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UNIVERSITY OF CALIFORNIA SAN DIEGO

Exosomes and cell-cell fusion in cancer

A dissertation submitted in partial satisfaction of the requirements for the degree of
Doctor of Philosophy

in

Biomedical Sciences

by

Stephen Curtis Searles

Committee in charge:

Professor Jack Bui, Chair
Professor John Chang
Professor Richard Klemke
Professor Maurizio Zanetti
Professor Dong-Er Zhang

2018

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Chair

University of California San Diego

2018

DEDICATION

To my dad, Bob Searles- you played a critically important role in shaping me into the man I am today, yet sadly, you are not alive to witness me complete this milestone. I think about you often, and I deeply miss the time we had together. I miss and love you more that you could ever know.

EPIGRAPH

“An ‘Alice in Blunderland’ approach led to the discovery of exosomes and their involvement in protein extrusion from maturing reticulocytes. Current studies have shown that the process of exosome formation extends to many mammalian cells.

Full knowledge of the contribution of exosomes to intercellular information transmission and the potential medical application of this knowledge will depend on the ingenuity of future investigators and their insight into biological processes”

-Dr. Rose Johnstone (discoverer of exosomes), 2005

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LIST OF ABBREVIATIONS

MVB	Multivesicular body
BAL	Bronchoalveolar lavage
TD	Tumor-derived
NK	Natural killer
DC	Dendritic cell
MAP2K1	Mitogen-activated protein kinase kinase 1
FDA	Food and Drug Administration
APC	Antigen presenting cell
MCA	3'methylcholantrene
s.c.	Subcutaneous
PBS	Phosphate-buffered saline
RAG1	Recombination activating gene 1
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
AU	Arbitrary units
GFP	Green fluorescent protein
PD-1	Programmed cell death protein 1
i.p.	Intraperitoneal
CFSE	Carboxyfluorescein succinimidyl ester
FACS	Fluorescent activated cell sorting
BMDM	Bone marrow-derived macrophage
HA	Hemagglutinin
WCL	Whole cell lysate

ECV	Extracellular vesicle
MEF	Mouse embryonic fibroblast
ADF	Adult dermal fibroblast
FSC	Forward scatter
PCR	Polymerase chain reaction

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VITA

2009 BA, Human Biology, University of Montana

2009-2012 Research Assistant, Montana State University

2018 PhD, Biomedical Sciences, University of California San Diego

Publications

Searles S, Di Giannatale A, Hatano M, Hoshino A, Matei I, Lyden D, and Bui J. "Exosomal MEK1 inhibits tumor growth by boosting anti-tumor immunity." Manuscript in preparation.

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* **equal contribution**

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ABSTRACT OF THE DISSERTATION

Exosomes and cell-cell fusion in cancer

by

Stephen Curtis Searles

Doctor of Philosophy in Biomedical Sciences

University of California San Diego, 2018

Jack D. Bui, Chair

The tumor microenvironment plays a critical role in dictating cancer outcome. Specifically, intercellular communication that occurs between transformed cancer cells and non-transformed host cells is increasingly recognized as a critical regulator of many processes related to cancer, including progression, regression, drug resistance, and metastasis. In this dissertation, we have investigated the role of 2 independent mechanisms of intercellular communication in cancer: exosomes and cell-cell fusion.

Exosomes are small membrane vesicles of endosomal origin that mediate intercellular communication via the transfer of bioactive cargo to target cells. Cancer cells abundantly produce exosomes, and tumor-derived (TD) exosomes are now appreciated as major forces capable of modulating the properties of host cells, including immune cells. Here, we discovered that the signaling molecule

MAP2K1 (MEK1) is enriched in exosomes from regressing tumors compared to exosomes from progressively-growing tumors. Moreover, we found that MEK1 protein in exosomes promotes anti-tumor immunity and is sufficient to delay the growth of both syngeneic and 3rd party tumors. These findings have clinical significance since MEK1 inhibitors currently used to treat cancer could have negative consequences by dampening the tumor-suppressive, immunestimulatory effects of MEK1 in exosomes.

Cell-cell fusion was first recognized to occur in cancer over 100 years ago, yet it remains debated whether this phenomenon is physiologically relevant to disease progression. Here, we serendipitously developed a model system that could observe and measure cell-cell fusion in cancer. We found that cancer cells spontaneously and rapidly fuse with non-cancer cells, and the resulting hybrid cells possess enhanced chemoresistance and a higher degree of heterogeneity/ clonal diversity compared to parental cells. These results imply that cell-cell fusion promotes malignant properties associated with cancer and could serve as an engine to drive cancer cell diversification, thus providing the substrate for cancer cell evolution.

The studies presented in this dissertation add to the growing body of evidence that intercellular communication within the tumor microenvironment has profound effects on many different aspects of cancer, and serve as a reminder that the cell-extrinsic communication occurring between cancer cells and non-cancer cells should be carefully studied to develop novel cancer treatments.

CHAPTER I: INTRODUCTION

1.1 Intercellular communication between tumor cells and host cells regulates cancer progression

At its simplest form, cancer is a disease of uncontrolled cellular proliferation regulated by intrinsic defects (gene mutations). However, it is now appreciated that extrinsic factors also play an important role in regulating tumor progression [1]. Indeed, interactions between transformed cells and healthy host cells are often critical determinants of whether a tumor will grow progressively, reject, resist chemotherapy or metastasize [2, 3]. Many different forms of intercellular communication between tumor and host cells have been shown to affect tumor progression. These include the release of tumor-derived cytokines (such as IL-17D [4] and TGF- β [5]) and the expression of cell-surface proteins on tumors (such as NKG2D ligands [6] and PD-L1 [7]). However, it has become increasingly clear that additional mechanisms of communication are also likely relevant to tumor progression. Specifically, exosome-mediated transfer of tumor-derived proteins to host cells and cell-cell fusion between tumor and host cells are two mechanisms of communication that are likely relevant in dictating cancer outcome, yet are still not fully understood [8, 9].

1.2 Exosomes: potent mediators of intercellular communication

Exosomes are small membrane vesicles derived from the endosomal pathway and secreted by most cell types, containing various bioactive molecules (proteins,

RNA, DNA and lipids) [10]. They form through the inward budding of the luminal membrane of the late endosome, resulting in a vesicle-filled organelle called the multivesicular body (MVB), which fuses to the plasma membrane and releases the exosomes into the extracellular environment [11]. Originally observed in reticulocytes, exosomes were initially thought to be simply a means by which unwanted molecules are removed from cells [12]. Subsequent studies, however, demonstrated that the cargo of exosomes remain biologically functional, and amazingly, can be transferred to distant recipient cells and alter the phenotype of target cells [13, 14]. This data, along with observations that exosomes are found in nearly every biological fluid (plasma [15], saliva [16], urine [17], breast milk [18], BAL fluid [19]) sparked renewed interest in the field, and it is now well appreciated that exosomes are potent mediators of both local and distal intercellular communication. Nearly all cells secrete exosomes, but transformed cells secrete more exosomes than non-transformed cells [20]. In addition, the serum of tumor-bearing patients contains more exosomes compared to healthy controls [21]. These observations led to the hypothesis that tumor-derived (TD) exosomes may be involved in regulating tumor outcome depending on how they affect host cells.

1.3 TD exosomes exert both pro- and anti-tumorigenic effects on immunity

It has been demonstrated that immune cells can uptake TD exosomes, and initial studies describing the functional consequences of TD exosomes on immune cells reported enhanced anti-tumor immune activity [22]. It was suggested that TD exosomes act as a source of tumor antigen, as mice injected with TD exosomes are

less susceptible to progressive growth of the tumor from whence the exosomes were derived and also harbor more tumor antigen-specific CD8⁺ T cells compared to control [14]. This observation fueled speculation that TD exosomes are capable of promoting immune responses directed against the tumor. Subsequent reports, however, described inhibitory effects of TD exosomes on anti-tumor immunity. These include inducing apoptosis of tumor-reactive CD8⁺ T cells via exosomal Fas-L [23-25], decreasing NK cell cytotoxicity [26], impairing dendritic cell (DC) maturation [27], and supporting the development of suppressive immune cells (Tregs [28] and myeloid-derived suppressor cells [29, 30]). Clearly it is evident that TD exosomes actively communicate with and can affect the function of host immune cells, ultimately stimulating or inhibiting anti-tumor immunity and affecting cancer outcome [22, 31]. However, more work is needed to better understand what exosomal proteins have the capacity to modulate anti-tumor immunity.

1.4 Cell-cell fusion is a common biological process

Cell-cell fusion occurs when two or more uni-nuclear cells combine their plasma membranes to become a single, multi-nuclear “hybrid” cell containing the complete genome of each parent cell [32]. It is a common biological process and has been well documented in plants [33], yeast [34], *C. elegans* [35], *D. melanogaster* [36], and mammalian cells [37]. In mammals, fusion is a rare but important biological event, and occurs in various tissues including during fertilization, in trophoblasts (during placenta formation), muscle (myoblasts) and hematopoietic cells (giant cells formed from fused macrophages and bone-resorbing osteoclasts) [32]. The functional

consequence of fusion between 2 diploid cells is the formation of a single tetraploid hybrid cell, after which nuclear fusion may or may not occur. In muscle, bone and placenta, fused cells retain separate nuclei and are considered heterokaryons. They are fully differentiated without replicative capacity [38]. Interestingly in plants it has been shown that heterokaryons provide a rich environment for the exchange of genetic elements (such as transposons) between two separate nuclei [39]. In contrast, spontaneous fusion events can be accompanied by nuclear fusion, where the genome of each parent cell is combined within the same nucleus to form a hybrid synkaryon [40]. Hybrids of this type will either maintain tetraploidy or undergo reductive division, creating 2 diploid daughter cells with DNA from each parent cell (**Figure 1.1**).

The molecular regulation of fusion in mammalian cell has been well characterized [41]. Proteins involved in cell fusion are called “fusogens” and include most importantly syncytins, a class of transmembrane glycoproteins derived from the envelope gene of an endogenous retrovirus [42]. Other proteins that have been implicated include annexins, Rab proteins, and SNAREs [43, 44]. Additional molecular cues have also been shown to influence fusion, including small ions/ molecules (such as calcium and cAMP) [45, 46], hormones [47] and lipid membrane components (like phosphatidylserine and cholesterol) [48].

1.5 Cell fusion occurs in cancer

Interestingly, cell fusion has been reported to also occur in cancer and was first noticed over 100 years ago [49]. After repeatedly observing what appeared like

fused cells in cancer biopsies, Otto Aichel proposed as a potential mechanism for cancer cells to gain migratory/ metastatic abilities fusion with a non-transformed “normal” cell already endowed with migratory capabilities (i.e. macrophage). He postulated that hybrid cells would adopt properties of both parent cells and be capable of indefinite replication and tissue migration, and thus could form metastatic lesions. Since this early observation, several different lines of evidence have emerged to suggest that cell fusion does indeed occur in vitro and in vivo. For example, human breast cancer cells have been shown to fuse with endothelial cells in vitro [50], and this may be mediated by syncytin-1 [51]. Fusion has been reported in many types of cancer, including melanoma [52, 53], glioblastoma [54], and renal [55] and ovarian [56] carcinomas. In some instances, human tumors are grown in either mice or hamsters and fusion is determined by the presence of species-restricted DNA. Many studies have observed characteristics associated with increased malignancy in hybrid cells, like chemoresistance. These include studies showing acquired drug resistance when two mammary tumor cell lines were fused [9]. In addition, resistance has been observed in melanoma-derived hybrids [53, 57]. It has also been hypothesized that cell fusion generates the “recurrent cancer stem cell” that repopulates a cancer following chemotherapy due to the expression of stem cell-associated antigens on hybrid cells [58, 59]. However, most of these studies relied on artificially induced fusion methods (such as polyethylene glycol, electroporation, or virus infection) to generate hybrid cells and therefore may not accurately reflect hybrid cells generated in truly physiological conditions.

1.6 Cell-cell fusion as a driver of genetic diversity in cancer

It is believed that cell fusion may be a driving force in cancer progression by providing a mechanism to robustly increase genetic diversity amongst daughter cells [9, 59]. According to this hypothesis, fusion is a more reasonable explanation for any individual cell acquiring the appropriate “mutation signature” necessary for progressive growth, compared to one cell accumulating all of the mutations on its own, cumulatively. For example, mutations could be generated independently in separate cells and shuffled together to generate daughter cells with different combinations of mutations. Alternatively, if fusion occurs between a cancer cell and a non-cancer cell, the epigenetic signature of the non-cancer cell can be preserved, co-opting the “healthy” genes from the non-transformed cell. This theory is intriguing, and would provide a mechanistic basis for the generation of massive genetic diversity within a population of cancer cells, but due to the difficulty of studying this process in vivo it remains debated if cell fusion is physiologically relevant in the context of human cancer.

1.7 Significance

In summary, a variety of different extrinsic mechanisms are involved in regulating tumor progression. Tumor cells communicate with their environment in part via release of exosomes. Exosomes have been shown to transfer proteins to target cells, alter target cell function and affect tumor outcome. However, the specific exosomal proteins capable of stimulating anti-tumor immunity have yet to be fully explored. Tumor cells also can fuse with normal cells or other cancer cells, a process

that was observed over 100 years ago. It occurs in many different cancer types and is regulated by proteins, small molecules and lipids. This phenomenon may increase the malignant behavior of hybrid cells, including inducing chemoresistance and potentially stem-cell formation. However, most work in this field has focused on non-physiologic models and therefore may not represent how cell fusion affects tumor outcome in vivo.

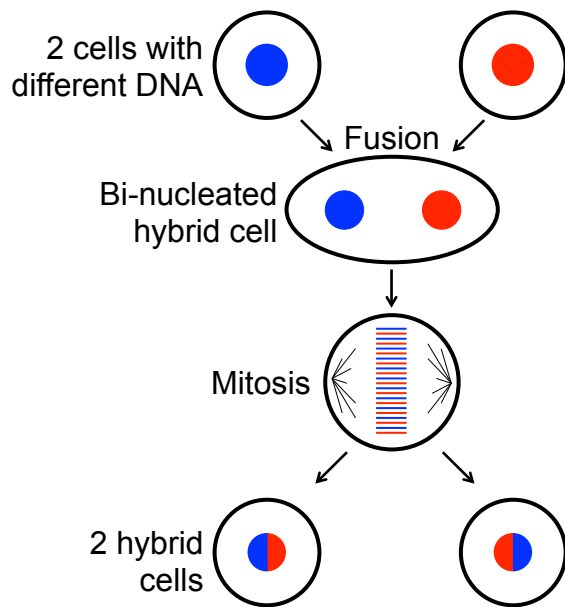


FIGURE 1.1 Cell-cell fusion increases the heterogeneity of progeny cells. Schematic representation depicting how cell-cell fusion could lead to the diversification of daughter cells via DNA pooling and subsequent reductive division.

CHAPTER II: EXOSOMAL MEK1 INHIBITS TUMOR GROWTH BY BOOSTING ANTI-TUMOR IMMUNITY

2.1 Abstract

Exosomes mediate intercellular communication in many processes, including cancer. The cargo of exosomes can be physically transferred to cells both locally and distally, affecting the functionality of the target cell. Transformed cells are known to produce more exosomes than non-transformed cells, which has encouraged investigation into how tumor-derived (TD) exosomes affect cancer outcome. In this manuscript, we have studied how exosomes derived from immunogenic regressor tumor cells promote anti-tumor immune activation and tumor regression. Using a proteomic approach, we found that the signaling molecule MAP2K1 (MEK1) is enriched in immunogenic regressor relative to poorly immunogenic progressor exosomes. We forced expression of MEK1 in progressor tumor cells and found that MEK1 content in progressor exosomes was concomitantly upregulated. MEK1-hi exosomes were found to inhibit tumor growth by indirectly promoting adaptive immunity in both syngeneic and 3rd party tumors. This effect was dependent on MEK1 protein within the exosomes, and could occur by promoting M1-type polarized macrophages within the tumor. These findings have exceptional therapeutic consequences, since MEK inhibitors have been approved by the FDA for the treatment of some cancers. Our data indicate that, in some instances, MEK inhibition may be deleterious to cancer treatment, since MEK1 activity seems to play an important cell-extrinsic, tumor-suppressive role within exosomes. Moreover, the

delivery of MEK1 to tumor-associated macrophages, either by exosomes, nanoparticles, or some other means, could be a useful strategy to treat cancer via the activation of anti-tumor immunity.

2.2 Introduction

Although initially considered cellular “garbage disposals”, exosomes are now appreciated as major forces of intercellular communication [12, 60]. Exosomes are small membrane vesicles derived from the late endosomal pathway. They contain bioactive cargo that can include lipids, nucleic acids (both RNA and DNA), and proteins [10]. The cargo of exosomes can be physically transferred between cells, resulting in functional changes in the target cell [13, 14]. Over the past two decades, numerous studies have examined the role of exosomes in a variety of different physiological processes, and it has become increasingly obvious that exosomes can exert powerful effects on target cells both locally and distally, and in both healthy and disease conditions [61].

Exosomes in cancer have been studied extensively in recent years, especially after it was noticed that cancer cells, on average, secrete more exosomes than non-transformed cells of the same type [20, 21]. This observation led investigators to hypothesize that exosomes could be important regulators of cancer, and subsequent studies have revealed that this is indeed true [62]. Tumor-derived (TD) exosomes can affect numerous cell types, including other cancer cells in an autocrine/paracrine manner [63-65] and non-cancer host cells [8, 66-70].

TD exosomes have been shown to induce profound effects on immune cells [22, 31, 71, 72]. Early reports suggested that TD exosomes exert immune-stimulatory effects, and it was suspected that exosomes could act as a source of shed tumor antigen that boost CD8⁺ T cell responses against the tumor [14]. Accordingly, highly immunogenic antigens have been detected in exosomes derived from melanoma cells (MelanA/ Mart-1 and gp100) and colon carcinoma cells (CEA and HER2) [20]. Heat-shock proteins, which are known to augment APC activation by providing danger signals [73], are also present within TD exosomes [74, 75]. These results supported the hypothesis that TD exosomes are immune-stimulatory in nature and could act as an anti-cancer vaccine. However, further investigation revealed that exosomes also exert inhibitory effects on immune cells and can promote tumor growth [76, 77]. For example, TD exosomes laden with Fas-ligand were shown to induce apoptosis of tumor-specific CD8⁺ T cells directly via FasL-death receptor interaction [23-25]. In another example, TD exosomes were shown to prevent the maturation of circulating monocytes into DCs in a TGF- β -dependent manner [30]. Furthermore, MET protein within melanoma exosomes has even been shown augment metastasis by permanently “educating” bone marrow progenitor cells to promote metastatic niche formation [8]. Therefore, it has become apparent that exosomes are potent modulators of immunity, and that this modulation can subsequently affect cancer progression in both negative and positive terms, depending on the specific molecules involved.

Over the past decade, immune therapies for cancer have emerged as some of the most promising and efficacious therapies in decades, and are even curative in

some instances [78, 79]. For this reason, interest in the discovery of novel immune stimulatory mechanisms and pathways has recently undergone great expansion. While much attention has been paid to adaptive immunity and how this impacts tumor growth and development, less attention has been paid to innate immune mechanisms that may also be relevant to cancer. Here, we propose a novel mechanism by which innate immune cells, specifically macrophages, may be manipulated via exosomal MAP2K1 (MEK1) to provide enhanced therapeutic efficacy against cancer.

The studies presented here describe a novel, cell-extrinsic tumor-suppressive role for the signaling molecule MEK1 mediated by exosomes, likely acting directly on macrophages within tumors and indirectly promoting adaptive anti-tumor immunity. We have discovered that MEK1 is enriched in exosomes derived from tumors that undergo immune-mediated rejection relative to those from progressively growing tumors, and further demonstrate an immune-mediated tumor-suppressive role for MEK1 protein in exosomes against both syngeneic and 3rd party tumors. We propose that MEK1 activity in macrophages can be augmented by tumor-derived exosomes, promoting their development toward the anti-tumor M1-type polarization, which boosts adaptive immunity to inhibit tumor growth.

2.3 Results

2.3.1 Secreted factors from regressor tumors inhibit tumor growth via adaptive immunity

We previously generated and characterized a set of immunogenic 3-methylcholanthrene (MCA)-induced “regressor” fibrosarcoma cell lines that activated the immune system [80], in part by secreting relatively high levels of the cytokine IL-17D [4]. Matched MCA-induced “progressor” cell lines derived under similar conditions were poorly immunogenic and expressed low levels of IL-17D. Although the activation by IL-17D proceeded via local recruitment of innate cells, we wished to also test whether regressor tumors had systemic effects on tumor growth. Therefore, we developed a model system (**Figure 2.1A**) whereby regressor cells (5×10^6 in 200 μ l) or PBS (200 μ l) were injected subcutaneously (s.c.) into the right flank of syngeneic C57BL/6 (B6) mice, representing an “instigator” tumor. Three days later, 1×10^6 progressor cells were injected s.c. into the contralateral (left) flank of both groups of mice (“indicator” tumors), and the growth rate of the progressor (indicator) tumor was monitored over 15 days. We found that after 8 days, indicator tumors grown in mice with a contralateral regressor tumor were approximately 69% smaller in area than tumors grown in control mice that had been injected with PBS (13.2 mm^2 vs. 43 mm^2 , $p < 0.05$ **Figure 2.1B**). After 15 days of growth, indicator tumors grown with a contralateral regressor were still smaller than control indicator tumors by about 44% (57.4 mm^2 vs. 103 mm^2 , $p < 0.05$ **Figure 2.1B**). Since regressor tumors begin to reject after about day 7 in wild-type mice, this likely explains why the difference in size of indicator tumor between the two groups is maximal at day 7, and is gradually reduced as the regressor rejects.

When we performed the same experiment in mice lacking adaptive immunity via deletion of the RAG1 gene ($\text{RAG1}^{-/-}$), we observed no significant difference in the

size of indicator tumors grown with a contralateral regressor compared to those grown in control mice (108 mm² vs. 118 mm² at day 13, **Figure 2.1C**). Taken together, we interpret these results to signify that a secreted factor (or factors) derived from regressor tumors act in a systemic manner to inhibit the growth of the contralateral progressor tumor, and that this effect is mediated (either directly or indirectly) through adaptive immunity.

2.3.2 MEK1 is enriched in regressor exosomes

What could the regressor-derived secreted factor(s) be that promote tumor rejection? Our lab has previously focused on soluble proteins as facilitators of immune-mediated tumor rejection, and in fact discovered that some regressor tumors secrete the cytokine IL-17D to induce rejection via local recruitment of innate immune cells [4]. However, we recognize that other factors may also play a role, so we sought to expand our focus to exosomes. Exosomes have recently been the subject of remarkable interest, and have been shown to exert powerful modulatory effects on immune cells both locally and distally [31, 72]. Moreover, previous publications have implicated specific proteins within exosomes as mediators of immune modulation [8, 25, 68], so we considered that this could be occurring in our model system as well. Therefore, we hypothesized that regressor exosomes contain different proteins than progressor exosomes and the presence of these proteins in regressor exosomes stimulates anti-tumor immunity and promotes tumor rejection.

To test this hypothesis, we performed proteomic analysis on a panel of exosomes isolated from 8 independently derived MCA sarcomas, 4 progressors (2

generated in B6 mice, 2 generated in 129 mice) and 4 regressors (2 generated in B6 mice, and 2 generated in 129 mice). The workflow for this analysis is presented in **Figure 2.2A**. Briefly, each of the 8 cell lines were grown in culture for 72 hrs in media that had been depleted of exosomes, after which conditioned media was harvested and subjected to differential ultracentrifugation to isolate exosomes [81]. The isolates were then subjected to proteomic analysis via LC-MS/MS, which resulted in a list of total proteins identified in each of the 8 isolates.

We first created a list of proteins that were detected in all 4 of the progressor isolates (“progressor common signature”, 205 proteins) and all 4 of the regressor isolates (“regressor common signature”, 254 proteins). We then compared the progressor common and regressor common signature, and proteins that were detected in both signatures were termed the “fibrosarcoma exosome signature”. This list contains proteins that were detected in 8 out of the 8 samples analyzed, and consisted of 155 proteins (**Figure 2.3** and **Table 2.1**). To validate that our isolates were exosomal in nature, we cross-referenced this list with proteins most commonly identified in “ExoCarta”, an online database that catalogues –omic datasets performed on exosomes [82]. We found that of the 25 proteins most commonly identified in the ExoCarta database, 72% (18) belonged to our fibrosarcoma exosome signature (CD9, PDC6I, GAPDH aka G3P, ANXA2, CD63, SDCB1, TS101, KPXM, LDHA, EEF1A1, PGK1, EF2, HS90B, ANXA5, FAS, 14-3-3E, CLH1, and CD81), meaning that they were detected in all 8 of our samples. Furthermore, the other 10 most commonly identified proteins were detected in 7 out of 8 (HSP7C, ENOA, 14-3-3Z, and ALDOA), 6 out of 8 (HS90A) or 5 out of 8 samples (ACTB)

(**Figure 2.4**). Interestingly the 25th most commonly identified protein, ALBU (albumin), was only identified in 1 of our 8 samples. However, since albumin is a common contaminating protein in exosomes isolated from serum, its presence on the list of 25 most commonly identified exosomal proteins is likely an artifact. Taken together, these results gave us confidence that our isolates were in fact exosomal in origin, and allowed us to move forward with our analysis to find regressor-specific exosomal proteins.

We next performed a comparative analysis of our 8 lists of proteins in order to identify proteins that were present in regressor exosomes and absent in progressor exosomes. We established a stringent criteria for “regressor-specific proteins” stipulating that a protein must be present in 4 out of 4 regressor samples and absent in 3 or 4 out of 4 progressor samples, OR present in at least 3 out of 4 progressor samples and absent in 4 out of 4 progressor samples in order to be considered a regressor-specific candidate. These criteria resulted in a list of 9 candidate proteins (**Figure 2.2B**). Interestingly, only a single protein, MAP2K1 (MEK1), showed complete regressor-restricted specificity, meaning it was present in 4 out of 4 regressor exosomes, and absent in 4 out of 4 progressor exosomes. Since MEK1 showed the highest degree of specificity to regressor exosomes, we selected it as our candidate regressor protein to study further.

We next validated our proteomic findings with western blotting. We found that at the whole cell level, there was no difference in MEK1 level between a panel of 3 progressor and 2 regressor cell lines (**Figure 2.2C**). Within the exosomal compartment, however, we observed a significant enrichment of MEK1 in regressor

compared to progressor exosomes, by 540% ($p < 0.01$ **Figure 2.2D**). We also found that the activated form of MEK1, phosphorylated on serine residue 298 (pMEK1), was similarly enriched in regressor relative to progressor exosomes, by 350% ($p < 0.05$ **Figure 2.2D**), but not at the whole cell level (**Figure 2.2C**). We used the exosome marker CD63 as a confirmation that the isolates we measured were exosomal in origin.

We were intrigued that MEK1 showed this expression pattern, since when we initially conceived this project, we had anticipated finding tumor-suppressive proteins in regressor exosomes. We were surprised to find MEK1, a proto-oncogene that is overexpressed in several different cancers, to be restricted to regressor exosomes. Therefore, we decided to further investigate the function of this regressor candidate protein in exosomes.

2.3.3 Forced expression of MEK1 in progressor tumors increases the MEK1 content in progressor exosomes

In order to study the role of MEK1 in exosomes, we sought to develop a model system whereby we could isolate the role of exosomal MEK1, independent of other factors found within exosomes. We considered comparing progressor exosomes (with endogenously low MEK1 in exosomes) to regressor exosomes (with endogenously high MEK1 in exosomes), but since progressor and regressor exosomes have many other proteins differentially expressed beyond MEK1, and also come from independently derived cell lines that would each contain a unique set of mutations, this would not be a suitable model to investigate exosomal MEK1.

Instead, we sought to engineer a cell line that endogenously expresses low MEK1 in exosomes (i.e. any progressor) to express high levels of MEK1 in exosomes. We accomplished this by overexpressing MEK1 in the progressor cell line 9609 (which has very low MEK1 expression in exosomes, **Figure 2.2D**) via retroviral transduction. 9609 cells were also transduced with GFP for control. Using this method, we were able to achieve approximately a 10-fold overexpression of MEK1 protein in 9609 cells (**Figure 2.5A**). Importantly, this overexpression extended to the exosomal compartment (**Figure 2.5A**). Therefore, by simply using retroviral transduction, we were able to engineer the 9609 cell line to produce MEK1-hi exosomes, creating “matched” MEK1-low and MEK1-hi cell lines and exosomes.

We then performed nanoparticle tracking analysis on MEK1-low and MEK1-hi exosomes to determine the size distribution and production level of exosomes from tumor cells. We found no significant difference in size between MEK1-low and MEK1-hi exosomes (mean: 145.7 nm vs. 149.1 nm; mode: 106.5 nm vs. 102.4 nm, **Figure 2.6A**). Furthermore, we found no significant difference in exosome production level between MEK1-low and MEK1-hi tumor cells over 48 hrs (6.15e11 particles/ml vs. 5.54e11 particles/ml, **Figure 2.6A**). We also performed electron microscopy on MEK1-low and MEK1-hi exosomes to further evaluate their size and morphology. We found that MEK1-low and MEK1-hi exosomes appeared similar in both size, with most particles from both isolates around 100 nm in diameter, and morphology, with both exosomes exhibiting the classic “cup-shaped” morphology that is traditionally associated with exosomes (**Figure 2.6B**).

Next, we performed semi-quantitative proteomic analysis of MEK1-hi vs. MEK1-low exosomes to determine if MEK1 overexpression affects other proteins within exosomes. We first confirmed that MEK1 was indeed overexpressed in MEK1-hi relative to MEK1-low exosomes: western blot analysis showed a 777% increase in MEK1 protein in MEK1-hi compared to MEK1-low exosomes (1.00 AU vs. 8.77 AU, **Figure 2.7A**; see also Figure 2.5A), and semi-quantitative proteomics showed a 918% increase (6.27e7 AU vs. 6.39e8 AU, **Figure 2.7A**). Next, we compared the total proteins identified in MEK1-low vs. MEK1-hi exosomes. This analysis identified 1,526 proteins in MEK1-low exosomes, and 1,441 in MEK1-low exosomes. Of these, 372 (21%) were exclusive to MEK1-low exosomes, 287 (15%) were exclusive to MEK1-hi exosomes, and 1,154 (64%) were shared (**Figure 2.7B**). Due to the highly sensitive nature and very low detection limit of LC-MS/MS, this list includes proteins that are expressed at extremely low levels in exosomes and likely are not even present in all exosomes. So, we refined our analysis to include only the most abundantly expressed proteins within the exosomes. We identified the 244 most abundantly expressed proteins in MEK1-low exosomes and asked if any of these were significantly changed (greater than 10-fold difference) in MEK1-hi exosomes. Under these criteria, we found that 222 proteins (91%) were unchanged in MEK1-low vs. MEK1-hi exosomes, 18 (7.4%) were overexpressed in MEK1-hi exosomes, and 4 (1.6%) were overexpressed in MEK1-low exosomes (**Figure 2.8**). Therefore, we conclude that the overexpression of MEK1 in exosomes only minimally affects other exosomal proteins beyond MEK1.

2.3.4 Forced expression of MEK1 in progressor tumors delays tumor growth via adaptive immunity

We next sought to determine how MEK1 overexpression affected the growth of 9609 tumors. Since MEK1 is an oncogene, and in fact inhibitors of MEK1 are used to treat cancer, we expected MEK1 overexpression in tumor cells to augment their growth. Strikingly, we found that in immunocompetent mice, MEK1-hi tumors grew significantly slower than control, MEK1-low tumors. After 15 days of growth, MEK1-hi tumors were approximately 72% smaller in area than MEK1-low tumors (30.6 mm² vs. 108.8 mm², $p < 0.01$ **Figure 2.5B**). When we performed the same experiment in severely immunodeficient NSG mice, we did not observe a significant difference in the area of MEK1-low and MEK1-hi tumors (286 mm² vs. 303 mm² at day 18), suggesting an immune-mediated component was responsible for the delayed growth phenotype of MEK1-hi tumors (**Figure 2.5C**). Furthermore, when we grew MEK1-low and MEK1-hi tumors in mice lacking adaptive immunity (RAG1^{-/-}), we again observed no significant difference in area of MEK1-low and MEK1-hi tumors (151 mm² vs. 154.4 mm² at day 18, **Figure 2.5D**). This result indicates that the immune-mediated component that contributes to the reduced growth of MEK1-hi tumors (either directly or indirectly) is part of the adaptive immune system.

2.3.5 Secreted factors from MEK1-hi tumors delays tumor growth via adaptive immunity

To determine the mechanism by which MEK1 overexpression in tumor cells retarded their growth, we first asked if *any* secreted factor (exosomes or otherwise)

was responsible for delaying growth of MEK1-hi tumors. To answer this question, we performed a contralateral flank experiment (similar to Figure 2.1) where we compared the growth of the parental 9609 tumor (“indicator”) grown in the presence of a contralateral 9609-MEK1-low or a 9609-MEK1-hi tumor (“instigator”). The workflow for these experiments is shown in **Figure 2.9A**. We found that after 14 days, indicator tumors grown with a contralateral MEK1-hi tumor were, on average, 63% smaller in area than indicators grown with a contralateral MEK1-low tumor (35.2 mm² vs. 96 mm², $p < 0.05$, **Figure 2.9B**). This result shows that a secreted factor from MEK1-hi, but not MEK1-low, tumors is capable of reducing growth of the parental 9609 tumor. When we performed the same experiment in mice lacking adaptive immunity (RAG1^{-/-} mice), we saw no significant difference in the area of indicator tumors grown with a contralateral MEK1-low compared to MEK1-hi tumor (221 mm² vs. 180.3 mm² **Figure 2.9C**). Based on this result, we conclude that the secreted factor derived from MEK1-hi tumors exerts its anti-tumor effect through adaptive immune cells, either directly or indirectly.

2.3.6 MEK1-hi exosomes are sufficient to delay tumor growth via adaptive immunity

Having demonstrated that a secreted factor from MEK1-hi tumors has at least moderate anti-tumor activity, we next sought to determine if this secreted factor was exosomes. To this end, we directly administered MEK1-low and MEK1-hi exosomes as therapeutic compounds. The purpose of these experiments was to determine if MEK1-hi exosomes, and specifically MEK1 in exosomes, were sufficient to delay tumor growth in our tumor model.

We co-transplanted parental 9609 tumor cells with PBS (vehicle control), MEK1-low, or MEK1-hi exosomes into immunocompetent mice and monitored tumor growth for 17 days. Over the 17 day timecourse, we further treated tumors with PBS, MEK1-low, or MEK1-hi exosomes via intratumoral injection on days 7, 12, and 15, in order to mimic the persistent release / presence of exosomes from tumors (**Figure 2.10A**). We found that after 17 days, compared to tumors treated with PBS, tumors that were treated with MEK1-hi exosomes were 61% smaller in area (77.8 mm^2 vs. 200.8 mm^2 , $p < 0.05$ **Figure 2.10B**) and 55% smaller in weight (68 mg vs. 150 mg, $p < 0.05$ **Figure 2.10B**). This result shows that MEK1-hi exosomes inhibit the growth of 9609 tumors. We further found that, compared to MEK1-low exosomes, tumors treated with MEK1-hi exosomes were 65% smaller in area (77.8 mm^2 vs. 221.8 mm^2 , $p < 0.05$ **Figure 2.10B**) and 50% smaller in weight (68 mg vs. 136 mg, $p = 0.064$ **Figure 2.10B**) after 17 days of growth. Importantly, this result strongly suggests that it is MEK1 within the exosome that mediates their anti-tumor effect, since MEK1-low and MEK1-hi exosomes both come from the same parental tumor (9609) and have very few differences apart from MEK1 expression level. In accordance, we found no significant difference in area (200.8 mm^2 vs. 221.8 mm^2 **Figure 2.10B**) or weight (150 mg vs. 136 mg **Figure 2.10B**) of tumors treated with MEK1-low exosomes compared to PBS. Together, we interpret this to mean that MEK1-hi exosomes, and specifically exosomal MEK1, mediate the anti-tumor effect observed with MEK1-hi tumors.

When we preformed the same experiment in $\text{RAG1}^{-/-}$ mice, we found no significant difference in area (205.8 mm^2 vs. 213.8 mm^2 vs. 229 mm^2 **Figure 2.10C**) or weight (171.7 mg vs. 194.8 mg vs. 163.6 **Figure 2.10C**) of tumors treated with

PBS, MEK1-low exosomes, or MEK1-hi exosomes. These results demonstrate that the anti-tumor effect of MEK1-hi exosomes and exosomal MEK1 are mediated (either directly or indirectly) through adaptive immunity.

2.3.7 MEK1-hi exosomes delay 3rd party tumor growth and enhance efficacy of checkpoint blockade therapy

Having demonstrated therapeutic efficacy of 9609-MEK1-hi exosomes in a syngenic (9609) tumor model, we next sought to determine if MEK1-hi exosomes and exosomal MEK1 could provide a therapeutic benefit against 3rd party tumors. To this end, we co-transplanted B16.F10 melanoma cells together with PBS (vehicle control), MEK1-low, or MEK1-hi exosomes into immunocompetent mice and monitored tumor growth for 17 days. Over the 17 day timecourse, we further treated the tumors with PBS, MEK1-low, or MEK1-hi exosomes via intratumoral injection on days 7, 12, and 15, mimicking the workflow used in the previous experiment and outlined in **Figure 2.11A**. We found that after 17 days of growth, compared to PBS, tumors that were treated with MEK1-hi exosomes were 51% smaller in area (166.7 mm² vs. 342.5 mm², p<0.05 **Figure 2.11B**) and 44% smaller in weight (533.8 mg vs. 958.3 mg, p=0.063 **Figure 2.11B**). Furthermore, we found that compared to MEK1-low exosomes, MEK1-hi exosomes reduced B16 tumor area by 46% (166.7 mm² vs. 308.3 mm², p<0.05 **Figure 2.11B**) and weight by 45% (533.8 mg vs. 967.3 mg, p=0.076 **Figure 2.11B**). We found no significant difference in area (342.5 mm² vs. 308.3 mm² **Figure 2.11B**) or weight (958.3 mg vs. 967.3 mg **Figure 2.11B**) of B16 tumors treated with MEK1-low compared to PBS. Therefore, we have demonstrated

that MEK1-hi exosomes and specifically, exosomal MEK1, have a positive therapeutic effect in both syngenic and also 3rd party tumors, thus broadening the applicability of the concept that MEK1 in exosomes provides anti-cancer benefit.

We next tested the therapeutic effect of MEK1-hi exosomes in combination with anti-PD1. We co-transplanted B16 tumor cells with PBS (vehicle control) or MEK1-hi exosomes, and treated tumors with PBS or MEK1-hi exosomes on days 8, 11, and 15 as in the previous experiments. In addition, we treated mice with the checkpoint blockade inhibitor anti-PD-1 (clone J43, BioXCell) on days 6, 8, 11, 13, and 15, via intraperitoneal (i.p.) injection, according to established protocol [83, 84]. We monitored tumor growth regularly over 17 days (**Figure 2.11C**). We found that anti-PD-1 therapy alone reduced tumor area by 49% (152 mm² vs. 298.8 mm², $p < 0.05$ **Figure 2.11D**) and weight by 54% (435.2 mg vs. 951.8 mg, $p < 0.05$ **Figure 2.11D**) compared to control conditions. When MEK1-hi exosomes were used in concert with checkpoint blockade, the anti-tumor effect was the greatest. Compared to control-treated tumors, tumors treated with both checkpoint blockade and MEK1-hi exosomes were 69% smaller in area (91.4 mm² vs. 298.8 mm², $p < 0.01$ **Figure 2.11D**) and 79% smaller in weight (195.4 mg vs. 951.8 mg, $p < 0.05$ **Figure 2.11D**) than checkpoint blockade alone. Moreover, compared to checkpoint blockade alone, tumors treated with both checkpoint blockade and MEK1-hi exosomes were 40% smaller in area (91.4 mm² vs. 152 mm², $p = 0.193$ **Figure 2.11D**) and 55% smaller in weight (195.4 mg vs. 435.2 mg, $p = 0.215$ **Figure 2.11D**), demonstrating the additive effect of MEK1-hi exosomes on the efficacy of checkpoint blockade. These results

show that MEK1-hi exosomes provided an added effect to checkpoint blockade therapy.

2.3.8 MEK1 inhibition promotes the growth of MEK1-hi, but not MEK1-low, tumors via adaptive immunity

Next, we sought to establish if the growth delay phenotype attributable to MEK1-hi exosomes was dependent on MEK1 *activity*. To this end, we grew MEK1-low and MEK1-hi tumors in immune competent mice and treated the mice with the MEK1-specific inhibitor PD98059. Mice were treated intraperitoneally (i.p.) with 10 mg/kg PD98059 three times weekly (MWF) starting 3 days after tumor inoculation, according to previously published methods [85] **Figure 2.12A**). We found that treatment with PD98059 had no effect on MEK1-low tumors, as they were not significantly different in size after 17 days of growth in mice treated with the MEK1-inhibitor compared to vehicle control (129.8 mm² vs. 150 mm² **Figure 2.12B**). On the other hand, MEK1-hi tumors were 130% larger in area when grown in mice treated with PD98059 compared to vehicle control (143 mm² vs. 62.3 mm², p=0.114 **Figure 2.12C**). We ruled out that this effect could be due to cell-intrinsic effects, since both MEK1-low and MEK1-hi tumors are equally susceptible to MEK1 inhibition in vitro (**Figure 2.13**). This demonstrates that the reduced growth phenotype observed in MEK1-hi tumors required MEK1 activity. We were very intrigued by this result, since MEK1 inhibitors are used as first line therapy to control several tumor types, especially those with BRAF activating mutations [86]. Therefore, it was surprising that we found the MEK1 inhibitor actually *increased* tumor growth. However, since this

effect was only observed in MEK1-hi tumors, this supports our hypothesis that MEK1 activity transferred to immune cells via exosomes could be responsible for mediating the growth delay we observed in this tumor model.

When we repeated the experiment in mice lacking adaptive immunity, we found that the PD98059 no longer promoted growth of MEK1-hi tumors like it did in immune competent mice. Both MEK1-low tumors (150.5 mm² vs. 148.3 mm² **Figure 2.12D**) and MEK1-hi tumors (148.5 mm² vs. 130 mm² **Figure 2.12E**) were not significantly different in area after 17 days of growth in RAG1^{-/-} mice treated with the MEK1-inhibitor compared to vehicle control (**Figure 2.12D and E**) This result illustrates that adaptive immune cells are responsible for mediating the effect seen when MEK1 inhibition is applied to MEK1-hi tumors, and further supports the hypothesis that MEK1-hi tumors increase MEK1 activity in immune cells to delay tumor growth in a manner dependent on adaptive immunity.

2.3.9 Myeloid cells uptake progressor exosomes and contain exosomally-delivered MEK1

In order to better understand the mechanism by which MEK1-hi exosomes could contribute to tumor rejection, we first sought to determine which cell types can uptake exosomes. We started in vitro, and used a single cell suspension of whole spleen to model the cells within the tumor microenvironment. We administered exosomes that had been labeled with CFSE according to previously published methods [87] to allow for the tracking of their uptake in a single cell suspensions of whole spleen for 24 hours, and then analyzed the cells by FACS for the presence of

CFSE. We found that NK cells and T cells had very low/ no CFSE signal, whereas about 30% of macrophages were CFSE⁺ (**Figure 2.14A**). This shows that macrophages, but not T cells or NK cells, can uptake 9609 exosomes. We next confirmed the uptake of exosomes by macrophages using a different source of macrophages and a different methodology for measuring uptake. We generated bone marrow-derived macrophages (BMDM) in vitro, added CFSE-labeled exosomes for 24 hrs, and then imaged the cells by fluorescent microscopy to visualize exosome uptake. We found that a high frequency of BMDM were CFSE⁺, indicating that they had taken up the labeled exosomes (**Figure 2.14B**). These results show that macrophages can abundantly uptake exosomes, which is congruent with the known phagocytic abilities of macrophages. Therefore, these in vitro results provide evidence to suggest that the anti-tumor effect induced by our MEK1-hi exosomes could be mediated by macrophages.

Since we hypothesized that the transfer of MEK1 to immune cells is responsible for mediating the anti-tumor properties of MEK1-hi exosomes, we next asked if MEK1 from exosomes can be physically transferred to and maintained within the cells that uptake exosomes. To this end, we sought to generate a cell line that produces exosomes containing MEK1 tagged to an immunologically unique epitope, such as hemagglutinin (HA). Exosomes from this cell line could then be used to track the transfer of exosomal MEK1 between cells via detection of the HA epitope. We transduced 9609 cells with a retrovirus expressing HA-tagged MEK1 (HA-MEK1) or GFP control and found that HA-MEK1 was expressed at the whole cell level (**Figure 2.15A**). Importantly, we also found that HA-MEK1 expression extended to the

exosomal compartment (**Figure 2.15B**). In both WCL and exosomes, the HA signal was approximately 48 kDa in size, corresponding to the size of MEK1 (45 kDa) plus the HA tag (3 kDa). To test for transfer and perseverance of exosomal MEK1 to macrophages, we treated BMDM with PBS (vehicle control), GFP exosomes, or HA-MEK1 exosomes for 24 hrs in vitro. After 24 hrs, the cells were thoroughly washed and lysed with RIPA lysis buffer containing protease and phosphatase inhibitors. These BMDM lysates were then analyzed for the presence of the HA epitope as a readout for transfer of exosomal MEK1 by western blot. We found a dose-dependent increase in HA signal in BMDM that were treated with 5 or 25 $\mu\text{g/ml}$ of HA-MEK1 exosomes (**Figure 2.14C**). By contrast, no HA signal was detected in BMDM treated with vehicle control or 25 $\mu\text{g/ml}$ exosomes from 9609-GFP cells, as expected (**Figure 2.14C**). These results demonstrate that not only do macrophages uptake exosomes, but that proteins with the exosomes, and specifically MEK1, are transferred to and maintained within macrophages at least for 24 hrs.

We next sought to determine if we can observe and quantify exosome uptake in vivo. To this end, we injected unlabeled or CFSE-labeled exosomes into established, day 14 9609 tumors via intratumoral injection. After 24 hrs, we harvested the tumors, created a single-cell suspension, and analyzed these cells for CFSE expression (**Figure 2.14D**). Expectedly, we found that in a tumor that was injected with unlabeled exosomes, 0% of cells were CFSE⁺. On the other hand, in a tumor treated with CFSE-labeled exosomes, almost 1% of all cells in the single-cell suspension were CFSE⁺. This result demonstrated that we can indeed observe and quantify exosome uptake in our in vivo model.

Next, we asked: what are the cells that uptake 9609 exosomes in vivo? To this end, we analyzed the surface marker expression pattern of CFSE⁺ cells in the single-cell suspension of 9609 tumor cells treated with CFSE-labeled exosomes (**Figure 2.14E**). We found that the vast majority of CFSE⁺ cells (>97%) expressed CD45, demonstrating that immune cells are the predominate target of exosomes in vivo. Upon further analysis, we found that CFSE⁺ cells largely expressed CD11b (99%), F4/80 (90.2%), Ly6C (95.8%), and to a lesser degree, MHCII (18.3%) (**Figure 2.14E**). Conversely, we found very little/ no expression of CD4, CD8, or NK1.1 on cells expressing CFSE (**Figure 2.14E**). Based on these results, we can conclude that the cells that uptake exosomes in vivo are myeloid (based on expression of CD11b), and are either late monocytes or early macrophages (based on the expression of Ly6C and F4/80). Furthermore, we can conclude that T cells and NK cells do not uptake exosomes in vivo, similar to our in vitro findings. Taken together, the results from these in vitro and in vivo exosome uptake experiments prove that some myeloid cell, likely macrophages, uptake 9609 exosomes, and strongly suggest that this cell could be mediating the anti-tumor effect of MEK1-hi exosomes.

2.3.10 MEK1-hi exosomes boost anti-tumor immunity

To gain further insight to the mechanisms mediating the anti-tumor effect of MEK1-hi exosomes and exosomal MEK1, we analyzed the infiltrating immune cells in 9609-MEK1-low and 9609-MEK1-hi tumors. To this end, we created single-cell suspensions of MEK1-low and MEK1-hi tumors that were grown in immune competent mice for 15 days and analyzed surface marker expression by FACS to

quantify the relative frequencies of various immune cells within the tumors (**Figure 2.16A**). We found that MEK1-hi tumors had a 15% increase in the relative frequency of CD45⁺ cells compared to MEK1-low tumors (66.8% vs. 76.8%, $p<0.05$), suggesting an overall boost in cancer immunity. Furthermore, we observed a 95% increase in CD8⁺ T cells (4% vs. 7.8%, $p=0.075$) and a 100% increase in NK cells (2.2% vs. 4.4%, $p<0.05$) in MEK1-hi compared to MEK1-low tumors, showing that specific anti-tumor effector cells were increased in MEK1-hi tumors. We also observed a striking change in macrophage polarization between MEK1-low and MEK1-hi tumors. While the overall frequency of macrophages in MEK1-low vs. MEK1-hi tumors was not significantly different (44% vs. 49.9%), we observed a striking shift toward M1-type macrophage polarization in MEK1-hi compared to MEK1-low tumors. Specifically, we saw that M1-type macrophages were increased 35% (33.4% vs. 45.1%, $p<0.05$) and M2-type macrophages were decreased by 55% (10.6% vs. 4.8%, $p<0.05$) in MEK1-hi compared to MEK1-low tumors. This manifested in a 247% increase in the M1:M2 macrophage ratio in MEK1-hi compared to MEK1-low tumors (3.6 vs. 12.5, $p<0.05$). Together, these results clearly demonstrate that MEK1-hi tumors have a significantly improved “anti-cancer immune” profile when compared to MEK1-low tumors. Specifically, the dramatic increase in M1-type macrophage polarization, combined with knowledge gained from the exosome uptake experiment showing that macrophage-like cell uptakes exosomes in vivo, suggest to us that MEK1-hi exosomes could be involved in altering macrophage polarization as an anti-tumor mechanism.

Since the changes in immune cells described above cannot be definitively attributed to exosomes, we next sought to establish how infiltrating immune cells are affected by MEK1-hi exosomes and specifically exosomal MEK1. To this end, we characterized the infiltrating immune cells in 9609 tumors that were co-injected and treated with vehicle control, MEK1-low exosomes, or MEK1-hi exosomes for 17 days as depicted in the schematic in Figure 2.10A. After day 17, tumors were digested into single-cell suspensions and analyzed by FACS to characterize and quantify infiltrating immune cells (**Figure 2.16B**). The results of each immune cell subset are described below.

2.3.10.1 Effect of MEK1-hi exosomes on general immunity (CD45⁺ cells)

We found that compared to control conditions, MEK1-low exosomes slightly increased the frequency of CD45⁺ cells by 10% (56.2% vs. 62.4%, $p=0.329$) in 9609 single-cell suspensions. Moreover, MEK1-hi exosomes further increased CD45⁺ cells by 7% relative to MEK1-low exosomes (62.4% vs. 66.6%, $p=0.222$) and 16% over vehicle control (56.2% vs. 66.6%, $p<0.05$). These results show that even MEK1-low exosomes moderately increase infiltrating immune cells in 9609 tumors, but this effect is bolstered by the increased expression of MEK1 in exosomes, suggesting an immune stimulatory role for exosomal MEK1.

2.3.10.2 Effect of MEK1-hi exosomes on CD8⁺ T cells

The frequency of CD8⁺ T cells was increased 93% with MEK1-low exosomes compared to vehicle control (4.2% vs. 8.1%, $p<0.05$). MEK1-hi exosomes also

increased the frequency of CD8⁺ T cells compared to control conditions, by 81% (4.2% vs. 7.6%, $p<0.05$). However, there was not a significant difference in the frequency of CD8⁺ T cells when tumors were treated with MEK1-low compared to MEK1-hi exosomes (8.1% vs. 7.6%). This suggests that the increase in CD8⁺ T cells observed in tumors treated with exosomes is due an exosomal factor *other than MEK1*, since the overexpression of MEK1 in exosomes induces no additive effect over MEK1-low exosomes.

2.3.10.3 Effect of MEK1-hi exosomes on NK cells

We found that MEK1-low exosomes increased the frequency of NK cells by 46% compared to vehicle control (2.6% vs. 3.8%, $p=0.222$), and that MEK1-hi exosomes increased NK cell frequency by 100% compared to vehicle control (2.6% vs. 5.2%, $p<0.05$). This shows that MEK1-hi exosomes more potently promote NK cell recruitment and/or proliferation with the tumor microenvironment compared to MEK1-low exosomes. Accordingly, we observed a 37% increase in the frequency of NK cells in tumors treated with MEK1-hi exosomes compared to MEK-low exosomes (3.8% vs. 5.2%, $p=0.197$). Together, these results suggest that 9609 exosomes can slightly promote NK cells within tumors, but the presence of MEK1 within exosomes acts to further strengthen this response.

2.3.10.4 Effect of MEK1-hi exosomes on tumor-associated macrophages

Perhaps the most dramatic changes observed were with the polarization state of tumor-associated macrophages. Specifically, we saw slight increases in the

frequency of M1-type macrophages in tumors treated with MEK1-low compared to vehicle control (6% increase, 39.4% vs. 41.6%) and MEK1-hi exosomes compared to vehicle control (13% increase, 39.4% vs. 44.5%). More impressively, we saw a 43% decrease in the frequency of M2-type macrophages in tumors treated with MEK1-low exosomes compared to vehicle control (12.1% vs. 6.9%, $p=0.095$), and a 52% decrease with MEK1-hi exosomes compared to vehicle control (12.1% vs. 5.8%, $p<0.05$). The frequency of M2-type macrophages was 16% lower in tumors treated with MEK1-hi compared to MEK1-low exosomes (6.9% vs. 5.8%, $p=0.310$). These changes manifested in increased M1:M2 ratios in tumors treated with MEK1-low or MEK1-hi exosomes compared to vehicle control. Specifically, the M1:M2 ratio was 50% higher in tumors treated with MEK1-low exosomes compared to vehicle control (4.0 vs. 6.0, $p=0.151$) and 113% higher with MEK1-hi exosomes compared to vehicle control (4.0 vs. 8.5, $p=0.056$). Treatment with MEK1-hi exosomes increased the M1:M2 ratio 42% compared to MEK1-low exosome treatment (6.0 vs. 8.5, $p=0.310$). Together, these results demonstrate that exosomes (regardless of MEK1 level) can exert immune modulatory effects on tumor-associated macrophages, trending toward promoting M1/ inhibiting M2 macrophage polarization. However, since the only significant changes were observed with MEK1-hi exosomes, we conclude that the presence of MEK1 within the exosomes is required for this effect, and therefore that exosomal MEK1 promotes the polarization of macrophages toward the anti-tumor, M1-type phenotype.

2.4 Discussion

We have discovered that MEK1 protein is enriched in exosomes from tumors that undergo immune-mediated rejection. This was a surprising finding since we initially anticipated that tumor-suppressive proteins would be enriched in exosomes from regressor tumors. Our discovery suggests that the role of MEK1 in cancer could have opposing functions if acting in a cell-intrinsic vs. cell-extrinsic manner. Cell-intrinsically MEK1 is oncogenic, as constitutively active MEK1 can induce cellular transformation and promote solid tumor growth [88, 89]. Moreover, over-activation of MEK1 signaling is known to occur in many cancers [90]. Cell-extrinsically, however, we propose that MEK1 acts to stimulate anti-tumor immunity and inhibit tumor growth in a manner that is dependent on both innate and adaptive immune cells. Our data suggest that this activity is mediated by exosomes acting on macrophages to promote their polarization toward the M1-type phenotype, which subsequently promotes T- and B cell-mediated anti-tumor immunity and inhibits tumor growth (**Figure 2.17**). The crosstalk that occurs between macrophages and adaptive immune cells and other innate cells found in tumors is known to be a critical regulator of anti-tumor immune responses [91]. Our data showing that anti-tumor effector cells such as CD8⁺ T cells and NK cells are enriched in tumors treated with MEK1-hi compared to MEK1-low exosomes supports that exosomal MEK1 could provide a general boost to anti-tumor immunity via promotion of M1-type macrophage polarization. The therapeutic implications of our findings are far reaching- not only do they support the further investigation of delivery of MEK1 to macrophages as a form of cancer therapy, they also call into question the use of MEK1 inhibitors to treat cancer.

Macrophages are known to play important roles in cancer. They are highly plastic cells that can adopt many different phenotypes depending on the extracellular signals they receive [92]. For instance, M1-type polarized macrophages are known to exert anti-tumor effects, whereas M2-type polarized macrophages function in a pro-tumor manner [93]. This paradigm has inspired investigators to explore ways in which the polarization state of macrophages can be manipulated to provide anti-cancer benefit [94]. Since macrophage polarization is regulated by the extracellular signals received by the cell, signaling pathways within macrophages are logical targets to influence polarization. Here, we propose that MEK1 protein from exosomes is transferred to macrophages to alter their polarization state via the augmentation of MEK1 activity within the cell. This is supported by our observation that tumor-associated macrophages, but not other cells, abundantly uptake labeled exosomes both in vitro and in vivo, and that we see changes in the polarization status of tumor-associated macrophages from tumors that were treated with MEK1-hi compared to MEK1-low exosomes.

Many different intracellular signaling pathways have been demonstrated to affect macrophage polarization [95]. For example, the PI3K/ Akt pathway has recently been implicated in influencing macrophage polarization in the context of cancer [96, 97]. Specifically, it has been shown that PI3K γ signaling in macrophages promotes immune suppression and tumor growth via Akt and mTor-mediated inhibition of NF κ B and activation of C/EBP β . Moreover, Notch signaling can regulate the functional state of macrophages in cancer, as it has been shown that M2-type macrophages in tumors had lower activation of the Notch pathway, and forced

activation of Notch signaling promoted M1-type macrophages and inhibited tumor growth [98]. JAK/ STAT signaling can also have profound effects on macrophages, since this pathway mediates responses to cytokines that influence macrophage polarization. For example, the JAK/STAT pathway promotes M1-type macrophage polarization, since the M1-inducing cytokine IFN γ signals through STAT1 [99]. Clearly, numerous different signaling pathways can mediate the polarization of macrophages toward an M1- or M2-type phenotype. Our hypothesis that exosomal MEK1 transferred to macrophages to alter their phenotype fits neatly into this paradigm, and supports that MAP signaling may also play a role in influencing macrophage polarization status.

Indeed, recent studies have shown that the MAP signal cascade can impact macrophage polarization and functionality. For example, it was shown that TSC1 regulates M1- and M2-type polarization in a mouse model of allergy, and that the effect was mediated by MAP kinase signaling. Specifically, TSC1 was shown to inhibit M1-type polarization via inhibition of the MAP kinase cascade. This result suggests that MAP kinase activity drives M1-type polarization [100]. More recently, it was demonstrated that pharmacological and genetic inhibition of MEK1/2 increases expression of M2-type genes in macrophages. These transcriptional changes were accompanied by functional consequences, as mice treated with a MEK inhibitor sustained less immunopathology following LPS-induced lung injury compared to untreated mice, further suggesting that MEK activity promotes the inflammatory M1-type polarization [101]. Together, these reports clearly demonstrate that inhibition of MEK1 activity in macrophages correlates with M2-type polarization. Or, put another

way, MEK1 activity drives M1-type polarization. These conclusions fit with our hypothesis that MEK1 protein from exosomes transferred to macrophages could promote their polarization toward the M1-type phenotype. Alternatively, lack of exosomal MEK1 could drive M2-type polarization.

Therefore, based on preexisting knowledge in the literature and data from this manuscript, we propose that MEK1 delivered to macrophages could be a strategy to boost anti-tumor immunity and treat cancer. In fact, many groups have proposed engineering exosomes for the purpose of cancer therapy [102]. While several studies over the past two decades have examined the potential use of exosomes derived from immune cells, specifically DCs, to treat cancer [103-105], of greater relevance to our research would be the manipulation of tumor-derived exosomes for the purpose of immune modulation. For instance, it was demonstrated that mouse cancer-derived exosomes engineered to express human MUC1 (hMUC1) stimulated anti-tumor immunity and inhibited the growth of hMUC1-expressing tumors [106]. Another study using exosomes engineered to express IL-18 found that the exosomes were sufficient to promote PBMC proliferation and Th1 cytokine secretion in the context of colon cancer [107]. In another report, it was shown that lung cancer-derived exosomes engineered to express RAB27a could inhibit the growth of established syngeneic tumors while concomitantly inducing general anti-tumor immunity [108]. Based on the data presented here, we feel that MEK1 is an attractive candidate to engineer into exosomes, nanoparticles, or some other carrier for the intention of influencing macrophage polarization to promote anti-tumor immunity and inhibit tumor growth. Since manipulating the polarization status of macrophages is also

under investigation as a strategy to treat cancer [94], we believe that the delivery of MEK1 protein to macrophages via exosomes to promote M1-type polarization could be a suitable approach to treat cancer, and requires further investigation.

Our data also supports that the use of macrophage-targeted delivery of MEK1 could be used in combination with immune checkpoint blockade. We show here that MEK1-hi exosomes provide additional benefit to anti-PD1 therapy against B16 melanoma. Indeed, blockade of immune cell checkpoints is now being tested with countless other immune stimulatory and anti-cancer agents [109]. For example, chemotherapeutic agents and radiotherapy have both been shown to potentiate the efficacy of checkpoint blockade by acting as an “in vivo vaccine” via the exposure of immunogenic proteins on the surface of dead and dying cells that act to promote APC activation and subsequent T cell cross presentation [110-113]. In another example, recent studies have combined checkpoint blockade therapy and cancer neoantigen vaccines with great success [114]. In particular, two clinical studies showed that patients with progressive disease following either peptide- or RNA-based cancer vaccine achieved complete responses with anti-PD1 therapy, illustrating how multiple immunomodulatory therapies could complement each other to cure disease [115, 116]. Along these same lines, we believe that MEK1-containing exosomes, or MEK1 delivered directly to macrophages via alternative delivery strategies such as nanoparticles, could provide additional benefit to checkpoint blockade therapy, likely acting as an adjuvant to boost general anti-tumor immunity by promoting M1-type macrophage polarization.

Interestingly, recent studies have shown that MEK inhibitors can actually augment the efficacy of checkpoint blockade [117]. In a recent study, combination of MEK inhibitor plus anti-PD-L1 antibodies showed favorable activity against colorectal cancer [118]. In particular, MEK inhibitors have been shown to upregulate tumor-associated antigens and promote T cell infiltration and production of pro-inflammatory cytokines [119, 120]. These results seem to contradict our finding that MEK1-hi exosomes promote the effects of checkpoint blockade. However, it is possible that the effects seen in these studies are due to mechanisms that are independent from MEK1 activity in macrophages. Moreover, it is possible that manipulating MAP signaling in cancer cells and immune cells has different outcomes in different cancer settings. In this case, more care will need to be taken to determine in which contexts MAP signaling augments checkpoint blockade (in which case MEK1-containing exosomes or nanoparticles would be a preferred combination partner) and in which contexts MAP signaling negatively impacts checkpoint blockade efficacy (in which case MEK1 inhibitors should be the combination partner).

Another profound therapeutic implication for the findings presented in the manuscript relate to the use of MEK inhibitors for cancer treatment. Over-activation of the MAP signal cascade is estimated to occur in nearly 30% of all cancers [121]. As a result, numerous inhibitors directed against this pathway, particularly MEK1, have been developed over the years. Although MEK1 plays an extremely important role in MAP signaling, inhibitors directed against it have shown only modest success in the clinic [86]. In fact, only 1 MEK inhibitor, trametinib, has been approved by the FDA for clinical use in recent years [122]. There are several hypotheses as to why MEK

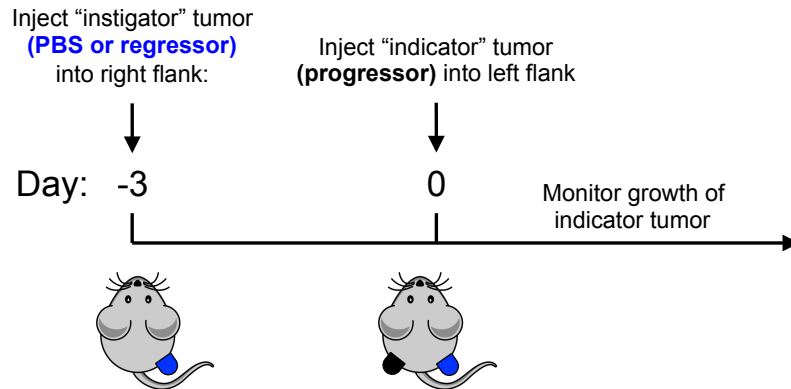
inhibitors failed to produce robust efficacy as was initially expected. For example, RAS/ RAF could signal through other effector molecules besides MEK1, so alternative downstream pathways could compensate for MEK1 activity when it is inhibited. Moreover, the level of MEK1 inhibition required to induce cancer cell death remains unknown, so it is possible that MEK1 was not inhibited enough to provide durable benefit [123]. Finally, MEK1 activity may not be a driver of tumorigenesis in all cancers. Without predictive biomarkers to delineate which tumors are driven by MAP signaling and those that are not, lack of efficacy could be due to unselected patient populations in clinical studies [124]. Based on the data presented in this manuscript, we propose that another reason why MEK1 inhibitors may not be highly efficacious against all cancers could be the cell-extrinsic tumor-suppressive effects of MEK1 in exosomes. Specifically, in tumors that have high MEK1 expression in exosomes, MEK1 inhibition could prevent the anti-tumor effect of these exosomes, thus actually promoting tumor growth instead of inhibiting it. Therefore, we propose that MEK1 inhibitors may only be useful in the context where MEK1 is absent from tumor-derived exosomes. If clinicians could screen cancer patient exosomes for the presence of MEK1, this could be a useful form of “personalized medicine” that could screen and select patient populations that would be most likely to respond to MEK inhibitors without the deleterious effects caused by inhibiting MEK1 activity in exosomes. Certainly, however, more work is needed to fully establish the anti-tumor role of MEK1 in exosomes before this can take place.

The results presented in this manuscript detail a novel tumor-suppressive property of MEK1 within tumor-derived exosomes. We believe this effect is caused by

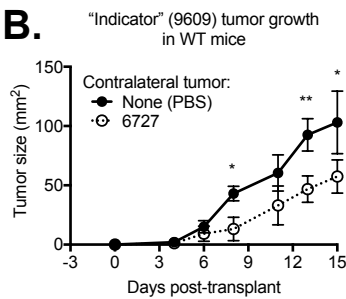
macrophage uptake of exosomes containing MEK1, and subsequent crosstalk with adaptive immune cells. The findings from this manuscript bring to light several therapeutic consequences, including the potential use of macrophage-delivered MEK1 as a form of cancer immune therapy and the possibility that MEK inhibitors may have reduced efficacy due to the anti-tumor role of MEK1 in exosomes. Therefore, future studies should aim to better understand exactly how macrophages are affected by tumor-derived exosomes and exosomal MEK1, with the intent of developing novel therapies to treat cancer based on immune activation.

Chapter II, in full, is currently being prepared for submission for publication of the material. Searles, S, Di Giannatale A, Hatano M, Hoshino A, Matei I, Lyden D, and Bui J. "Exosomal MEK1 inhibits tumor growth by boosting anti-tumor immunity." Manuscript in preparation. The dissertation author was the primary author of all material.

A.



B.



C.

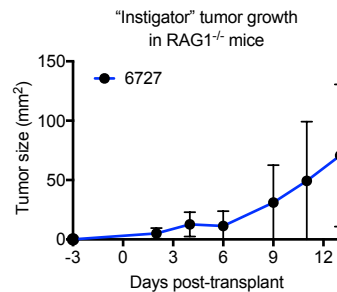
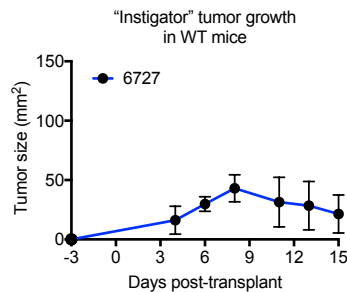
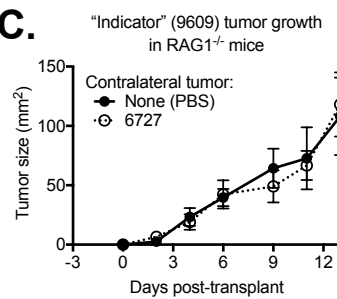


FIGURE 2.1 Secreted factors from regressor tumors inhibit progressor tumor growth via adaptive immunity. (A) Schematic representation of workflow for contralateral flank experiment. "Instigator" tumor cells (5e6 6727 cells) or PBS (vehicle control) were injected s.c. into the right flank of mice at day -3. At day 0, "indicator" tumor cells (1e6 9609 cells) were injected s.c. into the left flank, and tumor growth was monitored over time. **(B and C)** Growth curves of indicator progressor tumors (9609) grown contralaterally with instigator regressor tumors (6727) or vehicle control (PBS) in **(B)** WT and **(C)** RAG1^{-/-} mice. n= 5 animals per group. Data are represented as mean ± SD.

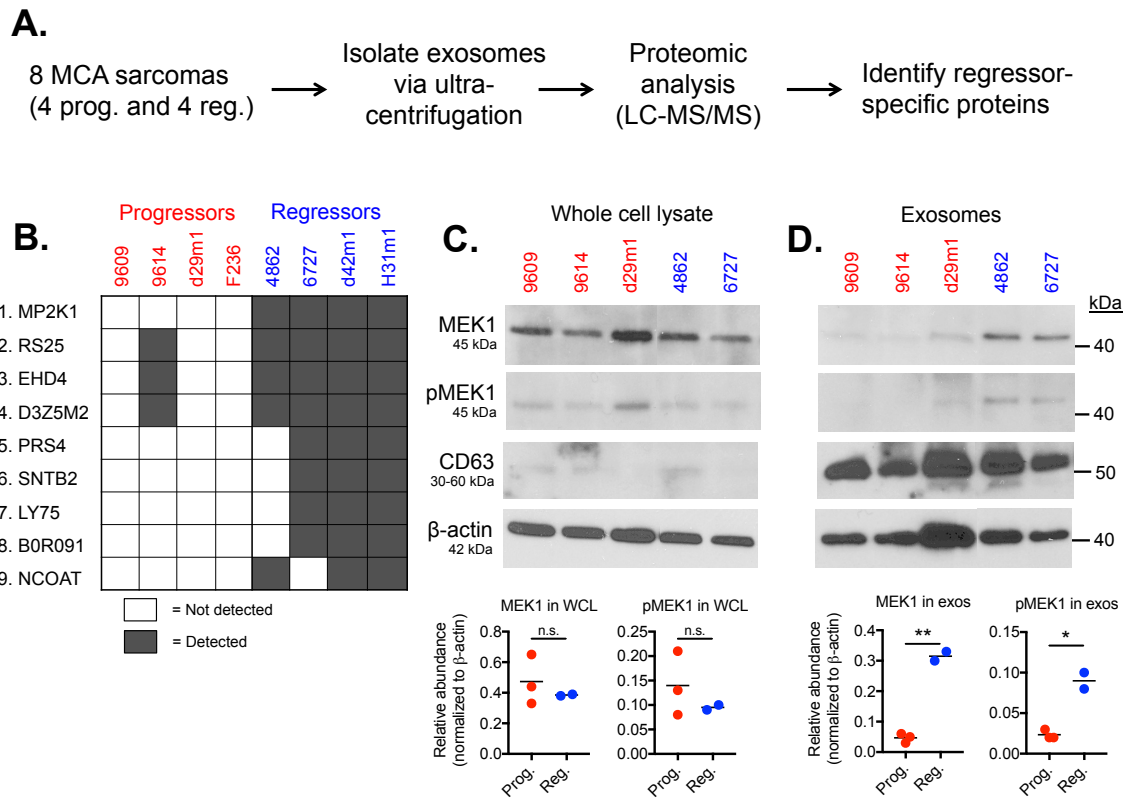


FIGURE 2.2 MEK1 (MP2K1) is enriched in regressor exosomes. (A) Schematic representation depicting workflow for proteomic analysis of progressor and regressor exosomes. (B) Table showing the top differentially expressed proteins in regressor exosomes. (C and D) Western blot analysis of MEK1, phosphorylated MEK1 (pMEK1, Ser298), CD63, and β-actin in (C) whole cell lysate (WCL) and (D) exosomes (exos) from 3 progressor and 2 regressor cell lines. CD63 was used to validate the identity of the exosomal fraction. 10 μg of protein was loaded in each lane. Also shown is quantification of MEK1 and pMEK1 abundance in (C) WCL and (D) exosomes, normalized against β-actin. Each symbol represents a single progressor or regressor cell line, and the horizontal line represents the mean of each group. Data is representative of two independent experiments.

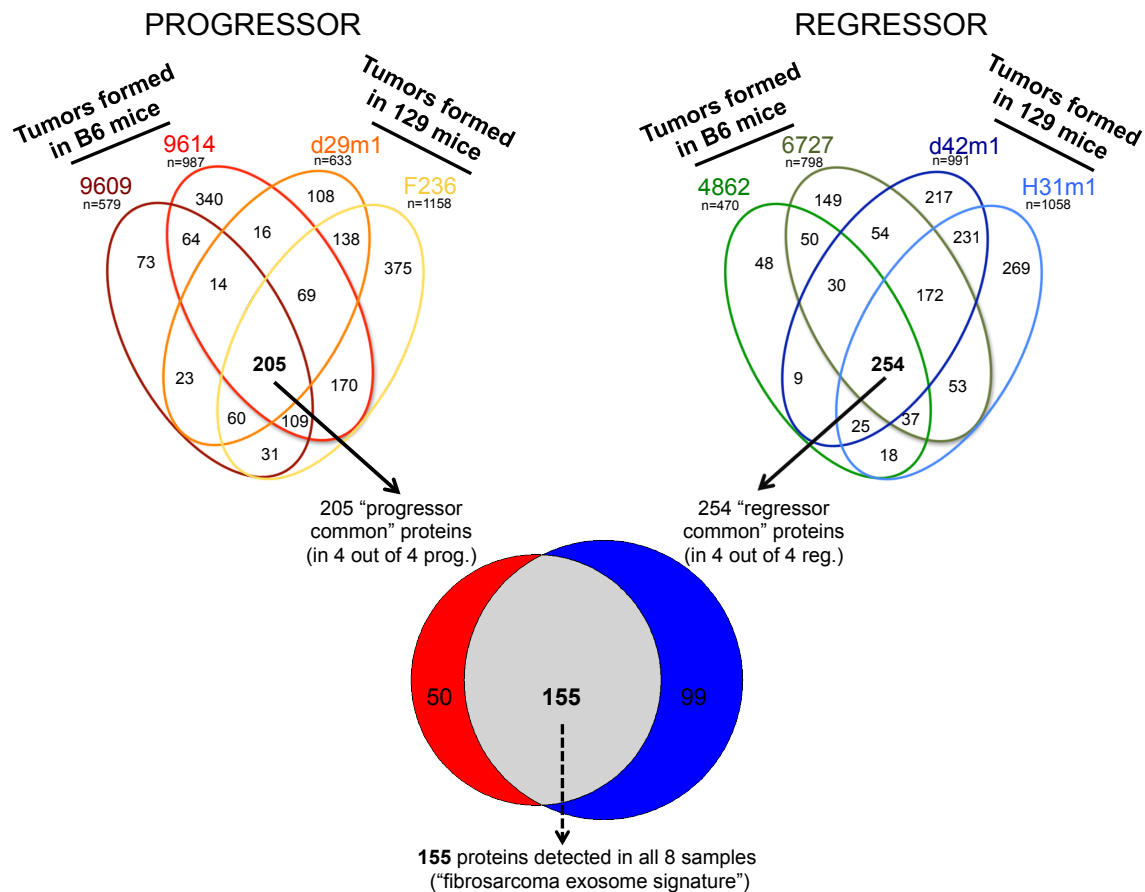


FIGURE 2.3 Schematic representation showing the how the fibrosarcoma exosome signature was defined. Proteomic analysis was performed on exosomes from 4 progressor tumors (9609, 9614, d29m1, F236) and 4 regressor tumors (4862, 6727, d42m1, H31m1). The number of proteins detected in each sample is shown below the name of each cell line. Proteins that were detected in 4 out of 4 progressor exosomes ("progressor common") and 4 out of 4 regressor exosomes ("regressor common") were compared, and proteins that were present in both the progressor common signature and the regressor common signature were termed the "fibrosarcoma exosome signature". A full list of the 155 proteins that comprise the fibrosarcoma exosome signature can be found in Table 2.1.

Detection of the 25 proteins most commonly identified in ExoCarta database in 8 fibrosarcoma exosomes analyzed by proteomics

RANK	No. times ID (ExoCarta)	Gene symbol	Progressors				Regressors			
			9609	9614	d29m1	F236	4862	6727	d42m1	H31m1
1	98	CD9								
2	97	HSP7C								
3	96	PDC6I								
4	95	G3P								
5	93	ACTB								
6	83	ANXA2								
7	82	CD63								
8	78	SDCB1								
9	78	ENOA								
10	77	HS90A								
11	76	TS101								
12	72	KPYM								
13	72	LDHA								
14	71	EF1A1								
15	69	14-3-3Z								
16	69	PGK1								
17	69	EF2								
18	69	ALDOA								
19	67	HS90B								
20	67	ANXA5								
21	66	FAS								
22	65	14-3-3E								
23	64	CLH1								
24	64	CD81								
25	64	ALBU								

 = Not detected
 = Detected

FIGURE 2.4 List of the 25 proteins most commonly identified in the ExoCarta database, and their detection in fibrosarcoma exosomes. The 25 proteins most commonly identified in ExoCarta were detected in nearly every fibrosarcoma exosome sample analyzed for this study. Proteins that were detected in a given sample are indicated by a grey box, and those that were not detected are indicated by a white box. The number of times each protein has been identified in the ExoCarta database is also shown. 18 out of the 25 most commonly identified proteins were detected in 8 out of 8 fibrosarcoma exosomes, and are shown in bold.

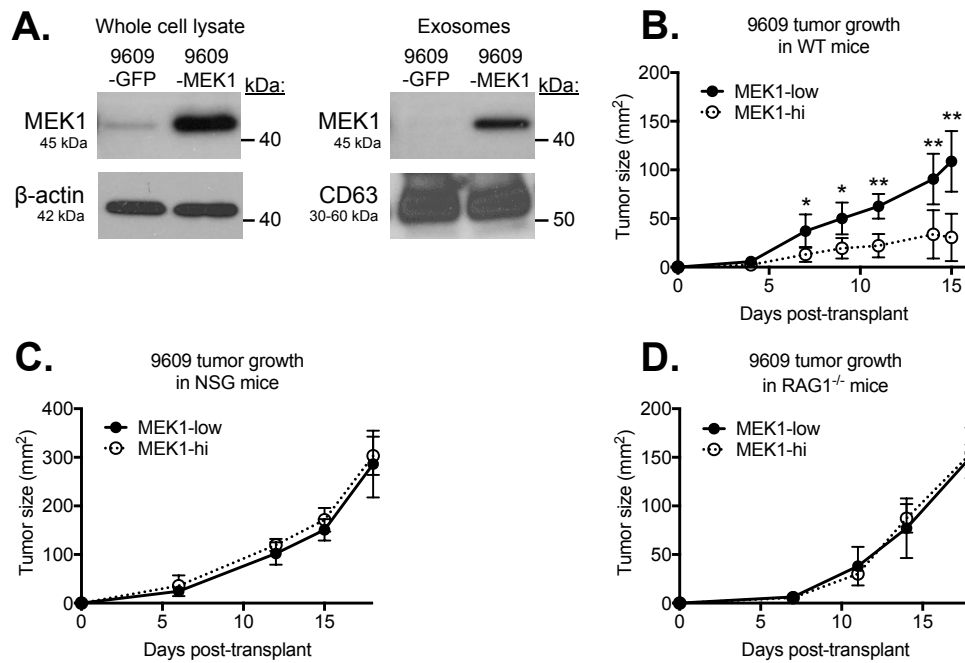


FIGURE 2.5 Forced expression of MEK1 in progressor tumors delays tumor growth via adaptive immunity. (A) Western blot analysis of whole cell lysate and exosomes from the progressor cell line 9609 transduced with GFP- or MEK1-expressing retroviruses. MEK1 overexpression extended to the exosomal compartment. CD63 was used to validate the exosomal fraction. 10 µg of protein was loaded in each lane. (B, C, and D) Growth curves of 9609-GFP (MEK1-low) or 9609-MEK1 (MEK1-hi) tumors grown in (B) WT, (C) NSG, and (D) RAG1^{-/-} mice. For each experiment, 1e6 cells were transplanted s.c., and tumor growth was monitored over time. n= 5 mice per group. Data are represented as mean ± SD.

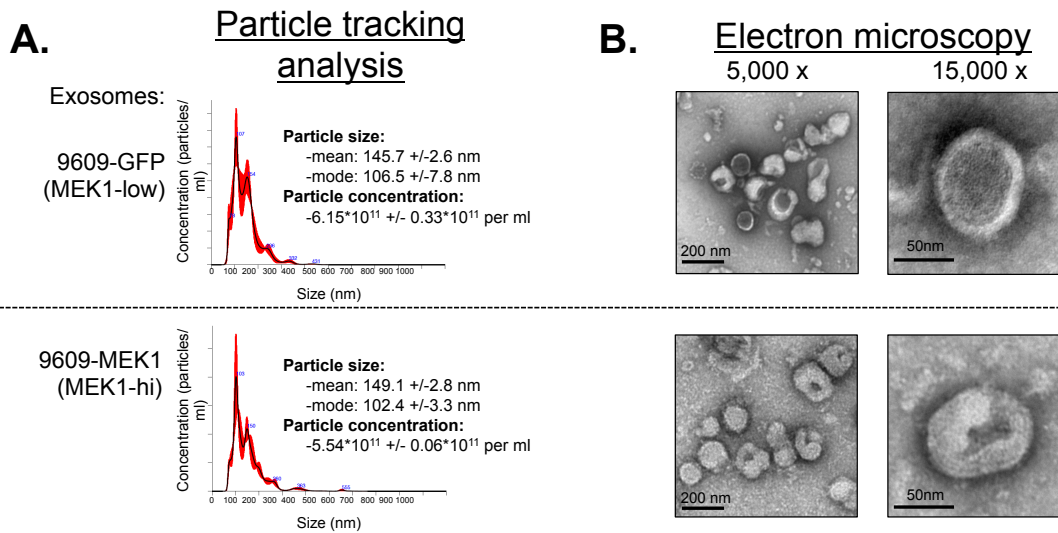
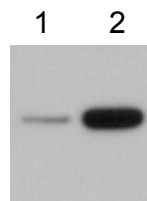


FIGURE 2.6 9609-GFP (MEK1-low) and 9609-MEK1 (MEK1-hi) exosomes are similar in size, production level, and morphology. (A) Nanoparticle tracking analysis was used to measure the size distribution and concentration of exosomes from the conditioned media of MEK1-low and MEK1-hi 9609 tumor cell lines. The mean and mode particle size and particle concentration for each sample is indicated. **(B)** Electron microscopy was used to compare the size and general morphology of MEK1-low and MEK1-hi exosomes and 5,000x and 15,000x magnification.

A.

Western blot



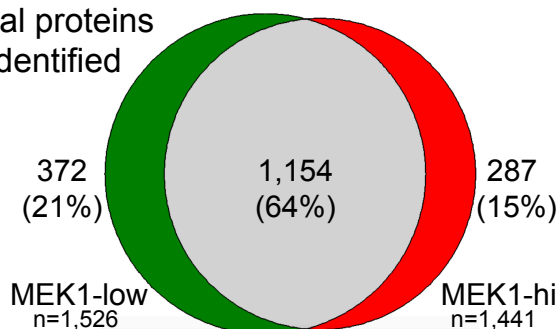
Relative MEK1 expression:
 9609-GFP (lane 1) **1.00**
 9609-MEK1 (lane 2) **8.77**

Semi-quantitative LFQ scores:

	Coverage (%)	MEK1-low (area)	MEK1-hi (area)
Phosphatase-like protein 3 OS=Mus musculus GN=PDCL3 PE=1 SV=1 [PDCL3_MOUSE]	13.73	6.273E7	9.770E7
Dual specificity mitogen-activated protein kinase kinase 1 OS=Mus musculus GN=Map2k1	34.61	6.272E7	6.388E8
Isotype 2 of E3 ubiquitin-protein ligase UBR4 OS=Mus musculus GN=Ubr4 [UBR4_MOUSE]	1.76	6.355E7	6.000E8

10.2-fold
higher in
MEK1-hi

B. Total proteins identified



of proteins identified:

	Total	Exclusive (%)
MEK1-low	1,526	372 (21)
MEK1-hi	1,441	287 (15)

FIGURE 2.7 MEK1-low and MEK1-hi exosomes share a similar proteome. (A) Semi-quantitative proteomic analysis was performed on MEK1-low and MEK1-hi exosomes. Western blot showed a 8.77-fold upregulation of MEK1 in MEK1-hi relative to MEK1-low exosomes, and semi-quantitative proteomic analysis showed a 10.2-fold increase of MEK1, thus validating the system. **(B)** Comparison of all proteins identified in MEK1-low and MEK1-hi exosomes show that they shared a similar proteome, with 64% of proteins shared between the two samples. 21% of proteins identified were exclusive to MEK1-low exosomes and 15% of proteins were exclusive to MEK1-hi exosomes.

Semi-quantitative proteomic analysis of the most abundant proteins in 9609-MEK1-low vs. 9609-MEK1-hi exosomes

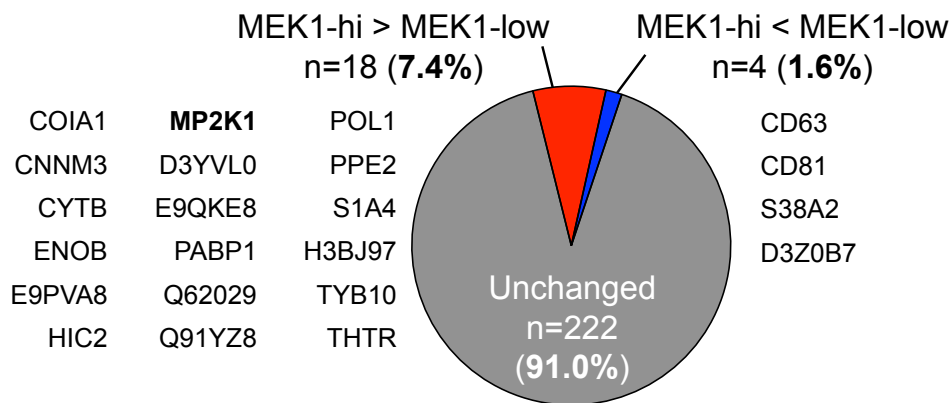


FIGURE 2.8 The most abundant proteins in MEK1-low and MEK1-hi exosomes are highly similar. Pie chart showing the proteins that are differentially expressed in MEK1-low vs. MEK1-hi exosomes based on semi-quantitative proteomic analysis. The 244 proteins most abundantly expressed were analyzed. A threshold of 10-fold different was used to classify proteins as differentially expressed.

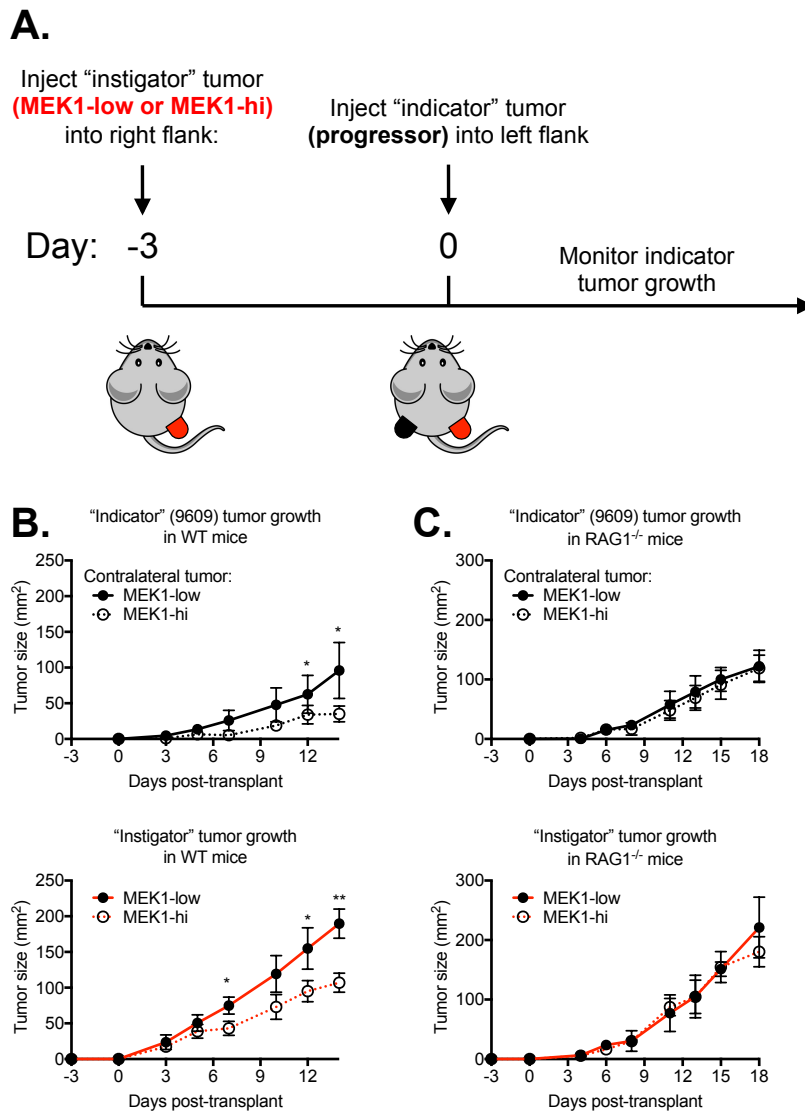


FIGURE 2.9 Secreted factors from MEK1-hi tumors delay tumor growth via adaptive immunity. (A) Schematic representation of workflow for contralateral flank experiment. “Instigator” tumor cells (1e6 9609-GFP or 9609-MEK1 cells) were injected s.c. into the right flank of mice at day -3. At day 0, “indicator” tumor cells (1e6 9609 cells) were injected s.c. into the left flank, and tumor growth was monitored over time. (B and C) Growth curves of indicator progressor tumors (9609) grown contralaterally with instigator tumors (9609-GFP or 9609-MEK1) in (B) WT and (C) RAG1^{-/-} mice. n= 5 animals per group. Data are represented as mean ± SD.

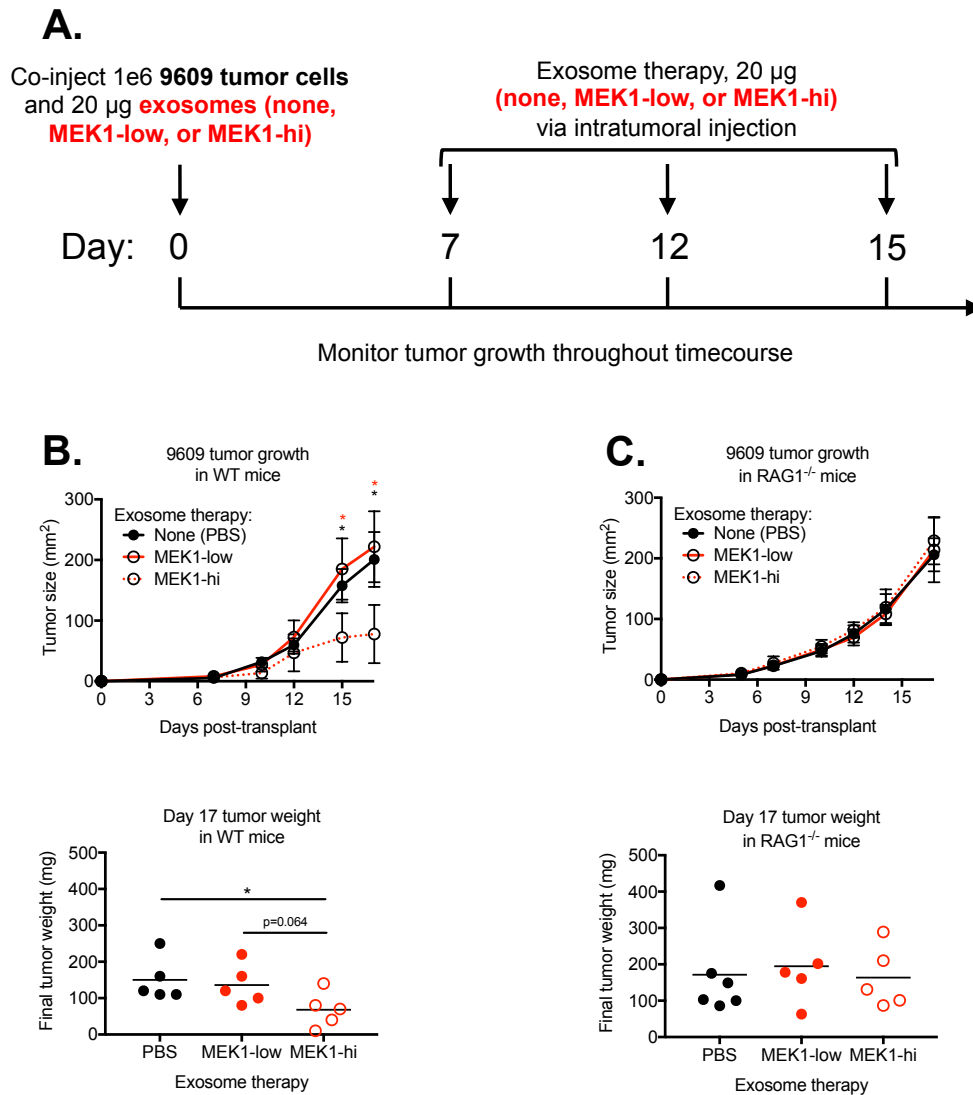


FIGURE 2.10 MEK1-hi exosomes are sufficient to delay tumor growth via adaptive immunity. (A) Schematic representation depicting workflow for experiment using MEK1-low and MEK1-hi exosomes as therapeutic compounds against syngeneic 9609 tumors. 9609 tumor cells (1e6) were co-transplanted with PBS (vehicle control), MEK1-low, or MEK1-hi exosomes. Tumors were subsequently treated with PBS, MEK1-low, or MEK1-hi exosomes via intratumoral injection on days 7, 12, and 15. Tumor growth was monitored over time. (B and C) Growth curves and final tumor weight of 9609 tumors (9609) grown in (B) WT and (C) RAG1^{-/-} mice and treated with PBS, MEK1-low, or MEK1-hi exosomes as described above. n= 5 or 6 mice per group. Growth curve data are represented as mean $\pm$ SD. Final tumor weight data are plotted as individual values, with each symbol representing an individual tumor, and the horizontal line represents the mean of each group.

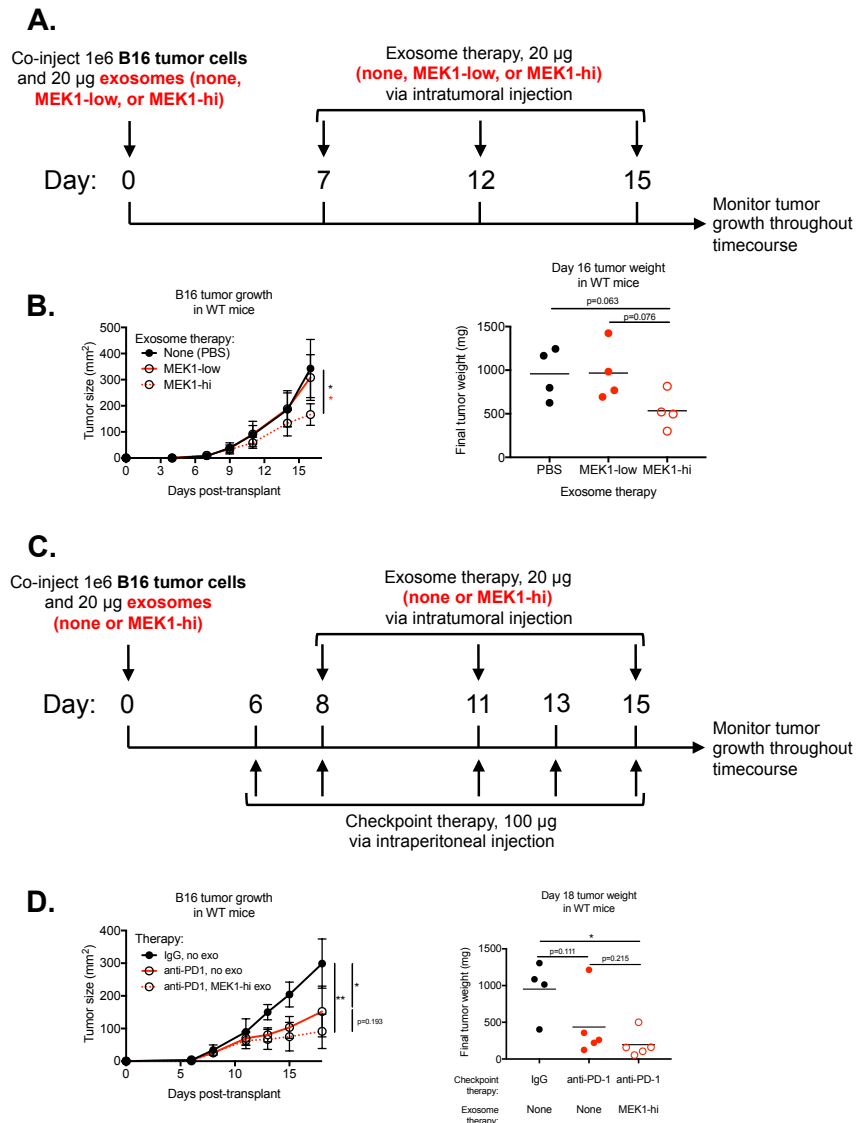


FIGURE 2.11 MEK1-hi exosomes delay 3rd party tumor growth and enhance efficacy of checkpoint blockade therapy. (A) Schematic representation depicting workflow for experiment using MEK1-low and MEK1-hi exosomes as therapeutic compounds against 3rd party tumors. B16 melanoma cells (1e6) were co-transplanted s.c. with PBS (vehicle control), MEK1-low, or MEK1-hi exosomes. Tumors were subsequently treated with PBS, MEK1-low, or MEK1-hi exosomes via intratumoral injection on days 7, 12, and 15. Tumor growth was monitored over time. (B) Growth curves and final tumor weight of B16 tumors grown in WT mice and treated with PBS, MEK1-low, or MEK1-hi exosomes as described above. $n = 4$ mice per group. (C) Schematic representation depicting workflow for experiment using MEK1-hi exosomes in combination with anti-PD-1 therapy against B16 melanoma. MEK1-hi exosomes or PBS were co-injected with B16 melanoma cells, and tumors were subsequently treated with PBS or exosomes on days 8, 11, and 15 via intratumoral injection. Mice were also treated with 100 µg anti-PD-1 antibody or IgG control on days 6, 8, 11, 13, and 15 via i.p. injection. (D) Growth curves and final tumor weight of B16 tumors grown in WT mice and treated with MEK1-hi exosomes and anti-PD-1 as described above. $n = 4-5$ mice per group. Growth curve data are represented as mean $\pm$ SD. Final tumor weight data are plotted as individual values, with each symbol representing an individual tumor, and the horizontal line represents the mean of each group.

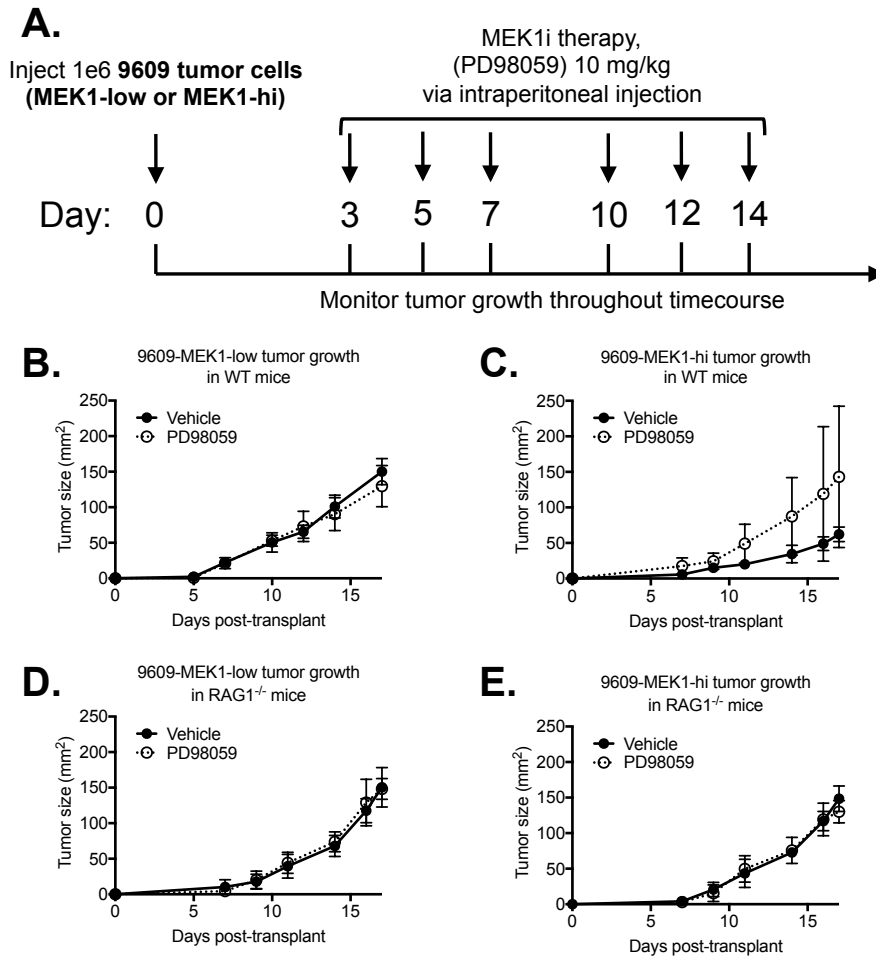


FIGURE 2.12 MEK1 inhibition promotes the growth of MEK1-hi, but not MEK1-low, tumors, via adaptive immunity. (A) Schematic representation depicting the workflow for MEK1 inhibition experiment using the MEK1-specific inhibitor PD98059. MEK1-low or MEK1-hi tumor cells (1e6) were transplanted s.c. into mice, which were subsequently treated with PD98059 (10 mg/kg) on days 3, 5, 7, 10, 12, and 14 via i.p. injection. (B and C) Growth curves of (B) 9609-MEK1-low and (C) 9609-MEK1-hi tumors grown in WT mice and treated with PD98059 or vehicle control as described above. (D and E) Growth curves of (D) 9609-MEK1-low and (E) 9609-MEK1-hi tumors grown in RAG1^{-/-} mice and treated with PD98059 or vehicle control as described above. n= 4-6 mice per group. Data are represented as mean $\pm$ SD.

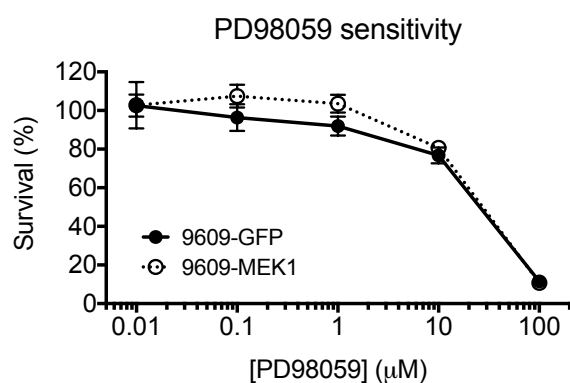


FIGURE 2.13 MEK1-low and MEK1-hi tumor cells are equally sensitive to PD98059, a MEK1-specific inhibitor, in vitro. Survival curves for 9609-MEK1-low and 9609-MEK1-hi tumor cells treated with the indicated concentrations of the MEK1-specific inhibitor PD98059 for 24 hrs in vitro. Survival data is plotted as mean of three replicates $\pm$ SD, and is representative of two independent experiments.

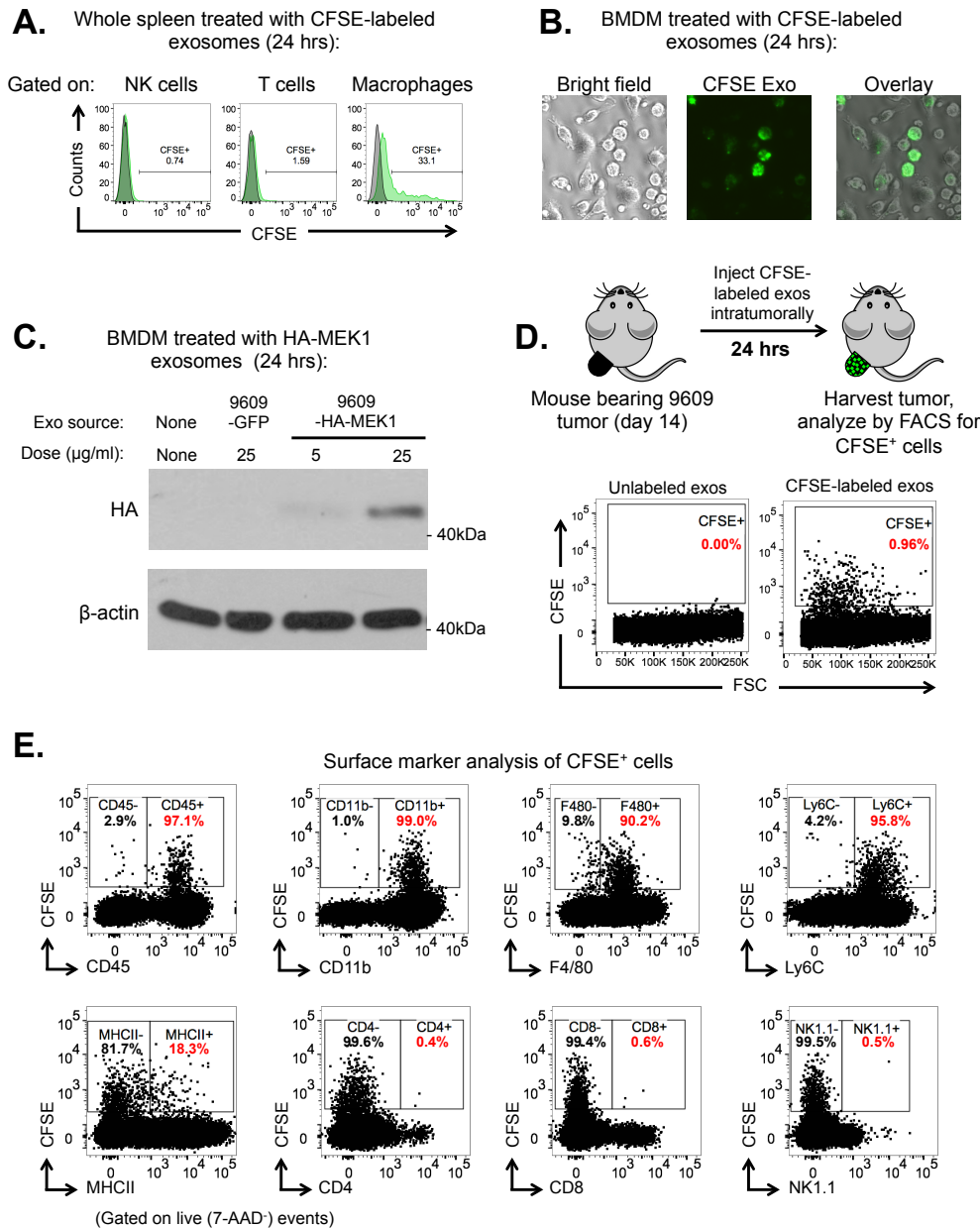


FIGURE 2.14 Myeloid cells uptake 9609 exosomes and contain exosomally-delivered MEK1. (A) Histograms showing CFSE expression in NK cells (CD45⁺, CD3⁻, NK1.1⁺), T cells (CD45⁺, CD3⁺), and macrophages (CD45⁺, Ly6C⁻, CD11b⁺, F4/80⁺) gated from whole spleen that was treated with unlabeled or CFSE-labeled 9609 exosomes for 24 hrs in vitro. Grey histograms represent cell treated with unlabeled exosomes, and green histograms represent cells treated with CFSE-labeled exosomes. (B) Brightfield and fluorescent images of BMDM treated with CFSE-labeled 9609 exosomes for 24 hrs in vitro. (C) Western blot analysis showing HA and β-actin expression in BMDM that were treated with HA-MEK1-containing 9609 exosomes for 24 hrs in vitro. (D) Schematic representation depicting workflow to detect in vivo exosome uptake, and dot plots showing CFSE expression in cells from single cell 9609 tumor suspensions that were treated with 20 µg of unlabeled or CFSE-labeled 9609 exosomes for 24 hrs. (E) Dot plots showing expression of CFSE vs. CD45, CD11b, F4/80, Ly6C, MHCII, CD4, CD8, and NK1.1 in single cell suspensions of 9609 tumors treated with 20 µg of CFSE-labeled 9609 exosomes.

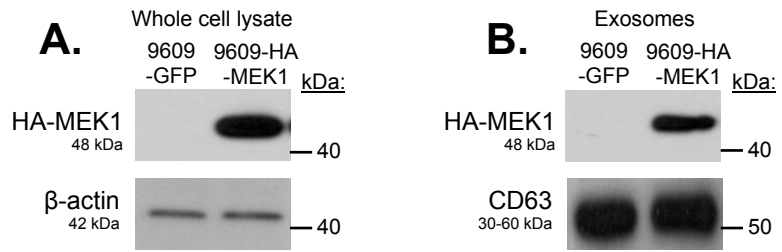
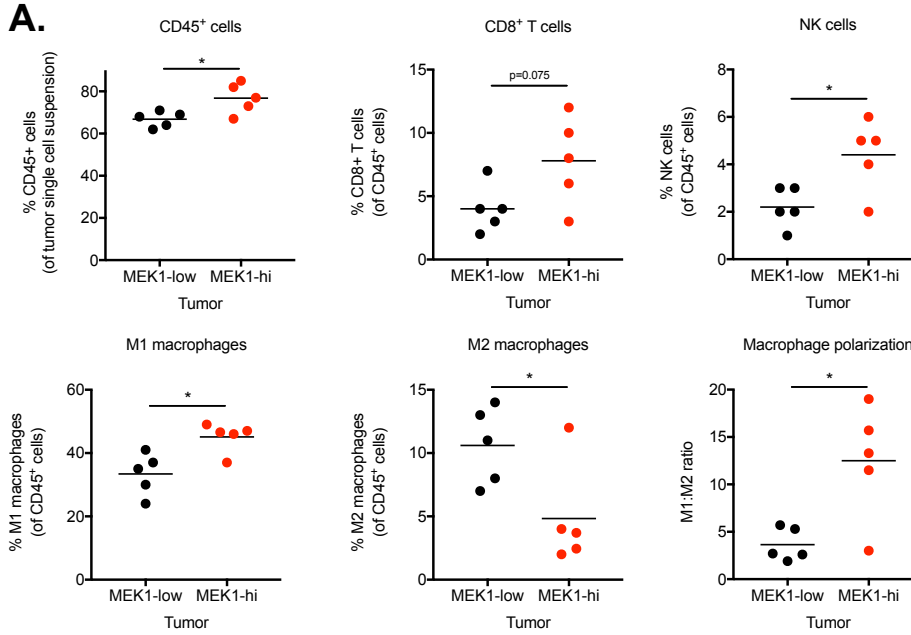


FIGURE 2.15 Forced expression of HA-tagged MEK1 in 9609 tumor cells causes HA-MEK1 to be included in exosomes. Western blot analysis of the HA epitope in 9609 cells transduced with GFP or HA-MEK1 retroviruses. Whole cell lysate (WCL) and exosomes (Exo) were analyzed. β -actin and CD63 were used as loading controls for WCL and exosomes, respectively. 10 μ g of protein was loaded per lane.

Immune cell analysis of 9609-MEK1-low and 9609-MEK1-hi tumors (day 15)



Immune cell analysis of 9609 tumors treated with MEK1-low or MEK1-hi exos (day 17)

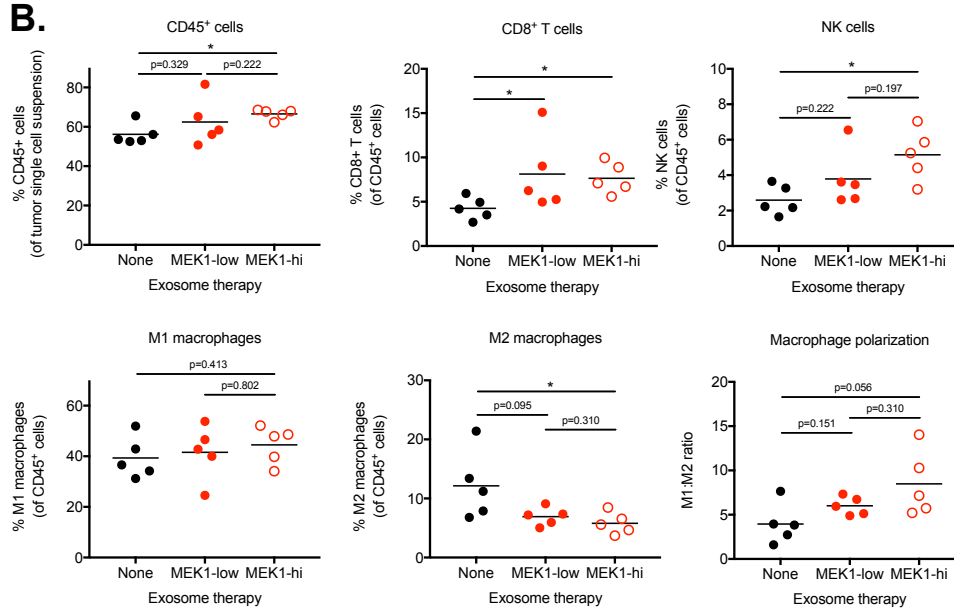


FIGURE 2.16 MEK1-hi exosomes boost anti-tumor immunity. (A) Quantification of relative frequencies of CD45⁺ cells, CD8⁺ T cells (CD45⁺, CD3⁺, CD8⁺, CD4⁻), NK cells (CD45⁺, CD3⁻, NK1.1⁺), M1-type macrophages (CD45⁺, Ly6C⁻, F4/80⁺, MHCII^{hi}) and M2-type macrophages (CD45⁺, Ly6C⁺, F4/80⁺, MHCII^{low}), and M1:M2 ratios in 9609-MEK1-low or 9609-MEK1-hi tumors grown in WT mice for 15 days. (B) Quantification of relative frequencies of CD45⁺ cells, CD8⁺ T cells, NK cells, M1-type macrophages, and M2-type macrophages, and M1:M2 ratios in 9609 tumors grown in WT mice and treated with exosomes at days 0, 7, 11, and 15 (as in Figure 2.10A) for 17 days. n=5 mice per group. Data are plotted as individual values, with each symbol representing an individual tumor, and the horizontal line represents the mean of each group.

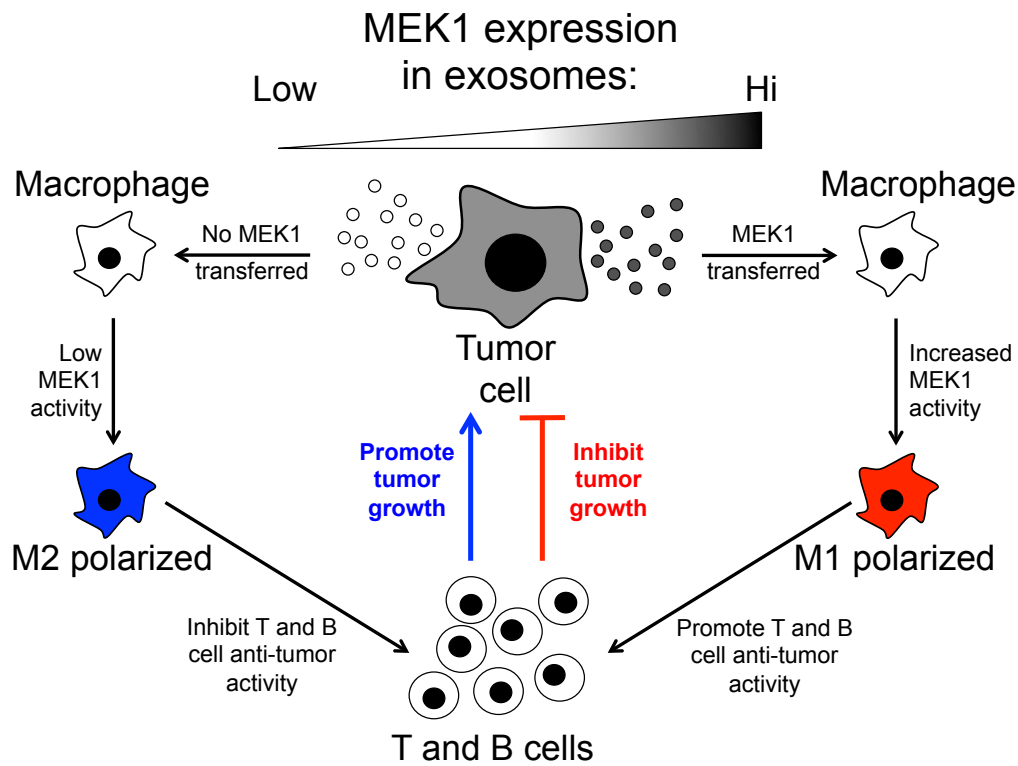


FIGURE 2.17 Proposed model depicting a cell-extrinsic, tumor suppressive role for MEK1 mediated by exosomes. Tumors that secrete MEK1-low exosomes transfer very little MEK1 to macrophages, resulting in M2-type polarization that inhibits adaptive anti-tumor immunity and supports tumor growth. On the other hand, tumors that secrete MEK1-hi exosomes transfer significantly more MEK1 to macrophages, increasing MEK1 activity and promoting M1-type polarization, which stimulates anti-tumor adaptive immunity and inhibits tumor growth.

TABLE 2.1 List of proteins comprising the “fibrosarcoma exosome signature”. Proteins that are also among the top 25 proteins identified in the ExoCarta online database are shown in bold

List of proteins identified in 8 out of 8 fibrosarcoma exosomes, also termed the “fibrosarcoma exosome signature”					
14-3-3E	CD9	F6WKE4	IF5A1	PPCE	STOM
14-3-3F	CDC42	F8VPU2	ILK	PPIA	SYAC
14-3-3G	CLH1	F8WIT2	IMB1	PPID	SYEP
2AAA	CLIC1	FA10	IQGA1	PRDX6	SYK
6PGD	CLIC4	FAS	ITB1	PRS7	TBA4A
A1BN54	CNDP2	FINC	K6PL	PSMD2	TBB4B
A2M	D3YW48	FPRP	KPRB	PSMD5	TBB5
ABP1	D3YYM6	G3P	KPYM	PUR4	TCPB
ACLY	D3Z0B7	G5E839	LAT1	PUR9	TCPE
ACOC	DDX6	G5E8Y7	LDHA	RAB10	TCPH
ACTG	DNJA1	GALK1	LEG1	RAB21	TCPZ
ADA10	DNJA2	GBB1	LKHA4	RAB2A	TFR1
ADK	DYHC1	GBLP	MBB1A	RAB7A	TLN1
AL1L1	E9QAZ2	GDIR1	MFGM	RAN	TS101
ANXA2	E9QPG8	GFPT1	MOES	RAP1A	TSP1
ANXA5	EDH1	GNAI2	MYL6	RAP1B	UB2D3
ANXA7	EDH2	GNAI3	NID1	RAP2B	UBA1
ARF4	EDH3	GNAS2	NSF	RAP2C	UGPA
ARP3	EF1A1	GRP78	PARVB	RHOA	URP2
AT1A1	EF1G	GTR1	PCBP1	RIR1	VINC
B1B0C7	EF2	H32	PDCD6	RL4	VP37B
C1TC	EIF3C	H4	PDC6I	RL7	VPS28
CAB39	ENPL	HBA	PDIA3	S10A6	VPS35
CALM	EXT1	HS90B	PGK1	SDCB1	WDR1
CD63	F2Z4A3	HTRA1	PGM1	SERA	XPP1
CD81	F6SVV1	IF4A1	PLXA1	SERPH	

CHAPTER III: CELL-CELL FUSION AS A MECHANISM OF DNA EXCHANGE IN CANCER

3.1 Abstract

Cell-cell fusion describes the process by which two cells combine their plasma membranes and become a single cell, possessing and retaining certain genetic information from each parent cell. Here, using a Cre-*loxP*-based method initially developed to investigate extracellular vesicle targeting, we found that cancer cells spontaneously and rapidly deliver DNA to non-cancer cells *in vitro* via a cell-cell fusion event. The resulting hybrid cells were aneuploid and possessed enhanced clonal diversity and chemoresistance compared to non-hybrid cancer cells. We also observed cell-cell fusion to occur *in vivo* between melanoma cells and non-cancer cells of both hematopoietic and non-hematopoietic lineages. These findings suggest that cell-cell fusion occurs during the natural progression of cancer and show that this mechanism has the potential to cause massive genomic alterations that are observed in cancer. Furthermore, these findings somewhat contradict recent publications suggesting that the Cre-*loxP* method measures only extracellular vesicle-mediated intercellular communication.

3.2 Introduction

Cell-cell fusion refers to the process by which two or more cells combine their plasma membranes to become a single hybrid cell containing DNA from each parent cell [32]. This fundamental biological process has been well documented in many

organisms, including plants [33], yeast [34], *C. elegans* [35], *D. melanogaster* [36], and higher eukaryotes [37]. The functional consequence of cell-cell fusion is the formation of a hybrid cell that can maintain genotypic and phenotypic properties of both parent cells. In this sense, cell-cell fusion is a robust mediator of cellular reprogramming that can lead to the creation of cells with novel properties [125].

In the context of cancer, it has been hypothesized that cell-cell fusion may act to increase the genotypic and phenotypic diversity of daughter cells [9]. This mechanism of DNA exchange, via “sexual reproduction” (fusion and subsequent reductive division), is thought to be a more efficient way to generate populational heterogeneity as opposed to simply relying on the accumulation of oncogenic mutations in a single cell (“asexual reproduction”). Based on this hypothesis, hybrid cells are more likely to possess characteristics that would allow for the progressive growth of cancer compared to non-hybrid cells. These characteristics include rapid proliferation [59], cancer stem-cell formation [53, 59], resistance to chemotherapeutics [57, 126, 127], and metastasis [49, 128], among others. Fusion has been reported to occur in many types of cancer, including breast, melanoma, sarcoma, glioblastoma, renal cell carcinoma, and ovarian carcinoma [129, 130]. However, only few studies have quantified cell-cell fusion *in vivo* [131], and to our knowledge, none have clearly identified which non-cancer cells are capable of fusing with cancer cells *in vivo*. While definitive evidence linking cell fusion to cancer progression in humans is lacking, it has become increasingly clear using animal models that cell fusion plays a physiologically relevant role in the progression of

cancer [132], especially as it relates to metastasis [133, 134], drug resistance [126, 127], and cancer stem cell formation [59, 127].

Extracellular vesicles (ECVs) have recently been recognized as major mediators of intercellular communication in numerous physiological processes, including cancer [10, 135]. ECVs encompass both secreted exosomes as well as small vesicles that are shed directly from the plasma membrane [136]. The molecular contents of ECVs remain biologically active and can be transferred to cells locally and distally, resulting in the cellular reprogramming of targeted cells. ECV cargo includes proteins [24], lipids [137], and nucleic acids [13], all of which have been shown to possess biological activity. Recent publications have elegantly demonstrated the profound role of tumor-derived ECVs in modulating tumorigenic processes, including immune-evasion, angiogenesis [138], pre-metastatic niche formation [8], and metastatic organotropism [68]. In particular, it appears that tumor-derived ECVs are able to modulate and reprogram host cells to provide a more hospitable environment for cancer cells to grow. Despite this recent flood of new information regarding this process, the identity of which specific cells uptake ECVs in truly physiological conditions remains unresolved.

In this study we developed a *Cre-loxP* model system initially to investigate how molecular information is transferred out of cancer cells via ECVs. We unexpectedly found that cancer cells and non-cancer cells spontaneously and rapidly combine DNA via a fusion event that could affect cancer cell ploidy, heterogeneity, and fitness. These studies document and quantify cell-cell fusion *in vitro* and *in vivo* using

transplantable murine tumor models and show that this process could serve as an engine to drive cancer aneuploidy and heterogeneity.

3.3 Results

3.3.1 Cancer cells rapidly transfer Cre to fibroblasts and macrophages *in vitro*

We initially sought to identify which healthy host cells are capable of receiving cancer-derived molecular information, and then to determine how this communication affects the behavior of these cells. To this end, we established a Cre-*loxP* system consisting of cancer cells that express Cre recombinase and non-cancer cells that contain a reporter locus consisting of a floxed stop codon preceding tdTomato (*loxP*-STOP-*loxP*-tdTomato, or LSL-tdTomato) under control of the ROSA promoter. In this model system, if a non-cancer cell receives cancer-derived Cre, the reporter will be activated and the non-cancer cell will turn red via expression of tdTomato. (**Figure 3.1A**)

As an initial proof-of-concept that Cre transfer occurs between cancer and non-cancer cells, we co-cultured mouse embryonic fibroblasts (MEFs) derived from reporter mice (B6.Cg-Gt(ROSA)26Sortm9(CAG-tdTomato)Hze/J) with B16.F10 melanoma cells expressing GFP-Cre (B16-GFP-Cre) for 24 and 48 hours and then measured tdTomato fluorescence by FACS. We could detect tdTomato⁺ cells after 24 hours, indicating that Cre transfer occurred rapidly between B16 and reporter MEF cells *in vitro* (**Figure 3.1B**). The percentage of fused cells was 0.55% at 24 hours and 0.63% at 48 hours, indicating that the fusion occurred quickly and continued to occur. The apparent decrease in rate of fusion (0.08% between 24 and 48 hours) was likely

due to the rapid proliferation of B16 tumor cells, which are included in the denominator of the calculation. B16-derived Cre was transferred to other cell types derived from reporter mice, including adult dermal fibroblasts (ADF), bone marrow-derived macrophages (BMDM), and peritoneal macrophages, albeit with differing levels of efficiency (0.5-5%) (**Figure 3.1C**). We expressed GFP-Cre in an expanded panel of cancer cell lines encompassing 4 MCA sarcomas (4862, 6727, 9609, 9614 [80]) and 2 breast cancer cells (MDA-MB-231 and Py117 [139]) and then co-cultured these cells with reporter MEF and BMDM for 48 hours. We found that some of the MCA sarcoma cell lines could induce low levels of reporter activation in target MEF and BMDM, but none were as efficient as transferring Cre to target cells than B16 cells. We also found that neither of the breast cancer cell lines tested could induce reporter activation in MEF or BMDM during the 48 hour co-culture period to any significance (**Figure 3.1D**). These data demonstrate that melanoma cells seem particularly well adept at transferring molecular information to non-cancer target cells in the conditions tested here, but also show that this phenomena can occur with other types of cancer cells, albeit at lower efficiency.

3.3.2 B16-GFP-Cre ECVs contain Cre RNA

Previous reports have demonstrated that Cre activity can be transferred between cells via ECVs [140-142]. We therefore sought to determine if ECVs alone are responsible for mediating the rapid Cre transfer we observed in our model system. We purified ECVs from the conditioned media of B16-GFP-Cre cells via differential ultracentrifugation and verified their identity using electron microscopy.

Our isolates consisted of vesicles that were around 100 nm in diameter with a “cup-shaped morphology” typically associated with small vesicles (**Figure 3.2**). We next examined Cre protein and transcript in the ECVs. We found that while the amount of Cre protein in ten µg of B16-GFP-Cre ECVs was below the detection level of western blotting (**Figure 3.3A**), Cre transcript was highly enriched in B16-GFP-Cre ECVs relative to ECVs from control B16 cells (**Figure 3.3B**). This result matches previously published reports showing that Cre RNA, but not protein, can be detected in ECVs derived from Cre-expressing cancer cells [141].

3.3.3 ECVs do not mediate the rapid transfer of Cre between B16-GFP-Cre cells and MEFs

Next, we sought to determine if B16-GFP-Cre ECVs contain Cre *activity*. We administered varying concentrations of ECVs onto reporter MEFs for up to three days and measured tdTomato expression by FACS. In any condition tested, we were unable to detect tdTomato⁺ cells, suggesting that Cre⁺ ECVs alone are not sufficient to activate the reporter locus (**Figure 3.3C**). This was not due to the inability of MEFs to uptake ECVs, since CFSE-labeled exosomes were taken up by MEFs in a dose dependent manner (**Figure 3.3D**). Despite the transfer of B16-GFP-Cre ECVs into MEFs, we were unable to detect an increase in Cre transcript in ECV-treated MEFs to any significance (**Figure 3.3E**). These results suggest that even though ECVs can be taken up by MEF and contain Cre RNA, they do not transfer *enough* Cre RNA to generate bioactive Cre protein in the target cell, and as a result, they alone are not sufficient to activate the reporter locus.

Since it is possible that the bulk addition of ultracentrifugation-isolated ECVs does not recapitulate physiological ECV release, we sought to establish if *physiologically secreted* ECVs were sufficient to activate the reporter locus in MEF. We co-cultured reporter MEF and B16-GFP-Cre cells for up to four days with the cells separated by a transwell insert with 0.4 μ m pores so that ECVs could pass through the membrane but cells could not. In these conditions, we were unable to detect an increase in tdTomato⁺ MEF compared to control conditions (**Figure 3.3F**). Based on these cumulative results, we conclude that in our model system, the rapid exchange of Cre between cancer cells and non-cancer cells is not mediated by ECVs but instead by some other mechanism.

3.3.4 The rapid transfer of Cre between B16-GFP-Cre cells and MEF occurs via cell-cell fusion

Two intriguing observations led us to hypothesize that Cre transfer may occur via cell-cell fusion. First, we noticed that in all B16 co-cultures tested, nearly all tdTomato⁺ cells also expressed GFP (**Figure 3.4A**). Second, we saw that tdTomato⁺ cells from B16:MEF co-cultures had a significantly higher forward scatter (FSC), which is a read-out for cell size, than B16 cells (1.43-fold, $p < 0.01$) and MEF (1.24-fold, $p < 0.05$), indicating that they are larger in size (**Figure 3.4B**). In fact, tdTomato⁺ cells from every B16 co-culture tested had a higher FSC than both the reporter cells and B16-GFP-Cre cells (**Figure 3.5**). Based on these observations, we hypothesized that Cre transfer between cancer and non-cancer cells may occur via cell-cell fusion.

To investigate this hypothesis further, we performed live-cell confocal imaging on B16-GFP-Cre cells co-cultured with CellTracker Blue-labeled reporter MEF, allowing us to definitively identify the origin of tdTomato⁺ cells. B16 cells were added to adherent MEF, and video recording was initiated after 2 hours. As seen in **Supplemental Video 3.1** and **Figure 3.4C**, we observed a MEF (outlined in a solid white line) interacting with a B16 cell (outlined in a dashed white line) at 6:20 hrs. Over the course of less than 20 minutes, the MEF suddenly attained GFP expression, while the intensity of the GFP signal from the B16 cell diminished, suggesting that at this moment, the cytosol of each cell physically connected, allowing the GFP from the B16 cell to diffuse into the cytosol of the MEF. Following this event, the cells remained physically connected for the next several hours (indicated by the white arrows), and by 12:50 hrs, they joined together and appeared to fuse into a single cell, noticeably larger than those around it. By 18:00 hrs, the heterokaryon began expressing tdTomato (**Figure 3.4C**) demonstrating that a tdTomato⁺ cell is *both* a MEF and a B16 cell.

We observed many other instances of cell-cell fusion mediating Cre transfer between MEF and B16 cells. In one example, the fusion event happened nearly immediately after initiating video recording (**Supplemental Video 3.2**). In another, the cells had already fused prior to starting the video, evident by the appearance of a GFP⁺/CellTracker Blue⁺ cell at the beginning of the video that eventually turned red (**Supplemental Video 3.3**). We also observed a large tdTomato⁺ cell undergoing what appeared to be programmed cell death after approximately 32 hrs, suggesting that these hybrid cells may be unstable (**Supplemental Video 3.4**). Finally, using a

different experimental setup where MEF were left unlabeled, we observed a tdTomato⁺ cell originate from a GFP⁺ cell (**Supplemental Video 3.5**).

3.3.5 B16xMEF hybrids contain B16-restricted DNA but do not maintain expression of B16-restricted GFP

Spontaneously fused cancer-cell:normal cell hybrids have not been studied extensively. Having established a simple and robust model of cell fusion, we further characterized the fused cells by generating clonal cell lines. Limiting dilution cloning of sorted tdTomato⁺ cells was performed from a 24 hour B16:MEF co-culture. After the 4th passage (about 6-8 weeks in culture), we extracted DNA from each of the clones and probed for the presence of Cre DNA by PCR. We found that 100% (20/20) of the clones contained Cre DNA, which is restricted to B16 cells and absent in MEF (**Figure 3.6A**). Since each clone also maintained high expression of MEF-restricted tdTomato (data not shown), this strongly suggests that the tdTomato⁺ clones originated from B16xMEF hybrid cells. Interestingly, despite the fact that 100% of the tdTomato⁺ clones contained B16-restricted DNA, only 50% (10/20) maintained expression of B16-restricted GFP (**Figure 3.6B**). In addition, 15% (3/20) of the clones lost expression of tdTomato in a small sub-population of cells (**Figure 3.6C**). These results illustrate how despite inheriting DNA from two different cells, the hybrid clones do not necessarily express the same genes as both parent cells. This suggests that genetic silencing and/or deletion likely occurred during the clonal expansion of the hybrid clones. Indeed, epigenetic reprogramming is a common hallmark of hybrid cells [143]. The silencing and/or loss of genes [144] and even

whole chromosomes [145] have been documented to occur following a cell fusion event, so this result matches previous findings.

3.3.6 B16xMEF hybrids are hyperploid

Next, we quantified the DNA content of the tdTomato⁺ clones and B16-GFP-Cre cells using two independent techniques. By FACS, we observed that 60% (12/20) of the tdTomato⁺ clones contained significantly more DNA than B16-GFP-Cre cells (which are already known to be hyperploid [146]), only 5% (1/20) contained significantly less, and 35% (7/20) were not significantly different (**Figure 3.6D** and **Figure 3.7**). We independently generated ten B16-GFP-Cre clonal cell lines and found that the average DNA content of the twenty tdTomato⁺ clones was significantly higher (1.13-fold) than the ten B16-GFP-Cre clones (**Figure 3.6E**). Similarly, by karyotype analysis we observed that 100% (8/8) of the tdTomato⁺ clones tested contained more chromosomes than B16-GFP-Cre cells. In fact, one clone (#B1) averaged 128.1 chromosomes per cell, which is 3.20-fold higher than a non-transformed cell and 1.88-fold higher than B16 (**Figure 3.6F**). These results clearly show that tdTomato⁺ clones contain more DNA than B16 and provide strong evidence that DNA is pooled between MEF and B16 cells during a cell fusion event.

3.3.7 B16xMEF hybrids express both B16- and MEF-restricted genes

To determine whether the combination of DNA resulting from cell fusion events could lead to functional changes in gene expression, we examined expression of candidate “MEF genes” (*Bmp4*, *Fgf2*) and “B16 genes” (*Met*, *Mitf*) in the tdTomato⁺

clones versus the B16-GFP-Cre clones and MEF by qPCR. Among the tdTomato⁺ clones, the variability in expression of both MEF- and B16 genes was very high: some clones expressed as much as or more of a given gene than B16 cells and MEF, while in other clones the genes were barely expressed (**Figure 3.8A**). By contrast, the ten B16-GFP-Cre clones homogenously expressed B16 genes and lacked expression of MEF genes (**Figure 3.8B**). On average, the expression of the MEF genes was significantly higher in the tdTomato⁺ clones than the B16 clones (*Bmp4*: 4.49-fold, $p>0.001$ and *Fgf2*: 9.02-fold, $p>0.0001$), and the expression of the B16 genes was at least as high in the tdTomato⁺ clones than the B16 clones (**Figure 3.8C**). This suggests that the hyperploidy resulting from DNA mixing allowed for gene expression patterns of both parent cells (in this case, MEF and B16) to be maintained in some daughter cells over many generations. We observed similar results using an expanded panel of genes specific for MEF (*Cd24a*, *Gas1*, *Sca1*) and B16 (*Trmp1*, *Tyr*) (**Figure 3.9**).

We found that the variability in expression level of *Bmp4*, *Fgf2*, *Met*, and *Mitf* was significantly higher among the tdTomato⁺ clones compared to the B16-GFP-Cre clones at the $\alpha=0.01$ confidence level. This result implies that cell-cell fusion diversifies the gene expression profile of hybrid cells, increasing clonal heterogeneity at the populational level. Interestingly, we observed a strong correlation between the expression levels of each of the four genes among the twenty tdTomato⁺ clones (**Figure 3.8D**). This shows that the expression of MEF- or B16 genes is not mutually exclusive in hybrid cells, since certain tdTomato⁺ clones (like #C4, D3, and D6) express high levels of both B16- and MEF genes. This result provides evidence that

cell-cell fusion not only acts as a mechanism of DNA exchange but also a modulator of gene expression in cancer.

3.3.8 Cell-cell fusion promotes resistance against chemotherapeutics in B16 melanoma cells in vitro

Previous reports have demonstrated that cell-cell fusion induces chemoresistance in cancer [126, 132, 147, 148]. However, many studies have used artificial fusion methods (electroporation or fusion-inducing chemicals) to generate hybrid cells and therefore may not reflect true physiology. We sought to employ our more physiological model to ask if cell-cell fusion promotes chemoresistance in melanoma. We grew co-cultures of B16-GFP-Cre cells with reporter target cells (either MEF or BMDM) for 24 hours, treated the co-cultures with various concentrations of paclitaxel for 24 hours, and then measured the frequency of tdTomato⁺ cells by FACs. We found that the frequency of tdTomato⁺ cells was significantly enriched in both B16:MEF and B16:BMDM co-cultures that were exposed to paclitaxel relative to control conditions (**Figure 3.10A**) suggesting that B16xMEF and B16xBMDM hybrids are more resistant to paclitaxel than B16 cells. Alternatively, it is possible that chemotherapy induces cell fusion, as has previously been reported [149].

We also tested the sensitivity of each of the twenty tdTomato⁺ clones to paclitaxel and compared against B16-GFP-Cre. In the presence of 10 μ M paclitaxel for 24 hours, we found that 30% (6/20) of the tdTomato⁺ clones had a significantly higher survival rate than B16-GFP-Cre, and 70% (14/20) were not significantly

different (**Figure 3.10B**). On average, the twenty tdTomato⁺ clones were significantly more resistant (1.72-fold, $p<0.01$) to paclitaxel than the ten B16-GFP-Cre clones (**Figure 3.10C**). We also found that the variability in resistance to paclitaxel was significantly higher in the tdTomato⁺ clones than the B16-GFP-Cre clones at a confidence level of $\alpha=0.01$. We performed the same experiment using a second chemotherapeutic, doxorubicin. In the presence of 10 μ M doxorubicin for 24 hours, we found that 30% (6/20) of the tdTomato⁺ clones had a significantly higher survival rate than B16-GFP-Cre, 15% (3/20) had a significantly lower survival rate, and 55% (11/20) were not significantly different (**Figure 3.10D**). On average, the twenty tdTomato⁺ clones were significantly more resistant (2.32-fold, $p<0.05$) to doxorubicin than the ten B16-GFP-Cre clones (**Figure 3.10E**). Similar to paclitaxel, we found that the variability in doxorubicin resistance was significantly higher in the tdTomato⁺ clones than the B16-GFP-Cre clones at a confidence level of $\alpha=0.01$. Together, these results demonstrate that cell-cell fusion promotes chemoresistance at both the single cell- and population level, and show that clones derived from hybrid cells can vary greatly in terms of survival against paclitaxel and doxorubicin.

3.3.9 Cre transfer from B16 melanoma cells to non-cancer cells occurs in vivo within the tumor microenvironment

Having demonstrated that Cre transfer occurs *in vitro*, we sought to establish whether this phenomenon also occurs *in vivo*. We injected B16-GFP-Cre melanoma cells (1e6 cells, s.c.) into reporter mice, and after tumors reached 10x10 mm in size (about 18-21 days), we harvested the tumors for analysis of tdTomato⁺ cells by

microscopy and FACS. In sections of flash-frozen B16-GFP-Cre tumors, we observed tdTomato⁺ cells (asterisks), demonstrating that Cre transfer does occur *in vivo* (**Figure 3.11A**). We noticed that some of the tdTomato⁺ cells also expressed GFP (arrows), an observation that supports the hypothesis that *in vivo* Cre transfer may occur via cell-cell fusion. By FACS, we found that the frequency of tdTomato⁺ cells was significantly higher in single-cell suspensions of B16-GFP-Cre tumors compared to control B16 tumors that did not express Cre, indicating that FACS is sensitive enough to detect and quantify Cre transfer *in vivo* (**Figure 3.11B**). However, it must be noted that the frequency of tdTomato⁺ cells in B16-GFP-Cre tumors was very low (<0.02% of all cells), indicating that *in vivo* Cre transfer in B16 melanoma is a rare phenomenon or that tdTomato⁺ cells do not remain viable after receiving B16-derived Cre.

We next sought to identify which cells had received B16-derived Cre by characterizing the surface markers expressed by tdTomato⁺ cells in single-cell suspensions of B16-GFP-Cre tumors. By FACS, we found that a majority (but not all) of tdTomato⁺ cells expressed CD45, indicating that both hematopoietic and, to a lesser degree, non-hematopoietic cells can uptake Cre. The majority of tdTomato⁺ cells expressed F4/80, suggesting that the immune cells that uptake Cre are macrophages. This result matches previous results showing macrophages are capable of fusing with cancer cells [49, 57, 126, 150]. A small portion of tdTomato⁺ cells expressed additional immune-related markers (MHCII, CD11c). We noticed a small population of tdTomato⁺ cells expressing the stem cell marker Sca-1, suggesting that progenitor cells within the tumor microenvironment can uptake B16-

derived Cre. We did not detect expression of the endothelial marker CD31 on any tdTomato⁺ cells (**Figure 3.11C**).

To determine if Cre can be transferred to sites distal from the tumor, we used FACS to quantify the frequency of tdTomato⁺ cells in a variety of different tissues (blood, bone marrow, lymph nodes, lung, spleen) from control, non-tumor bearing reporter mice or reporter mice bearing B16-GFP-Cre tumors for 18-21 days. In all tissues tested, we were unable to detect significantly more tdTomato⁺ cells in tumor-bearing compared to control mice (**Figure 3.11D**). This result demonstrates that in our model system, the amount of Cre transfer to distal sites is below the detection limit of FACS, suggesting it is an even rarer phenomena than local Cre transfer or does not occur at all.

3.3.10 Cell-cell fusion between B16 melanoma cells and non-cancer cells occurs in vivo

Since we demonstrated that cell-cell fusion can mediate Cre transfer *in vitro*, we sought to determine if this phenomenon occurs *in vivo*. We generated an “*in vivo*-derived” tdTomato⁺ clonal cell line using limited dilution cloning on tdTomato⁺ cells that were sorted from a twenty day B16-GFP-Cre tumor single-cell suspension. After four passages (6-8 weeks in culture), we performed karyotype analysis to quantify the number of chromosomes in the *in vivo*-derived clone, B16-GFP-Cre cells, and a non-transformed cell, adult dermal fibroblasts (ADF). Per cell, ADF had 39.7 ± 0.7 chromosomes, B16-GFP-Cre cells had 67.1 ± 1.3 , and the *in vivo*-derived clone had 81.7 ± 27.1 . Cells from the *in vivo*-derived clone contained between 21 to 105

chromosomes, and we observed 3 different groups within this clone based on chromosome number. Most cells (group I, 68%) had greater than 90 chromosomes per cell, 17% of cells had 40-90 chromosomes (group II), and 19% of cells had less than 40 chromosomes (group III) (**Figure 3.11E**). These results not only demonstrate that the *in vivo*-derived clone has more DNA than B16 and non-transformed cells, but also highlight the immense variability in the genome size of this clone. This is likely a reflection of genomic instability, which is a hallmark of hybrid cells with high amounts of DNA [145, 151, 152]. Together, these observations show that the *in vivo*-derived clone is likely a hybrid cell and support the hypothesis that cell-cell fusion can occur *in vivo* and induce clonal heterogeneity. In addition, we observed that a sub-population of the *in vivo*-derived tdTomato⁺ clone expressed GFP, demonstrating that the tdTomato⁺ clone originated from a cell that also contained B16-restricted GFP (**Figure 3.12A**). We also saw that the *in vivo*-derived clone had a higher FSC MFI than B16-GFP-Cre cells, demonstrating that these cells are larger in size (**Figure 3.12B**).

3.3.11 Cre transfer between B16 melanoma cells and non-cancer cells promotes survival in the presence of paclitaxel in vivo

To demonstrate that the presumed fused cells displayed higher chemoresistance *in vivo*, we injected reporter mice with B16-GFP-Cre cells (1e6 cells s.c.) and treated them with paclitaxel or HBSS (control) for 15 days. By day 17, paclitaxel treatment had reduced the size of tumors by an average of 60% (**Figure 3.13**). After reaching a size of 10x10 mm, we harvested tumors and analyzed tumor

single-cell suspensions for the frequency of tdTomato⁺ cells. We found that tdTomato⁺ cells were enriched 7.4-fold in paclitaxel-treated compared to control tumors ($p = 0.054$, **Figure 3.11F**). This result demonstrates that tdTomato⁺ cells are more resistant than B16 cells *in vivo* and supports the general hypothesis that chemoresistance in cancer is enhanced by cell-cell fusion.

3.4 Discussion

Cancer cell heterogeneity forms the substrate for chemoresistance, metastasis, and cancer progression [153, 154]. Cancers possess abundant clonotypes of cells that can be selected by external forces including chemotherapy, radiotherapy, and immune therapy. Although the driving force of cancer heterogeneity has been suggested to derive from accumulated spontaneous and random mutagenic events [155], it is also clear that during the process of transformation, whole-scale genomic alterations have occurred [156]. Indeed, nearly all cancers display aneuploidy, a result that has been attributed to a “crisis event” that occurs early during carcinogenesis [157, 158]. It is not clear what the “crisis” entails, and indeed, the mechanism by which cancers become aneuploid has still not been fully defined [159]. Here, we have found that cancer cells spontaneously fuse with normal cells to form hybrid hyperploid cells that we postulate contributes to some of the heterogeneity observed in cancer [152, 160, 161].

We observed cell-cell fusion using a Cre-*loxP* system that we had originally developed to track the exchange of molecular information between cancer and non-cancer cells. In this model system, we found that cancer cells expressing Cre could

induce *loxP* recombination in normal reporter cells after direct co-culture, rendering the reporter cells red via expression of a tdTomato reporter gene. Surprisingly, the red cells all displayed hyperploidy and were found to result from a cell fusion event. The most efficient Cre transfer occurred between melanoma cells and fibroblasts, where 2-6% of cells expressed tdTomato after 48 hours. We observed *in vivo* Cre transfer from melanoma cells to non-cancer cells within the tumor microenvironment, but only at a very low frequency (~0.02% of cells). These numbers are in line with reports that suggest only about 1% of tumor cells fuse *in vivo* [131] and the hypothesis that only about 1% of fused cells actually survive/ proliferate [162], so in total only about 0.01% of tumor cells are hybrids. We did not observe Cre transfer to sites distal from the tumor, including spleen, lymph nodes, lung, bone marrow, or blood. This result, although negative, infers that ECVs were not mediating Cre transfer in our model system.

Two recent publications using a similar Cre-*loxP* system concluded that Cre was transmitted to reporter cells via ECVs [141, 142]. We took care to demonstrate that this was not occurring in our experimental system. We showed that purified ECVs could not transfer enough Cre to target cells to induce *loxP* recombination. This finding cannot be attributed to a lack of uptake of ECVs by target cells, since we observed uptake of CFSE-labeled ECVs by MEF, nor by an absence of Cre in ECVs, since we detected Cre transcript by PCR. Rather, it appears that not enough Cre is transferred from ECVs to target cells in order to induce reporter activation. This could be because our Cre expression system lacks the “ECV targeting motif” used by [141], or because we expressed Cre bi-cistronically with GFP, making it a larger transcript

than if Cre were expressed alone. However, since we do see Cre transcript significantly enriched in ECVs from Cre expressing cells, we do not think these are likely explanations. Moreover, when Zomer et al. attempted this experiment, they also could not demonstrate functional Cre transfer via direct administration of ECVs to any significance [142]. Therefore, we conclude that the rapid Cre transfer we observed is not mediated by ECVs.

We report here that Cre exchange is mediated by cell-cell fusion, an observation that is supported by the fact that 100% of the tdTomato⁺ clonal cell lines were hyperploid and contained DNA from both cancer and non-cancer cells. Our gene expression data also back up this hypothesis by showing that our hybrid clones express genes from both parent cells (B16 and MEF), mirroring previous studies and showing that there are functional consequences of DNA exchange that occurs during cell-cell fusion [134, 163]. Furthermore, these findings support the broad hypothesis that cell-cell fusion could mediate aneuploidy in cancer, since all of the hybrid-derived clonal cell lines created in this study contained atypical numbers of chromosomes [152, 164]. This result highlights the efficiency of cell-cell fusion as a mechanism by which cancer cells can attain aneuploid numbers of chromosomes in the absence of cytokinesis failure. However, it must be noted that the process of cell-cell fusion is distinct from entosis, which has also been shown to induce aneuploidy in human cancers [165].

Importantly, we also show here that cell-cell fusion increases the phenotypic heterogeneity of cancer cells. In this sense, fusion acts as a driver of clonal diversification in cancer. This result matches previous reports highlighting the unique

involvement of cell-cell fusion in enhancing phenotypic diversification of cancer [9, 132]. Evidence for this comes from comparing variance among hybrid vs. non-hybrid B16 clones in terms of DNA content, gene expression, and resistance to chemotherapeutics. For each parameter tested, we observed significantly more variance in the hybrid clones compared to non-hybrid clones. This result clearly illustrates that the process of cell-cell fusion imparts a higher degree of phenotypic variability to daughter cells. These observations support the hypothesis that cell-cell fusion is useful to an evolving cancer cell population by more easily creating a cell with novel properties that can survive a given selective pressure. In fact, we speculate that cell fusion is perhaps the most efficient way to create heterogeneity in a cancer cell population.

Previous reports have shown that cell-cell fusion can modulate various properties of cancer cells including resistance to chemotherapy. A recent study using metastatic colon carcinoma showed that cell-cell fusion occurs *in vivo* and is involved in the appearance of tumor cells that were resistant to both 5-fluorouracil (5-FU) and oxaliplatin [148]. Another study using breast cancer cells showed that cell lines derived from breast cancer/epithelial cell fusion exhibited increased resistance to several chemotherapeutics (5-FU, doxorubicin, etoposidase, and paclitaxel) compared to parental breast cancer cell lines [147]. Similarly, we found that our hybrid B16 cell lines were more resistant to two different chemotherapeutics compared to non-fused B16 cells *in vitro*. Further, we found a higher relative frequency of tdTomato⁺ cells in B16 tumors that were treated with chemotherapy compared to untreated control tumors. This finding mirrors a recent report showing

that chemotherapy increased tumor cell hybridization *in vivo* [149]. Together, these data support the hypothesis that cell-cell fusion contributes to tumor progression at least in part by allowing for a higher degree of heterogeneity, which in the presence of a selective pressure like chemotherapy, results in a higher likelihood that at least one cell will have mutated around it. In this regard, our data adds to the growing list of evidence that cell-cell fusion could have functional relevance in cancer progression.

Chapter III, in full, is a reprint of the material as it appears in *Oncotarget*. Searles SC, Santosa EK, and Bui JD. “Cell-cell fusion as a mechanism of DNA exchange in cancer.” *Oncotarget*. Jan 2018. 9(5): 6156-6173. The dissertation author was the primary author of all material.

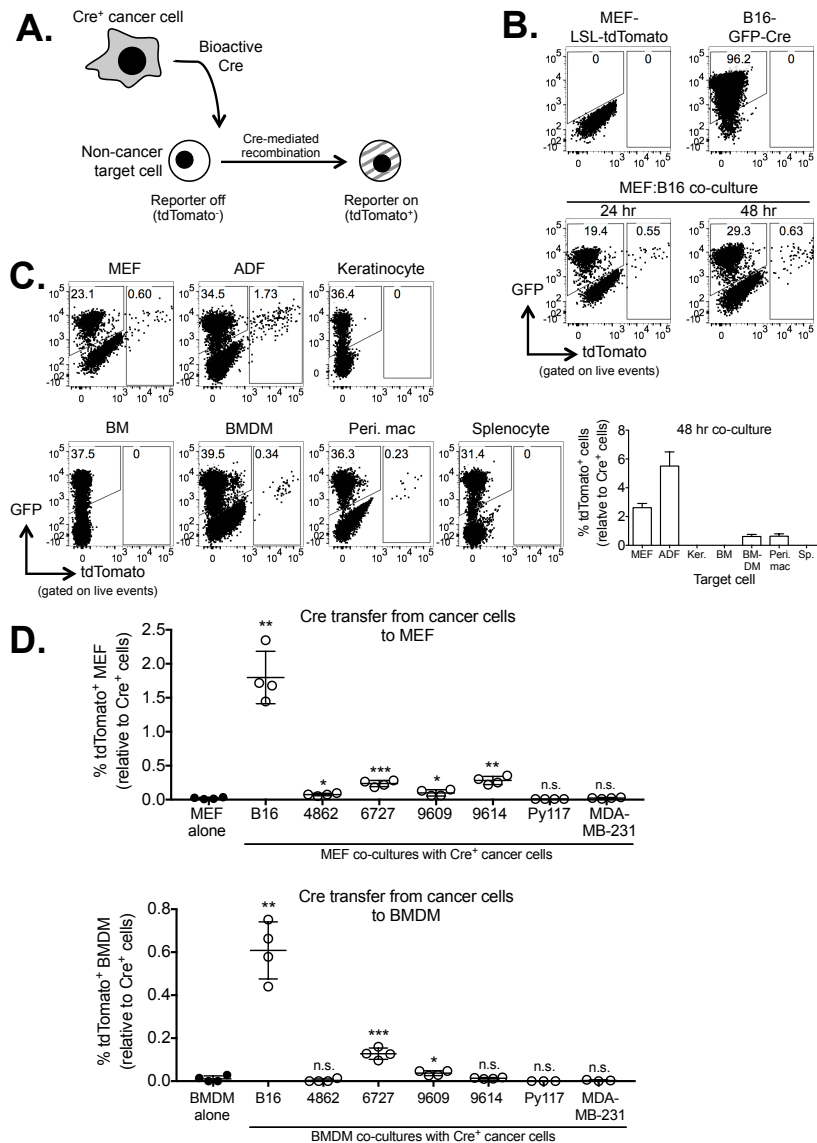


FIGURE 3.1 Bioactive Cre is rapidly transferred from cancer cells to non-cancer cells in vitro. (A) Schematic representation depicting the Cre-loxP model system used to investigate the exchange of molecular information between cancer cells and non-cancer cells. (B) FACS plots showing GFP and tdTomato expression in reporter MEF (LSL-tdTomato), B16-GFP-Cre cells, and 24- and 48 hr B16:MEF co-cultures. (C) Representative FACS plots and quantification of tdTomato expression in 48 hr co-cultures of B16-GFP-Cre and different reporter cells including MEF, adult dermal fibroblasts (ADF), keratinocytes (Ker.), bone marrow (BM), BM-derived macrophages (BMDM), peritoneal macrophages (Peri. mac), and splenocytes (Sp.) (n= 3 or 4 independent experiments). The relative percentage of tdTomato⁺ cells is shown, and was calculated by dividing the frequency of tdTomato⁺ cells by the frequency of GFP-Cre⁺ cells in each co-culture. Data is represented as mean $\pm$ SEM. (D) Quantification of tdTomato expression in 48 hr co-cultures of various different GFP-Cre-expressing cancer cell lines (B16 melanoma, 4862, 6727, 9609, and 9614 MCA sarcoma, Py117 and MDA-MB-231 breast cancer) with reporter MEF or BMDM (n= 3 or 4 independent experiments). The relative percentage of tdTomato⁺ cells is shown, and was calculated by dividing the frequency of tdTomato⁺ cells by the frequency of GFP-Cre⁺ cells in each co-culture. Data is represented as mean $\pm$ SEM. Symbols represent statistically significant increases in tdTomato⁺ cells compared against reporter cells alone.

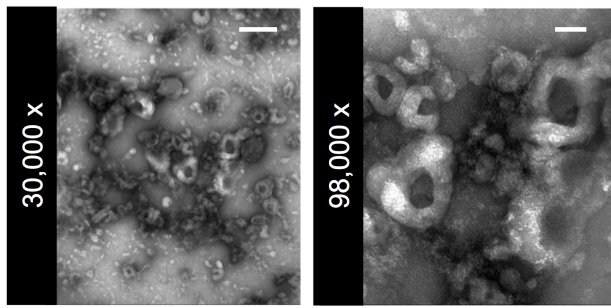


FIGURE 3.2 Size and morphology of B16-GFP-Cre ECVs. Electron micrographs showing size and morphology of vesicles isolated from the conditioned media of B16-GFP-Cre cells via differential ultracentrifugation. Size bar equals 200 nm (30,000x) or 50 nm (98,000x).

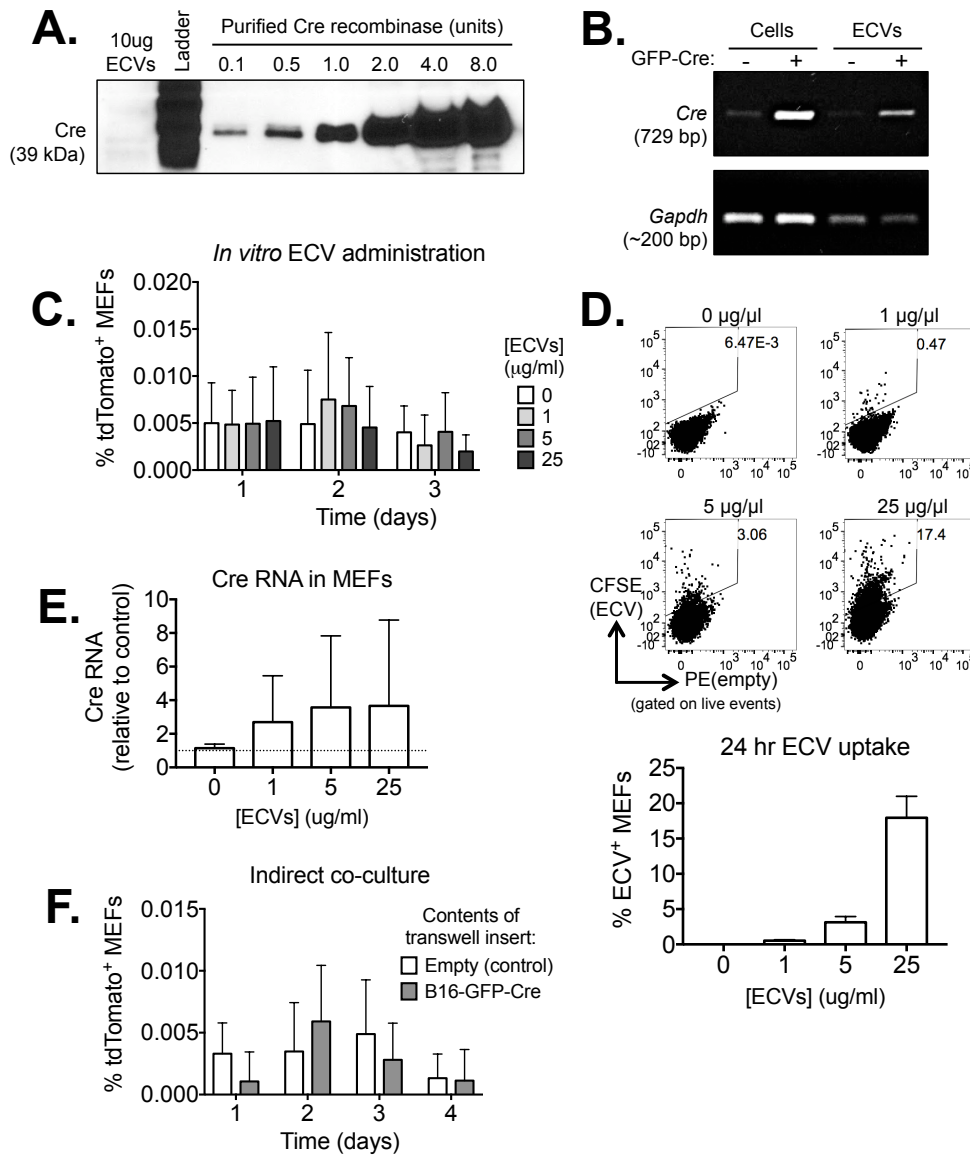


FIGURE 3.3 The rapid transfer of Cre from B16 melanoma cells to MEF is not mediated by ECVs. (A) Analysis of Cre protein in 10 μ g of B16-GFP-Cre ECVs by western blotting. (B) Analysis of Cre RNA in B16-GFP-Cre ECVs by PCR. (C) Quantification of the frequency of tdTomato⁺ MEF after treatment with B16-GFP-Cre ECVs for up to three days ($n = 4$ independent experiments). Data is represented as mean $\pm$ SEM. (D) Representative FACS plots and quantification showing the frequency of MEF that uptake CFSE-labeled ECVs in 24 hrs *in vitro* ($n = 4$ independent experiments). Data is represented as mean $\pm$ SEM. (E) Analysis of Cre RNA in MEF that were treated with B16-GFP-Cre ECVs for 24 hrs by qPCR. Data were normalized against *Hprt* ($n = 3$ independent experiments). Data is represented as mean $\pm$ SEM. (F) Quantification of tdTomato expression in reporter MEF that were cultured alone (control) or indirectly with B16-GFP-Cre cells (separated by a membrane with 0.4 μ m pores) for up to 4 days ($n = 4$ independent experiments). Data is represented as mean $\pm$ SEM.

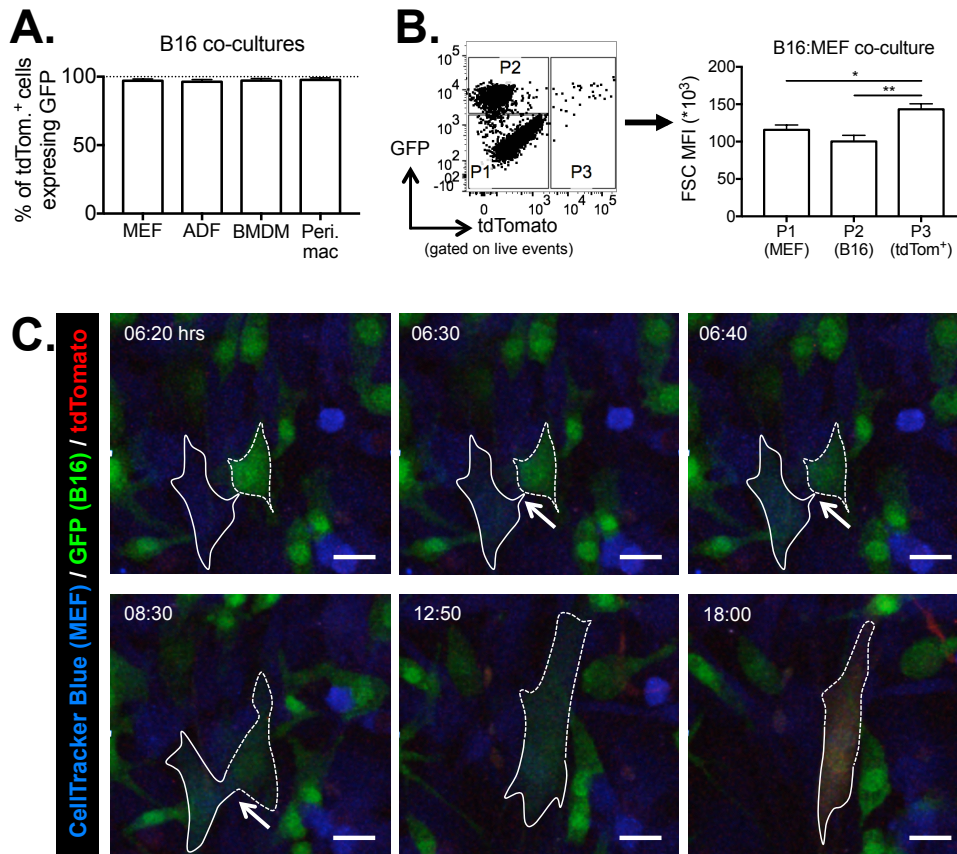


FIGURE 3.4 Cell-cell fusion mediates the rapid transfer of bioactive Cre from B16 melanoma cells to non-cancer cells in vitro. (A) Quantification of GFP expression in tdTomato⁺ cells from 48 hour co-cultures of B16-GFP-Cre cells with various reporter cells (n= 3-4 independent experiments). Data is represented as mean $\pm$ SEM. (B) Quantification of FSC MFI of three populations of cells from a 24 hour B16:MEF co-culture: P1= GFP⁻, tdTomato⁻ (MEF); P2= GFP⁺ (B16 cells); P3= tdTomato⁺ (MEF that received bioactive Cre) (n= 7 independent experiments). Data is represented as mean $\pm$ SEM. (C) Stills from confocal imaging movie (Video 1) of B16:MEF co-culture showing a CellTracker Blue-labeled reporter MEF (outlined in solid white line) turn green and then red after fusing with a B16-GFP-Cre cell (outlined in dashed white line). Arrows indicate the area of contact between the MEF and B16 cell that ultimately fuse and start expressing tdTomato at 18:00 hrs.

See also Supplemental Videos 3.1, 3.2, 3.3, 3.4, and 3.5.

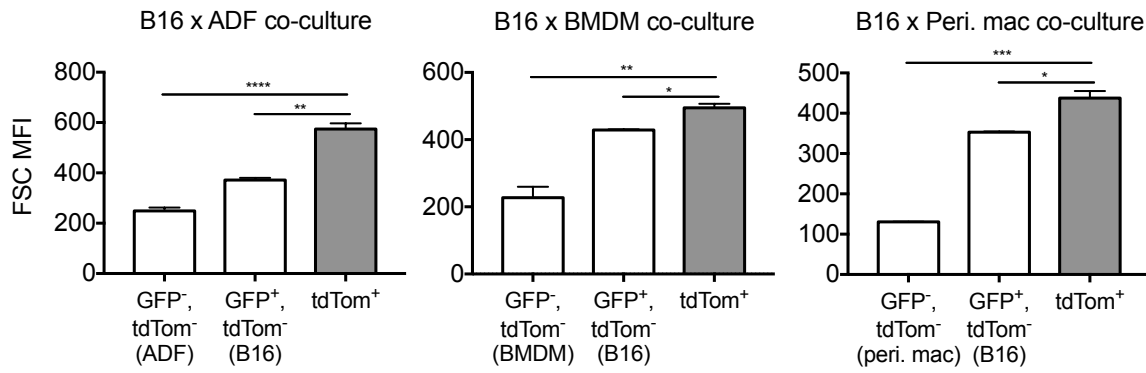


FIGURE 3.5 Reporter⁺ cells are larger than B16 cells and reporter⁻ cells. FSC analysis of three populations of cells (GFP^{-/-}/tdTomato^{-/-}, GFP⁺/tdTomato^{-/-}, tdTomato⁺) from 48 hr co-cultures of B16-GFP-Cre cells with various reporter cells (indicated).

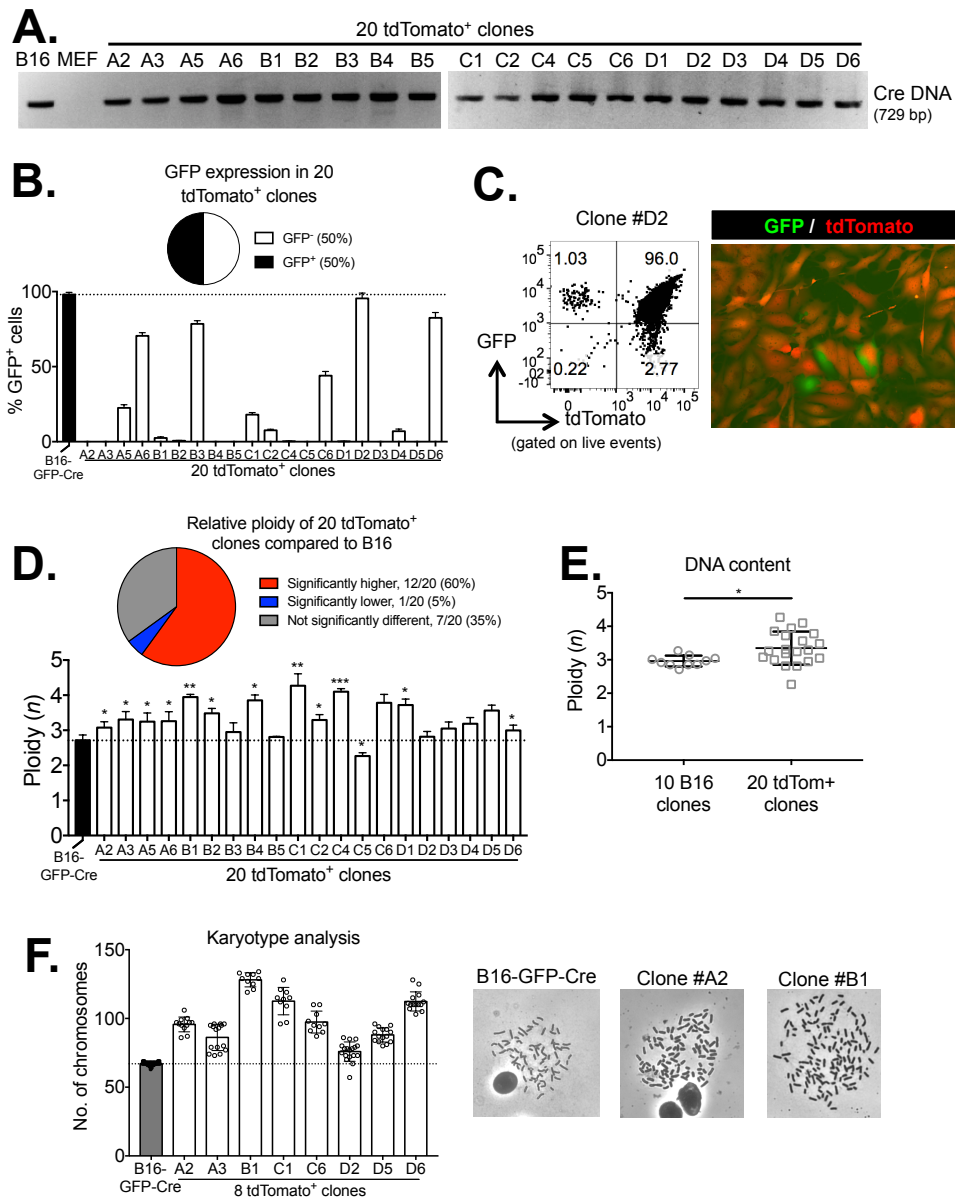


FIGURE 3.6 B16xMEF hybrids stably maintain B16-restricted DNA, do not maintain expression of B16-restricted GFP, and are hyperploid. (A) Analysis of Cre DNA in B16-GFP-Cre, MEF, and twenty tdTomato⁺ clones by PCR. (B) Quantification of GFP expression in B16-GFP-Cre and twenty tdTomato⁺ clonal cell lines measured by FACS (n=3 independent experiments). Data is represented as mean $\pm$ SEM. (C) FACS plot and fluorescent micrograph showing GFP and tdTomato expression in one of the three tdTomato⁺ clones (#D2) that showed loss of tdTomato expression. (D) Quantification of DNA content of B16-GFP-Cre and twenty tdTomato⁺ cell lines by FACS. Ploidy was determined by normalizing the 7-AAD MFI of each cell line relative to MEF, which was set at $2n$ (n= 3 independent experiments). Data are represented as mean $\pm$ SEM. (E) Comparison of the average DNA content in ten B16-GFP-Cre clones and twenty tdTomato⁺ clones as determined by FACS (n=2-3 independent experiments per data point). Data is represented as mean $\pm$ SEM. (F) Quantification of chromosome number in B16-GFP-Cre cells and eight tdTomato⁺ clonal cell lines by karyotyping (n= 15-20 metaphase spreads per group). Shown is mean $\pm$ SD. Representative metaphase spreads of B16-GFP-Cre and two tdTomato⁺ clones are shown on the right.

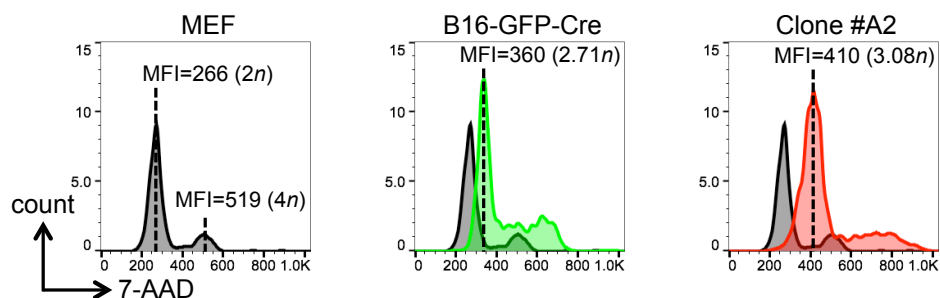


FIGURE 3.7 FACS-based quantification of DNA content. Representative histograms showing DNA content of MEF, B16-GFP-Cre, and one of the twenty tdTomato⁺ clones (#A2). The two peaks in each histogram correspond to cells in the G1 (left peak) or G2 (right peak) phase of the cell cycle. For B16-GFP-Cre and each of the twenty tdTomato⁺ clones, relative ploidy was calculated by normalizing the MFI of the G1 peak to MEF, which were set as $2n$.

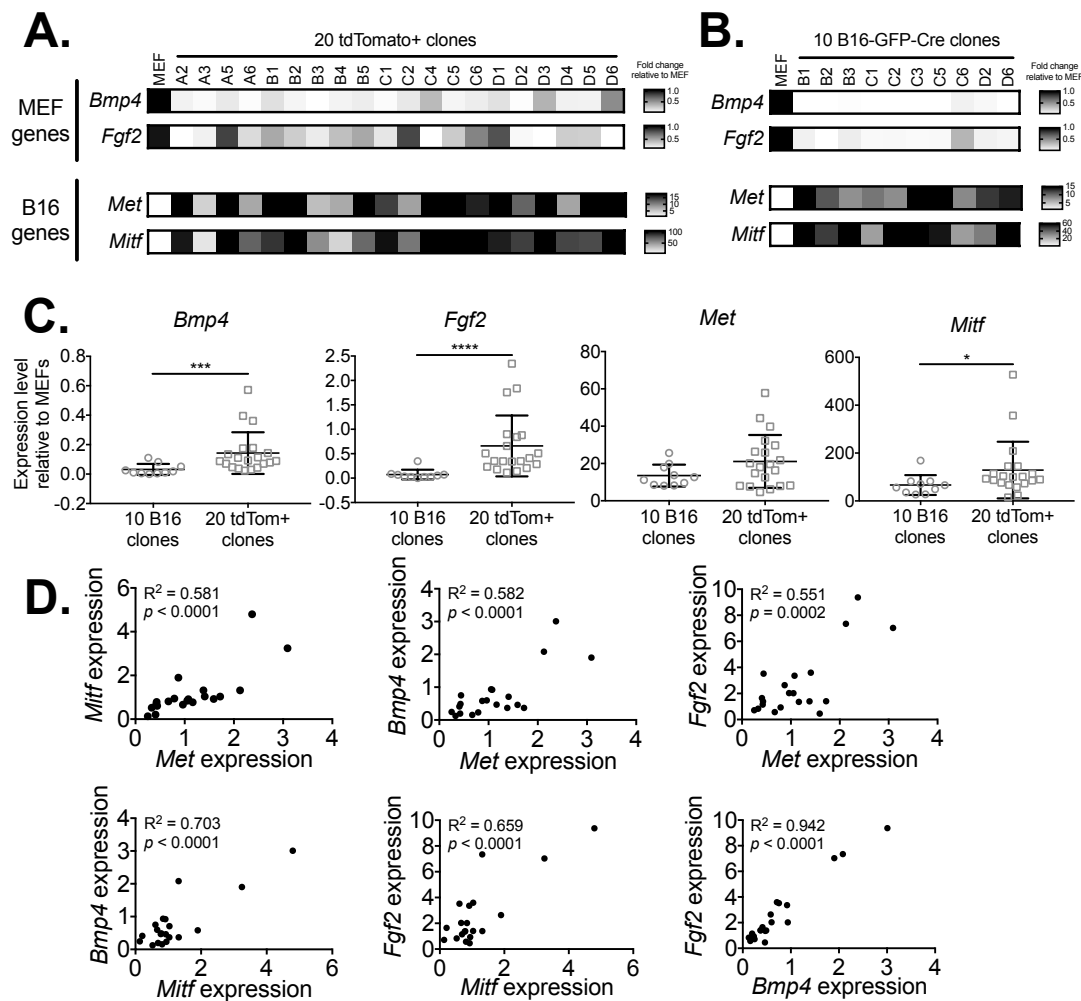


FIGURE 3.8 B16xMEF hybrids express B16- and MEF-restricted genes. (A) Heat map showing the relative expression level of two candidate “MEF genes” (*Bmp4*, *Fgf2*) and two candidate “B16 genes” (*Met*, *Mitf*) in MEF and twenty tdTomato⁺ clones. The expression level of each gene is shown as relative to MEF and normalized against *Hprt* (n = 2 independent experiments). (B) Heat map showing the expression level of *Bmp4*, *Fgf2*, *Met*, and *Mitf* in ten B16-GFP-Cre clones relative to MEF and normalized against *Hprt* (n = 2 independent experiments). (C) Comparison of the average expression level of *Bmp4*, *Fgf2*, *Met*, and *Mitf* in ten B16-GFP-Cre clones and twenty tdTomato⁺ clones (n=2-3 independent experiments per data point). Data is represented as mean ± SEM. (D) Dots plots comparing the expression level of *Bmp4*, *Fgf2*, *Met*, and *Mitf* (relative to B16-GFP-Cre) in twenty tdTomato⁺ clones.

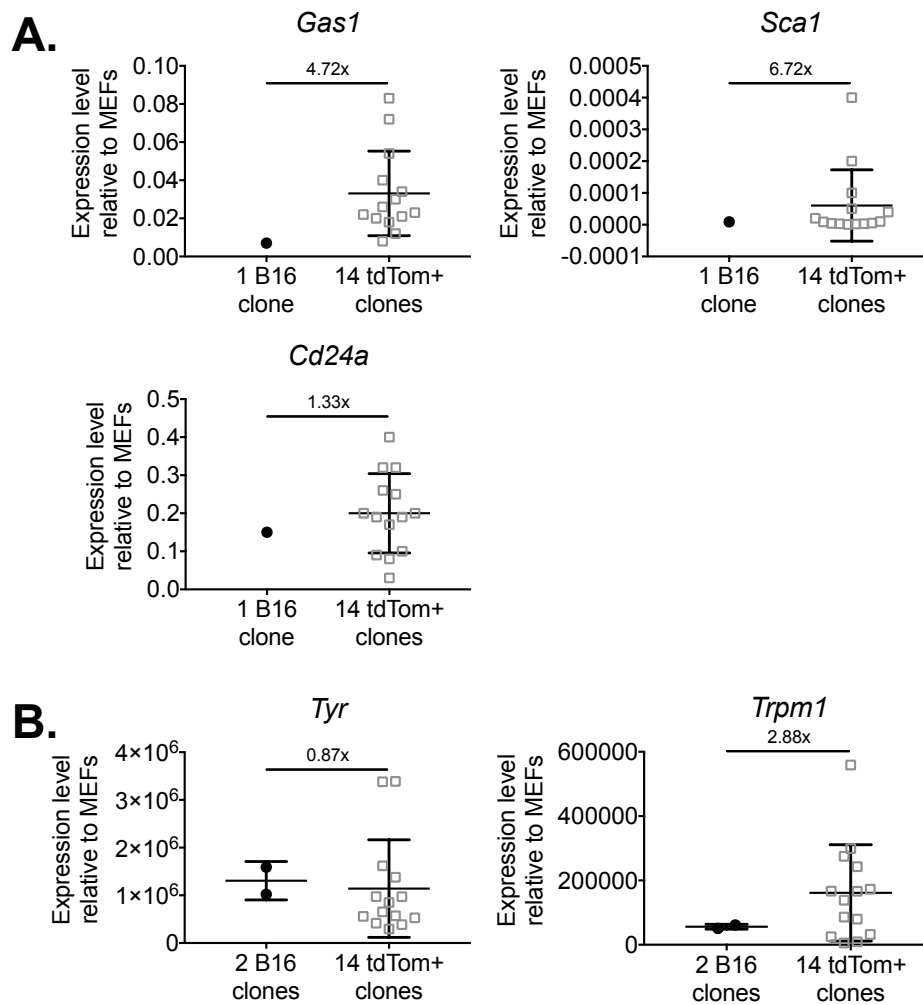


FIGURE 3.9 Expanded panel of B16- and MEF-restricted genes that are expressed by B16xMEF hybrid clones. (A) Comparison of the average expression level of three candidate “MEF genes” in B16-GFP-Cre cells and fourteen tdTomato+ clones (n= 2 independent experiments per data point). For the tdTomato+ clones, data is represented as mean +/- SEM. **(B)** Comparison of the average expression level of two candidate “B16 genes” in two B16-GFP-Cre clones and fourteen tdTomato+ clones (n= 2 independent experiments per data point). Data is represented as mean +/- SEM.

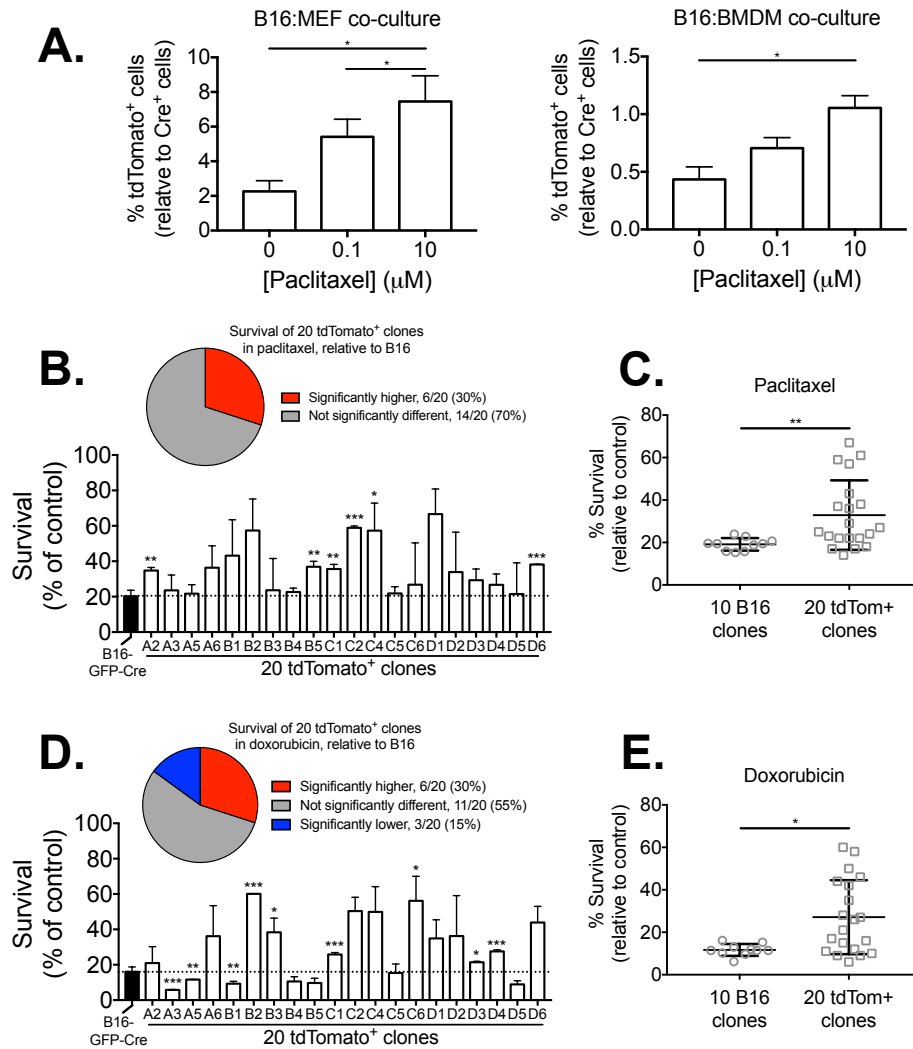


FIGURE 3.10 Cell-cell fusion promotes resistance to chemotherapeutics in B16 melanoma cells in vitro. (A) Quantification of tdTomato⁺ cells in B16:MEF and B16:BMDM co-cultures grown together for 24 hrs and then treated with 0, 0.1, or 10 μM paclitaxel for 24 hrs (n= 3 independent experiments). Data is represented as mean ±SEM. (B) Bar graph showing survival of B16-GFP-Cre and twenty tdTomato⁺ clones grown in the presence of paclitaxel (10 μM) for 24 hrs relative to control (n= 2-3 independent experiments). Data are represented as mean ±SEM. (C) Comparison of the average survival rate of ten B16-GFP-Cre clones and twenty tdTomato⁺ clones in the presence of 10 μM paclitaxel for 24 hrs (n= 2-3 independent experiments). Data are represented as mean ±SEM. (D) Bar graph showing survival of B16-GFP-Cre and twenty tdTomato⁺ clones grown in the presence of doxorubicin (10 μM) for 24 hrs relative to control (n= 2-3 independent experiments). Data are represented as mean ±SEM. (E) Comparison of the average survival rate of ten B16-GFP-Cre clones and twenty tdTomato⁺ clones in the presence of 10 μM doxorubicin for 24 hrs (n= 2-3 independent experiments). Data are represented as mean ±SEM.

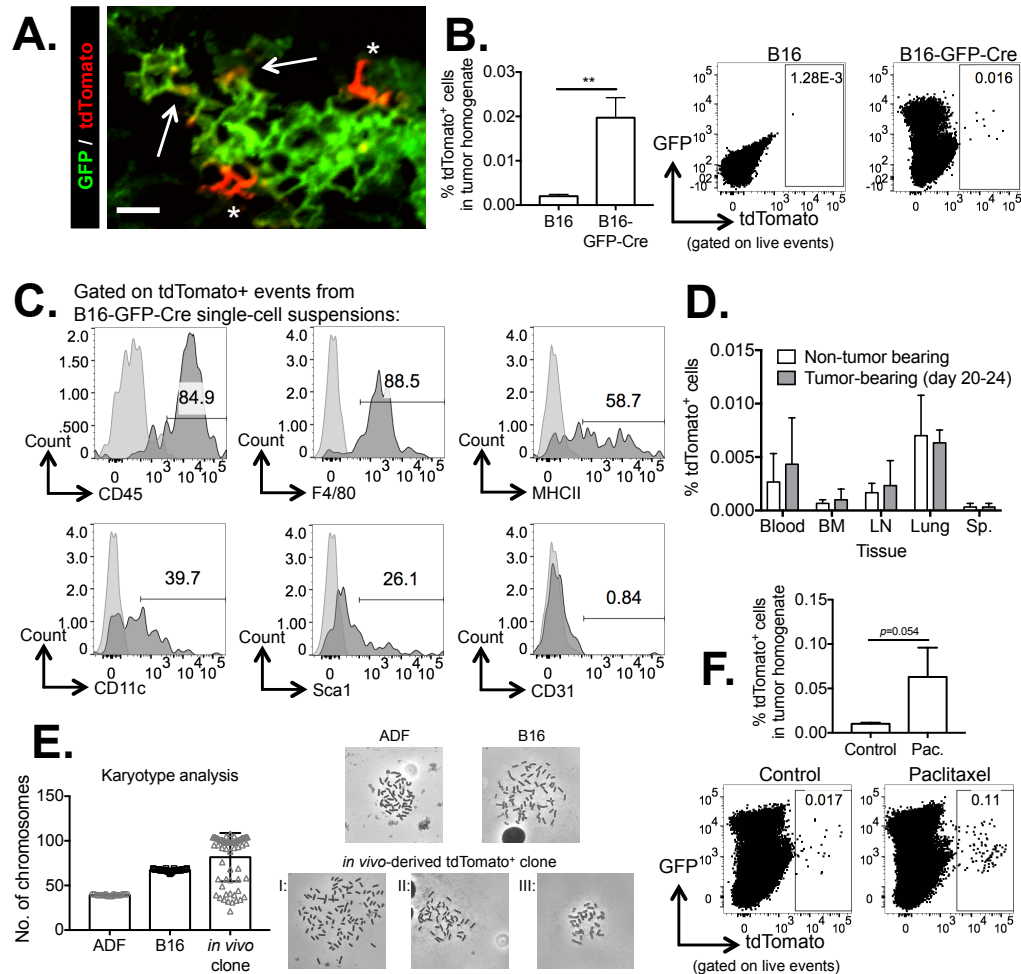


FIGURE 3.11 Cre transfer from B16 melanoma cells to non-cancer cells occurs in vivo and promotes survival in the presence of paclitaxel. (A) Representative confocal micrograph showing GFP and tdTomato expression in a flash-frozen B16-GFP-Cre tumor grown in a reporter mouse for 21 days. Asterisks indicate cells expressing tdTomato, and arrows indicate cells expressing both tdTomato and GFP. Scale bar equals 50 μ m. (B) Quantification and representative FACS plots of tdTomato expression in single-cell suspensions of B16 or B16-GFP-Cre tumors that were grown in reporter mice for 18-21 days. $n = 4$ (B16) or 10 (B16-GFP-Cre) mice. Data are represented as mean $\pm$ SEM. (C) FACS analysis of CD45, F4/80, MHCII, CD11c, Sca-1, and CD31 expression on tdTomato+ cells from B16-GFP-Cre single-cell suspensions. Histograms in black were obtained using fluorescently-labeled antibodies against each of the antigens listed, and histograms in light grey were obtained using isotype control antibodies. (D) Quantification of tdTomato expression in single-cell suspensions of various tissues including blood, bone marrow (BM), lymph nodes (LN), lung, and spleen (Sp.) from reporter mice bearing B16-GFP-Cre tumors for 0 or 20-24 days ($n = 4$ mice per group). Data are represented as mean $\pm$ SEM. (E) Karyotype analysis of adult dermal fibroblasts (ADF), B16-GFP-Cre, and an *in vivo*-derived tdTomato+ clonal cell line. $n = 10$ (ADF and B16) or 66 (*in vivo* clone) metaphase spreads per group. Shown is mean $\pm$ SD. Representative metaphase spreads of ADF, B16-GFP-Cre and the three types of karyotypes observed in the *in vivo* clone are shown on the right. (F) Representative FACS plots and quantification of tdTomato expression in single-cell suspensions of B16-GFP-Cre tumors grown in reporter mice that were treated with HBSS or paclitaxel. Tumors were harvested after reaching a size of 10x10 mm² ($n = 6$ mice per group). Data are represented as mean $\pm$ SEM.

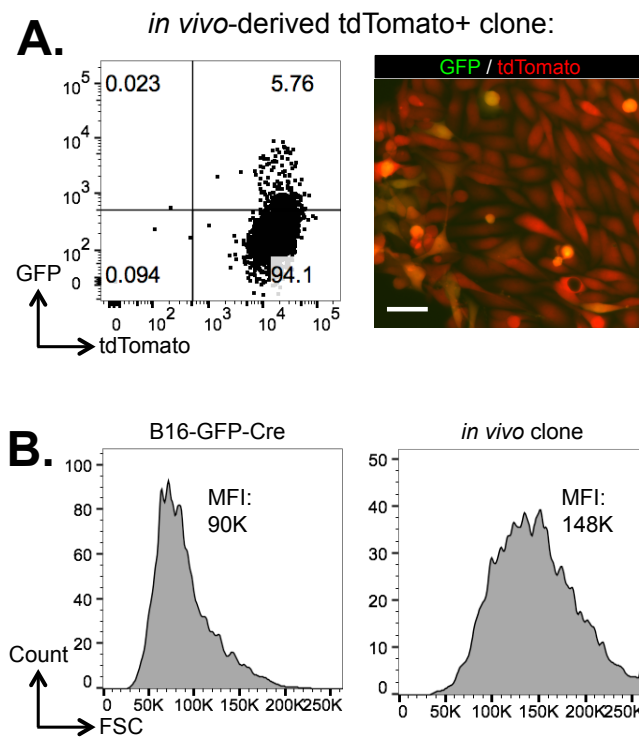


FIGURE 3.12 Cell-cell fusion occurs in vivo. (A) Representative FACS plot and fluorescent micrograph showing GFP and tdTomato expression in the *in vivo*-derived tdTomato⁺ clone after four passages. Scale bar = 100 μ M. (B) Representative histograms showing FSC (as a read-out for cell size) of B16-GFP-Cre and the *in vivo*-derived clone.

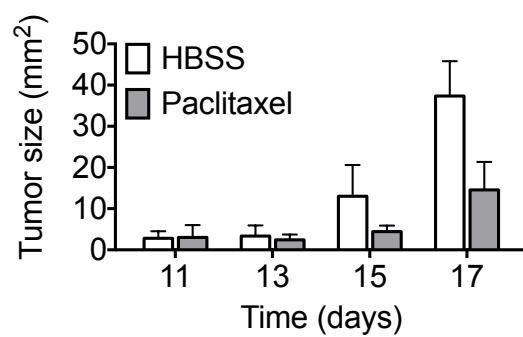


FIGURE 3.13 Paclitaxel treatment shrinks B16-GFP-Cre tumors in vivo. Bar graph showing the size of B16-GFP-Cre tumors that were grown in mice treated with paclitaxel or HBSS (control). Data are represented as mean $\pm$ SEM (n=6 mice per group).

TABLE 3.1 List of primers for PCR

Table of Primer Sequences	
Name	Sequence (5' to 3')
<i>Bmp4</i> (forward)	GGCTGGCCATTGAGGTGACT
<i>Bmp4</i> (reverse)	TCGGCTGATTCTGACATGCTG
<i>Cd24a</i> (forward)	CTGCTGGCACTGCTCCTAC
<i>Cd24a</i> (reverse)	GGTGGTGGCATTAGTTGGAT
<i>CRE</i> (forward)	GCCAGCTAAACATGCTTCATC
<i>CRE</i> (reverse)	ATTGCCCTGTTTCACTATCC
<i>Fgf2</i> (forward)	TCCAGTTGGTATGTGGCACTGA
<i>Fgf2</i> (reverse)	CAGTATGGCCTTCTGTCCAGGTC
<i>Gapdh</i> (forward)	GGAGCCAAAAGGGTCATCAT
<i>Gapdh</i> (reverse)	GTGATGGCATGGACTGTGGT
<i>Gas1</i> (forward)	CCCTCTTCTGTGCGGTTTTTC
<i>Gas1</i> (reverse)	CACCGTTCAGCACTCTGAGTC
<i>Hprt</i> (forward)	GCTTGCTGGTGAAAAGGACCTCTCGAAG
<i>Hprt</i> (reverse)	CCCTGAAGTACTCATTATAGTCAAGGGCAT
<i>Met</i> (forward)	GCATGTCAGCATCGCTCAA
<i>Met</i> (reverse)	TGCAGGCCAGCTGTTTC
<i>Mitf</i> (forward)	GCCTTGTTTATGGTGCCTTC
<i>Mitf</i> (reverse)	GTCCTCCTCCCTCTACTTTCTGT
<i>Sca1</i> (forward)	CTCTGAGGATGGACACTTCT
<i>Sca1</i> (reverse)	GGTCTGCAGGAGGACTGAGC
<i>Trmp1</i> (forward)	GAGCTGAAGGAGGCTAGGCTG
<i>Trmp1</i> (reverse)	CTTGGTGTCTCTCTCTGTTGT
<i>Tyr</i> (forward)	CCTCCTGGCAGATCATTTGT
<i>Tyr</i> (reverse)	ATCGCATAAAACCTGATGGC

CHAPTER IV: CONCLUSIONS

The studies presented here compromise a new look at how intercellular communication that occurs within the tumor microenvironment affects cancer development. Specifically, we have analyzed the ways in which two independent mechanisms of communication, exosomes and cell-cell fusion, are involved in tumorigenesis. Our exosome data reveals a novel role for MEK1 in exosomes, and could change the paradigm of how MEK1 activity is thought to control tumor growth. In addition, we show how delivery of MEK1 to macrophages could be an efficacious strategy to treat cancer based on the overall stimulation of anti-tumor immunity. On the other hand, our cell-cell fusion data adds to the growing list of reports showing how the phenomena of fusion can promote the malignant properties of cancer cells, in this instance, chemoresistance. Moreover, we show how fused cells have enhanced diversity compared to parental clones, thus providing a mechanism by which cancer cells can evolve more rapidly than relying on the accumulation of specific mutations within a single cell. These results provide critical evidence that the interactions that occur between cancer cells and non-cancer cells are important determinants of many different aspects of cancer.

The role of exosomes in cancer can be both pro-and anti-tumor. Many of the mechanisms by which exosomes affect cancer growth are exerted by their effects on immune cells. Specifically, it appears that TD exosomes can both promote and inhibit anti-tumor immunity, so specific knowledge of which exosomal factors exert which effects is needed to be able to leverage this to fight cancer. We have demonstrated

here that, contrary to its cell-intrinsic role, MEK1 in exosomes actually inhibits tumor growth via the stimulation of anti-tumor immunity. We believe this is of significant clinical value, as the targeted delivery of MEK1 to macrophages could be a useful way to promote immune responses directed against cancer. Moreover, our data suggest that MEK1 inhibitors should be used cautiously in the treatment of cancer, since the tumor-suppressive role of MEK1 in exosomes could account for some of the lack of efficacy that has been observed from MEK1 inhibitors in clinical trials. We propose that determining the MEK1 content of exosomes from patient tumors could be a useful way to determine how an individual would respond to MEK1 inhibition for cancer treatment, although more work is needed to fully determine the effect of exosomal MEK1 on macrophage functionality.

Cell fusion has been known to occur in cancer for over a century, but if it is actually relevant in disease progress is still debated. Here, we have provided more evidence that cell fusion in cancer can have significant effects that could ultimately aid tumor progression. This includes promoting resistance against chemotherapeutics and, perhaps most importantly, increasing heterogeneity of individual tumor cells, allowing a population of cancer cells a higher likelihood of evolving around a given selective pressure. Therefore, we propose that finding ways to inhibit cell fusion in cancer could be a novel treatment strategy when other options fail. However, certainly more knowledge on how the process of cell fusion affects other aspects of cancer is needed before this can be explored in human patients. Regardless, we feel confident that we have demonstrated here the potential for

fusion to promote malignant properties associated with cancer, and therefore it should be on the radar as a potential therapeutic target to treat the disease.

To conclude, this dissertation has demonstrated the important role for intracellular communication in many different aspects of cancer. In particular, the role for MEK1 protein in exosomes in stimulating anti-tumor immunity to prevent tumor growth and the role of cell fusion to promote tumor growth via increasing chemoresistance and augmenting clonal heterogeneity are novel outcomes that highlight the critical role that interactions between cancer cells and non-cancer cells play in tumorigenesis. The work presented here could serve as a foundation to further examine mechanisms by which intercellular interactions that occur during cancer are involved in either promoting or inhibiting the disease, specifically as it relates to exosomes and cell-cell fusion.

APPENDIX A: MATERIALS AND METHODS

Mice

For all experiments, 8-12 week old age- and sex-matched mice were used. The following mouse strains were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and bred in house: Black 6 (C57BL/6J), RAG1^{-/-} (B6.129S7- *Rag1*^{tm1Mom}1J), NSG (NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ), and LSL-tdTomato reporter (B6.Cg-*Gt(ROSA)26Sor*^{tm9(CAG-tdTomato)Hze}/J). All experiments involving mice were conducted under the animal protocol approved by the University of California, San Diego Institutional Animal Care and Use Committee (IACUC protocol #s06201).

For experiments using exosomes as therapeutic compounds, tumor cells were co-injected with 20 µg of exosomes. Tumors were subsequently treated with 20 µg exosomes via intratumoral injection of 100 µl of exosomes at a concentration of 0.2 µg/ µl at the time points indicated in the figures.

For experiments using the MEK1-specific inhibitor PD98059 (Cell Signaling Technology, Danvers, MA, USA), mice were treated at 10 mg/kg via intraperitoneal injection according to previously established methods [85]. Mice were treated at the time points indicated in the figures.

For experiments measuring the uptake of exosomes in vivo, mice bearing 14-day 9609 tumors were treated with 20 µg exosomes that were either unlabeled or labeled with CFSE (as described below). Exosomes were administered via intratumoral injection of 100 µl of exosomes at a concentration of 0.2 µg/ µl. After 24 hours, tumors were removed from mice, processed into a single-cell suspension (as described below), and analyzed by FACS (as described below).

Tumor transplantation

Tumor cell lines were grown in vitro (as described below), harvested by trypsinization, washed three times, and resuspended at a final concentration of either 5×10^6 cells/mL or 25×10^6 cells/mL (for studies using the regressor cell line 6727) in HBSS with Ca^{2+} and Mg^{2+} . Two hundred microliters of cells (1×10^6 or 5×10^6 total cells) were injected subcutaneously into the flank of mice. After tumors reached approximately 15x15 mm in size, mice were sacrificed. For studies analyzing tumor-infiltrating immune cells, after sacrificing mice, tumors were processed into a single cell suspension by mechanical dicing/ collagenase digestion as previously described [80] and analyzed by FACS. Antibodies used for FACS analysis are described below.

Cell lines and culture conditions

MCA sarcoma cells and B16 melanoma cells were cultured in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, 0.0375% sodium bicarbonate, 5% (v/v) MEM Non-essential amino acids, 2 mM L-glutamine, 10 µg/ml ciprofloxacin, and 56 µM 2-mercaptoethanol. B16.F10 cells were kindly provided by Dr. David Lyden and MCA sarcoma cell lines (4862, 6727, d42m1, H31m1, 9609, 9614, d29m1, and F236) were generated previously in our laboratory [80]. MDA-MB-231 breast cancer cell were grown in DMEM supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin, and were kindly provided by Dr. Steve Gonias. Py117 breast cancer cells were grown in Ham's F12K medium supplemented with 5% fetal calf serum, 2.5 µg/mL fungizone, 50 µg/mL

gentamycin, and MITO+ and were generated previously in our laboratory [139]. All cells were grown and maintained in standard humidified tissue culture conditions (37°C with 5% CO₂).

Viral transduction of cancer cell lines

For GFP, MEK1, and HA-MEK1 expression in 9609 cells, retroviruses generated from 293T cells were used to transduce parental 9609 cells. Retroviral plasmids for MEK1 (pLZRS-Mek1-wt1 #21196) and HA-MEK1 (pBABEpuro-HA-MEK1 #53195) were purchased from AddGene (Cambridge, MA, USA). The retroviral plasmid for GFP (pMSCV-GFP) was provided by Dr. Robert Schreiber [166]. HA-MEK1-expressing cells were selected on puromycin supplemented media for 1 week.

For GFP-Cre expression, cancer cell lines were transduced with a lentivirus encoding a bi-cistronic GFP-Cre expression cassette (GenTarget Inc., San Diego, CA, USA). After 48 hrs, GFP⁺ cells were sorted with a BD Aria II Cell Sorter (BD, Franklin Lakes, NJ, USA) and cloned using the limited dilution method. For each cell line, the clone with the highest expression of GFP was used.

In vitro exosome/ ECV uptake

For studies investigating the in vitro uptake of exosomes/ ECVs, whole spleen was used to model the cells of the tumor microenvironment. Spleens were excised from wild-type mice, pushed through a cell-strainer, and resuspended in media containing 50 units/ml human IL-2 (Biolegend, San Diego, CA, USA) to promote the expansion and survival of T cells. BMDM were generated from BM cells cultured in

the presence of 10 ng/ml M-CSF (Biolegend, San Diego, CA, USA) for 6-7 days. MEF cells were generated as previously described [167]. For all experiments using Cre-containing ECVs, cells were derived from transgenic mice harboring the “ROSA-LSL-tdTomato” reporter locus.

Cell fusion co-culture experiments

Reporter cells used in co-culture experiments were all derived from tdTomato reporter mice. BMDM were derived from BM cells cultured in the presence of 10 ng/ml M-CSF (Biolegend, San Diego, CA, USA) for 6 days. ADF and keratinocytes were generated as previously reported [168]. MEF were generated as described previously [167]. To generate tdTomato⁺ clonal cell lines, tdTomato⁺ cells were FACS sorted from a 48 hr B16:MEF co-culture using a BD Aria II Cell Sorter (BD, Franklin Lakes, NJ, USA) and cloned using the limited dilution method.

For B16 co-culture experiments, cells were seeded at a ratio of 1:10 (Cre-expressing cell:reporter cell). For co-culture experiments involving MCA sarcomas and breast cancer cells, cells were seeded at a ratio of 1:1 due to the lower expression of GFP-Cre in these cell lines. Co-culture experiments involving splenocytes were performed in the presence of human IL-2 (50 units/ml, Biolegend, San Diego, CA, USA). For transwell experiments, reporter MEF were seeded in the well of a 24-well plate and B16-GFP-Cre cells were seeded in the transwell insert with 0.4µM pores (Corning Inc., Corning, NY, USA). The B16:MEF ratio was the same for the transwell experiment as it was for the direct co-culture (1:10).

Isolation of exosomes/ ECVs

Exosomes/ ECVs were isolated by differential ultracentrifugation as previously described [81]. Briefly, cells were grown for 48-72 hrs in media containing serum that had been depleted of ECVs by centrifugation for 70 minutes at 100,000 x g. The conditioned media was harvested and subjected to serial differential centrifugation steps to clear large and small debris as follows: 10 minutes at 500 x g (to remove large debris/ dead cells) followed by 20 minutes at 20,000 x g (to remove small debris/ apoptotic bodies). Next, the cleared conditioned media was spun for 70 minutes at 100,000 x g to pellet exosomes/ ECVs. The exosome/ ECV pellet was resuspended in a large volume of HBSS and spun again for 70 minutes at 100,000 x g to wash soluble proteins from the exosomes/ ECVs. Finally, the washed pellet was resuspended in HBSS in a volume approximately 1/500th of the starting volume of conditioned media. The concentration of exosomes/ ECVs was determined by BCA assay (Thermo Fisher, Waltham, MA, USA). All ultracentrifugation steps were performed using a Beckman Avanti J-30I ultracentrifuge with a JA-30.50 Ti fixed-angle rotor (Beckman Coulter, Carlsbad, CA, USA).

Proteomic analysis of exosomes

Proteomic analysis of exosomes via mass spectrometry was performed as previously described [67, 68]. Briefly, each exosome sample (10-20 µg of exosomal protein) was denatured, reduced, and alkylated, followed by proteolytic digestion with endoproteinase LysC (overnight at room temperature) and then trypsin (5 hrs at 37°C). After quenching the digestion with formic acid, the resulting peptide mixtures

were desalted, dried, and then solubilized in loading buffer that contained acetonitrile and formic acid. For analysis by reverse phase liquid chromatography-tandem mass spectrometry (LC-MS/MS), approximately 3-5 µg of each sample was loaded onto a C18 trap column with 5 µm beads, and peptides were separated using a 75-µm inner diameter C18 column with 3 µm beads. Each spectra obtained was extracted and searched against the Uniprot mouse proteome database based on a 1% false discovery rate. To quantify protein amounts in each sample, the average area of the three most abundant peptides for each matched protein was used. For each analysis, data from three technical replicates was used.

Immunoblotting

For western blot analysis, MCA sarcoma cells were lysed in RIPA buffer containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and phosphatase inhibitor cocktail (Roche, Basel, Switzerland) used according to manufacturer's protocol. Exosomes/ ECVs were lysed by boiling in Laemmli loading buffer containing 10% BME for 5 minutes. 10 µg of WCL or exosomal protein was loaded for each gel. Samples were resolved using SDS-PAGE in reducing conditions, transferred onto a PVDF membrane, and imaged using the ECL method. For Cre western blot, purified Cre recombinase (New England Biolabs, Ipswich, MA, USA) was used as a positive control. Primary antibodies directed against the following epitopes were used: MEK-1 (clone #30C8, Cell Signaling Technology, Danvers, MA, USA), phospho-MEK1 (polyclonal, Cell Signaling Technology), CD63 (polyclonal, Santa Cruz Biotechnology, Dallas, TX, USA), β -actin (clone #AC-15, Sigma-Aldrich,

St. Louis, MO, USA), HA (clone #C29F4, Cell Signaling Technology), and Cre (polyclonal, Cell Signaling Technology). All primary antibodies were used at a concentration of 1 µg/ml. The following HRP-conjugated secondary antibodies were used: goat-anti-rabbit (polyclonal, Cell Signaling Technology), and goat-anti-mouse (polyclonal, Sigma-Aldrich). Secondary antibodies were diluted at a concentration of 1:10,000.

Exosome/ ECV characterization via particle tracking analysis and electron microscopy

The size distribution and production amount of exosomes in conditioned media was determined by nanoparticle tracking analysis using a NanoSight. The conditioned media from each cell line grown for 48 hrs in media containing serum that had been depleted of exosomes was used for these analyses. A NanoSight NS300 (Malvern Instruments, Malvern, United Kingdom) was used for acquisition with light scatter from a red laser, and NTA 2.3 Analytical software was used for analysis.

The size and morphology of exosomes/ ECVs was evaluated by transmission electron microscopy using a previously described method [81]. Exosomes/ ECVs were stained with 2% uranyl acetate for 1 minute, and grids were viewed using a JEOL 1200EX II (JOEL, Peabody, MA, USA) transmission electron microscope and photographed using a Gatan digital camera (Gatan, Pleasanton, CA, USA).

Flow cytometry and antibodies

For cell surface staining of tumor single-cell suspensions, $1-2 \times 10^6$ total cells were incubated for 20 minutes at 4°C with the following antibodies: APC-Cy7-conjugated anti-CD45 (clone #30-F11, Biolegend, San Diego, CA, USA), PE-conjugated anti-CD45 (clone #104, Biolegend), PE-Cy7-conjugated anti-F4/80 (clone #BM8, Biolegend), APC-conjugated anti-MHCII (clone #M5/114.15.2, Biolegend), PE-Cy7-conjugated anti-CD11b (clone #M1/70, eBioscience, San Diego, CA, USA), APC-conjugated anti-Ly6C (clone #HK1.4, Biolegend), PE-conjugated anti-CD4 (clone #GK1.5, Biolegend), APC-conjugated anti-CD8 (clone #53-6.7, Biolegend), APC-conjugated anti-NK1.1 (clone #PK136, Biolegend), PE-Cy7-conjugated anti-CD3 (clone #17A.2, Biolegend), PE-Cy7-conjugated anti-CD11c (clone #N418, eBioscience), PE-Cy7-conjugated anti-Sca1 (clone #D7, Biolegend), and AlexaFluor647-conjugated anti-CD31 (clone #390, Biolegend). Surface staining was performed in the presence of 1 µg/ml F_c blocking anti-CD16/32 antibody. 7-aminoactinomycin D (7-AAD, Calbiochem, San Diego, CA, USA) was added immediately before FACS analysis at a final concentration of 1 µg/ml to stain and exclude dead cells. FACS was performed using a BD FACSCanto (BD, Franklin Lakes, NJ, USA) and data were analyzed using FlowJo software (Treestar, Ashland, OR, USA).

For cell fusion studies, reporter activation was measured by analysis of tdTomato expression in reporter cells by FACS. For analysis of in vitro co-cultures, cells were trypsinized, washed, and resuspended in FACS staining buffer (1X PBS with 1% FCS and 0.05% NaN₃). For analysis of tumors grown in vivo, tumors were harvested after reaching approximately 15x15 mm and processed into a single-cell

suspension as described previously [80]. 7-AAD) was added immediately before FACS analysis at a final concentration of 1 µg/ml to stain and exclude dead cells from analysis.

Fluorescent labeling of exosomes/ ECVs

As described [87], exosomes/ ECVs were incubated with CFSE at a final concentration of 25 µM for 30 minutes at 37°C in the dark. Specifically, 2.5 µL 5 mM CFSE was added to 497.5 µL exosomes/ ECVs. After 30 minutes of labeling, excess CFSE was washed out by spinning the exosomes/ ECVs in a large volume of HBSS for 70 minutes at 100,000 x g. After this wash step, CFSE-labeled exosomes/ ECVs were resuspended in a volume approximately 1/500th the original starting volume of conditioned media.

PCR for Cre transcript in ECVs

Total RNA was extracted from purified ECVs or cells using the Trizol reagent (Life Technologies, Carlsbad, CA, USA) and then subjected to cDNA synthesis using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. A ND100 spectrophotometer (Nanodrop, Wilmington, DE, USA) was used to assess the concentration and purity of RNA prior to cDNA synthesis. PCR was performed on cDNA using Cre-specific primers (see **Table 3.1**) that amplify a 729 bp-sized fragment under the following thermal cycle conditions: 10 minutes at 94°C, followed by thirty cycles consisting of: 45 seconds at 94°C, 45 seconds at 62°C, and 45 seconds at 72°C. PCR reactions

were concluded with incubation for 10 minutes at 72°C. Primers specific for *Gapdh* were used as control. After completion, the PCR reactions were loaded on to a 1% TAE agarose gel, electrophoresed, and imaged with ethidium bromide (Sigma, St. Louis, MO, USA).

To probe for Cre DNA in tdTomato⁺ clonal cell lines, total genomic DNA was isolated from each cell line using the ethanol precipitation method and assayed for concentration and purity using a ND100 spectrophotometer (Nanodrop, Wilmington, DE, USA). DNA was subjected to PCR using Cre-specific primers and the following thermal conditions: [45 seconds at 94C; 45 seconds at 62C; 45 seconds at 72C] repeated thirty times. After PCR was completed, each PCR reaction was loaded on to a 1% agarose gel, electrophoresed, and imaged with ethidium bromide (Sigma, St. Louis, MO, USA).

Imaging

For live cell imaging of in vitro Cre transfer, B16-GFP-Cre cells were added to adherent reporter MEF that had been labeled with 5 uM of CellTracker Blue (Molecular Probes, Eugene, OR, USA) according to the manufacturer's protocol. The video recording was initiated after 2 hours. Images were collected every 3–4 minutes with xyz-t acquisition mode using an Axio Observer.Z1 microscope with the LSM 700 scanning module (Zeiss, Jena, Germany). Cultures were maintained at 37°C, 5% CO₂ using a Heating Insert P Lab-Tek S1 with an Incubator PM S1 (Zeiss, Jena, Germany).

For imaging of in vivo Cre transfer, B16-GFP-Cre tumors were grown in reporter mice as described above. After 18-20 days, mice were sacrificed, and tumors were harvested, coated in OCT, and flash frozen in liquid nitrogen. Cryosectioning was performed to generate tumor sections 15 μm thick, which were imaged with a Nikon D-Eclipse C1TE2000 confocal microscope (Nikon, Tokyo, Japan).

DNA content analysis by flow cytometry

DNA content of B16-GFP-Cre and tdTomato⁺ clonal cell lines was measured using flow cytometry as previously described [169]. 1×10^6 cells were resuspended in 0.5 ml HBSS, to which was added 4.5 ml 70% ethanol (dropwise). After incubation for 60 minutes at 4°C, the cells were washed three times with HBSS and incubated in the presence of 7-AAD (2 $\mu\text{g}/\text{ml}$) for 20 minutes at 4°C. Finally, the cells were washed, resuspended in FACS staining buffer, and analyzed in the PerCP channel using linear voltage setting. These analyses reveal two peaks that represent cells in the G1 (left peak) or G2 (right) phase of the cell cycle. The MFI of the G1 peak was determined for each cell line, and the relative ploidy was calculated by normalizing the G1 MFI of each cell line against MEF, which was set at a ploidy of “2n”.

Karyotype analysis

For karyotype analysis, adherent cells were treated with 0.1 $\mu\text{g}/\text{ml}$ of KaryoMAX Colcemid Solution (Life Technologies, Carlsbad, CA, USA) for 4 hours. Cells were then harvested, treated with a hypotonic solution of 0.8% sodium citrate

for 10 mins at room temperature, washed, treated with Carnoy's fixative (75% MeOH, 25% glacial acetic acid) for 10 minutes at room temperature, and washed again. These four steps (hypotonic solution, wash, fix, wash) were repeated two additional times. After fixation, a drop of cells in fixative was released onto a slide and allowed to sit until dry. The slide was then stained with Giemsa for 20 minutes and mounted in mounting medium for analysis. At least 15 cell-karyotypes were counted for each cell line.

Quantitative RT-PCR

RNA was extracted from cell lines using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and measured with a ND100 spectrophotometer (Nanodrop, Wilmington, DE, USA) for concentration and purity. RNA was then subjected to cDNA synthesis using High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Foster City, CA, USA) according to the manufacturer's protocol. qPCR was performed using SYBR Green PCR Master Mix (Applied Biosystem, Foster City, CA, USA) and the following thermal cycle conditions: 10 minutes at 95°C, followed by forty cycles consisting of: 10 seconds at 95°C, 60 seconds at 60°C using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Irvine, CA, USA). Gene expression was analyzed with the $2^{-\Delta\Delta C_t}$ method normalized against *Hprt*. Primer sequences are listed in **Table 3.1**.

In vitro chemoresistance assays

To measure chemoresistance of tdTomato⁺ cells from *in vitro* co-cultures, B16-GFP-Cre cells were co-cultured with reporter MEF or BMDM for 24 hrs at a ratio of 1:10 and then treated with 0.1 or 10 μ M paclitaxel (TEVA Pharmaceuticals, Petah Tikva, Israel) or vehicle control for 24 hrs. Then the frequency of tdTomato⁺ cells in each co-culture was measured using FACS.

To measure chemoresistance of tdTomato⁺ and B16-GFP-Cre clonal cell lines, each cell line was seeded in a 24 well plate, grown for 24 hrs, and then treated with 10 μ M paclitaxel, 10 μ M doxorubicin (Bedford Laboratories, Bedford, OH, USA), or vehicle control for 24 hours. Each condition was performed in triplicate. Then cells were harvested and counted, and the relative survival of each cell line in the presence of each drug was calculated by dividing the number of cells in the drug treated condition by the number of cells in the control condition. Viability was determined based on the exclusion of 7-AAD as measured by flow cytometry.

In vivo chemoresistance assay

To determine if *in vivo*-derived tdTomato⁺ cells exhibited increased resistance to chemotherapy, 1×10^6 B16-GFP-Cre cells were injected into reporter mice that were then treated with a chemotherapy regime that shrunk tumors by approximately 50%. The chemotherapy regimen consisted of intraperitoneal injections of paclitaxel (15 mg/kg) or vehicle control on days 6, 8, 10, 12, 14, and 16 post-tumor cell injection. Once tumors reached 10x10 mm, mice were sacrificed and tumors were harvested/ prepared into a single cell suspension and analyzed by FACS to calculate the frequency of tdTomato⁺ cells.

Quantification and Statistical Analysis

GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA) was used to analyze all datasets. Pairwise comparisons were generated with two-tailed t tests. Variance between groups was calculated with the F test using a confidence level of $\alpha=0.01$. Definitions of center/ dispersion measurements and n values are all indicated in the associated figure legends for each figure. P-values are represented as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

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