Tumorigenesis and Neoplastic Progression

Myosin II Co-Chaperone General Cell UNC-45 Overexpression Is Associated with Ovarian Cancer, Rapid Proliferation, and Motility

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Both tumor cell proliferation and metastasis are dependent on myosin II. Because UNC-45 is required to chaperone the assembly of a functional myosin II motor, we examined the expression of the general cell (GC) UNC-45 isoform in ovarian tumors. Serous carcinoma expressed elevated levels of GC UNC-45 compared with normal ovarian surface epithelium and benign cystadenoma. High-stage exhibited greater GC UNC-45 expression than low-stage serous carcinoma. Similarly, GC UNC-45 transcripts and protein levels were higher in ovarian cell lines than in immortalized ovarian surface epithelial cells. Elevation of GC UNC-45 levels by ectopic expression enhanced the rate of ovarian cancer cell proliferation, whereas siRNA knockdown of GC UNC-45 suppressed proliferation without altering myosin II levels. GC UNC-45 and myosin II were diffuse within the cytoplasm of confluent interphase cells, but both accumulated together at the cleavage furrow during cytokinesis. GC UNC-45 and myosin II also trafficked to the leading edges of ovarian cancer cells induced to move in a scratch assay. Knockdown of GC UNC-45 reduced the spreading ability of ovarian cancer cells whereas it was enhanced by GC UNC-45 overexpression. In sum, these findings implicate elevated GC UNC-45 protein expression in ovarian carcinoma proliferation and metastasis. (*Am J Pathol* 2007; 171:1640–1649; DOI: 10.2353/ajpath.2007.070325)

among women in the United States. Approximately 70% of ovarian cancer patients are diagnosed with disease that has spread beyond the ovaries when, despite aggressive surgery and chemotherapy, the 5-year survival rate is less than 30%. Ovarian carcinoma rapidly seeds the peritoneal cavity with highly proliferative and invasive cancer cells, and new therapies targeting both of these properties are urgently needed.^{1,2}

Actomyosin contractility plays a central role in both cell division and motility. Genetic studies in lower organisms (*Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, and *Drosophila melanogaster*) and mammalian cells show an evolutionarily conserved and central role for the myosin II motor protein in both cytokinesis and cell migration.^{3–6} Studies in lower organisms and human cells also demonstrate that functional assembly of myosin II requires both a heat shock protein (Hsp) and a highly conserved member of the UCS (UNC-45/CRO1/She4p) protein family to act together to chaperone motor assembly.^{7–10} The UCS proteins are a family of co-chaperone molecules that interact with the well-established chaperone molecule Hsp90 and with both conventional and non-conventional myosin heads to ensure their proper activity during cytokinesis, cell motility, cell contraction, as well as organelle trafficking within the cellular compartment.^{7,11–14}

Elevated expression of one or more Hsps frequently occurs in hematological and solid malignancies.¹⁵ Hsp90 in particular is highly expressed in ovarian cancer, and its expression correlates with FIGO stage.^{16,17} Moreover, mounting evidence links Hsp90 with cancer cell migration, and myosin II and Hsp90 inhibitors show promise for cancer treatment.^{18–22} There are two UCS orthologs expressed in mammalian cells: SM UNC-45 (striated muscle UNC-45) whose expression is restricted to skeletal muscle and heart, and GC UNC-45 (general cell UNC-45

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Epithelial ovarian cancer is the most lethal gynecological malignancy and the fifth major cause of cancer death

comprising 20 exons of chromosome 15), which is expressed in several organs including ovaries where GC mRNA levels are particularly abundant.^{8,23,24}

Interestingly, microarray expression data indicates that elevated expression of GC UNC-45 strongly associates with the malignant phenotype as well as the severity of disease in several cancers, including melanoma and lung cancer.^{25,26} This study addresses changes in the expression level of GC UNC-45 in ovarian carcinogenesis and examines the potential involvement of GC UNC-45 in ovarian cancer cell proliferation and motility. We find that GC UNC-45 expression is elevated in ovarian carcinoma both *in vitro* and *in vivo* as compared with ovarian surface epithelium, and its level correlates with disease stage. Furthermore, GC UNC-45 concentrates with myosin II at the cleavage furrow during cytokinesis, and modulation of GC UNC-45 levels influences the proliferation rate of ovarian cancer cells. Finally, GC UNC-45 accumulates in the leading edges of interphase ovarian cancer cells and is necessary for ovarian cancer cell migration.

Materials and Methods

Human Specimens

Studies using human tissue were performed with the approval of the Johns Hopkins institutional review board. Fresh and archival tissues were obtained from the Department of Pathology of the Johns Hopkins Hospital and the latter assembled in tissue microarrays by a core facility.

Cell Culture

IOSE-29, IOSE-397, and IOSE-386 cell lines were kindly provided by Nelly Auesperg (University of British Columbia, Vancouver, BC, Canada) and cultured in Medium 199 and MCDB105 (1:1) with 10% fetal bovine serum and 50 μ g/ml gentamicin (Invitrogen, Carlsbad, CA). OVCAR-3, SKOV-3, and ES-2 were obtained from American Type Culture Collection (Manassas, VA). The JH514 cell line was derived from a low-grade serous carcinoma of the ovary and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (100 IU/ml), and streptomycin (100 μ g/ml) at 5% CO₂.

Expression and Purification of Recombinant GC UNC-45 Protein

Full-length human GC UNC-45 coding sequence (GenBank ID BC006214) was subcloned from MGC-999 (American Type Culture Collection) into pProExHTc vector (Invitrogen). After transformation the bacteria were grown to an OD₆₀₀ of 0.6 and induced with 1 mmol/L isopropylthio- β -D-galactoside (1 mmol/L) for 6 hours. 6His-tagged protein was purified on nickel-nitriloacetic acid resin columns under denaturing conditions according to the manufacturer (Qiagen, Valencia, CA).

Generation of Rabbit Antisera

Rabbits were immunized with 250 μ g of antigen in Freund's complete adjuvant and boosted with 125 μ g of antigen in

Freund's incomplete adjuvant on days 14, 28, and 35. Rabbits were inoculated with the following antigens: full-length 6His-tagged human GC UNC-45 and keyhole limpet hemocyanin-coupled peptide CLDKAVEYGLIQPNQDGE of human GC UNC-45. Immunizations and peptide affinity purification were performed by Proteintech Laboratories (Chicago, IL) using their standard protocol.

Antibodies and Western Blot Analysis

Total cellular protein (10 to 20 μ g) from each sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and subjected to Western blot analysis. Antibodies were used at the concentration recommended by the manufacturer: rabbit polyclonal anti-myosin V and mouse anti-myosin VI (Sigma, St. Louis, MO), rabbit anti-myosin II (Covance, Berkeley, CA), anti- β -actin (Sigma), and peroxidase-linked anti-rabbit or anti-mouse IgG (GE Healthcare, Piscataway, NJ). Rabbit antisera to GC UNC-45 were used at 1:1000 dilution. The above conditions are within the linear quantitative range of immunoreactivity.

Immunohistochemistry of Tissue Microarrays

Immunohistochemical analysis of paraffin-embedded tissues was performed and scored as previously described.² In brief, 5- μ m tissue microarray sections were deparaffinized and rehydrated. Antigen retrieval was performed and slides were incubated for 5 minutes with 3% hydrogen peroxide, washed, and then incubated in antibody dilution 1:250 for 60 minutes at room temperature. The avidin-biotin-peroxidase complex method (DAKO, Glostrup, Denmark) was used to visualize antibody binding, and slides were subsequently counterstained with hematoxylin. The staining was scored by consensus of three observers blind to specimen identity. Staining intensity was scored as negative (0), weak (1), intermediate (2), or strong (3). The GC UNC-45 staining, when present, was homogeneous (see Figure 2), and therefore the percentage of positive cells was not included in the algorithm.

Generation of GC UNC-45 Transient Transfectants

Full-length GC UNC-45 cDNA (GenBank ID BC006214) was cloned into pcDNA3.1 (Invitrogen) and pEGFP-C1 (Clontech, Mountain View, CA). Subconfluent cultures of ES-2 or SKOV-3 cells were transfected with plasmid DNA by using Lipofectamine 2000 reagent (Life Technologies, Carlsbad, CA).

Transfection of Ovarian Carcinoma Cells with Short Interfering RNA (siRNA) Oligomers

Sequences of siRNA oligomers (synthesized by Qiagen) were as follows: GC UNC-45 siRNA-1, 5'-GCUGGAA-GAUUACGACAAAdTdT-3'; GC UNC-45 siRNA-2, 5'-AC-CUCAAGCUGGAAGAUUAdTdT-3'. ES-2 cells were trans-

fected with oligomers by using Lipofectamine 2000 reagent (Life Technologies). Efficacy of GC UNC-45 knockdown was tested by using oligomers at a range of concentration (20, 50, 100 nmol/L) throughout a period of 24, 48, and 96 hours by Western blot analysis. As a control, ES-2 cells were transfected with nonsilencing fluorescein-labeled siRNA (Qiagen) under the same conditions used for GC UNC-45 siRNAs.

Proliferation Assay

Fluorescent SKOV-3 cells were imaged and manually counted after 12, 24, and 48 hours from the transfection in a $\times 200$ power field under an Eclipse TE2000 inverted fluorescence microscope (Nikon, Melville, NY). Six fields per each condition were counted. Alternatively, cells were seeded at the concentration of 1000 cells per well in 100 μ l of medium in a 96-well plate; after the indicated periods, cells were incubated with 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's protocol. Formazan dye was quantified using a spectrophotometric plate reader to measure the absorbance at 405 nm (ELISA Reader 190; Molecular Devices, Sunnyvale, CA). All experiments were done in triplicate.

Cellular Localization of GC UNC-45

SKOV-3 cells were seeded 12 hours after transfection at 2000 cells/500 μ l per well in Lab-Tek II chambered coverglass (Nalge Nunc Int., Rochester, NY). Cells were fixed and permeabilized with methanol and incubated for 1 hour with the indicated primary antibodies. Fluorescent secondary antibodies were used to visualize protein localization, and nuclei were visualized by 4,6-diamidino-2-phenylindole (DAPI) staining. Mounted samples were viewed under a Nikon Eclipse TE 2000E inverted microscope and images captured with Spot 3.5.8 acquisition software (Diagnostics Instruments, Sterling Heights, MI).

In Vitro Cell-Spreading Assay

Scratch assays were performed after transfection of ES-2 cells for 24 hours with negative control siRNA and GC UNC-45 siRNA-1. Confluent cultures were scratched with a sterile pipette tip and examined by phase-contrast microscopy at the time of scratching and 5 hours later, and images were captured with Spot 3.5.8 acquisition software. For ectopic expression experiments, scratch assays were performed after transfection of SKOV-3 cells for 24 hours with p-EGFP-C1 or p-EGFP-C1-GC UNC-45. The transfection efficiency was $\sim 80\%$. Confluent cultures were scratched by using a sterile pipette tip and examined by phase-contrast microscopy at the time of scratching and 6 hours later. Images were captured as previously described.

Statistical Analysis

Results are reported as mean $\pm$ SD. Unless otherwise indicated, statistical significance of difference was assessed by two-tailed Student's *t*-test using Prism (V.4; GraphPad, San Diego, CA) and Excel (Microsoft, Redmond, WA). The level of significance was set at $P < 0.05$.

Results

GC UNC-45 Expression Is Associated with Aggressive Behavior of Ovarian Carcinomas in Vivo

To investigate GC UNC-45 protein expression in ovarian carcinomas, we generated two rabbit antisera, one against a synthetic peptide comprising the C-terminal 18 residues of GC UNC-45 and a second against the full-length GC UNC-45 produced in *Escherichia coli*. The GC UNC-45 peptide antibody was affinity-purified on a peptide column and its specificity examined by Western blot (Figure 1). Figure 1A shows that the affinity-purified GC UNC-45 peptide antibody detects a major ~ 100 -kDa band corresponding to the full-length protein and a minor band of ~ 50 kDa in human tissues. In cell lines several minor reactive bands of varying abundance (Figure 1A, shown for ES-2) were observed. Ectopic expression of GC UNC-45 cDNA led to dramatically elevated levels of the full-length product and smaller increases in the lower molecular weight species. Alternative splicing is a possible source of the low molecular weight species because GC UNC-45 is encoded on 20 exons, and several splice variants have been described. Alternatively, the minor reactive species are normal degradation products generated as part of cell metabolism of GC UNC-45 or during cell lysis (although lysis was performed on ice in buffer containing the Roche Complete protease inhibitor cocktail). Importantly, siRNA silencing of GC-UNC-45 expression led to coordinated decrease in full-length GC-UNC-45 and all of the low molecular weight species (Figure 1B). Thus, we conclude that the affinity-purified GC-UNC-45 antibody is monospecific for GC UNC-45 polypeptides. Although we have used the antiserum raised to recombinant GC UNC-45 protein to verify independently the data obtained with affinity-purified anti-peptide antibody, all of the primary data presented in this report are generated using the affinity-purified anti-peptide antibody. However, as noted throughout the text, consistent data are obtained with both reagents. We also confirmed the linearity of the immunoblot analysis used in this study. Figure 1C shows that the protein amounts used for immunoblot analysis (10 to 20 μ g of cell lysate) are within the linear range for the anti-GC UNC-45 peptide antisera.

To address the question of whether GC UNC-45 expression levels change during ovarian carcinogenesis, we performed immunohistochemical staining of microarrays of ovarian tumors and normal tissues using affinity-purified peptide antibody (although similar results were obtained with the antiserum to full-length GC UNC-45,

Figure 1. GC UNC-45 antisera characterization. **A:** Affinity-purified antibodies to GC UNC-45 peptide were tested by Western blot analysis for reactivity to endogenous and ectopically expressed GC UNC-45 in the ES-2 ovarian cancer cell line as well as in clinical specimens of ovarian cancer. Equal loading was verified by using an antibody directed against β -actin. **B:** Rabbit antisera to GC UNC-45 peptide was tested by Western blot analysis for reactivity to endogenous GC UNC-45 in an ES-2 ovarian cancer cell line transfected with GC UNC-45 siRNA oligomer (GC UNC-45-siRNA-1) or with nonsilencing siRNA (neg. control-siRNA). GC UNC-45 expression level was tested by immunoblot analysis at 48 hours after transfection. Equal loading was verified by using an antibody directed against β -actin. **C:** Serial dilution of ES-2 cell lysate was immunoblotted for GC UNC-45. The density of immunoreactive bands was quantified by using Scion Image software using automatic background subtraction. The values were plotted and linear regression was calculated. Naphthalene Blue-Black-stained blot confirmed the loading of twofold dilutions of total cell lysate (not shown).

data not shown). A total of 64 clinical specimens representing, normal ovarian surface epithelia ($n = 14$), benign cysts of the ovary ($n = 7$), low-grade ovarian carcinomas ($n = 14$), and high-grade ovarian carcinomas ($n = 32$) were subjected to immunohistochemical staining for GC UNC-45 expression (Figure 2A), which was scored as no

staining = 0, weak staining = 1, moderate staining = 2, and intense staining = 3. Using this scoring system, we found that GC UNC-45 was only weakly expressed in normal ovarian surface epithelium and in benign ovarian cysts. In contrast, serous carcinoma exhibited significantly ($P < 0.02$) more intense staining than the nonma-

Figure 2. Immunohistochemical staining of GC UNC-45 in clinical specimens. **A:** Representative examples of intense GC UNC-45 staining in high-grade serous carcinoma, moderate staining intensity in low-grade serous carcinoma, and weak staining intensity in benign cyst and normal ovarian surface epithelium. **B:** Staining intensity for each case was graded as 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (intense staining). $**P < 0.02$ statistical significance in staining intensities among indicated groups (Mann-Whitney U -test). Error bars indicate SD. Original magnifications: $\times 40$.

lignant counterparts, normal ovarian surface epithelium, and benign cysts. Furthermore, there was a statistically significant ($P < 0.02$) difference in GC UNC-45 expression in low-grade versus high-grade carcinomas (Figure 2B, top). Moreover, there was a statistically significant ($P < 0.02$) difference in GC UNC-45 expression in high-grade early-stage versus high-grade high-stage ovarian carcinomas, with the latter expressing higher levels of GC UNC-45 protein. (Figure 2B, bottom). To corroborate further the finding of GC UNC-45 aberrant expression in ovarian carcinoma, GC UNC-45 expression levels were measured by Western blot analysis in human specimens of serous cystadenoma and serous carcinoma (Figure 3A). In line with the immunohistochemical analysis, GC UNC-45 protein expression was found to be higher ($P < 0.02$) in ovarian carcinomas versus nonmalignant coun-

terparts. A semiquantitative analysis of the ratio between GC UNC-45 and β -actin is given in Figure 3B based on the linearity of the immunoblot analysis (Figure 1C).

CG UNC-45 Is Aberrantly Expressed in Ovarian Cancer Cell Lines and Regulates Cell Proliferation

To establish a suitable *in vitro* model for investigating the biological significance of GC UNC-45 overexpression in ovarian cancer, a panel of immortalized, but nontumorigenic, ovarian surface epithelial cells (IOSE-29, IOSE-386, and IOSE-397) and a panel of ovarian cancer cell lines (SKOV-3, OVCAR-3, ES-2, and JH-514) were subjected to Western blot analysis for GC UNC-45 (Figure 3C). The ovarian cancer cell lines exhibited higher levels of GC UNC-45 as compared with IOSE cell lines. This suggests that aberrant expression of GC UNC-45 associated with malignancy is maintained in established ovarian cancer cell lines. A semiquantitative analysis of the ratio between GC UNC-45 and β -actin is given in Figure 3D. Similar results were obtained by using both antisera, but Western blot analysis using the affinity-purified peptide antibody is shown.

Aberrant expression of GC UNC-45 in ovarian carcinoma and genetic studies in model systems suggest a possible role for GC UNC-45 in cell proliferation.^{23,24} To address the significance of the elevated levels of GC UNC-45 present in ovarian cancer, we investigated whether modulation of GC UNC-45 expression levels via ectopic overexpression or siRNA knockdown reciprocally regulate cell proliferation. The ovarian cancer cell line SKOV-3 was chosen for ectopic overexpression because Western blot analysis shows that SKOV-3 had expression level of GC UNC-45 that is between IOSEs and the ovarian cancer cell lines (Figure 3, C and D). SKOV-3 cells were transiently transfected with vectors expressing GFP alone (pEGFP-C1) or GFP-GC UNC-45 fusion protein (pEGFP-C1 GC UNC-45). Transfection efficiency was more than 70% (data not shown). The number of fluorescent cells was counted at 12, 24, and 36 hours after transfection. The results show significantly increased proliferation by the cells transfected with pEGFP-C1-GC UNC-45 compared with pEGFP-C1 vector alone ($P < 0.02$ at both 24 and 48 hours after transfection) (Figure 4A). To exclude the possibility that GFP fusion affects the function of GC UNC-45 or potential toxic effects of GFP expression, we compared the proliferation rate of SKOV-3 cells transiently transfected with pEGFP-C1 plus pcDNA3 (vector control) versus pEGFP-C1 plus pcDNA3-GC UNC-45. Transfection efficiency was more than 70% (data not shown). The proliferation rates were followed by counting the number of fluorescent cells at 12, 24, and 36 hours after transfection. Again, ectopic expression of GC UNC-45 was associated with an increased cell proliferation in SKOV-3 cells ($P < 0.02$, Figure 4B). Similar results were also obtained with ectopic overexpression of GC UNC-45 in both HeLa and 293T cells, suggesting that this phenomenon is not restricted to ovarian cancer cells (not shown).

Figure 3. Western blot analysis of GC UNC-45 in clinical specimens and cell lines. **A:** Lysates of serous cystadenoma (lanes 1 to 4) and serous carcinoma (lanes 5 to 10) were probed using GC UNC-45 peptide-specific rabbit antibody by Western blot. Equal loading was verified by using an antibody directed against β -actin. **B:** Ratio between GC UNC-45 and β -actin in tissue lysates from serous cystadenoma (four cases) and serous carcinoma (six cases). $**P < 0.02$. **C:** Lysates of immortalized ovarian surface epithelial cell lines (IOSE-29, IOSE-386, and IOSE-397) and ovarian cancer cell lines (SKOV-3, OVCAR-3, ES-2, and JH-514) were probed with anti-GC UNC-45 peptide rabbit antibody by Western blot. Equal loading was verified by using an antibody directed against β -actin. **D:** Ratio between GC UNC-45 and β -actin in tissue lysates from IOSEs and ovarian cancer cell lines. $**P < 0.02$. Error bars indicate SD.

Figure 4. GC UNC-45 level influences ovarian cancer cell proliferation. **A:** Proliferation rate of SKOV-3 cells ectopically expressing either GFP (pEGFP-C1 vector) alone or GFP-tagged GC UNC-45 (pEGFP-C1-GC UNC-45) was determined by counting the number of fluorescent cells 12, 24, and 36 hours after transfection. *** $P < 0.001$. **B:** Proliferation rate of SKOV-3 co-transfected with either pEGFP-C1 and pcDNA3 or pEGFP-C1 and pcDNA3-GC UNC-45 was determined by counting the number of fluorescent cells 12, 24, and 36 hours after transfection. *** $P < 0.001$. **C:** Efficiency of GC UNC-45 knockdown in ES-2 cells transfected with one of two different GC UNC-45 siRNA oligomers (GC UNC-45-siRNA-1 or -2) or with nonsilencing siRNA (neg. control-siRNA) at the indicated concentration was tested by immunoblot analysis at 48 hours after transfection. Equal loading was verified by using an antibody directed against β -actin. **D:** Proliferation rate of ES-2 cell line transfected with nonsilencing siRNA, GC UNC-45-siRNA-1, or GC UNC-45-siRNA-2 was measured by XTT assay starting from 12 hours after transfection. Results are expressed in terms of daily proliferation rate. ** $P < 0.02$, *** $P < 0.001$. Error bars indicate SD.

To determine whether higher basal levels of GC UNC-45 contribute to the rapid proliferation of ovarian cancer cells, we performed GC UNC-45 knockdown experiments using siRNA in the ES-2 ovarian cancer cell line. The GC UNC-45 expression was unaffected on transient transfection of nonsilencing control siRNA (negative control siRNA), whereas both GC UNC-45 siRNA-1 and GC UNC-45 siRNA-2 considerably reduced the protein expression levels within 24 hours of siRNA treatment (Figure 4C). After assessing the protein knockdown conditions, the proliferation rate was determined using the XTT assay. A significant reduction was observed in the proliferation rate of ES-2 cells transfected with either GC UNC-45-specific siRNA-1 or -2 compared with the proliferation rate of the cells transfected with the negative control siRNA (Figure 4D). This is consistent with anti-sense experiments reporting that the proliferation of murine myoblasts is severely compromised on reduction of GC UNC-45 expression.²⁴

Co-Localization of GC UNC-45 with Myosin II and the Effect of GC UNC-45 Levels on Myosin Expression

Genetic and biochemical studies reveal that UCS domain proteins have chaperone activity during the assembly and maturation of conventional and nonconventional myosins. Specifically, GC UNC-45 orthologs in yeast and nematodes act on myosin types II and V and thereby play a fundamental role during cytokinesis.⁸ To address the hypothesis that GC UNC-45 plays a similar role in ovarian cancer cells, we performed co-localization studies by immunofluorescence microscopy. Although there was partial overlap between GC UNC-45 and myosin V immunostaining (Figure 5A), GC UNC-45 and myosin II consistently co-localized within the cytoplasm of interphase ovarian cancer cells (Figure 5B). Myosin II is also known to accumulate at the cleavage furrow and thereby drive cytokinesis.^{27–29} Immunohistochemical staining also revealed the co-localization of GC UNC-45 with myosin II at the cleavage furrow in mitotic ovarian cancer cells during both metaphase and anaphase (Figure 5, C and D), consistent with the role of GC UNC-45 as a chaperone that facilitates the correct assembly of myosin II during cytokinesis.

Cytokinesis is very sensitive to appropriate levels and folding of myosin.³⁰ Thus the inhibition in cell proliferation of ovarian cancer cell lines after siRNA knockdown of GC UNC-45 might occur via unfolding and/or destabilization of myosins. To address this hypothesis we examined whether knockdown of GC UNC-45 expression would destabilize and reduce the levels of conventional (myosin II) and nonconventional (V and VI) myosins. For this purpose GC UNC-45 expression levels were knocked down by siRNA in ES-2 ovarian cancer, and the expression levels of myosins II, V, and VI were assessed by immunoblot analysis. As shown in Figure 5E, reduction of GC UNC-45 expression levels did not have obvious effects on the levels of myosin II, V, or VI in siRNA-treated ES-2 cell lines at 48 hours after transfection. At 96 hours after

Figure 5. Co-localization of GC UNC-45 with myosin II during cytokinesis and its effect on myosin expression levels. **A:** SKOV-3 cells were transfected with p-EGFP-C1-GC UNC-45 and stained with the DNA stain DAPI and anti-myosin V antibody. **B:** SKOV-3 cells were transfected with p-EGFP-C1-GC UNC-45 and stained with the DNA stain DAPI and anti-myosin II antibody. **C:** Mitotic SKOV-3 cells stained with the DNA stain DAPI, anti-GC UNC-45 peptide and anti-myosin II. **A:** The cellular localization of GC UNC-45 only partially overlaps myosin V. In particular, whereas significant myosin V staining is present over the nuclei, very little GC UNC-45 is present over the nuclei. In contrast, there is a significant and robust co-localization of GC UNC-45 with myosin II in interphase (**B**), metaphase (**C**), and telophase (**D**) of mitotic cells (indicated by the **arrow** pointing toward the cleavage furrow). **E:** Lysate of ES-2 cells transfected with negative control siRNA or GC UNC-45-siRNA-1 (100 nmol/L) for the time indicated was immunoblotted with antibodies against myosin II, myosin V, or myosin VI. Equal protein loading was verified by using an antibody directed against β -actin. Original magnifications, $\times 630$ (**D**).

siRNA treatment, the levels of myosin V was notably decreased. Because the effect of the GC UNC-45 down-regulation on proliferation of ovarian cancer cells was already evident between 24 and 48 hours after transfection with GC UNC-45 siRNA, it is not likely that the decrease in myosin levels is responsible for the slowed cell proliferation after GC UNC-45 down-regulation.

Localization of GC UNC-45 in Leading Edges of Moving Cells and Its Effect on Cell Motility

Migration of cancer cell occurs as a result of forces generated by the contraction of cytoskeletal proteins. Whereas myosin II is widely recognized to play a role during posterior retraction of moving cells,^{30–34} recent

Figure 6. Localization of GC UNC-45 on the leading edges and its effect on cell motility. **A:** Microchambers of confluent SKOV-3 ovarian cancer cells were scratched, and migrating cells were stained with anti-myosin II and anti-GC UNC-45 peptide antibody 5 hours after scratch. **Arrow** indicates overlapping staining between myosin II and GC UNC-45 in the leading edges of moving cells. **B:** Confluent (**left**) and subconfluent (**right**) SKOV-3 ovarian cancer cell lines were stained with anti-GC UNC-45. **Arrows** indicate GC UNC-45 perinuclear localization in confluent cultures and GC UNC-45 localization in leading edges of spreading cells. **C:** Plates of confluent ES-2 cells were examined by phase-contrast microscopy at the time of removal by scratching ($t = 0$ hours) and 6 hours later ($t = 6$ hours). Fewer ES-2 cells transfected with GC UNC-45-siRNA spread across the plastic substratum compared with ES-2 cells transfected with neg. control-siRNA. **D:** Analysis of the number of cells spreading across the wound, results are means $\pm$ SD of three independent experiments. $***P < 0.001$. **E:** Plates of confluent SKOV-3 p-EGFP-C1- or p-EGFP-C1-GC UNC-45-transfected cells were examined by immunofluorescent microscopy at the time of removal by scratching ($t = 0$ hours) and 6 hours later ($t = 6$ hours). SKOV-3 cells transfected with p-EGFP-C1-GC spread more rapidly across the plastic substratum into the wounded area compared with SKOV-3 p-EGFP-C1-transfected cells. **F:** Analysis of the number of cells spreading across the wound. Results are means $\pm$ SD of three independent experiments. $**P < 0.02$. Original magnifications, $\times 630$ (**B**).

studies also indicate a role for myosin II in the leading edges of spreading cells.^{20,35} Thus, given the role for myosin II in cell motility and GC UNC-45 in its functional assembly, we investigated a possible role for GC UNC-45 in ovarian cancer spreading. First, we investigated whether GC UNC-45 co-localizes with myosin II at the leading edges of ovarian cancer cell lines. For this purpose, immunostaining of GC UNC-45 and myosin II was performed in SKOV-3 ovarian cancer cell lines during a scratch wound assay. As shown in Figure 6A, myosin II and GC UNC-45 are found to co-localize partially at the leading edges of moving cells, consistent with a role for GC UNC-45 during cell spreading. To explore further the localization of GC UNC-45 during cell movement, immunostaining of GC UNC-45 was compared between contact-inhibited and scratched SKOV-3 monolayers. Whereas endogenous GC UNC-45 was mainly localized around the nuclei in nonmigrating, confluent cells (Figure 6B, left), GC UNC-45 was found to localize in the leading edges of migrating ovarian cancer cells (Figure 6B, right). Finally, to investigate directly the potential functional role for GC UNC-45 during cell movement, the effect of GC UNC-45 knockdown and the effect of ectopic expression of GC UNC-45 on cell spreading was tested by scratch assay. Knockdown experiments showed that ES-2 cells transiently transfected with GC UNC-45-siRNA-1 spread across plastic substratum at a significantly reduced rate ($P = < 0.001$) compared with ES-2 cells transfected with a negative control siRNA (Figure 6, C and D). In contrast to the delayed spreading of SKOV-3 cells across the plastic substratum on GC UNC-45

knockdown, ectopic overexpression of GC UNC-45 in SKOV-3 cells significantly enhanced the rate ($P < 0.02$) of spreading across the wound edge in p-EGFP-C1-GC UNC-45 cells as compared with the vector-transfected cells (Figure 6, E and F).

Discussion

In this study, we found that GC UNC-45, a member of the UCS co-chaperone family, is aberrantly overexpressed in ovarian cancer cell lines as compared with immortalized ovarian surface epithelium and in serous carcinoma tissues compared with benign tumors and normal ovarian epithelium. GC UNC-45 expression is significantly higher in high- compared with low-stage serous carcinoma, even when examining only high-grade disease.

To address the significance of GC UNC-45 overexpression in ovarian cancer and its biological function, we examined both the consequences of manipulating its level and the localization of GC UNC-45 with respect to known interacting proteins. Here, we show that ectopic overexpression or knockdown of GC UNC-45 increased and reduced the proliferation rate of ovarian cancer cells, respectively, suggesting that GC UNC-45 overexpression may both contribute to and be necessary for rapid ovarian cancer expansion. We also found that overexpression of GC UNC-45 in HeLa cells and 293 cells enhances their rate of proliferation (not shown). This suggests that potential elevation of GC UNC-45 should be

explored in other cancer types and that GC UNC-45 functions similarly in different cell types.

GC UNC-45 orthologs in fungi and nematodes act as chaperone molecules for the assembly and folding of conventional and nonconventional myosins, including myosins II and V. Furthermore, the function of GC UNC-45 paralogs and myosins II and V are critical during cytokinesis in these model organisms.⁸ Immunofluorescence studies in ovarian cancer cells suggest that GC UNC-45 co-localizes with myosins, myosin II in particular. This co-localization occurs during interphase but is most obvious during cytokinesis whereupon GC UNC-45 and myosin II accumulate and co-localize within the cleavage furrow. Gene dosage studies in model organisms suggest that cytokinesis is extremely sensitive to the levels of myosin. Further, studies have shown that UCS protein binds directly to and prevents thermal aggregation of the myosin head domain and are necessary for its activity.^{7,36} Consistent with the genetic studies, our study shows that knockdown of GC UNC-45 slows the rate of ovarian cancer cell proliferation within 48 hours, although levels of myosins II, V, and VI do not change during this period. This suggests that continued chaperone function of GC UNC-45 is required to maintain correct folding of myosins and rapid proliferation of ovarian cancer cells. Conversely, ectopic overexpression of GC UNC-45 in SKOV-3 cells increases the rate of cell proliferation, suggesting that GC UNC-45 levels may be limiting for the proliferation of SKOV-3 cells.

High-grade ovarian cancer typically invades and metastasizes rapidly.³⁷ Metastasis is associated with changes in cancer cell morphology and motility, processes regulated by the cytoskeleton and myosin motors. Immunofluorescence studies in ovarian cancer cells suggest that GC UNC-45 co-localizes with myosins II during ovarian cancer cell spreading. Myosin II is an important determinant of force generation in cellular motility, and the myosin II-specific inhibitor blebbistatin inhibits pancreatic adenocarcinoma cellular invasiveness.^{21,38} Myosin II accumulates at the lamellipodia leading edges in cancer cells and interacts with the S100A4 metastasis factor.²⁰ A higher level of GC UNC-45 expression was observed in advanced versus early-stage disease suggesting a hypothesis that elevated GC UNC-45 expression may also contribute to ovarian cancer metastasis by facilitating myosin II assembly.

UCS proteins act in concert with Hsp90 to chaperone the assembly of myosin II. Interestingly the Hsp90 inhibitor geldanamycin and its analogs are active against ovarian cancer and inhibit cytokinesis in model organisms.^{18,39–41} Indeed, Hsp90 inhibitors reduce motility in *C. elegans*, consistent with a role in myosin assembly with UNC-45. Furthermore numerous studies indicate that Hsp90 inhibitors block cell migration and angiogenesis.^{42–44} However, it is important to note that Hsp90 acts as a chaperone for the folding of many proteins besides the myosins, and these agents may affect other cellular functions. Thus, future study is required to determine whether reduced GC UNC-45 function contributes to the cytotoxic effect of Hsp90 inhibition in ovarian cancer cells and to determine whether GC-UNC-45 represents a useful target for developing drugs to slow ovarian cancer progression.

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