An Alternatively Spliced Cadherin-11 Enhances Human Breast Cancer Cell Invasion

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ABSTRACT

Although reduced levels of the epithelial cell adhesion molecule E-cadherin are often associated with poorly differentiated breast cancers, recent studies show that expression of other cadherins such as N-cadherin, P-cadherin, and the mesenchymal cadherin-11 is actually elevated in invasive breast cancers and cell lines. Cadherin-11 is unique among cadherins in that it exists as two alternatively spliced forms that are expressed together in the same cell. We now show that expression of wild-type cadherin-11, with or without coexpression of the COOH-terminal truncated splice variant, promotes epithelial differentiation of the cadherin-negative SKBR3 cell line. Exogenous wild-type cadherin-11 association with and membrane recruitment of β-catenin and p120 are unaffected by coexpression of the truncated variant. Cadherin-11-expressing cells exhibit modest changes in cell proliferation and no change in anchorage-independent growth. However, coexpression of wild-type cadherin-11 and the splice variant promotes a dramatic increase in the ability of SKBR3 cells and E-cadherin-positive MCF7 cells to traverse Matrigel-coated filters. Biochemical studies indicate that the truncated variant may be secreted from the cell and/or enters a detergent-insoluble compartment. These data suggest that the presence of the cadherin-11 splice variant promotes invasion of cadherin-11-positive breast cancer cells.

INTRODUCTION

The cadherins are a superfamily of transmembrane glycoproteins that mediate cell to cell adhesion in a variety of tissues (reviewed in Refs. 1–3). Members of the classic cadherin subfamily possess five EC domains and a conserved cytoplasmic region. These molecules are responsible for formation of the adherens junction in most cell types. Upon calcium ion binding by the extracellular region, the EC domains change conformation, allowing lateral dimerization and subsequent cross-association between dimers on adjacent cells, generally in a homotypic fashion (i.e., E-cadherin to E-cadherin; Ref. 4). The cytoplasmic tail interacts directly or indirectly with “linker proteins,” including β-catenin, plakoglobin or p120, α-catenin, α-actinin, and vinculin. These proteins are thought to couple cadherins to the actin cytoskeleton to strengthen the adhesive force of the entire junctional complex (5).

The best-characterized classic cadherin is E-cadherin. Differential expression of E-cadherin has been implicated in several aspects of development, including cell sorting during gastrulation and tissue morphogenesis as well as the establishment of differentiated cell identity (for example, in the intestinal lumen (6, 7)). In addition, E-cadherin has been studied extensively with respect to its role as a putative tumor suppressor gene. Decreased E-cadherin expression has been correlated with metastasis and decreased survival in several different cancers, including breast carcinoma (8). However, E-cadherin loss is not an absolute predictor of tumor invasion or metastasis. Interestingly, increased expression of other cadherins, such as N-cadherin and P-cadherin, may also be associated with development or progression of breast carcinoma (9, 10).

Cadherin-11, or OB-cadherin, was originally identified in mouse osteoblasts (11); it was later found to be expressed in a variety of normal tissues of mesodermal origin, including areas of the kidney and brain (12). Although a type II cadherin (lacking a conserved HAV sequence), cadherin-11 is otherwise similar in structure to the type I cadherins N- and P-cadherin. In addition, the genomic structure of cadherin-11 exhibits a unique mRNA splice site, allowing for two forms of the protein to be expressed: cadherin-11; and a COOH terminus-truncated variant (Fig. 1A; Ref. 11). The variant has an extracellular domain identical to that of cadherin-11, but a frameshift event adds a unique cytoplasmic domain with no homology to the cytoplasmic domain of any known cadherin (13). The function of the variant protein is unknown, but its sequence shows slight similarity to the src family of tyrosine kinases. In all situations thus far examined, cadherin-11 and its variant form are expressed coincidentally (14, 15).

Cadherin-11 mRNA is expressed in several types of cancer cells, including colon, gastric, renal cell, and breast cancer and osteosarcoma (14–18). In many instances, cadherin-11 expression has been associated with more aggressive, dedifferentiated cancers, such as the signet ring cell subtype of gastric carcinoma (18). In addition, we have previously shown cadherin-11 to be differentially expressed in more aggressive breast cancer cell lines (14).

However, no studies have examined the function of cadherin-11 in carcinoma cells. In the present study we find that, in contrast to cadherin-11, which localizes to the cell membrane (14), the truncated variant is found in a detergent-insoluble compartment. In addition, cadherin-11, in the presence or absence of the truncated variant, can mediate the formation of a functional adherens junction complex, recruiting β-catenin, α-catenin, and p120 to the membrane. Finally, although expression of cadherin-11 and variant does not dramatically alter anchorage-independent growth or cellular proliferation rates, it does cause significant changes in the invasive capacity of both cell types.

MATERIALS AND METHODS

Production of Fusion Protein Vectors. Portions of cadherin-11 cDNA were subcloned into the pGEX-2TK fusion protein expression vector (Amerham Pharmacia Biotech) as follows. Using RT-PCR,1 intracellular (WTIC) domains of wild-type cadherin-11 were amplified from total RNA derived from the MRC5 human embryonic lung cell fibroblast cell line (14). Oligonucleotide primers (For-5′-GGGCGCGGATCCGGGGAAGAACAGACAGAAGAC-3′; Rev-5′-CGGCCGGAATTCCTTGAGAAGCAGACAC-3′) were designed to incorporate BamHI and EcoRI restriction enzyme digest sites into the 5′ and 3′ ends, respectively, of the resulting cDNA fragments. RT-PCR reactions were performed with the following parameters:

1 The abbreviations used are: RT-PCR, reverse transcription-PCR; PBS, fetal bovine serum; CAT, chloramphenicol transferase; PBS, phosphate buffered saline; LDS, lithium dodecyl sulfate; TCF, T-cell enhancer binding factor.

Received 4/17/02; accepted 9/18/02.

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1 Supported by grants from the NIH and Komen Foundation (to S. W. B.) and from the Department of Defense (to S. W. B. and C. M. F.).

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fragments were digested with 30° C for 1.5 min, and 72° C for 1ha t3 7 PCR cycles (94°

virus reverse transcriptase (Life Technologies, Inc.) for1ha t3 7° C for 1.5 min, and 72

N-cadherin or E-cadherin, the two cadherins most closely related to cadherin-11.

cadherin-11. Note that the antibody does not recognize the variant form or cross-react with

polyclonal antibody WTID1 was characterized by analyzing lysates from MDA-MB-231

and bleeding three times, 2 days apart, and sacrificed. For purification, each

booster injection, bled once more 10 days later, and then given another

booster injection 4 days later. Serum was tested by Western blot to identify high-titer

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CADHERIN-11 AND BREAST CANCER INVASION

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Fig. 1. A, the genomic sequence of cadherin-11 is depicted at the junction between exons 13 and 14, which corresponds to amino acid 632 in the transmembrane region of wild-type cadherin-11. Cadherin-11 is produced when the intron between exons 13 and 14 is removed, and the subsequent mRNA is translated. The variant protein arises when a portion of the intervening intronic sequence is alternatively spliced between exons 13 and 14 and subsequently translated. The splice event produces a frameshift and early truncation of the protein due to a stop codon 179 bp after the splice site. The resulting variant protein is therefore identical to wild-type cadherin-11 in its extracellular region and the 5’ transmembrane region (1). However, 3’ to the splice site, the variant (3) exhibits no homology to the intracellular domain of cadherin-11 (2, adapted from Ref. 13). B, NP40-soluble lysates from different breast cancer cell lines expressing endogenous or exogenous cadherins were evaluated by Western analysis to determine the specificity of monoclonal 5B2H5 antibody. SKBR3 parental cells express no known cadherins, MCF7 cells express only E-cadherin, MDA-MB-231 cells express cadherin-11, HS578T cells express N-cadherin and cadherin-11, and MDA-MB-435 cells express N-cadherin alone. In addition, SKBR3 and MCF7 cells were transiently transfected with cDNA encoding cadherin-11 or variant. A distinct M, 120,000 band is recognized in those cells expressing cadherin-11. Note that the antibody does not recognize the variant form or cross-react with N-cadherin or E-cadherin, the two cadherins most closely related to cadherin-11. C, the polyclonal antibody WTID1 was characterized by analyzing lysates from MDA-MB-231 cells (cadherin-11), MCF7 cells (E-cadherin), and MDA-MB-435 cells (N-cadherin) and found to recognize all three cadherins.

production and isolation of polyclonal and monoclonal antibodies, pGEX-2TK-WTIC was transformed into DH5a bacterial cells, and glutathione S-transferase fusion proteins were made and purified using standard procedures. Two rabbits were immunized and given four injections 1 week apart and then a fifth injection 2 weeks later. Rabbits were bled 10 days after the fifth booster injection, bled once more 10 days later, and then given another injection 4 days later. Serum was tested by Western blot to identify high-titer animals. After the 10th immunization, rabbits were bled by collecting 25 ml of total RNA using mouse mammary tumor virus reverse transcriptase (Life Technologies, Inc.) for 1 h at 37°C with 0.2

µM reverse primer was followed by the addition of 0.2 µM forward primer and 30 PCR cycles (94°C for 1 min, 55°C for 1.5 min, and 72°C for 2 min). After isolation of appropriate PCR products by gel electrophoresis, the cDNA fragments were digested with BamHI and EcoRI, gel-purified, and ligated overnight into a BamHI/EcoRI-cut pGEX-2TK vector using T4 DNA ligase (Life Technologies, Inc.). Clones were isolated, digested to ensure correct orientation of the insert, and subsequently sequenced to verify that no mutations occurred during amplification.

Production and isolation of polyclonal and monoclonal antibodies, pGEX-2TK-WTIC was transformed into DH5a bacterial cells, and glutathione S-transferase fusion proteins were made and purified using standard procedures. Two rabbits were immunized and given four injections 1 week apart and then a fifth injection 2 weeks later. Rabbits were bled 10 days after the fifth booster injection, bled once more 10 days later, and then given another injection 4 days later. Serum was tested by Western blot to identify high-titer animals. After the 10th immunization, rabbits were bled by collecting 25 ml and bleeding three times, 2 days apart, and sacrificed. For purification, each two bleedings (25 ml each) following a single booster were pooled. An affinity column was made by conjugating the same peptide used for immunization to aminoethyl gel by sulfo-SMCC. Serum was incubated with the gel, and the column was washed extensively. Purified antibodies were first eluted with KSCN and then with glycine. The two fractions of antibodies were dialyzed, concentrated if necessary, and then tested by immunoblot and/or immunoprecipitation and/or immunocytochemistry.

For production of monoclonal antibodies, Balb/C mice (8 weeks old) were immunized i.p. with recombinant fusion proteins mixed with complete Freund’s adjuvant and then boosted several times with the same antigen mixed with incomplete Freund’s adjuvant. Blood samples were collected and tested by ELISA and Western blot. Mouse splenocytes from the best responders were fused with mouse myeloma cells (P3 × 63Ag8.653) using polyethylene glycol. All hybridoma supernatant samples from the 96-well culture plates were screened by ELISA, using plates coated with the appropriate fusion protein. After expansion into 24-well plates, the positive clones were further examined by Western blot. All single-cell clones (subclones) were examined by ELISA and Western blot to confirm their production of the antibody of interest. Final subclones were isotypied by Mouse MonoAb ID Kit (catalogue number 90-6550; Zymed). Ascites from clone 5B2H5 were produced in Balb/C mice and purified using a protein A column.

Characterization of antibodies. Characterization of antibodies was performed by Western blot analysis of several cell lines that expressed varying combinations of exogenous or endogenous cadherin-11 and variant, E-cadherin, and N-cadherin. Briefly, SKBR3 cells (which express no known cadherins) and MCF7 cells (which express E-cadherin) were transiently transfected with cadherin-11 or variant cDNA. These cells, as well as parental SKBR3 and MCF7 cells, MDA-MB-231 cells (which express cadherin-11 and variant), HS578T cells (which express cadherin-11, variant, and N-cadherin), and MDA-MB-435 cells (which express N-cadherin but not cadherin-11), were lysed in NP40, 150 mM NaF, 50 mM Tris (pH 8.0), 1 mM sodium vanadate, 50 mM NaF, and protease inhibitors (Boehringer Mannheim). The soluble fractions were isolated by centrifugation at 12,000 x g for 10 min at 4°C, diluted in sample buffer, and run under denaturing conditions on 4–12% Tris-glycine gels (Novex Corp.). After transfer to nitrocellulose and blocking for 1 h at room temperature in 5% nonfat milk in phosphate buffered saline (2% Tween), membranes were incubated with each antibody at varying concentrations, rinsed, and incubated with appropriate secondary antibody conjugated to peroxidase (Jackson ImmunoResearch). Blots were visualized using enhanced chemiluminescence (Amer sham). All cadherin-11 antibodies were also used in immunocytochemistry, immunoprecipitation, and immunohistochemistry assays to further determine specificity as well as efficacy in these techniques.

Cell culture and production of stable transfectants. SKBR3 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured at 37°C, 5% CO2 in DMEM (Life Technologies, Inc.) plus 10% FBS (Biofluids). Cells were transfected with pCXN2-cadherin-11 and/or pCXN2-variant or with pCDNA3-CAT (as a control), and equal amounts of the porcine resistance plasmid pH262pur (a gift from Dr. H. te Riele, Netherlands Cancer Institute) using the calcium phosphate method. Resistant pools were subsequently selected in 1 µg/ml puromycin for 4–6 weeks, further enriched by differential trypsinization, and characterized by Western blot, RT-PCR, and/or immunocytochemistry.

MCF7 cells were obtained from American Type Culture Collection and cultured at 37°C, 5% CO2 in DMEM (Life Technologies, Inc.) plus 5% FBS (Biofluids). Cells were transfected with pCXN2-cadherin-11 and/or pCXN2-variant or with pCDNA3-CAT (as a control) using LipofectAMINE Plus (Life Technologies, Inc.) and selected in 0.5 µg/ml G418 for 4–6 weeks. Clonal populations of cells were obtained by selecting with a limiting dilution in 0.5 µg/ml G418; subsequent clonal populations were screened by immunocytochemistry and Western blot analysis.

Antibodies. The following primary antibodies were used for immunocytochemistry, immunoprecipitation, and immunoblotting: (a) monoclonal anti-cadherin-11 clone 5B2H5 and polyclonal anti-cadherin-11 (WTID; Zymed), both of which were raised against the intracellular domain of cadherin-11 and hence recognize the wild-type cadherin-11 alone; (b) monoclonal anti-cadherin-11 (a gift from M. J. Bussemakers; 228) and monoclonal anti-cadherin-11 113H (a gift from the ICOS Corp.), both of which recognize the extracellular domain of cadherin-11 and variant; (c) monoclonal anti-β-catenin (Transduction Laboratories); (d) polyclonal anti-β-catenin SHB7 (a gift from

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D. Rimm); (e) monomolar anti-p120\(^{\text{TM}}\) (Transduction Laboratories); and (f) monomolar anti-p120\(^{\text{TM}}\) clone 12F4 (a gift from A. B. Reynolds).

**Immunocytochemistry and Microscopy.** Cells were plated on sterile 18-mm glass coverslips and allowed to adhere for at least 4 h before fixation in 2% paraformaldehyde (20 min), with subsequent permeabilization in 0.2% Triton X-100-PBS (5 min). Cells were blocked for 1 h at room temperature in 3% ovalbumin-PBS and then incubated with the appropriate antibody diluted in 6% normal goat serum-PBS for 1 h at room temperature. After three 3-min washes in PBS, cells were incubated with the appropriate secondary antibodies conjugated to either Texas Red (Jackson ImmunoResearch) or FITC (Kirkegaard & Perry) for 1 h at room temperature in the dark. All secondary antibodies were diluted 1:200 in 6% normal goat serum-PBS. For double staining, polyclonal primary and antirabbit secondary antibodies were applied first, followed by monomolar primary and antimouse secondary antibodies. After the final three 3-min washes in PBS, coverslips were mounted on slides with Vectorshield. All fluorescence and Nomarski interference contrast images were digitally captured using an Olympus Fluoview confocal microscope.

**Immunoblotting and Immunoprecipitation.** Cells were solubilized in ice-cold 1% NP40 buffer solution [1% NP40, 1250 mm NaCl, and 50 mm Tris (pH 8.0)] containing 1 mm sodium vanadate, 50 mm NaF, and complete protease inhibitors (Boehringer Mannheim). Lysates were centrifuged at 14,000 rpm for 15 min at 4°C to remove the NP40-insoluble material. After the addition of 2× sample buffer [4% SDS, 120 mm Tris (pH 6.8), and 20% glycerol] to the NP40-soluble fraction and 1× sample buffer to the insoluble pellet, samples were boiled, and Bio-Rad protein assays were performed to determine total protein content. After the addition of reducing agent, the samples were again boiled, and equal total protein was loaded on 3–8% NuPage Tris-acetate gels (Invitrogen), unless otherwise indicated. Proteins were blotted to nitrocellulose (Schleicher & Schuell) and blocked for 1 h at room temperature or overnight at 4°C in 5% milk-PBST. After incubation with appropriate primary and secondary antibodies (each for 1 h at room temperature), blots were treated with enhanced chemiluminescence reagent (Amer-Sham) and exposed to film. Blots were sometimes stripped [62.5 mm Tris (pH 7.5), 2% SDS, and 1.7% B-mercaptoethanol for 30 min at 50°C], reblotted, and reprobed with new primary and secondary antibody. Alternatively, cells were lysed in ice-cold 1× LDS sample buffer (Invitrogen), scraped, passed through a 27-gauge needle, and boiled in the presence of a reducing agent for 30 min before gel electrophoresis and immunoblotting as described.

For immunoprecipitation, lysates were obtained as described above. Lysates were first precleared with 50 µl of protein G-Sepharose beads (Zymed) alone for 1 h at 4°C. The beads were centrifuged and removed, and appropriate precipitating antibodies were added to lysates for 1 h with rocking at 4°C; 50 µl of new beads were then added with rocking for an additional 2 h at 4°C. After three 10-min washes with ice-cold lysis buffer, sample buffer and reducing agent were added to precipitated proteins and beads and boiled for 10 min. Samples were subsequently analyzed by immunoblotting as described above.

**Reporter Assays.** Twelve-well plates were seeded at 10\(^5\) cells/well 24–48 h before transfection. Cells were transfected with TOPFLASH reporter (indicates lymphocyte enhanced binding factor reporter activity), pCXN2-cadherin-11 and pCXN2-variant or pCDNA3-CAT (control), and the thymidine kinase Renilla luciferase plasmid (Promega) to control for variations in transfection efficiency. After lysis, luciferase and Renilla activities were read on a standard luminometer using the Dual-Reporter Luciferase Assay Kit (Promega). Luciferase values were normalized to Renilla values and plotted using Sigma Plot. Each experiment was performed in triplicate at least three independent times, with error bars representing SD.

**Proliferation Assays.** WST-1 assays (Boehringer Mannheim) were performed as indicated by the manufacturer. Briefly, 1000–3000 cells of each population were plated in triplicate in 96-well plates on day 1. WST-1 readings were taken on alternate days beginning with day 0 on an optical densitometer. For analysis (Sigma Plot), data for each population were plotted relative to the mean day 0 value to account for variance in plating efficiency, with error bars representing SD. All experiments were performed independently at least three times.

**Soft Agar Assays.** Soft agar assays were performed as described previously (19). Briefly, cells were plated in 6-well plates at 5 x 10\(^3\) cells/well in a 0.3% agar suspension (SeaKem) on a 0.6% agar cushion, with 1 ml of DMEM + 5% FBS covering the cells. The cells were incubated at 37°C in 5% CO\(_2\), and media were carefully changed every 3–4 days. After 2 or 3 weeks, colonies greater than 140 µm in diameter were scored by an Omnicon 3600 Colony Counter; data were subsequently analyzed and graphed on Sigma Plot. All experiments were performed in triplicate at least three independent times; error bars represent SD.

**In Vitro Invasion Assays.** Invasion assays were performed as described previously (9, 20). Standard Boyden chambers were prepared by placing NIH3T3-conditioned media (24 h, DMEM +50 µg/ml ascorbic acid) in the bottom well of the chamber as a chemoattractant. After coating a 12-µm pore polycarbonate filter (Poretics, Inc.) with 10 µg of Matrigel, 3 x 10\(^3\) cells in DMEM with 0.1% BSA were placed in the upper chamber and incubated for 16 h at 37°C. Membranes were then removed, cells were fixed in 25% methanol with 0.5% crystal violet, and the remaining cells were wiped from the upper surface of the membrane with a damp cotton swab. Quantification of cells on the bottom of the membrane was performed by counting the number of cells/field in 5 random fields/membrane; the fields were then averaged. Bars represent the mean of each population over three membranes, with error bars representing SD. Each experiment was performed at least three independent times.

**RESULTS**

**Cadherin-11 Antibody Production and Characterization.** To characterize the monomolar and polyclonal antibodies raised against cadherin-11, we first determined their specificity by Western analysis. Lysates from MDA-MB-231 cells were evaluated because these cells express both forms of cadherin-11 (14). In addition, we examined lysates from MCF7, HS578T, and MDA-MB-435 cells, which express E-cadherin, N-cadherin and cadherin-11, and N-cadherin, respectively (14, 20). Cadherin-11 is relatively homologous to E- and N-cadherin; anti-cadherin-11 antibodies, especially those raised against the intracellular domain, were therefore most likely to cross-react with these proteins. Finally, to determine whether antibodies could recognize exogenously expressed cadherin-11, we evaluated lysates from both SKBR3 cells and MCF7 cells transiently transfected with cDNA encoding the wild-type cadherin-11 or variant protein.

One monomolar antibody (5B2H5) to the intracellular domain of cadherin-11 was reactive by Western blot, indicated by the M\(_r\) ~115,000 species (Fig. 1B). 5B2H5 did not react with the variant form or with any proteins in the MCF7 or MDA-MB-435 lanes (E-cadherin and N-cadherin, respectively), indicating that it was specific to the wild-type form of cadherin-11. In addition, the only band seen in the HS578T lane is M\(_r\) 115,000, indicating that it is cadherin-11; N-cadherin runs at approximately M\(_r\) 140,000. The 5B2H5 clone was found to be useful for immunocytochemistry, immunoprecipitation, and immunohistochemistry and is available commercially as Zymed 32-1700 (clone 5B2H5).

One polyclonal antibody raised against the intracellular domain of wild-type cadherin-11 was also analyzed and found to recognize not only cadherin-11 but also E-cadherin and N-cadherin (Fig. 1C). This antibody (pWTID1) is also useful for immunocytochemistry; its utility in immunoprecipitation or immunohistochemistry was not investigated due to its cross-reactivity with E- and N-cadherin. This pan-cadherin antibody is available commercially as Zymed 71-7600 (cadherin-11 WTID1). We were not successful in generating monomolar or polyclonal antibodies specific to the variant intracellular domain.

**Characterization of Stable Transfectants Expressing Cadherin-11.** Both SKBR3 and MCF7 breast cancer cells are relatively well differentiated and moderately invasive in in vitro invasion assays. Because SKBR3 cells have a homozygous deletion of the E-cadherin gene (21) and do not express other cadherins, they represent a suitable system for determining whether or not cadherin-11 could act as a functional adherens junction molecule in addition to whether or not it could induce a phenotypic change. The MCF7 cell line, which ex-
presses E-cadherin alone, is an appropriate model to determine whether exogenous cadherin-11 expression interferes with E-cadherin function as well as induces a more proliferative or invasive phenotype in the presence of E-cadherin.

Cells were cotransfected with vectors containing the full-length cDNA coding for human wild-type cadherin-11 and/or human variant cadherin-11 (pCXN2-cadherin-11 and pCXN2-variant) or empty vector containing the CAT cDNA for control, with or without a second puromycin resistance plasmid (pHA262pur). Pooled stable populations expressing cadherin-11, both cadherin-11 and the variant, or CAT were obtained either by selection with G418 or puromycin. These cell populations will be referred hereafter as SKBR3-cad11, SKBR3-cad11+var, and SKBR3-ctrl, respectively.

MCF7 cells were transfected with cadherin-11 and variant or CAT as described above; selection with G418 resulted in pooled populations 40–90% positive for cadherin-11. To obtain populations in which 100% of cells expressed the appropriate protein(s), we selected clones by limiting dilution in G418 with subsequent screening by immunocytochemistry (Fig. 2A). Further characterization by RT-PCR analysis revealed production of the cadherin-11 variant in all clones (Fig. 2B). These cell populations will be referred hereafter as MCF7-cad11+var and MCF7-ctrl, respectively.

Expression of Cadherin-11 Results in Morphological Changes in SKBR3 Cells. SKBR3 cells were cotransfected with the puromycin resistance plasmid pHA262pur and cadherin-11, cadherin-11 and variant, or CAT cDNA. Selection with puromycin produced populations of SKBR3 cells transfected with cadherin-11 and variant that were 90–100% positive without the need for further sorting (Fig. 2A). Because we had no antibody specific to the variant protein, cells were further characterized by RT-PCR analysis (Fig. 2C). Using primers specific for the variant mRNA species, it was clear that only SKBR3 cells transfected with the variant cDNA expressed the variant mRNA (MDA-MB-231 cells were used as a positive control). SKBR3 cells transfected with only wild-type cadherin-11 and selected in puromycin were approximately 40% positive for cadherin-11; this was further enriched to ~100% by differential trypsinization because SKBR3 cells expressing cadherin-11 adhered to plastic much more efficiently than control cells. Neither CAT-transfected nor parental SKBR3 cells express cadherin-11.

In SKBR3 cells, the expression of cadherin-11 alone or with the variant produced a profound change in morphology (Fig. 3A). Parental SKBR3 cells (which express no known cadherins) are weakly attached to one another, resulting in loose aggregates of cells that detach easily from one another and from tissue culture-treated plastic, especially when grown to high density. Cells expressing cadherin-11 or cadherin-11 and the variant formed closely adherent islands of cells; some of these islands grew quite large, incorporating hundreds of cells (Fig. 3A). These foci were significantly more difficult to dislodge from plastic with trypsinization than parental or control cells, suggesting an increased ability to adhere to another and to the extracellular substrate. No differences were observed in the morphology or trypsinization properties of cells expressing wild-type cadherin-11 compared with those expressing both cadherin-11 and the variant.

MCF7 clones expressing cadherin-11 and the variant did not exhibit any noticeable changes in morphology when compared with control cells (data not shown). This is probably because MCF7 cells already express E-cadherin, which confers strong cell-cell adhesion; it is unlikely that cadherin-11 expression would further increase this adhesion (20).

Cadherin-11 Expression Results in the Assembly of Adherens Junction Components. As discussed earlier, cadherin-11 is generally expressed in mesenchymal cells or cells with a mesenchymal phenotype, such as invasive cancer cells. These types of cells do not usually form stable adherens junctions. However, the morphological changes that accompany the expression of cadherin-11 in SKBR3 cells suggest that this cadherin, like E-cadherin, can promote homotypic cell-cell adhesion in addition to mediating the formation of adherens junctions. We previously showed that β-catenin interacts with cadherin-11 in cells expressing both proteins endogenously (14). In contrast, Thoreson et al. (22) suggested that another adherens junction component and member of the catenin family, p120catn, does not interact with cadherin-11. They demonstrated that p120catn does not localize to the membrane in MDA-MB-231 cells, which express only one known cadherin, cadherin-11, in the membrane at sites of cell-cell

![Image](https://example.com/image.png)

**Fig. 2.** A. SKBR3 cells were cotransfected with cadherin-11 without or with variant (SKBR3-cad11 or SKBR3-cad11+var, respectively) or pCDNA-CAT as a control (SKBR3-ctrl) and a puromycin resistance plasmid; selection resulted in pooled populations expressing the protein(s) of interest. MCF7 cells were transfected with cadherin-11 and variant (MCF7-cad11+var) or pCDNA-CAT (MCF7-ctrl) and selected with G418 before selecting clonal populations. To verify protein expression, cells were stained for cadherin-11. B. RT-PCR was performed to verify production of variant mRNA. Note that control cells do not express the 194-bp PCR product. MDA-MB-231 cells (+), which express cadherin-11 and variant mRNA (14), were used as a positive control. C. RT-PCR was performed to ensure that the stably transfected SKBR3 cells expressed cadherin-11 variant mRNA.
contact (22). We independently confirmed these results (data not shown). The localization of p120ctn in the cytoplasm of MDA-MB-231 cells, instead of at the membrane with cadherin-11, suggests that these molecules might not interact with one another. We therefore tested the ability of cadherin-11 to recruit members of the adherens junction complex to the membrane of SKBR3 cells.

Cadherin-11-expressing SKBR3 cells were stained for cadherin-11, p120ctn, and β-catenin and visualized by confocal microscopy (Fig. 3B). These experiments were carried out on puromycin-selected pooled stable expressers before final enrichment (see “Materials and Methods”). Consequently, not all cells express cadherin-11, allowing the negative cells to act as internal controls. First, we analyzed the expression pattern of cadherin-11. It was noted that SKBR3-cad11 cells localize the protein to the membrane and only do so at sites of cell-cell contact. Note that there is no cadherin-11 protein at the edges of cells unless they contact other cells. Next, we examined the expression of p120ctn in these cells. We found a marked relocation of p120ctn to the membrane only in those cells concomitantly expressing cadherin-11. Merged image analysis indicates that cadherin-11 and p120ctn colocalize, suggesting that cadherin-11 does indeed have the capability to interact with and recruit p120ctn. Staining for β-catenin (Fig. 3B) shows that cadherin-11 also recruits this protein to the membrane. Colocalization of the two proteins is clear in the merged image. Finally, concurrent expression of the variant form of cadherin-11 did not appear to alter the localization of either catenin (Fig. 3C).
levels are markedly elevated in cells expressing cadherin-11 compared to the SKBR3-cad11 expressing both cadherin-11 and variant, this is because the SKBR3-cad11 population is more enriched for cadherin-11 than the SKBR3-cad11 population of cadherin-11 and variant. Although β-catenin appears to be more stable in cells expressing cadherin-11 than in cells expressing both cadherin-11 and variant, this is because the SKBR3-cad11 population is more enriched for cadherin-11 than the SKBR3-cad11+var population at the time of this experiment. SKBR3 cells were transiently transfected with a β-catenin-Luc reporter construct, TOPFLASH, or with pCDNA-CAT (control). β-Catenin increased TOPFLASH activity roughly 20-fold, and cadherin-11 reduces β-catenin signaling 5-fold, indicating that cadherin-11 effectively removes a majority of β-catenin from the signaling pool, likely by stabilizing it at the membrane.

Experiments were also performed in MCF7 cells. These cells already express E-cadherin; coexpression of cadherin-11 and variant did not alter the distribution of β-catenin or p120ctn (data not shown).

Parental SKBR3 cells normally exhibit barely detectable levels of cytoplasmic β-catenin, similar to the cadherin-11-negative cells in the margins of the image in Fig. 3, B and C (20). Our findings suggested that cadherin-11 might be stabilizing β-catenin (i.e., preventing its degradation) by recruiting it to the cell membrane. To confirm this, a Western blot analysis for β-catenin protein was performed on control SKBR3 cells, cells expressing cadherin-11, and cells expressing both cadherin-11 and the variant. Fig. 4A reveals that β-catenin protein levels are markedly elevated in cells expressing cadherin-11 compared with control cells. Additionally, it has been shown previously in other systems that expression of E-cadherin can recruit β-catenin to the membrane, thus reducing its ability to activate TCF reporters (23).

Experiments were performed (Fig. 5). Both β-catenin and p120ctn were present in cadherin-11 immunoprecipitations from cadherin-11-expressing SKBR3 cells. It was also noted that the β-catenin that precipitated with cadherin-11 has a slower electrophoretic mobility than the β-catenin found in the cell lysate. Finally, reprobing of these blots determined that a third catenin, α-catenin, is present in the complex and also appears to be of a slightly higher molecular weight than the protein found in the cell lysate (Fig. 5A).

To verify that cadherin-11 exists in a complex with both β-catenin and p120ctn, instead of simply colocalizing in the same vicinity, immunoprecipitation experiments were performed (Fig. 5). Both β-catenin and p120ctn were present in cadherin-11 immunoprecipitations from cadherin-11-expressing SKBR3 cells. It was also noted that the β-catenin that precipitated with cadherin-11 has a slower electrophoretic mobility than the β-catenin found in the cell lysate. Finally, reprobing these blots determined that a third catenin, α-catenin, is present in the complex and also appears to be of a slightly higher molecular weight than the protein found in the cell lysate (Fig. 5A).

We also wished to determine whether the expression and activity of cadherin-11 and variant proteins might affect or be affected by the presence of E-cadherin. We therefore immunoprecipitated either cadherin-11 or E-cadherin from parental MCF7 cells and cadherin-11-expressing MCF7 cells and blotted for the same catenins described above (Fig. 5, B and C). We found that neither cadherin-11 nor E-cadherin prevented the other from associating with p120ctn, β-catenin, or α-catenin. In addition, p120ctn appeared to immunoprecipitate more readily with cadherin-11 than with E-cadherin. Finally, expression of cadherin-11 does not appear to alter the steady-state levels of E-cadherin expression.

Effects of Exogenous Cadherin-11 on Cellular Proliferation. To determine whether exogenous expression of cadherin-11 might alter the cellular phenotype, we evaluated several indicators of cellular behavior, including proliferation rate, anchorage-independent growth, and invasive activity. The rate of cell proliferation was assessed by plating cells at varying densities and then monitoring their growth using the WST-1 assay, which measures cellular metabolic activity. Expression of wild-type cadherin-11 in SKBR3 cells led to a moderate change in the proliferation rate of cells (Fig. 6A). At the peak of the proliferation log phase, SKBR3-cad11 cells exhibited approximately 150% the growth rate of controls. In SKBR3-cad11+var cells, the growth rate was approximately 60% that of control cells. The difference in cell proliferation between the two cadherin-11-expressing cell populations at log phase was significant (P < 0.001). However, coexpression of cadherin-11 and variant proteins did not alter the peak of the proliferation log phase for these cells. Instead, it took them longer to reach log phase than controls (14 days versus 6 days). These data suggest that expression of cadherin-11 alone may confer a slight proliferative advantage to SKBR3 cells, which appears to be reversed by concomitant expression of the variant protein. It should be noted that although the observed changes in proliferation rate assessed by the WST-1 assay were repeatable and statistically significant, they were not large. For example, we did not notice in the daily management of the cells that they needed to be passaged at different times. In MCF7 cells, no consistent differences in the rate of proliferation could be observed among the various control and cadherin-11-expressing cells (data not shown).

Exogenous Cadherin-11 Does Not Affect Anchorage-independent Growth. The ability of cells to grow in soft agar is not always related to changes in proliferation. Consequently, it is possible that although cadherin-11 expression does not have a major effect on cell growth, it may affect the ability of cells to grow in an anchorage-independent manner. The effect of cadherin-11 expression on anchorage-independent growth was examined in both SKBR3 and MCF7 cells by culturing them on a cushion of soft agar for 2 weeks. We found that the ability of SKBR3 and MCF7 cells to grow in an anchorage-independent fashion was not affected by expression of cadherin-11 or by coexpression of cadherin-11 and variant proteins (Fig. 6, B and C).

Exogenous Cadherin-11 Affects Invasive Activity. We reported previously that cadherin-11 is expressed in breast cancer cell lines displaying a more aggressive and invasive phenotype. We therefore wished to determine whether cadherin-11 expression might actually have a causal role in the acquisition of invasive capacity and tested the ability of cadherin-11-expressing cells to invade a Matrigel-coated membrane in a standard Boyden chamber assay. Equal numbers of control and cadherin-11-expressing cells were trypsinized and then plated onto Matrigel-coated filters above a well filled with chemotactrant-enriched media. Cells were then incubated for 16 h to assess their ability to move through the Matrigel toward the chemotactrant on the other side of the membrane. We found that expression of cadherin-11 in SKBR3 cells led to a 5-fold reduction in invasive capacity (compared with control or parental cells) that was statistically significant (P < 0.05; Fig. 7). In contrast, cells coexpressing...
cadherin-11 and the variant protein invaded at twice the rate of control cells ($P < 0.05$) and 10 times more than cells expressing cadherin-11 alone ($P < 0.001$). Despite these clear differences in invasive behavior, both populations of cells were morphologically indistinguishable when viewed by phase-contrast microscopy (Fig. 3).

Nieman et al. (24) showed previously that expression of cadherin-11 in the E- and P-cadherin-expressing cell line BT20 slightly increased the invasive activity of the cells. It was not clear whether both cadherin-11 and the variant protein were expressed in these experiments, but it is possible that the effects of cadherin-11 on invasion might be different in cells that express other cadherins. To confirm that cadherin-11 may affect invasion by cells already expressing other cadherins, we repeated these experiments in MCF7 cells. Coexpression of cadherin-11 and variant increased the invasive capacity of MCF7 cells 7-fold compared with parental cells or control (Fig. 7). This increase in invasive capacity occurs despite expression of E-cadherin in these cells, indicating that cadherin-11 may in some way alter the tumor-suppressive activities of E-cadherin or that its ability to bring about an invasive phenotype is independent of E-cadherin.

**Localization of the Cadherin-11 Variant.** The marked effects of variant protein expression on the invasive behavior of SKBR3 and MCF7 cells prompted us to reexamine its cellular localization. Because we could not generate antibodies that specifically recognized the variant, we were forced to rely on antibodies that detected both cadherin-11 forms (i.e., wild-type and variant). In earlier experiments, we found that a protein corresponding to the predicted size of the variant ($M_r \approx 85,000$ versus $M_r 115,000$ for wild-type cadherin-11) was present in cells expressing endogenous cadherin-11 (14). However, in the present study, we found that cells transfected with wild-type cadherin-11 cDNA but not variant cDNA also expressed a protein of similar molecular weight (Fig. 8A). Because the variant form is a product of alternative splicing of the primary RNA transcript, it cannot have been present in these cells; we hypothesize that the $M_r \approx 85,000$ protein is instead a protein degradation product of wild-type cadherin-11.

Interestingly, we found that it was difficult to detect the variant in transfected cells by either immunocytochemistry or Western blot of NP40-soluble extracts, despite abundant expression of variant mRNA. Analysis of the NP40-insoluble fraction confirmed that whereas wild-type cadherin-11 is predominantly in the NP40-soluble fraction as published previously (14), the variant is located in the NP40-insoluble, LDS-soluble fraction (Fig. 8B). This cellular fraction contains intracellular cytoskeletal and associated proteins as well as extracellular matrix.

However, the fact that the variant could not be detected within the cell by immunocytochemistry led us to speculate that it must instead be outside the cell. We hypothesized that the loss of two-thirds of the...
transmembrane domain in the variant might cause it to be secreted from the cells. To test this, SKBR3-cad11 cells were transiently transfected with variant or CAT cDNA. Twenty-four h after transfection, cells were incubated for an additional 24 h at 22°C to allow for protein secretion (25) and then stained for cadherin-11. We could clearly detect the variant protein inside the variant-transfected cells by immunocytochemistry (Fig. 8C). Because we could not detect the variant in cells in which secretion was not blocked, we interpreted this result as suggesting that the variant form may indeed normally be secreted.

We therefore attempted to isolate variant protein from media conditioned by cells transiently transfected with variant cDNA. Twelve h after transfection, cells were placed in low-serum media (0.1%) for 72 h. Media were then collected and concentrated 40-fold in the presence of protease inhibitors. Immunoprecipitation of the variant from media was attempted using two antibodies that recognize the extracellular domain; neat media were also analyzed for variant protein by Western blot. In addition, cell lysates were examined to ensure adequacy of transfection. Despite adequate transfection, we were unable to detect variant in the conditioned media (Fig. 8D).

**DISCUSSION**

The acquisition of an invasive or metastatic phenotype by tumor cells is a complex process involving changes in a number of cellular characteristics and behaviors. Changes in cell adhesion are thought to be particularly important (26, 27). In the present study, we show that cadherin-11 with or without expression of the variant form can recruit components of the adherens junction to the membrane and result in epithelial differentiation of SKBR3 cells. Expression of cadherin-11 and its variant has little effect on cell proliferation and anchorage-independent growth but does markedly alter the invasive ability of breast cancer cells, even in the presence of E-cadherin. Our results also suggest that the variant protein may be secreted and/or deposited into a NP40-insoluble intra- or extracellular compartment.

**Cadherin-11 Recruits Components of the Adherens Junction and Promotes Epithelial Differentiation of SKBR3 Cells.** Cadherin-11 was originally identified in osteoblasts and subsequently found in many cells of mesodermal origin as well as in certain invasive tumors (11, 12). Because most of these cadherin-11-expressing cells do not form stable adherens junctions, it is quite surprising that cadherin-11 expression in SKBR3 breast cancer cells results in the recruitment of adherens junction proteins to the membrane. We found that cadherin-11 brings adherens junction components such as β-catenin, α-catenin, and p120<sup>catenin</sup> to the membrane in both cells lacking endogenous cadherins and cells possessing endogenous cadherins (SKBR3 cells and MCF7 cells, respectively). Additionally, this recruitment is not affected by concomitant expression of the variant protein. Finally, β-catenin protein levels are themselves stabilized by cadherin-11 expression in SKBR3 cells, which...
Fig. 8. A, MCF7 parental cells were transiently transfected with cDNA encoding CAT (control), cadherin-11, or variant. Twenty-four h later, cells were incubated at 22°C for an additional 24 h before lysis in sample buffer. Whole cell lysates (LDS) were Western blotted and incubated with an antibody that recognizes both the wild-type and variant forms. Lysate from the variant lane (Var) exhibits a strong band at approximately M, 80,000 (arrow). There is no band at M, 120,000 corresponding to wild-type cadherin-11. The cadherin-11 lane (Cad11) does exhibit a strong M, 120,000 band as well as an additional band at approximately M, 80,000, a presumptive breakdown product. B, NP40-insoluble fractions were isolated from cells transiently transfected with the variant or wild-type cadherin-11 cDNA and analyzed by Western blot. The variant remains in the insoluble fraction, which contains cytoskeletal elements and associated proteins, as well as extracellular matrix components. C, SKBR3 cells stably expressing wild-type cadherin-11 were transiently transfected with cDNA encoding the variant or CAT (control). Twenty-four h after transfection, cells were trypsinized, plated on coverslips, and subsequently incubated at 22°C for another 24 h to reduce protein secretion. Cells were then fixed, permeabilized, and stained using an antibody that recognizes both cadherin-11 and variant proteins. Analysis by confocal microscopy revealed the presence of variant protein in the cytoplasm of SKBR3-cad11-Var cells, but not SK-cad11-CAT cells. This expression was so strong it saturated the confocal image (saturation is indicated by red in the field). To accommodate this, we decreased the sensitivity threshold when visualizing the SKBR3-cad11-Var cells. Consequently, the membrane staining appears to be decreased. D, MCF7 cells were transiently transfected with CAT, cadherin-11, or variant cDNA and placed in low-serum media to determine whether the variant protein might be secreted. Media were collected 72 h after transfection and concentrated 40-fold in the presence of protease inhibitors. Western blot analysis of conditioned media failed to detect the variant (top blot). Whole cell lysates of transfected cells verify the presence of the variant protein (bottom blot).

normally exhibit barely detectable levels of the protein. This is further proven by analysis of β-catenin signaling in these cells in both the absence and presence of cadherin-11. These data represent the first indication that cadherin-11 can act as a functional adherens junction molecule, actively recruiting catenins to a membrane complex and, in the case of β-catenin, stabilizing its expression and affecting its signaling function.

In addition, we found that cadherin-11 can interact with p120<sup>ctn</sup>. Thoreson et al. (22) showed previously that p120<sup>ctn</sup> did not associate with membrane complexes in MDA-MB-231 cells, which only express cadherin-11, suggesting that cadherin-11 and p120<sup>ctn</sup> could not interact. Although we also found that p120<sup>ctn</sup> was not present at the membrane of MDA-MB-231 cells, our experiments clearly demonstrate that cadherin-11 can form complexes with the catenins in general and with p120<sup>ctn</sup> in particular. This finding is consistent with the fact that the p120<sup>ctn</sup>-binding region of cadherin-11 is almost perfectly conserved with that of the other cadherins. The failure of cadherin-11-expressing MDA-MB-231 cells to recruit p120<sup>ctn</sup> to the membrane must therefore be a consequence of some other aspect of their transformed phenotype. Elucidation of the mechanism responsible for the lack of interaction between cadherin-11 and p120<sup>ctn</sup> might even explain in part the aggressive phenotype of this cell line because p120<sup>ctn</sup> interaction with cadherins has previously been associated with regulation of adhesion (28).

Effects of Cadherin-11 on Breast Cancer Cell Proliferation, Anchorage-independent Growth, and Invasion. After establishing that cadherin-11 was capable of interacting with catenins and forming an apparently functional adherens junction, we wished to determine whether its expression could alter the phenotype of breast cancer cell lines by assaying proliferation rate, anchorage-independent growth, and invasive activity. Expression of cadherin-11 in SKBR3 cells increased their proliferation rate modestly compared with control cells. Concomitant expression of variant resulted in cells whose proliferation rate was modestly reduced compared with control. Although the difference between the two cell populations was significant, it is important to note that the overall change in proliferation was fairly small. These findings suggest that cadherin-11 alone can act as a mitogenic factor in SKBR3 cells, likely by establishing adhesive contacts between cells. Moreover, no consistent changes in proliferation were observed in cadherin-11-expressing MCF7 cells. Examination of anchorage-independent growth in the presence of exogenous cadherin-11 with or without the variant revealed no significant changes in either SKBR3 cells or MCF7 cells.

Finally, we investigated the ability of cadherin-11 to induce changes in the invasive capacity of both epithelial cell lines. In keeping with its ability to promote formation of the adherens junction, expression of cadherin-11 markedly reduced the invasive activity of SKBR3 cells. Remarkably, concomitant expression of the variant protein led to cells that were 2.5 times more invasive than control and 10 times more invasive than those expressing cadherin-11 alone. In E-cadherin-expressing MCF7 cells, coexpression of cadherin-11 and variant increased invasion 7-fold. In an earlier study (24), expression of cadherin-11 in the E- and P-cadherin-expressing BT20 cells also led to an increase in their invasive capacity, although it is not clear if both cadherin-11 and its variant form were transfected in this experiment.

As discussed earlier, in all systems thus far examined, both cadherin-11 and its variant form are coexpressed. Nevertheless, changes in the relative amounts of the two forms of cadherin-11 do occur; an increase in the relative amount of the variant form is associated with more aggressive osteosarcoma cells (13). Taken to-
gether, these studies indicate that even though the splice variant does not directly alter the ability of cadherin-11 to assemble junctional complexes, under appropriate circumstances (e.g., a migratory stimulus) it can allow cells to become more invasive. The fact that cadherin-11 and the variant are always coexpressed strongly suggests that both are necessary for the observed functional changes. Finally, cadherin-11- and N-cadherin-induced changes in invasive activity occur regardless of the presence of E-cadherin, indicating that the invasion-suppressing function of E-cadherin is not always predomi-

ACKNOWLEDGMENTS

We thank Dr. Al Reynolds for the p120 antibody and for helpful comments.

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