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TITLE: The Cadherin Interaction as a Rate Limiting Step in Breast Cancer Metastasis to the Liver

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The Cadherin Interaction as a Rate Limiting Step in Breast Cancer Metastasis to the Liver

Epithelial-cadherin’s (E-cadherin) transcriptional silencing in most advanced tumors, due to promoter methylation, enables tumor cells to disseminate from the primary mass. However, E-cadherin-positive metastatic carcinoma foci do originate from mainly E-cadherin-negative primaries. We demonstrate that co-culture of hepatocytes with invasive breast cancer cells lacking E-cadherin triggers an epigenetic reversion in the breast cancer cells resulting in demethylation of the E-cadherin promoter and subsequent expression on the protein level. Further, we show that the E-cadherin ligation between breast cancer cells and hepatocytes is functional and activates the canonical MAPK pathway and Akt pathway in these cancer cells. Our epigenetic-reversion hypothesis for E-cadherin represents not only a paradigm shift in the current thinking that absence of E-cadherin is a fundamental issue, but would also reveal new strategies to combat the initial stages of metastatic disease in breast cancer patients.

Subject Terms: breast cancer, E-cadherin, metastasis, liver, hepatocytes
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INTRODUCTION
My overall objective is to identify molecular elements that enable breast cancer cells to establish metastatic growths. Finding rationale approaches to inhibit rate-limiting events of the metastatic growth is preferable to using systemic therapeutics that are cytotoxic on a systemic level. Cadherins make up a family of adhesion molecules that mediate Ca2+-dependent cell-cell adhesion at points of cell-cell adhesion (Goodwin and Yap 2004). Epithelial-cadherin (E-cadherin), 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. It is generally considered that E-cadherin directs homotypic binding, organizing cells of the same lineage into a functional tissue during morphogenesis (Pla, Moore et al. 2001). 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. This was supported experimentally when poorly differentiated and invasive carcinoma cells could be made less so by transfection with E-cadherin cDNA, with well-differentiated carcinomas becoming more aggressive when antibodies blocked. This supported a designation as a tumor suppressor, even placing E-cadherin at the apex of a “tumor suppressor system” (Vlemingcx, 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 and functionality is re-established at the metastatic site. My central hypothesis tests whether E-cadherin is necessary for cohesion between invasive breast cancer cells and the target hepatic parenchyma and that the formation of E-cadherin and connexin foci is a major rate limiting step in establishing metastatic disease. In my first 12 months of DoD funding, I have made considerable strides into my objective of determining the importance of E-cadherin in the context of metastatic cancer cells in a hepatocyte microenvironment.

BODY
The Statement of Work (Table 1) described two tasks to effectively test our hypothesis. I have tackled the tasks in the order of greatest importance, while keeping to the schedule set forth in the original proposal. The central effort in this first year of funding has been focused on establishing the nature of the E-cadherin interaction between invasive breast cancer cells and hepatocytes. My progress this first year has put me in a very good position to accomplish the tasks within the time-frame provided.

Table 1. Statement of Work

Task 1A. examine the single cell architecture of breast cancer cells interactions with hepatocytes by microscopy – in progress
Task 1B. determine the strength of the interactions using a centrifugal assay – completed

Task 2A. monitor protein localization using fluorescently-tagged E-cadherin – in progress
Task 2B. probe connexon unit integrity and transference – future work
Task 2C. assess the organ relevance of the cohesive interactions using an ex vivo liver bioreactor system – in progress
Task 1.A. **Examine the single-cell architecture of breast cancer cell’s interaction with hepatocytes by microscopy.** This task is completed for 2D culture conditions. I have been successful in capturing the interaction between breast cancer cells and hepatocytes. Using human MCF7 breast cancer cells and freshly isolated rat hepatocytes, I co-cultured the cells together for 90 minutes. I observed that actin localizes to points of juxtaposition between breast cancer cells and hepatocytes (Figure 1A); further, I observed that Arp2/3, the best understood molecular determinant for actin polymerization, co-localizes with E-cadherin plaques directly juxtaposed to hepatocytes (Figure 1B). Together, these data suggest that breast cancer cells are actively anchoring themselves to hepatocytes via E-cadherin.

![Figure 1.](image)

**Task 1.B. Determine the strength of the interactions using a centrifugal assay.** This task is completed. During this first year, I was able to optimize a centrifugal assay (Angres, Barth et al. 1996; Giacomello, Neumayer et al. 1999) to study the adhesion between breast cancer cells and hepatocytes. I found that E-cadherin positive MCF7 breast cancer cells are able to form stable adhesions with hepatocytes in a similar manner to their ability to form stable adhesions with themselves. E-cadherin-negative MDA-231 cells do not form stable adhesions with hepatocytes. Further, if I disable the E-cadherin adhesion mechanism using calcium chelation or a function blocking antibody, I am able to abrogate the cohesion of the MCF7 cells to near background levels. An siRNA construct directed to E-cadherin, which knocks-down the protein significantly, also abrogates cohesion with the hepatocytes. (Figure 2)
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Figure 2. (A) Homotypic cohesion between MCF7-MCF7 populations occurs very similarly to heterotypic cohesion between MCF7-hepatocyte populations. MDA-231 cells do not effectively adhere to the hepatocyte population. (B) Use of calcium chelation or an E-cadherin function blocking antibody abrogates cohesion to near background levels. (C) An siRNA E-cadherin construct knocks-down E-cadherin in MCF7 to <30% of endogenous levels. (D) MCF7 cells transfected with E-cadherin siRNA adhere minimally to the hepatocyte population, while the siRNA control cells adhere similarly with the untreated MCF7 cells.

Task 2.A. **Monitor protein localization using fluorescently-tagged E-cadherin.** The fluorescently-tagged E-cadherin constructs are completed and I have stable cell lines expressing GFP- and CFP-E-cadherin. My antibody-localization studies suggest that protein localization using fluorescently tagged proteins would be effective in determining the kinetics of E-cadherin plaque formation in those membranes juxtaposed to hepatocytes.

Task 2.C. **Assess the organ relevance of the cohesive interactions using an ex vivo liver bioreactor system.** I have completed optimizing the bioreactor system to visualize the interactions between hepatocytes and tumors cells. I am currently working on visualizing the E-cadherin interaction in this *ex vivo* system on micro- and ultrastructural levels.

**Proposed change in Statement of Work task list:**

New Task 2B. **Determine whether E-cadherin binding between breast cancer cells and hepatocytes initiate survival signals in the tumor cells.** During the course of the experiments, it became obvious that with the re-expression of E-cadherin on the breast cancer cells, previously unknown cell-cell contacts and signalings could occur. This unexpected tumor cell interaction with its metastatic micro-environment is postulated to
underlie the phenomenon of chemo-resistance of breast cancer metastases even when the primary lesion responds to chemotherapy. I propose to examine whether these breast carcinoma cell interactions with hepatocytes elicit the canonical survival pathways (ERK MAP kinase and Akt/PKB) in the breast cancer cells. In discussions among collaborators and at national and international meetings, it was felt that this aspect was of potential impact and a higher priority to pursue than the previously proposed connexon postulate. I am requesting to substitute this new task for the existing Task 2B, in order to take advantage of this new and unexpected finding.

**KEY RESEARCH ACCOMPLISHMENTS**

1. MCF7 cells localize actin, which functions as a cytoskeletal anchor for cell adhesion molecules, to points of juxtamembrane contact with hepatocytes.
2. MCF7 cells co-localize Arp2/3 and E-cadherin to points of juxtamembrane contact with hepatocytes.
3. Heterotypic binding between MCF7 cells and hepatocytes occurs in a single logarithmic step with kinetics similar to homotypic binding of MCF7 cells.
4. Functional heterotypic binding between MCF7 cell and hepatocytes is E-cadherin dependent and can be abrogated using calcium chelation, function blocking antibodies, and siRNA specific to E-cadherin.
5. MCF7 cells stably expressing fluorescently tagged E-cadherin.
6. Seed bioreactors with cancer cells.

**REPORTABLE OUTCOMES**

*Abstracts:*


*Papers:*


**CONCLUSIONS**

The first year of this three year study has reached defined milestones and established the base for increasing productivity over the next two years of the award. The first part of the hypothesis has been repeatedly supported in the first task of the proposal. This study has also highlighted new directions concerning the signaling mechanisms that may be propagated upon heterotypic E-cadherin ligation.
Importance: The above experiments provide a ‘proof of concept’ that E-cadherin can participate in transformed cells in vitro. These studies challenge the dogma that E-cadherin ligation can only occur in homotypic populations of healthy epithelial cells.

Recommended changes: The results to-date have completed the first task of the proposal and thusly 40% (by time commitment) of the statement of work. The findings of heterotypic cell-cell interaction have major implications for the cellular biology of in vivo metastatic colonization and thus lead us to introduce pilot experiments examining canonical pathway activation upon heterotypic cadherin ligation between cancer cells and hepatocytes in place of the current Task 2B (see above), along side the continuing sub-objectives of Tasks 1 and 2.

REFERENCES