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PRINCIPAL INVESTIGATOR: Margaret J. Wheelock, Ph.D.

CONTRACTING ORGANIZATION: University of Toledo
Toledo, Ohio 43606-3390

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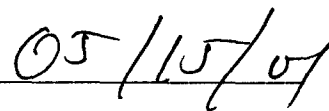
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13. ABSTRACT (Maximum 200 Words) E-cadherin is a transmembrane glycoprotein that mediates calcium-dependent, homotypic cell-cell adhesion and plays a role in maintaining the normal phenotype of epithelial cells. Decreased expression of E-cadherin has been correlated with increased invasiveness of breast cancer. In other systems, inappropriate expression of a non-epithelial cadherin, such as N-cadherin, by an epithelial cell has been shown to down-regulate E-cadherin expression and to contribute to a cell motility. During the first 2 years of this study we showed that N-cadherin promotes cell motility and invasion in breast cancer cells; that decreased expression of E-cadherin does not necessarily correlate with invasion in breast cancer cells; that forced expression of E-cadherin in invasive, N-cadherin positive cells does not reduce their motility or invasive capacity; that forced expression of N-cadherin in non-invasive, E-cadherin-positive cells produces an invasive cell even though these cells continue to express high levels of E-cadherin; and that N-cadherin-dependent motility may be mediated by fibroblast growth factor receptor signaling. This year we report that the extracellular domain of N-cadherin is responsible for this activity and map the domain of N-cadherin that is active in mediating cell motility to a 70 amino acid portion of extracellular repeat 4.				
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Introduction

This is the third year report for my four year Career Development Award. The purpose of this award was to provide me with additional time and support necessary to become versed in the biology of breast epithelial cells and the transformation of these cells into a cancerous state. The Chair of our department agreed to relieve me of my formal teaching responsibilities (which constituted 40% of my effort) once I received the award in order to permit me to focus my efforts on breast cancer research. I was indeed relieved of all of my formal teaching responsibilities. I continued, during the course of the year, to train graduate students. 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 4 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 was a masters student in the lab who spent 2 years working with Marvin on a survey of a large number of breast cancer cell lines for expression of cadherins. Ryan finished his master's research and is now a medical student at Ohio University. Both Marvin Nieman and Ryan Prudoff graduated and their work will be continued by Young Kim and Emhonta Johnson. Dr. Jani Lewis was hired by the department to do my teaching. In addition to her responsibilities as a teacher, Jani will spend time in my laboratory doing research on a project of her choice. In summary, the award of this grant has accomplished its goal which was to provide me with the time to establish a focus in breast cancer research.

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 3 years of this project and have indicated in green those tasks which were completed during the first 2 years of the project and in red those tasks which have been completed this year. In dark blue I have indicated what I intend to do during the next year.

1. Introduction to the study

Cadherins comprise a family of calcium-dependent cell-cell adhesion proteins that play important roles in embryonic development and in maintenance of normal tissue architecture. As the transmembrane component of cellular junctions, the cadherins are composed of three segments: an extracellular domain comprised of five homologous repeats that mediates adhesion, a single pass transmembrane domain, and a conserved cytoplasmic domain that interacts with catenins to link cadherins to the actin cytoskeleton (reviewed in Wheelock et al., 1996). The catenins were first identified as proteins that co-immunoprecipitated with cadherins and were termed α -, β - and γ -catenin according to their mobility on SDS-PAGE. Either β - or γ -catenin binds directly to the cadherin and to α -catenin, while α -catenin associates directly and indirectly with actin filaments (Stappert and Kemler, 1994; Knudsen et al., 1995; Rimm et al., 1995; Nieset et al., 1997). The ability of cadherins to simultaneously self-associate and link to the actin cytoskeleton mediates both the cell recognition required for cell sorting and the strong cell-cell adhesion needed to form tissues.

In addition to their structural role in the adherens junction, catenins are thought to regulate the adhesive activity of cadherins. For example, phosphorylation of β -catenin in Src transformed cells may contribute to the non-adhesive phenotype of these cells (Matsuyoshi et al., 1992; Hamaguchi et al., 1993). In its capacity as a signaling molecule, β -catenin plays a critical role in patterning during development and in maintenance of the normal cellular phenotype during tumorigenesis (Miller et al., 1999; Cadigan and Nusse, 1997; Polakis et al., 1999). The signaling functions of β -catenin are due to its interactions with transcription factors of the LEF/TCF family and with receptor tyrosine kinases. In addition, p120^{ctn}, originally identified as a Src substrate and subsequently shown to bind to the cytoplasmic domain of cadherins, has been suggested to play a role in regulating the adhesive activity of cadherins (Reynolds et al., 1994; Shibamoto et al., 1995; Daniel and Reynolds, 1995). P120^{ctn} binds to the juxtamembrane domain of cadherins, a domain that has been implicated in cadherin clustering and cell motility (Finnemann et al. 1997, Navarro et

al., 1998; Yap et al., 1998; Chen et al., 1997). It is thought that p120^{cas} influences the strength of cadherin-mediated adhesion, perhaps by influencing the organization of the actin cytoskeleton (Aono et al., 1999; Ohkubo and Ozawa, 1999; Thoreson et al., 2000). Thus, a number of studies have shown that the cytoplasmic domain of cadherins interacts with proteins that likely regulate adhesive function.

The extracellular domain of classic cadherins is involved in the interactions that mediate adhesion. The earliest evidence for this came from studies demonstrating that antibodies produced against the extracellular domain of cadherins inhibit cell adhesion. The extracellular domain of cadherins can be divided into 5 "extracellular cadherin structural domains" (EC) each of which consists of approximately 110 amino acids and contains the conserved motifs LDRE, DXNDN and DXD (Oda et al., 1994). EC-1 is the most N-terminal domain and is responsible for adhesive activity (reviewed in Takeichi, 1990). The binding sites for most mAbs that block the adhesive function of E, P and N-cadherin have been mapped to EC-1 (reviewed in Takeichi, 1990), a domain that contains an HAV tripeptide that has been implicated in adhesion. Synthetic peptides containing an HAV sequence inhibit cadherin-mediated adhesion, mimicking the activity of antibodies directed against EC-1 (Blaschuk et al., 1990). Structural studies have shown that the HAV tripeptide and surrounding residues mediate self association by interacting with a separate set of amino acids within EC-1 of the interacting cadherin on the adjacent cell (Shapiro et al., 1995). In addition, mutations in the N-terminus of classical cadherins or deletion of EC-1 results in molecules that do not mediate cell adhesion (Ozawa and Kemler, 1990; Nose et al., 1990; Ozawa et al., 1990; Shan et al., 1999).

It was observed that cells expressing different members of the classical cadherin family segregate from one another when mixed together in culture (reviewed in Takeichi, 1990). It has been suggested that this preferential binding of cadherins plays an important role in the sorting activities of embryonic cells. Interestingly, the binding specificity of cadherin molecules also maps to EC-1. When the amino-terminal regions of E-cadherin were replaced with those of P- or N-cadherin, the chimeric molecules displayed P- or N-cadherin specificity, respectively (Nose et al., 1990; Shan et al., 1999). Thus, EC-1 of the classical cadherins is responsible not only for cadherin binding activity but also for cadherin specificity.

A number of studies have implicated E-cadherin in maintenance of the normal phenotype of epithelial cells (reviewed in El-Bahrawy and Pignatelli, 1998; Behrens, 1999). For example, invasive, fibroblast-like carcinoma cells could be converted to a non-invasive phenotype by transfection with a cDNA encoding E-cadherin (Frixen et al., 1991) and forced expression of E-cadherin in rat astrocytoma cells suppressed motility (Chen et al., 1997). Likewise, transfection of invasive E-cadherin-negative cell lines with E-cadherin resulted in cells that were less invasive in *in vitro* assays (Frixen et al., 1991; Luo et al., 1999). It has been suggested that, unlike E-cadherin, N-cadherin may promote motility and invasion in carcinoma cells. For example, Hazan et al. (1997) 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 cancer cells and stromal cells. Studies from our laboratory suggest that N-cadherin plays a direct role in invasion. Expression of N-cadherin by squamous epithelial cells resulted in a scattered phenotype accompanied by an epithelial to mesenchyme transition. In this study, forced expression of N-cadherin in cultured cells resulted in down-regulation of the expression of E-cadherin (Islam et al., 1996). Thus it was difficult to separate the characteristics due to decreased expression of E-cadherin from those due to increased expression of N-cadherin. In a second study we showed that expression of N-cadherin by BT-20 human breast epithelial cells converted the cells to a motile and invasive phenotype. In this case increased motility was not accompanied by decreased E-cadherin expression, suggesting that N-cadherin plays a direct role in epithelial cell motility (Nieman et al., 1999). The results of this second study were included in last year's progress report and have now been published in the Journal of Cell Biology. A copy of this paper is included in this year's appendix. Hazan et al. (2000) confirmed our results using the MCF7 human breast carcinoma cell line. Importantly, these authors extended their studies to show that

N-cadherin expression increased metastasis when the transfected cells were injected into nude mice. Thus, there is evidence that expression of an inappropriate cadherin may alter cellular behavior suggesting that cadherins function as more than just cell-cell adhesion molecules.

The present study was designed to determine which domains of N-cadherin are responsible for both the epithelial to mesenchymal transition that we have seen in squamous epithelial cells and the increased motility seen in breast cancer cells. To address this question, we made use of chimeric cadherins constructed between N-cadherin and E-cadherin. The chimeras were transfected into the SCC1 oral squamous epithelial cell line to determine their effect on cell morphology and into the BT20 breast cancer cell line to investigate influences on cell behavior. We found that a 70 amino acid portion of EC-4 of N-cadherin was both necessary and sufficient to promote both activities. This study makes two important points: 1) it shows that cadherins promote differential cellular behavior and 2) it identifies a novel activity that maps to the extracellular domain of N-cadherin.

2. Materials and Methods

Antibodies and Reagents

Mouse mAbs against the cytoplasmic domain of human N-cadherin (13A9), α -catenin (1G5) and β -catenin (6E3) have been described (Knudsen et al., 1995; Johnson et al., 1993). Mouse mAbs against the extracellular amino acids 92-593 of human N-cadherin (8C11) and the cytoplasmic domain of human E-cadherin (4A2) were prepared as described (Johnson et al., 1993). Mouse mAb against the myc-epitope (9E10.2) was a gift from Dr. K. Green (Northwestern University, Chicago, IL). All reagents were from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

Cell Culture

The human squamous carcinoma cell line UM-SCC-1 (SCC-1) and the human breast cancer cell line BT20 were maintained in MEM 10% FCS (Hyclone Laboratories, Logan, UT). A cadherin-negative derivative of A431 called A431D was described previously (Lewis et al. 1997) and was maintained in DME 10% FCS.

Molecular Constructs

Human N-cadherin (GenBank S42303; a gift of Dr. A. Ben Ze'ev; Weizmann Institute, Rehovot, Israel) and human E-cadherin (Lewis et al., 1997) were used for construction of chimeric cadherins using recombinant PCR (Higuchi et al., 1988). Each recombinant PCR product was subcloned, and a clone was identified that encoded the complete, correct amino acid sequence. Each full-length construct was assembled and moved into a derivative of pLKneo (Hirt et al., 1992). Amino acid sequences across the chimeric junctions are given in Table I.

Chimera	Junction	Details
E/N	L L F L / K R R D	joins E-cad 731 to N-cad 747
N/E and N/E myc	V V W M / R R R A	joins N-cad 746 to E-cad 732
N/E5a myc	A G F P / T A E L	joins N-cad 637 to E-cad 627
N/E5 myc	D N A P / I P E P	joins N-cad 603 to E-cad 594
N/E4 myc	N I R Y / R I W R	joins N-cad 534 to E-cad 524
N/E3 myc	N A V Y / T I L N	joins N-cad 420 to E-cad 414
N/E2 myc	M L R Y / T I L S	joins N-cad 306 to E-cad 303
E/N/E myc	5' K I T Y / T K L S	joins E-cad 523 to N-cad 535
	3' D N A P / I P E P	joins N-cad 603 to E-cad 594
N/E/N myc	5' N I R Y / R I W R	joins N-cad 534 to E-cad 524
	3' D N A P / Q V L P	joins E-cad 553 to N-cad 604

Table I. In our N-cadherin cDNA, there is an additional leucine (CTG) following amino acid 11. The entire open reading frame is thus 906 codons. The numbers in the table reflect this change to S42303. The E-cadherin cDNA has an open reading frame of 882 codons.

The E/N-chimera has the extracellular and transmembrane domains of E-cadherin and the cytoplasmic domain of N-cadherin, while the N/E-chimera has the extracellular and transmembrane domains of N-cadherin and the cytoplasmic domain of E-cadherin.

To make the N/E-myc construct, the cytoplasmic domain of E-cadherin was amplified such that the stop codon was replaced with a restriction site. The PCR product was inserted into a modified pSPUTK (Falcone and Andrews, 1991) to add a C-terminal 2X-myc tag (Nieman et al., 1999a). A fragment from the N/E-chimera was moved into the above construct to make the full-length N/E-myc cDNA. To make N/E5a-myc, N/E5-myc and N/E4-myc, recombinant PCR fragments were used to replace portions of the N/E-myc construct. To make N/E3-myc, a recombinant PCR fragment was used to replace a portion of the N/E4-myc construct. In a similar fashion, the N/E2-myc construct was made by replacing a portion of the N/E3-myc construct with a recombinant PCR fragment. Each of these full-length cadherins was then inserted into pLKpac (Islam et al., 1996).

The E/N/E-myc chimera was generated by substituting nucleotides encoding N-cadherin amino acids 535-603 for the corresponding E-cadherin sequence. Recombinant PCR was performed to create the 5' junction between E- and N-cadherin. The product of this reaction was used in a second recombinant PCR step to create the 3' junction between N- and E-cadherin. The resulting PCR product was used to replace a portion of E-cadherin-2X-myc (Nieman et al., 1999b). The N/E/N-myc construct was prepared similarly. In this case, the final PCR product was used to replace a portion of the N-cadherin sequence in an N-cadherin-2X-myc construct. The chimeras were inserted into pLKpac.

Transfections

SCC1 and A431D cells were transfected using calcium phosphate and BT20 by electroporation as previously described (Nieman et al., 1999a). Stable clones were selected by growth in puromycin (1 µg/ml) or G418 (1 mg/ml). Clones were screened for transgene expression by immunoblot analysis. Clones that showed homogenous expression by immunofluorescence were selected. For morphological studies, at least three clones from each transfection were examined.

Microscopy

Cells were grown on glass coverslips, fixed with Histochoice (Amresco, Solon, OH), blocked using PBS 10% goat serum and stained with primary antibodies for 1 h followed by secondary antibody (Jackson Immuno Research Laboratories, West Grove, PA). Photos were taken with a Zeiss Axiophot microscope (Carl Zeiss, Thornwood, NY) equipped with a SPOT CCD camera (Spot Diagnostic, Sterling Heights, MI).

Cell Fractionation and Protein Assays

Confluent monolayers were washed with PBS and extracted on ice with TNE buffer (10 mM Tris-acetate, pH 8.0, 0.5 % Nonidet P-40, 1 mM EDTA, 2 mM PMSF). Extracts were mixed at 4 °C for 30 minutes and centrifuged at 15,000 rpm for 15 minutes. Protein determinations were done using a Bio-Rad kit (Bio-Rad, Richmond, CA).

Immunoprecipitations, Electrophoresis and Immunoblot analysis

A 300 µl aliquot of cell extract was incubated with 300 µl mAb for 30 minutes at 4 °C. Protein A-beads were added and incubation was continued for 30 minutes. Immune complexes were washed with TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Tween 20) 5X at 4 °C. Pellets were resolved by SDS-PAGE and immunoblotted as described (Johnson et al., 1993).

Aggregation and Motility Assays

Hanging drops of 5000 cells in 20 µl were applied to tissue culture lids and analyzed after 24 h at 37 °C. Motility assays were done as described (Nieman et al., 1999a).

3. Results

Previous studies from our laboratory showed that expression of N-cadherin by squamous epithelial cells or breast cancer cells altered cellular behavior. In oral squamous epithelial cells, expression of N-cadherin produced a scattered phenotype with an epithelial to mesenchymal transition (Islam et al., 1996). In breast cancer cells, expression of N-cadherin did not alter the morphology of the cells but did induce cell motility and invasion (Nieman et al., 1999a). In the current study we sought to determine how N-cadherin functioned to alter the phenotype of epithelial cells. We predicted that the cytoplasmic domain of N-cadherin was capable of initiating a signal transduction pathway that resulted in increased cellular motility. To determine if this were indeed the case, we engineered two chimeric cadherins. The first E/N-cadherin consisted of the extracellular and transmembrane domains of E-cadherin joined to the cytoplasmic domain of N-cadherin. The second chimera consisted of the extracellular and transmembrane domains of N-cadherin joined to the cytoplasmic domain of E-cadherin (N/E-cadherin). A schematic of these two chimeric cadherins is presented in Fig. 1A.

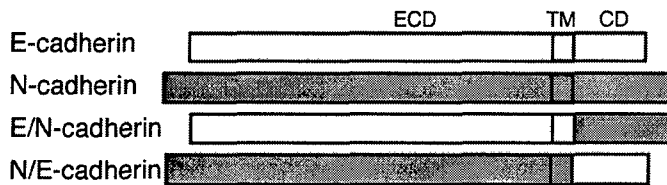


Figure 1A. Diagram of chimeric cadherins. Chimeric cadherins consisting of the extracellular and transmembrane domains of E-cadherin (white) and the cytoplasmic domain of N-cadherin (gray) or consisting of the extracellular and transmembrane domains of N-cadherin and the cytoplasmic domain of E-cadherin were cloned into pLK-neo2.

The extracellular domain of N-cadherin influences epithelial cell behavior

Our goal was to test E/N-cadherin and N/E-cadherin for effects on cellular morphology and behavior using two model systems we had already established. In the first model system the oral squamous epithelial cell line SCC1 undergoes a significant and readily discernable morphological change from a typical epithelial cell to a fibroblastic cell when transfected with N-cadherin. In the second model system, the human breast cancer cell line BT20 changes from a relatively non-motile to a highly motile cell when transfected with N-cadherin. Interestingly, the BT20 cells do not undergo a morphological change when they are transfected with N-cadherin, suggesting that the effects of N-cadherin differ somewhat between these two different types of epithelial cells. Before testing the effect our chimeric cadherins had on the morphology and behavior of cells, it was important to show that each chimera was a functional adhesion molecule. To determine if the chimeras were functional, we transfected them into the cadherin-negative A431D cell line which has been previously described by our laboratory (Lewis et al., 1997, Thoreson, et al., 2000). Figs. 1 B and C show that the chimeric cadherins were expressed by the A431D cells at the expected size (Fig. 1B) and that they associated with catenins in an immunoprecipitation assay (Fig. 1C).

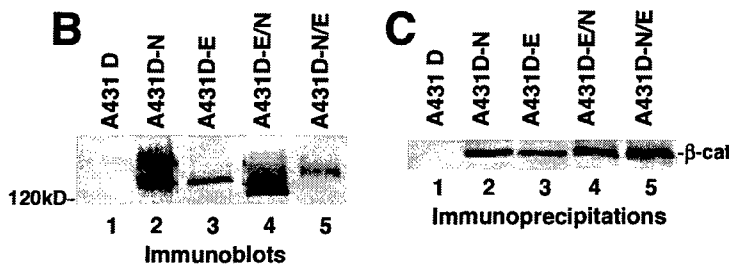


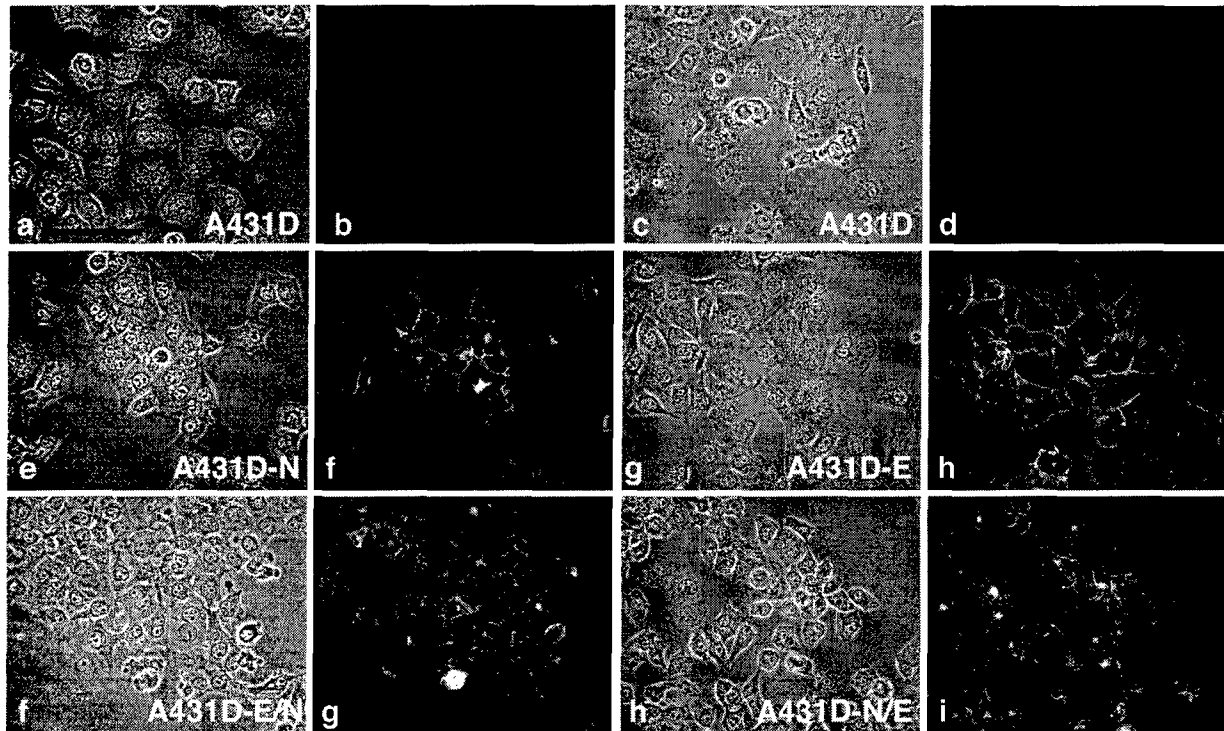
Fig. 1 B and C. Expression of E/N- and N/E-cadherin in A431D cells. B. A431D cells were transfected with E-cadherin, N-cadherin, E/N-cadherin or N/E-cadherin and examined for transgene expression by immunoblotting with antibodies against the cytoplasmic domain of N-cadherin (lanes 1, 2 and 4), the

extracellular domain of N-cadherin (lane 5) or the extracellular domain of E-cadherin (lane 3).

Note: In some cases we observed a number of processing variants when transfected cadherins were overexpressed in cells. C. Extracts were immunoprecipitated, resolved by SDS-PAGE and immunoblotted for β-catenin.

In addition, we showed that the chimeric cadherins were located at the cell surface (Fig. 1D) and that they mediated cell aggregation (Fig. 1E). These data demonstrate that both E/N-cadherin and N/E-cadherin function as adhesion molecules in a manner similar to E-cadherin or N-cadherin.

D



E

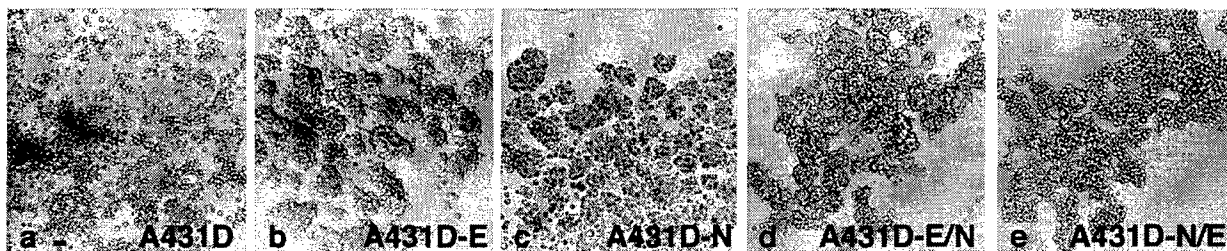


Fig 1 D and E. Expression of E/N- and N/E-cadherin in A431D cells. *D.* Untransfected A431 D cells (Panels a-d) or A431D cells expressing N-cadherin (Panels e and f), E-cadherin (Panels g and h), E/N-cadherin (Panels f and g) or N/E-cadherin (Panels h and i) were processed for immunofluorescence microscopy using the appropriate cadherin antibody. Corresponding phase and fluorescence micrographs are shown. *E.* Untransfected A431 D cells (Panel a) or A431D cells expressing E-cadherin (Panel b), N-cadherin (Panel c), E/N-cadherin (Panel d) or N/E-cadherin (Panel e) were tested for their ability to aggregate in a hanging drop aggregation assay. Bar 10 μ m.

E/N-cadherin and N/E-cadherin were transfected into SCC1 cells and analyzed for their ability to induce an epithelial to mesenchymal transition. Each chimera was highly expressed (Fig. 2A), co-immunoprecipitated with β -catenin (Fig. 2B) and was localized at the cell surface (Fig. 2C, panels f and h). To our surprise, the N/E-cadherin (Fig. 2C, panel g) produced a change in morphology similar to that seen with intact N-cadherin (Fig. 2C, panel c), whereas the E/N-cadherin did not effect the morphology of these cells (Fig. 2C, panel e).

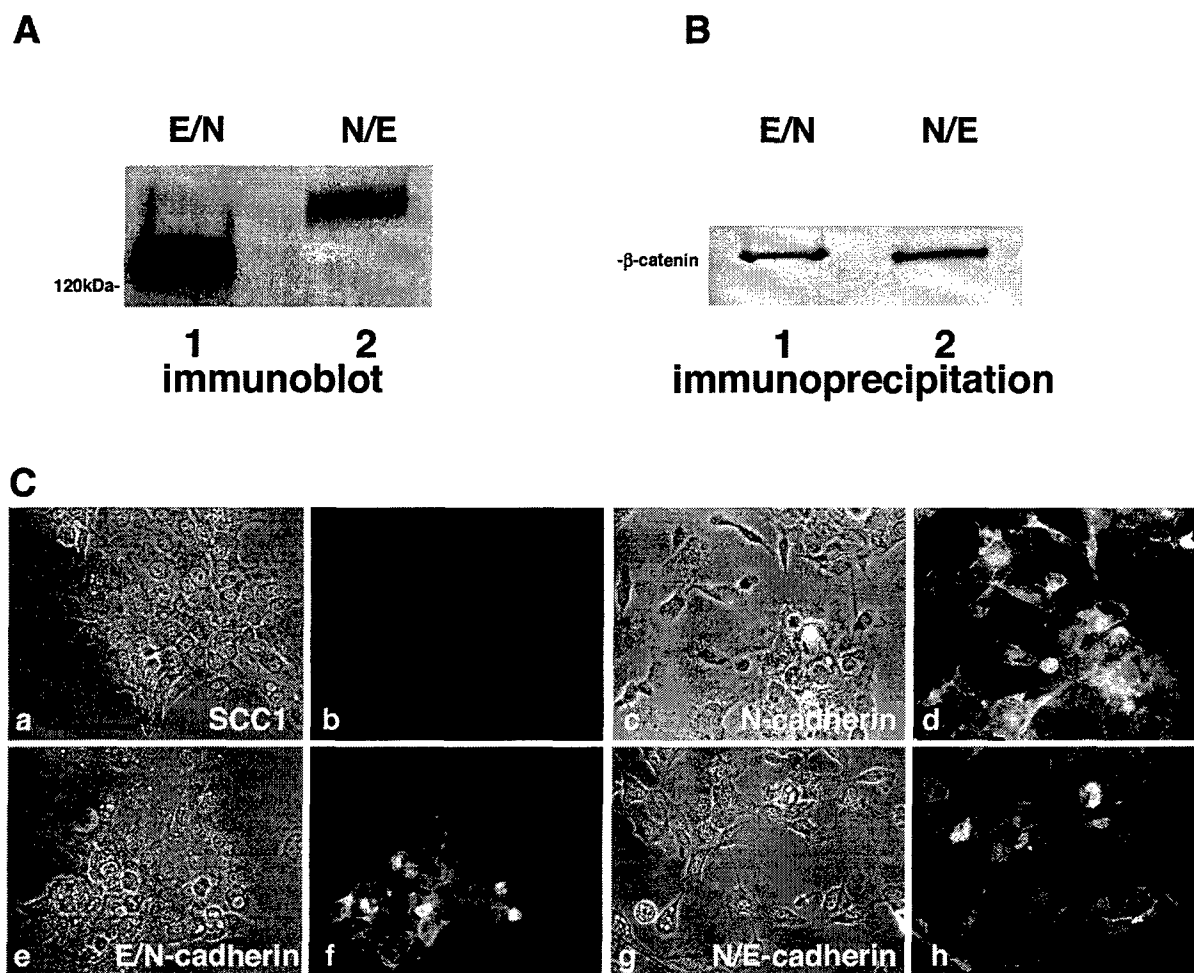


Fig 2. Expression of E/N- and N/E-cadherin in SCC1 cells. A. SCC1 cells were transfected with E/N- or N/E-cadherin and examined for transgene expression by immunoblotting with antibodies against the cytoplasmic (lane 1) or extracellular (lane 2) domain of N-cadherin. B. Extracts were immunoprecipitated, resolved by SDS-PAGE and immunoblotted for β -catenin. C. Untransfected SCC1 cells (Panels a and b) or SCC1 cells expressing N-cadherin (Panels c and d), E/N-cadherin (Panels e and f) or N/E-cadherin (Panels g and h) were processed for immunofluorescence microscopy using the appropriate cadherin antibody. Corresponding phase and fluorescence micrographs are shown. Bar 10 μ m.

To determine if the extracellular domain of N-cadherin was also responsible for the change in motility of BT20 cells, we transfected N/E-cadherin and E/N-cadherin into these cells. Fig. 3A shows that both chimeric cadherins were expressed at the cell surface and that neither chimera produced an effect on the morphology of these cells. This is consistent with our previous studies showing that N-cadherin did not effect the morphology of BT20 cells (Nieman et al., 1999a; Fig. 3A, panel c). Fig. 3B shows that N/E-cadherin was as efficient as intact N-cadherin at inducing motility in BT20 cells whereas E/N cadherin did not significantly alter the motile characteristics of BT20 cells. Thus, our hypothesis that the cytoplasmic domain of N-cadherin initiates a signaling pathway resulting in increased cell motility was not substantiated. Rather, it appeared that the extracellular domain of N-cadherin was responsible for the epithelial to mesenchymal transition in squamous epithelial cells and for increased motility in breast cancer cells. The remainder of this study was aimed at determining which part of the extracellular domain of N-cadherin influenced cellular morphology and behavior.

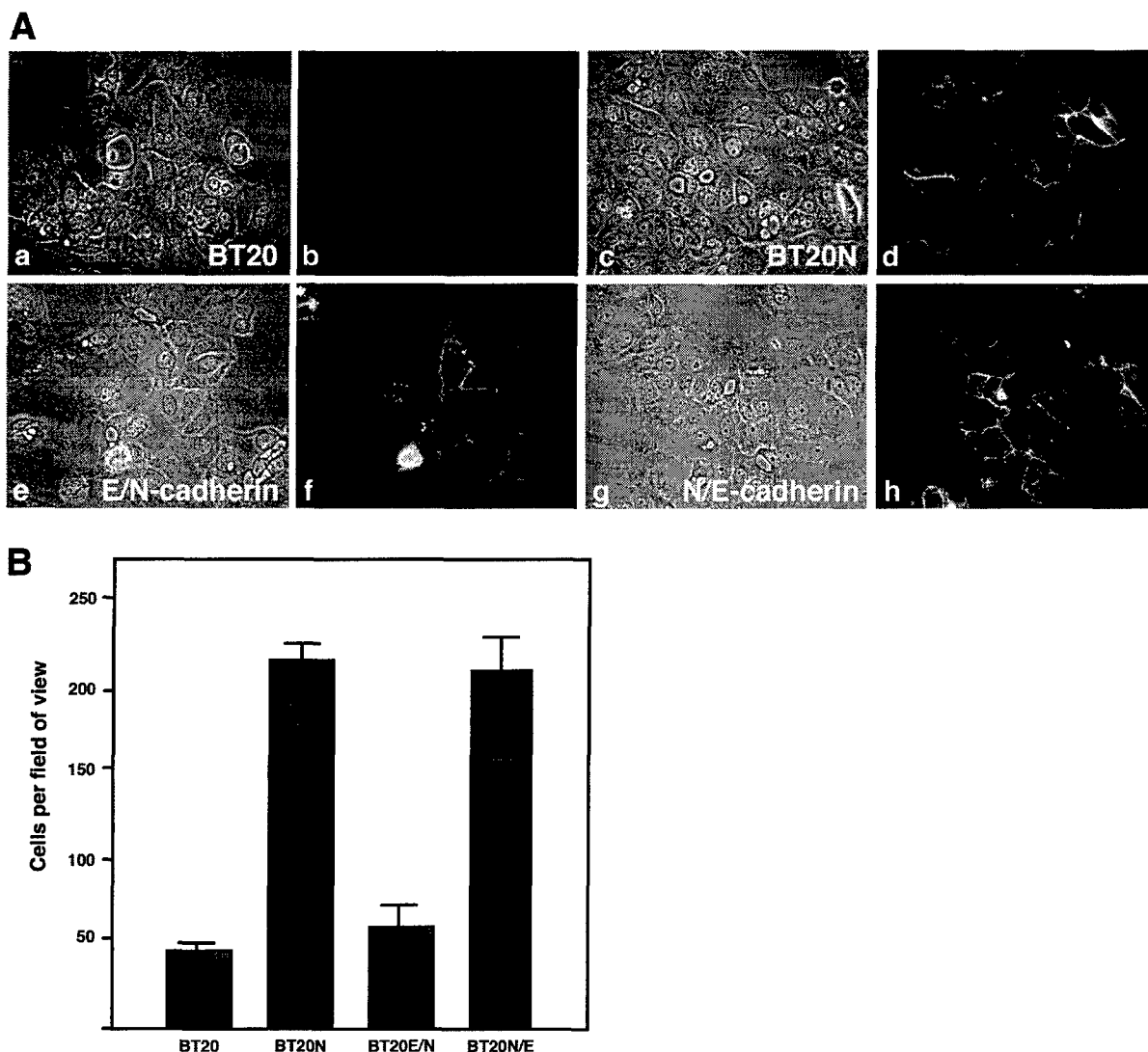


Fig 3. Expression of E/N- and N/E-cadherin in BT20 cells. *A. BT20 cells were transfected with full-length N-cadherin (BT20N), E/N-cadherin or N/E-cadherin. Untransfected BT20 cells (Panels a and b) or BT20 cells expressing N-cadherin (Panels c and d), E/N-cadherin (Panels e and f) or N/E-cadherin (Panels g and h) were processed for immunofluorescence microscopy using the appropriate cadherin antibody. Corresponding phase and fluorescence micrographs are shown. Bar 10 μ m. B. Cells were plated on membranes for motility assays, incubated for 24 h and the number traversing the membrane was determined by averaging 10 random fields at. Data are expressed as the number of cells/field. Each experiment was done 3X and error bars indicate SD.*

Extracellular domain 4 of N-cadherin confers a motile phenotype on epithelial cells

To investigate further the extracellular domain of N-cadherin, we constructed additional chimeric cadherins. We started with N/E-cadherin and moved the boundary between E- and N-cadherin progressively towards the N-terminus (Fig. 4A). We added a myc tag to the C-terminus of the chimeras so that we could use the identical antibody to detect each chimera. We also constructed a chimeric N/E-cadherin with a myc tag (N/E-myc) to ensure addition of the tag did not alter the ability of N/E-cadherin to confer a motile phenotype on human epithelial cells. The chimeric cadherin that included approximately one third of EC5 of E-cadherin was designated N/E5a-myc; the chimeric cadherin that included EC5 of E-cadherin was designated N/E5-myc; the chimeric

cadherin that included EC5 and most of EC4 of E-cadherin was designated N/E4-myc; the chimeric cadherin that included EC5, EC4 and most of EC3 of E-cadherin was designated N/E3-myc; and the chimeric cadherin that included EC5, EC4, EC3 and most of EC2 of E-cadherin was designated N/E2-myc (Fig. 4A).

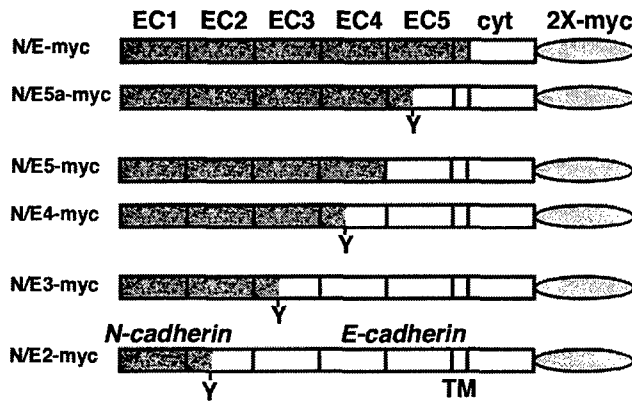


Fig 4A. Generation of additional cadherin chimeras. A. Chimeric cadherins consisting of E-cadherin (white) and N-cadherin (gray) were cloned into pLK-pac with a 2X-myc tag at the C-terminus.

Each chimera was transfected into the cadherin-negative A431D cells to determine if it functioned properly as an adhesion molecule. The N/E-cadherin with a 2X-myc tag (N/E-myc-cadherin) behaved exactly like N/E-cadherin indicating that the myc tag did not influence the function of the chimeric cadherin. Chimeras N/E-myc, N/E5-myc, N/E4-myc, and N/E3-myc were each expressed at a high level as indicated by immunoblot analysis using anti-myc antibodies (Fig. 4B). The proteins were processed to the predicted size although there was more unprocessed protein than was seen for endogenous cadherins and more than we saw for E/N-cadherin or N/E-cadherin. Each chimera efficiently associated with β -catenin as demonstrated by co-immunoprecipitation (Fig. 4C).

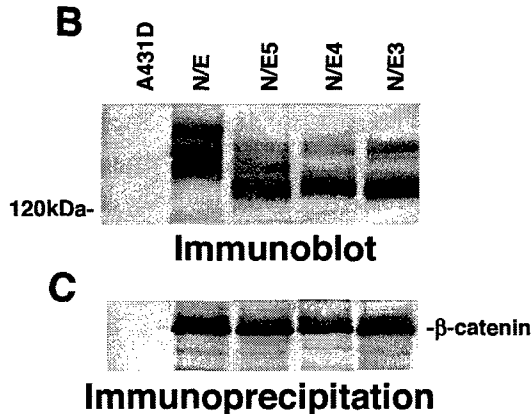


Fig. 4 B and C. Expression of the transgenes in A431D cells. B. A431D cells were transfected and examined for transgene expression by immunoblotting with anti-myc. Note: In some cases we observed a number of processing variants when transfected cadherins were overexpressed in cells. C. Extracts were immunoprecipitated using anti-myc, resolved by SDS-PAGE and immunoblotted for β -catenin.

In addition, each chimera mediated cell aggregation (Fig. 4D).

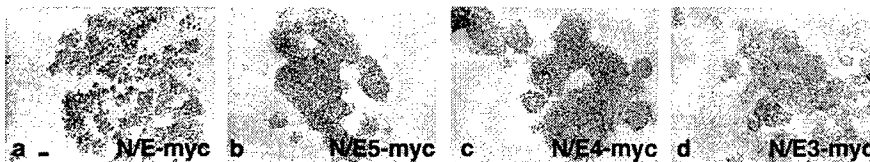


Fig 4 D. Aggregation assays. A431D cells expressing N/E-myc-cadherin (Panel a), N/E5-myc-cadherin (Panel b), N/E4-myc-cadherin (Panel c) or N/E3-myc-cadherin

(Panel d) were tested for their ability to aggregate in a hanging drop aggregation assay.

Chimeras N/E5a-myc and N/E2-myc were not processed properly and were not expressed on the surface of the A431D cells so we did not use them in assays to map the domain of N-cadherin that functions to induce motility in epithelial cells.

When N/E-myc, N/E5-myc, N/E4-myc and N/E3-myc chimeric cadherins were transfected into SCC1 cells, they were highly expressed (Fig. 5A) and co-immunoprecipitated with β -catenin (Fig. 5B).

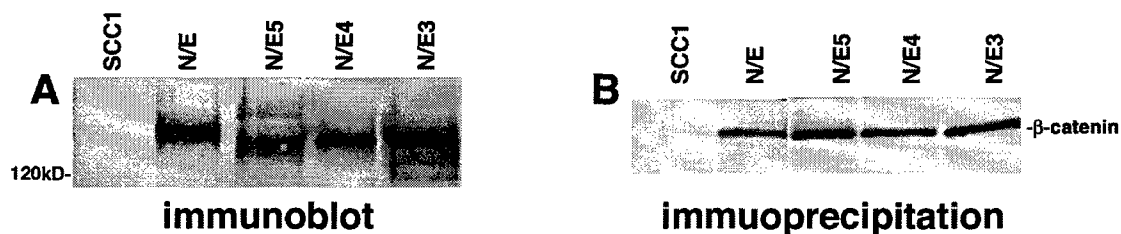


Fig 5 A and B. Expression of additional N/E-cadherin chimeras. A. SCC1 cells were transfected with N/E-myc-cadherin, N/E5-myc-cadherin, N/E4-myc-cadherin or N/E3-myc-cadherin and examined for transgene expression by immunoblotting with anti-myc. B. Extract were immunoprecipitated using anti-myc, resolved by SDS-PAGE and immunoblotted for β -catenin.

The N/E-myc and N/E5-myc chimeras produced the same morphological change in SCC1 cells that was seen with N/E-cadherin (Fig. 5C, panels a and c). In contrast, the N/E4-myc and N/E3-myc chimeras had no effect on the morphology of SCC1 cells (Fig. 5C, panels e and g).

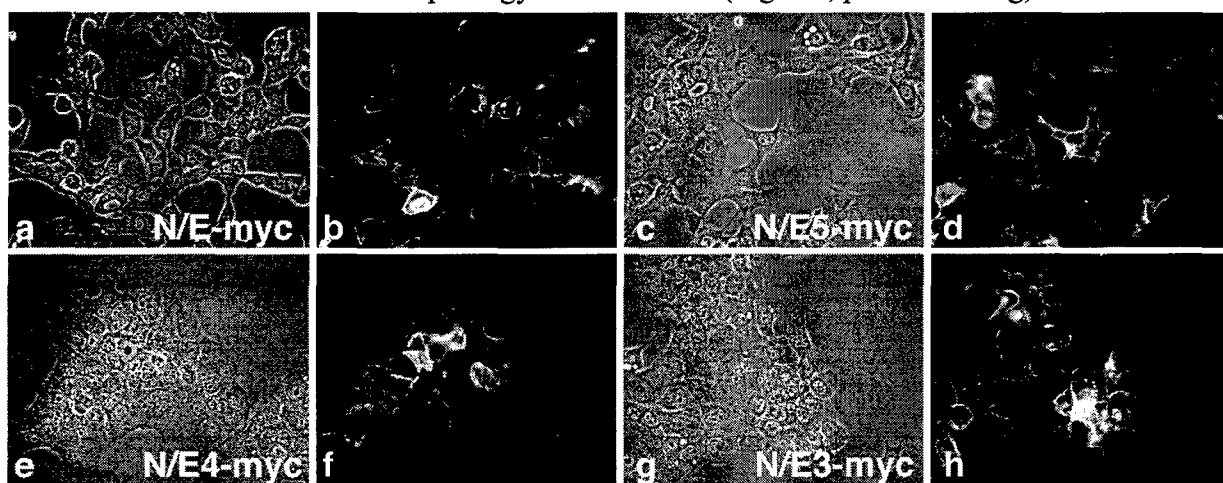


Fig 5 C. Expression of chimeras in Scc1 cells. SCC1 cells transfected with N/E-myc-cadherin (Panels a and b) N/E5-myc-cadherin (Panels c and d), N/E4-myc-cadherin (Panels e and f) or N/E3-myc-cadherin (Panels g and h) were processed for immunofluorescence microscopy using anti-myc. Corresponding phase and fluorescence micrographs are shown. Bar 10 μ m.

We were equally interested in the ability of these additional chimeric cadherins to influence cellular motility. We typically use the BT20 cells for this assay since we have established a clear difference between N-cadherin expressing and non-expressing BT20 cells. In addition, we wanted to be sure we were looking at the same phenomenon we had previously published (Nieman et al, 1999a). However, the BT20 cells grow slowly in culture and are difficult to transfect. Therefore, we established a motility assay that made use of the already transfected A431D cells. We first showed that A431 D cells transfected with N-cadherin were more motile than untransfected A431D cells or A431D cells transfected with E-cadherin (Fig. 5D). In addition, we showed that A431D cells transfected with E/N-cadherin behave similarly to A431 cells transfected with intact E-cadherin,

and that A431D cells transfected with N/E-cadherin behave like A431D cells transfected with intact N-cadherin. Thus, we believe we are testing the same N-cadherin-mediated effect on motility whether we use the BT20 system or the A431D system. A431D cells transfected with the N/E5 chimera were as motile as those transfected with full-length N-cadherin or with the N/E chimera while the motility rates of cells transfected with the N/E4 and N/E3 chimeras were similar to the motility rates of cells transfected with E-cadherin or with the E/N chimera.

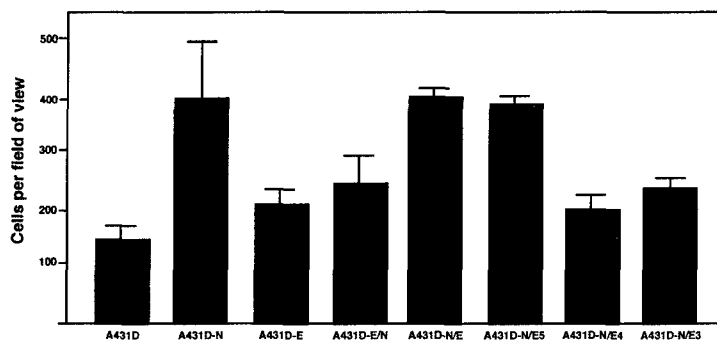


Fig 5D. Motility assays. A431D cells either non-transfected or transfected with N-cadherin (A431D-N), E-cadherin (A431D-E), E/N-myc-cadherin (A431D-E/N), N/E-myc-cadherin (A431D-N/E), N/E5-myc-cadherin (A431D-N/E5), N/E4-myc-cadherin (A431D-N/E4) or N/E3-myc-cadherin (A431D-N/E3) were plated on membranes for motility assays, incubated for 24 h and the

number traversing the membrane was determined by averaging 10 random fields. Data are expressed as the number of cells/field. Each experiment was done 3X and error bars indicate SD.

Thus, we determined that the domain of N-cadherin that is responsible for the epithelial to mesenchymal transition when expressed in squamous epithelial cells is most likely the same domain that increases cell motility when N-cadherin is expressed in epithelial cells. This domain probably resides in EC4, most likely between amino acids 533 and 602.

Extracellular domain 4 is sufficient to confer a motile phenotype.

To confirm that extracellular domain 4 of N-cadherin alone was responsible for altering the behavior of epithelial cells, we constructed two additional chimeric cadherins. The first was E-cadherin except that amino acids 535-603 of N-cadherin replaced the corresponding portion of E-cadherin and was called E/N/E-cadherin (Fig. 6A). The second chimera was N-cadherin except that amino acids 535 to 603 of N-cadherin were replaced by the corresponding amino acids of E-cadherin (E/N/E-cadherin). Both chimeras included a 2X-myc tag.

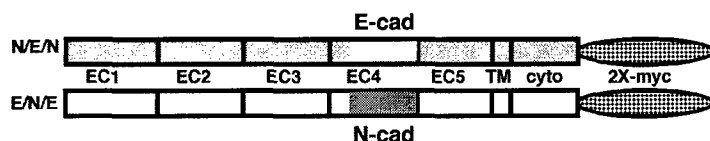


Fig 6A. Chimeric cadherins. Chimeric cadherins consisting of E-cadherin (white) and N-cadherin (gray) were constructed.

When transfected into the cadherin-negative A431D cells, both the E/N/E-cadherin and the N/E/N-cadherin were highly expressed, co-immunoprecipitated with β -catenin (Fig. 6B) and efficiently mediated cell aggregation (Fig. 6C).

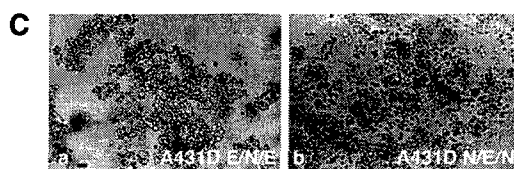
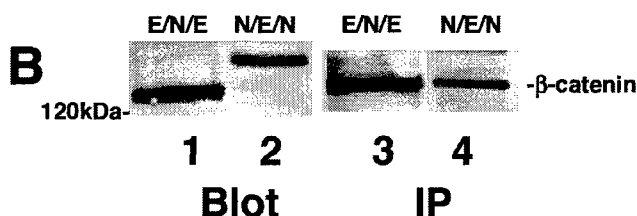


Fig 6 B and C. Expression of chimeric cadherins. A431D cells were transfected and immunoblotted with anti-myc (lanes 1 and 2). Extracts were immunoprecipitated using anti-myc, resolved by SDS-PAGE and immunoblotted for β -catenin (Lanes 3 and 4). C. A431D cells expressing E/N/E-myc-cadherin (Panel a) or N/E/N-myc-cadherin (Panel b) were tested for their ability to aggregate in a hanging drop aggregation assay.

In addition, each chimera was expressed at cell borders in SCC1 cells (Fig. 6D). The E/N/E chimera produced the epithelial to mesenchymal transition seen with full length N-cadherin (Fig. 6D, panel a) while the N/E/N chimera did not (Fig. 6D, panel c).

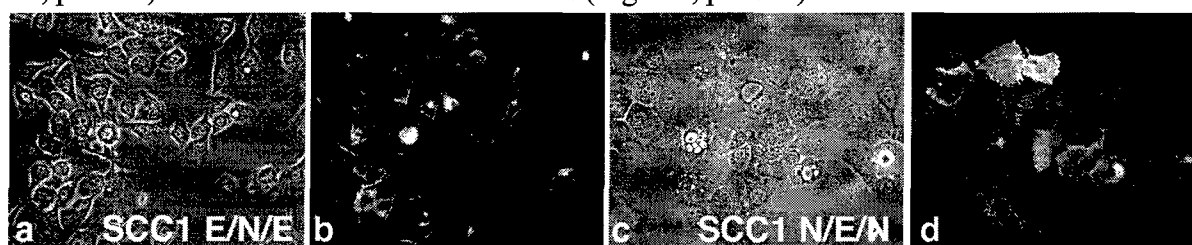


Fig. 6D. Expression of E/N/E and N/E/N-cadherins in SCC1 cells. SCC1 cells transfected with E/N/E-myc-cadherin (Panels a and b) or N/E/N-myc-cadherin (Panels c and d) were processed for immunofluorescence microscopy using anti-myc. Corresponding phase and fluorescence micrographs are shown. Bar 10 μ m

When A431D cells were transfected with the E/N/E-cadherin they showed motility rates similar to that seen when the cells were transfected with full-length N-cadherin while the N/E/N transfected cells showed motility rates similar to those seen with E-cadherin transfected cells (Fig. 6E). Thus, this short 70 amino acid domain of N-cadherin was both necessary and sufficient to cause the morphological and behavioral changes seen in epithelial cells.

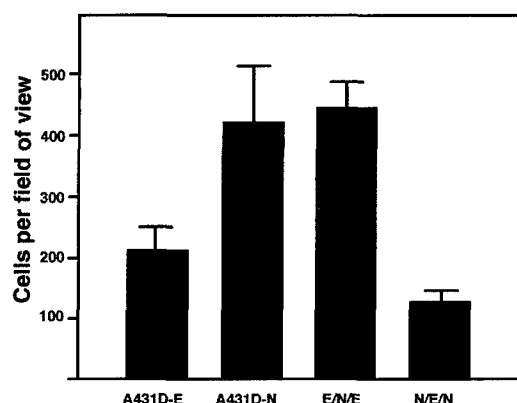


Fig 6E. Motility assays. A431D cells transfected with E-cadherin (A431D-E), N-cadherin (A431D-N), E/N/E-myc-cadherin (E/N/E) or N/E/N-myc-cadherin (N/E/N) were plated on membranes for motility assays, incubated for 24 h and the number traversing the membrane was determined by averaging 10 random fields at. Data are expressed as the number of cells/field. Each experiment was done 3X and error bars indicate SD.

Antibodies directed against the extracellular domain of N-cadherin inhibit motility in epithelial cells

The domain of classical cadherins that is responsible for cell adhesion resides in EC1. Antibodies directed against EC1 inhibit cadherin-mediated cell-cell interactions. Thus, we sought to determine if the ability of N-cadherin to influence cellular behavior could be inhibited by antibodies that bind to EC4. We immunized mice with the entire extracellular domain of human N-cadherin and chose those antibodies that mapped near EC4 for these studies. Fig. 7 A shows that one antibody, 8C11, bound to chimeric cadherins N/E-myc, N/E5a-myc, N/E5-myc, and N/E4-myc but did not bind to N/E3-myc or N/E2-myc. The control anti-myc antibody recognized each chimeric cadherin.

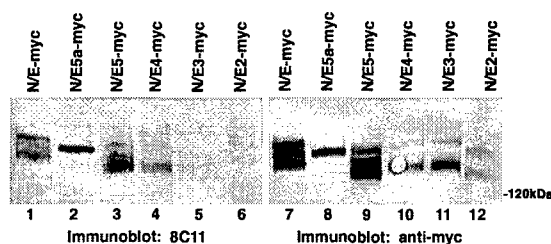


Fig 7A. Antibodies against N-cadherin EC4. Extracts of A431D cells expressing N/E-myc-cadherin (1 & 7), N/E5a-myc-cadherin (2 & 8), N/E5-myc-cadherin (3 & 9), N/E4-myc-cadherin (4 & 10), N/E3-myc-cadherin (5 & 11) or N/E2-myc-cadherin (6 & 12) were immunoblotted with mAb 8C11 (1-6) or anti-myc (7-12). Note: In some

cases we observed a number of processing variants.

When 8C11 was added to BT20N cells in a motility assay, it inhibited motility in a dose-dependent manner, indicating that this antibody did, indeed, bind near the domain of N-cadherin that was responsible for altering the behavior of these cells (Fig. 7B). We used the antibody at a dilution of 1:10 to repeat the experiment and to determine if it had any effect on N-cadherin-negative cells. For this experiment, we used smaller filters and counted the number of cells traversing the entire filter. The 8C11 antibody had no effect on the motility of N-cadherin-negative cells (Fig. 7C). In addition, an irrelevant ascites (4A2) used at a dilution of 1:10 had no effect on the motility of BT20N or on the motility of untransfected BT20 cells (Fig. 7C). Importantly, even at a 1:10 dilution, the mAb 8C11 did not inhibit cell aggregation in N-cadherin-expressing cells.

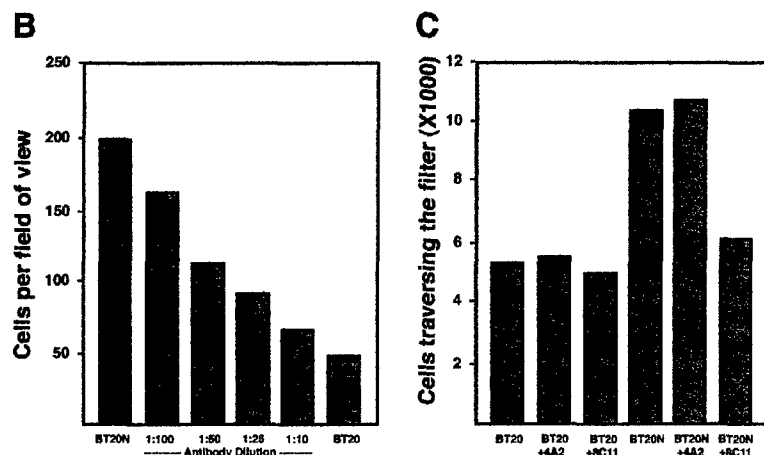


Fig. 7 B and C. Motility assays. B. BT20 cells transfected with N-cadherin (BT20N), were plated on membranes for motility assays in the presence of no antibody or 8C11 ascites at a dilution of 1:10 to 1:100. Untransfected BT20 cells in the absence of antibody were included as a control. After 24 h the number of cells traversing the membrane was determined by averaging 10 random fields at 100X magnification. Data are expressed as the number of cells/field.

C. Untransfected BT20 cells or BT20 cells transfected with N-cadherin (BT20N), were plated on membranes for motility assays in the presence of no antibody, irrelevant ascites 4A2 at a dilution of 1:10 or 8C11 ascites at a dilution of 1:10. After 24 h the number of cells traversing the membrane membrane was determined by counting the entire membrane. Data are expressed as the number of cells traversing the filter.

4. Relationship to the approved Statement of Work

I have copied the Statement of Work from the original proposal and have indicate in green those tasks which have been completed in the first two years of the project and in red those tasks which were completed this year. In dark blue is our plan for the next year.

Technical objective 1. Survey cell lines and biopsies:

Task 1. Surveying breast cancer cell lines for E-cadherin expression. Part of this was reported last year and part is presented in Table I and figure 1 above. **This task is complete.**

Task 2. Survey E-cadherin negative cell lines for expression of N-cadherin, P-cadherin, R-cadherin and Cadherin 5. Part of this was reported last year and part is presented in Table I and figure 1 above. We have analyzed all the cell lines for R-cadherin and cadherin 5 and have not found expression of either of these cadherins. **This task is complete.**

Task 3. Survey frozen histological sections for expression of the cadherin identified in Task 2. We have initiated a collaborative project with a pathologist, Dr. David Rimm at Yale University, to survey frozen sections. We have extended our collaborative efforts to include Dr. Jean-Paul Thiery of the Curie Institute, Paris France.

Task 4. 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. We have almost a full length clone now and will continue to characterize it during the next year.

Task 5. Prepare antibodies against the newly identified cadherin (X-cadherin) if necessary. A fusion protein has been made in *E. coli*, is being injected into mice. We hope to have an antibody within the next year.

Technical objective 2. Determine if the expression of inappropriate cadherins contributes to tumorigenesis.

Task 6. Obtain normal breast cell lines from the Michigan Tissue Bank. Transfect them with X-cadherin and observe the morphology of the transfected cells. We have transfected BT-20 cells with N-cadherin and reported the results of this experiment in The Journal of Cell Biology (see appendix, Nieman et al., 1999). **This task is complete.**

Task 7. Transfect the tumor cells that express X-cadherin with antisense X-cadherin and observe the morphology of the transfected cells. We have determined that this is not a feasible experiment. The anti-sense has been transfected and is not effective in down-regulating N-cadherin. **This task is complete.**

Task 8. Assay the normal cells, the transfected normal cells from task 6, the tumor cells, the transfected tumor cells from task 7 for motility and invasive characteristics. This has been accomplished and is reported in The Journal of Cell Biology (see appendix, Nieman et al., 1999). **This task is complete.**

Technical objective 3. Explore the mechanisms that regulate the expression of cadherins in breast tumor cells.

Task 9. Transform normal breast epithelial cells with ras and determine the levels of expression of E-cadherin and the inappropriate cadherin(s) found in technical objectives 1 and 2 above. Our ideas on this topic have changed since the submission of the original grant. We are pursuing the idea that transformation to the tumorigenic phenotype may be regulated by the FGF receptor. This is discussed above and some of it is published in The Journal of Cell Biology (see appendix, Nieman et al., 1999). **This task is complete.**

Task 10. Survey the cell lines that express X-cadherin for expression of erbB-2, EGF receptor and p53. Determine if there is a correlation between any of these markers and expression of X-cadherin or down-regulation of E-cadherin. Our ideas on this topic have changed since the submission of the original grant. We decided to pursue the determination of which part of N-cadherin influences motility. This is presented above and has been submitted for publication. We will continue to work on this next year.

Task 11. Transfect normal breast cells with markers identified in task 10 to determine if overexpression of this marker results in down-regulation of E-cadherin or increased expression of X-cadherin. We have found that the extracellular domain of N-cadherin influences cell behavior and will continue to characterize this domain.

Task 12. Treat normal breast epithelial cells with estrogen and progesterone to determine if these hormones have an effect on the expression of E-cadherin or other cadherins. Treat normal breast epithelial cells with TGF β and other TGF β family members to determine if these hormones have an effect on the expression of E-cadherin or other cadherins. Treatment with hormones did not influence the behavior of these cells. **This task is complete.**

Task 13. Analyze the data from task 12 and propose a mechanism for regulation of cadherin expression that can be further explored during the final 6 months of this project. This will be pursued during the next year.

Educational and training objectives:

Task 1. Analyze the literature on breast cancer. Pull together information relevant to this project. This was accomplished during the first year and was included in that year's report. **This task is complete.**

Task 2. Spend some time meeting with Dr. Fearon's lab group to discuss our current collaborative project. Establish new collaborative efforts between our laboratories. Our lab and Dr. Fearon's lab have gone in different directions. However, we have initiated a collaborative effort with the lab of Dr. David Rimm as noted above which should be very fruitful. In addition, we are working with Drs. Mark Day and Steve Ethier at University of Michigan on experiments that will be useful to the Breast Cancer project. We have extended our collaborative efforts to include Dr. Jean-Paul Thiery of the Curie Institute, Paris France. **This task is complete.**

Task 3. Apply for grant support from The NIH to continue our work on breast cancer. This will be done during the next year.

Key Research Accomplishments

- ◆ We constructed several chimeric cadherins between N-cadherin and E-cadherin.
- ◆ The chimeras were transfected into the SCC1 oral squamous epithelial cell line to determine their effect on cell morphology.
- ◆ The chimeras were transfected into the BT20 breast cancer cell line to investigate influences on cell behavior.
- ◆ We found that a 70 amino acid portion of EC-4 of N-cadherin was both necessary and sufficient to influence cell morphology and to promote cell motility.
- ◆ We showed that cadherins promote differential cellular behavior.
- ◆ We identified a novel activity that maps to the extracellular domain of N-cadherin.

Reportable Outcomes

- ◆ A paper has been published in the Journal of Cell Biology. The citation is: Nieman, M.T., Prudoff, R.S., Johnson, K.R. and Wheelock, M.J. 1999. N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression. J. Cell Biol. 147:631-643.
- ◆ A second paper has been submitted to the Journal of Cell Biology and is currently under revision.
- ◆ This work was presented as a platform talk at the American Society for Cell Biology Meeting in 1999.
- ◆ This work was presented as an invited talk at the International Bat-Sheva de Rothschild Seminar Weizmann Institute, Rehovot, Israel
- ◆ This work was presented at the Era of Hope DOD Breast Cancer Meeting in 2000.
- ◆ Several cell lines were generated by transfecting cadherins into breast cancer cells.
- ◆ Ryan Prudoff completed his masters degree based on work supported on this project.
- ◆ Dr. Jani Lewis (who's salary is supported on this grant) has submitted a proposal to the NIH that is currently under review.

Conclusions

We and others have shown that N-cadherin influences the morphology and behavior of epithelial cells (Islam et al., 1996; Hazan et al., 1997; Li et al., 1998; Hazan et al., 2000). These studies implicate N-cadherin in an epithelial to mesenchymal transition in some cells but not in others. In squamous epithelial cells, expression of N-cadherin results in down-regulation of E-cadherin, which is most likely responsible for the change in cellular morphology. In other cells such as breast cancer cells, expression of N-cadherin does not alter cell morphology but does alter cellular behavior by inducing a motile phenotype. In breast cancer cells, expression of E-cadherin is not decreased when the cells are forced to express N-cadherin. This suggests that even in cells that express abundant E-cadherin, N-cadherin influences cell behavior. N-cadherin is often expressed by motile cells such as fibroblasts, and a switch from E-cadherin expression to N-cadherin expression occurs when some cells become motile and/or invasive during normal developmental processes (Huttenlocher et al., 1998; Edelman et al., 1983; Hatta and Takeichi 1986; Zhou et al., 1997). Thus, it is not unexpected that expression of N-cadherin by tumor cells alters cellular morphology and/or behavior.

The extracellular domain of a cadherin promotes cell-cell adhesion while the cytoplasmic domain serves to link the cadherin to the cytoskeleton via interactions with catenins. These cytosolic interactions are critical to the adhesive function of the cadherin. Linkage to the cytoskeleton is necessary to promote strong cell-cell adhesion and to allow organization of the junction itself. In addition, the catenins have been implicated in signaling events that are thought to regulate the strength of the adhesive activity of the cadherin (reviewed in Gumbiner, 2000). This led us to propose that the cytoplasmic domain of N-cadherin was responsible for increasing the motility of epithelial cells. When we prepared two chimeric cadherins, one comprised of the extracellular domain of N-cadherin linked to the cytoplasmic domain of E-cadherin (N/E-cadherin) and the other comprised of the extracellular domain of E-cadherin linked to the cytoplasmic domain of N-cadherin (E/N-cadherin) we were surprised to find that it was the extracellular domain of N-cadherin that promoted cell motility. The extracellular domain of cadherins is comprised of 5 repeat regions with EC1 being the most N-terminal. Most of the known activities of cadherins have been mapped to EC1. The best understood examples are those where cadherin molecules interact with other cadherin molecules. Structure determinations (Shapiro et al., 1995; Nagar et al., 1996; Tamura et al., 1998; Pertz et al., 1999) and biochemical characterization (Ozawa et al., 1990; Ozawa and Kemler, 1990; Nose et al., 1990; Koch et al., 1997; Shan et al., 2000) have demonstrated that EC1 is the site of the adhesion interface. Data from several laboratories have suggested that cadherins are displayed on the surface of cells as dimers (Shapiro et al., 1995; Briher et al., 1996; Chitaev and Troyanovsky, 1998; Takeda et al., 1999; Shan et al., 2000). Although several differing pictures exist as to how these cis (also called lateral) dimers form and are maintained, the data point to EC1 and EC2 of the cadherins as playing major roles.

In some instances, it has been shown that cadherins can promote cell-cell adhesion via heterophilic interactions, for example N-cadherin can bind to R-cadherin (Inuzuka et al., 1991), B-cadherin can bind to L-CAM (Murphy-Erdosh et al., 1995) and cadherin-6B can bind to cadherin-7 (Nakagawa and Takeichi, 1995). Recently, Shimoyama et al. (2000) examined 8 different type II cadherins and frequently observed interactions between L cells transfected with different cadherins. Another recent study showed that, in L cells expressing both N- and R-cadherins, the two cadherins formed cis heterodimers that functioned in cell adhesion (Shan et al., 2000). In this latter case, it was the N-terminus of the cadherins that played a role in the formation of the cis heterodimers. It will be interesting to determine if other pairs of cadherins shown to mediate heterophilic cell-cell adhesion are able to form cis heterodimers and what parts of the cadherins are involved. In this study we have shown that the ability of N-cadherin to promote cell motility resides in EC-4. Thus, this activity is distinct from the adhesive function of the cadherin.

In addition to the interaction of cadherins with themselves, a number of other interacting proteins have been described. The bacterium *Listeria monocytogenes* has been shown to use E-cadherin as a receptor. InlA, a surface protein on the bacterium, binds to E-cadherin. Lecuit et al. (1999)

showed that changing a single amino acid in EC1 of E-cadherin (proline-16 of EC1) eliminated the binding of InlA and dramatically compromised internalization of *Listeria* by cells. In addition to being a target for *Listeria*, E-cadherin is the only cadherin that is known to be an integrin ligand. Integrin $\alpha_5\beta_1$ binds EC1 of E-cadherin; glutamate-31 of EC1 plays a critical role in the interaction (Karecla et al., 1996). Since EC1 of cadherins has been shown to play a major role in their biological activities, all of the chimeras used in this study retained the intact EC1 of N-cadherin.

Although most activities have been mapped to the N-terminal domains, there are several reports suggesting roles for EC3, EC4 and EC5 in cadherin adhesion. Zhong et al. (1999) have characterized a mAb (AA5) recognizing EC5 of C-cadherin that activates adhesion, perhaps by changing the cadherin's organization or altering its interaction with other cellular factors. Sivasankar et al. (1999) have studied the biophysical characteristics of adhesion mediated by layers of oriented recombinant C-cadherin ectodomains. They concluded that complete interdigitation of antiparallel ectodomains (i.e., where EC1 of one molecule interacted with EC5 of the antiparallel partner, EC2 interacted with EC4 of the partner, etc.) gave the strongest interactions. Their data also suggested that ratcheting the molecules one EC domain further apart (such that EC1 interacted with EC4 of its antiparallel partner, etc.) also resulted in an adhesive interaction. In addition, Troyanovsky et al. (1999) have reported that EC3 and EC4 of E-cadherin can mediate cis dimerization under some conditions.

A series of papers from Lilien's laboratory (reviewed in Lilien et al., 1999) have suggested that, in neural retina cells, the ectodomain of N-cadherin is stably associated with and is a substrate for the cell surface enzyme N-acetylgalactosaminylphosphotransferase (GalNAcPTase). The interaction of neurocan, a chondroitin sulfate proteoglycan, with GalNAcPTase results in inhibition of N-cadherin mediated cell adhesion. However, the site(s) on N-cadherin where this interaction takes place is unknown.

Investigators have suggested that N-cadherin can interact with and activate FGF receptors (FGFR) both in neurons (Doherty and Walsh, 1996) and in ovarian surface epithelial cells (Peluso, 2000). In the ovarian surface epithelial cell system, it has been reported that N-cadherin and FGFR co-immunoprecipitate. Our laboratory recently showed that N-cadherin-mediated cell motility of breast cancer cells can be decreased by an inhibitor of the FGF-mediated signal transduction pathway that has been characterized by the Walsh and Doherty labs (Nieman et al., 1999a). In addition, Hazan et al. (2000) showed that FGF caused a dramatic increase in motility in N-cadherin-expressing cells. The FGFRs contain an HAV sequence (Byers et al., 1992) that has been proposed to interact with EC4 of N-cadherin. It is interesting to note that the 70 amino acid segment of N-cadherin we have identified in this study includes the sequences proposed by Doherty and Walsh to interact with the FGFRs. The structure of a portion of FGFR1 bound to FGF2 has been determined (Plotnikov et al., 1999). The histidine and valine side chains of the HAV sequence in FGFR1 were involved in intradomain contacts and thus appear to be unavailable for interacting with partner molecules. Thus, the precise role the FGFR plays in N-cadherin-dependent cell motility is still unknown and it is still not clear at this time whether N-cadherin and the FGFR directly interact with one another.

Since the 70 amino acid portion of N-cadherin can influence epithelial cell morphology and motility, we compared this portion of human N-cadherin to other cadherins. In this region, mouse and rat N-cadherin are identical to human N-cadherin while 78% of the amino acids in human R-cadherin are identical. The corresponding region of human E-cadherin contains 71 amino acids and is only 54% identical to N-cadherin. To further investigate the role this portion of N-cadherin plays in cell motility, we produced a mAb that binds near EC-4 of N-cadherin. When applied to cells in a motility assay, this antibody inhibited cell motility in N-cadherin-expressing cells but not in N-cadherin-negative cells. In addition, this antibody inhibited motility without inhibiting cell-cell aggregation, providing further evidence that adhesion and motility are two separate properties of the extracellular domain of N-cadherin. It is important to remember that all the chimeras used in

this study were full-length cadherins. Studies are in progress to determine if truncated cadherins can influence cell motility.

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Appendices

1. Abstract from an invited talk at the American Society for Cell Biology Meeting in 1999.
2. Abstract from an invited talk at the International Bat-Sheva de Rothschild Seminar Weizmann Institute, Rehovot, Israel.
3. Abstract from a poster presented at the Era of Hope DOD Breast Cancer Meeting in 2000.
4. Copy of : Nieman et al.1999. N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression. *J. Cell Biol.* 147:631-643.

1. Abstract from an invited talk at the American Society for Cell Biology Meeting in 1999.

E-cadherin is a transmembrane glycoprotein that mediates calcium-dependent, homotypic cell-cell adhesion and plays a role in maintaining the normal phenotype of human breast epithelial cells. Decreased expression of E-cadherin has been correlated with increased invasiveness of breast cancer. In other systems, inappropriate expression of a non-epithelial cadherin, such as N-cadherin, by an epithelial cell has been shown to down-regulate E-cadherin expression and to increase the invasive potential of the cell. In this study we explored the possibility that expression of non-epithelial cadherins may be correlated with increased invasion in human breast cancer cells. We showed that up-regulation of inappropriate cadherins, rather than down-regulation of E-cadherin, correlates with increased motility and invasion. In most cases breast epithelial cells with reduced E-cadherin expression have turned on the expression of an inappropriate cadherin. However, we found examples of cells with reduced E-cadherin that did not express an inappropriate cadherin. In these cases, the cells were non-motile and non-invasive.

2. Abstract from an invited talk at the International Bat-Sheva de Rothschild Seminar Weizmann Institute, Rehovot, Israel

E-cadherin is a transmembrane glycoprotein that mediates calcium-dependent, homotypic cell-cell adhesion and plays a role in maintaining the normal phenotype of human breast epithelial cells. Decreased expression of E-cadherin has been correlated with increased invasiveness of breast cancer. In other systems, inappropriate expression of a non-epithelial cadherin, such as N-cadherin, by an epithelial cell has been shown to down-regulate E-cadherin expression and to contribute to a scattered phenotype. In this study we explored the possibility that expression of non-epithelial cadherins may be correlated with increased cellular motility and invasion in human breast cancer cells. We present data showing that N-cadherin promotes cell motility and invasion in breast cancer cells; that decreased expression of E-cadherin does not necessarily correlate with invasion in breast cancer cells; that N-cadherin expression correlates both with invasion and motility in breast cancer cells and likely plays a direct role in promoting motility; that forced expression of E-cadherin in invasive, N-cadherin positive cells does not reduce their motility or invasive capacity; and that forced expression of N-cadherin in non-invasive, E-cadherin-positive cells produces an invasive cell even though these cells continue to express high levels of E-cadherin.

3. Abstract from a poster presented at the Era of Hope DOD Breast Cancer Meeting in 2000.

E-cadherin is a transmembrane glycoprotein that mediates calcium-dependent, homotypic cell-cell adhesion and plays a role in maintaining the normal phenotype of epithelial cells. Decreased expression of E-cadherin has been correlated with increased invasiveness of breast cancer. In other systems, inappropriate expression of a non-epithelial cadherin, such as N-cadherin, by an epithelial cell has been shown to down-regulate E-cadherin expression and to contribute to a scattered phenotype. In this study we explored the possibility that expression of non-epithelial

cadherins may be correlated with increased motility and invasion in breast cancer cells. We show that N-cadherin promotes motility and invasion; that decreased expression of E-cadherin does not necessarily correlate with motility or invasion; that N-cadherin expression correlates both with invasion and motility and likely plays a direct role in promoting motility; that forced expression of E-cadherin in invasive, N-cadherin positive cells does not reduce their motility or invasive capacity; that forced expression of N-cadherin in non-invasive, E-cadherin-positive cells produces an invasive cell even though these cells continue to express high levels of E-cadherin; that N-cadherin-dependent motility may be mediated by fibroblast growth factor receptor signaling; and that cadherin 11 promotes epithelial cell motility in a manner similar to N-cadherin.

4. On the following 13 pages is a copy of Nieman et al.1999. N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression. J. Cell Biol. 147:631-643.

N-Cadherin Promotes Motility in Human Breast Cancer Cells Regardless of their E-Cadherin Expression

Marvin T. Nieman, Ryan S. Prudoff, Keith R. Johnson, and Margaret J. Wheelock

Department of Biology, University of Toledo, Toledo, Ohio 43606

Abstract. E-cadherin is a transmembrane glycoprotein that mediates calcium-dependent, homotypic cell-cell adhesion and plays a role in maintaining the normal phenotype of epithelial cells. Decreased expression of E-cadherin has been correlated with increased invasiveness of breast cancer. In other systems, inappropriate expression of a nonepithelial cadherin, such as N-cadherin, by an epithelial cell has been shown to downregulate E-cadherin expression and to contribute to a scattered phenotype. In this study, we explored the possibility that expression of nonepithelial cadherins may be correlated with increased motility and invasion in breast cancer cells. We show that N-cadherin promotes motility and invasion; that decreased expression of E-cadherin does not necessarily correlate with motil-

ity or invasion; that N-cadherin expression correlates both with invasion and motility, and likely plays a direct role in promoting motility; that forced expression of E-cadherin in invasive, N-cadherin-positive cells does not reduce their motility or invasive capacity; that forced expression of N-cadherin in noninvasive, E-cadherin-positive cells produces an invasive cell, even though these cells continue to express high levels of E-cadherin; that N-cadherin-dependent motility may be mediated by FGF receptor signaling; and that cadherin-11 promotes epithelial cell motility in a manner similar to N-cadherin.

Key words: N-cadherin • E-cadherin • breast cancer • motility • fibroblast growth factor receptor

CADHERINS constitute a family of transmembrane glycoproteins that mediate calcium-dependent homotypic cell-cell adhesion and play an important role in the maintenance of normal tissue architecture. The cadherin intracellular domain interacts with several proteins, collectively called catenins, that link cadherins to the actin cytoskeleton (reviewed in Wheelock et al., 1996). This linkage is required for full cadherin adhesive activity. Either β -catenin or plakoglobin binds directly to the cadherin and to α -catenin, whereas α -catenin links directly and indirectly to actin (Aberle et al., 1994; Nagafuchi et al., 1994; Stappert and Kemler, 1994; Knudsen et al., 1995; Rimm et al., 1995; Nieset et al., 1997; Watabe-Uchida et al., 1998). Their ability to simultaneously self-associate and link to the actin cytoskeleton enables cadherins to mediate both the cell recognition required for cell sorting and the strong cell-cell adhesion needed to form tissues.

In addition to their structural role in the adherens junction, catenins are thought to regulate the adhesive activity of cadherins. For example, phosphorylation of β -catenin in Src transformed cells may contribute to the nonadhesive phenotype of these cells (Matsuyoshi et al., 1992;

Hamaguchi et al., 1993). In addition, p120^{cas}, originally identified as a Src substrate and subsequently shown to bind to the cytoplasmic domain of cadherins, has been suggested to play a role in regulating the adhesive activity of cadherins (Reynolds et al., 1994; Daniel and Reynolds, 1995; Shibamoto et al., 1995; Aono et al., 1999; Ohkubo and Ozawa, 1999).

Numerous studies have demonstrated the importance of the E-cadherin/catenin complex in maintaining the normal phenotype of epithelial cells. Early studies showed that inhibiting E-cadherin activity with function-perturbing antibodies altered the morphology of MDCK cells and conferred upon them the ability to invade both collagen gels and embryonic chicken heart tissue (Behrens et al., 1989; Chen and Öbrink, 1991). In addition, invasive, fibroblast-like carcinoma cells could be converted to a noninvasive phenotype by transfection with a cDNA encoding E-cadherin (Frixen et al., 1991). Moreover, E-cadherin expression is downregulated or lost in epithelial tumors from various tissues, including stomach, colon, head and neck, bladder, prostate, and breast (Schipper et al., 1991; Bringuier et al., 1993; Dorudi et al., 1993; Mayer et al., 1993; Oka et al., 1993; Umbas et al., 1994).

It has been suggested that alterations in cadherin function may be a critical step in the development of breast cancers. A survey of 18 cell lines derived from breast carci-

Address correspondence to Margaret J. Wheelock, Department of Biology, University of Toledo, Toledo, OH 43606. Tel.: (419) 530-1555. Fax: (419) 530-7737. E-mail: mwheelo@uoft02.utoledo.edu

nomas showed that ten lines failed to express detectable levels of E-cadherin, and two other lines failed to express α -catenin (Pierceall et al., 1995). Other studies have identified breast tumor cell lines with mutations in the E-cadherin gene (Berx et al., 1995), or with changes in the levels of expression or in the phosphorylation state of β -catenin or plakoglobin (Sommers et al., 1994). Surveys of breast cancer tissue make an equally compelling case for the involvement of E-cadherin in the formation or progression of breast tumors, and clinical studies have shown that loss of E-cadherin correlates with metastatic disease and poor prognosis (Gamello et al., 1993; Moll et al., 1993; Oka et al., 1993; Rasbridge et al., 1993; Berx et al., 1996; Guriec et al., 1996).

In vitro studies support the role of E-cadherin as an invasion suppressor gene. For example, forced expression of E-cadherin in rat astrocytoma cells suppressed motility (Chen et al., 1997). Likewise, transfection of invasive E-cadherin-negative breast or prostate cell lines with mouse E-cadherin resulted in cells that were less invasive in in vitro assays (Frixen et al., 1991; Luo et al., 1999). When treated with function blocking E-cadherin antibodies, the transfected cells returned to an invasive phenotype, thus implicating E-cadherin as an invasion suppressor (Frixen et al., 1991).

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 were shown to be noninvasive (Sommers et al., 1991). These authors suggested that in order for E-cadherin-negative cells to be invasive, they must also express vimentin.

In another study, Sommers et al. (1994) showed that transfection of E-cadherin into the invasive breast cancer cell lines, BT549 and HS578t, altered neither the morphology nor the invasive behavior of these cells. These authors speculated that the transfected E-cadherin may not be fully functional in these cells, due to altered posttranslational modification of the cadherin-associated proteins β -catenin, α -catenin, or plakoglobin.

It has been suggested that, unlike E-cadherin, N-cadherin may promote motility and invasion in carcinoma cells. For example, Hazan et al. (1997) 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. A study conducted in our laboratory suggested that N-cadherin may play a more direct role in the process of invasion and may actually promote invasion by inducing a scattered phenotype when expressed by oral squamous cell carcinoma-derived cells (Islam et al., 1996). In this study, forced expression of N-cadherin resulted in downregulation of endogenous E- and P-cadherins, making it impossible to separate the motility-promoting effects of N-cadherin from the motility-suppressing activity of E-cadherin. In contrast, it has been suggested that N-cadherin promotes contact inhibition in normal skeletal muscle myoblasts and, in so doing, inhibits migration upon contact, but does not suppress motility in subconfluent cells (Huttenlocher et al., 1998).

Thus, the information in the literature concerning the role cadherins may play in tumor cell invasion is inconclusive and even contradictory, prompting us to revisit the question using new reagents generated by our laboratory to examine both previously studied and newly derived breast cancer cell lines. The data presented in this paper indicate: decreased expression of E-cadherin does not necessarily correlate with invasion in breast cancer cells; N-cadherin expression correlates both with invasion and motility in breast cancer cells, and likely plays a direct role in promoting motility; forced expression of E-cadherin in invasive, N-cadherin-positive cells does not reduce their motility or invasive capacity; forced expression of N-cadherin in noninvasive, E-cadherin-positive cells produces an invasive cell, even though these cells continue to express high levels of E-cadherin; the data suggest that N-cadherin-mediated cell motility may be stimulated by FGF receptor signaling; and other cadherins, such as cadherin-11, may promote motility in epithelial cells in a manner similar to N-cadherin.

Materials and Methods

Cells

Breast carcinoma cell lines were obtained from American Type Culture Collection (ATCC) and maintained in DME with 10% FBS (SKBr3, MDA-MB-435, MDA-MB-436, BT-549, and HS578t) or MEM with 10% FBS (MDA-MB-453 and BT-20). The cell lines MCF-7 and MDA-MB-231 were obtained from Dr. Mary J.C. Hendrix (University of Iowa, Iowa City, IA) and maintained in DME with 10% FBS. The cell lines SUM 159PT and SUM 149 were kindly provided by Dr. Steve Ethier and generated by the University of Michigan Human Breast Cell/Tissue Bank and Data Base. They were maintained in Ham's F-12 with 5% FBS supplemented with insulin (5 mg/ml) and hydrocortisone (1 mg/ml). The cell line SUM 1315 was obtained from the same source and maintained in Ham's F-12 with 5% FBS supplemented with insulin (5 mg/ml) and EGF (10 ng/ml). HT1080 cells were obtained from ATCC and maintained in DME with 10% FBS.

Transfections

To transfect MDA-MB-435 with E-cadherin, the calcium phosphate transfection kit (Stratagene) was used, according to manufacturer's protocol. For electroporations (BT-20 cells), 10^6 cells were washed with PBS and resuspended in electroporation buffer (120 mM KCl, 0.15 mM CaCl_2 , 10 mM K_2HPO_4 , 10 mM KH_2PO_4 , 25 mM Hepes, 2 mM EGTA, 5 mM MgCl_2) supplemented with 2 mM ATP and 5 mM glutathione. After a 5 min incubation on ice, the cells were electroporated at 500 μF and 380 V in a Bio-Rad gene pulser. Cells were immediately plated in a 100-mm dish in complete medium. Floating cells were removed and fresh medium was added 24 h after electroporation; puromycin was added to the culture for selection of clones 48 h after electroporation.

Clones and Vectors

For transfection of N-cadherin, a restriction fragment containing nucleotides 442-3362 (GenBank/EMBL/DDBJ accession number S42303; a kind gift of Dr. Avri Ben-Ze'ev, The Weizmann Institute of Science, Israel) was ligated into the expression vector pLK-pac (Islam et al., 1996). The E-cadherin construct has been described previously (Lewis et al., 1997). The human cadherin-11 cDNA was provided by Drs. S. Takashita and A. Kudo (Tokyo Institute of Technology, Japan; accession number D21254; Okazaki et al., 1994).

Antibodies and Reagents

Unless otherwise stated, all reagents were from Sigma Chemical Co. Rabbit polyclonal antibodies (Jelly) against human E-cadherin extracellular domain (Wheelock et al., 1987), and mouse mAbs against E-cadherin

(HECD1: a kind gift of Dr. Masatoshi Takeichi, Kyoto University, Kyoto, Japan) and N-cadherin (13A9; Knudsen et al., 1995; Sacco et al., 1995), have been described previously. The mouse mAb against β -catenin (6E3) was made as described by Johnson et al. (1993). The mouse mAbs against cadherin-11 were kindly provided by Dr. Marion Bussemakers (University Hospital Nijmegen, The Netherlands). The diacylglycerol lipase inhibitor, RHC80267, was purchased from BIOMOL.

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% NP-40 (BDH Chemicals Ltd.), 1 mM EDTA, and 2 mM 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. Cell extracts were resolved on 7% SDS-PAGE as described (Lewis et al., 1994), transferred electrophoretically to nitrocellulose, and immunoblotted as described (Wheelock et al., 1987) using primary antibodies followed by ECL, according to the manufacturer's protocol (Pierce Chemical Co.). For the purpose of loading equal amounts of protein onto SDS-PAGE, quantification was done using the BioRad protein assay reagent according to the manufacturer's protocol.

Immunofluorescence and Microscopy

Cells were grown on glass coverslips, fixed with Histochoice (Amresco), washed three 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 three times with PBS, and exposed to species-specific antibodies conjugated to FITC or rhodamine for 1 h. Cells were viewed using a Zeiss Axiophot microscope equipped with the appropriate filters, and photographed using Kodak T-MAX 3200 film. Living cells were viewed using a Zeiss Axiovert microscope and photographed using Kodak T-MAX 400 film.

In Vitro Invasion Assays and Motility Assays

For motility assays, 5×10^5 cells were plated in the top chamber of non-coated polyethylene terephthalate (PET) membranes (6-well insert, pore size 8 mm; Becton Dickinson). For in vitro invasion assays, 3×10^4 cells were plated in the top chamber of Matrigel-coated PET membranes (24-well insert, pore size 8 mm; Becton Dickinson). In motility and invasion assays, 3T3 conditioned medium was used as a chemoattractant in the lower chamber. The cells were incubated for 24 h and those that did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. Cells transverse the membrane were stained with Diff-Quick (Dade). Cells in ten random fields of view at 100 $\times$ were counted and expressed as the average number of cells/field of view. Three independent experiments were done in each case. The data were represented as the average of the three independent experiments with the SD of the average indicated. When cells were induced with dexamethasone to express a transgene, the control cells were treated with the same level of dexamethasone. To inhibit FGF receptor signaling, cells were treated with RHC80267 (which inhibits the activity of diacylglycerol lipase) at a concentration of 10–40 μ g/ml 3T3 conditioned culture medium during the 24 h of the assay.

Results

Expression of Cadherins by Breast Cancer Cells

E-cadherin has been termed a tumor suppressor, mainly because cells derived from E-cadherin-negative epithelial tumors tend to be invasive, whereas cells derived from E-cadherin-positive tumors tend not to be. In the case of cells derived from breast carcinomas, the majority of E-cadherin-negative cells are invasive (Sommers et al., 1991, 1994; Pierceall et al., 1995). However, an increasing number of exceptions to this rule are becoming evident. Our laboratory has recently shown that expression of an inappropriate cadherin by an oral squamous carcinoma cell line influences expression of E-cadherin and the cellu-

Table 1. Cadherin Expression in Breast Carcinoma Cell Lines

Cell line	E-cadherin	N-cadherin	P-cadherin	Cadherin-11	β -Catenin	Motility
MCF-7	+* [‡]	—	—	—	+* [‡]	No* [‡]
BT-20	+*	—	+*	—	+*	No*
SUM149	+*	—	+*	—	+*	No*
SKBr3	—* [‡]	—	—	—	—* [‡]	No* [‡]
MDA-MB-453	—* [‡]	—	—	—	—* [‡]	No [‡]
SUM1315	—	—	+*	±*	+*	No*
MDA-MB-435	—* [‡]	+*	—	—	+* [‡]	Yes [‡]
MDA-MB-436	—* [‡]	+*	—	—	+* [‡]	Yes [‡]
BT549	—* [‡]	+*	+*	—	+* [‡]	Yes [‡]
Hs578t	—* [‡]	+*	—	—	+* [‡]	Yes* [‡]
SUM159PT	—	+*	—	—	+*	Yes*
MDA-MB-231	—	—	—	+* [‡]	+*	Yes* [‡]

*Current study; [‡]Sommers et al., 1991; [‡]Frixen et al., 1991; [‡]Sommers et al., 1994; [‡]Pierceall et al., 1995; [‡]Pishvaian et al., 1999.

lar phenotype (Islam et al., 1996). This observation led us to hypothesize that the invasiveness of some breast cancer cells may be due to an increase in the expression of an inappropriate cadherin, possibly N-cadherin, rather than to a decrease in the expression of E-cadherin. To test this hypothesis, we surveyed a large number of cell lines, many of which had been characterized previously, for expression of E- and N-cadherin. The data, which are summarized in Table 1, supported our notion that invasiveness is correlated with N-cadherin expression, rather than lack of E-cadherin expression.

Fig. 1 is an immunoblot of extracts of the cell lines presented in Table 1. Equal amounts of protein were loaded in each lane. The samples were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for E-, N-, or P-cadherin, cadherin-11, and β -catenin. Fig. 2 presents phase micrographs of the living cells to compare the

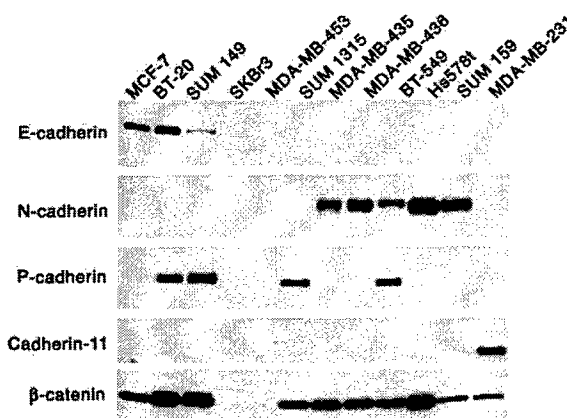


Figure 1. Cadherin and β -catenin expression in breast carcinoma cell lines. Confluent monolayers of MCF-7, BT-20, SUM 149, SKBr3, MDA-MB-453, SUM 1315, MDA-MB-435, MDA-MB-436, BT-549, Hs578t, SUM 159PT, or MDA-MB-231 were extracted with NP-40. 20 μ g total protein from each cell extract was resolved by SDS-PAGE, transferred to nitrocellulose, and blotted with antibodies against E-cadherin (HECD1), N-cadherin, P-cadherin, cadherin-11, or β -catenin.

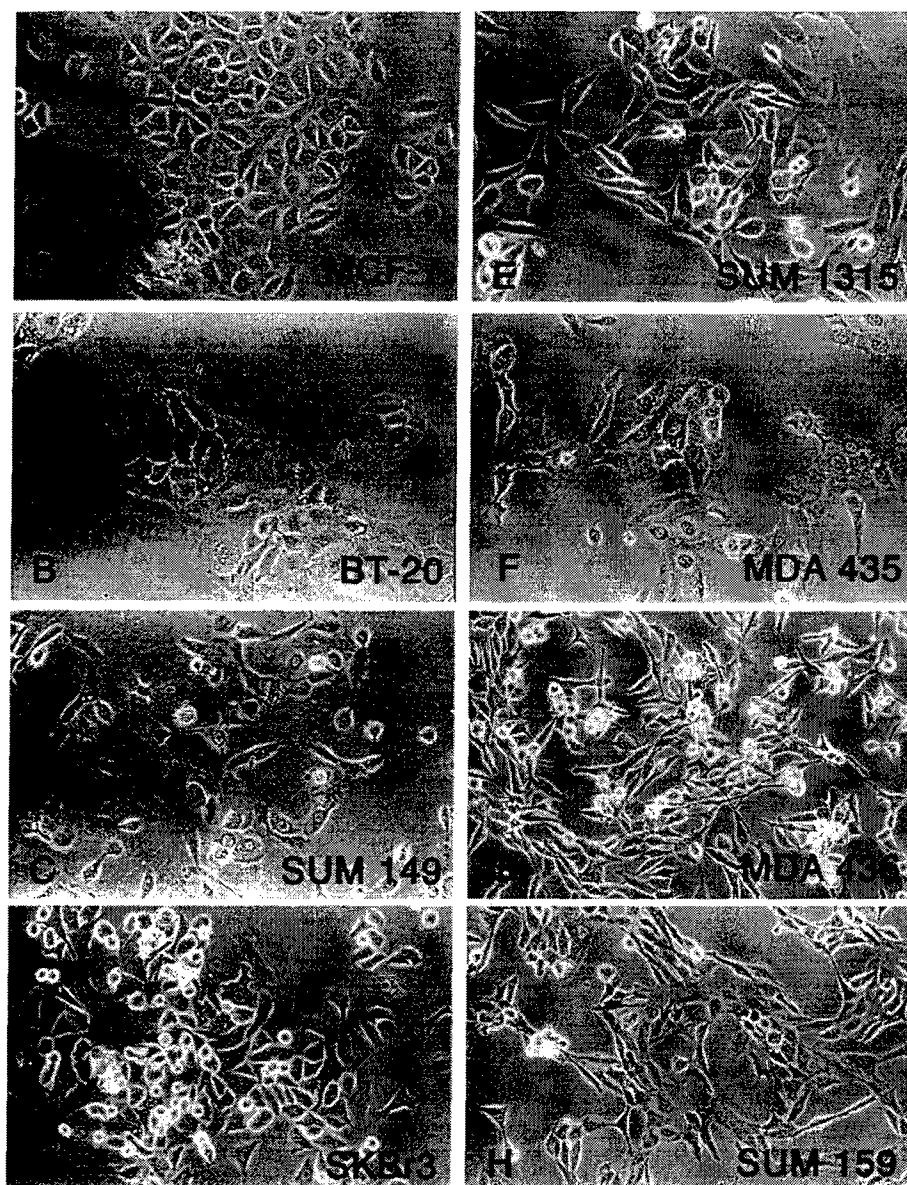


Figure 2. Morphological analysis of breast cancer cell lines. Living monolayers of MCF-7 (A), BT-20 (B), SUM 149 (C), SKBr3 (D), SUM 1315 (E), MDA-MB-435 (F), MDA-MB-436 (G), or SUM 159PT (H) cells were photographed using an inverted Zeiss microscope at 200 $\times$. Bar, 10 μ m.

morphologies of breast cancer cells expressing the various members of the cadherin family. MCF-7 cells expressed E-cadherin, had low invasion rates, and presented an epithelial-like morphology. BT-20 cells expressed both E- and P-cadherin, had low invasion rates, and presented an epithelial-like morphology. In contrast, E-cadherin-negative cell lines did not present an epithelial morphology, but rather appeared as fibroblast-like cells with less obvious cell-cell interactions. Even the SUM149 cell line that expressed a small amount of E-cadherin, along with substantial amounts of P-cadherin, did not have the epithelial appearance typified by the MCF-7 and BT-20 cell lines. SUM1315 cells, which expressed P-cadherin, along with a small amount of cadherin-11, also had a fibroblastic appearance with minimal cell-cell interactions. However,

these fibroblastic, N-cadherin-negative cell lines had low motility and invasion rates (Table I and Fig. 3). The N-cadherin-expressing cell lines all displayed a fibroblastic phenotype, as typified by MDA-MB-435, MDA-MB-436, and SUM159 (Fig. 2). Cell lines that did not express any cadherin, as typified by SKBr3, displayed a fibroblastic phenotype much like the N-cadherin-positive cells, however, they were less adhesive to the substratum than were cadherin-expressing cells. In addition, they tended to float in the medium upon reaching confluency and when undergoing mitosis.

A Role for N-Cadherin in Cell Motility

In this study, we hypothesized that the invasive behavior

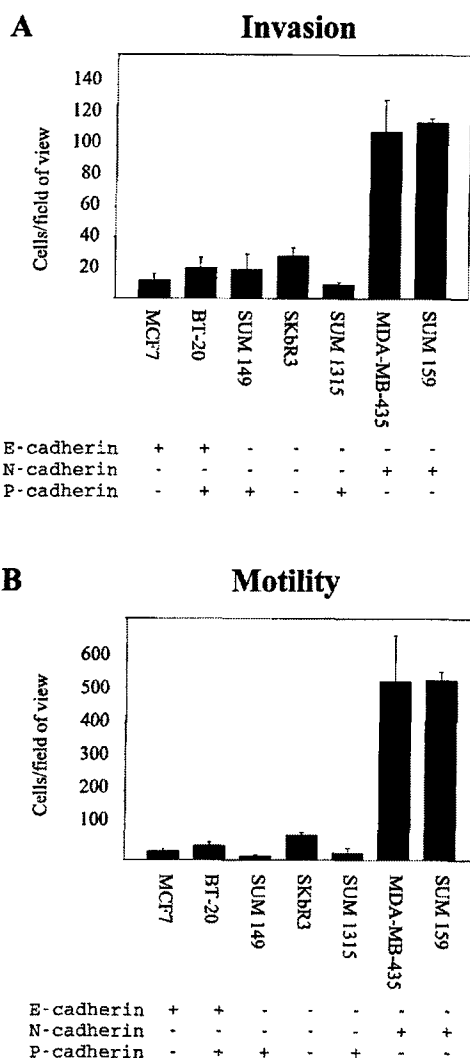


Figure 3. N-cadherin expression correlates with increased invasiveness and motility in breast carcinoma cell lines. Cells were plated on Matrigel-coated or noncoated membranes for invasion assays or motility assays, respectively. The cells were incubated for 24 h, and those that did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. The remaining cells were stained, and the number transverse the membrane was determined by averaging ten random fields of view at 100 $\times$. The data are expressed as the number of cells/field of view and is the average of three independent experiments. Error bars indicate SD of the average.

of some breast cancer cell lines may be due to expression of N-cadherin, rather than to lack of expression of E-cadherin. To test this hypothesis, we performed invasion assays on Matrigel-coated membranes and motility assays on uncoated membranes. Fig. 3 presents data from representative cell lines. The N-cadherin-expressing cell lines, SUM159 and MDA-MB-435, were substantially more invasive and more motile than the E-cadherin-expressing line (MCF-7), the E/P-cadherin-expressing cell lines (BT-20

and SUM149), and the P-cadherin-expressing line (SUM1315). The cell line that did not express any cadherins, SKBr3, was no more motile nor invasive than were the E-cadherin-expressing cell lines BT-20, MCF-7, and SUM149. Together, these data suggest that, in these cells, N-cadherin acts to promote motility and invasion, rather than E-cadherin acting to suppress these activities.

Since the cell lines in this study were derived from separate tumors and, thus, are likely to be descendants of different cell types, we sought to manipulate expression of specific cadherins in representative cell lines to determine if the invasive phenotype was due to N-cadherin or to other cellular aspects. We chose two cell lines for these studies: BT-20, which expresses E- and P-cadherin and has a low rate of invasion, and MDA-MB-435, which expresses N-cadherin and is highly invasive. When BT-20 cells were transfected with N-cadherin (BT-20N), they expressed levels of N-cadherin that were comparable to MDA-MB-435; however, they did not undergo a morphological change (compare Fig. 2 B with Fig. 4 A), nor did they downregulate the expression of E-cadherin to any significant level. Fig. 4, B and C, show that E- and N-cadherin colocalized at cell-cell borders, suggesting that both cadherins are active at the cell surface. When equal amounts of protein from extracts of BT-20 and BT-20N cells were resolved by SDS-PAGE and immunoblotted for cadherin expression, it could be seen that the BT-20N cells slightly downregulated E-cadherin, that the two cell lines expressed equal levels of P-cadherin, and that the BT-20N cells expressed levels of N-cadherin that were comparable to the invasive N-cadherin-expressing cells depicted in Fig. 1. In addition, β -catenin coimmunoprecipitated equally well with either E- or N-cadherin in these cells (Fig. 4 E). BT-20 cells were unusual in that they expressed high levels of both E- and N-cadherin and, thus, were an ideal cell line in which to test the hypothesis that it is the expression of N-cadherin, not the lack of E-cadherin, that promotes cell motility and invasion in some breast cancer cells. As predicted, motility and invasion rates for BT-20N were five- to eightfold higher than the rates for nontransfected BT-20 cells (Fig. 5). Although BT-20N cells were not as motile as the N-cadherin-expressing MDA-MB-435 cells (Fig. 5 B), they were almost as invasive (Fig. 5 A).

E-Cadherin Does Not Suppress Motility in N-Cadherin-expressing MDA-MB-435 Cells

Since the BT-20N cells expressed high levels of E-cadherin, and were highly motile and invasive, we had good evidence that E-cadherin did not inhibit invasion in these cells and, thus, does not act as an invasion suppressor in all breast cancer cells. However, to further test this idea, we transfected N-cadherin-expressing MDA-MB-435 cells with E-cadherin (MDA-MB-435E) to see if E-cadherin would decrease the invasive nature of these cells. In this experiment, we sought to obtain clones that expressed high levels of E-cadherin, but still retained a significant level of N-cadherin. Fig. 6 D shows the levels of expression of E- and N-cadherin in several clones. Clone 2 was chosen for subsequent studies because it expressed the highest level of E-cadherin and, in addition, showed a two- to threefold reduction in N-cadherin expression, compared

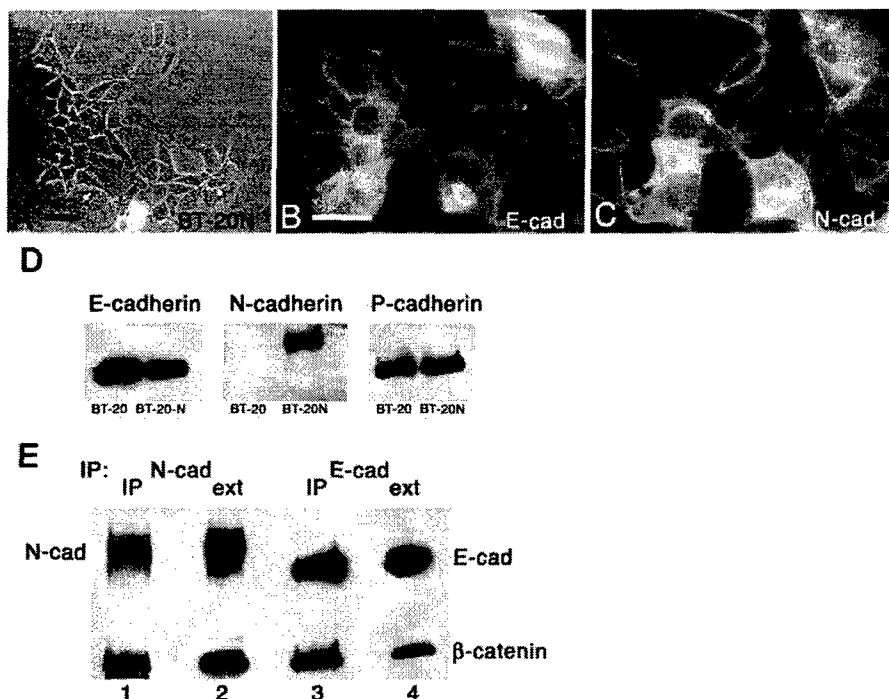


Figure 4. Expression of N-cadherin by BT-20 cells. BT-20 cells were transfected with N-cadherin (BT-20N) and expression induced with dexamethasone. A, Phase-microscopy of living BT-20N cells. Bar, 10 μ m. B and C, Cells were grown on glass coverslips and processed for coimmunofluorescence localization with antibodies against E-cadherin (Jelly; B) and N-cadherin (C). D, BT-20 and BT-20N cells were extracted with NP-40 and 20 μ g protein from each extract was resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for E-cadherin (HECD1), N-cadherin, or P-cadherin. E, Extracts of BT-20N cells were immunoprecipitated with antibodies against N-cadherin or E-cadherin (HECD1). The immunoprecipitation reactions, as well as cell extracts, were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for N-cadherin and β -catenin (lanes 1 and 2) or E-cadherin (HECD1) and β -catenin (lanes 3 and 4).

with the parental cells. Although these cells expressed very high levels of E-cadherin, they did not display a typical epithelial morphology, and closely resembled the parent cell line (compare Figs. 6 A with 2 F). Both E- and N-cadherin were localized to regions of cell-cell contact (Fig. 6, B and C). When the MDA-MB-435E cells were tested for motility and invasion, they were not significantly different from the parental MDA-MB-435 cells (Fig. 5), even though β -catenin was associated with the transfected E-cadherin, as well as the endogenous N-cadherin (Fig. 6 E).

BT-20N Cells Effectively Segregate from HT1080 Fibroblasts

Hazan et al. (1997) suggested that N-cadherin-expressing breast cancer cells invade the stroma because they associate with the N-cadherin-expressing stromal cells. In our studies, we employed an in vitro invasion assay in which the cells invade an extracellular matrix that does not include any stromal cells. Thus, we can make the important statement that, in our studies, N-cadherin actively promotes invasion and motility. In Hazan et al. (1997), the investigators showed that N-cadherin-expressing breast cancer cells coaggregated with N-cadherin-expressing fibroblast-like cells. Since it has been suggested that it is the entire complement of cadherins expressed by a cell that determines its ability to associate with other cells, and that even cells expressing different levels of the same cadherin

can sort from one another (Steinberg and Takeichi, 1994), we sought to determine if the BT-20N cells that express N-, E-, and P-cadherin would segregate from an N-cadherin-expressing fibroblast cell line, HT1080. Equal numbers of BT-20 cells and HT1080 cells, or BT-20N cells and HT1080 cells, were mixed together and allowed to settle on glass coverslips. They were then prepared for immunofluorescence analysis using antibodies against E- or N-cadherin. In the immunofluorescence analysis of the BT-20/HT1080 cocultures, E-cadherin stained only the BT-20 cells and N-cadherin stained only the HT1080 cells. Fig. 7, A and B, show that these two cell lines effectively segregated from one another as expected. In the immunofluorescence analysis of the BT-20N/HT1080 cocultures, antibodies against E-cadherin stained only the BT-20N cells, whereas antibodies against N-cadherin stained both the BT-20N cells and the HT1080 cells. Fig. 7, C and D, show that the BT-20N cells and the HT1080 cells effectively segregated from one another, even though both cell lines express N-cadherin. Thus, epithelial cells that express N-cadherin along with other cadherins have not necessarily gained the ability to intermix with stromal cells.

Cadherin-11 Promotes Motility in Breast Epithelial Cells

In the course of our studies on breast tumor cell lines, we characterized one atypical line (MDA-MB-231) that did

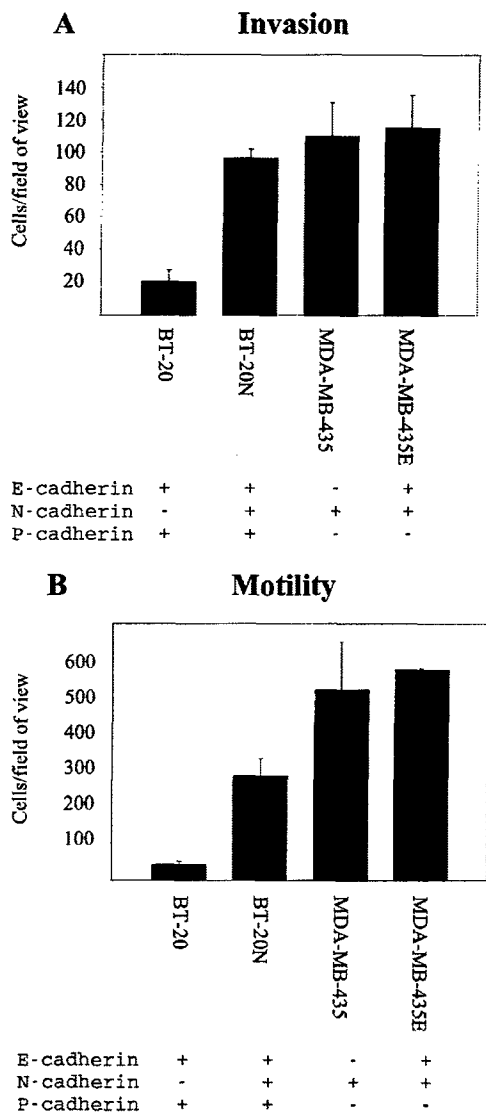


Figure 5. Exogenous expression of N-cadherin by BT-20 cells (BT-20N) increases their invasiveness, whereas exogenous expression of E-cadherin by MDA-MB-435 cells (MDA-MB-435E) does not effect their behavior. Cells were plated on Matrigel-coated or noncoated membranes for invasion assays or motility assays, respectively. The cells were incubated for 24 h, and those that 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 transverse the membrane was determined by averaging ten random fields of view at 100 $\times$. The data are expressed as the number of cells/field of view and is the average of three independent experiments. Error bars indicate SD of the average.

not express E-, P-, or N-cadherin, but nonetheless was invasive (Table I). Since MDA-MB-231 cells expressed significant levels of β -catenin, a protein that is not stable in cadherin-negative cells, we suspected that this cell line expressed another member of the cadherin family of pro-

teins, possibly one that is closely related to N-cadherin. We therefore analyzed RNA from this line with degenerate PCR primers designed to amplify all cadherins and found that it expressed cadherin-11 mRNA. Expression of cadherin-11 protein was confirmed by immunoblotting data with a cadherin-11-specific mAb, in agreement with recent data (Pishvaian et al., 1999). Like N-cadherin, cadherin-11 is expressed by some mesenchymal cells (Simonneau et al., 1995). Interestingly, cadherin-11 is expressed in some epithelial cells of the human placenta, and it has been suggested that cadherin-11 plays a role in mediating trophoblast-endometrium interactions as the cytotrophoblasts invade the uterine wall (MacCalman et al., 1996). Thus, one idea is that cadherin-11 could act in a manner similar to N-cadherin in promoting cell motility and invasion in breast cancer cells. To test this idea, we transfected cadherin-11 into BT-20 cells (BT-20Cad-11 cells). Like the BT-20N cells, BT-20Cad-11 cells retained the morphology of their parent line, even though they expressed high levels of cadherin-11 at cell-cell borders (Fig. 8, A-C). As predicted, cadherin-11-expressing BT-20 cells were more invasive and motile than the parental BT-20 cells (Fig. 8, D and E). Interestingly, the cadherin-11-expressing cells were not as invasive or motile as the N-cadherin-expressing cells. For example, the MDA-MB-231 cells were not as motile as the MDA-MB-435 cells (Figs. 5 and 8). More significantly, the BT-20 cells transfected with cadherin-11 did not become as motile as they did when transfected with N-cadherin. This may be due to differences between the two cadherins, or differences in expression levels of the transfected cadherins. It is reasonable to speculate that the level of expression of the inappropriate cadherin is relevant since the cell line SUM1315 expresses a small amount of cadherin-11, yet is not invasive.

N-Cadherin May Promote Cell Motility through a Fibroblast Growth Factor Receptor Signal Transduction Pathway

The laboratories of Frank Walsh and Patrick Doherty have shown that N-cadherin promotes neurite outgrowth from cerebellar neurons (Williams et al., 1994a). In addition, they showed that N-cadherin-mediated neurite extension was dependent on FGF receptor signaling, but was independent of ligand (Williams et al., 1994b). Walsh and Doherty thus proposed a model whereby the FGF receptor was induced to dimerize in the absence of FGF via interaction with N-cadherin (Doherty and Walsh, 1996). Dimerization of the FGF receptor results in receptor cross phosphorylation that initiates a number of signal transduction pathways. The pathway relevant to N-cadherin-dependent neurite outgrowth involves the generation of arachidonic acid from diacylglycerol, by the action of diacylglycerol lipase. The Walsh and Doherty laboratories showed that the diacylglycerol lipase inhibitor, RHC 80267, prevented neurite extension on N-cadherin-transfected 3T3 cells, thus implicating this type of FGF receptor signaling in N-cadherin-dependent neurite extension (Meiri et al., 1998). We hypothesized that the N-cadherin-mediated cell motility we observed in epithelial cells may also be acting through FGF receptor signaling. To test this hypothesis, we treated MDA-MB-435 cells, BT-20 cells,

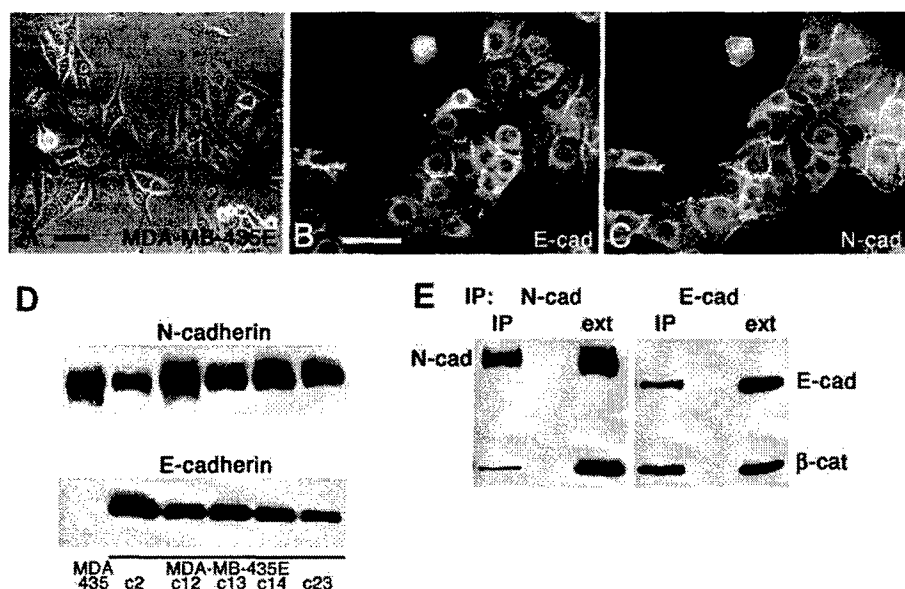


Figure 6. Expression of E-cadherin by MDA-MB-435 cells. MDA-MB-435 cells were transfected with E-cadherin (MDA-MB-435E) and expression was induced with dexamethasone. A, Phase-contrast microscopy of MDA-MB-435E cells. Bar, 10 μ m. B and C, Cells were grown on glass coverslips and processed for coimmunofluorescence localization with antibodies against E- (Jelly; B) and N-cadherin (C). D, MDA-MB-435 and several clones of MDA-MB-435E cells were extracted with NP-40 and 20 μ g protein from each extract was resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for E- (HECD1) and N-cadherin. Clone 2 (cl2) expressed the highest level of E-cadherin

and was chosen for subsequent studies. E, Extracts of MDA-MB-435 and MDA-MB-435E cells were immunoprecipitated with antibodies against N- or E-cadherin (HECD1). The immunoprecipitation reactions, along with cell extracts, were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for N-cadherin and β -catenin (lanes 1 and 2), or E-cadherin (HECD1) and β -catenin (lanes 3 and 4).

and BT-20N cells with varying levels of RHC80267 to determine if it would influence the motility of these cells in the transwell assay. RHC80267 inhibited cell motility in both N-cadherin-expressing cell lines in a dose-dependent manner (Fig. 9 A). Importantly, this inhibitor had no effect on the motility of the N-cadherin-negative BT-20 cells. Although these data are consistent with the hypothesis that N-cadherin dependent cell motility is mediated

through FGF receptor signaling in a manner similar to N-cadherin-dependent neurite outgrowth, additional experiments must be done to further support this notion. Thus, we are continuing to investigate the mechanism whereby N-cadherin mediates motility in epithelial cells. To determine if cadherin-11 and N-cadherin promote cell motility through a similar pathway, we treated MDA-MB-231 and BT-20cad11 cells with RHC80267, and compared

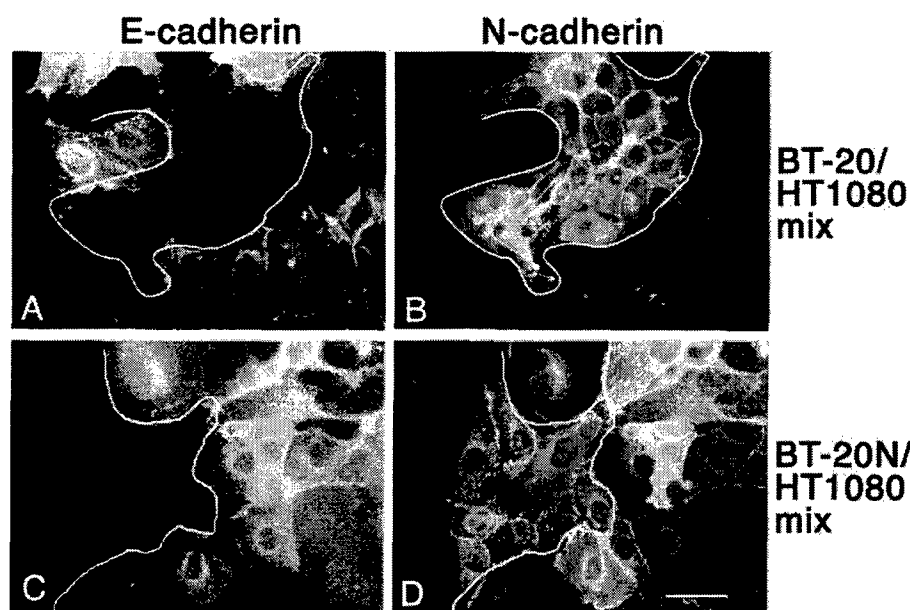


Figure 7. BT-20N cells do not mix with HT1080 cells. 5×10^4 BT-20 or BT-20N cells were mixed with an equal number of HT1080 cells, allowed to settle on coverslips, and processed for immunofluorescence with an mAb against N- (13A9) or E-cadherin (Jelly). A and B are a mix of BT-20 and HT1080 cells stained for E- and N-cadherin, respectively. The encircled cells are a group of E-cadherin-negative, N-cadherin-positive HT1080 cells. C and D are a mix of BT-20N and HT1080 cells stained for E- and N-cadherin, respectively. The encircled cells are a group of E-cadherin-negative, N-cadherin-positive HT1080 cells.

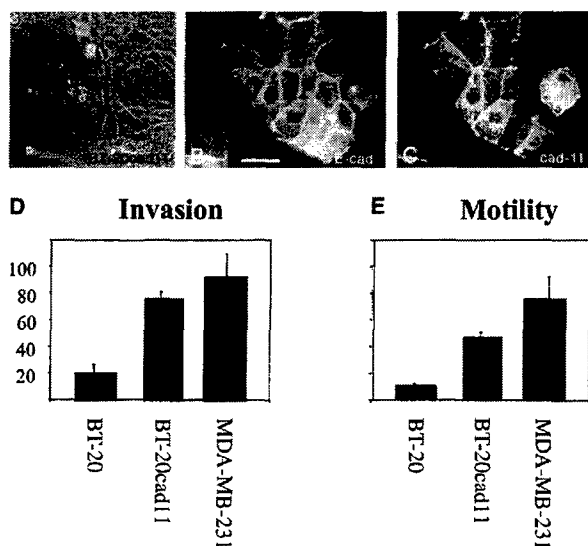


Figure 8. Exogenous expression of cadherin-11 by BT-20 cells (BT-20cad11) increases their invasiveness. BT-20 cells were transfected with cadherin-11 (BT-20cad11) and expression induced with dexamethasone. A, Phase-microscopy of living BT-20cad11 cells. Bars, 10 μ m. B and C, Cells were grown on glass coverslips and processed for coimmunofluorescence localization with antibodies against E-cadherin (Jelly; B) and cadherin-11 (C). D and E, Cells were plated on Matrigel-coated or noncoated membranes for invasion assays or motility assays, respectively. The cells were incubated for 24 h, and those that 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 transverse the membrane was determined by averaging ten random fields of view at 100 $\times$. The data are expressed as the number of cells/field of view and is the average of three independent experiments. Error bars indicate SD of the average.

motility rates between treated and nontreated cells (Fig. 9 B). The diacylglycerol lipase inhibitor decreased the motility of cadherin-11-expressing cells in a dose-dependent manner. Cadherin-11-expressing cells are less motile than MDA-MB-435, and the inhibitor is less effective in decreasing the motility of the cadherin-11 expressing cells, suggesting there may be some differences in the respective signal transduction pathways, possibly in growth factor receptor levels or isoforms.

Discussion

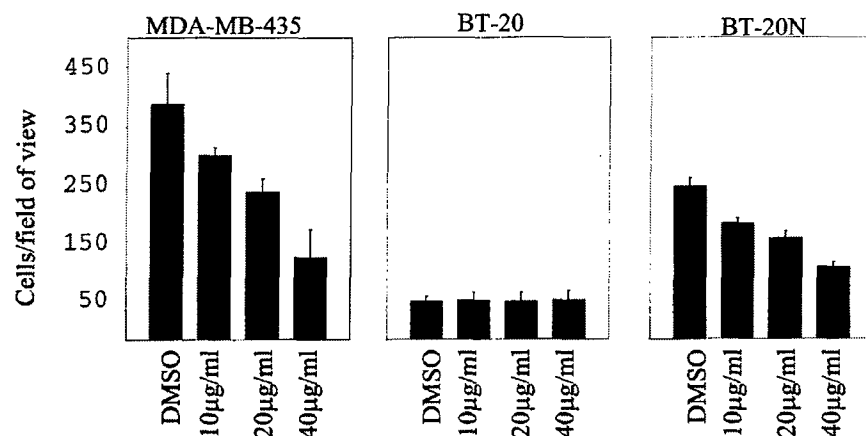
Previously, our laboratory showed that expression of different cadherin family members by squamous epithelial cells markedly effected morphology (Islam et al., 1996), i.e., when oral squamous epithelial cells expressed N-cadherin, they converted to a fibroblastic phenotype concurrent with decreased cell-cell adhesion. Thus, when we turned our attention to breast cancer cells for the present study, we were interested not only in the expression of various cadherins by these cells, but also in whether these cadherins influenced the morphology of the cells. We were not surprised to find that breast cancer cells endogenously

expressing N-cadherin displayed a fibroblastic phenotype with tenuous cell-cell contacts, whereas breast cancer cells endogenously expressing E-cadherin displayed a typical epithelial morphology. We were, however, surprised to find that transfection of N-cadherin into the E-cadherin-expressing BT-20 breast cancer cell line had no effect on morphology, even though it had a dramatic effect on cell behavior. Equally surprising was the fact that forced expression of E-cadherin had no effect on the morphology of the fibroblastic N-cadherin-expressing MDA-MB-435 cells. Thus, the breast cancer cell lines examined in this study behaved very differently from the oral squamous epithelial lines that we characterized previously. Interestingly, the oral squamous epithelial cells downregulated E-cadherin when they were forced to express N-cadherin, suggesting an inverse relationship between these cadherins. In contrast, the breast cancer cells continued to express their endogenous cadherin(s) when transfected with a different cadherin. The continued expression of endogenous cadherin may account for the lack of morphological change in the transfectants. Thus, the breast cancer cells differ from the oral squamous epithelial cells in two very important ways: first, the oral squamous epithelial cells appear to coregulate cadherins in an inverse manner, whereas these cadherins are independently regulated in breast cancer cells; and second, expression of E-cadherin by the oral squamous epithelial cells is sufficient for epithelial morphology, whereas epithelial morphology in the breast cancer cells appears to depend on other factors, in addition to E-cadherin.

In the present study, we have demonstrated that N-cadherin (or cadherin-11) expression in human breast carcinoma cells promotes an invasive phenotype. By transfecting the BT-20 cells with these nonepithelial cadherins, we have provided evidence for a direct role of these cadherins in cell motility and invasion. Previous studies have correlated the expression of N-cadherin or cadherin-11 with invasion in breast cancer cells. However, in this study, we took the important next step and used transfection studies to show that a cell line that has a low invasion rate could be converted to a highly invasive cell by expression of N-cadherin or cadherin-11. The BT-20 breast cancer cell line provided an important tool for these studies since they did not downregulate E-cadherin when forced to express N-cadherin. Thus, we can conclude that, even in cells expressing high levels of E-cadherin, N-cadherin (or cadherin-11) can promote motility, suggesting that, in this regard, both N-cadherin and cadherin-11 are dominant over E-cadherin. A study by Sommers et al. (1994) supports this idea. These authors showed that transfection of E-cadherin into the E-cadherin-negative breast cancer cell lines, BT549 and HS578, did not decrease the invasive capacity of these cells. These authors suggested that the transfected E-cadherin was not functional; however, these authors were unaware of the fact that the BT549 and HS578 cell lines express N-cadherin.

A previous study using MDA-MB-435 cells showed that transfection of E-cadherin into these cells reduced their capacity to form tumors when injected into the foot pads of nude mice (Meiners et al., 1998). In contrast to our study, these authors showed that E-cadherin-transfected clones of MDA-MB-435 cells underwent a morphological

A



B

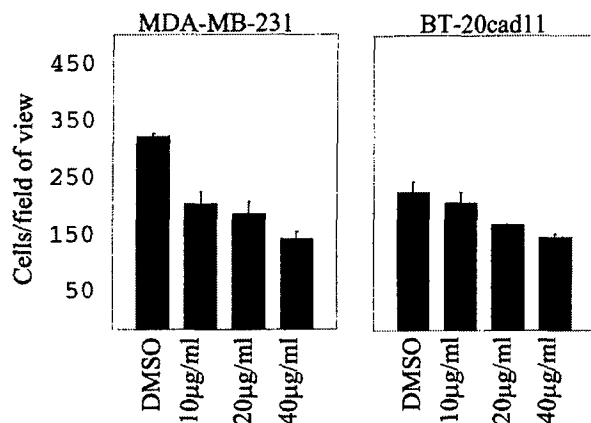


Figure 9. The diacylglycerol lipase inhibitor RHC80267 decreases motility of N-cadherin- and cadherin-11-expressing cells. Cells were plated on noncoated membranes for motility assays. The cells were incubated for 24 h in the presence of RHC80267 at varying concentrations, and those that did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. The remaining cells were stained, and the number transverse the membrane was determined by averaging ten random fields of view at 100 $\times$. The data are expressed as the number of cells/field of view and is the average of three (A) or two (B) independent experiments. Error bars indicate SD of the average.

change upon E-cadherin expression. In addition, they showed that E-cadherin-transfected clones were less tumorigenic in their assay than the parental cells. One difference in the study of Meiners et al. (1998) and ours is that they did not assay for N-cadherin expression in their E-cadherin-positive clones of MDA-MB-435 transfectants. Our study clearly demonstrates that N-cadherin influences the behavior of the cells, and that cells retaining N-cadherin do not undergo a morphological or behavioral change upon expression of E-cadherin. Thus, one possible explanation for the difference between these two studies is that the cells in the Meiners' study did not express N-cadherin. The point of our study was to determine if N-cadherin was capable of influencing the behavior of epithelial cells, even if they expressed E-cadherin, thus, we were particularly careful to select cell lines that retained N-cadherin expression after transfection with E-cadherin (Fig. 6).

One puzzling aspect of cell lines derived from metastatic

tumors is that they often express E-cadherin and appear to be relatively normal epithelial cells. A possibility suggested by the present study is that such cells may have up-regulated the expression of N-cadherin during the process of metastasis. Our results suggest that expression of N-cadherin would confer on these cells the capacity to invade, even though they continued to express E-cadherin. In this regard, expression of an inappropriate cadherin like N-cadherin (or other related cadherins) may be a better gauge of the clinical state of a tumor than is decreased expression of E-cadherin.

Some of the E-cadherin-negative breast cancer cells expressed endogenous P-cadherin. These cells had a fibroblastic morphology similar to that of the N-cadherin-expressing cells; however, they were not highly invasive, suggesting that P-cadherin confers upon breast cancer cells characteristics different from those conferred by either E- or N-cadherin. P-cadherin is expressed in the myo-

epithelial cells surrounding the luminal epithelial cells of the mammary gland. Radice et al. (1997) recently showed that P-cadherin deficient mice develop age-dependent hyperplasia and dysplasia of the mammary epithelium, and suggested that P-cadherin may play a role in maintaining the normal phenotype of breast epithelial cells. One possibility is that the P-cadherin-expressing tumor cells were derived from the myoepithelium, rather than from the true epithelium.

E-cadherin has been termed an invasion suppressor because transfection of this protein into some E-cadherin-negative invasive carcinoma cells resulted in decreased invasive capacity. Our prediction is that at least some of these cell lines expressed a cadherin, like N-cadherin or cadherin-11, and overexpression of E-cadherin resulted in downregulation of the endogenous cadherin, as we saw with the oral squamous epithelial cells. Thus, we hypothesize that the invasion suppressor role of E-cadherin arises in part from its ability to decrease the level of N-cadherin in certain, but not all, tumors. In the present study, cell lines that did not express any classical cadherins, as evidenced by lack of β -catenin protein, as well as lack of detectable cadherin, had low invasion rates. Our hypothesis, that loss of E-cadherin alone does not necessarily increase invasive capacity in breast carcinoma cells, is supported by the observation that function-blocking antibodies against E-cadherin did not confer a highly motile, invasive phenotype on MCF-7 cells, a breast cancer cell line that is E-cadherin-positive and N-cadherin-negative (Sommers et al., 1991). The current study suggests that, in some carcinoma cells, expression of N-cadherin, or a similar cadherin such as cadherin-11, may actually be necessary for increased motility and invasion. A recent clinical study suggested that inactivation of E-cadherin is an early event in the progression of lobular breast carcinomas (Vos et al., 1997). We might suggest that a subsequent event would be activation of the expression of an inappropriate cadherin, such as N-cadherin or cadherin-11.

Understanding the mechanism by which N-cadherin promotes motility in epithelial cells is important if we are to develop treatments that will decrease the invasiveness of tumor cells. A number of studies have shown that epithelial cells can be induced to scatter in response to growth factors, such as hepatocyte growth factor and members of the FGF, EGF, and TGF families (Blay and Brown, 1985; Vallés et al., 1990; Behrens et al., 1991; Geimer and Bade, 1991; Gherardi and Stoker, 1991; Rosen et al., 1991; Miettinen et al., 1994; Savagner et al., 1994, 1997). Walsh, Doherty, and coworkers have established, through extensive studies on FGF receptor and cell adhesion molecules, that N-cadherin and the FGF receptor cooperate to induce neurite outgrowth in cerebellar neurons (reviewed in Doherty and Walsh, 1996; Walsh and Doherty, 1997). These authors have proposed a scheme for activation of the kinase activity of the FGF receptor through cis interactions with N-cadherin, via an HAV domain in the FGF receptor and an HAV interaction domain in the fourth extracellular domain of N-cadherin (Doherty and Walsh, 1996). In addition, it has been proposed that the cadherins form lateral dimers in the plane of the membrane (Shapiro et al., 1995; Takeda et al., 1999), which could result in dimerization of the FGF receptor, and subsequent activa-

tion of the signal transduction pathway. We based the studies presented herein on the model presented by Walsh and Doherty, and proposed that interaction of N-cadherin with the FGF receptor in N-cadherin-expressing epithelial cells may result in increased motility, similar to that seen by treating epithelial cells with growth factors. To test this hypothesis, we interfered with the N-cadherin-dependent FGF receptor signal transduction pathway proposed by Walsh and Doherty by inhibiting a downstream enzyme, diacylglycerol lipase. We showed that inhibiting diacylglycerol lipase decreased motility of N-cadherin-expressing cells in a dose-dependent manner while having no effect on the motility of N-cadherin-negative cells. Thus, our data strongly support the notion that N-cadherin promotes motility in breast cancer cells by activating growth factor receptor signal transduction pathways. Continued efforts in our laboratory are aimed at further defining the signal transduction pathway(s) that mediate cadherin-dependent motility in epithelial cells.

At first glance, it might seem unlikely that expression of an additional cell adhesion molecule would confer a motile and invasive phenotype upon an epithelial cell. However, motile cells, such as fibroblasts and myoblasts, express N-cadherin (Knudsen et al., 1995; Huttenlocher et al., 1998) and a switch from E- to N-cadherin occurs in the chick embryo when epiblast cells ingress through the primitive streak to form the mesoderm (Edelman et al., 1983; Hatta and Takeichi, 1986). Another interesting cadherin switch occurs during establishment of the human placenta, where fetal cytotrophoblast cells invade the vasculature of the uterus. During this invasive process, the cytotrophoblast cells downregulate the expression of E-cadherin and upregulate vascular/endothelial (VE) cadherin (Zhou et al., 1997). Thus, it is feasible to suggest that increased expression of a nonepithelial cell cadherin, such as N-cadherin, could increase the invasive potential of tumor cells. Ongoing studies in our laboratory are designed to determine how N-cadherin differs from E-cadherin in its ability to induce cell motility. We hypothesize that E-cadherin does not have the ability to interact with the relevant growth factor receptors, and we are preparing chimeric molecules between E- and N-cadherin to test this hypothesis.

An important message from the present studies is that cadherins may not function identically in different cell types. The fact that cadherins may act differently in different cell types is particularly evident when comparing the current study with earlier studies showing that mouse L cells or S180 fibroblasts attained an epithelial morphology when transfected with either E- or N-cadherin (Nagafuchi et al., 1987; Hatta et al., 1988; Matsuzaki et al., 1990). It will be important in future studies to consider the cellular makeup, as well as the complement of cadherin family members, when interpreting data on cellular morphology and behavior.

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DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

23 May 02

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for Grant DAMD17-97-1-7298. Request the limited distribution statement for Accession Document Number ADB267138 be changed to "Approved for public release; distribution unlimited." This report should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management