

Original Article

MUC16 mucin (CA125) regulates the formation of multicellular aggregates by altering β -catenin signaling

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Abstract: After shedding from the primary tumor site, ovarian cancer cells form three-dimensional multicellular aggregates that serve as vehicle for cancer cell dissemination in the peritoneal cavity. MUC16 mucin (CA125) is aberrantly expressed by most advanced serous ovarian cancers and can promote proliferation, migration and metastasis. MUC16 associates with E-cadherin and β -catenin, two proteins involved in regulation of cell adhesion and the formation of multicellular aggregates. However, the role of MUC16 in the formation of multicellular aggregates remains to be defined. Here, we show that MUC16 alters E-cadherin cellular localization and expression. Consistent with this, MUC16 knockdown inhibited the formation of multicellular aggregates and, conversely, forced expression of MUC16 C-terminal domain (CTD) enhanced the formation of multicellular aggregates. MUC16 knockdown induces β -catenin relocation from the cell membrane to the cytoplasm, decreases its expression by increasing degradation and decreases β -catenin target gene expression. MUC16 CTD inhibits GSK-3 β -mediated phosphorylation and degradation of β -catenin, leading to increased β -catenin levels. Importantly, knockdown of β -catenin inhibited multicellular aggregation. These findings indicate that MUC16 promotes the formation of multicellular aggregates by inhibiting β -catenin degradation.

Keywords: MUC16, membrane-bound mucin, ovarian cancer, multicellular aggregates, B-catenin, E-cadherin

Introduction

Current evidence suggests that ovarian carcinomas (OC) may develop from the single layer of ovarian surface epithelium (OSE) that covers the surface of the ovary and/or epithelium of oviductal fimbriae [1, 2]. Because OC are mostly asymptomatic, patients are diagnosed at a late stage with extensive abdominal metastasis [3]. In contrast to many solid tumors, OC mainly spreads by direct extension, through exfoliation of tumor cells from the ovary into ascites, in which tumor cells survive and proliferate, and later implant on the mesothelium lining of the peritoneal cavity. In ascites, OC cells exist as multicellular aggregates. The formation of these cell aggregates is necessary for OC to survive and metastasize after shedding from the original tumor site [4]. Thus, these aggregates serve as vehicles for dissemination in the peritoneal cavity, protecting cells from anoikis.

E-cadherin is a membrane glycoprotein that mediates calcium-dependent cell-cell adhesion and is located at the adherens junctions [5, 6]. Cell-cell adhesion is facilitated by the assembly of a multimolecular bridge that spans from the cytoplasmic tail of E-cadherin to the actin cytoskeleton. Although most normal epithelia express E-cadherin, mesenchymally derived normal OSE cells do not usually express E-cadherin. In contrast, pre-neoplastic OSE-derived inclusion cysts and well-differentiated OC express E-cadherin [7]. E-cadherin expression appears to be maintained during OC progression and metastasis, although reduced staining is usually observed in metastatic lesions [8]. In addition, the down-regulation of E-cadherin promotes OC metastasis [9]. One important component of the adhesion complex is β -catenin. β -catenin is found predominantly in association with the E-cadherin cytoplasmic domain at cell-cell junctions, and the levels are maintained at low concentration in the cytoplasm by a phos-

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phorylation-dependent degradation of β -catenin [10]. Cytoplasmic β -catenin is targeted to a complex comprised of Axin, adenomatous polyposis coli (APC), casein kinase 1 and glycogen synthase kinase-3 β (GSK-3 β), resulting in its phosphorylation that targets β -catenin for degradation through the ubiquitin-proteasome pathway. When it is not targeted for proteasomal degradation, a small fraction of the free, non-junctional β -catenin is capable of translocating to the nucleus where it binds to members of the TCF/LEF transcription factor family to regulate the expression of genes that can be oncogenic including cyclin D1, c-Myb and MMP7 among others [11]. Phosphorylation of GSK-3 β inhibits its ability to phosphorylate and target β -catenin for degradation [12]. A dysregulation of β -catenin-dependent LEF-1 signaling has been noted in ovarian carcinoma, particularly in the endometrioid sub-type [13]. Therefore, changes in adherens junction status that alter the free and junctional pools of β -catenin could, potentially, influence ovarian carcinogenesis.

MUC16 mucin (CA125) is a high molecular weight membrane-associated glycoprotein (200-2000 kDa) that is not expressed by OSE cells but aberrantly expressed in the majority of OC on the entire cell surface [14-19]. Although MUC16 serum levels correlate with disease progression and remission, its tissue expression as a prognostic factor is more controversial [20-24]. MUC16 is composed of an enormous, heavily glycosylated, N-terminal domain of more than 12,000 amino acid residues, a central domain containing up to 60 glycosylated repeat sequences constituting the tandem repeats characteristic of mucins and a C-terminal domain composed of a short cytoplasmic tail of 31 amino-acid, a transmembrane domain and the extracellular portion that remains on the cell surface after cleavage [25-28]. Although the functions of MUC16 are still unclear and probably cell context dependent, recent data suggest that MUC16 expression is associated with an increased malignant behavior of OC cells, with increased tumorigenicity and with increased resistance to drug-induced apoptosis [29-32]. MUC16 binds E-cadherin and β -catenin and MUC16 knockdown has been shown to promote epithelial to mesenchymal transition (EMT) suggesting a potential role for MUC16 in regulating cell-cell adhesion [33]. This is further supported by the recent findings that MUC16 cytoplasmic tail directly binds c-src [34]. Other

membrane-bound mucins such as MUC1 have been involved in the regulation of cell-cell adhesion by promoting E-cadherin/ β -catenin complex formation [35, 36]. Indeed, the cytoplasmic domain of MUC1 directly interacts with β -catenin through a SXXXXXSSL motif. However, this motif is notably absent in MUC16 cytoplasmic tail [37, 38].

The objective of this study was to investigate the effects of altered MUC16 expression on the formation of multicellular aggregates, and regulation of E-cadherin and β -catenin localization and function. Stable down-regulation of MUC16 in OVCAR3 cells was achieved using single-chain antibodies (scFvs) and ectopic expression of MUC16 was created by transfecting MUC16 C-terminal domain (CTD) vector in SKOV3 cells. Both systems have previously been described [29, 31-33]. Our data suggest that MUC16 regulates E-cadherin-mediated multicellular aggregation by altering β -catenin levels in tumor cells. Therefore, the expression of MUC16 could be important for the survival of tumor cells in ascites by promoting cell-cell aggregation.

Material and methods

Cell lines

The OVCAR3 and SKOV3 human ovarian cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). OVCAR3 cells overexpress MUC16 whereas there is no detectable MUC16 expression in SKOV3 cells. OVCAR3 cells were grown in RPMI 1640 (Wisent, St-Bruno, QC, Canada) supplemented with 20% heat-inactivated FBS (Wisent), 2 mM L-glutamine (Wisent), 100 units/ml penicillin, 100 μ g/ml streptomycin and 10 μ g/ml insulin, and maintained at 37°C in a humidified 5% CO₂ incubator. The SKOV3 cell line was maintained at 37°C in a humidified 5% CO₂ incubator in DMEM/F12 (Wisent) supplemented with 10% heat-inactivated FBS and antibiotics. The construction and validation of the anti-MUC16 scFv has been previously described in detail [29, 31-33]. Two independent stable OVCAR3 clones expressing anti-MUC16 scFvs (MUC16 scFv-7, MUC16 scFv-9), and a control scFv (Ctrl scFv), which does not bind MUC16, were generated. This MUC16 knockdown model has been previously described [31-33]. The derivation of the empty vector (EV) and MUC16 CTD expressing

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SKOV3 cells has also been described previously [29]. Briefly, MUC16 CTD cDNA contains an IgK leader sequence, a unique 229 a.a. extracellular domain and the transmembrane and cytosolic domains of MUC16 tagged at the C-terminal with His₆ peptide, which is separated by a small linker with a c-myc peptide tag. Pooled colonies were expanded to generate the SKOV3 EV (empty vector) and SKOV3 MUC16 CTD cell lines. Ovarian surface epithelial (OSE) cells were obtained and cultured as previously described [33]. The tetracycline-inducible pLenti-sh β -catenin plasmid was kindly provided by Pr F. Boudreau (Université de Sherbrooke, QC, Canada). Lentiviruses produced in 293T cells were used for cell infection according to Life Technologies recommendation (ViraPower Lentiviral Expression System).

Antibodies and reagents

Anti-CA125 M11 antibodies were obtained from Dako (Burlington, ON, Canada) and anti-tubulin from Sigma (Oakville, ON, Canada). Anti-E-cadherin antibody (clone 67A4) was from Chemicon International Inc. (Billerica, MA). HRP-conjugated anti-mouse and -rabbit antibodies were purchased from GE Healthcare (Baie d'Urfé, QC, Canada) and Cell Signaling (Beverly, MA) respectively. Anti-c-myc antibody (clone 9E10) was purchased from Bioshop (Burlington, ON, Canada). Anti-phospho β -catenin (S33/37), anti-phospho GSK-3 β (S9) and anti-cyclin D1 were from Cell Signaling. Anti- β -catenin and anti-GSK-3 β were from BD Transduction Laobratouires (Mississauga, ON, Canada). Anti-MMP-7 and anti-cMyb antibodies were from Santa Cruz Biotechnology (Dallas, TX). Propidium iodide and cycloheximide were from Sigma.

Immunoblot analysis

Cell were lysed in lysis buffer (50 mM Tris-HCL, pH 7.5, 0.1% SDS, 150 mM NaCl, 0.5 mM EGTA, 1% Triton X100) with freshly added protease inhibitors (1 μ g/ml 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 2 μ g/ml aprotinin, 0.7 μ g/ml pepstatin and 0.5 μ g/ml leupeptin) (Sigma) and proteins were quantified by Bradford assay (Bio-Rad, Mississauga, ON).

Cells lysates (equal amounts of proteins) were submitted to SDS-PAGE electrophoresis (10%) and transferred onto PVDF membrane (Roche, Laval, Canada). The membranes were blocked

with 5% non-fat milk and probed with indicated antibodies. The immunoblots were developed with chemiluminescence using the ECL system according to the manufacturer's instruction (GE healthcare, Baie d'Urfe, QC). For the detection of phosphoproteins, cells were lysed in Nonidet P-40 isotonic lysis buffer (283 mM KCl, 10 mM MgCl₂, 50 mM HEPES, pH 7.2, 4 mM EGTA, 0.5% NP-40, 10 mM sodium fluoride, 100 μ M sodium pyrophosphate, 400 μ M sodium orthovanadate with freshly added protease inhibitors.

Immunofluorescence

Cells were grown on glass slides until a 50-70% confluence was reached. Glass slides were then washed in cold PBS and cells fixed in 3.7% formaldehyde (Fisher) for 10 min. Glass slides were next rinsed 5 min in PBS and quenched (Glycin 0.1 M) during 30 min. Depending on the experiment, cells were permeabilized in PBS containing 0.2% TritonX-100 for 20 min at room temperature. Slides were rinsed twice in PBS and blocked overnight in PBS/2% goat serum at 4°C. Slides were washed 3 times in PBS, incubated with primary antibodies in blocking buffer at room temperature for 1 h. Slides were washed 3 times in PBS, incubated for 45 min at room temperature with Alexa Fluor 594 or F(ab')₂ fragment of goat anti-mouse or goat anti-rabbit IgG from Life Technologies (Burlington, ON). After washing, slides were incubated for 2 min in 4',6' Di amidino-2-phenyl indole (DAPI) to visualize nuclei, washed again in PBS and mounted for visualization by fluorescence microscopy with an Olympus IX70 (Olympus, Hamburg, Germany). Expression of MUC16 CTD was detected using anti-c-myc sc-789 antibody (1:200).

Cell aggregation assay

The bottom of 6-well plates was covered with 1% bacto-agar (in 1X complete media) to prevent cell-substratum adhesion. To each well, 1.5 X 10⁴ EDTA-detached cells were deposited as a cell suspension and incubated in complete medium. Cells were dissociated by 10 times pipetting before plating. The formation of multicellular aggregates was evaluated for up to two days by phase contrast microscopy (Nikon Eclipse TS100) at 10X magnification and representative photographs were taken using a Nikon Coolpix 4500 digital camera. Then, the number of isolated and aggregated cells was

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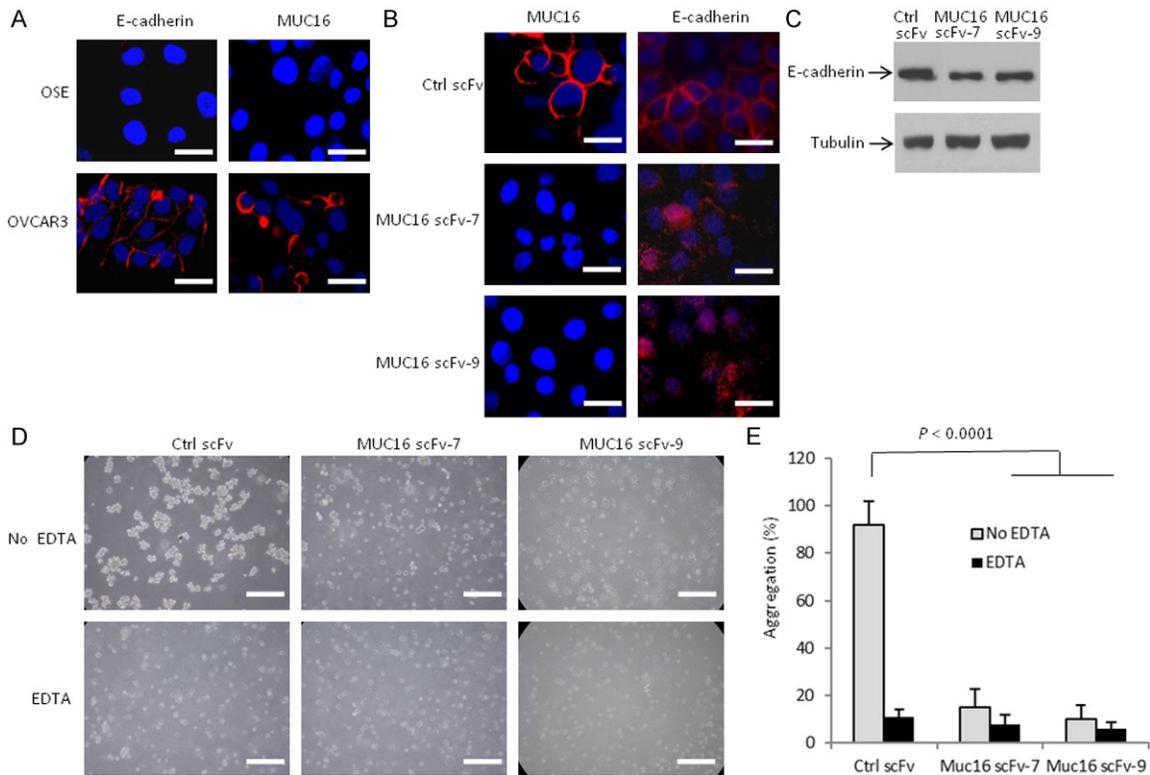


Figure 1. MUC16 knockdown alters E-cadherin dynamic and decreases cell aggregation. **A.** Cellular localization of MUC16 and E-cadherin in normal OSE cells and OVCAR3 cancer cells by immunofluorescence. Cell nuclei were stained with DAPI. *Scale bars* 20 μ m. **B.** Immunofluorescence staining for MUC16 and E-cadherin in control (Ctrl scFv) and MUC16 knockdown (MUC16 scFv-7 and scFv-9) OVCAR3 transfectants. *Scale bars* 20 μ m. **C.** Immunoblot analysis of E-cadherin expression. Tubulin was used as a loading control. **D.** Control and MUC16 knockdown OVCAR3 cells were seeded in suspension for this aggregation assay in the presence or absence of EDTA. A representative picture is shown at 48 h. *Scale bars* 200 μ m. **E.** Quantification of the percentage of aggregating cells from three independent experiments. *P* value is indicated for control versus knockdown cells in the absence of EDTA.

calculated. The percentage of aggregation was defined as follow: $100 - \text{number of isolated cell at } T_n / \text{number of isolated cells at } T_0 \times 100$. Data were obtained from at least two independent experiments.

Statistical analyses

Statistical analysis was done using the BMDP statistical analysis software (UCLA, Los Angeles, CA). The threshold for statistical significance is a probability of < 0.05. The two-sided Student's *t* test was used to assess statistical differences between groups.

Results

MUC16 knockdown alters E-cadherin expression and junctional localization

OC cells often express E-cadherin and MUC16 whereas normal human OSE cells, from which

ovarian cancer may develop, do not. As shown in **Figure 1A**, the OVCAR3 cell line, a well-differentiated OC cell line with low migratory and tumorigenic potential expressed high levels of E-cadherin and MUC16 that are predominantly localized at the cell membrane. In contrast, human OSE cells are negative for E-cadherin and MUC16 staining. To evaluate whether MUC16 expression may alter E-cadherin dynamic, we knocked down MUC16 in OVCAR3 cells using single-chain antibodies (scFv). This system has been previously described and validated [29, 31-33]. The knockdown of cell surface expression of MUC16 resulted in a loss of junctional E-cadherin staining in the two independent MUC16-scFv expressing clones compared to the control (Ctrl scFv) cells (**Figure 1B** and **1C**). In MUC16 knockdown cells, we observed a redistribution of E-cadherin immunoreactivity from a junctional to a diffuse cytoplasmic localization (**Figure 1B**). The down-reg-

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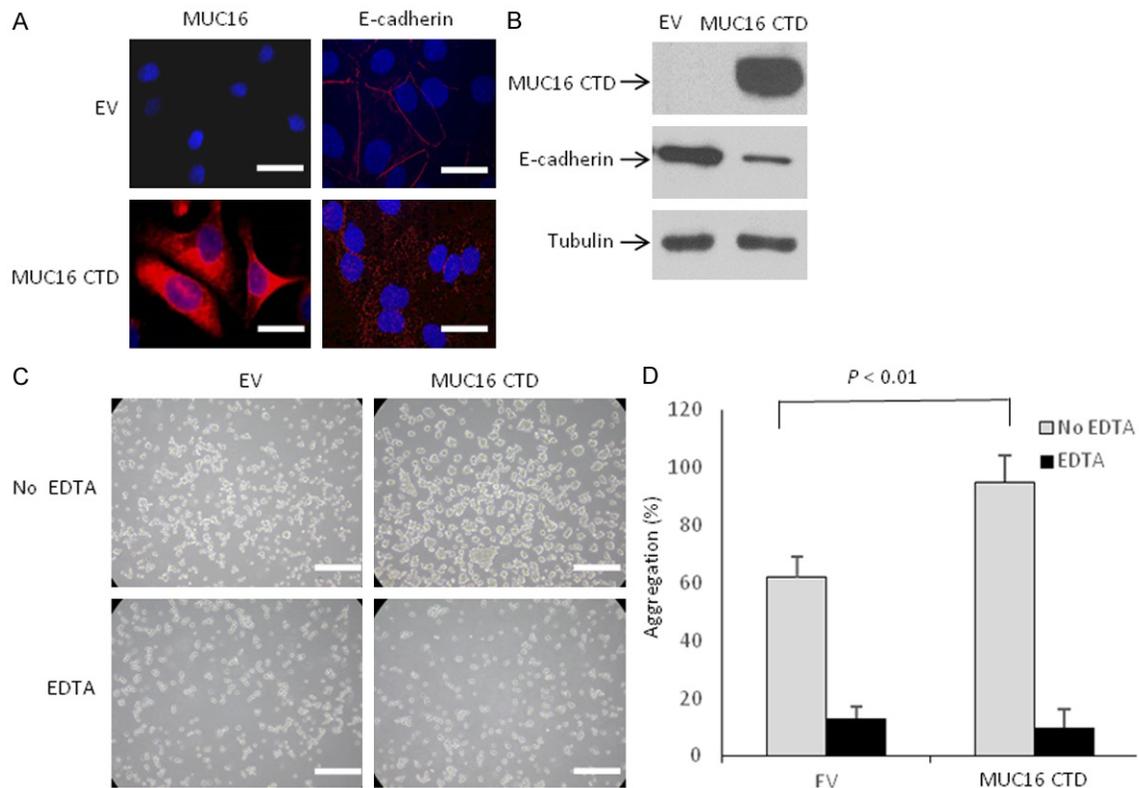


Figure 2. Expression and localization of E-cadherin in MUC16 CTD- and EV-expressing SKOV3 cells. **A.** Immunofluorescence staining of E-cadherin in control (EV) and MUC16 CTD SKOV3 transfectants. Scale bars 20 μ m. **B.** Immunoblot analysis of MUC16 CTD and E-cadherin expression in SKOV3 transfectants. Tubulin was used as a loading control. **C.** Control EV and MUC16 CTD SKOV3 cells were seeded in suspension for this aggregation assay in the presence or absence of EDTA. A representative picture is shown at 48 h. Scale bars 200 μ m. **D.** Quantification of the percentage of aggregating cells from three independent experiments. *P* value is indicated for MUC16 CTD versus control cells in the absence of EDTA.

ulation of MUC16 also decreased the expression of E-cadherin as shown by immunoblot (**Figure 1C**).

OC cells exist as multicellular aggregates in ascites and the formation of these aggregates promotes tumor cell survival and metastasis after shedding from the primary site [4]. As shown in **Figure 1D** and **1E**, the knockdown of MUC16 substantially decreases the ability of OVCAR3 cells to form multicellular aggregates in anchorage-independent conditions. Because E-cadherin-mediated adherent junction formation is Ca^{2+} -dependent, the presence of EDTA strongly inhibited multicellular aggregate formation in both control and MUC16 scFv-expressing OVCAR3 cells as expected (**Figure 1D** and **1E**). These data suggest that MUC16 knockdown inhibits cell-cell aggregation in suspension by altering E-cadherin localization and expression.

Ectopic expression of MUC16 C-terminal domain promotes cell-cell aggregation

E-cadherin expression is abundant in well-differentiated ovarian carcinomas. However, in poorly and undifferentiated ovarian carcinomas, reduced E-cadherin staining is often observed [7]. The SKOV3 cell line, which does not express MUC16, is a more aggressive cell line (as compared to OVCAR3) with higher migratory potential. When compared to OVCAR3 cells, SKOV3 cells express lower level of E-cadherin [39]. Nonetheless, both of these cell lines are able to mimic the progression of OC. The effect of ectopic and stable expression of MUC16 CTD in SKOV3 cells on E-cadherin localization and multicellular aggregate formation was evaluated. Although not as drastic as the knockdown of MUC16 in OVCAR3, MUC16 CTD expression in SKOV3 cells induced a partial relocation of E-cadherin from the cell surface to

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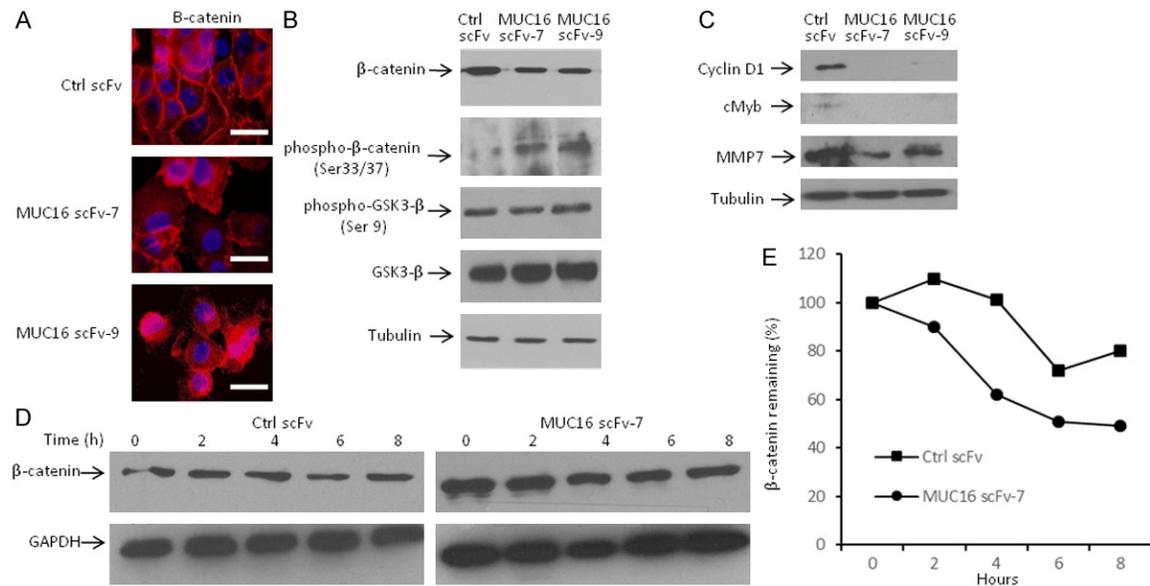


Figure 3. MUC16 knockdown alters β -catenin dynamic. A. Immunofluorescence staining for β -catenin in control (Ctrl scFv) and MUC16 knockdown (MUC16 scFv-7 and scFv-9) OVCAR3 transfectants. Scale bars 20 μ m. B. Immunoblot analysis of total and phospho β -catenin and GSK-3 β protein expression in control and MUC16 knockdown OVCAR3 cells. Tubulin was used as a loading control. C. Expression of β -catenin target gene cyclin D1, cMyb and MMP-7 by immunoblot. D. Control (Ctrl scFv) and MUC16 knockdown (MUC16 scFv-7) OVCAR3 cells were treated with cycloheximide to block protein synthesis and chase for β -catenin expression for the indicated times. Immunoblots with anti- β -catenin antibody. E. Intensity of β -catenin signals was determined by densitometric scanning and plotted as the percentage of β -catenin remaining compared with the baseline (0 hour).

the cytoplasm (**Figure 2A**). MUC16 CTD expression also decreased E-cadherin expression as shown in **Figure 2B**. Despite the partial loss of E-cadherin junctional localization and E-cadherin reduced expression, SKOV3 cells expressing MUC16 CTD formed more abundant and larger cell-cell aggregates in anchorage-independent conditions (**Figure 2C**). These data suggest that MUC16 CTD expression promotes cell-cell aggregation despite altering E-cadherin localization and expression.

MUC16 knockdown decreases β -catenin expression and diminishes β -catenin target gene expression

β -catenin is commonly found in association with the E-cadherin cytoplasmic domain at cell-cell junction [40]. Furthermore, it has been shown that MUC16 associates with the E-cadherin/ β -catenin complex [33, 34]. We therefore examined β -catenin expression and localization in MUC16 knockdown OVCAR3 cells and MUC16 CTD-expressing SKOV3 cells. Following MUC16 knockdown, a relocation of junctional (E-cadherin-associated) β -catenin was observed when compared control-scFv expressing OVCAR3 cells (**Figure 3A**). As shown

in **Figure 3B** (top panel), we also observed a decreased β -catenin expression in MUC16 knockdown cells. Cytosolic β -catenin can be targeted for degradation or translocated to the nucleus. GSK-3 β phosphorylates β -catenin on Ser-33/37 and targets it for ubiquitination and degradation, preventing translocation to the nucleus [11, 41]. Phosphorylation of GSK-3 β on Ser-9 inhibits its activity and prevents targeting of β -catenin for degradation [11, 12]. Whole cell lysates were examined for Ser-33/37-phosphorylated β -catenin in MUC16 knockdown cells. As shown in **Figure 2B**, β -catenin phosphorylation was increased in MUC16 knockdown cells whereas phosphorylation of GSK-3 β on Ser-9 remained unchanged. Consistent with a decreased pool of transcriptionally active β -catenin in MUC16 knockdown cells, we found that expression of β -catenin target genes cyclin D1, cMyb and MMP-7 were decreased (**Figure 3C**). We tried to confirm these results by transfecting control scFv- and MUC16 knockdown OVCAR3 cells with TOPflash luciferase reporter construct and with control FOPflash construct to evaluate changes in β -catenin-regulated promoter activation. However, because of the low level of TCF/Lef promoter activity

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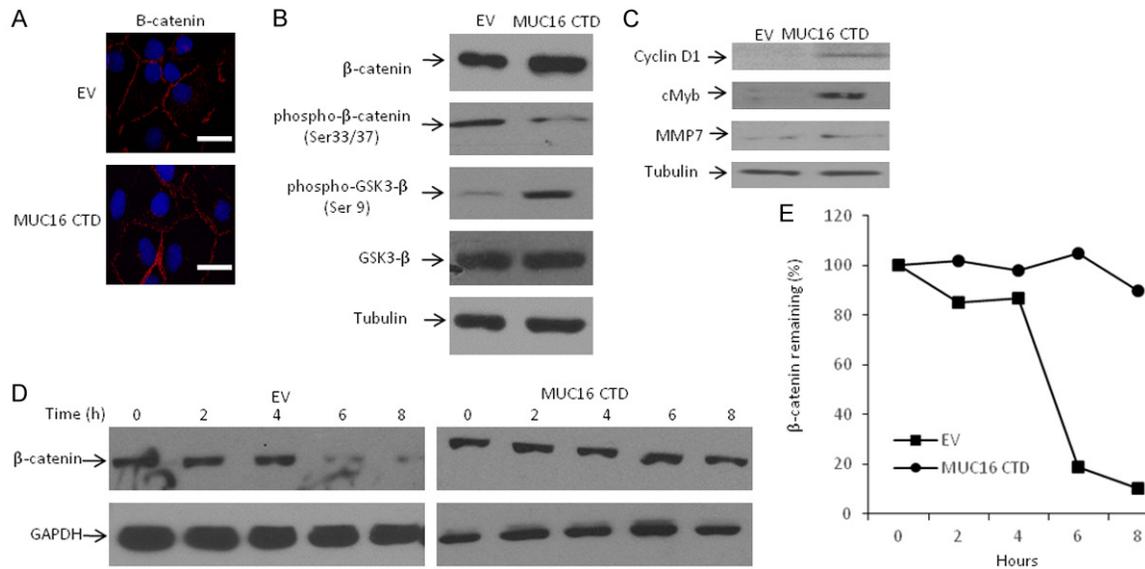


Figure 4. MUC16 CTD stabilizes β -catenin levels. A. Immunofluorescence staining for β -catenin in control (EV) and MUC16 CTD SKOV3 transfectants. Scale bars 20 μ m. B. Immunoblot analysis of total and phospho β -catenin and GSK-3 β protein expression in control and MUC16 CTD SKOV3 cells. Tubulin was used as a loading control. C. Expression of β -catenin target gene cyclin D1, c-Myb and MMP-7 by immunoblot. D. Control (EV) and MUC16 CTD SKOV3 cells were treated with cycloheximide to block protein synthesis and chase for β -catenin expression for the indicated times. Immunoblots with anti- β -catenin antibody. E. Intensity of β -catenin signals was determined by densitometric scanning and plotted as the percentage of β -catenin remaining compared with the baseline (0 hour).

in OVCAR3 cells [39], there was no significant change in the ratio TOP/FOP flash between control and MUC16 knockdown cells (data not shown). Because our data demonstrate that MUC16 knockdown increases the phosphorylation of β -catenin and decreases the expression of β -catenin target genes, we assessed whether MUC16 knockdown affects β -catenin stability. Control and MUC16 knockdown OVCAR3 cells were pretreated with cycloheximide to block protein synthesis and cell lysates were obtained at different time points. Data showed that MUC16 knockdown increased the turnover rate of β -catenin when compared to control MUC16 expressing cells (Figure 3D). Densitometric scanning of the signals showed that the half-lives of β -catenin in Ctrl scFv and MUC16 scFv cells are > 8 and 6 hours, respectively (Figure 3E). Taken together, these data suggest that MUC16 knockdown decreases E-cadherin (membrane)-bounded β -catenin as well as the overall pool of β -catenin, by promoting its degradation, leading to diminish expression of β -catenin targets genes.

MUC16 CTD stabilizes β -catenin

We further examined whether the expression of MUC16 CTD in SKOV3 cells affects β -catenin expression and localization. Immunofluorescence

analyses revealed that β -catenin localization at the cell-cell contacts were mostly comparable to mock-transfected cells (Figure 4A). Interestingly, β -catenin expression was increased in cells expressing MUC16 CTD (Figure 4B). In agreement with this observation, the phosphorylation of β -catenin was decreased and correlated with increased GSK-3 β phosphorylation in MUC16 CTD expressing cells (Figure 4B). The increase expression of β -catenin was associated with increases of cyclin D1, cMyb and MMP7 expression suggesting more β -catenin translocation to the nucleus (Figure 4C). Consistent with these observations, we noted a stabilization of β -catenin in MUC16 CTD expressing cells (Figure 4D). β -catenin half-life was around 5 h in EV cells and > 8 h in MUC16 CTD cells (Figure 4E). These data suggest that MUC16 CTD stabilizes β -catenin by inhibiting GSK-3 β -mediated phosphorylation of β -catenin, which augments β -catenin-mediated expression of cyclin D1, cMyb and MMP7.

Down-regulation of β -catenin in OVCAR3 cells inhibits cell-cell aggregation

The present studies suggest that MUC16-mediated regulation of β -catenin expression and localization may affect the formation of

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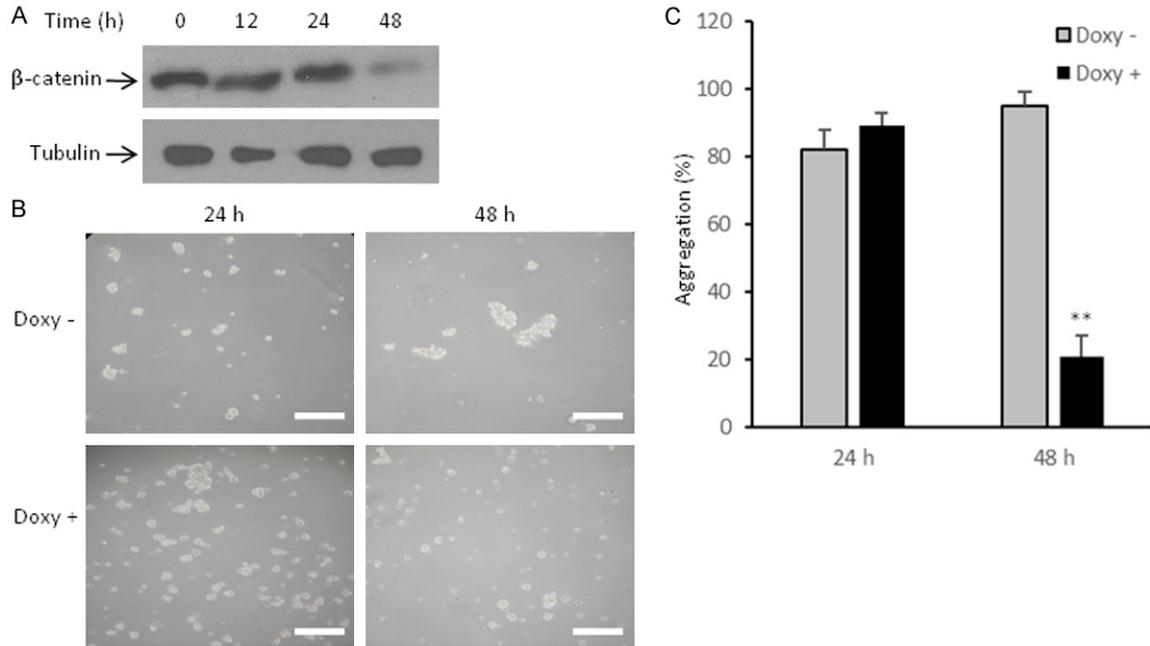


Figure 5. β -catenin regulates cell-cell aggregation in OVCAR3 cells. A. Lysates of OVCAR3 cells stably expressing a tetracycline-induced β -catenin shRNA were subjected to immunoblotting. Cells were treated with doxycycline (1 μ g/ml) and β -catenin protein expression was assessed for the indicated times. B. Cell-cell aggregation was assessed in the presence or absence of doxycycline at 24 h and 48 h. Representative pictures are shown at 24 h and 48 h. Scale bars 200 μ m. C. Quantification of the percentage of aggregating cells from three independent experiments. $P < 0.001$ for presence of doxycycline (Doxy +) versus absence of doxycycline (Doxy -) at 48 h.

multicellular aggregates. To further support the role of β -catenin in OVCAR3 cell-cell aggregation, we down-regulated its expression using a tetracycline-inducible β -catenin shRNA. As shown in **Figure 5A**, there was a substantial decrease of β -catenin expression by 48 h following the addition of doxycycline in the culture medium of OVCAR3 cells stably expressing the β -catenin shRNA. Analysis of multicellular aggregates showed that the percentage of cell-cell aggregation remained unaffected 24 h after the addition of doxycycline (**Figure 5B**), which is consistent with the observation that, at this time point, β -catenin expression remained mostly unchanged (**Figure 5A**). In contrast, at 48 h there was a marked decrease of cell-cell aggregation in the presence of doxycycline compared with absence of doxycycline (**Figure 5B**) consistent with the decreased expression of β -catenin seen by immunoblotting. These data suggest that β -catenin is critical for the formation of multicellular aggregates in OVCAR3 cells

Discussion

Growing evidence functionally implicate MUC16 in cancer development and progression [29-

34, 42, 43]. Furthermore, most advanced OC overexpress MUC16. In addition to MUC16 expression, E-cadherin and β -catenin are also involved in OC progression [7, 9, 44]. Many β -catenin target genes regulate tumor progression [41, 45]. Shedding from the primary tumor site and intraperitoneal OC metastasis is mediated by multicellular aggregates that survive in ascites and later implant on the peritoneal lining by adhesion to mesothelial cells [46]. High E-cadherin expression has been associated with larger and tighter OC multicellular aggregates [47]. Given the critical role of multicellular aggregates in OC progression, there is a need for more detailed understanding of factors that regulate cell-cell aggregation.

The objective of the present study was to evaluate the impact of alterations in MUC16 expression on cell-cell aggregation and on E-cadherin and β -catenin expression and localization in OC cells. To achieve this goal, we used two well-validated systems that rely on scFv-mediated MUC16 knockdown and on stable expression of MUC16 CTD [29, 31-33]. MUC16 protein undergoes post-translational proteolytic cleavage to create a small subunit that contains a short extracellular domain, a transmembrane

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region and a cytoplasmic tail, which explains why the larger N-terminal domain is found in serum of OC patients [25, 26]. Interestingly, E-cadherin and β -catenin are co-immunoprecipitated with MUC16 CTD suggesting that the cytoplasmic tail of MUC16 could bind to β -catenin despite lacking β -catenin binding motif [33, 34]. This is unclear however because it was reported that a MUC16 construct lacking the cytoplasmic domain still associated with E-cadherin/ β -catenin [34]. In this study, the role of MUC16 in cell-cell aggregation was demonstrated by showing that the percentage of multicellular aggregates was significantly decreased by MUC16 knockdown and, conversely, by showing that expression of MUC16 CTD enhances cell-cell aggregation. This is consistent with previous studies showing decreased cell adhesion in MUC16 knockdown OC cells [30, 33]. However, our observations contrast with the study by Akita et al. which found that enforced MUC16 CTD expression in breast cancer cells attenuated cell-cell aggregation [34]. The discrepancy between these observations may reflect the fact that the effect of MUC16 CTD on cell aggregation could be cell-context dependent. This is further supported by the observation that in the study by Akita, β -catenin expression remained unchanged by MUC16 CTD expression whereas in our study, MUC16 CTD increased β -catenin expression (**Figure 4**). Alternatively, the difference between the two studies could be related to the size of the extracellular domain in the MUC16 CTD constructs. In Akita's study, the MUC16 CTD construct contains the 54-56th sea urchin sperm protein (SEA) domains whereas our construct contains a short unique extracellular domain that lacks the SEA domains.

β -catenin regulates the formation of adherens junctions. GSK-3 β -mediated phosphorylation of β -catenin is critical for targeting β -catenin for ubiquitination and degradation [12]. The present studies show that MUC16 knockdown decreases β -catenin expression and diminishes β -catenin target gene expression. MUC16 knockdown also induced relocalization of β -catenin from the membrane to the cytoplasm. Consistent with these results, MUC16 knockdown was associated with increase phosphorylation and increase degradation of β -catenin. In the SKOV3 cell model, MUC16 CTD was associated with increased phosphorylation of GSK-3 β and decreased β -catenin phosphorylation lead-

ing to increase β -catenin stability and overall expression. These findings collectively indicated that MUC16 regulates β -catenin stability and β -catenin target gene expression. Thus, MUC16 appears to regulate the available pools of free β -catenin. This is consistent with previous studies showing that MUC1 oncoprotein blocks GSK-3 β -mediated phosphorylation and degradation of β -catenin [48, 49].

The disruption of junctional E-cadherin usually decreases cell-cell aggregation, increases the pool of free β -catenin and promotes β -catenin-regulated transcription. Interestingly, we found that although the knockdown of MUC16 and the expression of MUC16 CTD were both associated with the loss of junctional E-cadherin and decreased E-cadherin expression, MUC16 knockdown inhibited cell-cell aggregation whereas MUC16 CTD promotes cell-cell aggregation. The mechanism by which MUC16 regulates E-cadherin expression and localization remains unclear. It is possible that in ovarian cancer cells the extracellular domain of MUC16 interacts with the extracellular domain of E-cadherin, as shown for other proteins such EGFR [50], and this interaction stabilizes E-cadherin at the cell membrane. In contrast, MUC16 knockdown or deletion of most of the extracellular domain – as in MUC16 CTD – induce at least a partial E-cadherin relocalization. This is supported by the observation that E-cadherin associates with MUC16 mutants that lack the cytoplasmic domain [34]. Despite the loss of junctional E-cadherin, the expression of MUC16 CTD appears to be sufficient to maintain certain pools of β -catenin at the cell membrane as well as increasing the pools of free β -catenin. Interestingly, integrin-mediated aggregation increases internalization of E-cadherin concomitant with GSK-3 β inhibition, consequently promoting β -catenin-mediated transcription [44]. A recent report also point out to β -catenin as an important pathway activated in multicellular aggregates [51].

In summary, the current data demonstrate that MUC16 alters E-cadherin and β -catenin dynamics and enhances cell-cell aggregation. MUC16 may therefore play a critical role in OC progression by promoting the formation of multicellular aggregate in ascites. Further work is necessary to gain a better understanding of the specific functions of the different MUC16 domains.

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Disclosure of conflict of interest

None to disclose.

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