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13. ABSTRACT <i>(Maximum 200 words)</i> Cadherins are cell-cell adhesion proteins that have been shown to be important in the progression of various tumors. When the gene encoding E-cadherin is mutated in a tumor cell such that it is non-functional, the resulting tumor becomes invasive and the prognosis is unfavorable. In addition, when there are inactivating mutations in the genes encoding the catenins, proteins that serve to hook the transmembrane cadherin up to the cytoskeleton, the result is similar. The cadherins comprise a large family of proteins and it is expression of the various family members that allows cells to recognize one another as "like" or "different". This recognition promotes cell segregation events during the morphogenesis of tissues in a developing embryo. In our laboratory, we have shown that, on occasion, tumor cells turn on the expression of an inappropriate cadherin, that is, one that is different from the surrounding cells. Our hypothesis is that expression of an inappropriate cadherin allows the cells to sort out from their surrounding neighbors and invade the surrounding tissues, thus promoting tumor invasion. Our goal in this project is to determine if this is indeed the case in some breast carcinomas.			
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FOREWORD

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Margaret J. Whitham 6/30/99
PI - Signature Date

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5. Introduction

This is the first year report for my three year IDEA Award. The purpose of this award was to initiate a breast cancer project in my lab. My lab has been focused for many years on the role of cadherin cell adhesion molecules in cellular behavior. Cadherins are cell-surface proteins that play important roles in normal cell-cell interactions. Members of the cadherin family of transmembrane glycoproteins mediate calcium-dependent, homotypic cell-cell adhesion. Numerous studies have implicated E-cadherin as a tumor suppresser protein in carcinomas; i.e., decreased E-cadherin adhesion correlates with a tumor phenotype. The mechanism by which the adhesive function is decreased varies amongst different tumors, but disruption of the function of E-cadherin, regardless of the mechanism, is thought to aid in the formation of a tumor. With this award, I have been able to focus some of my efforts on studying similar questions with regard to human breast cancer cells. Two graduate students focused their efforts on getting the breast cancer project off the ground. Marvin Nieman has been a Ph.D. student in my lab for 3 years and has been studying the effect of dominant-negative cadherins on squamous epithelial cells. He finished up that project and moved on to the breast cancer project. Ryan Prudoff is a masters student in the lab who spent the past year working with Marvin on a survey of a large number of breast cancer cell lines for expression of cadherins. In addition, technical help has been provided first by Indu Palliwal and then by Christine Trapp. The effort of these individuals has contributed to our understanding of how cadherin-mediated cell interactions may influence the behavior of breast cancer cells. In particular, our lab has shown that N-cadherin, which is not normally expressed by epithelial cells, promotes motility and invasion when expressed by human breast cancer cells.

6. Body

At the end of this section, I have copied the portion of the Statement of Work from the original proposal that is appropriate for the first year of this project and have indicate in red which tasks have been completed.

A. Introduction to the study

E-cadherin expression is down regulated or lost in epithelial tumors from various tissues, including stomach, colon, head and neck, bladder, prostate and breast (1-6). In breast carcinoma, clinical studies have shown that loss of E-cadherin expression correlates with a poor prognosis and an invasive phenotype (6, 7).

In vitro studies support the role of E-cadherin as an invasion suppressor gene. Frixen et al. transfected an invasive E-cadherin-negative breast cell line (MDA-MB-435) with mouse E-cadherin and showed that the transfected cells were less invasive in *in vitro* assays (8). When treated with function blocking E-cadherin antibodies, the transfected cells returned to an invasive phenotype thus implicating E-cadherin as an invasion suppressor. Although a number of studies with breast carcinoma cell lines have shown that loss of E-cadherin generally results in an invasive phenotype, important exceptions have been reported. In one study, two E-cadherin-negative cell lines (SKBr3 and MDA-MB-453) were shown to be noninvasive (9). These authors suggested that in order for E-cadherin-negative cells to be invasive they must also express vimentin.

Hazan et al. reported that expression of N-cadherin by breast carcinoma cells correlated with invasion and suggested that invasion was potentiated by N-cadherin-mediated interactions between the breast cancer cells and stromal cells (10). Data from our laboratory suggests that N-cadherin plays a more direct role in the process of invasion and actually promotes invasion by increasing cell motility when it is expressed by oral squamous cell carcinoma-derived cells (11, Islam, S., Kim, J.-B., Johnson, K.R., and Wheelock, M.J. unpublished).

Thus, the information in the literature concerning the role of cadherins in tumor cell invasion is unclear. This prompted us to revisit the question using new reagents generated by our laboratory to examine previously reported, as well as newly derived, breast cancer cell lines. In the present study we show the following: 1) Decreased expression of E-cadherin does not always correlate

with invasion in breast cancer cells; 2) N-cadherin expression does correlate both with invasion and motility in breast cancer cells and likely plays a direct role in promoting invasion; 3) Vimentin expression does not correlate with invasion in cadherin-negative breast cancer cells.

B. Materials and Methods

Cells: Breast carcinoma cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (SkBr3, MDA-MB-435, MDA-MB-436, BT-549, Hs578t) or minimal essential medium (MEM) with 10% fetal bovine serum (MDA-MB-453). The cell lines MCF-7 and MDA-MB-231 were obtained from Mary Hendrix (University of Iowa, Iowa City, IA) and maintained in DMEM with 10% fetal bovine serum. The cell lines SUM159PT and SUM149 were obtained from the University of Michigan Human Breast Cell/Tissue Bank and Data Base and maintained in Ham's F-12 with 5% fetal bovine serum supplemented with insulin (5 mg/ml) and hydrocortisone (1 mg/ml). The cell line SUM1315m02 was obtained from the University of Michigan Human Breast Cell/Tissue Bank and Data Base and maintained in Ham's F-12 with 5% fetal bovine serum supplemented with insulin (5 mg/ml) and epidermal growth factor (10 ng/ml).

Antibodies and reagents: Unless otherwise stated, all reagents were from Sigma Chemical Co. (St. Louis, MO). The rat monoclonal antibody (E9) against the extracellular domain of human E-cadherin and mouse monoclonal antibodies against β -catenin (5H10) and N-cadherin (13A9) have been previously described (12-14).

Extraction of cells: Monolayers of cells were washed with PBS at room temperature and extracted on ice with 2.5 ml/75 cm² flask 10 mM Tris acetate, pH 8.0, containing 0.5% Nonidet P-40 (BDH Chemicals Ltd., Poole, United Kingdom), 1mM EDTA and 2mM PMSF. The cells were scraped followed by vigorous pipetting for 5 min on ice. Insoluble material was removed by centrifugation at 15,000 g for 10 min at 4° C.

Immunofluorescence: Cells were grown on glass coverslips, fixed with Histochoice (Amresco, Solon, OH), permeabilized with methanol for 3 min, washed 3 times with PBS and blocked for 30 min with PBS supplemented with 10% goat serum. Coverslips were exposed to primary antibodies for 1 h, washed 3 times with PBS and exposed to species specific antibodies conjugated to FITC for 1 h. Cells were viewed using a Zeiss Axiophote microscope equipped with the appropriate filters and photographed using Kodak T-MAX 3200 film.

***In vitro* invasion assays and motility assays:** Cells (5×10^5) were plated in the top chamber of Matrigel coated or noncoated transwell membranes (pore size 8 mm; Becton Dickinson, Research Triangle Park, N.C.) for invasion assays or motility assays respectively. In the lower chamber, 3T3 conditioned medium was used as a chemoattractant. The cells were incubated for 24 hours and those which did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. Cells transversing the membrane were stained with Diff-Quick (Dade, Aquada, P.R.). Cells in random fields of view at 100x magnification were counted and expressed as the average number of cells/field of view. Three independent experiments were done except where noted.

C. Results

Previous studies showed that, in the majority of cases, E-cadherin-negative breast cancer cells were invasive (9, 15, 16). However, in some cases E-cadherin-negative cells were non-invasive. These studies did not examine the expression of N-cadherin. A recent study from our laboratory showed that expression of N-cadherin by oral squamous cell carcinoma-derived cells resulted in decreased cell-cell adhesion (11). This study also showed that expression of E-cadherin and N-cadherin was mutually exclusive. We therefore hypothesized that for breast cancer cells to be invasive they must not only down-regulate E-cadherin but also upregulate expression of N-cadherin. To test this, we surveyed a large number of cell lines, many of which had been previously characterized, for expression of E-cadherin, N-cadherin and β -catenin.

The data, which are presented in Table I, supported our hypothesis. The only exception was the cell line MDA-MB-231 which did not express E-cadherin or N-cadherin but, none-the-less, was invasive. This cell line expressed levels of β -catenin that were consistent with expression of a

cadherin (see Fig. 1). Therefore, we examined it using degenerate PCR primers designed to amplify all cadherins and found that it expressed cadherin 11, a cadherin that is expressed by some mesenchymal cells (17). Interestingly, cadherin 11 replaces E-cadherin in cytotrophoblast cells when they invade the uterine wall (18). Thus, we propose that cadherin 11 can act in a manner similar to N-cadherin in promoting cell motility and invasion in breast cancer cells.

Table I

Cell Line	E-cadherin	N-cadherin	β -catenin	Vimentin	Invasion
MCF-7	+ ^{ab}	- ^a	+ ^{ade}	- ^b	no ^{ab}
SUM 149	+ ^a	- ^a	+ ^a	- ^a	no ^a
SKBr3	- ^{ab}	- ^a	- ^{ade}	- ^b	no ^{ab}
MDA-MB-453	- ^{ab}	- ^a	- ^{ade}	- ^b	no ^b
SUM 1315m02	- ^a	- ^a	- ^a	- ^a	no ^a
Hs578t	- ^{ab}	+ ^a	+ ^{ade}	+ ^b	yes ^{ab}
BT549	- ^{ab}	+ ^a	+ ^{ade}	+ ^b	yes ^b
MDA-MB-435	- ^{ab}	+ ^a	+ ^{ae}	+ ^f	yes ^c
MDA-MB-436	- ^{ab}	+ ^a	+ ^{ade}	+ ^b	yes ^b
SUM 159PT	- ^a	+ ^a	+ ^a	+ ^a	yes ^a
MDA-MB-231	- ^{ab}	- ^a	+ ^{ade}	- ^b	yes ^b

^a current study; ^b ref (9); ^c ref (8); ^d ref (15); ^e ref (16); ^f Nieman, M.T., Johnson, K.R. and Wheelock, M.J. unpublished observations

Fig. 1 is an immunoblot of representative cell lines presented in Table 1. In the newly derived SUM cell lines we saw examples of each phenotype: SUM149 expressed E-cadherin and β -catenin; SUM159PT expressed N-cadherin and β -catenin; SUM1315m02 did not express E-cadherin, N-cadherin or β -catenin. To compare the newly established SUM cell lines with well characterized

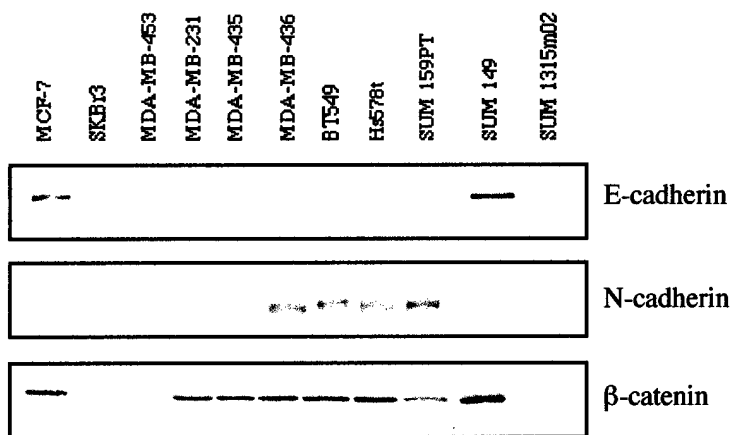


Fig. 1. Cadherin and β -catenin expression in breast carcinoma cell lines. Confluent monolayers of MCF-7, MDA-MB-453, SKBr3, MDA-MB-231, MDA-MB-435, MDA-MB-436, BT-549, Hs578t, SUM159PT, SUM149 or SUM1315m02 were extracted with NP-40. Equal amounts of total protein from each cell extract was resolved by SDS-PAGE, transferred to nitrocellulose and blotted with antibodies against E-cadherin (E9), N-cadherin (13A9) or β -catenin (5H10). Molecular weight

breast cancer cell lines, we performed invasion assays on matrigel-coated membranes and motility assays on uncoated membranes. Fig. 2 presents data from representative cell lines. We included the previously characterized E-cadherin-positive, non-invasive MCF7 cell line and the N-cadherin-positive, invasive Hs578t cell lines as controls. The N-cadherin expressing cell lines, SUM159PT and Hs578t were substantially more invasive and more motile than the E-cadherin expressing cell lines or the cell lines lacking a cadherin (Fig. 2 and Table 1). Surprisingly, the cell lines that did not express any cadherins, SKBr3 and SUM1315m02, were no more motile or invasive than were the E-cadherin expressing cell lines, MCF-7 and SUM149, suggesting that N-cadherin actively promotes motility and invasion.

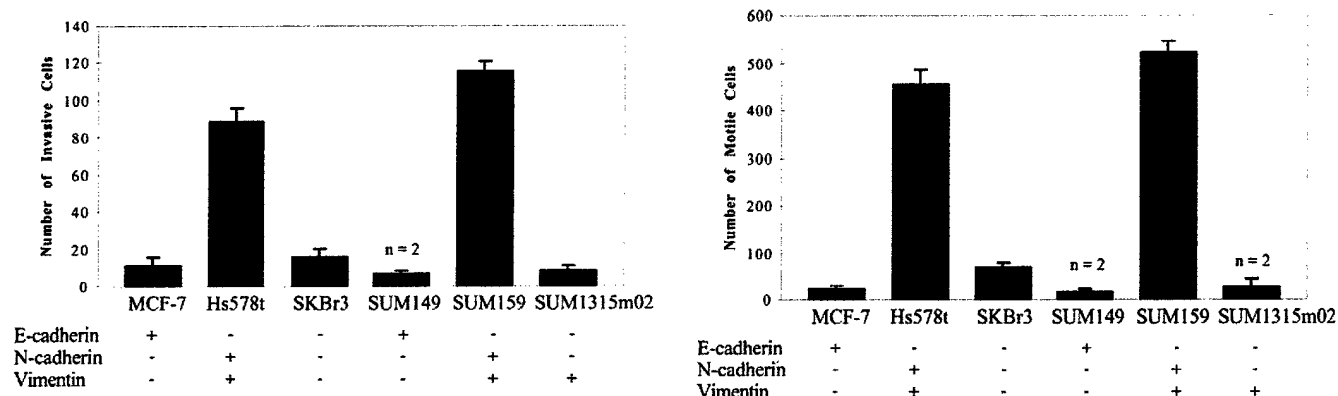


Fig. 2. N-cadherin expression correlates with increased invasiveness and increased motility in breast carcinoma cell lines. Cells (5×10^6) were plated on Matrigel coated or noncoated membranes for invasion assays or motility assays respectively. The cells were incubated for 24 hours and those which did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. The remaining cells were stained and the number of cells which had migrated through the pores was determined by averaging 10 random fields of view at 100x magnification. The data is expressed as the number of cells/field of view and is the average of three independent experiments except where noted. Error bars indicate standard deviation.

To confirm that the N-cadherin expressed by the breast cancer cell lines was functional, we examined cellular localization using immunofluorescence. Results from two representative cell lines (Hs578t and SUM159) are shown in Fig. 3.

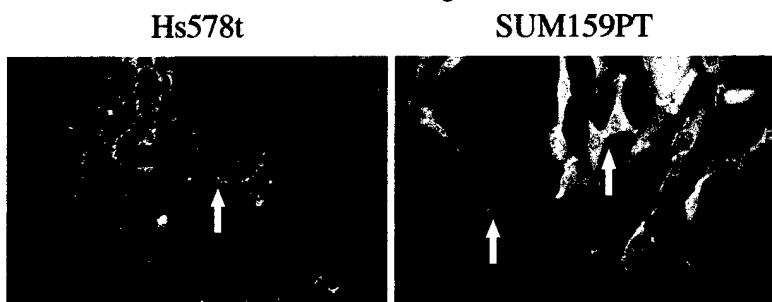


Fig. 3. Localization of N-cadherin in breast cell lines. Cells were grown on glass coverslips and processed for immunofluorescence with a monoclonal antibody against N-cadherin (13A9). Arrows point out regions of cell-cell contact.

Although these cells do not have extensive cell-cell contacts, N-cadherin staining is localized to the region of cell contact as indicated by an arrow (Fig. 3). Since the cytoplasmic domain of cadherins must interact with the catenins to function in cell adhesion, we used co-immunoprecipitation experiments to show that N-cadherin expressed in each of the

cell lines was associated with α -catenin, β -catenin and plakoglobin (data not shown). In addition, the catenins co-localized with N-cadherin in immunofluorescence (data not shown).

Sommers et al. suggested that vimentin expression correlated with an invasive phenotype in E-cadherin-negative breast cancer cell lines. (9, 15). Vimentin is typically expressed by mesenchymal cells which frequently are N-cadherin-positive (14). Since our previous studies had implicated N-cadherin in invasion, and studies by others showed that transfection of vimentin into E-cadherin-positive MCF7 cells only slightly increased invasion and did not convert these cells to a malignant phenotype (20), we suspected that the relevant molecule correlating with invasion in the Sommers studies may have been N-cadherin, not vimentin. To determine if our suspicion was correct, we examined several cell lines for expression of E-cadherin, N-cadherin and vimentin and correlated these findings with invasion. SUM1315m02 was an example of a cell line that was E-cadherin-negative, N-cadherin-negative, vimentin-positive and was not invasive (see Fig. 4 and Fig. 1) suggesting that vimentin alone was not sufficient to induce invasion in E-cadherin-negative cells. Fig. 4 also includes an example of noninvasive cells that were negative for vimentin and positive for E-cadherin (SUM149) and an example of invasive cells that were positive for vimentin and positive for N-cadherin (SUM 159). Each of the N-cadherin-positive cells in this study were also vimentin-positive so we could not examine the invasiveness of vimentin-negative, N-cadherin-positive cells.

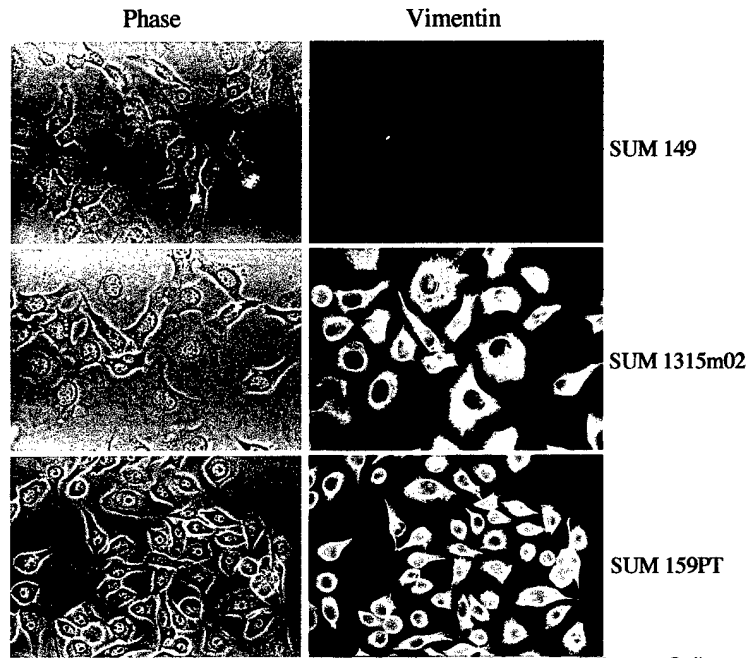


Fig. 4. Morphology and vimentin expression in breast cell lines. Cells were grown on glass coverslips and processed for immunofluorescence with a monoclonal antibody against vimentin (Sigma). Note the extensive network of vimentin filaments in 1315m02, a cell line which is negative for E-cadherin, N-cadherin and b-catenin.

D. Statement of Work from the original grant proposal

Listed below are the technical objectives for the first year of this proposal. In red I have indicated our accomplishments to date.

Technical objective 1. Survey cell lines and biopsies:

Task 1. Months 1-6: Surveying breast cancer cell lines for E-cadherin expression; survey E-cadherin negative cell lines for expression of N-cadherin, P-cadherin, R-cadherin and Cadherin 5. This has been accomplished and is presented in Table I and figure 1 above.

Task 2. Months 1-12: Prepare monoclonal antibodies against the cadherin identified in the preliminary studies. We have prepared an antibody against this cadherin and are in the process of characterizing it.

Task 3. Months 7-8. Survey frozen histological sections for expression of the cadherin identified in Task 2. Frozen sections have been ordered and will be stained with antibodies against the cadherins as soon as they arrive.

Task 4. Months 9-12. If we do not identify one specific cadherin in task 2 we will perform PCR using degenerate primers to identify the cadherin of interest. This is irrelevant at this point as we have identified N-cadherin as expressed by invasive breast carcinoma cells. In addition, we have identified a new, previously unreported cadherin that shares some homology with cadherin 11. The characterization of this cadherin will be a component of the next funding period.

Task 5. Months 12-18. Prepare antibodies against any other newly identified cadherins if necessary. We have prepared antibodies against the newly identified cadherin. The other cadherins we feel are relevant at this point are N-cadherin and cadherin 11. We have already prepared antibodies against N-cadherin and antibodies are commercially available against cadherin 11.

7. Key Research Accomplishments

- We have shown that human breast cancer cells express N-cadherin, cadherin-11 and a newly identified, as yet uncharacterized cadherin.
- We have shown that N-cadherin correlates with an invasive phenotype in human breast cancer cells.
- We have shown that vimentin expression does not correlate with an invasive phenotype in human breast cancer cells.
- We have identified a new cadherin that is expressed in human breast cancer cells and have prepared an antibody against this cadherin.

8. Reportable Outcomes

The data discussed above was presented at the 89th annual meeting of The American Association for Cancer Research in the form of a poster entitled "**N-cadherin expression promotes invasive phenotype in breast carcinoma cell lines**" by Nieman, M.T., Ethier, S.P., Johnson, K.R., and Wheelock, M.J. The data will be submitted as a full paper once we have completed an experiment to show that N-cadherin is indeed responsible for the altered phenotype of these cells.

In addition, the work was presented as a poster at the Cell Contact and Adhesion Gordon Conference held in June 1999.

My student was selected to receive a travel award to attend the 89th meeting of the American Association for Cancer Research in order to present this work.

9. Conclusions

In the current study, we have demonstrated that N-cadherin expression in human breast carcinoma cells correlates with an invasive phenotype. Cell lines that did not express any classical cadherins, as evidenced by lack of β -catenin protein, had motility and invasion characteristics similar to cells that expressed E-cadherin. Our hypothesis that loss of E-cadherin alone is not sufficient to increase invasion in breast carcinoma cells is supported by the observation that function blocking antibodies against E-cadherin did not confer a motile, invasive phenotype on E-cadherin-expressing MCF-7 cells (9). In fact, the current study suggests that expression of N-cadherin, or a similar cadherin such as cadherin 11, may be necessary for increased motility and invasion in breast cancer cells. Hazan et al. suggested that tumor cells expressing N-cadherin may be more invasive due to interactions with N-cadherin-expressing stromal cells (10). Our *in vitro* invasion and motility assays, which do not depend on interactions with stromal cells, suggest that N-cadherin plays a more direct role in cell motility. A recent clinical study suggested that inactivation of E-cadherin is an early event in the progression of lobular breast carcinomas (21). We might suggest here that a subsequent event would be activation of N-cadherin expression.

Previous work suggested that vimentin correlated with an invasive phenotype in breast carcinoma cell lines. Here, we have shown that vimentin alone does not confer an invasive phenotype as the cell line SUM1315m02 expresses vimentin but lacks cadherin expression and is not invasive. Rather, we would suggest that co-expression of vimentin and N-cadherin may correlate with an invasive phenotype in human breast carcinoma cells. We were not able to find an N-cadherin positive cell line that did not express vimentin so we cannot say that N-cadherin expression in the absence vimentin would correlate with invasion. We are currently investigating this idea by attempting to transfect N-cadherin into the vimentin-negative SKBr3 cell line.

At first glance it might seem unlikely that expression of a cell adhesion molecule would confer a motile and invasive phenotype. However, motile cells such as fibroblasts express N-cadherin (14) and a switch from E-cadherin to N-cadherin occurs during gastrulation when epiblast cells ingress through the primitive streak to form the mesoderm (22, 23). In addition, N-cadherin has been shown to correlate with a motile, fibroblastic phenotype in squamous cell carcinomas of the head and neck (11) and in melanomas (24). Thus, it is feasible to hypothesize that an increase in N-cadherin expression would increase the invasive potential of breast carcinoma cells.

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11. Appendix

Below is the abstract of this work that was presented at the 89th meeting of the American Association for Cancer Research.

N-cadherin expression promotes invasive phenotype in breast carcinoma cell lines. Nieman, M.T.¹, Ethier, S.P.², Johnson, K.R.¹, Wheelock, M.J.¹ ¹The University of Toledo, Department of Biology, Toledo, OH 43606, ²The University of Michigan Medical School, Department of Radiation Oncology, Ann Arbor, MI 48109

The E-cadherin/catenin complex is important in maintaining an epithelial phenotype and normal tissue architecture. E-cadherin expression is decreased or absent in invasive epithelial tumors from various tissues. This has led to the hypothesis that down regulation of E-cadherin allows a cell to disassociate from the surrounding cells and to invade the surrounding tissues. In vitro data has shown that not all E-cadherin negative breast carcinoma cell lines are invasive. In the present study, we examine the expression of N-cadherin in breast carcinoma cell lines. The E-cadherin negative cell lines that have previously been described as invasive (Hs578t, MDA-MB-435, MDA-MB-436 and BT-549) as well as a new invasive cell line (SUM159) expressed N-cadherin. In contrast, E-cadherin negative cell lines which were not invasive (SKBr-3, MDA-MB-453 and SUM1315m02) did not express N-cadherin. The N-cadherin in these breast cell lines is functional in that it forms a complex with α -catenin and β -catenin as determined by coimmunoprecipitation experiments. These observations suggest that the loss of E-cadherin is not sufficient to confer an invasive phenotype in breast carcinoma cell lines and that the expression of a mesenchymal cadherin (N-cadherin) in breast epithelial cells promotes invasiveness.