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**PRINCIPAL INVESTIGATOR:** Carrie W. Rinker-Schaeffer, Ph.D.

**CONTRACTING ORGANIZATION:** The University of Chicago  
Chicago, IL 60422

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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b>  In order to control ovarian cancer metastasis formation, there is significant interest in identifying the tissue microenvironments involved in cancer cell colonization of the omentum. Omental adipose is a site of prodigious metastasis in both ovarian cancer models and clinical disease. It is unusual as it contains milky spots, structures consisting of immune cells, stromal cells and structural elements surrounding glomerulus-like capillary beds. Contrary to studies reporting that omental colonization is adipocyte-driven, work presented herein shows that milky spots and adipocytes play distinct, complementary roles in omental metastatic colonization. Specifically, <i>in vivo</i> assays showed that ID8, CaOV3, HeyA8 and SKOV3ip.1 cancer cells preferentially lodge and grow within omental and splenoportal fat, which contain milky spots, as compared to other peritoneal fat depots. Similarly, media conditioned by milky spot-containing adipose tissue caused 75% more cell migration than media conditioned by milky spot-deficient adipose. Studies using a panel of immune-deficient mice showed that the mouse genetic background does not alter omental milky spot number and size, nor does it affect ovarian cancer colonization. Finally, consistent with the role for lipids as an energy source for cancer cell growth, <i>in vivo</i> time-course studies found an inverse relationship between metastatic burden and omental adipocyte content. Our findings provide new insights into the critical role milky spots play in omental metastatic colonization, the critical first step in the development of widespread peritoneal disease.					
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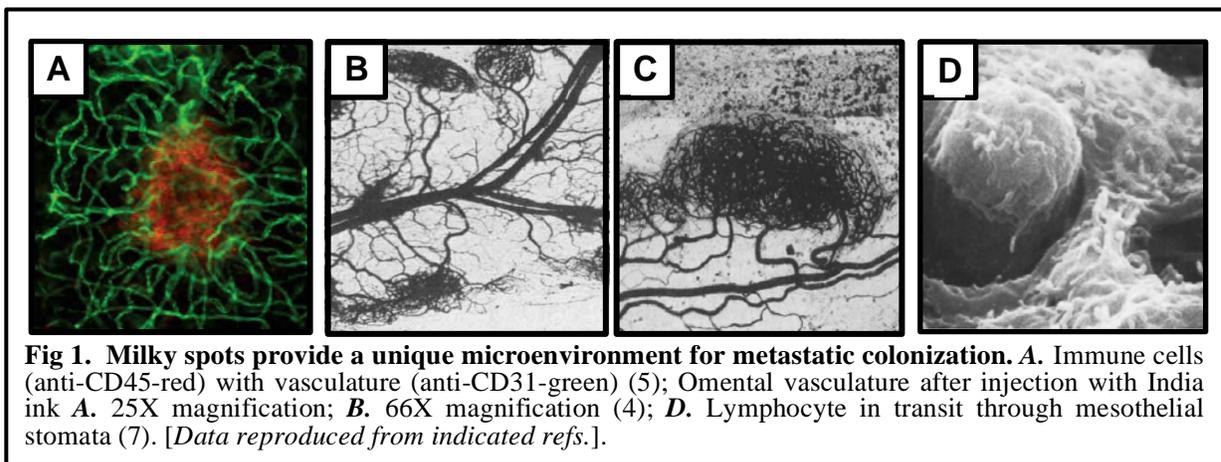
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## Introduction:

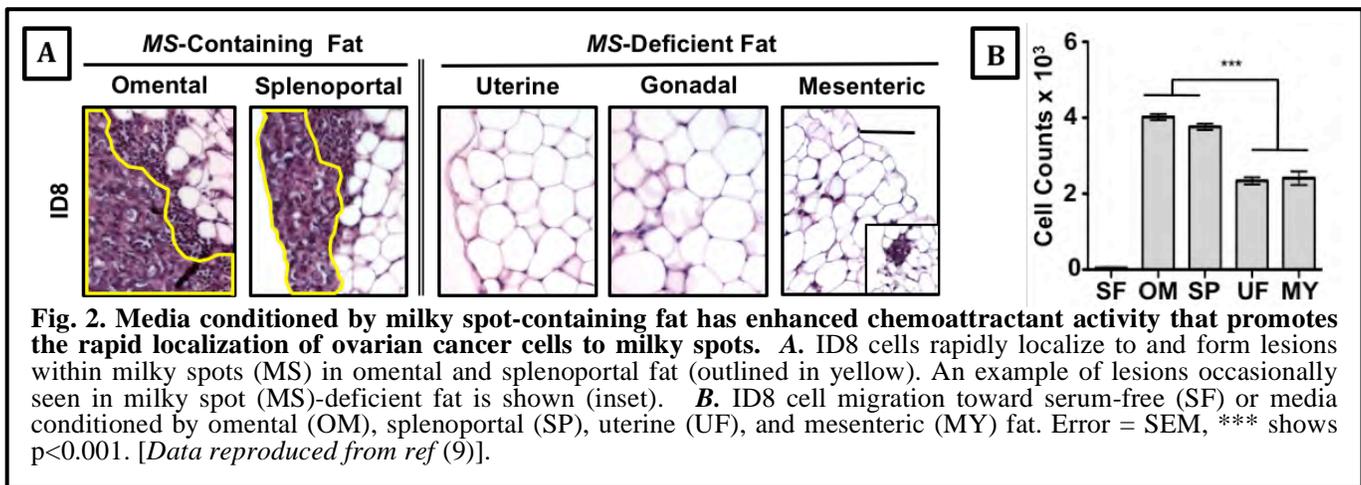
As described in the 2012 progress report, we have recently demonstrated a requirement for immune cell-containing structures known as *milky spots* in metastatic colonization of peritoneal adipose by ovarian cancer cells. Further, *in vivo* studies using a panel of immunodeficient mice lacking T-, B- and/or NK- cells suggested that macrophages may play a functional role in promoting the localization of ovarian cancer cells to these structures. In support of this notion, data presented herein found that depletion of peritoneal macrophages via clodronate treatment abrogated both ovarian cancer cell localization to the omentum and the ability of omentum-conditioned media to promote ovarian cancer cell migration *in vitro*. As described in the next section of this report, these findings prompt the hypothesis that the milky spot macrophages play a functional role in the unique microenvironment that supports the survival and growth of disseminated ovarian cancer cells and their subsequent progression to widespread peritoneal disease.

## Body:

**Milky spots provide a niche for metastatic colonization.** Disseminated cells require a receptive tissue microenvironment in order to engraft and grow into clinically relevant metastases. Cancer cells can create niches by secreting factors that prompt their formation, or utilize/adapt pre-existing physiologic niches to support their growth (1, 2). Taken together, data from our laboratory and others raised the possibility that ovarian cancer cells use milky spots as a niche for metastatic colonization. A literature review found similarities between milky spots and pre-metastatic niches (1, 2). Like pre-metastatic niches, milky spots contain progenitor (mesenchymal) cells, immune cells, and fibroblasts that are localized to capillary nests [Fig. 1, A-C, (3, 4-6)]. Capillary endothelial cells and the overlying mesothelium are adapted for immune cell migration [Fig.1D, (7, 3)].



**Omental tissues secrete one or more factors that promote ovarian cancer cell migration.** After intraperitoneal (ip) injection, ovarian cancer cells rapidly localize to milky spots in omental and splenoportal fat, but not uterine, gonadal, or mesenteric fat depots, which lack milky spot structures [Fig. 2A; (8, 9)]. This suggested that a cell(s) within milky spots produce(s) a factor(s) promoting cancer cell localization; however, previous studies have examined only the contribution of isolated adipocytes (10). To address this, conditioned media from tissue equivalents of omental, splenoportal, uterine, and mesenteric fat were used as chemoattractants in cell migration assays (9). Media conditioned by omental and splenoportal fat caused a 95-fold increase in migration as compared to serum-free media (Fig. 2B). In contrast, milky spot-deficient uterine and mesenteric fat showed a 75% reduction in the migration-stimulatory activity in their conditioned media (Fig. 2B).



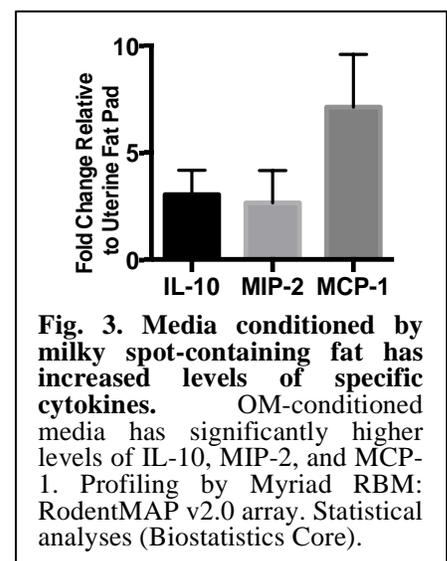
### Key Research Accomplishments:

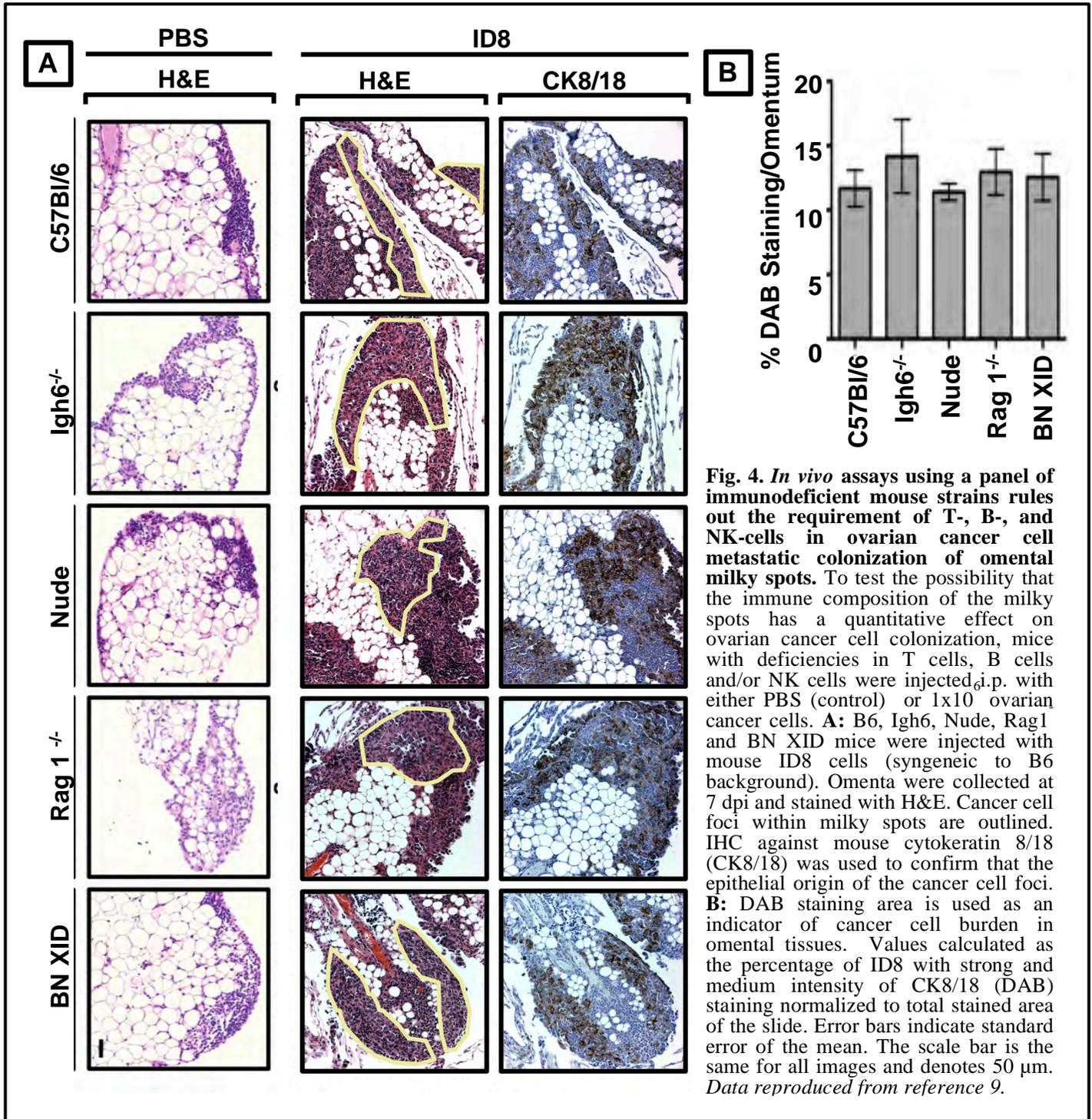
1. Demonstrated that omentum-conditioned media contains increased level of macrophage-associated cytokines.
2. Showed that omental adipose tissue macrophages (ATMs) are required for ovarian cancer cell colonization of milky spots.
3. Demonstrated that omental ATMs are required for the enhanced ability of omentum-conditioned media to promote cancer cell migration *in vitro*.
4. Optimized a protocol for the isolation of ATMs from omental and gonadal fat.
5. Demonstrated that media conditioned by ATMs isolated from omental fat promotes significantly more cancer cell migration than media conditioned by ATMs isolated from gonadal fat.
6. Established new collaborations with Dr. Victoria Seewaldt (Duke University), Dr. Patricia Shaw (Univ. of Toronto), and Dr. Lev Becker (Univ. of Chicago) to conduct future studies prompted by our research findings.

### Reportable Outcomes:

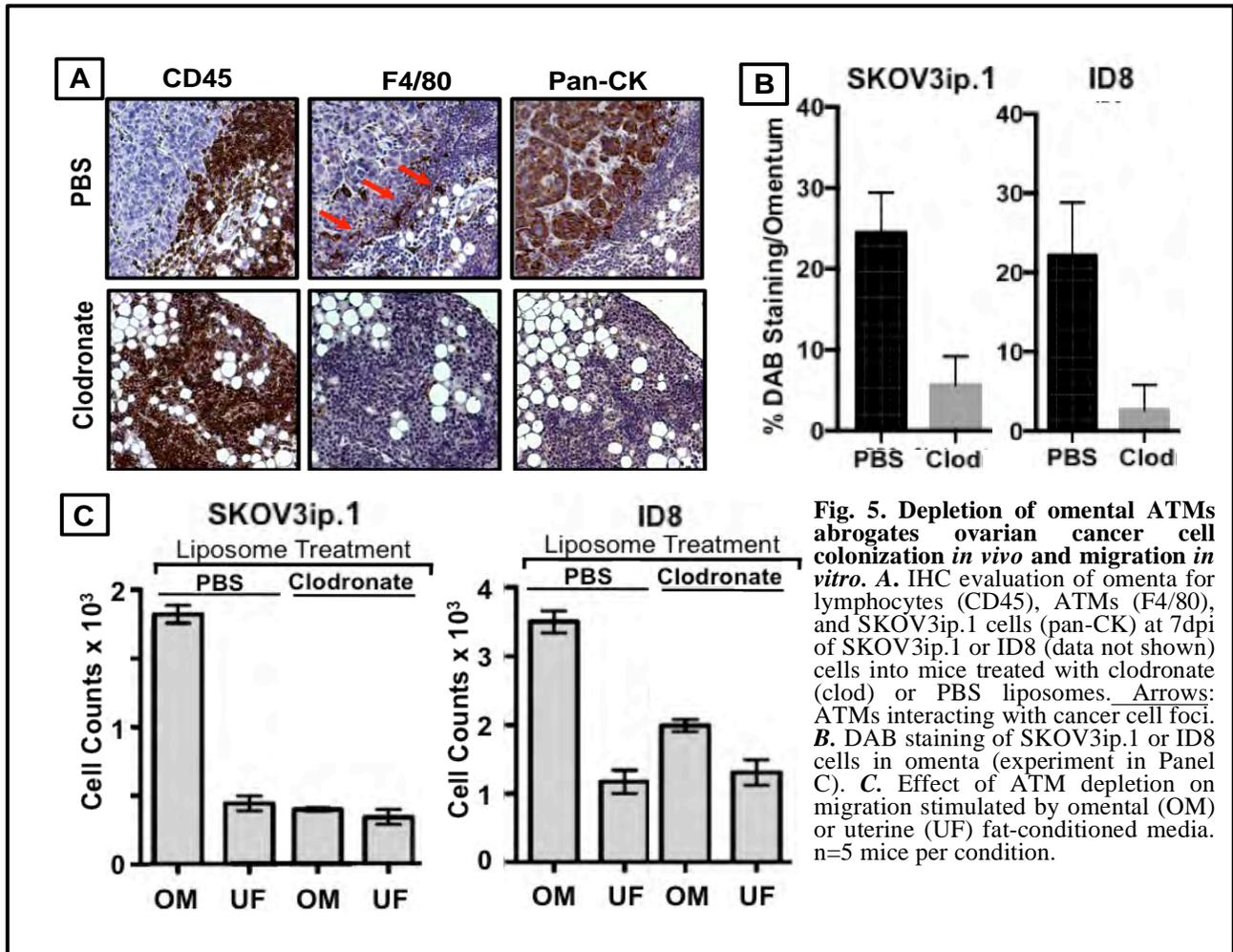
**Omental adipose tissue macrophages (ATMs) are required for ovarian cancer cell colonization of milky spots.** Results from the quantitative cell migration assays presented in Fig. 2 suggested that milky spots provide a soluble factor(s) that increases the chemoattractive activity of peritoneal fat. In support of this, profiling found increased levels of macrophage-associated cytokines IL-10, MCP-1, and MIP-2 in omentum-conditioned media (Fig. 3). This is consistent with the role of the omentum in supplying macrophages to the peritoneal cavity (3, 11-14).

Finding increased levels of macrophage-associated cytokines in omentum-conditioned media prompted us to test the contribution of omental ATMs, which reside within milky spots, to ovarian cancer cell localization to these structures. ID8 cells were intraperitoneally (ip) injected into immunocompetent (C57/Bl6) and immunodeficient (Igh6<sup>-/-</sup>, Nude, Rag1<sup>-/-</sup>, and BN XID) mice. There was no difference in the incidence of cancer cell foci in the omentum across these strains, ruling out a requirement for T, B, and/or NK cells colonization (Fig. 4).





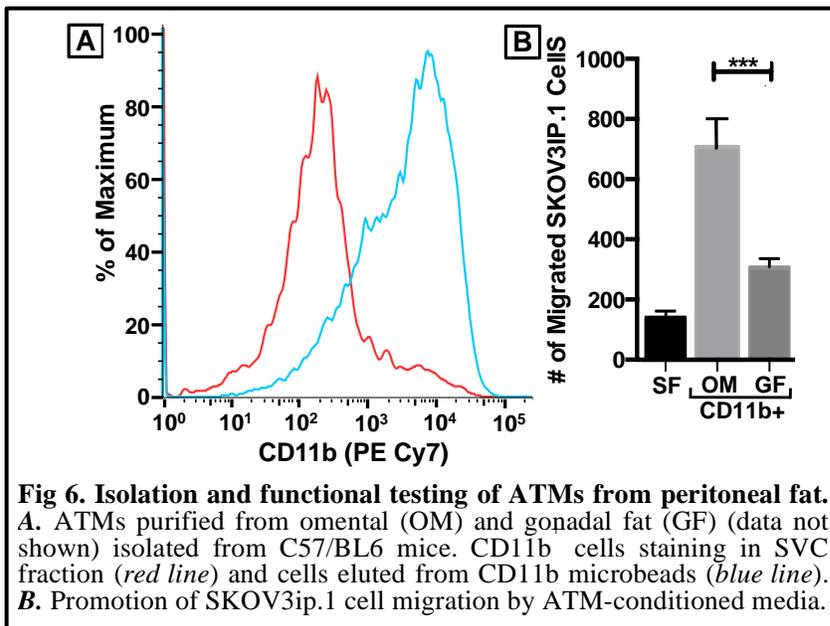
We next tested the possibility that omental ATMs, which are present in all these mouse strains, play a functional role in milky spot colonization (9). Depletion of ATMs prior to ip injection of SKOV3ip.1 cells abrogated cancer cell localization to milky spots in both omental (Fig. 5A, B) and splenoportal fat (data not shown). Quantitative *in vitro* assays show migration stimulated by conditioned media from ATM-depleted omenta (OM) is on a par with migration induced by media conditioned by uterine fat (UF), which lacks milky spots (Fig. 5C).



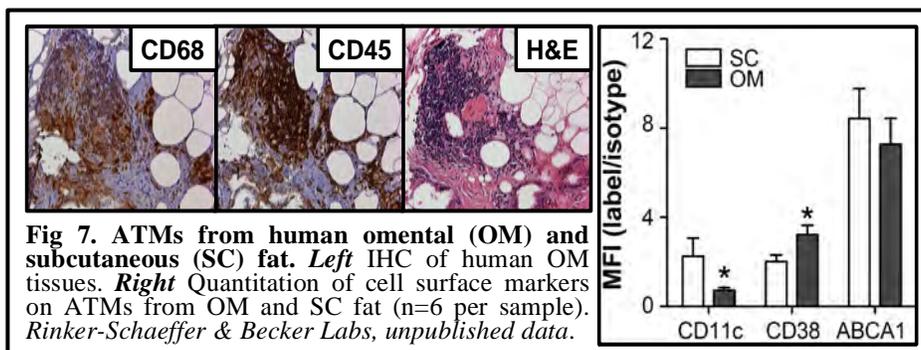
**Fig. 5. Depletion of omental ATMs abrogates ovarian cancer cell colonization *in vivo* and migration *in vitro*.** A. IHC evaluation of omenta for lymphocytes (CD45), ATMs (F4/80), and SKOV3ip.1 cells (pan-CK) at 7dpi of SKOV3ip.1 or ID8 (data not shown) cells into mice treated with clodronate (clod) or PBS liposomes. Arrows: ATMs interacting with cancer cell foci. B. DAB staining of SKOV3ip.1 or ID8 cells in omenta (experiment in Panel C). C. Effect of ATM depletion on migration stimulated by omental (OM) or uterine (UF) fat-conditioned media. n=5 mice per condition.

The requirement for omental ATMs in ovarian cancer cell localization to milky spots suggests that in addition to the well-characterized role of tumor-associated macrophages in supporting tumor growth and development (15, 16), ATMs may participate in the initial phases of metastatic colonization. As resident tissue macrophages, omental ATMs play a key role in tissue maintenance and omental function (17, 18). The specific physiology and function(s) of ATMs is determined by signals from their immediate microenvironment, which activate the cellular pathways needed for the required function (17). In the case of the omentum, ATMs are located in milky spots, providing a microenvironment differing vastly from that experienced by ATMs in gonadal, uterine, and mesenteric fat, which lack milky spots (Figs. 1 & 2). This raised the possibility that ATMs from milky spot-containing and -deficient fat have different physiologic patterns of protein expression, which correspond to activation of pathways required for their tissue-specific functions (19, 20).

**Omental ATM-conditioned media has an enhanced ability to promote migration.** ATMs isolated from omental and gonadal fat confirm that they are responsible for the difference in the migration-promoting activities of media conditioned by milky spot-containing and -deficient fat (Figs. 2B & 5C). ATMs were isolated from stromal and vascular cells (SVC) [Fig. 6 left]. Using *in vitro* migration assays, omental ATM-conditioned media promoted significantly more migration than media conditioned by ATMs from gonadal fat [Fig. 6, right]. These data suggest that omental ATMs have a different functional phenotype and secrete factor(s) that increases the chemo-attractive activity of omental fat.



**Isolation and evaluation of ATMs from clinical samples.** The structure of human milky spots is analogous to that of rodents and other mammals, with ATMs being a significant portion of the immune cell population (3,14,21-24). As a first step toward translational studies, we confirmed our ability to detect and isolate ATMs in human omentum. Milky spots were evaluated for expression of CD45 (lymphocyte) and CD68 (macrophage) using standard immunohistochemical (IHC) techniques (Fig. 9, left). Similarly, we successfully isolated ATMs from human omental and subcutaneous fat (as described in Fig. 6). After purification, the expression of cell surface markers on ATMs was assessed by flow analysis (Fig. 7, right). This showed that omental ATMs expressed a significantly lower level of CD11c (integrin  $\alpha$  X chain protein) and a significantly higher level of CD38 (cyclic ADP ribose hydrolase) than ATMs isolated from subcutaneous fat. These findings suggest that in humans, ATMs in milky spot-containing and -deficient fat also have distinct PM protein patterns. In sum, these data show our ability to: 1) identify ATMs in human milky spots; 2) isolate ATMs from human fat; and, 3) quantitate cell surface markers on ATMs from these tissues.



### Conclusions and Future Directions

To develop therapies targeting metastatic colonization, we must first identify the mechanisms that control this process. Progress toward this goal has been stymied for three main reasons. **First**, is the lack of animal models and experimental tools needed to study metastatic colonization. **Second**, is the failure to consider omental contributions to metastasis formation. **Finally**, is the lack of model cell lines that mimic disease and comprehensive human tissue resources with clinical data. Findings from

this DOD award have served as a foundation for establishment of new collaborations that leverage our team's expertise, unique resources, and innovative tools to overcome these hurdles.

As a result of our work on this DOD grant we developed optimized xenograft models that are enabling us to dissect cellular and molecular events over the time course of omental metastatic colonization. Assays using these models allow the quantitative assessment of both early and late steps in the process. Omental colonization assays evaluate localization, survival, and proliferation, while metastasis formation assays evaluate growth, progression, and formation of overt metastases (15,17). We can now use these models to further dissect the interactions between disseminated cells and milky spot macrophages that play a functional role in metastatic colonization. To achieve this goal we are employing innovative tools identify the pathway(s) regulating omental ATM function.

Going forward, we will use a novel mass spectrometric approach focused on plasma membrane proteomics that has been used to successfully interrogate a variety of macrophage phenotypes (19, 20). Developed by Dr. Lev Becker (University of Chicago), our new collaborator, this approach affords important advantages. Plasma membrane proteins are well positioned for flow cytometric analysis, which is widely used to isolate and interrogate macrophage populations. It also provides insights into signal transduction pathways, since such proteins recruited to the cell surface via protein-protein interactions are readily co-purified in our plasma membrane preparations. Finally, bioinformatics analysis generates unbiased functional hypotheses that can be tested experimentally.

Finally, we have assembled the expertise and resources needed to efficiently and effectively translate our findings into the clinical setting. Dr. Patricia Shaw (University of Toronto)<sup>1</sup> is internationally recognized as a leader in efforts to understand the pathobiology and development of high-grade serous cancer from fallopian tube epithelial cells. She has developed a repository of samples that will allow the development of fimbrial tubal epithelial-derived cell lines that model ovarian cancer precursor cells (which are now believed to give rise to high grade serous cancers). Such model cell lines will enable us to evaluate the early events in omental metastatic colonization and subsequent progression to widespread peritoneal metastases.

We have also established a collaboration with Dr. Vicky Seewaldt and Dr. Anne Ford (both of Duke University) who run a clinic for patients at high risk for development of ovarian cancer (BRCA1/2 mutation carriers). We will leverage the high-risk clinical cohort (BRCA mutation carriers) developed by Drs. Seewaldt and Ford to validate pathways that regulate milky spot ATM functions identified in our studies. As a result, this effort will also generate the largest data set ever created on milky spot number, structure, and composition in adult women and will enhance the ability to pursue larger scale studies that target women at high risk for ovarian cancer.

In summary, work completed during DOD funding has now launched new collaborative translational studies that we believe are poised to have a significant impact on high-grade serous ovarian cancer (HGSC), the major cause of peritoneal disease. Since its early detection is rarely successful and prevention strategies are limited, the majority of patients have advanced disease at the time of diagnosis (28). Women with inherited mutations in the BRCA1 or BRCA2 genes are at increased risk for developing ovarian cancer (29-33). Such individuals often undergo prophylactic removal of the ovaries and fallopian tubes to reduce cancer risk (34, 35). Unfortunately, many patients still develop HGSC due to the outgrowth of precursor ovarian cancer cells present within metastatic sites at the time of surgery. Our team is in a unique position to study mechanisms regulating this process. We appreciate the support of the DOD, which enabled the work that is the foundation for our future efforts.

## References

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<sup>1</sup> Dr. Shaw is part of the DOD Ovarian Cancer Research Program Consortium

1. Psaila B, Lyden D. The metastatic niche: adapting the foreign soil. *Nat Rev Cancer*. Nature Publishing Group; 2009;9:285–93.
2. Peinado H, Lavotshkin S, Lyden D. The secreted factors responsible for pre-metastatic niche formation: old sayings and new thoughts. *Semin Cancer Biol*. 2011;21:139–46.
3. Usunoff KG, Michailova KN. The milky spots of the peritoneum and pleura: structure, development and pathology. *Biomedical Reviews*. Berlin/Heidelberg: Springer Berlin Heidelberg; 2006;183:1–17.
4. Simer PH. On the Morphology of the Omentum with Especial Reference to its Lymphatics. *American Journal of Anatomy*. Springer-Verlag; 2013;54:203–28.
5. Gerber SA, Rybalko VY, Bigelow CE, Lugade AA, Foster TH, Frelinger JG, et al. Preferential attachment of peritoneal tumor metastases to omental immune aggregates and possible role of a unique vascular microenvironment in metastatic survival and growth. *Am J Pathol*. 2006;169:1739–52.
6. Sorensen EW, Gerber SA, Sedlacek AL, Rybalko VY, Chan WM, Lord EM. Omental immune aggregates and tumor metastasis within the peritoneal cavity. *Immunol Res*. Humana Press Inc; 2009;45:185–94.
7. Mironov VA, Gusev SA, Baradi DAF. Mesothelial stomata overlying omental milky spots: Scanning electron microscopic study. *Cell Tissue Res*. Springer-Verlag; 2004;201:327–30.
8. Khan SM, Funk HM, Thiolloy S, Lotan TL, Hickson J, Prins GS, et al. In vitro metastatic colonization of human ovarian cancer cells to the omentum. *Clin Exp Metastasis*. 2010;27:185–96.
9. Clark R, Krishnan V, Schoof M, Rodriguez I, Theriault B, Chekmareva M, et al. Milky Spots Promote Ovarian Cancer Metastatic Colonization of Peritoneal Adipose in Experimental Models. *Am J Pathol*. American Society for Investigative Pathology; 2013;183:576–91.
10. Nieman KM, Kenny HA, Penicka CV, Ladanyi A, Buell-Gutbrod R, Zillhardt MR, et al. Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. *Nature Medicine*. Nature Publishing Group; 2011;17:1498–503.
11. Shimotsuma M, Simpson-Morgan MW, Takahashi T, Hagiwara A. Activation of Omental Milky Spots and Milky Spot Macrophages by Intraperitoneal Administration of a Streptococcal Preparation, OK-432. *cancer resewarch*. 2006;52:5400–2.
12. Krist L, Eestermans IL, steenbergen J, hoefsmit E, cuesta M, meyer S, et al. Cellular Composition of Milky Spots in the Human Greater Omentum: An Immunochemical and Ultrastructural Study. *The Anatomical Record*. 2004;241:163–74.
13. Cranshaw ML, Leak LY. Milky spots of the omentum: a source of peritoneal cells in the normal and stimulated animal. *Arch Histol Cytol*. 1990;53 Suppl:165–77.
14. Mixer RL. On macrophagal foci (“milky spots”) in the pleura of different mammals, including man. *American Journal of Anatomy*. Wiley Subscription Services, Inc., A Wiley Company;

1941;69:159–86.

15. Ruffell B, Affara NI, Coussens LM. Differential macrophage programming in the tumor microenvironment. *Trends Immunol.* 2012;33:119–26.
16. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. *Nat Rev Cancer.* Nature Publishing Group; 2009;9:239–52.
17. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nature Reviews Immunology.* Nature Publishing Group; 2008;8:958–69.
18. Davies LC, Jenkins SJ, Allen JE, Taylor PR. Tissue-resident macrophages. *Nature Immunology.* Nature Publishing Group; 2013;14:986–95.
19. Becker L, Gharib SA, Irwin AD, Wijsman E, Vaisar T, Oram JF, et al. A macrophage sterol-responsive network linked to atherogenesis. *Cell Metab.* 2010;11:125–35.
20. Becker L, Liu N-C, Averill MM, Yuan W, Pamir N, Peng Y, et al. Unique proteomic signatures distinguish macrophages and dendritic cells. *PLoS ONE.* Public Library of Science; 2012;7:e33297.
21. Wilkosz S, Ireland G, Khwaja N, Walker M, Butt R, Giorgio-Miller A, et al. A comparative study of the structure of human and murine greater omentum. *Anat Embryol.* 2005;209:251–61.
22. Krist LFG, Koenen H, Calame W, van der Harten JJ, van der Linden JC, Eestermans IL, et al. Ontogeny of milky spots in the human greater omentum: An immunochemical study. *The Anatomical Record.* Wiley Subscription Services, Inc., A Wiley Company; 1997;249:399–404.
23. Shimotsuma M, Kawata M, Hagiwara A, Takahashi T. Milky spots in the human greater omentum. *Cells Tissues Organs.* Karger Publishers; 1989;136:211–6.
24. Shimotsuma M, Takahashi T, Kawata M, Dux K. Cellular subsets of the milky spots in the human greater omentum. *Cell Tissue Res.* Springer-Verlag; 1991;264:599–601.
25. Lotan T, Hickson J, Souris J, Huo D, Taylor J, Li T, et al. c-Jun NH2-terminal kinase activating kinase 1/mitogen-activated protein kinase kinase 4-mediated inhibition of SKOV3ip.1 ovarian cancer metastasis involves growth arrest and p21 up-regulation. *Cancer Res.* American Association for Cancer Research; 2008;68:2166–75.
26. Hickson JA, Huo D, Vander Griend DJ, Lin A, Rinker-Schaeffer CW, Yamada SD. The p38 kinases MKK4 and MKK6 suppress metastatic colonization in human ovarian carcinoma. *Cancer Res.* American Association for Cancer Research; 2006;66:2264–70.
27. Bainer RO, Veneris JT, Yamada SD, Montag A, Lingen MW, Gilad Y, et al. Time-dependent transcriptional profiling links gene expression to mitogen-activated protein kinase kinase 4 (MKK4)-mediated suppression of omental metastatic colonization. *Clin Exp Metastasis.* 2012;29:397–408.
28. Vaughan S, Coward JI, Bast RC, Berchuck A. Rethinking ovarian cancer: recommendations for improving outcomes. ... *Reviews Cancer.* 2011;11:719–25.

29. D F Easton DFDTB. Breast and ovarian cancer incidence in BRCA1-mutation carriers. Breast Cancer Linkage Consortium. American Journal of Human Genetics. Elsevier; 1995;56:265.
30. Foulkes WD, Narod SA. Ovarian cancer risk and family history. The Lancet. Elsevier; 1997;349:878.
31. Antoniou A, Pharoah PDP, Narod S, Risch HA, Eyfjord JE, Hopper JL, et al. Average Risks of Breast and Ovarian Cancer Associated with BRCA1 or BRCA2 Mutations Detected in Case Series Unselected for Family History: A Combined Analysis of 22 Studies. The American Journal of Human Genetics. Elsevier; 2003;72:1117–30.
32. King M-C, Marks JH, Mandell JB, Group TNYBCS. Breast and ovarian cancer risks due to inherited mutations in BRCA1 and BRCA2. Science. 2003.
33. Sjøgaard M, Krüger Kjær S, Gayther S. Ovarian cancer and genetic susceptibility in relation to the BRCA1 and BRCA2 genes. Occurrence, clinical importance and intervention. Acta Obstet Gynecol Scand. 2006;85:93–105.
34. Rebbeck TR, Lynch HT, Neuhausen SL, Narod SA, van't Veer L, Garber JE, et al. Prophylactic Oophorectomy in Carriers of BRCA1 or BRCA2 Mutations. N Engl J Med. 2002;346:1616–22.
35. Olivier RI, Van Beurden M, Lubsen MAC, Rookus MA, Mooij TM, van de Vijver MJ, et al. Clinical outcome of prophylactic oophorectomy in BRCA1/BRCA2 mutation carriers and events during follow-up. British Journal of Cancer. 2004;90:1492–7.

## **Appendices**

### **i. Publications**

### **ii. Collaborative Resources (for future studies)**



## This Month in *AJP*

### New Mouse Model for Systemic Autoimmune Disease

The lack of successful mouse models for mutation-activated JAK1-induced diseases hampers the understanding of disease pathology related to signaling downstream of cytokine receptor activation. Sabrautzki et al (*Am J Pathol* 2013, 183:352–368) isolated a dominant *Jak1* mouse line carrying a new nonsynonymous point mutation (*Jak1*<sup>S645P+/-</sup>). The morphological, histological, clinical chemical, and hematological phenotypes of *Jak1*<sup>S645P+/-</sup> mice correlate with systemic autoimmune diseases. This new model may be useful for the development of JAK kinase inhibitors to treat arthritis, psoriasis, lupus, colitis, and multiple types of cancer.

### DMA Prevents Preterm Birth

Preterm birth (PTB) causes more than 70% of perinatal morbidity and mortality in the United States. Using an established mouse model, Sundaram et al (*Am J Pathol* 2013, 183:422–430) investigated the effect of pro-inflammatory cytokines in inflammation-associated PTB pathogenesis. *In vitro* and *in vivo* analyses showed that the common organic solvent *N,N*-dimethylacetamide (DMA) can prevent endotoxin-induced preterm birth in timed pregnant mice and rescue their pups from spontaneous abortion at doses many-fold lower than those currently used clinically and in a dose-dependent fashion. DMA may be a promising novel anti-inflammatory agent not only in PTB but also in a broad spectrum of inflammatory disorders.

### C3 Protects the Retina in Age-Related Macular Degeneration

Complement component C3 is a key inflammatory protein activated in age-related macular degeneration (AMD), a

leading cause of blindness in the elderly in the Western world. Kam et al (*Am J Pathol* 2013, 183:480–492) examined retinal integrity in aged mice deficient in various complement pathway components. Data show that both uncontrolled C3 activation (via complement factor H deletion) and complete absence of C3 negatively impact aged retinas. Data are consistent with a major role for C3 in maintaining retinal health and also indicate an additional role for complement factor H in this process that is not related to its role in C3 regulation.

### Human Oligodendrocyte Progenitor Injury in MS

Remyelination in multiple sclerosis (MS) is often incomplete, and the source of cells and the basis for their limited effectiveness remains unclear. Cui et al (*Am J Pathol* 2013, 183:516–525) compared the relative susceptibility of adult human oligodendrocyte progenitor cells (OPCs) and mature oligodendrocytes (OLs) to injury in actively demyelinating MS lesions and under *in vitro* stress conditions. Results suggest that vulnerability of human OPCs to conditions that induce injury of mature OLs in MS lesions contribute to the limited remyelination observed in both acute and chronic stages of MS.

### Ovarian Cancer Colonizes Milky Spots

Omental adipose, which contains milky spots—structures consisting of immune cells—is a site of prodigious metastasis in ovarian cancer models and in clinical disease. Clark et al (*Am J Pathol* 2013, 183:576–591) examined the milky spot-driven model of omental colonization using both *in vivo* and *in vitro* assays. Their findings support a two-step model wherein the localization of disseminated cancer cells is first dependent upon milky spots, and then adipocytes are required for progressive growth and subsequent spread to other sites within the peritoneal cavity.



TUMORIGENESIS AND NEOPLASTIC PROGRESSION

# Milky Spots Promote Ovarian Cancer Metastatic Colonization of Peritoneal Adipose in Experimental Models

Robert Clark,<sup>\*</sup> Venkatesh Krishnan,<sup>†</sup> Michael Schoof,<sup>†</sup> Irving Rodriguez,<sup>†</sup> Betty Theriault,<sup>‡</sup> Marina Chekmareva,<sup>§</sup> and Carrie Rinker-Schaeffer<sup>†</sup>

From the Department of Pathology—Molecular Pathogenesis and Molecular Medicine,<sup>\*</sup> the Section of Urology,<sup>†</sup> Department of Surgery, and the Animal Resources Center,<sup>‡</sup> The University of Chicago, Chicago, Illinois; and the Department of Pathology,<sup>§</sup> Robert Wood Johnson Medical School, New Brunswick, New Jersey

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Address correspondence to  
Carrie Rinker-Schaeffer, Ph.D.,  
Section of Urology, Department  
of Surgery, MC6038, The  
University of Chicago, 5841  
S. Maryland Ave., Chicago,  
IL 60637. E-mail: [crinkers@uchicago.edu](mailto:crinkers@uchicago.edu).

The goal of controlling ovarian cancer metastasis formation has elicited considerable interest in identifying the tissue microenvironments involved in cancer cell colonization of the omentum. Omental adipose is a site of prodigious metastasis in both ovarian cancer models and clinical disease. This tissue is unusual for its milky spots, comprised of immune cells, stromal cells, and structural elements surrounding glomerulus-like capillary beds. The present study shows the novel finding that milky spots and adipocytes play distinct and complementary roles in omental metastatic colonization. *In vivo* assays showed that ID8, CaOV3, HeyA8, and SKOV3ip.1 cancer cells preferentially lodge and grow within omental and splenoportal fat, which contain milky spots, rather than in peritoneal fat depots. Similarly, medium conditioned by milky spot-containing adipose tissue caused 75% more cell migration than did medium conditioned by milky spot-deficient adipose. Studies with immunodeficient mice showed that the mouse genetic background does not alter omental milky spot number and size, nor does it affect ovarian cancer colonization. Finally, consistent with the role of lipids as an energy source for cancer cell growth, *in vivo* time-course studies revealed an inverse relationship between metastatic burden and omental adipocyte content. Our findings support a two-step model in which both milky spots and adipose have specific roles in colonization of the omentum by ovarian cancer cells. (*Am J Pathol* 2013, 183: 576–591; <http://dx.doi.org/10.1016/j.ajpath.2013.04.023>)

It is estimated that 22,240 women will be diagnosed with and 14,030 women will die of cancer of the ovary in 2013 ([http://seer.cancer.gov/csr/1975\\_2009\\_pops09](http://seer.cancer.gov/csr/1975_2009_pops09), last accessed June 18, 2013). The majority of patients present with metastases or eventually die of metastatic disease within the abdominal cavity. After escape from the primary tumor, ovarian cancer cells in the peritoneal fluid have access to and

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R.C. and V.K. contributed equally to this work.

Current address of M.S. and I.R., Department of Natural Sciences, Stanford University, Stanford, CA.

can potentially lodge within a variety of tissues.<sup>1,2</sup> In both clinical disease and experimental models, however, the omentum is the site of prodigious metastasis formation.<sup>3–6</sup> Thus, attachment of ovarian cancer cells to the omentum represents an early step in the development of widespread peritoneal disease.<sup>7,8</sup> Although the importance of the omentum is widely acknowledged, there still is no consensus on its role in metastasis formation. This raises the question of what components of the omental tissue microenvironment participate in, or facilitate, ovarian cancer metastatic colonization.

Studies of omental function date back to the early 19th century. Jobert de Lamballe, a 19th-century surgeon in France, was reportedly the first to recognize the curious ability of this organ to fight infection and form adhesions to help control injuries.<sup>9</sup> After nearly two centuries of investigation, a great deal is known about the physiology and surgical applications of the omentum.<sup>9–12</sup> As the central regulator of peritoneal homeostasis, its functions include regulating fluid and solute transport, sensing and repairing injuries, promoting angiogenesis, fighting infection, serving as a source of stem cells, producing regulatory molecules, and storing and supplying lipids. These diverse functions are conferred by the cellular composition and architecture characteristic of human omenta.

Aside from the clear collagenous membrane that acts as a scaffold for the organ, the majority of the omentum is composed of bands of adipose tissue that contain adipocytes, blood and lymph vessels, immune cells, stromal cells, and connective matrix components that lie beneath an irregular mesothelium.<sup>13</sup> In general, adipocytes have a variety of functions, ranging from lipid storage to production of endocrine molecules, and can serve as an integrating hub for inflammation, metabolism, and immunity.<sup>2,14–23</sup> A distinctive feature of the omental vasculature is the presence of numerous branching blood vessels ending in tortuous glomerulus-like capillary beds near the tissue periphery.<sup>24–28</sup> Immune cells aggregate around and within these capillary beds to form milky spots, which are the major immune structure for host defense of the peritoneal cavity.<sup>24,29–36</sup> In milky spots, both the endothelial lining of the capillaries and the overlying mesothelium are specially adapted to facilitate transmigration of immune cells.<sup>24,37,38</sup> Additional structural elements include plasmocytes, fibroblasts, and mesenchymal cells, as well as collagen and reticular and elastic fibers.<sup>29,34,37,39</sup>

A comprehensive literature review showed that studies examining the role of the omentum in metastasis focus on the contribution of its individual components, and not on the tissue as a whole. In our view, results from the majority of studies support models in which ovarian cancer metastatic colonization is driven either purely by milky spots or purely by adipocytes. The milky spot–driven model is based on a large body of *in vivo* data showing that, on intraperitoneal injection, cancer cells rapidly and specifically localize, invade, and proliferate within omental milky spots.<sup>3,6,24,28,40–44</sup> In contrast, the adipocyte-driven model is based on the observation that, in

its resting state, the omentum is composed predominantly of adipose and that cultured adipocytes can produce adipokines capable of promoting ovarian cancer cell migration and invasion *in vitro*.<sup>45</sup> Adipocytes can also provide a proliferative advantage by transferring fatty acids to ovarian cancer cells.<sup>45</sup> Although both models have clear strengths, neither addresses the intimate and dynamic interaction among milky spots, surrounding adipocytes, and other components of omental tissues.

Taking tissue architecture and function as a guide, we propose that an alternative, more fully integrated model of metastatic colonization is needed. To test this idea, we identified peritoneal fat depots (omentum, mesentery, and uterine, gonadal, and splenoportal fat) that are accessible to ovarian cancer cells after intraperitoneal injection.<sup>2</sup> Of these, the omentum and splenoportal fat are reported to contain milky spot structures.<sup>24,46</sup> We reasoned that a comparison of peritoneal adipose that either contains or lacks milky spots could be used to determine the contributions of adipocytes and milky spots to the lodging and progressive growth of ovarian cancer cells in physiologically relevant tissues. *In vivo* studies using a panel of ovarian cancer cell lines showed that milky spots dramatically enhance early cancer cell lodging on peritoneal adipose tissues. Consistent with this finding, conditioned medium from milky spot–containing adipose tissue had a significantly increased ability to direct cell migration, compared with conditioned medium from milky spot–deficient adipose tissue. Studies using a panel of immunodeficient mice showed that the number and size of omental milky spots is not dependent on the mouse genetic background and, similarly, that ovarian cancer cell colonization does not depend on the immune composition of the milky spot. Finally, consistent with the role for lipids as an energy source for ovarian cancer cell growth, *in vivo* time-course studies revealed an inverse relationship between metastatic burden and adipocyte content in the omentum. Our findings support a two-step model in which both milky spots and adipose have specific roles in colonization of the omentum by ovarian cancer cells.

## Materials and Methods

### Cell Lines

The SKOV3ip.1 human ovarian carcinoma cell line<sup>47</sup> was generously supplied by Dr. Gordon Mills (MD Anderson Cancer Center, Houston, TX). These cells were maintained in standard growth medium [Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L D-glucose, 584 mg/L L-glutamine, and 110 mg/L sodium pyruvate (Mediatech, Manassas, VA), supplemented with 5% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), and 1% penicillin/streptomycin (P/S) solution (a mixture of 5000 IU/mL penicillin and 5000 µg/mL streptomycin), 1× nonessential amino acids, and 2× minimum essentials medium vitamin solution (all from Mediatech)]. The HeyA8 human ovarian carcinoma cell line (ATCC, Manassas, VA) was maintained in standard growth medium [DMEM supplemented with 5% fetal bovine

serum, 1% P/S, 1× nonessential amino acids, and 1× minimum essentials medium vitamin solution]. The CaOV3 human ovarian carcinoma cell line (ATCC) was maintained in standard growth medium [DMEM supplemented with 8% fetal bovine serum and 1% P/S]. The ID8 mouse ovarian carcinoma cell line, derived from and syngeneic to mice of the C57BL/6 background,<sup>4</sup> was generously provided by Dr. Katherine Roby (University of Kansas Medical Center, Kansas City, KS). These cells were maintained in a standard growth medium [DMEM supplemented with 4% fetal bovine serum, 1% P/S solution, and 5 µg/mL insulin-transferrin-sodium selenite (Roche Diagnostics, Indianapolis, IN)]. ID8 cells that stably express tdTomato (ID8-tdTomato) were constructed by lentiviral delivery of pLVX-tdTomato expression vector (Clontech, Mountain View, CA) followed by selection for puromycin resistance. In brief, 3 µg of pLVX-tdTomato and 9 µg of ViraPower lentiviral packaging mix (Life Technologies—Invitrogen, Carlsbad, CA) was transfected into HEK293T cells to generate the viral conditioned medium. The ID8 cells were transduced with the viral medium and established by selection in medium containing 0.6 µg/mL puromycin. Fluorescence-activated cell sorting using a BD FACSAria II system (BD Biosciences, San Jose, CA) at the University of Chicago Flow Cytometry Core Facility was used to select for high tdTomato-expressing cells. All cells were maintained under standard tissue culture conditions (ie, in a humidified incubator at 37°C supplemented with 5% CO<sub>2</sub>).

## Mice

All mice were housed, maintained, and euthanized according to Institutional Animal Care and Use Committee guidelines and under the supervision of the University of Chicago Animal Resource Center. Outbred CD1 mice were obtained from Charles River Laboratories International (Wilmington, MA). Inbred B6 (C57BL/6NHsd; immunocompetent), Nude (Athymic Nude-*Foxn1*<sup>nu</sup>; T-cell deficient), and BN XIX (Hsd:NIHS-*Lys1*<sup>bg</sup>*Foxn1*<sup>nu</sup>*Btk*<sup>xid</sup>; NK-cell and T-cell deficient) mice were obtained from Harlan Laboratories (Indianapolis, IN). Rag1 (B6.129S7-*Rag1*<sup>tm1Mom</sup>/J; with no mature T or B cells, no CD3-positive cells, and no T cell receptor  $\alpha$ - $\beta$ -positive cells) and Igh6 (B6.129S2-*Ighm*<sup>tm1Cgn</sup>/J; lacking mature B cells) mice were obtained from Jackson Laboratories (Bar Harbor, ME).

## Harvesting Mouse Tissues

The locations of the well-defined adipose depots used for the present studies have been described previously.<sup>2</sup> Omenta were harvested as described by Khan et al.<sup>6</sup> Splenoportal fat bands were collected by isolating the thin fatty band of tissue connecting the hilum of the spleen to the pancreas,<sup>46</sup> releasing it from the pancreas, and then carefully dissecting it from the spleen. Uterine fat was excised from the uterine horns using dissecting scissors; gonadal fat was similarly

excised from the ovaries. Mesenteries were collected by first cutting the junction between the small intestine and the pylorus, firmly gripping the free end of the small intestine with forceps, peeling the small intestine from the mesentery by pulling the tissue slowly, and finally releasing the mesentery from the mesentery root using dissecting scissors.

## Preparation of Tissues for Standard Histological Evaluation

Tissues were processed for histological evaluation immediately after harvest or *ex vivo* culture. Larger tissues (whole gonadal fat, uterine fat, and mesentery) were fixed in 10% neutral buffered formalin (Sigma-Aldrich, St. Louis, MO) for 48 hours at 4°C. Smaller tissues (whole omentum, splenoportal fat, and tissue equivalents of uterine fat and mesentery) were fixed overnight (12 to 16 hours) in 5% neutral buffered formalin (Sigma-Aldrich) at 4°C. Fixed tissue was stored in 70% ethanol at 4°C until paraffin embedding. Tissues were sectioned (4 µm thick) and stained with H&E at the University of Chicago Human Tissue Resource Center. Histological evaluation of tissues was performed by a pathologist (M.C.) under masked conditions.

## Immunohistochemistry

Tissue sections were deparaffinized in xylenes and rehydrated through serial dilutions of ethanol to distilled water. Sections were subsequently incubated in antigen retrieval buffer (Dako, Carpinteria, CA) and heated in a steamer to 97°C for 20 minutes. Primary antibodies were diluted in Tris-buffered saline and Tween 20 with 0.025% Triton X-100. Pancytokeratin (clone H-240; sc-15367; Santa Cruz Biotechnology, Santa Cruz, CA) and CD45 (clone H130; 14-0459-82; eBioscience, San Diego, CA) were applied to tissue sections at a 1:100 dilution and incubated for 1 hour at room temperature. After application of primary antibody, slides were rinsed in Tris-buffered saline, and then biotinylated secondary antibody diluted in Tris-buffered saline and Tween 20 (with 10% mouse serum; Jackson ImmunoResearch, West Grove, PA) was applied to the slides. For pancytokeratin staining, sections were incubated with goat anti-rabbit IgG (1:200 dilution; BA-1000; Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature. For CD45 staining, sections were incubated with anti-mouse IgG (1:100 dilution; BA-2001; Vector Laboratories) for 30 minutes at room temperature. The biotinylated secondary antibody was detected using a Vectastain Elite ABC kit (Vector Laboratories) and 3,3'-diaminobenzidine (DAB; Dako) peroxidase substrate. Cytokeratin 8/18 (TROMA-1; Developmental Studies Hybridoma Bank, University of Iowa, Iowa) was applied undiluted on tissue sections for 1 hour. Antigen-antibody binding was detected using a Bond Polymer Refine Detection system (DS9800; Leica Biosystems, Buffalo Grove, IL).

Cytokeratin 8/18–stained slides were scanned using an Aperio ScanScope XT instrument (Aperio Technologies, Vista, CA) at  $\times 20$  magnification. An Aperio color deconvolution algorithm (Color Deconvolution version 9.1; Aperio Technologies) was used for analyzing each omentum for oxidized DAB intensity. The input parameters for each channel (hematoxylin and DAB) were separately calibrated before the analysis. Digital scan of each omentum was selected using the pen tool within the ImageScope viewing software version 11.1.2.752 (Aperio) and analyzed for intensity levels on a four-point scale (3 = strong staining, 2 = medium staining, 1 = weak staining, and 0 = no staining). Intensity was reported as percentage of cells with strong plus medium DAB staining, normalized to the total stained area.

### Peritoneal Metastasis Assay

Exponentially growing SKOV3ip.1, HeyA8, CaOV3, or ID8 cells were trypsinized and prepared as a single-cell suspension at a concentration of  $2 \times 10^6$  cells/mL in ice-cold PBS. Animals were injected intraperitoneally with 500  $\mu$ L of the cell suspension ( $1 \times 10^6$  cells) at a point equidistant between the inguinal papillae. For all experiments, 500  $\mu$ L of ice-cold PBS was injected as a negative control in a parallel group of control mice. At the experimental endpoint of each assay, mice were sacrificed via CO<sub>2</sub> asphyxiation and vital organs were removed. Tissues were then harvested, processed, sectioned, and stained as described above.

### Tissue Dissociation and Flow Cytometry

B6 mice were injected intraperitoneally with either PBS or  $1 \times 10^6$  ID8–tdTomato cells. At 7 days post injection (dpi), animals were sacrificed and peritoneal fat depots were excised and immediately placed in ice-cold PBS. Tissues were subsequently transferred to separate 5-mL Eppendorf tubes containing 3 mL of serum-free (SF) DMEM containing 0.2% (w/v) collagenase I (Worthington Biochemical, Lakewood, NJ) and 0.1% (w/v) bovine serum albumin (Sigma-Aldrich). The tissues were then minced using surgical scissors, and the tissue suspension was incubated at 37°C for 30 minutes with rotational mixing. To enhance tissue dissociation, the digested tissue was transferred to a microstomacher bag and masticated in a Stomacher 80 Biomaster system (Seward, Worthing, UK) on low for 10 minutes, rotating bags after 5 minutes.

To ensure a sufficient yield of cells for flow cytometry, cell suspensions prepared from tissues harvested from three independent mice were pooled to form one sample. Pooled samples were filtered through a nylon mesh filter (60- $\mu$ m pore) to remove larger debris. To collect the cellular component of the digestion, the filtrate was centrifuged at  $250 \times g$  for 5 minutes; the supernatant fraction was removed and discarded. To lyse RBCs, the cell pellet was resuspended in 100  $\mu$ L PBS combined with 900  $\mu$ L ACK

lysing buffer (Invitrogen) and was incubated at room temperature for 1 minute. Cells were collected for flow cytometric analysis by centrifugation at  $250 \times g$  for 5 minutes; the supernatant fraction was removed and discarded. The remaining pelleted cells were resuspended in 250  $\mu$ L ice-cold PBS. Finally, to ensure that no clumped cells remained, the cell suspension was passed through a filter (60- $\mu$ m pore) and the filter was rinsed with 250  $\mu$ L ice-cold PBS. The cell suspension was analyzed for the presence of tdTomato-labeled cells using an LSRII flow cytometer (BD Biosciences) equipped with a 561-nm yellow-green laser and a 585 nm/15 nm bandpass filter. The tdTomato-positive gate was based on analysis of parental ID8 cells. Data are expressed as fold change in ID8–tdTomato cell-injected mice relative to PBS-injected control mice ( $n = 3$  pooled samples for control mice and  $n = 5$  pooled samples for ID8–tdTomato cell–injected mice).

### Preparation of Tissue-Conditioned Medium

Tissues harvested under sterile conditions were immediately placed in ice-cold, sterile PBS. After collection, tissue equivalents were prepared by first weighing the omentum from an individual mouse and then excising a strip of uterine fat or branch of mesentery of equivalent weight. In our studies, a single band of splenoportal fat weighed  $2.90 \pm 0.62$  mg (means  $\pm 2$  SD). Given that this is approximately one half the weight of an omentum ( $6.72 \pm 2.82$  mg), two fat bands were combined to make one tissue equivalent. Each tissue equivalent was placed in its own well of a polystyrene 12-well plate containing 1.25 mL DMEM/F-12 medium supplemented with 20% fetal bovine serum and 1% P/S and was incubated at 37°C in a 5% CO<sub>2</sub>–enriched atmosphere, as described by Khan et al.<sup>6</sup> Tissues were allowed to acclimate to these *ex vivo* conditions for 24 hours, after which the serum-containing medium was removed; the tissues were then carefully rinsed with PBS and placed in 1.25 mL SF DMEM/F-12 medium (containing 1% P/S). After 24 hours of conditioning, the SF medium was collected and used for Transwell migration assays (described below).

For longer *ex vivo* culture, the medium was changed to fresh SF DMEM/F-12 medium at subsequent 24-hour intervals, such that the tissues were fed for the first 24 hours and starved for the remainder of the assay. Conditioned medium from each 24-hour interval was collected. To ensure that stimulatory activity in the conditioned medium was due to secretion of chemotactic factors and not to cellular breakdown, tissue integrity was assessed by H&E staining and light microscopy, as described by Khan et al.<sup>6</sup> Tissue function was also confirmed by measuring IL-6 levels in the conditioned medium. IL-6 is produced by adipose, mesothelial cells, and immune cells and is immediately secreted into the microenvironment.<sup>48</sup> Specifically, enzyme-linked immunosorbent assays (ELISA) for IL-6 were performed on

tissue-conditioned medium using a Mouse IL-6 Platinum ELISA kit (eBioscience, San Diego, CA) according to the manufacturer's instructions.

### Transwell Migration Assay

All migration assays used the same physical setup, with 1 mL of conditioned or control medium (unconditioned SF culture medium) to be tested for chemoattractant activity placed in a well of a 12-well plate acting as the lower chamber. Cancer cells were placed in the upper chamber consisting of a PET 8  $\mu$ m pore membrane in a suspended from an insert scaffold (Corning Life Sciences, Lowell, MA). Exponentially growing SKOV3ip.1 or ID8 cells were trypsinized and resuspended in SF DMEM at a concentration of  $3.33 \times 10^5$  cells/mL or  $2.50 \times 10^5$  cells/mL, respectively. In each case, 600  $\mu$ L of cell suspension (SKOV3ip.1,  $2.0 \times 10^5$  total; ID8,  $1.5 \times 10^5$  total) was added to the upper chamber. Cells were allowed to migrate in response to signals from conditioned or control medium for 6 hours under standard tissue culture conditions.

After incubation, medium was aspirated from both chambers, and the upper and lower surfaces of the membrane were washed once with ice-cold PBS. The membrane was fixed in 10% buffered formalin for 10 minutes at room temperature. Residual formalin was removed by washing with ice-cold PBS. Both sides of the membrane were subsequently stained with 0.05% crystal violet solution (Thermo Fisher Scientific, Waltham, MA) for 30 minutes at room temperature and then washed once with tap water. Cells that had not migrated through the membrane were removed by gently wiping the upper membrane surface with a cotton swab; the membrane was then allowed to air-dry overnight. Finally, the membrane was removed from the Transwell scaffold using a scalpel and was mounted with the top surface facing up onto a slide using Permout mounting medium (Thermo Fisher Scientific). Five random images were captured per slide at  $\times 100$  magnification with a Zeiss Axiovert 200M inverted microscope (Carl Zeiss, Jena, Germany) at the University of Chicago Integrated Microscopy Core Facility, and the number of cells that had migrated through the membrane was quantified. ImageJ 64 software version 1.45s (NIH, Bethesda, MD) was used to quantitate the total number of migrated cells present in each image. The counts from each of the five images were summed to give the number of migrated cells for each sample ( $n = 5$  for all conditions).

### Milky Spot Identification Using Carbon Nanopowder Uptake

Carbon nanopowder was used to visualize omental milky spots, essentially as described previously.<sup>29</sup> In brief, carbon nanopowder (<50-nm particle size; Sigma-Aldrich) was mixed with PBS at a concentration of 5 mg/mL and was sonicated for 20 minutes to produce a homogenous

suspension. The suspension was autoclaved, allowed to cool to room temperature, and resonicated for 20 minutes immediately before injection. Mice were intraperitoneally injected with 800  $\mu$ L of the carbon suspension. At 14 dpi, mice were euthanized. Omenta were harvested and fixed in 10% formalin for 2 hours at room temperature. Whole fixed tissue was dehydrated through increasing concentrations of ethanol (70%, 90%, 100%, and 100%; 10 minutes each) and was cleared in xylene for 10 minutes. Tissues were then whole-mounted between a slide and coverslip using Permout medium. Images of the whole-mounted tissues were captured using a CRi Panoramic Scan Whole Slide Scanner at the University of Chicago Light Microscopy Core Facility, and images were processed with Panoramic Viewer software version 1.15.2 (3DHitech, Budapest, Hungary).

### Milky Spot Identification Using Giemsa Staining

Excised omenta were prepared for standard histological evaluation, with careful orientation of the tissues during paraffin embedding to produce longitudinal sections. The whole omentum was serially sectioned at 4- $\mu$ m thickness, and every third section was Giemsa stained (Fluka; Sigma-Aldrich). Slides were deparaffinized in xylenes and rehydrated through serial dilutions of ethanol to water. Slides were stained in 5% Giemsa solution (prepared in tap water) for 4 minutes, rinsed in tap water for 60 seconds, allowed to air-dry, and mounted using Permout. Images of the stained slides were captured using a CRi Panoramic Scan Whole Slide Scanner and were processed with Panoramic Viewer software (3DHitech).

### Three-Dimensional Rendering of Giemsa-Stained Omentum

ImageJ software was used to render a three-dimensional image of a B6 omentum. Imaged, Giemsa-stained omentum sections from an entire omentum were aligned using the StackReg plug-in.<sup>49</sup> The resulting stack was inverted and the 3D Project tool, with the Interpolate function set to a slide spacing of 4  $\mu$ m, was used to render a three-dimensional image. This was converted to 8-bit grayscale and was false-colored using the Union Jack color scheme.

### Milky Spot Identification and Quantitation in Giemsa-Stained Omentum

ImageJ software was used to quantify milky spot volume in Giemsa-stained omental sections. Images were converted to 8-bit grayscale and inverted. The color balance was adjusted to increase the contrast between the milky spots and surrounding tissue. The image was converted to binary format, to reduce background noise, and then was inverted again. The threshold was set using the Auto function, resulting in an image in which milky spots are pure black and the rest of the tissue is pure white. The immune

aggregates were measured using the Analyze Particles function. This was repeated for each of the Giemsa-stained sections from each omentum. For each omentum, milky spot areas per section were multiplied by 4  $\mu\text{m}$  and summed to yield a total milky spot volume.

The whole omentum volume was measured by adjusting the brightness and contrast of the grayscale image to darken the immune aggregates. The threshold was set to include the entire tissue, and the image was converted to a pure black mask. To make a cohesive border for the omentum, the Find Edges tool was applied, and the image was again converted to a mask and the Fill Holes function was selected. Again, the omentum was quantified using the Analyze Particles function. Particles smaller than 25 pixels were excluded from the analysis, to reduce background noise. Areas for each omentum were multiplied by 4  $\mu\text{m}$  and then were summed to yield a total whole omentum volume ( $n = 5$  omenta per mouse strain).

### Quantitation of Adipocyte Area in Omentum Over the Time Course of Metastatic Colonization

Omenta were harvested from mice at 1, 3, 6, and 9 weeks after ID8 cancer cell injection into B6 mice, and tissues were prepared for standard histological evaluation. H&E-stained sections were imaged using a CRi Panoramic Scan Whole Slide Scanner. With ImageJ software, section images were converted to 8-bit grayscale, the brightness and contrast were adjusted to darken the nonadipocyte tissue, and the image threshold was set (using the Auto function) such that only nonadipocyte structures were transformed to pure black. These structures were measured using the Analyze Particles function, with particles smaller than 25 pixels excluded. Resulting areas were summed to produce a total nonadipocyte area. The whole omentum was measured by adjusting the threshold of a brightness- and contrast-adjusted grayscale image such that the whole area was converted to pure black. Again, the omentum was quantified using the Analyze Particles function, excluding particles smaller than 25 pixels and with the Include Holes option selected. Resulting areas were summed to yield a total omentum area. The percentage of adipocytes was calculated by subtracting the total nonadipocyte area from the total omentum area and dividing that difference by the total omentum area. Three sections were quantified per mouse, with  $n = 5$  mice per time point.

Macros for the several image processing steps are provided in [Supplemental Table S1](#).

### Statistical Analysis

All statistical analyses were performed using GraphPad Prism software version 5.0f (GraphPad Software, La Jolla, CA). Statistical significance was determined using one-way analysis of variance followed by Tukey–Kramer multiple comparison tests. Data are expressed as means  $\pm$  SEM of biological replicates.

## Results

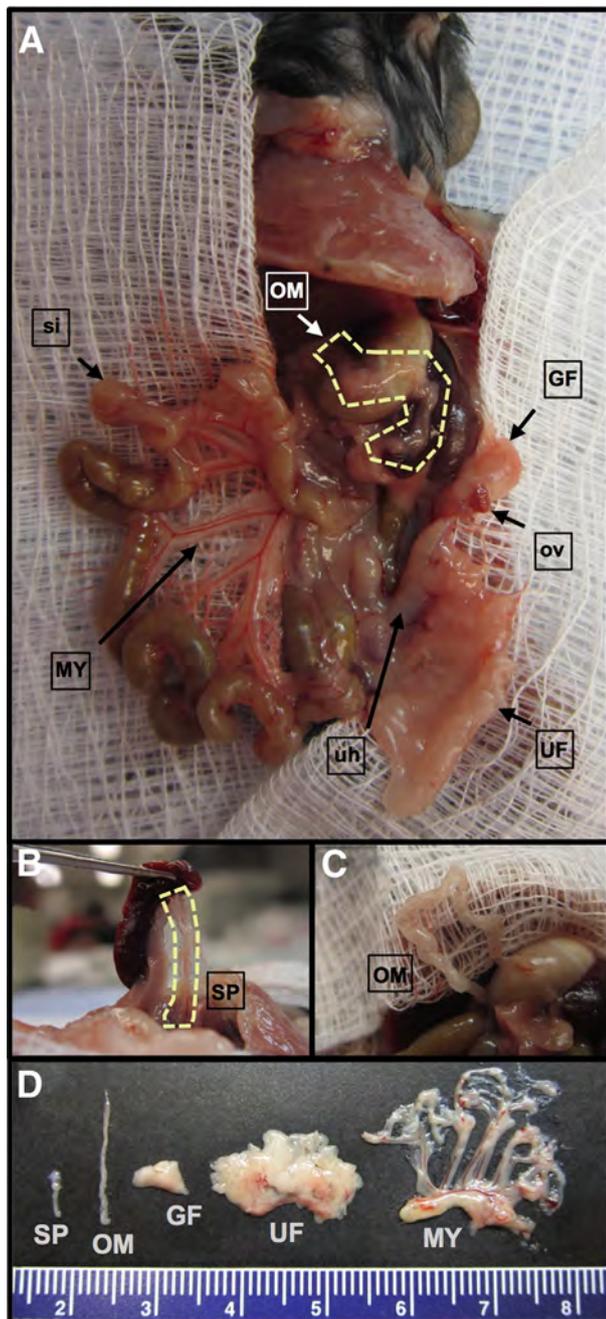
### Several Sources of Adipose Tissue Are Accessible to Ovarian Cancer Cells in the Peritoneal Cavity

The organ-specificity of ovarian cancer metastasis is often explained by the anatomical location and tissue composition of the omentum.<sup>5,44</sup> To our knowledge, however, these assertions have not been tested *in vivo* by evaluating ovarian cancer cell lodging or growth in other adipose depots that are also accessible during peritoneal dissemination. There are five major adipose depot structures in the peritoneal cavity.<sup>2</sup> A lateral view of a ventral dissection allows visualization of the omentum, gonadal fat, uterine fat, and mesentery ([Figure 1A](#)). Further dissection allows for clear visualization of the splenoportal fat, which surrounds the splenic artery and connects the hilum of the spleen to the celiac artery ([Figure 1B](#)), and of the omentum, isolated from the pancreas ([Figure 1C](#)). The gross structure and relative size of these tissues can be seen in [Figure 1D](#).

Interestingly, in 1995 Takemori et al<sup>46</sup> reported the presence of milky spots in the splenoportal fat of New Zealand Black mice that are similar in structure and composition to omental milky spots previously reported in the literature. The authors did not, however, examine cancer cell localization to these structures. Our histological evaluation showed that both the omentum and splenoportal fat have archetypal milky spot structures, but these structures were not observed in uterine, gonadal, or mesenteric fat ([Figure 2A](#)). Furthermore, at 7 days after intraperitoneal injection of SKOV3ip.1 cells, similar cancer cell lesions were observed in both omental and splenoportal fat ([Figure 2B](#)), but not in other fat depots. IHC using a human pancytokeratin antibody showed that the lesions were composed of epithelial (SKOV3ip.1) cells intermingled with the immune cells. The specificity of IHC staining was confirmed using an IgG control for the pancytokeratin antibody ([Figure 2B](#)).

### Ovarian Cancer Cells Preferentially Colonize Peritoneal Adipose That Contains Milky Spots

A review of the literature showed that the predilection of ovarian cancer for omental metastasis formation can be ascribed to either adipose-driven or milky spot–driven mechanisms. However, these models are based on studies focusing on either structural features (ie, milky spots) or cellular components (ie, adipocytes) of omental tissue.<sup>28,42,45</sup> The availability of peritoneal fat depots that contain or lack milky spot structures enabled us to distinguish experimentally between the two models in the physiologically relevant setting of the peritoneal cavity. We reasoned that if colonization were purely adipocyte-driven, then ovarian cancer cells would colonize the various peritoneal adipose depots to a similar extent after intraperitoneal injection. In contrast, if milky spots drive this process, then the omentum and splenoportal fat would have cancer cell foci



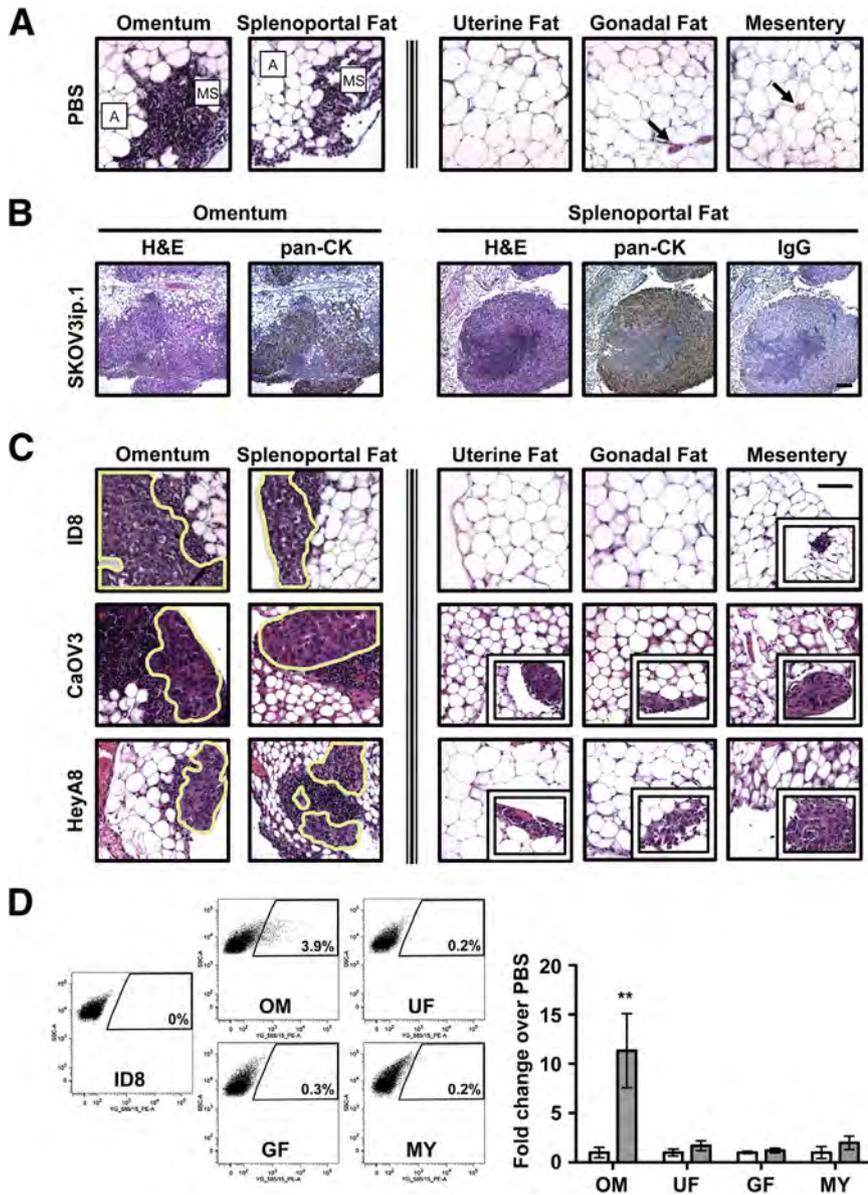
**Figure 1** Relative locations of the main peritoneal adipose depots, with ovary, uterine horn, and small intestine indicated as points of reference. **A:** Left view of the peritoneal cavity of a B6 mouse, exposed via a ventral incision. This gross anatomical dissection shows the relative location of four of the five primary sources of peritoneal fat: the omentum (OM; **outlined**), located above the stomach and spleen; the gonadal fat (GF), surrounding the left ovary (ov); the uterine fat (UF), attached to the left uterine horn (uh); and the mesentery (MY), attached to the small intestine (si). **B:** The fifth source of peritoneal adipose is the splenoportal fat, which can be exposed by lifting the spleen with forceps (SP; **outlined**). **C:** To improve visualization, the mouse omentum here is dissected free from the pancreas. Although analogous to the human omentum in composition and tissue architecture, the mouse omentum consists of a single ribbon of fat attached to the pancreas. **D:** The five sources of peritoneal fat are excised, to show relative size. The mesentery is shown with attached mesenteric root. The scale is marked in centimeters.

in their numerous milky spots, whereas uterine, gonadal, and mesenteric fat would essentially be free of cancer cell colonies. Although our studies using SKOV3ip.1 cells support this latter model, determining the generalizability of these findings requires further study using additional ovarian cancer cell lines. To this end, the ability of ID8, CaOV3, and HeyA8 cells to form cancer cell foci in the five distinct peritoneal fat depots after intraperitoneal injection into B6 mice (ID8) or Nude mice (CaOV3, HeyA8) was assessed by histology. The average number of cancer cells in a representative section of tissue was determined and expressed as foci per slide (Supplemental Table S2). At 7 dpi, numerous large foci of ID8 cells were seen within the milky spots of both the omentum (mean, 48 foci per slide) and splenoportal fat (mean, 5 foci per slide) (Figure 2C). No ovarian cancer cells were detected in the uterine or gonadal fat (Figure 2C). In the mesentery, small clusters (<10 cells) of cancer cells were observed (mean, 2 foci per slide) on the tissue periphery (Figure 2C). In accord with these findings, CaOV3 cells showed similar pattern and extent of foci formation in the peritoneal adipose (Figure 2C and Supplemental Table S2). Interestingly, HeyA8 cells had a greater ability to form surface foci on uterine fat, with a mean of 8 foci per slide (Figure 2C and Supplemental Table S1).

Finally, to complement our findings from our histological analyses and to enable further studies, we developed a protocol to quantitate the number of cancer cells present in peritoneal adipose depots. ID8-tdTomato cells were prepared and injected intraperitoneally into B6 mice. At 7 dpi, the adipose organs were harvested and dissociated into a single-cell suspension. The number of tdTomato cells present in the population of remaining cells (eg, fibroblasts and immune, endothelial, and mesothelial cells) was quantified via flow cytometry. Splenoportal fat was excluded because its small size prohibited reliable cell recovery. Omental tissue preparations contained a significant population of tdTomato-positive cells (Figure 2D). The omentum showed an approximately 12-fold increase in the number of tdTomato-positive events, relative to PBS-injected controls (Figure 2D), but there was no significant increase in cell preparations from the gonadal fat, uterine fat, or mesentery. These data support our histological finding that ovarian cancer cells preferentially colonize milky spot-containing adipose and provide an additional quantitative method for further studies by our research group and others.

### Omental Tissues Secrete a Factor or Factors That Can Promote Ovarian Cancer Cell Migration

Ovarian cancer cells specifically localize to the omentum within minutes after intraperitoneal injection.<sup>3,6,40</sup> This suggests that omental tissue produces a factor or factors that promote cancer cell homing; however, previous studies have examined only the contribution of isolated adipocytes.<sup>45</sup> To address this gap in knowledge, we first tested the ability of



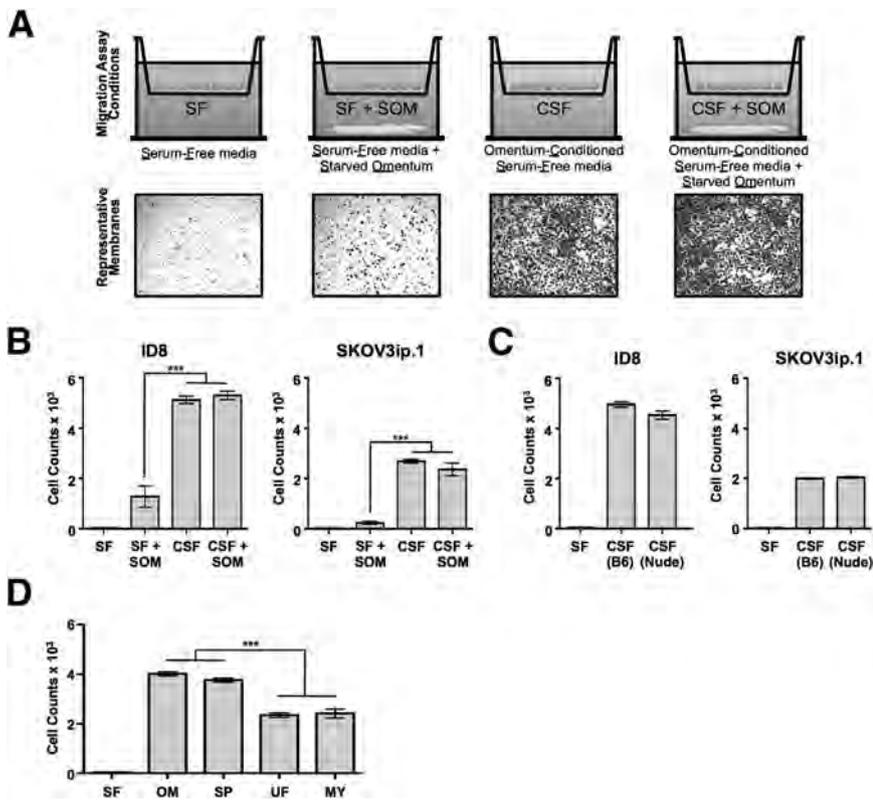
**Figure 2** Ovarian cancer cells preferentially colonize peritoneal adipose that contains milky spots. **A:** Milky spots (MS) are observed in the adipose (A) of the omentum and splenoportal fat of PBS-injected and naïve mice. In contrast, no milky spots were detected in the uterine fat, gonadal fat, or mesentery (each of which is composed mostly of adipocytes). Images are representative of PBS-injected B6 mice. Blood vessels are indicated by **arrows**. **B:** Examination of tissues by both standard histology (H&E) and IHC [pancytokeratin (pan-CK)] shows similar colonization of milky spots in both omentum and splenoportal fat (after injection of  $1 \times 10^6$  SKOV3ip.1 cells into Nude mice). Sections were first evaluated by H&E staining. The presence of epithelial (cancer) cells within the lesions was confirmed by IHC detection of cytokeratin using a pancytokeratin antibody. IHC using an IgG isotype antibody for pancytokeratin was used as a control for staining specificity. **C:** Evaluation of ID8, CaOV3, and HeyA8 ovarian cancer colonization of peritoneal fat depots at 7 dpi. Large cancer cell lesions in the milky spots of both the omentum and splenoportal fat are outlined. Representative examples of the cancer lesions occasionally seen in uterine, gonadal, and mesenteric fat are shown in the corresponding insets. **D:** Flow cytometric analyses of omentum, uterine fat, gonadal fat, and mesentery harvested from mice at 7 dpi of ID8-tdTomato cells. The quantified flow cytometry data are expressed as means  $\pm$  SEM for fold change increase of tdTomato-positive events (grey bars) relative to PBS-injected mice (white bars). **\*\*** $P < 0.01$  versus PBS controls. Original magnification:  $\times 400$  (A and C);  $\times 100$  (B).

omentum-conditioned medium to promote directed cancer cell migration. Using a modification of our published method for *ex vivo* organ culture,<sup>6</sup> omenta were excised and allowed to normalize in DMEM/F12 medium containing 20% fetal calf serum for 24 hours. Tissues were then rinsed with PBS, placed in SF DMEM/F-12 medium, and maintained for up to 5 days *ex vivo*. Tissue integrity was assessed both histologically, by visually evaluating intact (round, continuous cell membrane) versus necrotic (stellate, discontinuous cell membrane) adipocytes, and functionally, by measuring the level of IL-6 in the conditioned medium every 24 hours. In accord with our previous studies,<sup>6</sup> omental tissues did not show loss of integrity or function under these conditions (Supplemental Figure S1).

After normalization in DMEM/F-12 medium containing 20% fetal calf serum, omenta were rinsed with PBS and allowed to condition SF DMEM/F-12 medium for 24 hours (referred to as conditioned SF medium). Omentum

maintained in SF medium is termed starved omentum. The combinations of omentum and medium used as chemoattractants for the 6-hour migration assay and representative membranes from the migration assays are summarized in Figure 3A. The number of cells that migrated to the lower side of the membrane was determined by summing the number of cells in each of five independent fields observed at  $\times 100$  magnification.

Initial studies tested the ability of omenta harvested from CD1 mice to promote migration of both mouse ID8 and human SKOV3ip.1 cells (Figure 3B). The CD1 conditioned SF medium served as a strong chemoattractant for both ID8 and SKOV3ip.1 cells, resulting in a greater than 150-fold increase in migration relative to SF medium controls. To ensure that these results are not specific to omenta harvested from CD1 mice, conditioned SF media from B6 and Nude mouse omenta were also tested in their ability to promote ID8 and SKOV3ip.1 cell migration. Conditioned



**Figure 3** Milky spot-containing adipose tissues show enhanced ability to stimulate directed migration. Transwell migration assays are used as an indicator of soluble factors that promote the directed migration of ovarian cancer cells in tissue-conditioned medium. **A, top row:** Migration assay setup. Cancer cells are placed in the upper chamber of the Transwell apparatus. The chemoattractant medium, with or without starved tissue, is placed in the lower chamber. **A, bottom row:** Representative membranes from ID8 migration assays. **B:** Quantitation of ID8 and SKOV3ip.1 cell migration in response to factors produced by omenta harvested from CD1 mice under the four conditions shown in panel A. **C:** Quantitation of ID8 and SKOV3ip.1 cell migration in response to SF medium conditioned by omentum from either B6 mice [CSF (B6)] or Nude mice [CSF (Nude)]. **D:** Migration assay of ID8 cells toward SF medium conditioned for 24 hours by tissue equivalents of omenta, splenoportal fat, uterine fat, and mesentery harvested from B6 mice.  $n = 5$  for all conditions. Data are expressed as means  $\pm$  SEM.  $***p < 0.001$ . CSF, omentum-conditioned SF medium; SOM, starved omentum.

SF media prepared from B6 and Nude mouse omenta were a strong chemoattractant for ID8 cells (Figure 3C), stimulating migration on par with CD1-conditioned medium (Figure 3B). Consistent with this finding, B6 and Nude conditioned SF media also stimulated equivalent levels of migration in SKOV3ip.1 cells. Interestingly, SKOV3ip.1 cells showed a consistently lower level of migration than ID8 cells in response to medium conditioned by omenta from CD1, B6, and Nude mice. Taken together, these experiments showed that intact omental tissue can be used as a starting point for efforts to identify the secreted factor or factors that promote ovarian cancer cell homing to omental tissues.

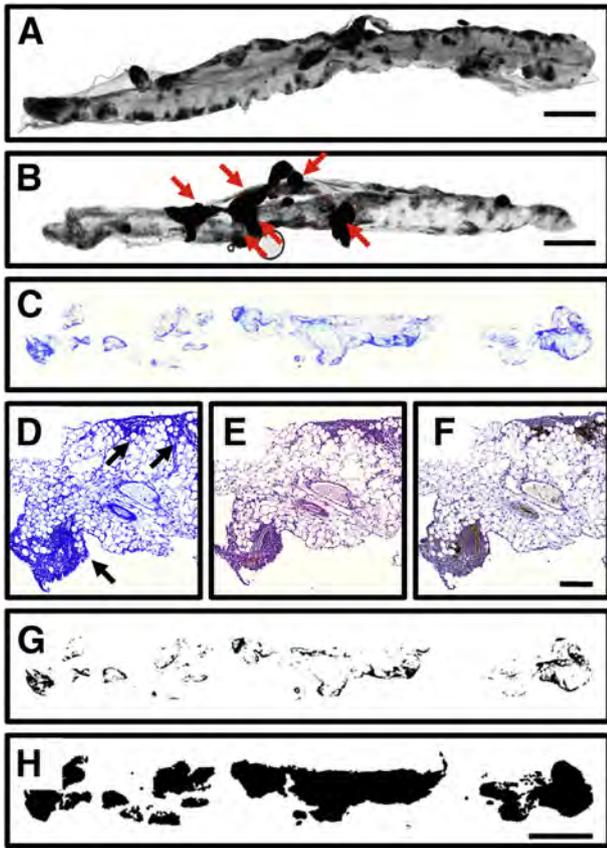
### Milky Spot-Containing Tissues Show Enhanced Ability to Stimulate Directed Migration

Nieman et al<sup>45</sup> showed that adipocytes cultured *in vitro* secreted cytokines that can promote migration of SKOV3ip.1 ovarian cancer cells. This raised the possibility that migration of cancer cells toward omentum-conditioned medium could be a strictly adipose-driven process. If that were the case, medium conditioned by adipose tissue lacking milky spots should promote migration of ovarian cancer cells to the same extent as medium conditioned by milky spot-containing adipose tissue. Alternatively, if milky spots play a key role in organ-specific homing, conditioned medium from tissues containing milky spots should have an enhanced ability to promote migration.

To distinguish between these possibilities, conditioned SF medium prepared using weight-matched tissue equivalents of omentum, splenoportal fat, uterine fat, and mesentery was used as a chemoattractant in Transwell migration assays. Tissues did not show loss of integrity or function for the duration of the migration assay; however, IL-6 production of the cultured uterine fat dropped significantly at 3 days in culture (Supplemental Figure S1). The migration-promoting activity of conditioned SF medium prepared from each of these tissues is summarized in Figure 3D. The medium conditioned by omentum or by splenoportal fat caused a 95-fold increase in cell migration, compared with control SF medium. In contrast, the absence of milky spots in uterine fat and mesentery corresponded with a 75% reduction in the migration-stimulatory activity in their conditioned medium (Figure 3D). Taken together, these functional studies bridge the adipocyte-driven and milky spot-driven models and argue that the presence of milky spots increases the chemoattractive potential of peritoneal fat depots.

### The Number and Size of Omental Milky Spots Is Not Dependent on the Mouse Genetic Background

Immune cells, including macrophages, lymphocytes, and mast cells, are integral to both the composition and function of omental milky spots.<sup>29,34,37,39</sup> There is a common view that omenta from immunodeficient mice will either have no milky spots or significantly fewer milky spots than omenta from immunocompetent mice. To test this notion, we sought



**Figure 4** An alternative protocol for labeling milky spots in mouse omenta. **A:** A rare example of a whole mount of a B6 omentum with clear milky spot labeling after carbon staining. **B:** A more typical case of a B6 omentum ineffectively stained, with several carbon plaques (arrows) obstructing milky spot visualization. **C:** As an alternative to carbon labeling, we developed a method in which naïve mouse omenta were paraffin-embedded, sectioned at 4  $\mu\text{m}$ , and stained with Giemsa. Dark staining regions indicate dense areas of immune aggregates. **D:** Image of omental tissue section stained with Giemsa. Milky spots are indicated with arrowheads. **E:** Image of omental tissue section adjacent to that shown in panel **D**, stained with H&E. **F:** Image of omental tissue section adjacent to that shown in panel **E**, evaluated by IHC using anti-CD45 antibody to identify lymphocytes within the milky spot structure. **G:** Mask of the omentum section shown in panel **C**, processed so that milky spots are specifically converted to pure black pixels. **H:** Mask of the omentum section shown in panel **C**, processed so that the entire area of the omentum is converted to pure black pixels. Scale bars: 1.0 mm (**A–C**, **G**, and **H**); 100  $\mu\text{m}$  (**D–F**).

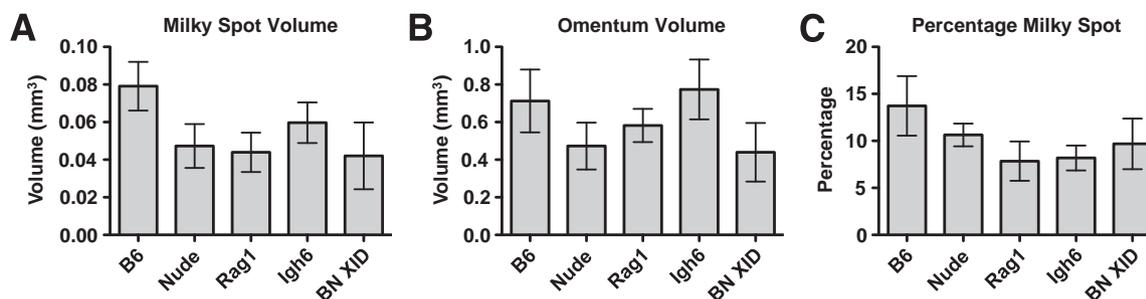
to quantitate the number and/or size of milky spots in omenta from a panel of immunodeficient mice. The majority of published studies use two standard methods to identify milky spots. The first uses a nonspecific esterase stain, which colors macrophages and T-lymphocytes dark red.<sup>29</sup> In our hands, this method yielded highly variable results that were not of sufficient quality or resolution for quantitative analyses (data not shown). The second method uses intraperitoneal injection of a carbon nanopowder suspension, which is phagocytosed by peritoneal macrophages over a period of days and thus highlights milky spots.<sup>29</sup> Tissues are subsequently harvested, whole-mounted, and

the number and/or area of the black-staining macrophages are used as a measure of milky spot number and size. When this method works correctly, the milky spot structures are crisp and well defined, and they can be visualized and counted under light microscopy (Figure 4A). Nonetheless, this technique can give inconsistent results, because carbon plaques often form on various peritoneal surfaces (Figure 4B). This plaque formation prevents both precise isolation (excision) of the omentum and accurate evaluation of milky spot area in the whole-mounted tissue.

To circumvent this difficulty, we developed a novel technique to visualize the milky spots on a whole-mount scale using Giemsa staining. In this approach, excised omenta were paraffin-embedded and the entire organ was serially sectioned at 4  $\mu\text{m}$  (resulting in approximately 30 to 60 sections). Every third section was stained in a 5% Giemsa solution, and images of the stained tissues were captured using a CRi Panoramic Scan Whole Slide Scanner (Figure 4C). Milky spots appear as regions staining dark blue (Figure 4D). The identity of these regions as milky spots was confirmed in serial sections by both H&E staining and IHC for cells positive for CD45, a common lymphocyte marker (Figure 4, E and F, respectively). Milky spots from B6, Nude, Rag1, Igh6, and BN XIX mice were successfully stained with Giemsa using this method (Supplemental Figure S2). By stacking and aligning all of the Giemsa-stained images from one omentum, we were able to produce a three-dimensional rendering of a mouse omentum that accurately depicts the tissue architecture and presents a novel view of the location and structure of milky spots (Supplemental Video S1).

We used ImageJ software to process the Giemsa-stained omentum slices to produce images that display both milky spots (Figure 4G) and the whole omentum area (Figure 4H) as pure black pixels. (The processing steps are detailed under *Materials and Methods*.) These black pixels were quantified using the Analyze Particles function of ImageJ software, producing a milky spot area and a whole omentum area for each slice. For each omentum, the milky spot and whole omentum areas were summed for all sections and the result was multiplied by 4  $\mu\text{m}$  (the section thickness), yielding milky spot and total volume for each omentum. Surprisingly, we found no difference in the milky spot volumes (Figure 5A) or the omentum volumes (Figure 5B) for the five mouse strains. Furthermore, no significant difference was found when the milky spot volume was expressed as a percentage of the total omentum area (Figure 5C). We therefore conclude that there is no significant difference in the milky spot volume in omenta from C57BL/6, Nude, Rag1, Igh6, and BN XIX mice.

*In vivo* colonization of omental milky spots by ovarian cancer cells is not dependent on their immune cell composition. As a first step toward understanding the effect of the immune cell composition of milky spot structures on ovarian cancer cell colonization, experimental metastasis assays were performed using immunocompetent (B6) and



**Figure 5** The milky spot volume of the omentum is not affected by the immune status of the host. Giemsa-stained sections of omenta harvested from B6, Nude, Rag1, Igh6, and BN XID mice were processed, sectioned, Giemsa stained, and imaged to determine the area of milky spots and the whole omenta area on each section. **A:** Milky spot volume per omentum, calculated by multiplying the area of each section by  $4 \mu\text{m}$  and summing the sections. **B:** Total volume of the whole omentum. **C:** Milky spot volume as a percentage of the total omentum volume. For each mouse strain, milky spot and omental volumes were determined for five independent animals. No measurements differed statistically among any of the mouse strains (one-way analysis of variance). Data are expressed as means  $\pm$  SEM.

immunodeficient (Igh6, Nude, Rag1, and BN XID) mice. Specifically,  $1 \times 10^6$  ID8 ovarian cancer cells were injected intraperitoneally into mice of each of the five different strains. Cancer cell foci were observed within milky spots in each of these mouse strains at 7 dpi (Figure 6A) and were confirmed to be epithelial via positive staining for mouse cytokeratin 8/18. To determine whether the missing immune-cell types in the various immunodeficient mice alters cancer localization to milky spots, DAB area was quantified in cytokeratin 8/18–stained sections. ID8 cancer cells colonized omenta from each strain to a statistically equivalent degree (Figure 6B). In parallel,  $1 \times 10^6$  SKOV3ip.1 human ovarian cancer cells were injected into Nude, Rag1, and BN XID mice, and cancer foci were again observed in each mouse strain (Figure 6C). SKOV3ip.1 lesions were stained for human pancytokeratin and found to be cytokeratin-positive. Thus, ovarian cancer cell colonization of omental milky spots is not affected by deficiency or absence of T cells, B cells, and/or NK cells in these mouse strains.

### During Progressive Growth, Ovarian Cancer Cells Replace Omental Adipose

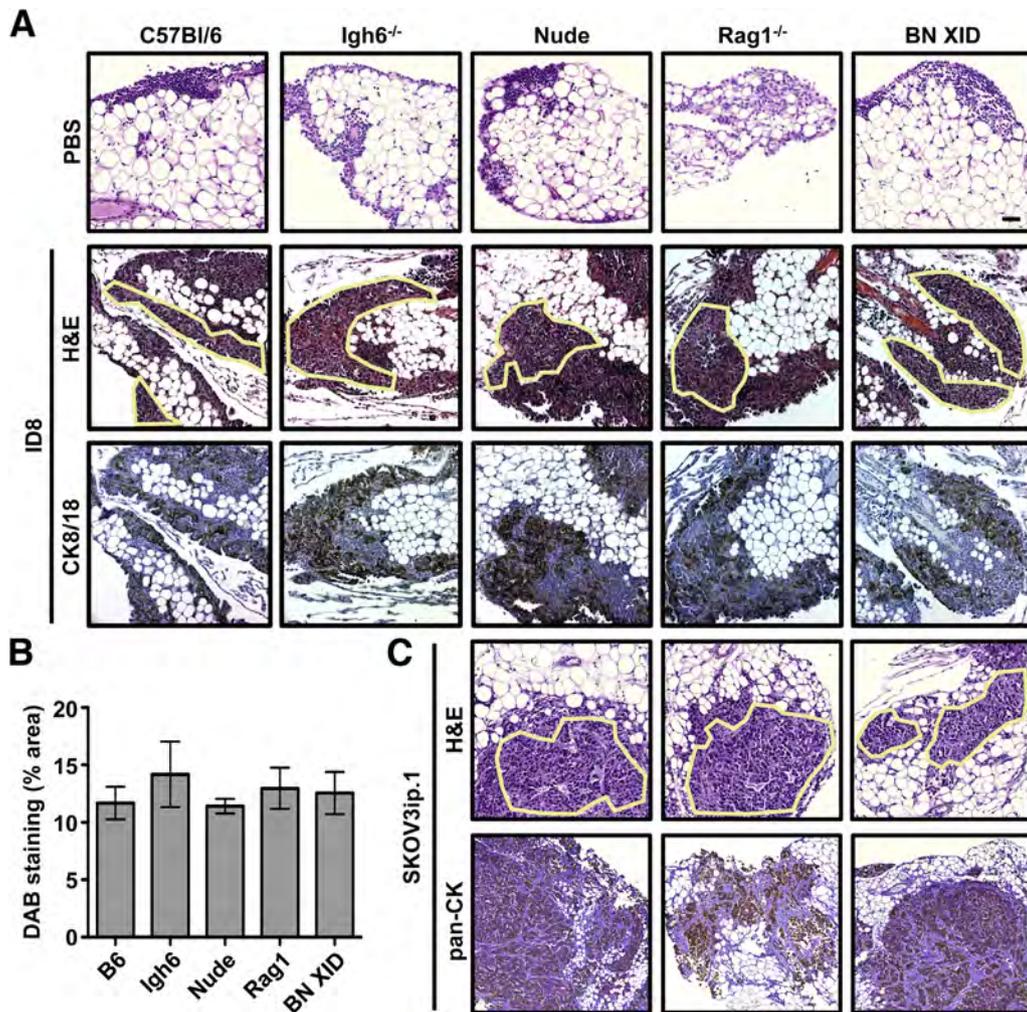
Mechanistic studies by Nieman et al<sup>45</sup> indicated that ovarian cancer cells can use adipocytes as an energy source for tumor growth. If this holds true *in vivo*, we predicted that as cancer cells proliferate they interact with and consume adipocyte lipids. The ultimate outcome of this inverse relationship between cancer cell area and adipocyte area would be that, at the experimental endpoint, the omental adipose would be replaced completely with cancerous tissue. To test this notion,  $1 \times 10^6$  ID8 ovarian cancer cells were injected intraperitoneally into a cohort of B6 mice. Groups of five mice were euthanized and tissues were collected for histological analysis at 1, 3, 6, and 9 weeks after injection (Figure 7A). Consistent with an inverse relationship between ovarian cancer cell growth and adipocyte depletion, there was a marked reduction in the

adipocyte area over time. To quantify this change, we used a pixel-based image processing protocol similar to milky spot quantification (detailed under *Materials and Methods*) to calculate the adipocyte area in omenta over time. This showed a linear decrease in the percentage of adipocytes in the omentum, corresponding to the expansion of ID8 cancer cell lesions (Figure 7B). These data are consistent with cancer cell utilization of lipids stored in adipocytes as an energy source for their continued growth.

## Discussion

There is now considerable literature on the structure and function of milky spots in both the omentum and extra-omental sites. Beginning in the 1970s, investigators noted that ascites tumors have a proclivity for these structures,<sup>41</sup> and subsequent studies have confirmed and refined these findings.<sup>3,6,40</sup> The strength of this work is that it implies a functional role for milky spots in the early steps of omental colonization. The weakness of the milky spot–driven model is the fact that the studies on which it is based do not consider the potential contributions of adipocytes and other cells within the omentum. Although the failure to consider the contribution of omental adipose in cancer metastasis is consistent with the now-antiquated view of adipocytes as an inert component of connective tissues,<sup>14,19,20</sup> it is a fundamental oversight that must be addressed if we are to understand the organ specificity of ovarian cancer cells.

The adipocyte-driven model was prompted by the findings of Nieman et al,<sup>45</sup> who showed that in omental metastases the ovarian cancer cells at the interface with adipocytes contain abundant lipids. Their *in vitro* studies showed that the adipocytes transfer to ovarian cancer cells lipid droplets containing fatty acids,<sup>45</sup> which can be used as an energy source. The strength of their studies is that they focused on human ovarian cancers and identified a novel function for adipocytes in the progressive growth of ovarian cancer lesions, and the weakness lies in the effort to show that adipocytes drive, and are solely responsible for, early

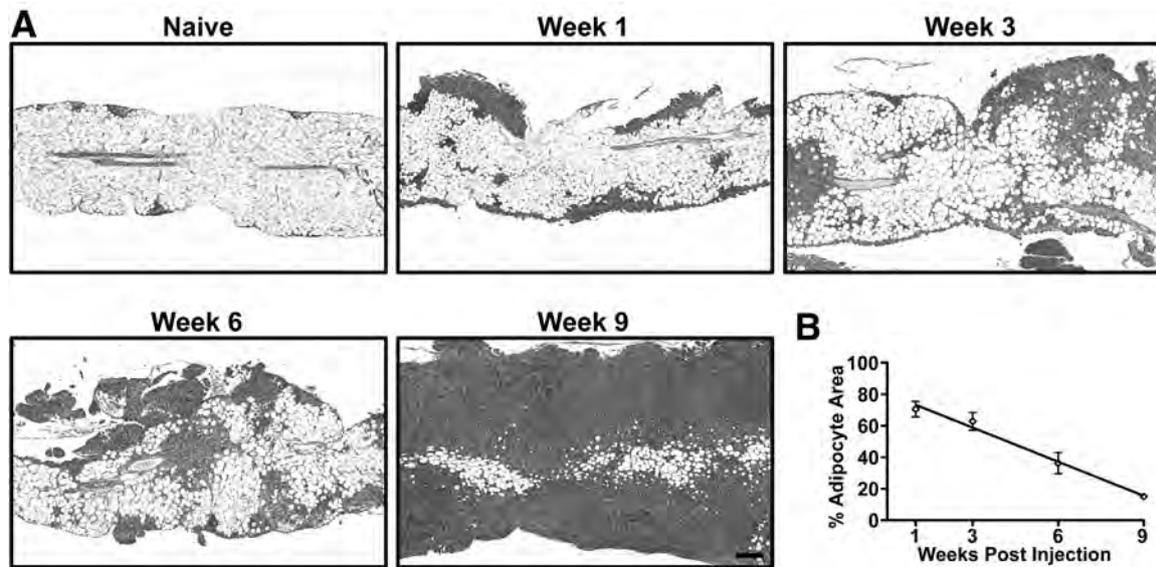


**Figure 6** Colonization of omental milky spots by ovarian cancer cells is not dependent on the immune status of the host. To test the possibility that the immune composition of the milky spots has a quantitative effect on ovarian cancer cell colonization, mice with deficiencies in T cells, B cells, and/or NK cells were injected intraperitoneally with either PBS (control) or  $1 \times 10^6$  ovarian cancer cells. **A:** B6, Igh6, Nude, Rag1, and BN XID mice were injected with mouse ID8 cells (syngeneic to B6 background). Omenta were collected at 7 dpi and stained with H&E. Cancer cell foci are outlined. Immunohistochemistry (IHC) against mouse cytokeratin 8/18 (CK8/18) was used to confirm the epithelial origin of the cancer cell foci. **B:** DAB staining area was used as an indicator of cancer cell burden in omental tissues. Values were calculated as the percentage of area with strong plus medium intensity of cytokeratin 8/18 (DAB) staining, normalized to total stained area of the slide. **C:** Human SKOV3ip.1 cells were injected into Nude, Rag1, and BN XID mice. Omenta were collected at 7 dpi and stained with H&E. IHC against human pancytokeratin was used to confirm the epithelial origin of cancer cell lesions. Samples from five independent animals were evaluated for each condition of each test. Data are expressed as means  $\pm$  SEM. Original magnification:  $\times 200$ .

steps in omental colonization. The case for adipose as the sole determinant of the organotropic metastasis of ovarian cancer is based on an incomplete examination of the literature and on a biased approach to experimental design. As a result of the focus on adipocytes, important clues in the data were overlooked, and the potential contributions of milky spots, vasculature, or other unique characteristics of the omentum were neither tested nor discussed. Thus, like the milky spot–driven model, the adipocyte-driven model also is limited by the narrow focus of the studies in which it is based.

Despite their strengths, neither the milky spot–driven nor the adipocyte-driven model addresses the intimate and dynamic interactions among milky spot structures, adipocytes, and other omental components. Integration of these

two models requires a fresh look from a different perspective. Thus, rather than taking the more traditional omentum-focused approaches, we sought to understand why ovarian cancers do not colonize other sources of peritoneal fat as extensively as they do the omentum. This led to our novel strategy of comparing colonization of peritoneal adipose that either contains or lacks milky spots. The report by Takemori et al<sup>46</sup> showing the presence of milky spots in the splenoportal fat of New Zealand Black mice was key to our approach. To our knowledge, the splenoportal fat band has not been studied in other mouse strains, nor has its colonization by any type of cancer cells been examined previously. Our *in vivo* studies using a panel of ovarian cancer cell lines (ID8, SKOV3ip.1, CaOV3, and HeyA8) yielded the most comprehensive assessment of ovarian cancer cell



**Figure 7** Adipocyte area of the omentum decreases during the time course of ovarian cancer growth. **A:** A representative H&E-stained section of an omentum from a naïve B6 mouse. Milky spots are seen within adipose at the tissue periphery. Images are representative of omental tissues harvested from B6 mice at 1, 3, 6, and 9 weeks after injection. **B:** Quantitation of adipocyte area from H&E images; the percent adipocyte area was normalized to the whole omental area. Data are expressed as means  $\pm$  SEM ( $n = 5$  independent animals per time point). A linear regression of the data points indicates a slope significantly deviant from zero ( $P < 0.0001$ ;  $R^2 = 0.8145$ ) Original magnification:  $\times 50$ .

lodging in peritoneal adipose and provided clear data showing that milky spots dramatically enhance early cancer cell lodging.

To dissect the mechanism or mechanisms by which milky spots promote colonization, we made use of the observation that, after intraperitoneal injection, ovarian cancer cells rapidly localize to omental milky spots, which suggests involvement of one or more tissue-secreted factors. Development of a quantitative Transwell migration approach (Figure 3) enabled us to assess the ability of fat tissue-conditioned medium to stimulate directed migration of ovarian cancer cells. Although milky spot-deficient tissues (uterine fat, gonadal fat, and mesentery) secrete one or more factors that promote directed migration, results from *in vivo* assays indicate that this signal is not sufficient for ovarian cancer cells to achieve the high level (both in number and size) of foci formation that is seen in the omentum and splenoportal fat. This suggests that colonization requires additional chemotactic signals and/or tissue structures. In support of this notion, our *in vivo* assays showed that ovarian cancer cells efficiently colonize milky spots in the omentum and splenoportal fat. In addition, using medium conditioned by milky spot-containing adipose yielded the novel finding that the presence of these structures caused a significant enhancement in the ability of the medium to promote directed cancer migration.

Although milky spot structures comprise a number of cell types, their importance in multiple facets of immune defense prompted us to ask what effect the host immune status has on the number of milky spots or on their ability to be colonized by cancer cells. Ostensibly, it would seem

that immunodeficient mice would have fewer or smaller milky spots (because of their immunodeficiency) and that these structures would therefore be poorly colonized by cancer cells. Use of a novel virtual whole-mount technique enabled the quantitation of milky spot volume for the first time in various mouse strains. These data showed that the immune status of the host affects neither milky spot volume (in naïve omenta) nor its colonization by cancer cells (cancer burden) after intraperitoneal injection. Finally, the role of adipocytes in supplying energy for cancer cell growth is supported by the direct relationship between cancer cell growth and adipocyte depletion. Interestingly, this finding is in accord with breast cancer models in which cancer growth causes a reduction in adipocyte number and size (reducing the ratio of adipocyte-to-cancer area), implying lipolysis and possible adipocyte dedifferentiation.<sup>50</sup>

A large body of evidence has established the critical importance of milky spots to ovarian cancer cell lodging and initial colonization of peritoneal adipose<sup>3,6,24,28,40–44</sup> and provides a foundation for studies to identify milky spot components involved in cancer cell homing and invasion. As a first step toward this goal, the use of immunodeficient mouse strains allowed us to rule out a requirement for B cells, T cells, or NK cells for ovarian cancer cell lodging within milky spots, thus confirming and expanding on the findings of Lotan et al.<sup>8</sup> Previous studies have shown that mast cells and macrophages are frequently observed in the milky spots.<sup>30,33,34,36</sup> Macrophages are an intriguing candidate, because they have been shown to assist the survival and growth of established tumors.<sup>51–53</sup> Furthermore, the depletion of peritoneal macrophages has been

shown to decrease ovarian cancer tumor burden on the diaphragm at the experimental endpoint.<sup>54</sup> Milky spot macrophages are thus possible contributors to the rapid and specific colonization of omental milky spots. Another possible source of the chemotactic properties of the omentum is the abundance of endothelial cells found in the milky spots. The vessels within the dense and tangled capillary bed of the milky spots have been shown to undergo a constitutive level of active vascular remodeling.<sup>28,42</sup> The activated endothelial cells associated with angiogenic vessels are known to support and promote metastatic disease.<sup>55,56</sup> Either or both of those cell types could be responsible for the prolific omental metastases and warrant further study.

The growing emphasis on the role of the host tissue microenvironment in metastasis formation stems from the seminal work of Stephen Paget, who in 1889 showed that certain tumor cells (metaphorically thought of as the seed) have a proclivity for specific organ microenvironments (the soil).<sup>57–59</sup> A powerful but often underappreciated aspect of studies by Paget and other pioneers of metastasis research was their innate appreciation of the unique tissue architecture, physiology, and function of the target organ that is essential to understanding metastatic organ specificity.<sup>60–63</sup> With our present studies, we have sought to integrate milky spot and adipocyte function in the omentum.

We propose a two-step model for omental colonization in which the localization of disseminated cancer cells is dependent on milky spots. Adipocytes are then required for progressive growth and subsequent spread of cancer cells to other sites within the peritoneal cavity. This model is likely a more accurate representation of the overall process of ovarian metastatic colonization. It is our hope that both our findings and our discussion of the larger literature will serve as a framework for studies that will continue to refine our understanding of omental colonization. Ultimately, it is our goal to use this information to extend the duration of metastatic suppression and significantly increase the quality of life for patients diagnosed with ovarian cancer.

## Acknowledgments

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## Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2013.04.023>.

## References

1. Naora H, Montell DJ: Ovarian cancer metastasis: integrating insights from disparate model organisms. *Nat Rev Cancer* 2005, 5:355–366
2. Cinti S: The adipose organ. Edited by Fantuzzi G, Mazzone T. *Adipose Tissue and Adipokines in Health and Disease*. Totowa, NJ, Humana Press, 2007, pp 3–19
3. Hagiwara A, Takahashi T, Sawai K, Taniguchi H, Shimotsu M, Okano S, Sakakura C, Tsujimoto H, Osaki K, Sasaki S, Shirasu M: Milky spots as the implantation site for malignant cells in peritoneal dissemination in mice. *Cancer Res* 1993, 53:687–692
4. Roby KF, Taylor CC, Sweetwood JP, Cheng Y, Pace JL, Tawfik O, Persons DL, Smith PG, Terranova PF: Development of a syngeneic mouse model for events related to ovarian cancer. *Carcinogenesis* 2000, 21:585–591
5. Lengyel E: Ovarian cancer development and metastasis. *The Am J Pathol* 2010, 177:1053–1064
6. Khan SM, Funk HM, Thiolloy S, Lotan TL, Hickson J, Prins GS, Drew AF, Rinker-Schaeffer CW: In vitro metastatic colonization of human ovarian cancer cells to the omentum. *Clin Exp Metastasis* 2010, 27:185–196
7. Kenny HA, Krausz T, Yamada SD, Lengyel E: Use of a novel 3D culture model to elucidate the role of mesothelial cells, fibroblasts and extra-cellular matrices on adhesion and invasion of ovarian cancer cells to the omentum. *Int J Cancer* 2007, 121:1463–1472
8. Lotan T, Hickson J, Souris J, Huo D, Taylor J, Li T, Otto K, Yamada SD, Macleod K, Rinker-Schaeffer CW: c-Jun NH2-terminal kinase activating kinase 1/mitogen-activated protein kinase kinase 4-mediated inhibition of SKOV3ip.1 ovarian cancer metastasis involves growth arrest and p21 up-regulation. *Cancer Res* 2008, 68: 2166–2175
9. Liebermann-Meffert D: The greater omentum. *Anatomy, embryology, and surgical applications*. *Surg Clin North Am* 2000, 80: 275–293. xii
10. Collins D, Hogan AM, O'Shea D, Winter DC: The omentum: anatomical, metabolic, and surgical aspects. *J Gastrointest Surg* 2009, 13:1138–1146
11. Platell C, Cooper D, Papadimitriou JM, Hall JC: The omentum. *World J Gastroenterol* 2000, 6:169–176
12. Goldsmith HS: *The Omentum: Research and Clinical Application*. 1st ed. New York, Springer Verlag, 1990
13. Wilkosz S, Ireland G, Khwaja N, Walker M, Butt R, de Giorgio-Miller A, Herrick SE: A comparative study of the structure of human and murine greater omentum. *Anat Embryol (Berl)* 2005, 209: 251–261
14. Wozniak SE, Gee LL, Wachtel MS, Frezza EE: Adipose tissue: the new endocrine organ? A review article. *Dig Dis Sci* 2008, 54: 1847–1856
15. Trayhurn P, Beattie JH: Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ. *Proc Nutr Soc* 2001, 60:329–339

16. Hauner H: The new concept of adipose tissue function. *Physiol Behav* 2004, 83:653–658
17. Scherer PE: Adipose tissue: from lipid storage compartment to endocrine organ. *Diabetes* 2006, 55:1537–1545
18. Kershaw EE, Flier JS: Adipose tissue as an endocrine organ. *J Clin Endocrinol Metab* 2004, 89:2548–2556
19. Wang P, Mariman E, Renes J, Keijer J: The secretory function of adipocytes in the physiology of white adipose tissue. *J Cell Physiol* 2008, 216:3–13
20. Conde J, Scotece M, Gómez R, López V, Gómez-Reino JJ, Lago F, Gualillo O: Adipokines: biofactors from white adipose tissue. A complex hub among inflammation, metabolism, and immunity. *Biofactors* 2011, 37:413–420
21. Frühbeck G, Gómez-Ambrosi J, Muruzábal FJ, Burrell MA: The adipocyte: a model for integration of endocrine and metabolic signaling in energy metabolism regulation. *Am J Physiol Endocrinol Metab* 2001, 280:E827–E847
22. Tilg H, Moschen AR: Adipocytokines: mediators linking adipose tissue, inflammation and immunity. *Nat Rev Immunol* 2006, 6:772–783
23. Rajala MW, Scherer PE: Minireview: the adipocyte—at the crossroads of energy homeostasis, inflammation, and atherosclerosis. *Endocrinology* 2003, 144:3765–3773
24. Michailova KN, Usunoff KG: The milky spots of the peritoneum and pleura: structure, development and pathology. *Biomed Rev* 2004, 15:47–66
25. Simer PH: On the morphology of the omentum, with especial reference to its lymphatics. *Am J Anat* 1934, 54:203–228
26. Ackermann PC, De Wet PD, Loots GP: Microcirculation of the rat omentum studied by means of corrosion casts. *Acta Anat (Basel)* 1991, 140:146–149
27. Shimotsuna M, Shields J, Simpson-Morgan M, Sakuyama A, Shirasu M, Hagiwara A, Takahashi T: Morpho-physiological function and role of omental milky spots as omentum-associated lymphoid tissue (OALT) in the peritoneal cavity. *Lymphology* 1993, 26:90–101
28. Gerber SA, Rybalko VY, Bigelow CE, Lugade AA, Foster TH, Frelinger JG, Lord EM: Preferential attachment of peritoneal tumor metastases to omental immune aggregates and possible role of a unique vascular microenvironment in metastatic survival and growth. *Am J Pathol* 2006, 169:1739–1752
29. Shimotsuna M, Kawata M, Hagiwara A, Takahashi T: Milky spots in the human greater omentum. Macroscopic and histological identification. *Acta Anat (Basel)* 1989, 136:211–216
30. Shimotsuna M, Simpson-Morgan MW, Takahashi T, Hagiwara A: Activation of omental milky spots and milky spot macrophages by intraperitoneal administration of a streptococcal preparation, OK-432. *Cancer Res* 1992, 52:5400–5402
31. Litbarg NO, Gudehithlu KP, Sethupathi P, Arruda JAL, Dunea G, Singh AK: Activated omentum becomes rich in factors that promote healing and tissue regeneration. *Cell Tissue Res* 2007, 328:487–497
32. Rangel-Moreno J, Moyron-Quiroz JE, Carragher DM, Kusser K, Hartson L, Moquin A, Randall TD: Omental milky spots develop in the absence of lymphoid tissue-inducer cells and support B and T cell responses to peritoneal antigens. *Immunity* 2009, 30:731–743
33. Wjffels JF, Hendrickx RJ, Steenbergen JJ, Eestermans IL, Beelen RH: Milky spots in the mouse omentum may play an important role in the origin of peritoneal macrophages. *Res Immunol* 1992, 143:401–409
34. Shimotsuna M, Takahashi T, Kawata M, Dux K: Cellular subsets of the milky spots in the human greater omentum. *Cell Tissue Res* 1991, 264:599–601
35. Cranshaw ML, Leak LV: Milky spots of the omentum: a source of peritoneal cells in the normal and stimulated animal. *Arch Histol Cytol* 1990, 53 Suppl:165–177
36. Beelen RH, Fluitsma DM, Hoefsmit EC: The cellular composition of omentum milky spots and the ultrastructure of milky spot macrophages and reticulum cells. *J Reticuloendothel Soc* 1980, 28:585–599
37. Krist LF, Eestermans IL, Steenbergen JJ, Hoefsmit EC, Cuesta MA, Meyer S, Beelen RH: Cellular composition of milky spots in the human greater omentum: an immunochemical and ultrastructural study. *Anat Rec* 1995, 241:163–174
38. Mironov VA, Gusev SA, Baradi AF: Mesothelial stomata overlying omental milky spots: scanning electron microscopic study. *Cell Tissue Res* 1979, 201:327–330
39. Dux K, Janik P, Szaniawska B: Kinetics of proliferation, cell differentiation, and IgM secretion in the omental lymphoid organ of B10/Sn mice following intraperitoneal immunization with sheep erythrocytes. *Cell Immunol* 1977, 32:97–109
40. Krist LF, Kerremans M, Broekhuis-Fluitsma DM, Eestermans IL, Meyer S, Beelen RH: Milky spots in the greater omentum are predominant sites of local tumour cell proliferation and accumulation in the peritoneal cavity. *Cancer Immunol Immunother* 1998, 47:205–212
41. Green JA, Williams AE: The relationship between inflammatory responses and WBP1 tumour cell attachment to the rat omentum. *Eur J Cancer* 1978, 14:1153–1155
42. Sorensen EW, Gerber SA, Sedlacek AL, Rybalko VY, Chan WM, Lord EM: Omental immune aggregates and tumor metastasis within the peritoneal cavity. *Immunol Res* 2009, 45:185–194
43. Tsujimoto H, Takahashi T, Hagiwara A, Shimotsuna M, Sakakura C, Osaki K, Sasaki S, Shirasu M, Sakakibara T, Ohyama T: Site-specific implantation in the milky spots of malignant cells in peritoneal dissemination: immunohistochemical observation in mice inoculated intraperitoneally with bromodeoxyuridine-labelled cells. *Br J Cancer* 1995, 71:468–472
44. Tsujimoto H, Hagiwara A, Shimotsuna M, Sakakura C, Osaki K, Sasaki S, Ohyama T, Ohgaki M, Imanishi T, Yamazaki J, Takahashi T: Role of milky spots as selective implantation sites for malignant cells in peritoneal dissemination in mice. *J Cancer Res Clin Oncol* 1996, 122:590–595
45. Nieman KM, Kenny HA, Penicka CV, Ladanyi A, Buell-Gutbrod R, Zillhardt MR, Romero IL, Carey MS, Mills GB, Hotamisligil GS, Yamada SD, Peter ME, Gwin K, Lengyel E: Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. *Nat Med* 2011, 17:1498–1503
46. Takemori N, Hirai K, Onodera R, Saito N, Namiki M: Light and electron microscope study of splenoportal milky spots in New Zealand black mice: comparison between splenoportal milky spots and aberrant spleens. *J Anat* 1995, 186:287–299
47. Yu D, Wolf JK, Scanlon M, Price JE, Hung MC: Enhanced c-erbB-2/neu expression in human ovarian cancer cells correlates with more severe malignancy that can be suppressed by E1A. *Cancer Res* 1993, 53:891–898
48. Kishimoto T: Interleukin-6 (IL-6). *The Cytokine Handbook*. 4th ed. Boston, Academic Press, 2003, 1:281–304
49. Thévenaz P, Ruttimann UE, Unser M: A pyramid approach to sub-pixel registration based on intensity. *IEEE Trans Image Process* 1998, 7:27–41
50. Dirat B, Bochet L, Dabek M, Daviaud D, Dauvillier S, Majed B, Wang YY, Meulle A, Salles B, Le Gonidec S, Garrido I, Escourrou G, Valet P, Muller C: Cancer-associated adipocytes exhibit an activated phenotype and contribute to breast cancer invasion. *Cancer Res* 2011, 71:2455–2465
51. Qian BZ, Pollard JW: Macrophage diversity enhances tumor progression and metastasis. *Cell* 2010, 141:39–51
52. Tlsty TD, Coussens LM: Tumor stroma and regulation of cancer development. *Annu Rev Pathol* 2006, 1:119–150
53. Coussens LM, Werb Z: Inflammation and cancer. *Nature* 2002, 420:860–867
54. Robinson-Smith TM, Isaacsohn I, Mercer CA, Zhou M, Van Rooijen N, Husseinzadeh N, McFarland-Mancini MM, Drew AF: Macrophages mediate inflammation-enhanced metastasis of ovarian tumors in mice. *Cancer Res* 2007, 67:5708–5716

55. Orr FW, Wang HH, Lafrenie RM, Scherbarth S, Nance DM: Interactions between cancer cells and the endothelium in metastasis. *J Pathol* 2000, 190:310–329
56. Chouaib S, Kieda C, Benlalam H, Noman MZ, Mami-Chouaib F, Rüegg C: Endothelial cells as key determinants of the tumor microenvironment: interaction with tumor cells, extracellular matrix and immune killer cells. *Crit Rev Immunol* 2010, 30:529–545
57. Paget S: The distribution of secondary growths in cancer of the breast. *Lancet* 1889, 133:571–573, [reproduced in *Cancer Metastasis Rev* 1989, 8:98–101]
58. Kenny PA, Bissell MJ: Tumor reversion: correction of malignant behavior by microenvironmental cues. *Int J Cancer* 2003, 107:688–695
59. Bissell MJ, Radisky DC, Rizki A, Weaver VM, Petersen OW: The organizing principle: microenvironmental influences in the normal and malignant breast. *Differentiation* 2002, 70:537–546
60. Nicolson GL: Organ specificity of tumor metastasis: role of preferential adhesion, invasion and growth of malignant cells at specific secondary sites. *Cancer Metastasis Rev* 1988, 7:143–188
61. Fidler IJ: The pathogenesis of cancer metastasis: the ‘seed and soil’ hypothesis revisited. *Nat Rev Cancer* 2003:453–458
62. Hart IR, Fidler IJ: Role of organ selectivity in the determination of metastatic patterns of B16 melanoma. *Cancer Res* 1980, 40:2281–2287
63. Sugarbaker EV: Cancer metastasis: a product of tumor-host interactions. *Curr Probl Cancer* 1979, 3:1–59

## Unique Collaborative Resources of Our Team.

**Clinical and Translational Resources:** Work in this proposal leverages the extensive clinical and translational resources established by Dr. Seewaldt and Dr. Ford as part of the Duke University High Risk Clinic. In addition to the patient volume and active participation of our research-oriented surgical (Dr. Ford) and medical oncology (Dr. Seewaldt) collaborators, our studies will also benefit directly from the seamless infrastructure that they have established to conduct translational studies (please see description on subsequent pages). Indeed, we already have established a collaborative protocol to rigorously define the number and composition of milky spots in the omenta of women. We developed this protocol to provide additional baseline information for our work and to provide a more complete understanding of omental milky spots in a large cohort. This protocol will be amended to address specific needs of studies herein as needed. With collaborators at the University Health Network, Dr. Shaw has established a cohort of 1000 snap-frozen and formalin-fixed paraffin embedded (FFPE) normal fallopian tube and ovarian cancer tissue samples containing different histotypes (mucinous, clear cell, endometrioid, and serous). In order to gain a functional understanding of the molecular changes seen in the progression of fimbrial tubal epithelial cells to serous tubal intraepithelial carcinomas, her laboratory has also developed an extensive repository containing more than 500 fresh-processed fallopian tube epithelia with multiple primary and immortalized fimbrial epithelial cell lines including BRCA1 mutation carriers. These resources are key to the functional studies proposed in this application. Taken together, these resources provide our team with the ability to develop clinically relevant cell lines that model precursor cells shed from serous tubal intra-epithelial carcinomas as an extensive repository of clinical samples that will enhance our ability to effectively and rapidly translate findings from the laboratory findings to the clinic.

**Personnel Resources:** We have assembled a team with the unique expertise and resources needed to efficiently and effectively test our hypotheses and take our findings to the clinic. **Dr. Patricia Shaw** is internationally recognized as a leader in efforts to understand the pathobiology and development of high-grade serous cancer from fallopian tube epithelial cells. As described above, she has developed a repository of samples that will allow the development of fimbrial tubal epithelial-derived cell lines that are a unique and powerful feature of this proposal. **Dr. Rinker-Schaeffer** is internationally recognized for her work on metastasis suppressor proteins, metastatic colonization, and more recently the omental tissue microenvironment in ovarian cancer metastasis formation. As a result of 20 years of experience focusing on metastatic colonization, she has unparalleled tools for conducting the *in vivo* work critical to the mechanistic studies described herein. Having identified a key role for macrophages in milky spot colonization, the team was extremely fortunate to recruit **Dr. Lev Becker** as a collaborator for this work. His novel proteomics-based approach to defining the function of macrophages in both tissue- and disease-specific settings is a game-changer. Specifically, it will enable the design of rational, mechanism-based studies to dissect the macrophage-ovarian cancer cell interactions involved in milky spot homing and growth, which are novel features of the proposed work. **Dr. Marina Chekmareva** brings expertise and experience in gynecologic pathology, milky spot histology, and the histologic assessment of metastatic colonization of murine tissues. She will evaluate both experimental tissues and clinical tissues from these studies. Finally, **Dr. Seewaldt** and **Dr. Ford** are leaders in the clinical management of and translational research in high-risk breast and ovarian cancers. They bring the tools, expertise, and infrastructure needed to validate our findings in clinical cohorts, and ultimately translate them into the clinical setting.

## CLINICAL RESOURCES FOR TRANSLATIONAL AND CLINICAL RESEARCH



The **Duke University Breast Cancer Prevention Clinic** is a multi-disciplinary research-focused clinic, led by *Victoria Seewaldt, M.D.* that aims to 1) evaluate genetic modifiers of breast cancer risk in high-risk women, 2) offer pharmacological-, nutritional-, and immunological-based risk reduction strategies coupled with biological markers of response, and 3) evaluate short-term breast cancer risk using a combination of RPFNA, epigenetic profiling, and state-of-the-art breast imaging. This is a state-of-the-art translational clinic where women at high-risk for breast cancer can undergo risk assessment, be offered genetic counseling (*Kelly Marcom, Joellen Schildkraut, Trish Moorman*), have access to novel methods of breast imaging (*Jay Baker, Ed Coleman, Nimmi Ramanujam, Martin Tornei*), and undergo breast Random Periareolar Fine Needle Aspiration (RPFNA) to assess early cytologic breast changes. Reception to this clinic has been extremely enthusiastic and 3-5 patients per week are being recruited for RPFNA. The ability to repeatedly evaluate cytologic field effects in the breasts of high-risk women allows investigators to validate risk-markers developed in the laboratory and test whether these markers can predict 1) short-term breast cancer risk and 2) response to pharmacological, immunological, nutritional, and complementary breast cancer prevention strategies. This program has become a focal point to recruit and train women and minority scientists in state-of-the-art bench to bedside research.

**Duke University Prevention Cohort:** Over 103 new high risk women are seen in consultation per year in the Duke University Breast Cancer Prevention Center, plus 879 per year in follow-up. Standard prevention options such as tamoxifen are discussed, as well as clinical trial prevention options are presented to a woman at the time of her initial consultation or follow-up visit. For those women not wishing to take tamoxifen, a clinical trial is offered where appropriate. Over 550 RPFNAs are performed per year, and more than 50 women are enrolled per year as subjects on Phase II chemoprevention trials. There is no charge to



a woman for an RPFNA done for risk stratification or screening eligibility for a clinical trial. Women have the option of entering in a wide range of clinical trials including 1) experimental imaging, 2) Yoga and meditation trials to reduce menopausal symptoms, and 3) genetic risk assessment trials. In the past 12 months 120 women underwent 3.0 T MRI in combination with RPFNA.

**BRCA1/2:** The clinic follows over 48 families with known BRCA1/2 mutations.

**Minority Recruitment:** The High Risk Breast Clinic routinely sees over 35% minority women with approximately those percentages being enrolled on various clinical trials. Recruiting minority women to our research will continue to be a high priority. Currently over 25-71% of the patients entering in our prevention and risk assessment trials are African American and 4% are Latina. Approximately 51% of high-risk women entering experimental imaging trials are African American.

### SUMMARY OF RECENTLY COMPLETED CLINICAL TRIALS BY THE DUKE PREVENTION GROUP

#### 1) **Nutrition and Pharmacological Based Prevention Trials:**

STRENGTH: Survivor TRaining for ENhancinG Total Health Study (Wendy Demark), a clinic-based and a distance medicine based program aimed at reducing the problem of sarcopenic obesity resulting from chemotherapy

FRESH START: (Wendy Demark) Promoting Health Among Cancer Survivors to reduce diabetes and breast cancer recurrence by improving lifestyle behaviors.

Cox-2 inhibitors as modulators of breast cancer risk in women with DCIS (Carol Fabian, UAB Breast SPORE/University of Kansas.), Duke site (Seewaldt) funding: *UAB Breast SPORE*.

Flax seed in high-risk women (Seewaldt, Seo) *NCI/NIH funding*.

## **2) Biomarker Trials:**

Identification of polymorphisms in DNA repair genes as modifiers of BRCA1/2 penetrance (Joellen Schildkraut) funding: *P50-A-086438*.

Discovery and validation of circulating breast cancer markers (Jeffery Marks) funding: *UO1-CA84955*.

Modulation of methylation based markers by chemopreventive agents in women at high-risk for breast cancer (Seewaldt), (Fabian, University of Kansas), (Yee, Ohio State)

Retinoid metabolites as intermediate markers of breast cancer risk. (Seewaldt, Yee) funding: Komen.

Cancer Genetics Network (CGN) (Schildkraut) funding, *U24-CA78157*

## **3) Imaging Trials**

Breast cancer imaging using non-parallel beam SPECT (Tornai) funding: *R01-CA076006* and *R01-CA96821* (Tornai).

Reducing benign breast biopsies with computer modeling (Baker) funding: *R01-CA0956*.

PET imaging of breast cancer using F-18 labeled choline analogs (Soo) funding: *DAMD17-01-0515*.

Positron emission mammography to determine local extent and distribution of breast cancer (Rosen) funding: *DAMD17-01-10517*.

Integrating 3T MRI and RPFNA to predict short-term breast cancer risk in high-risk women (Seewaldt, Baker) funding: *DCCC pilot project*.

## **4) Behavioral Trials**

Effects of Supportive and Nonsupportive Behaviors on the Quality of Life of Breast Cancer Patients and Their Spouses (Lipkus) Assessed the relationships among perceptions of illness uncertainty, perceptions of supportive and non-supportive behavior, and quality of life within and between breast and prostate cancer patients and their spouses.

Effects of Information Displays in Decisions about Tamoxifen Use for Breast Cancer Chemoprevention (Lipkus) funding *CAMD17-03-1-03820*

Finding the M.I.N.C for mammography maintenance (Rimner, Lipkus) The goal of this study is the use of a stepped care approach to maintain mammography adherence through the use of reminder systems, barriers counseling and having women think about the benefits of getting mammograms or the losses associated by not getting mammograms. Funding *NIH/NCI CA105786*.

Yoga to reduce menopausal symptoms in high-risk women (Cohen, Seewaldt) *Funding Susan G. Komen*

### **ADDITIONAL RESOURCES FOR TRANSLATIONAL RESEARCH:**

Duke University Medical Center provides an outstanding environment for the support of translational investigation, especially in breast cancer related research. Specific examples of the local Duke resources include the following:

**a) Clinical Research Training Program:** This program is a two year, part-time commitment for clinical investigators. The curriculum provides enrollees with training in the quantitative methodology of clinical research, and successful trainees receive a Master's Degree in Health Sciences. This program is also available to all fellows at the National Institutes of Health through collaboration with Duke University Medical Center.

**b) General Clinical Research Center (GCRC):** The clinical portion of this trial will take place in the NIH sponsored General Clinical Research Center (GCRC) directed by Louise Markert, M.D., Ph.D. The Duke GCRC is one of approximately 75 General Clinical Research Centers (GCRC) funded by the National Center for Research Resources, National Institutes of Health. Our GCRC was one of the twelve original centers opened in 1960, and we are currently in our 37th consecutive year of funding.

**c) Duke University Clinical Research Institute (DCRI):** The DCRI represents clearly the high level of institutional commitment to clinical investigation. The DCRI houses 41 faculty members and 700 personnel for the design, implementation and analysis of clinical research. The focus of the DCRI was originally in cardiovascular diseases, but now the DCRI has greatly broadened its clinical research portfolio, including cancer.

**d) Cancer Specific Clinical Research Environment:** A leading focus of clinical investigation at Duke University Medical Center is cancer related research. In-toto these resources create an extremely productive research environment which catalyzes exchange between basic and clinical investigators, and leads to the development of translational, multidisciplinary research projects. Specific examples of these resources include the following:

**d1) Duke Program in Molecular Therapeutics:** The Program in Molecular Therapeutics, directed by Dr. Lyerly has become a model of translational research. Investigators are able to see patients five days per week and occupy exam and consultation rooms. This includes cancer clinicians in Medicine, Surgery, Pathology, and Pediatrics to operate simultaneously and in contiguous space. In an era of contracting expenditures for medical care, Duke University is continuing to provide the institutional support for expansion of both research and clinical care in the area of cancer.

**d2) Duke Comprehensive Cancer Center:** The Duke Comprehensive Cancer Center (DCCC), directed by Dr. Kim Lyerly (Director) and Dr. Michael Colvin (Director Emeritus), is a matrix of administrative and laboratory facilities, which integrates and facilitates basic laboratory research and clinical trials on a university wide basis. The Cancer Center is funded by an NCI Core Support Grant approved through 2008. The Center is composed of 12 established Programs of Research, which serve to align the 270 members of the DCCC in programmatic and collaborative areas. Leadership in the Programs is composed of nationally and internationally recognized scientists, many of whom will participate in this project. In addition, the DCCC supports 19 shared core laboratories which provide essential large scale services and equipment to members of the DCCC.

Statistical support and computing will be coordinated by the Biostatistics Group at Duke:

1. Resource sharing - Database servers are available to the Duke Breast Cancer SPORE through its association with the Cancer Center.
2. Data sharing - As databases are integrated with other Cancer Center sources, data can be accessed and shared by the program easily, reducing duplicate data entry processes.
3. Programmer support - The Cancer Center provides support to the Duke Breast Cancer SPORE by interactions with the Cancer Center's programming group. This group provides database administration services and database infrastructure assistance.
4. Access to institutional resources - The Cancer Center will support an HL7 interface to institutional systems which will provide electronic data access to the Duke Breast Cancer SPORE. Again, this will reduce duplicate data entry processes.

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## BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.  
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

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NAME Rinker-Schaeffer, Carrie W.	POSITION TITLE Professor with Tenure/Director of Urologic Research		
eRA COMMONS USER NAME (credential, e.g., agency login) crinker			
EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)			
INSTITUTION AND LOCATION	DEGREE (if applicable)	MM/YY	FIELD OF STUDY
University of Cincinnati, Cincinnati, OH	B.S	1986	Chemistry/Biochemistry
University of Kentucky, Lexington, KY	Ph.D.	1992	Biochemistry
The Johns Hopkins School of Medicine, Baltimore, MD	Post-Doc	1992-94	Tumor Biology; Metastasis

### A. Personal Statement

Dr. Rinker-Schaeffer is recognized internationally for her expertise in metastasis research, especially in the areas of metastasis suppressors/signaling proteins and mechanism-based studies of metastatic colonization. More recently she has leveraged her laboratory's unique expertise in quantitative model systems to determine how the omental microenvironment promotes ovarian cancer metastasis formation. To this end her team recently published the novel finding that milky spots, not adipocytes, drive ovarian cancer metastatic colonization of omental fat. (Clark and Krishnan *et al.*, *Am. J. Path.* 183: 576-591). In this application, her laboratory proposes to conduct experiments evaluating the mechanistic contributions of both milky spot macrophages and ovarian cancer precursor cells to the formation of high-grade serous ovarian cancers.

### B. Positions and Honors

- 1994-2001 Assistant Professor, Department of Surgery, The University of Chicago School of Medicine, Chicago, IL
- 1994-present Director of Urological Research, Section of Urology; Department of Surgery, The University of Chicago School of Medicine, Chicago, IL
- 1994-present The University of Chicago Cancer Research Center (UCCRC), Chicago, IL
- 1994-present The University of Chicago Committee on Genetics, Chicago, IL
- 1994-present The University of Chicago Committee on Cancer Biology, Chicago, IL
- 1996-1999 Associate Director of the Prostate Cancer Program of the UCCRC, Chicago, IL
- 1999-2003 Director of the Prostate/Genitourinary Cancer Research Program of The University of Chicago Comprehensive Cancer Research Center, Chicago, IL
- 2001-2011 Associate Professor with Tenure, Department of Surgery, The University of Chicago School of Medicine, Chicago, IL
- 2001-2006 Associate Professor, Department of Medicine, The University of Chicago
- 2003-2011 Associate Professor, Department of Obstetrics and Gynecology, The University of Chicago
- 2011-present Professor with Tenure, Departments of Surgery and Obstetrics and Gynecology, University of Chicago

**Honors:** Sigma Xi Grants-in-Aid of Research Award, 1990-1991; Sigma Xi Award for Outstanding Research at the University of Kentucky, 1992; Special Program of Research Excellence (SPORE) Post-Doctoral Fellow, Laboratory of Dr. John Isaacs, Department of Oncology and the Brady Urological Institute, The Johns Hopkins School of Medicine, 1992-1994; Trainee, Urological Research Training Grant, Johns Hopkins School of Medicine, 1992-1994; Edwin Beer Award, New York Academy of Medicine, 1995; Huggins Award, Department

of Surgery, University of Chicago, 1995; University of Chicago Cancer Research Foundation Young Investigator Award, 1995; First Prize in the AUA/CaPCURE Contest, American Urological Association, 1996; CaPCURE Award for Research, CaPCURE Association for the Cure of Cancer of the Prostate, 1996; Society for Basic Urology Research/Merck Young Investigator Award, 2001; Subcommittee A, Cancer Centers Review, NCI; Subcommittee C, Basic and Preclinical Studies, NCI; Editorial Board Member, *Clinical and Experimental Metastasis*, *The Prostate*, and *Cancer Biology and Therapy*, ongoing; Permanent Member, Metabolic Pathology Study Section, 2001-2003; Fletcher Scholar Award, Cancer Research Foundation, Chicago, IL, 2002; Member, Tumor Progression and Metastasis Study Section, NCI, 2003-2005; Assoc. Editor, *Can.*

*Research*, 2003-2011; Adjunct Member, Metastasis Working Group of the NCI, 2003; Invited Opponent, Cancer Center Karolinska, Karolinska Institute, Stockholm, Sweden, 2003; Chair-elect, Women in Cancer Research Council (WICR), American Association for Cancer Research (AACR), 2006; Chair, WICR, AACR, 2007; VP, Society for Basic Urology Research, 2007; Ad Hoc Member, DOD Prostate Cancer Integration Panel, 2007; Member, Cell Biology and Metastasis Study Section of American Cancer Society, 2008-2011; President Society for Basic Urology Research, 2008; Past Chair, WICR, AACR, 2008, Organizing Committee AUA Education Workshop 2009; Organizing Committee AUA Foundation Leadership conference, 2009; Co-organizer WICR Professional Development Workshop AACR Annual Meeting, 2009, Chair, Cell Biology and Metastasis Study Section of the American Cancer Society, Jun 2010; Vice President, Metastasis Research Society, 2010; President, Metastasis Research Society, 2012.

### **C. Selected Peer-Reviewed Publications (of 74).**

#### **Most Relevant to the Current Proposal**

1. Vander Griend, D.J., Kocherginsky, M., Hickson, J.A., Stadler, W.M., Lin, A., **Rinker-Schaeffer, C.W.** Suppression of Metastatic Colonization by the Context-Dependent Activation of JNK Kinases JNKK1/MKK4 and MKK7. ***Cancer Res* 65**:10984-10991, 2005.
2. Hickson, J.A. Huo, D., Vander Griend, D.J., Lin, A., **Rinker-Schaeffer, C.W.**, Yamada, S.D. The p38 Kinase MKK4 and MKK6 Suppress Metastatic Colonization in Human Ovarian Cancer. ***Cancer Res* 66**:2264-70, 2006.
3. **Rinker-Schaeffer, C.W.**, O'Keefe, J.P., Welch, D.R., Theodorescu, D. Metastasis Suppressor Proteins: Discovery, Molecular Mechanisms, and Clinical Application. ***Clin Cancer Res* 12** 3882-9, 2006. PMID: PMC1525213
4. Lotan, T., Lyon, M., Huo, D., Taxy, J.B., Brendler, C., Foster, B., Stadler, W., **Rinker-Schaeffer, C.W.** Up-regulation of MKK4, MKK6, and MKK7 during prostate cancer progression: an important role for SAPK signalling in prostatic neoplasia. ***J Pathol* 212** 386-94, 2007.
5. Robinson, V.L., Shalhav, O., Otto, K., Kawai, T., Gorospe, M., **Rinker-Schaeffer, C.W.** Mitogen-activated protein kinase kinase 4/c-Jun NH2-terminal kinase kinase 1 protein expression is subject to translational regulation in prostate cancer cell lines. ***Mol Cancer Res* 6** 501-8, 2008. PMID: PMC2435180
6. Lotan, T., Hickson, J., Souris, J., Huo, D., Taylor, J., Li, T., Otto, K., Yamada, S.D., Macleod, K., **Rinker-Schaeffer, C.W.** c-Jun NH2-terminal kinase activating kinase 1/mitogen-activated protein kinase kinase 4-mediated inhibition of SKOV3ip.1 ovarian cancer metastasis involves growth arrest and p21 up-regulation. ***Cancer Res* 68** 2166-75, 2008. PMID: PMC3484373
7. Marasa, B.S., Srikantan, S., Masuda, K., Abdelmohsen, K., Kuwano, Y., Yang, X., Martindale, J.L., **Rinker-Schaeffer, C.W.**, Gorospe, M. Increased MKK4 abundance with replicative senescence is linked to the joint reduction of multiple microRNAs. ***Sci Signal* 2**(94):ra69, 2009. PMID: PMC2770878
8. Hickson J., Yamada S.D., Berger J., Alverdy J., O'Keefe J., Bassier B., **Rinker-Schaeffer C.W.** Societal interactions in ovarian cancer metastasis: a quorum-sensing hypothesis. ***Clin Exp Metastasis* 26**(1);67-76, 2009.
9. Khan, S.M., Funk, H.M., Thiolloy, S., Lotan, T.L., Hickson, J., Prins, G.S., Drew, A.F., **Rinker-Schaeffer, C.W.** *In vitro* metastatic colonization of human ovarian cancer cells to the omentum. ***Clin Exp Metastasis***

27:185-96, 2010.

10. Khan S., Taylor J.L, **Rinker-Schaeffer C.W.**, Disrupting ovarian cancer metastatic colonization: insights from metastasis suppressor studies. **J Oncol.** 2010:286925, 2010. PMID: PMC2838371
11. Thiolloy S., **Rinker-Schaeffer C.W.** Thinking outside the box: using metastasis suppressors as molecular tools. **Semin Cancer Biol.** Apr;21(2):89-98, 2011.
12. Shoushtari, A.N., Szmulewitz, R.Z. and **Rinker-Schaeffer, C.W.** Metastasis-suppressor genes in clinical practice: lost in translation? **Nat Rev Clin Oncol** 8: 333-342, 2011.
13. Szmulewitz, R.Z., Clark, R., Lotan, T., Otto, K., Taylor Veneris, J., Macleod, K. and **Rinker-Schaeffer, C.W.** MKK4 suppresses metastatic colonization by multiple highly metastatic prostate cancer cell lines through a transient impairment in cell cycle progression. **Int J Cancer** 130: 509-520, 2012. PMID: PMC3465713
14. Bainer, R.O., Veneris, J.T., Yamada, S.D., Montag, A., Lingen, M.W., Gilad, Y. and **Rinker-Schaeffer, C.W.** Time-dependent transcriptional profiling links gene expression to mitogen-activated protein kinase kinase 4 (MKK4)-mediated suppression of omental metastatic colonization. **Clin Exp Metastasis** 29: 397-408, 2012.
15. Krishnan V., Stadick N., Clark R., Bainer R., Veneris J.T., Khan S, Drew A, **Rinker-Schaeffer C.** Using MKK4's metastasis suppressor function to identify and dissect cancer cell-microenvironment interactions during metastatic colonization. **Cancer Metastasis Rev.** Dec;31(3-4):605-13, 2012.
16. Souris, J.S., Hickson, J.A., Msezane, L., **Rinker-Schaeffer, C.W.**, Chen, C.T. Flexible peritoneal windows for quantitative fluorescence and bioluminescence preclinical imaging. **Mol Imaging** Jan-Feb; 12(1):28-38, 2013.
17. Clark, R., Krishnan, V., Schoof, M., Rodriguez, I., Theriault, B., Chekmareva, M., **Rinker-Schaeffer, C.W.** Milky spots promote ovarian cancer metastatic colonization of peritoneal adipose in experimental models. **American J. Path** Aug;183(2):576-91, 2013. PMID: PMC3730760

#### **D. Research Support**

##### **Active**

2 R01CA089569 (PI: Rinker-Schaeffer)

12/01/08-11/30/2013

NCI/NIH

“Prostate Cancer Metastatic Colonization: Role of MKK4.”

The goal of this project was to determine the mechanism through which MKK4/SEK1 suppresses metastatic colonization and to establish identify the upstream and downstream events involved in this process.

Role: PI

W81XWH-09-1-0449/DOD (PI: Rinker-Schaeffer)

08/01/2009-07/31/2014

Prostate Cancer Research Program Idea Development Award

“Identifying the Mechanism(s) Responsible for the Translational Regulation of the Stress Kinase MKK4.”

The goal of this project is to examine a mechanism by which MKK4 proteins levels are regulated in prostate cancer cells.

Role: PI

HERA Women's Cancer Foundation (PI: Rinker-Schaeffer)

4/1/2011-01/31/2014

“Harnessing the power of materials science for studies of metastasis biology: Development of tunable models of ovarian cancer omental colonization.”

The goal of this project is to develop *in vitro* models of ovarian cancer microenvironment interactions.

Role: PI

##### **Completed (last three years)**

Marsha Rivkin Center (PI: Rinker-Schaeffer)

4/1/2011-3/31/2012

For Ovarian Cancer Research

Milky Spot Macrophages: Co-Conspirators in Omental Metastasis

The goal of this project is to test the hypothesis that macrophages promote ovarian cancer metastatic colonization.

Role: PI

Department of Defense DOD/OCRP Pilot Award

05/15/2011-05/14/2013

(PI: Bellis/UAB and Co-Investigator: Rinker-Schaeffer)

“The Role of Receptor Sialylation in the Ovarian Tumor Cell Phenotype.”

The goal of this project is to define the role of receptor sialylation in regulating tumor cell invasion and survival.

Role: Co-Investigator

W81XWH-09-1-0127/DOD (PI: Rinker-Schaeffer)

08/01/2009-07/31/2013

Ovarian Cancer Research Program Idea Development Award

“The Role of the Omental Microenvironment in Ovarian Cancer Metastatic Colonization.”

The goal of this project is to determine whether ovarian cancer cells interact with milky spot structures on the omentum and if so determine the effect of these interactions on cell growth.

Role: PI

## 1. BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.  
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME Victoria Seewaldt	POSITION TITLE Professor		
eRA COMMONS USER NAME seewa001			
EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)</i>			
INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	YEAR(s)	FIELD OF STUDY
Cornell University	B.A.	1976-1980	Chemistry
University of California, Davis	MD	1986-1989	Medicine
Fred Hutchinson Cancer Research Center	PDF	1992-1998	Molecular Medicine

### A. Statement of Expertise and Contribution:

**Victoria Seewaldt, MD** is an internationally recognized expert in biomarker development, translational research, and breast cancer initiation. Dr. Seewaldt leads a bench to community research program focusing on identifying molecular targets for early detections and biomarker development. Dr. Seewaldt has extensive basic/translational research expertise in 3-D models of early mammary carcinogenesis, apoptotic signaling, and biomarker development. Dr. Seewaldt's clinical/translational research involves multi-disciplinary, multi-institutional collaborations with basic, translational, and clinical scientists, with the goal of integrating novel functional imaging strategies with risk-marker to evaluate both environmental and epigenetic markers of risk. The proposed study will draw heavily on Dr. Seewaldt's ability to span from lab to the community. Dr. Seewaldt established a cohort of over 600 women who are at high-risk for breast cancer. Her outreach project works with community members to evaluate environmental risk factors for triple-negative breast cancer. The unique feature of Dr. Seewaldt's program is that biomarkers identified in the laboratory can be immediately tested in the high-risk women in her cohort as predictors of short-term breast cancer risk.

### B. Professional Experience

1982-1984 **Research Assistant**, Cancer Biology Research Lab., Stanford University (Dr. Henry Kaplan).  
1984-1985 **Research Assistant**, Department of Virology, Stanford University (Dr. Edward J. Mocarski).  
1989-1990 **Intern**, Department of Obstetrics and Gynecology, University of Washington.  
1990-1992 **Intern and Resident**, Department of Medicine, University of Washington.  
1992-1999 **Postdoctoral Fellow**, Molecular Med., Fred Hutchinson Cancer Research Center, Seattle, WA.  
2000-2001 **Assistant Professor, Medical Oncology**, Duke University, Durham, NC.  
2001-2009 **Associate Professor, Medical Oncology**, Duke University, Durham, NC.  
2004-2012 **Leader, Breast and Ovarian Cancer Program**, Duke Comprehensive Cancer Center.  
2004-2011 **Associate Professor, Pharmacology and Cancer Biology**, Duke University, Durham, NC.  
2005-2010 **Vice Chair Research**, Division of Medical Oncology, Duke University, Durham, NC  
2006 **Tenure**, Duke University, Durham, NC  
2009-pres. **Professor**, Department of Medicine, Duke University, Durham, NC

**Honors and Awards:** 1989 Robert Stowell Award; 1992 Robert S. Evans Award; 1993-1997 American Board of Internal Medicine Clinical Investigator Pathway; 1994-1998 Susan G. Komen Postdoctoral Fellow, 2000 V-Foundation Scholar; 2001-2004 Section Head, Susan G. Komen Postdoctoral Fellowship Committee; 2003 Joklik Award in Basic Science Research; 2002-2004 Innovators in Breast Cancer; 2003-2009; 2003-2009 NIH/NCI TPM, Charter Member 07/04; 2005 NIH/NCI ZRG1 ONC-J; 2006 NIH/NCI TCB *ad hoc*; 2005-pres. NCI/SPORE AVON Achievement Award; 2007 ELAM; 2007-pres Best Doctors in America; 2007-2011 Chair, Komen PDF Committee; 2008 Malek Award in Translational Cancer Research. 2008-pres NCI National Cancer Advisory Board, Breast Cancer Steering Committee; 2009-pres. Board of Advisors FORCE; 2008-pres Best Doctors in America; 2009 NCI Biospecimen TRC; 2010-pres. Board of Advisors Marsha Rifkin Foundation. 2010-pres Charter member NCI IRG Subcommittee A for Cancer Center Review (Parent Committee), 2011 NIH ZRG1 OTC-N Chair, 2011-pres Board Member International Association for Breast Cancer Research; 2011-pres. Charter Member NIH CDP; 2011 DoD Breast Cancer Integration Panel; 2012-pres ASCO Program Review Committee; 2012 NCI Provocative Question Workshop on Disparities and Cancer.

### C. Selected Publications (total 87):

1. Bowie M, Dietze EC, Bean G, and **Seewaldt VL**. Interferon regulatory factor-1 is critical for tamoxifen-mediated apoptosis in human mammary epithelial cells. *Oncogene*, 23:8743-55, 2004. PMID: PMC3458110.
2. Dietze EC, Bean GR, Bowie M, and **Seewaldt VL**. Tamoxifen and tamoxifen ethyl bromide induce apoptosis in acutely damaged mammary epithelial cells through modulation of AKT activity by plasma-membrane associated 17-beta-estradiol binding sites. *Oncogene*, 23: 3851-3862, 2005. PMID: 1207480.
3. Dietze E, Ibarra C, and **Seewaldt VL**. CBP modulates proliferation and resistance to extracellular matrix-induced apoptosis in human mammary epithelial cells. *J Cell Sci* 118: 5005-22, 2005. PMID: 16219677.
4. Bean GR, Kimler BF, and **Seewaldt VL**. Failure of Raloxifene chemoprevention in high-risk women. *New England Journal of Medicine*, 355:1620-1622, 2006. PMID: 17035661.
5. **Seewaldt VL**, Jones L, Zalles C, and Demark W. Increased BMI Predicts Atypia in RPFNA from High-Risk Postmenopausal Women, *Cancer Epi Biomarkers Prev* 16: 613-616, 2007. PMID:17016339.
6. Bowie, M, Dietze, EC, Bean, G, Ibarra, C, and **Seewaldt, VL**. CBP-mediated induction of interferon regulatory factor-1 promotes apoptosis in human mammary epithelial cells. *Oncogene*, 26:2017-2026, 2007. PMID: 17016442.
7. Ibarra C, Wilke L, Yee L, Kulkarni S, Wood M, Garber J, Stouder A, Grant T, Broadwater G, and **Seewaldt, VL**. Random Periareolar Fine Needle Aspiration is highly reproducible in a cooperative multi-institutional trial. *Cancer Epi Biomarkers Prev*, 18:1379-1384, 2009. PMID: 19258476.
8. Lu J, Guo H, Treekitkarnmongkol W, Li P, Zhang J, **Seewaldt V**, Muller W, Sahin A, Hung M-C, and Yu D. 14-3-3 $\zeta$  Cooperates with ErbB2 to Promote Progression of Ductal Carcinoma in Situ to Invasive Breast Cancer by Inducing Epithelial-Mesenchymal Transition. *Cancer Cell*. 16: 195-207, 2009. PMID: PMC3243533
9. Ostrander J, McMahon C, **Seewaldt V**, and Ramanujam N. Optical redox ratio differentiates breast cancer cell lines based on estrogen receptor status. *Cancer Res*, 70:4759-65 2010. PMID: PMC2957814.
10. Millon S, Ostrander JH, **Seewaldt VL**, and Ramanujam N. Uptake of 2-NBDG as a method to monitor glucose uptake and therapy response in breast cancer cells. *Breast Cancer Res.*, 2010. PMID: 20390344.
11. Pilie P, Ibarra-Drendall C, Broadwater G, Petricoin E, Liotta LA, Zalles C, Ford A Yu D, and **Seewaldt VL**. Protein Microarray Analysis of Mammary Epithelial Cells from Obese and Non-Obese Women at High-Risk for Breast Cancer. *Cancer Epi Biomarkers Prev*. 20:476-482, 2011 (cover article) PMID: 21647677.
12. Ibarra-Drendall C, Barry W, Petricoin E, Yu D, Zalles C, and **Seewaldt VL**. Activation of IL6/Akt-phosphoprotein network signaling during initiation of breast cancer in high-risk women. *Breast Cancer Res*, 2012. PMID: 21242333.
13. Henderson B\*, Lee N\*, **Seewaldt VL\***, Shen H\*, The influence of race and ethnicity on the biology of cancer. *Nature Reviews Cancer*, 12, 648-653, 2012. (\*all authors contributed equally). PMID: 22854838.
14. Ostrander J, D'Amato N, Cardiff RD, Simin K, Delrow J, Bachelder R, Ibarra C, and **Seewaldt VL**. Evidence for phenotypic plasticity in triple-negative breast cancer: Human biology is recapitulated by a novel model system. *PLoS One*; pone.0045684. Epub 2012 Sep 25. PMID: PMC3458110.
15. **Seewaldt, V.L.** Destiny from density. *Nature*, 490, 490–491, 2012. PMID: 23099400.
16. Peterson B, Broadwater G, Ibarra-Drendall, Wilke L, Ford A, and **Seewaldt VL**. Atypia in Random Periareolar Fine Needle Aspiration predicts short-term progression in high-risk women. Submitted *JNCI*, 2013.

## D. Active Support

**1R01CA155664-01** (Seewaldt, V.L.) 04/01/11-3/31/16  
NIH/NCI  
Role: P.I.  
Title: *KCNK9 imprinting and metastasis of triple-negative breast cancer in African American women*

**1R01CA158668-01A1** (Seewaldt, V.L.) 04/01/12-03/30/17  
NIH/NCI  
Role: P.I.  
Title: *Phosphoprotein network signaling during the initiation of triple-negative breast cancer*

**1R01CA170851-01** (Kuperwasser, C, Seewaldt, V.) 09/01/12-08/30/17  
NIH/NCI Provocative Questions (PQ-1)  
Role: Multi-PI  
Title: *PQ-1 Mechanisms of microenvironment signaling during initiation and progression of Triple-Negative Breast Cancer in obese and lean women*

### Disparities and Community Outreach funding for uninsured women:

**Susan G. Komen** (Seewaldt, V.L.) 1/01/07-04/31/14  
Susan G. Komen Triangle Affiliate – Disparities funds  
Role: P.I.  
Title: *Breast MRI Screening in African American and Caucasian High-Risk Women.*

**National Breast Cancer Foundation** (Seewaldt, V.L.) 07/01/10-12/31/13  
Free mammogram and ultrasound resources for uninsured women  
Role: P.I.  
Title: *Breast cancer screening resources for uninsured women*

### Cooperative Trials:

**ALLIANCE Intergroup Trial: A211102**  
Role: PI  
Title: *Role of metformin in inhibition of Akt/IL6 signaling during initiation of TNBC.*

### Completed Support

**5R01-CA088799-10** (Seewaldt, V.L.) 07/01/06 - 05/31/12  
National Institutes of Health  
Role: PI  
Title: *ECM-Mediated Apoptosis in p53 (-) HMECs*

**3R01-CA088799-07S1** (Seewaldt, V.L.) 07/01/00 - 05/31/11  
National Institutes of Health  
Role: PI  
Title: *ECM-Mediated Apoptosis in p53 (-) HMECs (Minority Supplement)*

**5R01-CA114068-05** (Seewaldt, V.L.) 08/01/06 – 07/31/12  
National Institutes of Health  
Role: PI  
Title: *Molecular Markers of Breast Cancer Risk and Prevention*

## BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.  
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME Anne C. Ford	POSITION TITLE Associate Clinical Professor Obstetrics and Gynecology		
eRA COMMONS USER NAME			
EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)</i>			
INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	YEAR(s)	FIELD OF STUDY
Duke University	B.A.	1980-1984	Spanish, Magna Cum Lade
University of North Carolina at Chapel Hill	M.D.	1984-1988	Medicine
University of North Carolina School of Public Health		1988-1989	Health Policy Administration

### A. Personal Statement

Anne Ford, MD is an Assistant Professor and Minority Investigator who runs our outreach clinic to underserved African American women. Dr. Ford will provide outreach efforts and recruit high-risk women to identify and recruit African American women to this trial.

### B. Positions and Honors

#### Positions and Employment

1998-1990 Internship, University of Alabama, Birmingham, Alabama  
1990-1993 Residency, OB/Gyn, Emory University, Atlanta, GA  
1993-1998 Athens Women's Clinic, Athens, GA  
1999-2012 Assistant Clinical Professor, Duke University  
2004-2008 Division Chief, Women's Health Specialist  
2009-present Director, Duke Women's Community Outreach Program  
2012-present Associate Clinical Professor, Duke University

#### Other Experience and Professional Memberships

Fellow, American College of Obstetricians and Gynecologists  
Diplomat, American Board of Obstetrics and Gynecology  
North Carolina Ob/Gyn Society  
Emory/Grady Society  
Carter Society

### C. Selected Publications

Ibarra, C., Ford, A., Wilke, L., Broadwater, G., and Seewaldt, V.L. Atypia in Random Periareolar Fine Needle Aspiration predicts short-term breast cancer risk in high-risk women. Submitted *JNCI*, 2010.  
Retrospective Analysis of the Evaluation and Follow-up on Ambisexual Infants in the Three Hospital Emory University System. Presented at the John D. Thompson Resident Research Day, 1993  
The Surgical Scrub and the Relationship between Fingernail Length and Subungual Bacteria. Presented at the John D. Thompson Resident Research day, 1992  
Retrospective Analysis of the Frequency and Accuracy of Clinical Breast Examination by Ob/Gyn Residents at Grady Memorial Hospital. Presented and Awarded Second Place at the John D. Thompson Resident Research Day, 1991. Presented at ACOG National Meeting, Las Vegas, NV 1992  
"Surrogate Motherhood - Sale, Service or Gift?" Thesis written to complete degree of M.D. with Honors, 1988

## **D. Research Support**

### **Ongoing Research Support**

5R01-CA155664-03 (Seewaldt)

01/06/11-12/31/15

National Institutes of Health

*KCNK9 Imprinting in Breast Cancer Progression and Metastasis*

The goal of this project is to investigate a novel signaling pathway that holds promise for early detection of triple-negative breast cancer.

Aim 1: Will test whether hypoxia transcriptionally activates TASK3 in triple-negative breast cancers that lack imprinting of the *KCNK9* DMR. We will perform *in vivo* multiparametric analysis of *KCNK9* HRE activity, TASK3 expression, tumor growth and metastasis, and hemoglobin saturation in normoxic and hypoxic conditions.

Aim 2: Will test whether the loss of normal *KCNK9* DMR imprinting promotes initiation, progression, and metastasis of triple-negative breast cancer.

33780/98088972 (Seewaldt)

09/25/09 – 09/24/14

UT MD Anderson Cancer Center

The Role of HER2 Signaling Networks in Early Stage Breast Cancer Initiation and Resistance to Tamoxifen Prevention

The goal is to understand the role of HER2 signaling network in early stage breast cancer initiation and resistance to Tam prevention.

Role: Investigator

NC-SGK Foundation 2013-NC101-DUMC 69(Seewaldt)

04/01/13 – 03/31/14

Susan G. Komen Breast Cancer Foundation

An Integrated Community/Academic Partnership for Breast Cancer Screening

Our goals are to:

1) Use our Patient Navigator program to provide trusted sources of community information and reduce barriers to screening

2) Link our Patient Navigator with community providers to coordinate referrals and follow-up

3) Provide free screening services for uninsured and underinsured women

Role: Investigator

### **Completed Research Support**

**None**

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## BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.  
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

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NAME Shaw, Patricia	POSITION TITLE Professor		
eRA COMMONS USER NAME (credential, e.g., agency login) PATRICIASHAW			
EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)			
INSTITUTION AND LOCATION	DEGREE (if applicable)	MM/YY	FIELD OF STUDY
University of Toronto, Faculty of Medicine	MD	1976	Medicine
University of Toronto, Anatomical Pathology	FRCPC	1985	Anatomical Pathology
American Board of Pathology, Anatomical Pathology	Diploma		Anatomical Pathology

### A. Personal Statement

**Patricia Shaw, MD** is recognized internationally for her expertise in gynecologic pathology, specifically the areas of ovarian and tubal serous tumor. Dr. Shaw's research focuses on the etiology and pathogenesis of high-grade serous carcinoma. She has established a cohort of 1000 snap-frozen and formalin-fixed paraffin embedded normal fallopian tube and ovarian cancer tissue samples, containing different histotypes (mucinous, clear cell, endometrioid and serous). Her research focuses on the molecular progression of fimbrial tubal epithelial cells to serous tubal intraepithelial carcinomas. In support of this research Dr. Shaw's laboratory has established an extensive repository of more than 500 fresh processed fallopian tube epithelia (FTE) including BRCA1 mutation carriers. In addition, her team has used portions of these clinical samples to establish multiple primary and immortalized FTE cell lines, including those from BRCA1 mutation carriers. Using an unprecedented cohort of over 300 fallopian tube samples Dr. Shaw's laboratory found that "high risk" tubal epithelium from carriers of germ line mutations of BRCA1 responds differently to microenvironmental cues. This cohort is being utilized to understand and characterize the earliest molecular changes in HGSC development and has also identified alterations characteristic of HGSC for potential utility in devising personalized treatment strategies. Taken together, Dr. Shaw's expertise and unique clinical resources enable her to make a critical contribution to the studies proposed in this application.

### B. Positions and Honors (selected)

#### Employment

2001- Active Staff, Department of Pathology, University Health Network (UHN)  
2011- Active Staff, Sunnybrook Health Sciences Centre  
2011- Active Staff, Lakeridge Hospital  
2012- Professor, Laboratory Medicine & Pathobiology, University of Toronto  
2012- Professor, Division of Gynecologic Oncology, Department of Obstetrics and Gynecology

#### Current Committee Membership (selected):

2004 - Editorial Board, International Journal of Gynecological Pathology  
2010 - National Cancer Institute of Canada (NCIC) Clinical Trials Working Group – Correlative Sciences/Tumour Biology Committee  
2010 - Canadian Association of Pathologists – Task Force  
2011- World Health Organization Sub-Committee – Classification of Ovarian and Tubal Tumours

### C. Selected Peer-reviewed Publications (Last 5 years; Lifetime total: >60)

1. H Risch, J McLaughlin, D Cole, B Rosen, L Bradley, I Fan, J Tang, S Li, S Zhang, **P Shaw**, S Narod. General Population Frequencies of BRCA1 and BRCA2 Mutations and their Penetrance for Cancer: Results of a Population-Based Kin-Cohort Study in Ontario, Canada. *JNCI* 98(23):1694-1706, 2006.
2. A Tone, H Begley, M Sharma, J Murphy, B Rosen, TJ Brown, **PA Shaw**. Gene Expression Profiles of Luteal Phase Fallopian Tube Epithelium from BRCA-Mutation Carriers Resemble High Grade Serous Carcinoma. *Clin Cancer Res* 14(13):4067-78, 2008.
3. **Shaw PA**, Rouzbahman M, Pizer E, Pintile M, Begley H. Candidate Serous Cancer Precursors in Fallopian Tube Epithelium of BRCA1/2 Mutation Carriers. *Mod Pathol*. Sep;22(9):1133-8, 2009.
4. Vicus D, **Shaw PA\***, Finch A, Rosen B, Armel S, Sun P, Narod SA\*. Risk factors for fallopian tube precursor lesions in BRCA mutation carriers. *Gynecologic Oncology* 2010 Sep;118(3):295-8.
5. May T, Sharma M, Milea A, Begley H, Rosen B, Murphy KJ, Brown T\* and **Shaw PA\***. Low malignant potential tumors with micropapillary features are molecularly similar to low-grade serous carcinoma of the ovary. *Gynecologic Oncology*. 2010 Apr;117(1):9-17.
6. Tone AA, Virtanen C, **Shaw PA\***, Brown TJ\*. Decreased progesterone receptor isoform expression in luteal phase fallopian tube epithelium and high-grade serous carcinoma. *Endocr Relat Cancer*. 2011 Feb 23;18 (2):221-34. PMID: PMC3043379
7. Zhang S, Royer R, Li S, McLaughlin JR, Rosen B, Risch HA, Fan I, Bradley L, **Shaw PA**, Narod SA. Frequencies of BRCA1 and BRCA2 mutations among 1,342 unselected patients with invasive ovarian cancer. *Gynecologic Oncology* 2011 May 1;121(2):353-7.
8. George SH, Greenaway J, Milea A, Clary V, Shaw S, Sharma M, Virtanen C, **Shaw PA** Identification of abrogated pathways in fallopian tube epithelium from BRCA1 mutation carriers. *J Pathol*. 2011 Sep;225(1):106-17.
9. Stewart J, **Shaw PA**, Gedye C, Bernardini M, Neel B, Ailles L. Phenotypic Heterogeneity and Instability of Human Ovarian Tumor-Initiating Cells. *PNAS* 2011 Apr 19;108(16):6468-73. PMID: PMC3081039
10. Visvanathan K, Vang R, **Shaw P**, Gross A, Soslow R, Parkash V, Shih IM, Kurman RJ. Diagnosis of serous tubal intraepithelial carcinoma based on morphologic and immunohistochemical features: a reproducibility study. *Am J Surg Pathol*. 2011 Dec;35 (12):1766-75.
11. Vang R, Visvanathan K, Gross A, Maambo E, Gupta M, Kuhn E, Li RF, Ronnett BM, Seidman JD, Yemelyanova A, Shih IM, **Shaw PA**, Soslow RA, Kurman RJ. Validation of an Algorithm for the Diagnosis of Serous Tubal Intraepithelial Carcinoma. *Int J Gynecol Pathol*. 2012 Apr 10. PMID: PMC3366037
12. E Kuhn, R Wu, G Wu, B Guan, J Zhang, Y Wang, L Song, X Yuan, L Wei, R Roden, KT Kuo, K Nakayama, Blaise Clarke, **Patricia Shaw**, Narciso Olvera, Douglas Levine, Robert Kurman, Tian-Li Wang, and le-Ming Shih. Identification of molecular pathway aberrations in uterine serous carcinoma by genome-wide analyses. *J Natl Cancer Inst*. 2012 Oct 3;104(19):1503-13. PMID: PMC3692380
13. AA Tone, C Virtanen, **PA Shaw\***, T J Brown\*. Prolonged post-ovulatory pro-inflammatory signalling in the fallopian tube epithelium may be mediated through a BRCA1/DAB2 axis. *Clin Cancer Research*. 2012 Jul 2.
14. George SH, Milea A, **Shaw PA**. Proliferation in the normal FTE is a hallmark of the follicular phase, not BRCA mutation status. *Clin Cancer Res*. 2012 Nov 15;18(22):6199-207
15. McLaughlin JR, Rosen B, Moody J, Pal T, Fan I, **Shaw PA**, Risch HA, Sellers TA, Sun P, Narod SA. Long-term ovarian cancer survival associated with mutation in BRCA1 or BRCA2. *J Natl Cancer Inst*. 2013 Jan 16;105(2):141-8. PMID: PMC3611851

## D. Research Support

### Peer reviewed

U.S. Office of Army Research, OCRF, Consortium Grant Kurman (PI) 2011-2016  
Prevention of Ovarian High Grade Serous Carcinoma by Elucidating its Early Changes.

The goal of this project is to carefully characterize the morphologic, molecular genetic, immunohistochemical (IHC) and epidemiologic features of the tubal/ovarian high grade serous cancer precursor lesions in the fallopian tube, with the long term aim of developing novel preventative strategies.

Role: Co- PI

## Personalized Medicine with Targeted and Genome-Wide Sequencing

The goal of this project is to

Role: Co-Investigator

Canadian Cancer Society, Operating Grant

Neel (PI)

2009-2014

Target cells and tumor-initiating cells in serous ovarian carcinoma

The goal of this study is the identification and characterization of tumour-initiating cells in high-grade serous cancer using a xenograft model derived from human tumour tissues.

Role: Co-Investigator

NCIC CBCRA Operating Grant

Narod (PI)

2009-2014

Risk factor analysis of hereditary breast and ovarian cancer

This is a population-based molecular epidemiology study which includes the identification, and retrieval of paraffin blocks and slides for tumour classification and molecular analysis in 1800 Ontario women diagnosed with ovarian cancer.

Role: Co-Investigator

### **Non-peer reviewed**

Princess Margaret Hospital Foundation

Shaw (PI)

2008-present

Appel Fund: Advances in Pathology: Detecting Ovarian Cancer Earlier

The goal of this project is to determine the role of the fallopian tube in serous carcinogenesis

Role: Principle Investigator

NCI The Cancer Genome Atlas Project

Genomics and transcriptomics of endometrial cancer and thyroid cancer

Role: Co-PI (Funding to my account: \$1,000 per case accepted by TCGA – total to date \$30,000)

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## BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.  
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

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NAME Becker, Lev	POSITION TITLE Assistant Professor		
eRA COMMONS USER NAME (credential, e.g., agency login) levbecker			
EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)</i>			
INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	MM/YY	FIELD OF STUDY
Queen's University, Kingston, Ontario	BS	06/1997	Life Sciences
Queen's University, Kingston, Ontario	MS	09/1999	Biochemistry
Queen's University, Kingston, Ontario	PhD	09/2003	Biochemistry
Queen's University, Kingston, Ontario	Postdoc	09/2004	Biochemistry
University of Washington, Seattle, WA	Postdoc	09/2009	Proteomics

### A. Personal Statement

We are proposing to determine the mechanistic contribution of omental milky spot macrophage in ovarian metastatic colonization. As a co-investigator, my research will focus on using proteomic, immunological, and functional approaches to interrogate macrophages in various adipose tissue depots. My laboratory has well-documented expertise in combining immunological and proteomic methods to studying macrophage biology (Becker et al., Plos ONE, 2012; Kanter et. al. PNAS, 2012; Becker et al., Cell Metabolism, 2010) and have developed statistical and computational tools to facilitate proteomics data analysis (Heinecke et al., Bioinformatics, 2010).

### B. Positions and Honors

- 10/1/2003 Post-doctoral Fellow, Department of Biochemistry, Queen's University, Kingston, ON, Canada  
10/1/2004 Post-doctoral Fellow, Department of Medicine, Division of Metabolism, Endocrinology and Nutrition, University of Washington, Seattle, WA  
10/1/2009 Acting Instructor, Department of Medicine, Division of Metabolism, Endocrinology and Nutrition, University of Washington, Seattle, WA  
03/1/2011 Research Assistant Professor, Department of Medicine, Division of Metabolism, Endocrinology and Nutrition, University of Washington, Seattle, WA  
09/1/2011 Assistant Professor, Department of Pediatrics, Section of Sleep Medicine, Committee on Molecular Metabolism and Nutrition, University of Chicago, Chicago, IL

### Honors and Awards

- 1997-02 Queen's Graduate Award  
1998 Queen's Graduate Fellowship  
1999 E.G. Baumann Fellowship  
2000 - Canadian Lipoprotein Conference Doctoral Oral Presentation Award  
- Ontario Graduate Scholarship  
2001 Ontario Graduate Scholarship in Science and Technology  
2002 Heart and Stroke Foundation of Canada Doctoral Research Fellowship  
2003 Canadian Lipoprotein Conference Post-Doctoral Fellow Oral Presentation Award  
2004 - Ph.D. Dissertation nominated for Governor General's Gold Medal Award  
- PhD Dissertation awarded the Most Outstanding Thesis in the Life Sciences, Queen's University  
2004  
2005 Canadian Institutes of Health and Research Postdoctoral Research Fellowship (accepted)  
2008 Young Investigator Award, 3<sup>rd</sup> International Symposium - Integrated Biomarkers in Cardiovascular Disease  
2012 George M. Eisenberg Foundation for Charities Excellence Award for Pediatrics

## C. Selected Peer-Reviewed Publications

### Most relevant to the current application

1. Yurkovetskiy, L., Burrows, M., Khan, A.A., Graham, L., Volchkov, P., **Becker, L.**, Antonopoulos, D., Umesaki, Y., and Chervonsky, A.V. Gender bias in autoimmunity is influenced by microbiota. *Immunity*, **39**, 400-412.
2. Suzuki, M., **Becker, L.**, Pritchard, D.K., Gharib, S.A., Wijsman, E.M., Bammler, T.K., Beyer, R.P., Vaisar, T., Oram, J.F., & Heinecke, J.W. (2012). Cholesterol accumulation regulates expression of macrophage proteins implicated in proteolysis and complement activation. *Arterioscler. Thromb. Vasc. Biol.* **32**, 2910-2918. PMC3501207
3. **Becker, L.**, Liu, N.C., Averill, M.M., Yuan, W., Pamir, N., Peng, Y. Irwin, A.D., Fu, X., Bornfeldt, K.E., & Heinecke, J.W (2012). Unique proteomic signatures distinguish macrophages and dendritic cells. *PLoS ONE*. **7**, e33297. PMC3299764
4. Kanter, J.E., Kramer, F., Barnhart, S., Averill, M.M., Vivekanandan-Giri, A., Vickery, T., Li, L.O., **Becker, L.**, Yuan, W., Chait, A., Braun, K.R., Potter-Perigo, S., Sanda, S., Wight, T.N., Pennathur, S., Serhan, C.N., Heinecke, J.W., Coleman, R.A. & Bornfeldt, K.E. (2012) Diabetes promotes an inflammatory macrophage phenotype and atherosclerosis via acyl-CoA synthetase 1. *Proc. Natl. Acad. Sci.* **109**, E715-24. PMC3311324
5. Averill, M.M., Barnhart, S., **Becker, L.**, Li, X., Heinecke, J.W., LeBoeuf, R.C., Hamerman, J.A., Sorg, C., Kerkhoff, C., & Bornfeldt, K.E. (2011) S100A9 differentially modifies phenotypic states of neutrophils, macrophages, and dendritic cells: Implications for atherosclerosis and adipose tissue inflammation. *Circulation*. **123**, 1216-1226. PMC3072335
6. Suzuki, M., Pritchard, D.K., **Becker, L.**, Hoofnagle, A.N., Tanimura, N., Bammler, T.K., Beyer, R.P., Bumgarner, R., Vaisar, T., de Beer, M.C., de Beer, F.D., Miyake, K., Oram, J.F., & Heinecke, J.W. (2010) HDL suppresses the type I interferon response, a family of potent antiviral immunoregulators, in macrophages challenged with lipopolysaccharide. *Circulation*. **122**, 1919-1927. PMC2988582
7. Heinecke, N.L., Pratt, B.S., Vaisar, T., & **Becker, L.** (2010) PepC: Proteomics software for identifying differentially expressed proteins based on spectral counting. *Bioinformatics* **26**, 1574-1575. PMC2881356
8. **Becker, L.**, Gharib, S.A., Wijsman, E., Vaisar, T., Oram, J.F., & Heinecke, J.W. (2010) A macrophage sterol-responsive network linked to atherogenesis. *Cell Metab.* **11**, 125-135. PMC2893224

### Additional publications of importance to the field

1. **Becker, L.**, Gabel, B.R., Spencer, C.A., Marcovina, S.M. & Koschinsky, M.L.: Effect of low-density lipoprotein buoyant density and cholesterol content on the formation of lipoprotein(a) particles. *Clin Exp Med.* **1**: 121-125, 2001.
2. **Becker, L.**, McLeod, R.S., Marcovina, S.M., Yao, Z. & Koschinsky, M.L.: Identification of a critical lysine residue in apolipoprotein B-100 that mediates noncovalent interaction with apolipoprotein(a). *J Biol Chem.* **276**: 36155-36162, 2001.
3. **Becker, L.**, Webb, B.A., Chitayat, S., Nesheim, M.E. & Koschinsky, M.L.: A ligand-induced conformational change in apolipoprotein(a) enhances covalent Lp(a) formation. *J Biol Chem.* **278**: 14074-14081, 2003.
4. **Becker, L.**, Cook, P.M. & Koschinsky, M.L.: Identification of sequences in apolipoprotein(a) that maintain its closed conformation: A novel role for apo(a) isoform size in determining the efficiency of covalent Lp(a) formation. *Biochemistry* **43**: 9978-9988, 2004.
5. **Becker, L.**, Cook, P.M., Wright, T.G. & Koschinsky, M.L.: Quantitative evaluation of the contribution of weak lysine-binding sites present within apolipoprotein(a) Kringle IV types 6-8 to Lp(a) assembly. *J Biol Chem.* **279**: 2679-2688, 2004.
6. **Becker, L.**, Nesheim, M.E. & Koschinsky, M.L.: Catalysis of Covalent Lp(a) Assembly: Evidence for an extracellular enzyme activity that enhances disulfide bond formation. *Biochemistry.* **45**, 9919-9928, 2006.

## D. Research Support

### Ongoing Research Support

AHA 10SDG3600027

Becker (PI)

07/01/2010-06/30/2014

Dysregulation of the macrophage sterol-responsive network promotes atherogenesis.

The goal of this project is to interrogate how coordinated MSRN protein expression influences macrophage lipid metabolism and the development of atherosclerosis.

ROLE: PI

UM1HL119073-01

Goss (PI)

07/01/2013-06/30/2016

Proof of Principle Evaluation of IV Gallium Nitrate in Patients with CF.

This project is a phase 2, randomized, placebo-controlled proof of principal study of IV gallium nitrate in adults with cystic fibrosis (CF). As PI of an ancillary study, my studies will focus on combining immunological, functional, and proteomic methods to investigate the effects of gallium on airway macrophages derived from study subjects.

ROLE: CO-INVESTIGATOR

R01 HL110879

Singh and Bruce (PI)

06/01/2012-05/31/2015

Investigating bacterial-host interactions driving CF Pulmonary Exacerbations.

The proposed research will test the hypothesis that at the onset of exacerbations in patients with cystic fibrosis, changes in the composition of infecting *P. aeruginosa* populations elicit host responses leading to lung inflammation and injury. As co-investigator, my research will focus on interrogating airway macrophage responses in patients during exacerbations.

ROLE: CO-INVESTIGATOR

P30 DK089507

Greenberg and Ramsey (PI)

07/01/2010-05/31/2013

Translational Research Center to Expedite Novel Therapies in Cystic Fibrosis.

This P30 Research Center will support both basic and clinical studies directed towards advancing new therapies to improve and prolong the lives of patients with cystic fibrosis. The overall goal of my Pilot and Feasibility proposal is to determine mechanisms by which CFTR deficiency modulate macrophage and dendritic cell protein expression patterns, function, and phenotype *in vitro* and *in vivo*.

ROLE: PI (Pilot and Feasibility) – *no cost extension*.

Res Med Foundation

Gozal and Becker (PI)

01/01/2012-12/31/2013

Urine-based biomarkers in adult sleep apnea.

The proposed research will map the urinary proteomes to functionally coherent networks to gain mechanistic insights in to the systemic effects of sleep apnea and develop the first phase of a robust diagnostic classifier in OSA, consisting of identification of putative candidate biomarkers. As co-PI, my research will focus on performing proteomics and bioinformatics analyses.

ROLE: CO-PI – *no cost extension*

P50 HL107160-01

Gozal (PI)

05/01/2011-04/30/2013

Validation of Urinary Biomarkers in Diagnosis of Pediatric OSA.

The proposed research will allow for development of a non-invasive, reliable and potentially cheap method for screening and diagnosis of obstructive sleep apnea (OSA) in children, and therefore facilitate timely treatment and prevention of OSA-associated morbidities. As co-investigator, my research will focus on performing proteomics and bioinformatics analyses.

ROLE: CO-INVESTIGATOR – *no cost extension*

### **Completed Research Support**

None

## BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.  
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME Marina Anatolievna Chekmareva		POSITION TITLE Assistant Professor of Pathology and Laboratory Medicine	
eRA COMMONS USER NAME			
EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)</i>			
INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	YEAR(s)	FIELD OF STUDY
Novosibirsk Medical School Krasniy Prospekt 52, Novosibirsk, Russia 630091	M.D. with honors	09/85-06/91	Internal Medicine
Institute of Therapy, Siberian Branch of the Russian Academy of Medical Sciences, Vladimirovskiy Spusk 2a Novosibirsk, Russia 630003	Residency	07/91-08/94	Cardiovascular Disease
Robert Wood Johnson University Hospital, UMDNJ New Brunswick, NJ 08901	Residency	07/02-06/06	Pathology, Combined AP/CP Program
Weill Medical College of Cornell University, New York, NY 10065	Fellowship	07/06-06/07	Gynecologic Pathology

### A. Personal Statement

I am a surgical pathologist, with special interest and extensive training in gynecologic pathology. I also have significant laboratory research experience, gained prior my years of pathology residency, in a series of projects to identify the biochemical pathways and genes responsible for prostate cancer metastasis. For the present project I will examine the specimens to characterize tissue/ tumor histology and identify areas of interest for microdissection, and consult on the optimization of immunohistochemistry assays for the project.

### B. Positions and Honors

#### Positions and Employment

- 04/12 - present Co-Director, Biospecimen Repository Service, Rutgers Cancer Institute of New Jersey, Rutgers, The State University of New Jersey, 195 Little Albany Street, New Brunswick, NJ 08901
- 04/11 - present Associate Director, Histopathology & Imaging Shared Resource, Rutgers Cancer Institute of New Jersey, Rutgers, The State University of New Jersey, 195 Little Albany Street, New Brunswick, NJ 08901
- 07/07 - present Assistant Professor, Department of Pathology and Laboratory Medicine, Robert Wood Johnson Medical School, Rutgers, The State University of New Jersey, One Robert Wood Johnson Place, New Brunswick, NJ 08901
- 10/94 - 06/02 Senior Research Technologist, Department of Surgery, Section of Urology, University of Chicago, 5841 S. Maryland Ave. Chicago, IL 60637

#### Professional Memberships

- 2006 - present United States and Canadian Academy of Pathology
- 2006 - present College of American Pathologists
- 2006 - present American Society for Clinical Pathology

## Honors

1999	Travel Award and Podium Presentation, Prouts Neck Prostate Cancer Conference, Prouts Neck, ME
1998	Roberta and Irving Lewis Fellowship for Junior Scientists, University of Chicago Urology "RESCUE" (RESearchCure and Education) Fund"
1996	Travel Award, American Urological Association, Conference for Advances in Cell Biology, Houston, TX
1996	CaP CURE Award, Association for the Cure of Cancer of the Prostate, American Urological Association, Annual Meeting, Orlando, FL
1986 - 1991	Scholarship for Excellence in Study, Novosibirsk Medical School, Novosibirsk, Russia

## **C. Selected Peer-Reviewed Publications** (in chronological order)

1. Clark R, Krishnan V, Schoof M, Rodriguez I, Theriault B, **Chekmareva M**, Rinker-Schaeffer C. Milky spots promote ovarian cancer metastatic colonization of peritoneal adipose in experimental models. *Am J Pathol.* 2013 Aug;183(2):576-91.
2. Jin F, Irshad S, Yu W, Belakavadi M, **Chekmareva M**, Ittmann MM, Abate-Shen C, Fondell JD. ERK and AKT signaling pathways promote MED1 overexpression in prostate cancer cells in association with elevated proliferation and tumorigenicity. *Mol Cancer Res.* 2013 Mar 28. [Epub ahead of print].
3. Musa F, Frey MK, Im HB, **Chekmareva M**, Ellenson LH, Holcomb K. Does the presence of adenomyosis and lymphovascular space invasion affect lymph node status in patients with endometrioid adenocarcinoma of the endometrium? *Am J Obstet Gynecol.* 2012 Nov;207(5):417.e1-6. doi: 10.1016/j.ajog.2012.06.051.
4. Miletti-González KE, Murphy K, Kumaran MN, Ravindranath AK, Wernyj RP, Kaur S, Miles GD, Lim E, Chan R, **Chekmareva M**, Heller DS, Foran D, Chen W, Reiss M, Bandera EV, Scotto K, Rodríguez-Rodríguez L. Identification of function for CD44 intracytoplasmic domain (CD44-ICD): modulation of matrix metalloproteinase 9 (MMP-9) transcription via novel promoter response element. *J Biol Chem.* 2012 Jun 1;287(23):18995-9007. doi: 10.1074/jbc.M111.318774.
5. Sterling ME, **Chekmareva MA**, Barone JG. Benign testicular enlargement due to diffuse interstitial fibrosis associated with cryptorchid testis in 11-month-old boy. *Urology.* 2012 Feb;79(2):440-2. Epub 2011 Sep 21.
6. **Chekmareva, MA**, Ellenson, LH, Pirog, EC. Immunohistochemical differences between mucinous and microglandular adenocarcinomas of the endometrium and benign endocervical endometrium. *Int J Gynecol Pathol.* 2008 Oct;27(4):547-54
7. Ang, DC, **Chekmareva, MA**, Ke, Y. A 14-Year-Old Girl With a Retroperitoneal Soft Tissue Mass. *Arch. Pathol. Lab. Med.* 2005 Aug;129(8):e176-8.
8. Jaeger EB, **Chekmareva MA**, Tennant TR, Luu HH, Hickson JA, Chen SL, Samant RS, Sokoloff MH, Rinker-Schaeffer CW. Inhibition of prostate cancer metastatic colonization by approximately 4.2 Mb of human chromosome 12. *Int. J Cancer.* 2004 Jan 1;108(1):15-22.
9. Kim HL, Vander Griend DJ, Yang X, Benson DA, Dubauskas Z, Yoshida BA, **Chekmareva MA**, Ichikawa Y, Sokoloff MH, Zhan P, Karrison T, Lin A, Stadler WM, Ichikawa T, Rubin MA, Rinker-Schaeffer CW. Mitogen-activated protein kinase kinase 4 metastasis suppressor gene expression is inversely related to histological pattern in advancing human prostatic cancers. *Cancer Res.* 2001 Apr 1;61(7):2833-7.
10. Christiano AP, Palmer JS, **Chekmareva MA**, Brendler CB. Duplicated seminal vesicle. *Urology* 1999 July; 54(1):162.

11. Yoshida BA, Dubauskas Z, **Chekmareva MA**, Christiano TR, Stadler WM, Rinker-Schaeffer CW. Mitogen-activated protein kinase kinase 4/stress-activated protein/Erk kinase 1(MKK4/SEK1), a prostate cancer metastasis suppressor gene encoded by human chromosome 17. *Cancer Res.* 1999 Nov. 1;59(21):5483-7.
12. **Chekmareva MA**, Kadkhodaian MM, Hollowell CM, Kim H, Yoshida BA, Luu HH, Stadler WM, Rinker-Schaeffer CW. Chromosome 17-mediated dormancy of AT6.1 prostate cancer micrometastases. *Cancer Res.* 1998 Nov 1;58(21):4963-9.
13. Yoshida BA, **Chekmareva MA**, Wharam JF, Kadkhodaian M, Stadler WM, Boyer A, Watabe K, Nelson JB, Rinker Schaeffer CW. Prostate cancer metastasis-suppressor genes: a current perspective. *In Vivo.* 1998 Jan-Feb;12(1):49-58 (Review)
14. **Chekmareva MA**, Hollowell CM, Smith RC, Davis EM, LeBeau MM, Rinker-Schaeffer CW. Localization of prostate cancer metastasis-suppressor activity on human chromosome 17. *Prostate* 1997 Dec 1;33(4):271-80.
15. Rinker-Schaeffer CW, **Chekmareva MA**, Mohler JL. The role of motility proteins and metastasis-suppressor genes in prostate cancer progression. *Stem Cells.* 1996 Sep;14(5):508-16 (Review).