A hallmark biological response that is considered to distinguish normal epithelial cells from transformed ones is the cessation of proliferation upon treatment with TGF-β (Nelson, Vanduijn et al. 2006), though growth effects of transformed mammary epithelia upon TGF-β treatment has been subject to much debate (Roberts and Wakefield 2003). We treated both MCF7 and MDA-231 cells with TGF-β and saw a growth inhibitory effect at all concentrations in both cell types (Figure 11a and 11b), which is consistent with previous literature (Mazars, Barboule et al. 1995). Contrary to the currently accepted model, that as mammary cells progress toward malignancy the TGF-β response is more oncogenic (Wakefield and Roberts 2002), the response of MCF7 and MDA-231 resembled cells of the normal epithelium, where suppressor activities dominate in response to TGF-β treatment. These data suggest mammary tumor cells that retain elements of the epithelial lineage and are plastic in these aspects.

3.3.2 Epithelial markers vary quantitatively between breast cancer lines

The carcinoma-related EMT affects more than E-cadherin cohesiveness and TGF-β responsiveness. Many cytoskeletal and cell adhesion proteins change levels with the acquisition of a more mesenchymal phenotype (Gotzmann, Mikula et al. 2004). We queried how these

levels had changed in MCF7 and MDA-231 cells in comparison to the normal human mammary epithelial 184A-1 cell line using fluorescent microscopy. We also stably transfected the MDA-231 cells using a dominant negative fragment H-2kd-Ecad (referred to as 231/H2kd) that has an intact intracellular and transmembrane E-cadherin, but the extracellular domain is that of the class I major histocompatibility complex antigen (H-2kd) (Vizirianakis, Chen et al. 2002; Fedor-Chaiken, Hein et al. 2003), thereby producing a stable 231/H2kd cell line that can bind intracellular catenins but not form cell-cell adhesions. We postulated that expressing the cytosolic tail of E-cadherin may allow for a partial MErT, because the intracellular domain of the E-cadherin molecule serves as a stable docking station of many effector protein in epithelial cells that is absent in mesenchymally transformed cells. Our three-pronged analysis included the signaling molecule (EGF receptor tyrosine kinase (EGFR)), cell adhesion protein and effector molecules (cadherins and catenins), and cytoskeletal proteins.

EGFR positive feedback and autocrine activation is a hallmark of aggressive carcinoma cells (Kim, Kassis et al. 1999). Such is the case with MDA-MB-468 cells, an invasive cell line with amplified EGFR (Ennis, Valverius et al. 1989). Surprisingly, we observed similar levels of the EGFR in the 184A-1 HMEC, MDA-231, and 231/H2kd cell lines (Figure 11A). This is not in conflict with the initial characterization of the MDA-231 ATCC cell line (Bates, Valverius et al. 1990). ER-positive MCF7 cells expressed EGFR at only 22% of the levels of the 184A-1 HMEC cell line, which is also in accordance with studies that ER positivity is inversely correlated to EGFR status (Neskovic-Konstantinovic, Nikolic-Vukosavljevic et al. 1999).

When compared to the normal nontransformed 184A-1 HMEC cell line, the cell adhesion protein and effector molecules also shared striking similarities and differences (Figure

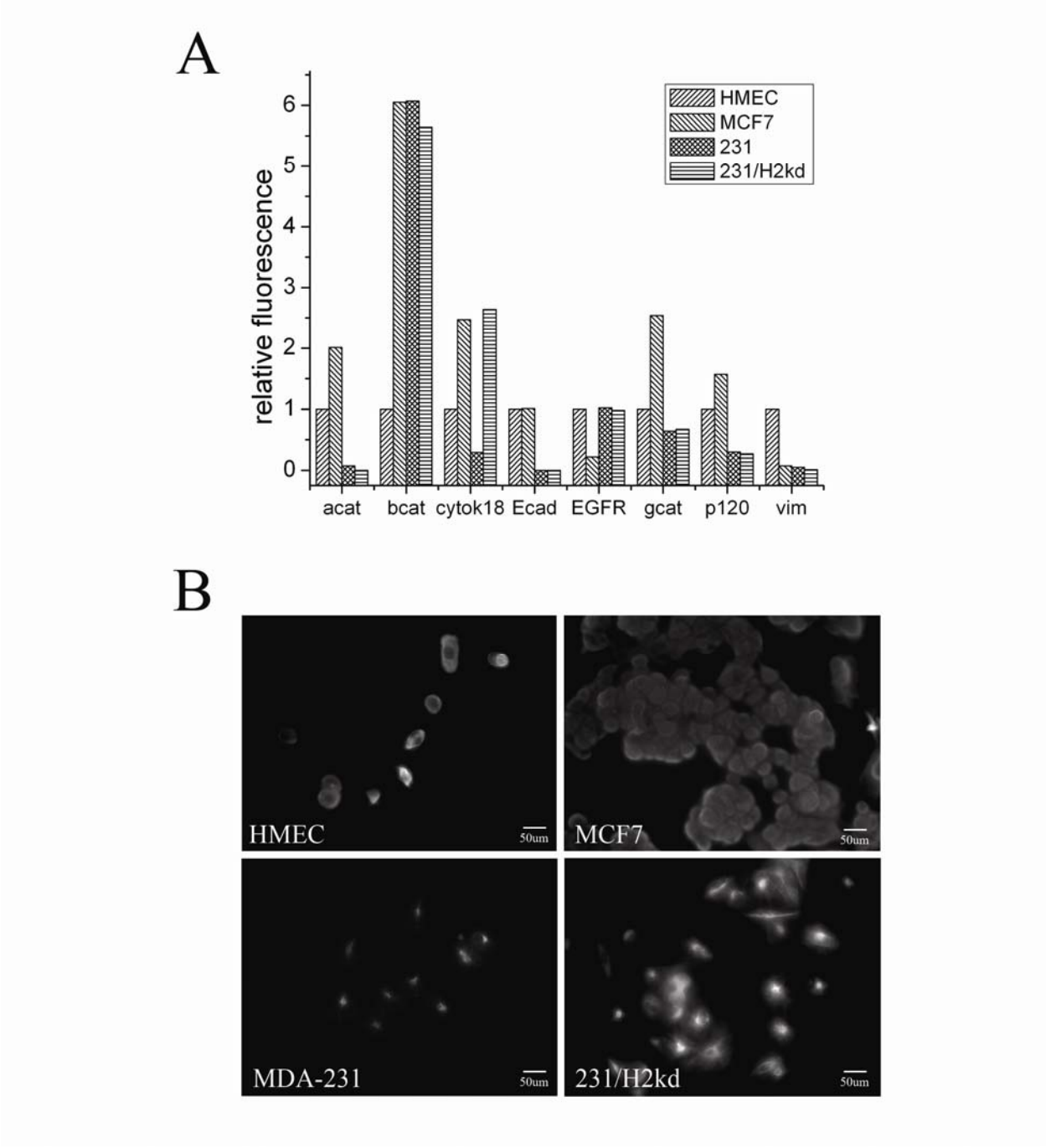

Figure 11. Invasive cancer cells exhibit a very skewed protein profile when compared with untransformed HMECs. A) Cells were examined by immunofluorescence and the histograms of the acquired pictures were analyzed for pixel intensity. B) 231/H2kd cells exhibit remarkably upregulated levels of cytokeratin-18 compared to their untransformed MDA-231 counterparts.

11A). Expectedly, E-cadherin was very similar in the MCF7 and 184A-1 HMEC cell lines, while MDA-231 and 231/H2kd cells completely lacked the protein. The expression levels of α-catenin, and E-cadherin effector molecule that couples the transmembrane adhesion protein to the actin cytoskeleton, were polar opposite in MDA-231 and MCF7 cells. The more epithelial MCF7 line had α-catenin levels over two-fold higher than in 184A-1 HMECs, while the MDA-231 line had much lower levels. β-catenin was expressed in both MCF7 and MDA-231 lines at levels almost 6-fold above the 184A-1 HMEC line; this is not surprising given the link between the canonical Wnt signaling pathway and transformation. β-catenin expression in 231/H2kd cells was similarily elevated. β-catenin's homologue, γ-catenin, was elevated in MCF7 cells and a little more than half that of 184A-1 HMEC expression. E-cadherin's other effector catenin, p120, was present in very high levels in MCF7 and at lower levels in MDA-231 and 231-derived lines.

The EMT is also characterized by a switch from cytokeratin-18 to vimentin intermediate filament expression. We did not observe the characteristic switch in intermediate filaments, as MCF7 and MDA-231 cells exhibited similar amounts of vimentin below 10% that of the model epithelial 184A-1 cells (Figure 11A), though some groups report that low vimentin expression is indicative of high tumor grade *in vivo* (Willipinski-Stapelfeldt, Riethdorf et al. 2005). Our most significant observations were those of cytokeratin-18 expression and how the expression changed upon introduction of the dominant negative H-2kd-Ecad. Consistent with a mechanism that restores epitheliality, MCF7 cells display 2.5-fold more cytokeratin-18 than the HMEC counterparts, while this epithelial marker was decreased in MDA-231 cells (Figure 11A). Most interestingly, in the 231/H2kd cells, cytokeratin-18 was restored to levels similar to MCF7 (Figure 11A and 11B), though the cellular localization of cytokeratin-18 in 231/H2kd cells was

starkly different, being punctate rather than as a cytosolic scaffold such as with the normal cells. This suggests that the cytosolic tail of E-cadherin acts to affect an increase in cytokeratin-18 levels. In total, while the MCF7 cell line is much more epithelial than its MDA-231 counterpart, there are similar mesenchymal markers that the two lines share; conversely, the MDA-231 cell line has features shared by the normal 184A-1 HMEC line, such as untransformed levels of EGFR. Though the dominant negative construct imparted the ability on MDA-231 cells to upregulate cytokeratin-18, a robust epithelial marker, we observed the other proteins to stay similar to those levels of MDA-231.

3.3.3 Intermediate mesenchymal-epithelial phenotypes can be engineered

Upon seeing the varied protein profiles of the MCF7,MDA-231, and 231/H2kd cells, we suspected that we had engineered an epithelial to mesenchymal reverting transition in the more invasive MDA-231 cells by controlling the localization of β-catenin and p120. Upon co-immunoprecipitation of H-2kd from total cell lysate of 231/H2kd cells, we were able to co-precipiate β-catenin and p120, a result of their sequestration onto the cytoplasmic domain of E-cadherin (Figure 12A). This engineering also resulted in a change of cell morphology in the 231/H2kd cells to a more flattened morphology and the 231/H2kd cells having punctuated lamellipodia instead of a defined leading edge as in the MDA-231 cells (Figure 12A, inset). It is important to note that this engineering was performed without establishing cell-cell connections, and therefore suggests that outside-in signaling by E-cadherin is not necessary for at least a partial switch to epithelial morphology.

Testing the functional implications of H-2kd-Ecad in MDA-231 cells using the invasion assay, we found that sequestration of E-cadherin effector catenins abrogated the invasiveness of

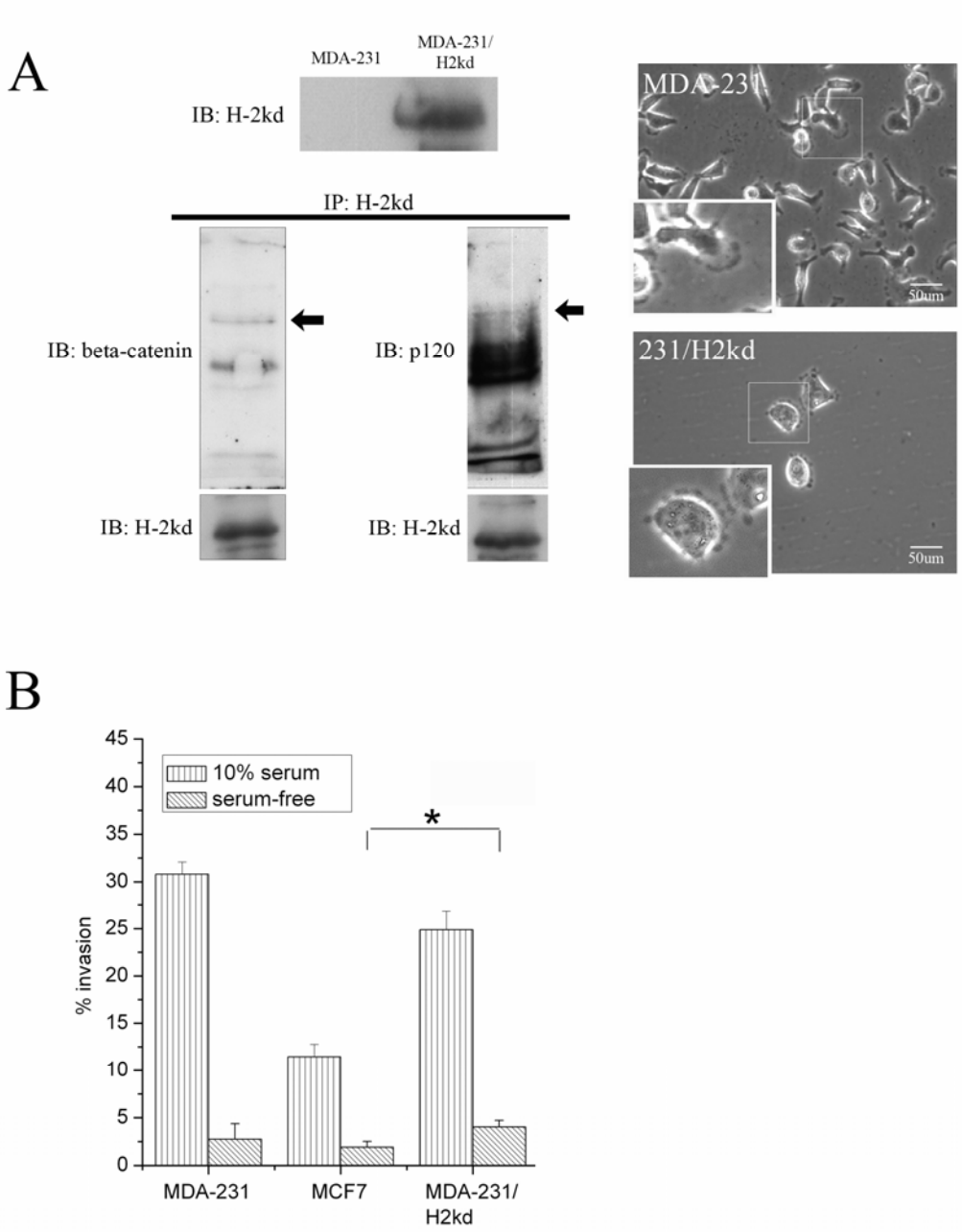

Figure 12. Cancer cells can be engineered to be more epithelial by introducing a dominant negative E-cadherin which sequesters catenins and mitigates the invasive phenotype. A) In whole cells lysates, the H2kd fragment is detected in MDA-231 cells transfected with a dominant negative E-cadherin (H-2kd-Ecad). β-catenin and p120 can be co-immunoprecipitated with H2kd in 231/H2kd cells. 231/H2kd cells have a flattened morphology with punctuated lamellipodia rather than the apparent leading edge seen in the untransformed MDA-231 counterparts (insets). B) Invasion assay comparing wild-type MDA-231 cells, epithelial MCF7 cells, and 231/H2kd cells. Asterisks denotes statistical significance by Student t-test (*$p<0.08$).

these cells by almost almost one-third when comparing the difference between the untransfected MDA-231 cells and the basal MCF7 cells (Figure 12B). Though we were able to mitigate invasiveness in MDA-231 cells by sequestering β-catenin and p120, we found that the baseline invasiveness without a chemotactic signal ("serum free" in the bottom chamber) was still greater than the MCF7 cells ($p < 0.08$), in which E-cadherin is expressed in high amounts both limiting the signaling of the catenin proteins and establishing cell-cell connections. Though β-catenin and p120 have been the most studied proteins to date regarding their ability to regulate epitheliality with their association in the E-cadherin complex, the expression of the cytosolic tail of E-cadherin could have sequestered or effected the signaling of other uncharacterized effector proteins that could have contributed to these results. In total, this suggests that changes to the localization of key signaling proteins during the mesenchymal to epithelial transition can have profound effects mitigating the mesenchymal nature of an invasive cell.

3.4 DISCUSSION

Metastatic transformation follows a sequence of ordered events, during which normal epithelial cells become metastatic by both the gain and loss of functions that perturb the cell from its epithelial equilibrium. The hallmark imbalance of the epithelial to mesenchymal transition begins at the primary tumor, where E-cadherin silenced (Foty and Steinberg 2004). The silencing of the cell adhesion molecule not only contributes to the decoupling of the epithelial sheet, which allows malignant cells to break away from the primary mass, but also to increased activity of E-cadherin's effector catenins, β-catenin and p120 (Brennan and Brown 2004; Sarrio, Perez-Mies et al. 2004). In epithelial equilibrium, the catenin signaling is limited by

sequestration on the intracellular tail of the E-cadherin transmembrane protein; when E-cadherin is no longer present, β-catenin and p120 are free to transit throughout the cytoplasm of the cell, signaling to their downstream pathways. When this signaling is not balanced by E-cadherin sequestration, it most likely results in a metastatic cascade, further perturbing the equilibrium until the full mesenchymal nature of the cell is reached. The present study provides proof-of-principle that even invasive carcinoma cells can regain some epithelial phenotypes by modulating this catenin signaling.

In previous studies, we documented how normal epithelial cells can drive the re-expression of E-cadherin in breast cancer cells in a process described as the mesenchymal to epithelial reverting transition (MErT) (Yates, Shepard et al. 2007). In the prostate cancer cell lines DU-145 and PC-3, E-cadherin expression could be induced by inhibition of the EGFR, indicating that a post-transcriptional pathway was responsible for E-cadherin downregulation. Several intermediate pathways linking tyrosine kinase activation to E-cadherin recycling and/or destruction have been reported (Pece and Gutkind 2002). As EGFR-family member amplification is a frequent occurrence in invasive cancers, increased signaling through this receptor family is a likely cause for loss of epithelial equilibrium and progression towards a mesenchymal phenotype. Inhibition of EGFR signaling demonstrated epithelial phenotype plasticity in highly invasive carcinomas, leading us to query how else this equilibrium could be affected.

In the MDA-231 breast cancer line, in which E-cadherin is silenced on the genetic level by promoter methylation, we saw that coculturing with epithelial cells triggered a demethylation of the promoter region and subsequent expression of E-cadherin that could functionally ligate with juxtaposed epithelial cells and transduce signals by canonical pathway activation (Shepard

and Wells 2007). In these studies, we observed that the epithelial interaction altered the MET equilibrium on the epigenetic level, allowing re-expression of E-cadherin and relocalization of β-catenin and p120 to the cytosolic tail of E-cadherin. Our observations of mesenchymal-epithelial equilibrium shifts raised the question of what aspect of E-cadherin expression was responsible – the cell-cell connections or the altered signaling from relocalization of the attendant catenins.

First, by examining phenotypic and proteomic patterns in both the aggressive MDA-231 and the nearly nontumorigenic MCF7 cells, we found that there is variation in breast cancer cell lines such that this no 'pure' signature for either end of the EMT spectrum; and that these characteristics are plastic. In MCF7 cells, which are non-invasive *in vivo*, a highly differentiated phenotype can be realized simply by sorting for those cells at the tail-end of the E-cadherin expression distribution; in this way, an equilibrium shift changing the amount of E-cadherin expression in the MCF7 population would restore the epithelial phenotype of the cell population. We also observed biological responses, thought only present in normal epithelial cells, to be preserved in the invasive cancer cell line MDA-231; responsiveness to TGF-β1 mediated growth suppressive effects is a property of development-associated transitions involving branching morphogenesis. Our data suggests that invasive cells can still be responsive to TGF-β in a growth-suppressive manner. This is such a hallmark characteristic, one of the defining ones in mammary bud development, that the possibility for MDA-231 cells' ability to redifferentiate to a pseudo-normal mammary epithelium cannot be ignored.

After observing these variations at odds with a one-way EMT theory, we hypothesized that we could engineer a small phenotypic equilibrium shift in MDA-231 cells by sequestering the E-cadherin-associated catenins with a non-binding E-cadherin construct. After transfecting the MDA-231 cells with the cytosolic domain of E-cadherin linked to the MHC external domain, we

saw that the dominant negative protein sequestered both 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, Hein et al. 2003). We compared the transfectants with the parental MDA-231 cells and the MCF7 cells. Not only did the 231/H2kds expression levels of cytokeratin-18 seen in the epithelialoid MCF7 cells, but when the invasiveness was measured by invasion chamber assay, we found that MDA-231/H2kd cells were less invasive than their MDA-231 counterparts. The MDA-231/H2kd cells were still more invasive than the MCF7 cells even at basal levels without a chemoattractant present. Thus, we provide proof-of-principle that cancer cells can be engineered to approach a mesenchymal-to-epithelial reverting transition by altering the functions of E-cadherin expression. Malignant carcinoma reversion to epitheliality *in vivo*, whether in the natural history of the disease or by pharmaceutical intervention, may be a double edged sword. The result of returning invasive cells back into their epithelial counterparts will mitigate the pathology of invasive cells. However, this reversion may provide other behaviors that may make the cells more likely to persist and survive attempts to exterminate the tumor. More research into how invasive cells undergo the MErT is necessary to formulate a pharmaceutical intervention strategy.

3.5 MATERIALS AND METHODS

3.5.1 Cell lines and reagents

The human breast adenocarcinoma cell lines MCF7 and MDA-231 were originally derived from pleural effusions. Cells were maintained in RPMI-1640 with 10% FBS as previously described (Yates, Shepard et al. 2007). 184A-1 HMECS were a kind gift from Lauffenburger *et al* and cultured as previously described (Wolf-Yadlin, Kumar et al. 2006). High E-cadherin expression MCF7 cells were sorted by FACS using a human E-cadherin-specific (67A4) antibody (Santa Cruz Biotechnology; Santa Cruz, CA) and cultured as above. TGFβ-1 (Peprotech; Rocky Hill, NJ) was used at stated concentrations in serum-supplemented media. MDA-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, Chen et al. 2002). H-2kd-Ecad cells were selected by FACS using the H-2kd (SF1-1.1) antibody (BD Pharmingen; San Jose, CA) and were maintained in 600μg/ml G418 until used for experimentation.

3.5.2 Immunofluorescence staining and analysis

Cells were plated in Nunc Lab-Tek 8-well Chamber Slides (Fisher; Houston, TX) and allowed to attach overnight. The next day cell were fixed in 4% paraformaldehyde then permeabilzed using 1% Triton in PBS for 4 minutes. Cells were incubated with primary antibody for 60 minutes, washed, and then secondary antibody for 60 minutes, washed, then mounted using Vectashield with DAPI (Vector Labs; Burlingame, CA). Images were acquired using an Olympus 1X70 inverted microscope (Center Valley, PA). Primary antibodies were purchased from Santa Cruz

Biotechnology, unless otherwise stated: E-cadherin (67A4), p120 (H-90), β-catenin (C-18), vimentin (J144), cytokeratin-18 (RCK106), γ-catenin (C-20), α-catenin (H297), EGFR (Ab-3) (Oncogene; Uniondale, NY). Raw 8-bit color depth images were analyzed using the Photoshop (Adobe; San Jose CA) pixel histogram tool using channel-specific parameters. Enlarged insets were sharpened using Photoshop's "Smart Sharpen" tool; image manipulations were applied equally to all comparable figures.

3.5.3 Immunoprecipitations and western blots

MDA-231/H2kd cells were quiesced for 24 hours and immunoprecipitation was performed using the H-2kd antibody from sodium-dodecyl-sulfate (SDS)-sample buffer cell lysate as previously described (PMID: 14747473). Immunoprecipiated proteins were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membrane/Immobilon-P (Millipore; Beford MA). Blots were probed with given primary antibody above before visualizing with the Enhanced Chemiluminescence Kit (Amersham Biosciences; Piscataway, NJ).

3.5.4 Invasion assay

Invasive potential was determined *in vitro* by migration through an artificial ECM (Zhou, Grandis et al. 2006). 1.5e4 cells were challenged in growth-factor reduced matrigel invasion chambers (Becton Dickinson/Biocoat; Bedford, MA) as previously described. Cells were plated in 1% BSA for the first 24 hours; after, media in the top chamber was replaced with serum-free media and media containing 10% serum was added to the lower chamber for the remainder of the assay. After 48 hours, the remaining cells and ECM in the top chamber were removed by cotton

swab and the cells that invaded through the matrix and reached the bottom of the filter were counted visually. Individual experiments were performed in triplicate. Negative controls were performed by using serum-free media in the bottom chamber for the duration of the experiment. Measurements were recorded as the fraction of total cells seeded at the beginning of the assay.

3.5.5 Statistical Analysis

All quantitative data are presented as mean ± sd obtained from at least three independent experiments. P-value significance was determined using a two-tailed unpaired Student t-test. All images were representative of at least three independent observations.

3.6 ACKNOWLEDGEMENTS

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4.0 NORMAL EPITHELIAL CELLS OF THE METASTATIC NICHE DRIVE FUNCTIONAL RE-EXPRESSION OF E-CADHERIN BREAST CANCER CELLS

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4.1 ABSTRACT

Epithelial-cadherin downregulation enables cancer cells to escape from the primary mass; however, E-cadherin has been found to be expressed on metastatic foci, bringing into question the role of this molecule in tumor progression. We define a novel role for the cellular adhesion molecule E-cadherin, in which the protein's re-emergence promotes carcinoma-parenchymal interactions in ectopic sites. Non-metastatic E-cadherin positive MCF7 breast cancer cells form heterotypic cohesions mediated by E-cadherin, and in invasive and metastatic MDA-MB-231 cells, the E-cadherin promoter hypermethylation that prevents endogenous E-cadherin expression is reversed when these cells are cultured with hepatocytes. The function of this re-expression is suggested by the E-cadherin-dependent sustained activation of Erk-MAP kinase and Akt in these breast carcinoma cells. Thus, we propose that E-cadherin expression and subsequent heterocellular interactions direct cell fate decisions that may ultimately enable colonization of a secondary site by an invasive cancer cell.

4.2 INTRODUCTION

Cadherins make up a family of adhesion molecules that mediate Ca2+-dependent cell-cell adhesion at points of cell-cell contact (Nose, Nagafuchi et al. 1988; Takeichi 1991). Epithelial-cadherin (E-cadherin, CDH1), the prototype classical cadherin present on the surface of most

epithelial cells, has a cytoplasmic domain that anchors the cell adhesion molecule to the actin cytoskeleton via catenin-based complexes (Aberle, Schwartz et al. 1996). It is generally considered that E-cadherin directs homotypic binding, organizing cells of the same lineage into a functional tissue during morphogenesis (Takeichi 1991). Thus, E-cadherin is central to epithelial cell differentiation and suppression of proliferation and migration.

Finding E-cadherin downregulated or even lost in invasive and metastatic carcinomas buttressed this role of E-cadherin in modulating the epithelial phenotype (Hirohashi 1998). It has been hypothesized that loss of E-cadherin allows individual tumor cells to break from the primary tumor mass at the same time as enabling autocrine pro-proliferative and –migratory signaling to ensue from receptors and ligands physiologically separated by cell polarity and the E-cadherin-based tight junctions (Hazan and Norton 1998). This supported a designation as a tumor suppressor, even placing E-cadherin at the apex of a "tumor suppressor system" (Vleminckx, Vakaet et al. 1991). More recent reports of E-cadherin being expressed at the site of metastatic foci in the liver, lung and lymph nodes (Kowalski, Rubin et al. 2003) have caused reconsideration of E-cadherin downregulation as required for tumor dissemination. The key question is whether downregulation of E-cadherin is not required for dissemination, or rather, as we posit here, that E-cadherin expression is re-established at the metastatic site.

We previously reported that co-culture of DU145 human prostate carcinoma cells with normal rodent hepatocytes causes an increase in E-cadherin at both the mRNA and the protein level (Yates, Shepard et al. 2007). This re-expression could also be accomplished by LHRH receptor negative attenuation of the stimulatory autocrine EGFR signaling loop extant in most carcinomas (Kim, Turner et al. 1999; Wells 2000). We thus proposed that the re-expression of E-cadherin on invasive prostate cancer cells was described by a mesenchymal-to-epithelial reverting transition

(MErT) (Yates, Shepard et al. 2007). In this study, we test this hypothesis in a cell line in which the E-cadherin promoter is hypermethylated, the aggressive breast carcinoma line MDA-MB-231. Herein, we report that hepatocytes drive the re-expression of E-cadherin in these breast carcinoma cells and in some primary breast carcinoma explants. These are functional in that ligation with cell heterotypic E-cadherins elicits intracellular signaling related to tumor cell survival. Thus, we propose that a stimulus originating from the hepatocytes drives the demethylation of the E-cadherin promoter region in breast cancer cells and results in a functional adhesion between invasive cancer cells and the parenchymal hepatocyte, suggesting that E-cadherin may not only be a tumor-supressor in the locale of the primary tumor, but also act as a metastasis-specific oncogene promoting survival in ectopic organs.

4.3 RESULTS

4.3.1 Distant soft tissue micrometastases that originate from E-cadherin-negative MDA-MB-231 primary tumor xenografts re-expression E-cadherin

Our working model proposes two changes in E-cadherin expression that promote tumor dissemination. First, E-cadherin downregulation or loss in the primary tumor site enables emigration, as has been shown in numerous studies (Mareel, Behrens et al. 1991; Shiozaki, Tahara et al. 1991; Vleminckx, Vakaet et al. 1991; Bracke, Van Roy et al. 1996; Hirohashi 1998; Hazan, Qiao et al. 2004). Second, E-cadherin expression is upregulated in metastatic sites to provide for survival in the ectopic environments; we show evidence for the re-expression phenomena herein. We evaluated metastases of the highly aggressive, E-cadherin deficient

MDA-MB-231 human breast carcinomas from orthotopic xenografts in the inguinal fat pads. After four weeks to allow for dissemination from the primary tumor, the mice were sacrificed and lung micrometastases examined (Figure 13). Our use of human breast cancer cells and a mouse host allowed for a human-specific E-cadherin antibody to discern between the cancer cells and the epithelial mouse parenchyma. The primary xenograft tumor was similar to *de novo* occurring *in situ* human breast cancers, exemplified by nuclear atypia and cellular disorganization. At the periphery of the tumor, cancer cells invaded the neighboring adipocytes indicative of their invasive ability. We first confirmed that the primary xenograft transplants in the inguinal fat pads did not express E-cadherin (Figure 13A). The lack of E-cadherin expression was expected in the primary xenograft, since the E-cadherin promoter region in the MDA-MB-231 cells is highly methylated (Graff, Herman et al. 1995). There was no change in E-cadherin status of the invading cells in the primary xenograft, as we observed that in both the center and periphery areas of the tumor that E-cadherin was not detectable by immunoperoxidase staining (Figure 13A, middle and right). The early micrometastases in the lung showed a markedly different pattern of E-cadherin expression; these tumor cell nodules were less than 2mm in diameter fitting the definition of a micrometastase as stated in the AJCC staging manual (Figure 13B, left) (Huvos, Hutter et al. 1971). When immunoperoxidase labeling was performed on these sections, we found that isolated islands expressed E-cadherin (Figure 13B, top). When the image was captured at higher resolution (Figure 13B, top adjacent), we found that the E-cadherin

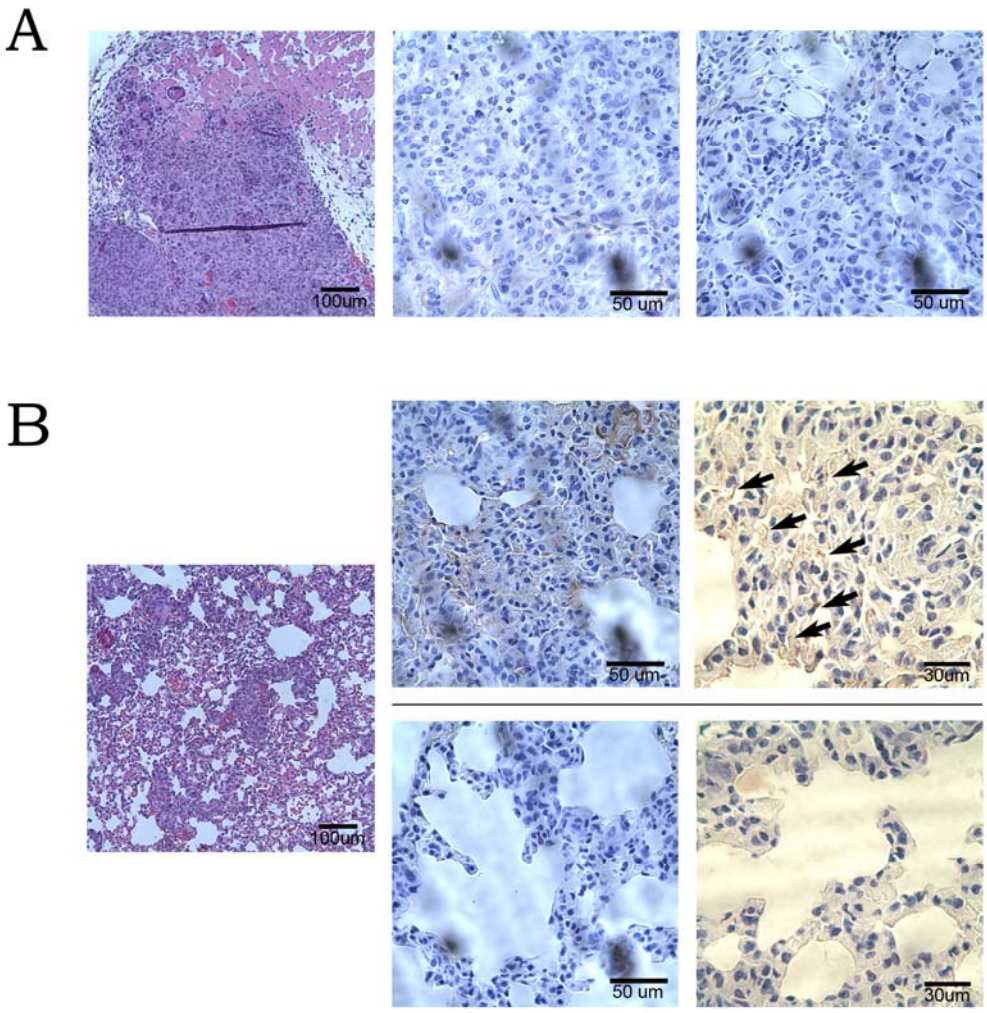

Figure 13. E-cadherin positive metastatic foci originate from E-cadherin negative primary tumors. A) Left, human MDA-MB-231 breast cancer cell xenograft in a mouse inguinal fatpad (H&E); middle, immunoperoxidase labeling with a human-specific E-cadherin antibody indicates the absence of E-cadherin expression in the center of the primary tumor; right, immunoperoxidase labeling of a field at the periphery of the tumor indicates the homogeneity of the absence of E-cadherin in all fields of the primary tumor. B) Left, islands of micrometastases in the lung originating from the primary xenograft in *A* (H&E); top adjacent, immunoperoxidase staining using a human-specific antibody of diseased portions of the mouse lung indicate the presence of E-cadherin positive human MDA-MB-231 cancer cells (arrows); bottom adjacent, mouse epithelial alveolar cells in a portion of the unaffected lung do not exhibit labeling with the human-specific E-cadherin antibody.

staining in the micrometastases was localized to the membrane of the cells, and that E-cadherin positive cancer cells occurred in islands and were not disseminated throughout the entire tumor field. Other fields of the same lung, unaffected and clear of metastatic lesions, had no staining (Figure 13B, bottom). The heterogeneity of the secondary micrometastases was distinct from the homogeneous solid tumor at the primary xenograft transplant, and we queried this re-expression in the remainder of the study.

4.3.2 E-cadherin protein and message expression in MDA-MB-231 cells is driven by hepatocyte co-culture

The initial finding of E-cadherin expression being upregulated in the early micrometastases of MDA-MB-231 cells, while consistent with our model of re-expression at the ectopic site, leaves open the possibility of preferential dissemination of E-cadherin-positive cells. We had earlier shown that prostate cancer cells undergo a program of mesenchymal to epithelial reverse transition (MErT) that includes upregulation of E-cadherin when these cells are co-cultured with primary hepatocytes (Yates, Shepard et al. 2007). Therefore, we determined whether the individual breast cancer cells also would undergo this reverse transition as best determined *in vitro*. This is of particular interest as the E-cadherin is silenced by promoter hypermethylation in the MDA-MB-231 cells, similar to breast cancers and most invasive carcinomas (Graff, Herman et al. 1995), while in prostate cancer cell lines, including DU-145 and PC3, E-cadherin is downregulated at the post-transcriptional stage (Mitchell, Abel et al. 2000).

We co-cultured MDA-MB-231 cells with freshly isolated rodent hepatocytes, as liver is a common site of breast cancer metastasis (Goldhirsch, Gelber et al. 1988; O'Reilly, Richards et al.

1990; Wyld, Gutteridge et al. 2003) and isolation of highly enriched parenchymal cells is more readily accomplished in liver than lung. Upon whole cell lysis of both hepatocytes and cancer cells on day 6 we noted E-cadherin immuno-reactivity using a human-specific antibody (Figure 14A), suggesting that this expression occurred in the MDA-MB-231 cells. Control experiments were performed to confirm the minimal cross-reactivity of the antibody with E-cadherin of rat origin (data not shown). To both quantify the level of E-cadherin upregulation on individual cells and further define it is the breast cancer cells that express E-cadherin, we determine cell reactivity by quantitative flow-cytometry (Figure 14B). Side and forward scatter as well as hepatocyte-specific autofluorescence gating were optimized to exclude the hepatocyte population. MDA-MB-231 cells has a unimodal level of background fluorescence. The same analysis of MDA-MB-231 cells after 6 days of co-culture with hepatocytes forms a bimodal distribution, with 22.32% of cells forming a distinct population in the second decade of the log scale. From this data, we observe that E-cadherin is translated or survives at the protein level in only a fraction of the breast cancer cells, which we further studied below. Importantly, we did not detect E-cadherin upregulation in MDA-MB-231 cells when cultured alone (Figure 14C). Therefore, hepatocytes drive the expression of E-cadherin in MDA-MB-231 invasive adenocarcinoma cell line.

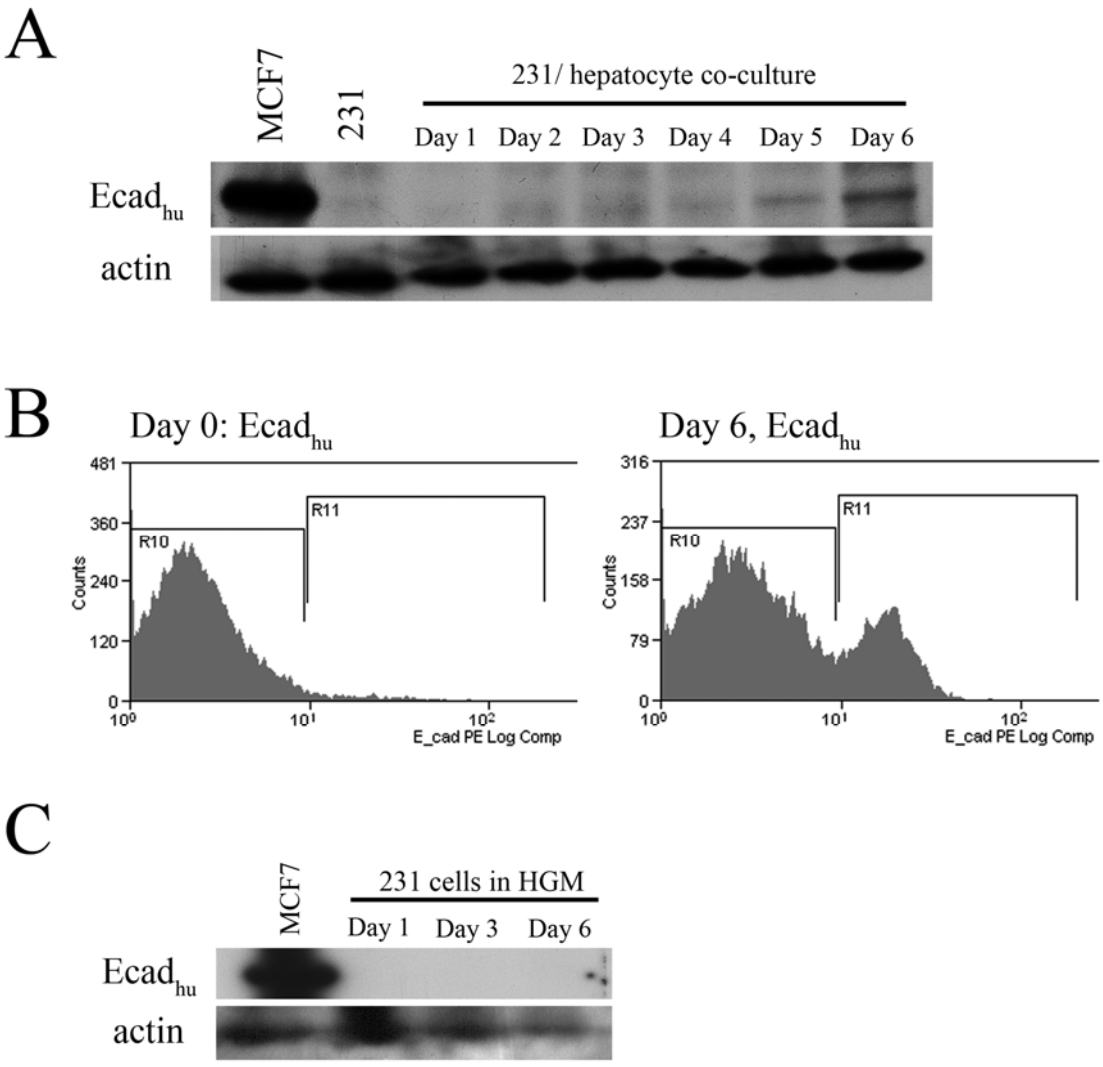

Figure 14. Hepatocytes drive the re-expression of E-cadherin in MDA-MB-231 breast cancer cells. A) Immunoblot of proteins lysates from MDA-MB-231/hepatocyte co-cultures using a human-specific antibody indicates that within 6 days of co-culture with hepatocytes, the cancer cells re-express E-cadherin. B) Flow cytometry analysis of the MDA-MB-231 population. Hepatocyte interference was excluded using a human-specific antibody as well as SS/FS gating. On Day 0, MDA-MB-231 cells have a homogenous level of background fluorescence with a coefficient of variation (CV) of 63.01 that falls within the first decade of the log scale, gated as R10. The E-cadherin negative MDA-MB-231 cells on day 6 have a similar CV of 66.91, which suggests the MDA-MB-231 population has become bimodal, with E-cadherin positive cells gated as R11.

4.3.3 Demethylation of the E-cadherin promoter region without global demethylation allows for protein re-expression

We explored an epigenetic mechanism to explain the re-expression of E-cadherin in MDA-MB-231 cells when in the presence of ectopic parenchymal cells, since the promoter region of MDA-MB-231 cells has been well characterized (Graff, Herman et al. 1995; Graff, Herman et al. 1997; Graff, Gabrielson et al. 2000) as being fully methylated, with this silencing E-cadherin transcription. We assayed a CpG island that was proximal to the E-cadherin transcription start site, whose methylation correlates with E-cadherin expression (Kallakury, Sheehan et al. 2001). Following the same co-culture protocol as above, we isolated genomic DNA for methylation specific PCR (MSP) analyses. 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 15A.c). It was determined in control experiments of hepatocytes alone in culture over the same period of time that the methylation-specific primers amplified only human E-cadherin DNA and not rat (Figure 15A.c). The stability of the E-cadherin promoter in MCF7 cells, an E-cadherin-positive, non-invasive breast epithelial cell line, was assessed over the same time-course and remained unchanged (Figure 15A.a) and MDA-MB-231 cells remained hypermethylated in the absence of hepatocytes (Figure 12A.b). These MSP data show that demethylation of the promoter region was antecedent to transcription of the E-cadherin message in MDA-MB-231 cells co-cultured with hepatocytes.

An open question is whether the loss of promoter methylation is specific or global in the presence of hepatocytes. The *H19* gene is a paternally imprinted gene whose methylation is modulated during gametogenesis (Lucifero, Mertineit et al. 2002), and does not change after terminal differentiation of a cell line. We performed bisulfite MSP analysis on MDA-MB-231 cells before and 1,3, and 6 days into co-culture with hepatocytes, examining a previously reported CpG site of *H19* (Figure 15B). Evaluation of the data revealed that the average methylation of *H19* remained unchanged at all time points. This suggests that MDA-231 cells initiate a specific program of demethylation involving the E-cadherin gene when co-cultured with hepatocytes, and demethylation of the E-cadherin promoter is not epiphenomenal to a global demethylation program.

Our data show a loss of nearly all methylation in the MDA-MB-231 promoter region, despite our flow cytometry data indicating only a subpopulation of cells expressed the protein. To confirm that demethylation of the promoter region allowed E-cadherin transcription, we quantified E-cadherin message in MDA-MB-231 using qRT-PCR; MCF7 cells served as the positive control. MDA-MB-231 cells presented undetectable levels of transcript (Figure 15A.d). However, after 6 days of coculture, the level of E-cadherin transcript was comparable to that in MCF7 cells (95 ± 2%, n = 4).

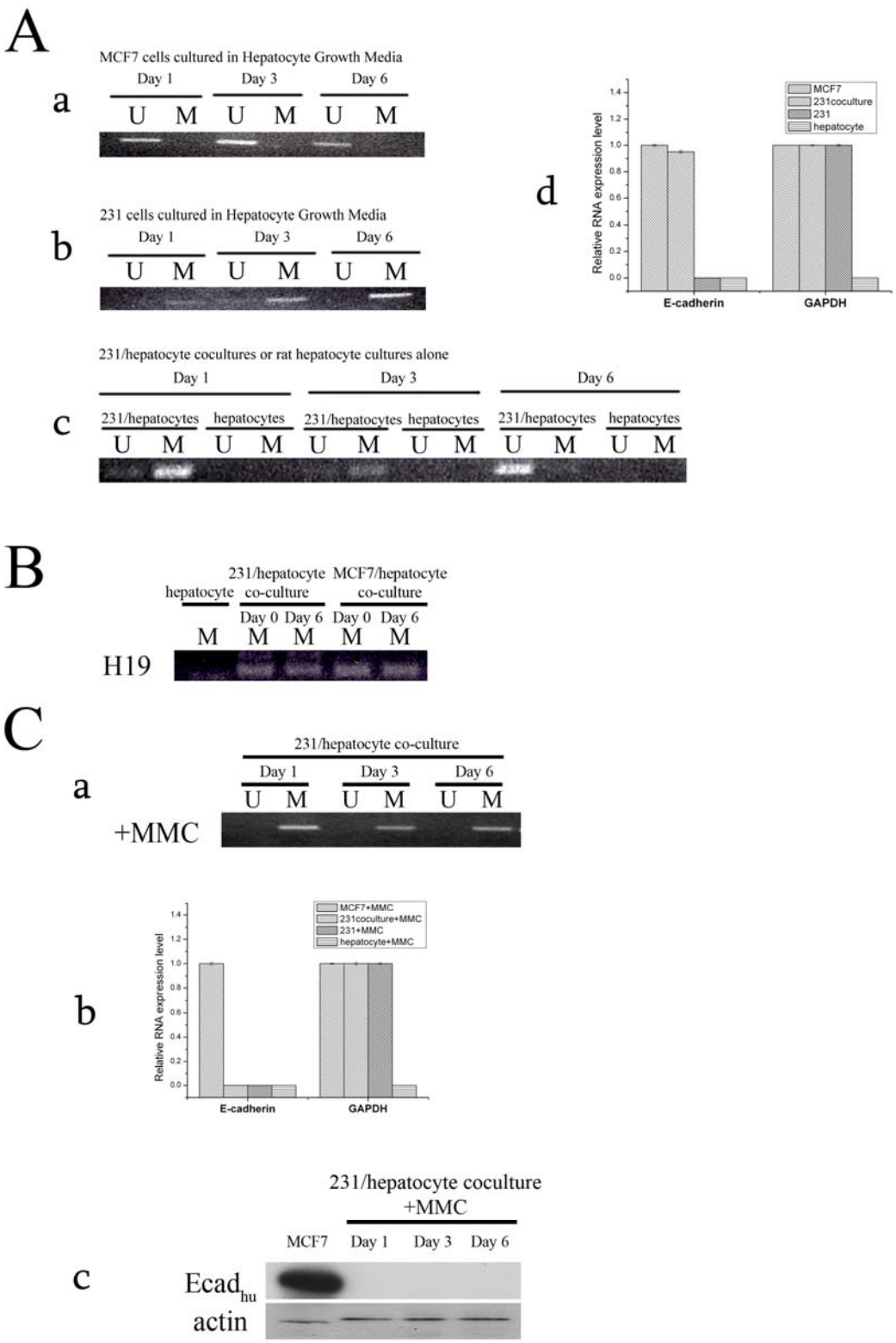
A
a
MCF7 cells cultured in Hepatocyte Growth Media
Day 1
Day 3
Day 6
U M U M U M
b
231 cells cultured in Hepatocyte Growth Media
Day 1
Day 3
Day 6
U M U M U M
c
231/hepatocyte cocultures or rat hepatocyte cultures alone
Day 1
Day 3
Day 6
231/hepatocytes
hepatocytes
231/hepatocytes
hepatocytes
231/hepatocytes
hepatocytes
U M U M U M U M U M U M
d
Relative RNA expression level
MCF7
231coculture
231
hepatocyte
E-cadherin
GAPDH
B
hepatocyte
231/hepatocyte co-culture
MCF7/hepatocyte co-culture
Day 0 Day 6 Day 0 Day 6
M M M M M
H19
C
a
231/hepatocyte co-culture
Day 1
Day 3
Day 6
U M U M U M
+MMC
b
Relative RNA expression level
MCF7+MMC
231coculture+MMC
231+MMC
hepatocyte+MMC
E-cadherin
GAPDH
c
231/hepatocyte coculture
+MMC
MCF7
Day 1
Day 3
Day 6
Ecad$_{hu}$
actin

Figure 15. Re-expression of E-cadherin follows a proliferation-dependent demethylation of the E-cadherin promoter. A) *a*-*b*, HGM does not affect the methylation status of E-cadherin positive MCF7 cells or E-cadherin negative MDA-231 cells; *c*, primers that amplify only the human E-cadherin promoter sequence indicate that hepatocytes drive the demethylation of the E-cadherin promoter region by 6 days of co-culture with hepatocytes; *d*, human specific RT-PCR primers indicate that E-cadherin message in MDA-MB-231 cells after 6 days of co-culture is comparable to MCF7. B) human specific primers that amplify the imprinted H19 gene indicate that a global hypomethylation phenomena is not occurring. C) a, upon addition of 50μg/ml MMC, demethylation of the MDA-MB-231 E-cadherin promoter does not occur; *b*, with the addition of MCC, E-cadherin message transcript in MDA-MB-231 cells is undetectable; *c*, human-specific E-cadherin is not detectable on the protein level with the addition of MMC. Shown are mean±sd (n=4).

4.3.4 Cancer cells undergo a proliferation-dependent demethylation of the E-cadherin promoter

Currently, there are no well-defined DNA demethylases, leaving either a passive mechanism, lack of maintenance methylation subsequent to mitosis, or an active mechanism, enzyme-mediated excision, to explain the loss of methylation on the E-cadherin promoter. Our MSP experiments suggested a passive process, reflected in both intermediate stages of promoter methylation on Day 3 and extended time period to unmethylated status (6 days). To test directly whether the demethylation was dependent on proliferation of the cancer cells, we inhibited proliferation of the cancer cells using mitomycin-C (50 ug/ml). This treatment completely prevented promoter demethylation as demonstrated by MSP (Figure 15C.a). Quantitative RT-PCR confirmed the levels of E-cadherin transcript in co-cultured MDA-MB-231 cells treated with mitomycin-C were undetectable (Figure 15C.b). Furthermore, this translated through to the protein level, wherein we could not detect protein if mitomycin-C was present during the induction period (Figure 15C.c). These findings further support E-cadherin demethylation as occurring by reported mechanisms in the literature (Razin and Riggs 1980; Turek-Plewa and Jagodzinski 2005).

4.3.5 E-cadherin on breast cancer cells can mediate functional heterotypic adhesion to hepatocytes

As we had established that re-expression of E-cadherin occurs, we speculated that E-cadherin on cancer cells may play the same role as on mesenchymal cells after dissemination from the neural crest, one of mediating interactions with the ectopic environment (Pla, Moore et al. 2001) . The primary physiological role of E-cadherin is one of cell-cell adhesion, as the molecule is the chief contributor to the architecture of epithelial sheets. Similarly, the functioning of the molecule on the cell surface depends on several factors not limited to the density of protein expression of the adhering cell types, the cadherin isotype, and amount of glycosylation on the protein. In order to show that E-cadherin on breast cancer cells can mediate an adhesive ligation with the E-cadherin on hepatocytes, we used a centrifugal assay that had been used in previous studies to quantify the kinetics of E-cadherin ligation in homocellular interactions; these studies have shown that E-cadherin ligation occurs in a single logarithmic step (McClay, Wessel et al. 1981; Angres, Barth et al. 1996; Giacomello, Neumayer et al. 1999).

The kinetics of adhesion between breast cancer cells and hepatocytes were measured over a period of 60 minutes. To determine whether breast cancer cells can make cell heterotypic interactions, we used the MCF7 cell line, which has high E-cadherin expression. These cells homotypically adhered to each other in a single logarithmic step, in agreement with the literature (Figure 8A) (Angres, Barth et al. 1996). Interestingly, MCF7 cells adhered to hepatocytes with similar kinetics during the initial stages of strengthening of the adhesion, though the half-maximal binding of cells in the heterotypic adhesion was slightly, though significantly less (Figure 16A). MDA-MB-231 cells did not adhere to hepatocytes (Figure 16A). To demonstrate that this cell adhesion was E-cadherin mediated, we targeted this mechanism specifically. The

CAFCA binding was abrogated by EDTA, a calcium chelator, or an E-cadherin blocking antibody (SHE78) (Figure 16B). Both of these interventions were consistent with E-cadherin-mediated cell-cell adhesion. However, to confirm that it is the breast cancer cell E-cadherin that is responsible for adhesion, we treated the MCF7 cells with siRNA constructs prior to CAFCA analysis. A human E-cadherin-specific siRNA reduced E-cadherin levels by well over half in MCF7 cells while a different, nontargeted siRNA had no effect on E-cadherin presentation (Figure 16C). The E-cadherin-specific siRNA reduced cell heterotypic adhesion down to background levels (Figure 16D). These data, for the first time, hint at the relevancy of heterotypic cohesion between two different lineages of cells, both with epithelial characteristics.

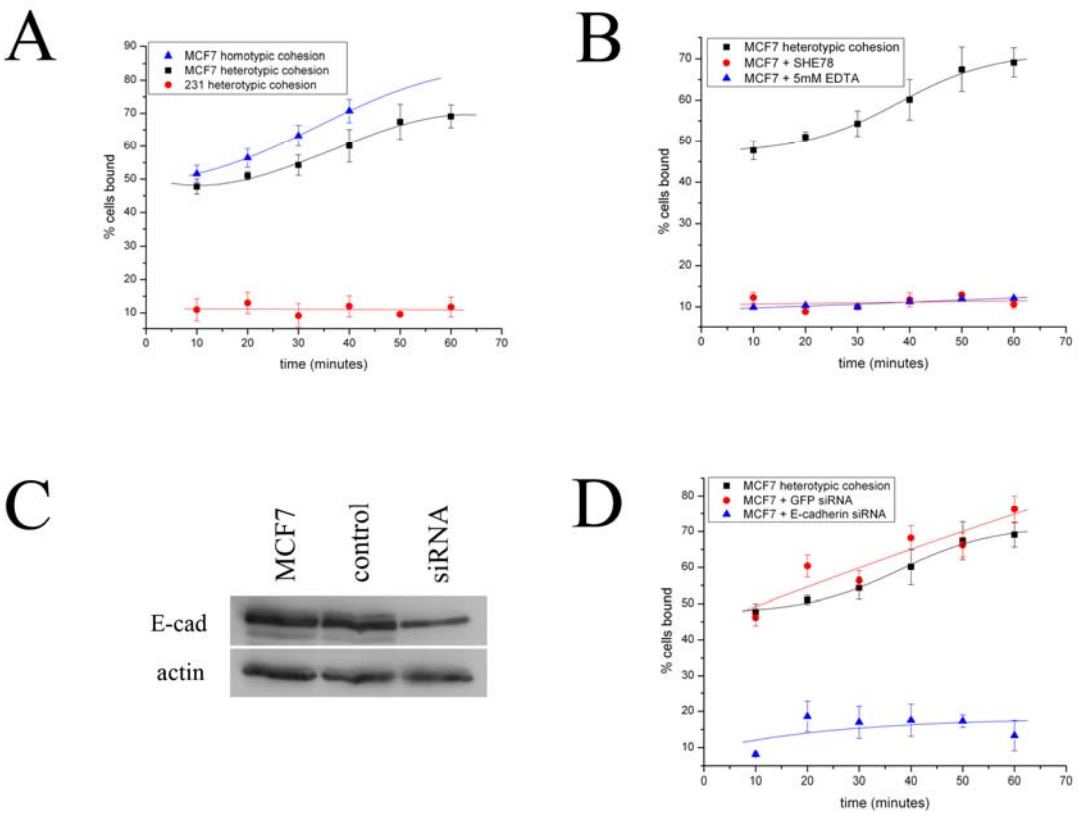

Figure 16. Heterotypic adhesion between cancer cells and hepatocytes exerts an E-cadherin-dependent functional mechanical force. A) Homotypic cohesion between MCF7-MCF7 cells develops in a single logarithmic step (triangles); heterotypic cohesion between MCF7-hepatocytes still develops in a single logarithmic step, though the half-maximal number of cells bound is significantly less (squares); heterotypic cohesion between 231-hepatocytes is negligible and indistinguishable from background levels (circles). B) Hetertypic MCF7-hepatocyte cohesion is E-cadherin dependent and can be abolished by either calcium chelation (triangles) or the E-cadherin function blocking antibody, SHE78 (circles). C) siRNA knock-down of E-cadherin in MCF7 cells. D) Heterotypic adhesion between MCF7-hepatocytes can be abolished to near-background levels with an E-cadherin-directed siRNA, but adhesion remains unaffected with a non-targeted siRNA. Shown are mean±sd(n=4).

To confirm that the E-cadherin in MCF7 cells was participating in a functional heterotypic ligation with the E-cadherin on hepatoytes, we localized the cytoskeleton and cytoskeleton assembly proteins to points of juxtaposed MCF7 cells and hepatocytes. That the E-cadherin ligation was functional in the CAFCA studies indicated of involvement of the actin cytoskeleton to anchor the heterotypic cellular-adhesion. We observed areas of focused actin polymerization where MCF7 cells came into contact with hepatocytes at early co-culture time points (Figure 17A). Arp2/3, a good indicator of focused actin polymerization (Machesky and Insall 1999; Machesky, Mullins et al. 1999), co-localized with these areas. To further show involvement of cytoskeletal anchoring, we set out to reproduce studies that determined Arp2/3 associates with E-cadherin and is necessary for efficient E-cadherin ligation (Kovacs, Goodwin et al. 2002; Verma, Shewan et al. 2004). After co-culturing E-cadherin positive MCF7 cells with hepatocytes for 90 minutes, we found that both Arp2 and E-cadherin localized to E-cadherin plaques at the edge of MCF7 cells that were directly juxtaposed with hepatocytes (Figure 17B). Hence, the adhesion machinery that mediates the contact between hepatocytes and breast cancer cells is mechanically functional and the molecules that mediate actin-based anchoring in cell-cell adhesion are present in plaques at places of cell juxtaposition.

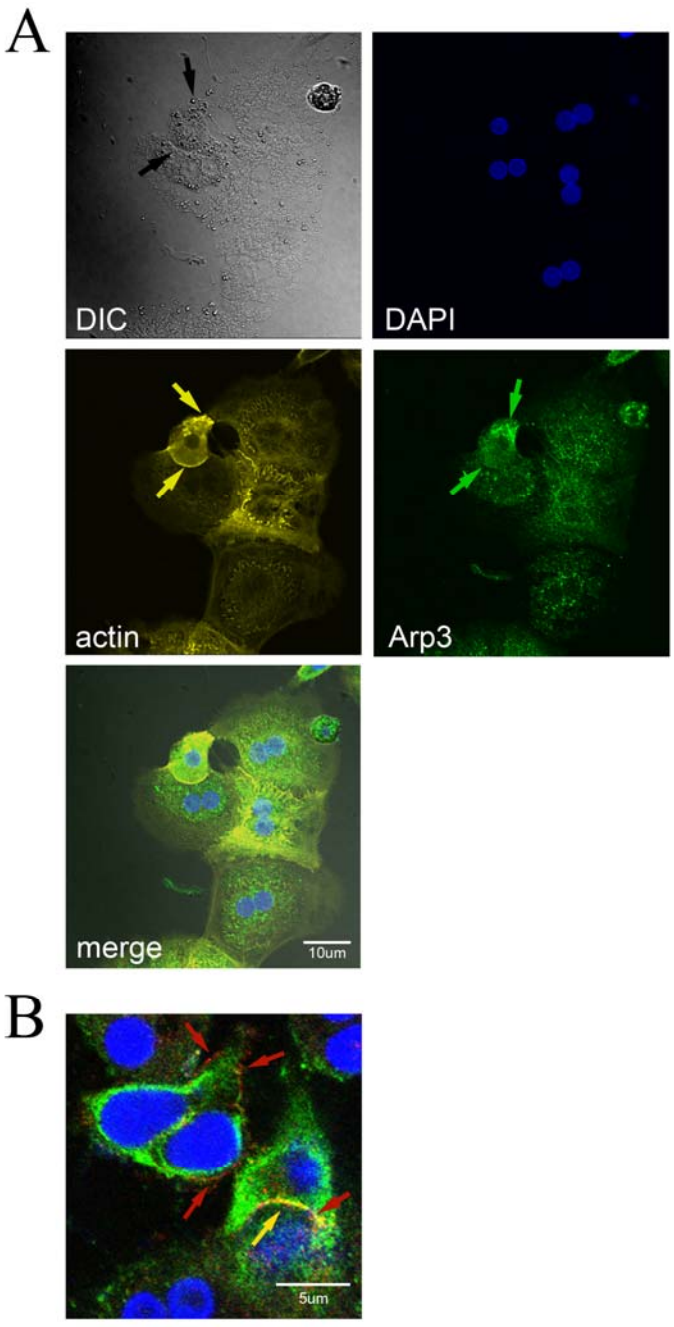

Figure 17. In E-cadherin positive MCF7 cells, the actin assembly complex Arp 2/3 is recruited to points of heterotypic cohesion to actively anchor the cancer cells to the hepatocytes. DAPI, blue; actin, yellow; Arp3, green; human specific E-cadherin, red. A) Cell interaction was observed 90 minutes after seeding of MCF7 cells into hepatocyte cultures. At points where well-differentiated hepatocytes and E-cadherin positive MCF7 cells are directly juxtaposed, focused actin polymerization occurs at the periphery of the cells that colocalizes with Arp3, while absent from the periphery not juxtaposed. B) A human specific antibody was used to show the colocalization of E-cadherin and Arp3. Colocalization in yellow.

Recent reports have documented E-cadherin ligation to activate canonical signaling pathways involved in mitogenesis, motility, and survival (Pece, Chiariello et al. 1999; Pece and Gutkind 2000). However, as soluble factors from hepatocytes might also signal the breast cancer cells, we isolated hepatocyte membranes using differential centrifugation and passively adsorbed them onto culture plates coated with poly-L-lysine. The membranes were characterized by immunoblot and shown to contain E-cadherin (data not shown). In this way, we could stringently assay kinase activation in the breast cancer cells without assaying the activity of pathways in the hepatocytes or those induced by soluble factors from hepatocytes. In MCF7 cells, both Erk and Akt are activated in an E-cadherin-dependent manner (Figure 18). This activation could be attenuated to background levels by either Ca2+ removal from the media or by an antibody that blocks E-cadherin function (Laur, Klingelhofer et al. 2002). Immunocomplex assays confirmed that these canonical pathways were being activated in MCF7 cells (Figure 18C). The kinase assay showed maximal phosphorylation of Erk was 65% of EGF treatment and maximal Akt activation was 45% of PDGF treatment. The ability of an E-cadherin functional blocking antibody or calcium chelation to abrogate pathway activation suggests that E-cadherin is directly propagating signals to the canonical Erk-MAPK and Akt pathways.

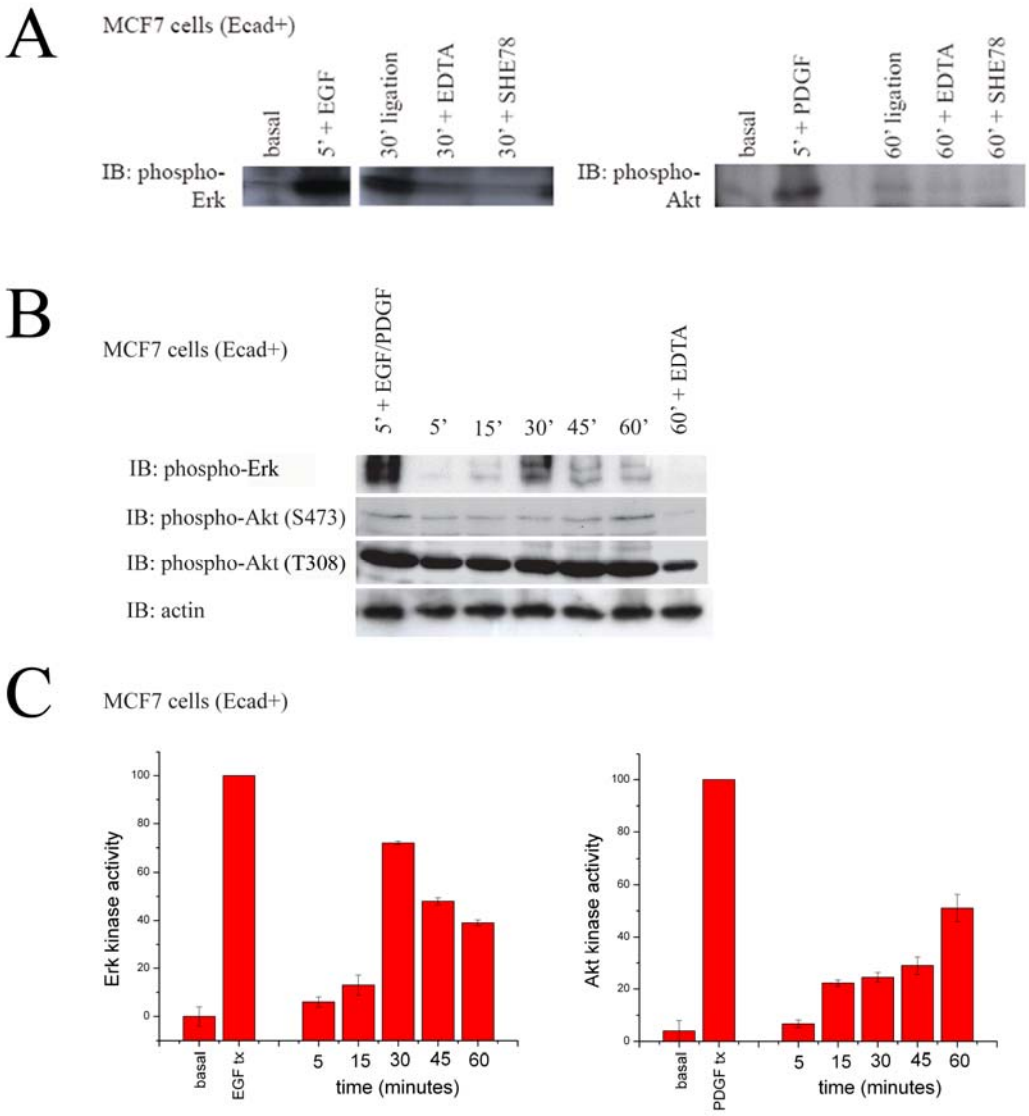

Figure 18. The canonical Erk-MAPK and Akt pathways are activated in E-cadherin positive MCF7 cells upon ligation with hepatocyte E-cadherin. A) Erk-MAPK activation peaks at 30min after ligation and Akt activation peaks at 60min after contact; activation of Erk and Akt can be attenuated with calcium chelation or the function blocking antibody, SHE78. B) 60min time-course of Erk-MAPK and Akt activation. Calcium chelation completely abrogates activation of Erk and Akt(pS473). C) Total Erk or Akt were immunoprecipitated and MBP was used as a substrate for in vitro kinase assays. Results are shown as fractions of maximal activation by 5min EGF or PDGF treatment. Shown are mean±sd (n=3).

4.3.6 E-cadherin trafficking to the surface requires EGFR attenuation but EGFR functionality is necessary for preserved E-cadherin-dependent MAPK activation in MDA-MB-231 cells

The invasive, mesenchymally-transitioned MDA-MB-231 cells did not adhere to hepatocytes in the CAFCA assay (Figure 17A). This was expected as these cells do not express E-cadherin. We postulated upon E-cadherin upregulation after co-culturing, these cells should adhere to hepatocytes in an E-cadherin dependent manner. After co-culturing, MDA-MB-231 cells did adhere to the hepatocytes, but not at the levels of the E-cadherin positive MCF7 cells (Figure 19A). This suggested that E-cadherin was present at the cell surface in sufficient amounts to allow for adhesion, but not in amounts that allowed the robust adhesion similar to MCF7. Upon examination by confocal microscopy, we found that the re-expressed E-cadherin was not stably presented on the surface of MDA-MB-231 cells, but much of the protein remained in intracellular locales (Figure 19B). This was postulated as due to autocrine EGFR signaling leading to E-cadherin internalization (Pece and Gutkind 2002). As we can restore E-cadherin surface expression and cell-cell adhesion in prostate carcinoma cells by inhibiting EGFR (Yates, Wells et al. 2005; Yates, Shepard et al. 2007), we exposed these co-cultured MDA-MB-231 cells to PD153035, an inhibitor of EGFR kinase activity. This resulted in E-cadherin re-localization to the cell surface that corroborated with increased beta-catenin localization (Figure 19C) and increased cell adhesion to hepatocytes (Figure 19A).

We were curious whether the E-cadherin dependent canonical activation we observed in the MCF7 cells was preserved in MDA-MB-231 cells that re-expressed E-cadherin. MDA-MB-

231 cells that were co-cultured with hepatocytes for 6 days, when exposed to hepatocytes, exhibited the same maximal Erk activation at 30 minutes and was prolonged through 60 minutes (Figure 19D). As our previous PD153035 experiment allowed E-cadherin to the surface, we questioned whether PD153035 treatment would either increase Erk signal activation or abrogate it, since it has been reported that E-cadherin activates the MAPK pathway through EGFR (Pece and Gutkind 2000). Upon treatment with PD153035, we observed a complete abrogation of Erk signaling (Figure 19E), suggesting that E-cadherin-mediated activation occurs via transactivation of EGFR as has been reported in the literature. To further support this data, we found that EGFR co-immunoprecipitates with E-cadherin in MCF7 cells and MDA-MB-231 cells after co-culture with hepatocytes. These data indicate that modulation of E-cadherin adhesion and signaling in invasive cancer cells by EGFR receptor tyrosine kinase signaling is a delicate balance between high EGFR signaling and complete destruction or remodeling of the receptor, or low but not absent EGFR signaling to allow cell fate decisions transduced by E-cadherin.

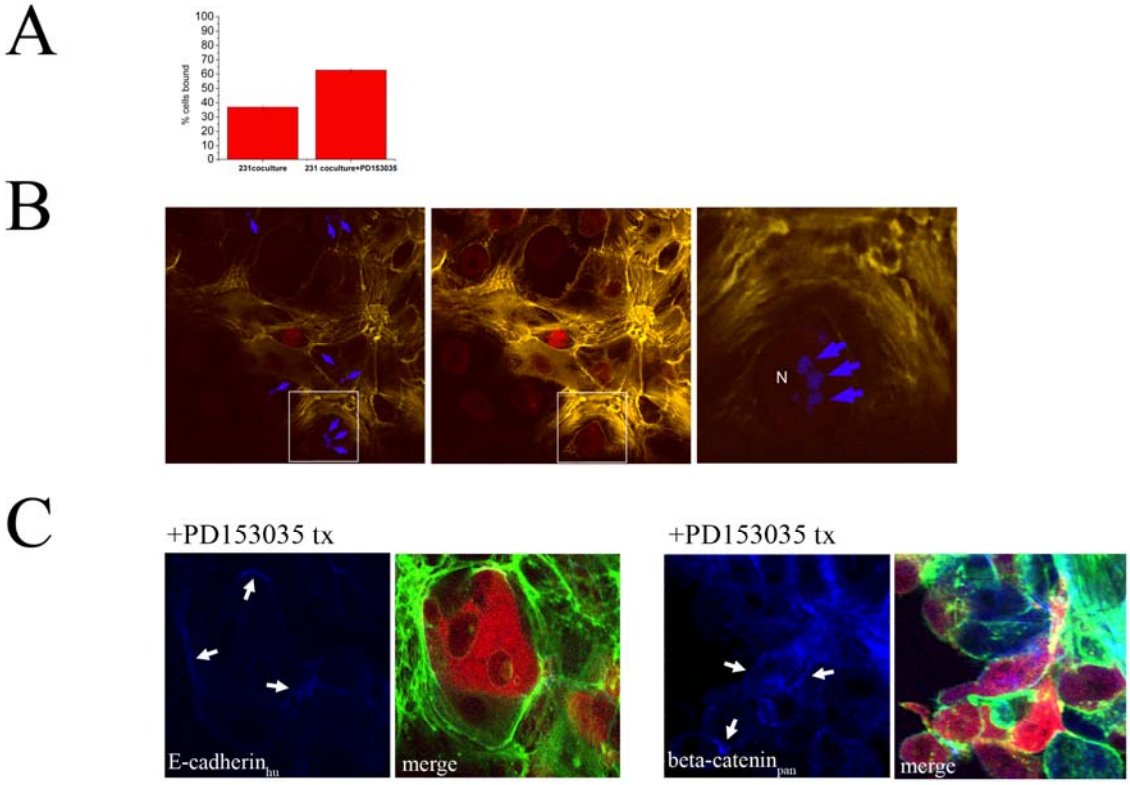
A
% cells bound
231coculture
231 coculture+PD153035
B
N
C
+PD153035 tx
E-cadherin
merge
+PD153035 tx
beta-catenin
merge
D
MDA-231 before co-culture
MDA-231 after co-culture
+EGF
0 5 15 30 45 60
0 5 15 30 45 60
+SHE78
IB: pErk
IB: Ecad
E
MDA-231 after co-culture
PD153035 tx
+EGF
0
+EGF
0 5 15 30 45 60
IB: pErk
IB: actin
F
total cell lysate
IP: EGFR
MDA-231
MCF7
MCF7
MDA-231
MDA-231 co-culture
IB: Ecad
IB: EGFR
IB: actin

Figure 19. Abrogation of EGFR signaling in MDA-MB-231 cells is sufficient to increase heterotypic binding with hepatocytes, but EGFR signaling is necessary for E-cadherin dependent Erk-MAPK activation. A) MDA-MB-231 cells were co-cultured with hepatocytes and subjected to heterotypic CAFCA. MDA-MB-231 cells that were treated with the EGFR inhibitor PE153035 increased adhesion 1.7 fold. Shown is mean±sd (n=4). B) Despite re-expression of E-cadherin in MDA-MB-231 cells, a large amount of E-cadherin does not traffick to the cell surface, but is rather seen in perinuclear organelles. C) Trafficking of E-cadherin to the cell surface can be recovered by abrogation of the EGFR with PD153035. D) E-cadherin that does traffick to the cell surface transduces the same pattern of Erk-MAPK activation as seen in MCF7 cells. Activation can be attenuated by a function blocking antibody directed towards E-cadherin. E) Treatment with PD153035 completely attenuates E-cadherin transduced Erk-MAPK activation. F) EGFR co-immunoprecipitates with E-cadherin in MDA-MB-231 cells that have been subjected to hepatocyte coculture. An E-cadherin antibody that reacts with a human-specific epitope was used for the immunoprecipitation.

4.3.7 Hepatocytes drive the upregulation of E-cadherin in a subset of cells from primary breast cancer explants

To make our in vitro results more germane to clinical cases of breast adenocarcinoma, we used explants from primary breast tumors to determine if patterns of E-cadherin expression occured when these primary cells were cultured with primary rat hepatocytes. All primary explants were used before the third passage. In total, we assayed four primary explants by flow cytometry and seven primary explants by immunofluorescence. Following the same protocol as in the previous co-culture experiments, we analyzed the cells by flow cytometry using side scatter and forward scatter gating in addition to using a human specific antibody to exclude interference from the hepatocytes. Our flow cytometry experiments indicated that one of the four explants we tested, designated 1570, expressed E-cadherin at 7-fold higher levels after co-culture with hepatocytes (Figure 20A). The delay in E-cadherin expression of the 1570 explant could be attributable to a delay in proliferation of the cancer cells, which would be consistent with passive E-cadherin promoter demethylation as the cause of protein expression as we observed in our *in vitro* findings. We observed a decrease in E-cadherin in the 1568 and 1569 explants, and no change in expression in the 1550 explant. (Figure 20A) We found these same patterns in a proportionate amount in seven other primary breast cancer explants that we observed by confocal microscopy.

When we imaged two explants that were E-cadherin negative when introduced to the hepatocyte population, after 6 days in co-culture with hepatocytes, the cancer cells expressed robust and well localized E-cadherin (Figure 20B). Five of the explants that we examined by confocal immunofluoresence already expressed E-cadherin that was well localized to the plasma

membrane in the cancer cells. We cultured these five lines with primary hepatocytes for 6 days in HGM. Interestingly, those lines that predominately expressed E-cadherin, T2410, T2391, and T2392, lost most of their E-cadherin expression, except for those cancer cells that were juxtaposed to the primary hepatocytes (Figure 20B, merge). Though the limited number of primary cells prevented us from performing quantitation of the number of cells that expressed E-cadherin alone or in hepatocyte co-culture, we measured the median and standard deviation of fluorescence intensity of the E-cadherin expression on the primary cells to stay the same, except in the T2410 primary cell line, in which it decreased. We were unable to run further experiments or controls due to the limited number and passage integrity of the primary breast cancer explants, nonetheless, this line of evidence strongly correlates to an E-cadherin-dependent hepatocyte interaction in clinically relevant breast carcinomas.

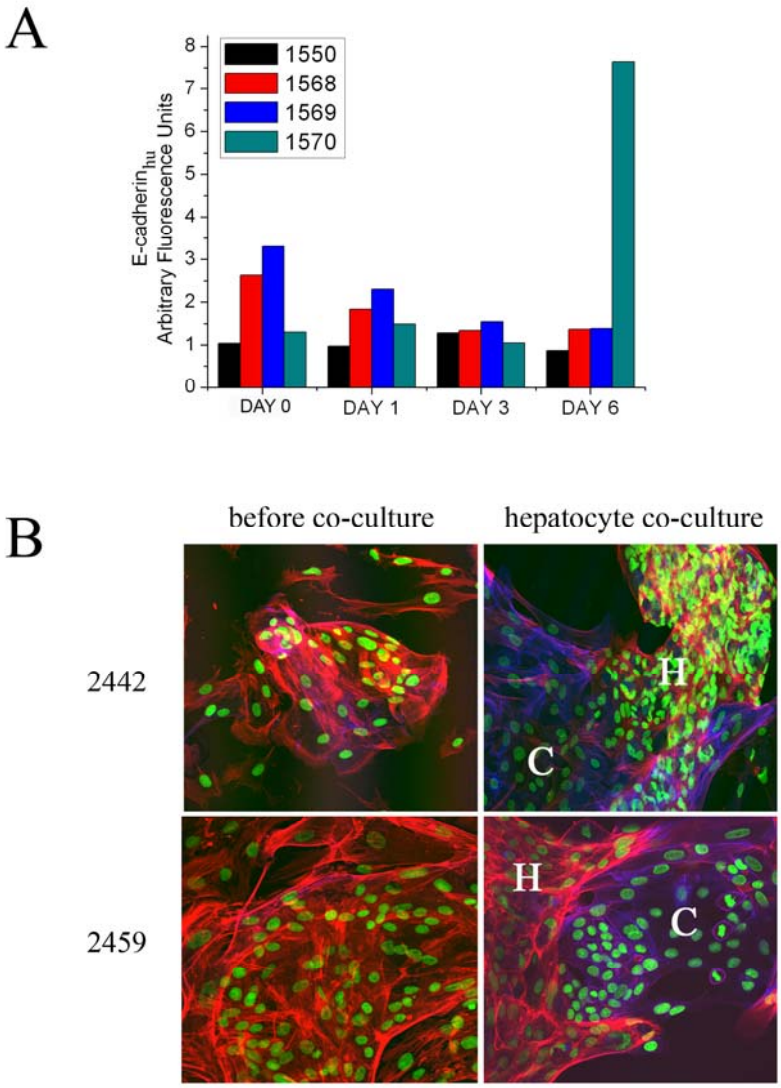

Figure 20. A subset of primary breast carcinoma explants re-express E-cadherin when cocultured with primary hepatocytes. A) Flow cytometry analysis of primary explants using the human-specific E-cadherin antibody. A fluorescence unit of 1 indicates that the fluorescence intensity was equal to the same gate performed without addition of antibody. B) Two explants, *C*, positive for E-cadherin upregulation when cocultured with hepatocytes, *H*, identified by confocal microscopy. In control experiments, hepatocytes were identified by endogenous autofluorescence excited by the 488-laser line.

4.4 DISCUSSION

Carcinomas are generally considered to undergo an epithelial to mesenchymal-like transition (EMT) as a prelude to dissemination. The key marker for this phenotypic shift is the downregulation of E-cadherin, a cell-adhesion molecule whose normal functioning is vital to the maintenance of epithelial tissue integrity and whose loss enables tumor cells to disseminate from the primary masszInterestingly E-cadherin expression has been noted in distant metastases of primary tumors suggesting a possible survival advantage as is noted during development with the reverse mesenchymal to epithelial transition (Larue and Bellacosa 2005). It is currently unknown whether these E-cadherin-positive tumor cells disseminate from the primary tumor and form secondary metastatic lesions, or if E-cadherin-negative cells regain E-cadherin expression in response to the ectopic environment. The present study provides proof-of-principle that, at least in the presence of hepatocytes, E-cadherin-negative invasive breast cancer cells undergo a mesenchymal to epithelial reverting transtion (MErT) secondary to loss of E-cadherin promoter hypermethylation, and that the hepatocyte-ligated E-cadherin on tumor cells can activate canonical survival pathways in the cancer cells.

E-cadherin is downregulated in carcinomas predominantly by epigenetic processes such as receptor tyrosine kinase signaling and promoter methylation (Becker, Atkinson et al. 1994; Kanai, Oda et al. 1994; Risinger, Berchuck et al. 1994). This is unusual among tumor suppressor in which loss or mutation appears to be the rule. However, it does allow for reversion of this phenotypic change. In MDA-MB-231 cells, that are representative of invasive breast

cancers and in which E-cadherin silencing is due to promoter hypermethylation (Graff, Herman et al. 1995), the CpG islands in the promoter region most proximal to the E-cadherin initiation site are fully methylated, and this exerts a profound effect on mesenchymal nature, as demethylation of these islands by a chemical agent causes re-expression of E-cadherin and loss of invasive ability (Nam, Ino et al. 2004). As we had previously found that co-culture of invasive prostate cancer cells with primary hepatocytes allowed stable re-expression of E-cadherin and that re-expressed E-cadherin participated in a cell-cell adhesive ligation in E-cadherin on hepatocytes (Yates, Shepard et al. 2007), we queried whether this phenomenon was relevant to breast cancer cells. The primary distinction is that in the prostate cancer cells, E-cadherin was downregulated only by receptor tyrosine kinase signaling (Yates et al., 2005), while in breast cancer there is also methylation induced silencing of E-cadherin (Graff et al, 1995). Indeed, culturing of MDA-MB-231 cells with primary hepatocytes resulted in lack of methylation of the E-cadherin promoter and expression of E-cadherin message and protein, thought at a longer time course than the prostate cancer cells. We observed that the loss of methylation was dependent on the proliferation of the cancer cells. This re-expression of E-cadherin on the surface requires also the secondary step of inhibition of receptor tyrosine kinase signaling, as hepatocyte coculture-induced E-cadherin compartmentalized to perinuclear organelles, with mass relocation to the membrane only upon further treatment with a tyrosine kinase inhibitor.

The consequence of this E-cadherin transcriptional upregulation is to allow for additional signaling from the surface. Using the MCF7 cells that retain E-cadherin expresion and epithelial characteristics (Soule, Vazguez et al. 1973), E-cadherin-mediated heterotypic interactions with hepatocytes are functional, inducing plaque formation with Arp2/3 colocalization, which had previously only been shown to occur in epithelial homotypic

interactions. The ability of E-cadherin on MCF7 cells to engage in a heterotypic interaction with hepatocytes was also documented in our centrifugal studies, the results of which showed that the E-cadherin ligation between MCF7 cells and hepatocytes was force-bearing, which is consistent with previous homotypical E-cadherin interaction and our own Arp2/3 localization experiments (Angres, Barth et al. 1996). Finally, we saw that ligation of E-cadherin between MCF7 cells and hepatocytes could transduce activation of both Erk-MAPK and Akt pathways with independent kinetics. These signaling pathways were also actuated in MDA-MB-231 cells after reversion to E-cadherin expression by coculture with hepatocytes. Though cadherin-mediated heterotypic interactions have been previously noted in cells made to express various cadherins, to our knowledge this is the first line of evidence that has shown cancer cells using heterotypic interactions to activate canonical pathways which may very well determine their fate in a foreign *in vitro* environment.

The foundation of our findings rest on the epigenetic reversion we observe when breast cancer cells are co-cultured 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 important. These observations in connection with clinical observations suggest that E-cadherin positive cells do not leave the tumor, but actually regain the protein at distant sites. In addition, a similar phenomenon has been observed in human tumor cells when cultured in either spheroid or transwell invasion chambers, during which E-cadherin promoter methylation is unstable during *in vitro* simulation of metastatic progression (Graff, Gabrielson et al. 2000). Our study brings those previous studies full circle with relevant parenchymal cell interaction and physiologically relevant pathway activation. We

found it especially interesting that the loss of methylation of the E-cadherin promoter in MDA-MB-231 cells was dependent on proliferation of the cancer cells. This dependency on proliferation suggests a passive loss of methylation of the E-cadherin promoter region, in which daughter strands do not participate in the maintenance methylation to which other methylated CpG islands are subjected after DNA replication. Our cursory observations of doubling times of MDA-MB-231 cells suggest that these cells undergo about three rounds of replication in about 6 days (unpublished data). A passive process would result in non-methylation of 87.5% of the alleles in three rounds of replication; this is consistent with our observations of a lack of amplification of methylated loci after 6 days of culture. PCR bias must be taken into account in that the geometric progression of the reaction will favor the template of higher frequency in the reaction, so the complete absence of methylated E-cadherin alleles may not be literal. In addition, it has been demonstrated that suboptimal melting temperature may also contribute bias to nonmethylated alleles. To ensure the efficacy of these results, we used a commercially marketed kit to evaluate the status of the E-cadherin promoter. To our knowledge, we are the first group to demonstrate that normal primary hepatocytes drive the demethylation of the E-cadherin promoter in invasive cancer cells.

Our *in vitro* data is consistent with the storyline that cancer cells respond in a series of tightly woven sequential steps to adapt in foreign environments. There are several possible outcomes or combinations of outcomes after a cell extravasates into a metastatic target tissue: 1) a single cell can succumb to metastatic inefficiencies, leading to necrosis or apoptosis; 2) dormancy has been reported, which may result in short-term remission with long-term recurrence; or 3) the cell could receive either a mitogenic or motogenic signal. We speculate with our current data that the re-expression of E-cadherin in invasive cancer cells may act to counter

apoptosis and either promote long-term malignant recurrence by cloaking the cancer cell with epithelioid-like characteristics, or re-expressed E-cadherin may act to transmit either mitogenic or motogenic signals resulting in proliferation and invasion of the target tissue. Indeed, the downstream proliferatory and migratory pathways of Erk-MAPK as well as the anti-apoptotic effects of Akt are well documented (Torii, Yamamoto et al. 2006; Dillon, White et al. 2007). Further supporting this are quantitative studies that show during the phases of formation and development of micrometastases in secondary organs, cells are particularly vulnerable to apoptosis (Luzzi, MacDonald et al. 1998), yet it is still unclear what gives metastatic cells competency to survive this phase. In the context of the cadherin-switch, evidence that E-cadherin, and not N-cadherin, can modulate EGFR may play a role (Fedor-Chaiken, Hein et al. 2003). E-cadherin re-expression has been speculated to play a central role in establishing metastases (Park, Karesen et al. 2007). In developmental mesenchymal to epithelial transition associated with the dorsal-lateral pathway dissemination from the neural crest, E-cadherin re-expression may enable melanoblasts to degrade the basement membrane locally by metalloproteinase activity, as the cytoplasmic domain of E-cadherin can induce the expression of stromelysin-1 (Delmas, Pla et al. 1999). This is relevant because it has been considered that cancer is merely an emulation of development-associated pathways. Finally, E-cadherin-dependent intercellular adhesion enhances chemoresistance (Nakamura, Kato et al. 2003). This line of data illustrated the chemo-protectant effects of E-cadherin, which is very suggestive with our result of canonical pathway activation. Collectively, we see the picture emerging of E-cadherin re-expression occurring during the micrometastatic stages of cancer formation in ectopic tissues, which not only allows cancer cells to evade metastatic inefficiencies and survive

in the foreign microenvironment, but re-expressed E-cadherin may also contribute to pharmaceutically refractive disease.

We demonstrate in this study that E-cadherin demethylation in breast cancer cells is modulated by co-culture with primary hepatocytes, and the protein expression is dependent on proliferation of the cancer cells. We have implicated that a gene-specific program is executed in the cellular cohesion machinery during co-culture, and E-cadherin is targeted preferentially converse to a global hypomethylation program. Finally, E-cadherin ligation activates canonical pathways in the cancer cells including the MAPK and Akt pathways, which may contribute to either the mitogeneis, motogenesis, or survival in metastasized cells; these different outcomes may be controlled by subtle regulatory factors such as cadherin affinity and clustering that preceeds activation of specific signaling cascades. The search for therapeutic targets that act on molecules specific to the process of metastatic colonization of distant sites, rather than reliance on systemic cytotoxic treatments, is ongoing. Upon further investigation of these mechanisms, E-cadherin may emerge as a molecule that is tumor-suppressant at the primary site but oncogenic at distant metastases.

4.5 MATERIALS AND METHODS

4.5.1 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.

4.5.2 Cell culture and co-culture

Human MDA-MB-231 and MCF7 cells were cultured as described previously (Kassis, Moellinger et al. 1999). Primary rat hepatocytes were isolated and cultured as described previously (Rosenberg, Strom et al. 1982; Yates, Shepard et al. 2007). Cancer cells were separated from hepatocytes using FACS.

4.5.3 Immunohistochemistry

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

4.5.4 SiRNA

Silencer Validated siRNA (#161135, Ambion, Austin, TX) was transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were gorwn in antibiotic-free media till 60% confluent and transfected with siRNA for 24h. The final concentration of siRNA in the experiment was 100nM. Cells were allowed to recover in media with antibiotics for 24h before further experimentation. The control GFP DNA (5'-ACCCGCGCCGAGGUGAAGTT-3', IDT, Skokie, IL) was that from another species.

4.5.5 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). TaqMan Gene Expression Assay Hs00170423_A1 CDHI probe was obtained from Applied Biosystems (Foster City, CA). Amplification and analysis in quadruplicate was run in an Applied Biosystems 7500 Real-Time PCR System. Relative values were normalized by using GAPDH levels as a reference using TaqMan Pre-Developed Human GAPDH Assay Reagent by Applied Biosystems.

4.5.6 Methylation specific PCR

DNA was isolated from co-culture using the DNeasy Blood and Tissue Kit (Qiagen, Velencia, CA). 500ng 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 using the CpG WIZ E-cadherin Amplification Kit per the manufacturer's instructions (Millipore, Temecula, CA). 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. The reactions were performed using Platinum Taq (Invitrogen).

4.5.7 Westernblots, flow cytometry, and immunofluorescence

Proteins were resolved on 7.5% SDS-PAGE and detected by Western analysis. Mouse-monoclonal human-specific E-cadherin (67A4 1:1000), pAkt1/2/3 (Ser473 1:1000), pAkt1/2/3(Thr308 1:1000), pErk (E-4 1:1000) were purchased from Santa Cruz Biotech (Santa Cruz, CA). Flow cytometry was performed using a DakoCytomation CyAN High Speed Analyzer. Co-cultures were non-ezymatically 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 PI-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 was collected on at least 10^6 cells in the appropriate SSC/FSC region. Immunofluorescence was performed similarly to flow cytometry, except primary antibody incubation was performed overnight. Arp3 (G-15 1:100), E-cadherin (67A4 1:100), beta-catenin (C-18 1:100) were purchased from Santa Cruz Biotech and Alexa 488-phalloidin was purchased from Molecular Probes (Carlsbad, CA). Secondary fluorphores included Alexa Fluor-488, -594, and -674 (Molecular Probes).

Visualization was performed on an Olympus Fluoview 1000 confocal microscope (Olympus, Center Valley, PA). Histograms of acquired pictures were widened equally on all comparable images.

4.5.8 Centrifugal assay for fluorescent cell adhesion (CAFCA)

This assay is a modification of the McClay and Giacolmello assays (McClay, Wessel et al. 1981). Cancer cells were non-enzymatically dissociated and labelled with 5 μM Calcein AM (Molecular Probes, Carlsbad, CA, USA). Labelled cancer cells were seeded at a density of 42 000 cells well^{-1} in 96-well plates containing a densely confluent hepatocyte monolayer. The plates were centrifuged for <60s at 50**g** to pellet the cancer cells onto the hepatic monolayer, then incubated at 37°C. At defined times, the plates were inverted and centrifuged at 600**g** for 5 min and then gently washed to remove unbound cells from the hepatocyte monolayer. Fluorescence was measured with a 494/517 bandpass filter set-up from the bottom of the plate by a TECAN Spectra-Fluor plate fluorometer. Absolute emission measurements were background subtracted.

4.5.9 Hepatocyte membrane assays

Heptocyte membranes were isolated as described (Forster and Kaltschmidt 1999). Culture plates were coated with poly-L-lysine per the manufacturer (mol wt 150000-300000 Sigma, St. Louis, MO). Hepatocyte membranes (2 mg protein/cm2) were passively adsorbed onto 6-well culture plates using poly-L-lysine (Sigma) for 10 minutes. MCF7 and MDA-MB-231 cells were quiesced in serum-free media for 3 hours before phosphorylation measurements were taken by

Western blot or *in vitro* kinase assay. MCF7 or MDA-MB-231 cells were seeded at 19E3 cells/cm2 onto 6-well plates coated with the membranes and briefly centrifuged at 50g for 1 minute to synchronize the cells with the membrane layer. At defined time-points, RIPA lysates were taken and for *in vitro kinase assay,* total Erk and Akt proteins were immunoprecipitated from the total protein using agarose conjugated Erk (K-23) and Akt1/2(N-19) antibodies, both purchased from Santa Cruz Biotech. Kinase activity was assayed using the MBP 96-well Assay Kit Chemiluminescence Detection from Upstate (Lake Placid, NY). Luminescence detection was performed with either a Tecan SpectraFluor or a Tecan GENios Pro apparatus.

4.5.10 Primary explants

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

4.6 ACKNOWLEDGEMENTS

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DISCUSSION

5.0 CARCINOMA DISSEMINATION FOLLOWS FROM AN EPITHELIAL-TO-MESENCHYMAL-LIKE TRANSITION (EMT)

The major part of cancer morbidity and mortality results from metastatic colonization of distant organs by carcinomas far from their original sites of development. In tumors of epithelial origin, a change in architecture usually denotes the first signs of neoplastic transformation. While neoplastic transformation is usually a precursor to a more motile phenotype, carcinomas are distinguished from their normal counterparts by a loss of tissue and cell coupling (Guarino, Rubino et al. 2007).

In untransformed tissues, the epithelial cells are polarized and hence the apical face of the cell interacts with the luminal compartment, and the basolateral face of the cell interacts with the stromal compartment. Epithelial cells are often arranged in sheets with adjacent cells tightly connected via tight junctions and gap junctions. At the apical surface in most tissues, the epithelial cells are in contact with body fluids that are either wholly or partly produced by these same cells. These fluids contain biologically factors, growth factors in particular, that are usually inert to the tissue as the receptors for such factors are sequestered on the basolateral surfaces. The tight junctions, constructed upon homotypic binding of E-cadherin, limit the access of the apical fluids to the basolateral spaces and surfaces and the underlying stromal compartment. Distinct and compartmentalized interactions occur solely because of the organized structure of both the cell and the tissue as a whole.

Upon neoplastic transformation, this orderly arrangement is lost. A host of genetic and epigenetic changes occur that lead to degradation and eventual loss of the tight junctions. What

follows contributes fundamentally to the change from neoplasm to carcinoma *in situ*. First, the epithelial cells are now exposed to potential autocrine factors, as the localization of ligand production and receptor expression are no longer segregated. Second, the epithelial cells are released from physical restraints with loss of contact inhibition, allowing for cell movement and even proliferation. And third, the stromal elements are now exposed to many epithelially derived bioactive factors promoting a stromal response. The latter event may change the profile of signals that derive from the stromal compartment as the composition and architectural profile of the stromal elements change. The net sum of these tissue alterations are that the epithelial cells are directed to assume a less differentiated state that converges towards the mesenchymal phenotype, characterized by motility and proliferation with amoeboidal architecture that has limited direct cell-cell communication. This is the so-called epithelial-to-mesenchymal-like transition (EMT) of carcinomas (Peinado, Portillo et al. 2004; Guarino, Rubino et al. 2007).

Carcinoma EMT is distinguished from normal developmental EMT by the fact that the cells do not actually reassume a fully functioning physiological role similar to an epithelially derived cell (Peinado, Portillo et al. 2004). Rather, the attributes of the cells converge on those noted in stromal cells, and the normal epithelial markers and functions are lost indefinitely.

Thus, the first steps in the dedifferentiation process of carcinoma progression is an EMT process very similar to the transformation of primitive epithelial that form the primitive trophoblastic germ layers in normal development. Despite the long-standing recognition of this carcinoma EMT, there is no accepted molecular definition of what constitutes this process (Peinado, Portillo et al. 2004; Thompson, Newgreen et al. 2005; Lee, Dedhar et al. 2006). The vastly heterogeneous etiology of cancer probably contributes to this ambiguity. A picture is emerging of common molecular findings that account for both the histopathological picture and

the cell biology findings. At the minimum, the carcinoma EMT is defined by a loss of normal epithelial architecture, namely both the homogeneous physiological symmetry of the cell, cell-cell contacts, and communications dependent on that architecture. At the molecular level, this is reflected by downregulation or loss of specific cytokeratins and E-cadherin. The latter is of particular importance, because it can be viewed as both a cause and effect of the EMT.

Loss of E-cadherin disrupts not only cell-cell junctions but also allows for loss of the normal organ architecture. The transformed cell's endowed properties allow them to move with many more degrees of freedom within the epithelial layer, as they are no longer constrained within a functional syncytium (Friedl, Zanker et al. 1998). This is a histopathological hallmark of neoplastic transformation. Furthermore, the absence of apical-basal barriers between the cells enables soluble factors secreted by the cells to reach cognate receptors normally segregated on the basolateral surfaces. This autocrine signaling often further reinforces the EMT phenotype because stimulation of most receptors with intrinsic tyrosine kinase activity, particularly the ubiquitous EGF receptor system, drive E-cadherin downregulation and epithelial dedifferentiation (Roura, Miravet et al. 1999; Cavallaro, Schaffhauser et al. 2002). Lastly, the E-cadherin-based cell connection can control global cell signaling, as the catenins that anchor these plaques possess signaling properties dependent upon whether that are in soluble or insoluble complexes (Aberle, Schwartz et al. 1996; Bracke, Van Roy et al. 1996). At an albeit simplistic level, the E-cadherin protein complex at the cell membrane sequesters α-, β- and p120-catenins in epithelial cells and prevents transformative effects on the cellular cytoskeleton and transcriptome. The extent of the global control on cell phenotype exerted by E-cadherin is inherent to its designation as a tumor suppressor; forced re-expression of E-cadherin in invasive carcinoma cells can revert the neoplastic phenotype (Chen and Obrink 1991; Frixen, Behrens et

al. 1991). For these reasons, E-cadherin expression on the cell surface has emerged as a molecular hallmark of the carcinoma EMT.

6.0 METASTATIC SEEDING IS RATE-LIMITING FOR METASTATIC COLONIZATION OF DISTANT ORGANS

Key to carcinoma pathology is the ability of carcinoma cells to survive and thrive in ectopic tissues after their escape from the primary tumor mass. As described in Chapter 5, the carcinoma EMT is critical to this initial escape by enabling individual cell migration and invasion through barrier matrices (Wells 2000). In order to establish metastatic foci, similar transition-like events occur (Luzzi, MacDonald et al. 1998). First, the cells arrest in the dissemination conduits (lymph and blood vessels) by size mismatch between the tumor cells and the diameter of capillaries or because of obstruction of rolling immune cells. The carcinoma cells then must penetrate between the endothelial cells and migrate into the ectopic tissue. This appears to be relatively efficient, as cells extravasate at high frequencies (Chambers, Naumov et al. 2000). The primary rate-limiting step after initial EMT and dissemination is the ability of invasive cells to successfully colonize the ectopic site.

Many years ago it was appreciated that tumor cells disseminate preferentially to ectopic tissues based mainly on the ability of that host tissue to support the survival and proliferation of those cells (Fidler 2001). This is referred to as the 'seed and soil' hypothesis. The stromal component of different tissues and organs produce and present overlapping but distinct matrix components and secreted factors that serve to hospitably accommodate the local cells. Disseminated tumor cells must find a host environment that provides the necessary signals or

these cells must provide the missing factors in an autocrine fashion or other manner that results from transformation. Some of these missing factors might be replaced by genetic mutations that alleviate a particular requirement or are provided by the autocrine signaling inherent in the carcinoma EMT. Still the transformation rarely allows for proliferation outside of a hospitable micro-environment (Bissell 2007).

The survival of these carcinoma cells in ectopic tissue is complicated by the question of tumor cell dormancy. While tumor masses need to approach a billion cells to be detectable as metastases, this should occur relatively quickly if the tumor cells continue to proliferate at the inherent cell-doubling rate quantifiable in most highly aggressive primary tumors. However, in many tumors, metastases appear only years after removal of the primary mass. During this time, the metastatic foci must remain minimal, being either in a state of balanced proliferation-necrosis or as a dormant cancer cell. The key difference between these two options is that in the former, the local milieu must present a full complement of factors for cell proliferation plus signals for death, whereas in the latter, only a partial complement of factors needs to be present to enable cell survival at a lower metabolic load. The question of ectopic site preference, survival, and proliferation reduces to that of the ectopic microenvironment. While there are nascent efforts to fully catalogue tumor microenvironments components, qualitative aspects can be deduced from *in situ* studies. What has been surprising is that many metastatic carcinomas appear more differentiated than the primary tumor from which they derive. In fact, it is well established that many breast cancer metastases to the liver seem to recreate the hepatic architecture without a desmoplastic reaction (Stessels, Van den Eynden et al. 2004). This is inconsistent with the prevailing view that carcinomas continue down a dedifferentiation EMT cascade as they get

more aggressive and disseminate. Rather these reports suggest that the metastatic foci may be more epithelialoid than the primary site.

Examining these metastases with immunohistochemistry confirmed this more differentiated phenotypes in many carcinomas. Interestingly, a number of reports related E-cadherin membrane staining in the metastases but not in the primary carcinoma sites (Putz, Witter et al. 1999; Rubin MA 2001; Kowalski, Rubin et al. 2003). It has been reported *in vitro* that cancer cells expressing E-cadherin are chemoresistant (Green 2004 ; Nakamura 2003). If E-cadherin re-expression is necessary for metastatic dormancy, then these micrometastases would escape chemotherapies that actively target proliferating cells, as is the case for cancer relapse many years after the primary tumor has been eliminated.

7.0 E-CADHERIN IN THE MESENCHYMAL-TO-EPITHELIAL REVERTING TRANSITION

The finding of membrane-expressed E-cadherin in carcinoma metastases raises two possibilities. The first is that the distant metastases formed from E-cadherin-positive cancer cells that disseminated from the primary mass. Even though many primaries are scored as E-cadherin-negative (either the protein is silenced completely or robustly downregulated), this possibility cannot be excluded as the larger primary tumors examined show heterogeneity of expression with some areas of primary tumor expressing the epithelial marker. Still, the clinicopathological correlation between low to absent E-cadherin and tumor dissemination is strong (Birchmeier and Behrens 1994; Cavallaro, Schaffhauser et al. 2002), and the cellular mechanisms supporting this model are logical and repeatedly demonstrated. Carcinoma cells that express E-cadherin show limited migration and invasiveness *in vitro*, and do not form distant metastatic tumors in animal models. However, given the rare nature of metastases despite relatively frequent shedding of cells from the primary mass (Cristofanilli, Budd et al. 2004), these metastases could arise from the unusual escape of an E-cadherin-positive cell in the primary mass, if E-cadherin expression provided a survival and proliferation advantage. This question in its current form cannot be addressed by human tumor surveys because of technical complications, but rather requires experimental probing using clonal carcinoma cell populations. In what appears to be the first report directly addressing this question, this thesis describes how the highly aggressive and E-

cadherin-negative MDA-MB-231 breast carcinoma cell line forms lung metastases from orthotopic primary tumors in the inguinal mammary fat pads of mice; these metastases express E-cadherin. While this is far from conclusive, it does present a proof a principle that E-cadherin-expressing metastases might arise from E-cadherin-negative cells. Re-expression of E-cadherin in the metastatic site, therefore, represents the second possibility. While this is consistent with the histopathological correlations, E-cadherin re-expression would be the result of tumor cell plasticity, that of reverting the carcinoma associated EMT that has not been demonstrated to-date. The nature of E-cadherin downregulation mechanisms suggests how this could be accomplished.

E-cadherin surface expression is downregulated by two known mechanisms, functioning separately at the post-translational and the transcriptional levels. Tyrosine kinase signaling, noted both in response to various growth factors and during neoplastic progression, can downregulate E-cadherin secondary to phosphorylation of β-catenin on the E-cadherin complex (Reynolds, Daniel et al. 1994; Hazan and Norton 1998). EGF and HGF/scatter factor lead to epithelial cell migration away from cohesive masses secondary to E-cadherin-complex dissocation (Birchmeier and Behrens 1994; Miura, Nishimura et al. 2001; Morkel, Huelsken et al. 2003). The endocytosis of E-cadherin after dissociation results in E-cadherin internalization and degradation. This may function in carcinomas, almost all of which have autocrine growth factor signaling loops most often that via the EGF receptor (Wells 2000). Previous reports have found in prostate carcinoma lines that inhibition of this autocrine EGF receptor loop (and likely the EGFR-induced HGF/c-met autocrine loop (Mamoune, Kassis et al. 2004)), either by direct disruption of the signaling loop or by trans-attenuation, results in E-cadherin re-expression and

cell-cell cohesion (Yates, Wells et al. 2005; Yates, Shepard et al. 2007; Yates, Shepard et al. 2007).

Thus, post-translational E-cadherin downregulation represents an available target for counter-regulation by other factors that might be present in the metastatic microenvironment. In most carcinomas, E-cadherin appears to be shut off at the transcriptional level by promoter hypermethylation (Graff, Herman et al. 1995; Jones and Baylin 2002; Strathdee and Brown 2002). This mode of generating a null phenotype differs from other tumor suppressors that are usually victims of other genetic anomalies such as deletions, mutations, or frame-shifts of the coding DNA. While these latter mechanisms are irreversible by their nature, promoter hypermethylation is readily reversible if only by maintenance methylation of the CpG site after daughter strand synthesis. One distinction between normally irreversible tumor suppressors from the E-cadherin tumor suppressor is that loss of the former often occurs early in neoplastic transformation, whereas E-cadherin is relevant throughout many of the sequential steps that result in metastatic colonization of secondary tissues. It has been noted that E-cadherin promoter methylation is unstable (Graff, Gabrielson et al. 2000). This thesis documents that E-cadherin promoter methylation can be selectively lost in breast cancer cells when proliferating in the presence of normal hepatocytes. Thus, there exist signals in the hepatic microenvironment that undo the epigenetic silencing of E-cadherin by promoter methylation.

The above discussion begs the question of what selective advantage does the re-expression of E-cadherin impart? Nascent investigation beyond this thesis is currently answering that question. The speculation can be easily cast that E-cadherin re-expression provides a survival advantage for these cells in a challenging ectopic environment.

Two key pathways activated upon E-cadherin binding are the survival-associated ERK MAP kinase and Akt/PKB cascades (Pece, Chiariello et al. 1999; Pece and Gutkind 2000). Complementing the seed-and-soil hypothesis, functional E-cadherin ligation, either between the secondary parenchymal-cancer cells or cancer cell-cancer cell, would provide necessary signals to endure in the ectopic soil. At the start of metastatic seeding, this requires cell-heterotypic E-cadherin binding, which is reported for the first time in this thesis. Later as the metastatic cells proliferate, E-cadherin ligation may occur between carcinoma cells. To support the possibility of carcinoma-parenchymal binding via E-cadherins, histopathological analyses of many tumors suggest close associations between metastatic carcinoma cells and the neighboring parenchymal cells (Putz, Witter et al. 1999; Rubin MA 2001; Kowalski, Rubin et al. 2003). Furthermore, in an *ex vivo* model of carcinoma metastasis to the liver, close connections are observed on the ultrastructural level (Yates, C et al. 2007a). Also, when prostate carcinoma cells are induced to re-express E-cadherin, both DU-145 and PC3 cells can form heterotypic adhesions with rat hepatocytes (Yates, C C et al. 2007b). These data provide proof of principle that carcinoma cells may re-express E-cadherin in response to the ectopic organ micro-environment so as to establish connections with the resident, non-neoplastic epithelial cells.

8.0 A MODEL FOR THE MESENCHYMAL-EPITHELIAL REVERTING TRANSITION

The following is a speculative model for the mesenchymal-epithelial reverting transition, with a focus on the results present in the thesis. It encompasses a model of tumor cell plasticity with E-cadherin playing a central role in the phenotypic differentiation in both the primary and metastatic sites.

At the primary site, ill-defined genetic and epigenetic changes lead to E-cadherin downregulation, via receptor tyrosine kinase signaling and/or, as is the case with the majority of invasive cancer, promoter hypermethylation (Figure 21, I). The loss of adherens and tight junctions in those cells that progress from neoplastic to carcinoma *in situ* allows physical freedom to move and reorganize, as well as the biological signals derived from autocrine signaling. It is unknown whether the loss of the epithelial barrier further alters the cancer field stroma in a manner that accelerates EMT.

Once free of the physical tethering and architectural restraints imposed by E-cadherin binding, the now mesenchymal-like carcinoma cells can actively migrate through barriers and enter vascular conduits for dissemination to distant sites. While the cell must counter many metastatic inefficiencies during travel to distant sites of the body, the major impediment to metastatic growth appears to be the survival and growth (albeit often delayed or slow) in the ectopic site. This is likely due to the incomplete match of factors and signals in different tissues.

The literature reports that many metastatic carcinoma foci do express E-cadherin, whose downregulation is considered critical if not required for dissemination. This suggests that a subset of the disseminated carcinoma cells receive signals from the ectopic organ microenvironment to revert the EMT process at least partially to enable E-cadherin re-expression (Figure 21, II). As E-cadherin binding elicits canonical survival signals and prevents anoikis (Conacci-Sorrell, Simcha et al. 2003; Reddy, Liu et al. 2005; Kang, Jenabi et al. 2007), the selective advantage is obvious. This thesis documents the process that is the so-called mesenchymal-epithelial reverting transition, or MErT.

Further investigations are required to establish the reverting transition can occur in *ex vivo* and *in vivo* model systems, as important for *de novo* disseminating cancers. Future investigations would include the abrogation and rescue of E-cadherin re-expression to limit metastatic seeding and also inducible E-cadherin re-expression to counteract metastatic inefficiency.

Still, the advantages of E-cadherin re-expression and seeming redifferentiation must be explored. The reversion of the general carcinoma cell phenotype to a less aggressive, less proliferative cell seems counterintuitive particularly in regards to the picture that emerges at the primary site. While activation of ERK-MAP kinase and Akt/PKB signaling pathways promote survival, these pathways are even more strongly elicited by other factors and external signals, without the suppression of proliferation that ensues from E-cadherin adhesions (Simcha et al. 1996) (Figure 21, III). The thesis herein does not answer those questions, but one can speculate that the combination of survival signals with limited proliferation and lowered metabolic requirements are precisely the keys to metastatic seeding. By and large, fast growing cells are most susceptible to death signals whether due to DNA damage, limited nutrients/metabolic

inefficiency, absent pro-survival signals, or apoptotic signals. It is well recognized in oncology that the slowest growing tumors generally are the most resistant to standard chemotherapies. In the ectopic metastatic environment, tumor cells not only carry damaged DNA but also face an absence of external factors, as reflected in the seed and soil hypothesis (Fidler 2003). It is also possible that entry of these foreign cells will elicit a localized inflammatory reaction, with the production of pro-death signals. Thus, not only would activation of the pro-survival ERK-MAP kinase and Akt/PKB pathways protect the cancer cell, but also proliferation and metabolic suppression would provide added survival advantages possibly equilibrated by secretion of TGFβ in the damaged liver (Figure 21, IV) This speculation can be tested in models of chemotherapeutic and apoptotic challenge.

Lastly, one must consider the implications of this proposed carcinoma-related MErT. Currently, E-cadherin is categorized as a tumor suppressor due to its protective role against the carcinoma-related EMT at the primary site. However, interventions to prevent E-cadherin downregulation may perversely promote seeding of disseminated cells as the re-expression phenomena appears to be highly inefficient in the natural course of cancer. Thus, any E-cadherin targeted therapies must be highly cognizant of carcinoma progression dynamics. Second, the formation of E-cadherin ligation to the metastatic target organ parenchyma may result in dormancy at the micrometastatic stage. This would result not only in resistance to chemotherapy with tumor re-emergence, but also have implications for what may lead to delayed relapses. It is tempting to speculate that if the proposed MErT leads to dormancy of early micrometastases, then a secondary insult to the local environment may be what induces renewed carcinoma cell proliferation and escape from E-cadherin-mediated contact inhibition. This, along with other many questions raised by this proposed MErT, awaits further experimentation.

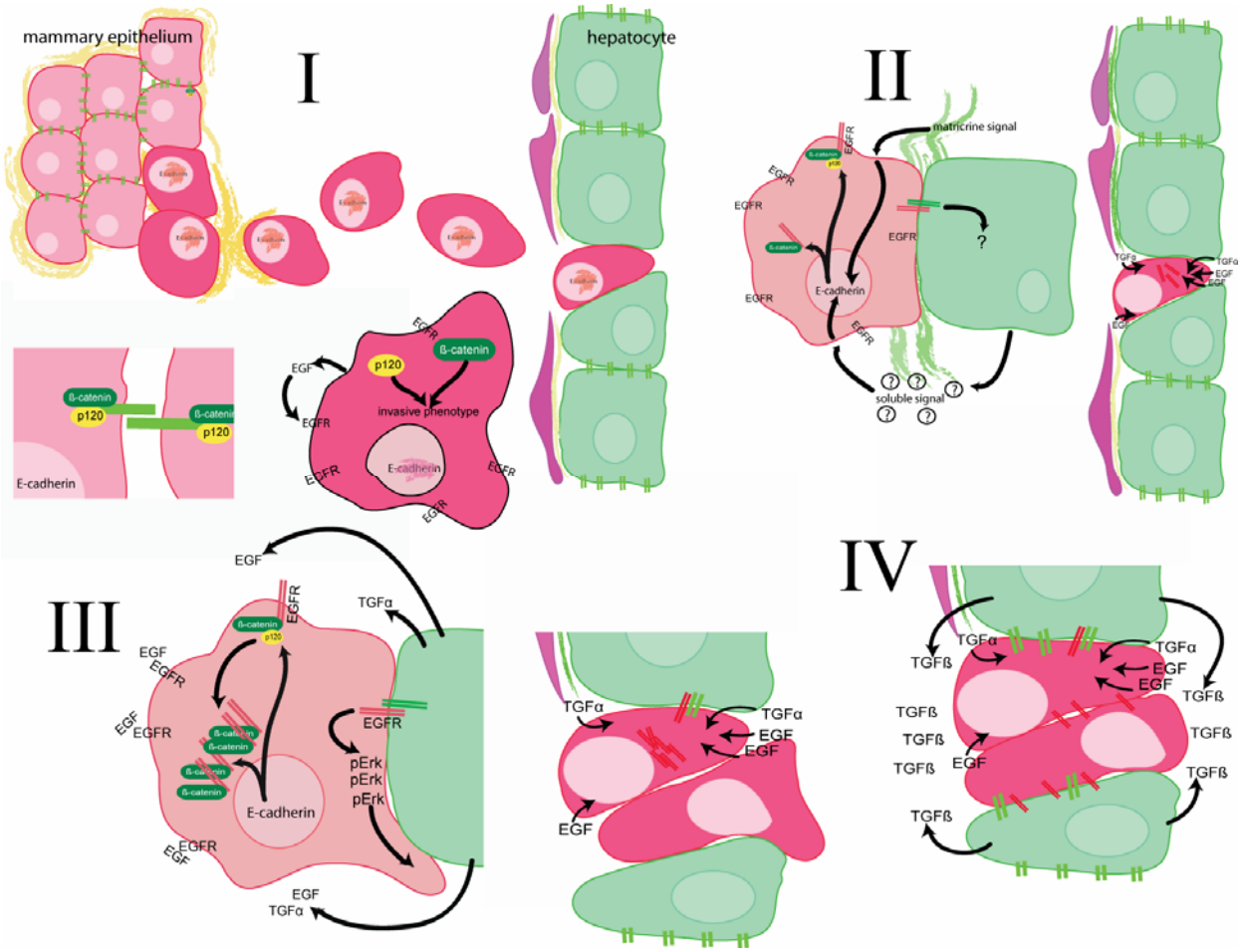

Figure 21. A model for the mesenchymal-epithelial reverting transition (MErT). *I*, The normal mammary epithelium (pink), which expresses E-cadherin (green bars), can become carcinoma *in situ* by EMT (red), which involves hypermethylation of the E-cadherin promoter. The cancer cell disseminates to the liver, extravasates from the sinusoid, and invades the hepatic plates. *II*, In the liver, cancer cells are juxtaposed to hepatocytes and in contact with the basement-like membrane in the liver parenchyma. An unknown signal causes E-cadherin promoter hypomethylation, which allows E-cadherin expression. EGFR ligands, such as EGF and TGFα, either from cancer cell autocrine loops or secreted by the hepatocytes, are present in the microenvironment. *III*, Sustained tyrosine kinase activation causes proliferation, while any E-cadherin participating in trans-ligation with hepatocyte E-cadherin transduces EGFR-dependent Erk-MAPK activation that remains close to the cell surface resulting in motility/invasion. A primary response to the damaged hepatic epithelium may result in release of TGFβ, which results in a stop-proliferation signal. *IV*, In a dormant micrometatasis, an equilibrium has been reached between E-cadherin signaling and TGFβ signaling. The cell has now undergone partial or complete MErT.

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