

# Lessons from border cell migration in the *Drosophila* ovary: A role for myosin VI in dissemination of human ovarian cancer

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**Dissemination of ovarian cancer is a major clinical challenge and is poorly understood at the molecular level due to a lack of suitable experimental models. During normal development of the *Drosophila* ovary, a dynamic process called border cell migration occurs that resembles the migratory behavior of human ovarian cancer cells. In this study, we found that myosin VI, a motor protein that regulates border cell migration, is abundantly expressed in high-grade ovarian carcinomas but not in normal ovary and ovarian cancers that behave indolently. Inhibiting myosin VI expression in high-grade ovarian carcinoma cells impeded cell spreading and migration *in vitro*. Optical imaging and histopathologic studies revealed that inhibiting myosin VI expression reduces tumor dissemination in nude mice. Therefore, using genetic analysis of border cell migration in *Drosophila* is a powerful approach to identify novel molecules that promote ovarian cancer dissemination and represent potential therapeutic targets.**

Ovarian cancer is the fifth major cause of cancer death among women in the U.S. Approximately 70% of ovarian cancer patients are diagnosed with disease that has spread beyond the ovaries (1). Despite advances in platinum-taxol regimens, the cure rate for women with disseminated ovarian cancer is dismal, with <30% of these patients surviving 5 years after initial diagnosis (1). *i.p.* dissemination is the most common pattern of spread of ovarian cancer. The lack of suitable experimental models has hampered efforts to systematically identify molecules that promote dissemination of ovarian cancer. However, there is increasing evidence that genetic analysis of border cell migration in the *Drosophila* ovary may be a useful starting point. During normal development of the *Drosophila* ovary, a dynamic process of epithelial cell migration occurs that is reminiscent of the migratory behavior of cancer cells. Each egg chamber in the *Drosophila* ovary is surrounded by a simple epithelium. During oogenesis, a pair of specialized cells differentiates at the anterior end of the egg chamber and recruits four to eight additional cells to form a border cell cluster (2, 3). At stage nine of development, the border cell cluster delaminates from the epithelium and migrates between neighboring nurse cells to the oocyte (2, 3). Several genes that control border cell migration are homologous to human genes that promote ovarian cancer progression. *slbo*, the first locus identified to control border cell migration, encodes the homolog of the transcription factor C/EBP (4). One downstream target of C/EBP identified in *Drosophila* is focal adhesion kinase (5). This kinase is thought to stimulate cell motility by promoting turnover of focal contacts and is overexpressed in ovarian cancers (6). *taiman*, another locus that controls border cell migration, encodes a steroid hormone receptor coactivator related to AIB1 (5). Amplification/overexpression of the *AIB1* gene has been detected in breast and ovarian cancers and is associated with poor prognosis (7, 8). Signaling through the epidermal growth factor receptor stimulates ovarian cancer cell migration (9) and guides migration of border cells (10). Given the similarities at the molecular and behavioral levels between

border cell migration and ovarian cancer progression, studying human homologs of other *Drosophila* genes that control border cell migration could provide new insight into the migratory behavior of ovarian cancer cells.

Geisbrecht and Montell (11) reported that the actin-based motor protein myosin VI is highly expressed in migrating border cells, and that depleting myosin VI from border cells severely retards their migration. Because border cells behave like cancer cells, we investigated whether myosin VI regulates ovarian cancer cell migration. Myosin VI was found to be absent from normal ovary and expressed in ovarian cancers at levels that strongly correlated with the aggressiveness of clinical behavior. Inhibiting myosin VI expression substantially impeded migration of ovarian cancer cells *in vitro* and reduced *i.p.* dissemination of tumor cells propagated in nude mice. This study supports the validity of a “cross-species” approach of using genetic analysis of border cell migration in *Drosophila* to identify novel mediators of ovarian cancer progression.

## Materials and Methods

### Tissue Microarray Construction and Immunohistochemical Analysis.

Two separate core biopsies were taken from paraffin-embedded specimens of 156 cases of epithelial ovarian tumors at the University of Texas M. D. Anderson Cancer Center. Microarrays were constructed by using a Beecher Instruments (Silver Spring, MD) tissue arrayer. Cases included the following: clear cell ( $n = 19$ ), high-grade serous ( $n = 27$ ), high-grade endometrioid ( $n = 17$ ), low-grade serous ( $n = 11$ ), and low-grade endometrioid ( $n = 14$ ) carcinomas; serous ( $n = 29$ ) and mucinous ( $n = 23$ ) borderline tumors; and serous ( $n = 8$ ) and mucinous ( $n = 8$ ) cystadenomas. Microarrays included biopsies taken from 10 specimens of normal ovaries. Microarray slides were stained by using a rabbit polyclonal antibody to myosin VI (1:200 dilution). The C-terminal tail of human myosin VI (AA1062–1284) was amplified from IMAGE clone 5285868 and cloned in-frame into the pMAL<sub>TEV</sub> vector. The fusion protein was expressed and purified as described (11). The C-terminal myosin VI was cleaved with tobacco etch virus protease and purified before antibody production (Covance, Princeton, NJ). An average myosin VI staining score was determined for biopsies of each case. Histopathologic diagnosis of specimens was made before and independently of immunohistochemical analysis to eliminate bias in scoring staining intensity. Staining intensity was graded on a 0–3 scale, where 0 = no staining as assessed by staining with anti-rabbit secondary antibody alone, 1 = weak, 2 = moderate, and 3 = intense staining.

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Abbreviations: Dab2, disabled-2; siRNA, short interfering RNA; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide.

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**Transfection of Ovarian Carcinoma Cells with Antisense Sequences.** A 295-bp sequence spanning the first 98 bp of coding sequences plus 197 bp of the 5' UTR of human myosin VI was amplified from a partial cDNA clone (IMAGE 4295486) and cloned in antisense orientation into the pIRES-EGFP expression vector containing the gene encoding GFP (Clontech). This construct (pIRES-EGFP-myoVI-AS) was stably transfected into cells of the human ovarian carcinoma cell line ES-2 (provided by Pat Morin, National Institute on Aging, Baltimore). ES-2 cells were cultured in McCoy5A medium (GIBCO) containing 10% FBS. Transfected clones, derived from single colonies, were selected by G418 (400  $\mu\text{g}/\text{ml}$ ) (Invitrogen) and screened for reduced myosin VI expression by Western blot analysis. Control cell lines were generated by stably transfecting ES-2 cells with the pIRES-EGFP vector. Myosin VI was also detected in fixed cells by using myosin VI antibody and Texas red-labeled anti-rabbit IgG antibody (Kirkegaard & Perry Laboratories).

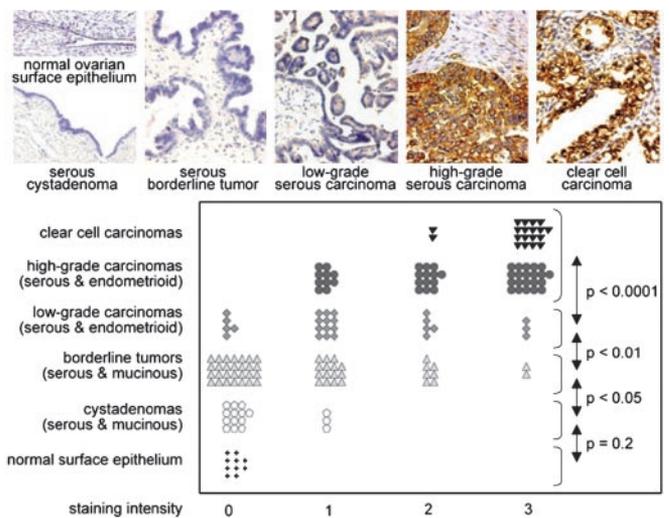
**Transfection of Ovarian Carcinoma Cells with Short Interfering RNA (siRNA) Oligomers.** Sequences of siRNA oligomers (synthesized by Qiagen-Xeragon, Germantown, MD) were as follows:

myoVI-siRNA-1, 5'-GCUGGCAGUUCAUAGGAAUd-(TT)-3'; myoVI-siRNA-2, 5'-CGUGCUCCAAAGUCUGUUAd(TT)-3'. ES-2 cells were transfected with oligomers by using RNAiFect reagent (Qiagen, Valencia, CA). Efficiency of myosin VI knockdown was tested by using oligomers at a range of concentrations (50, 100, and 200 nM) and over a period of days (1, 2, and 3 days) by Western blot analysis. Blots were also probed by using actin antibody (Santa Cruz Biotechnology). As controls, ES-2 cells were mock-transfected (without siRNA) and transfected with nonsilencing fluorescein-labeled siRNA (Qiagen) under the same conditions as myosin VI siRNAs.

**In Vitro Cell Spreading and Migration Assays.** Confluent cultures of stably transfected ES-2 cells in 35-mm dishes were scratched by using a sterile pipette tip and examined by phase-contrast microscopy at the time of scratching and 5 h later. Scratch assays were also performed after transfection of cells for 24 h with myosin VI siRNA, with nonsilencing control siRNA, and without siRNA. For migration chamber assays,  $3 \times 10^4$  cells of stably transfected lines were seeded in the upper well of 24-well transwell migration chambers (Becton Dickinson). Fibronectin was added to the lower well as a chemoattractant. One hour after seeding, the filter separating the chambers was removed and its upper surface swabbed to remove nonmigrating cells. Migrating cells on the lower surface were stained with Giemsa stain and counted. Migration assays were also performed by seeding  $3 \times 10^4$  ES-2 cells that had been transfected 24 h earlier with and without siRNA, and migrating cells counted at 2 h after seeding.

**Proliferation Assays.** Cell proliferation was measured by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche Diagnostics, Indianapolis, IN). One thousand ES-2 cells stably transfected with pIRES-EGFP vector and cells stably transfected with pIRES-EGFP-myoVI-AS were seeded per well in 96-well plates. Optical density at 570 nm after MTT addition was measured daily over a 5-day time course. MTT assays were also conducted in parallel for mock-transfected ES-2 cells and cells transfected with myosin VI siRNA and with nonsilencing control siRNA at days 1, 2, and 3 after transfection.

**Inoculation and Imaging of ES-2 Cells in Nude Mice.** One million ES-2 cells stably transfected with pIRES-EGFP vector were suspended in 100  $\mu\text{l}$  of PBS and injected i.p. into one group of nine 4-week-old female nude mice. A second group of mice was injected i.p. with  $10^6$  ES-2 cells stably transfected with pIRES-EGFP-myoVI-AS. At 3 weeks after injection, mice from both groups were killed by CO<sub>2</sub> asphyxiation. Ascites was collected



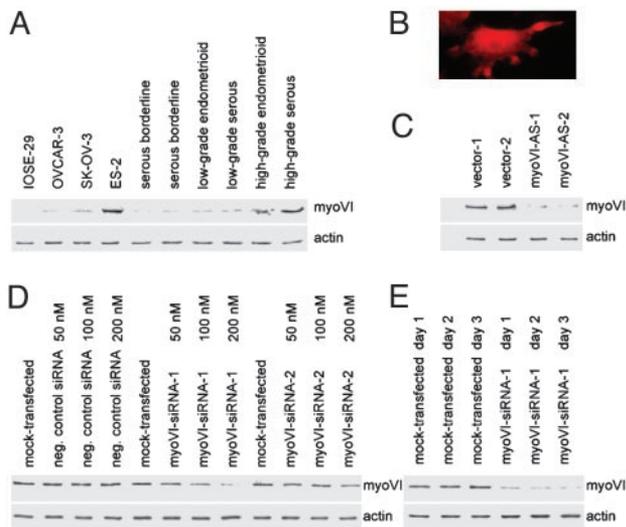
**Fig. 1.** Immunohistochemical staining of myosin VI in clinical specimens. Shown are representative examples of intense myosin VI staining in high-grade ovarian carcinomas, weak staining in low-grade carcinomas, and no staining in borderline ovarian tumor, cystadenoma, and normal ovary. Staining intensity for each case was graded as 0 = no staining, 1 = weak, 2 = moderate, or 3 = intense. Each symbol represents an individual case. Statistical significance of differences in staining intensities among indicated groups of cases was assessed by the Mann-Whitney *U* test.

and volume determined. The abdominal cavity was gently flushed with PBS to remove residual fluorescent ascitic cells that could interfere with visualizing nodules attached to host tissues. Abdominal cavities were viewed under a Leica MZ9.5 fluorescent stereoscope equipped with mercury lamp power supply and GFP filter set. Sections of tissues from both groups of mice were stained with hematoxylin/eosin for histopathologic analysis.

## Results

**Myosin VI Expression Is Associated with Aggressive Behavior of Ovarian Carcinomas.** Myosin VI expression levels were compared between normal ovary and epithelial ovarian tumors by immunohistochemical staining of tissue microarrays. Myosin VI was absent from or only weakly expressed in normal ovarian surface epithelium and benign ovarian cystadenomas (Fig. 1). Borderline (low malignant potential) ovarian tumors are distinguished from carcinomas by their lack of stromal invasion. Of the 52 borderline cases examined, 43 (83%) were myosin VI-negative or only weakly positive (Fig. 1). Myosin VI staining was higher in low-grade carcinomas compared to borderline tumors ( $P < 0.01$ ) and significantly stronger in high-grade compared to low-grade carcinomas ( $P < 0.0001$ ). Relative levels of myosin VI in tissue specimens were confirmed by Western blot analysis (Fig. 2A). Myosin VI expression was restricted to the epithelia (Fig. 1). Of the various forms of ovarian carcinoma, the clear cell subtype has a particularly unfavorable prognosis (12). Intense myosin VI staining was observed in almost all clear cell carcinoma cases studied (Fig. 1). These studies indicate that the level of myosin VI expression is strongly associated with aggressive behavior of ovarian carcinomas.

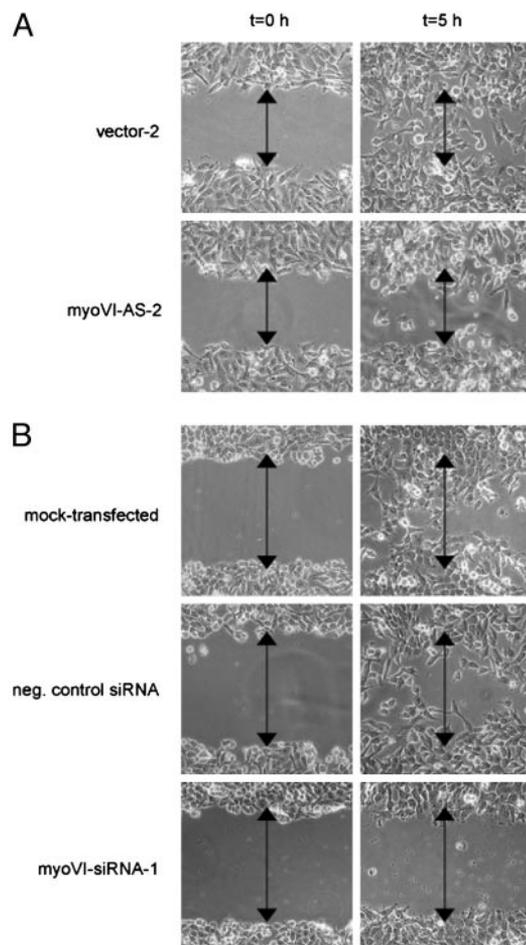
**Inhibition of Myosin VI Expression by Antisense and siRNA.** We investigated whether myosin VI regulates ovarian cancer cell migration by inhibiting its expression in ES-2 cells, a cell line established from a human clear cell carcinoma of the ovary (13). This cell line was chosen as a suitable model because clear cell carcinomas behave aggressively and strongly express myosin VI (Figs. 1 and 2A). Myosin VI was found to be distributed throughout the cytoplasm of ES-2 cells, with strong focal staining



**Fig. 2.** Myosin VI expression in ES-2 cells. (A) Western blot demonstrating levels of myosin VI in tissue specimens derived from different cases of borderline tumor and ovarian carcinoma, in nontumorigenic ovarian surface epithelial cells (IOSE-29) and ovarian carcinoma cell lines OVCAR-3, SK-OV-3, and ES-2. (B) Abundance of myosin VI in parental ES-2 cells throughout the cytoplasm and in membrane protrusions visualized by immunofluorescent staining. (C) Western blot confirming inhibition of myosin VI expression in two lines of ES-2 cells stably transfected with myosin VI antisense sequences (myoVI-AS-1, -2), compared to lines of ES-2 cells transfected with pIRES-EGFP vector (vector-1 and -2). (D) Efficiency of myosin VI knockdown in ES-2 cells with two different myosin VI siRNA oligomers (myoVI-siRNA-1, -2) was tested at concentrations of 50, 100, and 200 nM and assayed at 1 day after transfection (D) and at 1, 2, and 3 days after transfection by using oligomers at 200 nM (E). As controls, cells were treated with transfection reagent without siRNA (mock-transfected) and transfected with nonsilencing control siRNA (negative control siRNA).

in cellular extensions and protrusions (Fig. 2B). Two approaches were taken to inhibit myosin VI expression. First, ES-2 cells were stably transfected with pIRES-EGFP-myosin VI, a construct in which 197 bp of 5'-UTR and the first 98 bp of myosin VI coding sequences are expressed in antisense orientation. Western blot analysis confirmed that myosin VI expression was inhibited in ES-2 cell lines transfected with pIRES-EGFP-myosin VI-AS (myoVI-AS-1, -2), as compared to isolated lines of ES-2 cells stably transfected with pIRES-EGFP vector alone (vector-1 and -2) (Fig. 2C). In the second approach, myosin VI expression was inhibited by transfecting ES-2 cells with myosin VI siRNAs. Myosin VI expression was affected neither by the transfection reagent (mock-transfected) nor by nonsilencing control siRNA (negative control siRNA) (Fig. 2D). siRNA oligomers targeting different sites within the myosin VI gene were tested at a range of concentrations. The myoVI-siRNA-1 oligomer that targeted sequences within exon 16 exhibited efficient knockdown of myosin VI when used at 200 nM and for up to 3 days after transfection (Fig. 2D and E). In subsequent experiments, ES-2 cells were transfected with myoVI-siRNA-1 at a concentration of 200 nM and migration assayed at 1 day after transfection.

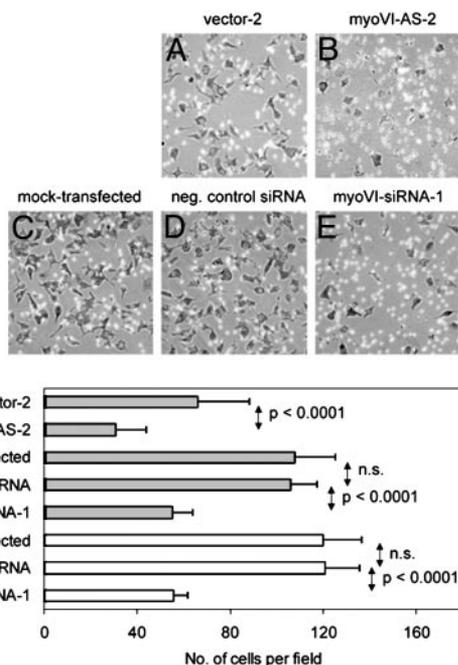
**Inhibiting Myosin VI Expression Impedes Cell Spreading and Migration of Ovarian Carcinoma Cells *in Vitro*.** The effect of inhibiting myosin VI on cell spreading was initially tested by the scratch assay. As shown in Fig. 3A, ES-2 cells stably transfected with myosin VI antisense sequences (myoVI-AS-2) spread across plastic substratum at a reduced rate compared to vector-transfected cells (vector-2). ES-2 cells transfected with myoVI-siRNA-1 oligomer also exhibited retarded cell spreading, as compared to mock-



**Fig. 3.** Inhibiting myosin VI expression impedes cell spreading. Plates of confluent ES-2 cells were examined by phase-contrast microscopy at the time of removal by scratching ( $t = 0$  h) and 5 h later. (A) Fewer ES-2 cells transfected with myosin VI antisense sequences (myoVI-AS-2) spread across the scratch than vector-transfected cells (vector-2). (B) Reduced cell spreading was also observed for ES-2 cells after transfection for 24 h with myoVI-siRNA-1 at 200 nM, as compared with mock-transfected cells and cells transfected with 200 nM negative control siRNA.

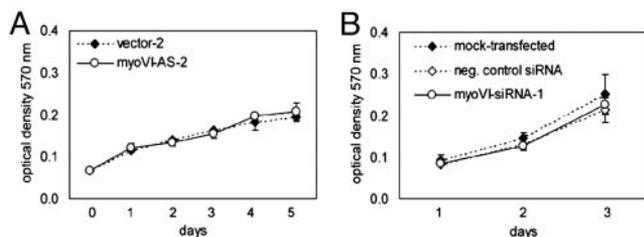
transfected cells and cells transfected with negative control siRNA (Fig. 3B). The effect of inhibiting myosin VI on cell migration was assayed in migration chambers. The ability of myoVI-AS-2 cells to migrate was reduced by 50% compared to vector-2 cells (Fig. 4A, B, and F). Migration was also significantly reduced when myosin VI expression was inhibited in ES-2 cells by myoVI-siRNA-1 oligomer (Fig. 4C–F). These observations were confirmed by using another ovarian carcinoma cell line SK-OV-3 (Fig. 4F).

**Inhibiting Myosin VI Expression Does Not Alter Cell Proliferation.** Studies of Geisbrecht and Montell (11) indicated that inhibiting myosin VI expression does not significantly alter border cell proliferation. MTT assays revealed no significant difference in proliferation of vector-2 and myoVI-AS-2 cells (Fig. 5A). Likewise, the proliferative activity of ES-2 cells transfected with myoVI-siRNA-1 was almost identical to that of mock-transfected cells and cells transfected with negative control siRNA (Fig. 5B). These studies eliminate the possibility that a decrease in the population of migrating cells in which myosin VI expression is inhibited is due to reduced proliferative activity, thus confirming a role for myosin VI in regulating cell migration.

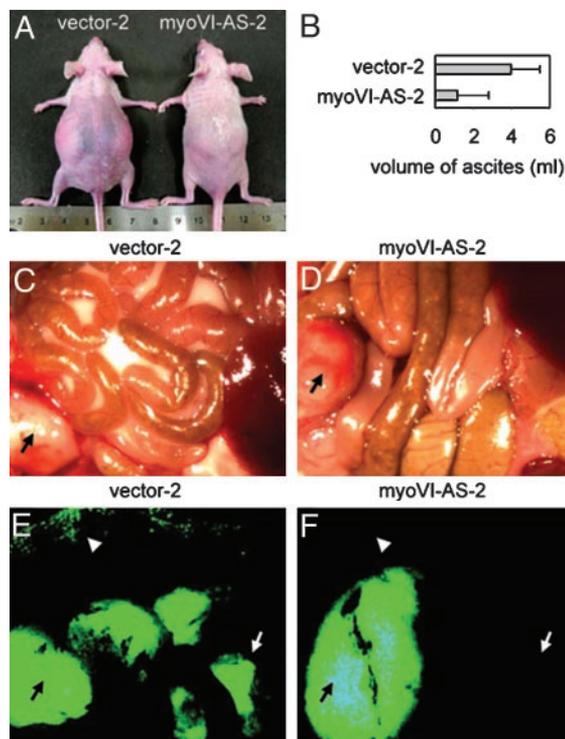


**Fig. 4.** Inhibiting myosin VI expression impedes cell migration. Equivalent numbers ( $3 \times 10^4$ ) of vector-2 (A) and myoVI-AS-2 (B) cells were seeded in migration chambers. After 1 h, cells that migrated through chamber filters were visualized by light microscopy. At 24 h after transfection with no oligomer (C), 200 nM neg. control siRNA (D) and 200 nM myoVI-siRNA-1 (E),  $3 \times 10^4$  ES-2 cells were seeded in chambers and migrating cells counted at 2 h thereafter. Migrating cells were counted in five different fields per assay at  $\times 100$ . Each assay was performed in triplicate. Shown are mean  $\pm$  SD of migrating cells per microscopic field (F, gray bars). Statistical analysis was performed by using Student's *t* test. *P* values  $> 0.05$  were considered not significant (n.s.). Migration assays were also performed by using SK-OV-3 cells under the same conditions as for ES-2 cells, except that migrating cells were counted at 4 h after seeding (F, white bars).

**Inhibition of Myosin VI Expression Impedes i.p. Dissemination of Ovarian Carcinoma Cells Propagated in Nude Mice.** We investigated the effect of inhibiting myosin VI expression on dissemination of ovarian carcinoma cells *in vivo* by comparing tumor progression in nude mice inoculated i.p. with GFP-expressing myoVI-AS-2 cells with mice inoculated i.p. with GFP-expressing vector-2 cells. At 3 weeks after inoculation, mice inoculated with myoVI-



**Fig. 5.** Inhibiting myosin VI expression does not affect cell proliferation. (A) Equivalent numbers ( $1 \times 10^3$ ) of myoVI-AS-2 cells (open circles) and vector-2 cells (solid diamonds) were seeded per well in 96-well plates. Proliferation was assayed by MTT assay at the time of seeding (day 0) and daily for 5 days thereafter and did not significantly differ between the two cell lines ( $P > 0.05$ ). (B) Proliferation was also measured in ES-2 cells at 1, 2, and 3 days after transfection with 200 nM myoVI-siRNA-1 (open circles) and did not significantly differ from that of cells transfected with 200 nM negative control siRNA (open diamonds) and with no oligomer (solid diamonds). Each assay was performed in triplicate. Shown are mean  $\pm$  SD of proliferative activity measured in terms of optical density at 570 nm on each given day.

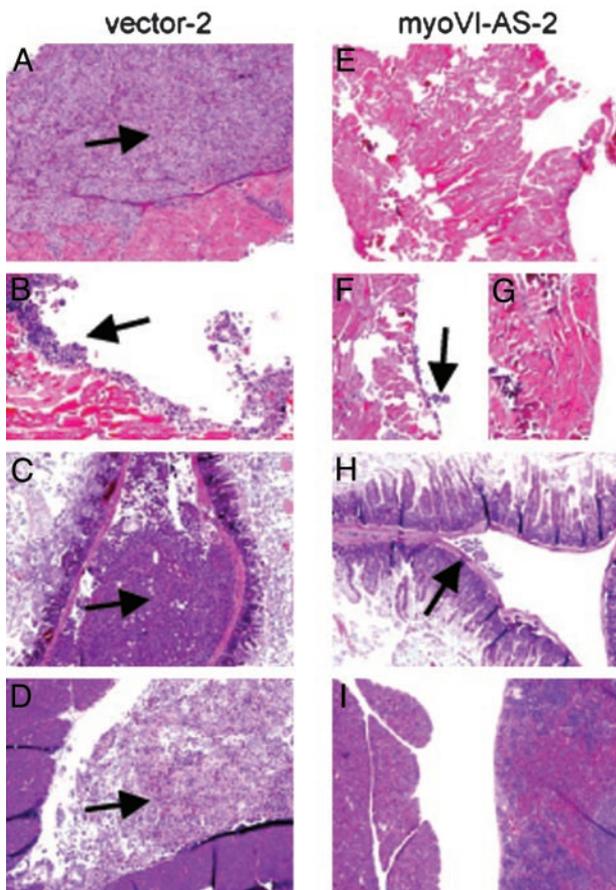


**Fig. 6.** Inhibiting myosin VI prevents dissemination of ES-2 tumors propagated in nude mice. One group of nude mice was inoculated i.p. with  $10^6$  GFP-expressing myoVI-AS-2 cells and another group with  $10^6$  GFP-expressing vector-2 cells. At 3 weeks after inoculation, mice inoculated with myoVI-AS-2 cells exhibited less abdominal distention (A) and significantly less ascites formation (ml) than mice inoculated with vector-2 cells ( $P < 0.05$ ) (B). Both groups of mice formed a tumor mass at the site of injection (black arrow) (C–F). However, i.p. dissemination in mice inoculated with myoVI-AS-2 cells was less extensive than in mice inoculated with vector-2 cells as visualized by a stereoscope under brightfield (C and D) and fluorescent (E and F) conditions. Dissemination to the omentum (white arrow) and to the abdominal cavity wall (white triangle) was observed only in mice inoculated with vector-2 cells (E).

AS-2 cells exhibited less abdominal distention and a significantly smaller amount of ascites than mice inoculated with vector-2 cells (Fig. 6A and B). Both groups of mice formed a solid tumor mass at the site of injection (Fig. 6C and D). However, dissemination of tumor throughout the bowel was substantially reduced in mice inoculated with myoVI-AS-2 cells (Fig. 6D). Inspection of the abdominal cavity of mice inoculated with vector-2 cells using a fluorescence stereoscope revealed extensive dissemination throughout the bowel and to the omentum and numerous small nodules attached to the abdominal cavity wall (Fig. 6E). Attachment of tumor to these tissues and other sites such as the pancreas and diaphragm was confirmed by histopathologic analyses (Fig. 7A–D). In contrast, dissemination throughout the bowel and to the omentum was very limited in mice inoculated with myoVI-AS-2 cells (Fig. 6F). Only minute focal deposits were detected on the bowel and abdominal cavity wall, and many regions of the wall were tumor-free (Fig. 7F–H). The diaphragm, pancreas, and spleen of mice inoculated with myoVI-AS-2 cells were tumor-free (Fig. 7E and I). These observations indicate that inhibiting myosin VI expression in ovarian carcinoma cells substantially impedes i.p. dissemination *in vivo*.

## Discussion

Genetic manipulation of lower organisms such as *Drosophila* has enabled identification of molecules that control basic biological



**Fig. 7.** Sections of tissues of mice killed at 3 weeks after inoculation with vector-2 cells (A–D) and with myoVI-AS-2 cells (E–I) were stained with hematoxylin/eosin. Attachment of tumor (black arrows) to the diaphragm (A), abdominal cavity wall (B), bowel (C), and pancreas (D) was observed in mice inoculated with vector-2 cells. In contrast, the diaphragm (E), pancreas, and spleen (I) of mice inoculated with myoVI-AS-2 cells were tumor free. Only minute focal deposits were detected on the abdominal cavity wall (F and G) and bowel (H).

processes in humans and that contribute to disease pathogenesis. Various tumor suppressor genes have been identified in *Drosophila* by loss-of-function mutations that lead to overgrowth of larval brains and imaginal discs (14–16). Border cell migration in the *Drosophila* ovary is a normal developmental process that resembles the migratory behavior of human ovarian cancer cells. Here, we found that inhibiting myosin VI, a motor protein that regulates border cell migration, in metastatic human ovarian cancer cells impedes their ability to migrate *in vitro* and *in vivo*. These findings strongly support the validity of using genetic analysis of border cell migration in *Drosophila* to identify related molecules that promote dissemination of human ovarian cancer.

Myosins are a large family of structurally diverse motor proteins. Each myosin utilizes energy from ATP hydrolysis to generate force for unidirectional movement along actin filaments. Myosin VI is only one of two myosins known to move toward the pointed (minus) end of the actin filament, whereas other myosins move toward the barbed (positive) end (17). Little is known regarding the role of myosins in promoting cancer cell migration. Cell migration through tissues results from a continuous cycle of coordinated interdependent steps that involve dynamic changes in actin filaments, cell adhesion, and membrane traffic. The first step involves extension of the leading edge of the cell and generation of a traction force that leads to forward

propulsion of the cell body (18). This step is followed by cell interactions with the extracellular matrix (ECM), formation of focal contacts, localized proteolysis of ECM components, disassembly of focal contacts, and detachment of the cell's trailing edge. The localization of myosin VI in cell extensions and membrane protrusions of parental ES-2 cells (Fig. 2B) is suggestive of a role for myosin VI in propulsion. Myosin VI is localized in membrane ruffles at the leading edge of fibroblasts (19). Border cells depleted of myosin VI exhibit considerably fewer membrane protrusions (11). It has been suggested that by moving toward the minus end of actin filaments (away from the plasma membrane), myosin VI could push actin filaments outward to form a protrusion (20, 21). Therefore, inhibiting myosin VI expression could impede the initial step in the migration process by disrupting the protrusive force in the leading edge required for propelling cell movement.

Geisbrecht and Montell (11) reported that border cells lacking myosin VI have reduced levels of E-cadherin and  $\beta$ -catenin, and that border cells lacking either of these adhesion molecules have reduced levels of myosin VI. Myosin VI interacts with  $\beta$ -catenin, suggesting that myosin VI promotes border cell migration by stabilizing E-cadherin and  $\beta$ -catenin (11). E-cadherin is required for border cell migration (22). However, high-grade aggressive carcinomas typically express little or no E-cadherin (23). Loss of cell adhesion through down-regulation of E-cadherin is thought to play an important role in metastasis (23). In preliminary studies, we observed no change in E-cadherin levels in ES-2 cells when myosin VI expression is inhibited (data not shown). It is possible that myosin VI promotes migration of ovarian cancer cells by a mechanism different from that in border cells and independent of E-cadherin. However, it has been speculated that E-cadherin is required to provide the traction necessary for epithelial cell migration and is rapidly turned over in migrating cells (2). Therefore, the possibility that myosin VI promotes ovarian cancer cell migration by an E-cadherin-dependent mechanism cannot be eliminated.

Various lines of evidence indicate that myosin VI also plays an important role in endocytosis. Receptor internalization by endocytosis is an integral step in the process of cell migration. After disassembly of focal contacts, integrins detach from the extracellular matrix and become internalized by endocytotic vesicles for recycling toward the cell's leading edge to reinitiate the process of propulsion, adhesion, and detachment (18). Cell migration mediated by the urokinase plasminogen activator receptor is likewise a dynamic process involving receptor internalization and recycling (24). Myosin VI associates with clathrin-coated vesicles and modulates clathrin-mediated endocytosis (25). Myosin VI could potentially function in cancer cell migration not only by providing the initial propelling force but also by promoting internalization and recycling of key mediators such as integrins and urokinase plasminogen activator receptor.

The mechanism by which myosin VI promotes receptor internalization is poorly understood but may involve its interaction with various molecules, including the phosphoprotein disabled-2 (Dab2) (26). Dab2 is expressed in normal ovarian surface epithelium but is lost in 85% of ovarian carcinomas (27). Loss of Dab2 appears to occur as an early step in epithelial ovarian neoplasia and correlates with loss of basement membrane of the ovarian surface before morphologic transformation (27, 28). The inverse correlation of Dab2 and myosin VI expression in normal and malignant ovarian tissues is consistent with the apparently opposing effects of these molecules on tumor progression. However, it has been reported that in the proportion of ovarian cancers that retain Dab2, metastatic lesions have higher levels of Dab2 than the primary tumor (28). Dab2-positive tumors were found to acquire the ability to deposit pseudobasement membrane around cells, suggesting that pseudobasement membrane may promote metastasis (28). The functional interactions be-

tween Dab2 and myosin VI in promoting ovarian cancer cell migration have yet to be determined. However, it is possible that the mechanism of action of myosin VI may differ depending on the relative level of Dab2 and/or the status of the surrounding basement membrane.

In addition to its interaction with  $\beta$ -catenin and Dab2, myosin VI interacts with various other molecules including the PDZ domain-containing proteins GLUT1CBP and SAP97 (29, 30). The multiple unique domains in myosin VI potentially represent ideal drug targets against which specific small molecule inhibitors could be designed. Another approach to block myosin VI would be to inhibit its activation. One likely candidate target is p21-activated kinase 1 (pak1). pak1 phosphorylates myosin VI in human epidermoid carcinoma cells (19) and promotes motility and invasiveness of various cell types (31, 32). *PAK1* copy number gains and overexpression are prevalent in high-grade ovarian carcinomas (33). Because high-grade ovarian carcinomas strongly express myosin VI (Fig. 1), pak1 could promote ovarian cancer metastasis through its activation of myosin VI.

Tumor grade, histologic subtype, and stage of disease are critical parameters for assessing prognosis of ovarian cancer. Among the common histologic subtypes, low-grade tumors more frequently present at earlier stages than high-grade tumors. Of the serous and endometrioid carcinomas we studied, 52% of low-grade but only 29% of high-grade cases were at stages I/II. Among serous and endometrioid carcinomas, we found myosin VI staining intensity not only to be strongly associated with high tumor grade but also to correlate with disease stage ( $R = 0.81$ ,  $P < 0.001$  determined by Spearman analysis). However, clear cell carcinoma has poorer prognosis stage for stage than the other more common subtypes but is more frequently detected at earlier stages (12). Of the clear cell cases we studied, 63% were stage I/II, but 89% exhibited intense myosin VI staining (Fig. 1). It therefore appears that the myosin VI level reflects the ability or potential of a tumor to disseminate rather than the actual

progression of disease. Tumor progression is controlled by other factors in addition to those that mediate cell motility. Several apoptosis regulators and proteases are differentially expressed in clear cell and serous carcinomas at different disease stages (34). Such differences in molecular signature could account for the paradoxical aggressive behavior and early presentation of clear cell carcinomas.

The relationship between ovarian cancer dissemination and ascites formation is not well defined. It has been proposed that accumulation of ascites involves increased vascular permeability (35) and blockage of outflow due to obstruction by ovarian cancer cells of the diaphragmatic lymphatics (36). In this study, we observed that ascites accumulation was markedly reduced in mice bearing tumors in which myosin VI expression is inhibited, and that diaphragms of these mice were tumor free. It is possible that by inhibiting myosin VI, migration of tumor cells to the diaphragm is impeded, thereby permitting outflow of ascites. Although the precise mechanism by which myosin VI promotes ovarian cancer cell migration has yet to be defined, this study provides a conceptual framework for identifying novel molecules that control the dissemination of human ovarian cancer. Further pursuit of this cross-species approach has strong potential to uncover better prognostic markers and promising therapeutic targets.

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