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The Role of the Omental Microenvironment in Ovarian Cancer Metastatic Colonization

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## INTRODUCTION:

The majority of ovarian cancer patients have advanced disease at the time of diagnosis. Patients undergoing surgical cytoreduction to control metastatic disease would be immediate candidates for therapies that target metastatic regrowth if they will be available. Such approaches require mechanistic knowledge of the process of metastatic colonization, the final step of metastasis in which ovarian tumor cells undergo progressive growth at the secondary sites [1,2]. Our current knowledge is limited to the fact that the omentum is the one of the most common sites of ovarian cancer metastasis [3,4] and consists of a fatty peritoneal fold that extends from the greater curvature of the stomach and covers most of the abdominal organs. The omentum is a highly vascularized tissue that comprises mainly adipocytes interspersed with structures known as “milky spots” which contain immune cells [5,6]. Studies from our laboratory and others have demonstrated that ovarian tumor cells preferentially seed to the milky spots suggesting that they are a favorable microenvironment for the establishment of ovarian metastases in the omentum [7,8,9]. The goal of this project is to increase our knowledge of the structure and function of milky spots in the mouse omentum, the interactions of ovarian tumor cells with these structures and identify specific cells and molecules involved in the interactions between ovarian tumor cells and the omental microenvironment.

## BODY:

Aim 1: To conduct cellular and molecular characterization of omental structures and their interactions with ovarian cancer cells.

- 1) Determination of the structure and composition of the omenta of females Athymic Nude, Beige XID (NIHIII) and C57Bl/6 mice using histology, IHC and transmission electron microscopy and following injection of activated carbon as described by Hagiwara *et al* (Hagiwara, cancer res, 1993).
- 2) Assessment of the structure and cellular composition of omental milky spots using IHC and fluorescence-activated cell sorting (FACS).
- 3) Determination of the interaction between ovarian cancer cells and the omentum during metastatic colonization using fluorescent microscopy
- 4) Development of an *ex vivo* omental organ culture model for metastatic colonization.

Using FACS analysis, we quantify the levels of different immune cell populations (monocytes, B and T-cells) present in the omentum of Nude and C57Bl/6 mice (**Figure 1**). Due to technical difficulties, we had to change some of the proposed immune cell markers for the FACS analysis. We are currently determining the localization of these different immune cell types using IHC. Furthermore, we are in the process of assessing the numbers of milky spots in omenta of Nude, Beige XID and C57B/6 females using intraperitoneal (i.p) injection of 1mg of activated carbon. We will also expand the cellular characterization of the milky spots using IHC for other immune cells beside B-cells and macrophages.

To investigate the interactions between ovarian cancer cells and the omentum during metastasis, female Nude Athymic mice were i.p injected with SKOV3ip.1-GFP tumor cells. At different time points after injection, tumor-bearing omentum was harvested, stained with Hoechst and imaged using 2-photon confocal microscopy. Representative pictures in **Figure 2** showed that as early as 30 minutes after i.p injection, ovarian tumor cells localize within the milky spot structures. More interestingly, we repeated this experiment in 3 independent occasions and we never once observed the presence of ovarian tumor cells within the adipocytes. This result suggested that the immune cells of the milky spots could contribute to the localization of the ovarian tumor cells in the omentum. Therefore, we quantified the different immune cell populations using FACS analysis (**Figure 3**). We observed a significant increase in the number of monocytes per omentum in both Nude and C57Bl/6 mice in presence of ovarian tumor cells compared to PBS injected mice. More interestingly, this increase in monocytes was transient and disappeared 10 days after injection.

With respect to macrophages, the effect of ovarian cancer cell localization by IHC for F4/80 (macrophage cell marker) was even more dramatic. Representative data from the macrophage localization in milky spots after i.p injection of SKOV3ip.1 are shown in **Figure 4**. However, IHC for B220 (B-cell marker) did not show any particular change in B-cell localization within the milky spots in presence of ovarian tumor cells (**Figure 4**). These findings suggest that ovarian tumor cells require the presence of macrophages but not B-cells to localize within the milky spots. To test this hypothesis and better understand the tumor-omental immune cell interactions, we will treat mice with clodronate liposomes or vehicle control prior to i.p injection of ovarian tumor cells. Clodronate is a compound that efficiently depletes macrophages [10,11]. We will assess if in absence of macrophages, ovarian tumor cells are still able to colonize omental milky spots.

We also developed a physiological relevant *in vitro* model to identify specific omental-ovarian tumor cell interactions during metastatic colonization. We were able to harvest and culture fresh mouse omentum for 13 days (**Figure 5**). Viability of the tissue was assessed by measuring the levels of IL-6 secreted by the omentum in the culture media (**Figure 5**). In order to utilize this *ex vivo* model for metastatic colonization experiments, we determined the ability of ovarian tumor cells to grow in the omental milky spots *ex vivo*. Nude mice were i.p injected with  $10^6$  SKOV3ip.1-GFP cells, 3 days post-injection, omenta were removed from one group of mice and maintained in culture *ex vivo* while the omenta from the other group of animals were maintained *in vivo* (**Figure 6**). After 8 days post-injection, omenta were imaged using the OV100 In Vivo Imaging System (**Figure 6**). We did not find any difference in terms of formation of tumor micro-colonies in omenta cultured *ex vivo*. The omenta cultured *ex vivo* present a qualitatively similar distribution and pattern of tumor micro-colonies.

To assess the possibility to use this novel *ex vivo* organ culture model to study metastasis, we determined the ability of ovarian tumor cells to adhere to cultured omenta. Harvested omentum is immobilized using a tissue adhesive (Cell-Tak) and SKOV3ip.1-GFP cells are allowed to adhere on the omentum for 6 h at 37°C. Co-cultured omenta with SKOV3ip.1 cells were compared to *in vivo* omenta harvested at 6 h post-injection (**Figure 7**). H&E stainings showed similar distribution of tumor cells within the milky spots (**Figure 7b, c**). Immunohistochemistry for human cytokeratin confirmed the presence of ovarian tumor cells in both *ex vivo* and *in vivo* omenta (**Figure 7d**). Furthermore, we determined that the tumor cells present in the milky spots

in both experimental conditions proliferate by staining for MCM2 (**Figure 7e**). In conclusion, we developed a novel *ex vivo* model where ovarian tumor cells can adhere to the omental metastatic site and proliferate to form metastases.

#### **KEY RESEARCH ACCOMPLISHMENTS:**

1. We started the cellular characterization of the omental milky spots.
2. Immune cells present in the milky spots contribute to the specific colonization of ovarian tumor cells to the milky spots.
3. Presence of ovarian tumor cells significantly increases the levels of macrophages in the milky spots.
4. We developed a novel *ex vivo* model to study ovarian cancer metastasis to the omentum where the tumor cells can adhere to a viable omentum and are able to proliferate to form metastatic foci.
5. Publication in a peer-reviewed journal (see appendices)

#### **REPORTABLE OUTCOMES:**

Khan SM, Funk HM, Thiolloy S, Lotan TL, Hickson J, Prins GS, et al. (2010) In vitro metastatic colonization of human ovarian cancer cells to the omentum. *Clin Exp Metastasis* 27: 185-196.

Thiolloy S and Rinker-Schaeffer, CW. (2010) Thinking outside the box: Using metastasis suppressors as molecular tools. *Seminars in Cancer Biol.* Dec 13. [Epub ahead of print]

#### **CONCLUSIONS:**

During the first year, we determined the optimal conditions to perform immunohistochemistry and FACS analyses for the different immune cell populations composing the milky spots. We will dedicate the second year of our study to precisely characterize the cellular composition of the milky spots both qualitatively and quantitatively.

We demonstrated that the immune cells present in the milky spots contribute to the specific localization of the metastatic ovarian tumor cells in the omentum. Using FACS analysis, we showed that tumor-bearing omenta present a transient significant increase in the level of monocytes compared to the control mice. Furthermore, by IHC staining, we established that macrophages are surrounding the ovarian tumor foci in the milky spots are early time points after injection. However, 10 days post-injection, the only few macrophages are present within the tumor. These data suggest a contribution of milky spot macrophages to ovarian cancer metastasis early in the metastatic process. During the second year of this funding, we will investigate the potential contribution of macrophages to ovarian cancer metastasis by assessing their potential role in tumor migration, proliferation and apoptosis using both *in vitro* and *in vivo* approaches.

During this first year, we also established a novel *ex vivo* model to study the molecular mechanisms underlying ovarian cancer metastasis to the omentum. We determined the optimal conditions for the culture of whole omentum for an extended period of time (up to 13 days). We also established that ovarian tumor cells do colonized the omentum and the milky spots in an *ex vivo* setting without major differences compared to an *in vivo* metastasis assay. The development

of this *ex vivo* organ explant led to the publication of a scientific manuscript in a peer-reviewed journal. We will utilize this model to further characterize the interactions between the ovarian tumor cells and the immune cells composing the omental milky spots.

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## **APPENDICES:**

## In vitro metastatic colonization of human ovarian cancer cells to the omentum

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**Abstract** Despite the potentially crucial contributions of the omentum in the regulation of ovarian cancer metastatic growth, it remains a poorly understood organ. Due to its anatomic location and structural fragility, the omentum presents inherent challenges to mechanism-based in vivo studies. Thus, the availability of an ex vivo omental model would, in part, address some of these difficulties posed. Here we describe a technique for identifying, isolating and maintaining ex vivo cultures of omenta from immune-

compromised and -competent mice. Ex vivo culture conditions were developed that maintain tissue viability, architecture, and function for up to 10 days. Further experiments demonstrate that the ex vivo culture conditions allow for the proliferation of ovarian cancer cells in vitro and support a similar pattern of microscopic lesions after either intraperitoneal injection of ovarian cancer cells or co-culture of ovarian cancer cells with the omentum. In agreement with previous studies from our laboratory, histologic evaluation of these specimens found that ovarian cancer cells, as well as other peritoneal cancer cells, preferentially accumulate in, and colonize, omental areas rich in immune cells. We now recognize that these are specific, functional structures referred to as milky spots. In sum, these are foundational studies of a readily accessible model, which is easily manipulated and can be immediately used to study the dynamic process of omental colonization. It is hoped that investigators will use the data herein as a starting point for refinements and modifications which will enable them to tailor the model to the specific needs of the experimental question(s) they wish to pursue.

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colonization · Milky spots · Immune aggregates

### Abbreviations

H&E Hematoxylin and eosin  
PBS Phosphate buffered saline  
CO<sub>2</sub> Carbon dioxide  
FCS Fetal calf serum  
GFP Green fluorescent protein  
ITS Insulin transferrin and selenium solution  
dpi Days post injection

## Introduction

The management of metastatic ovarian cancer continues to be a critical clinical problem. Ovarian cancer affects more than 20,000 women yearly in the United States alone [1], and most patients have extensive metastatic disease at the time of diagnosis [2]. The standard therapeutic approach is to surgically remove as much of the tumor(s) as possible, a process known as surgical cytoreduction, with subsequent administration of platinum based chemotherapy [2–5]. The nearly 80% recurrence rate after surgical cytoreduction and chemotherapy clearly illustrates the urgent need for methods to control the growth of minimal residual disease [2–5]. Improved treatment strategies require a greater and more robust understanding of the process of *metastatic colonization*, the final step in metastasis, in which cancer cells undergo progressive growth at secondary sites [6, 7].

In clinical ovarian cancer, the omentum is the primary site of metastasis formation [8, 9]. The human omentum is a peritoneal fold composed of layers of mesothelial cells that envelope adipose tissue [10]. This highly vascularized organ is a storage site for lipid, a regulator of peritoneal fluid transit, and a reservoir for immune cells in the peritoneal cavity [10–12]. It extends from the stomach and blankets most abdominal organs, which may partly explain its susceptibility to colonization by ovarian and peritoneal cancer cells within ascites [13, 14]. Despite its clinical importance, the omentum remains an understudied organ, and misconceptions of its structure, function, and composition abound. To date most studies examining its role in ovarian cancer metastasis take a reductionist view and focus on ovarian cancer cell-mesothelial cell interactions [15]. While this approach is beneficial in the study of interactions between individual cell types, studies of metastatic colonization require more complex model systems which ideally retain tissue composition and architecture.

Omenta from mice and humans are similar in their composition and function [10]. Both are made up primarily of adipocytes with focal areas of immune cells known as milky spots. Interestingly, in the mouse, the omentum is a thin, adipose rich strip of tissue interconnected with the pancreas leading to some confusion about its precise location. Although functions are conserved, such variations in the structural details of certain organs, such as the prostate, between species are not uncommon. In addition to the location of the omentum adjacent the pancreas and spleen, the depth and associated light scattering of fluorescent and/or bioluminescent signals originating from the omentum makes it challenging to employ optical imaging modalities, limiting many investigators to using end-point assays. An ex vivo model could enable real-time data collection by allowing investigators to employ methods such as the use of optical imaging devices. Studies of ovarian metastasis

would be greatly enhanced by the ability to manipulate and visualize the omentum ex vivo and could thus be expanded to include experiments on, for example, test therapeutic agents.

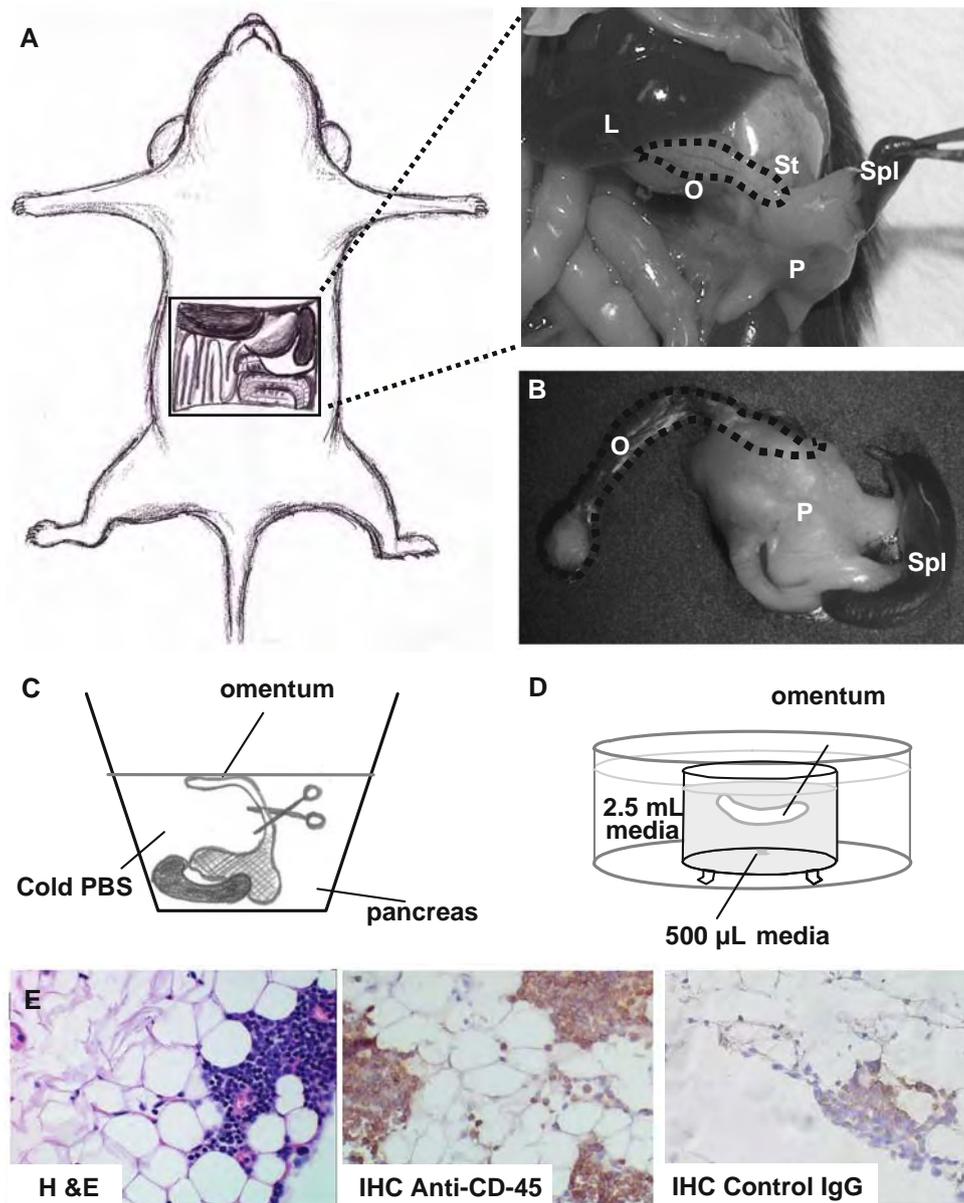
In this work we report our progress on the identification, development, and implementation of organ culture conditions which enable the maintenance of mouse omental tissue integrity for ex vivo studies. The long-term goal of this work is to develop methods that can be used to interrogate metastatic colonization of the mouse omentum by ovarian cancer cells in real time. Our current studies focus on the feasibility of the organ culture model. The advantages and limitations of the method are described and its potential utility in studies of the mechanism(s) by which ovarian cancer cells colonize the omentum is discussed.

## Methods

### Optimal conditions for isolation and ex vivo culture of the mouse omentum

After testing several procedures and verifying tissue identity by histology, the following was determined to be the most direct method to cleanly excise the omentum for ex vivo culture. Immune-competent CD-1-ChrRiv (Charles River) mice were euthanized and placed in a supine position. The skin over the abdomen was sterilized by washing with 70% ethanol and removed. An incision was made in the peritoneal wall exposing internal organs (Fig. 1, Panel a). The spleen was extended from the peritoneal cavity with forceps to reveal the omental/pancreatic complex (Fig. 1, offset Panel, right). The omentum and surrounding organs were excised by closely trimming all tissue connections to the stomach and intestines. The omentum, pancreas and spleen were removed en bloc (Fig. 1, Panel b) and immediately placed in sterile, ice-cold phosphate buffered saline (PBS; Fig. 1, Panel c). After a few seconds in cold PBS, the adipose-rich omentum floated to the surface of the well and the higher density pancreas and spleen remained submerged (Fig. 1, Panel c). The omentum was excised by cutting at its base, being careful not to cut into the pancreas. For ex vivo culture, the omentum was then placed upon one Millicell culture plate insert (Millipore cat # PICM01250) set within one well of a twelve-well tissue culture plate (Falcon, Becton–Dickinson, Cat # 353043; Fig. 1, Panel d). Routine histological processing and staining was performed to verify the identity of the omental tissue as well as to assess tissue integrity after various timepoints in culture.

Fourteen culture media combinations were tested based upon growth media used previously to maintain the four basic cell types that comprise the omentum—(i.e.



**Fig. 1** Location and strategy for isolation of the mouse omentum. **a** Diagram indicating the location of the murine omentum in the peritoneal cavity between the stomach, pancreas and the spleen. The *inset* to the *right* shows an enlarged view of the area with the omentum (*O*) indicated with a *dashed line*. The spleen (*Spl*) is distended to the *right* with forceps, and the liver (*L*), stomach (*St*) and pancreas (*P*) are indicated. **b** The excised complex of spleen, pancreas and omentum are displayed to indicate the conjoined nature of the omentum and pancreas. **c** Placement of the excised spleen–pancreas–omentum structure in ice cold PBS allows specific identification of the

omentum due to its buoyancy compared with the other organs. The omentum can be isolated by cutting the omental/pancreatic junction with a scissor. **d** The omentum is then cultured in the apparatus shown, consisting of a Millipore transwell insert containing 500 µl media, placed in a 12-well culture plate containing 2.5 ml media. **e** The presence of immune cells within milky spot structures was verified by IHC with anti-CD-45 antibodies. *Left* H&E of of omentum; *middle* IHC with anti CD-45 antibody, *right* IHC with IgG control antibody

mesothelial cells, adipocytes, and immune cells [16–24]. Modifications were made to some growth media as to produce a milieu that optimized the preservation of mouse omental tissue. A summary of the combinations of tissue culture media and growth supplements that were evaluated is given in Table 1. Organ cultures were maintained at 37°C

in a 5% CO<sub>2</sub> environment for varying lengths of time, after which tissue integrity was assessed by hematoxylin and eosin (H&E) staining and light microscopy. Tissue integrity was further assessed by manually counting the number of healthy and necrotic adipocytes on H&E sections and formulating a percentage of live tissue present. For each culture

**Table 1** Summary of media tested for ability to maintain viability and integrity of ex vivo tissues

Media <sup>a</sup>	Variation A	Variation B	Variation C	Growth condition rationale
RPMI 1640	10% FCS	10% FCS 0.5 µg/ml insulin 0.5 µg/ml Transferrin Selenium A 0.4 µg/ml hydrocortisone		Variation A is a modification of media used to successfully culture human omental mesothelial cells [15] Variation B is a modification of media used to maintain mouse peritoneal mesothelial cells in culture for 8 days [24]
Ham's F-12	10% FCS	10% FCS 0.5 µg/ml insulin 0.5 µg/ml Transferrin Selenium A 0.4 µg/ml hydrocortisone		Variation B is a modification of conditions capable of maintaining human omental tissues for ~18 days [17]
DME/F-12	20% FCS	20% FCS, P/S 0.5 µg/ml insulin 0.5 µg/ml transferrin Selenium A 0.4 µg/ml hydrocortisone	10% FCS	Variation B has been successfully used in the propagation of human, rat and rabbit peritoneal mesothelial cells for up to 90 days [22]
M199	10% FCS	10% FCS, 0.5 µg/ml insulin 0.5 µg/ml transferrin Selenium A 0.4 µg/ml hydrocortisone		Variations used successfully to maintain ovarian surface epithelium [19] and human omental mesothelial tissue [21]
DMEM	10% FCS	10% FCS 0.5 µg/ml insulin 0.5 µg/ml transferrin Selenium A 0.4 µg/ml hydrocortisone	20% FCS 0.5 µg/ml insulin 0.5 µg/ml transferrin Selenium A 0.4 µg/ml hydrocortisone	Variation A used successfully to maintain pancreatic tissue [18, 20]

<sup>a</sup> All media contained a final concentration of 100 U/ml penicillin and 100 mg/ml streptomycin

condition ~120 cells within a minimum of 5 independent sections of omental tissue were scored by this method.

#### Extended ex vivo culture of the mouse omentum

Both immune competent as well as immunodeficient mice were used in ex vivo culture studies. Omenta were collected from euthanized, immune competent, 10–12 week old female CD-1-ChrRiv (Charles River) and 10–12 week old, immunodeficient Athymic Nude-Foxn1nu (Harlan) female mice, as described above, and maintained in a total of 3 ml DME/F-12 supplemented with 20% fetal calf serum (FCS). The cultures were maintained at 37°C in a 5% CO<sub>2</sub> environment for 1, 7, 10, 15 and 20 days after which tissue integrity was assessed by H&E staining as described above. The efficacy of ex vivo culture conditions was assessed in triplicate.

#### Functional assays

Murine omenta were collected from euthanized, immune-competent, 8–12 week old female C57B16 mice and placed in PBS. Omenta were placed in separate wells of a 24-well plate containing 500 µl DME/F12 media with 20% FCS. IL-6 production was assessed by ELISA (eBioscience) and used as a measure of tissue function. Specifically, 250 µl of conditioned media was removed and replaced with the same volume of fresh DME/F12 complete media every other day until day 13. As a control for nonspecific secretion, one group of omenta were exposed to detergent (10% SDS; 3 incubations of 20 min each) then washed in 3 changes of PBS for 30 min each to remove the detergent, prior to incubation. Triplicate samples were stored at –80°C until the assay was performed.

## Cell culture and cell lines

The human ovarian carcinoma cell lines HEYA8 and SKOV3ip.1, were a generous gift from Dr. Gordon Mills (M.D. Anderson Cancer Center, Houston, TX, USA). SKOV3ip.1 cells are maintained in DMEM containing L-glutamine (584 mg/l) and glucose 4.5 g/l; (Mediatech Inc, Herdon, VA, USA), supplemented with 5% fetal calf serum (Atlanta Biological, Lawrenceville, GA), penicillin (100 units/ml)/streptomycin (100 µg/ml), 10 mmol/l pyruvate, 1× nonessential amino acids, and 2× MEM vitamin solution (Mediatech Inc, Herdon, VA, USA). Cells were GFP-tagged by transfection with 4 µg of pSEX-Flag-eGFP plasmid which encodes blasticidin<sup>R</sup> following the Qiagen Effectene Transfection Kit (301427) protocol. SKOV3ip.1-GFP cells, which had stable expression of the GFP protein, were then established by selection in media containing 2.5 µg/ml blasticidin (Invitrogen, Carlsbad, CA). The resulting cells were then sorted for high GFP expression by flow cytometry at the University of Chicago Flow Cytometry Core Facility.

## In vitro assessment of cancer cell growth curves

To establish growth curves,  $1.0 \times 10^5$  SKOV3ip.1 or HEYA8 cells were plated in triplicate on 6-well culture plates [25]. Cells were cultured under standard growth conditions (described in the preceding section) or the three organ culture media conditions which maintained optimal tissue viability: (1) DMEM supplemented with 10% FCS, 10 µg/ml insulin, 5.5 µg/ml transferrin, 0.5 µg/ml sodium selenite (ITS solution, Sigma St. Louis, MO), and 0.4 µg/ml hydrocortisone (Sigma, St. Louis, MO); (2) DME/F-12 supplemented with 20% FCS; or (3) F-12 supplemented with 10% FCS, 30 µl ITS solution per 3 ml media and 0.4 µg/ml hydrocortisone. Cells were trypsinized and viable cells were confirmed by trypan blue exclusion and counted using a hemocytometer every 2–3 days.

## Ex vivo culture of the mouse omentum with adherent SKOV3ip.1-GFP cells

SKOV3ip.1-GFP cells were grown to 80% confluence as previously described [25]. For intraperitoneal injections, the cells were harvested, washed twice with PBS, and resuspended at a concentration of  $2.0 \times 10^6$  cells per ml in cold, sterile PBS. Female immunodeficient, Athymic Nude-Foxn1nu (Harlan) mice (10–12 weeks old) were each injected with  $1.0 \times 10^6$  SKOV3ip.1-GFP cells shortly after preparation. The ability of the ex vivo culture conditions to support the formation of microscopic lesions on the omentum was assessed as summarized in Fig. 4b. In brief, 3 days post injection (dpi) mice were randomized to an “in

vivo growth” group (left) or and “ex vivo growth” group (right). In the case of in vivo growth, lesions were allowed to continue growth for an additional 5 days. In contrast, for ex vivo growth, omenta harvested at 3 dpi were maintained in ex vivo culture conditions for 5 days. At the experimental endpoint, the extent and distribution of SKOV3ip.1-GFP omental lesions were assessed using the Olympus OV-100 optical imaging system.

## Ex vivo co-culture of the mouse omentum with SKOV3ip.1-GFP cells

SKOV3ip.1-GFP cells were grown to 80% confluence and harvested as described above. The cells were resuspended in DME/F-12 supplemented with 20% FCS at a concentration of  $2 \times 10^6$  per ml. Omenta were excised from female Athymic Nude-Foxn1nu (Harlan) mice and immediately attached to the membrane of Millicel culture inserts using Cell-Tak Cell and Tissue Adhesive (BD Biosciences) as follows. A volume of 6 µl of Cell Tak was spread evenly on the transwell membrane. The membrane was air-dried at room temperature in a laminar flowhood and washed twice with 500 µl of sterile water. The membrane was again air-dried and the transwell was placed in one well of a 12-well culture plate. Omenta were excised and placed on the dried Cell-Tak coated surfaces (one omentum per transwell) without media for 1 min to allow for optimal adhesion. After adherence, 500 µl of the SKOV3ip.1-GFP cell suspension was transferred onto each omentum with no further agitation and 2.5 ml of DME/F-12 media was placed around the transwell. Omenta were co-cultured with the cell suspension for 6 h at 37°C in a 5% CO<sub>2</sub> environment. After 6 h, omenta were removed from culture, washed with ~10 ml of PBS and visualized for fluorescence using the OV100 In Vivo Imaging System. As a positive control, omenta harvested from mice 6 h post-injection with  $1 \times 10^6$  SKOV3ip.1-GFP cells were also examined. Experiments were performed in duplicate at two independent times. Tissues were subsequently fixed and stained by H&E and examined by light microscopy.

## Immunohistochemistry of ex vivo co-culture

Omenta subjected to the co-culture conditions, omenta harvested 6 h post injection, and omenta freshly excised from Athymic Nude-Foxn1nu female mice were fixed overnight in 10% formalin and subsequently processed and embed in paraffin. Paraffin sections were deparaffinized, subjected to antigen retrieval by proteinase K digestion diluted at 1:50 (Dako) and incubated for 5 min at room temperature. Endogenous peroxidase activity was quenched by incubation with 3% H<sub>2</sub>O<sub>2</sub> for 5 min. The sections were incubated at room temperature with polyclonal rabbit anti-cow wide

spectrum screening cytokeratin antibody (Dako) at a dilution of 1:500 in PBS for 1 h. In the case of MCM2 staining, the antibody (Abcam ab 31159) was diluted 1:200). Visualization of the immunohistochemical reaction was carried out according to the DAKO EnVision™ + System protocol (Dako).

## Results

The composition and physiology of the omenta in mice parallels that of humans, although its relative size and location differ somewhat between species. In mice, it is structurally distinct in that it is essentially a thin strip of adipose which lies between the stomach and the pancreas (Fig. 1, Panels a and b). In order to reproducibly identify and excise the omentum, the pancreas-omentum-spleen structure was removed en bloc (Fig. 1, Panel b) and placed into a reservoir of sterile, cold PBS (Fig. 1, Panel c). The omentum was identified by its buoyancy and isolated for ex vivo culture (see “Methods” section). Using this approach we were henceforth able to specifically and reproducibly dissect the omentum for use in subsequent experiments.

In order to determine the optimal organ culture conditions, omenta were maintained in the various media and conditions summarized in Table 1 for time periods of 24 h and 3 days using either 2 or 3 mls of media. The apparatus employed in this organ culture method is depicted in Fig. 1 (Panel d). The condition that maintained optimal tissue integrity was a total of 3 ml of DME/F-12 with 20% FCS. Omenta under these conditions exhibited healthy adipocytes with no obvious necrosis for up to 3 days while those maintained under other conditions showed significant tissue degradation, less than 50% viable tissue, after 1 day of culture (data not shown). A series of studies were conducted to assess our ability to maintain omental tissues in organ culture for extended periods of time. The integrity of ex vivo explant tissues was compared to a positive control of freshly excised omentum, which was immediately fixed in formalin and stained with H&E, is shown in Fig. 1, Panel e. The tissue shows rounded, well-formed adipocytes and foci of immune cells dispersed throughout. Immune cells were identified by evaluation by a pathologist (TLL). Specifically, examination of the tissues identified cells within milky spot structures as being a combination of a population of small mononuclear cells with scant cytoplasm and condensed chromatin, morphologically consistent with lymphocytes, and another population of cells centrally placed nucleus, small nucleoli and well-demarcated abundant foamy cytoplasm, consistent with macrophages. Rare cells had a spindle or mesenchymal morphology with elongated nuclei consistent with fibroblasts. These data were confirmed by IHC with antiCD45 antibody (Fig. 1, Panel e).

Assessment of omental tissues maintained in explant cultures showed that tissue integrity is qualitatively comparable to freshly excised tissue as long as there is no residual pancreatic tissue present (Fig. 2, Panel a upper row). Extended cultures for 7, 15 and 20 days, also showed apparent viability on a par with freshly excised tissue based on the absence of cytoplasmic vacuolization, eosinophilia or nuclear pyknosis (Fig. 2, Panel a lower row). As histologic examination indicated that cells in the ex vivo omental cultures remained viable, we next assessed the production of the cytokine IL-6 during the culture period. IL-6 is produced by adipose, mesothelial cells and immune cells, and immediately secreted into the microenvironment [26]. Ex vivo omentum cultures expressed abundant IL-6, with little decline during the experimental period (13 days, Fig. 2, Panel b). Omentum pretreated with detergent to kill cells, however, did not produce significant levels of IL-6 (Fig. 2, Panel b). These data indicate that cells of the omenta continue to secrete IL-6, in ex vivo explant culture, at a similar rate over a 13 day period.

## Assessment cancer cell proliferation in explant culture maintenance media

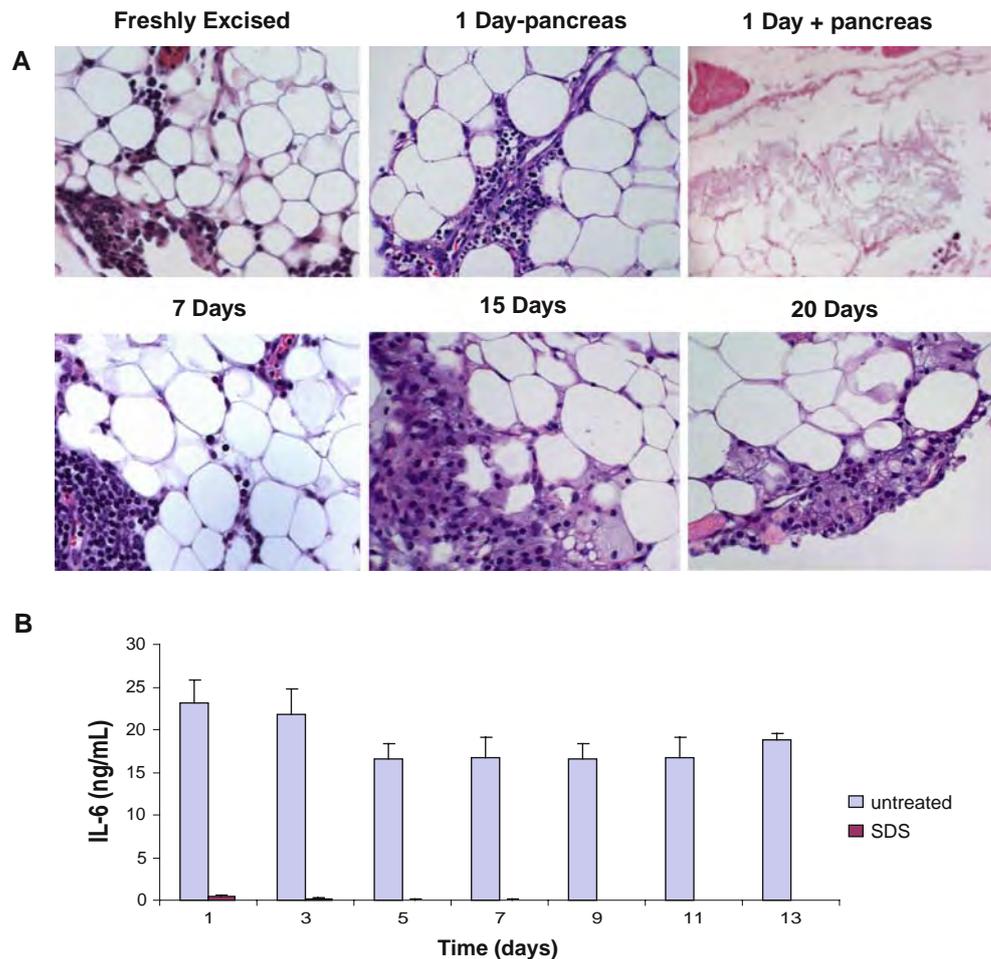
Having established conditions which could support omental tissue viability ex vivo, the question still remained whether these conditions could support the attachment and growth of human ovarian cancer cell lines which are routinely used for studies of ovarian cancer metastasis in immunocompromised mice. Since a key application of this omental culture model is to enable assessment of metastatic colonization in the ex vivo setting, it is imperative that human ovarian cancer cell growth be maintained under organ culture conditions. To test this, SKOV3ip.1 cells and HEYA8 cells were cultured under standard growth media, DMEM plus supplements (Table 1, Variation B), DME/F-12 (Table 1, variation A), and F-12 plus supplements (Table 1, Variation A). Supplemented DMEM and DME/F-12 supported the growth of SKOV3ip.1 cells and Hey-8 cells at a level indistinguishable from control media for at least 7 days (Fig. 3). Conversely, cells cultured in supplemented F-12 showed significantly decreased growth. These data showed that DME/F-12, the media condition that maintains omental tissue viability under organ culture conditions similarly supports the growth of ovarian cancer cell lines as well as the standard SKOV3ip.1 and HEYA8 growth media.

## Visualization of ovarian cancer cells attached to the omentum and ex vivo culture of adherent metastases

By injecting  $1 \times 10^6$  SKOV3ip.1-GFP cells intraperitoneally into Nude-Foxn1nu female mice in our standard

**Fig. 2** Ex vivo organ cultures of murine omentum are viable and functional for at least 13 days. **a** Histological assessment (H&E staining) of tissues maintained in ex vivo culture under varying conditions. In order to maintain tissue integrity *ex vivo*, the pancreas to be cleanly excised from the omentum. *Upper panels left* freshly excised omentum, *middle* 1 day ex vivo culture with clean excision of pancreatic tissue, *right* 1 day ex vivo culture with contaminating pancreatic tissues still attached to omentum. *Lower panels* tissue integrity was assessed after extended times in ex vivo culture *left*, 7 days, *middle* 15 days, and *Right* 20 days. In all cases the original magnification was 40 $\times$ .

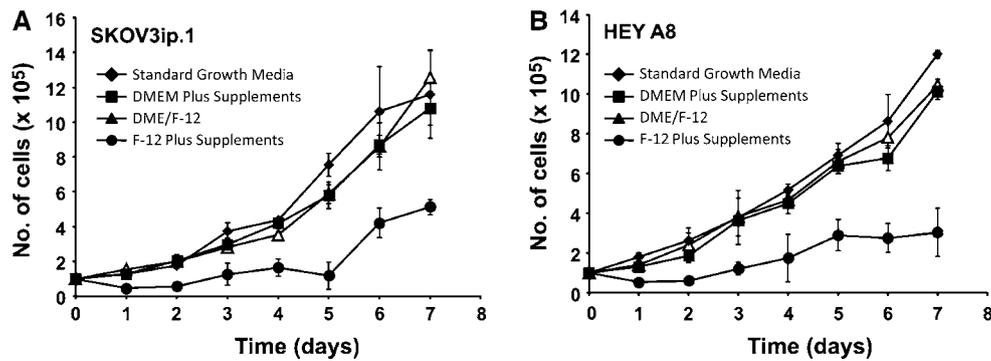
**b** Tissue viability was assessed by evaluating the secretion of mouse IL-6 into the media by the omenta over a period of 13 days by ELISA. Omenta pretreated with detergent were used as a control for nonspecific release of IL-6



metastasis assay, we showed that at 3 dpi, fluorescent cell microcolonies were remarkably restricted to the omentum and absent from the adnexal pancreas (Fig. 4 Panel a, *upper left*). The spleen-pancreas-omentum structure was excised, and SKOV3ip.1-GFP cell fluorescence was visualized by microscopy (Fig. 4, Panel a, *upper right*). Further, H&E staining showed that SKOV3ip.1-GFP cells are admixed with immune cells (Fig. 4, Panel a, *lower left*). We have previously reported that SKOV3ip.1 cells are always seen in the presence of an “immune infiltrate” during metastatic colonization [27]. We now recognize that these localized immune cells of the omentum are consistent with structures previously termed “milky spots” [28]. Assessment of pancreatic tissues by both fluorescence and light microscopy showed an absence of cancer cells (Fig. 4 Panel a, *lower right*), supporting previous findings that ovarian cancer cells, including SKOV3ip.1 cells, preferentially localize to the omentum.

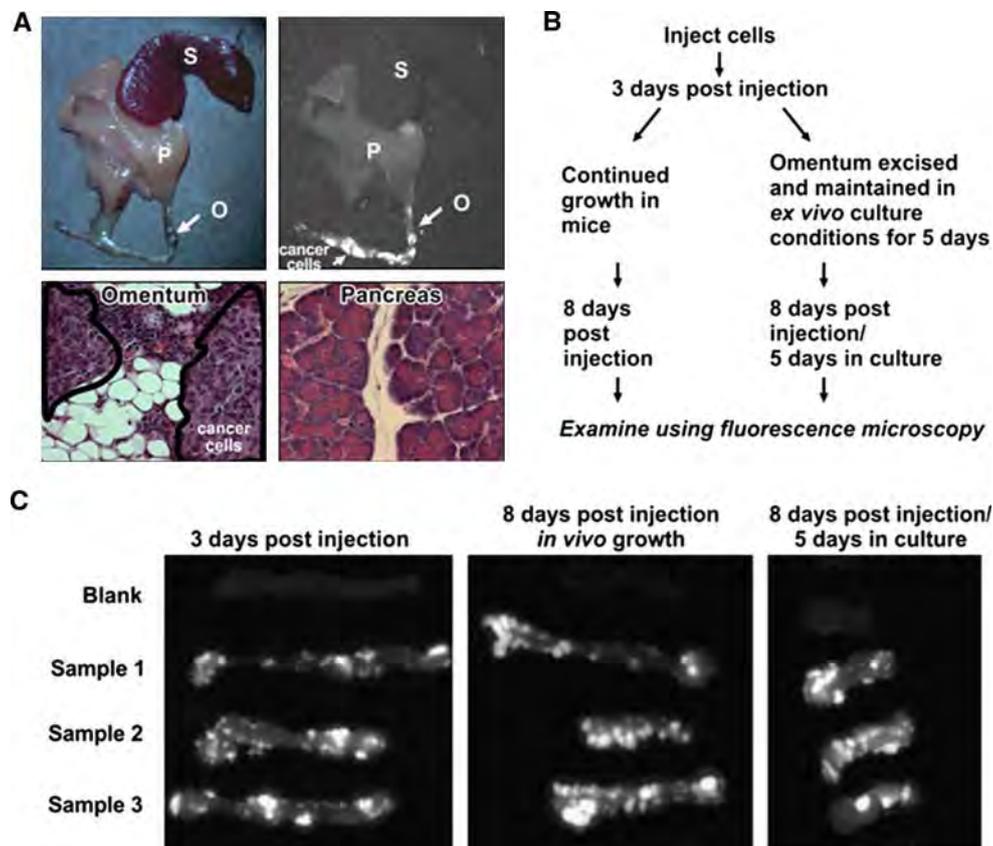
After establishing conditions that maintained the viability of both the omental tissue as well as cancer cells, a study was designed to test the ability of omental tissue explants to support microcolony formation by adherent

SKOV3ip.1-GFP cells *ex vivo* (Fig. 4, Panel b). Athymic nude mice were intraperitoneally injected with  $1.0 \times 10^6$  SKOV3ip.1-GFP cells which were allowed to adhere for 3 days and establish microcolonies on the omenta. Omenta from representative mice were examined by fluorescence microscopy to establish a baseline of colonization (Fig. 4c, *left panel*). The remainder of the injected mice was then randomized into two groups (Fig. 4, Panel b). In the first group, cells were allowed to continue *in vivo* attachment for five additional days. At 8 dpi, omenta were excised and assessed by fluorescence microscopy. In the second group, omenta were harvested at 3 dpi and then placed in organ culture conditions for 5 days, after which tissues were examined by fluorescence microscopy. The appearance of representative omenta from each of these groups is shown in Fig. 4c. At 3 dpi, discrete microcolonies can be seen along the omenta (Fig. 4 Panel c *left*). At 8 dpi, the omenta present with larger microcolonies (Fig. 4, Panel c *center*). The omenta cultured *ex vivo* have a qualitatively similar distribution and pattern of microcolonies, however, the overall length of the omentum was reduced (Fig. 4, Panel c *right*).



**Fig. 3** Assessment of growth of ovarian cancer cell lines in various explant organ culture media. SKOV3ip.1 and HEYA8 cells were plated at a density of  $1 \times 10^5$  in 9.6 cm<sup>2</sup> dishes. Triplicate samples were counted every 2 days. The *symbols* indicate the following types

of media: (*diamond*) standard growth media (*square*) DMEM plus supplements (*triangle*) DME/F-12, and (*circle*) F-12 plus supplements. *Error bars* represent standard deviation from the mean



**Fig. 4** Ovarian cancer cells preferentially localize to the omentum, and show continued growth in explant culture. **a** Cancer cell localization occurs preferentially to the omentum (*O*) after injection of SKOV3ip.1-GFP cells *i.p.*, by light microscopy (*left*) and by fluorescence imaging (*right*). The peritoneum (*P*) and stomach (*S*) are indicated. Histologic analysis (*lower panels*) confirms the colonization of tumor cells in the milky spots of the omentum (*left*) but not in

the pancreas (*right*). Original magnification: 40x. **b** Schematic depicting the experimental design for the data shown in Panel **c**. SKOV3ip.1-GFP cells were injected into groups of mice; after 3 days omenta were removed from one group and maintained in explant culture while the omenta from a parallel group of mice were maintained *in vivo*. **c** Cancer cell foci on omenta maintained under *in vivo* or *ex vivo* conditions were imaged by fluorescence microscopy

In sum, a qualitative assessment of the cellular fluorescence from the adherent cancer cells on the cultured omenta is similar to that found on the 8 dpi freshly excised omenta.

Adhesion of cancer cells to omenta *ex vivo*

In order to test the feasibility of developing a completely *ex vivo* experimental system, excised omenta were exposed

to SKOV3ip.1-GFP cells *ex vivo* to determine whether the ovarian cancer cells would adhere to omenta in a similar fashion as cells injected *in vivo*. Initially, development of this approach was challenging as the omenta, due to their buoyancy, float in the cell suspension thus decreasing the efficiency of tissue-cancer cell interactions (data not shown). To address this, omenta were immobilized using a tissue adhesive (Cell-Tak). SKOV3ip.1-GFP cells at 37°C for a period of 6 h. Co-cultured omenta were compared to *in vivo* omenta harvested 6 h post injection of 10<sup>6</sup> SKOV3ip.1-GFP cells. Both the *in vivo* and *ex vivo* co-culture tissues showed fluorescence concentrated in discreet foci (Fig. 5, Panel a). H&E staining showed remarkably similar patterns of cancer cell localization to immune aggregates with admixtures of immune cells and cancer cells (Fig. 5, Panels b and c). Immunohistochemistry further showed the cancer cells (brown) localizing with the immune cells (Fig. 5, Panel d). Comparatively, the adherent cancer cells in both the *ex vivo* and *in vivo* culture showed similar viability through H&E staining. These data confirm that SKOV3ip.1-GFP cells can adhere to the omentum to a similar extent and manner as cells injected *in vivo*. To determine whether cells, which have attached to the omentum *ex vivo* are viable for longer periods of time, SKOV3ip.1-GFP cells were co-incubated with the omentum *ex vivo* and maintained under *ex vivo* conditions for 24 h. Then the tissue was sectioned and viability of the cancer cells was assessed by IHC for the proliferation marker MCM2 (Fig. 5, Row e, *right*). Cancer cells which have adhered to the surface of the omentum stain positively for this proliferative marker, confirming their viability under our *ex vivo* conditions.

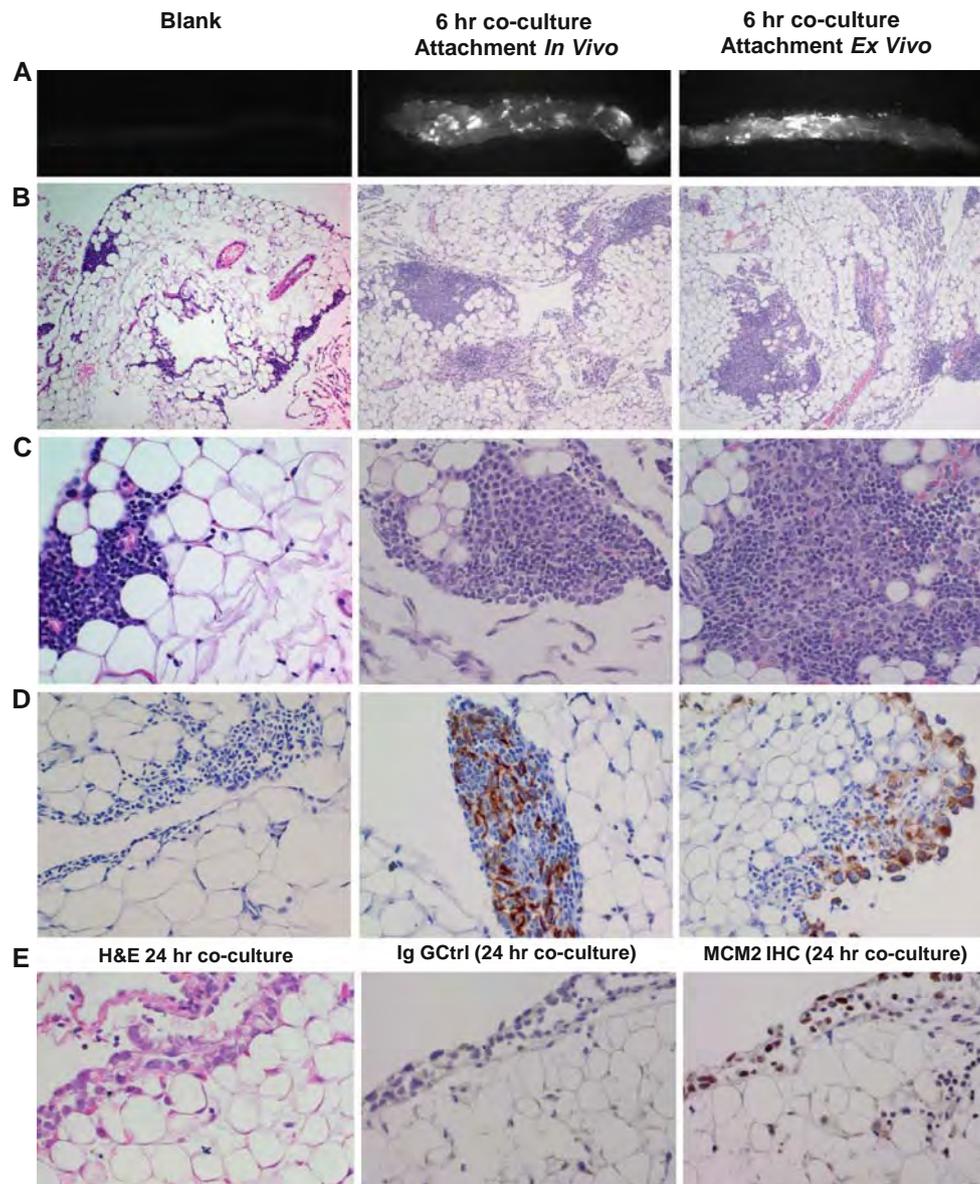
## Discussion

To enhance studies of ovarian cancer metastatic colonization, we need easily manipulatable, physiologically relevant models. At this time, there are several elegant models available for *in vitro* studies. Some incorporate two dimensional cultures employing a monolayer of mesothelial cells or extracellular matrix [29–31]. These models are vastly restricted by their simplicity as they lack the host of other cells that the omentum is composed of. Any influence these cells may have on the behavior of the cancer cells *in vivo* may thus not be seen *ex vivo*. A more sophisticated three dimensional model developed by Kenny et al. uses primary human fibroblasts extracted from normal human omentum, mixed with ECM and covered by a layer of primary human mesothelial cells, also from normal human omentum. After addition of ovarian cancer cells, the histological appearance of their culture recapitulated key aspects of microscopic metastases to the omentum from

patients with ovarian cancer [15]. Such models are powerful in that they use cellular components from clinical samples, are well-suited for mechanistic studies of adhesion and invasion, and recapitulate cellular attributes of *in vivo* systems. However, like all models, they have some limitations. Their preparation is laborious and requires fresh clinical resources which are not available to many researchers and are difficult to obtain on a routine basis. As mentioned previously, they lack certain cell types, for example, the immune cells found in milky spots. Further, they do not provide the structural information conferred by the intact organ and their *ex vivo* reconstitution design is based on assumptions about the key components of the omentum that are involved in metastatic colonization. We sought to complement currently available models by developing an approach to maintain the omentum in culture *ex vivo* while retaining tissue architecture. Further, our goal was to develop conditions that are amenable to a variety of research settings.

Herein we report a reproducible method for identifying and excising the omentum from mice. This first step is crucial as many investigators, including us, initially had difficulty identifying and cleanly excising the omentum. It should be noted that if even a small amount of pancreas remains attached to the omentum it will cause rapid tissue degradation under *ex vivo* conditions, presumably due to release of digestive enzymes from the exocrine pancreas. Our straightforward approach to distinguish the omentum from the pancreas on the basis of buoyancy eliminates this problem. Methodical evaluation of tissue culture media formulations and optimizing the physical conditions (i.e. well size, media volume, etc.) identified optimal conditions for the maintenance of omental tissue structures. Under these conditions, the highly-vascularized omentum continued to secrete IL-6 and maintained tissue architecture for more than 1 week, with only slight changes in histologic tissue appearance at 15 dpi. The prolonged viability of the omentum under *ex vivo* conditions may be due in part to the small size of the omentum (1–2 mm diameter) and the enhanced fluid permeability of this adipose-rich tissue.

The optimal conditions for maintaining omental tissue structure and function also supported growth of cancer cells *in vitro* and *ex vivo* after adhesion to the omentum. Ovarian cancer microcolonies were readily formed on the omentum either by injected cells *in vivo* or mixing the omentum with cancer cells *ex vivo*. Interestingly, we noted that the overall size of the omentum was reduced after *ex vivo* culture. We suspect that this was due to a gradual loss of tissue mass and not general tissue degradation as the tissue retained intact structures such as foci of immune aggregates and vessels, and adipocytes had a healthy appearance. We also noted that with each media change, a small film of lipid was observed on the surface of the



**Fig. 5** Tumor colonization of the omentum occurs in explant culture. The columns show either naive omenta (*left*), omenta from mice 6 h after being injected with  $1 \times 10^6$  SKOV3ip.1 cells (*middle*) or omenta incubated for 6 h with SKOV3ip.1-GFP cells in ex vivo culture conditions (*right*). Row **a** Fluorescence imaging shows similar patterns of cancer cell localization to omenta exposed to SKOV3ip.1-GFP cells either in vivo or ex vivo. Rows **b** and **c** Histologic analysis using H&E staining indicates similar cancer cell colonization occurring in the milky spots of the omentum after 6 h of in vivo or ex

vivo exposure to cancer cells (Row **b** 10× Magnification, Row **c** 40× Magnification). SKOV3ip.1-GFP cells are seen as large cells with abnormal nuclei which are located within milky spot structures. Row **d** the presence of cancer cells (*brown-staining cells*) within milky spots of omenta was also confirmed using cytokeratin staining. Row **e** the viability of cells which attach to the omentum ex vivo and are maintained in ex vivo culture for 24 h was examined by IHC. *Left* H&E, *middle* IHC with IgG control antibody, *right* IHC with anti-MCM2 antibody

culture, perhaps indicating a loss of intracellular lipid or of adipocytes. A second possibility is that the omental tissue contracted during ex vivo maintenance due to lack of tethering forces found in vivo. We are currently conducting studies to potentially distinguish between these two possibilities to further optimize experimental conditions to reduce or eliminate loss of tissue mass.

An intriguing outcome of these studies was the observation of structural components of the omentum. In previous work from our laboratory we noted that during metastatic colonization SKOV3ip.1 cells are invariably found in association with immune aggregates [27]. Similar findings in the current study prompted a rigorous evaluation of the literature. We found that omenta from a wide

variety of animals contain aggregates of immune cells which were first described by von Recklinghausen in 1863 [32, 33] and termed milky spots by Ranvier in 1875 [28]. In the omentum, these structures are specialized to enable mobilization of immune cells for migration into the peritoneal cavity. They may also facilitate reentry of immune cells from the peritoneum into the connective tissue (and therefore bloodstream) [34]. This implies that in addition to the localization of immune cells, milky spots have specialized cellular junctions that participate in regulating cell transit into and out of the peritoneal cavity. In agreement with our observations two recent papers showed the preferential localization of cancer cells to milky spots on the omentum [14, 35]. Remarkably, the physiologic functions of milky spots, or even their existence, have not been integrated into generally accepted models of ovarian cancer metastasis. This is a crucial oversight as it does not consider the possibility that ovarian cancer cells may exploit a highly specialized organ that filters peritoneal fluid and is the source of immune cells and growth factors in order to adhere, survive, and grow into metastases. Evidence for specific interactions of ovarian cancer cells with milky spot structures immediately identifies a target for mechanism-based studies of ovarian metastatic colonization. It is interesting that in our athymic nude mouse omentum, the milky spots retain their function. We hypothesize that macrophages within the milky spots are the key immune cells enhancing or enabling metastatic colonization and are currently testing this hypothesis with further studies. Thus, the maintenance of milky spot structures may provide a key advantage for our omental explant model.

The number of women who die from metastatic ovarian cancer has remained relatively constant. Because of the advanced nature of the disease at the time of initial diagnosis, targeting the process of metastatic colonization has been largely overlooked by the translational research community. While identifying the disease in the earlier stages is a high priority, controlling the disease in patients already afflicted *is equally important*. Women undergoing surgical cytoreduction are immediate candidates for therapies that target metastatic colonization. It is possible that such adjuvant therapies could prevent or delay relapse after local therapy. Studies of omental metastatic colonization would be expedited by the availability of simple and accessible physiologically relevant experimental tools. Work herein demonstrates the feasibility of an *ex vivo* approach and provides an experimental framework for mechanism-based studies. Our goal is to provide this model to investigators in order to facilitate work on omental metastatic colonization. As an example, our model could be extended to use omenta from transgenic and knock-out mice to study roles of specific molecules in ovarian cancer metastasis. It is our hope that rather than being limited by

endpoint assays, investigators will be able to gain access to real-time data by utilizing an *ex vivo* omental culture model such as the one presented herein.

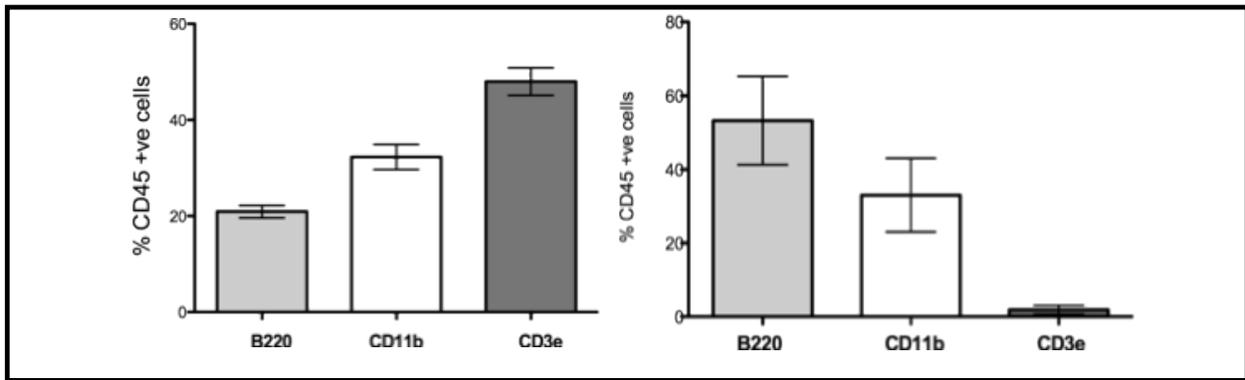
**Acknowledgments** We would like to thank Ms. Jennifer Taylor for her support and assistance in the conducting the intraperitoneal injections and for generously providing the SKOV3ip.1-GFP tagged cells for use in this study. We appreciate the supportive and enthusiastic input of Dr. Karl Matlin and Dr. Jerry Turner of the University of Chicago as we developed work reported herein. We appreciate the superb technical support and assistance of Dr. Lynnette Gerhold of the University of Chicago Optical Imaging Core Facility and Ms. Shirley Bond of the University of Chicago Biological Sciences Division Microscopy Core Facility. This work was made possible by the generous philanthropic support of The University of Chicago Section of Urology and grants from The Department of Defense Ovarian Cancer Research Program (W81XWH-09-0127) and the National Cancer Institute (2R01CA089569-06A2).

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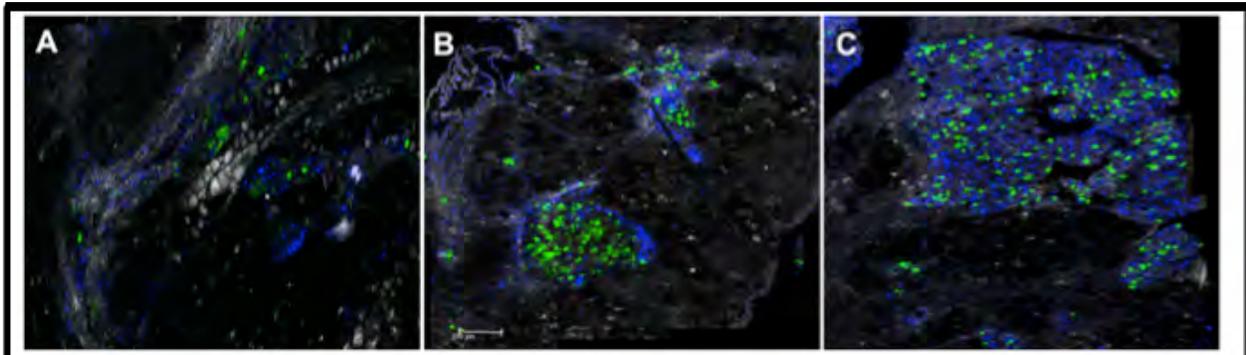
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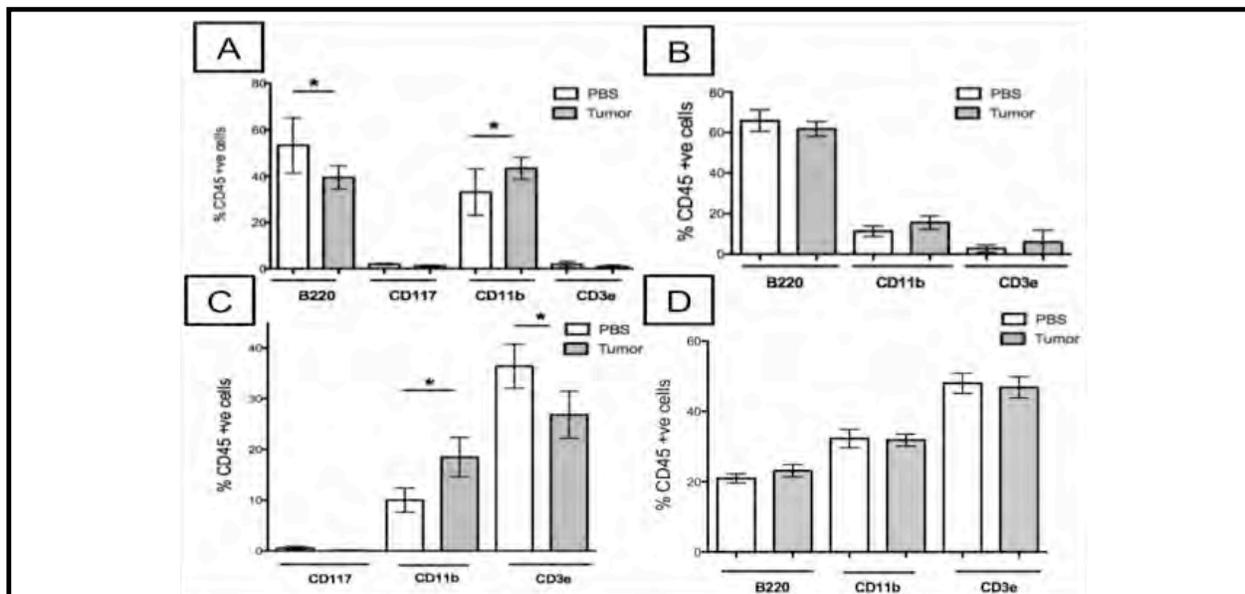
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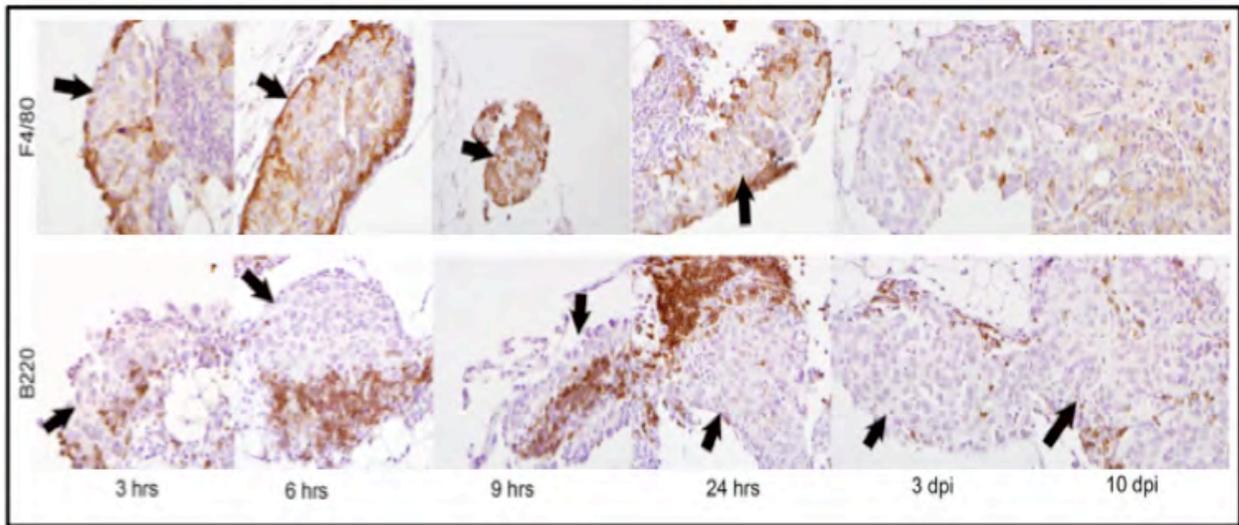
**Fig.1. Immunotype of naïve omentum of C57Bl/6 and Nude mouse.** Quantification of immune cells of naïve C57Bl/6 (left panel) and Nude (right panel) omentum was performed using FACS analysis of CD45-positive cells using the following fluorescent antibodies: B220 (B-cells), CD8 $\epsilon$  (T-cells), CD11b (monocytes). The relative abundance of each immune cell type is expressed as a percentage of the CD45 (leukocytes) population (n=11). Bars are standard deviation.



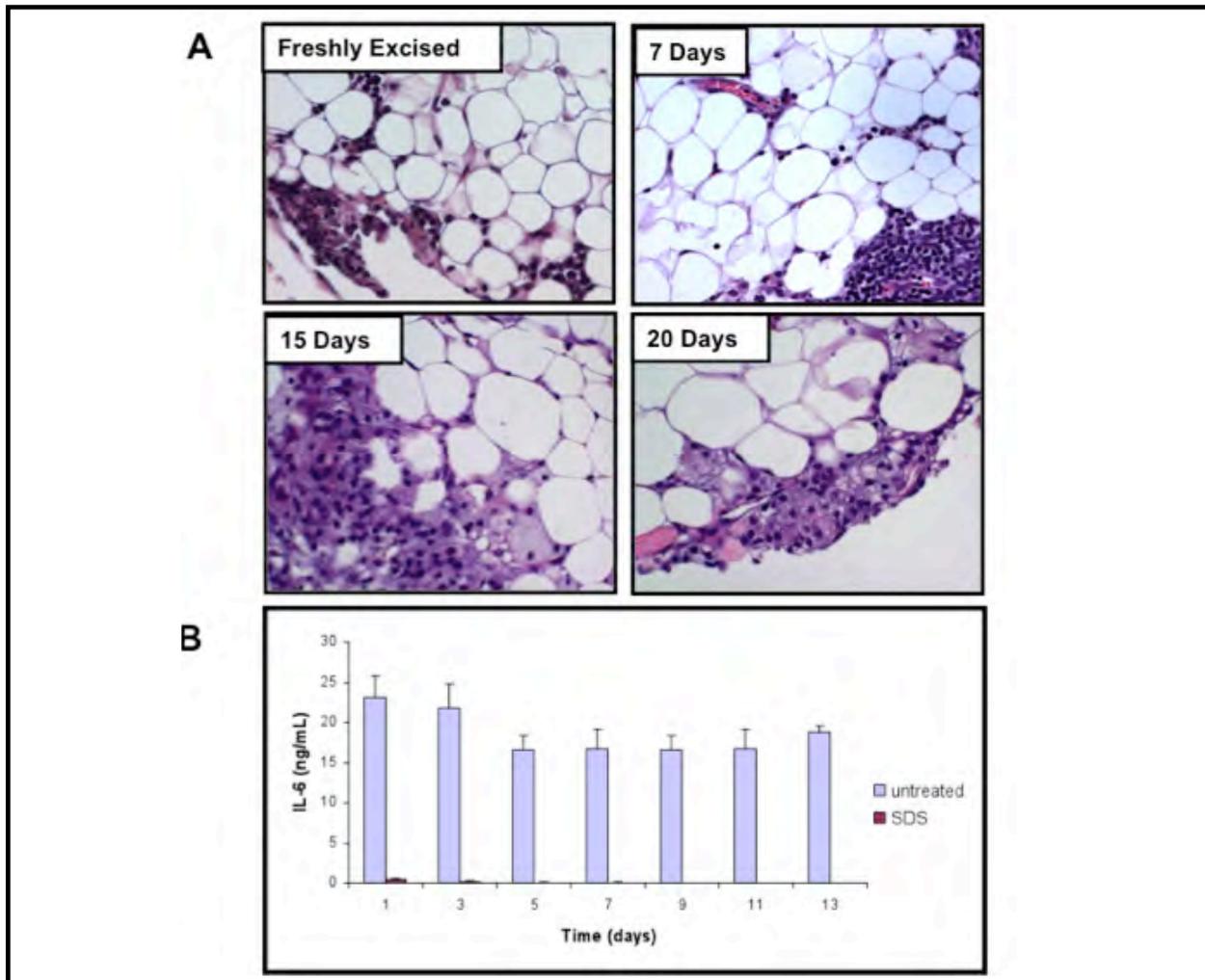
**Fig. 2. Ovarian tumor cell metastasis to omental milky spots.** Female CD1 mice were i.p injected with  $8 \times 10^5$  SKOV3ip.1-GFP cells and omentum was harvested at 30, 40 and 60 min post-injection and stained with 2 $\mu$ g/ml Hoechst for 30 min. Images were acquired using 2-photon confocal microscope (objective 20X).



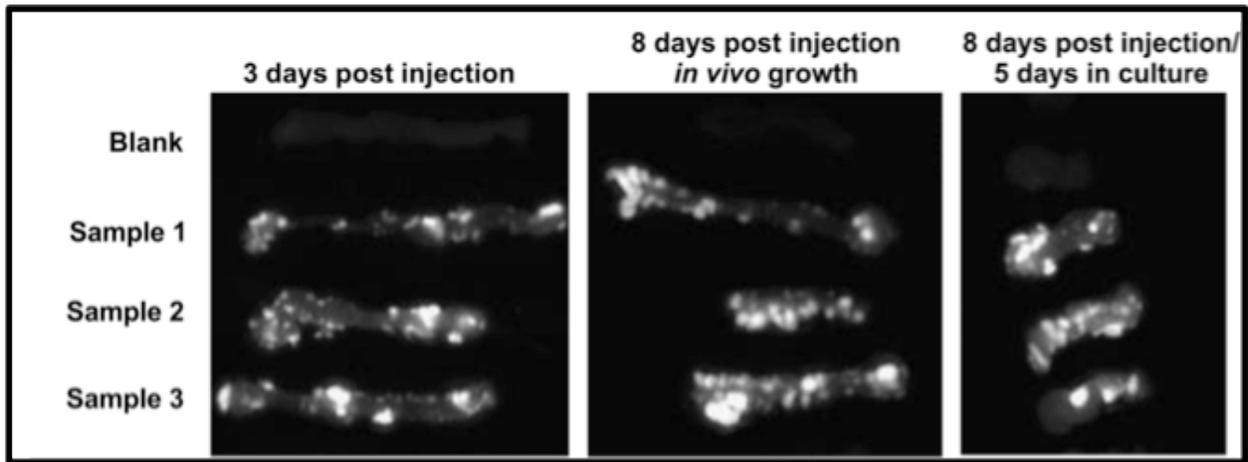
**Fig. 3. Immunotype of ovarian tumor-bearing omentum.** Nude or C56Bl/6 mice were injected i.p with PBS or SKOV3ip.1 (A and B) or ID8 (C and D) cells. At different time points after injection (A: 1 day, C: 5 days, B and D: 10 days) quantification of immune cell population was performed using FACS analysis of CD45-positive cells using the following fluorescent antibodies: B220 (B-cells), CD8 $\epsilon$  (T-cells), CD11b (monocytes) and CD117 (mast cells). The relative abundance of each immune cell type is expressed as a percentage of the CD45 (leukocytes) population (n=10).



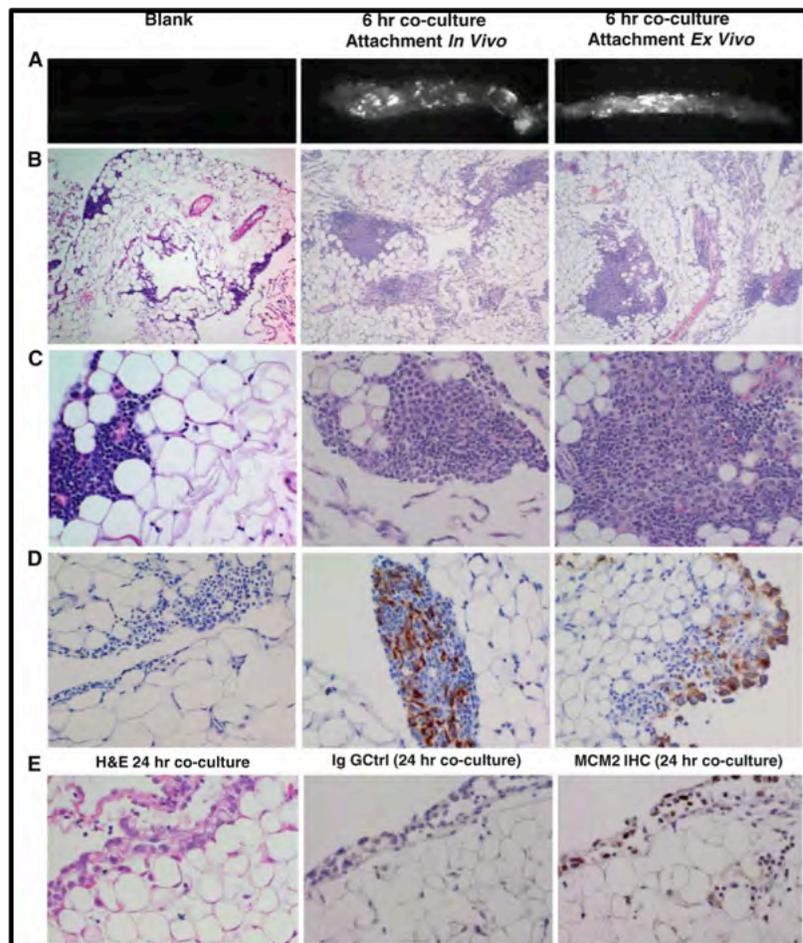
**Fig. 4. Localization of macrophages and B-cells in the omentum in presence of ovarian tumor cells.** Nude mice were i.p injected with human SKOV3ip.1 ovarian cancer cells and the omentum was harvested at different time points posty-injeciton. Immunohistochemistry for the macrophage marker F4/80 and the B-cell marker B220 (brown staining) was performed. Arrows indicate the presence of tumor cells.



**Fig. 5. Viability of *ex vivo* organ cultures.** (A) H&E staining of omenta cultured *ex vivo* for different length of time. (B) Quantification of secreted IL-6 levels in culture of *ex vivo* organ explant using ELISA.



**Fig. 6. Ovarian tumor cells can grow in the omentum cultured *ex vivo*.** Nude mice were i.p injected with  $10^6$  SKOV3ip.1-GFP cells, after 3 days post-injection, omenta were isolated from one group of animals and cultured *ex vivo* while omenta from the second group of mice were culture *in vivo*. After 8 days post-injection, the presence of ovarian tumor cells in the omentum was detected by imaging the omenta using OV100 In Vivo System Imaging for fluorescence.



**Fig. 7. Ovarian tumor colonization in *ex vivo* organ culture model.** Representative pictures of naïve omentum (left), omenta 6h after injection with SKOV3ip.1-GFP cell *in vivo* (middle) or omenta 6h after incubation with SKOV3ip.1-GFP cells *ex vivo* (right). (A) Fluorescent imaging showing similar pattern of distribution of SKOV3ip.1-GFP cells across the different conditions. (B-C) H&E staining pictures showing the same distributions of tumor cells within the milky spots in both the *in vivo* and *ex vivo* models. (D) Immunohistochemistry for human cytokeratin confirmed the presence of human SKOV3ip.1 tumor cells in the omental milky spots. (E) Immunohistochemical stain for MCM2, proliferation marker showing that tumor cells that adhered to the omentum are still proliferating. The middle panel represents the IgG control condition.