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Approaches toward Investigation of Metastatic Progression of Ovarian Tumor Cells

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We, the thesis committee for the above candidate for the

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Abstract of the Thesis

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Metastasis is a common cause of cancer death. Tumor cells metastasized in the peritoneal organs are associated with formation of ascites in patients with advanced stages of epithelial ovarian cancer (EOC). Previous studies show cellular clusters in ascites of EOC patients are enriched with disseminated ovarian tumor cells that exhibit invasive capability *in vitro,* and that cell lines derived from these cells are tumorigenic. However, little is known about the tumorigenic and metastatic capabilities of these ovarian tumor cells. To initiate the investigation into the metastatic progression in ovarian cancer, tumor cells enriched in clusters of ascites of EOC patients were isolated and used to generate xenografts in NOD-SCID mice. Ovarian tumor cells were tagged with green fluorescence protein (GFP) using lentivirus to track their path during metastastic progression in the mice. Judging from the increased GFP signal in ascites and peritoneal organ metastases, the ovarian cancer xenografts established in NOD-SCID mice can be useful in the investigation on the molecular mechanism of metastatic progression in ovarian cancer.

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A. Introduction

It is estimated that there will be 22,430 new cases and 15,280 deaths from ovarian cancer in the United States in 2007 (American Cancer Society, 2007). Epithelial ovarian cancer (EOC) is the most common type of ovarian cancer. Most patients with EOC have widespread metastases at the time of diagnosis and often result in poor prognosis. Current anti-EOC therapies eliminate the bulk tumor but EOC often relapses. However, little is known about the tumor cells that are responsible for recurrence of the cancer.

Metastasis in human EOC often occurs via the transcoelomic route (Tan, et al., 2006). Although the mechanism of transcoelomic metastasis in EOC has not been determined, tumor cells may detach from primary tumors and propagate in the peritoneal cavity. Then, ovarian tumor cells disseminate into the peritoneal cavity and invade the peritoneum and its underlying organs to form metastases (Fujimoto, et al., 1996). Blockage of lymphatic drainage of ascitic fluid by invasive tumor cells and secretion of pro-dilation factors by the tumor cells or peritoneal mesothelial cells may cause the development of ascites in ovarian cancer (Burghardt, et al., 1991, Tan, et al, 2006). Thus, accumulation of ascites may largely reflect invasive and metastatic potential of the ovarian tumor cells. Recent studies showed that tumor cells enriched in clusters in ascites of EOC patients were able to disaggregate and invade normal mesothelial cells *in vitro* (Burleson, et al., 2006). In addition, cell lines derived from these tumor cells were tumorigenic in immuno-deficient mice (Bapat, et al., 2005). However, little is known about the tumorigenic and metastatic capabilities of these ovarian tumor cells.

To initiate the investigation on metastatic progression in ovarian cancer, tumor cells enriched in clusters of ascites of EOC patients were isolated and used to generate

xenografts in NOD-SCID mice. Cells in the xenografts were identified as “tumor cells” by criteria that showed the cellular morphology and immuno-reactivity toward epithelial cell lineage markers including epithelial cell adhesion molecule (EpCAM), cytokeratins (CK), epithelial surface antigen (ESA) and epithelial membrane antigen (EMA) similar to the tumor cells in ascites of EOC patients.

Stable expression of GFP in tumor cells is an effective way to track their metastasis. Tracking cells that express GFP is sensitive and rapid, making it possible to determine the fate of injected tumor cells *in vivo* (Yang, et al., 2000). Lentiviral transfer vectors encoding GFP have been used to transduce normal or neoplastic human cells (Hope, et al., 2004; Micucci, et al., 2006). In this study, ovarian tumor cells were tagged with green fluorescence protein (GFP) using lentivirus to track their path during metastastic progression in the mice. GFP-labeled tumor cells in single cell suspension were isolated by fluorescence-activated cell sorting (FACS) and expanded in NOD-SCID mice. Cell clusters consisted of GFP-expressing cells were formed in ascites of the recipient mice, and they were isolated by size exclusion. The resulting enriched tumor cells with GFP were evaluated for their ability to propagate and metastasize in the EOC xenograft. Tumor growth was evaluated by estimating GFP signal in the peritoneal effusion (ascites) and organs by fluorescence microscopy and by Maestro *in vivo* imaging. Judging from the increased GFP signal in ascites and peritoneal organ metastases, the ovarian tumor xenografts established in NOD-SCID mice maybe useful in the investigation on the molecular mechanism of metastatic progression in ovarian cancer.

B. Materials and methods

B1.Tumor specimen transplantation

The human subject protection protocol for collection of tumor tissues and peritoneal effusion (ascites) from patients with ovarian cancer was reviewed and approved by the Institutional Review Board at Stony Brook University, NY. Primary tumors were first suspended in DMEM medium containing 10% FBS and minced with sterile scalpel blade over ice. Tumor fragments were treated with 0.05 % Trypsin/EDTA at 37 ºC for 30 min and frequently mixed with a 10-ml pipette. Dissociated tumor cells were separated from large fragments by filtering through a 35-μm Nylon screen mesh. Ascites obtained from EOC patients were first spanned down at 800 g for 5 minutes to obtain the cellular component. Large tissue fragments were removed with a 35-μm Nylon screen mesh. $1x10^4$ cells from each primary tumor or ascites sample were set aside for cell viability assay by incubating with a mixture of 1 mM ethidium homodimer-1 (EthD-1) and 5 mM Calcein AM (Invitrogen). $1x10^5$ cells were fixed, blocked, and then stained with a mixture (referred to as Epi-mix) of FITC-conjugated primary antibodies against epithelial surface antigen (ESA) (clone VU-1D9, Biomeda, Foster City, CA), epithelial membrane antigen (EMA) (clone GP1.4, Biomeda, Foster City, CA), and epithelial cell adhesion molecule (EpCAM) (clone Ber-Ep4, Dako, Carpinteria, CA), followed by 10 min staining with 10 μg/ml Hoechst 33342 (Invitrogen). The cells were then washed with PBS by repeated suspension and centrifugation. The concentration of Epi-mix$^+$Hoechst$^+$ cells was estimated under a Nikon TE300 fluorescence microscope (Nikon, Japan). Approximately 0.5 to1 x 10^7 viable Epi-mix$^+$ cells from each sample were re-suspended in DMEM with 15% FBS and injected i.p. to a 4-6 week old NOD-SCID mouse (Jackson

Labs, Bar Harbor, Maine). Tumor cells from 11 ascites samples and 1 primary tumor sample were transplanted. Five xenografts were created and maintained for multiple passages by *i.p.* injection of approximately 5 x 10^6 tumor cells from mouse ascites at each passage. Ascites from all EOC patients or xenografts used in this study were withdrawn in the presence of Anticoagulant Citrate Dextrose Solution (Baxter, Deerfield, IL) containing 0.2 U/ml of heparin.

B2. Immunocytochemistry

Cells isolated from ascites or primary tumors of EOC xenografts or patients were fixed with 3.7% paraformaldehyde for 5 min and blocked with 2% BSA for 1 hour. The cells were then stained with primary antibodies for 1 hour at room temperature or overnight at 4 ^{o}C. When a non-conjugated primary antibodies was used, the cells were incubated with a TRITC- or FITC-conjugated secondary antibody for 1 hour at room temperature, followed by a 10 min incubation with Hoechst 33342 (10 μg/ml final concentration). Between each step, the cells were washed with PBS for three times by repeated suspension and centrifugation. The primary antibodies used were all mouse antibodies, including FITC-anti-ESA (clone VU-1D9, Biomeda, Foster City, CA), FITC-anti-EMA (clone GP1.4, Biomeda, Foster City, CA), FITC-anti-EpCAM (clone Ber-Ep4, Dako, Carpinteria, CA), anti-CA125 (clone M11, Lab Vision, Fremont, CA), and anti-Pan-cytokeratins (CK) (clone C11, Sigma).

B3. Transduction of tumor cells with GFP-encoded lentivirus

The GFP-containing transfer vector plasmid (pRRL-CMV-GFP), the helper plasmids (pMDLg/pRRE, pRSV.Rev, and pMD.G), and the packaging cell line (293T) were all provided by Dr. Scott Lowe from Cold Spring Harbor Laboratory. All plasmids were amplified by transformation into competent *E-coli* cells and purified using a DNA isolation kit (Qiagen). To produce the lentiviruses, approximately 5 x 10^6 293T cells were first seeded in a 10-cm-dameter culture dish in DMEM medium containing 10% FBS without penicillin and streptomycin 24 hours prior to transfection. The culture medium was replenished 2 hours prior to transfection. 10 µg of pRRL-CMV-GFP, 6.5 µg of pMDLg/pRRE, 2.5 µg of pRSV.Rev, and 3.5 µg of pMD.G were mixed and incubated in 1 ml of DMEM for 5 minutes. The mixture of plasmids was then mixed with 1 ml of DMEM containing 40 µl of Lipofectamin 2000 (Invitrogen). After 20 minutes of incubation at room temperature, the mixture was added drop-wised to the 293T cells. After 16 hours of incubation, the medium was replaced with 10 ml of DMEM containing 10% FBS, 100 IU/ml of penicillin, and 100 µg/ml of streptomycin. After another 24 hours, the medium containing the lentivirus was collected and filtered with a 4.5-µm cellulose acetate filter. Fresh medium was added and collected for two more times at 24-hour intervals. The quality of lentivirus in the medium was tested on an ovarian cancer cell line (SB247) and a melanoma cell line (LOX) by examining GFP expression in these cells 48 hours after infection. The concentration of the lentivirus was determined in a serial dilution experiment with LOX cells using similar a method. To concentrate the virus, the medium was spanned down at 19,200 rpm for 2 hours in a Beckman SW28 rotor.

Tumor cells from ascites of SB329A and SB333A xenografts were infected with the GFP-encoded lentivirus. Approximately 2.5 x 10^5 tumor cells from ascites of each xenograft were first treated with 0.05% Trypsin/EDTA for 10 min to enhance accessibility of the viruses to cells inside the clusters. The cells were washed once with DMEM. The cells were then infected with the lentivirus for two cycles. At each cycle, the cells were suspended in 2 ml of medium containing approximately 1.5 x 10^7 viral particles (MOI = 75) and 8 μg/ml of polybrene. They were then seeded into 20 wells in a 96-well plate with 100 μl per well. The plate was spun at 1800 g for 45 min at room temperature and then incubated at 37 C for 3 hours. After the infection, the cells were collected into a 15-ml tube. Approximately 1 x 10^5 cells per mouse were then injected i.p. to two NOD-SCID mice. When bloody ascites developed in the recipient mice, ascites was withdrawn. Expression efficiency of GFP in tumor cells in the ascites were estimated . To estimate transduction efficiency of the lentivirus in vitro, approximately 1 x 10^3 cells were seeded in two wells in a 96-well plate in DMEM with 15% FBS.

B4. Flow cytometry

Ascites was withdrawn from SB329A or SB333A xenograft created by injection of tumor cells transduced with GFP-encoded lentivirus. Approximately 5 x 10^6 tumor cells from the ascites were placed into a 15-ml tube and were washed once with PBS. The cells were then treated with 0.2 % trypsin/EDTA for 45 minutes to obtain single cell suspension. After washing 3 times with PBS, cells were re-suspended in 2 ml of PBS with 3% FBS and 1% BSA. GFP^+ cells were sorted by FACS. The efficiency of sorting

was confirmed under fluorescence microscope. Approximately 1 x 10^6 sorted cells were then immediately injected i.p. to a NOD-SCID mouse.

B5. Isolation of tumor cells from ascites of mouse xenografts

Ascites were withdrawn from SB329A or SB333A xenograft created with GFP-expressing cells sorted by FACS. The ascites was first diluted 50 times with pre-warm PBS and then filtered through a 20-μm Nylon screen mesh. The follow-through was discarded while the residue cells were saved as enriched tumor cells, which are mainly consisted of cell clusters. A small sample of enriched tumor cells was set aside and treated with 0.02 % trypsin/EDTA for 45 min to obtain single cell suspension. The concentration of cells was estimated by counting the cells with a hemacytometer under a phase contrast microscope.

B6. Animal experiments

GFP-tagged tumor cells isolated from ascites of SB329A or SB333A xenograft were *i.p.* injected to NOD-SCID mice at two different doses, with 3 mice per dose. The cells were mostly in clusters, which were not dissociated with trypsin/EDTA prior to injection. The recipient mice were examined weekly for the presence of GFP signal by the Maestro *in vivo* imaging system (Cambridge Research & Instrumentation, Inc.) and for development of bloody ascites by observing for abdominal distension and blood accumulation. 12 weeks after injection, all mice were sacrificed. Ascites were withdrawn from each mouse and its volume was measured. The peritoneal cavity was then washed 3 times with PBS remove trapped tumor cells. The total number of tumor cells in the

ascites of each mouse was estimated by hemacytometry. Peritoneal organs in each mouse were dissected out. After washing three times with PBS to remove tumor cells from ascites, each organ was examined for the presence of GFP^+ solid nodules or lesions under a fluorescence microscope.

C. Result

C1. Engraftment of human EOC cells in immuno-diffident mice

Mouse xenografts have been created by transplantation *i.p.* of tumor cells isolated from ascites and primary tumors of EOC patients. Prior to injection, the cells were evaluated for viability and expression of a combination of common epithelial cell antigens (Epi-mix), including epithelial surface antigen (ESA), epithelial membrane antigen (EMA), and epithelial cell adhesion molecule (EpCAM). Approximately 5 to 10 millions viable Epi-mix^{+} cells from each of patient sample were injected *i.p.* to a NOD-SCID mouse (Figure 1A). Five xenografts were created, including SB329A, SB333A, SB333T, SB345A, and SB359A. "A" and "T" indicates the cells used for engraftment were from ascites and primary tumor, respectively. 10-12 weeks after injection, examination of each xenograft found accumulation of 2 to 5 ml of bloody ascites (Figure 1B). Cell viability in the ascites was estimated by a cell viability assay to be over 98% (Figure 1C). Most cells in mouse ascites were seen in clusters, with the morphology and immunoreactivity to epithelial markers similar to those in the corresponding patients (Figure 1D). Most cells in the clusters were stained positive with antibodies against antigens common to ovarian tumor cells including ESA, EMA, Ep-CAM, Pan-cytokeratins, and CA125 (Figure 1E). These observations suggested that tumor cells from ascites of late-stage human EOC could propagate in immuno-deficient mice.

C2. Proliferation of ovarian tumor cells *in vivo*

To track the fate of ovarian tumor cells in mice, approximately 2.5 x 10^5 cells from ascites of SB329A or SB333A xenograft were transduced with GFP-encoded

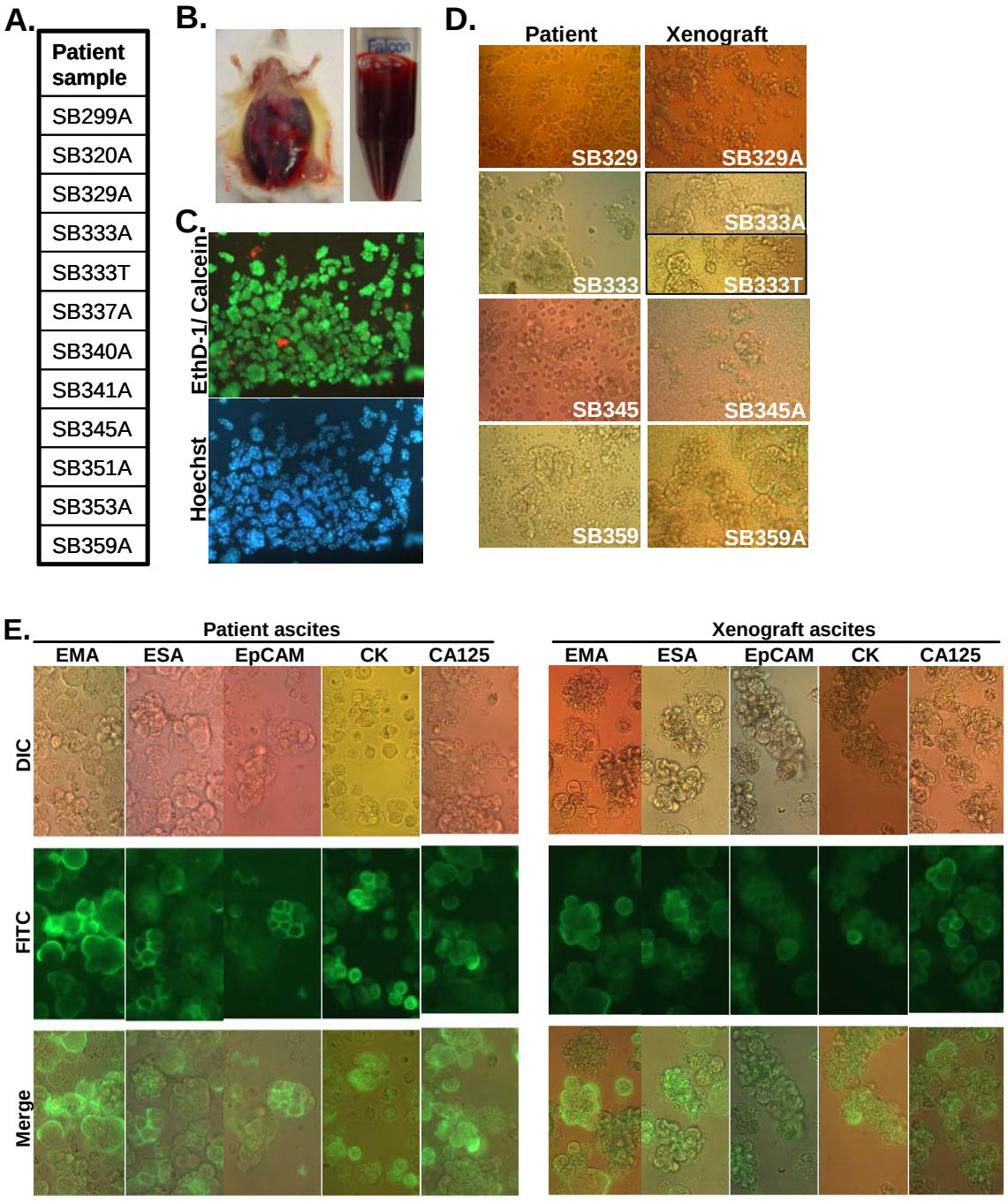

Figure 1. Propagation of human EOC cells in NOD-SCID mice. A). Tumor cells isolated from 11 ascites samples and 1 primary tumor sample of EOC patients were inoculated i.p. in NOD-SCID mice. "A" or "T" indicates the xenograft was created with primary cancer cells from ascites or primary tumor respectively. **B).** Bloody ascites was accumulated in the SB329A xenograft and was withdrawn into a 15-ml plastic tube. **C).** Viability of cells from ascites of SB329A xenograft was estimated under fluorescence microscope after reaction with EthD-1 (red) and calcein AM (green) (100x). Green indicates viable cells. Red indicates dead cells. Blue is Hoechst staining. **D).** Ascites cells from SB329A, SB333A, SB333T, SB345A, and SB359A xenografts (right panel) morphologically resembled those from ascites of the corresponding patients (200x, left panel). **E).** Cells in the ascites of SB329A xenograft (right panel) were similar to those in ascites of SB329 patient (left panel). Tumor-like cells were seen as cell clusters and expressed epithelial cell antigens such as EMA, ESA, EpCAM, CK, and CA-125 (400x).

lentivirus *in vitro*. A small sample of the tranduced cells was set aside for culture to estimate the efficiency of GFP expression. About 40% of the cells in culture expressed GFP (Figure 2A, left panel). Approximately 1 x 10^5 cells per mouse were immediately injected *i.p.* to two NOD-SCID mice. 12-16 weeks after injection, ascites was withdrawn from the recipient mice to estimate the efficiency of GFP expression *in vivo,* which was found similar to that *in vitro* (Figure 2A, middle panel). GFP-tagged cells from ascites of SB329A and SB333A xenografts were isolated by FACS. Approximately 1 x 10^6 sorted cells per mouse were i.p. injected to NOD-SCID mice for propagation. After 10 weeks, bloody ascites were detected in the recipient mice. The ascites were filled with GFP-tagged cells, mostly in clusters. (Figure 2A, right panel).

Mouse ascites often contain non-tumor mouse cells such as red blood cells, leukocytes, and mesothelial cells. To enrich tumor cells from ascites of SB329A and SB333A xenografts created with GFP-expressing cells, mouse ascites was filtered through a 20-um Nylon screen mesh to obtain tumor cells in clusters. To evaluate if the isolated tumor cell clusters can propagate in immunodeficient mice, two doses of tumor cells isolated from SB329A or SB333A xenograft were *i.p.* injected to NOD-SCID mice, with 3 mice per dose. The mice were monitored weekly for tumor growth for 12 weeks by Maestro *in vivo* imaging to detect of GFP signal in the peritoneal cavity. The mice were then sacrificed to estimate the number of tumor cells in the ascites by the presence of GFP^+ cells. GFP^+ cells were detected in ascites from five of the six mice injected with tumor cells from SB329A (Figure 2BA) or SB333A xenograft (Figure 2C). Approximately $2.4x10^8$ and $2.8x10^8$ of tumor cells per mouse were found in ascites of the mice received $5x10^2$ and $5x10^3$ of SB329A tumor cells (n=3), respectively (Figure 2D). Also,

approximately $0.1x10^7$ and $2.8x10^8$ of tumor cells per mouse were found in the ascites of the mice received $2x10^3$ and $2x10^4$ of SB333A tumor cells (n=3), respectively (Figure 2E). These results suggested that tumor cells isolated from ascites of SB329A and SB333A xenografts could proliferate in NOD-SCID mice.

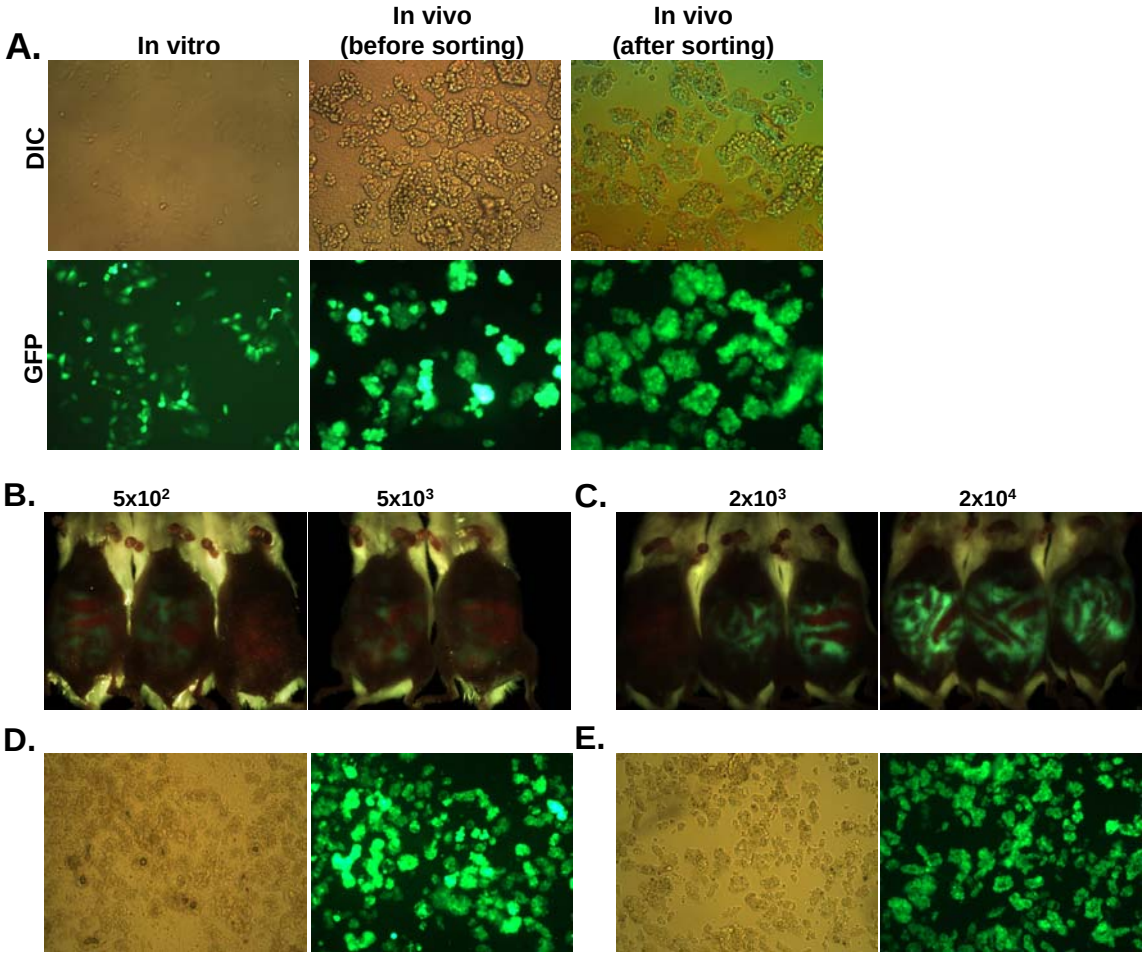

Figure 2. Proliferation of SB329A and SB333A tumor cells *in vivo*. A). Cells from ascites of SB333A xenografts were transduced with GFP-encoded lentivirus. The expression efficiency of GFP were examined under fluorescence microscope (200x). Approximately 40% of cells expressed GFP *in vitro* or *in vivo* (left and middle panels). GFP-tagged cells sorted by FACS propagated extensively in NOD-SCID mice. Almost all cells in the ascitess of the recipient mice express GFP (right panel). **B-C).** Maestro *in vivo* imaging was used to monitor tumor growth in the peritoneal cavity of the mice injected with tumor cells at two different doses: $5x10^2$ and $5x10^3$ cells per mouse from SB329A xenograft (**B**); $2x10^3$ and $5x10^4$ cells per mouse from SB333A xenograft (**C**). Showing here are images taken 10 weeks after injection. **D-E**). Ascites were withdrawn from the mice received tumor cells from the SB329A (**D**) or SB333A (**E**) xenograft and examined under fluorescence microscope(100x).

C3. Metastatic capability of ovarian tumor cells

The metastatic propensity of SB329A and SB333A tumor cells was evaluated by examining their ability to metastasize in NOD-SCID mice. The volume of ascites per mouse in the same recipient mice describe above was examined at the time of sacrifice. Bloody ascites were found in five of the six mice injected with tumor cells from SB329A (Figure 3A) or SB333A xenograft (Figure 3B, left panel). Approximately 3.7 ml and 4.3 ml of ascites per mouse were accumulated in the mice received $5x10^2$ and $5x10^3$ SB329A tumor cells (n=3), respectively. Additionally, approximately 0.1 ml and 2.0 ml of ascites per mouse were accumulated in the mice received $2x10^3$ and $2x10^4$ SB333A tumor cells (n=3), respectively. When the recipient mice were examined for GFP^+ nodule or lesions under fluorescence microscope, GFP^+ aggregates were detected in almost all

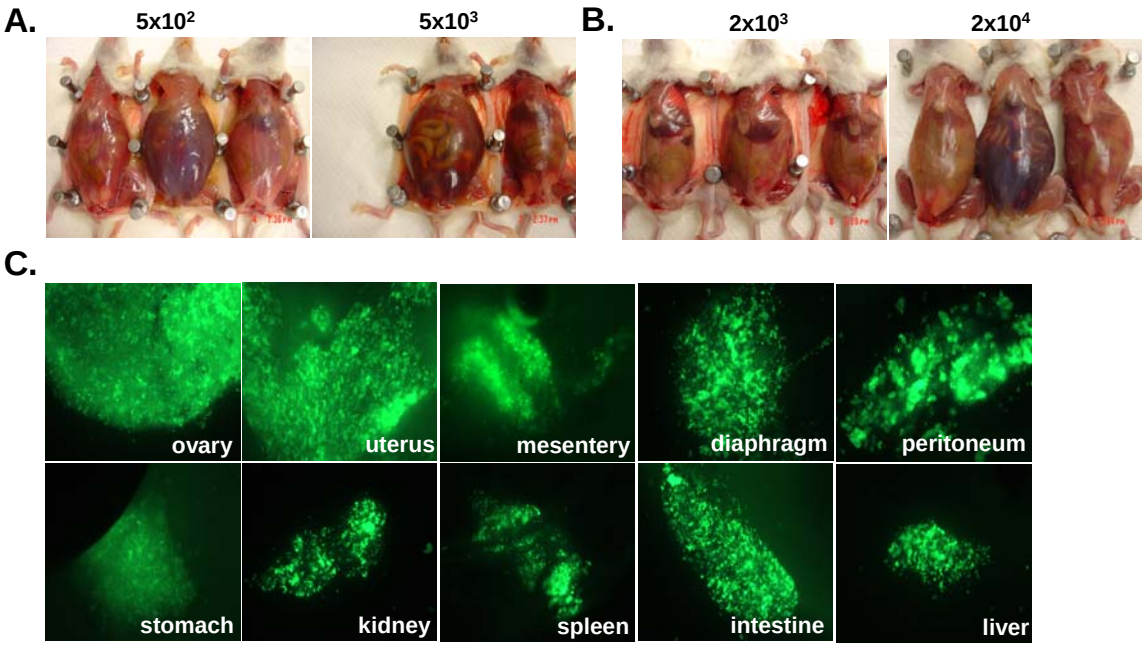

Figure 3. Metastatic capability of SB329A and SB333A tumor cells *in vivo*. A-B). Accumulation of bloody ascites was observed in 5 of the 6 mice 12 weeks after injection of ascites tumor cells from SB329A (**A**) or SB333A (**B**). Tumor cells isolated from ascites of each xenograft were injected at two different doses: $5x10^2$ and $5x10^3$ cells per mouse for SB329A; $2x10^3$ and $5x10^4$ cells per mouse for SB333A. Showing here are images taken 12 weeks after injection. **C).** Metastases were detected in peritoneal organs in the mice received SB333A tumor cells, most frequently in ovary, uterus, mesentery, diaphragm, and peritoneum (40x).

peritoneal organs of five of the six mice injected with SB329A or SB333A tumor cells, suggesting that GFP-tagged cells metastasize to various peritoneal organs (Figure 3C). There was a correlation between accumulation of bloody ascites and peritoneal metastases. The mice accumulated with more ascites were more likely to have more metastases.

D. Discussion

Animal models have been widely used to study cancer biology. To investigate the tumorigenic and metastatic capabilities of tumor cells in ascites of human epithelial ovarian cancer, we have created mouse xenograft models using tumor cells from patients' ascites. Tumor cells in ascites of the xenografts show morphologic and phenotypic properties similar to those in ascites of the patients. The mouse xenografts could not only be used as "animal culture" to expand and maintain primary cancer cells but also as an *in vivo* assay to evaluate the capability of these cells to initiate bloody ascites. Moreover, while patients' ascites were often consisted of largely leukocytes and dead tumor cells, ascites from the xenografts were filled with viable tumor cells and useful for molecular analysis of cancer progression.

Lentivirus is an effective transfer vector for transduction of primary cells. Here, we showed ovarian tumor cells in ascites of the xenografts can be efficiently and stably labeled with GFP by lentivirus, thus providing an effective means to track and evaluate their capability to metastasize. GFP-tagged cells isolated by FACS were able to cause bloody ascites and peritoneal metastases similar to those in the primary xenografts and patients, further suggesting that the *i.p.* ovarian tumor xenograft model described here is useful for molecular analysis of cancer progression. The results from this study are consistent with published data showing that clusters of tumor cells from ascites of EOC patients are invasive *in vitro* (Burleson, et al., 2006).

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