CELL FUSION POTENTIATES TUMOR HETEROGENEITY THROUGH ACQUISITION OF MACROPHAGE BEHAVIOR

By

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ALCAM/CD66	Activated leukocyte-cell adhesion molecule
APC^{min}	Adenomatous polyposis coli multiple intestinal neoplasia
APP	Amyloid precursor protein
BMDC	Bone marrow-derived cell
BMP2	Bone morphogenetic protein 2
BMP4	Bone morphogenetic protein 4
BMT	Bone marrow transplant
BRAF	v-Raf murine sarcoma viral oncogene homolog B
CAMLS	Cancer-associated macrophage-like cells
CCL3	Chemokine (C-C motif) ligand 1
CCR1	C-C chemokine receptor type 1
CCR2	C-C chemokine receptor type 2
CD11b	Integrin alpha M
CD11c	Integrin alpha X
CD163	Cluster of differentiation 163
CD19	Cluster of differentiation 19
CD206	Cluster of differentiation 206
CD3	Cluster of differentiation 3
CD4	Cluster of differentiation 4
CD45	Cluster of differentiation 45
CD47	Integrin associated protein
CD68	Cluster of differentiation 68
CD8	Cluster of differentiation 8
CDX2	Caudal-type homeobox transcription factor 2
CEA	Carcinoembryonic antigen
cfDNA	Cell-free DNA
cGAMP	Cyclic guanosine monophosphate-adenosine monophosphate
CIN	Chromosomal instability
CK	Cytokeratin
CRC	Colorectal cancer
CRLM	Colorectal cancer liver metastasis
CSF1	Colony stimulating factor 1
CSF1R	Colony stimulating factor 1 receptor
CTC	Circulating tumor cell
CXCR4	C-X-C chemokine receptor type 4
DMEM	Dulbecco's modified eagle medium
DR6	Death receptor 6
DTC	Disseminated tumor cell
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
Edu	5-ethynyl-2'-deoxyuridine
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition

EpCAM	Epithelial cell adhesion molecule
F4/80	EGF-like module-containing mucin-like hormone receptor-like 1
FABP2/IFABP	Intestinal fatty acid binding protein
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FFPE	Formalin-fixed paraffin-embedded
FISH	Fluorescence in situ hybridization
FOLFOX	Folinic acid, 5-FU, and oxaliplatin
FUDR	Floxuridine
G-CSF	Granulocyte colony stimulating factor
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
GVHD	Graft-vs-host disease
H&E	Hematoxylin and eosin
H2B	Histone-2B
HAI	Hepatic arterial infusion
HDAC	Histone deacetylase
HER2	Human epidermal growth factor receptor 2
HIF	Hypoxia-inducible factor
HNSCC	Head ad neck squamous cell carcinoma
HSC	Hematopoietic stem cell
IFNγ	Interferon gamma
IGF1	Insulin-like growth factor 1
IHC	Immunohistochemistry
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-17	Interleukin 17
IL-4	Interleukin 4
iMC	Immature myeloid cell
JAG1	Jagged 1
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LV	Leukovorin
Ly6C	Lymphocyte antigen 6 complex, locus C1
Ly6G	Lymphocyte antigen 6 complex, locus G
ME	Microenvironment
MEMA	Microenvironment microarray
MHCII	Major histocompatibility complex class II
MMP	Matrix metalloproteinase
MMR	Mismatch repair
MMTV	Mouse mammary tumor virus
MSI	Microsatellite instability
MΦ	Macrophage
NK cell	Natural killer cell
NRAS	Neuroblastoma RAS viral oncogene homolog

OS	Overall survival
p53	Tumor protein p53
PanIN	Pancreatic intraepithelial neoplasia
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD-L1	Programmed death-ligand 1
PD-L2	Programmed death-ligand 2
PDAC	Pancreatic ductal adenocarcinoma
PEG	Polyethylene glycol
PFS	Progression-free survival
PlGF	Placental growth factor
PSA	Prostate-specific antigen
PTX	Paclitaxel
PyMT	Polyoma virus middle T antigen
RANKL	Receptor activator of NFκB ligand
RB1	Retinoblastoma protein
RFP	Red fluorescent protein
RFS	Recurrence-free survival
SCC	Squamous cell carcinoma
SDF1	Stromal cell-derived factor 1
STR	Short tandem repeat
TAM	Tumor-associated macrophage
TDSF	Tumor-derived stromal factor
TF	Tissue factor
TGFβ	Transforming growth factor beta
T_H17	T helper 17
T_H2	T helper 2
TIE2	Angiopoietin-1 receptor
TNFα	Tumor necrosis factor alpha
VCAM1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VEGFR1	Vascular endothelial growth factor receptor 1
VHL	von Hippel-Lindau
YFP	Yellow fluorescent protein
αCSF1	Neutralizing antibody to CSF1
β-gal	Beta-galactosidase

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Abstract

Metastatic spread of disease accounts for the overwhelming majority of cancer-related deaths, as current therapies are largely ineffective in treating this phase of cancer. This highlights our lack of understanding of disease progression surrounding metastatic spread of disease. Metastatic cancer cells acquire the ability to invade the surrounding environment, enter the blood stream and/or lymphatics, survive circulation, extravasate into the parenchyma of a distant organ, and proliferate as a metastatic lesion, yet the mechanisms underlying acquisition of these supporting phenotypes are not completely clear. A multitude of ways to gain metastatic potential exists, including acquired mutations and interactions with the surrounding microenvironment. Research presented in this dissertation establishes a mammary cancer model system to study metastatic disease, and investigates a novel mechanism by which cancer cells can acquire pro-metastatic phenotypes, specifically by fusion with macrophages. Speculation around cancer cell fusion permeates the literature, but there is little evidence that spontaneous fusion actually occurs or leads to biologically relevant phenotypes. This dissertation provides experimental evidence that cell fusion is a mechanism for acquisition of metastatic phenotypes, and also presents the identification of a novel prognostic circulating biomarker that may guide treatment regimens in patients.

Chapter I: Background and Introduction

Metastasis is the major cause of cancer-related deaths, due in part to the limited depth of knowledge of how cancer cells gain metastatic behaviors, leading to a dearth of effective therapeutics targeting this phase of disease. Although advances exist in identifying mechanisms that contribute to metastatic spread of disease, including direct acquisition of specific prometastatic phenotypes (Hanahan & Weinberg, 2011), there are certainly multiple, undiscovered mechanisms by which cancer cells gain metastatic properties. It is established that key multi-step processes must successfully occur for cancer cell progression along the metastatic cascade (Chaffer & Weinberg, 2011) (Figure 1.1). These events include primary tumor growth and local invasion into the surrounding microenvironment (ME), tumor cell intravasation (entry into blood circulation), survival of tumor cells in circulation, adhesion to the endothelium followed by tumor cell extravasation into distant organ sites and finally survival/growth within the metastatic ME.

While cancer cells acquire these key metastatic attributes, mechanisms promoting cancer cell progression along the metastatic cascade are not limited to the neoplastic cell within the tumor. Rather, numerous cell types and extracellular proteins both within the tumor and at distant sites heavily influence tumor progression. This includes, but is not limited to, immune cells, fibroblasts, blood vessels, lymphatics, collagens, proteoglycans, growth factors and cytokines. There are complex and dynamic interactions between these various cell types as well as their influences on cancer cells. This complexity ultimately results in variable tumor composition and cell phenotypes, making each tumor unique.

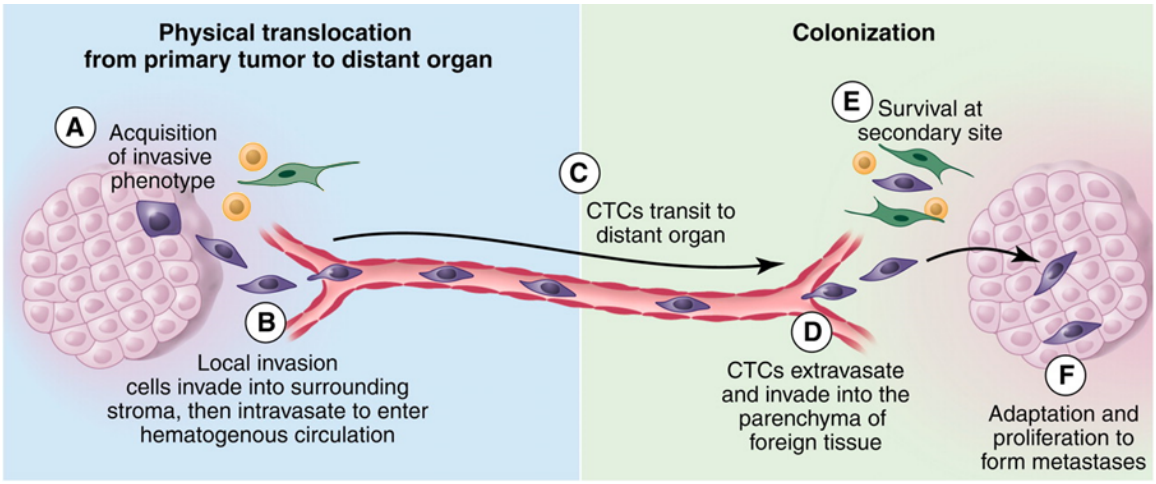

Figure 1.1. The metastatic cascade.
(A-B) Acquisition of an invasive phenotype allows for invasion and intravasation into circulation. (C) Cancer cells within circulation, termed circulating tumor cells (CTCs) survive in transit to distant organs. (D) Adhesion and extravasation into a distant organ parenchyma is followed by (E-F) survival and proliferation at a metastatic site. Adapted and reprinted from Science, 331, Chaffer et al., A Perspective on Cancer Cell Metastasis, 1559-1564, © 2011 with permission from The American Association for the Advancement of Science.

Regardless of this diversity, common themes within the tumor ME have been identified that support the “hallmarks of cancer” as shown in the Hanahan and Coussens comprehensive review of the tumor ME (Figure 1.2) (D. Hanahan & L. M. Coussens, 2012). Importantly, specific tumor cell types can have a multitude of functions that can contribute to tumor progression. The monocyte/macrophage (MΦ) lineage of immune cells, which is a focus of this dissertation, can promote angiogenesis, cancer cell invasion, evasion of the adaptive immune response, as well as anti-tumorigenic functions such as antigen presentation and phagocytosis of cancer cells (D. Hanahan & L. M. Coussens, 2012). The influence of MΦs on tumor progression is dynamic, interconnected, and can ultimately promote metastatic spread of cancer. Given that both cancer cell intrinsic properties promote tumor progression and MΦs influence metastatic spread of disease, a greater understanding of the interconnection between cancer cell intrinsic and extrinsic properties may reveal novel mechanisms underlying metastatic spread of disease.

Given the key phenotypes that contribute to progression along the metastatic cascade, mechanisms by which cancer cells acquire these phenotypes must be explored. Classically, attention has focused on cancer cells acquiring metastatic properties through mutations or through signals derived from the ME. It is now recognized that cancer cells can directly acquire protein and genetic components from supporting stromal cells or other cancer cells (Q. Chen et al., 2016; Pasquier et al., 2013; Zhao et al., 2016). Identification of small secreted vesicles, exosomes (Zhao et al., 2016), and gap junctions

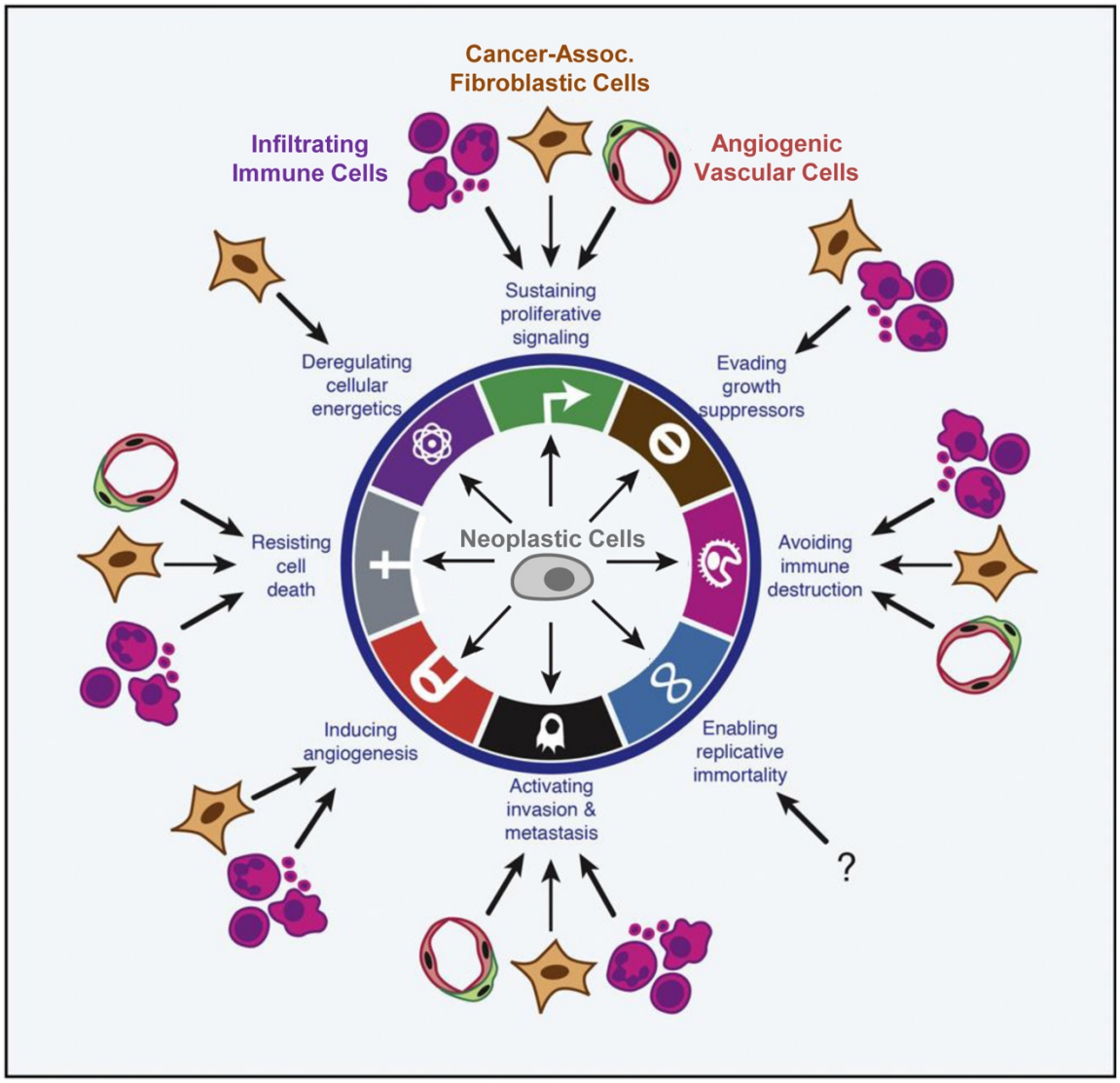

Figure 1.2. Influences on the "Hallmarks of Cancer".
The "Hallmarks of Cancer," originally described in Hanahan and Weinberg, 2011, have been shown to be closely associated with various cells within the distant and local tumor microenvironment. In addition to neoplastic cells directly contributing to the hallmarks of cancer, Hanahan and Coussens depict three broad classes of stromal cell types that have multiple and variable influences in tumor progression, with diverse compositions and functions depending on cancer cell type or organ location. Adapted and reprinted from Cancer Cell, 21, Hanahan et al., Accessories to the Crime: Functions of Cells Recruited to the Tumor Microenvironment, 309-322, © 2012, with permission from Elsevier.

(Q. Chen et al., 2016) provide examples of direct cell-cell communication that promote acquisition of factors that impact tumor progression. While these mechanisms have opened up new areas of study, one intriguing possible mechanism for acquisition of protein and/or genetic material is direct cell-cell fusion

Cancer cell-cell fusion as a mechanism for increased ploidy and tumorigenesis is a hypothesis that is over a century old (Lu & Kang, 2009), and multiple reviews or a limited number of case reports suggest cell fusion contributing to tumor progression. However, this mechanism has not been validated and its functional contribution to tumor progression has remained unexplored. Data supporting neoplastic cell-stromal cell fusion-derived hybrid cells, including from our laboratory, have led to the hypothesis that fusion underlies a mechanism for acquisition of new phenotypic properties that contribute to tumor progression (Harris, Miller, Klein, Worst, & Tachibana, 1969; A. E. Powell et al., 2011). The relevance of cell fusion remains controversial, as neoplastic cell fusion could result in non-neoplastic cell hybrids, where hybrid cells may reacquire tumor suppressor function, ameliorating the neoplastic drive from loss of tumor suppressor function. Therefore, understanding the relevance of *in vivo* cancer cell-cell fusion, as it relates to acquired pro-metastatic properties, has exciting potential to open new areas for development of treatment regimens or diagnostic/prognostic tools for cancer patients.

Tumor progression

Tumor cell intrinsic properties, as well as microenvironmental influences from the monocyte/ MΦ lineage, play integrated roles in the spread of cancer along the metastatic cascade (Figure 1.1).

Growth in the primary tumor setting

Primary tumor growth, represents the initial step of tumor progression. One of the classic hallmarks of cancer is the tumor's ability for sustained proliferation (Hanahan & Weinberg, 2011) (Figure 1.2). Non-neoplastic cells have safeguards to maintain homeostatic levels of proliferation, whereas cancer cells tip the balance in numerous ways including autocrine signaling, gain of growth factor-independence, and interaction with infiltrating immune cells (D. Hanahan & L. M. Coussens, 2012; Hanahan & Weinberg, 2011). Production of growth factors that act via autocrine signaling, is exemplified in small cell lung cancer producing Insulin-like growth factor-I (IGF-1) that induces proliferation through stimulation of its own IGF-1 receptor (Nakanishi et al., 1988). Similarly, proliferation can result from constitutive activation, or overexpression of receptor proteins that stimulate proliferation in the absence or relatively low concentrations of ligand respectively. A subset of breast cancer cells demonstrates this by overexpressing human epidermal growth factor receptor 2 (HER2), resulting in increased activation of mitogenic pathways (Harari & Yarden, 2000). Aberrant activation of downstream signaling pathways in the absence of growth factor stimulation can similarly sustain a proliferative signal. In addition to aberrant proliferation, cancer cells also bypass tumor suppressor signals that function to inhibit cell death and

suppress growth. The classic tumor suppressor p53, plays important roles as a gatekeeper for the cell cycle and evasion of apoptosis. In addition to playing a key role in carcinogenesis and primary tumor growth, driver mutations such as p53 can also alter cancer cells favoring pro-metastatic phenotypes (E. Powell, Piwnica-Worms, & Piwnica-Worms, 2014).

Recently, the immune system has been shown as a critical regulator of primary tumor growth (D. Hanahan & L. M. Coussens, 2012). While the adaptive immune system can recognize tumor-specific antigen to form an immune response against the neoplastic cells, chronic inflammation often results in an immune suppressive environment favorable for cancer cell survival. Neoplastic cells can express proteins that allow for evasion of the immune system such as programmed death-ligand 1 (PD-L1) or CD47 (Iwai et al., 2002; Poels et al., 1986; Van Niekerk, Ramaekers, Hanselaar, Aldeweireldt, & Poels, 1993). PD-L1 can be expressed on antigen-presenting immune cells as well as on numerous types of cancer cells, induced either in response to cytokines [e.g. interferon gamma (IFNγ)], or by oncogene-driven expression (Lastwika et al., 2016). PD-L1 suppresses an anti-tumor $CD8^+$ T cell response and is now recognized as an important component of tumor growth (L. Chen & Han, 2015; Lastwika et al., 2016). Highly effective treatment regimens targeting this pathway highlight the importance of this immune component to tumor progression (Postow et al., 2015). CD47, while having a physiological role in MΦ- MΦ cell fusion, also can be expressed on epithelial cells where it acts as an anti-phagocytic or self-tolerance signal on cancer cells (Han et al., 2000; Tseng et al., 2013). Inhibition

of CD47 can promote MΦ phagocytosis of cancer cells and induce an anti-tumor T cell response. With evasion of the anti-tumor immune response, neoplastic cells are capable of surviving in a complex environment, with the potential to progress along the metastatic cascade.

Additional cancer cell intrinsic mechanisms that promote tumor growth include the production of growth factors and cytokines that alter its ME, which in turn can promote tumor growth. From production of vascular endothelial growth factor (VEGF) that can stimulate angiogenesis to expression of colony stimulating factor 1 (CSF1) to recruit immune suppressive tumor-associated macrophages (TAMs), cancer cell production of cytokines and growth factors is vital to primary tumor growth (DeNardo et al., 2009; DeNardo et al., 2011; Fan et al., 2005; Ruffell et al., 2014). Thus, cancer cell intrinsic phenotypes are intertwined with microenvironmental influences that promote primary tumor growth.

Myeloid cell relevance and diversity in solid tumors

Of the microenvironmental influences on primary tumor growth, the monocyte/MΦ population has been studied extensively with respect to its pro-tumorigenic properties (Noy & Pollard, 2014). MΦs are abundant in primary tumors and correlate with worse prognosis for certain cancers (Campbell et al., 2011; DeNardo et al., 2011). While defining this population with cell surface markers like CD68 or F4/80, it is important to note that this myeloid population is quite diverse. Heterogeneity is in part, established by (1) cytokines, growth factors, and immune complexes that alter MΦ polarization states, (2) the location

of MΦs within the solid tumor, (3) the MΦ differentiation state, and (4) the developmental origin of different MΦ populations. These factors result in specialized subsets of monocytes and MΦs that have either variable or redundant influences on solid tumor progression.

Growth factors and cytokines contribute to MΦ heterogeneity

The monocyte/MΦ population exhibits certain phenotypes following exposure to external stimuli, including growth factors and cytokines, where they can enter a pro-tumorigenic state that has been termed more M2-like, in contrast to the pro-inflammatory M1-like state (Gordon & Mantovani, 2011). It should be noted however that within the M2-like polarization state, different functionally and molecularly-defined myeloid subsets can be identified within unique tumor MEs (Movahedi et al., 2010). After exposure to T_H2 cytokines such as IL-4 and IL-13, MΦs can produce IL-10, which suppress a $CD8^+$ T cell response through repression of IL-12 expression by dendritic cells (Ruffell et al., 2014). T_H2 cytokines are not the only signal that influence MΦ function as immunoglobulin-containing immune complexes from B cells have been shown to promote a pro-tumor ME through FcγR-expressing myeloid cells in both squamous cell carcinoma and pancreatic ductal adenocarcinoma (Affara et al., 2014; Gunderson et al., 2016). While MΦs can affect T cell response in specific ways, including expression of Arginase I (Rodriguez et al., 2004) and PD-L1 (Loke & Allison, 2003) that both result in inhibition of T cell receptor signaling, the immunosuppressive mechanisms of MΦs are quite extensive and affect various other immune components. These include direct inhibition of natural killer cell

(NK cell) lytic function (Borrego, Ulbrecht, Weiss, Coligan, & Brooks, 1998) as well as recruitment of regulatory T cells (Curiel et al., 2004; Liu et al., 2011). These varied phenotypes can be strongly influenced by the presence of various growth factors, including GM-CSF and CSF1 which can not only influence myeloid proliferation and recruitment, but also drive MΦ expression to a more M1- and M2-like signature respectively (Fleetwood, Lawrence, Hamilton, & Cook, 2007).

MΦs also support primary tumor angiogenesis and tumor progression by production of VEGF (E. Y. Lin et al., 2007; E. Y. Lin & Pollard, 2007) which can be induced by exposure to the cytokine stromal cell-derived factor 1 (SDF1) (Sanchez-Martin et al., 2011). In mice depleted of MΦs, tumor progression was restored when regulated expression of VEGF was introduced in mammary epithelial cells (E. Y. Lin et al., 2007). Thus, a link between developing supportive vasculature and presence of VEGF-producing MΦs was made with primary tumor development and progression.

Location within the tumor contributes to MΦ heterogeneity

In addition to heterogeneity of the monocyte/MΦ population driven by various cytokines and growth factors, the specific location within a solid tumor can also influence MΦ function. One considerable factor that exemplifies the importance of location influencing MΦ phenotype is proximity to vasculature; where hypoxia may play a critical role (Casazza et al., 2013). Casazza and colleagues determined that neuropilin-1 expression on MΦs was important for trafficking to hypoxic regions to promote a pro-tumorigenic phenotype. Gene

deletion of neuropilin-1 in MΦs resulted in localization of MΦs in normoxic regions where they displayed a more M1-like phenotype. MΦs are also commonly identified at the invasive tumor margin and density of this population in certain cancers is associated with improved survival and decreased metastasis, indicating these MΦ may foster an anti-tumorigenic phenotype (Forssell et al., 2007; Zhou et al., 2010). These studies indicate that location of the MΦ population can be closely associated with, or promote, specific phenotypes.

Differentiation state contributes to MΦ heterogeneity

While differentiated MΦs are identified in solid tumors through specific markers, other myeloid populations lacking these markers can also be identified within the tumor parenchyma, indicating a more “immature” myeloid or monocytic differentiation state. It is likely these populations in the tumor are not merely bystanders destined for further differentiation into a MΦ or dendritic cell, as monocytes can express many immune suppressive enzymes, including nitric oxide synthase that generate nitric oxide (Movahedi et al., 2008). Importantly, monocyte populations have also been identified to reduce metastasis, as enriched monocyte populations in the lung promote NK cell recruitment and activation (R. N. Hanna et al., 2015). Thus, various differentiation states of the monocyte/MΦ lineage can have a number of different influences on tumor progression.

Site of developmental origin contributes to MΦ heterogeneity

MΦ heterogeneity is also evident by identifying various subpopulations with different developmental origins. While monocyte/MΦ populations are

recruited to a tumor ME from the bone marrow, various organ sites contain tissue resident MΦs, including the liver, lung and brain, where MΦs were established at distant organ sites early in embryogenesis followed by self-maintaining proliferation at the organ site (Hashimoto et al., 2013; Yona et al., 2013). One example of a tissue resident MΦ influencing tumor progression is the alveolar MΦ within the lung, where this population can suppress a T_H1 immune response, facilitating lung metastasis (Sharma et al., 2015).

Collectively, cytokines, growth factors, location within the solid tumor, differentiation state, and site of origin, contribute to the heterogeneity of the tumor-associated monocyte/MΦ population. This complexity must be recognized when identifying conditions and myeloid populations that may participate in cell fusion, as discussed in Chapters II and III. Additionally, a comprehensive understanding of the various MΦ populations is necessary when considering therapeutic strategies targeting MΦ function. For example, inhibition of the CSF1/CSF1R axis may have variable influences with respect to bone marrow-derived myeloid cell migration or recruitment into a solid tumor, polarization state, and resident MΦ proliferative capacity. Ruffell and Coussens highlight the importance of these approaches as numerous clinical trials have been implemented with the rationale of targeting MΦ recruitment, polarization, function and activation, based on efficacious results from preclinical models (Ruffell & Coussens, 2015). An additional component to understanding MΦ influence on tumor progression and implementing MΦ-targeted therapies, is determining MΦ influence on tumor progression in the context of administering standard of care

therapeutic regimens like chemotherapy and radiation therapy. MΦ polarization toward a more immune-suppressive phenotype was identified following radiation therapy and MΦ depletion in combination with radiation therapy delayed tumor growth (Shiao et al., 2015). Further, the chemotherapeutic Paclitaxel (PTX) increases expression of CSF1 in neoplastic mammary cancer cells and induces tumor infiltration of MΦs (DeNardo et al., 2011). DeNardo and colleagues further went on to demonstrate that combined neoadjuvant MΦ inhibition with PTX reduced primary tumor growth, inducing an anti-tumor T cell response and inhibited metastasis (DeNardo et al., 2011). These studies have important biological and translational implications as MΦ-targeted therapeutics in patients are, and will continue to be, integrated with standard of care treatment regimens

Together, these MΦ extrinsic mechanisms, in concert with neoplastic cell intrinsic mechanisms, play important roles in supporting primary tumor growth, which is necessary for cancer progression along the metastatic cascade. With enhanced proliferation, evasion of growth suppressive, apoptotic, and/or senescence signals, cancer cells can invade into the surrounding environment, a next step to promote spread of disease to distant sites.

Invasion and intravasation

An epithelial cancer that has not invaded through the basement membrane into the surrounding stroma is considered carcinoma *in situ*. Prognosis for carcinoma *in situ* is generally favorable. For example, ductal carcinoma *in situ* of the breast has a disease-free survival at 10 years of 83 to 94

percent depending on pathologic classification (Di Saverio et al., 2008). This association underlies an effort to improve methods for early detection and highlights the importance of understanding how a cancer cell becomes invasive either through intrinsic mechanisms or through interactions with stromal cells such as MΦs.

Neoplastic cell dispersal contributes not only to promoting intratumoral heterogeneity (Waclaw et al., 2015), but also allows for relocation of a neoplastic cell toward vasculature, allowing for subsequent intravasation into the blood stream. This process requires neoplastic cell motility, where reorganization of the actin cytoskeleton is regulated by Rho family small GTPases (Yamazaki, Kurisu, & Takenawa, 2005). This motile phenotype then allows for directed movement toward a given stimulus. Chemotaxis and haptotaxis are processes by which directed movement is facilitated by soluble or substrate-bound chemoattractants respectively. Yet in order for a neoplastic cell to become invasive, it must also pair ECM proteolytic activity with directed movement toward a given stimulus.

Cancer cells can gain an invasive phenotype through conversion to a more mesenchymal-like cell (Parri & Chiarugi, 2010). This alteration in phenotype is termed epithelial-mesenchymal transition (EMT) and is defined by loss of epithelial cell phenotype (e.g. down-regulation of E-cadherin responsible for stable epithelial cell-cell contacts between epithelial cells) and acquisition of mesenchymal phenotypes [e.g. expression of matrix metalloproteases (MMPs) that allow for matrix degradation and movement into the extracellular matrix (ECM)] (Sahai, 2005). Two key functions acquired during EMT are the cell's

response to chemokine gradients for directional movement and the ability to degrade ECM.

One of these chemokine gradients involved in cancer cell invasion is SDF1 (Balkwill, 2004b). CXCR4 is a receptor that binds to the ligand SDF1 and promotes EMT (Hu et al., 2014). While the SDF1/CXCR4 signaling axis plays important roles under homeostatic conditions such as neuron migration (Zhu, Matsumoto, Mikami, Nagasawa, & Murakami, 2009) and regulation of monocyte-MΦ differentiation (Sanchez-Martin et al., 2011), it is commonly expressed on numerous cancer cell types (Balkwill, 2004a) and the pathway is co-opted in disease, as cancer cells migrate toward SDF1 (Balkwill, 2004b). One approach to assess directed migration of a cell toward a specific ligand *in vitro* is to use a Boyden chamber that allows tracking of cultured cells across a chemokine gradient. Both directed migration (chemotaxis) alone and chemotaxis paired with matrix degradation (invasion) can be assessed with or without the addition of matrigel to the Boyden chamber respectively. Using the invasion assay, ovarian cancer cell lines expressing CXCR4 invaded toward SDF1 (Scotton et al., 2002). Directed migration of cancer cells toward SDF1 has been demonstrated extensively *in vitro*. Correspondingly, *in vivo* studies showed that cells transfected with CXCR4 resulted in larger tumors, muscle invasion, and increased metastasis (Balkwill, 2004b) supporting that migration toward SDF1 may be a key component explaining how CXCR4 promotes metastasis. While the mechanisms underlying how a cancer cell can acquire increased SDF1/CXCR4 signaling are diverse, including upregulation of CXCR4 by VEGF or TGFβ, there

is a clear role played by this pathway in promoting a more invasive/migratory phenotype (Balkwill, 2004b).

An additional example of chemokine/receptor signaling linked to EMT and promoting invasion is the CSF1/colony stimulating factor 1 receptor (CSF1R) axis. CSF1R is expressed on monocytes and MΦs and is thought to play a significant role in cancer cell movement and metastasis through paracrine interactions between the neoplastic cell and the MΦ (DeNardo et al., 2009). On the other hand cancer cells can also express the CSF1R (Filderman, Bruckner, Kacinski, Deng, & Remold, 1992; Ide et al., 2002; Kacinski et al., 1991), supporting autocrine signaling for its survival (Cioce et al., 2014). John Condeelis and colleagues determined that cancer cell expression of CSF1R promotes invasion in an *in vivo* system and that ablation of cancer cell-specific CSF1R reduced the number of circulating tumor cells (CTCs) and spontaneous metastasis (Patsialou et al., 2015).

Following invasion into the surrounding ME, cancer cells progress along the metastatic cascade by intravasating into circulation. Intravasation is the process by which cancer cells invade across either the endothelium and/or lymphatics into circulation. This can occur by paracellular movement between endothelial cell junctions. While factors produced locally by cancer cells such as transforming growth factor β (TGFβ) or VEGF can reduce endothelial barrier function (Reymond, d'Agua, & Ridley, 2013), intravasation has been difficult to study *in vivo*. Recently, intravital imaging has provided insight into these mechanisms and has identified stromal cells, such as MΦs associated with the

vasculature as a major component in facilitating cancer cell intravasation (Wyckoff et al., 2004).

MΦ have been shown to heavily influence invasion and intravasation of neoplastic cells (Noy & Pollard, 2014). Like neoplastic cells, MΦs are also shown to express MMPs and their expression of MMP2 has been implicated in promoting epithelial cell migration (Giannelli, Falk-Marzillier, Schiraldi, Stetler-Stevenson, & Quaranta, 1997). An additional MΦ-associated mechanism for invasion is through secretion of chemotactic growth factors. For example, CSF1 production by the neoplastic cell and MΦ production of epidermal growth factor (EGF) establish a cancer cell-MΦ paracrine loop to promote cancer cell invasion (DeNardo et al., 2009; Wyckoff et al., 2004). A stream of alternating MΦs and cancer cells moving toward vasculature driven by this relayed CSF1-EGF paracrine loop was visualized with intravital microscopy and *in vitro* studies (Dovas, Patsialou, Harney, Condeelis, & Cox, 2013). While this invasive event can be inhibited by blocking the function of either the EGF or CSF1 receptors, the precise mechanism of invasion may be variable as some cancer cells can also express the CSF1R (Kacinski et al., 1991; Patsialou et al., 2015). Thus, the complex interaction between cancer cells and MΦs may use a combination of both autocrine and paracrine signaling.

MΦs have also been shown to play a role in promoting cancer cell intravasation. The presence of Angiopoietin-1 receptor (TIE2)-expressing MΦs adjacent to endothelial cells and breast cancer cells in patient primary tumor biopsies was correlated with metastasis (Harney et al., 2015). These TIE2-

expressing MΦs promote transient vascular permeability that permits tumor cell intravasation. Thus, as with cancer cells, MΦs within a primary tumor have variable phenotypes and multiple mechanisms to promote metastatic invasion and entry into circulation.

Together, cancer cells undergo invasion and intravasation either directly by expressing numerous proteins such as the CSF1R or by support from the ME. Of the components of the primary tumor ME, MΦs play a crucial role in the invasion and intravasation steps along the metastatic cascade. Thus, an integrated understanding of both tumor cell-intrinsic function and MΦ-specific influences will lead to a more comprehensive understanding into the mechanisms of neoplastic cell invasion.

Circulating tumor cells

Entry into circulation provides a conduit for neoplastic cells to rapidly spread to various organ sites. In the blood, CTCs must overcome multiple challenges, including reverting to a more migratory phenotype, surviving in circulation, and evading the immune system to successfully navigate to a metastatic site. A subset of cancer cells undergo apoptosis within circulation (Kallergi et al., 2013; Larson et al., 2004; Rossi et al., 2010; Smerage et al., 2013). Yet cancer cell survival within circulation does not represent a bottleneck with respect to progression along the metastatic cascade (Cameron et al., 2000; Luzzi et al., 1998), as survival in circulation allows for efficient spread to distant metastatic sites. Importantly, the presence of these cells in circulation strongly

correlates with progression-free and overall survival in a number of different cancer patients, including those with breast cancer (Cristofanilli et al., 2004). CTC correlation with survival is observed in a limited number of other cancers, including colorectal and prostate cancer (S. J. Cohen et al., 2008; de Bono et al., 2008). For prostate cancer specifically, CTC numbers post-treatment better predicted overall survival than decreases in prostate-specific antigen (PSA), a commonly used FDA-approved test. This indicates a stronger association between CTCs and prognosis, however it relies on identifying cells expressing specific cancer-related epithelial markers. Accepted detection assays use the identification of cytokeratin (CK) and/or epithelial cell adhesion molecule (EpCAM) as a marker for the epithelial cancer cell. Further, these assays stipulate that CTCs must not express markers of circulating leukocytes (i.e. the pan-leukocyte marker CD45), as this is viewed as artifact. Alternative assays for CTCs are based on size, density, or charge (C. Alix-Panabieres & K. Pantel, 2014). These restrictions result in biased subsets of CTCs and ignore other, possibly more biologically relevant subpopulations. For example, CellSearch® is currently an FDA-approved test to detect CTCs by magnetic separation of $EpCAM^+$ cells followed by positive staining for CK and negative staining for CD45. Yet Dario Marchetti and colleagues have reported the high metastatic capability in mouse xenografts of an $EpCAM^-$ CTC population isolated from breast cancer patients (L. Zhang et al., 2013). These assays do not account for the possibility that CTCs gain mesenchymal features and may express CD45.

EMT is a process by which cancer cells reduce expression of epithelial proteins to take on more mesenchymal and migratory behaviors. These cells do not express EpCAM and thus can be missed when identifying cells based on positive selection of a specific marker (Gorges et al., 2012). CTCs that have undergone EMT acquire phenotypes that drive metastasis. For example, the coagulation factor, tissue factor (TF) was identified in the majority of CK^+ CTCs in metastatic breast cancer patients as well as in EMT-positive cancer cell lines (Bourcy et al., 2016). EMT-positive cells displayed enhanced seeding and survival in mouse lungs and this was abrogated by inhibition of TF expression in the cancer cells. While coagulation factors are not conclusively linked to early metastasis, CTCs that have acquired EMT-associated pro-metastatic phenotypes may be excluded by current CTC detection methodologies, including CellSearch®. Thus, current biased approaches for CTC detection limits analysis to a subpopulation of CTCs and may exclude those with biologic relevance to disease progression. I will explore a novel CTC population (CK^+CD45^+) derived by MΦ-cancer cell fusion in Chapter III.

Detection of CTCs expressing CD45 has been reported by three groups (Clawson et al., 2012; M. B. Lustberg et al., 2014; Sheng et al., 2014). In addition, it was reported that some CTCs also expressed the MΦ marker CD68 (Clawson et al., 2015). However, from these brief descriptions it is unclear if these cells were associated with cancer or if they were detected in healthy controls. Additionally, it is unknown how these cancer cells acquired CD45 or CD68 expression. Importantly, studies identifying this double-positive population

by flow cytometry did not rule out the possibility that this reported cell population was actually neoplastic cell-immune cell clusters. The relationship of circulating MΦs and CTCs is relatively unexplored. Recent reports describe CTCs and MΦs as circulating cell clusters, however the biological relevance of these clusters is still unknown (Sarioglu et al., 2015). While rare, relative to single CTCs, oligoclonal clusters of CTCs have been shown to have increased metastatic potential, however their contribution to tumor progression is not known (Aceto et al., 2014). In addition, reports describing circulating cancer-associated macrophage-like cells or CAMLS bound to CTCs in cancer patients but not in healthy patient blood describe a novel population that express both MΦ and epithelial markers and contain multiple nuclei (Adams et al., 2014). Despite this, the identification of a novel CTC population may be critical for understanding underlying biology of metastatic spread of disease.

Extravasation and growth at a distant site

One of the bottlenecks of productive metastatic cell growth is the neoplastic cell's transition from circulation to colonization (i.e. by extravasation), formation of micrometastases, and successful outgrowth. This is evident by the disproportionate number of detectable CTCs compared to the infrequent number of overt metastatic foci (Nagrath et al., 2007). Murine models of experimental metastasis support this clinical data, as a high percentage of cells injected directly into circulation die within days and do not form metastases (Minn et al., 2005). While the process is clearly inefficient, it is important to identify cancer cell

intrinsic and extrinsic mechanisms by which subpopulations possess the ability to effectively adhere, extravasate, and proliferate to form overt metastasis.

An initial important step for cancer cell seeding at a distant site is its ability to adhere to the endothelium and initiate the process of extravasation into the distant organ parenchyma. Vascular permeability and transendothelial migration, similar to leukocyte extravasation, are primary mechanisms by which cancer cells cross the endothelial layer. In addition, recent reports identified direct contact between cancer cells and endothelial cells facilitated by amyloid precursor protein (APP) and its ligand death receptor 6 (DR6) to induce endothelial necroptosis (a regulated form of necrosis) and promote subsequent seeding of cancer cells at metastatic sites (Strilic et al., 2016). It is possible endothelial cell death provides cancer cells direct access to the distant organ stroma or downstream components of endothelial cell death could promote metastasis by altering tumor cell or surrounding endothelial cell phenotypes. Once a cancer has accessed the stroma, it must adhere and survive in order to become a clinically detectible metastatic lesion.

Matrix remodeling activity in cancer cells can play a role in cancer-cell adhesion at a metastatic site. Ovarian cancer cells cleave ECM proteins at the metastatic site, allowing for increased cancer cell adhesion and seeding (Kenny, Kaur, Coussens, & Lengyel, 2008). Pretreatment of cancer cells with a protease inhibitor reduced metastases and improved survival in mice, indicating matrix remodeling by cancer cells can be important for adhesion and establishing metastases.

Cancer cells that colonize metastatic sites enter a dormant state and can exist in the distant organ parenchyma without being grossly detectible. This is consistent with the observation that patients can form detectible metastases years after surgical resection of the primary tumor. This latent state in tumor progression can be explained by a cancer cell remaining in a quiescent state and/or remaining in an immunogenic/angiogenic homeostatic balance (Sosa, Bragado, & Aguirre-Ghiso, 2014). Little is known about what cancer sub-clones are involved in this latent state, the ME that supports dormancy, or how cancer cells are stimulated into a proliferative state to produce overt metastases. There is evidence that the immune response may be a key factor in this process. Organ transplant studies from donors who were previously “cured” have shown that donor-derived tumors can arise in the immunosuppressed recipient. This indicates latent metastatic cancer cells from the organ donor may be held in check by the immune system and that disruption of the immune response may support metastatic outgrowth (Collignon, Holland, & Feng, 2004; Strauss & Thomas, 2010). Thus, the immune ME is a key component of metastatic growth.

The seed and soil hypothesis (Fokas, Engenhart-Cabillic, Daniilidis, Rose, & An, 2007), where cancer cells spread to a specific ME that is permissive for cancer cell survival and growth, led to studies that identified monocytes and MΦs can promote cancer cell extravasation and survival. Myeloid cells have been shown to mobilize to distant organs sites and are detected prior to arrival of neoplastic cells (Sceneay, Smyth, & Moller, 2013). The first description of this established ME, termed the pre-metastatic niche, was by detection of tumor-

derived stromal factors such as VEGF and placental growth factor (PlGF) which promoted vascular endothelial growth factor receptor (VEGFR1)$^{+}$ hematopoietic cell recruitment to distant organs and metastasis (Kaplan et al., 2005). While, controversy remains around whether or not these pre-metastatic niches are necessary for metastasis, many groups have identified tumor-derived stromal factor (TDSF)-mediated hematopoietic cell trafficking to distant organs to promote metastases in multiple cancer models (Sceneay et al., 2013). Further, studies have demonstrated that monocyte/MΦ depletion in mice prior to injection of cancer cells into circulation resulted in a significant decrease in the percentage of cancer cells identified in the lung extravascular space and development of fewer metastatic foci (B. Qian et al., 2009). It was further identified that CCR2^{+} monocytes can be recruited to the lung and promote pulmonary seeding of cancer cells in part by producing VEGF (B. Z. Qian et al., 2011). MΦ differentiated from CCR2^{+} monocytes express CCL3 and when this ligand, or its receptor CCR1 was deleted in MΦs, a reduced retention of MΦs at the metastatic site and reduced number of metastatic foci was found (Kitamura et al., 2015).

Following extravasation into a distant organ parenchyma, MΦs support cancer cell survival within the metastatic ME. MΦs expressing α4-integrin can promote pro-survival signals through vascular cell adhesion molecule 1 (VCAM1) signaling in cancer cells in a juxtacrine fashion (Q. Chen, Zhang, & Massague, 2011). While there are likely a multitude of additional roles MΦs play at metastatic sites such as creating an immune suppressive ME similar to primary tumors, these mechanisms support the notion that metastatic seeding and

growth of cancer cells at a metastatic site is propagated by the monocyte/MΦ population.

The biological intricacies of cancer complicate understanding tumor progression along the metastatic cascade. One approach to understanding the metastatic process is by determining **how** specific phenotypes are acquired. This approach is highlighted in Chapter III of this dissertation.

Mechanisms underlying acquisition of metastatic phenotypes

It is widely accepted that progression along the metastatic cascade is regulated by alterations in genetic and/or epigenetic properties in cancer cells, enabling acquisition of key behaviors. Certainly, selection of advantageous behaviors drive the process (Greaves & Maley, 2012). Investigation of tumor phylogenetics and the evolutionary history of cancer in patients have identified cancer cell sub-clones within primary tumors that share genetic characteristics with their corresponding metastases, supporting the notion of tumor cell evolution with respect to progression along the metastatic cascade (Naxerova & Jain, 2015). Acquisition of small incremental phenotypes that promote metastasis in a linear, step-wise manner, may underlie the process. However, it is also possible that very few alterations are needed for metastatic spread of disease. Relatively rapid alterations in a specific genotype/phenotype may influence a large number of processes that collectively contribute to progression of disease. One hypothesis, the focus of this dissertation, is that cancer cell fusion with a cell type that shares similar functions as a metastatic cell, underlies the rapid acquisition of metastatic properties. These mechanisms are likely not mutually exclusive.

Genetic mechanisms of acquired phenotypes

One mechanism for sequential acquisition of genetic alterations derives from the two-hit hypothesis. This posits that cancer develops as a result of sequential genetic mutations and was originally proposed by Carl Nordling in 1953 (Nordling, 1953). Evidence supporting this hypothesis includes heterozygous mutations in tumor suppressor genes such as retinoblastoma

protein (RB1), where tumors developed with acquired somatic mutations in the wild type copy, effectively inducing cancer through “two hits” (Knudson, 2001). The same hypothesis can apply to acquisition of metastatic properties where genetic or epigenetic alterations incrementally contribute to individual stages of metastasis. There may also be a link between acquired driver mutations that promote carcinogenesis and metastasis as well. One supporting observation identifies discrete somatic mutations associated with different colorectal cancer metastatic locations (Lipsyc & Yaeger, 2015; Yaeger et al., 2015). *KRAS* mutations were associated with lung, brain and bone metastasis, whereas *BRAF* mutations were associated with peritoneal and lymph node metastasis. Further, loss of p53 has also been shown to promote expression of genes involved in EMT and may promote acquisition of pro-metastatic phenotypes (E. Powell et al., 2014). Finally, mutations in the von Hippel-Lindau (VHL) tumor suppressor gene upregulate CXCR4 (Staller et al., 2003), a receptor that is regulated by hypoxia-inducible factor (HIF) and wild-type VHL (Schioppa et al., 2003). While the mechanisms of metastasis are likely a combination of acquired neoplastic cell behaviors and their interplay with its surrounding ME, it is clear that genetic mutations can influence metastasis.

Epigenetic mechanisms of acquired phenotypes

A second mechanism for acquisition of genetic alterations that promote metastasis is altered epigenetic regulation of cell function, including methylation of DNA, modification of histones, and microRNAs, all of which have been shown to be dysregulated in cancer (Esteller, 2006). Dysregulated epigenetic

mechanisms are implicated in carcinogenesis, where hypermethylation of tumor suppressors promotes cancer initiation. Further, alterations in epigenetic regulation that affect genes and pathways can promote metastatic spread of disease (Lujambio & Esteller, 2009). For example, TGFβ-mediated regulation of EMT in renal epithelial cells was found to be affected by histone deacetylase (HDAC) inhibition (Heerboth et al., 2015). HDACs are linked with regulation of cancer cell invasion and seeding distant organ sites (Tang et al., 2015). Further, dysregulation of epigenetic regulators that directly promotes metastasis has also been uncovered. One example is by over-expression of a chromatin remodeling non-coding RNA in metastasis (R. A. Gupta et al., 2010). Gupta and colleagues demonstrated that the non-coding RNA HOTAIR is systemically dysregulated throughout breast cancer progression, where metastatic lesions have the highest expression relative to primary tumors. Further, expression of HOTAIR promoted cancer invasion and metastasis, indicating a cancer cell can acquire epigenetic dysregulation that promotes metastasis (R. A. Gupta et al., 2010). Strong links between alterations in epigenetic regulation and tumor evolution add another component to the complexity of metastatic progression.

Microenvironmental mechanisms promoting acquisition of phenotypes

A third, and one of the most well studied, mechanism by which neoplastic cells acquire pro-metastatic phenotypes is through interactions with the tumor ME. From immune cells, cytokines, and extracellular proteins to hypoxic conditions and exposure to chemotherapy; these and other microenvironmental

components can promote cancer metastasis through paracrine or endocrine signaling as well as direct cell-cell interactions.

The acquired pro-metastatic phenotype from the ME may be transient and is therefore unique compared to acquisition of metastatic properties through genetic alterations. For example, neoplastic cells can acquire pro-metastatic function after exposure to a specific growth factor or cytokine. Expression of the CSF1R can be induced by TGFβ (Patsialou et al., 2015), a known inducer of EMT (Xu, Lamouille, & Derynck, 2009). Further, CSF1R signaling in neoplastic cells can down-regulate expression of tight junctions and reduce proliferation, key attributes of a mesenchymal-like phenotype (Patsialou et al., 2015).

Cell-cell contact facilitates acquisition of phenotypes that promote metastatic disease. Studies highlighting how cell-cell contact contributes to metastases have identified pro-survival or pro-self renewal properties acquired by cancer cells at distant metastatic sites. MΦs provide pro-survival signaling through integrin-VCAM1-mediated cell-cell interactions with breast cancer cells in the lung (Q. Chen et al., 2011). Additionally, reactive astrocytes in the brain express Jagged1 (JAG1), a Notch pathway ligand, and promote cancer stem cell self-renewal (Xing et al., 2013).

Direct cellular material exchange as a mechanism of acquired phenotypes

A recently developing mechanism for cancer cell gain of pro-metastatic phenotype is by directly acquiring DNA, RNA or protein from another cell mediated by vesicular transfer. Exosomes, which are vesicles secreted from various cell types that contain constituents of their cell of origin, are emerging as

a key mechanism (van Niel, Porto-Carreiro, Simoes, & Raposo, 2006). While many groups have focused on tumor-derived exosomes, more recent observations have identified that neoplastic cells have the ability to acquire protein and function from ME-derived exosomes (Zhao et al., 2016). Importantly, acquisition of exosomes by neoplastic cells can promote migration and invasion. For example, melanoma cancer cells receive exosomes from adipocytes containing proteins involved in fatty acid oxidation and acquire a more migratory/invasive phenotype that results in increased experimental metastases (Lazar et al., 2016). While questions arise, including if physiological levels of exosomes provide phenotypes demonstrated in these *in vitro* studies, this intriguing mechanism supports new ways of acquired phenotypes from another cell type. Membrane nanotubes, or small-diameter extensions that are capable of connecting two cells over a large distance, can also theoretically be a mechanism by which cancer cells gain metastatic capabilities (Davis & Sowinski, 2008). Neoplastic cells can acquire chemoresistance by forming nanotubes with endothelial cells (Pasquier et al., 2013). Nanotubes can transfer mitochondria from endothelial to cancer cells and are associated with chemoresistance (Pasquier et al., 2013). With the ability to directly donate cellular components from a benign cell type to a cancer cell, this mechanism has the potential to provide a conduit for cancer cells to acquire phenotypes that promote metastasis. A more recent example for how cancer cells can directly acquire metastatic function from another cell type is through gap junctions, specialized pores that allow for passage of ions and various molecules between two cells (Q. Chen et

al., 2016). Mammary and lung cancer cells form gap junction connections with astrocytes within the brain. cGAMP was identified to transfer from cancer cells to astrocytes, inducing inflammatory cytokine release from astrocytes that promoted metastatic outgrowth and resistance to chemotherapy in a paracrine manner. The unique interactions between cancer cells and their ME, where cellular components can be freely exchanged between cell types, is a newly emerging field with exciting implications in promoting disease progression.

An additional exciting but largely uncharacterized mechanism for sharing genetic material is cell-cell fusion, where neoplastic cells fuse with other cells, giving rise to a fusion-derived hybrid cell type. As with exosomes, nanotubes, or gap junctions, cell fusion may represent a mechanism by which cancer cells acquire a pro-metastatic phenotype.

Cell fusion

Cell-cell fusion is a functionally relevant and necessary cellular mechanism found throughout physiological processes, from cytotrophoblast fusion establishing placental syncytiotrophoblasts, to egg and sperm fusion forming a zygote. In some tissues, essential functions require cell-cell fusion. For example, myotube formation in skeletal muscle development and regeneration is necessary for the enhanced contractile strength when compared to muscle cells that do not undergo cell fusion, such as smooth muscle (Berendse, Grounds, & Lloyd, 2003). Many fusion events characterized to date have focused on homotypic cell-cell fusion, where the parental fusogenic cells share the same lineage (Figure 1.3a). For example, MΦs are known to fuse with each other to form either osteoclasts or giant cells, multinucleated cells necessary for bone resorption and response to certain infections respectively (Vignery, 2000). While the functional relevance and defined phenotypes of cell types derived from homotypic cell fusion are well-established, heterotypic cell fusion has been less studied and, in many cases, more controversial.

Heterotypic cell-cell fusion, or fusion between parental cell types of different developmental lineages, has been well-documented in only a few biological processes, such as egg and sperm fusion (Figure 1.3b). Yet more recent evidence supports the notion that heterotypic cell fusion occurs in numerous settings, including between bone marrow-derived cells (BMDCs) and various cell types (Figure 1.3c) such as epithelial cells under homeostatic and inflammatory conditions

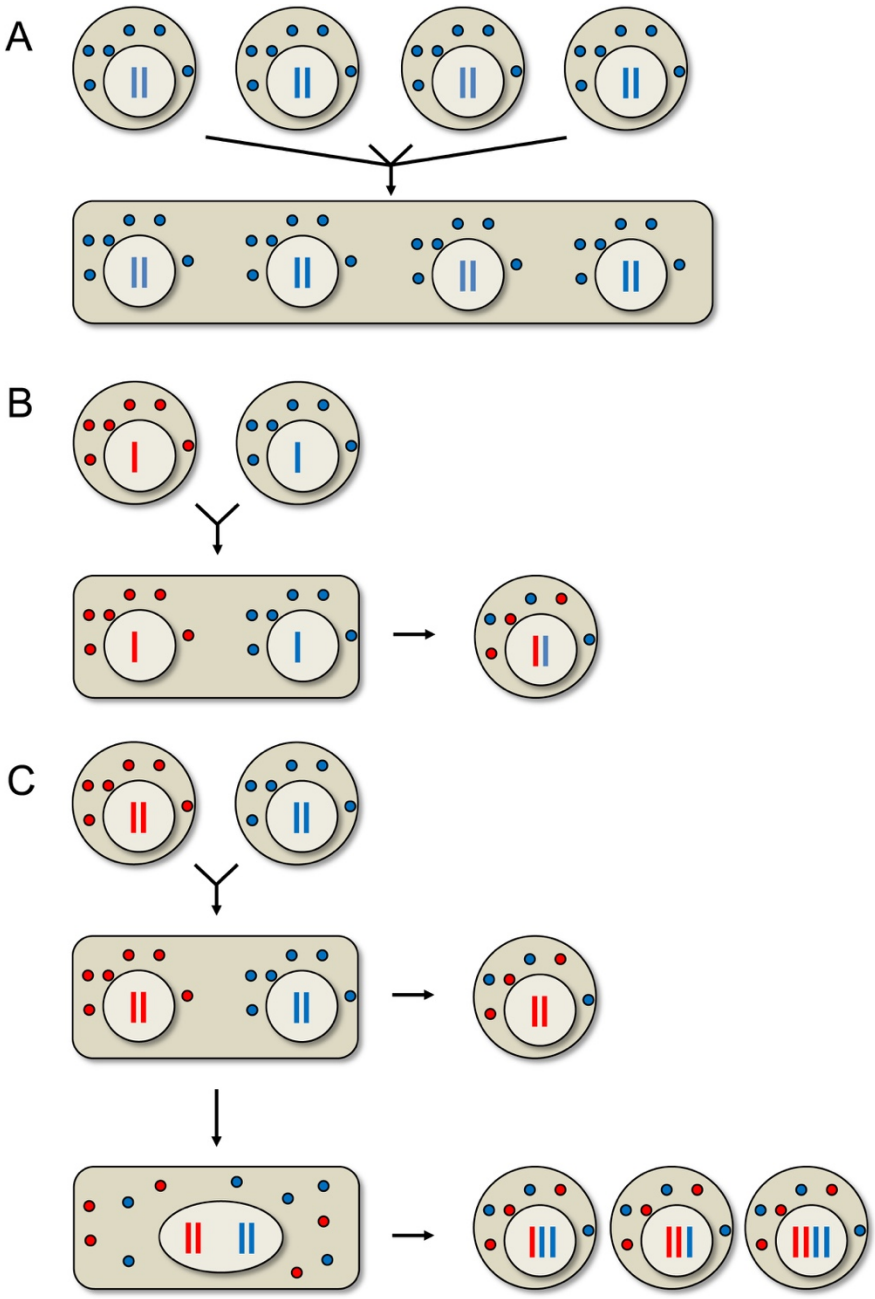

Figure 1.3. Cells derived by fusion.
(A) Cell fusion among cells of the same lineage (homotypic cell fusion) occurs both during development (placenta and muscle) and in adults (multinucleated-giant cells), resulting in a cell with multiple nuclei, termed a syncytium. Cell fusion among cells of the different lineages (heterotypic cell fusion) results in a cell with multiple nuclei, termed a heterokaryon that can result in a cell with a single nucleus called a synkaryon. Synkaryons may retain genetic material from both parental lineages such as egg and sperm fusion (B), or may transiently or permanently harbor varying degrees of genetic material from both parental lineages (C).

(Davies, Powell, Swain, & Wong, 2009; Oikawa et al., 2012). Unlike osteoclasts or giant cells, the relevance of such examples of heterotypic cell fusion is unknown. Questions regarding the functional consequence of heterotypic cell fusion include: do hybrid cells transiently or permanently acquire new phenotypes? Do these phenotypes play a biological role under homeostatic conditions and disease? Is heterotypic cell fusion a regulated biological process or is it “random”, a defect or error in another biological process? These questions have become even more relevant with the observation that cancer cells may also undergo spontaneous heterotypic cell fusion. While heterotypic cancer cell fusion driving acquisition of phenotypes aligned with progression is a hypothesis that has been around for over a century (Lu & Kang, 2009), there is limited conclusive evidence that such fusion occurs.

Homotypic cell fusion

Homotypic cell-cell fusion and the phenotypes of the resulting progeny have been well-characterized in different tissues and developmental states. Fusion and underlying mechanisms have been studied extensively in *C. elegans*. In this model, cell-cell fusion occurs early in development with one third of all somatic cells fusing to form multinucleated cells to drive the development of vital organs (E. H. Chen & Olson, 2005). Additionally, myoblast fusion to form myotubes in *Drosophila* is necessary for skeletal muscle differentiation and function (Abmayr & Pavlath, 2012). Cell fusion is not just restricted to lower organisms. In mammals, placental trophoblast fusion is vital to forming a layer

separating maternal and fetal blood (Gerbaud & Pidoux, 2015). Perhaps one of the most well studied homotypic fusion events is between MΦs.

Monocyte and MΦ homotypic fusion mechanisms and its functional relevance are well elucidated (Helming & Gordon, 2009). Osteoclasts are efficient bone resorbing cells, where their efficiency is largely attributed to increased size. Osteoclasts have increased surface area for bone resorption, which is crucial for normal bone homeostasis. One of the crucial factors that induce formation of osteoclasts is CSF1, which is produced by osteoblasts (Novack & Teitelbaum, 2008). Of the multiple mechanisms by which CSF1 promotes osteoclast formation, one is the recruitment of osteoclast precursor monocytes. In addition to other chemoattractants such as SDF1, this chemotactic function allows for recruitment of osteoclast precursors to a specified ME conducive for homotypic cell fusion, where osteoclasts can then function in their designated niche (Kikuta & Ishii, 2013). Importantly, the resulting mature osteoclast progeny maintains this chemotactic ability to migrate toward CSF1, indicating a hybrid may acquire specific phenotypes from parental cell lineages (Itokowa et al., 2011).

Molecular mechanisms of macrophage fusion

The biologic mechanisms involved in membrane fusion have been well studied in the context of cell infection by certain viruses, including influenza. Hemagglutinin is a surface glycoprotein expressed by influenza viruses and mediates attachment and fusion of the virus to the cell host (Russell et al., 2008). Through acidification of the endosome, hemagglutinin unfolds to expose a

hydrophobic portion of the protein, allowing for association to the endosome membrane (Stegmann, Booy, & Wilschut, 1987), and through refolding of the protein, membranes are brought together and a fusion pore is created (Skehel & Wiley, 2000). Mechanisms of viral-cell mediated fusion may extend to cell-cell fusion as groups identified cell-cell fusion after cell infection with certain viruses such as polyoma virus (Harris et al., 1969). Yet in a non-infectious setting, MΦs undergo homotypic fusion through a number of defined steps: chemotaxis, cell-cell attachment, cytoskeletal rearrangements and fusion.

In the context of osteoclast formation, osteoclast myeloid precursors are first recruited. In addition to osteoblast production of CSF1 and SDF1 mentioned above, the receptor activator of NFκB ligand (RANKL), CCL2, and Ca^{2+} gradients contribute to cell recruitment (Zaidi et al., 1991). Once the osteoclast precursors arrive at the destined site for fusion, they become fusion competent through exposure to specific cytokines and cell-cell interactions. Exposure to CCL2, in addition to cell-cell contact, triggers SYK signaling and induction of genes associated with a fusion-competent osteoclast precursor, including DC-STAMP, E-cadherin, and MMP9 (Kyriakides et al., 2004; MacLauchlan et al., 2009; Miyamoto et al., 2009).

Once fusion competent, Oikawa and colleagues eloquently demonstrated cell-cell fusion is promoted by the formation of actin-rich protrusions termed podosomes/invadopodia (Oikawa et al., 2012), similar to myoblast fusion (Shilagardi et al., 2013). Initiation of podosome formation on one of the two parental cells is induced by polarization of the plasma membrane with regions

enriched with phosphatidylinositol and activated TKS5 protein. Circumferential podosomes are then formed through actin polymerization. While the final steps of membrane-membrane fusion have yet to be elucidated in the context of osteoclast precursor fusion, it is possible cortical tension, derived in part by induction of podosome invasion into the recipient cell, promotes fusion pore formation similar to events demonstrated in myotube formation (Kim et al., 2015).

The formation of monocyte/MΦ hybrids, however is not limited to one specifically defined microenvironmental cue. For example, MΦs can fuse together to form what are termed multinucleated giant cells in response to inflammatory cytokines. Giant cells are classically associated with infections resulting in granuloma formation as well as other chronic inflammatory conditions. These inflammatory environments include tumors, where giant cells exist in numerous cancer types and may play a functional role in tumor progression (Hatano, Nakahama, Isobe, & Morita, 2014). Experiments have shown that T_H2 and T_H17 cytokines can induce giant cell formation such as IL-4, IL-13, and IL-17 as well as granulocyte-macrophage colony stimulating factor (GM-CSF) and IFNγ (Helming & Gordon, 2009). Because tumor MEs contain many of these cytokines in the presence of tumor-associated MΦs, it is possible a tumor may provide an environment primed for MΦ cell fusion. Remarkably, IL-4 induces expression of fusion mediators such as E-cadherin on MΦs, which promotes MΦ-MΦ adhesion. Successful MΦ fusion also requires inhibition of phagocytosis by expression of the cell surface receptor CD47. What makes this mechanism of cell fusion intriguing, is that many of these receptors are found in

other cell types including non-neoplastic and neoplastic epithelial cells where E-cadherin is crucial for cell-cell adhesion and epithelial cell survival and CD47 is expressed to prevent MΦ phagocytosis. Thus, it is possible that MΦ s may fuse with other cell types such as epithelial cells under discrete microenvironmental conditions.

Heterotypic cell fusion in non-neoplastic cells

It is widely accepted that heterotypic cell-cell fusion (two parental cells of different lineages) occurs both under homeostatic conditions and in pathologic states (Stein, Primakoff, & Myles, 2004; Vassilopoulos, Wang, & Russell, 2003). This, however, has been limited to only a few specific biological processes such as egg and sperm during fertilization or liver regeneration (Vassilopoulos et al., 2003). Heterotypic fusion was identified in tissues not classically believed to support cell fusion such as leukocyte-epithelial cell fusion in adult tissues (Nygren et al., 2008; Vassilopoulos et al., 2003). Further, neoplastic cells undergo heterotypic cell fusion with the hypotheses that this drives malignancy or altered phenotypes.

Egg sperm fusion

Fertilization of a mature oocyte via fusion with a sperm is a classical heterotypic cell fusion event. The genomic consequences of fertilization are acquisition of homologous chromosomes, resulting in a zygote with retained DNA from both parental cells. This process leads to acquiring a new phenotype, more broadly defined as initiation of replication and differentiation leading to a fully developed organism. This translates to long-term acquisition of specific

phenotypes from a specific parental lineage and can be clearly demonstrated by autosomal dominant diseases or gender. While this seems trivial today, this example clearly highlights heterotypic cell fusion as a mechanism for acquiring novel phenotypes from a specific parental lineage.

Fusion with hematopoietic cells.

While changes in genotype and phenotype were detected in specific examples of heterotypic cell fusion with clear biological significance, other physiologic and pathologic processes have been largely unexplored and remain controversial. Examples of heterotypic cell fusion have identified hematopoietic cell fusion with cardiomyocytes, skeletal muscle, hepatocytes and Purkinje neurons (Nygren et al., 2008). One crucial finding that identified hematopoietic-epithelial heterotypic cell fusion, was the observation of intestinal epithelial cells expressing both β-galactosidase (β-gal) and green fluorescent protein (GFP) from β-gal-expressing mice that had received a bone marrow transplant (BMT) from a GFP donor Rizvi et al. (2006). This novel finding was further confirmed to occur under normal tissue homeostasis (in the absence of total body irradiation) using a parabiosis approach (Davies et al., 2009). By surgically joining GFP- and β-gal-expressing mice, a shared circulating blood supply was established and β-gal-expressing intestinal epithelial cells expressed GFP from the hematopoietic lineage. It was also identified that BMDC-intestinal epithelial cell-cell fusion was mediated by an increase in inflammation and proliferation (Davies et al., 2009), suggesting heterotypic cell fusion, in addition to normal homeostasis, may play a role in pathogenic processes by either contributing to, or resolving, an

inflammatory pathology. Similar results were reported with hematopoietic cell-Purkinje neuron cell fusion via BMT and parabiosis approaches (Johansson et al., 2008). Induced inflammatory states resulted in an increase in BMDC-neuron cell fusion hybrids, indicating BMDC heterotypic cell fusion in the pathologic state is likely not specific to one parental cell type. To determine the functional relevance for heterotypic fusion of BMDCs with epithelial cells in the pathologic state, whether a chronic inflammatory disease or cancer, it was important that the primary hematopoietic fusion partner was identified. Powell and colleagues determined that the MΦ population was the primary fusogenic partner underlying heterotypic fusion with intestinal epithelial cells (A. E. Powell et al., 2011). These fusion-derived hybrids were shown to express the MΦ marker F4/80 (Davies et al., 2009). Given that MΦs have a well-established homotypic fusogenic potential, it is not surprising this is the predominant hematopoietic lineage fusing with intestinal epithelial cells; however, it is remarkable that hybrids derived from MΦ-epithelial fusion retain an expression profile and phenotype shared by MΦs (A. E. Powell et al., 2011). This observation supports the notion that fusion-derived hybrid cells have acquired intrinsically unique functional properties. These functional properties and their relevance are currently unknown; however if heterotypic-cancer cell fusion can be validated, cancer cells may similarly have the ability to acquire unique phenotypes. Thus, the relevance of heterotypic-cancer cell fusion in tumor progression must be determined.

Cancer cell fusion

The hypothesis that heterotypic cell fusion contributes to cancer progression is over a century old; it was first suggested by a German pathologist Otto Aichel who proposed somatic cells fuse with leukocytes, resulting in malignancy (Lu & Kang, 2009). This hypothesis was based on the observation that leukocytes were detected in tumors and that neoplastic cells had chromosomal abnormalities and increased ploidy. It was not until decades later that evidence began to identify heterotypic cell fusion in the context of cancer. However, the physiologic relevance of cell fusion has remained controversial. For example, in 1969 Henry Harris reported that malignant properties of cancer cell lines can be suppressed by fusion with non-neoplastic cells such as fibroblasts (Harris et al., 1969). Here, mouse carcinoma and sarcoma cell lines fused spontaneously with a fibroblast cell line *in vitro* and the majority of hybrids failed to form tumors when injected *in vivo*. This supported that heterotypic cell fusion resulted in loss of malignant properties. In this case, malignant properties driven by loss of a tumor suppressor would be compensated upon fusion with a cell harboring wildtype alleles. Alternatively, the progeny of a cell fusion hybrid could lose sufficient expression of an oncogene resulting in loss of malignant properties. Importantly, reports have demonstrated that cancer cell fusion with fibroblasts do not always result in loss of malignancy (Harris et al., 1969). For example, an activated NRAS oncogene-driven fibrosarcoma was shown to remain tumorigenic after fusion with a non-neoplastic fibroblast and the ability to form tumors appeared to be gene dosage dependent (Benedict, Weissman,

Mark, & Stanbridge, 1984). This data indicates that a subset of hybrids have the capacity to remain malignant and acquire genetic material from both parental cell lineages. Because of this, a heterogeneous population of hybrids that spontaneously form *in vivo* could theoretically result in hybrid subsets with a selective advantage. This selective advantage may not be limited to oncogene-driven tumors and could contribute to disease progression in cancer driven by loss of functional tumor suppressor genes. Hybrid populations losing chromosomes over time, supported by experimental evidence in Chapter III of this dissertation, would support the notion that a hybrid progeny may be able to maintain or re-acquire the malignant phenotype. Thus, cell fusion may play a role in tumor diversity and a mechanism by which cancer cells acquire new phenotypes. The overall contribution of cell fusion on disease progression remains to be determined and is likely variable depending on cancer type, oncogene/tumor suppression expression, and the lineage of the non-neoplastic parental cell.

Initial experiments demonstrated that MΦs fuse with pre-cancerous intestinal polyps *in vivo* (Davies et al., 2009), as well as with malignant carcinoma cell lines *in vitro* (Davies et al., 2009; Shabo et al., 2015). However, extensive genotypic and phenotypic characterization of these hybrids is currently lacking or have considerable limitations. For example, one report of cell fusion used polyethylene glycol (PEG), a dehydrating polymer that promotes fusion between two lipid bilayers to artificially fuse MΦs with cancer cells (Ding, Jin, Chen, Shao, & Wu, 2012). In some reports, these artificially-derived hybrids were evaluated in

mouse models of metastasis (Rachkovsky et al., 1998) with claims of fusion driving local metastasis. Pawelek went on to pursue identifying acquired phenotypes that may play a role in gaining metastatic potential by cell fusion. He determined artificial hybrids acquired increased expression of specific integrin subunits that promote chemotactic migration toward fibroblast conditioned medium or fibronectin (Chakraborty, Funasaka, Ichihashi, & Pawelek, 2009). However, these experiments fall short of validating fusion in cancer.

The most convincing evidence to date for spontaneous MΦ cancer cell fusion *in vitro* is co-culture of GFP MΦs and a colon cancer cell line expressing Histone H2B-tagged red fluorescent protein (RFP), as published by our laboratory (A. E. Powell et al., 2011). Cell fusion hybrids were detected by identifying cells expressing cytoplasmic GFP with an RFP-expressing nucleus. More recently, it was similarly reported that a GFP expressing breast cancer cell line acquired expression of the MΦ proteins CD163 and CD45 after co-culture with MΦs (Shabo et al., 2015). Short tandem repeat (STR) analysis also identified DNA from both cancer cells and MΦs. While these examples conclusively demonstrate cells have the ability to spontaneously fuse, many questions remain. In addition to determining if hybrids acquire a transient or persistent biologically relevant phenotype, it is unknown if hybrid populations are a heterogeneous population and if this could potentially contribute to tumor heterogeneity. Additionally, *in vivo*-derived cell fusion hybrid cells have yet to be detected or isolated directly from mice.

One of the most compelling examples to date for spontaneous, *in vivo*-derived cancer cell fusion is from Kerbel and colleagues, who showed that major histocompatibility complex subtypes from the mouse host (H-2^K or H-2^b) were present in a cancer that was originally H-2^d after culturing the excised tumor cells (Kerbel, Lagarde, Dennis, & Donaghue, 1983). Importantly, bone marrow chimera experiments implicated a hematopoietic origin for the fusogenic parental cell that fuses with cancer cells, leading to progeny with higher ploidy (Kerbel et al., 1983). Another compelling example that cell fusion occurs *in vivo* was reported by Halaban and colleagues (Halaban, Nordlund, Francke, Moellmann, & Eisenstadt, 1980). A female-derived melanoma cell line defective in a metabolic enzyme that was unable to grow in specified selection media was injected subcutaneously into a syngeneic male mouse, resulting in the identification of Y chromosome positive tumor subclones that could successfully grow in culture. While this is compelling evidence for fusion hybrids, it cannot be ruled out that the fusion event occurred in culture after tumor resection and that hybrids were selected in culture given that MΦs and cancer cells are known to fuse *in vitro*.

Yet with isolation and partial analysis of a single hybrid clone a limited number of times, it remains unclear what frequency or functional relevance hybrids play in tumorigenesis or disease progression. Similar to artificial *in vitro*-derived hybrids, one attempt to assess phenotypic alterations was conducted by injecting the parent or hybrid cell clones subcutaneously into the tail of mice and "local" metastasis to the tail was determined by identifying melanotic regions of the tail immediately proximal to the injection site (Rachkovsky et al., 1998). Using

this approach, it was concluded that *in vivo*-derived hybrid cells were more metastatic. Despite the number of reports describing fusion, phenotypic assessment of spontaneously fused hybrid cells has not been performed to date. This will be addressed in this dissertation.

Evidence for cancer hybrids in patients

One of the greatest challenges in determining the functional significance of MΦ-cancer cell fusion in patients is identifying cell fusion in humans. Many of the limitations have centered around the inability to confirm biparental lineage of a hybrid cell type. While murine models allow labelling and tracking of specific lineages through reporters, these approaches in patients are not possible. One approach to identifying hybrids in patient samples is to identify cancer cells that express leukocyte markers or leukocytes expressing epithelial or cancer markers. This approach, however does not rule out scenarios whereby cancer cells intrinsically express a leukocyte marker in response to environmental cues or acquire these markers though alternative mechanisms such as exosomes. One of the most convincing approaches to identifying cell fusion hybrids in patient cancer biopsies utilized a sex-mismatched BMT approach. This approach first identified female patients who had previously received a BMT from a male donor, whereby leukocytes contain a Y chromosome. Following transplant, the female patient acquired a secondary solid tumor and fusion was evaluated by identifying cancer cells with a Y chromosome. The most convincing example of this approach to date is a single case report from a female renal cell carcinoma patient that previously received a BMT from a male donor (Yilmaz, Lazova,

Qumsiyeh, Cooper, & Pawelek, 2005). Tumor cells had trisomy 17 by fluorescence in-situ hybridization (FISH) and karyotype analysis and one Y chromosome by FISH. While fairly convincing that these cells are hematopoietic-cancer hybrids, karyotype analysis failed to detect cells with both three chromosome 17s and one Y chromosome. The inconsistency between FISH and karyotype analysis, and the assessment of a single patient were limitations to the study, however the data remains strong evidence for cell fusion in humans.

A second approach examined a cancer patient with a previous BMT from his brother and forensic STR analysis to distinguish genomic DNA from BMDCs versus cancer cells (Lazova et al., 2013). A tissue section from a melanoma brain metastasis was stained with antibodies for the leukocyte marker CD45 by immunohistochemistry (IHC). Regions of the tumor section without identifiable CD45 staining were selected and microdissected for STR analysis. The rationale was that CD45 negative tumor regions would contain both the donor and recipient genomic DNA to support cell fusion. One of the limitations of this study is that this approach cannot rule out leukocyte contamination in their microdissected samples, yet cell fusion remains a viable explanation for identification of recipient DNA in these tumor regions.

A third example by Cogle and colleagues is of a case study assessing the potential for cell fusion in patient biopsies also utilizing a sex-mismatched BMT approach. In this study, a female patient with a previous BMT from a male donor developed a colonic adenoma and a single image is shown of a Y chromosome-positive cell with dual epithelial-specific CK and mucin expression. While this

adenoma is pre-cancerous, this example convincingly identifies an epithelial cell with a Y chromosome. As with the previous example, this approach can be a result of either cell fusion or differentiation of an immune cell into an epithelial cell and there is much controversy regarding which mechanism is the explanation for donor DNA within epithelial cells. Cogle makes the claim that these cells are a result of bone marrow cells mimicking adenoma cells based on the following mouse experiment. Similar to the identification of *in vivo*-derived hybrids in tumors by Powell et al. (A. E. Powell et al., 2011), Cogle performed a BMT from a male donor into an APC^{min} mouse with intestinal tumors. Because Y chromosome positive epithelial cells in the tumor did not have more than one X chromosome, the conclusion was presented that these were not derived by cell-cell fusion. Cell fusion between BMDCs and non-neoplastic epithelium was shown to have an increase in ploidy but did not display tetraploidy (Powell, unpublished). This indicates that ruling out cell fusion as a mechanism due to chromosome numbers alone may not be warranted. Additionally, results presented in Chapter II of this dissertation identify Y-chromosome positive epithelial cells with multiple X chromosomes in human intestinal epithelial cells, however the majority have less than two X chromosomes, highlighting the possibility that hybrids may lose chromosomes over time. Additionally, temporal analysis of *in vitro*-derived hybrids, as discussed in Chapter III, shows loss of chromosomes over time. Thus, the Y chromosome positive human intestinal adenoma may be a result of cell fusion and is consistent with mouse studies supporting cell fusion.

Collectively, these case studies provide intriguing support for the claim that cell fusion between hematopoietic cells and cancer cells can be detectible in patients, however all of these studies were based in singular patients and some showed only a single cell supporting cell fusion. Because of the lack of rigor, more comprehensive studies, including multiple patients with multiple examples of cell fusion are needed to support the field. I present more in depth analysis of human patient samples from graft-vs-host disease (GVHD) biopsies from sex mismatched BMT patients and in secondary solid cancers of various origins (in Chapters II and III).

Rationale and hypothesis

For a cancer cell to metastasize, it must acquire specific phenotypes, either intrinsically or through support from the ME. This process includes invading into the surrounding stroma, intravasation and survival in circulation, extravasation at a distant organ and growth at the metastatic site. Although there is clear evidence that tumor progression and metastasis are influenced by both acquired tumor intrinsic properties as well as extrinsic influences from the local and distant ME, such as MΦs, the precise mechanisms are complex and diverse. In addition, the mechanisms by which cancer cells acquire these phenotypes are equally diverse. From mutations and changes in epigenetic regulation, to directly acquiring cellular components from another cell via exosomes or nanotubes, cancer cells have found multiple strategies to gain metastatic potential. Because a limited number of studies identify cell fusion between cancer cells and MΦs, it is possible fusion is another mechanism promoting gain of metastatic phenotypes. Addressing this possibility is crucial, given that monocytes and MΦs inherently possess prometastatic phenotypes such as migration and invasion, intravasation, survival in circulation, and extravasation into multiple organ types. Therefore, I hypothesize that MΦ-cancer cell fusion potentiates acquisition of MΦ-like behavior that can contribute to tumor progression and heterogeneity.

Chapter II: Fusion between hematopoietic and epithelial cells in adult human intestine

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Abstract

Following transplantation of hematopoietic lineage cells, genetic markers unique to the transplanted cells have been detected in non-hematopoietic recipient cells of human liver, vascular endothelium, intestinal epithelium and brain. The underlying mechanisms by which this occurs are unclear. Evidence from mice suggests it is due in part to fusion between cells of hematopoietic and non-hematopoietic origins; however, direct evidence for this in humans is scant. Here, by quantitative and statistical analysis of X- and Y-chromosome numbers in epithelial and non-epithelial intestinal cells from gender-mismatched hematopoietic cell transplant patients, we provide evidence that transplanted cells of the hematopoietic lineage incorporate into human intestinal epithelium through cell fusion. This is the first definitive identification of cell fusion between hematopoietic cells and any epithelial cell type in humans, and provides the basis for further understanding the physiological and potential pathological consequences of cell fusion in humans.

Personal contribution

CEG's contribution to this chapter are as follows:

Table 2.1.
Experiments were not designed nor were patients identified by CEG.

Figure 2.1.
Immunofluorescence and FISH was performed on samples by CEG followed by microscopy and identification of Y-chromosome positive epithelium.

Table 2.2, Figure 2.2.
Quantification was performed by CEG and analysis was performed by both CEG and ADS.

Figure 2.3
Immunofluorescence and FISH was performed on samples by CEG and PSD.

CEG wrote the introduction of this manuscript.

Introduction

In patients who received hematopoietic cell transplantation, genetic markers specific to transplanted hematopoietic lineage cells have been found in fully differentiated cells of multiple non-hematopoietic tissues, including liver, brain, vascular endothelia, intestinal epithelia and cancerous tissue (Jiang et al., 2004; Okamoto et al., 2002; Theise et al., 2000; Weimann, Charlton, Brazelton, Hackman, & Blau, 2003; Yilmaz et al., 2005). Despite this overwhelming evidence, it is not currently known how it occurs in the vast majority of cases (Weimann, Charlton, et al., 2003). Indeed, there is considerable debate as to the mechanisms underlying the presence of hematopoietic-specific genetic markers in non-hematopoietic human tissue. One possibility is that transplanted hematopoietic stem cells (HSCs) retain a high degree of plasticity, and after homing to non-hematopoietic cellular compartments undergo transdifferentiation into cell types outside of the recognized hematopoietic lineage. The alternative is that transplanted HSCs or HSC-derived cells undergo direct cell fusion with non-hematopoietic cell types, producing hybrids carrying genetic material from both parental cells. It is probable that these two mechanisms have distinct consequences for tissue physiology. Hematopoietic transdifferentiation, involving the reprogramming of a single genome, is more likely to produce cells that are phenotypically similar to resident differentiated cells within a given tissue. Cell fusion, by virtue of combining two differentially regulated genomes within a single cell, has a greater potential to produce cells that show fundamentally different behaviors relative to surrounding tissue-resident cells. While there have been

repeated demonstrations in humans that genetic markers specific to hematopoietic cells can be found in non-hematopoietic cell types, there have been very few attempts to conduct quantitative analysis at the single-cell level to definitively identify whether this occurs via hematopoietic transdifferentiation or cell fusion. Distinguishing between these mechanisms is necessary in order to guide subsequent investigation towards the plasticity of hematopoietic progenitor cells or the phenotypic outcomes of fusion between different cell types. One obvious feature that distinguishes cells derived from fusion relative to transdifferentiation as a mechanism for the origin of non-hematopoietic cells carrying hematopoietic-specific genetic markers is that cell fusion results in a direct and immediate increase in cellular chromosomes content, while transdifferentiation does not.

In the human brain, there is strong support for cell fusion as one mechanism by which markers from transplanted hematopoietic cells incorporate into non-hematopoietic recipient tissue. There is an abnormally high number of X-chromosomes in Y-chromosome-containing Purkinje neurons in female recipients of gender-mismatched bone marrow transplantation; consistent with hematopoietic-Purkinje fusion in the cerebellum (Weimann, Charlton, et al., 2003). These observations are supported by studies in mice, which demonstrate that bone marrow-derived cells (BMDCs) undergo fusion with cerebellar Purkinje neurons (Johansson et al., 2008; Weimann, Johansson, Trejo, & Blau, 2003). By contrast, incorporation of hematopoietic-specific genetic markers into endothelium appears to occur exclusively by transdifferentiation, in both humans

and mice (Bailey et al., 2006; Jiang et al., 2004). However, in other human tissues it is not known how genetic markers unique to hematopoietic cells come to exist in non-hematopoietic cell types. For example, while fusion between hepatocytes and hematopoietic lineage cells has been conclusively demonstrated in mice, there is conflicting evidence as to whether it also occurs in humans (Fujino et al., 2007; Newsome et al., 2003; Wang et al., 2003). Similarly, while spontaneous cell fusion between hematopoietic and non-hematopoietic cells in a wide variety of other tissues is supported by multiple studies in mice, there has been considerable debate as to whether it occurs in humans at all (Alvarez-Dolado et al., 2003; Camargo, Green, Capetanaki, Jackson, & Goodell, 2003; Rizvi et al., 2006).

In the human gastrointestinal tract, it is clear that donor-specific markers are found within the epithelium of hematopoietic cell transplant patients (Korbling et al., 2002; Okamoto et al., 2002). Evidence from mice suggests that cell fusion plays a part in this process and transcriptional analysis of fusion-derived cells indicates that these cells share transcriptional characteristics of both epithelial and bone marrow-derived myeloid cells (A. E. Powell et al., 2011; Rizvi et al., 2006). In addition, there is a detectable basal level of hematopoietic-epithelial fusion in the mouse intestine in the absence of irradiation-induced injury, indicating that fusion occurs endogenously and independent of cellular transplantation (Davies et al., 2009). These results suggest that hematopoietic-epithelial cell fusion may contribute to aspects of intestinal pathophysiology or potentially the replacement of epithelial cells lost by continuous sloughing of the

epithelial cell layer, but previous studies have failed to find evidence of cell fusion in the human intestine (Spyridonidis et al., 2004). Here, by quantitative and statistical analysis of X- and Y-chromosome numbers in individual epithelial and non-epithelial nuclei of gender-mismatched hematopoietic cell transplant patients, we demonstrate that cell fusion is one mechanism by which hematopoietic lineage cells incorporate into the human gastrointestinal epithelium.

Materials and methods

Human tissues samples and ethics statement

Screening the Oregon Health & Science University (OHSU) hematopoietic transplantation registry identified one-hundred and ninety-five female patients who had received hematopoietic cell transplant from male donors between 1994 and 2011. Of these, thirty-six were diagnosed with acute graft-versus-host disease (GVHD) and underwent skin and intestinal biopsies for confirmation of GVHD diagnosis by medical pathologists. Patients without suspected GVHD are not biopsied. In each biopsy sample pathology reports confirmed the GVHD diagnosis. We acquired intestinal tissue sections from ten of these patients for analysis in this study and classified GVHD as mild, moderate or severe in each patient depending on the degree of crypt necrosis, confluent apoptosis, and/or heavy inflammatory infiltrate. Two patients were excluded due to moderate or severe GVHD with disorganized intestinal histology, making the epithelial cell population difficult to identify. Fluorescence in situ hybridization (FISH) and immunohistochemical staining failed on tissues from two additional patients, and therefore these were not included in our analysis. Human tissue samples were collected according to the ethical requirements and regulations of the OHSU institutional review board (IRB; FWA00000161) with written consent provided by patients. Approval to use de-identified archived tumor tissues in this study was provided by the OHSU IRB under approved protocol number IRB5169.

Fluorescence in situ Hybridization

X- and Y-chromosome FISH probes were hybridized to 5μm paraffin intestinal tissue sections. Briefly, tissue sections were deparaffinized, treated with Retrievagen A (BD Biosciences, CA) and processed with the Tissue Digestion Kit II reagents (Kreatech, Netherlands) according to the manufacturer's protocol. CEP X (DXZ1 locus) and Y (DYZ1 locus) probes (Abbott Molecular, IL) were hybridized to samples at 80°C for 5 min, followed by incubation at 37°C for 12h. Samples were washed in 2×SCC (30mM sodium citrate, 300mM sodium chloride, pH 7.0) + 0.1% NP-40 at 24°C for 2 min, 2×SCC + 0.3% NP-40 at 72°C for 5 min, and 2×SCC + 0.1% NP-40 at 24°C for 1 min. Tissue sections were dehydrated with a series of graded alcohols, air dried, and washed twice in phosphate buffered saline (PBS) for 5 min prior to antibody staining.

Immunofluorescence Staining and Microscopy

FISH-processed intestinal tissues were incubated in Blocking Buffer (PBS + 10% Donkey Serum, 5% bovine serum albumin + 0.3% TritonX-100) for 30 min at 24°C followed by incubation with goat-anti-human Lamin B1 antibodies (1:200, Santa Cruz Biotechnology, CA) in a humidified chamber at 4°C for 12h. The Lamin B1 antibody was visualized by incubation with a Cy5-conjugated anti-goat antibody (1:2000; Jackson, PA) at 24°C for 1h. Coverslips were mounted with ProLong Gold antifade reagent (Invitrogen, NY). Staining for cytokeratin was performed by removing coverslips from FISH and Lamin B1 stained sections, and incubating with guinea pig anti-Cytokeratin 14 antibody (1:100, Fitzgerald, MA) followed by an Alexa 488- conjugated anti-guinea pig secondary antibody (1:200, Life Technologies, NY). Tissue sections were imaged using a Zeiss LSM780

confocal microscope mounted on a fully motorized AxioObserver Z1 inverted microscope stand, controlled by ZEN2009 software (Carl Zeiss, NY). 1μm-thick optical sections were captured in 1.5μm intervals spanning the entire thickness of each tissue section. Maximum intensity projections of Lamin B1-positive planes were generated for manual counts of X- and Y-chromosome signals.

Immunohistochemical Staining

For detection of FABP2/IFABP, tissue sections were deparaffinized and subjected to 50 minutes of boiling in 10mM Sodium Citrate pH 6 with 1mM EDTA, followed by 5 minutes in 2% H2O2 in methanol. Slides were incubated for 30 minutes in Blocking Buffer and then overnight with a rabbit-anti-FABP2 antibody (1:200, Sigma, MO), followed by incubation with a biotinylated goat anti-rabbit secondary antibody (1:500, Jackson ImmunoResearch, PA). Diaminobenzidine staining was carried out with the Vectastain ABC and Peroxidase Substrate DAB kits (Vector Laboratories, CA). Hematoxylin (Vector Laboratories, CA) was applied as a counterstain.

Image Scoring, Quantification and Statistics

More than 20,000 Lamin B1-stained epithelial cells from female GVHD patients who had previously received male hematopoietic cell transplant were screened for the presence of a Y-chromosome. The total number of epithelial cells screened was calculated by determining the average number of epithelial cells in eight acquired microscope fields of view (≈300 cells per field), including at least one field from each patient, and multiplying by the total number of fields examined (n=74). The number of X-chromosomes was counted in each intestinal

cell nucleus with a contiguous Lamin B1-stained nuclear envelope and a single Y-chromosome. We classified cells as epithelial or non-epithelial using histological criteria, based on the spatial organization of nuclei within the intestine, as conventionally histologically defined (Ham & D.H., 1979). We also determined the number of X-chromosomes in Y-chromosome positive cells from epithelial and adjacent non-epithelial compartments in sections of normal male intestinal tissue. Quantification of chromosome numbers in cells from GVHD and control tissues was validated by blinded re-count. Fisher's exact test was performed to test for significant differences between groups, regarding the incidence of cells with 2 or 3 X-chromosomes; a p-value of less than 0.05 was considered statistically significant.

Results

In this study we demonstrate that cell fusion is one mechanism by which genetic markers of hematopoietic cells are incorporated into human intestinal epithelium. To do this, we analyzed intestinal biopsies from female patients who had received gender-mismatched peripheral blood stem cell or bone marrow transplantation (Table 2.1). Fusion between hematopoietic cells and intestinal epithelial cells is known to occur in mice but has not yet been identified in humans (Davies et al., 2009; A. E. Powell et al., 2011; Rizvi et al., 2006). We chose to analyze samples from male-into-female gender-mismatched transplant patients because this situation provides a genetic marker—the Y-chromosome—which uniquely identifies putative fused or transdifferentiated hematopoietic cells with single cell resolution. All of our samples were from patients diagnosed with GVHD because only patients with suspected cases of GVHD underwent intestinal biopsy; GVHD was confirmed by medical pathologists in each case. No intestinal biopsy samples were available from gender-mismatched transplant patients that did not also have a confirmed GVHD diagnosis. We sub-classified GVHD in each case as mild, moderate or severe based on the degree of inflammation and disruption of normal intestinal tissue architecture. We conducted analysis only on patient samples with mild or moderate GVHD in which epithelial and non-epithelial intestinal compartments could be readily distinguished. Fluorescent in situ hybridization (FISH) for X- and Y-chromosomes was used to identify Y-chromosome-containing nuclei within the epithelial and non-epithelial compartments of the intestine and quantify the number of X-

Table 2.1. Patient characteristics and detection of Y-chromosome positive epithelial cells.

Patient	Gender	Donor gender	Reason for transplant*	Type of trans-plant**	Days from transplant to biopsy	Biopsy Site	Pathological diagnosis***	number of epithelial cells analyzed	with Y-chromosome Total	with Y-chromosome With ≥2 X chromosomes
1	F	M	ALL	PBSC	86	duodenum	GVHD	4200	10	3
2	F	M	NHL	PBSC	49	colon	GVHD	2100	1	1
3	F	M	AML	PBSC	30	duodenum	GVHD	3300	17	3
4	F	M	MDS	PBSC	25	small bowel	GVHD	3900	25	1
5	F	M	CML	BM	60	sigmoid colon	GVHD	2700	10	1
6	F	M	AML	PBSC	28	sigmoid colon	GVHD	3900	2	1

*ALL, acute lymphoblastic leukemia; NHL, non-Hodgkin lymphoma; AML/CML, acute/chronic myeloid leukemia; MDS, myelodysplastic syndrome.
**PBSC, peripheral blood stem cells; BM, bone-marrow.
***GVHD, graft versus host disease.

chromosomes in each of these nuclei. Nuclear boundaries were identified by the presence of Lamin B1. The epithelium of the intestine comprises a histologically distinct compartment defined by basal localization and positioning of nuclei within each tissue section (Ham & D.H., 1979). Epithelial classification of cells was guided by comparison with Hematoxylin and Eosin (H&E) stained near or adjacent sections (Figures 2.1A and 2.1B). This simple classification scheme allowed for an unbiased analysis of all Y-chromosome-containing cells within the epithelial and non-epithelial compartments. Retrospective staining for cytokeratin (CK), a recognized epithelial-specific marker, confirmed the selectivity of our unbiased compartmentalization strategy (Figure 2.1B, inset). We quantified the number of X-chromosomes present within Y-chromosome-positive, Lamin B1-circumscribed nuclei in both epithelial and non-epithelial compartments in intestinal epithelial biopsies from gender-mismatched transplant patients and normal male controls (Figure 2.1 and Table 2.2).

Validation of staining and scoring strategy

In normal male intestinal sections the majority of epithelial and non-epithelial nuclei harbored a single Y-chromosome and a single X-chromosome, as expected (Table 2.2). Approximately one-third of Y-chromosome-containing nuclei did not contain an X-chromosome, likely an artifact of analysis within 5μm tissue sections, which include partial nuclei. Rare instances of nuclei with one Y-chromosome and two X-chromosomes were also detected (Table 2.2). In these normal male intestinal sections, we observed that epithelial and non-epithelial nuclei had similar distributions of sex karyotypes, consistent with the expectation

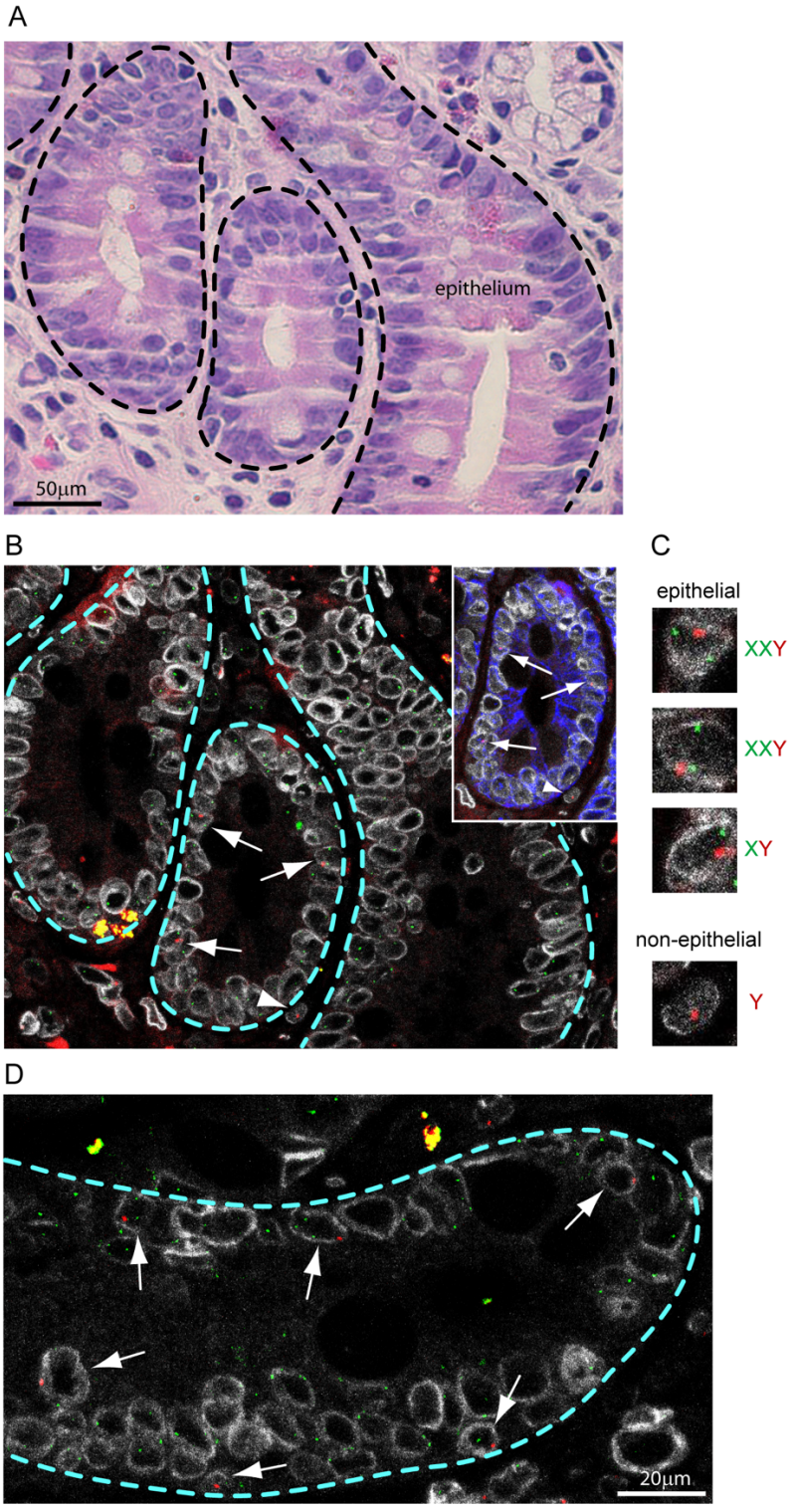

Figure 2.1. Epithelial compartmentalization and sex-karyotyping of intestinal cells.
(A) Hematoxylin and Eosin stained intestinal biopsy; epithelial compartment is labeled. (B) Adjacent tissue section to that from panel A stained for X- (green)

and Y- (red) chromosomes and Lamin B1(white). Arrows indicate Y-chromosome-positive epithelial cells and the arrowhead points to a Y-chromosome-positive non-epithelial cell. Inset shows a sub-region stained for cytokeratin (blue); arrows and arrowhead serve as positional references. (C) Enlarged views of cells indicated in panel B by arrows and arrowhead; sex-karyotype is indicated for each. (D) Independent patient sample also stained for X- (green) and Y- (red) chromosomes and Lamin B1 (white). Arrows indicate Y-chromosome-positive epithelial cells. Dashed lines in all panels indicate boundaries of epithelial and non-epithelial compartments.

Table 2.2. Sex karyotypes of epithelial and non-epithelial cells.

	# of cells with observed sex karyotype			
	Y	**XY**	**XXY**	**XXXY**
normal male epithelium	25	47	2	0
normal male non-epithelium	25	53	1	0
transplant epithelium	15	40	9	1
transplant non-epithelium	31	36	1	0

that the overwhelming majority of cells in each compartment were diploid (Table 2.2). To evaluate FISH probe specificity, we also stained intestinal tissue sections from two female patients with GVHD that had received gender-matched hematopoietic cell transplant, and observed no nuclear Y-chromosome signals in more than 7,000 cells (not shown). These experiments demonstrate the efficiencies of X- and Y-chromosome FISH staining and establish a baseline X-chromosome number distribution in normal male intestinal epithelial and non-epithelial cells.

Increased X-chromosome number in donor-marker-carrying intestinal epithelial nuclei

To examine the occurrence of cell-fusion between hematopoietic and epithelial cells in human intestine, we quantified the number of X-chromosomes in Y-chromosome-positive nuclei of intestinal tissue from female patients who had received gender-mismatched hematopoietic cell transplant. We scored only nuclei in which X- and Y-chromosomes were clearly contained within a well demarcated contiguous nuclear envelope, as marked by Lamin B1 staining (Figure 2.1B-D). In the non-epithelial compartment, Y-chromosome-positive nuclei represent donor-derived blood cells within the intestinal mesenchyme, their expected histological location. While the frequency of donor-derived cells in the mesenchyme varied between transplant patients (not shown), only a small fraction of these nuclei had more than a single X-chromosome (1.5%), a similar proportion as observed in normal male epithelial (2.7%) or non-epithelial populations (1.3%) (Table 2.2 and Figure 2.2). This result is consistent with the

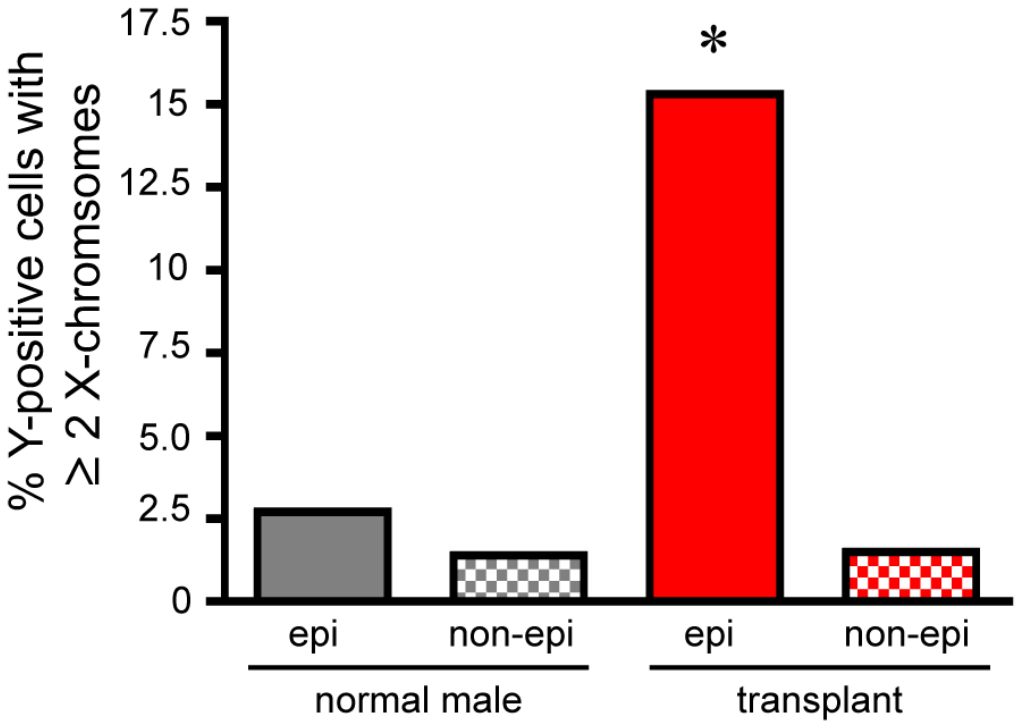

Figure 2.2. Frequency of nuclei with 2 or 3 X-chromosomes.
Percent of Y-chromosome-positive nuclei with two or three X-chromosomes, observed in epithelial (epi) and non-epithelial (nonepi) compartments of normal male and male-into-female gender mismatched bone marrow transplant patients (transplant). *, p = 0.0016, Fisher's exact test.

identity of these cells as normal diploid donor-derived blood cells. By contrast, analysis of more than 20,000 nuclei in the epithelial compartment of six female patients that had received male bone marrow identified sixty-five nuclei carrying Y-chromosomes (Table 2.1) and ten of these (15%) also had two or more X-chromosomes (Table 2.2 and Figure 2.2). Nine of these nuclei had two X-chromosomes and one nucleus had three X-chromosomes (examples in Figure 2.1C and 2.1D); nuclei with two or more X-chromosomes were identified in all patient samples (Table 2.2). Statistical comparison of the incidence of nuclei with 2 or more X-chromosomes between cell populations yielded a p-value of 0.0016, indicating a much higher incidence of nuclei with 2 or more X-chromosomes in Y-chromosome positive epithelial cells of transplant patients than in any other cell population (Figure 2.2). These results support the hypothesis that donor-marker-carrying cells within the epithelium of hematopoietic cell transplant recipients comprise a karyotypically distinct cell population, and are highly consistent with their origin as a result of fusion between hematopoietic lineage cells and intestinal epithelial cells.

Differentiation status of epithelial regions carrying fusion-derived cells

Studies in mice suggest that hematopoietic lineage cells fuse directly with stem or long-lived proliferating progenitors in the intestine(Rizvi et al., 2006). To determine whether this was also the case in humans, we analyzed adjacent tissue sections to the FISH-stained sections for the expression of the intestinal fatty acid binding protein (Fabp2/Ifabp), a marker of differentiated intestinal epithelial cells (Uhlen et al., 2010) (Figure 2.3A). Ifabp expression was low or

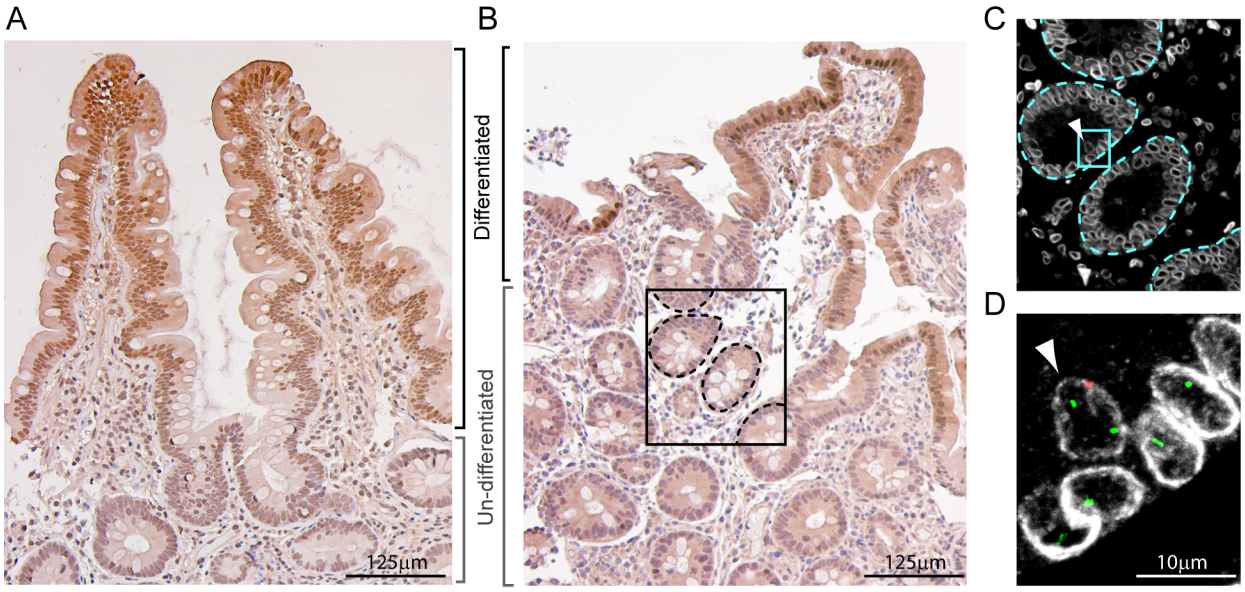

Figure 2.3. Differentiation status of cells with abnormal sex-karyotypes. (A) FABP2/IFABP expression in a control and (B) transplant patient sample with an example of an XXY cell. Brackets indicate differentiated (high Fabp2/Ifabp expression, black brackets) and undifferentiated (low Fabp2/Ifabp expression, gray brackets) regions of epithelium within each sample. (C) Enlarged view of boxed region from B, in an adjacent tissue section stained for Lamin B1.(D) Enlarged view of boxed region from panel C, showing X- (green) and Y- (red) chromosomes and Lamin B1 (white). Arrowhead indicates the same nucleus in panels C and D. Dashed lines indicate boundaries of epithelial and non-epithelial compartments.

absent in epithelial regions containing cells with abnormal sex-karyotypes—cells likely derived from fusion—indicating that hematopoietic-lineage cells had fused with undifferentiated and proliferating epithelial progenitors (Figure 2.3).

Discussion

Previous work has demonstrated that genetic markers specific to transplanted hematopoietic cells can often be found in non-hematopoietic tissue of transplant recipients. Using an unbiased and direct quantitative and statistical approach to evaluate the Y-chromosome-containing population of cells in female gender-mismatched hematopoietic cell transplant patients, we now provide evidence that cell fusion is one mechanism by which this occurs. In these patients, a genetic marker which should be exclusively found within hematopoietic lineage cells—the Y-chromosome—is detectable in cells of the epithelium. If this resulted exclusively from hematopoietic transdifferentiation or from intra-epithelial invasion of lymphocytes, as previously suggested, these cells should have a similar number of X-chromosomes as normal diploid male cells (Spyridonidis et al., 2004). However, within this population we find a statistically significant increase in the number of cells with 2 or more X-chromosomes. This indicates that these cells are derived from hematopoietic-epithelial cell fusion, since only fusion—and neither transdifferentiation nor intra-epithelial lymphocytic invasion—directly produces cells with an increased chromosome number. Many of the Y-chromosome-containing epithelial nuclei we identified in hematopoietic cell transplant patients had either zero or one X-chromosome. These cells could either be intra-epithelial donor lymphocytes, examples of hematopoietic transdifferentiation, fusion-derived cells that lost X-chromosomes during mitosis, or partial nuclei of fused cells that were incompletely analyzed due to tissue sectioning limitations.

It is important to note that our analysis was focused on determining whether hematopoietic-epithelial fusion occurs in human intestinal tissue, independent of the phenotypic outcomes of fusion. Similar to an analysis of fusion between hematopoietic cells and Purkinje neurons, we used a robust molecular marker independent method to classify cellular compartments (Weimann, Charlton, et al., 2003). Previously published studies have analyzed intestinal tissue for evidence of cell fusion by relying on cell type specific marker expression. However, restricting the identification of fusion events to cells that express specific markers—either hematopoietic or epithelial—may inadvertently exclude fusion-derived cells, biasing analysis. Of course, retrospective analysis of protein levels or gene expression in fusion-derived cells identified by more direct methods is important for understanding phenotypes of these cells and their impact on tissue physiology. Notably, we observed that at least a subset of XXY cells expressed the epithelial marker CK, suggesting that fusion-derived cells are likely phenotypically similar to adjacent epithelial cells (Figure 2.1B, inset). It remains unclear whether the combination of hematopoietic and epithelial genomes by cell fusion results in cells that are phenotypically identical to other epithelial cells. In mice, cells derived from fusion between hematopoietic and intestinal epithelial cells have altered gene expression patterns relative to normal epithelium (A. E. Powell et al., 2011). Similarly, hepatocytes derived from hematopoietic-hepatocyte cell fusion also are transcriptionally distinct from normal hepatocytes (Quintana-Bustamante et al., 2012). In the intestine, these transcriptional differences could result in altered levels of traditional epithelial

proteins, complicating cell identification based on molecular markers. Although we have previously performed sequence based transcriptional analysis of the cellular products formed by in vivo hematopoietic-epithelial fusion in mice, there is insufficient data at the single-cell level to predict the fates of these cells and the degree to which they have altered expression of lymphocyte or epithelial cell surface markers (A. E. Powell et al., 2011).

While the presence of donor cells with supernumerary X-chromosomes in the epithelium of hematopoietic cell transplant patients almost certainly reflects cell fusion, there are several less probable explanations. Our data could reflect the preferential epithelial invasion or transdifferentiation of hematopoietic cells with an increased chromosome number. Alternatively, it is also possible that lymphocytes which invade into the epithelium are prone to high rates of chromosome segregation errors, resulting in the gain of additional X-chromosomes. However, there is neither experimental support nor mechanistic basis for either of these possibilities. By contrast, there is strong evidence for cell fusion between hematopoietic and non-hematopoietic cells in multiple tissue types in mice and also for a degree of hematopoietic cell fusion with neurons in humans (Alvarez-Dolado et al., 2003; Camargo et al., 2003; Johansson et al., 2008; Rizvi et al., 2006; Vassilopoulos et al., 2003; Weimann, Charlton, et al., 2003). Therefore, our data and analyses demonstrate the occurrence of hematopoietic-epithelial fusion in the human intestine, providing the first definitive evidence for hematopoietic cell fusion with any non-neoplastic epithelial cell type in humans.

In mice, hematopoietic cells can fuse with proliferating intestinal stem cells, and our data suggest that the same occurs in humans (Davies et al., 2009; Rizvi et al., 2006). Specifically, the detection of individual cells with XXY and XXXY sex-karyotypes is highly consistent with these cells arising from the proliferation of fused cells. If hematopoietic fusion occurred with terminally differentiated epithelial cells, then this would produce binucleated cells. Each nucleus would remain diploid since these cells are post-mitotic and the only recognized path from binucleation to mononucleation is mitosis. The identification of a significant increase in the incidence of cells with 2 or more X-chromosomes per nucleus in Y-chromosome-positive epithelial cells in gender mismatched transplant patients therefore indicates that fusion occurs between hematopoietic lineage and progenitor cells within the proliferative zone of the intestine. Consistent with this, Y-chromosome positive epithelial cells with supernumerary X-chromosomes were detected within the proliferative crypt compartment as determined by lack of Ifabp expression, a marker of epithelial differentiation.

Establishing hematopoietic cell fusion with non-hematopoietic cell types in humans provides the basis for subsequent analysis of the physiological function and pathological potential of fusion between different cell types. In mice, hematopoietic fusion with non-hematopoietic cell types occurs in the absence of overt inflammation or tissue injury associated with hematopoietic transplant, and hematopoietic fusion with non-hematopoietic cell types in humans therefore likely occurs endogenously in the absence of disease (Davies et al., 2009; Johansson et al., 2008; Nygren et al., 2008). Currently, the phenotypes of fusion-derived

cells are poorly understood and therefore the physiological consequences of fusion between different cell types remain unclear. In the brain, fusion-derived neurons are rare and non-proliferative and so there is limited potential for the expansion of a population of these cells (Weimann, Charlton, et al., 2003; Weimann, Johansson, et al., 2003). It remains untested whether hematopoietic cell fusion with Purkinje neurons alters normal Purkinje function, and observations of an increased numbers of fusion-derived Purkinje neurons in ageing mice and in pathologic mouse and human brains provide the impetus for additional study (Diaz, Recio, Weruaga, & Alonso, 2012; Kemp, Gray, Wilkins, & Scolding, 2012; Weimann, Johansson, et al., 2003). In the intestine, there is high potential for a physiological impact of cell fusion. In mice, hematopoietic lineage cells fuse with intestinal epithelial progenitors, resulting in the long-term production of progeny of a single fusion event (Rizvi et al., 2006). We find that the same occurs in humans. Further, the gene expression profile of fusion-derived cells shows ongoing transcription of hematopoietic genes, suggesting these cells may be phenotypically unique (A. E. Powell et al., 2011). It is now critically important to understand the phenotypic consequences of spontaneous fusion between hematopoietic lineage and epithelial cells in order to understand the physiological relevance of hematopoietic-epithelial cell fusion in the intestine and other human tissues.

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Chapter III: Cell fusion potentiates tumor heterogeneity though acquisition of macrophage behavior

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Summary

The high fatality associated with metastatic cancer highlights the lack of biologic understanding of how cancer cells gain aggressive behaviors and contribute to tumor heterogeneity. Our data from in vitro and in vivo models provide definitive evidence that cell fusion between cancer cells and macrophages underlies one mechanism contributing to metastatic behavior of cancer cells. *In vitro*-derivation of macrophage- cancer cell fusion hybrids harbored shared genomes and displayed functional acquisition of macrophage phenotypes. *In vivo*-generation of fusion hybrids in mice and humans were readily detectible and resulted in the identification of a novel, unappreciated circulating tumor cell population that correlated with stage and overall survival. This exciting, paradigm-shifting discovery opens an innovative view of tumor evolution in the face of pressure from the microenvironment, leading to tumor diversity that can ultimately result in treatment failure.

Personal contribution

CEG's contribution to this chapter are as follows:

Figure 3.1, Supplementary Figure 3.1.
CEG analyzed stained sections of patient primary tumor samples to identify Y-chromosome-positive epithelium.

Figure 3.2.
CEG performed, co-culture experiments designed by ADS for imaging and sorting. Immunoblot and Edu-labelled co-culture experiments were performed by ADS.

Supplementary Figure 3.2.
Co-culture experiments were performed and imaged by CEG and identification of hybrids was performed by CEG and JG.

Supplementary Figure 3.3.
Co-culture experiments were performed and imaged by CEG. Experimental metastasis assays were performed by CEG. Transwell migration assays were performed by CEG. Boyden chamber assay was performed by SI and SC.

Supplementary Figure 3.4.
Karyotype analysis and microarray were performed by ADS. GO analysis was performed by JB.

Supplementary Figure 3.5.
Differential growth and cytokine response was performed by ADS. MEMA was performed by SW and JG. Scratch assay was performed by CEG.

Figure 3.3.
Tumor doubling time was performed by ADS. Experimental metastasis assays were performed by CEG. Transwell migration assays were performed by CEG. Boyden chamber assay was performed by SI and SC. Motility was assessed by CEG and BO. GO Chord analysis was performed by JB and CEG.

Figure 3.4.
In vivo mouse experiments and PCR were performed by CEG.

Figure 3.5.
In vivo mouse experiments were performed by CEG. Human CTC experimental design was performed by CEG. Human CTC quantification was performed by LZ and VP. Correlation with stage and survival was performed by CEG and JB.

CEG and MHW wrote the manuscript

Introduction

Metastatic cancer fails to respond to effective therapies utilized for primary tumors and therefore accounts for the vast majority of cancer-related deaths (Coghlin & Murray, 2010; NCI, 2016; Nguyen, Bos, & Massague, 2009; Talmadge & Fidler, 2010). It is generally accepted that primary tumor cells acquire behaviors that permit them to escape the primary tumor site, navigate circulation, and colonize in a metastatic site (Fidler, 2003; G. P. Gupta & Massague, 2006), but the underlying mechanism for this is not fully understood (D. Hanahan & L. M. Coussens, 2012; Nguyen et al., 2009; Talmadge & Fidler, 2010). Further, research is only beginning to define how, during the act of metastasis, cancer cells gain heterogeneous properties that complicate treatment effectiveness for late-stage cancer (Heppner, 1984; Marusyk & Polyak, 2010). Clearly, acquired mutations and altered epigenetic regulation rank as top mechanistic candidates contributing to metastatic behaviors, but this knowledge has not yet resulted in development of reliably effective therapeutics (D. Hanahan & L. M. Coussens, 2012). This may be due to the complex nature of tumors and the myriad of undiscovered microenvironmental influences that shape tumor behavior.

One possible microenvironmental impact on tumorigenesis concerns macrophage (MΦ)-cancer cell fusion. Cell fusion between immune and cancer cells is a century-old hypothesis (Carter, 2008; Pawelek, 2005) that has been circumstantially implicated (Lorico, Corbeil, Pawelek, & Alessandro, 2015; Pawelek, 2005; A. E. Powell et al., 2011), but not definitely demonstrated to

functionally affect tumor cell behaviors. Early in vitro studies revealed that cell fusion hybrids harbored more rapid cell cycling times when compared to either of their parental cell lines.(Islam, Meirelles Lda, Nardi, Magnusson, & Islam, 2006; Xue et al., 2015) Further, metastatic cancer cells expressing genes from immune MΦs (Lorico et al., 2015) was highlighted as evidence that aggressive metastatic cells resulted from fusion. Reports of cells that contained components of both immune cells and cancer cells have been increasingly frequent (Clawson et al., 2012; Orsolya Giricz, 2015; Patsialou et al., 2015; Sheng et al., 2014), but there lacks definitive evidence for physiologic relevance of these fusion hybrids in neoplastic disease. Despite this, fusion between a cancer cell and a migratory MΦ provides an attractive mechanism for how cancer cells can rapidly gain discrete cellular behaviors attributed to aggressive MΦ-like phenotypes that may facilitate metastatic spread of cancer, including extracellular matrix (ECM) remodeling, survival in circulation and seeding of distant metastatic sites. It could also explain one mechanism by which tumors gain cellular heterogeneity that contributes to treatment resistance. Here, we present the first systematic analysis of MΦ-cancer cell fusion. We also provide evidence that these cells may be physiologically relevant to tumor evolution and progression.

Cell fusion in human tumors

To establish that cell fusion occurs in human cancers, we exploited a system amenable to identifying a blood cell marker in the context of the tumor epithelium (Silk et al., 2013). Specifically, we analyzed tumor biopsies from female patients who previously underwent a sex-mismatched bone marrow transplant (BMT) but subsequently developed a secondary solid tumor. Tumor epithelia were identified with pan cytokeratin (CK) antibodies and interrogated with fluorescence *in-situ* hybridization (FISH) probes to the Y-chromosome to reveal fusion between the tumor cell and the transplanted male hematopoietic cell (Figure 3.1, Supplementary Figure 3.1). In a patient biopsy of pancreatic ductal adenocarcinoma (PDAC), cancer cells that contained nuclei harboring a Y-chromosome were readily detectible in regions of pancreatic cancer (Figure 3.1a-e, Supplementary Figure 3.1a, b), as well as in pre-neoplastic areas of pancreatic intraepithelial neoplasia (PanIN; Supplementary Figure 3.1c). Confocal analyses of these cells confirmed that the Y-chromosome was located in the nucleus of a CK-positive tumor cell (see higher magnifications in Figure 3.1). These Y-chromosome-positive tumor epithelial cells are not unique to PDAC, as they were also detected in various other solid tumors, including renal cell carcinoma, head and neck squamous cell carcinoma (HNSCC), and lung adenocarcinoma (Supplementary Figure 3.1d-f). These observations are consistent with previous reports of cell fusion in human cancer that used various detection methods (Lazova, Chakraborty, & Pawelek, 2011; Lorico et al., 2015), as well as our report of MΦ-cancer cell fusion in a mouse model of intestinal cancer

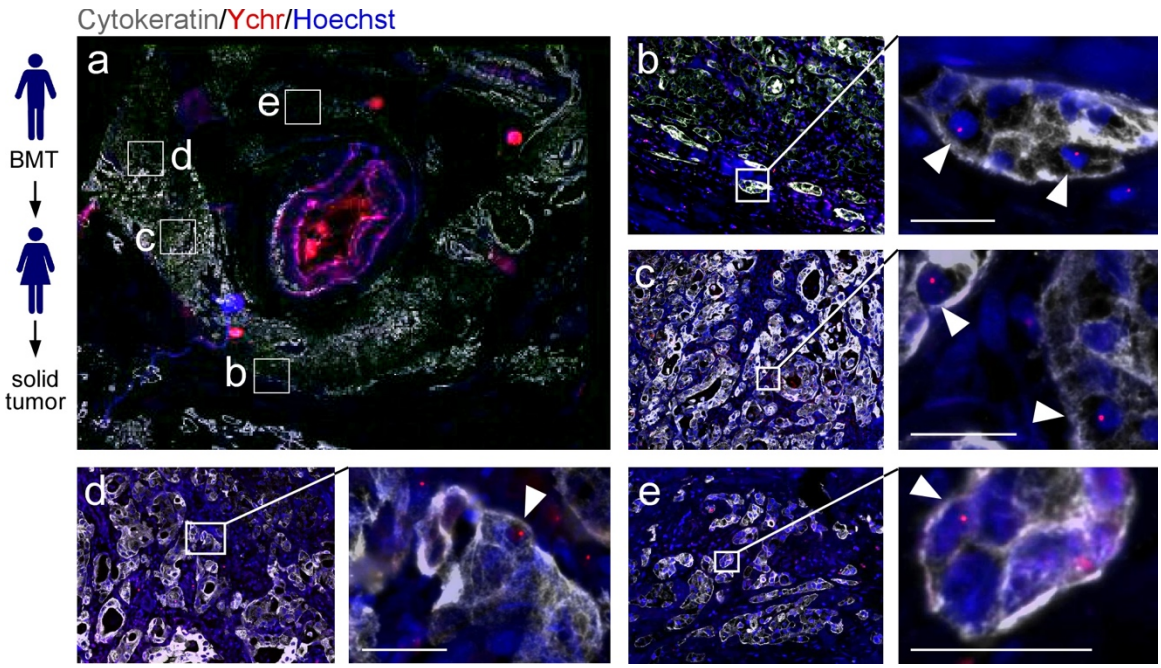
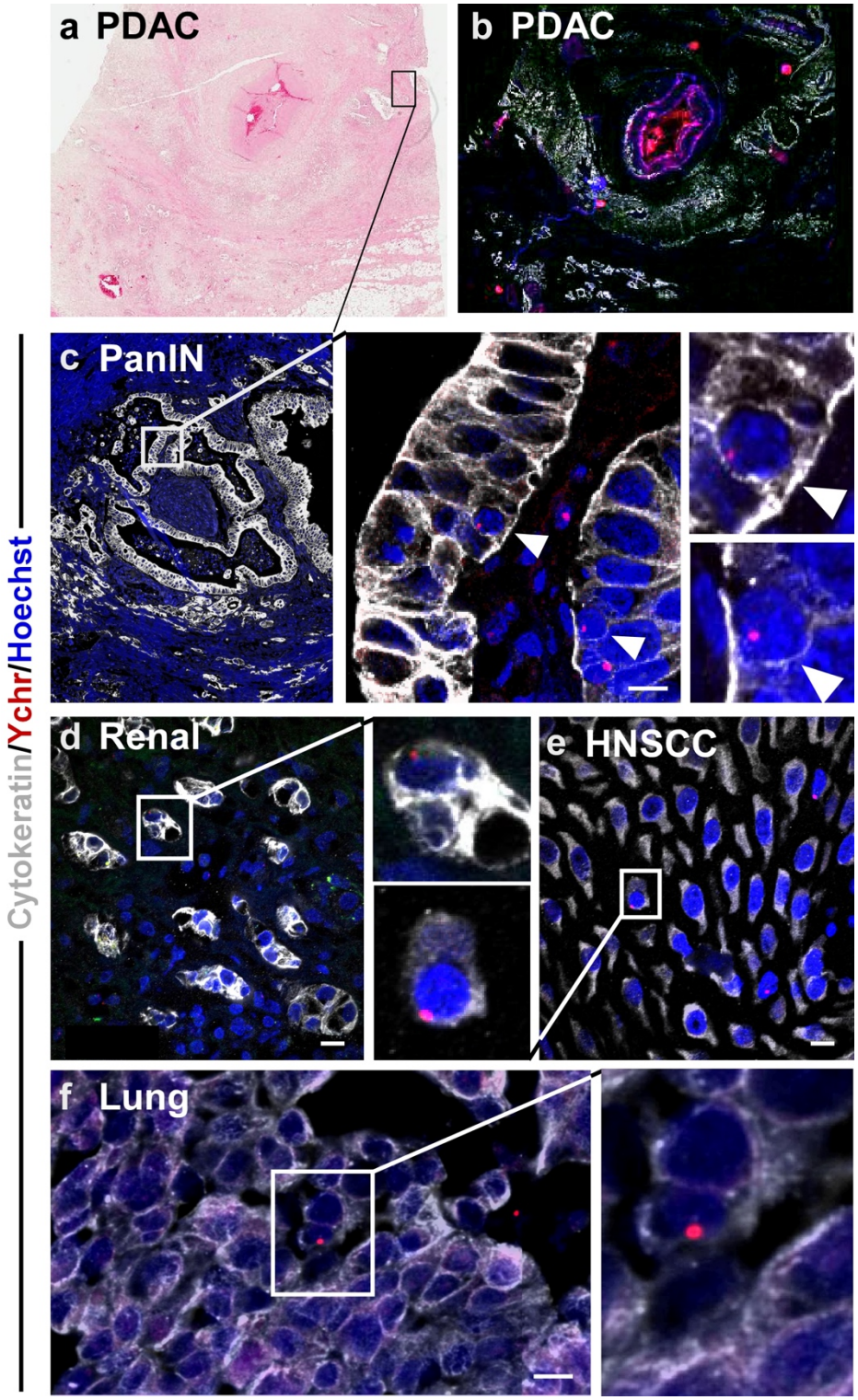


Figure 3.1. Cell fusion in human tumors.
Solid tumors from women with previous sex-mismatched bone marrow transplantation (BMT) permits analysis of cell fusion. (a) Pancreatic ductal adenocarcinoma tumor section with cytokeratin (gray), the Y-chromosome (Ychr, red) and Hoechst (blue) detection revealed areas of cytokeratin-positive cells with Y-chromosome-positive nuclei, white arrowhead. Representative areas boxed enlarged in b-e. Bar = 25 μm



Supplementary Figure 3.1. Cell fusion in PanIN and tumors from other organ sites.
Solid tumors from women with previous sex-mismatched bone marrow transplantation permits analysis of cell fusion. (a) Hematoxylin and Eosin stain of

pancreatic ductal adenocarcinoma (PDAC) section, (b) with cytokeratin (gray), the Y-chromosome (Ychr, red) and Hoechst (blue) detection. Boxed region enlarged in (c) contains pancreatic intraepithelial neoplasia (PanIN). (c-f) Cytokeratin-positive cells with Y-chromosome-positive nuclei, white arrowhead. Representative areas boxed in white are enlarged. Bar = 10 μm.

(A. E. Powell et al., 2011). This extensive analysis in human disease provides the foundation to explore the physiologic relevance of these intriguing fused cells in tumorigenesis.

***In vitro*-derived MΦ-tumor cell fusion hybrids display bi-parental lineage**

Based upon our previous discovery that MΦs are the most prominent fusogenic immune cell partner for the epithelial cell (A. E. Powell et al., 2011), we conducted *in vitro* validation and analyses of MΦ-cancer cell fusion hybrids. Two mouse cancer cell lines (colon adenocarcinoma, MC38, and melanoma, B16F10) were engineered to stably express a Cre recombinase and histone 2B fused to red fluorescent protein (H2B-RFP). In co-cultures, these cancer cells spontaneously fused with bone marrow-derived MΦs isolated from transgenic mice expressing Actin-GFP (Okabe, Ikawa, Kominami, Nakanishi, & Nishimune, 1997) or the Yellow fluorescent protein (YFP) Cre reporter (Srinivas et al., 2001) to produce MΦ-cancer fusion hybrids. Fusion hybrids were identified by their co-expression of nuclear RFP and cytoplasmic GFP, or YFP (Figure 3.2a, b, Supplementary Figure 3.2, Supplementary Figure 3.3a). Hybrid cells could be FACS- isolated with high purity based upon YFP expression (Figure 3.2c). To demonstrate the biparental lineage of these hybrid cells, we used three different approaches. First, MΦs labeled with 5-ethynyl-2'-deoxyuridine (EdU) prior to co-culture with cancer cells produced MΦ-cancer cell fusion hybrids that initially harbored two nuclei, one labelled with EdU, of MΦ origin, and the other expressing H2B-RFP, of cancer cell origin (Figure 3.2d). Importantly, upon first mitotic division, bi-nucleated hybrids underwent nuclear fusion and contained a single nucleus with both EdU-labelled and H2B-RFP-labelled DNA (Figure 3.2d). A second approach, using karyotype analysis of sex-chromosomes in male-isolated MΦs (XY) fused to cancer cells (XO), revealed that fusion hybrids

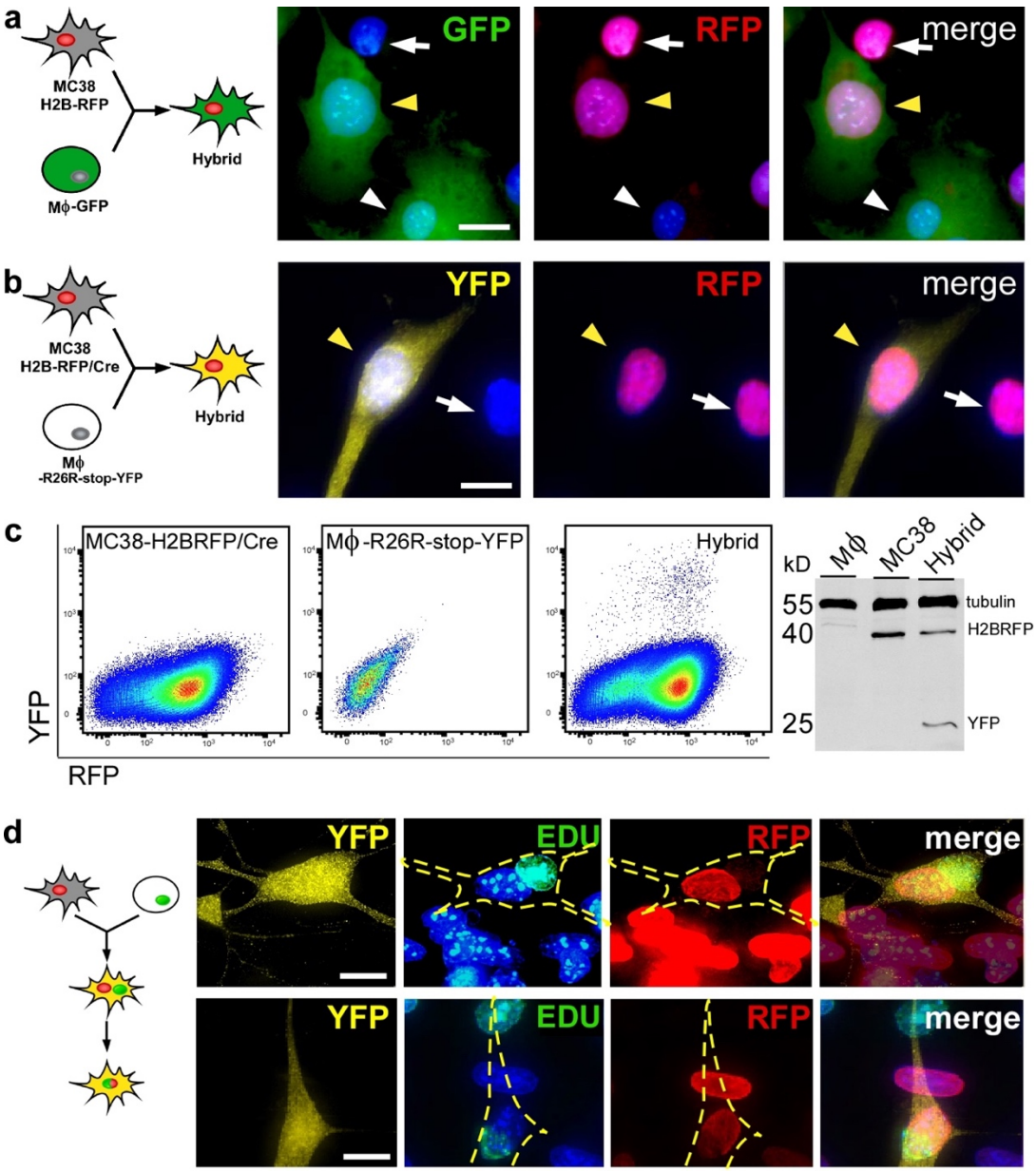
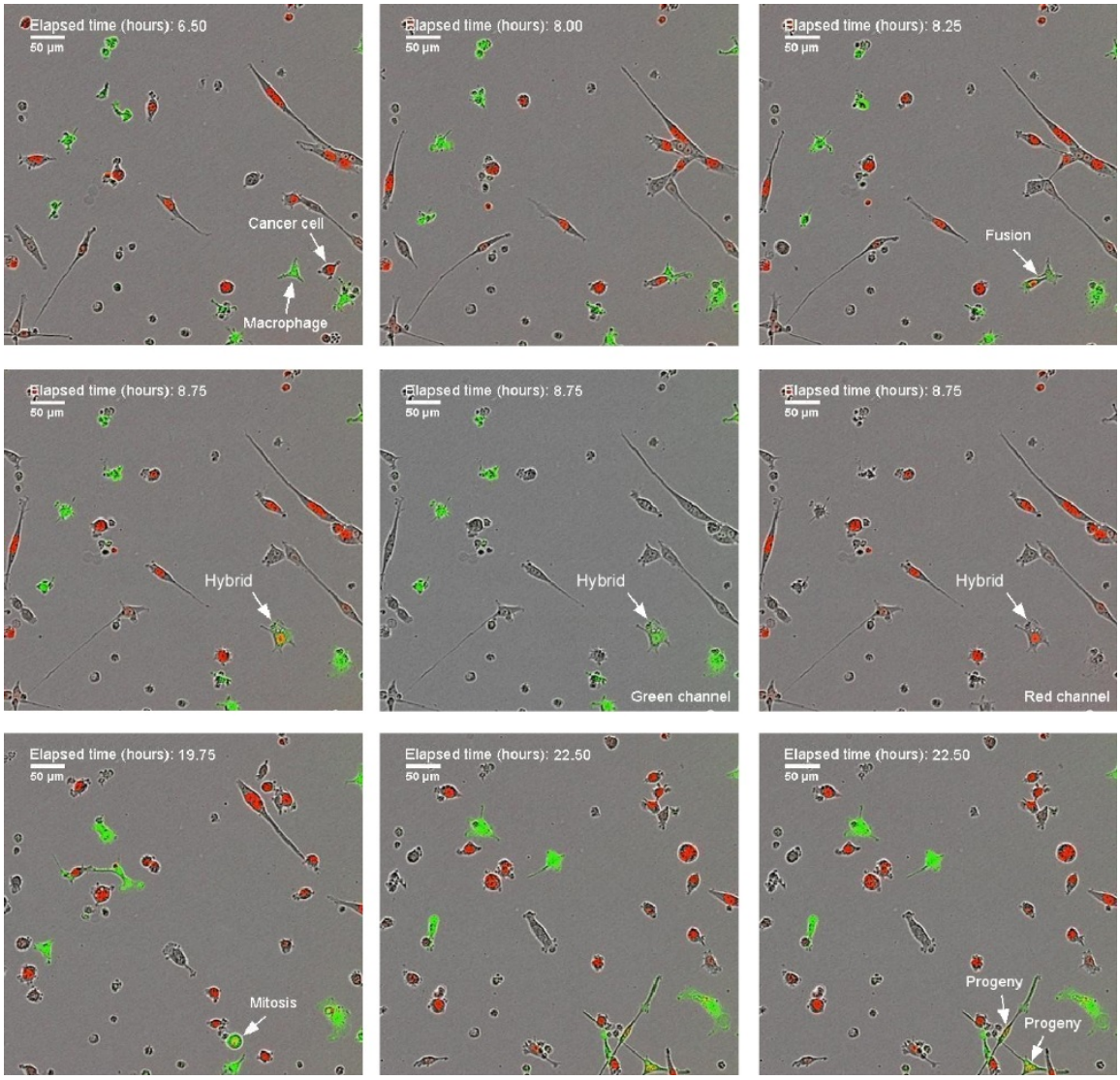
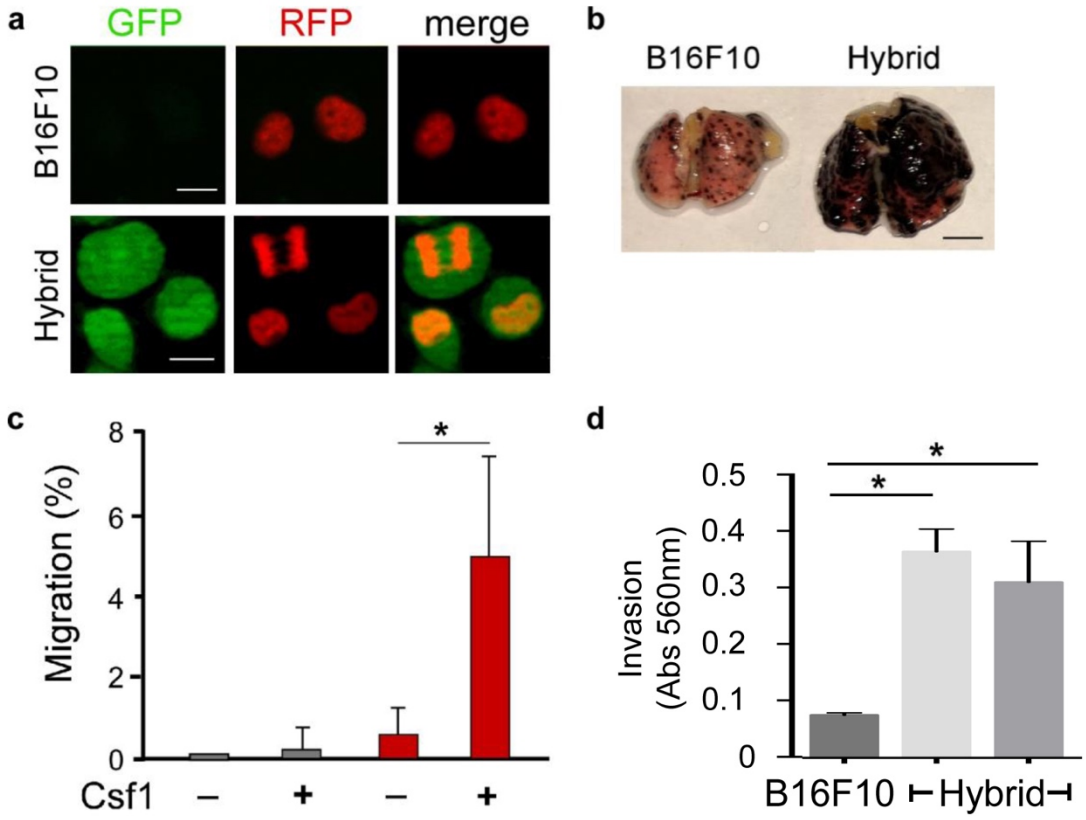


Figure 3.2. *In vitro*-derived MΦ-cancer cell fusion hybrids.
(a) MC38 (H2B-RFP) cancer cells co-cultured with GFP-expressing MΦs result in hybrid cells with RFP nuclei and GFP-expressing cytoplasm (yellow arrowhead) among unfused cancer cells (white arrow) and macrophages (white arrowhead). (b) MC38 (H2B-RFP/Cre) cancer cells co-cultured with MΦs expressing the cre reporter, R26R-stop-YFP results in YFP-expressing hybrid cells (yellow arrowhead). (c) YFP-expressing hybrids can be FACS-isolated to purify YFP-expressing hybrid cells confirmed by Immunoblot. (d) Co-cultured MΦs labeled with EdU (green) and MC38 (H2B-RFP/Cre) cancer cells produce YFP-expressing hybrids that initially harbor two nuclei—one from each parent, but upon mitotic division undergo nuclear fusion resulting in a single nucleus with EdU-labeled and RFP-expressing DNA. Hybrid cell outlined in yellow. Bar = 10 μm.

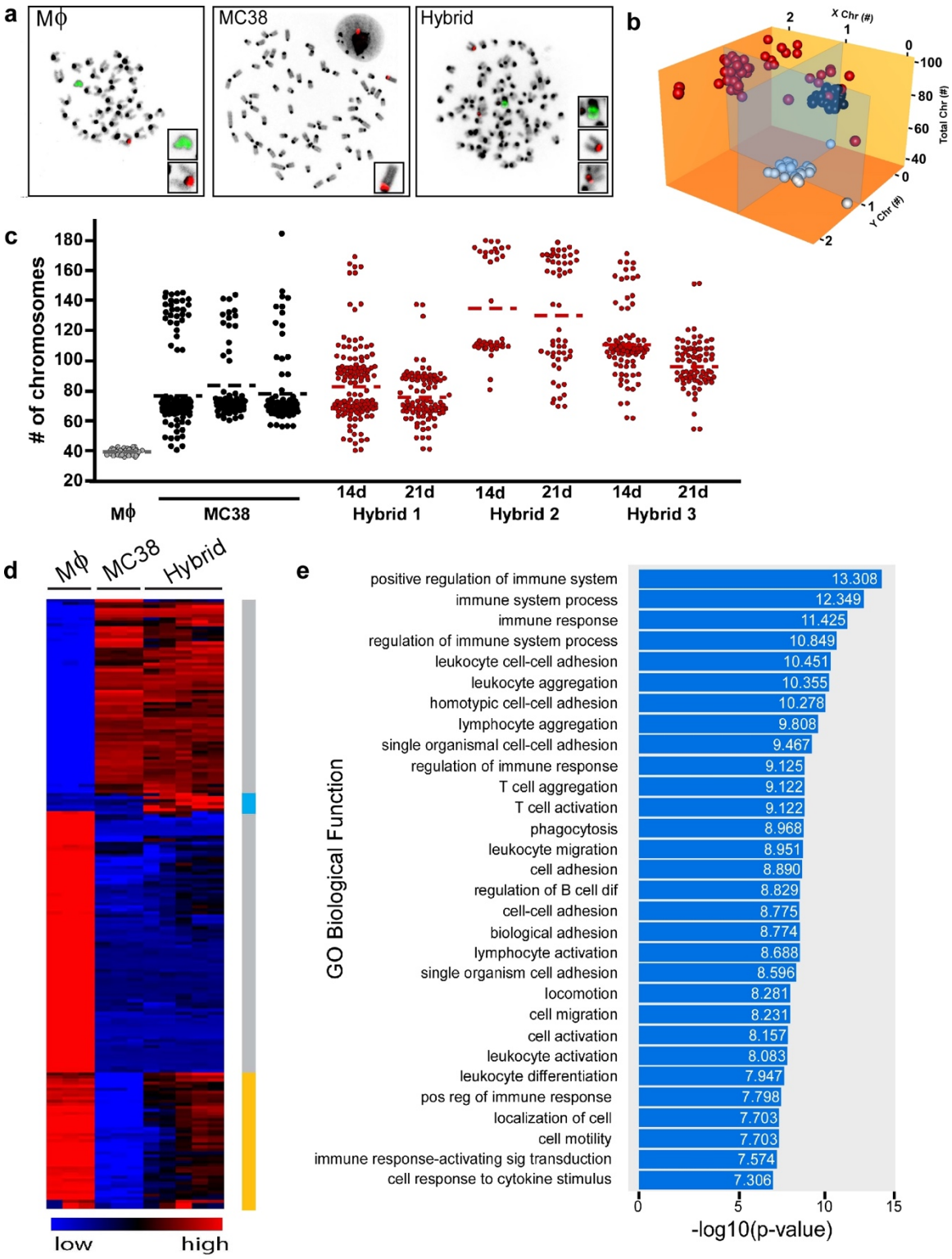


Supplementary Figure 3.2. Still images from cell fusion movie.
Nine panels taken from a video file displays an MC38 cell (red nucleus) fusing with a GFP- expressing MΦ, then undergoing mitotic division.



Supplementary Figure 3.3. B16F10-derived hybrid characterization.
(a) B16F10 (H2B- RFP) cancer cells co-cultured with GFP-expressing MΦs result in hybrid cells with RFP nuclei (red) and GFP-expressing cytoplasm (green). Unfused cancer cells only express RFP. Bar = 10 μm. (b) Lungs from mice injected with B16F10 cells and B16F10-derived hybrids. (c) Chemotaxis assays towards CSF1 ligand. Hybrid chemotaxis towards CSF1 is statistically significant relative to unfused B16F10 cells and cells without ligand after 36h (*$p < 0.037$). (d) Boyden chamber invasion assay into matrigel. Fixation and crystal violet staining was performed at 19h incubation. Invasion of B16F10 cells and two different hybrid isolates was quantified by Abs 560nm (*$p < 0.05$).

contained three sex chromosomes (XXY; Supplementary Figure 3.4a), consistent with fusion between the MΦ and cancer cell. Chromosome counting demonstrated that fusion hybrids clustered as a unique cell population defined by total chromosome number and sex-chromosome content (Supplementary Figure 3.4b). Interestingly, loss of chromosomes was observed in many of the fusion hybrids (Supplementary Figure 3.4b, c), suggesting fusion as a mechanism of rapidly amplifying tumor heterogeneity. Consistent with this observation, fusion hybrids analyzed after the fusion event contained chromosome numbers representing complements of both parent cells, but with continued passaging, the hybrids lost chromosomes before settling in a hyperdiploid state (Supplementary Figure 3.4c). Finally, transcriptome analysis of MΦ-cancer cell fusion hybrids revealed that these cells predominantly displayed cancer cell transcriptional identity, but also notably retained a MΦ gene expression signature (Supplementary Figure 3.4d). Interestingly, 5,827 genes were differentially expressed in MC38 cancer cells relative to both MΦ and cell fusion hybrids (Supplementary Figure 3.4d) and were clustered into GO Biologic functions that were attributed to MΦ behavior (Supplementary Figure 3.4e). Together, these findings support the notion that cell fusion between MΦs and cancer cells produces a distinct hybrid cell type that shares characteristics of both parental derivatives.



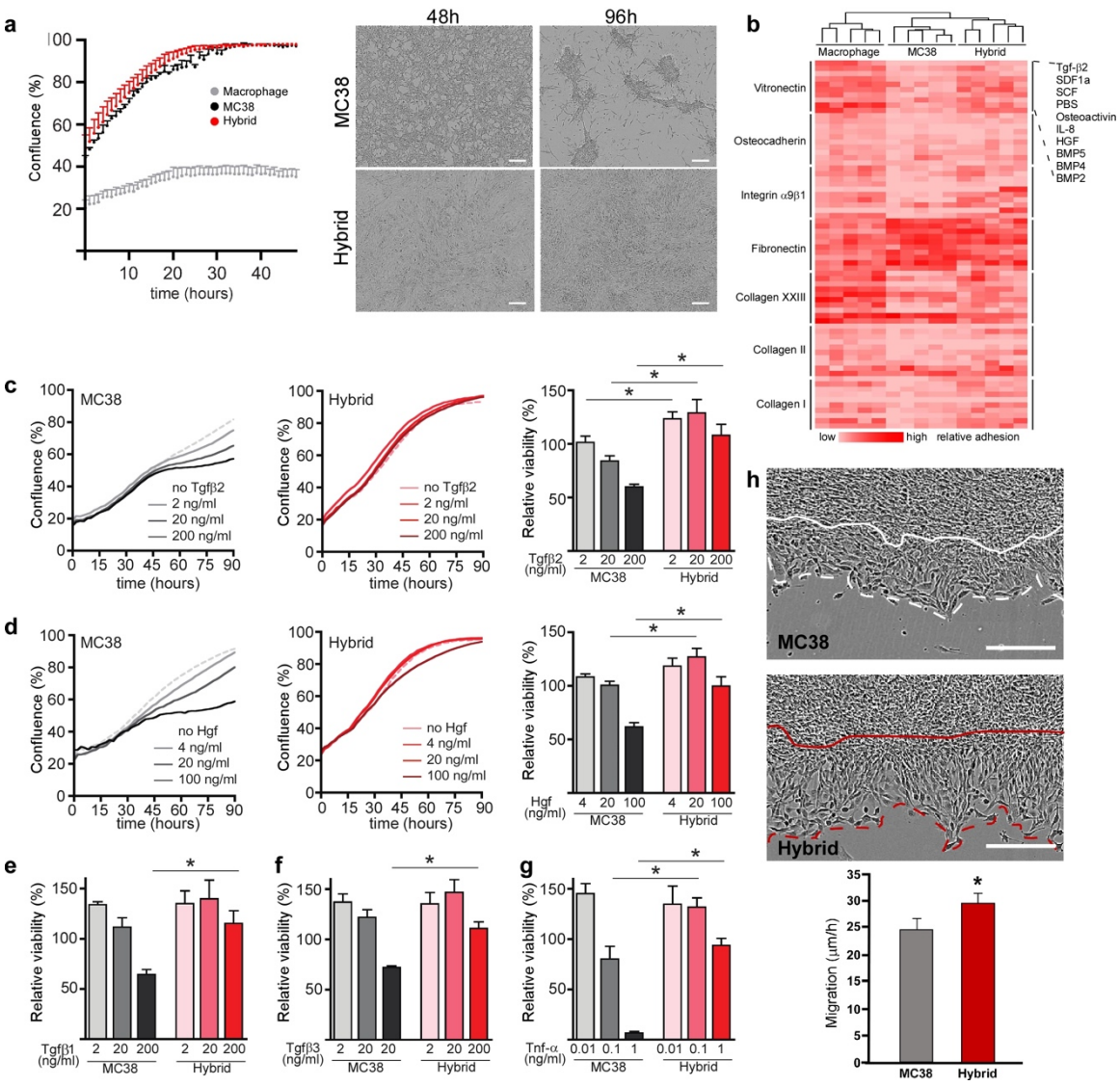
Supplementary Figure 3.4. Characterization of MC38-derived fusion hybrids.

(a) Karyotype and X- (red) and Y-chromosome (green) FISH analyses of parental macrophages (MΦ), unfused MC38 cancer cells and fusion hybrids. (b) Fusion

hybrids (red sphere) cluster as a unique population based on their chromosome number and sex chromosomes, relative to MΦs (white sphere) and MC38s (black sphere). (c) Karyotype analyses in MΦ, MC38 cells and 14d and 21d hybrids. Three hybrid isolates are shown. (d) Microarray analyses. Gray side bar marks hybrid gene expression that is similar to MC38 cancer cells, while blue and yellow bars denote gene expression unique from MC38 cells. Yellow bar marks hybrid gene expression that is similar to that in MΦs. (e) GO Analyses of differentially expressed genes in fusion hybrids versus MC38 cells that are similar to MΦ gene expression. Top 30 GO terms are displayed.

Fusion hybrids acquire MΦ-associated phenotypes

Despite acquiring a MΦ gene expression profile, MΦ-cancer cell fusion hybrids retained in vitro proliferative capacity similar to unfused cancer cells (Supplementary Figure 3.5a), dividing like cancer cells rather than like MΦs. However, with prolonged growth--past confluence--unfused cancer cells formed cellular aggregates, whereas the MΦ-cancer fusion hybrids remained sheet-like, reminiscent of a fibroblast growth pattern (Supplementary Figure 3.5a). This suggested that these hybrids might also have differential growth properties in an *in vivo* environment. Therefore, *in vitro*-derived hybrids from MC38 or B16F10 cells were injected into the flank or the dermis, respectively, of immune-competent mice. Indeed, fusion hybrids retained their tumorigenicity. Further, MC38 hybrids grew faster than unfused cancer cells (Figure. 3.3a), supporting the observation that these cells gained properties for differential growth in a physiologic environment. Further, when MC38-derived MΦ-cancer cell fusion hybrids were injected into the spleen, they trafficked to the liver and seeded metastatic foci at greater numbers than unfused cancer cells (Figure 3.3b), suggesting that fusion with MΦs provided an *in vivo* growth advantage. Likewise, B16F10-derived fusions injected retro-orbitally trafficked and grew more abundantly in the lung (Supplementary Figure 3.3). These findings align with our data identifying the fusion-associated increased expression of GO pathway genes implicated in metastatic spread (Figure 3.3c), in particular those pathways that contribute to tumor invasion (attachment, matrix dissolution and migration) as well as pathways involving response to specific microenvironmental



Supplementary Figure 3.5. Differential growth, adhesion and cytokine response in hybrids.

(a) Cell confluence relative to time for MC38, MΦ, and hybrid cells. Still images from 48 and 96 h timepoints. (b) Heatmap of relative adhesive preference for replicate MC38, MΦ and independent hybrid isolates determined by microenvironment microarray assay; hierarchical clustering according to relative preference for adhesion under 70 different microenvironmental conditions. (c) Mean cell confluence over time, and mean viability relative to untreated cells, for replicate MC38 and independent hybrid populations in the presence of increasing concentrations of Tgfβ2, (d) Hgf, (e) Tgfβ1, (f) Tgfβ3, and (g) Tnfα. *p<0.05, Student's t-test. (h) Scratch assay for relative migration of confluence MC38 and hybrid cultures. Quantification of migration over time. *p<0.024.

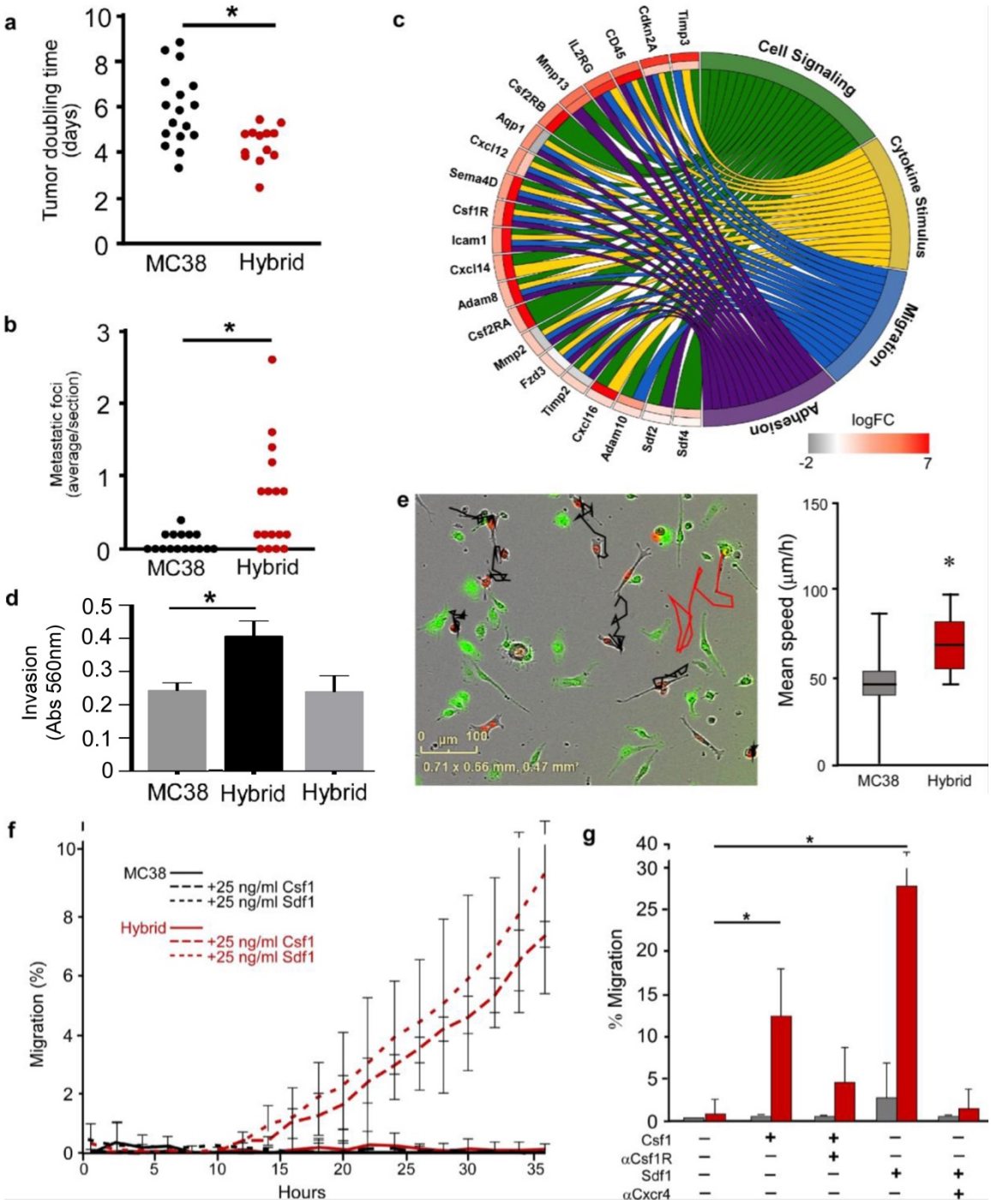


Figure 3.3. *In vitro*-derived fusion hybrid characterization.
(a) Proliferative analyses of MC38 cells and MC38-derived hybrids injected into the flank of an immune competent mouse. (b) Analyses of metastatic seeding of hybrids and MC38 cells injected into the spleen and analyzed in the liver. (c) GoChord display of key metastatic GO pathways and selected differentially regulated genes from the significantly enriched in hybrid versus MC38 comparison (Log2 fold-change). Outer ring denotes hybrid-MC38 and inner ring represents MΦ-MC38 comparisons. (d) Boyden chamber invasion assay into

matrigel. Fixation and crystal violet staining was performed at 19h incubation. Invasion of MC38 cells and two different hybrid isolates was quantified by Abs 560nm ($p < 0.05$) (e) Static portrayal of migration tracks from unfused MC38s (black) and a MC38-derived fusion hybrid (red) generated from live-imaged co-cultures. Mean speed of hybrids (red bar) relative to MC38s (gray bar) is statistically significant, $*p < 1.1\times10^{-9}$. (f) Chemotaxis assays towards CSF1 and SDF1 ligands. Hybrid chemotaxis towards CSF1 and SDF1 is statistically significant relative to unfused MC38 cells after 24h ($p < 0.05$). (g) Incubation of cells with antibodies to CSF1R and CXCR4 reduce migration of hybrids towards their ligand. $p < 0.05$ and 0.01 respectively. (Hybrid=red bar, MC38=gray bar).

cues (Bissell & Hines, 2011; Hoshino, Branch, & Weaver, 2013; Massague, 2008).

These observations led us to test the impact of different microenvironments (MEs) on tumor hybrid growth, because the evolving tumor ME provides discrete niches for a context-dependent selective advantage. Therefore, to directly test whether cell fusion altered a cancer cell's ability to respond to microenvironmental interactions, we evaluated adhesion phenotypes and cytokine-dependent growth responsiveness of MC38-derived fusion hybrids and parental cells on a microenvironment microarray (MEMA) (C. H. Lin, Lee, & LaBarge, 2012). This high throughput assay specifically measures cellular behavior in different MEs—ECM and growth factors spotted in combinatorial rows and columns—permitting the comparison of adhesion phenotypes of parental cancer cells, MΦs, and hybrids. Analysis of ME-specific adhesion showed that MC38 cells had a distinct growth factor-independent adhesive preference for discrete ECMs, such as fibronectin (Supplementary Figure 3.5b), and they also displayed enhanced Bone morphogenetic protein 2 (BMP2)- and Bone morphogenetic protein 4 (BMP4)-specific adhesion to Collagen II and Collagen XXIII (Supplementary Figure 3.5b). MΦs, by contrast, had a higher adhesion to Collagen XXIII and the ECM component vitronectin, and more uniform adhesion across all MEMA conditions relative to parental cancer cells (Supplementary Figure 3.5b). Interestingly, fusion hybrids displayed a combination of adhesion biases, reflecting properties of both parental cells. Further analysis, using hierarchical clustering, distinguished hybrids from

parental cancer cells with respect to adhesion on independent MEs (Supplementary Figure 3.5b).

To extend these observations, and to more directly test whether MΦ fusion could provide cancer cells with a selective growth advantage, we directly analyzed the growth effects of >90 different cytokines and soluble factors on MC38 and hybrid cells (not shown). A number of growth factors displayed differential influence on MC38 compared to hybrid cells, including Tgfβ1-3, which displayed a clear dose-dependent suppression of MC38 proliferation but had no effect on hybrids (Supplementary Figure 3.5c, e, f). Likewise, a moderate, dose-dependent growth-suppressing effect of Hepatic growth factor (Hgf) was apparent on MC38 cells but not on hybrids (Supplementary Figure 3.5d). More strikingly, hybrids were resistant to Tnfα, which had a profound inhibitory effect on the growth of MC38 cells (Supplementary Figure 3.5g). Resistance of hybrids to cytokine concentrations that suppressed MC38 growth clearly demonstrates the capacity of fusion to influence selectable phenotypes. These results confirm that MΦ fusion can alter cancer cell phenotypes and demonstrate that, under specific growth conditions, spontaneous fusion with MΦ provides cancer cells with a selective growth advantage.

To determine if cell fusion provides a mechanism by which cancer cells acquire MΦ phenotypes, we analyzed the acquisition of upregulated MΦ genes identified in fusion hybrids (Supplementary Figure 3.4d) from key pathways and biologic processes defined by GO terms that are associated with the metastatic process (Figure 3.3c). One upregulated gene set identified is involved in

modulation of the ECM and includes matrix metalloproteases (MMP2 and MMP9) that are capable of degrading type IV collagen, the most abundant component of the basement membrane (Kessenbrock, Plaks, & Werb, 2010; Sahai, 2005; Zeng, Cohen, & Guillem, 1999). Notably, degradation of the basement membrane in combination with directed chemotaxis, or invasion, is an essential step for tumor progression (Liotta et al., 1980). To determine if MΦ-cancer cell fusion hybrids gain functional invasion activity, we performed a boyden chamber invasion assay (Figure 3.3d). Subsets of hybrid cells displayed increased invasion relative to their unfused parental cancer cell lines (Figure 3.3d, Supplementary Figure 3.3d). Further, consistent with upregulated GO pathways related to cellular migration, *in vitro*-derived MC38-derived fusion hybrids migrated faster than unfused MC38 cells when analyzed in MΦ cancer cell co-cultures, and in scratch assays comparing MC38-derived fusion hybrids and their parental cell lines (Figure 3.3e, Supplementary Figure 3.5f). Together, these data demonstrate that MΦ fusion underlies one mechanism by which a cancer cell can gain functional cell behaviors commonly attributed to a MΦ and related to key behaviors--cell attachment, matrix dissolution and migration--that are associated with cancer cell invasion and metastasis.

GO genes involved in “response to stimulus” that are expressed at high levels in MΦs were also upregulated in MΦ-cancer fusion hybrids (Figure 3.3c). In particular, fusion hybrids harbored elevated expression of the MΦ-associated gene colony stimulating factor 1 receptor (CSF1R), which promotes differentiation and function of MΦs (Sherr & Rettenmier, 1986) —as well as

facilitates metastasis (DeNardo et al., 2011). Additionally, hybrids exhibited high expression of CXCR4, the receptor for the strongly chemotactic lymphocyte cytokine, stromal cell-derived factor 1 (SDF1). To determine if acquisition of gene expression translated to a functional migratory response to their ligand, we used a transwell chemotaxis assay coupled to live-imaging technology (Incucyte Chemotaxis, Essen). Under these conditions, fusion hybrids migrated towards the Colony stimulating factor 1 (CSF1) or SDF1 ligand at various concentrations (shown 25 ng/ml), whereas unfused MC38 cancer cells were incapable of responding to the chemoattractant, and B16F10 cancer cell hybrids had low response (Figure 3.3f, g, Supplementary Figure 3.3c). Notably, presence of ligand did not change proliferative dynamics of either fusion hybrids or unfused cancer cells (not shown); however, incubation with anti-CSF1R or anti-CXCR4 antibodies prevented the chemotactic response in the fusion hybrid (Figure 3.3g). Interestingly, some hybrid lines expressed both CSF1R as well as the CSF1 ligand. CSF1 over-expression in lung cancer has increased tumor cell proliferation and invasion (Hung et al., 2014) and its inhibition correlated with decreased tumor metastasis. Further, aggressive metastatic breast cancer frequently gains CSF1R expression (Patsialou et al., 2015). How tumor cells gain chemotactically responsive receptor expression is not entirely clear and there may be multiple mechanisms that underlie this change in transcriptional profile.

In vivo **generation of tumor cell fusion hybrids**

While our *in vitro*-derived fusion hybrids allowed for in-depth functional association of MΦ behaviors and the FISH analysis of human tumors demonstrate that cell fusion occurs *in vivo*, these studies do not provide insight into the role of fusion hybrids in the metastatic cascade. Therefore, to definitively demonstrate cell fusion in a mouse model of tumorigenesis, we subcutaneously injected MC38 cancer cells into the flank of R26R-YFP Cre reporter mice. In this system, fusion hybrids were identified as RFP^+/YFP^+ cells; which were detected among unfused tumor cells (RFP^+) by immunohistochemical analyses of the primary tumor (not shown). Orthotopic injection into the cecum, MΦ- MC38 fusion hybrids, however, resulted in pervasive peritoneal seeding and limited the utility of this model. We therefore opted to establish a more tractable system that allowed ease of tumor growth monitoring at an orthotopic site, specifically a melanoma model. Here, B16F10 melanoma cells injected into the dermis of a recipient mouse grew a 1 cm tumor (Figure 3.4a). Fluorescence analysis of the primary tumor revealed the presence of RFP^+/GFP^+ fusion hybrids (Figure 3.4b) and RFP^+/YFP^+ fusion hybrids (Supplementary Figure 3.6a). Primary tumor dissociation from B16F10 (H2B-RFP/Cre) cells injected into YFP-reporter mice followed by FACS-isolation (Supplementary Figure 3.6b) and quantification of YFP^+/RFP^+ fusion hybrids identified a rare presence of fusion hybrid cells among unfused tumor cells (<0.48%, Figure 3.4d). To determine the tumorigenicity and relative growth property of the fusion hybrids, 100

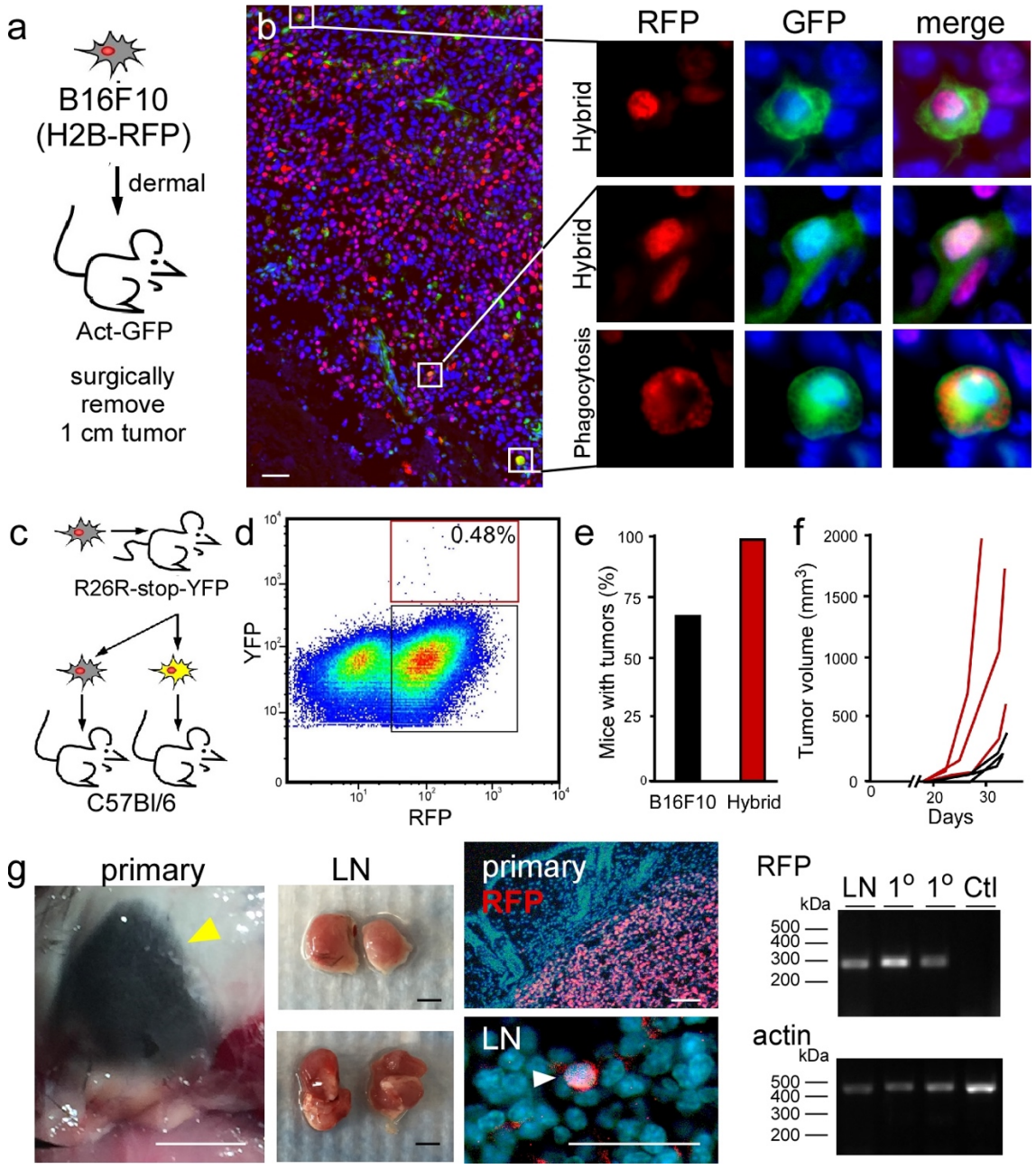
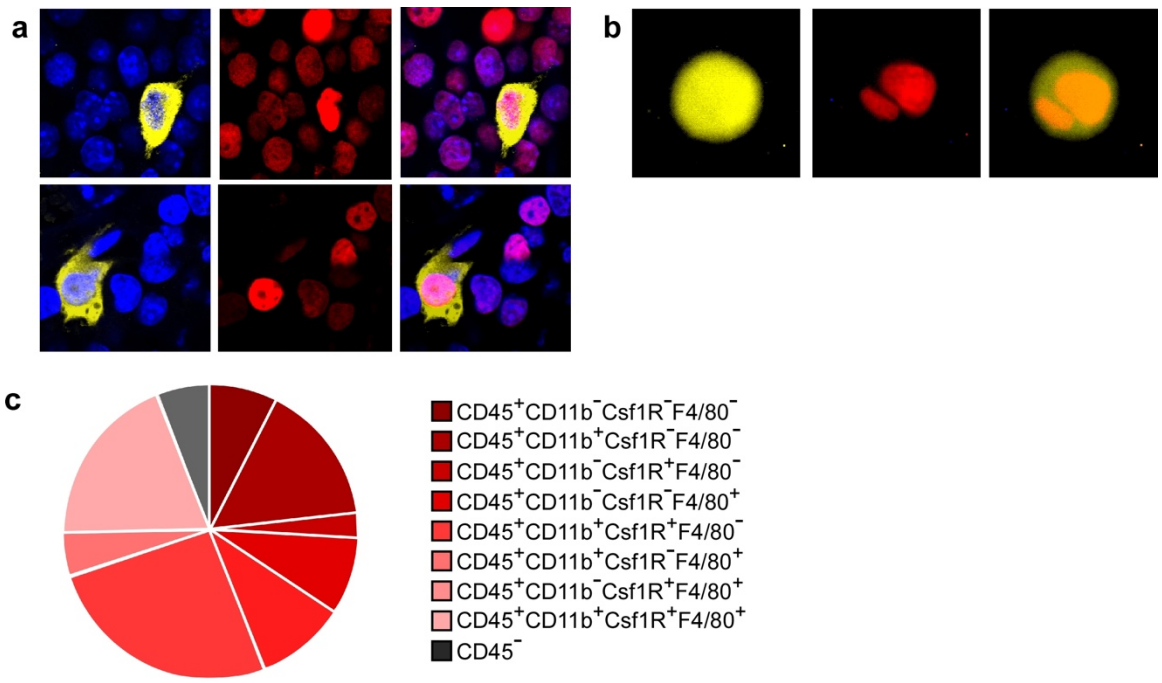


Figure 3.4. B16F10 *in vivo*-derived fusion hybrids.
(a) B16F10 (H2B-RFP) cells intradermally injected into GFP-expressing mice were harvested at 1 cm of growth. (b) Fluoresence analyses of tumor for RFP (red) and GFP (green) reveal double-positive hybrids and phagocytosed cancer cells with different nuclear morphology. Bar = 25 μm. (c) B16F10 (H2B-RFP/Cre) cells injected into R26R-stop-YFP transgenic mice. (d) Hybrid and unfused cancer cells from a dissociated tumor subjected to FACS--hybrids (red box) and unfused (gray box) cancer cells. (e) 100 FACS-isolated cells injected into wildtype secondary recipient mice analyzed for tumor growth at 40 days, and (f) 3,000 FACS- isolated cells injected into recipient mice and temporally monitored for growth. (g) Primary tumor (subcutaneous view, yellow arrowhead) and

metastatic lymph node (LN) growth of hybrid-derived-primary tumor. Both primary tumor and lymph nodes express RFP hybrid cells (white arrowhead) by fluorescence and PCR. Bar in gross = 5 mm, Bar in tissue section = 50 μm.



Supplementary Figure 3.6. Characterization of *in vivo*-derived B16F10 fusion hybrids.
(a) Representative confocal micrograph of B16F10-derived fusion hybrid in a primary tumor visualized for YFP (yellow), RFP (red). (b) Isolated hybrid circulating tumor cells from B16F10 injected mouse blood visualized for YFP and RFP expression. (c) FACS analyses of cell surface antigens on hybrid circulating tumor cells ($GFP^+/RFP^+/CD45^+$; red scale). Gray wedge denotes $GFP^+/RFP^+/CD45^-$ cells.

(RFP^+/YFP^+) *in vivo*-derived hybrid cells were reinjected into the dermis of each secondary recipient mice (Figure 3.4c), demonstrating that B16F10 hybrid cells retained tumorigenecity (Figure 3.4e). Of these injected mice, one mouse injected with B16F10-derived hybrids developed metastatic spread of disease (Figure 3.4g). To encourage tumor growth and assess tumor heterogeneity we collected sufficient numbers of fusion hybrids to allow robust tumor growth in additional animals. Surprisingly, the *in vivo*-derived fusion hybrids appeared to grow more rapidly than the unfused tumor cells (Figure 3.4f), but more importantly, the three mice, each injected with 3,000 cells, displayed a level of heterogeneous growth patterns, suggesting that MΦ cell fusion contributes to diverse tumor growth. Collectively, these data indicate that hybrid cells develop spontaneously *in vivo*, retain tumorigenic capacity, may exhibit accelerated tumor growth and can result in metastasis.

MΦ-tumor cell fusion hybrids are enriched in circulation

Detectible fusion hybrids in both primary and metastatic sites supported the possibility that fused cancer cells have gained the ability to traffic from the primary tumor to a distant metastatic site. To explore this biologic hallmark of the metastatic cascade, we revisited our experimental melanoma model, and collected blood from mice with established isogenic tumors (Figure 3.5a). Peripheral blood was subjected to flow cytometry for quantification of circulating tumor cells (CTCs). RFP^+/GFP^+ fusion hybrids were easily detectible, representing 90.1% of the CTCs, dramatically out-numbering unfused RFP^+ CTCs (Figure 3.5b). Fusion hybrid CTCs were still present in the circulation of tumor free animals, following surgical removal of the primary tumor, suggesting that these hybrid CTCs have long-term survival or that they were seeded by undetectable metastatic foci (data not shown). Imaging of collected individual fused CTCs confirmed their fusion identity and morphologically distinguished them from MΦs that had phagocytosed or adhered to a cancer cell (Figure 3.5b).

Importantly, the classical definition of CTCs in human cancer is a cell that expresses a tumor antigen (typically EpCAM or CK for epithelial cancers) and does not express the pan-leukocyte antigen CD45 (Fehm et al., 2002; Racila et al., 1998). MΦs normally express CD45, therefore we reasoned that MΦ-cancer cell fusion hybrids would also express this cell surface epitope and be excluded from conventional CTC isolation. Indeed, the majority of RFP^+/GFP^+ fusion hybrids expressed CD45, while unfused RFP^+ cancer cells largely did not (Figure 3.5c). Notably, isolated fusion hybrids displayed a diverse cell surface

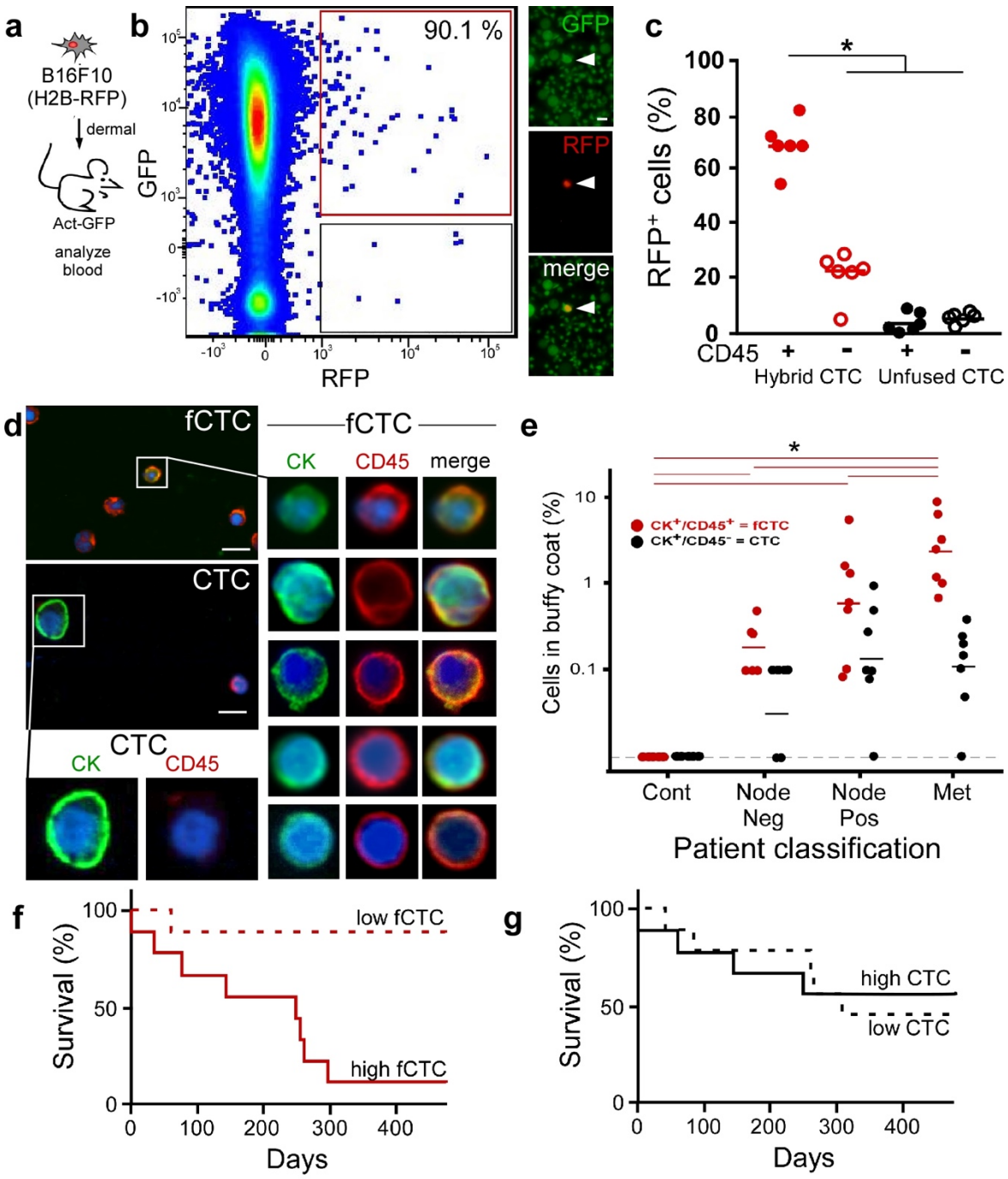


Figure 3.5. Circulating tumor cells.
(a) B16F10 (H2B-RFP) cells intradermally injected into a GFP-expressing mouse. (b) Blood collected at time of tumor resection, analyzed by flow cytometry for GFP and RFP expression. RFP^+GFP^+ cells were detectible in pre- sorted cell preparations by immunofluorescence. (c) Percentages of fusion hybrids (RFP^+/GFP^+) and unfused CTCs (RFP^+/GFP^-) expressing the leukocyte antigen CD45, * $p < 0.000002$. (d) Human pancreatic cancer patient peripheral blood analyzed for $Cytokeratin^+$ (green) and $CD45^+$ (red) expression using in situ

analyses and digital scanning. (e) $CK^+/CD45^+$ and $CK^+/CD45^-$ cells quantified in patient blood across cancer stage, *ANOVA $p < 0.023$. (f,g) Kaplan-Meier Curve of dichotomized biomarkers (fCTC and CTC) was associated with statistically significant increased risk of death for fCTC ($p = 0.0029$) but not for CTCs ($p = 0.95$). Bar = 50 μm.

expression of MΦ antigens (Supplementary Figure 3.6c). This prompted us to translate the identification of this novel tumor marker^{+}, CD45^{+} CTC population—which comprises the majority of the tumor cells in circulation—to human patients.

To evaluate CTCs in human cancer patients diagnosed with various tumor stages, we collected peripheral blood from patients with node-negative, node-positive or metastatic pancreatic cancer. We then performed *in situ* antibody staining (CD45, CK) on isolated leukocytes followed by digital image analyses (Figure 3.5d). This allowed us to validate the double-positive expression of CD45 and CK on "fused" CTCs and exclude doublets or clusters of cells. We found that the percentage of fused CTCs expressing CD45^{+}/CK^{+} significantly correlated with advanced disease (Figure 3.5e) and with overall survival (Figure 3.5f). Notably, conventionally defined CTCs (CD45^{-}/CK^{+}) did not correlate with stage or survival (Figure 3.5e,g) and were detected at an order of magnitude lower than fused CTCs in metastatic disease. These data identify a unique, under-appreciated population of tumor cells analogous to the MΦ-cancer cell fusion hybrid cells observed in our mouse models. Significantly, fused CTCs were indicators of disease stage in pancreatic cancer, indicating an avenue for the development of biomarkers for this aggressive disease.

Discussion

Together, our *in vitro* and *in vivo* data demonstrate that cancer cells fuse spontaneously with primary MΦs. They also indicate that this fusion process influences cancer cell genotypes in a manner that alters their physical behaviors in cellular processes that impact successful navigation along the metastatic cascade. We demonstrate that cell fusion produces tumorigenic cells that have increased MΦ-associated behaviors, specifically that fusion hybrids express functional levels of the MΦ gene, CSF1R. This finding has important implications for how cell fusions are generated and for how fusion hybrids may respond in the context of chemotherapy or combination treatment with inhibitors to CSF1R (DeNardo et al., 2011; Ngiow et al., 2016).

We also provide evidence that MΦ-cancer cell fusion hybrids are differentially modulated by their ME, as specific extracellular conditions provided a selective growth advantage to hybrids but not unfused cancer cells. These discoveries have implications for cancer progression, indicating that MΦ fusion with cancer cells provides a level of tumor cell heterogeneity that allows greater opportunity for positive- selection based on the evolving ME during tumor growth or in response to therapeutic treatment. Thus, MΦ-cancer cell fusion provides a previously unappreciated mechanism by which phenotypic diversity can be achieved within a population of cancer cells, increasing the chances that for any given selection pressure, highly fit subclones will be present within a tumor. Recent evidence strongly supports the occurrence of heterotypic fusion between hematopoietic lineage and cancer cells in humans (NCI, 2016); and although the

frequency of cell fusion in human cancers is unknown, we have demonstrated that this mechanism has clear potential to drive clonal expansion in the face of specific selective pressures, thereby contributing to the processes of tumor evolution and cancer progression.

Our studies have now provided an evaluation of MΦ-cancer cell fusion hybrids along the metastatic cascade; most significantly, we identified hybrid cells in peripheral circulation in mouse models of tumor progression and in human patients. Fused CTCs outnumbered unfused, conventionally isolated CTCs in both mice and humans. Notably, the extent of fusion-derived CTCs was highly correlated with tumor stage and overall survival.

It is not currently known whether MΦ-cancer cell fusions more efficiently leave the primary tumor site, as our data suggest. It is possible that fusion hybrids escape the primary tumor at rates equal to that of unfused tumor cells, but have enhanced survival in the circulation due to their immune heritage promoting immune evasion or other mechanisms. Regardless, these possibilities provide intriguing insights for future examination into immune surveillance of MΦ-tumor cell fusion hybrids, which could impact effectiveness of immune therapy.

Our studies demonstrate acquisition of biologic phenotypes of MΦ-tumor cell fusion hybrids that are consistent with properties of metastatic tumor cells, supporting that cell fusion is one mechanism that drives metastatic spread of disease. Further, we identified a novel population of CTCs that has largely been overlooked. We demonstrate biologic phenotypes of these fused hybrids and suggest their development as potent biomarkers linked to important physiologic

hallmarks of aggressive cancer. Overall, cell fusion as a mechanism for imparting heterogeneous behaviors linked to metastatic features, opens an exciting new area of biology of the metastatic cancer cell.

Contributions

C.E.G. and A.D.S. designed and performed experiments, analyzed data, and wrote the manuscript. L.R., L.Z., J.R.G., B.O., V.P., M.S., J.S., P.S.D., P.F., and S.W. designed and performed experiments and analyzed data. J.B. and J. B. performed biostatistics and analyzed data. B.S., J.G., and L.M.C. designed experiments and analyzed data. M.H.W. designed experiments, analyzed data, and wrote the manuscript.

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Extended methods

Human samples and ethics statement

All human blood and tissue samples were collected and analyzed with approved protocols in accordance with the ethical requirements and regulations of the OHSU institutional review board. Informed consent was obtained from all subjects. Peripheral blood was obtained from cancer patients diagnosed with pancreatic ductal adenocarcinoma at various stages and treated at Oregon Health & Science University (OHSU), as well as from healthy controls. Identification and acquisition of solid tumor biopsies from female patients that previously received a gender-mismatched bone marrow transplantation was conducted by screening of the Center for International Bone Marrow Transplant Registry (CIBMTR).

Fluorescence *in-situ* hybridization and immunohistochemical analyses of solid tumors

X- and Y-chromosome FISH probes were hybridized to 5 μm formalin-fixed paraffin embedded primary human tumor sections using CEP X (DXZ1 locus) and Y (DYZ1 locus) probes (Abbott Molecular, IL) following manufacturer's protocols. Briefly, tissue was treated with Retrievagen A solutions (BD Biosciences, CA), Tissue Digestion Kit II reagents (Kreatech, Netherlands) then hybridized with probe at 80 °C for 5 mins and 37 °C for 12 hr. Tissue sections were permeabilized with graded detergent washes at 24 °C, then processed for

immunohistochemical staining. Tissue was incubated with antibodies to pan-cytokeratin (Fitzgerald) and counterstained with Hoechst dye (1 μg/mL). Two slides were analyzed for each tumor section. Slides were digitally scanned and quantified by two independent investigators. Areas with Y-chromosome positivity were analyzed by confocal microscopy. Hematoxylin and eosin stain was conducted on adjacent sections.

In situ analyses of human peripheral blood

Patient peripheral blood was collected in heparinized vacutainer tubes (BD), then lymphocytes and peripheral mononuclear cells were isolated using density centrifugation and LeucoSep™ Centrifuge Tubes (Greiner Bio-One) according to manufacturer's protocol. Cells were then adhered to Poly-D-Lysine-coated slides, fixed with 4% paraformaldehyde for 5 min, then stained for CD45 and cytokeratin expression using antibodies to CD45 (eBioscience) and human pan-cytokeratin (Fitzgerald). Tissue was developed with fluorescent-conjugated secondary antibodies (anti-mouse Cy3; Jackson ImmunoResearch and goat anti-guinea pig 488; Invitrogen) then was stained with Hoechst (1μg/mL). Slides were digitally scanned with a Leica DM6000 B microscope and analyzed using Ariol® software. Manual quantification by three independent investigators of randomly selected regions containing 2,000 cells evaluated CD45 and cytokeratin status of Hoescht^{+} cells. Percentages of fused circulating tumor cells (fCTCs) in the buffy coat correlate with disease stage with significance determined by overall ANOVA post-test, $p < 6.3x10^{-8}$, (p-values: no nodal-met (0.00035), nodal-met (0.05), no

nodal-nodal (0.15), while none of the conventional circulating tumor cells (CTC; i.e. $CD45^-$) comparisons across stage were statically significant, p-values for no nodal-met (0.31), nodal-met (0.9). Survival analysis was conducted on 18/20 pancreatic patients (two were lost to follow-up) to correlate CTCs with time to death using Kaplan-Meier curve and log rank test using dichotomized biomarkers based on median value. High $CK^+/CD45^+$ (> 0.808, median) was associated with a statistically significant increased risk of death ($p = 0.0029$) with a hazard ratio of 8.31, but high $CK^+/CD45^-$ (> 0.101, median) did not have a statistically significant effect on time to death ($p = 0.95$).

Mice

All mouse experiments were performed in accordance to the guidelines issued by the Animal Care and Use Committee at Oregon Health & Science University, using approved protocols. Mice were housed in a specific pathogen-free environment under strictly controlled light cycle conditions, fed a standard rodent Lab Chow (#5001 PMI Nutrition International), and provided water *ad libitum*. The following strains were used in the described studies: C57BL/6J (JAX #000664), $Gt(ROSA)26Sor^{tm(EYFP)Cos}$/J (R26R-stop-YFP; JAX#006148)(Srinivas et al., 2001), Tg(act-EGFP)Y01Osb (Act-GFP; JAX #006567) (Okabe et al., 1997). Mice of both genders were randomized and analyzed at 8-10 weeks of age. When possible, controls were littermates housed in the same cage as experimental animals. The investigators were not blinded to allocation during experiments and outcome assessment.

Cell culture

MC38 mouse intestinal epithelial cancer cells were kindly provided by Jeffrey Schlom, (NCI, MD) and B16F10 mouse melanoma cells were obtained from the ATCC. Validation of cell lines were confirmed by PCR and by functional metastasis assay for the later. Cell lines and hybrids were negative for mycoplasma. Cell lines, both derived from C57BL/6J mice, were cultured in DMEM + 10% serum (Life Technologies, NY). Stable cancer cell lines, MC38(H2B-RFP), MC38(H2B-RFP/Cre, B16F10(H2B-RFP), and B16F10(H2B-RFP/Cre), were generated by retroviral transduction using pBABE-based retroviruses, and polyclonal populations were selected by antibiotic resistance and flow-sorted for bright fluorescence as appropriate. Primary MΦ derivation was conducted from the bone marrow of R26R-stop-YFP or Act-GFP mice. To elicit MΦs, cells were cultured for six days in DMEM + 15% serum supplemented with sodium pyruvate, non-essential amino acids (Life Technologies, NY) and 25 ng/ml CSF1 (Peprotech, NJ).

Cell fusion hybrid generating co-cultures were established in MΦ-derivation media without CSF1 for four days. MC38 or B16F10 cells and MΦs were co-seeded at a 1:2 ratio at low density. Hybrid cells were FACS-isolated for appropriate fusion markers on a Becton Dickinson InFlux or FACSVantage SE cell sorters (BD Biosciences, CA). FACS plots are representative of at least 20 independent MC38 or B16F10 hybrid isolates (technical replicates). Low passage hybrid isolates were established; functional experiments were conducted on

passage 8-20 hybrid isolates. Live-imaging of co-cultured cells were performed using an Incucyte Zoom automated microscope system and associated software (Essen Bioscience, MI). Technical triplicates generated 36 movies that covered 77.4 mm^2 and were screened for hybrid generation and division. Movie represents fusion event captured in one of 21 movies containing hybrids.

EdU-labeling and karyotype analysis

During hybrid generation. Cultured cells were fixed in 4% formaldehyde in PBS and processed for immunohistochemical analyses with antibodies against GFP (1:500; Life Technologies, NY) or RFP (1:1000; Allele Biotechnology, CA). 5-ethynyl-2'deoxyuridine- (EdU) labeling and detection was performed according to manufacturer directions (Life Technologies, NY). Briefly, MΦ DNA was labeled with 10 μM EdU supplemented in media for 24h prior to hybrid generation co-culture. 10 μM EdU was also used for determination of S-phase indices. N=6 biologic and technical replicates were conducted and screened for bi-parental hybrids.

For karyotype analyses. Chromosome spreads from cells in S-phase were prepared using standard protocols, from cells treated for >12 hours with 100 ng/ml Colcemid (Life Technologies, NY) to induce mitotic arrest. DNA was visualized by staining with DAPI; X- and Y- chromosomes were identified using fluorescently labeled nucleotide probes (ID Labs, Canada) as directed by the manufacturer. Images of stained fixed cells and chromosome spreads were acquired using a 40x1.35 UApo oil objective on a DeltaVision-modified inverted

microscope (IX70; Olympus) using SoftWorx software (Applied Precision, LLC), and represent maximum intensity projections of deconvolved z-stacks unless otherwise indicated. Experiments were replicated 8 times. Each biologic replicate was analyzed in an independent experiment. A minimum of n=20 cells were analyzed in each experiment. Chromosomes were counted manually by two independent investigators.

Gene expression analysis

Microarray analysis was performed with Mouse 430.2 gene chips (Affymetrix, CA) at the OHSU Gene Profiling Shared Resource and data were analyzed using GeneSifter software (Geospiza, WA) to identify relative expression differences between cell types (Replicates: MΦ, n=3; MC38, n=3; hybrids, n=5 independent isolates) and produce Gene Ontology analyses. Gene ontology category enrichment was calculated using the GOstats R package (Falcon & Gentleman, 2007) and visualized using functions from the GOplot R package (Walter, Sanchez-Cabo, & Ricote, 2015).

Code availability

Source code used to generate figures and corresponding tables is available for download from our public repository (Burkhart, 2016).

PCR

DNA was extracted from frozen formalin fixed melanoma primary tumor and lymph node sections by 40 min incubation in lysis buffer (25 mM NaOH, 0.2 mM EDTA pH 12) at 95 °C followed by neutralization with equal volumes of neutralization buffer (40 mM Tris-HCl pH 5). RFP primers: fwd 5'-CAGTTCCAGTACGGCTCCAAG-3' and rev 5'- CCTCGGGGTACATCCGCTC-3'. Actin primers: fwd 5'-GAAGTACCCCATTGAACATGGC-3' and rev 5'-GACACCGTCCCCAGAATCC-3'. Reactions were run with a 60 °C annealing temperature.

Microenvironment arrays

Recombinant proteins (R&D Systems, MN) (Millipore, MA) were diluted to desired concentrations in print buffer (ArrayIt, CA) and pair-wise combinations of extracellular matrix proteins and growth factors or cytokines were made in a 384 well plate. A Q-Array Mini microarray printer (Genetix, CA) was used to draw from the 384 well plate and print protein combinations onto Nunc 8-well chambered cell culture plates (Thermo Scientific, NY). Each combination was printed in quintuplicate in each array, and arrays were dried at room temperature. Printed MEMAs were blocked for 5 mins using 0.25% w/v F108 copolymer (Sigma-Aldrich, MO) in PBS, and then rinsed with PBS and media prior to plating cells. Cells were trypsinized, filtered to exclude cell clumps and counted; 10^5 cells were plated on each array in 2 ml of DMEM + 2.5% serum and incubated for 30 minutes in a humidified tissue culture incubator. Unbound cells were gently removed, and fresh media added; after 12 hours, arrays were fixed with 4%

formaldehyde in PBS for 10 mins and stained with DAPI. Adhesion was measured as relative cellular preference: the number of cells occupying a given microenvironment condition relative to the average cell number over all occupied microenvironmental spots across the entire MEMA for each sample. Five replicate samples each for MC38 cells and MΦ, and five independent MC38-derived hybrid isolates were analyzed. Standard two tailed t-tests were performed with $p < 0.05$ reported as significant. Error bars represent S.E.M.

In vitro-derived hybrid proliferation

For phenotypic profiling growth responsiveness to cytokines and soluble factors, 95 different cytokines or soluble signaling molecules were distributed at high, medium and low concentrations in 384 well plates, in 25µl of RPMI (Life Technologies, NY) supplemented with 1% FBS; and 25µl of a $1.2x10^4$ cells/ml suspension of hybrid or MC-38 cells in DMEM + 4% FBS was added to each well. 99 wells of each plate were left cytokine-free and no cells were added to two of these wells, which served to provide measurements of background signal. Plates were cultured in a humidified incubator for 72 hours, after which 5ul of MTS reagent was added to each well. Two hours later, absorbance at 490nm was read with a 384-well plate reader. For each plate, absorbance values for each cytokine-treated well were normalized to the mean absorbance of the cytokine-free wells on that plate, and expressed in terms of standard deviations from the cytokine-free mean. Three independent hybrid isolates and three MC38 replicates were analyzed. Cytokines or factors that showed a potential differential

effect on growth of MC38 and hybrid cells were re-tested in 96-well plates. In these experiments, $2.5x10^4$ hybrid or MC38 cells were plated in the presence of three different concentrations for each soluble factor, or in media alone (DMEM + 2.5% FBS), in triplicate for each condition. Plates were imaged every two hours for 90 hours, and then cell viability was assessed.

Chemotaxis assay

Chemotaxis assays were performed using IncuCyte™ Chemotaxis Cell Migration Assay (Essen) with at least three technical replicates of triplicate samples. Briefly, 1000 cancer cells were plated in the top wells in DMEM + 0.2% FBS after incubation in serum-free media for 20 h. CSF1 or SDF1 ligand (25 ng/mL) was added to the bottom well and cells were incubated at 37 °C for at least 36 hours with live-imaging. The neutralizing antibodies to the CSF1R (eBioscience), CXCR4 (Biolegend) and isotype control antibody were added to the top and bottom well (2.5 ng/μL). Migration was quantified by measuring phase contrast area of the top and bottom wells for each timepoint using IncuCyte ZOOM® software. Triplicates of each condition were performed, and the means and standard deviations were calculated. $p < 0.02$ for hybrids treated with CSF1 or SDF1 relative to hybrids without CSF1 or SDF1 by unpaired t-test. Two independent hybrid isolates were analyzed. Technical octupulicates (MC38) or sextuplicates (B16F10) with biologic quadruplicates or triplicates were analyzed. For inhibitor studies technical duplicates with biologic triplicates were analyzed.

Scratch Wound Assay

Cells were grown to confluence in 96-well plates and individual scratch wounds were made using an Essen® 96-well WoundMaker ™. Wound closure was monitored by live imaging from 2 to 14 hours post scratch and migration rate was determined with IncuCyte ZOOM® software. At least two technical replicates of triplicate samples was performed. $p < 0.024$ by unpaired t-test. Error bars represent s.d.

Migration Analysis

From IncuCyte live imaging of co-cultured MΦs and cancer cells, 24 to 48 h image series containing a cancer-MΦ fusion event was cropped and exported as two separate uncompressed Audio Video Interleave (AVI) files: one containing only the red channel for TrackMate analysis and another containing both red and green channels with a sizing legend. Red channel AVI files were imported into FIJI and converted to 8-bit image series with a mean filter of 1.5 pixels applied. TrackMate analysis was then performed on nuclei with an estimated diameter of 10 pixels and a tolerance of 17.5. Using the Linear Assignment Problem (LAP) Tracker, settings for tracking nuclei were as follows: 75.0 pixel frame to frame linking, 25.0 pixel and 2 frame gap track segment gap closing. Tracks segments were not allowed to split or merge. Using the analysis function in TrackMate, track statistics were exported to an excel file and tracks containing 11 or fewer frames were excluded from analysis. A total of 9 hybrid cells and 536 unfused

cells were analyzed with a $p < 1.1\times10^{-9}$ by unpaired t-test. Error bars represent s.d.

Boyden chamber invasion assay

Matrigel-containing Boyden transwell chambers (BD Biosciences) were preheated for 6 h prior to cell application. 3 x 10^5 cells (MC38-derived hybrids, B16F10-derived hybrids, MC38, and B16F10) were plated per transwell in 0.1 % FBS in the upper chamber with media containing 10% FBS in the bottom chamber. Cells which passed through the matrigel membrane 19h after plating were fixed with 0.4% Paraformaldehyde in PBS followed by staining with 0.09% crystal violet/10% ethanol. Optical density was measured at 560nm. All assays were conducted in quadruplicate.

***In vivo* analyses of *in vitro*-derived cell fusion hybrids**

For tumor growth, 8-12 week old C57BL/6J mice (Jackson, ME) were injected with 5×10^4 cells (MC38, MC38-derived hybrids) or $5x10^5$ cells (B16F10, B16F10-derived hybrids) subcutaneously or intradermally, respectively. Length (L) and width (W) of palpable tumors were measured three times weekly with calipers until tumors reached a maximum diameter of 2 cm. Tumors were surgically removed in survival surgery or animals were sacrificed during tumor removal in accordance with OHSU IACUC guidelines. Animals were observed for at least six months for detection of tumor growth. For each tumor, volume (V) was calculated by the formula $V=½(L\times W^2)$; volume doubling time for each tumor was extracted

from a curve fit to a plot of log tumor volume over time. Curves with R2 values of less than 0.8 were excluded from analysis, as were tumors with six or less dimension measurements; these exclusion criteria were established in response to the unanticipated early ulceration of some tumors, which precluded accurate measurements of length and width, $p < 0.05$, by Mann-Whitney U test. At least 13 animals per group were used to identify a difference in tumor doubling time between groups with an α=0.05 to confer a power=79% For growth of tumor at metastatic sites, 1×10^6 MC38 cells were injected into the spleen. Livers were analyzed 3 weeks later for tumor burden by Hematoxylin and Eosin stain. Hybrids formed metastatic foci more readily with a $p < 0.008$ by Mann-Whitney U Test. N=16 (MC38) and n=17 (MC38-derived hybrids) were injected in four different technical replicate experiments. At least 16 animals per group were used to identify a difference in number of metastatic foci between groups with an α=0.05 to confer a power=85%. For B16F10 cells, $2.5x10^5$ cells were retro-orbitally injected and lungs were analyzed 16 days post-injection. Melanin marked tumor metastasis were visualized. Duplicate studies of n=3 (B16F10 and B16F10-derived hybrids) were analyzed.

***In vivo*-derived cell fusion hybrids** For isolation of *in vivo*-derived hybrids or assessment of circulating tumor cells, 5×10^5 B16F10(H2B-RFP with or without Cre) cells were injected intradermally into R26R-YFP or Actin-GFP mice respectively. Once tumors reached 1-2 cm in diameter, it was surgically removed for immunohistochemical analyses or for FACS/flow analyses.

Immunohistochemical analysis of *in vivo*-derived tumors.

B16F10 (H2B-RFP, Cre) primary tumors in Act-GFP or R26R-stop-YFP mice were fixed in 10% buffered formalin, frozen in OCT and 5μm sections were obtained. Tumors from R26R-stop-YFP mice were incubated with antibodies for GFP (1:500; Life Technologies, NY) followed by detection with fluorescent secondary antibody (1:500, Alexa488, Jackson Immuno Research). Nuclei were counterstained with Hoechst (1 μg/mL). Slides were digitally scanned with a Leica DM6000 B microscope and analyzed using Ariol® software. Confocal images were acquired with a FluoView™ FV1000 confocal microscope (Olympus).

FACS-isolation and flow cytometric analyses of fusion hybrids. Tumors were diced, and digested for 30 minutes at 37 °C in DMEM + 2 mg/mL Collagenase A (Roche) + DNase (Roche) under stirring conditions. Digested tumor was filtered through a 40 μm filter and washed with PBS.

For FACS-isolation, hybrid and unfused cells were isolated by direct fluorescence on a Becton Dickinson InFlux sorter.

For flow cytometric analysis, blood was collected retro-orbitally using heparinized micro-hematocrit capillary tubes (Fisher) into K_2EDTA-coated tubes (BD). RBC lysis was performed by a 1 minute incubation in 0.2% NaCl followed by addition of the equivalent volume of 1.6% NaCl. Cells were washed and resuspended in FACS Buffer (PBS, 1.0 mM EDTA, 5% FBS). Cells were incubated in PBS containing Live Dead Aqua (1:500, Invitrogen) with Fc Receptor Binding Inhibitor (1:200, eBioscience). Cells were then incubated in FACS buffer for 30 min with

CD45-PeCy7 (1:8000, Biolegend), CSF1R-BV711 (1:200, Biolegend), F4/80-APC (1:400 Biolegend), CD11b-AF700 (1:200, eBioscience). BD Fortessa FACS machine was used for analyses. Statistical significance of $p < 2.2\times10^{-6}$ by unpaired t-test was determined for $CD45^+$ hybrid CTCs relative to $CD45^-$ hybrid, $CD45^+$ unfused, and $CD45^-$ unfused CTCs. Technical duplicates of n=5 or 6 mice were analyzed.

Tumorigenic analyses of FACS-isolated *in vivo*-derived hybrids. A total of 100 or 3,000 FACS-isolated hybrids and unfused B16F10 cells were reinjected intradermally into C57BL/6J mice. Technical octuplicates were performed. Biologic duplicates, triplicates or quadruplicates were analyzed, dependent upon the number of hybrids isolated from the primary tumor.

Statistical analyses and graphical displays

Dotplots, bar charts and line charts were generated in GraphPad Prism or Excel, which was also used for statistical analyses of these data, including ensuring that data met assumptions of the tests used and comparisons of variance between groups when appropriate. Microscoft Excel was used to perform 2-tailed t-tests. A three-dimensional scatterplot was generated in R using the rgl package. Flow cytometry data were prepared for display using FlowJo software. Microarray gene expression data were displayed as a heatmap prepared using Genesifter software. Heatmap of MEMA data was generated in R using the standard heatmap function and default parameters.

Chapter IV: Surgical procedures and methodology for a preclinical murine model of de novo mammary cancer metastasis

This manuscript was resubmitted to the *Journal of Visualized Experiments.* Gast CE, Shaw AK, Wong MH, Coussens LM. September 29, 2016.

Short abstract

Pre-clinical models evaluating adjuvant therapy targeting breast cancer metastasis are lacking. To address this, we developed a murine model of de novo pulmonary mammary adenocarcinoma metastasis, wherein therapies administered in the adjuvant setting (post surgical resection of primary tumors) can be evaluated for efficacy in impacting previously seeded pulmonary metastases.

Long abstract

A rate-limiting aspect of transgenic mouse models of mammary adenocarcinoma is that primary tumor burden in mammary tissue typically defines study end-points. Thus, studies focused on elucidating mechanisms of late-stage de novo metastasis are compromised, as are studies examining efficacy of anti-cancer therapies targeting mediators of metastasis in the adjuvant setting. To address these deficiencies, we developed a murine model of de novo mammary cancer metastasis, wherein primary mammary tumors are surgically resected, and metastatic foci subsequently develop over a 115 day post-surgical period. This long latency provides a tractable model to identify functionally significant regulators of metastatic progression in mice lacking primary tumor, as well as a model to evaluate preclinical therapeutic efficacy of agents aimed at blocking functionally significant molecules aiding metastatic tumor survival and growth.

Numerous murine mammary cancer models have been developed via targeted expression of dominant oncoproteins to mammary epithelial cells yielding models variably mimicking histopathologic and transcriptome-defined breast cancer subtypes common in women (Fantozzi & Christofori, 2006). While much has been learned regarding the biology of mammary carcinogenesis with these models, their utility in identifying molecules regulating growth of late-stage metastasis are compromised as mice are typically euthanized at earlier time points due to significant primary tumor burden. Moreover, that a significant percentage of women diagnosed with breast cancer receive adjuvant therapy

after surgical resection of primary tumors and prior to presence of detectable metastatic disease, preclinical models of de novo metastasis are urgently needed as platforms to evaluate new therapies aimed at targeting metastatic foci. To address these deficiencies, we developed a murine model of de novo mammary cancer metastasis that mimics human breast cancer metastasis, and as such, also provides a preclinical platform for evaluating efficacy of therapies delivered in the adjuvant setting.

Personal contribution

CEG's contribution to this chapter are as follows:

Figure 4.1, Supplementary Figure 4.1.
CEG designed and implemented the post-surgical resection of primary tumors and development of de novo pulmonary metastasis model.

Figure 4.2.
CEG designed and implemented isolation, perfusion and fixation of the lung.

CEG wrote the manuscript.

Introduction

Women in the North America have a ~12% lifetime risk of developing breast cancer (Howlader N); a majority of these individuals will have primary tumors removed via surgery, and depending on cancer subtype, will then receive targeted, endocrine, chemo- and/or radiation therapy in the adjuvant setting (NCCR). For example, women diagnosed with hormone receptor-positive cancers receive anti-estrogen therapies to block estrogen-regulating signaling or aromatase inhibitors, whereas women with human epidermal growth factor receptor 2 (HER2)-positive tumors are typically given various HER2-targeted therapies with radiation/chemotherapy, whereas no targeted therapies are yet available for triple negative tumors (NCCR). Despite advances in radiation, chemotherapy, personalized and hormone-based therapies that supplement surgical resection, disease recurs in 30-70% of women diagnosed with stage II or III disease (Kataja, Castiglione, & Group, 2008), as current therapies are largely ineffective in eradicating metastatic disease in distant organs, including lung, bone, brain and/or liver . This is especially significant given that when metastatic disease occurs in the absence of primary tumor regrowth, this implies that disseminated malignant cells were likely already present in secondary organs at the time of definitive surgery. Thus therapies able to eradicate or slow growth of metastatic tumors are urgently needed.

While de novo mouse models of mammary carcinogenesis have been remarkably informative in revealing mechanisms regulating neoplastic progression (Fantozzi & Christofori, 2006), existing models also have several

limitations. One of these is the fact that de novo transgenic models typically develop primary tumors in multiple mammary glands, wherein primary tumor burden limits duration of studies. While primary tumor cell escape and metastatic seeding likely occur early in neoplastic progression in these models, frank development of metastatic tumors occurs late, and depending on the mouse model and strain background, is often partially penetrant (Fantozzi & Christofori, 2006). This further limits the utility of de novo models for discovery of molecules regulating metastasis in secondary organs, and for evaluating preclinical efficacy of therapeutics in the adjuvant setting.

To circumvent these limitations, we developed a de novo autochthonous model of mammary carcinoma metastasis to lungs. Parental transgenic females (e.g., MMTV-PyMT on the FVB/n strain background for studies described herein) bearing late-stage de novo mammary tumors are aged to ~100 days (Guy, Cardiff, & Muller, 1992), at which point their primary tumors surgically resected and enzymatically dissociated into single cell suspensions. Suspensions ($1x10^6$ cells) are in turn orthotopically explanted into 6-7 week old recipient syngeneic female mice, where single primary mammary tumors develop over a 38 to 60 day period (Figure 4.1A). At a defined tumor size (172 to 450 mm^3), recipient mice are anesthetized and primary tumors surgically resected such that tumor regrowth at the surgical site is minimized, consistent with surgery in women (Supplementary Figure 4.1). On the FVB/n strain background, mice develop histologically-detectible metastatic foci in lungs with 45% penetrance by ~115 days post-surgery (Figure 4.1B). With this extended latency of metastatic tumor

growth, the model is uniquely positioned for adjuvant therapy delivery, and for elucidating and evaluating underlying biology influencing metastatic progression following surgical removal of primary tumors.

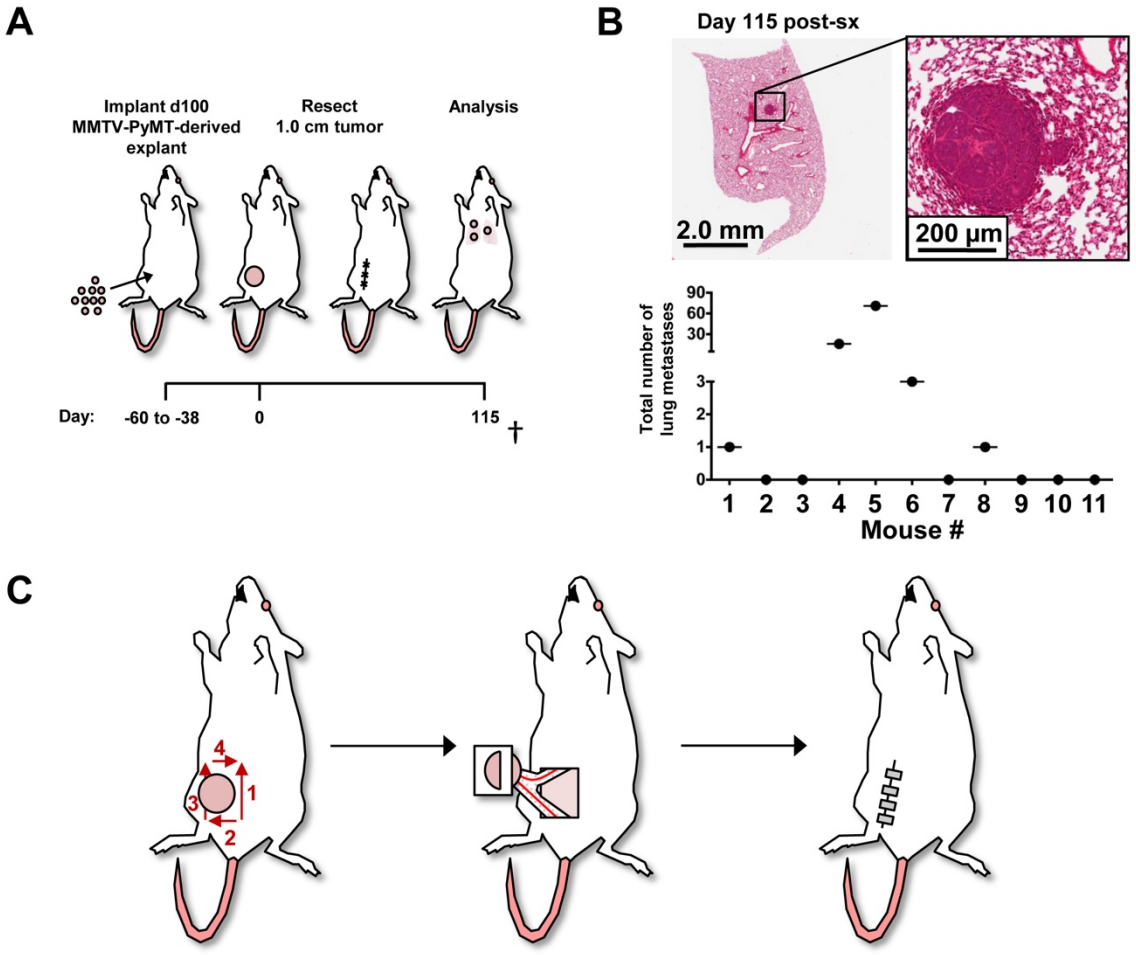
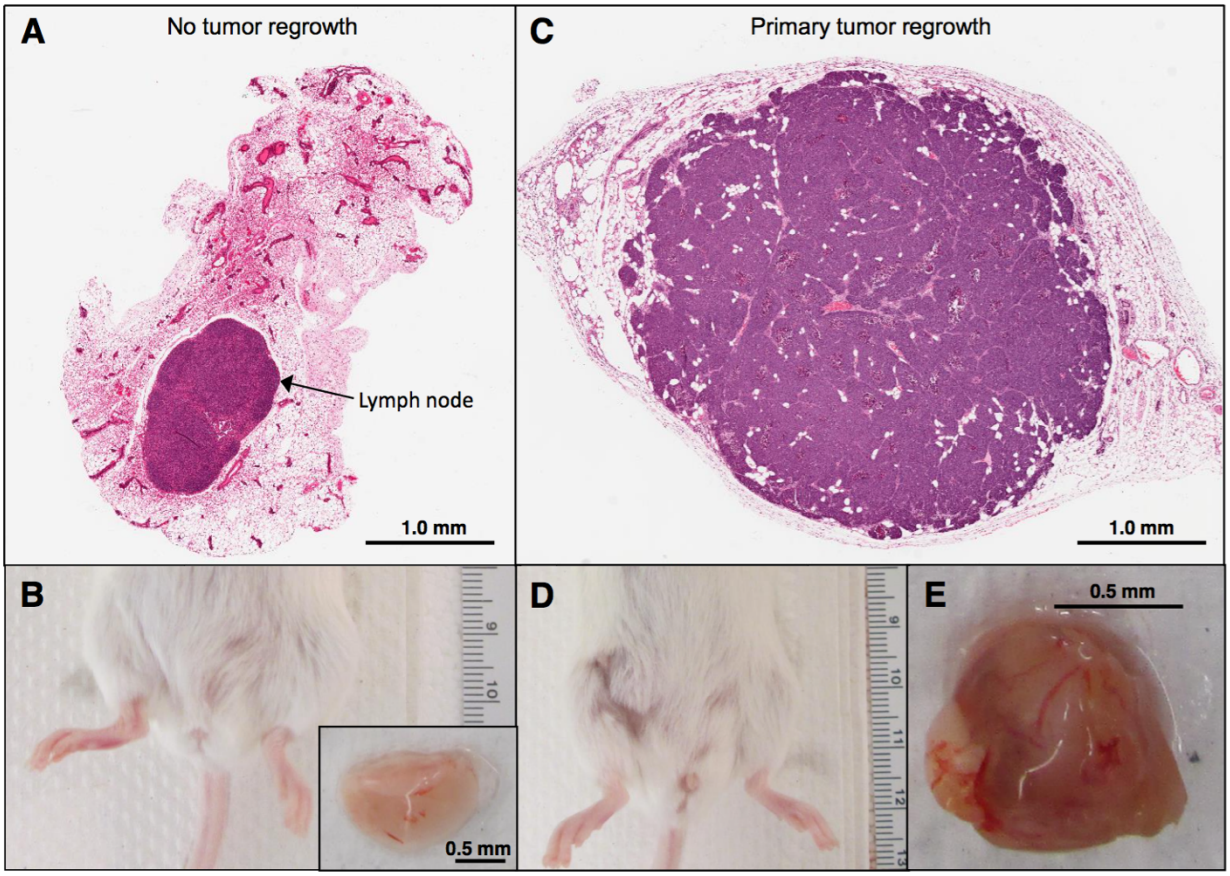


Figure 4.1. Post-surgical resection of primary tumors and development of de novo pulmonary metastasis.
(A) Experimental schema of murine mammary adenocarcinoma metastasis model. † denotes that all mice were cardiac perfused and injected with BrdU on the day of sacrifice. (B) Representative H&E where detection of metastatic foci was assessed by serial sectioning of FFPE lung tissue with lobes separated. Metastatic foci (>5 cells) were determined by H&E staining every 100 µm reflecting 1,300 µm of tissue. Lungs from 11 mice were analyzed. (C) Schema of surgical resection of primary mammary tumor. Red numbers and arrows denote the order and direction of skin incisions bordering the primary tumor (left). The right 4th and 5th mammary glands with major vessels are shown attached to the primary tumor (middle) followed by wound closure with wound clips (right).



Supplementary Figure 4.1. Primary tumor regrowth at surgical site. Representative H&E (top) and gross (bottom) images of the remaining right 4th and 5th mammary gland post-surgery, showing absence of tumor regrowth with inguinal lymph node (A-B) and mammary gland with primary tumor regrowth (C-E).

Protocol

Note: Refer to Table 4.1 for a list of reagents and equipment.

1. Isolation and preparation of single cell suspensions from primary mammary tumors:

1.1) Anesthesize donor female 100-day old transgenic MMTV-PyMT (FVB/n) mice under continuous sedation by administering 2% isofluorane via an anesthesia mask.

1.2) In a sterile setting, resect primary mammary tumors from 100-day old transgenic female MMTV-PyMT (FVB/n) mice.

1.3) Separate mammary tumor from overlying skin and surrounding adipose tissue and/or lymph nodes. With sterile scissors or a scalpel, mince primary tumors manually into small pieces (~ 1.0 mm^3). Place tumor pieces in collagenase A 3.0 mg/mL and 4.0 U/mL DNase I dissolved in DMEM. Volume for digestion medium is ~10 mL per 1.0 cm diameter tumor. Digestion is performed in a sterile 25 mL bottle with sterile stir bar at ~125 rpm and 37°C for 40 minutes.

1.4) Stop the digestion by adding fetal bovine serum (FBS) to a final dilution of 10% and place the entire mixture on wet ice where it is maintained.

1.5) Filter the digested tumor suspension through a 0.7 μm nylon strainer into a 50 mL conical tube. Centrifuge at 300 RCF at 4°C.

1.6) Resuspend pellet in 10 mL DMEM per 1.0 cm tumor and re-filter through a 0.7 μm nylon strainer. Count cell concentration followed by centrifugation at 300 RCF at 4°C.

Table 4.1. List of reagents and equipment.

Name of Material/ Equipment	Company	Catalog Number	Comments/ Description
Isofluorane	Piramal Healthcare	N/A	Prescription order
Collagenase A	Roche	11088793001	
DNase I	Roche	10104159001	
DMEM	ThermoFisher	12634010	
25 mL Pyrex bottle	Sigma-Aldrich	CLS139525	
Fetal Bovine Serum	Atlanta Bio	S11150	
0.7 µm nylon strainer	Corning	352350	
50 mL conical tube	VWR	89039-658	
Dimethyl sulfoxide	Sigma-Aldrich	D2650	
Growth factor-reduced Matrigel	BD	354230	
Poly(vinylpyrrolidone)–Iodine complex	Sigma-Aldrich	PVP1	
29 gauge 0.3 mL insulin syringe	BD	324702	
Small Vessel Cauterizer Kit	FST	18000-00	
Wound clips	Texas Scientific	205016	
AutoClip wound clip applier	BD	427630	
AutoClip wound clip remover	BD	427637	
Bromodeoxyuridine	Roche	10280879	
Heparinized capillary tubes	Fisher	22362566	
Microtainerâ tubes with dipotassium EDTA	BD	365974	
20 mL syringe	BD	309661	
DPBS	Thermo-Fisher	14190-250	
OCT-freezing medium	VWR	25608930	

1.7) Resuspend pellet in 10% dimethyl sulfoxide 90% FBS at a concentration of 2×10^7 live cells/mL. Store single-cell suspensions of whole primary tumor at -80°C.

2. Orthotopic injection of mammary tumor

2.1) Partially thaw frozen primary tumor suspensions at 37°C until frozen pellet can be released from the cryotube into 20 mL DMEM and count cells. Centrifuge at 300 RCF at 4°C and resuspend cells in a 1:1 DMEM:growth factor-reduced Matrigel at a concentration of 1×10^7 cells/mL.

2.2) Place anesthesized recipient female syngeneic mice ventral side up under continuous sedation by administering 2% isofluorane via an anesthesia mask.

2.3) Sterilize the right 4^{th} mammary gland injection site with aerosolized 70% ethanol followed by administration of Poly(vinylpyrrolidone)–Iodine with a sterile cotton swab.

2.4) Inject 100 µL (1×10^6 live cells) bevel-side up into uncleared right 4^{th} mammary gland of 6 to 10-week-old female FVB/n mice using a 29 gauge 0.3 mL insulin syringe.

3. Surgical resection of orthotopic mammary tumor

3.1) 38-60 days following tumor cell injection, cull mice not exhibiting orthotopic tumor volumes ranging between 172 to 450 mm^3 in volume [length×(width^2)/2].

3.2) Place anesthesized tumor-bearing mice ventral side up under continuous sedation by administering 2% isofluorane via an anesthesia mask.

3.3) Spray with 70% ethanol to sterilize the surgical area surrounding the primary tumor, followed by application of Poly(vinylpyrrolidone)–Iodine with a sterile cotton swab.

3.4) As shown in Figure 4.1D, an initial skin incision is made using blunted scissors medial-caudal to the tumor.

3.5) Next, a superior excision of the skin (Figure 4.1C) is made medial to the tumor, paying attention to the need to cauterize any vasculature feeding the tumor located on the skin before extending the incision.

3.6) Continue the skin incision laterally (posterior to the tumor), followed by a superior skin excision (lateral to tumor), and medial excision (superior to tumor) (Figure 4.1C).

3.7) After the skin has been excised circumferentially around the tumor (Figure 4.1C), lift overlying skin attached to the tumor using forceps while blunt dissecting the tumor away from the abdominal wall musculature keeping the mammary glands intact.

3.8) Identify, by blunt dissection, and cauterize large vessels running through the 4^{th} and 5^{th} mammary glands.

3.9) Excise ~half of the 4^{th} and 5^{th} mammary glands at the cauterization site to free the tumor, overlying skin, and segments of the mammary glands (Figure 4.1C).

3.10) In the event of bleeding, identify actively bleeding vessels and immediately cauterize. If more than 250 µl of blood is lost, the mouse is excluded from study and euthanized.

3.11) Excision sites are closed with wound clips using an AutoClip wound clip applier (Figure 4.1C), that are subsequently removed 10 days post-surgery with an AutoClip wound clip remover.

4. Isolation and processing of blood and lung for flow cytometry and histology

4.1) At the study endpoints, mice are prepared for various histpathologic assessments if desired. 90 minutes before sacrifice, mice are given an intraperitoneal injection of bromodeoxyuridine (50 µg/g mouse weight) at a concentration of 6.25 µg/µL in 1X PBS. Frozen stocks of dissolved bromodeoxyuridine are used within 1 month after preparation.

4.2) 10 minutes before sacrifice, retroorbital blood is collected (>500 µL) using heparinized capillary tubes, and subsequently transferred to Microtainer® tubes with dipotassium EDTA and held on ice.

4.3) To remove lungs and remaining mammary tissue, a midline incision is made with scissors from the lower abdomen to the mouth to expose the thoracic and peritoneal cavities (Figure 4.2A), peeling skin laterally to also expose remaining right 4^{th} and 5^{th} mammary glands.

4.4) Remaining mammary gland tissue is excised and examined to rule out primary tumor regrowth by assessing serial sectioned formalin-fixed paraffin-embedded (FFPE) tissue by hematoxylin and eosin (H&E) staining (Supplementary Figure 4.1).

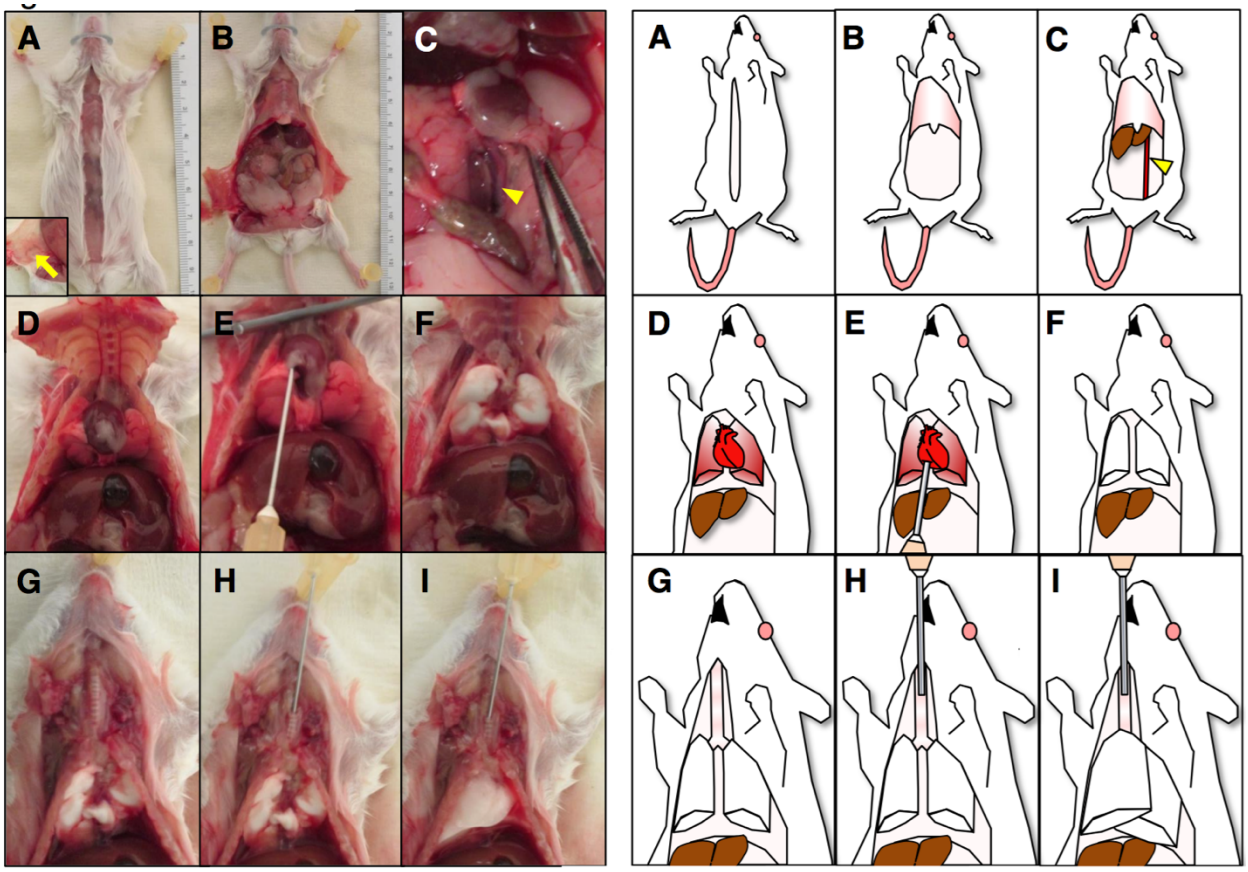


Figure 4.2. Isolation, perfusion and fixation of lung.
Picture (left) and corresponding cartoon (right) are shown of lung isolation, perfusion and fixation. (A) A midline incision is shown with inset image displaying reflected skin exposing the right 4th and 5th mammary glands (arrow). (B) The abdominal wall is shown opened to the diaphragm. (C) After reflection of intestine, the abdominal aorta (arrowhead) is identified and cut open. (D) The diaphragm and lateral sides of the rib cage are cut to expose the thoracic cavity. (E) The lung is perfused through the right ventricle of the heart until the lungs turn entirely white (F). (G) The exposed trachea is identified followed by injecting formalin (H) into the trachea until the lungs have expanded (I).

4.5) To remove lungs, the abdominal wall is opened to the diaphragm with scissors, followed by cutting of the abdominal aorta to drain blood prior to perfusion of lungs (Figure 4.2B-C).

4.6) Cut the diaphragm along the rib cage from an abdominal approach making sure to avoid the lung and heart, followed by exposing the thorax by cutting through the lateral sides of the rib cage (Figure 4.2D).

4.7) Using a 23 gauge needle on a 20 mL syringe, perfuse lungs with ~5.0 ml DPBS (~10 ml/min) through the right ventricle of the heart until the lungs turn entirely white (Figure 4.2E-F). Immediately cut off the heart from the main vessels so blood does not re-perfuse into the lung.

4.8) For lung tissue to be fixed and processed for histopathologic assessments, inject ~1.0 mL 10% formalin at 4°C into the exposed trachea bevel side up toward the lungs using a 23 gauge needle (Figure 4.2G-H). Cease injection once lungs are completely expanded and filled with fixative (Figure 4.2I).

4.9) Excise lung lobes from trachea, and emersion fix lung tissue in neutral-buffered formalin for subsequent paraffin embedding or OCT-freezing medium, per standard histopathologic procedures.

4.10) Metastatic burden in lungs is then quantitatively evaluated by serial sectioning of FFPE lung tissue and microtome sections evaluated every 100 μm thirteen times, by H&E staining (Figure 4.1B).

Representative results

Greater than 75% of recipient mice receiving 1×10^6 cells from primary mammary tumors derived from MMTV-PyMT mice, develop single mammary adenocarcinomas ranging from 172 to 450 mm^3 within 38-60 days (data not shown). Mice eligible for randomization are then enrolled into study groups following surgical resection of primary tumors as shown (Figure 4.1C). Primary tumor regrowth was identified in less than 2% of mice that underwent surgical resection of primary tumor (Supplementary Figure 4.1). For studies described herein, 45% of recipient mice evaluated by this protocol developed histologically detectible metastatic foci by day 115 post-tumor resection (Figure 4.1B). To affirm histology of metastases in areas identified containing metastatic cells by H&E staining, adjacent tissue sections were evaluated by PyMT PCR (data not shown).

Discussion

Mouse models of human cancer mimicking stages of disease progression, kinetics and histopathology provide invaluable tools within which to identify and evaluate new targets for therapy, as well as potential efficacy of new therapeutic agents targeting those molecules/pathways. While tail-vein and/or cardiac injection of established cancer cell lines are often used as experimental models of metastasis, these fail to recapitulate critical steps in the metastatic process, and instead reflect ectopic organ colonization assays where aspects of tumor cell survival can be evaluated. Moreover, whereas some existing transgenic mouse models of de novo mammary carcinogenesis development do provide model systems enabling study of steps involved in metastasis, significant primary tumor burden typically limits durations of study.

Regarding evaluating efficacy of therapeutics in these models, because primary tumors typically develop in all mammary glands, surgical resection of all primary tumors and adjuvant evaluation of therapies aimed at minimizing growth of metastatic colonies is not possible. Because of these issues, we developed an autochthonous model of metastatic dissemination wherein metastatic dissemination of tumor cells occurs de novo, and following surgical resection of primary tumor, an extended latency period is established that allows for identification of metastasis in the lung. Thus, this model mirrors human breast cancer metastasis and affords a unique system to evaluate efficacy of adjuvant delivered therapies for impact on regulating disease-free survival and/or overall survival with defined endpoints per IACUC guidelines. Whereas other

investigators have reported presence of fluorescently-labeled single metastatic cells disseminated to liver, kidney, spleen and brain, in addition to lung, following reimplantation of mammary terminal end buds derived from MMTV-PyMT mice (Kouros-Mehr et al., 2008), aside from lung, we observed several mice with metastatic foci in liver, the penetrance of which has yet to be determined.

Because a large proportion of women with breast cancer are treated by surgical resection of primary tumors, and for those that progress subsequently develop distal metastasis, this implies that dissemination and seeding had occurred prior to surgical resection. Given that distal organ microenvironments provide unique niches for surviving and/or proliferating metastatic cells, it is imperative that model systems mimic these facets such that molecules and pathways operative in secondary sites, that are likely distinct from primary tumors, can be identified, studied, and therapies targeting them accurately evaluated for efficacy; the model developed herein provides these aspects for study.

Acknowledgements

The authors thank Jo Hill for histopathology assistance, Dr. John Gleysteen for instruction in surgical technique, Tessa Diebel for videography assistance, all members of the Wong and Coussens laboratories for critical insight and discussions, and the OHSU Knight Cancer Institute for financial support. The authors acknowledge support from T32GM071388-10 and T32CA106195-11 to CEG, the NCI/NIH, the Department of Defense Breast Cancer Research Program, the Susan G Komen Foundation, the Breast Cancer Research Foundation, and a Stand Up To Cancer – Lustgarten Foundation Pancreatic Cancer Convergence Dream Team Translational Research Grant (SU2C-AACR-DT14-14) to LMC, a Women's Health Circle of Giving Foundation Award to MHW, and the Brenden-Colson Center for Pancreatic Health to MHW and LMC.

Chapter V: CSF1/CSF1R inhibition in the adjuvant setting reduces the metastatic niche

This body of work is planned for publication along with additional PD-L1 adjuvant therapy studies that are in progress (conducted by Tiziana Cotechini and Charles Gast). This work also provides a platform for the analysis of the role of cell fusion in disease progression within the early stages of dissemination at a distant organ site.

Abstract

Major advances in early detection of breast cancer and personalized hormone-based therapies to supplement mastectomy/lumpectomy, radiation and chemotherapy have significantly improved survival in this patient population; however, these treatment regimens remain ineffective in the metastatic setting (Richard G. Margolese, 2003), evident by the high mortality rate of late stage breast cancer. A key barrier to designing therapies targeting late stage disease lies in our incomplete understanding of metastatic propagation. The concept of an immune microenvironment (ME) educated by the primary tumor that is conducive for metastasis, a “premetastatic” niche (Kaplan et al., 2005), provides an intriguing mechanism for spread of disease. Yet, the fact that many patients exhibit metastases years after mastectomy or lumpectomy indicates additional biological mechanisms are involved; notably, undetectable disseminated tumor cells (DTCs) at metastatic sites could be responsible for priming immune cells within, or recruited to, the metastatic ME. Understanding how residual DTCs sculpt immune MEs within metastatic sites, and how immune cells support metastases, represent both critical insight and an exciting opportunity for therapeutic intervention. Here, we demonstrate reduction of neutrophils and an increase in monocytes in the lung ME after surgical resection of the primary tumor. Further, inhibition of the colony stimulating factor 1 (CSF1)/colony stimulating factor 1 receptor (CSF1R) axis in the adjuvant setting reduces the metastatic niche. Additionally, adjuvant treatment of the CSF1R inhibitor

PLX3397 with Paclitaxel (PTX) increases the number of PD-L1^{+} alveolar macrophages, warranting exploration of checkpoint inhibitor combination therapy.

Personal contribution

CEG's contribution to this chapter are as follows:

Figure 5.1.
CEG designed, performed and analyzed the lung immune profile.

Figure 5.2.
CEG designed, performed and analyzed lungs for immune infiltrates containing PyMT positive cells.

Figure 5.3.
CEG designed, performed and analyzed adjuvant therapy experiments.

Figure 5.4, 5.5.
CEG designed and analyzed peripheral blood samples. Experiments were performed by CEG and PF.

Figure 5.6, 5.7.
CEG designed, performed and analyzed the adjuvant therapy lung immune profile.

CEG wrote the manuscript.

Introduction

The average American woman has a 12.2% risk of being diagnosed with breast cancer during her lifetime (Howlader N). While there is a robust 5-year survival rate with early diagnoses, this plummets to a dismal 23% for patients who develop metastatic and recurrent disease, often appearing in the lung (Howlader N). The overwhelming majority of patients are candidates for surgical intervention (NCCR, 2014), yet despite surgery, cancer progression occurs in 30-70% of stage II and III patients (Kataja et al., 2008). The majority of breast cancer patients who develop recurrence are diagnosed multiple years after surgery (Karrison, Ferguson, & Meier, 1999) and the pathophysiology of tumor progression between surgery and detectible metastasis is largely unknown. This represents a crucial window for therapeutic treatment. It is likely cancer cells disseminate early, as disseminated cancer cells are detectible even in patients diagnosed with ductal carcinoma *in situ* (Banys et al., 2014). Because of this, understanding the metastatic ME during this biological window may provide valuable insight into disease progression and tailor treatment strategies.

Current therapeutic paradigms are ineffective in treating metastatic or recurrent breast cancer . Yet therapeutic approaches targeting the immune ME in breast cancer patients are actively underway in clinical trials based on pre-clinical work assessing the pro-tumorigenic, pro-metastatic role macrophages (MΦs) play in breast cancer (DeNardo et al., 2009; DeNardo et al., 2011; Ruffell et al., 2014). The small molecule inhibitor PLX3397, designed to target the CSF1R, has been shown to reduce metastasis in combination with chemotherapy in the neo-

adjuvant setting. Thus, it is possible monocytes and MΦs play an important role in metastasis in the adjuvant setting as well.

Much of what is known with regard to the metastatic ME is that the immune cell composition is heavily influenced by the presence of a primary tumor. The concept of a “pre-metastatic” niche was established with the observation that bone marrow-derived cells (BMDCs) migrate to lungs prior to detection of metastases (Kaplan et al., 2005) and tumor-conditioned medium can recruit immature myeloid cells (iMCs), a heterogeneous cell population including monocytes and both pro-tumorigenic and anti-tumorigenic granulocytes (Sceneay et al., 2012). However, this ME was not sustained, indicating that its maintenance was dependent on presence of continual secretion of factors from primary tumors (Deng et al., 2012) rather than factors secreted from newly established metastatic foci. Importantly, a large proportion of patients with breast cancer undergo surgical removal of primary tumors and subsequently develop lung metastases; therefore it is possible that residual DTCs, in local metastatic sites, such as the lung, and/or other sites such as lymph nodes or bone, contribute to maintenance and/or propagation of a pro-metastatic ME after surgery. While, some groups have reported differences in myeloid populations between directly-seeded (tail-vein injection) mammary tumor cell lines into WT mice versus transgenic models (B. Qian et al., 2009) (supporting differences of local versus distant tumor cells in shaping the lung ME), it is still unclear what myeloid cell populations are in the lung post-surgical removal of the primary tumor. Here, our group has identified alterations in the immune profile of lungs

following primary tumor resection. Within the lung, total leukocytes increase, yet myeloid populations, such as neutrophils, decrease in frequency after primary tumor removal. Additionally, an increase in Ly6C$^{low/-}$ monocytes is detected and persists up to 60 and 115 days following primary tumor removal, when peri-vascular/bronchial metastatic niches become detectible. Further, we demonstrate that inhibition of the monocyte/MΦ population in the adjuvant setting with or without the chemotherapeutic PTX, reduces the number of metastatic niche clusters as well as Ly6C$^{low/-}$ monocytes in the lung. Last, our findings indicate that PLX3397+PTX therapy can result in increased PD-L1^{+} alveolar MΦs, indicating an additional checkpoint inhibitor in the adjuvant setting may be beneficial.

Materials and methods

Animal care and use

Primary tumors were resected from 100-day-old MMTV-PyMT mice in the FVB/n background. Primary tumors were minced manually with a scalpel followed by a 40 minute incubation in collagenase A 3.0 mg/ml (Roche) and DNase I (Roche) dissolved in DMEM (Invitrogen) at 37°C. Digestion was stopped by adding DMEM + 10% FBS and filtered through 0.7 μm nylon strainers (Falcon). Single-cell suspensions of whole primary tumor was frozen and stored at -80°C in 10% DMSO (Invitrogen) 90% FBS. Frozen primary tumor suspensions were thawed at 37°C and washed with DMEM. Cells were resuspended in a 1:1 DMEM:Growth factor reduced Matrigel (BD Pharmingen) at a concentration of 1×10^7 cells/mL. 100μl (1×10^6 cells) were injected into uncleared right 4th mammary glands of 6 to 10-week-old FVB/n mice. 28 days after tumor cell injection, mice were randomized by orthotopic tumor volume with a median tumor volume of 300mm^3. Orthotopic tumor was surgically removed with surrounding mammary gland and overlying skin followed by closure with wound clips (MikRon) that were removed 10 days post-surgery. Mice were fed PLX3397 formulated in mouse chow (300mg/kg) or control chow (provided by Plexxikon Inc) from day 5 to 40 or day 60 to 95 post-tumor resection. PTX (Pfizer) was administered from day 10 to 35 or day 65 to 90, every 5 days by intravenous injection into the retro-orbital plexus. 2mg/ml PTX diluted in PBS was given at 10 mg/kg mouse weight. 90 minutes prior to sacrifice, mice received intraperitoneal injections of bromodeoxyuridine (BrdU; Roche Diagnostics) dissolved in PBS (50 μg per g of

mouse body weight). Mice were cardiac perfused with PBS prior to tissue collection. PBS-perfused lungs were inflated with 10% formalin followed by formalin fixation and paraffin embedding at 60 or 115 days post-tumor resection. PBS-perfused lungs were also isolated at 60 or 115 days post-tumor resection for flow cytometry. Metastatic niche burden was assessed by serial sectioning of formalin-fixed paraffin-embedded lung tissue whereby the entire lung was sectioned and the number of metastatic niche foci (>5 cells) was determined on 8 sections taken every 100 μm beginning 500 μm into tissue following H&E staining. All mice were maintained within the OHSU barrier facility.

Flow cytometry analysis

Lungs were diced and digested for 20 minutes at 37 °C in DMEM + 2 mg/mL Collagenase A (Roche) + DNase (Roche) under stirring conditions. Digestion was stopped by adding DMEM + 10% FBS and filtered through a 40 μm filter and washed with PBS. Retro-orbital collection of blood was performed with heparinized micro-hematocrit capillary tubes (Fisher) and K_2EDTA-coated tubes (BD). Red blood cells were osmotically lysed with a 1 minute incubation in 0.2% NaCl followed by addition of the equivalent volume of 1.6% NaCl. Cells were resuspended in FACS Buffer (PBS, 1.0 mM EDTA, 5% FBS). Cells were incubated in PBS containing Live Dead Aqua (1:500, Invitrogen) with Fc Receptor Binding Inhibitor (1:200, eBioscience). Cells were then incubated in FACS buffer for 30 min with various conjugated antibodies: CD45-PeCy7 (1:8000, Biolegend), CSF1R-BV711 (1:200, Biolegend), F4/80-APC (1:400

Biolegend), CD11b-AF700 (1:200, eBioscience), MHCII-ef450 (1:800, eBioscience), CD3-BV785 (1:100, Biolegend), CD19-BV650 (1:200, Biolegend), CD11c-PE Dazzle (1:200, Biolegend), CD4-BV605 (1:200, Biolegend), CD8-BV711 (1:200, Biolegend), Ly6C-PerCP-Cy5.5 (1:400, eBioscience), Ly6G-APC-Cy7 (1:400, Biolegend). BD Fortessa FACS machine was used for analyses.

PCR

DNA was extracted from metastatic microenvironment regions microdissected from frozen formalin fixed lung sections by 40 min incubation in lysis buffer (25 mM NaOH, 0.2 mM EDTA pH 12) at 95 °C followed by neutralization with equal volumes of neutralization buffer (40 mM Tris-HCl pH 5). A total of 77 metastatic microenvironment regions were microdissected from 16 serial sections 100μm apart, covering about 43 metastatic niche regions. PyMT primers: fwd 5'-GGAAGCAAGTACTTCACAAGGG-3' and rev 5'-GGAAAGTCACTAGGAGCAGGG-3'. Reactions were run with a 59°C annealing temperature.

Quantitation of metastatic niche burden

Following perfusion and resection, lungs were inflated with neutral buffered formalin intratracheally and incubated at 4°C overnight in formalin. The lobes were separated and underwent ethanol dehydration and paraffin embedding. Lungs were sectioned and starting 500μm into lung tissue, hematoxylin and eosin staining of sections every 100μm for a total of eight levels was performed.

Number and size of metastatic niche foci were quantified using Aperio ScanScope CS Slide Scanner (Aperio Technologies).

Results

The lung microenvironment is altered by surgical removal of the primary tumor

Primary tumor-derived secreted factors heavily influence the immune composition of various distant organs that subsequently promote metastasis (Erler et al., 2009; Kaplan et al., 2005); therefore there is a strong rational for surgical resection of the primary tumor. Yet because patients can acquire overt metastases decades after surgery, little is known about what comprises the metastatic ME post-surgery and how this environment supports or inhibits metastatic progression. To address the questions, (i) what immune cell types in the lung are dependent on the presence of the primary tumor, and (ii) what immune cell types are increased in the lung post-surgery, we utilized a variation of the murine model (see Chapter IV) where surgical resection of MMTV-PyMT primary tumor explants in syngeneic wild type mice was performed. After four weeks, primary tumors 0.8-1.4 cm were surgically excised and lung immune profiles were assessed 60 and 115 days post-surgery by flow cytometry (Figure 5.1A). Mice 60 and 115 days post-surgery had an increased percentage of $CD45^+$ leukocytes relative to tumor bearing mice (Figure 5.1B). Yet compared to tumor-bearing mice, neutrophil frequency was significantly lower 60 days post-surgery, providing evidence that a given distant organ ME is dependent on the primary tumor (Figure 5.1C).

In contrast to neutrophils, the inflammatory monocyte population was increased relative to wild type lungs (Figure 5.1D,E). Additionally, the

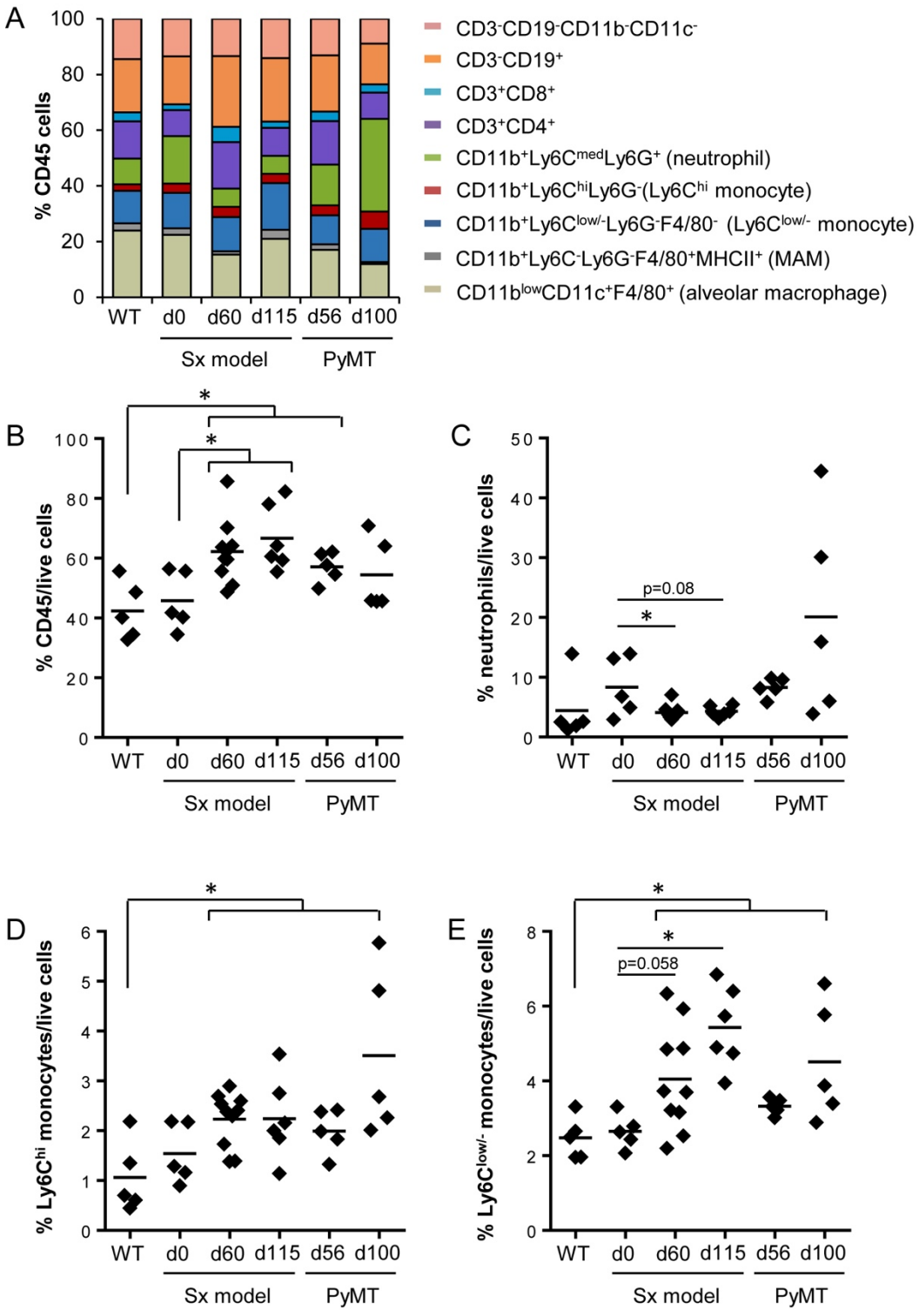


Figure 5.1. Immune profile of lungs post-surgical resection of primary tumor.

(A) Analysis of individual leukocyte populations in lungs by flow cytometry as a mean percentage of $CD45^+$ cells. MAM denotes metastasis-associated

macrophages. (B) Frequency of $CD45^+$ leukocytes as a percentage of total live cells. (C-E) Individual neutrophil and monocyte populations as a percentage of total live cells (WT=wild type, n=5; d0=orthotopic PyMT tumor bearing mouse, n=5; d60=60 days post-surgery, n=10; d115=115 days post-surgery, n=6; d56=56 day old MMTV-PyMT mice, n=5; d100=100 day old MMTV-PyMT mice). Asterisk denotes $p<0.05$ by unpaired t test.

Ly6C$^{low/-}$ monocyte population was found to increase in frequency after primary tumor surgical resection (Figure 5.1E), when histologically detectible metastatic niche regions (defined as peribronchial/perivascular immune cell clusters where at least 7% contain PyMT^{+} tumor cells) become detectible by H&E (Figure 5.2A-E) (Erler et al., 2009; Kaplan et al., 2005). The presence of this monocyte population indicates a potential role for this immune population in metastatic disease progression post-surgery. Given that monocytes and pro-metastatic MΦs both express the CSF1R, we rationalized inhibition of the CSF1/CSF1R axis may reduce metastatic disease in the adjuvant setting.

CSF1/CSF1R inhibition in the adjuvant setting reduces the metastatic niche

To determine if CSF1R^{+} cells promote metastasis post-surgical resection of the primary tumor, we treated mice in the adjuvant setting with either a neutralizing antibody to CSF1 (αCSF1) or the small molecule CSF1R inhibitor PLX3397 with or without the chemotherapeutic PTX. Because the metastatic niches that were identified 60 days post-surgery were minimal in tumor-bearing mice (Figure 5.2B-C), we set to assess presence of this niche after inhibition of CSF1R^{+} cells 5 to 40 days post-surgery by administering either αCSF1 or PLX3397 (Figure 5.3A, Schemas 1 and 2). At 60 days post-surgery, the average number of metastatic niche regions per lung section were significantly reduced after treatment with either αCSF1 or PLX3397 with or without PTX (Figure 5.3B). To determine if CSF1R^{+} cell inhibition reduces metastasis long-term, providing a durable response, mice given the same treatment regimen of PLX3397 with or

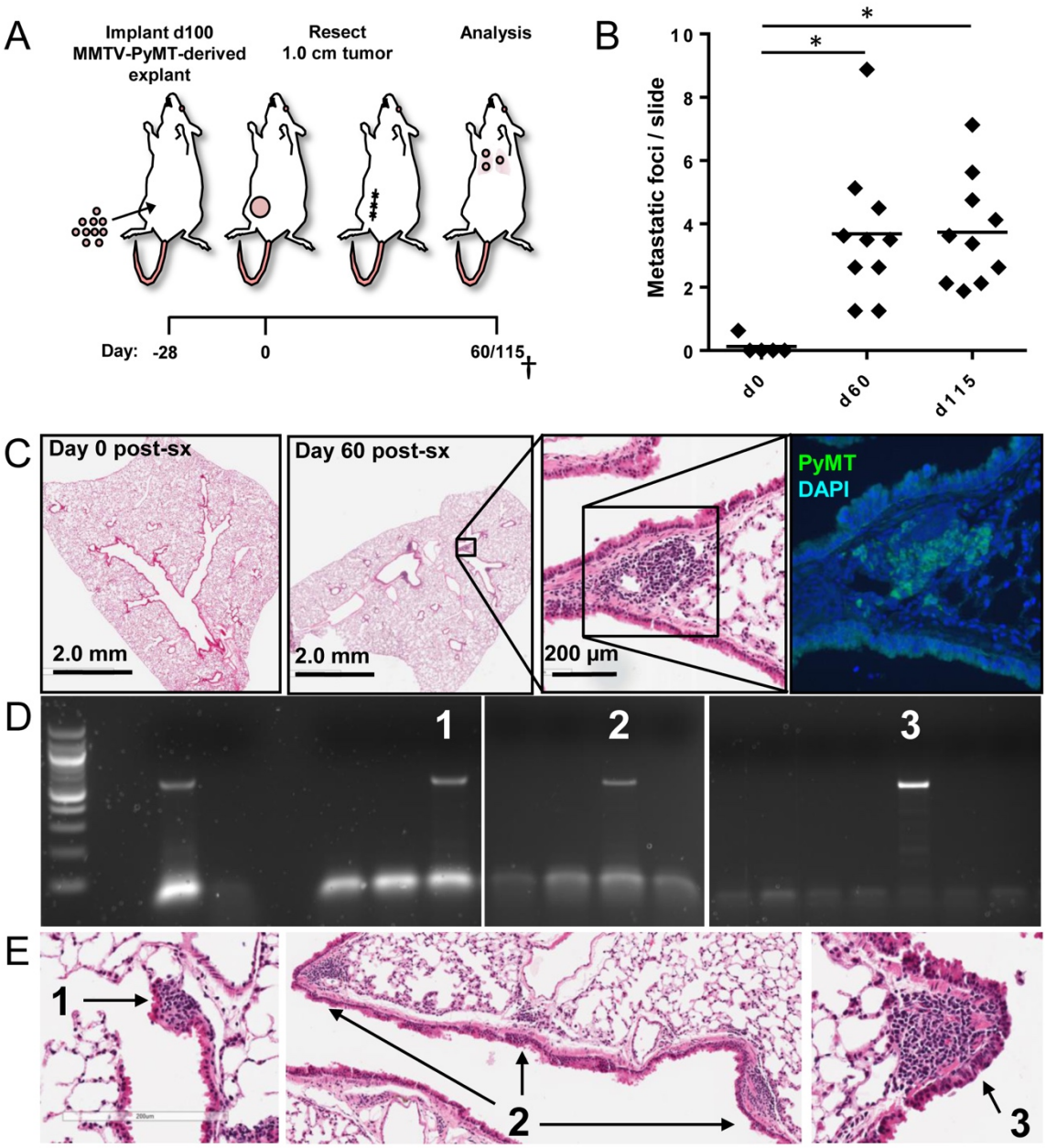


Figure 5.2. Metastatic microenvironments can contain PyMT tumor cells. (A) Experimental schema of murine mammary adenocarcinoma metastasis model. † denotes that all mice were cardiac perfused and injected with BrdU on the day of sacrifice. (B) The average number of metastatic niches per section was assessed by serial sectioning of FFPE lung tissue with lobes separated where after facing into 500 µm of lung, the number of metastatic niche regions (>5 cells) was determined by H&E staining every 100 µm reflecting 800 µm of tissue. Lungs from 5-10 mice/group were analyzed. Asterisk denotes $p<0.05$ by unpaired t test. (C) Representative H&E staining and immunofluorescent staining of adjacent tissue section of lung 0 or 60 days post-surgery. Blue: DAPI, Green: PyMT. (D) PyMT PCR (556bp) of metastatic niches microdissected from FFPE

lung sections 60 days post-surgery. (E) Corresponding H&E images (numbered 1-3) of regions microdissected that resulted in positive PyMT PCR. 7% of the metastatic niches tested resulted in a $PyMT^+$ PCR product.

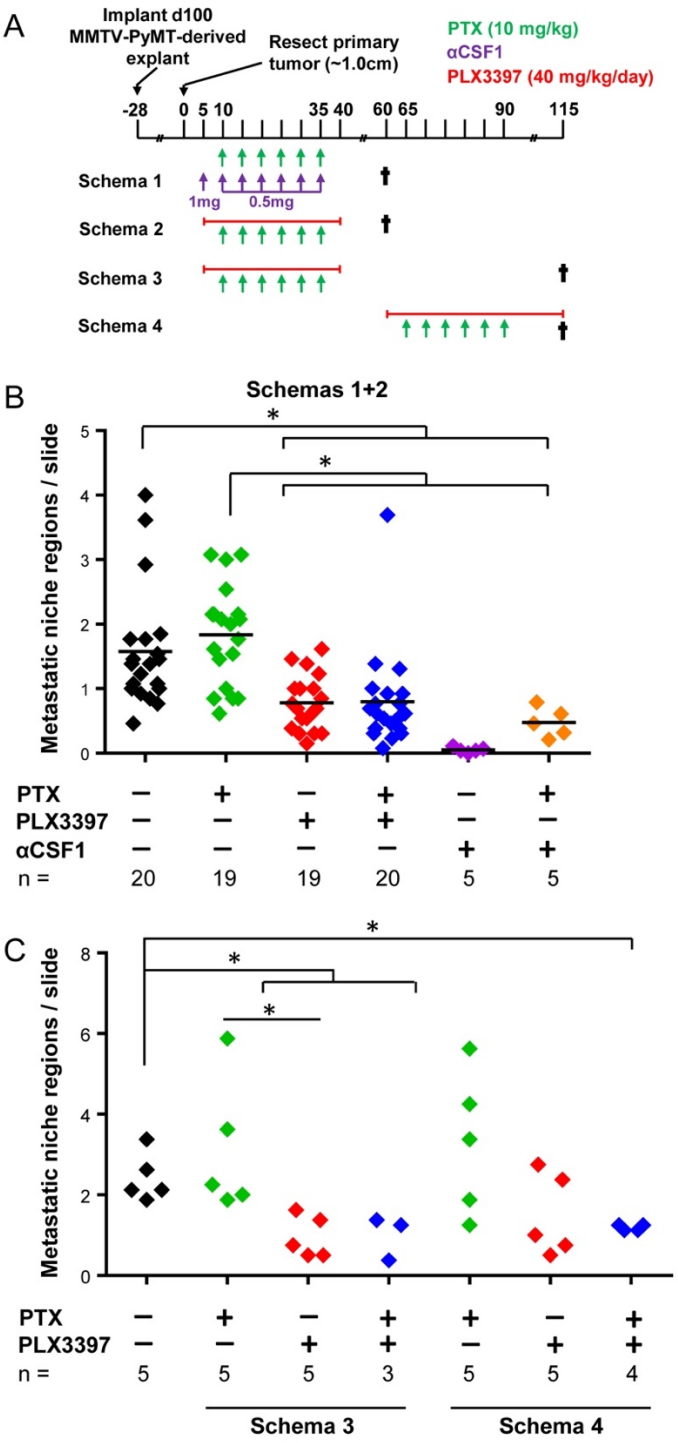


Figure 5.3. Adjuvant CSF1/CSF1R inhibition reduces metastatic niche regions.

(A) Experimental schemas in days with treatment regimens outlined below. Arrows indicate day of administration and bars indicate days mice were provided with either PLX3397-containing or control chow. † denotes day lungs were

collected. After facing into 500 μm of lung, the average number of metastatic niche regions (>5 cells) per section was quantified by H&E staining every 100 μm reflecting 800 μm of tissue for schemas 1-2 (B) and schemas 3-4 (C). Asterisk denotes $p<0.05$ by unpaired t test.

without PTX were analyzed at 115 days post-surgery (Figure 5.3A, Schema 3). The average number of metastatic niche regions were reduced in both PLX3397 and PLX3397+PTX treated groups relative to control, indicating a durable response (Figure 5.3C). Last, to investigate if $CSF1R^{+}$ cell inhibition reduces already established metastatic niches, PLX3397 and/or PTX treatment was initiated 60 days post-surgical removal of the primary tumor (Figure 5.3A, Schema 4). Similar to mice enrolled in schemas 2 and 3, metastatic niche regions were less prevalent in PLX3397+PTX treated mice relative to controls, indicating either elimination or a reduction in size of a subset of metastatic niches. Throughout treatment with the various schemas, peripheral blood was obtained to assess alterations in immune profile (Figure 5.4A-D). PLX3397 efficacy was determined by a reduction of peripheral blood patrol monocytes while on PLX3397 (Figure 5.5A-D). Additionally, $Ly6C^{low/-}$ monocyte frequency was reduced in lungs in mice treated with PLX3397 and PTX 115 days post-surgery (Figure 5.6). The specific role the monocyte/MΦ lineage plays in promoting overt metastasis remains unclear. One of the limitations of this model is that only a small subset of mice develop overt metastasis (data not shown) and this model may only represent a latent period common in many patients between mastectomy/lumpectomy and detection of overt metastasis. Nonetheless, these findings give insight to the biology of the metastatic ME at a crucial, previously unexplored time point in tumor progression.

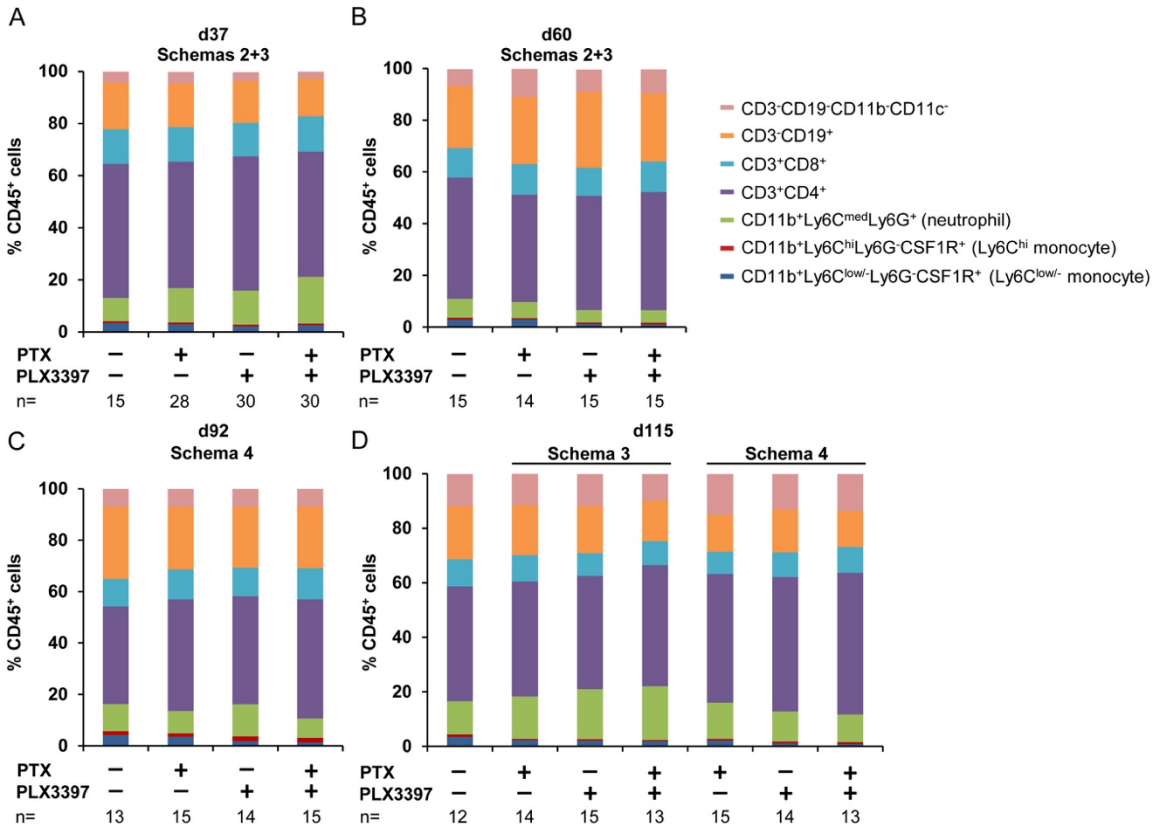


Figure 5.4. Peripheral blood immune profile.
Longitudinal analysis of peripheral blood immune profile from schemas 2-4 at days 37 (A), 60 (B), 92 (C) and 115 (D) post tumor resection as a percentage of total live $CD45^+$ cells.

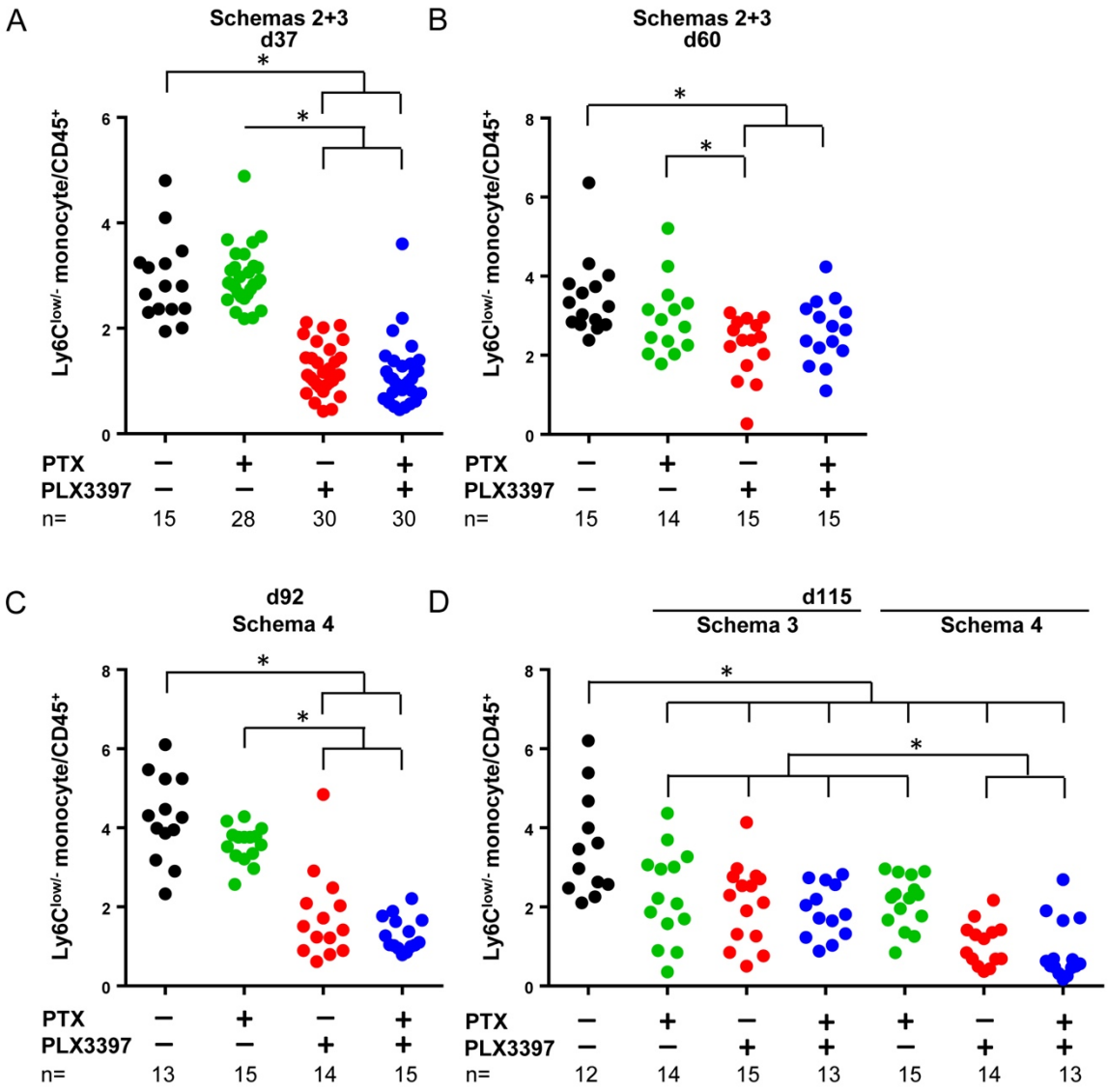


Figure 5.5. Peripheral blood Ly6C$^{low/-}$ monocytes are reduced by PLX3397. Longitudinal analysis of peripheral blood Ly6C$^{low/-}$ monocytes (CD11b^{+}Ly6C$^{low/-}$ CSF1R^{+}Ly6G^{-}) from schemas 2-4 at days 37 (A), 60 (B), 92 (C) and 115 (D) post tumor resection as a percentage of total live CD45^{+} cells. Asterisk denotes $p<0.05$ by unpaired t test.

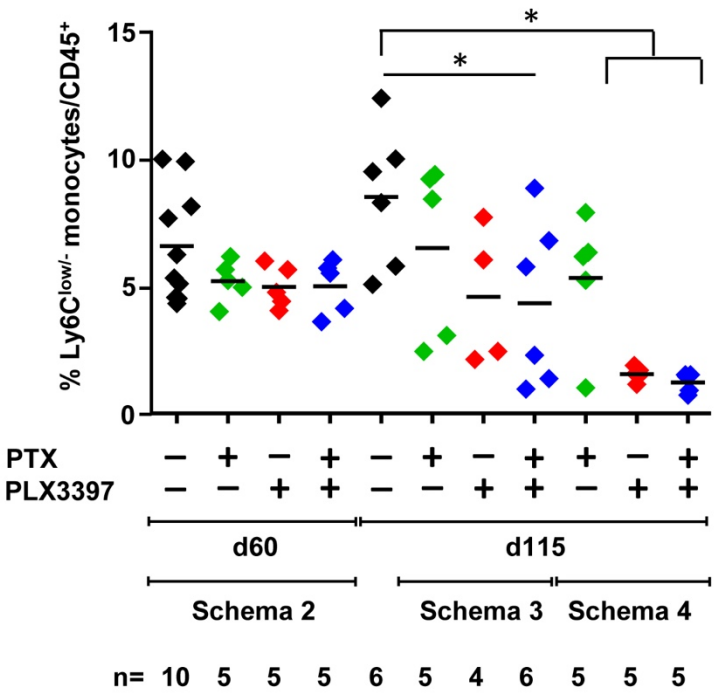


Figure 5.6. PLX3397 reduces Ly6C$^{low/-}$ monocytes in the lung.
Longitudinal analysis of lung Ly6C$^{low/-}$ monocytes (CD11b^{+}Ly6C$^{low/-}$CSF1R^{+}Ly6G^{-}) from schemas 2-4 at days 60 and 115 post tumor resection as a percentage of total live CD45^{+} cells. Asterisk denotes $p<0.05$ by unpaired t test.

PLX3397 Paclitaxel combination therapy increases PD-L1^{+} alveolar macrophages

While reduction of the number of metastatic niche regions was observed following adjuvant therapy of PLX3397 in combination with PTX, there was not complete elimination of this ME, indicating further combination therapies may prove beneficial. Given that the efficacy of PLX3397+PTX combination therapy in the neo-adjuvant setting was determined to be CD8-dependent and produced increased interferon gamma (IFNγ) levels (DeNardo et al., 2011; Ruffell et al., 2014), there may be mechanisms by which tumor cells can escape the cytotoxic T cell response. Because the co-inhibitory ligand PD-L1 is induced by IFNγ and inhibits T cell function, we reasoned potential increases in PD-L1 in the lungs of mice treated with PLX3397 and PTX may be observed. While an increase in expression levels of PD-L1 was not observed on alveolar macrophages, we identified increased numbers of PD-L1^{+} alveolar macrophages after treatment with PLX3397 and PTX (Figure 5.7).

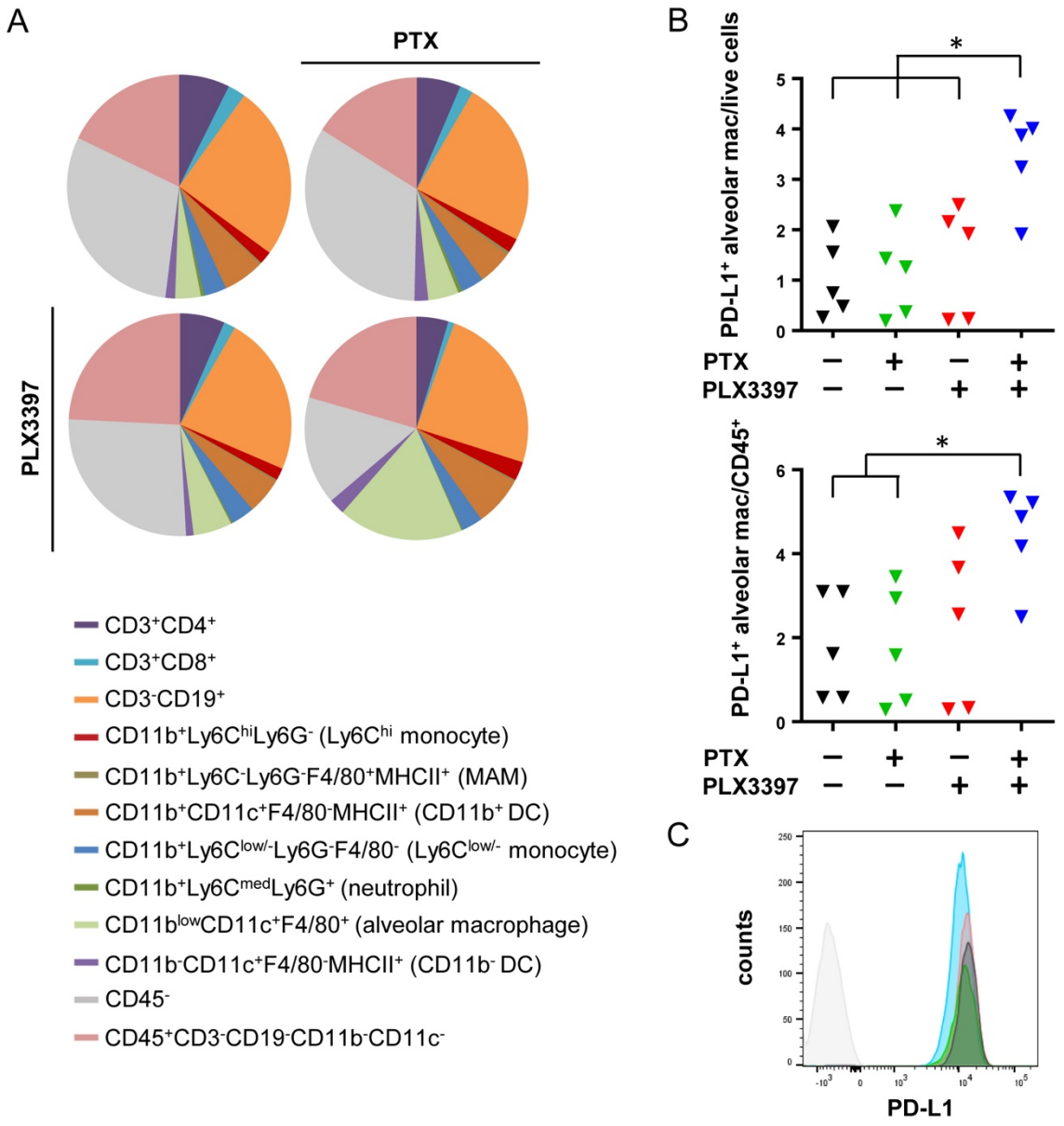


Figure 5.7. PLX3397+Paclitaxel increases PD-L1$^+$ alveolar macrophages in the lung.
(A) Immune profile of PD-L1$^+$ cells from lungs of mice in experimental schema 2. (B) PD-L1$^+$ alveolar macrophages as a percentage of live cells or CD45$^+$ cells from the lungs of mice in experimental schema 2. n=5 per treatment group. Asterisk denotes $p<0.05$ by unpaired t test. (C) Representative histograms of PD-L1 expression on alveolar macrophages (light gray: PD-L1 FMO, black: control, green: PTX, red: PLX3397, blue: PTX+PLX3397).

Discussion

The presence of myeloid cells in the lungs, including the monocyte population, are a crucial component of the pro-metastatic lung ME (Kowanetz et al., 2010; Sceneay et al., 2012). The heterogeneous population of myeloid cells can include monocytes and granulocytes expressing Ly6C and/or Ly6G. $Ly6G^{+}Ly6C^{low/med}$ cells are recruited to the lung in MMTV-PyMT mice (Kowanetz et al., 2010) and display functional immunosuppressive behavior when present in primary tumors (Gabrilovich & Nagaraj, 2009), however, their influence on metastatic tumor growth remains controversial. For example, administration of Ly6G neutralizing antibodies enhanced the number of mammary tumor lung metastases by likely targeting anti-tumorigenic neutrophils (Granot et al., 2011), but administration of antibodies to granulocyte colony stimulating factor (G-CSF), a secreted factor that mobilizes granulocytic iMCs from bone marrow, reduces lung metastases (Kowanetz et al., 2010). Whether pro-metastatic or anti-metastatic, our data indicates that neutrophils in the lung appear to be dependent of the presence of the primary tumor as lungs 60 and 115 days post-surgery have neutrophil levels similar to wild type mice, indicating surgery may be a sufficient treatment for any potential pro-metastatic phenotypes neutrophils display.

In addition to neutrophils, it is likely that other myeloid cell populations in the lung harbor what appear to be opposing tumor-influencing properties. A cell population may exert different phenotypes given a specific ME cue or may represent multiple immune cell subsets with different phenotypes that cannot be

distinguished with limited cell surface markers. Because of this, immune populations such as Ly6C$^{low/-}$ monocytes may also appear to either promote or inhibit metastasis. Patrol monocytes have only recently been recognized to influence tumor progression. For example, patrol monocytes were found to be enriched in metastatic lungs and in certain models, appear to have an anti-metastatic phenotype possibly by recruiting NK cells (R. N. Hanna et al., 2015). However, patrol monocytes are also known to promote immune tolerance through expression of PD-L1 and IL-10, which can be reversed with patrol monocyte/MΦ depletion (B. S. Hanna et al., 2016). Thus, it is crucial we understand the roles of specific monocyte and MΦ populations given defined locations (such as the lung) and treatment regimens (such as primary tumor resection).

In our adjuvant therapy murine model, we identified an increase in Ly6C$^{low/-}$ monocytes in the lungs of mice post-tumor resection that are effectively depleted by PLX3397. While future studies are warranted to determine the phenotype of this myeloid population, the reduction of the number of metastatic niche regions correlating with depletion of Ly6C$^{low/-}$ monocytes indicates that they may have a pro-metastatic phenotype. Alternatively, metastasis-associated MΦs may display a pro-metastatic phenotype that overrides an anti-metastatic Ly6C$^{low/-}$ phenotype, similar to reports by Hanna and colleagues (R. N. Hanna et al., 2015). While a greater understanding of these complex phenotypes is needed, these studies do provide pre-clinical evidence indicating inhibition of the

CSF1/CSF1R axis results in reduction of the metastatic niche in the adjuvant setting.

We have intriguing data that supports the notion that treatment with neutralizing antibodies to CSF1 or PLX3397 as monotherapy, without addition of the chemotherapeutic agent PTX, reduces the metastatic niche. This is in contrast to results in MMTV-PyMT transgenic mice harboring primary tumors that required combination CSF1/CSF1R-blockade and PTX to reduce overt metastatic burden (DeNardo et al., 2011). Thus, further studies are warranted to determine if a reduction in $PyMT^+$ cells are also found in concert with a reduction in the metastatic niche regions, possibly by qPCR of genomic DNA from whole lung tissue. It is feasible a PLX3397 monotherapy is only reducing the ME with unchanged disseminated tumor cell burden and only a combination of PTX+PLX3397 reduces metastatic cancer cells, yet this remains unknown.

Further, it is also unclear what biologic influence these peribronchial/perivascular immune cell clusters play in disease progression. It is possible these regions promote seeding of cancer cells into the lung that had previously disseminated to other distant organ regions at the time of surgery, such as lymph nodes (LNs) or bone marrow. This niche may harbor an anti-apoptotic environment for neoplastic cells, allowing for long-term survival. Alternatively, these regions may display a more anti-tumorigenic phenotype, holding DTCs in equilibrium with the immune system at a metastatic site. These possibilities must be investigated to understand tumor progression during this

important biologic window and may influence how we design new adjuvant, metastasis-specific therapeutic strategies.

It is also not known what mechanisms underlie why PD-L1^{+} alveolar macrophages are found in higher numbers following PTX+PLX3397 treatment in the adjuvant setting. One possibility can be inferred from observations found in glioblastoma where MΦs are capable of surviving CSF1R inhibition by the presence of secreted factors such as granulocyte-macrophage colony stimulating factor (GM-CSF) and IFNγ (Pyonteck et al., 2013). Growth factors such GM-CSF are known inducers of alveolar macrophage proliferation and this may be a possible mechanism by which increased alveolar macrophages are observed. Alternatively, PLX3397 and PTX may result in differentiation of other myeloid populations to express alveolar macrophage markers or may have direct effects on alveolar macrophage proliferation. Regardless, this observation provides a rationale for a potential therapeutic benefit in combining PD-L1 inhibition with PLX3397+PTX in the adjuvant setting.

Chapter VI: Conclusions, limitations and future directions

Heterotypic cell fusion in the context of cancer remains controversial. First, it has not been conclusively demonstrated cell fusion occurs *in vivo*, nor has cell fusion been directly observed. Additionally, there is little evidence that supports functional biological significance of cancer cell fusion. There are also limited studies supporting the notion that cancer cells undergo fusion in cancer patients. Given these limitations and previously unanswered questions, the concept of cancer cell fusion remains contentious and debatable within the field.

Within this dissertation, MΦ-cancer cell fusion was demonstrated to occur spontaneously in cell culture and in murine models. Further, hybrids displayed enhanced migration, chemotaxis and metastatic seeding in multiple organ sites, highlighting that cell fusion must be included as a mechanism for acquisition of metastatic phenotypes. Additionally, a more comprehensive assessment of cell fusion in patients was performed, including analysis of numerous patients with several instances of cell fusion from graft-vs-host disease (GVHD) biopsies and in multiple secondary solid cancers. Further, a novel circulating tumor cell (CTC) population expressing the surrogate cell fusion hybrid marker CD45 was determined to correlate strongly with stage and survival.

Taken together, in concert with a newly developed mouse model for understanding early stages of the metastatic microenvironment (ME), a relatively unexplored field of tumor biology that combines both the tumor ME and cancer cell-intrinsic biology is now primed for further investigation.

Limitations to models and alternative explanations

Cell fusion in patient tissue biopsies

Identification of Y chromosomes in non-neoplastic human epithelial cell nuclei, while supporting BMDC-epithelial cell fusion, is not conclusive evidence for this process as other explanations can account for this observation. Given an increased frequency of X chromosomes in Y chromosome positive epithelia relative to male controls, it is possible intraepithelial lymphocytes with increased ploidy or chromosomal aberrations are present in the context of GVHD and cytokeratin immunofluorescence is not sensitive to distinguish epithelial cells from intraepithelial lymphocytes. Further, BMDCs with increased numbers of X chromosomes may differentiate into epithelial cells. With respect to both GVHD and adenocarcinoma biopsies, alternative explanations for Y chromosome positive epithelial cells include differentiation of BMDCs into epithelial cells as well as fetal microchimerism, as these female patients may have had sons (Chan et al., 2012). While these alternative explanations have not been excluded, this human data is consistent with murine models demonstrating BMDC-epithelial cell fusion (Davies et al., 2009; A. E. Powell et al., 2011; Rizvi et al., 2006).

Cell fusion using *in vitro* and *in vivo* murine models

Utilization of dual fluorescence markers for identification of spontaneous fusion between cancer cells and MΦs has limitations. For expression of histone tagged RFP from cancer cells and cytoplasmic GFP from MΦs, an alternative explanation for co-expression of these reporters rather than fusion is by cancer

cell acquisition of GFP protein through an alternative mechanism, including exosomes and/or nanotubes. This could account for transient expression of GFP and could therefore be temporarily indistinguishable from a hybrid using these markers. Yet, as these cells divide, GFP protein would eventually be lost and progeny may not contain GFP. While FACS isolated, *in vitro*-derived hybrids retain GFP, it is possible the *in vivo*-derived RFP^+GFP^+ cells arose from one of these alternative mechanisms. Alternatively, a MΦ phagocytosing an RFP^+ cancer cell may give the appearance of a single cell expressing GFP with nuclear RFP. Direct visualization by live imaging can exclude this possibility as GFP can be seen entering the cytoplasm of the cancer cell at the moment of fusion followed by mitosis, resulting in two hybrid progeny with GFP cytoplasm and RFP nuclei.

With hybrid detection through a Cre recombinase-Cre reporter system, these alternative explanations remain a possibility. With respect to exosomes, it cannot be ruled out that exosomes containing Cre recombinase from a cancer cell enter a MΦ to activate the Cre reporter YFP, followed by exosome trafficking of YFP from MΦs into RFP^+ cancer cells. Similarly, nanotubes trafficking both Cre recombinase and the activated YFP can also result in a RFP^+YFP^+ cell. In these scenarios, transient expression of YFP would be expected in the RFP^+ cancer cells. While this is a possibility for a subset of cells in our *in vitro* co-culture experiments, we also observe cells with long-term expression of both RFP and YFP. Regardless, because of these possibilities, karyotype analysis

paired with X and Y chromosome FISH, as well as EdU-labelled DNA experiments were performed to confirm DNA of biparental origin.

While previous studies have identified macrophages as the main hematopoietic lineage fusing with intestinal epithelium by lineage-limited transplant studies (A. E. Powell et al., 2011), it is unclear what specific lineage fuses with neoplastic cells. While the majority of hybrid CTCs in our murine models express the myeloid cell surface antigens CD11b, CSF1R, and/or F4/80 in addition to CD45 as shown in Supplementary Figure 3.6, it has not been definitively demonstrated that a particular myeloid lineage is the main fusogenic partner with the neoplastic cell. Lineage limited transplant studies and/or experiments with Cre recombinase driven by promoters specific for a given lineage, may shed light into various populations fusing with neoplastic cells. This may lead to additional biological phenotypes acquired by fusion, and may result in more targeted approaches to inhibit cell-cell fusion.

Hybrid CTCs in pancreatic cancer patients

Because the vast majority of CTCs expressing the leukocyte marker CD45 in murine models were hybrid cells, we reasoned CD45 is a reliable surrogate marker for hybrids in patient peripheral blood. This provided us with the rationale that $CK^{+}CD45^{+}$ cells may be detected in patient peripheral blood. While this population showed a strong, statistically significant correlation with disease stage and overall survival, we have not conclusively determined this cell population's identity. While it is possible these $CK^{+}CD45^{+}$ cells are hybrids derived from

macrophages and malignant cells, there are other possibilities. First, these cells may represent an immune cell with induced expression of CK. Alternatively, this could represent an epithelial cell with induced expression of CD45. While possibly derived from cell fusion, it is also unknown if this epithelial cell type was derived from the neoplastic tumor cell. To address this possibility, sequencing of KRAS mutation status of $CK^{+}CD45^{+}$ cells is currently underway. It is possible that these cells arise from a non-malignant epithelial cells fusing with an immune cell an entering circulation. Regardless of these alternative explanations, the observation that this cell population correlates with disease stage and survival, implicates a potential biologic role and has exciting possibilities for clinical utility.

Detection of a metastatic niche after primary tumor resection

One of the interesting observations found in lungs of mice injected orthotopically with primary tumor from transgenic MMTV-PyMT mice is the increase in immune cell infiltrate 60 and 115 days post surgery, specifically increased monocytes, relative to tumor bearing mice (d0) (Figure 5.1). While we identify corresponding peribronchial/perivascular inflammatory clusters that also appear 60 and 115 days post surgery, it is also possible the increase in immune infiltrate in the lungs is not represented by these inflammatory clusters and instead is more diffuse across the entire lung parenchyma. To obtain a stronger correlation, CD11b, Ly6C, Ly6G and F4/80 staining should be performed on these inflammatory regions to determine if monocytes, the population increased in the lungs post-surgery, are located within the inflammatory clusters.

Regardless, inhibition of the CSF1/CSF1R axis reduces the number of these foci, indicating that cell types responsive to CSF1, such as monocytes or MΦs, may be present within these regions.

Because a subset of the peribronchial/perivascular inflammatory clusters contain $PyMT^+$ cells by IF and PCR, and are morphologically consistent with what have been termed premetastatic niches (Erler et al., 2009; Kaplan et al., 2005), these clusters were termed metastatic niches. This niche, however may not be specific to a neoplastic state. While $PyMT^+$ cells were identified in these areas, and these regions may have important biological roles in disease progression, they may also be present in additional pathological states, including infection or various chronic inflammatory diseases. Regardless of their potential non-specificity, this niche may influence metastatic progression and therefore, may lead to novel adjuvant therapeutic strategies.

Evidence of cell fusion in humans

The scientific community's acceptance of the concept that bone marrow-derived cells (BMDCs) fuse with non-neoplastic and neoplastic epithelial cells has been hampered by major barriers in the field that have yet to be overcome. In addition to determining biological relevance of cell fusion hybrids, one key remaining hurdle is a comprehensive approach to identifying cell fusion in humans.

Many of the limitations to validating cell fusion in humans is identifying and utilizing a reliable marker that can distinguish BMDCs from epithelial cells. By taking a genetic approach where patients received a bone marrow transplant (BMT), BMDCs can be distinguished from all host-derived cells. We first used this approach to comprehensively assess and validate the presence of cell fusion hybrids in female Graft-vs-host disease (GVHD) patients who had received bone marrow transplantation from male donors by identifying numerous intestinal epithelial cells that contained a Y chromosome with multiple X chromosomes. We determined that cell fusion in patients is not a rare event, but is observed frequently in the context of GVHD.

A similar approach was employed to identify evidence for cell fusion in patient solid tumors. While sample numbers were limited, we were able to identify multiple cancer epithelial cells per sample that contained Y chromosomes in female cancer patients with a previous sex-mismatched BMT. As with non-neoplastic intestinal epithelium, the identification of multiple Y chromosome-positive cancer epithelial cells supports the notion that cell fusion is not a rare

event, can be conclusively identified in patient tumors, and is not limited to one specific cancer type.

There are however, key limitations to this study. For example, limited access to tissue did not allow for large scale quantification of X chromosome frequency in Y chromosome positive cancer epithelial cells. This additional assessment would exclude alternate explanations, including immune cell differentiation into an epithelial cell within the tumor, as well as fetal-maternal microchimerism where rare male fetal cells can be detected in mothers long-term post-partum (Bianchi, Zickwolf, Weil, Sylvester, & DeMaria, 1996; Chan et al., 2012), neither of which have been reported in the context of cancer. While cell fusion is a mechanism that explains the presence of these cells and is consistent with mouse studies, more extensive approaches can more definitively identify such cells as hybrids. For example, single cell genome or exome sequencing of cancer cells from patients that have previous BMTs would be ideal to assess bi-parental DNA and may allow for further characterization of patient-derived cell fusion hybrids. What remains unexplored is the functional biological significance of cell fusion in humans. By identifying potential markers or gene expression signatures for patient-derived cell fusion hybrids, hybrids could subsequently be isolated and directly assessed for altered genotype and acquired phenotypes, similar to the approaches taken with murine models presented in this dissertation.

Why does cell fusion occur?

A key question that still remains in the field are why epithelial-MΦ cell fusion hybrids form in the first place. If similar to murine models, increased cell fusion may be promoted by highly proliferative and inflammatory environments (Davies et al., 2009). This indicates that there may be a regenerative role for cell fusion. For example, if epithelial stem or progenitor cells are impaired in a damage setting, epithelial-MΦ fusion may be a mechanism by which a differentiated cell gives rise to a stem-like cell, as resulting hybrids give rise to differentiated intestinal epithelial cells (Rizvi et al., 2006). Alternatively, cell fusion may have various other roles, from creating an anti-inflammatory ME to possibly promoting an inflammatory response.

How does cell fusion occur?

One recurring hypothesis is that fusion is merely an error in a potentially unrelated biological process such as phagocytosis. Given the structured mechanisms of both homotypic and certain examples of heterotypic cell fusion, this is likely not the case. Thus, understanding the mechanism(s) that induce cancer-MΦ fusion are important to determine the physiologic role of fusion and may present opportunities for inhibition of fusion with therapeutic intent. One of the more promising cellular structures likely involved in cancer-MΦ cell fusion is an invasive, actin-rich protrusion, termed invadopodia in cancer cells and podosomes in normal cells (Gimona, Buccione, Courtneidge, & Linder, 2008; Weaver, 2006). Podosome-like structures have been determined to play a role in myoblast fusion (Sens et al., 2010). More importantly, Oikawa and colleagues

report that melanoma cancer cells can be stimulated to fuse with myeloid-derived osteoclasts and is mediated by Tks5, a protein required for podosome or invadopodia formation (Oikawa et al., 2012). Given osteoclasts are derived from precursors f the myeloid lineage, a similar mechanism is likely involved. Knockout of Tks5 in either the neoplastic cell or the MΦ, followed by *in vitro* co-culture of the two populations would be an initial approach to determine the role of invadopodia in MΦ-cancer cell fusion. A reduction or absence of *in vitro*-derived hybrids would indicate invadopodia play an important role in facilitating fusion. It is likely that interference with invadopodia formation on the neoplastic parental cell will reduce cell fusion as this approach inhibited melanoma-osteoclast fusion, an event that is likely similar to MΦ-cancer cell fusion (Oikawa et al., 2012). Followup *in vivo* experiments using Tks5-knockout murine models may provide additional support for fusion facilitated by invadopodia. Assessment of podosome/invadopodia function in epithelial-MΦ and cancer-MΦ fusion is warranted and may give biological insight into the physiologic role of cell fusion.

Regardless of these gaps in our knowledge, my studies provide the most in depth evidence supporting presence of cell fusion hybrids in patient intestinal epithelial cells and cancer, providing a rationale for assessing the pathogenic role hybrids play in tumor progression.

Evidence and relevance of a $CD45^+$ CTC population

We report an exciting cytokeratin $(CK)^+CD45^+$ peripheral blood cell population that is consistent with fused CTCs in murine models. While subsets of this CTC population have been incidentally identified by various groups, it has

remained unknown if these cells are biologically relevant (Clawson et al., 2012; M. B. Lustberg et al., 2014; Sheng et al., 2014). Our studies reveal that the circulating CK^+CD45^+ population correlates with stage and survival in pancreatic cancer patients, whereas conventional CTCs do not. One limitation to this study is that the CK^+CD45^+ cells have not been confirmed to be derived from the PDAC. Future studies must be performed to establish genetic analysis of this circulating population, compare KRAS mutation status with corresponding primary tumor biopsies, and/or include additional markers more specific to PDAC. Importantly, the possibility exists that these CK^+CD45^+ cells are indeed not neoplastic, do not represent a CTC, and instead may be a result of an inflammatory state whereby non-neoplastic epithelial cells expressing CD45 enter the bloodstream or immune cells induce expression of CK. Regardless, CK^+CD45^+ cells in circulation may provide a reliable, non-invasive diagnostic and/or prognostic indicator, independent of its neoplastic state.

A more in depth assessment of immune and cancer-specific markers is also warranted. First, this may provide insight into what specific myeloid population(s) result in the CK^+CD45^+ cell population. For example, these cells could express CD163 or CD206, indicating a more phenotypically M2-like macrophage fuses with cancer cells. Second, further assessment of these populations may shed light on the function of these cells. For example, expression of CSF1R may indicate a more invasive phenotype consistent with murine models (Patsialou et al., 2015) and/or injection of CK^+CD45^+ subsets into a recipient mouse could assess varied tumorigenic potential. Last, identification

of additional immune or cancer-specific markers may yield a more specific diagnostic or prognostic indicator.

Can $CD45^+$ CTCs guide treatment strategies?

In addition to diagnosis and prognosis, a more valuable application for quantifying CK^+CD45^+ CTCs or specific subsets of these CTCs is either correlation with response to treatment or ideally predict response to treatment. This could influence decision points such as maintaining a patient on a given therapeutic or govern which specific treatment regimens a patient should receive. While still in its infancy, longitudinal assessment of these CTC populations will determine their potential for clinical utility. Given the non-invasive nature of acquiring these biomarkers and the fact that multiple samples can be longitudinally acquired, it is possible a highly sensitive and specific test that governs a patient's treatment regimen can come to fruition with further investigation into this novel CTC population.

Cancer cells acquire metastatic phenotypes via cell fusion

As shown in Supplementary Figure 3.3, this dissertation provides the first direct evidence that cell fusion between MΦs and cancer cells results in hybrid cells. Cell fusion was identified where the resulting hybrid retained proliferative potential as mitosis was subsequently observed. Because hybrid progeny can proliferate, we were able to acquire populations of hybrid cells. While multiple subsets of hybrid isolates acquired pro-metastatic phenotypes, there are many questions that remain. First, the significance of heterogeneity within the hybrid population is not fully appreciated as not all isolates shared acquired phenotypes,

indicating functional heterogeneity. Second, the immunologic role of cell fusion has yet to be explored. Last, it is unclear if cell fusion is necessary for any component of tumor progression.

What is the significance of cell fusion hybrid heterogeneity?

While confirming acquisition of MΦ genetic material via Edu-labelling and sex chromosome analysis, one of the observations made was that cell fusion resulted in hybrids with a heterogeneous chromosome composition. Selection of various independent hybrid populations yielded cancer cells in a hyper-diploid state with genetic heterogeneity both within a hybrid population and between different hybrid populations isolated. Further, different isolated hybrid populations did not all share acquired phenotypes. The significance of this heterogeneity can be addressed through multiple approaches. One that is of urgent need of further study is resistance to therapeutics, such as chemotherapy. Our approaches to assess hybrid population genetics and phenotypes relied on selection for their ability to proliferate *in vitro* and as such, likely resulted in selection of highly proliferative populations. Yet it is possible subsets of hybrid cells derived either *in vitro* or *in vivo* may provide a cancer cell subset with a less proliferative phenotype and may resist anti-mitotic chemotherapeutic regimens. In addition to therapeutic resistance, additional selective pressures could favor survival or proliferation of specific hybrid subpopulations, including resistance to an immunologic response. Thus, future studies must address cell fusion hybrid function in the context of the immune ME.

What is the immunologic role of cell fusion hybrids?

One intriguing component to tumor biology that warrants investigation is the immune function of resulting hybrid cells. Hybrids could theoretically perform MΦ-like functions within a tumor and may even have varied polarization states. It is possible that, like MΦs, hybrids contribute to an immunosuppressive ME by secreting cytokines such as IL-10 under certain conditions. On the other hand, hybrids may play an anti-tumor role where hybrids may engulf neighboring neoplastic cells. Additionally, hybrids may present tumor antigens to T cell populations similar to a classical antigen-presenting cell.

Instead of performing the roles of an immune cell, hybrids may display altered immunogenicity. While murine hybrid CTCs did not have increased expression of PD-L1 or CD47 relative to unfused primary tumor cells (data not shown), it is possible other mechanisms reduce the immunogenic response, including expression of additional immune checkpoints such as PD-L2, which is more classically associated with expression on leukocytes or hematologic malignancies (Pardoll, 2012). Both *in vitro*- and *in vivo*-derived hybrids should be evaluated for their ability to develop subsets of cells resistant to immune clearance. Insight into the immunological function of hybrid cancer cells and their interaction with the immune system will likely lead to a more comprehensive understanding of hybrid cell function and their role in tumor progression.

Is cell fusion necessary for tumor progression?

While we have conclusively shown that hybrid cells acquire many pro-metastatic phenotypes, including increased migration and seeding/growth at

distant organ sites, a crucial question remains: in certain contexts, is cell fusion necessary for metastatic spread of disease? Given the multitude of mechanisms by which cancer cells can acquire pro-metastatic function (from genetic/epigenetic changes to uptake of exosomes from the ME), the answer is likely no. Nonetheless, efforts should be taken to determine how much metastatic burden in various models is dependent on cell fusion. Specifically, highly metastatic Cre-expressing cancer cells can be injected into a diphtheria toxin Cre reporter mouse. With this system, all hybrids should theoretically activate expression of diphtheria toxin and undergo cell death. To assess the necessity of cell fusion on progression of disease in a model not reliant on a cell line but rather a heterogeneous spontaneously-derived tumor, MMTV-PyMT mice were crossed with the diphtheria toxin Cre reporter mice and can now be injected into various Cre-expressing mice, including mice with Cre under the promoter for the CSF1R. This approach merges techniques, methodology and expands on the biology from all chapters presented in this dissertation. While this approach would address the relative contribution of cell fusion with respect to acquisition of metastatic phenotype, it would also address the relative contribution of cell fusion hybrids with respect to macrophage-like function. Given that macrophages can produce an anti-inflammatory microenvironment or present antigen to induce an adaptive immune response, it is possible hybrids contribute similarly. Through ablation of *in vivo*-derived hybrids, these questions could be addressed. It is likely, in the context of the primary tumor ME, the relative contribution of immunosuppressive cytokines such as IL-10 would be low, compared to unfused

MΦs. Yet hybrids may have enhanced ability to present antigens, which may contribute to an anti-tumor response that either inhibits tumor growth or leads to activation of inhibitory checkpoints such as PD-L1.

Collectively, determining cell fusion as a mechanism by which cancer cells acquire pro-metastatic phenotypes revolutionizes the fields of both tumor cell intrinsic biology as well as the tumor ME. Merging what are often segregated fields of cancer biology now opens new exciting avenues for understanding progression of disease. Future studies on cell fusion and optimization of clinical biomarkers hold great potential for prognostic indicators and future longitudinal studies may lead to tailored treatment strategies. These studies reveal how cell fusion promotes metastatic spread of disease, which have exciting potential to result in the design of new interventional therapeutic approaches targeting metastasis, as well as lead to improved early detection and prognostic indicators.

Appendix: Metastatic Colorectal Cancer: Rational Treatment, Surveillance, and Decreasing the Risk of Recurrence

Components of this dissertation's introduction are adapted and included in this review. Submitted to *Cellular and Molecular Gastroenterology and Hepatology*. Zarour LR, Anand S, Billingsley KG, Bisson WH, Cercek A, Clarke MF, Coussens LM, Gast CE, Geltzeiler CB, Hansen L, Lopez CD, Ruhl R, Tsikitis VL, Vaccaro GM, Wong MH, and Mayo SC. October 3, 2016.

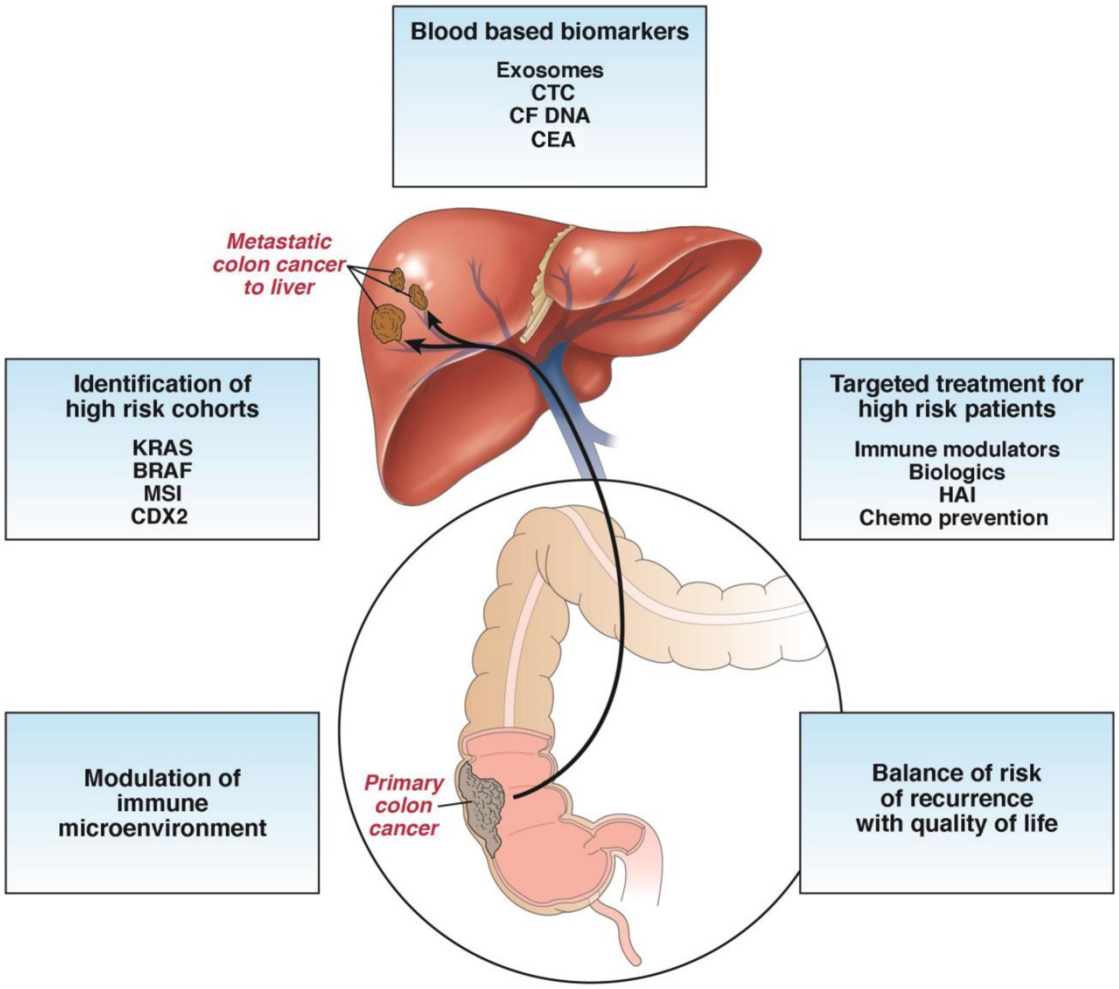


Figure A.1. Targeted treatment, surveillance, and decreasing the risk of recurrence for metastatic colorectal cancer

Abstract

In patients with colorectal cancer metastatic to the liver, key goals for improving outcomes include early detection, effective prognostic indicators of treatment response, and accurate identification of patients with high-risk for recurrence. While new therapeutic regimens developed over the past decade have increased survival, there is substantial room for improvement in effectively targeting regimens to patients who will derive the most benefit. Recently, there have been exciting developments in identifying high-risk patient cohorts, refinements in the understanding of systemic versus localized drug delivery to metastatic niches, new liquid biomarker development, and dramatic advances in tumor immune therapy, all of which promise new and innovative approaches to tackling the problem of detecting and treating metastatic spread of CRC to the liver. Our multidisciplinary group held a state of the science symposium to review advances on this front and herein we present a discussion around the issues facing treatment of metastatic colorectal cancer to the liver, including correlations of discrete gene signatures and expressions with prognosis. We also discuss the latest advances to maximize regional and systemic therapies to decrease intrahepatic recurrence, new insights into the tumor microenvironment, and a summary of advances in non-invasive multi-modal biomarkers for early detection of primary and recurrent disease. As our clinical and technological advances expand in the field of tumor biology, especially colorectal tumor biology and microenvironment, we aim to refine our predictive and prognostic studies to

decrease intrahepatic recurrence after curative resection and minimize toxicity through a tailored approach multidisciplinary to cancer care.

Introduction and background

Colorectal cancer (CRC) is the third most common cancer worldwide ranking as high as the 2nd leading cause of cancer-related deaths in developed countries.(Ferlay et al., 2015; Sameer, 2013; Siegel, Miller, & Jemal, 2016) The liver is recognized as the most common site of CRC metastasis since the majority of the intestinal mesenteric drainage enters the hepatic portal venous system. Over 50% of patients with CRC will develop metastatic disease to their liver, which ultimately results in death for over two-thirds of these patients.(House et al., 2011; Tomlinson et al., 2007) Currently, hepatic resection of colorectal cancer liver metastasis (CRLM) in patients with isolated liver metastasis remains the only option for potential cure. However, even when combined with modern adjuvant regimens, resection is curative in only 20% of patients(Fong, Fortner, Sun, Brennan, & Blumgart, 1999; House et al., 2011; Tomlinson et al., 2007), with 70% developing recurrence, primarily in the liver.(Tomlinson et al., 2007) Efforts to prevent recurrence are limited by the cumulative side effects of systemic therapy, development of chemoresistant cancer clones, and the ability to detect progression of radiographically occult micrometastatic disease. In an updated analysis of the largest randomized controlled trial (RCT) to date that examined the role of peri-operative systemic therapy in patients with resectable CRLM before and after curative hepatic resection, there was no improvement in 5-year overall survival (OS) compared to patients treated with hepatic resection alone (51% vs. 48%; $P = 0.34$).(Nordlinger et al., 2008; Nordlinger et al., 2013) While perioperative systemic therapy remains the standard of care for patients

with resected CRLM, there is significant room to better identify patients with a molecular high-risk signature who will benefit from adjuvant treatment aimed to decrease intrahepatic recurrence. In addition, for patients with liver-only metastatic CRC treated with curative intent surgery, detecting disease recurrence at the earliest stage and monitoring response to treatment are paramount to moving the field forward. Herein, we review modern approaches for treating patients with CRLM and ongoing work to molecularly risk-stratify patients to direct systemic treatment and surveil for intrahepatic recurrence (Figure 1).

Scope of the clinical problem for colorectal cancer liver metastasis

Detecting primary CRC and CRLM at an early stage results in better outcomes.(UK, 2016) At a molecular level, CRC consists of a heterogeneous group of diseases. Chromosomal instability (CIN), mismatch repair (MMR) with resultant microsatellite instability (MSI), aberrant DNA methylation, as well as altered molecular signaling pathways have all been described in the transformation from normal mucosa to adenocarcinoma.(Boland & Goel, 2010; Goel et al., 2007; MacDonald, Tamai, & He, 2009; Pino & Chung, 2010; Shaw & Cantley, 2006) Furthermore, these hallmarks have been associated with cancer sensitivity and resistance to both systemic therapy and biologic agents in the primary and metastatic setting.(Colussi, Brandi, Bazzoli, & Ricciardiello, 2013; Markowitz & Bertagnolli, 2009; Messersmith & Ahnen, 2008) Given the extensive molecular and clinical heterogeneity of the disease, it is essential to individualize therapy on the basis of molecular profiling to avoid treatment related toxicities without a realized survival benefit. Some of the strongest data to support the need for identification of high-risk cohorts amongst patients with CRLM come from adjuvant trials for primary CRC. The 2004 adjuvant MOSAIC trial(André et al., 2004) assessed the impact of an oxaliplatin containing systemic regimen (FOLFOX) for patients with resected CRC compared to fluorouracil (5-FU) alone in patients with stage II and III disease, a significant survival benefit for patients with stage III disease was found and has been maintained in recently updated 10-year results.(André et al., 2015) However, these benefits come with significant patient morbidity affecting quality of life. For patients with stage III CRC treated

with FOLFOX instead of 5-FU and leucovorin (LV) there is a consequent 4% decrease in mortality.(André et al., 2015) However, to achieve this 4% reduction in mortality with oxaliplatin, 92% of those patients will suffer from treatment-associated peripheral neuropathy, with 15% experiencing permanent neuropathy.(André et al., 2009) It is clear that even amongst patients with stage III disease there is an underappreciated disease heterogeneity that at present is being treated with a homogenous systemic treatment approach. These data in the primary CRC setting underscore the need for molecularly-driven systemic treatment to avoid both the financial and quality of life costs to patients with liver-only metastatic CRC. Work is ongoing to identify molecular subsets of CRLM with the ultimate goal to personalize targeted interventions to maximize therapeutic interventions. In this review, we describe the role of liquid biopsies and novel cancer and immunologic cell populations to both surveil and assess treatment response in patients with CRLM. We also propose using this information to guide the design and development of therapeutic strategies for liver-directed treatments.

Parallels between patients with high-risk primary colorectal cancer and insights into directing peri-operative treatment in patients with liver-only metastases

For patients with liver-only metastatic CRC, there is a pressing need for a more robust molecular characterization of the primary and metastatic lesions to direct peri-operative management of patients at highest risk for disease recurrence.(Benson & Hamilton, 2011) In the primary setting, there has been great interest in patients with high-risk stage II CRC—those patients with negative lymph nodes but other high-risk features such as T4 lesions, obstruction or perforation, cancers with lymphovascular invasion, and poorly differentiated histology. It is worth reflecting upon the impact of the uniform approach of adjuvant treatment of patients with primary CRC to better understand the challenges of directing peri-operative treatment for patients with CRLM. One of the early trials to investigate the impact of adjuvant treatment on stage II CRC was the 2007 QUASAR trial where patients with stage II CRC were randomized to treatment with adjuvant 5-FU/LV or observation after curative resection.(Group, 2007) The results of this trial demonstrated an approximate 3% improvement in outcome when 5-FU/LV was given in the adjuvant setting. Said another way, 97% of patients were exposed to chemotherapy without any benefit. Given that standard stage II patients do not benefit as shown in the QUASAR, MOSAIC, and other trials, it is currently at the discretion of the treating clinician to weigh the high-risk features of the primary CRC to decide to offer adjuvant treatment in this setting.(André et al., 2004; André et al., 2015; Group,

2007) According to the most current National Comprehensive Cancer Network (NCCN) guidelines, "…the current definition of high-risk stage II colon cancer is clearly inadequate, because many patients with high-risk features do not have a recurrence while some patients deemed to be average-risk do. Furthermore, no data point to features that are predictive of benefit from adjuvant chemotherapy, and no data correlate risk features and selection of chemotherapy in patients with high-risk stage II disease."((NCCN), 2016) These data are remarkably similar to and parallel the challenges of directing peri-operative treatment in patients with CRLM.

Aside from the relatively macro descriptions of the primary lesion for patients with high-risk stage II CRC, several investigators have sought a correlation between discrete gene signatures and a higher-risk patient population in order to molecularly stratify patients to better direct adjuvant therapy. In 2016, Dalerba et al. reported on the expression of the caudal-type homeobox transcription factor 2 (CDX2), a critical regulator of intestinal development and oncogenesis, as a prognostic biomarker in patients with stage II CRC.(Dalerba et al., 2016) The group used a combination of insights from basic science discoveries around normal colon stem cells and cancer stem cells, the availability of public databases of sequenced tumors (National Center for Biotechnology Information Gene Expression Omnibus, NCBI-GEO, and NCI-CDP) and the power of bioinformatics to query over 2329 human samples. They identified 16 genes that were not expressed in colorectal epithelia expressing high levels of the stem cell marker ALCAM/CD166 (Activated leukocyte-cell adhesion

molecule). Of these genes, they focused on CDX2, a marker already used in standard surgical pathological assessments of resected CRC specimens. The authors went on to demonstrate in validation datasets that patients with stage II and III CRC who were CDX2-negative had a worse 5-year disease-free survival (DFS) as compared to patients that had CDX2-positive cancers (49% among 15 patients with CDX2-negative tumors vs. 87% among 191 patients with CDX2-positive tumors, *P*=0.003). In a further validation study using datasets from two different clinical trials, the authors again found an association between DFS in patients with CDX2-negative stage II cancers who received adjuvant chemotherapy, regardless of their age, gender or cancer grade.(Dalerba et al., 2016) In the final pooled analysis including the test and validation database of all patient cohorts, the 5-year DFS was higher among the 23 patients with stage II CDX2-negative tumors who were treated with adjuvant chemotherapy compared to those patients not treated with adjuvant systemic therapy (91% vs. 56%, *P*=0.006).(Dalerba et al., 2016) This work is one example that illustrates the existence of subgroups of patients with CRC with a discrete biology who are likely to achieve a survival benefit from adjuvant treatment that will outweigh the treatment-associated morbidity. The ability to expand our understanding of the underlying biology driving disease recurrence in diverse subsets of patients will provide clarity for the therapeutic roadmap to effectively treat patients at all stages of disease. To date, this work has most recently been updated in the metastatic CRC population (Zhang et al., 2016) where patients with CDX2-negative metastatic CRC were found to have a median OS of 8 months versus

39 months for those with CDX-2-positive metastatic CRC (HR 4.04, 95% CI 2.49-6.54, p<0.0001). Those patients were also more likely to have right-sided primary tumors, have poorly-differentiated cancers, distant lymphatic metastasis, and to be women. This work will pave the way to further define a subgroup set of patients with liver-limited metastatic CRC that would derive a RFS benefit from adjuvant treatment after a curative hepatic resection of their disease.

Recurrence after a hypothetically curative hepatic resection of CRLM occurs in the majority of patients. Historically, several clinicopathologic factors that were independent predictors of poor outcome in patients with resected CRLM (nodal status of the primary cancer, preoperative carcinoembryonic antigen [CEA] level, size of the largest liver lesion, and number of hepatic metastases) were used to predict the risk of intrahepatic recurrence of disease (Fong et al., 1999). Similar to the tumor characteristics in patients with clinically high-risk stage II CRC, these factors paint a relatively broad description of the disease. Recognizing the prognostic limits of these clinical risk scores, these prediction models are now often used in conjunction with targeting of select cancer mutations such as in the epidermal growth factor receptor (EGFR) pathways (KRAS mutation status(N. E. Kemeny et al., 2014) or BRAF mutation status(Yaeger et al., 2014)) in order to treat patients who are most likely to respond to a given regimen. Recent work has explored deriving cancer genetic expressions as prognosticators of recurrence and survival for patients with CRLM. Balachandran et al. reported on gene signatures predictive of disease-specific survival as well as liver recurrence-free survival (RFS) in patients with

resected CRLM.(Balachandran et al., 2016) Using gene expression microarray on resected CRLM the authors were able to identify and validate 20 genes that were associated with OS. Importantly, this so-called “molecular risk score” was also prognostic of RFS and remained independently prognostic of RFS on multivariate analysis, unlike the traditional clinical risk score. Taken together, these data identifying patients with high-risk primary CRC and resected CRLM who are most likely to recur and therefore most likely to benefit from further treatment, allowing us to direct the selection and duration of adjuvant treatment. The continued identification of molecular subsets of CRLM that underlie discrete tumor biology and are predictive of treatment response is critical for bridging the gap between diagnoses and survival in order to best direct peri-operative treatment with both biologic and cytotoxic therapy.

Maximizing regional treatment of colorectal cancer liver metastasis to decrease intrahepatic recurrence

In patients with CRLM that undergo a hepatic resection with curative intent, it is estimated that approximately 75% of all recurrences—both intrahepatic and extrahepatic—occur within the first two years after operation.(Fong et al., 1997) Efforts over past decades have sought to address risk of recurrence, which is possibly the result of treatment-resistant micrometastatic disease. One avenue to aiming to obliterate micrometastatic disease in the liver focuses on maximizing hepatic locoregional therapy by exploiting basic tumor biology. Cancer cells from gastrointestinal malignancies, especially CRC, hematogenously spread via the portal circulation, often making the liver the first site of metastasis. Once hepatic metastases grow above 2 mm in size, they derive their blood supply from the hepatic artery, while normal hepatocytes are perfused mostly from the portal circulation.(Ensminger & Gyves, 1983) Exploitation of this biologic difference has led to treating select CRLM patients with hepatic arterial infusion (HAI) therapy, which is based upon the extraction of chemotherapy from the hepatic arterial circulation, resulting in high local drug concentrations with the goal of minimizing systemic toxicity. The ideal agent should have a high dose-response curve, high extraction, and rapid total body clearance once the infusion is discontinued. Of the various agents studied, HAI-delivered floxuridine (FUDR) approximates this ideal with a short half-life (<10 minutes) and >90% hepatic extraction, resulting in a 16-fold higher concentration in hepatic tumors as compared with venous administration.

(Ensminger & Gyves, 1983; Kelly, Kemeny, & Leonard, 2005) Using FUDR in combination with dexamethasone, patients with CRLM can have their liver disease maximally treated with modest side effects compared to standard systemic treatment.(N. Kemeny et al., 1992) Several prospective trials(N. Kemeny et al., 1987; Kerr et al., 2003; Lorenz & Muller, 2000; Rougier et al., 1992) have investigated using HAI alone in order to circumvent the toxicity associated with systemic treatment of CRLM, to maximize hepatic response in an effort to improve both OS and PFS, and potentially improve patient quality of life.(Allen-Mersh, Earlam, Fordy, Abrams, & Houghton, 1994; N. E. Kemeny et al., 2006)

The efficacy of HAI was initially tested without concurrent systemic therapy, which at the time of the initial trials did not include modern systemic agents such as oxaliplatin and irinotecan. To date, there have been no prospective RCTs comparing adjuvant HAI with current systemic therapy versus current systemic therapy alone in patients with resected CRLM. In 2016, Kemeny et al. reported on an analysis of four consecutive HAI adjuvant trials for patients with resected CRLM from 1991-2009 (n=287).(Nancy E. Kemeny et al., 2016) The patients were divided into two groups: those treated before and after 2003, corresponding to the incorporation of modern systemic oxaliplatin or irinotecan containing regimens. With a median follow-up time of 11 years, the authors reported that patients treated after 2003 had 5 and 10-year OS of 78% and 61% respectively, with the median survival not being reached. Patients treated before

2003 had 3 and 5-year RFS of 42% and 41%, respectively.(Nancy E. Kemeny et al., 2016)

Taken together, these data support that properly selected patients with CRLM can have hepatic resection of their disease followed by adjuvant systemic therapy plus HAI and achieve 5-year survival as high as 78%. However, similar to toxicity associated with systemic therapy, treatment with HAI has risks including biliary sclerosis in less than 5% of patients that needs to be balanced with anticipated benefit of treatment.(Nancy E. Kemeny et al., 2016) For treatments such as HAI that seek to maximally treat the liver, it is imperative to begin integrating pre-operative prognostic indicators that are predictive of a patient's risk of intra-hepatic recurrence after hepatic resection. Ultimately, we must integrate a non-invasive test to determine risk for both local and distant recurrence of disease with monitoring of response to systemic therapy as well as an early signal for intrahepatic recurrence. In addition to discrete gene expression profiles that identify patients at high risk of recurrence, blood based biomarkers for non-invasive monitoring of early detection of recurrent disease that are actively in development may serve as a more reliable marker to monitor response to treatment, and ultimately surveil patients for recurrence of disease after curative treatment.

Non-invasive liquid biomarkers for early detection of primary and recurrent disease

While newly identified gene signatures may identify at risk patient populations, a second front for informing biologically-driven treatment of cancer is the temporal analysis of disease response across treatment. Novel modes of "liquid biopsies" for solid tumors are on the forefront of discovery and promise to provide a monitor for early detection of recurrent disease, or even a series of accurate snapshots of the treatment-mediated evolution of the disease to allow flexible, tailored therapy. Solid tumors are known to shed cells, cell-free DNA (cfDNA), and exosomes into circulation—each provides opportunities to survey cancer behavior as a liquid biopsy. Conventionally isolated circulating tumor cells (CTC), defined by cell surface expression of Epithelial Cell Adhesion Molecule (EpCAM), cytokeratin (CK) and absence of the pan-leukocyte marker, CD45 expression, have been shown to correlate with PFS and OS in patients with breast cancer (Cristofanilli et al., 2004), as well as in colorectal and prostate cancer. (Steven J. Cohen et al., 2008; de Bono et al., 2008) While these data indicate utility for prognosis, CTCs, in general, have suffered from difficulty in surveying them, due to their rarity in circulation, and more importantly, that they have failed to provide biologic insights into the tumor that may guide informed treatment. One possible rejuvenation of the CTC field, is in the discovery of novel CTC populations, most that have previously been discarded as artefactual. For example, standard detection methods of CTCs rely on expression of specific epithelial markers, CK and/or EpCAM, and the exclusion of leukocyte specific

markers, typically CD45. CTCs have also been isolated based on size, density, charge or various other properties that positively or negatively enrich a specific cell population.(Catherine Alix-Panabieres & Klaus Pantel, 2014) These existing approaches bias the subsets of CTCs that are being evaluated, and may be excluding biologically relevant subpopulations. CellSearch® is the FDA-approved test to detect CTCs by magnetic separation of $EpCAM^+$ cells followed by positive staining for CK and negative staining for CD45. Yet Zhang et al. demonstrated the high metastatic capability in of an $EpCAM^-$ CTC population isolated from patients with breast cancer in a mouse xenograft assay (Lixin Zhang et al., 2013). This $EpCAM^-$ CTC population might represent cancer cells that have undergone epithelial-to-mesenchymal transition, thereby losing expression of $EpCAM^-$, but may represent a more migratory and invasive cell. Therefore, a biased approach to CTC exploration may exclude unique biology that can be appreciated in liquid biospies.

An additional population of CTCs that has been largely ignored by the field, those that express the leukocyte marker CD45. Peripheral blood cells from cancer patients, isolated by differential centrifugation and size exclusion were found to harbor CTCs that expressed CK *and* CD45, yet conferred robust growth in culture. (Clawson et al., 2015) Additionally, $CD45^+CK^+$ CTCs were identified in patients with metastatic pancreatic cancer from an $EpCAM^+$ enriched population (Gao et al., 2016), and in metastatic breast cancer patients, even with partial $CD45^+$ depletion with magnetic beads.(Maryam B. Lustberg et al., 2014) Interestingly, the breast cancer $CD45^+$ CTCs also expressed the macrophage

marker CD68, indicating CTC populations may acquire proteins typically expressed by macrophages, possibly through a cell fusion mechanism.(A. E. Powell et al., 2011) To fully appreciate these $CD45^+$ CTCs, direct visualization would rule cancer cell-immune cell clusters that could be construed as a $CD45^+$ CTC by flow cytometry. If these cells arise from leukocyte-cancer fusion, a novel tumor biology may provide important insights that may more effectively guide therapy. How untapped and uninvestigated populations of CTCs contribute to our overall knowledge of disease should be developed in parallel with the rapid advancements in other biomarker fields, such as that of cfDNA.

Cell free DNA (cfDNA), is hypothesized to arise from cells that die, whether by necrosis, cell lysis, or apoptosis, and their residual unique fingerprint and release naked DNA into the circulation. While this was first detected in healthy individuals in the late 1940's, it was not until the 1970's and 1980's that their neoplastic characteristics were identified and that cfDNA existed in higher concentrations in cancer patients relative to controls. (Leon, Shapiro, Sklaroff, & Yaros, 1977; Stroun et al., 1989) While quantification of cfDNA was useful in some disease states when used alongside classic blood tests (e.g., CEA) (Shapiro, Chakrabarty, Cohn, & Leon, 1983), the more important and specific role for cfDNA is in the identification of gene mutations and microsatellite instability. Detection of cancer-associated alleles in the blood represents a significant obstacle; however, with current technological advancements, this task has become achievable. In 1997, de Kok et al. analyzed 14 samples from patients with CRC for point mutations in KRAS then correlated the positive

samples with serum cfDNA amplified by PCR, validating this highly specific method for mutation detection.(de Kok et al., 1997) This also proved that cfDNA were at least partially derived from cancer cells. Now, microsatellite abnormalities have been detected in patient blood from breast cancer, head and neck cancer, lung cancer, melanoma, and CRC.(Bruhn et al., 2000; Mayall, Fairweather, Wilkins, Chang, & Nicholls, 1999; Nakayama et al., 2000; Nawroz, Koch, Anker, Stroun, & Sidransky, 1996) Isolated cfDNA has many characteristics of tumor DNA including presence of oncogenes as well as other global molecular classifiers such as MSI, *CpG island methylator phenotype* (*CIMP*)(Kloten et al., 2014), and CIN.(Crowley, Di Nicolantonio, Loupakis, & Bardelli, 2013) El Messaoudi et al. set out a multiparametric analysis of cfDNA evaluating the OS of n=97 patients with metastatic CRC. Higher cfDNA levels were associated with statistically significant decrease in OS (18.07 months vs. 28.5 months, *P*=0.0087). Furthermore, on multivariate analysis the authors demonstrated that a higher cfDNA level is an independent prognostic factor (*P*=0.034) and the levels of cfDNA fragmentation were correlated with decreased OS in the mutant KRAS/BRAF population whereas no correlation was found with the wild-type KRAS/BRAF patients.(El Messaoudi et al., 2016) Newer technologies under development such as PlasmaSelect assay (Parpart-Li et al., 2016) now afford identification of multiple mutations and genetic alterations thereby allowing an increased comprehensive genomic analysis. Those genetic alterations along with novel CTC populations have great potential to provide novel, non-invasive, commercially available approaches to cancer diagnosis, allowing for early

detection of recurrent disease, assessment of the evolving tumor biology, and provide a foundation for tailored treatment.

Immune reprogramming as a therapeutic strategy

Over the last five years, there has been an incredible reinvigoration in the interest and relevance of exploiting the tumor immune microenvironment as a viable and effective treatment option for many cancers that had previously been recalcitrant to treatment. It has become increasingly clear that the tumor microenvironment plays a key role in tumor progression and response to therapies across many different cancer types.(D. Hanahan & Lisa M. Coussens, 2012) Immune check-point inhibitors such as ipilimumab, pembrolizumab, and nivolumab are being widely studied in prospective trials in a variety of cancers, including in patients with liver-only metastatic CRC. Several recent studies have highlighted the role of non-neoplastic cells, particularly stromal cells and immune cells as prognostic markers in human CRC.(Calon et al., 2015; Isella et al., 2015; B. Mlecnik et al., 2016; Bernhard Mlecnik et al., 2016) Immunologically, it has been shown that MSI-high cancers harbor infiltrating tumor lymphocytes that are actively suppressed by immune-inhibitory signals such as the programmed death ligand-1 (PD-L1) and PD-1 complexes.(Calon et al., 2015) In 2015, Le et al. reported the results of a phase II trial of patients treatment-refractory progressive metastatic cancer treated with the anti-PD-1 antibody pembrolizumab. The results for n=32 patients with metastatic CRC were stratified by MMR-deficient (e.g., MSI-high) CRC compared to patients with MMR proficient tumors. For patients with MMR-deficient CRC, the immune-related objective response and immune-related PFS were 40% and 78%, respectively, as compared to 0% and 11% for patients with MMR-proficient CRC.

Specific features of the tumor microenvironment such as an abundance of T-helper 2(T_H2) cytokines, pro-inflammatory molecules, pro-angiogenic, and pro-fibrotic molecules are immunosuppressive and considered pro-tumorigenic. In contrast, an abundance of T_H1 cytokines, angiostatic factors, immunostimulatory molecules, along with the mobilization and reinvigoration of the CD8 T cells are all characteristic of a robust anti-tumorigenic microenvironment. Therefore, understanding the recruitment and function of leukocytes in the cancer will enable the development of both targeted therapies and biomarkers that can predict emergence of treatment resistance and recurrence of cancer.(Palucka & Coussens, 2016) Interestingly, there are several nuances that dictate the function of even the same leukocyte subsets in different cancers.(Affara et al., 2014; Ruffell et al., 2014) For example, pro-tumorigenic macrophages are regulated by a T_H2-$CD4^+$ T-cells in mammary carcinomas and B cells in pancreatic adenocarcinomas and squamous cell carcinomas (SCC). Similarly, the soluble mediators and signaling pathways that regulate this cellular cross-talk are also different with IL-4/IL-13 and Colony Stimulating Factor-1 (CSF-1) playing key roles in driving macrophage function in mammary carcinomas whereas Bruton tyrosine kinase (BTK) and phosphoinositide 3′-kinase (PI3K) regulating the macrophage function in pancreatic and SCC. As such, it is possible that the immune checkpoint pathways are also regulated differently in different tissues necessitating the development of tailored approaches to immunotherapy across different cancers.(Topalian, Taube, Anders, & Pardoll, 2016) In this context, we propose that a multi-modal biomarker based approach would be optimal for

immune-mediated cancer control and assessing treatment response. Such an approach would rely on biomarkers that measure the pro and anti-tumorigenic factors elaborated earlier and provide multiple avenues to mobilize and reinvigorate the cytotoxic T cell responses. This could include a combination of strategies such as neutralizing the T_H2 responses, cytotoxic and targeted agents, immune checkpoint blockade, vaccines, and chimeric antigen receptor T cells. This multi-modal approach will also sample the microenvironment whenever the cancers escape and recalibrate the specific reprogramming strategy, specific immune checkpoints that can be targeted and specific pathways that drive the microenvironment so cancer regression and control can be reestablished. In summary, an approach that dynamically engages the immune system by constantly sampling the microenvironment to detect recurrence and relapse is essential to incorporate into the management of patients with advanced CRC and direct novel immunologic therapies.

Summary, novel molecules, and future clinical trial directions

Ultimately, the biology of a tumor—both cell intrinsic and cell extrinsic—underlies clinical outcome for patients with metastatic CRC. Indeed, the concept of liver-only metastatic disease by definition implies a different biologic subtype. This is clinically apparent and our attempts to exploit this biology are the basis of all liver-directed therapy. Future directions in treating this CRC liver-only subtype therefore must revolve around a better molecular characterization and the development of improved therapeutic approaches. As liver-directed therapy continues to evolve with the development of other local treatment modalities—including microwave ablation, irreversible electroporation, and transarterial radioembolization (e.g., Y-90)—our ability to identify and understand this biology becomes paramount. Currently, clinicians use the biologic "test of time" to ascertain if a hepatic-only metastatic state can be maintained while first-line systemic agents are used—and hence provide the rationale to attempt intensive liver-directed approaches, including hepatic resection and HAI. Banking tissue from these patients and correlating these clinicopathologic specimens with high-quality clinical data is essential and has become an integral aspect of modern prospective clinical trials. Tissue samples across the continuum of patient treatment are mandatory to facilitate discovery-based approaches. Samples taken at several time points (e.g., pre-treatment, following each cycle, and after completion of systemic therapy) in addition to the surrounding hepatic parenchyma are essential for an in-depth interrogation of the local tumor microenvironment. Data support that properly selected patients with CRLM can

have hepatic resection of their disease followed by adjuvant systemic therapy plus HAI and achieve 5-year OS as high as 78% with a hepatic RFS of 62% at 5 years.(Nancy E. Kemeny et al., 2016) However, similar to toxicity associated with systemic therapy, treatment with HAI has risks including biliary sclerosis, underscoring the need for optimal patient selection. The role of HAI in the adjuvant treatment of patients with resected CRLM additionally offers the unique opportunity to deliver novel agents in a liver-directed fashion. While FUDR has been used for decades and represents a pharmacokinetically ideal agent, our rapidly expanding knowledge of CRC tumor biology and microenvironment begs for the development and study of novel agents coupled with the rational design of clinical trials that can exploit this knowledge in a liver-targeted fashion. Given the extensive molecular and clinical heterogeneity of the liver-only metastatic CRC, it is of great importance to individualize targeted therapy on the basis of molecular profiling through logical implementation of biomarker assessment in liquid biopsies for early metastasis and to surveil for intrahepatic recurrence. We are charged with moving beyond a blunt “one-size fits all” approach in treating patients with CRLM and only molecularly driven treatments will ultimately improve OS, reduce intrahepatic recurrence, and minimize the toxicity of peri-operative therapies.

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Biographical Sketch

OMB No. 0925-0001 and 0925-0002 (Rev. 10/15 Approved Through 10/31/2018)

BIOGRAPHICAL SKETCH

NAME: Charles E. Gast

eRA COMMONS USER NAME (credential, e.g., agency login): charliegast

POSITION TITLE: MD/PhD Student

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
Lewis & Clark College, Portland OR	BA	2002-2006	Biochem/Molecular Bio
Oregon Health & Science University, Portland, OR	MD-PhD (in progress)	2011-present	Medicine/Cancer Bio

A. Personal Statement

My desire to become a physician scientist is rooted in my early exposure to science in college, which stimulated me to become more academically focused, excited about learning and provided my first experience with the thrill of discovery from basic science research. This led to my first research position at Oregon Health and Science University where I joined the laboratory of a clinician, Dr. Brian Wong. As I determined key aspects of virulence and drug resistance in multiple fungal pathogens and developed an interest in having a career encompassing research, I also started volunteering in the department of emergency medicine and a free health clinic, which led to my interest to work with patients. During my five years of researching fungal pathogens and diversifying my exposure to various aspects of clinical medicine, I developed a sense of how tightly linked knowledge of disease and its clinical management and scientific discovery are—it confirmed my aspirations to become a physician scientist.

As a medical student, I found aspects of wound healing, cancer, and immunology particularly interesting. In my preclinical years of medical school, I rotated in the laboratories of Drs. Melissa Wong and Lisa Coussens, where I was engaged in exploration of a novel mechanism underlying metastatic spread of cancer and identifying immunotherapies that are efficacious in a metastatic mammary cancer mouse model. My work in Dr. Wong's laboratory resulted in a publication (Silk AD, **Gast CE**, Davies PS, Fakhari FD, Vanderbeek GE, Mori M, Wong MH. Fusion between hematopoietic and epithelial cells in adult human intestine. *PLoS One*. 2013;8(1):e55572) and work in Dr. Coussens' laboratory resulted in development of the novel a mammary cancer mouse model that develops lung metastasis after surgical removal of the primary tumor. As a result of the close interests of Drs. Wong and Coussens, I was able to engage in a collaborative research project in both laboratories and co-mentorship with two outstanding and complementary investigators.

I have passed my USMLE Step 1 with a score of 250 (6/13), comprehensive exam (7/14) and qualifying exam (10/14), and have now focused my research interests on elucidating myeloid cell-related mechanisms within the lung microenvironment that supports breast cancer metastasis. I am particularly interested in specific cell populations that appear to be involved in either the initial seeding or subsequent outgrowth of mammary cancer cells in the lung, cells dependent on CSF1. With my optimized mouse model that closely mimics the course of patient disease, I have preliminary data that CSF1-dependent myeloid cells promote metastasis in the lung after surgical removal of the primary tumor. My proposed research strategy has clear translational implications relating to the treatment or prevention of breast cancer metastasis.

Having a foundation in science as a research assistant, publishing numerous papers, including one first author manuscript, and making rapid advances in my research as a graduate student, demonstrate my technical expertise, ability to thoughtfully design experiments, and work as a successful team to productively drive research projects. My choice of joining the MD/PhD program at OHSU and working on tumor immunology will provide a solid foundation for becoming a successful clinician scientist by continually improving my skills in both the laboratory and in the clinic.

B. Positions and Honors

ACTIVITY/OCCUPATION	BEGINNING DATE	ENDING DATE	FIELD	INSTITUTION/COMPANY	SUPERVISOR/ EMPLOYER
Laboratory Assistant	2006	2011	Microbiology	OHSU	Dr. Brian Wong
Laboratory Assistant	May 2005	August 2005	Chemistry	Lewis & Clark College	Dr. William Randall
Clinic Assistant	May 2004	August 2004	Orthopedics	RMA Orthopedics	Dr. Kenneth Pettine

Academic and Professional Honors

2014-15 Ruth L. Kirchstein T32 Training Grant Recipient, Program in Molecular and Cellular Biosciences
2015 Best Poster Award: FASEB, Steamboat Springs, CO
2015-16 Ruth L. Kirchstein T32 Training Grant Recipient, Training in the Molecular Basis of Skin/Mucosa Pathobiology
2015 Travel Award: Critical Issues in Tumor Microenvironment, Angiogenesis and Metastasis Course
2016 American Skin Association Medical Student Grant Targeting Melanoma and Skin Cancer
2016 Winner of the Student Oral Presentation Award: Research Week, Portland, OR

Memberships in professional societies

2014-15 American Gastroenterology Association
2014-15 Society for Immunotherapy of Cancer

C. Contribution to Science

1. My early publications identified signals that regulate GPI-protein trafficking to the cell wall in *Candida albicans*. These results provide a basis for targeting a pathway that could inhibit function of virulence factors as a potential therapeutic strategy.

a) Mao Y, Zhang Z, **Gast C**, Wong B. C-terminal signals regulate targeting of glycosylphosphatidylinositol-anchored proteins to the cell wall or plasma membrane in Candida albicans. Eukaryot Cell. 2008 Nov;7(11):1906-15. PMCID: PMC2583546.

2. In addition to the work described above, my other publications that were focused on the *Candida* and *Cryptococcus* fungal pathogen species identified mechanisms of drug resistance to the azole antifungal group of drugs. These works focused mainly on identifying and evaluating antifungal efflux pumps as well as determining the role of the antifungal target protein Erg11p.

a) Basso LR Jr, **Gast CE**, Mao Y, Wong B. Fluconazole transport into Candida albicans secretory vesicles by the membrane proteins Cdr1p, Cdr2p, and Mdr1p. Eukaryot Cell. 2010 Jun;9(6):960-70. PMCID: PMC2901649.

b) Basso LR Jr, Bartiss A, Mao Y, **Gast CE**, Coelho PS, Snyder M, Wong B. Transformation of Candida albicans with a synthetic hygromycin B resistance gene. Yeast. 2010 Dec;27(12):1039-48. PMCID: PMC4243612.

c) **Gast CE**, Basso LR Jr, Bruzual I, Wong B. Azole resistance in Cryptococcus gattii from the Pacific Northwest: Investigation of the role of ERG11. Antimicrob Agents Chemother. 2013 Nov;57(11):5478-85. PMCID: PMC3811322.

d) Basso LR Jr, **Gast CE**, Bruzual I, Wong B. Identification and properties of plasma membrane azole efflux pumps from the pathogenic fungi Cryptococcus gattii and Cryptococcus neoformans. J Antimicrob Chemother. 2015 May;70(5):1396-407. PMCID: PMC4398472.

3. My work has also identified cell-cell fusion between hematopoietic and intestinal epithelial cells in human Graft vs. Host Disease patients. This work links human observations with previous evidence in mouse models demonstrating epithelial and cancer cells fusing with macrophages. This project also has currently unpublished data indicating cancer-macrophage hybrids acquire phenotypes attributed to disease progression.

a) Silk AD, **Gast CE**, Davies PS, Fakhari FD, Vanderbeek GE, Mori M, Wong MH. Fusion between hematopoietic and epithelial cells in adult human intestine. PLoS One. 2013;8(1):e55572. PMCID: PMC3559593.

D. Scholastic Performance

YEAR	COURSE TITLE	GRADE	YEAR	COURSE TITLE	GRADE
	Undergraduate Courses - Lewis and Clark College			**Medical School Courses - OHSU**	
2002	Investig. Ecology/Env. Sci.	B-	2011	Gross Anat/Imag/Emb	H
2002	Inventing America I	C	2011	Cell Structure & Function	NH
2002	Intermediate German	C	2011	Principles of Clin Med - Yr 1	S
2002	Calculus I	C+	2012	System Processes & Homeostasis	NH
2003	Investig. Genetics/Evol. Bio.	B-	2012	MD/PhD Journal Club	P
2003	Calculus II	D	2012	Biological Basis of Disease	NH
2003	Sound and Sense: Music	B	2012	MD/PhD Research (MS1 & MS2)	P
2003	Inventing America II	C	2012	Circulation	NH

YEAR	COURSE TITLE	GRADE	YEAR	COURSE TITLE	GRADE
2003	General Chemistry I	A	2012	Metabolism	NH
2003	Physics I	B	2012	Principles of Clin Med - Yr 2	S
2003	Introduction to World Music	B+	2012	MD/PhD Journal Club	P
2003	Weight Training	P	2013	Neuroscience and Behavior	H
2004	General Chemistry II	B+	2013	Human Growth and Development	S
2004	Investig. Cell/Molecular Bio.	B	2013	Blood	H
2004	Physics II	B	2014/16	Transition to Clerkship	P
2004	Drawing I	A-	2014-15	MD-PhD Longitudinal Clerkship	In prog.
2004	Organic Chemistry I	A-		**Graduate school courses - OHSU**	
2004	Molecular Biology	B	2013	Practice and Ethics of Science	P
2004	Molecular Biology Lab	B	2013	Structure & Function of Bio Mol.	A
2004	Evolution	B-	2013	Genetic Mechanisms	B+
2005	Making Modern China	B	2014	Bioregulation	B+
2005	Organic Chemistry II	A	2014	Molecular Cell Biology	A
2005	Weight Training	P	2014	Devl/Differentiation & Disease	A
2005	Cell Biology	A-	2014	Advanced Immunology	B
2005	Introduction to Psychology	A	2014	PMCB Comprehensive Exam	P
2005	Structural Biochemistry	A	2014	PMCB Qualifying Exam	P
2005	Astronomy	A	2015	Current Topics/Tissue Biology	A
2005	Ceramics	A-	2015	Intro. Biostatistics	Aud
2006	Microbiology	B	2015	Adv. Cancer Biology	A-
2006	Physical Chemistry	A			
2006	Metabolic Biochemistry	B			
2006	Biochemistry Lab	A-			

Undergraduate grading: A-F; Medical School Grading: Honors (H), Near-honors (NH), Satisfactory (S), Marginal (M), Fail (F); Graduate School Grading: A-F, Audit (Aud); Some courses graded Pass/Fail

Standardized Test Scores
Combined MCAT score: 30N
US Medical Licensing Exam I (taken 6/21/2013): PASS 250

E. Research Support

Current support:
2016
American Skin Association Medical Student Grant Targeting Melanoma and Skin Cancer

7/1/15–6/30/16
Grant number/Funding source: 2T32CA106195-11 (Kulesz-Martin, Molly)
Title: NIH Ruth L Kirschstein T32 Training in the Molecular Basis of Skin/Mucosa Pathobiology

7/1/14–6/30/15
Grant number/Funding source: T32 GM071388-10 (Maslen, Cheryl)
Title: NIH Ruth L Kirschstein T32 PMCB training grant

2014
Tartar Trust Grant Award