Integrin Regulation of β-Catenin Signaling in Ovarian Carcinoma*1

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Reversible modulation of integrin-regulated cell-matrix adhesion and epithelial (E)-cadherin-mediated cell-cell adhesion plays a critical role in the establishment of ovarian cancer metastases. In contrast to most epithelial cell-derived tumors that down-regulate E-cadherin expression during progression, acquisition of E-cadherin expression accompanies malignant transformation of the ovarian surface epithelium and is maintained in peritoneal metastases. Metastatic epithelial ovarian cancer cells are disseminated intraperitoneally and preferentially adhere via integrins to interstitial collagens in the peritoneal cavity. This study was undertaken to determine whether integrin engagement influences E-cadherin and β-catenin localization and function. The data demonstrate that multivalent integrin engagement results in increased internalization of E-cadherin, inhibition of GSK-3β, elevated levels of nuclear β-catenin, increased β-catenin-regulated promoter activation, and transcriptional activation of Wnt/β-catenin target genes. Blocking β-catenin transcriptional control with inhibitor of β-catenin and Tcf-4 reduces cellular invasion, suggesting a key role for β-catenin nuclear signaling in EOC invasion and metastasis. These studies support a model wherein cell-matrix engagement regulates the functional integrity of cell-cell contacts, leading to increased β-catenin nuclear signaling and enhanced cellular invasive activity. Furthermore, these results provide a mechanism for activation of Wnt/β-catenin signaling in the absence of activating mutations in this pathway.

Epithelial (E)-cadherin is a single-span transmembrane glycoprotein that mediates calcium-dependent cell-cell adhesion via interaction with the extracellular domains of cadherins on the surface of neighboring cells (1, 2). Key functions of E-cadherin include the regulation of cell polarity and the maintenance of epithelial organization (3). Although most normal epithelia express high levels of E-cadherin, this cadherin is absent in the mesenchymally derived normal ovarian surface epithelium, which expresses neural (N)-cadherin. In most carcinomas, E-cadherin expression is down-regulated or lost, facilitating cellular dispersal, invasion, and metastasis (4). However, a unique feature of EOC is that E-cadherin becomes more abundant in primary differentiated carcinomas, with all histotypes displaying strong immunoreactivity (reviewed in Refs. 5, 6). There is less clarity regarding relative E-cadherin levels during ovarian tumor progression and metastasis. Although complete loss of E-cadherin is uncommon, reduced staining is often detected in late stage tumors and in ascites-derived tumor cells (7–9), and negative E-cadherin is predictive of poor overall survival (10, 11).

β-Catenin is found predominantly in association with the E-cadherin cytoplasmic domain at cell-cell junctions (12). In the absence of cell-cell contact and Wnt signaling, cytosolic β-catenin forms a complex with adenomatous polyposis coli, Axin/conductin, casein kinases 1ε and 1ε, and glycogen synthase kinase-3β (GSK-3β). CK1 and GSK-3β phosphorylate β-catenin and target the protein for ubiquitination and proteasomal degradation (13). When Wnt signaling is active, Dishevelled prevents complex formation and phosphorylation by GSK-3β, enabling β-catenin to translocate to the nucleus, bind TCF/LeF-1 family transcription factors, and transcriptionally regulate Wnt/β-catenin target genes (reviewed in Refs. 14, 15). The transcriptional regulatory activity of β-catenin is also controlled by factors other than Wnt signaling. For example, ectopic E-cadherin expression can sequester β-catenin, thereby depleting the pool that binds TCF (16). Phosphorylation of GSK-3β by protein kinases A, B, and C, Akt/PI3K, and MAPK inhibits its ability to phosphorylate and target β-catenin for degradation (17–22).

Many Wnt/β-catenin target genes regulate tumor progression (14, 15); however, gene mutations in the Wnt signaling pathway are uncommon in ovarian cancer with the exception of some β-catenin mutations in endometrioid histotype EOC (23). Nevertheless, emerging data implicate dysregulated Wnt signaling in EOC progression in the absence of activating mutations in either adenomatous polyposis coli, Axin, or β-catenin (23–26). Furthermore, both E-cadherin ectodomain shedding (27) and decreased net E-cadherin expression (28) can promote...
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β-catenin-mediated transcription, suggesting that β-catenin is released from E-cadherin following disruption of the junctional complex. This study demonstrates that multivalent integrin engagement results in adherens junction disruption, internalization of E-cadherin, inhibition of GSK-3β, and increased levels of active β-catenin. Nuclear translocation of β-catenin and activation of the TOPflash TCF/Lef-1 promoter/reporter is observed following integrin engagement, together with enhanced expression of β-catenin target genes. Furthermore, cellular invasion is blocked by inhibiting β-catenin transcriptional control with inhibitor of β-catenin and Tcf-4 (ICAT). These studies describe a novel mechanism by which enhanced expression of E-cadherin, inhibition of GSK-3β, and increased levels of active β-catenin translocation to the nucleus, leading to increased β-catenin nuclear signaling and enhanced invasive activity.

EXPERIMENTAL PROCEDURES

Cells and Plasmids—OVCA433 and OVCA429 cells were generously provided by Dr. Robert Bast (M.D. Anderson Cancer Center, Houston, TX) and were maintained in minimal essential medium, 10% fetal bovine serum, penicillin/streptomycin, amphotericin B, nonessential amino acids, and sodium pyruvate at 37 °C in 5% CO2. TOPflash (TCF reporter plasmid) and FOPflash (TCF mutant reporter plasmid) were generously provided by Dr. Hans Clevers (Hubrecht Laboratory and Utrecht University, Utrecht, The Netherlands). The Renilla luciferase vector, pRL-CMV, was purchased from Promega (Madison, WI). The pFLAG-CMV-2 control vector and pFLAG-CMV-2-ICAT vector were generated according to the protocol described previously (16).

Antibodies and Antibody Absorption to Microbeads—Mouse anti-E-cadherin (clone HEC-1D-1) was purchased from Invitrogen. Mouse anti-human integrin β1 (clone P5D2), mouse anti-human integrin α3 (clone P1B5), mouse anti-human integrin α2 (clone P1E6) monoclonal antibodies, and purified mouse IgG were purchased from Chemicon International (Temecula, CA). The TS2/β1 integrin antibody was purified from conditioned media of TS2/16.2.1 hybridoma cells (American Type Culture Collection, Manassas, VA) according to the protocol described previously (29). Anti-active β-catenin (clone 8E7) recognizes β-catenin that is dephosphorylated on Ser-37 and/or Thr-41 and was purchased from Upstate Biotechnology (Lake Placid, NY). Purified mouse anti-β-catenin monoclonal antibody was purchased from BD Transduction Laboratories. Phospho GSK-3β (Ser9) antibody and GSK-3β (27C10) rabbit mAb were purchased from Cell Signaling Technology (Danvers, MA). GSK-3β activity is inhibited following phosphorylation of Ser-9 (13). Mouse monoclonal anti-tubulin, peroxidase-conjugated anti-mouse IgG, and peroxidase-conjugated anti-rabbit IgG were purchased from Sigma. AlexaFluor 488 goat anti-mouse IgG (H+L) was purchased from Molecular Probes (Eugene, OR). Antibodies to α2, α3, or β1 integrin and control IgG were passively absorbed onto polyelectrolyte 3.0-μm microspheres (5.95% solids latex; Polysciences, Inc., Warrington, PA) as described previously (30). Antibodies to β1 integrin were also passively absorbed onto Dynabeads M-450 epoxy magnetic beads (Invitrogen) using the procedure for coating of antibodies/proteins provided by the manufacturer.

Immunoﬂuorescence—Cells were plated on 22-mm² glass coverslips coated by passive adsorption with type I collagen (from rat tail, BD Biosciences, San Jose, CA) and placed in 6-well tissue culture plates for up to 5 days. Control cells were plated on uncoated glass coverslips. Cells were gently washed in phosphate-buffered saline (PBS), fixed in paraformaldehyde (10%) at room temperature, washed in PBS, and blocked in PBS, 1% bovine serum albumin (BSA) followed by the addition of anti-E-cadherin (HECD-1 clone) (1:500 dilution in PBS, 1% BSA) at 37 °C. After two washes in PBS, coverslips were incubated with Alexa Fluor 488 goat anti-mouse IgG (1:500 dilution in PBS, 1% BSA) in the dark at room temperature. Coverslips were washed in PBS twice and in distilled water once, fixed using Gelvatol, and visualized using fluorescence microscopy (Nikon Microphot FXA).

Biotin Surface Labeling and Analysis of Internalized E-cadherin—Cells were treated for 60–90 min with microbead-immobilized anti-integrin antibodies prior to surface biotinylation to label remaining cell surface proteins. Cells were incubated with the non-cell-permeable biotin analog Sulfo-NHS-LC-Biotin (1 mg/ml) (Pierce) on ice for 25 min and then were lysed in modified RIPA buffer (mRIPA) (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA) (29). The protein concentration of lysates was determined using a detergent- or non-competitive protein assay kit (Bio-Rad), and an equal concentration of lysates (50 μg) was incubated with NeutraAvidin (Pierce, 30 μl) overnight at 4 °C. NeutraAvidin/biotin-labeled proteins complexes were then isolated by centrifugation and washed five times in mRIPA buffer. For Western blotting, samples were loaded onto SDS-polyacrylamide gels, electrophoresed, and then transferred onto polyvinylidene fluoride (PVDF) microporous membranes (Millipore). After blocking nonspecific binding to membranes in 3% BSA in TBST for 1 h at room temperature, membranes were incubated with primary antibodies for 3 h at room temperature or overnight at 4 °C and then with HRP-conjugated secondary antibodies. Immunoreactivity was determined by SuperSignal West Dura extended duration substrate kit (Thermo Scientific).

Analysis of the internalized pool of E-cadherin was performed as described previously (31). Briefly, cells were surface-labeled on ice with a sulphydryl-cleavable biotin derivative (Sulfo-NHS-SS-Biotin at 1 mg/ml; Pierce), shifted to 37 °C in the presence of bead-immobilized β1 integrin antibody or control IgG to induce integrin clustering for 1 h, and then treated with MESNA (100 mM) to remove any remaining surface-associated (i.e., noninternalized) biotin and with iodoacetamide (5 mg/ml) to block nonreactive sites. The cells were then lysed; protein concentration of lysates was determined as above, and an equal concentration of lysates (50 μg) was incubated with NeutraAvidin (Pierce) to isolate the labeled surface proteins that had been internalized following integrin engagement. Lysates were subjected to Western blot analysis as above. In control experiments, the MESNA step was not included, allowing analysis of total labeled protein (internalized and cell surface pool), and cells were also kept on ice during the course of the experiment to determine the efficiency of surface stripping with MESNA (MESNA control).
**Cell Fractionation**—Nuclear proteins were isolated using the NE-PER nuclear and cytoplasmic reagents from Pierce, according to the manufacturer’s specifications. Alternatively, cells were fractionated following a 90-min incubation with bead-immobilized β1 integrin antibody (control cells were incubated with bead-immobilized IgG antibody) as described previously (32). Briefly, cells were washed twice with cold PBS and lysed with a cold hypotonic lysis buffer (10.0 mM NaCl, 20.0 mM HEPES, pH 7.9, 1.0 mM EDTA, 2.0 mM MgCl₂, 20.0 mM β-glycerophosphate, 1.0 mM Na₃VO₄, 1.0 mM PMSF, 1.0 mM DTT, 200 mM sucrose, 0.5% Nonidet P-40, and 10 μg/ml of each aprotinin, pepstatin A, and leupeptin). Lysate was collected by scraping, passed through a 26-gauge syringe, and centrifuged at 16,000 × g for 1 min at 4 °C after a 10-min incubation on ice. The cytoplasmic fraction was collected (supernatant), and the pellet was washed twice with hypotonic lysis buffer before treatment with nuclear extraction buffer (420.0 mM NaCl, 20.0 mM HEPES, pH 7.9, 1.0 mM EDTA, 2.0 mM MgCl₂, 20.0 mM β-glycerophosphate, 1.0 mM Na₃VO₄, 1.0 mM PMSF, 1.0 mM DTT, 25% glycerol, and 10 μg/ml of each aprotinin, pepstatin A, and leupeptin). Following a 10–15 min incubation and a 5–min centrifugation (16,000 × g at 4 °C), the nuclear fraction was collected. Western blot analysis was done as described previously (30). Western blots were quantified using ImageJ (National Institutes of Health, Bethesda). p values were determined using the t test function (two sample, unequal variance, one-tailed distribution) on Excel (Microsoft Corp., Redmond, WA).

**Isolation and Immunofluorescence Analysis of Nuclei**—Cells treated with either bead-immobilized IgG antibody (control) or bead-immobilized β1 integrin antibody were lysed with hypotonic lysis buffer (see above) in the absence of Nonidet P-40. Lysate was quickly centrifuged and then spun onto poly-1-lysine-coated coverslips using a Cytopro 7620 centrifuge (Wescor, Logan, UT) with acceleration to 2000 rpm for 10 min. Anti-β-catenin (total) antibody was purchased from BD Transduction Laboratories. Active anti-β-catenin antibody was purchased from Upstate (Billerica, MA). Each primary antibody was labeled using DyLight antibody Labeling kits (Pierce) according to manufacturer’s instructions. Anti-active β-catenin was conjugated with AlexaFluor 649 and anti-β-catenin with AlexaFluor 488. Immunofluorescence was performed as described above, omitting fluorescent secondary antibody.

**TOPflash Assay**—OVCA433 cells were plated at 40–50% confluence in 6-well plates and cotransfected with a Renilla luciferase reporter construct (pRL-CMV) and either the firefly luciferase TOPflash TCF reporter plasmid or the FOPflash TCF mutant reporter plasmid using FuGENE 6 transfection reagent according to the manufacturer’s instructions (Roche Diagnostics). Approximately 18 h after transfection, cells were cultured with low calcium minimal essential medium with serum (0.1 mM CaCl₂, Invitrogen) for 1 h before the addition of latex beads coated with TS2/β1 integrin antibody or control IgG for an additional 30 h. Cells were then lysed in passive lysis buffer (Promega). Both Renilla and Firefly luciferase readings were taken on a Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA) using the reagents and protocol provided in the Dual-Luciferase reporter assay system (Promega). Firefly luciferase readings were first normalized to the reading for the corresponding Renilla luciferase reading to account for transfection efficiency. The adjusted TOPflash reading was then normalized to the corresponding adjusted FOPflash reading to account for background reading of the TOPflash construct.

**Quantitative Real Time RT-PCR**—Total RNA was extracted from treated and control cells using TRIzol reagent (Invitrogen) according to manufacturer’s instructions. cDNA was synthesized from 1 μg of RNA using the RT² First Strand cDNA synthesis kit (SA Biosciences, Frederick, MD). Amplification was performed during using iCycler (Bio-Rad) for 40 cycles, with each cycle consisting of a 15-s denaturation at 95.0 °C followed by 1 min of annealing at 60.0 °C. SYBR Green Master Mix and primer sets for RAC1, VIM, LRP6, AP-1 (c-Jun), and WNT5A were purchased from SA Biosciences (Frederick, MD). GAPDH was used as an internal control in each reaction. The RT² Profiler PCR arrays specific for the Wnt signaling pathway (PAHS-043A) was purchased from SA Biosciences and used according to manufacturer’s instructions.

**Matrigel Invasion Assay**—OVCA433 cells were transiently transfected with either pFLAG-CMV-2 control vector (FLAG) or pFLAG-CMV-2-ICAT vector (ICAT) using FuGENE 6 transfection reagent according to the manufacturer’s specifications. Twenty four hours after transfection, OVCA433 cells were serum-starved overnight and added at a concentration of 500,000 cells/ml to the upper chamber of a Matrigel-coated Boyden chamber with serum-containing minimal essential medium in the bottom chamber as a chemottractant (33, 34). Invasion was quantified by enumerating the total number of cells per filter.

**RESULTS**

**Clustering of Collagen-binding Integrins Alters E-cadherin Functional Localization**—To model cellular responses to integrin engagement during intraperitoneal metastasis, cells were cultured on type I collagen surfaces, and E-cadherin distribution was evaluated by immunofluorescence microscopy. Although overall changes in E-cadherin expression were not apparent (Fig. 1A and supplemental Fig. 1), re-distribution of E-cadherin immunoreactivity from junctional (Fig. 1A, left panel) to a diffuse cytoplasmic localization (right panel) was observed following integrin engagement by collagen. To address the role of integrin engagement in E-cadherin redistribution, cells were incubated with integrin subunit-specific antibodies immobilized on microbeads to mimic matrix-induced integrin aggregation, followed by surface biotinylation with a non-cell-permeable biotin derivative and avidin precipitation to evaluate changes in cell surface proteins. Results show that E-cadherin cell surface levels were significantly decreased following engagement of integrins α2, α3, and β1 (Fig. 1B) but not α5 or αv control antibodies (data not shown).

To verify that E-cadherin internalization is induced by integrin clustering, cells were first surface-labeled at 4 °C with a sulfhydryl-cleavable biotin derivative, then shifted to 37 °C, and subjected to integrin clustering using microbead-immobilized anti-integrin antibodies. Cells were then treated with MESNA to remove any remaining surface-associated (i.e. noninternalized) biotin and with iodoacetamide to block nonreactive sites.
Engagement of collagen-binding integrins alters E-cadherin localization. A, cells were cultured on coverslips coated with BSA (left panel) or type I collagen (right panel) for 5 days and processed for immunofluorescence using anti-E-cadherin (HECD-1) (1:300 dilution) and FITC-conjugated IgG, as described under “Experimental Procedures” (×20 magnification). In control experiments in which primary antibody was omitted, no positive fluorescent signal was observed (data not shown). B, cells were treated with microbead-immobilized anti-integrin α2, α3, β1, or control IgG (as indicated) for 1 h. To evaluate the changes in surface E-cadherin induced by integrin aggregation, cells were surface-biotinylated (Sulfo-NHS-LC-Biotin, 1 mg/ml for 25 min at 4 °C) and lysed. Lysates (60–100 μg) were incubated with NeutrAvidin. Following incubation, the NeutrAvidin-conjugated lysates and resulting supernatant were electrophoresed on a 9% SDS-polyacrylamide gel, transferred to a PVDF membrane, and immunoblotted with anti-E-cadherin (HECD-1) (1:4000), followed by peroxidase-conjugated secondary antibody (1:5000) and enhanced chemiluminescence detection. A representative Western blot is shown. Results were normalized against the densitometric reading for untreated cells and represent four independent experiments (*, p < 0.02 relative to cells treated with control IgG beads). C, to evaluate E-cadherin internalization, cells were labeled at 4 °C with a sulphydryl-cleavable form of biotin (Sulfo-NHS-SS-Biotin at 1 mg/ml for 25 min) and then shifted for 1 h to 37 °C by the addition of warm culture medium together with 20 μl of magnetic microbeads containing immobilized β1 integrin antibody to induce integrin aggregation. Cells were then treated with MESNA and iodoacetamide at room temperature to strip off any remaining cell surface biotin and block nonreactive sites. The cells were then lysed, and lysates (50–100 μg) were incubated with NeutrAvidin to isolate the labeled surface proteins that were internalized following integrin engagement. In control experiments, the MESNA step was not included to allow analysis of total labeled protein (internalized and cell surface pool). Cells were kept on ice during the course of the experiment to determine the efficiency of surface stripping with MESNA (MESNA control). A representative Western blot is shown. Results were normalized against the densitometric reading for the MESNA control and the corresponding densitometric reading for total labeled E-cadherin population. These results represent the percentage of internalized E-cadherin relative to total labeled E-cadherin and include three independent experiments (*, p = 0.05 relative to IgG bead control).

The cells were then lysed, and labeled proteins present in the cell lysate (representative of the pool of internalized cell surface proteins) were isolated using avidin precipitation and subjected to Western blot analysis for E-cadherin. E-cadherin internalization was increased following β1 integrin engagement (Fig. 1C). Recycling of E-cadherin to the cell surface could not be determined, as cells lost viability following the additional temperature shifts necessary for this experimental protocol. Alternatively, integrins were engaged in the presence of proteasome inhibitor MG-132 to determine whether internalized E-cadherin was degraded via a proteasomal pathway. E-cadherin levels remained constant in the presence of MG-132 (data not shown and supplemental Fig. 2), suggesting that proteasomal degradation of E-cadherin following internalization is not a major mechanism of E-cadherin regulation in these cells. In addition, it was previously shown that long term integrin engagement (20 h) did not result in a net decrease of E-cadherin expression (29), suggesting that E-cadherin is likely either recycled or rapidly replenished if degraded via another degradation pathway.

Integrin Aggregation Alters β-Catenin Dynamics—β-Catenin is commonly found in association with the E-cadherin cytoplasmic domain at cell-cell junctions (35). Following integrin clustering, a decrease in surface-associated β-catenin was observed (Fig. 2A) suggesting that junctional (E-cadherin-associated) β-catenin is lost from this pool. Cytosolic β-catenin can be targeted for degradation or translocated to the nucleus. In the absence of Wnt signaling activation, GSK-3β phosphorylates β-catenin when it is complexed with adenomatous polyposis coli and Axin and targets it for ubiquitination and degradation, preventing translocation to the nucleus (14, 15). As phosphorylation of GSK-3β on Ser-9 inhibits its activity and prevents targeting of β-catenin for degradation (13, 36), whole cell lysates were examined for Ser-9-phosphorylated GSK-3β fol-
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Following integrin clustering. Although total GSK-3β expression levels were unaffected (Fig. 2B, middle panel), GSK-3β inhibition (Ser-9 phosphorylation) was enhanced following integrin clustering (Fig. 2B, top panel).

To examine whether integrin clustering may expand the pool of transcriptionally active β-catenin, whole cell lysates were also examined for the presence of active β-catenin that is dephosphorylated at residue Ser-37 or Thr-4 (37). A significant increase in the active (de-phospho) pool of β-catenin was observed following integrin engagement (Fig. 3A, top panel), although total β-catenin levels were unaltered (Fig. 3A, middle panel). Nuclear extracts were then isolated by differential centrifugation and analyzed for the presence of β-catenin. Results show that integrin clustering enhances nuclear accumulation of β-catenin (Fig. 3B). This was confirmed using cytopsin/immuno-fluorescence analysis of isolated intact nuclei from cells subjected to integrin clustering, which demonstrated an increase in nuclear β-catenin fluorescence (Fig. 3C).

Integrin Aggregation Activates Transcription of Wnt/β-Catenin Target Genes—As the data above indicated that integrin clustering altered β-catenin dynamics, resulting in enhanced nuclear β-catenin levels, cells were transfected with the TOPflash luciferase reporter construct to evaluate changes in β-catenin-regulated promoter activation. TOPflash activity was significantly increased following engagement of β1 integrins (Fig. 4A), demonstrating that integrin clustering can induce transcriptional activation of β-catenin/TCF/Lef-regulated target genes. This was confirmed by quantitative PCR profiling of Wnt target genes (supplemental Fig. 3). Validation of select genes from the PCR profiling data demonstrates significantly enhanced expression of PTGS2 (COX2), PLAUI (uPAR), VIM (vimentin), LRIP6, WNT5A, and MMP9 (p < 0.05) and increased expression of SNAI1 (p = 0.06) (Fig. 4B). To assess the functional contribution of β-catenin-regulated transcriptional activity to the invasive phenotype, cellular invasion was assessed in the presence or absence of ICAT, a polypeptide inhibitor of β-catenin nuclear signaling that binds β-catenin and competes its interaction with TCF (35, 38). Following transient transfection with ICAT, invasive activity was significantly reduced relative to vector control cells (Fig. 4C), demonstrating that β-catenin/TCF-regulated target genes contribute to ovarian cancer cell invasion.

DISCUSSION

 Seventy-five percent of women with ovarian cancer present at diagnosis with established metastases throughout the peritoneal cavity (39), indicating a need for more detailed understanding of factors that promote ovarian cancer cell dissemination. Modulation of cell-cell and cell-matrix adhesion are key events in ovarian cancer metastasis, as intraperitoneal adhesion of malignant cells and multicellular aggregates combined with localized integrin-mediated invasion of the collagen-rich submesothelial matrix are necessary to anchor secondary lesions (5, 40). Intraperitoneal ovarian cancer metastasis is mediated by adhesion via integrins α2β1 and α3β1 to peritoneal mesothelial cells displaying surface expression of collagen and the exposed interstitial (types I and III) collagen-rich submesothelial matrix, and antibodies directed against these integrins block collagen binding (41–47). Integrin engagement by a multivalent matrix ligand results in receptor aggregation, functionally coupling the extracellular environment to specific signal transduction pathways that modulate distinct cellular responses, including gene transcription, cell migration, and survival (48). Integrins thereby signal cellular responses by regulating the formation of signal transduction complexes on a cytoskeletal framework, and this integration of signaling and cytoskeletal events is dictated by the physical nature of the integrin-ligand interaction (48).

Recent studies have shown that disruption of E-cadherin can promote β-catenin-regulated transcription. For example, disruption of E-cadherin junctions in squamous carcinoma cells using lysophosphatidic acid treatment resulted in enhanced TCF/Lef-dependent transcriptional activity using the TOPflash reporter (49). Similarly, in a murine lung cancer model using mice with conditional disruption of E-cadherin function, enhanced VEGF-driven angiogenesis was shown to require β-catenin (50). Although integrin aggregation does not promote a loss in net E-cadherin expression in ovarian cancer cells (29), the current results show that engagement of collagen-binding integrins increases internalization of E-cadherin concomitant with GSK-3β inhibition, enabling β-catenin activation, nuclear translocation, and transcriptional regulation. Inhibition of β-catenin transcriptional activity with ICAT down-regulates invasion, supporting a functional role for integ-
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![Image](https://via.placeholder.com/150)

**FIGURE 3. Engagement of β1 integrins increases nuclear β-catenin.** A, cells were cultured for 1 h in medium containing serum and 0.1 mM CaCl₂ prior to treatment with microbead-immobilized anti-integrin β1 (T52) or control IgG (as indicated) for 90 min. Whole cell lysates (15–40 μg) were electrophoresed on a 9% SDS-polyacrylamide gel, transferred to a PVDF membrane, and immunoblotted with anti-active β-catenin (dephospho form) (1:1000), anti-total β-catenin, or anti-tubulin followed by peroxidase-conjugated secondary antibody (1:5000) and enhanced chemiluminescence detection. Proliferating cell nuclear antigen (PCNA) was used as a control nuclear protein, and E-cadherin was used to show absence of non-nuclear proteins. **B,** cells were treated with microbead-immobilized anti-integrin β1 or control IgG for 90 min and then immediately lysed by hypotonic shock. Intact nuclei were collected by cytospin and evaluated for nuclear β-catenin-AlexaFluor 649; green, anti-β-catenin-AlexaFluor 488; blue, DAPI. **C,** cells were treated with microbead-immobilized anti-integrin β1 or control IgG for 90 min and then immediately lysed by hypotonic shock. Intact nuclei were collected by cytospin and evaluated for nuclear β-catenin-AlexaFluor 649; green, anti-β-catenin-AlexaFluor 488; blue, DAPI. Right panel shows quantitation of positive nuclei from three different experiments (p = 0.001).

It has been proposed that Wnt/β-catenin target genes can be divided into two groups as follows: a “stemness/proliferation group” that is transiently expressed in late stage tumors predominately at the tumor-host interface (51). Disseminating ovarian cancer cells encounter collagen in the peritoneal cavity, thereby suggesting that a temporary EMT or transient loss of junctional E-cadherin may occur to facilitate cancer cell migration and invasion. Integrin-induced E-cadherin internalization may play an important role in the breaking and reforming of cell-cell junctions during ovarian cancer cell dissemination, while also contributing to intraperitoneal anchoring through activation of β-catenin-regulated target genes.

In contrast to endometrioid ovarian carcinomas, which often harbor mutations in the β-catenin gene (23, 24, 52), activating mutations of Wnt signaling pathway components are rare in serous, clear cell, and mucinous ovarian carcinomas (53, 54). Nevertheless, nuclear β-catenin has been observed in serous ovarian cancers, and coimmunoprecipitation of Lef-1 and β-catenin from serous tumors has been reported (53, 55–57). It should be noted that Wnt signaling is not constitutively activated in the majority of individual cells in cases positive for nuclear β-catenin; however, current approaches do not enable detection of transient activation of Wnt signaling, such as may occur following initial engagement of tumor cell integrins with submesothelial collagens.

Increased β-catenin nuclear signaling enhances transcription of many genes that contribute to tumor progression (14, 15), including genes that modulate invasion and metastasis (51). Detection of Wnt target gene expression provides additional evidence in support of Wnt pathway activation. Similar results were obtained in studies using ovarian cancer cells cultured in three-dimensional collagen gels, demonstrating altered expression of genes, including dickkopf homolog 1 (Dkk1), connective tissue growth factor, and membrane type 1 matrix metalloproteinase (MMP-14) that are known to be regulated by Wnt/β-catenin signaling (47, 58, 59). Enhanced expression of PTGS2 (COX2), PLAUR (uPAR), VIM (vimentin), LRP6, WNT5A, MMP9, and SNAI1 (Snail) was demonstrated in this study. Immunohistochemical analysis has shown Cox-2 immunoreactivity in 63–85% of human ovarian cancer speci-
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In summary, the current data describe a mechanism for integrin-dependent activation of Wnt/β-catenin signaling that may play a role in metastatic progression even in tumor cells devoid of Wnt pathway mutations. Integrin clustering alters E-cadherin and β-catenin dynamics, inducing nuclear translocation of β-catenin and transcriptional activation of Wnt/β-catenin target genes. Although some of these genes may also be activated by other pathways, the current data support the hypothesis that matrix-induced integrin clustering may expand the pool of transcriptionally active β-catenin and provide a novel mechanism for the dysregulation of Wnt signaling observed in ovarian tumors lacking mutations in the Wnt signaling pathway (23–26).

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FIGURE 4. β-Catenin transcriptional activity and cellular invasion. A, cells were transiently cotransfected with a Renilla luciferase reporter construct (pRL-CMV) and either the firefly luciferase TOPflash TCF reporter plasmid or TOPflash TCF mutant reporter plasmid prior to treatment with microbeads containing immobilized anti-integrin β1 (TS2) or control IgG for 30 h. Firefly luciferase readings were first normalized to the reading for the corresponding Renilla luciferase to normalize for transfection efficiency. The adjusted TOPflash reading was then normalized to the corresponding adjusted FOPflash reading to account for background reading of the TOPflash construct. The results are representative of five separate experiments (p = 0.022 relative to cells treated with IgG microbeads). B, cells were treated with microbead-immobilized anti-integrin β1 or control IgG prior to isolation of RNA and evaluation of Wnt target gene expression by real time RT-PCR as described under “Experimental Procedures.” Results represent the mean of a minimum of four independent experiments. (p < 0.05; #, p = 0.1). C, cells were transiently transfected with either ICAT or a control FLAG-tagged vector, and 2.5 × 10^4 cells were added to each porous polycarbonate filter (8-μm pore) coated with Matrigel. Serum-containing cell culture medium was added to the bottom well to serve as a chemoattractant. After 24 h, the filters were collected and stained, and cells were enumerated. Data represent the total number of cells per filter for three experiments performed in triplicate (p = 0.005 relative to cell transfected with control FLAG-tagged vector).
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