Fibroblast recruitment as a tool for ovarian cancer detection and targeted therapy

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Metastatic ovarian cancer, the most lethal of gynecologic malignancies, is typically managed by debulking surgery, followed by chemotherapy. However, despite significant efforts, survival rate remains low. We have previously demonstrated, in mouse models, a specific systemic homing of labeled fibroblasts to solid ovarian tumors. Here, we demonstrate the feasibility of utilizing this specific homing of genetically modified fibroblasts for detection and targeted therapy of orthotopic metastatic ovarian carcinoma model in immune-deficient mice. Using an in vivo metastatic mouse model for ovarian cancer, we demonstrated that fibroblasts expressing fluorescent reporters injected intra-peritoneally, were specifically recruited to peritoneal tumor nodules (resulting in 93-100% co-localization). We further used fibroblasts over expressing the soluble receptor variant of VEGFR1 (s-Flt1). Mice bearing tumors were injected weekly with either control or s-Flt1 expressing fibroblasts. Injection of s-Flt1 expressing fibroblasts resulted in a significant reduction in the ascites volume, reduced vascularization of adherent metastases, and improved overall survival. Using fluorescently labeled fibroblasts for tumor detection with readily available intra-operative fluorescence imaging tools may be useful for tumor staging and directing biopsies or surgical efforts during exploratory or debulking surgery. Fibroblasts may serve as a beacon pointing to the otherwise invisible metastases in the peritoneal cavity of ovarian cancer patients. Utilizing the recruited fibroblasts also for targeted delivery of antiangiogenic or antitumor molecules may aid in controlling tumor progression. Thus, these results suggest a novel approach for targeting ovarian tumor metastases for both tumor detection and therapy.

Key words: ovarian carcinoma, cell based therapy, s-FLT1, image guided therapy, fibroblasts

Abbreviations: bFGF: basic fibroblasts growth factor; CAF: cancer associated fibroblasts; MRI: magnetic resonance imaging; sFLT-1: soluble receptor variant of VEGFR1; VEGFA: vascular endothelial growth factor A; VEGFC: vascular endothelial growth factor C; VEGFR: vascular endothelial growth factor receptor

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Ovarian cancer is the most lethal gynecologic malignancy1,2 with a mean five year survival after diagnosis of only 43.2%.3 The poor prognosis may be due to the late stage at the time of diagnosis, when metastases are already widely spread.4,5 Currently, available treatment options are limited, patients diagnosed with ovarian cancer are treated by debulking surgery followed by cytotoxic chemotherapy (platinum and taxane analogues).6 Complete surgical removal of malignant tissue is critical for outcome and thus improved intraoperative imaging can have a significant benefit in surgical success.7 Magnetic resonance imaging (MRI) and computed tomography combined with ultrasound are being used for pre-operative assessment of malignancies in patients, but are not useful for real time surgical guidance, and manytimes cannot detect very small metastasis. Fluorescence imaging however, has advantages in preclinical and clinical trials for real time imaging with high sensitivity and specificity using a variety of probes.7,12 The first clinical trial in image guided surgery of ovarian cancer was conducted by van Dam et al. using intravenously administered folate-FTTC. This study demonstrated an increase in tumor detection.10,13 However there is still a need for improved specificity and sensitivity for targeting of fluorescent agents for guided surgery. The use of folate receptor for the detection of tumor lesions resulted in some false positive discovery rate of
23% in patients. This can significantly effect the surgical procedure and cause more extensive and unnecessary intervention. In addition, folate receptor is expressed in 72% of primary tumors in ovarian cancer and in 81.5% of recurrent ovarian tumors and there is a need to find supplementary method to match all patients with additional probes to other molecular or cellular targets. False positive detection can be dramatically reduced by targeting a unique probe or by taking advantage of a native biological process for the signal localization.

Ovarian tumors are characterized by relatively high contribution of stromal cells including fibroblasts, immune cells, endothelial cells and others, with a median of 50% of the tissue composed of stromal cells, not depending on stage or histotype. In both clinical specimens and mouse models, stroma is the major component of the ovarian tumor. Ovarian cancer is also characterized by the accumulation of ascitic fluid in the abdominal cavity and metastatic spread to the peritoneal surfaces. More than a third of ovarian cancer patients will develop ascites during their disease which may involve tumor cells, fibroblasts, macrophages, mesothelial cells and white and red blood cells.

Cancer associated fibroblasts (CAF) are a dominant compartment in the tumor stroma and have been shown to be involved in tumor progression and metastasis. Fibroblasts have a dual role in tumor progression. Normal fibroblasts inhibit preneoplastic growth in normal tissues. However, in response to oncogenic stimuli, fibroblasts can also act to promote tumor progression by leading and stabilizing tumor angiogenic vasculature and facilitate invasion and migration of cancer cells.

We have previously demonstrated in a subcutaneous tumor xenograft model, that exogenously administered fibroblasts are recruited specifically to the ovarian carcinoma vasculature. These recruited fibroblasts were activated to form myofibroblasts (namely, expressing α-SMA). The ability to use normal and abundant cells such as fibroblasts, to target tumors, opens possibilities for targeted imaging and therapy.

VEGF signaling was shown to regulate ascites formation in ovarian cancer. Mesiano et al. demonstrated inhibition of ascites formation in a mouse model by immunoneutralization with anti-VEGF antibody. They also showed that VEGF activity is necessary for ascites formation, but not for tumor growth. VEGF has a higher affinity towards VEGFR1 compared with VEGFR2, but VEGFR1 activation has a lower impact on signal transduction due to a weak tyrosin kinase activity. Through alternative splicing, VEGFR1 encodes a soluble variant (sFLT-1), which consists of an extra cellular domain of VEGFR1. sFLT-1 is more efficient than membrane bound VEGFR1 in binding VEGF, and therefore can serve as a VEGF trap. Importantly, sFlt-1 expression by ovarian cancer tumor cells, in a mouse model, resulted in suppression of ascites accumulation and prolonged survival.

Therefore in this study, we aimed to use the specific recruitment of fibroblasts as a multifunctional tool, not only to highlight tumors, but also for localized delivery of sFLT-1 to the tumor. We hypothesized that local inhibition of VEGF activity by sFlt-1 expression in peri-vascular fibroblasts at the tumor sites could provide an effective avenue to control ascites formation and angiogenesis in ovarian cancer while decreasing the systemic exposure to suppression of VEGF signaling. Here we demonstrate that fibroblasts expressing sFlt-1 are specifically recruited to tumor sites, and indeed significantly suppress the amount of ascites accumulated in the mice, reduced tumor angiogenesis and improved overall survival.

Material and Methods

Cell culture

CV1, normal monkey kidney fibroblasts (ATCC) and ES2, human ovarian epithelial carcinoma cells (ATCC) were grown in DMEM supplemented with 10% FBS. SKOV3 cells (ATCC) were cultured in RPMI supplemented with 10% FBS. MRC5sv, SV40-transformed human lung fibroblasts (kindly provided by Prof. Zvi Livneh from the Weizmann Institute and originally obtained from Dr. Alan R. Lehmann) were cultured in minimum essential medium (MEM Eagle; Biological industries) supplemented with 15% FBS (Biological industries). All media were also supplemented with 1% pen-strep solution and 1% l-glutamine (Biological industries) and cells were maintained in 5% CO₂, 95% air at 37°C.

Generation of ES2 spheroids was done by seeding 3x10^6 cells on agar-coated 75cm² flasks for 4 days. Prior to inoculation into mice, spheroids (~100 µm in diameter) were collected, washed in PBS and counted.

SKOV3 spheroids were created by seeding the cells in hanging drops (8,000 cells/25 µl) in culture medium containing 2.4% (final concentration) carboxymethylcellulose (Methocel,
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Sigma) as described by Laib et al. All the spheroids were collected and washed in PBS prior to injection into mice.

For conditioned medium experiments, tumor and fibroblast cells were seeded at $10^6$ cells/10 cm plate. The following day medium was changed with fresh medium. Forty-eight hours later the conditioned medium was collected, centrifuged to remove cell debris and used as growth media for fibroblasts or cancer cells for additional 48 hr.

RNA preparation, qRT-PCR

RNA was prepared using RNeasy kits according to manufacturer protocol (Qiagen). One microgram of RNA was reverse transcribed using High capacity reverse transcription kit according to manufacturer protocol (Applied Biosystems). qRT-PCR was performed using SYBR fast Master mix (Roche) on LightCycler480 instrument (Roche).

The following primers (Sigma) were used (from 5’ to 3’): GAPDH AGGCTGTCTTATACTCTGTG and CCCACCTT GATTTTGAGAGGA, B2M TTCTGGCTGGAGCTATC and TCAGGAAATTTGACTTTCATC, VEGF-A CCAGG CCCCCTGTCATTG and AAGGAGGGCGCAATTGAT, VEGF-C TGCCAGACACTCACAAG and GTGATTAT TCCCACATGTAATTGGTG, SDF1 TCGATGGTAGCCCC GCCTGAAAGA and AGCGAAAGTCCTTTTGCTGTG, bFGF CACATTAGAGAAGGAGTAAATCT and CCCGAC GGCTGAAGA and AGCGGAAAGTCCTTTTTGGCTGT, TCCCTCGTCATTG and AAGGAGGAGGGCAGAATCAT, VEGF-A CCAGGAAGGCAGACATCGTCATC and TCGTCATCGTCGGCTGAAGA, Mmp9 CCCAGTACTGA, Mmp2 GGGACAAGAACCAGATCACAATC and TCCAGATCGGTGTGAAGCCAAT, Mmp9 GGGACGCAAGATCGTCATC and TCGTCATCGTCGAAAATGGCC uPA CACGCAAGGGGAGATGAA and CACG CAAGGGGAGATGAA. The target messenger RNA (mRNA) expression level was calculated as the ratio of the target mRNA to GAPDH and B2M mRNA for each sample.

Expression vectors and stable transfections

ES2, CV1, SKOV3, and MRC5 cells were transfected to express fluorescent reporter proteins (GFP or Tomato) on a backbone of pIRES vector, under the EF-1a promoter. Stable transfections were carried out with Lipotamine 2000 reagent (Invitrogen), and 48 hr after transfection, puromycin (2.5 mg/ml; Sigma, St. Louis, MO) was added to initiate selection. Cells were maintained on same concentration of antibiotics after selection in order to maintain reporter gene expression. Selection was confirmed by fluorescence microscopy at all stages.

MRC5 cells were transfected to express sFlt1. The sFlt1 sequence (GenBank: D88690.1) was reverse-transcribed from mouse placenta mRNA and PCR-amplified using Phusion high-fidelity DNA polymerase (Finnzymes, Espoo, Finland). The following forward and reverse primers (Sigma) were used, ACCATGTCAGCTGGGAC and TTAATGTTTGGCATGACTTGG. The fragments were ligated into PCR-BluntII-TOPO (Invitrogen), and their sequence fidelity was confirmed by DNA sequence analysis. Inserts were restricted and ligated into pIRES expression vector containing the human EF-1a promoter.

Animal model

All animal procedures were approved by the Weizmann Institutional Animal Care and Use Committee. 7- to 8-week-old CD-1 nude female mice (Harlan laboratories, Israel) were used in all experiments.

For in vivo fluorescence imaging, the regular chow provided to the mice was replaced with chlorophyll free chow (Harlan laboratories, Israel) for at least 3 days prior to imaging, in order to reduce background auto-fluorescence.

Prior to tumor cell inoculation, mice were anesthetized by intra peritoneal injection of a mixture of 100 mg/kg ketamine and 20 mg/kg xylazine (Fort Dodge Animal Health); and 100–300 ES2 or SKOV3 spheroids were injected intra peritoneally35,36 to the left side of the abdomen using a 21G needle. Fibroblasts (2-4x10^6) were administered 5–7 days later by intra peritoneal injection to the contra-lateral abdominal side. For co-localization analysis of tumor and fibroblasts, all tumor nodules and fibroblasts in the mice were counted and marked ex vivo using fluorescence stereomicroscope.

For s-Flt1 treatment, mice bearing intraperitoneal tumors were injected weekly with s-Flt1 expressing fibroblasts or control fibroblasts. For ES2 the experiment was terminated 3 weeks after tumor initiation and ascites volume was collected and measured with a 1-ml syringe. Tumor burden in peritoneal cavity was scored with fluorescence stereo-microscope by an observer blinded to the treatment groups. For overall survival (ES2 and SKOV3), the animals were monitored regularly until morbidity or euthanized according to institutional ethical guideline.

For subcutaneous tumors 4x10^6 ES2 cells were injected alone or mixed with MRC5 fibroblasts cells. Tumor size was measured with caliper after 21 days.

Window chamber/dorsal skin fold chamber

Titanium dorsal window chamber was used for intravitral fluorescence microscopy (Research Instruments, Durham, NC). Mice were surgically implanted with the window chamber under anesthesia according to standard procedure as previously described. The procedure was performed on a heated platform, under sterile conditions. Mice were given 24-48 hr to recover in a heated environment (30°C) before tumor inoculation into the chamber. Subcutaneous chamber tumors were generated by inoculation of 2–5 x10^6 cells suspended in a total volume of 30 μl into skin in the window area of the mouse under anesthesia. CV-1 Tomato fibroblasts were injected IP (2x10^6 cells in 30 μl of PBS) 5–7 days following tumor inoculation.
**Blood vessel labeling in chamber experiments**

DiD is a lipophilic dye (molecular probes; Excitation – 644 nm, Emission 665 nm). DiD was prepared as 2 mg/ml stock solution in 100% ethanol. Dilution in mix of ethanol: Cremophor EL (Sigma), PBS at 1:1:18 volume ratio, resulted in final concentration of 0.1 µg/ml. A total volume of 200 µl was injected into IV and imaged with two photon microscope.

**Histological analysis**

Diaphragm tissues retrieved from mice, were fixed at the end of the experiment with 2.5% PFA for 48 hr followed by 48 hr in 1% PFA and then 70% ethanol and embedded in paraffin blocks. Sections (3–5 µm) were stained for haematoxylin and eosin and immune stained using CD34 (Cedarlane Laboratories, 1:100) antibody for blood endothelial cells, antibody for s-FLT1 (Invitrogen, 1:100). Images were acquired with Zeiss Axio observer microscope equipped with a fluorescence illuminator and an Olympus DP72 camera. Acquired images were processed using ImageJ (National Institutes of Health; http://rsb.info.nih.gov/ij/). For CD34 analysis images of area from the diaphragm containing mainly tumor mass was imaged, CD34 coverage area was analyzed and compared between groups.

**Imaging**

*Ex vivo* stereomicroscope imaging of peritoneal cavity was performed on a fluorescent zoomstereo microscope SZX12 (Olympus, Japan) coupled with a CCD camera PIXELFLY QE, 12 bit (PCO, Germany).

**Two photon microscopy**

Two photon microscopy was performed using an LSM 510 META NLO microscope (Zeiss, Germany) equipped with a Broad Band Mai Tai – HP – Femtosecond single box tunable Ti-Sapphire oscillator, with automated broad band wavelength tuning 700–1020 nm from Spectraphysics, USA, for two photon excitation. Imaging was performed using a plan-Apochromat 20X/0.8 and plan-Apochromat 59 lens. Excitation – 850/890 nm, Emission beam was split by beam splitters, main beam splitter KP-650 and NDD_DBS1: FT:560, and collected by normal or non-descanned detectors BP 500–550 (GFP) BP 575–640 (Tomato) and BP390-465 (Second Harmonic). Imagery area – x: 420.84 µm, y: 420.84 µm.

*Ex vivo* two photon microscopy - Tumors were excised from the peritoneal cavity.

*In vivo* two photon microscopy - Mice bearing window chambers were placed in a fixed position on a xy motorized stage and imaged through the chamber.

**Patient-derived ascites samples**

Ascites samples were collected with the approval of the IRB committee of Meir Medical Center, Kfar-Saba, Israel. Fresh ascites was collected from patients (n = 4) with confirmed diagnosis of high-grade serous ovarian cancer during debulk-
of the significance of difference between groups. t-tests were conducted in Excel. The survival analysis was conducted with a Cox proportional hazards model, using the “survival” package in R (reference https://cran.r-project.org/web/packages/survival/citation.html). The data were considered to indicate a significant difference when p values were less than 0.05 and indicated in the text.

Results

Fibroblast recruitment to tumor cells was associated with blood vessels in vitro and in vivo

Fibroblast recruitment to tumors in subcutaneous models of ovarian cancer was previously reported. Intravital microscopy showed that fluorescently labeled fibroblasts were recruited mainly to the ES2, human epithelial ovarian carcinoma, tumor rim and were aligned along blood vessels (Fig. 1a, arrows, Supporting Information Movie 1). The tumor region and fibroblast region seemed separated by a border marked by the fibrotic (collagen) “capsule” formed around the tumor. This recruitment was consistent with our previous studies showing that the systemic recruitment is specific and unique to the tumor area.

Non-cell-autonomous signaling between tumor and stroma cells was evaluated using conditioned medium. Fibroblast cells were cultured in medium collected from ovarian tumor cells or medium conditioned by fibroblasts as a control. Measurement of gene expression revealed significant induction of angiogenic signaling. We found that culturing fibroblasts in medium collected from tumor cells induced transcription of VEGFA, VEGFC and basic fibroblast growth factor (bFGF) (Fig. 1b, p values < 0.05). Conditioned medium induce the expression of α-SMA a hallmark gene for activation of fibroblasts by tumor cells (Fig. 1c, p values < 0.05), the upregulation of α-SMA goes in line with the expression of α-SMA in recruited fibroblasts as previously reported.

Stromal cell-derived factor-1 (SDF1) is a secreted factor involved in different aspects of the tumorigenic process including proliferation, survival, motility, migration, invasion...
and metastasis and recruiting endothelial cells and angiogenesis. In response to conditioned medium we found that fibroblasts down regulated the expression of SDF1 (Fig. 1d, p values < 0.05). We also measured changes in genes involved in ECM remodeling and found matrix metalloproteinase 1 (MMP1) to be upregulated in response to conditioned medium, and no change in the expression of MMP2, MMP9 and uPA (Fig. 1e, p values < 0.05). Tumor conditioned medium did not change the proliferation rate or migration of fibroblasts (Supporting Information Fig. S1).

Specific fibroblasts recruitment to intraperitoneal ovarian ES2 micro metastases

In view of the recruitment of fibroblasts to solid subcutaneous ovarian tumor xenografts, we wanted to better model the clinical manifestation of ovarian tumor, and further test whether they could be recruited to the tumor in an orthotopic model of metastatic ovarian carcinoma. For that end, female CD-1 nude mice were inoculated intra-peritoneally with fluorescent GFP-expressing human ES2 ovarian carcinoma spheroids. Seven days after tumor initiation, tomato-expressing fluorescent CV1 fibroblasts were injected intra-peritoneally (to the contra-lateral side of the abdomen). The mice were sacrificed and imaged one day after fibroblasts injection (Fig. 2). The fluorescence generated by the two reporter genes was used to detect tumor and fibroblast cells respectively. Injected tumor spheroids attached to the abdomen in multiple locations, and specific homing of the fibroblasts to tumor nodules and aggregates could be detected (Figs. 2a and 2b). In control animals in which no tumor was injected, very low residual fibroblasts signal could be detected, suggesting the effective clearance of non-adherent fibroblasts in the absence of tumor (Fig. 2c). No fibroblasts could be detected in other organs (including liver, lungs, omentum). Similar results were obtained in an additional fibroblast cell line, MRC5 (Supporting Information Fig. S2). The specific recruitment of fibroblasts to tumor nodules was also seen in SKOV3 tumor spheroids injected to mice (Supporting Information Fig. S3) and in solid tumors of both ES2 and MLS cells. Higher magnification of a tumor nodule with recruited fibroblasts, showed the attachment of the fibroblasts to the tumor cells and infiltration into the tumor nodule (Fig. 2d).

The fibroblasts highlighted almost all of the tumor nodules with high selectivity and specificity (Table 1).
Quantification of fibroblast recruitment demonstrated that 93–100% of the total detectable fibroblasts were co-localized with tumor nodules (Table 1). The fibroblast cells could be detected at the tumor site as early as 1 day after their injection and were still visible 4 days after injection (data not shown). The detection of tumors using the fibroblasts gave low false negative (tumors with no adhered fibroblasts; 8.5%) and very low false positive (fibroblasts attached in the absence of a tumor; 2.4%). Thus, we achieved very high selectivity and high specificity for detection of tumors, by intra-operative fluorescence imaging, including very small, avascularized clusters of tumor cells, through the use of exogenously labeled fibroblasts.

Fibroblasts are recruited to fresh mouse ascites and human ascites in vitro

Fibroblasts affinity towards ovarian carcinoma was also tested using fresh patient-derived carcinoma specimens. Ascitic fluid specimens were collected from four ovarian cancer patients during de-bulking surgery or ascitic fluid drainage. The fluid was maintained in suspension culture for 24 h after collection. Fluorescently labeled CV1 fibroblasts were then added to the culture medium and the co-cultured cells were observed by fluorescence microscopy (Fig. 3). Cultured ascitic fluid formed tumor clusters with a spheroid like morphology. The labeled fibroblasts adhered to the tumor clusters and remained viable and adherent throughout the experiment, 5–7 days of co-culture (Fig. 3). In order to compare the adherence of the fibroblasts to tumor cell aggregates from human-derived ascites to that of ascites of mouse origin, mouse ascitic fluid was collected 14 days after intraperitoneal injection of fluorescently-labeled ES2 cells. The adherence in both ascitic sources was comparable (Fig. 4). Suspension culture and co-culturing with fibroblasts did not affect spheroid morphology of the ascites-derived ES2 clusters, and again, adherence of fibroblasts to ovarian carcinoma cells was observed (Fig. 4).

s-Flt1-expressing fibroblasts attenuate ascitic fluid formation and improve survival

We further explored the feasibility of therapeutic utilization of the specific homing of labeled fibroblasts to ovarian tumors. First, we validated the contribution of fibroblasts to tumor growth. Endogenous fibroblasts are recruited by tumor cell from the surrounding neighborhood and mesenchymal stem cells are recruited from the bone marrow to become cancer associated fibroblasts. As previously reported,
exogenously administered fibroblasts add only a small contribution to the endogenous tumor stroma (Granot Cancer Res21). Due to the difficulty in assessing tumor burden for peritoneal metastasis model we used the subcutaneous model. Co injection of fibroblasts with tumor cells subcutaneously to mice did not change tumor volume compared with tumor cells injected with no fibroblasts (Supporting Information Fig. S4). No inhibitory or stimulatory effect of fibroblasts on tumor growth rate was also reported in PC3 prostate cancer-derived cells tumor model.42

The association of the recruited fibroblast with blood vessels in the dorsal tumor model (Fig. 1a) prompted us to utilize the fibroblasts for suppression of angiogenesis by local targeting of VEGFA around the tumors. For that purpose we have generated fibroblasts cells that over express s-Flt1. The expression of s-Flt1 was validated by ELISA assay (Supporting Information Fig. S5). Mice bearing ES2 tumors were injected weekly (at Day 7 and Day 14) with either control or s-Flt1 expressing MRC5 fibroblasts. In order to assess the influence of s-Flt1 expressing fibroblasts on angiogenesis in infiltrating metastasis, we analyzed the tumor region in the diaphragm of treated mice, and measured the coverage area of newly formed blood vessels as stained with CD34 antibody (Figs. 5a and c). We found a significant decrease in the amount of newly formed blood vessels in the group treated with s-Flt1 expressing fibroblasts (quantified in Fig. 5c, p values 0.014). The presence of fibrotic areas in the ES2 tumor could be identified in tumor section (Figs. 5d and 5e).

Ascites volume was measured and tumor burden was assessed and scored according to amount and spread of tumor nodules (Fig. 5i, Supporting Information Table 1). Injection of sFlt expressing fibroblasts resulted in a significant reduction in the ascites volume in the mice, (p values 0.0049). Although s-Flt1 expressing fibroblasts injection did not reduce the amount of peritoneal attached tumors, the overall survival of the mice was improved for both ES2 (Fig. 1f, p values 0.0216) and SKOV3 peritoneal tumor xenografts (Supporting Information Fig. S3, p values 0.0126).

The systemic level of s-Flt1 was measured in the blood, showing no difference between mice injected with control or
s-Flt expressing fibroblasts (Supporting Information Fig. S6). On the other hand, staining of the diaphragm region for s-Flt1 confirmed that locally it is more abundant following injection of s-Flt1 expressing fibroblasts (Figs. 5 g and 5 h).

In summary, we show that s-Flt1 expression by recruited fibroblasts remains localized to the recruiting tumor sites and affect VEGF activity, resulting in reduction of both ascites accumulation and formation of new blood vessels. These results also confirm the possible utility of fibroblasts for cell-based, site-specific delivery of therapeutics.

Discussion

The use of cell-based therapy in cancer was proposed long ago and is a topic of many preclinical and clinical trials. These trials often use immune cells to target the tumor or to activate an autoimmune response. In this study we demonstrate the feasibility of using fibroblasts cells that specifically home to a preclinical model of ovarian tumor nodules. Endogenous fibroblasts are recruited and activated by ovarian carcinoma tumors in a large amount. As fibroblasts are recruited to the tumor endogenously from local environment and from the bone marrow, exogenous fibroblasts are only fraction of the total amount of fibroblasts in the tumor. However, the mechanism by which fibroblasts are recruited to the tumor nodules is yet unknown. Mesenchymal stem cells (MSC) were reported to be recruited to tumor microenvironment by inflammatory signals, hypoxia, and various growth factor and cytokines. These signaling pathways may give some clues as to the recruitment of exogenous fibroblasts, however further work is still needed.

Addition of exogenously labeled fibroblasts in our subcutaneous model did not have an inhibitory or stimulatory effect on tumor growth. Similarly, Scherz et al. showed that coinjection of fibroblasts with tumor cells in a solid tumor model did not change tumor progression, as is the same in our model. These findings support the possibility of using fibroblasts cells as a tool in ovarian cancer treatment and detection.

The emerging field of fluorescence guided surgery holds a great promise to be used as a complimentary tool to traditional white light reflectance guided surgery. Fluorescence imaging in surgery has so far been implemented with the use of indocyanine green (ICG) and fluorescein as non-specific...
dyes to visualize blood vessels, lymphatic and other structures. Such low MW targeted fluorescent imaging probes can aid in evaluation of surgical margins and identification of residual disease during surgical procedures. In this context, the primary treatment offered to ovarian cancer patients is surgery, aimed to remove as much tumor mass as possible, so as to improve treatment outcome. The initial surgery is also the basis for tumor staging and for determining future treatments (chemotherapy regime etc.). In the absence of readily visible lesions, as typical to ovarian carcinoma, random biopsy samples are taken. The ability to enhance the accuracy of visualization of small, possibly otherwise undetected, metastatic nodules by using labeled fibroblasts could therefore improve staging and assist in debulking of tumor and guidance of biopsies, leading to better postoperative selection of therapy for the patient.

In this study, we used two ovarian cell lines (ES2 and SKOV3) in intraperitoneal model for ovarian cancer, and in subcutaneous model previously published the recruitment was demonstrated in MLS and ES2 cell lines. These results suggests that fibroblasts recruitment might be applicable in various types of ovarian cancer.

Tumor angiogenesis has a critical role supporting the growth of solid tumors and is associated with the accumulation of malignant ascites in ovarian carcinoma. Angiogenic inhibitors were shown to significantly reduce the accumulation of ascites. Increase in the peritoneal microvessels permeability was reported to correlate with ascites accumulation both in pre-clinical and clinical data. 

We have demonstrated that recruited fibroblasts are associated with blood vessels in vitro, they can up regulate the expression of pro-angiogenic factors in response to secreted signals from tumor cells. In this study we utilized the specific fibroblasts recruitment to tumor in metastatic model and demonstrated that they can be used efficiently to prevent ascites fluid accumulation by secretion of sFlt-1. The expression of sFlt-1 directly by ovarian cancer cells was previously reported to suppress ascites accumulation in a mouse model, showing the importance of VEGF to ascitic development. Adenovirus infection of muscle to express sFlt-1 was also used as a method to systemically deliver sFlt-1. The local expression of sFlt-1 in the tumor area and not in the circulation reported here indicate that using fibroblasts as a delivery vehicle provides a novel approach for the sustained localized delivery of sFlt-1. This approach can effectively suppress ascites accumulation and may assist in ovarian cancer treatment, while reducing the detrimental side-effects associated with systemic suppression of VEGF such as bowel perforation that may occur in the peritoneal cavity.

**Significance**

Fibroblasts may be enlisted for ovarian cancer therapy during staging and debulking surgery. We have demonstrated fibroblast recruitment to ovarian cancer spheroids and nodules in vitro and in vivo. The cancer nodules are mostly of a size which is undetectable by the naked eye. The co-localization of ovarian cancer cells and fibroblasts is very specific with low false negative and false positive events. This may be useful for both staging of cancer level in patients and during debulking surgery. The utility of available fluorescence detection tools for surgery guidance will be enhanced by improved labeling of tumors. Fluorescently labeled fibroblasts may serve as a beacon pointing to the otherwise invisible metastases in the peritoneal cavity of women with ovarian cancer. Moreover, by endowing these cells with expression and release of sFlt-1, these cells can aid in control of angiogenesis and formation of ascitic fluid.

These results and the system described in this study, using pre-clinical mice model for ovarian tumors and human tissue in vitro, provide a novel tool to target ovarian tumor metastases both for tumor detection and therapy. The recruited fibroblasts act as delivery vehicles, allowing for the sFlt-1 to be expressed only at the tumor site, inducing localized effect and reducing systemic effects. The presented study serves as a proof of concept of utilization of fibroblast as a biological tool for specific targeting labeling and delivery to tumor metastasis, which can be modified relatively easily to a range of labels and cargo. Further work is needed in order to develop this method to clinical application.

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19. Schauer IG, Sood AK, Mok S, et al. Cancer-associated fibroblasts and their putative role in potentiating the initiation and development of ovarian carcinoma. *Int. J. Cancer*: 2008;121:335–48.

20. Ahmed N, Stenvers K. Getting to know ovarian cancer ascites: opportunities for targeted therapy and research. *Nat Rev Cancer* 2013;13:273–82.

21. Granot D, Addadi Y, Kalchenko V, et al. In vivo imaging of the systemic recruitment of fibroblasts to the angiogenic rim of ovarian carcinoma tumors. *Cancer Res* 2007;67:9180–9.

22. Vandesburger MH, Radoul M, Addadi Y, et al. Ovarian carcinoma: quantitative biexponential MR imaging relaxometry reveals the dynamic recruitment of ferritin-expressing fibroblasts to the angiogenic rim of tumors. *Radiology* 2013; 268:790–801.

23. Joyce JA. Therapeutic targeting of the tumor microenvironment. *Cancer Cell* 2005;7:513–20.

24. Miek P, Ostman A. Tumour-stroma interaction: cancer-associated fibroblasts as novel targets in anti-cancer therapy? *Lung Cancer* 2004;45 (Suppl 2):S163–75.

25. Francia G, Emmenegger U, Kerbel RS. Tumor-associated fibroblasts as “Trojan Horse” mediators of resistance to anti-VEGF therapy. *Cancer Cell* 2009;15:3–5.

26. Mesiano S, Ferrara N, Jaffe RB. Role of vascular endothelial growth factor in ovarian cancer: inhibition of ascites formation by immunoneutralization. *Am J Pathol* 1998;153:1249–56.

27. Hirotsuka S, Minowa O, Kuno J, et al. Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *Proc Natl Acad Sci USA* 1998;95:5349–54.

28. Kearney JB, Ambler CA, Monaco KA, et al. Vascular endothelial growth factor receptor Flt-1 negatively regulates developmental blood vessel formation by modulating endothelial cell division. *Blood* 2002;99:2397–407.

29. Sela S, Itin A, Natanson-Yaron S, et al. A novel human-specific soluble vascular endothelial growth factor receptor 1: cell-type-specific splicing and implications to vascular endothelial growth factor homeostasis and preeclampsia. *Circ Res* 2008;102:1566–74.

30. Fischer C, Mazzone M, Joncks B, et al. FLT1 and its ligand VEGF receptor Flt-1: potential targets for anti-angiogenic therapy? *Nat Rev Cancer* 2008;8:942–56.

31. Hasumi Y, Mizukami H, Urabe M, et al. Soluble FLT-1 expression suppresses carcinomatous ascites in nude mice beating ovarian cancer. *Cancer Res* 2002;62:2019–23.

32. Laib AM, Bartol A, Alajati A, et al. Spheroid-based human endothelial cell microvesSEL formation in vivo. *Nat Protocols* 2009;4:1202–15.

33. Hobbs S, Ittrapakdee S, Wallace JC. Development of a bicistronic vector driven by the human polypeptide chain elongation factor 1-alpha 2-beta 2 promoter for creation of stable mammalian cell lines that express very high levels of recombinant proteins. *Biochem Biophys Res Commun* 1998;252:368–72.

34. Wiznerowicz M, Fong AZ, Hawley RG, et al. Development of a double-copy bicistronic retroviral vector for human gene therapy. *Adv Exp Med Biol* 1998;451:441–7.

35. Qian X, Wang X, Jin H. Cell transfer therapy for cancer: past, present, and future. *J Immunol* 2014;2015:529513.

36. Kershaw MH, Westwood JA, Darcy PK. Gene-engineered T cells for cancer therapy. *Nat Rev Cancer* 2013;13:525–41.

37. Cohen B, Addadi Y, Sapoznik S, et al. Transcriptional regulation of vascular endothelial growth factor C by oxidative and thermal stress is mediated by lens epithelium-derived growth factor/p75. *Neoplasia* (New York, NY) 2009;11:921–33.

38. Vandoorne K, Addadi Y, Neeman M. Visualizing the angiogenic rim of tumors. *Cell* 2009;139:1375–85.

39. Kobayashi M, Chiba A, Irawa H. The DCVogat-1/SoICT, et al. The feasibility and clinical effects of dendritic cell-based immunotherapy targeting synthesized peptides for recurrent ovarian cancer. *J Ovarian Res* 2014; 7:48

40. Cirri P, Chiarugi P. Cancer associated fibroblasts: the dark side of the coin. *Am J Cancer Res* 2011; 1:482–97.

41. Gelmini S, Mangoni M, Serio M, et al. The critical role of SDF-1/CXCR4 axis in cancer and cancer stem cells metastasis. *Endocrinol Invest* 2014;31:809–19.

42. Addadi Y, Moskovits N, Granot D, et al. p53 Status in stromal fibroblasts modulates tumor growth in an SDF1-dependent manner. *Cancer Res* 2010;70:9650–8.

43. Spaeth E, Klopp A, Dembinski J, et al. Inflammation and tumor microenvironments: defining the migratory itinerary of mesenchymal stem cells. *Gene Ther* 2008;15:730–8.

44. Scherr-Showall R, Santagata S, Mendillo Marc L, et al. The reprogramming of tumor stroma by HSFI is a potent enabler of malignancy. *Cell* 2014;158:564–78.

45. Alander JT, Kaartinen I, Laakso A, et al. A new of indocyanine green fluorescent imaging in surgery. *Int J Biomed Imaging* 2012:2012: 940585.

46. Orosco RK, Tsiem YJ, Nguyen QT. Fluorescence imaging in surgery. *Biomed Eng IEEE Rev* 2013:6:178–87.

47. Tran Cao HS, Kaushal S, Methrth CA, et al. Tumor-specific fluorescence antibody imaging enables accurate staging laparoscopy in an orthotopic model of pancreatic cancer. *Hepato-gastroenterology* 2012;59:1994–9.

48. Heuser LS, Taylor SH, Folkman J. Prevention of carcinomatosis and bloody malignant ascites in the rat by an inhibitor of angiogenesis. *J Surg Res* 1984;36:244–50.

49. Marme A, Strauss G, Bastert G, et al. Intraperitoneal bisepecific antibody (HEA125xOT3) therapy inhibits malignant ascites production in advanced ovarian carcinoma. *Int J Cancer Int Du Cancer* 2002;101:183–9.

50. Senger D, Galli S, Dvorka A, et al. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* 1983;219:983–5.

51. Garrison RN, Galloway RH, Heuser LS. Mechanisms of malignant ascites production. *J Surg Res* 1987;42:126–32.

52. Garrison RN, Kaclin LD, Galloway RH, et al. Malignant ascites. Clinical and experimental observations. *Annals Surg* 1986;203:644–51.

53. Sherr DM, Eliaikim R, Abulafia O. The role of angiogenesis in the accumulation of peritoneal fluid in benign conditions and the development of malignant ascites in the female. *Gynecol Obstetric Invest* 2000;50:217–24.

54. Takei Y, Mizukami H, Saga Y, et al. Suppression of ovarian cancer by muscle-mediated expression of soluble VEGFR-1/Flt-1 using adeno-associated virus serotype 1-derived vector. *Int J Cancer Int Du Cancer* 2007;120:278–84.