Expression and Functions of Transmembrane Mucin MUC13 in Ovarian Cancer

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Abstract

MUC13, a transmembrane mucin, is normally expressed in gastrointestinal and airway epithelium. Its aberrant expression has been correlated with gastric colon and cancer. However, the expression and functions of MUC13 in ovarian cancer are unknown. In the present study, the expression profile and functions of MUC13 were analyzed to elucidate its potential role in ovarian cancer diagnosis and pathogenesis. A recently generated monoclonal antibody (clone PPZ0020) was used to determine the expression profile of MUC13 by immunohistochemistry using ovarian cancer tissue microarrays and 56 additional epithelial ovarian cancer (EOC) samples. The expression of MUC13 was significantly (P < 0.005) higher in cancer samples compared with the normal ovary/benign tissues. Among all ovarian cancer types, MUC13 expression was specifically present in EOC. For the functional analyses, a full-length MUC13 gene cloned in pcDNA3.1 was expressed in a MUC13 null ovarian cancer cell line, SKOV-3. Here, we show that the exogenous MUC13 expression induced morphologic changes, including scattering of cells. These changes were abrogated through c-Jun NH2 kinase (JNK) chemical inhibitor (SP600125) or JNK2 siRNA. Additionally, a marked reduction in cell-cell adhesion and significant (P < 0.05) increases in cell motility, proliferation, and tumorigenesis in a xenograft mouse model system were observed upon exogenous MUC13 expression. These cellular characteristics were correlated with up-regulation of HER2, p21-activated kinase 1, and p38 protein expression. Our findings show the aberrant expression of MUC13 in ovarian cancer and that its expression alters the cellular characteristics of SKOV-3 cells. This implies a significant role of MUC13 in ovarian cancer.

Introduction

Ovarian cancer is the most lethal gynecologic malignancy. Nearly 25,000 women are diagnosed, and ~16,000 women die due to ovarian cancer every year in the United States (1–3). During early stages, ovarian cancer can be effectively treated with surgery and chemotherapy (4, 5). The mortality rate of ovarian cancer could be lowered by identification of novel molecular markers of early ovarian cancer and understanding the molecular mechanisms of the early events of ovarian cancer.

Mucins are generally known for providing protection to the luminal epithelial tissues under normal physiologic conditions (6–8). However, alterations in the expression of mucins and/or their glycosylation patterns are often associated with the development of cancer via influencing cellular growth, differentiation, transformation, adhesion, invasion, and immune surveillance (6, 9–13). The aberrant expression of certain membrane-anchored mucins in different histologic types and grades of ovarian tumors has recently been shown (13). These observations suggest important roles of mucins in ovarian cancer. In the present study, we report the expression profile and functions of a transmembrane mucin, MUC13, in ovarian cancer.

MUC13 is a recently identified membrane-bound mucin, and its gene is localized onto chromosome band 3q13.3 (14). Among normal tissues, MUC13 is expressed in the large intestine, trachea, kidney, small intestine, and gastric epithelium. In malignancy, MUC13 mRNA has been detected in colorectal, esophageal, gastric, pancreatic, and lung cancers (15). Recent studies have shown aberrant expression of MUC13 in gastric cancer (16, 17). The full-length MUC13 cDNA (~1.5 kb) was cloned recently, and the deduced amino acid sequence and predicted apoprotein structure suggest the presence of three epidermal growth factor (EGF)-like domains, a large 151–amino acid tandem repeat domain (TD) and a sea urchin sperm protein enterokinase arginine (SEA) domain within the extracellular component, a short transmembrane domain, and a 69–amino acid cytoplasmic domain (CD; refs. 14, 17). It has also been suggested that, within the CD of MUC13, there are several potential phosphorylation sites (eight serine and two tyrosine residues) and a protein kinase C consensus phosphorylation motif. The most common structural feature of all the mucins is the presence of a TD that provides a scaffold on which cells build oligosaccharide structures (6). The overexpression of the membrane-bound mucins can greatly affect cell-cell adhesion properties. The ectodomain of mucins may protrude >200 to 2,000 nm above the cell surface and can block the interaction (6, 18) and regulation of cell adhesion molecules in cancer cells (19–21).

In the present study, we have shown aberrant expression and localization of MUC13 mucin in ovarian cancer samples by immunohistochemical analyses using a recently generated anti-MUC13 monoclonal antibody (mAb; ref. 17). Additionally, we have elucidated the functions of MUC13 in ovarian cancer. Our results show the overexpression of MUC13 in ovarian cancer samples. The
exogenous expression of MUC13 induces remarkable changes in cell morphology while increasing cellular motility, cell proliferation, and tumorigenesis in SKOV-3 cells. These results show, for the first time, the direct association of MUC13 with ovarian cancer and that its overexpression influences cellular characteristics.

Materials and Methods

Tissue specimens. The tissue microarray (TMA) slides containing malignant and nonneoplastic ovarian tissues (n = 94) were procured from AccuMax Array (ISU Abaxis Co., Ltd.). Additionally, 56 archived ovarian cancer specimens were procured from Sanford Health. The utilization of archived cancer samples used in this study was approved by the University of South Dakota Institutional Review Board.

Immunohistochemistry. The TMA slides were stained with antihuman MUC13 mAb (17) by heat-induced epitope retrieval immunohistochemistry technique using a Vector ABC kit (Vector Laboratories), as described earlier (13, 22). Briefly, the TMA slides were deparaffinized, rehydrated, and incubated in 0.3% H2O2. After antigen retrieval, slides were incubated in Vectastain normal serum. The slides were then probed with anti-MUC13 mAb (clone PPZ0020). After washing with PBS containing 0.05% Tween-20 (PBS-T), slides were incubated with secondary antibody and washed again with PBS-T before incubation with ABC solution. The immunohistochemical reaction color was developed by treating the tissue sections with 3,3-diaminobenzidine (DAB) substrate (DAB substrate kit, Vector Laboratories). The slides were washed, counterstained, dehydrated, and mounted with VectaMount permanent mounting media (Vector Laboratories). All slides were analyzed using an Olympus BX 41 Microscope (Olympus Corporation). The intensity of immunoreactivity of the MUC13 was scored by a pathologist (M.K.), as described earlier (13, 22).

Human cell culture, transfection procedure, antibodies, and reagents. Human ovarian cancer cells were cultured in growth medium (SKOV-3/McCOY’S 5A/Ham’s F12, OVCAR-3/RPMI 1640, CaOV-3, PA1, OMC-3/DMEM/Ham’s F12) supplemented with 10% FCS (HyClone Laboratories). The human MUC13 cloned in pcDNA3.1 (17) or the empty vector was transfected in SKOV-3 cells by using Lipofectamine (Invitrogen). Stable clones were selected in a medium containing (200 μg/mL) G418 (Invitrogen). Scrambled control siRNA (siSC) and smart pool siRNA targets for MUC13, p38 mitogen-activated protein kinase (MAPK), and c-Jun NH2-terminal kinase antibodies were from Santa Cruz Biotechnology, Inc., and expressed in cells as per the manufacturer’s procedure. Anti-JNK1, anti-JNK2, and anti-p38 MAPK mouse mAbs and anti-HER2 rabbit polyclonal antibodies were from Santa Cruz Biotechnology, Inc. Anti-p21-activated kinase 1 (PAK1) and β-actin mAbs were from Cell Signaling Technology and Sigma, respectively. Chemical inhibitors SB203580, SP600125, LY294002, and SU6656 (Calbiochem) were used at 10 μM/L concentration.

RNA isolation and reverse transcription–PCR. Total RNA was isolated from the ovarian cancer cell lines by using RNA isolation kit (Qiagen, Inc.). RNA samples (2 μg) were reverse transcribed with the Superscript II RNase H-Reverse Transcriptase System (Invitrogen) using 5 pmol oligo-dT and 250 μM/L deoxynucleotide triphosphate (dNTP). The resulting cDNA samples were subjected to amplification using 10 pmol of gene MUC13-specific and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)—specific primers (MUC13 forward CTTCCGGTGTTGATTATTTGCG, reverse-GCATCGTGCTTCTCCTGGAG and GAPDH forward TGAAAGTCGGAGCTA-ACGTTTATG, reverse CATGTTGGGCGATTAGTTCCAC) in a final volume of 50 μL containing 250 μM/L dNTP, 1.5 mM/L MgCl2, and 1.25 units of Taq DNA polymerase (MasterMix, Eppendorf). PCR cycling parameters included initial denaturation at 94°C for 4 min, followed by 35 cycles (30 cycles for GAPDH) of denaturation at 94°C for 60 s, annealing at 58°C for 60 s, and extension at 72°C for 2 min, followed by a final extension at 72°C for 10 min. PCR products were resolved on a 1% agarose gel.

Confocal microscopy. Cells were grown on sterilized coverslips for 20 h and processed for confocal microscopy, as described earlier (23, 24). Briefly, cells were fixed and blocked by 10% goat serum containing 0.05% Tween-20 for 30 min. Cells were incubated with the anti-MUC13 mAb, washed, and then incubated with FITC-conjugated goat anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.). After washing, cells were mounted in Vectashield mounting medium (Vector Laboratories). Laser confocal microscopy was performed by using an Olympus Fluoview FV 1000 confocal laser microscope (Olympus Corporation). Photomicrographs of MUC13 staining were taken using the green channel. For the filamentous actin (F-actin) staining, cells were fixed using 3.75% formaldehyde-PBS solution, and permeabilized with PBS-T. Cells were blocked and incubated with rhodamine (TRITC)-conjugated phalloidin (1:500; Molecular Probes, Inc.) for 1 h. Cells were washed and mounted on glass slides in an antifade Vectashield mounting medium. F-actin staining was analyzed and photographed under an Olympus Fluoview FV 1000 Laser confocal microscope using the red channel.

Detergent extraction, SDS-PAGE, and immunoblot analyses. Monolayers were washed with PBS and extracted on ice with radioimmunoprecipitation buffer. The protein concentration was determined using a Bio-Rad kit, resolved on SDS-PAGE, and immunoblotted, as described earlier (23–25).

Aggregation assay. Aggregation assays were done as described earlier (26). In brief, cells were trypsinized and resuspended at 2.5 × 106/mL in the appropriate medium containing 10% fetal bovine serum (FBS). Medium containing 5,000 cells (25 μL) were pipetted onto the inner surface of the lid of a Petri dish. The lid was then placed on the Petri dish so that the drops were hanging from the lid with the cells suspended within them. To avoid evaporation, the 10-mL serum-free culture medium was placed in the bottom of the Petri dish. After overnight incubation at 37°C, the lid of the Petri dish was inverted, pipetted to disrupt the loose aggregates, and photographed using a phase-contrast microscope.

Cell migration assay. Cellular motility was determined by an agarose bead–based cell motility assay. Cells (1.0 × 105) were trypsinized and mixed into a 0.2% low melting point agarose solution. About 30 μL of cells/agarose suspension was then plated onto fibronectin/bovine serum albumin (BSA)—coated six-well plates. The agarose-beaded plates were placed at 4°C for 7 min to set the agarose beads. Complete growth medium was added to the wells so that the beads were covered, and then the plates were placed into a cell culture incubator at 34°C with 5% CO2. At 24, 48, 72, and 96 h, the plates were removed and photographed under a phase-contrast microscope.

Cell proliferation assay. Cells (2.0 × 105) were seeded on six-well plates in a 2-mL growth medium. At 24, 48, 72, and 96 h, cells were trypsinized and counted using an automated cell counter (Coulter Particle Counter, Beckman Coulter). Doubling time (Td) of cells was calculated from the growth rate during the exponential growth phase (0–96 h) using the formula, Td = 0.693/t (lnN0/Nt), wherein t is time in days, N0 is cell number at time t, and Nt is cell number at initial time (25).

Flow cytometry analyses. Cells (5 × 104) were grown on 25 cm2 dishes for 48 h. Cells were trypsinized, centrifuged, and washed in PBS. After fixing with cold ethanol for 15 min, cells were stained with Telford reagent at 4°C overnight. Flow cytometry analyses were performed using the FACS Vantage Flow Cytometer (BD Bioscience), and data from at least 10,000 cells were collected using ModFit LT version 2.0 for cell cycle analysis.

In vivo tumor xenograft study. Six-week-old athymic nude (nu/nu) mice (Charles River Laboratories) were maintained in pathogen-free conditions. Mouse studies were carried out following procedures approved by the University of South Dakota Institutional Animal Care and Use Committee. For in vivo tumorigenesis, SKOV-3 clones were mixed with Matrigel (BD Bioscience) and injected (5 × 105 cells per mouse in 200 μL) s.c. into the left flank and tumors were allowed to develop. On day 20 after injection, tumor volume was calculated by digital Vernier caliper measurements according to the ellipsoid volume formula, tumor volume (mm3) = π/6 × L × W2, wherein L is length, W is width, and H is height (in millimeters).

Statistical analysis. Statistical analysis was performed using SPSS Version 10.0 for Windows. A paired t test and χ2 test were used to analyze the MUC13 staining in ovarian cancer samples and functional assay data. P values of <0.05 were considered significant.
Results

Immunostaining profile of MUC13 in normal/benign ovary and ovarian cancer samples. The overexpression of mucins is known to occur in ovarian cancer (12, 13). However, the expression profile of MUC13 is not understood. We examined the expression pattern of MUC13 in normal/benign ovary and ovarian cancer tissue samples by immunohistochemistry. MUC13 expression was undetectable in normal and benign ovarian samples, whereas ovarian cancer samples showed a significantly higher MUC13 expression (Fig. 1A). MUC13 was predominantly localized on the apical membrane (Fig. 1A, c) in the majority of cases with cytoplasmic localization (Fig. 1A, d) in some cases. We further expanded our MUC13 expression study to 56 additional clinically confirmed epithelial ovarian cancer (EOC) and 10 benign/normal ovarian samples. In this study, we observed overexpression of MUC13 in 66.0% of EOC samples (Fig. 1B). Control immunoglobulin did not show any immunoreactivity (Supplementary Fig. S1B and D). To determine the correlation of MUC13 expression with cancer type and cancer stage, all cancer samples were grouped into histologic types (serous, mucinous, clear cell, Brenners, and endometroid) and early and advanced stage samples. MUC13 expression was significantly ($P < 0.05$) higher in mucinous and Brenners type of samples compared with other histologic types of ovarian cancer samples and adjacent normal ovary samples. The correlation between MUC13 expression and cancer stage was not found to be significant ($P > 0.05$).

MUC13 expression in ovarian cancer cell lines. The expression of MUC13 was investigated in a panel of five cell lines at the mRNA level by reverse transcription–PCR (RT-PCR) to select suitable cell lines for functional assays. Of these cell lines, CaOV-3 and OMC-3 cells showed a high-MUC13 expression and PA-1 showed a faint expression. SKOV-3 and OVCAR-3 cells did not show a detectable MUC13 expression (Fig. 2A, top). SKOV-3 cell line, therefore, was selected for exogenous expression of MUC13 to determine the MUC13 functions.

Exogenous expression of MUC13 in SKOV-3 cells. To investigate MUC13 functions, full-length MUC13 was exogenously expressed in SKOV-3 cells.
expressed in SKOV-3 cells via stable MUC13F-pcDNA3.1 plasmid transfection. Stable MUC13-expressing clones were obtained by G418 antibiotic selection. Four clones showed stable MUC13 expression, namely, SKOV-3-M13A, SKOV-3-M13C, SKOV-3-M13H, and SKOV-3-M13E (Fig. 2A, bottom). The expression of MUC13 was further examined by confocal microscopy to select homogeneous MUC13 expressing clone(s). To analyze the effect of MUC13 expression, we selected one high-MUC13–expressing clone (SKOV-3-M13A) and one low-MUC13–expressing clone (SKOV-3-M13E). In these clones, >90% of cells showed MUC13 expression (Fig. 2A, bottom and B, d and f). The SKOV-3-V6 vector-only clone (a mixture of two pooled clones) did not show expression of MUC13 (Fig. 2B, b) and was used as a control.

**Exogenous expression of MUC13 alters morphologic characteristics in SKOV-3 cells through JNK pathway.** The effect of MUC13 expression on cellular morphology was investigated by phase-contrast microscopy in SKOV-3 cells. The MUC13-expressing cells (SKOV-3-M13A and SKOV-3-M13E) exhibited scattering of cells and a marked increase in the numbers of actin-containing projections, such as lamellipodia, filopodia, and microspikes (Fig. 3A, b and c) compared with the vector-transfected (SKOV-3-V6) cells (Fig. 3A, a). The SKOV-3-V6 cells showed a growth pattern with close cell-cell contacts (Fig. 3A, a), whereas SKOV-3-M13A and SKOV-3-M13E cells showed fewer cell-cell contacts (Fig. 3A, b and c). To determine the signaling pathways involved in MUC13-induced alterations in cellular morphology, we examined potential pathways using inhibitors of the p38 MAPK (SB203580), the JNK (SP600125), the phosphatidylinositol 3-kinase (PI3K; LY294002), and the src family kinases (SU6656) pathway. Among all, the JNK inhibitor (SP600125) was the only compound to effectively suppress the MUC13-induced alterations in cellular morphology and cell scattering (Fig. 3B, c). JNK inhibitor SP600125 suppresses the expression/activation of both JNK1 and JNK2 isoforms (27); therefore, we determined the expression of both JNK/phosphorylated JNK forms by Western blot analysis in our clones. This analysis showed that MUC13 expression induced up-regulation of the JNK2 isoform and phosphorylated JNK in SKOV-3-M13A and SKOV-3-M13E cells (Fig. 3C). To further confirm the role of JNK2 in MUC13-induced alterations in cellular morphology and cell scattering, we expressed JNK2 siRNA in SKOV-3-M13A cells, which effectively inhibited JNK2 expression (Fig. 3C). Knockdown of JNK2 prevented MUC13-induced changes in cellular morphology and cell scattering (Fig. 3C, b).

**MUC13 expression modulates cell-cell aggregation in SKOV-3 cells.** Formation of appropriate cell-cell contacts is a characteristic that critically affects the growth pattern of cultured cells. MUC13...
Figure 3. Phase-contrast micrographs to determine morphologic and cell-cell adhesion characteristics. A, cell morphology assay. Cells (1 × 10^6) were seeded in cell culture dishes and grown in growth media, and photographs were taken at ~70% confluence. MUC13-negative cells (SKOV-3-V6) grew as a compact colony (a). MUC13-expressing cells (b, c) grew with relatively more cellular projections and with scattered morphology. Magnifications, 100× (a–c). B, inhibitor assay. The JNK inhibitor SP600125 suppresses MUC13-induced cellular morphology and scattering in SKOV-3-M13A cells. MUC13-overexpressing SKOV-3-M13A cells were cultured in the presence of the vehicle DMSO (a), the p38 MAPK inhibitor SB203580 (10 μmol/L; b), the JNK inhibitor SP600125 (10 μmol/L; c), the PI3K inhibitor LY294002 (10 μmol/L; d), or the src family kinase SU6656 (10 μmol/L; e) pathway inhibitor for 48 h. Phase-contrast pictures were taken at 100×. C, immunoblot assay. The expression of JNK1 and JNK2 in MUC13-positive and MUC13-negative clones (left). Cells were extracted, and 20 μg protein were resolved by SDS-PAGE and immunoblotted for JNK1, JNK2, and β-actin. The effect of JNK2 siRNA on JNK2 expression and cellular morphology and scattering in SKOV-3-M13A cells (right). The SKOV-3-M13A cells treated with JNK2 siRNA or transfection media (TM) or scrambled control siRNA (siSC) were grown in a six-well culture dish for 48 h, and protein extracts were made, resolved by SADS-PAGE, and immunoblotted for JNK2 and β-actin (top). Phase-contrast pictures of the cells treated with siSC (a) or siJNK2 (b) were taken before protein extraction. Original magnifications, 100×. D, aggregation assay. Drops of media containing 5,000 cells were pipetted onto the inner surface of the lid of a Petri dish. The lid was then placed on the Petri dish to form hanging drops from the lid with cells suspended within it. After overnight incubation at 37°C, the lid of the Petri dish was inverted and cells were gently pipetted five times to break loose the cell-cell aggregates and then photographed under phase contrast microscope. MUC13-expressing cells exhibited reduced cell-cell aggregation with smaller and loose cell-cell aggregates (b, c) in contrast to larger and tight cell-cell aggregates in MUC13-negative cells (a). Original magnifications, 100×.
expression in SKOV-3 cells was correlated with morphologic changes, including dispersed growth pattern and loose cell-cell contacts. Therefore, cell aggregation characteristics were analyzed in MUC13-expressing and MUC13-nonexpressing SKOV-3 derived sublines. Both MUC13-expressing clones (SKOV-3-M13A and SKOV-3-M13E) showed smaller cell aggregates (Fig. 3D, b and c), whereas the vector control SKOV-3-V6 cells exhibited larger cell aggregates (Fig. 3D, a). These results show an inhibitory effect of MUC13 on cell-cell aggregation. The inhibition in cell-cell aggregation caused by MUC13 in SKOV-3 cells may be due to the presence of a heavily glycosylated extracellular domain and/or interference of the MUC13 with the cell adhesion molecules.

MUC13 expression increases cellular migration and induces F-actin remodeling via up-regulation of HER2 and PAK1. The enhanced cellular migration ability is required for a cancer cell to become competent for metastasis. Because the morphologic changes induced by MUC13 involve the structures that are important for cell movement, cellular motility was assayed in

Figure 4. Cellular migration and F-actin staining in SKOV-3-derived MUC13-expressing and MUC13 null cells. A, cellular migration assay. SKOV-3-derived clones were mixed into agarose solution and dropped onto fibronectin/BSA-coated plates. MUC13-expressing cells (SKOV-3-M13A and SKOV-3-M13E) showed an increased cellular migration (d–i) compared with the MUC13-negative (SKOV-3-V6) cells (a–c). Black arrows and lines indicate migrated cells. AB, agarose bead; MC, migratory cells. Original magnification, 100×. B, quantitative analysis of cell migration assays. The cells migrated from agarose beads were counted in five representative photographs and compared. Columns, mean; bars, SE. n = 5. * P < 0.05. C, F-actin staining. Cells were grown on glass coverslips for 18 h. After washing with PBS, fixing in 3.75% formaldehyde-PBS solution, and blocking, cells were incubated with rhodamine (TRITC)-conjugated phalloidin (1:500) for 1 h. Stained cells were observed and photographed under an Olympus Fluoview FV 1000 confocal laser microscope. MUC13 null cells (SKOV-3-V6) exhibited F-actin staining throughout the cells (top). In contrast, in MUC13-expressing cells (SKOV-3-M13A and SKOV-3-M13E), F-actin staining was predominantly localized on the cell membranes and leading edges of the cells (middle and bottom). Original magnification, 400×. D, immunoblot assay. HER2, PAK1, and p38 expression in MUC13 null (SKOV-3-V6) and in MUC13-expressing (SKOV-3-M13A and SKOV-3-M13E) cells was determined. Cells were extracted, and 20 μg protein were resolved by SDS-PAGE and immunoblotted for HER2, PAK1, p38, and β-actin.
SKOV-3 transfectants. Cellular motility was analyzed by using agarose bead assays. MUC13-expressing clones (SKOV-3-M13A and SKOV-3-M13E) showed a higher cellular motility compared with the vector control SKOV-3-V6 cells (Fig. 4A and B). There was a significant difference in the cellular motility of high-MUC13–expressing (SKOV-3-M13A) and MUC13-nonexpressing (SKOV-3-V6) cells. The low-MUC13–expressing cell line, SKOV-3-M13E, had a relatively lower cellular motility compared with the high-expressing cell line SKOV-3-M13A. However, the difference between these two MUC13-expressing clones was not significant (Fig. 4A and B).

In general, cellular motility is dependent on localized actin polymerization at the leading edge of the cells. Polymerization of globular actin leads to the formation of long fibrous molecules, F-actin. In eukaryotic cells, cell migration requires the formation of cell membrane extensions containing actin filaments (28). Because overexpression of MUC13 in SKOV-3 cells caused a marked increase in the cellular motility, we sought to analyze the alterations in the pattern and distribution of the F-actin in MUC13-expressing cells. In MUC13-expressing clones (SKOV-3-M13A and SKOV-3-M13E), F-actin staining was predominantly localized in the cellular outgrowth and projections. In contrast, in vector control SKOV-3-V6 cells, F-actin staining was observed throughout the cytoplasm (Fig. 4C). These results suggest that MUC13 can modulate cellular dynamics by reorganization of the actin-cytoskeleton. The role of HER2 and PAK1 has been suggested in cytoskeleton reorganization and cell migration (29–32). We, therefore, determined the expression of HER2, PAK1, and p38 MAPK in our clones. Our immunoblot analysis showed a marked up-regulation of HER2, PAK1, and p38 in MUC13-expressing cells compared with the MUC13 null cells (Fig. 4D). These data suggest the involvement of HER2, PAK1, and p38 in MUC13-induced cellular motility.

**Exogenous expression of MUC13 enhances cellular proliferation and tumorigenesis in nude mice.** Enhanced plating efficiency and cell proliferation are measures of higher metastatic and tumorigenic potential of cancer cells. Highly metastatic and aggressive cancer cells, in general, have higher colony-forming and proliferative capabilities compared with less metastatic and normal cells. Therefore, the effect of MUC13 expression on colony formation and cell proliferation abilities was investigated. The colonic ability of SKOV-3–derived sublines was assessed by seeding 1,000 cells in a 60-mm cell culture dish and counting the number of colonies formed in 2 weeks. The expression of MUC13 significantly ($P < 0.05$) increased the colony-forming ability in SKOV-3-M13A (79.1%) and SKOV-3-M13E (68.2%) cells compared with vector-only SKOV-3-V6 (50.2%) cells (Fig. 5A).

The cell proliferation ability of these cells was assayed by cell counting using a Coulter Counter. When the cell numbers were compared at 96 h, SKOV-3-M13A and SKOV-3-M13E cells showed a 60% and 47% increase, respectively, in cell proliferation compared with SKOV-3-V6 vector-only cells (Fig. 5B). Additionally, cell doubling times were calculated during the exponential phase. Both SKOV-3-M13A and SKOV-3-M13E cells displayed significantly ($P < 0.05$) lower cell doubling times of 29 and 30 h, respectively, compared with 36 h for vector control cells (Fig. 5C). In cell cycle analyses, SKOV-3-M13A and SKOV-3-M13E clones showed an increased number of cells in S phase compared with the SKOV-3-V6 control cells (Supplementary Fig. S2). To determine the signaling pathways involved in the increase in MUC13-induced cellular proliferation, we examined potential pathways using inhibitors of the p38 MAPK, the JNK, the PI3K, and the src family kinases pathway. Of these inhibitors, the p38 MAPK inhibitor (SB203580) was the only compound to effectively repress the MUC13-induced cellular proliferation in SKOV-3-M13A cells (Fig. 5D). A marked inhibition in cell proliferation was also noticed upon suppression of p38 MAPK expression by p38 MAPK siRNA (Supplementary Fig. S3). To determine if MUC13 enhances tumorigenesis in vivo, MUC13-expressing and MUC13-nonexpressing cells were injected s.c. into the flank of athymic nude mice. SKOV-3 cells expressing MUC13 formed significantly larger tumors than MUC13 null SKOV-3 cells (Fig. 6B). To further confirm the role of MUC13 in cellular proliferation, we expressed MUC13 siRNA in MUC13-positive ovarian cancer cell lines, OMC-3 and CaOV-3, which effectively inhibited MUC13 expression (Fig. 6B and Supplementary Fig. S4). Knockdown of the MUC13 significantly ($P < 0.05$) suppressed cellular proliferation in OMC-3 and CaOV-3 cells (Fig. 6C). These data suggest the role of MUC13 in cell proliferation and tumorigenesis.

**Discussion**

In spite of making considerable progress in cancer research, the mortality rate of ovarian cancer has remained unchanged in the last several decades (3). This is primarily due to (a) the lack of a sensitive screening method for early-stage disease diagnosis and (b) poor treatment outcomes at advanced stages when the majority (>70%) of ovarian cancer patients are diagnosed (33). These facts accentuate the urgent need for the identification of novel molecular markers for early diagnosis of ovarian cancer. These newly identified molecular markers can be used alone or in combination with conventional diagnostic methods to achieve greater sensitivity without compromising specificity. Herein, we have investigated the expression profile of a newly identified mucin, MUC13, in ovarian cancer by immunohistochemistry using a recently developed anti-MUC13 mAb (17). Initially, TMA slides were used for immunohistochemical analyses to determine the overall expression profile of MUC13 in various types of ovarian cancers and in normal ovary. TMA is a powerful tool to investigate the expression profile of a protein using consistent immunohistochemical conditions on a large number of samples. Our TMA analyses show the expression of MUC13 specifically in EOC samples with no expression in normal ovary, including ovarian surface epithelium (OSE; Fig. 1). In addition to normal ovary, the expression of MUC13 was not detectable in benign and non-epithelial types of ovarian carcinomas. The TMA study was further expanded in a set of EOC samples obtained from Sanford Health. Overall, our immunohistochemical analyses show aberrant expression of MUC13 in EOC being maximally (100%) expressed in mucinous types of samples, in which CA 125, a currently used ovarian cancer marker, has least sensitivity. These data suggest that a combined panel of MUC13 and CA 125 will significantly improve the sensitivity of CA 125 for ovarian cancer screening procedures. Domain structure analyses show the presence of a cleavage site in the SEA domain of MUC13 (14). These data and our extracellular luminal staining data suggest that MUC13 may be released into the secretions and/or blood after shedding from the ovarian cancer cells. Therefore, there is a strong possibility that a MUC13 serum immunoassay can be developed as a screening method or diagnostic indicator of ovarian cancer. Additionally, an intense membrane-bound staining also suggests that MUC13 may be an excellent target for antibody-guided therapy of ovarian cancer.
Other studies have also described the deregulated expression of MUC13 in gastric cancer tissues and gastric cancer cell lines (16, 17).

In addition to the identification of novel biomarkers for early diagnosis, a better understanding of the molecular mechanisms involved in ovarian cancer progression is desirable for developing effective therapeutic modalities. To investigate the role of MUC13 in ovarian cancer, we used a full-length MUC13 construct, which has all the elements of the MUC13 gene. The expression of MUC13 in SKOV-3 ovarian cancer cells resulted in marked changes in the cell morphology with scattering of cells. When we investigated signaling downstream of MUC13-induced morphologic changes, the JNK inhibitor completely prevented MUC13-induced cell morphology and scattering. Knowing this involvement of the JNK pathway in MUC13-induced morphologic alterations, we determined the expression of JNK1 and JNK2 isoforms in our clones. In MUC13-expressing clones, we observed up-regulation of JNK2/ phosphorlated JNK, and JNK2 knockdown by siJNK2 prevented morphologic changes and cell scattering. Thus, it seems that MUC13-induced morphologic changes are mediated through the JNK2 pathway. In addition, MUC13 expression reduced cell-cell aggregation with a remarkable increase in cellular motility and induced F-actin reorganization. For migration, a cell requires the formation of cell membrane protrusions containing actin filaments with a continuous process of actin polymerization near the leading edges (28). Because the expression of HER2 and PAK1 has been associated with cytoskeleton reorganization and cell migration (29–31), we examined their expression in our clones. The up-regulation of HER2 and PAK1 in MUC13-expressing clones suggests that MUC13 induces cytoskeleton reorganization and enhanced cellular motility via altered HER2 and PAK1 expression. These observations show the direct association of MUC13 with increased cellular motility.

The human MUC13 mucin belongs to the subfamily of mucin genes encoding transmembrane mucins (6, 8). The aberrant expression of transmembrane mucins like MUC1, MUC4, and...
MUC13 in cancer cells over the entire cell surface may promote metastasis via the antiadhesive effects of the extracellular TD, which is usually heavily glycosylated (6). Aberrant expression of MUC13 in ovarian cancer was investigated by our immunohistochemical analysis. The results of our cell aggregation assays clearly suggested an antiadhesive function of MUC13 in ovarian cancer. MUC13 usually localized in the apical region of the cells, but its localization was also observed in the cytoplasm, including the basal region close to the basement membrane in some cases (Fig. 1A, c). Localization of MUC13 in the basal region and near the basement membrane might facilitate the detachment of the ovarian cancer cells from the primary site, as well as invasion of the tumor cells into the ovarian stromal tissue. Studies on rat SMC and human MUC4, which are also transmembrane mucins, showed that their overexpression reduced cell-matrix and cell-cell adhesion and potentiated tumor growth and metastasis in rat mammary carcinoma cells (34, 35) and pancreatic cancer cells (25), respectively.

The domain structure of MUC13 has been deduced from a full-length cDNA sequence (14, 17). The NH₂ terminus of the MUC13 protein is composed of a tandem repeat mucin domain. The COOH terminus of MUC13 consists of a transmembrane domain with three EGF-like domains, suggesting that MUC13 may play an important role as a signaling molecule (14, 36). With three EGF domains, MUC13 may potentially interact with EGF receptors (EGFR), such as HER2, which could modulate EGFR signaling pathways. It is conceivable that this interaction may facilitate ovarian cancer cell proliferation through altered cellular signaling mediated by tyrosine phosphorylation of EGFRs. Therefore, we sought to determine the proliferative characteristics of MUC13 transfectants to investigate the role of MUC13 in ovarian cancer development and progression. Our MUC13-expressing cells displayed higher colony-forming efficiency and cell proliferation with a reduced cell doubling time compared with vector control cells. These cellular changes were correlated with increased number of cells in S phase of the cell cycle. When we investigated signaling downstream of MUC13-induced cellular proliferation, the p38 MAPK inhibitor and siRNA prevented MUC13-induced cell proliferation. These data suggest that MUC13-induced cell proliferation is mediated through p38 MAPK pathway. The utilization of xenograft mouse model is a logical approach for...
tumorigenesis studies. Therefore, to determine the role of MUC13 in tumorigenesis, we implanted SKOV-3 clones s.c. into the athymic nude mice and monitored the tumor growth. In our in vivo mouse model study, MUC13 expression significantly increased tumor volume. In addition to exogenous gene expression, gene silencing is an alternative approach to investigate the biological functions of a gene. To further confirm the role of MUC13 in ovarian cancer, MUC13 expression was knocked down in SKOV-3-overexpressing ovarian cancer cell lines, OMC-3 and CaOV-3, by using siRNA technology. Knockdown of MUC13 expression markedly reduced cell proliferation in OMC-3 and CaOV-3 cells. These results provide strong evidence that MUC13 is associated with ovarian tumorigenesis.

In our present study, we observed potentiation of cell growth by exogenous MUC13 expression and up-regulation of HER2, PK1, p38, and JNK, so there is the possibility that MUC13 expression modulates the expression or stabilization of EGFRs in ovarian cancer cells. It is a subject of investigation whether MUC13 can influence ErbB2/HER2 translocation and phosphorylation in ovarian tumor cells. In addition to EGFR signaling, under these circumstances, HER2 can also bind β-catenin and potentially titrate it away from E-cadherin, resulting in a loss of cell-cell adhesion and an increase in the motile invasive behavior of the SKOV-3 cells. Exogenous overexpression of HER2 and its activation has been correlated with increased invasiveness and cellular motility (32, 37). These observations are helpful for predicting how exogenous MUC13 expression in SKOV-3 ovarian cancer cells may reduce cell-cell aggregation and increase cellular proliferation and motility. Further investigations on the MUC13 mucin are required to determine its role as a potential diagnostic marker for ovarian cancer screening and to explain its precise molecular mechanism of action in cancer progression/pathogenesis. However, considering the observations of the present study, we propose that MUC13 is overexpressed in ovarian cancer and induces alterations in cellular morphology, motility, and proliferation through modulation of JNK, PAK1, and p38 MAPK signaling pathways. This work is the first demonstration of the direct association of MUC13 with ovarian cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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