Loss of Betaglycan Expression in Ovarian Cancer: Role in Motility and Invasion

Nadine Hempel,[1] Tam How,[1] Mei Dong,[1] Susan K. Murphy,[2]
Timothy A. Fields,[3] and Gerard C. Blobe[1,4]

Departments of [1]Medicine, [2]Obstetrics and Gynecology, [3]Pathology, and [4]Pharmacology and Cancer Biology,
Duke University Medical Center, Durham, North Carolina

Abstract

The transforming growth factor-β (TGF-β) superfamily members, TGF-β, activin, and inhibin, all have prominent roles in regulating normal ovarian function. Betaglycan, or the type III TGF-β receptor, is a coreceptor that regulates TGF-β, activin, and inhibin signaling. Here, we show that betaglycan expression is frequently decreased or lost in epithelial derived ovarian cancer at both the mRNA and protein level, with the degree of loss correlating with tumor grade. Treatment of ovarian cancer cell lines with the methyltransferase inhibitor 5-aza-2-deoxy-cytidine and the histone deacetylase inhibitor trichostatin A resulted in significant synergistic induction of betaglycan message levels and increased betaglycan protein expression, indicating that epigenetic silencing may play a role in the loss of betaglycan expression observed in ovarian cancer. Although restoring betaglycan expression in Ovca429 ovarian cancer cells is not sufficient to restore TGF-β–mediated inhibition of proliferation, betaglycan significantly inhibits ovarian cancer cell motility and invasiveness. Furthermore, betaglycan specifically enhances the antimigratory effects of inhibin and the ability of inhibin to repress matrix metalloproteinase levels in these cells. These results show, for the first time, epigenetic regulation of betaglycan expression in ovarian cancer, and a novel role for betaglycan in regulating ovarian cancer motility and invasiveness. [Cancer Res 2007;67(11):5231–8]

Introduction

Ovarian cancer is the most lethal of all gynecologic cancers and the fifth leading cause of cancer death among women in the United States, with ∼ 22,000 new cases and 15,000 deaths reported annually (1). Without effective screening tests or early symptoms, most ovarian cancer patients are diagnosed with metastatic disease (1). Although effective local/regional approaches yield a 69% 5-year survival rate for patients with regional disease, the aggressive nature of this disease and a deficiency in effective treatments for the patients who present with metastatic disease results in a 30% 5-year survival rate for these patients (1). Investigation of the cellular origins and the early molecular events resulting in ovarian cancer have been hampered by the lack of identifiable precursor lesions, underscoring the need for further understanding of ovarian cancer disease progression.

Inhibin and activin are gonadal expressed members of the transforming growth factor-β (TGF-β) superfamily of cytokines that play an important role in modulating ovarian function. In particular, they regulate the synthesis of pituitary follicle-stimulating hormone (FSH), with activin stimulating its secretion and inhibin opposing this effect (2). Activin exerts its action by binding to the activin type II serine/threonine kinase receptor, ActRIIA or B, which then associates with and phosphorylates the type I receptor ActRIA (Alk4) or ActRIB (Alk2; refs. 3–6). This activates the ActRI serine/threonine kinase, which, in turn, phosphorylates and activates the Smad2 or 3 transcription factors. Smad2/3 bind Smad4 and translocate to the nucleus to regulate gene transcription, including the FSH promoter (7). Inhibin is thought to elicit its antagonistic action on activin by displacing its binding from ActRII, resulting in an inability of ActRII to dimerize with ActRI (8). To antagonize activin and inhibit its interaction with ActRII, inhibin requires binding to the ubiquitously expressed membrane-bound TGF-β superfamily coreceptor, betaglycan, also known as the type III TGF-β receptor (9).

In addition to binding inhibin, betaglycan has high affinity for all three TGF-β isoforms (9–11). Because betaglycan lacks a functional kinase domain, it has traditionally been characterized as a coreceptor, presenting ligand to the type II TGF-β receptor (TβRII), thereby enhancing TGF-β signaling (11). Betaglycan is particularly necessary for TGF-β2 binding and signaling because TβRII alone has very low affinity for this ligand (11–15). However, recent evidence suggests that the function of betaglycan may be more complex. Functionally, betaglycan can associate with β-arrestin 2 via its cytoplasmic domain following phosphorylation by TβRII, resulting in receptor complex internalization and down-regulation of TGF-β signaling (14). The cytoplasmic domain of betaglycan may also contribute to p38 signaling independent of TGF-β ligand stimulation (16). The importance of betaglycan is further highlighted by the embryonic lethality of betaglycan knock-out mice, with the embryos dying at day 16.6 due to heart and liver defects (17).

Although TGF-β functions as a regulator of epithelial homeostasis, by inhibiting proliferation and inducing differentiation or apoptosis, TGF-β exerts a dichotomous tumor suppressor/tumor-promoting role in cancer (18). Early during cancer progression, cancers derived from epithelial tissue develop resistance to these homeostatic effects, defining a tumor suppressor role for the pathway. However, at later stages, TGF-β signaling initiates cellular events that are advantageous to cancer progression, including promoting migration, invasion, epithelial to mesenchymal transition, and angiogenesis (19). In a breast cancer model, we recently showed that betaglycan can inhibit invasion and metastasis both *in vitro* and *in vivo* (20).

The role of the TGF-β ligands in ovarian cancer progression is poorly understood. Many ovarian cancer cells are unresponsive to TGF-β–mediated inhibition of proliferation, whereas the main

components of the signaling pathway, including the type I and II receptors and the Smads, are largely intact in human ovarian cancer cells (21–26). However, although many ovarian cancer cells seem to be unresponsive to TGF-β–mediated inhibition of proliferation, TGF-β can still stimulate invasion, suggesting that some TGF-β responsiveness remains (27). Although TGF-β levels increase significantly in primary and recurring ovarian cancer lesions compared with the normal ovary (21), the role of TGF-β ligands in ovarian cancer metastasis has not been extensively investigated.

Here, we investigate the role of betaglycan, a major inhibin and TGF-β coreceptor, in ovarian cancer.

Materials and Methods

Expression analysis on cDNA filter array. A cancer profiling filter array containing normalized cDNA from 14 ovarian cancers and corresponding normal tissues (Cancer Profiling Array I, Clontech) was probed with [^{32}P]-labeled cDNA probes for betaglycan following manufacturer's recommendations. The betaglycan cDNA probe was PCR amplified using the sense primer, 5′-GTAGTGGGTTGGCCAGATGGT-3′, and antisense primer, 5′-CTGCTGTCTCCCCTGTGTG-3′. Purified PCR products (25 ng) were labeled by random primed DNA labeling using [α-^{32}P]dCTP following the manufacturer's protocol (Roche Applied Science). Labeled cDNA probe was purified on Chroma Spin+STE-100 column (Clontech). Images were acquired using a phosphorimager, and subsequent data analysis was done using NIH Image J software. The densitometry units for the betaglycan probed array (with background subtracted) were normalized to the densitometry units for a control ubiquitin probed array (with background subtracted). These normalized densitometry units were then expressed as a ratio (normal/tumor). A ratio (normal/tumor) of 2 or higher was considered significant.

Immunohistochemistry. Immunohistochemical studies were done on a Tissue Microarray containing 40 ovarian cancer specimen (Protein Biotechnologies) and paraffin-embedded normal ovarian tissue specimen provided by the Duke University Research Foundation Tissue Bank. Tissues were probed with purified antihuman betaglycan antibody raised in rabbit.

Immunoreactivity and specificity of this antibody for betaglycan has been previously verified in immunohistologic studies in our laboratory (20). Following rehydration and blocking with 1% hydrogen peroxide, 10% goat serum/PBS, and avidin/biotin blocking kit (Vector Laboratories), tissue samples were incubated overnight at 4°C with betaglycan-specific antibody or preimmune serum at a dilution of 1:200 to 1:400 in 10% goat serum/PBS. Following secondary antibody incubation with biotinylated anti-rabbit immunoglobulin G at room temperature for 1 h, tissues were incubated with Vectastain ABC reagent (Vector) for 30 min, and immunoreactivity was visualized using the avidin-biotin complex immunoperoxidase system and diaminobenzidine (Vector). Slides were counterstained with hematoxylin, and immunoreactivity for betaglycan in the specimen was scored by staining intensity in a blinded manner, with 0 indicating no or trace staining, 1 low, 2 medium, and 3 high staining intensity, with two independent observers (N.H. and T.F.).

Cell culture. Human ovarian carcinoma cell lines Ovcar3, Ovca420, Ovca429, Ovca433, DOV13, and the normal spontaneously immortalized ovarian epithelial cell line Nose007 were cultured in RPMI 1680 (Life Technologies Invitrogen), supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified incubator. Two stable Ovca429 cell lines overexpressing HA-tagged rat betaglycan (Ovca429-betaglycan) or empty neomycin-resistant vector (Ovca429-neo) were created by transfection using LipofectAMINE 2000 (Invitrogen) and selection with G418.

5-Aza-2-deoxycytidine and trichostatin A treatment. Ovarian cancer cells were seeded at a density of 1×10^5 cells per well in a six-well plate and treated the next day for 96 h with 10 μmol/L 5-aza-2′-deoxycytidine (5-aza-2dC; Sigma) and/or 0.5 to 1 μmol/L trichostatin A (TSA; Sigma) for 24 h, followed by RNA isolation.

Reverse transcription-PCR and betaglycan protein visualization. RNA was isolated using RNeasy Mini Kit (Qiagen) and Superscript II reverse transcriptase (Invitrogen) with random primers used to reverse transcribe 0.2 to 0.5 μg of total RNA. Reverse transcription-PCR (RT-PCR) was carried out using the following primers pairs: human type I TGF-β receptor (TβRI), sense (s) 5′-ACGGCGTTACAGTGTTCTG-3′, antisense (a/s) 5′-GGTGTGGC-AGATATAGACC-3′; human TβRII, s 5′-GCAGGTGGGAACTGCAAGAT-3′, a/s 5′-GAAGGACTCAACATTCTCCAAATTC-3′; and human betaglycan, s 5′-CTGTTCACCCGACCTGAAAT-3′, a/s 5′-CGTCAGGAGGCACACACTTA-3′. The following PCR conditions were used: 10 min at 95°C, followed by
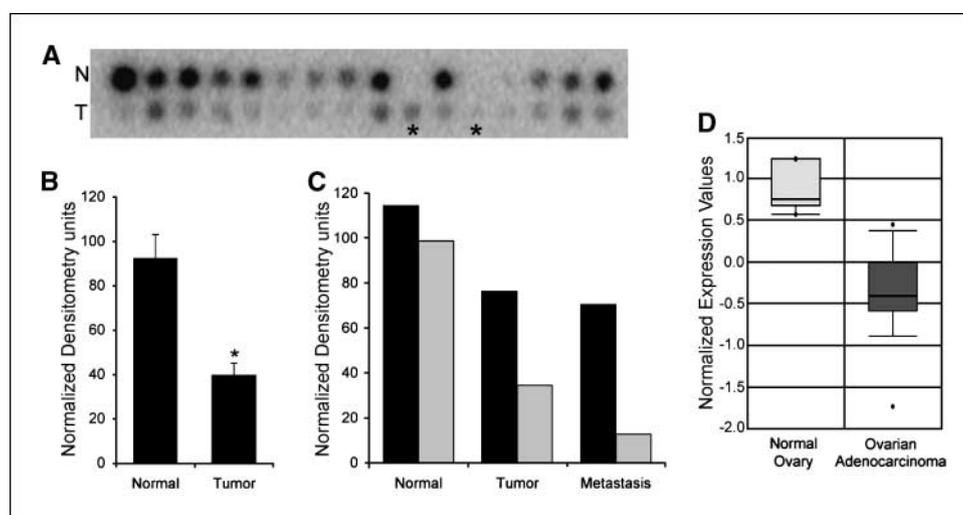

Figure 1. Loss of betaglycan expression at the mRNA level in human ovarian cancer. *A,* the Clontech cancer cDNA profiling array containing normal ovary cDNA (*N*) and matching tumor cDNA (*T*) spotted directly below was probed with a ^{32}P-labeled oligonucleotide probe directed against human betaglycan. Spots marked by asterisks represent cDNA from matching metastases of normal and primary tumors of patients immediately to their left. *B,* densitometry quantification of the cDNA Array indicating average densitometry ± SE, normalized to ubiquitin (*n* = 14); *, *P* < 0.0001, Student's *t* test. *C,* quantitative data from two matched normal, primary ovarian cancer, and metastatic ovarian cancer (marked by asterisks in Fig. 1*A*). *D,* data from a publicly available gene profiling study (28) was analyzed using the Oncomine gene microarray database (http://www.oncomine.org). This data set included mean betaglycan expression in normal ovary (*n* = 4) versus ovarian adenocarcinoma tissues (*n* = 28). *Boxes,* the interquartile range marking the 25th to 75th percentile; *whiskers,* 10th to 90th percent range, *points,* minimum and maximum; *bars,* median value. The difference between betaglycan expression in normal ovary and ovarian adenocarcinoma was significant (*P* < 0.001, independent two-tailed *t* test).
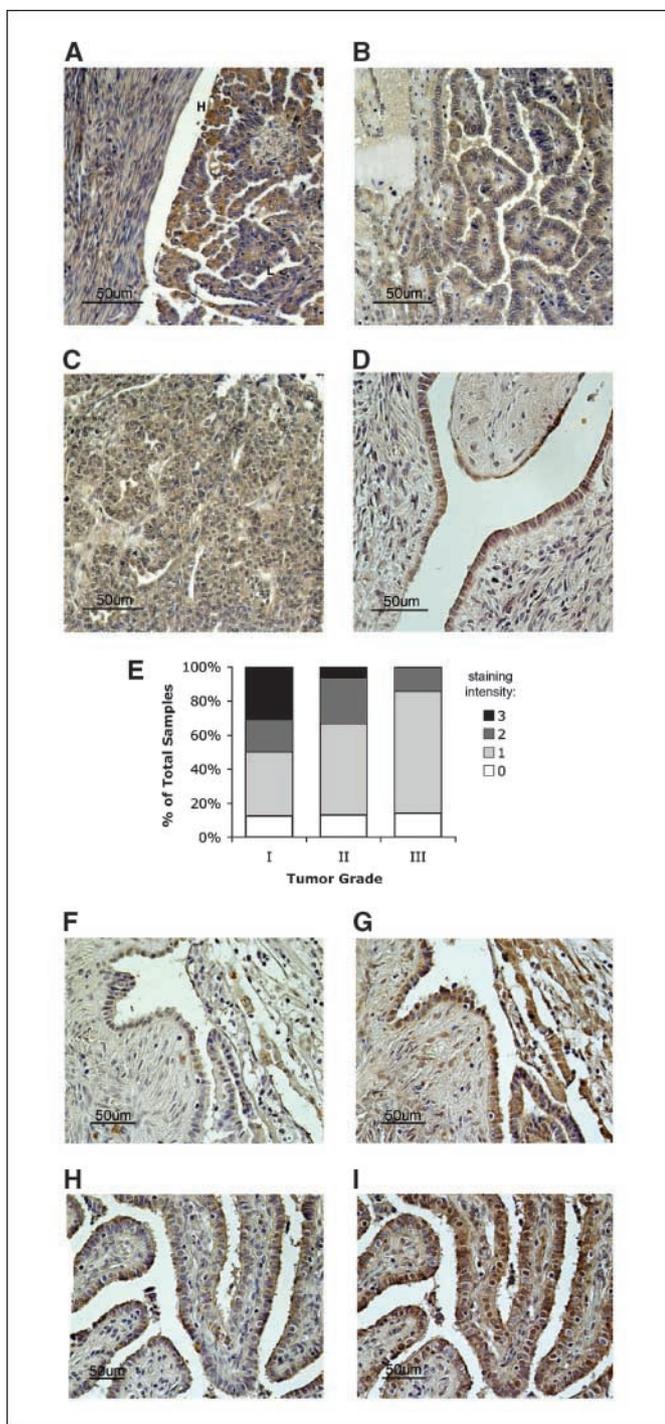
Figure 2. Loss of betaglycan protein in ovarian cancer. Betaglycan expression was analyzed using immunohistochemistry with a specific antibetaglycan antibody as described in Materials and Methods. Representative images from a human ovary cancer tissue microarray (Human Ovary Cancer Tissue Microarray, Protein Biotechnologies; 40×) of serous papillary adenocarcinoma grade I with high betaglycan staining (A); note areas of high (H) and low staining (L); a grade I adenocarcinoma with traces of betaglycan staining (B), a grade III adenocarcinoma with no betaglycan staining (C), and a specimen of normal ovarian surface epithelium on the tissue microarray with high betaglycan staining (D) are shown. E, immunoreactivity for betaglycan was scored as no or trace (0), low (1), medium (2), or high (3) staining and represented relative to tumor grade (I–III). F–I, expression of betaglycan in normal ovarian surface epithelium and fallopian tube. Representative images (40×) of normal ovarian tissue obtained from the Duke University Research Foundation Tissue bank, probed with preimmune serum (F) or antibody against betaglycan (G), and fallopian tube probed with preimmune serum (H) or betaglycan antibody (I) are shown.

30 cycles of 30 s denaturing at 95°C, annealing at 30 s at 58°C, and 30 s extension at 72°C. Real-time quantitative RT-PCR was carried out using SYBR Green (Bio-Rad) according to manufacturer's protocol on a Bio-Rad iCycler. After amplification, specificity of the reaction was confirmed by melt curve analysis. Relative quantitation was determined using the comparative CT method with data normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; s 5′-GAGTCAACGGATTTGGTCGT-3′, a/s 5′-TTGATTTTGGAGGGATCTCG-3′) and expressed relative to untreated controls. Betaglycan protein was visualized using binding and cross-linking of the receptor to ^{125}I-labeled TGF-β, following immunoprecipitation with a betaglycan-specific antibody, as described previously (13).

Proliferation and growth curve. Proliferation was assessed using [^{3}H]thymidine incorporation. Cells were grown in 24-well plates and treated with 0 to 200 pmol/L TGF-β1 for 48 h, followed by a 4-h incubation with 10 μCi of [^{3}H]thymidine (Amersham). Cells were washed with PBS and 5% trichloroacetic acid before harvesting with 0.1 N NaOH, and the amount of [^{3}H]thymidine incorporation was determined by scintillation counting. Growth of cells was assessed by trypsinizing and counting cells using a Coulter counter at times indicated.

Migration and invasion assays. Cells (7,500–25,000) were seeded in the upper chamber of a transwell filter, coated with either fibronectin to assess cell migration or with Matrigel (BD Bioscience) to determine the cell ability to invade this matrix. Cells were allowed to move for 12 to 24 h at 37°C, 5% CO_2 incubator through the fibronectin or Matrigel toward the lower chamber, containing media plus 10% FBS as the chemoattractant. In select experiments, cells were treated with 500 pmol/L inhibin A (Diagnostic Systems Laboratories) at the time of seeding onto the transwell filters. After incubation, the cells on the upper surface of the filter were gently removed with a cotton swab, and the cells that migrated to the underside of the filter were fixed and stained using the 3 Step Stain Set (Richard-Allan Scientific). The filters were mounted on slides, images were taken of representative fields of each filter, and the number of cells was quantified.

Matrix metalloproteinase activity assay and RT-PCR. Matrix metalloproteinase-2 (MMP-2) and MMP-9 activity in media from Ovca429-neo and Ovca429-betaglycan cells was assessed using gelatin zymography. A sample of the media, amount loaded being proportional to GAPDH levels of the cellular fraction to ensure equal protein loading, was electrophoresed on a nonreducing 8% acrylamide gel containing 0.1% gelatin, followed by washing of the gel in 2.5% Triton X and incubation at 37°C overnight in activation buffer [50 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 10 mmol/L $CaCl_2$, 0.02% Brij, 5 mmol/L phenylmethylsulfonyl fluoride] to renature and induce MMP activity. The cleaved gelatin by MMP-9 and MMP-2 was visualized using Coomassie staining and destaining. MMP message levels were assessed using RT-PCR following RNA extraction and cDNA synthesis as described above. RT-PCR was carried out as stated above, with an annealing temperature of 55°C for MMP-2 with sense 5′-CACCTACACCAAGAACTTCC-3′ and antisense 5′-AACACAGCC-TTCTCCTCCTG-3′ primers, and at an annealing temperature of 54°C for MMP-9 with sense 5′-GTATTTGTTCAAGGATGGGAAGTAC-3′ and antisense 5′-GCAGGATGTCATAGGTCACGTAG-3′ primers. Real-time RT-PCR for MMP-2 was carried out as described above at an annealing temperature of 55°C.

Results

Betaglycan expression is decreased or lost in human ovarian cancer. Because betaglycan has an emerging role in regulating inhibin and TGF-β signaling, and the role of TGF-β superfamily signaling in the regulation of ovarian cancer progression remains to be defined, we analyzed the expression profile of betaglycan in human ovarian cancer specimens. Using a cancer cDNA profiling array, we observed a significant decrease in betaglycan mRNA in 92.9% (13/14) of ovarian cancers (Fig. 1A), with an average 2.62 ± 0.90-fold decrease of betaglycan mRNA in ovarian tumor (T) compared with matched normal tissues (N; Fig. 1A and B;
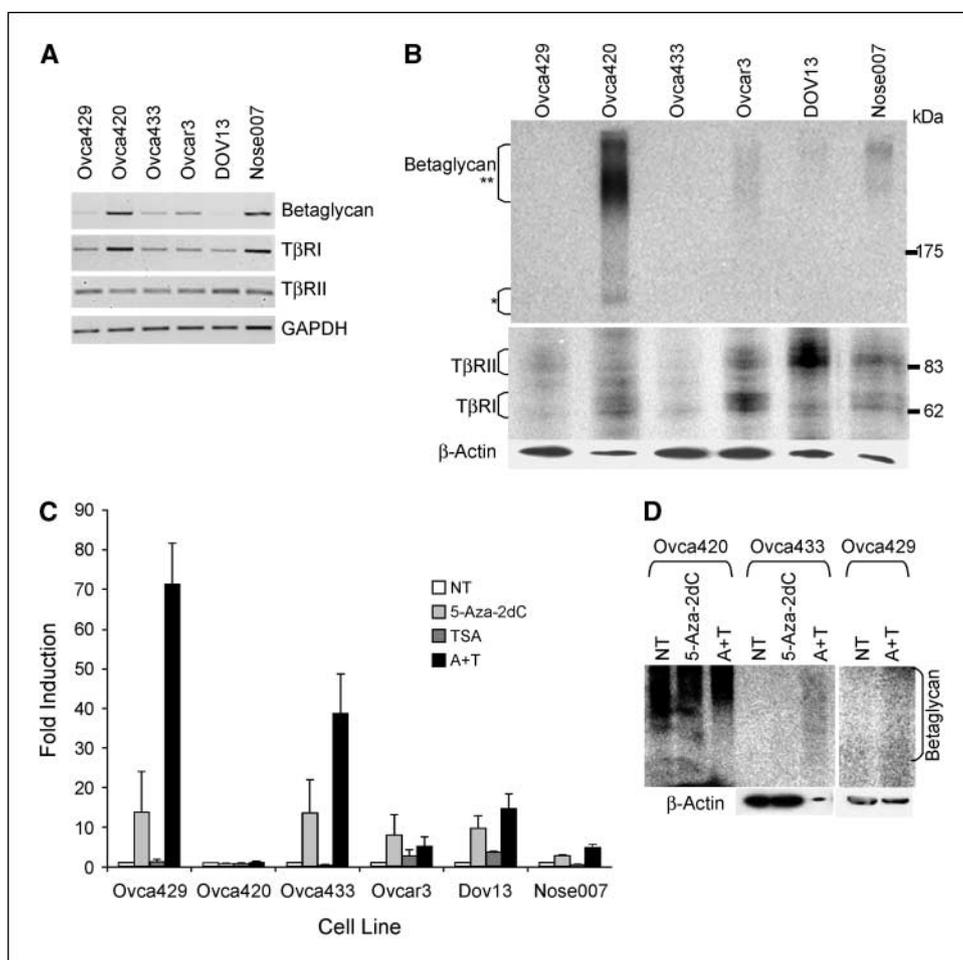
Figure 3. Epigenetic regulation of betaglycan in ovarian cancer. *A,* mRNA levels of betaglycan in ovarian cancer cell lines. RNA was isolated and reverse transcribed. RT-PCR of betaglycan, TβRI, TβRII, and GAPDH was carried out as stated in Materials and Methods and visualized on a 2% agarose gel. *B,* protein levels of betaglycan, TβRI, and TβRII in ovarian cancer cell lines were visualized following binding and cross-linking of ^{125}I TGF-β1 to cell surface receptors. Betaglycan protein was immunoprecipitated with antibetaglycan antibody (*top*), and TβRI and TβRII were resolved from whole cell lysate (*bottom*). Proteins were electrophoresed on 7.5% acrylamide gel and visualized by autoradiography. *, position of the betaglycan protein core; **, betaglycan protein containing glycosaminoglycan attachments. β-Actin was used as a loading control. *C,* ovarian cell lines were treated with 10 μmol/L 5-aza-2dC for 96 h and/or 500 nmol/L TSA for 24 h, or with vehicle (NT). RNA was isolated and reverse transcribed and SYBR Green real-time RT-PCR carried out with betaglycan-specific primers. Data are normalized against GAPDH levels and expressed relative to basal levels of betaglycan message levels from cell lines without treatment and represent the mean $\pm$ SE from three independent experiments. *D,* ovarian cancer cell lines were treated with 10 μmol/L 5-aza-2dC for 96 h and/or 500 nmol/L TSA for 24 h, followed by binding and cross-linking of ^{125}I TGF-β1 to cell surface receptors and immunoprecipitation with antibetaglycan antibody. Betaglycan proteins were resolved on 7.5% acrylamide gel and visualized by autoradiography. β-Actin was used as a loading control.

$P < 0.0001$). Two patient samples on the array contained RNA from a distant metastasis in addition to the primary tumor and normal tissue specimen (indicated by asterisks in Fig. 1*A*). In both cases, betaglycan expression progressively decreased from normal ovary to ovarian cancer to ovarian cancer metastasis (Fig. 1*C*). In addition, analysis of the betaglycan expression profile in normal and neoplastic ovarian tissue samples from a publicly available microarray database revealed a significant decrease in betaglycan message levels in adenocarcinoma samples compared with expression in normal ovarian tissues (Fig. 1*D*, $P < 0.001$; ref. 28).[5]

To confirm these results and assess whether betaglycan levels were also decreased at the protein level, betaglycan expression was analyzed by immunohistochemistry on an ovarian cancer tissue array using a specific polyclonal antibody to betaglycan (Fig. 2). Betaglycan expression was significantly decreased in ovarian cancer specimens, with complete loss or low immunostaining for betaglycan observed in 63.2% (24/38) of the specimens. Some grade I serous papillary adenocarcinomas exhibited high betaglycan staining overall, while still having areas with loss of betaglycan staining (indicated by *L*, Fig. 2*A*), and other low-grade ovarian cancers exhibited low betaglycan staining (Fig. 2*B*). Overall, 50% (8/16) of grade I tumors exhibited low to no staining for betaglycan, and loss of betaglycan correlated with increasing tumor grade (Fig. 2*E*), with very low or no betaglycan expression in

66.7% (10/15) and 85.7% (6/7) of grade II and grade III ovarian tumor specimen, respectively (Fig. 2*C* and *E*).

The cellular origin of ovarian cancers is controversial, with candidates including both the ovarian surface epithelial cells and cells that are embryologically related to the fallopian tubes, endometrium, and the cervix (29). The tissue microarray used in this study contained only one normal ovary specimen with a section of intact ovarian surface epithelium. In this sample, the surface epithelium had high betaglycan expression (Fig. 2*D*). High betaglycan expression was also observed in the surface epithelium of additional normal human ovarian specimens (Fig. 2*G*). Finally, high betaglycan expression was also observed in the fallopian tube (Fig. 2*I*), suggesting that betaglycan expression is high in several potential cellular origins of epithelial ovarian cancer. Taken together, these data support the loss of betaglycan expression as a frequent event in ovarian cancer, with loss of betaglycan expression correlating with worsening tumor grade and ovarian cancer progression.

Betaglycan expression in ovarian cancer cell lines. As observed in human ovarian cancer specimens, a similar lack of betaglycan expression was observed when screening a panel of human ovarian cancer cell lines. Three of five ovarian cancer cell lines (Ovca429, Ovca433, and DOV13) had markedly decreased or absent betaglycan expression at the mRNA level, whereas decreased betaglycan expression was observed in the Ovcar3 ovarian cancer cell line (Fig. 3*A*). In contrast, high betaglycan expression was observed in one ovarian cancer cell line, Ovca420,

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[5] http://www.oncomine.org

as well as a cell line derived from normal surface epithelium, Nose007 cells (Fig. 3A). This pattern of expression was also seen at the protein level (Fig. 3B). In contrast, the ovarian cancer cell lines had more consistent expression of both TβRI and TβRII at both the message (Fig. 3A) and protein (Fig. 3B) levels.

Methyltransferase and histone deacetylase inhibitors induce betaglycan expression in human ovarian cancer cell lines. Loss of gene expression during cancer progression can occur through a number of mechanisms, including mutation, loss of heterozygosity, and epigenetic silencing. Epigenetic transcriptional down-regulation can occur through hypermethylation and histone deacetylation of the gene promoter, resulting in tightly packed chromatin and decreased transcription factor access. Betaglycan protein expression in ovarian cancer cell lines correlated with betaglycan mRNA levels (Fig. 3A and B), suggesting regulation of betaglycan expression at the transcriptional level. To assess the role of epigenetic mechanisms regulating betaglycan expression, ovarian cancer cell lines were treated with the methyltransferase inhibitor 5-aza-2dC, and the histone deacetylase inhibitor, TSA. In all three ovarian cancer cell lines with low to absent betaglycan expression (Ovca429, Ovca433, and DOV13; Fig. 3A), treatment with 5-aza-2dC

resulted in a robust (>10-fold) induction of betaglycan mRNA levels (Fig. 3C). In addition, whereas TSA alone had little effect on betaglycan mRNA expression, in combination with 5-aza-2dC, betaglycan mRNA levels were now induced 15- to 70-fold in these cell lines (Fig. 3C). In the Ovcar3 ovarian cancer cell line with decreased betaglycan expression, treatment with 5-aza-2dC or with TSA and 5-aza-2dC resulted in a 5- to 10-fold induction (Fig. 3C). In contrast, in the ovarian cancer cell line (Ovca420) and the normal ovarian cancer epithelial cell line (Nose007) with robust betaglycan expression, treatment with 5-aza-2dC with or without TSA resulted in little to no induction of betaglycan expression (Fig. 3C). Thus, the ability of TSA and 5-aza-2dC to induce betaglycan expression was directly related to the basal level of betaglycan expression in these ovarian cancer cell lines.

To assess whether the ability of TSA and 5-aza-2dC to induce betaglycan expression was specific to betaglycan among TGF-β receptor family members, we assessed the effects on TβRII and TβRI mRNA expression. Treatment with 5-aza-2dC with or without TSA had no significant effect on TβRII levels (Supplementary Fig. S1). Although 5-aza-2dC induced TβRI message levels 12- and 6-fold in Ovcar3 and Nose007 cells, respectively, there was no
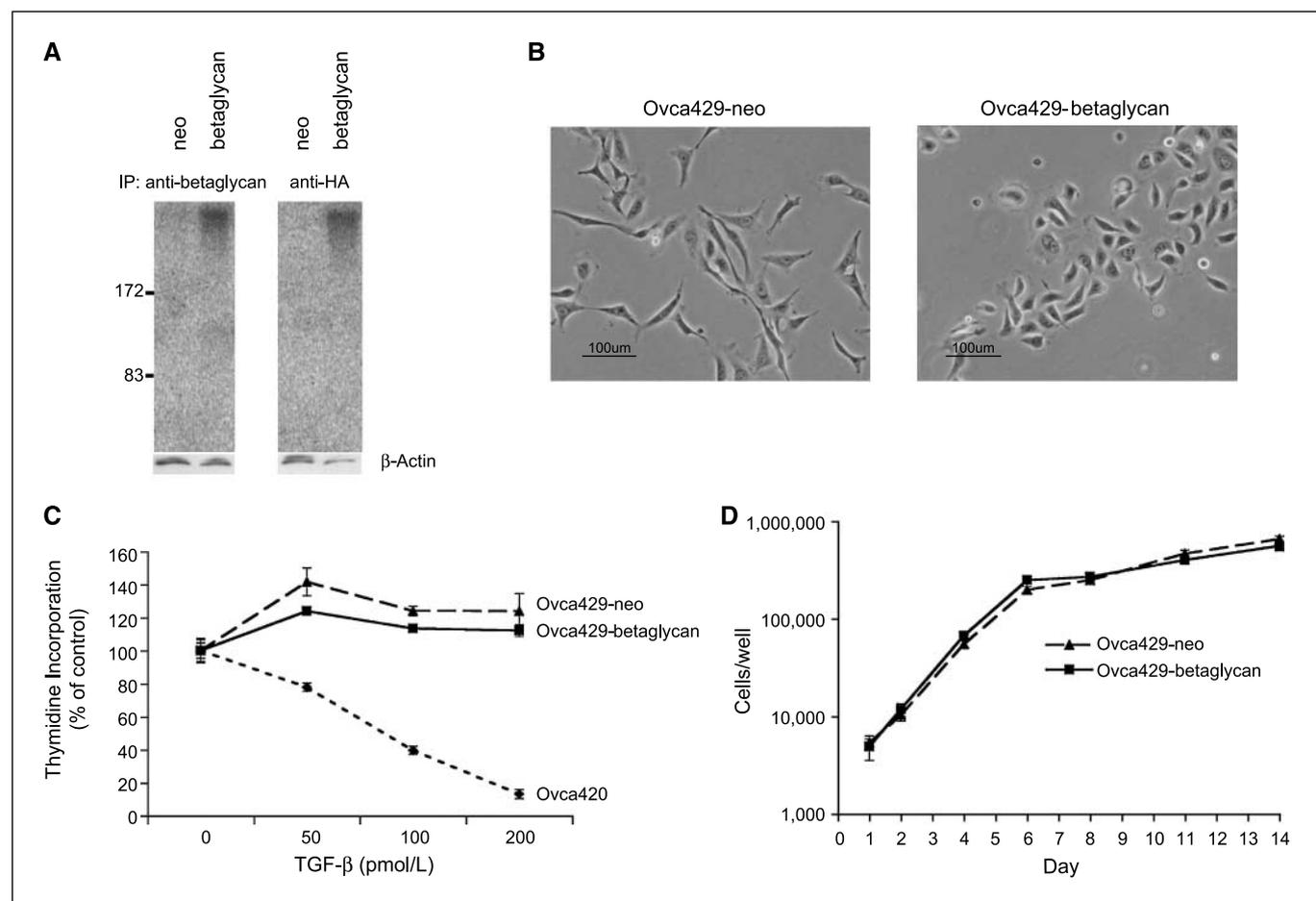

Figure 4. Betaglycan expression does not affect ovarian cancer cell proliferation in the presence or absence of TGF-β1. Betaglycan protein expression was visualized following binding and cross-linking of ^{125}I TGF-β1 to cell surface receptors, followed by immunoprecipitation with anti-betaglycan or anti-HA antibody. Proteins were resolved on 7.5% acrylamide gel and visualized by autoradiography. β-Actin was used as a loading control. *B,* morphology of Ovca429 cells stably expressing either empty vector neo or betaglycan. Approximately 200,000 cells were seeded into well of a six-well dish, and images were taken the following day at 20× magnification *C.* The indicated cells were treated with TGF-β for 48 h and proliferation was measured by [^{3}H] thymidine incorporation, relative to untreated cells (mean ± SE, *n* = 3). *D,* growth of the indicated cells of cells was assessed by trypsinizing cells from a six-well plate, and the cell number was counted in a standard Coulter counter (mean ± SE, *n* = 3).

synergistic effect with TSA; these effects were modest compared with the effects on betaglycan, and the observed effect did not correlate with baseline levels of expression (Supplementary Fig. S1). These studies suggest that of the three TGF-β receptor genes, the betaglycan gene is the most sensitive to epigenetic regulation in ovarian cancer cells.

We then investigated whether the resulting increases in mRNA level were sufficient to increase betaglycan protein levels. As expected, a corresponding increase in betaglycan protein levels was also observed after treatment of Ovca429 and Ovca433 cells with 5-aza-2dC and TSA (Fig. 3D). Note that the combined treatment with 5-aza-2dC and TSA resulted in significant toxicity, which was readily apparent upon inspection of the cells (data not shown) and was apparent by the decreased β-actin in these specimens (Fig. 3D). Taken together, these results suggest that betaglycan expression is regulated at the epigenetic level.

Betaglycan decreases motility and invasiveness of ovarian cancer cells. Frequent loss of betaglycan expression correlating with worsening tumor grade suggested that betaglycan loss during ovarian carcinogenesis may specifically promote tumor invasion and metastasis. To investigate the functional consequences of betaglycan in ovarian cancer, we analyzed the effects of restoring betaglycan expression in Ovca429 cells (Fig. 4A). Ovca429 cells normally lack betaglycan expression, yet retain high TβRI and TβRII levels and are highly motile and invasive (Fig. 3A; refs. 27, 30). Restoring betaglycan expression in Ovca429 cells altered their morphology, with Ovca429-neo cells appearing more spindle-like and Ovca429-betaglycan displaying a more cobblestone appearance (Fig. 4B). However, restoring betaglycan expression had no significant effect on the rate of cell division and did not restore Ovca429 cell responsiveness to TGF-β–induced growth inhibition (Fig. 4C and D). Similarly, restoring betaglycan expression in another ovarian cancer cell line with little endogenous betaglycan expression, the Ovca433 cell line (Fig. 3A and B) also had no significant effect on the rate of cell division and did not restore Ovca433 cell responsiveness to TGF-β–induced growth inhibition (data not shown). In contrast, Ovca420 cells, which express high levels of betaglycan, responded to TGF-β with growth inhibition, suggesting that the loss of betaglycan expression was not a mechanism for resistance TGF-β–mediated inhibition of proliferation in Ovca429 cells.

Subsequently, we tested the effects of betaglycan expression on cell motility and invasiveness. Ovca429-betaglycan cells were significantly less motile through a fibronectin matrix using 10% serum as a chemoattractant (Fig. 5A and B). Similarly, over-expression of betaglycan resulted in a less invasive phenotype, with Ovca429-betaglycan cells significantly less invasive through Matrigel than the Ovca429-neo cells (Fig. 5C and D).

Betaglycan enhances the antimigratory effects of inhibin. Ovca429 cells have been shown to lack responsiveness to the antimigratory properties of inhibin, a strong ligand of betaglycan and a major TGF-β ligand of the ovary (31). To assess whether a lack of betaglycan is responsible for this absence of inhibin response, we treated Ovca429-betaglycan and Ovca429-neo cells with inhibin and assessed their migration. Ovca429 cells lacking betaglycan expression did not respond to the antimigratory effects of inhibin, whereas the migration of Ovca429-betaglycan cells was significantly suppressed by inhibin (Fig. 6A). In contrast, we did not observe significant changes in migration in response to TGF-β1 treatment of either cell line (data not shown), suggesting that the effects of betaglycan on motility occur preferentially through the role of betaglycan as an inhibin coreceptor.

MMPs have an integral role in the increased metastatic and invasive potential of cancer cells, and recent studies have shown that inhibin negatively regulates MMP levels (32, 33). To determine whether betaglycan and inhibin were mediating their effects through regulating MMP levels, we assessed the levels of MMP-2 and MMP-9 at the protein level using gelatin zymography and at the mRNA level by RT-PCR. Ovca429-betaglycan cells had significantly less basal expression than Ovca429-neo cells of both enzymes at the protein level (Fig. 6B) and at the mRNA level (Fig. 6C). Moreover, inhibin treatment further decreased MMP-2 mRNA levels in Ovca429 cells expressing betaglycan in a dose-dependent fashion (Fig. 6D) while slightly inducing MMP-2 mRNA expression in Ovca429-neo cells (Fig. 6D). These results provide additional support for betaglycan functioning as an inhibin coreceptor to regulate ovarian cancer migration and invasion.
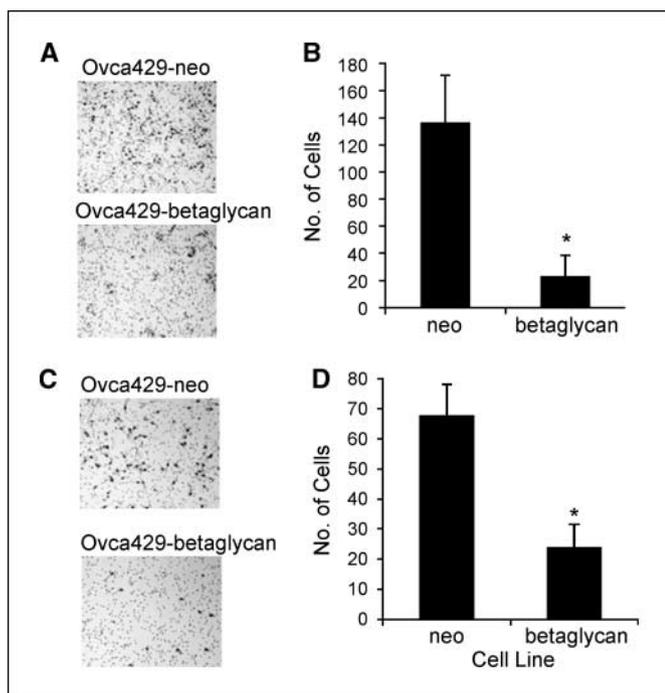

Figure 5. Betaglycan inhibits ovarian cancer cell migration and invasion. *A,* 25,000 Ovca429 cells stably expressing either betaglycan or empty vector (*neo*) were seeded in fibronectin-coated transwell filters in a 24-well plate and allowed to migrate for 24 h toward the lower chamber containing 10% FBS as a chemoattractant. Cells migrating through the fibronectin layer were visualized on the filter using H&E staining and cells quantified in (*B*), with each column representing the mean ± SE; *n* = 3; *, *P* < 0.05. *C,* 25,000 Ovca429 cells stably expressing either betaglycan or Neo were seeded in Matrigel-coated transwell filters in a 24-well plate and allowed to migrate for 24 h toward the lower chamber containing 10% FBS as a chemoattractant. Cells invading through the Matrigel were visualized on the filter using H&E staining and five images per filter quantified in (*D*), with each column representing the mean ± SE, *n* = 3; *, *P* < 0.05.

Discussion

Here, we show that expression of the inhibin and TGF-β coreceptor, betaglycan, is down-regulated in the majority of ovarian cancer specimens, and that this loss is progressive with increasing tumor grade. Loss of betaglycan expression in ovarian cancer occurs through a novel mechanism, epigenetic silencing. Functionally, betaglycan has a significant role in inhibiting ovarian
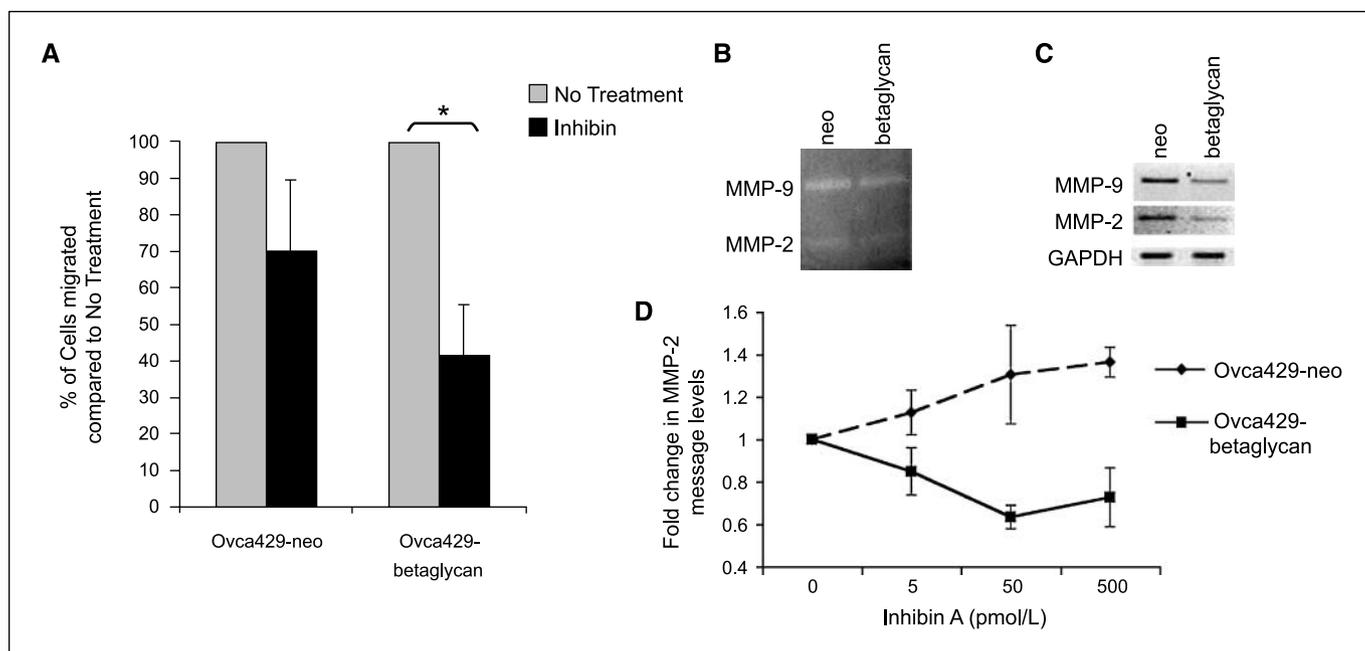
Figure 6. Betaglycan enhances the antimigratory and anti-invasive effects of inhibin. *A,* Ovca429-betaglycan (15,000 cells) or Ovca429-neo (7,500 cells) were seeded on fibronectin-coated filters in a 24-well plate and allowed to migrate for 24 h toward the lower chamber containing 10% FBS as a chemoattractant. At the time of seeding, cells were treated with 500 pmol/L inhibin A. Cells migrating through the fibronectin layer were visualized on the filter using H&E staining, and five images per filter were quantified, with each column representing the mean ± SE, *n* = 5; *, *P* < 0.05. *B,* media from Ovca429-neo and -betaglycan cells was collected and resolved on an 8% nondenaturing gel containing 0.1% gelatin. Cleavage of gelatin by MMP-2 and MMP-9 was visualized using Coomassie staining of the gel. *C,* RNA was isolated from Ovca429-neo and Ovca429-betaglycan cells, reverse transcribed and RT-PCR for MMP-2 and MMP-9 carried out as stated in Materials and Methods. *D,* Ovca429-neo and Ovca429-betaglycan cells were treated with indicated amounts of inhibin A for 24 h, followed by RNA extraction, cDNA synthesis, and real-time RT-PCR analysis for MMP-2 expression. Data are normalized against GAPDH levels and expressed relative to MMP-2 message levels from cell lines without treatment and represent the mean ± SE (*n* = 2).

cancer invasiveness and migration, specifically promoting the antimigratory action of inhibin.

Although the role of TGF-β in epithelial derived cancers has been well established, the role of TGF-β family members, such as inhibin, in the initiation and progression of epithelial derived ovarian cancer remains elusive. Given the abundant expression of inhibin throughout the ovary and its significant role in regulating normal ovarian function, inhibin seems poised to have a significant role in ovarian carcinogenesis. Although inhibin levels are maintained in many ovarian cancers, targeted deletion of the inhibin α gene results in high penetrance granulosa cell-derived ovarian tumors in mice (34). Although betaglycan is expressed in normal granulosa cells of the ovary and tumors derived from these cells (35), before this study, little was known of the expression and function of betaglycan in epithelial derived ovarian cancer, which represent >90% of human ovarian cancers. Inhibin has also recently been shown to play an important role in ovarian tumors of epithelial origin. Specifically, a recent study showed that more aggressive human epithelial derived ovarian cancer cell lines become inhibin resistant, and inhibin functioning to inhibit the ability of ovarian cancer cells to migrate through a Matrigel matrix, implicating inhibin as an antimigratory/anti-invasive factor (31). Although the authors report that expression of betaglycan is retained in the ovarian cancer cell lines studied, it seems that expression is relatively low compared with a control cell line (31), and we found little or no expression in some of the same lines (including Ovca429) in the present study. The current results suggest that the loss of the inhibin coreceptor, betaglycan, in ovarian cancer may be the cause for inhibin resistance in ovarian cancer cell lines.

How might betaglycan potentiate some of the tumor-suppressor effects of inhibin? Inhibin has been reported to decrease MMP levels (32, 33). As MMPs serve as gelatinases that degrade the basement membrane to aid invasion and their expression normally increases in ovarian cancer progression (36, 37), loss of betaglycan may decrease the ability for inhibin to suppress MMP levels. Indeed, restoring betaglycan expression in Ovca429 cells not only enhanced the antimigratory effects of inhibin in these cells, but also resulted in decreased MMP-2 and MMP-9 levels at the mRNA and protein levels and enhanced inhibin-mediated inhibition of MMP-2 message levels (Fig. 6). Thus, betaglycan seems to function as an inhibin coreceptor to enhance inhibin's tumor-suppressive effects.

In addition to a membrane-bound form, betaglycan also exists as a soluble form, generated by ectodomain shedding of the cell surface receptor. Although the membrane-bound form presents ligand, the soluble form is thought to sequester ligand from the serine/threonine TGF-β signaling receptors. This sequestering role may be of benefit in circumstances such as in later stage tumors, where excess TGF-β may aid in tumor progression by enhancing epithelial to mesenchymal transitions, MMP expression, and basement membrane degradation (20, 38). Indeed, whereas betaglycan enhanced the effects of inhibin on decreasing MMP levels (Fig. 6), our preliminary studies showed that betaglycan antagonized the ability of TGF-β to induce MMP-9 expression in Ovca429 cells (Supplementary Fig. S2). Similarly, soluble betaglycan has recently been shown to decrease MMP-9 levels in a prostate cancer model and reduce breast cancer cell migration and invasion (20, 39). Thus, in ovarian cancer progression, betaglycan may carry out a dual role; first by directly facilitating the tumor suppressor

effects of inhibin on migration; and second, by sequestering free TGF-β, through its soluble form, thereby preventing the positive effects of TGF-β on tumor progression. The dual effects of betaglycan in ovarian cancer warrants further investigation.

Loss of betaglycan expression in ovarian cancer seems to be due, at least in part, to epigenetic silencing. Although we could not assess whether loss of heterozygosity also had a role in the decreasing betaglycan expression in ovarian cancer due to a lack of matching normal samples, our laboratory has previously defined loss of heterozygosity as a mechanism for loss of betaglycan expression in breast cancer (20) and prostate cancer (40), suggesting that this may occur in ovarian cancer as well. Epigenetic regulation of betaglycan has both diagnostic/prognostic and therapeutic implications. For example, methylation of other tumor suppressors, including BRCA I, predicts outcome in ovarian cancer patients (41). Thus, screening for methylation of betaglycan may present a valuable tool with diagnostic or prognostic implications for ovarian cancer patients.

In addition, several clinical studies have shown the benefits of methyltransferase and histone deacetylase inhibitors in the treatment of ovarian cancer (42). Because such treatments would be expected to restore betaglycan expression, whether restoration of betaglycan expression represents a mechanism for their clinical activity, or if betaglycan expression can be specifically restored as a therapeutic strategy in ovarian cancer, remains to be explored.

Acknowledgments

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