UNIVERSITY OF CALIFORNIA, SAN DIEGO

Heterologous Cell-Cell Fusion as a Mechanism of DNA Exchange and Chemoresistance in Cancer

A Thesis submitted in partial satisfaction of the requirement for the degree

Master of Science

in

Biology

by

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2016

The Thesis of Endi Kusuma Pramudya Santosa is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2016

Dedication

I dedicate this thesis to my parents, and my brothers,

for their constant love and support.

Epigraph

“If we knew what it was we were doing, it would not be called research, would it?”

Albert Einstein

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Acknowledgements

Firstly, I would like to express my sincere gratitude to Dr. Bui for giving me the opportunity to grow and develop my love for science by allowing me to join his lab. His patience, support, and guidance help me grow both professionally and personally.

I would like to thank Steve Searles from the bottom of my heart. He has been a great mentor for the past two years. I could not have asked for a better mentor.

I would also like to thank all of current and former Bui lab members, namely, Emilie Gross, Ruth Seelige, Angela Meier, Allen Washington Jr., Yujin Jung, Calvin Lee, and Robert Saddawi-Konefka for their supports and friendships.

Lastly, I would like to thank my parents for supporting and encouraging me to pursue my goals in life. They always believe in me no matter what.

This thesis, in part, is an adapted version of material that is currently being prepared for publication. Searles, Stephen C.; Santosa, Endi K.; Bui, Jack D. The thesis author was a co-author of this material.

ABSTRACT OF THE THESIS

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Cell fusion is an important event that mediates various biological processes. However, the role of cell fusion in cancer has not been very well described due to technical limitations. Here I describe a Cre-Lox recombination

model system to study spontaneous heterotypic cell-cell fusion between tumor and non-tumor cells. This fusion event results in hybrid cells that are hyperploid and contain DNA from both parental cells. In addition, fusion-derived hybrids eventually adopt cancer gene profile, are more resistant to chemotherapy, and show increased tumorsphere-forming capacity. This model system can therefore be used to further our understanding of cell-cell fusion in the context of cancer.

I.

Introduction

Post-therapeutic cancer relapse describes the resurgence of cancer after initial treatments, such as surgery and chemotherapy, and is the major cause of cancer mortality (Grimes *et al*, 2012). Despite undetectable cancer after treatments, often times, cancer cells reemerge and invade the host in a more aggressive manner through various mechanisms including metastasis and chemoresistance (Shafee *et al*, 2008). To this end, many studies have focused on elucidating the molecular and biochemical basis of drug resistance and metastasis to develop treatments aimed at molecular targets known to be involved in these processes (Gottesman, 2002). However, despite a colossal list of candidates, the development of targeted treatments has only had a small impact on cancer mortality (Siegel *et al*, 2015). In addition, recent understanding and appreciation that cancer is not homogeneous, but rather, a mass of heterogeneous cells, adds to the complexity of finding effective treatments to limit cancer relapse. While there are many ways for cancer to develop drug resistance and metastasis, it is generally accepted that patients' irresponsiveness to specific treatments can be partly attributed to alteration of genomic and epigenetic landscape that results in aberration of normal cellular functions to acquire resistance and metastatic phenotypes (Hanahan and Weinberg, 2000). The mechanisms that induce genomic alterations that result in increased phenotypic fitness, however, are not well explored and/or understood.

One mechanism that has been proposed, but lacking definitive evidence due to technical limitations, is cell-cell fusion. Cell fusion is an important biological event that mediates various processes, such as fertilization and

tissue/organ development (Oren-Suissa and Podbilewicz, 2007). By definition, cell fusion is the process by which two cells combine their plasma membranes and become a single cell with twice the amount of DNA (Ogle *et al*, 2005). Fusion can occur between cells of the same type (homotypic), or between cells of two different types (heterotypic) (Duelli and Lazebnik, 2003). In pathology, cell-cell fusion in cancer is a well-documented occurrence, yet little attention has been paid to understand its significance and underpinning mechanisms. The ability of cell fusion to generate polyploid cells has been implicated to elicit genomic and chromosomal instability by destabilization of epigenetic landscape that increases the likelihood of carcinogenesis (Duelli and Lazebnik, 2007). In addition, it is also postulated that cell fusion increases the genotypic and phenotypic heterogeneity of hybrid cells (and their progeny) that can contribute to tumor malignancy (Lu and Kang, 2009).

Several studies have reported the contribution of cell fusion to chemoresistance and metastasis. A study using a mammary breast cancer model demonstrated that fusion between 5-fluorouracil resistant tumor cells and methotrexate resistant tumor cells not only results in hybrids that are resistant to both drugs, but also resistant to a different drug (Miller *et al*, 1989). Similarly, a study using colon carcinoma supports the hypothesis that cell fusion consolidates chemoresistance in hybrid cells (Carloni *et al*, 2013). In addition, spontaneous homotypic fusion between MDA-MB-231 breast cancer cell lines with two distinct metastatic potentials results in stable a hybrid that is not only resistant to chemotherapy, but also more metastatic than its parental cells (Lu and Kang,

2009). In humans, evidence of heterologous cell-cell fusion in the context of cancer has been obtained in patients who received gender mismatched allogeneic bone marrow transplantations prior to developing cancer. In such cases, chromosomes derived from tumor cells of these patients contain both donor and recipient chromosomes, suggesting hybridization events between donor and recipient cells (Pawelek, 2005).

However, despite a growing body of research that suggests the contribution of cell fusion in tumor malignancy, direct evidence is still lacking due to the lack of appropriate tools to directly track and study cells that have undergone fusion events *in vivo*. Most studies have relied on highly artificial fusion methods by subjecting cells to electrofusion or treating cells with fusogenic chemicals *in vitro*, such as polyethylene glycol, which may not reflect true physiology (Duelli and Lazebnik, 2003). Therefore, the physiological impacts of cell-cell fusion in tumor malignancy remain controversial.

In this study, by using the Cre-lox recombination system, we show that tumor cells are capable to spontaneously fuse with non-tumor cells both *in vitro* and *in vivo*. These heterologous fusion events result in hybrid cells that retain DNA from both tumor and non-tumor cells. DNA analyses by flow cytometry and chromosome spread demonstrate that hybrid cells contain, on average, more DNA than both parental cells, although a high degree of variability can be seen across hybrid cells. In addition, hybrid cells are eventually reprogrammed to adopt a cancer gene profile. Consequently, hybrid cells derived from heterologous cell-cell fusion are more resistant to chemotherapeutics than

parental cell, which can be potentially attributed to increase in cancer stemness. Moreover, contrary to what has previously been reported, hybrid cells seem to exhibit decreased metastatic behavior compared to parental cancer cells in *in vitro* transwell migration assay. Our study, therefore, provides a novel model system to study spontaneous heterologous cell-cell fusion in cancer and illustrates the role of heterologous cell-cell fusion as a mechanism of tumor heterogeneity.

II.

Results

Cre Recombinase Is Transferred Between Tumor Cells and Non-Tumor Cells

To study heterologous cell-cell fusion between tumor and non-tumor cells, we generated B16 melanoma and 9609 fibrosarcoma cell lines that stably express bicistronic construct of green fluorescent protein (GFP) and Cre recombinase (Cre). The reporter cells harbor a floxed stop site located upstream of tdTomato reporter locus under the control of a constitutive and ubiquitous ROSA26 promoter. Hence, upon Cre transfer from tumor cells into reporter cells, the floxed stop site in the reporter cells will be excised, which then allow tdTomato fluorescent protein to be expressed by the reporter cells. This system allows us to investigate the biological and physiological significance of cell-cell fusion in the context of cancer.

We first determine whether Cre recombinase can be transferred from tumor cells into reporter cells by co-culturing B16 Cre-GFP-expressing cells (hereinafter referred as $B16^{Cre\text{-}GFP}$) with various cell types harboring reporter locus for two days. Cre transfer occurred between $B16^{Cre\text{-}GFP}$ cells and mouse embryonic fibroblasts (MEF), adult fibroblasts (ADF), bone marrow-derived macrophages (BMDM), and peritoneal macrophages, but not keratinocytes or splenocytes (Figure 1A). Nearly all $tdTomato^{+}$ cells were also GFP^{+}, suggesting that GFP was also transferred into the reporter cells. Similar results, but to a lesser extent, was obtained using $9609^{Cre\text{-}GFP}$ cells in lieu of $B16^{Cre\text{-}GFP}$ cells (data not shown).

To further investigate whether Cre and GFP are also transferred *in vivo*, we subcutaneously injected B16 parent or $B16^{Cre\text{-}GFP}$ cells into either wild type or ROSA26-LsL-tdTomato reporter mice. As expected, $tdTomato^{+}$ cells were not detected in wild type mice injected with B16 parent or $B16^{Cre\text{-}GFP}$ cells, and reporter mice injected with B16 parent (Figure 1C). However, $tdTomato^{+}$ cells were only detected in proximal tumor milieu of reporter mice injected with $B16^{Cre\text{-}GFP}$ cells (Figure 1B and C), but not in distal tissues (Figure 1D), suggesting that Cre transfer occurs *in vivo* and requires spatial proximity between tumor cells and non-tumor reporter cells. These findings demonstrate that Cre recombinase can be transferred both *in vitro* and *in vivo*. In addition, *in vivo* transfer of Cre requires propinquity between tumor and reporter cells within the tumor milieu.

Cre Transfer Is Mediated By Cell Fusion, Not Extracellular Vesicles

Given that Cre recombinase can be transferred from tumor cells to non-tumor cells, we next sought to determine the mechanism of Cre transfer. Direct co-culture to allow for cell contact between $B16^{Cre\text{-}GFP}$ cells with reporter cells resulted in tdTomato-expressing cells in the culture after two days of co-culture (Figure 2A). However, separation of $B16^{Cre\text{-}GFP}$ and reporter cells using 400 nm transwell membrane did not induce tdTomato expression by the reporter cells (Figure 2A). Similarly, treatment of reporter cells with $B16^{Cre\text{-}GFP}$-derived exosomes resulted in undetectable amount of $tdTomato^{+}$ cells (Figure 2B), suggesting that Cre transference is cell-contact dependent and is not mediated

by extracellular vesicles. We next monitored direct co-culture of $B16^{Cre\text{-}GFP}$ with MEF harboring reporter locus ($MEF^{LsL:tdT}$) using time-lapse immunofluorescence microscopy. We found that GFP diffused from GFP-expressing cell into its neighboring cell that did not previously express GFP as early as 3 hours after seeding (Figure 2C, 3 hours). At 12 hours and 15 hours, a discernible pseudopodia-like structure can be seen extending from the donor tumor cell to the recipient cell, and at 21 hours, these cells fused together to produce a single cell with two nuclei and began to express tdTomato while maintaining GFP expression (Figure 2C, 21 hours). Eventually, this newly formed hybrid cell undergoes nuclear fusion that results in a single GFP^+ $tdTomato^+$ cell bearing one large nucleus (Figure 2C, 24 hours).

Additionally, after co-culturing $B16^{Cre\text{-}GFP}$ and $MEF^{LsL:tdT}$ for two days, we observed that $tdTomato^+$ cells are larger in size compared to $B16^{Cre\text{-}GFP}$ and $MEF^{LsL:tdT}$ based on forward scatter (FSC) mean fluorescent intensity (MFI) (Figure 3A). Furthermore, in addition to expressing B16-restricted marker, GFP, $tdTomato^+$ cells also express MEF-specific surface proteins, namely CD24 and Sca1 (Figure 3B), which are not expressed by $B16^{CreGFP}$. Together, these findings demonstrate that Cre can be rapidly transferred between tumor cells and non-tumor cells in cell-contact dependent manner. Moreover, time-lapse live imaging clearly shows that cell-cell fusion is the mechanism by which Cre is transferred between donor and recipient cells.

tdTomato^{+} Clonal Cell Lines Contain Both Tumor- and Reporter Cell-Restricted DNA and Are Hyperploid

Previous works have shown that tumor cells are capable of fusing with non-tumor cells (Rachovsky *et al*, 1998; Powell *et al*, 2011). By definition, the direct consequences of cell fusion between diploid somatic cells is a hyperploid cells that contain both parental DNA. To validate our hypothesis that Cre transference is mediated by cell-cell fusion, we generated 20 tdTomato^{+} clonal cell lines derived from B16$^{Cre\text{-}GFP}$ and MEF$^{LsL:tdT}$ co-culture (Figure 4A). Using these cell lines, we probed for B16-restricted DNA (Cre) and MEF-restricted DNA (tdTomato) by polymerase chain reaction and observed that 20/20 (100%) tdTomato^{+} cell lines contain both B16- and MEF-restricted DNA (Figure 4B and data not shown), which strongly supports the hypothesis that Cre transfer is mediated by cell-cell fusion.

Next, we assess the hyperploidy of these tdTomato^{+} clonal cell lines using DNA content analysis by flow cytometry. Consistent with what have been previously reported in the literature, we found B16 melanoma cells are hyperploid at quiescent state (Kendal *et al*, 1987) (Figure 4C). Interestingly, 19/20 tdTomato^{+} clonal cell lines are more hyperploid compared to either parental cells, namely MEF and B16 (Figure 4C). We validate our flow cytometry results by performing metaphase chromosome spread by randomly selecting 8 out of 20 tdTomato^{+} cell lines. As expected, freshly isolated adult fibroblasts isolated from the dermis layer of mouse skin contain exactly 40 chromosomes (Figure 4D and

E). Interestingly, immortalized MEF cells contain on average 50 chromosomes, and $B16^{Cre\text{-}GFP}$ consistently contain about 70 chromosomes (Figure 4D and E). Interestingly, across all 8 $tdTomato^+$ cell lines derived from MEF and B16 analyzed, high variability of chromosome numbers across all samples was observed. However, consistent with our DNA content analysis by flow cytometry, all of $tdTomato^+$ cell lines tested contain more chromosomes relative to either parental cell (Figure 4D and E). In addition, we managed to generate clonal cell lines derived from *in vivo* $tdTomato^+$ cells, B16 and bone marrow-derived macrophages co-culture (B16xBMDM), and B16 and adult fibroblast co-culture (B16xADF). Our analysis shows that there is a high variability within *in vivo*-derived $tdTomato^+$ cell line, while B16xBMDM and B16xADF cell lines consistently show hyperploidy (Figure 4E). These findings provide compelling evidence that Cre transference is indeed mediated by cell-cell fusion and generate cells with heterogeneous DNA content that potentially can affect their phenotypes.

$tdTomato^+$ Clonal Cell Lines Exhibit Variable Growth Kinetics and B16-dominant Gene Profile

To further characterize the properties of $tdTomato^+$ clonal cell lines, we assessed all 20 clonal cell lines growth kinetics and gene transcript profile. The growth kinetics of $tdTomato^+$ cell lines is highly variable, with 5/20 cell lines show greater kinetics, while 15/20 cell lines are slower than $B16^{Cre\text{-}GFP}$ (Figure 5A). In

addition, we also characterized tdTomato$^+$ clonal cell lines based on their gene expression profiles. We probed for genes that are highly expressed by the parental cells of our clonal cell lines, in this case, B16 melanoma and MEFs. To our surprise, 20/20 tdTomato$^+$ cell lines express B16-dominant gene profile, and not MEFs (Figure 5B). This is unexpected, as newly formed tdTomato$^+$ cells upon co-culture express both B16 and MEF proteins (Figure 3B). These findings suggest that fusion between cancer cells and non-cancer cells can give rise to progenies that are neoplastic with variable growth kinetics.

tdTomato$^+$ Hybrid Cells Are More Chemoresistant than B16 Melanoma

We next sought to determine the physiological significance of cell-cell fusion in cancer. Since cell fusion has previously been implicated in promoting resistance to chemotherapy, we sought to test this hypothesis (Miller *et al*, 1989). In order to test for chemoresistance property in our fusion-derived hybrid cells, we first assessed the survival of all 20 tdTomato$^+$ clonal cell lines when exposed to chemotherapy. We utilized two chemotherapeutics that have different mechanisms, namely Doxorubicin (Adriamycin), a DNA intercalating agent, and Paclitaxel (Taxol), a microtubule stabilizer. Our tdTomato$^+$ clonal cell lines, on average, are more resistant to both chemotherapeutics at two different doses (Figure 6A). However, some of the tdTomato$^+$ cell lines (4 out of 20) are highly resistant to both Doxorubicin and Paclitaxel. Since chemotherapeutics target fast growing cells, it is possible that these tdTomato$^+$ cell lines are more resistant to

chemotherapeutics solely due to their slower growth kinetics. To rule out this possibility, we performed correlation analyses the chemotherapeutics survival and growth kinetics of tdTomato^{+} hybrid clonal cell lines. Our correlation analyses suggest that there is no correlation between these cell lines resistance to chemotherapy and their growth kinetics (Figure 6B and C), which suggest that there are other mechanisms, apart from growth kinetics, that contribute to chemoresistance in these tdTomato^{+} clonal cell lines, as some of the cell lines that are highly resistant to chemotherapy are the ones that grow the fastest. In addition, treatment of co-culture of B16$^{Cre\text{-}GFP}$ with reporter MEF, BMDM, and ADF with Paclitaxel (Taxol) results in higher percentage of tdTomato^{+} cells compared to the control, which further corroborating the chemoresistance property of hybrid cells (Figure 6D).

To assess this chemoresistance phenomenon *in vivo*, we treated B16$^{Cre\text{-}GFP}$ tumor-bearing reporter mice with either HBSS or 15 mg/kg of Paclitaxel. Higher proportion of tdTomato^{+} cells in tumors that are treated with Paclitaxel indicates tdTomato^{+} ability to resist chemotherapy. Similar to our *in vitro* result, we found that tumors from mice that were treated with Paclitaxel showed enrichment in tdTomato^{+} cells compared to the control. Taken together, these data suggest that tdTomato^{+} cells derived from B16$^{Cre\text{-}GFP}$ are more resistant to chemotherapeutics compared to B16$^{Cre\text{-}GFP}$.

tdTomato^{+} Cells Exhibit Heterogeneity in Tumor-forming Capacity and Reduced Metastatic Potentials

Others have speculated that cell-cell fusion can lead to the development of cancer stem cells (Bjerkvig *et al*, 2005). In addition, some studies have posited that homotypic cell-cell fusion between cancer cells can generate hybrid cell progenies with increased metastatic potentials (Larizza, *et al*, 1984; Powell *et al,* 2011). Therefore, we would like to test whether our hybrid cells have both properties. To assess tdTomato^{+} cells stemness-like characteristic, we employed *in vitro* tumorsphere-forming assay. First, we co-cultured B16$^{Cre\text{-}GFP}$ with reporter MEF and after two days of co-culture, we sorted tdTomato^{+} cells and allowed them to grow in 3D tumorsphere media for seven days. To our surprise, newly sorted tdTomato^{+} cells are capable of forming GFP^{+} tdTomato^{-} cells (Figure 7A), something that was not observed when the cells were subjected to 2D culture (data not shown). In addition, we also investigate the ability of our tdTomato^{+} clonal cell lines in forming tumorsphere. Our analyses show that tdTomato^{+} clonal cell lines demonstrate high heterogeneity in tumorsphere-forming capacity (Figure 7B). Approximately, 30% of cell lines (6 out of 19) can form significantly higher number of spheres compared to B16, and the proportion of cell lines that are form lower number of spheres is lower (~25%, 5 out of 19) (Figure 7B). The other cell lines have either higher (6 out of 19 cell lines) or lower tumor-sphere forming capacity (2 out of 19 cell lines) although they did not reach statistical significance. Despite not reaching statistical significance, the higher proportion of

cell lines that are capable at forming higher number of spheres compared to B16 still suggest that cell-cell fusion tend to give rise to cells with higher tumorsphere-forming capacity, which is an indication of cancer stemness/tumor-initiating properties.

We next assessed the ability of tdTomato^{+} to form tumors *in vivo*. To do this, we injected tdTomato^{+} clonal cell lines into immunodeficient mice (Rag1$^{-/-}$, which lack adaptive immunity, namely B cells and T cells). Of all tdTomato^{+} cell lines that were randomly chosen, most are tumorigenic with different degree of penetrance and *in vivo* growth kinetics (Figure 7C and data not shown), suggesting that they are indeed tumorigenic. Lastly, we used *in vitro* transwell migration assay to determine the metastatic behavior of tdTomato^{+} clonal cell lines. To our surprise, in contrast to previous studies, all of our tdTomato^{+} cell lines show significantly lower metastatic potentials compared to B16$^{\text{Cre-GFP}}$ in *in vitro* transwell migration assay. Taken together, these findings suggests that although tdTomato^{+} are tumorigenic with increased stemness properties, they are, however, less metastatic than B16 melanoma parental cells.

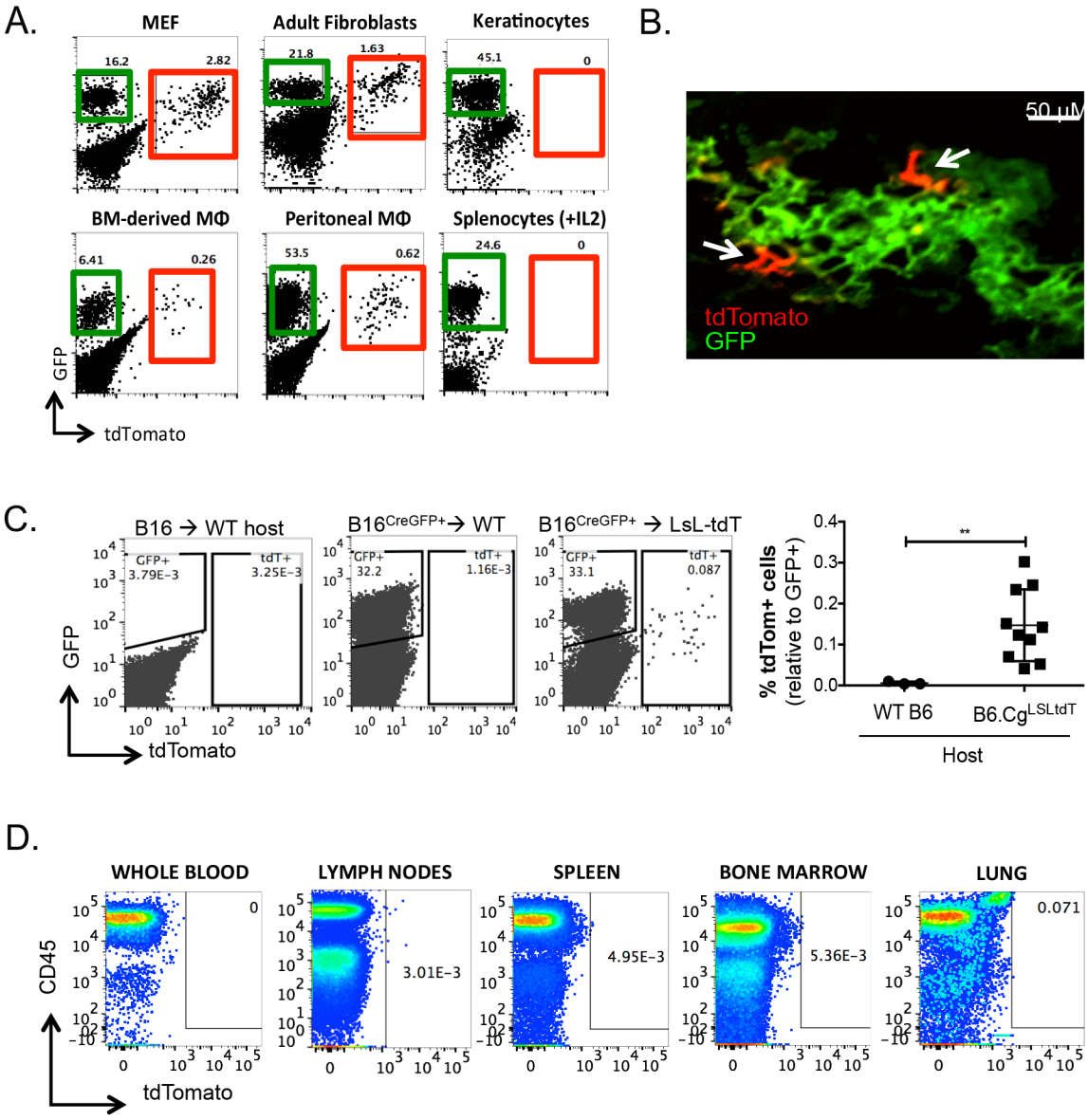


Figure 1. Cre transfer is detected both *in vitro* and *in vivo*. (A) Flow cytometry analyses of *in vitro* co-culture between B16 Cre-GFP$^+$ with different reporter cells harboring R26-LsL-tdTomato locus. (B) Image from confocal microscopy of flash-frozen B16 Cre-GFP$^+$ injected into R26-LSL-tdTomato reporter mice. (C) Flow cytometry analysis and quantification of tdTomato$^+$ cells within tumor tissue of reporter mice injected with B16 Cre-GFP$^+$ melanoma (D) Flow cytometry of distal tissues of B16 Cre-GFP$^+$ tumor-bearing reporter mice (day 22). Statistical analysis was performed using Student's t-test; * ($p<0.05$), ** ($p<0.01$), *** ($p<0.005$).

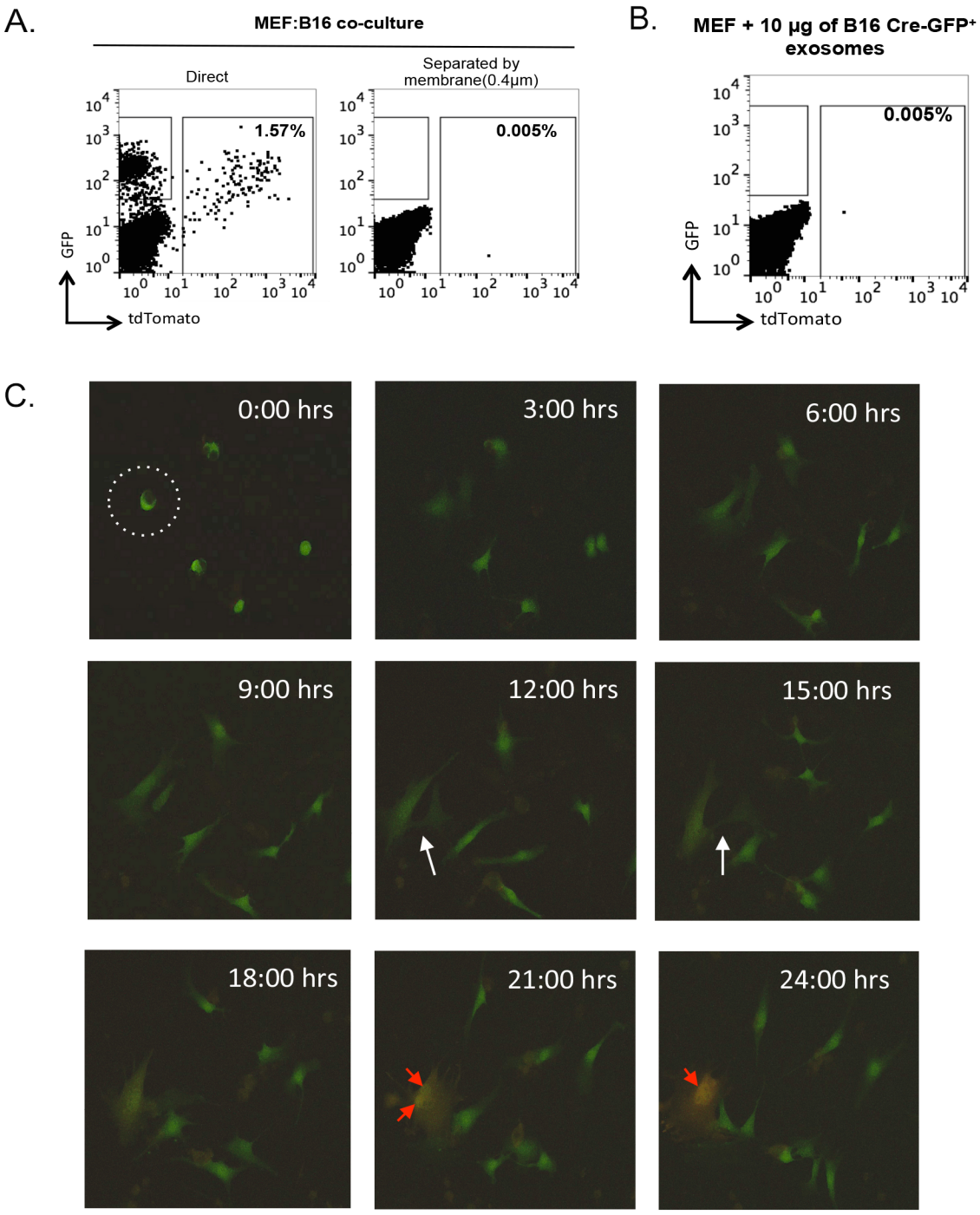


Figure 2. Cre transfer is cell-contact dependent through cell fusion and is not mediated by exosomes. (A) Flow cytometry analyses of *in vitro* co-culture of B16 Cre-GFP$^+$ with reporter MEF harboring LsL-tdTom reporter locus without or with transwell membrane seperation. (B) Flow cytometry of reporter MEF cells treated with exosomes derived from B16 Cre-GFP$^+$ cells. (C) Images from 24 hours live time-lapse microscopy of direct co-culture between B16 Cre-GFP$^+$ with reporter MEF cells. Circle at 0:00 hrs shows donor Cre-GFP tumor cell. White arrows at 12:00 and 15:00 hrs points to pseudopodia-like structure extending from donor tumor cell onto recipient cell. Nuclei are indicated by red arrows.

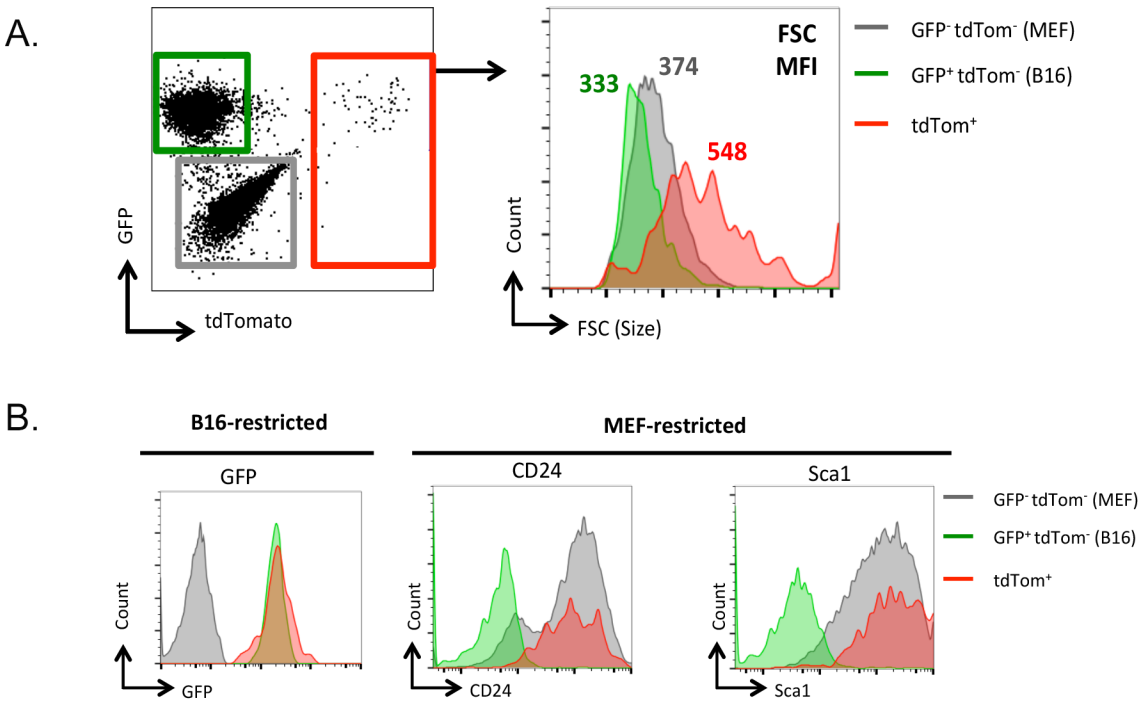


Figure 3. tdTomato⁺ cells are larger in size than parental cells and express both B16-restricted and MEF-restricted markers. (A) Analysis of Forward Scatter (FSC) mean fluorescent intensity (MFI) of MEF, B16, and tdTomato⁺ cells. (B) Expression of B16-restricted (GFP) and MEF-restricted (CD24 and Sca1) markers in MEF, B16, and tdTomato⁺ cells by flow cytometry.

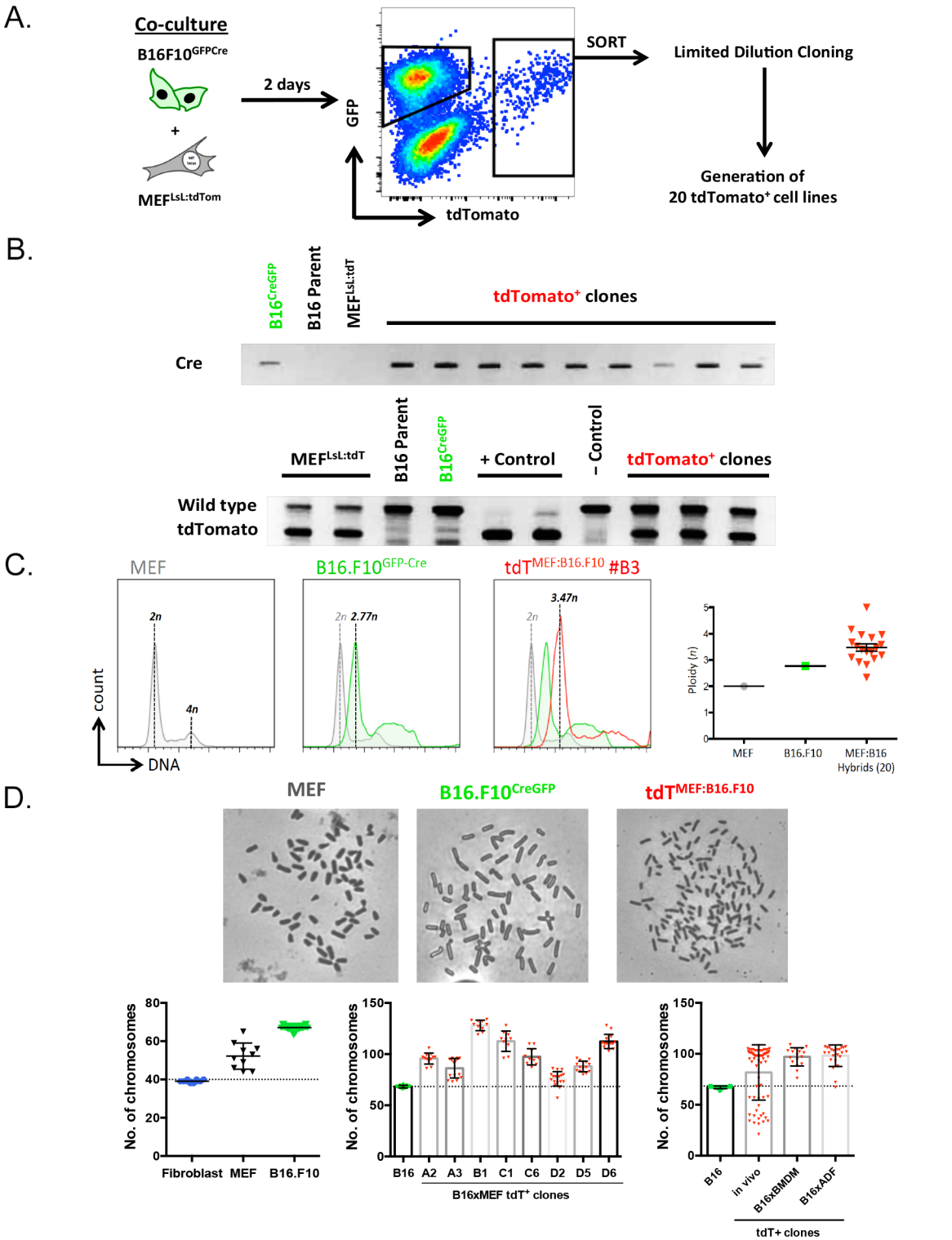
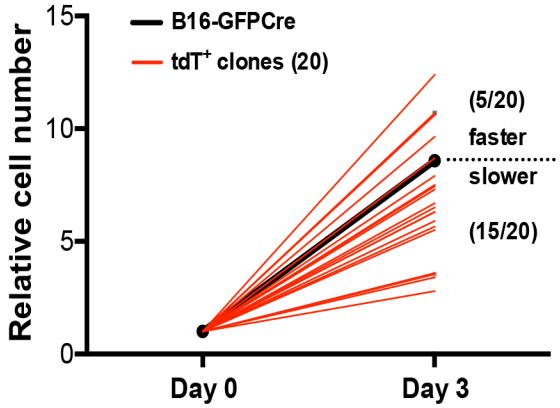


Figure 4. tdTomato⁺ clonal cell lines contain both B16- and MEF-restricted DNA and are hyperploid. (A) Schematic of tdTomato⁺ clonal cell lines generation. (B) Polymerase Chain Reaction (PCR) analysis probing for Cre DNA (B16-restricted) and tdTomato (MEF-restricted) DNA. (C) DNA content analysis by flow cytometry. (D) Images and quantification of karyotype analysis across different cells and clonal cell lines.

A.



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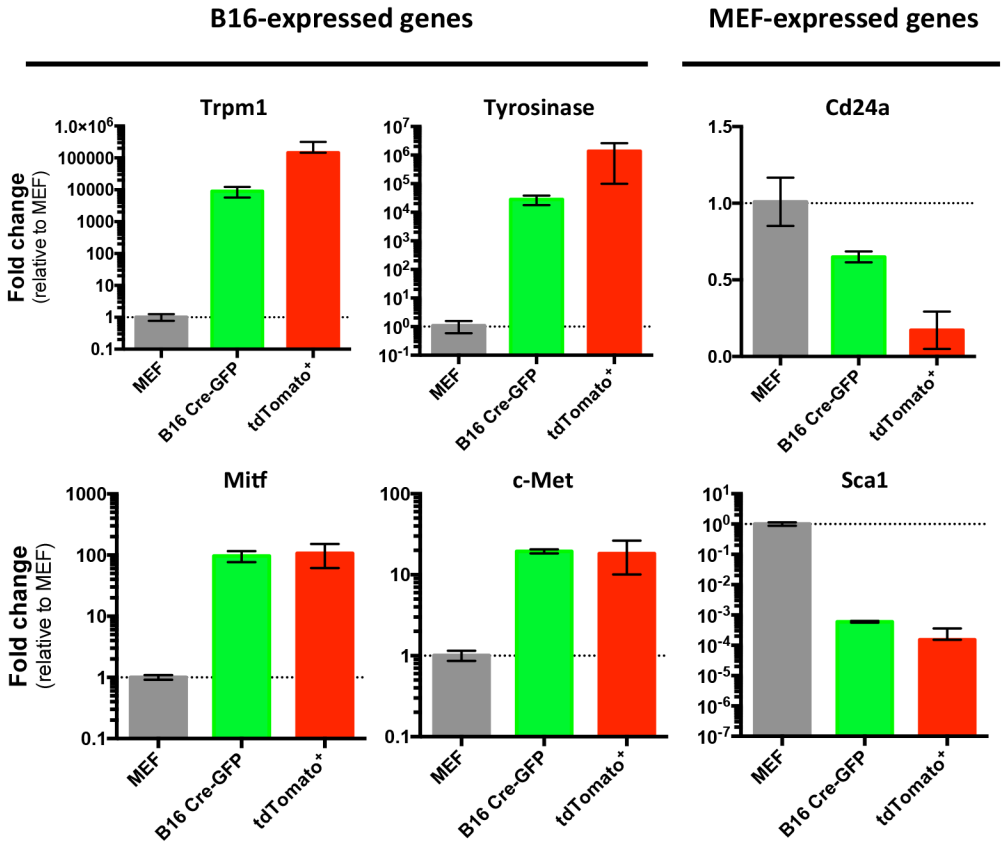


Figure 5. tdTomato⁺ clonal cell lines display variable growth kinetics and adopt B16 gene profile. (A) Growth kinetics of tdTomato⁺ clonal cell lines. (B) qRT-PCR to assess tdTomato⁺ clonal cell lines gene profile by probing for B16-expressed genes (Trpm1, Tyrosinase, Mitf, and c-Met) and MEF-expressed genes (Cd24a and Sca1).

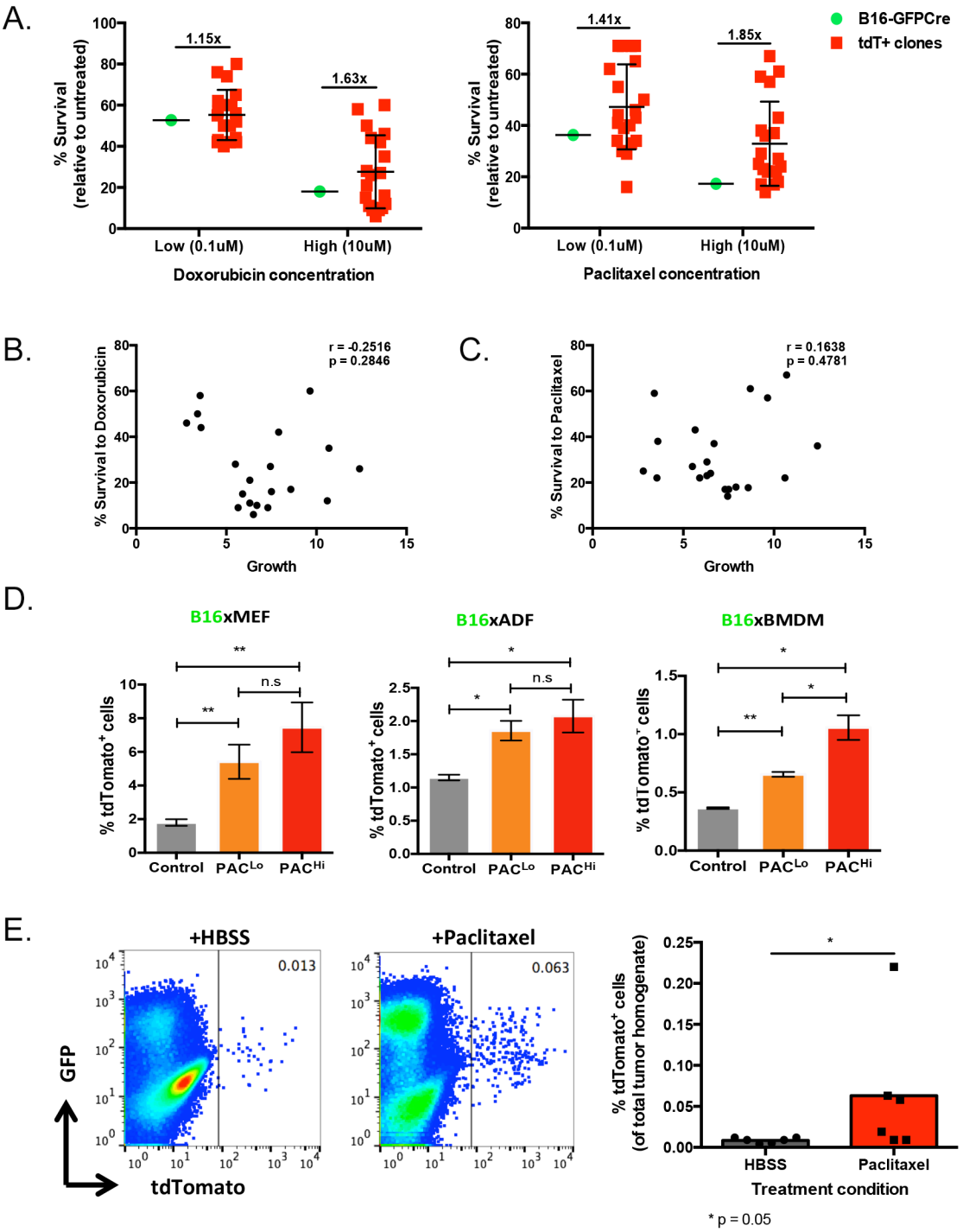
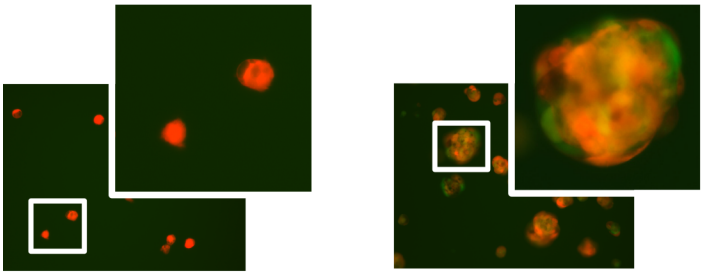


Figure 6. tdTomato⁺ cells are more resistant to chemotherapy than B16 melanoma both *in vitro* and *in vivo*. (A) Survival of tdTomato⁺ clonal cell lines in response to chemotherapy (Doxorubicin and Paclitaxel) at two different doses. (B & C) Correlation plots between growth and survival to chemotherapy of tdTomato⁺ clonal cell lines. (D) Percentage of tdTomato⁺ cells in *in vitro* direct co-culture treated with Paclitaxel (0.1 μM and 10 μM). (E) Flow cytometry plots and quantification of tdTomato⁺ cells on B16 CreGFP⁺ tumor-bearing reporter mice treated with either HBSS or 15 mg/kg Paclitaxel. Statistical analysis was performed using Student's t-test; * (p<0.05), ** (p<0.01), *** (p<0.005).

A.



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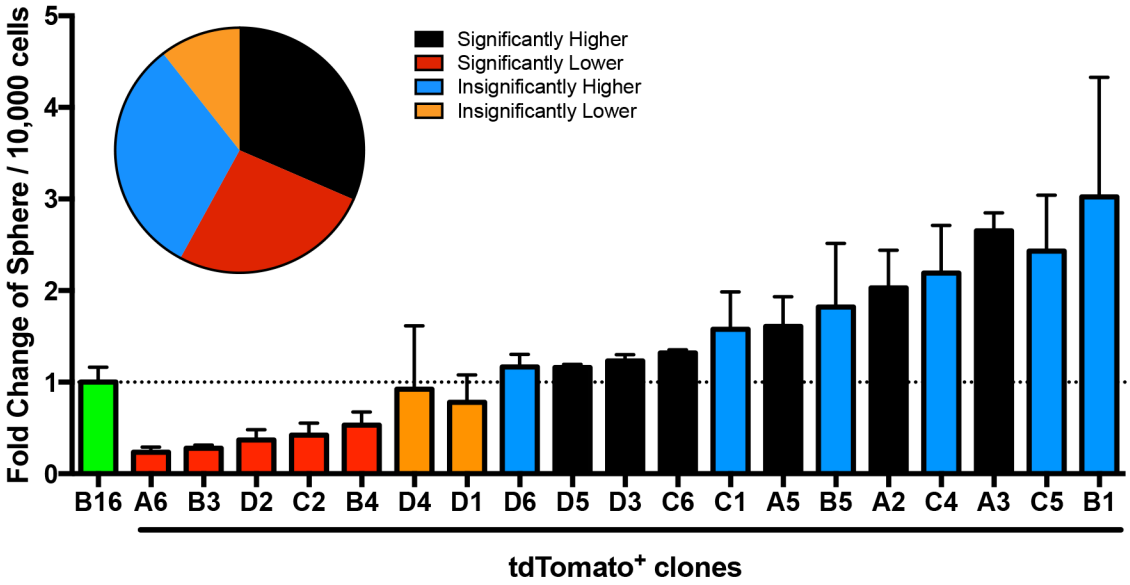

C.

Cell lines	Tumorigenic? (Y/N)	Penetrance rate (%)
$tdT^{MEF:B16}$ #A5	Y	100
$tdT^{MEF:B16}$ #B2	Y	100
$tdT^{MEF:B16}$ #C1	Y	100
$tdT^{MEF:B16}$ #C6	Y	67
$tdT^{BMDM:B16}$	Y	100
$tdT^{ADF:B16}$	Y	100
$tdT^{in\ vivo}$	N	0

D.

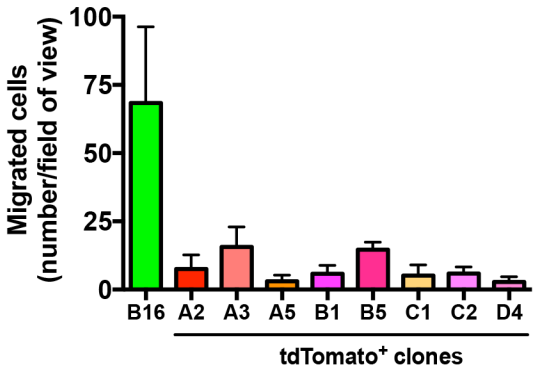

Figure 7. tdTomato⁺ cells are capable of forming tumorsphere, tumorigenic, and less metastatic than B16 melanoma. (A) Images of newly sorted tdTomato⁺ cells on day 0 and day 7. (B) Fold change quantification of tumorsphere from tdTomato⁺ clonal cell lines compared to B16. (C) *In vivo* tumorigenicity assay where $Rag1^{-/-}$ mice were injected with tdTomato⁺ cell lines. (D) Transwell migration assay of $B16^{CreGFP}$ and tdTomato⁺ hybrid cell lines.

III.

Discussion

In this thesis, we presented a model system that utilized the Cre-Lox recombination to study heterologous cell-cell fusion in cancer that mimics true physiological condition. A similar system has been used to study mRNA exchange between tumor cells via extracellular vesicles (EVs) (Zomer *et al*, 2015). Despite similarity in experimental conditions, our observation strongly suggests that Cre transfer from tumor cells to non-tumor cells is mediated by cell-cell fusion, rather than EVs. However, we acknowledge that there is still a possibility that a small fraction of Cre might be transferred through cell contact-independent mechanisms, such as uptake of apoptotic bodies, exosomes, and/or microvesicles, by the reporter cells.

We also demonstrate that not all cells are capable of fusing with tumor cells, which suggests that this process is non-random and selective. In our study, macrophages and fibroblasts, both of which are found in abundance in the tumor microenvironment (Solinas *et al*, 2009; Kalluri and Zeisberg, 2006), fuse with tumor cells *in vitro*, but splenocytes and keratinocytes do not. However, the molecular mechanism that regulate which cells types are capable of fusing with tumor cells is still not fully understood.

The ability of macrophages to fuse is not a new concept. Spontaneous homotypic fusion is known to occur in both mouse and human macrophages expressing high level of purinergic P2Z/$P2X_7$ receptors to form multinucleated giant cells (Chiozzi *et al*, 1997), although the role of these receptors in heterotypic fusion has never been explored. In tumors, fusion between macrophages and tumor cells, such as melanoma, lymphoma, carcinoma, and

others, have been reported and is implicated in metastasis (Rachovsky *et al*, 1998; Larizza *et al*, 1984; Powell *et al*, 2011). Yet, the enhanced resistance to chemotherapy we observed in macrophage-tumor hybrid cells has never been demonstrated before.

Our data show that hybrid cells derived from heterologous cell-cell fusion between B16 melanoma and macrophages, or fibroblasts, are more resistant to chemotherapy than parental tumor cells. However, how heterologous cell-cell fusion contributes to chemoresistance is not understood. Cell fusion allows for the rapid exchange of genetic material and alteration of the epigenetic landscape on a large scale (Bhutani *et al*, 2010; Su *et al*, 2015). This process can therefore be a potent inducer of molecular transformation to increase phenotypic and genotypic diversity of hybrid cells (and their resulting progeny) that can ultimately promote resistance to chemotherapy (Duelli and Lazebnik, 2003). Despite being widely accepted, concrete evidence to support this idea is still lacking. Detailed genomic, epigenetic, and transcriptome analyses on hybrid cells could provide significant insights on the dynamic of molecular transformation and help identify molecular signatures that contribute to chemoresistance.

More recently, with the emerging concept of cancer stem cells (CSCs), some also have postulated that this rare population of cells might have originated from cell-cell fusion (Bjerkvig *et al*, 2005). Our data supports this hypothesis by showing that hybrid cells have CSC properties. We observed that newly fused hybrid cells are capable of forming a heterogeneous population (based on GFP and tdTomato expression) in 3D tumorsphere culture. We also found that

B16xMEF hybrid cells form more tumorspheres than B16 melanoma. In support of our observation, several studies have also provided evidence for this hypothesis. In human melanoma, CSCs are marked by the expression of ABCB5 (Schatton *et al*, 2008). Interestingly, $ABCB5^+$ cells are polyploid, are generated by cell-cell fusion of melanocyte progenitor cells, and can give rise to both $ABCB5^+$ and $ABCB5^-$ progenies (Frank *et al*, 2003 and Schatton *et al*, 2008). Studies from the same group have also indicated that in addition to act as drug efflux transporter protein, hence mediating resistance to chemotherapy, ABCB5 is also a regulator of cell fusion process (Frank *et al*, 2003, 2005). Furthermore, similar observation has also been reported in human ovarian cancer (Zhang *et al*, 2014). Therefore, examination of different tumor types is crucial to establish whether fusogens play a role in CSCs generation, and whether they can be potential markers of CSCs. Taken together, cell-cell fusion might be an important cellular mechanism to generate CSCs that are resistant to chemotherapy and consequently cause cancer relapse.

Although we demonstrate that heterotypic cell-cell fusion can promote chemoresistance, our data also show that this is not true for all hybrid cells. Moreover, characterization of hybrid cells in terms of growth kinetics and ploidy levels show great variability despite a seemingly homogeneous gene transcript profile. The contribution of cell-cell fusion to differential gene expression, fate determination, and cellular reprogramming has been reported in bone marrow progenitor cells and embryonic stem cells (Terada *et al*, 2002; Nygren *et al*, 2004; Ying *et al*, 2002; Cowan *et al*, 2005). However, further examination of this

phenomenon in the context of cancer is warranted.

In conclusion, our findings show that heterologous cell-cell fusion has the capacity to generate a heterogeneous population of cells that are, on average, more resistant to chemotherapy than their parental cells. This phenotype can partly be attributed to increase in cancer stemness properties and cannot be ascribed to difference in growth kinetics. Future studies should focus on elucidating the molecular mechanisms of heterologous cell-cell fusion in cancer and how cell-cell fusion promotes chemoresistance. Understanding these elements can be potentially useful for cancer therapy to prevent relapse.

This thesis, in part, is an adapted version of material that is currently being prepared for publication. Searles, Stephen C.; Santosa, Endi K.; Bui, Jack D. The thesis author was a co-author of this material.

IV.

Materials and Methods

Cell culture and generation of cell lines

All cells were cultured in RPMI 1640 Medium (GIBCO, Life Technologies) supplemented with 10% (v/v) fetal bovine serum (GIBCO, Life Technologies), 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. B16F10 melanoma cells were transfected with bi-cistronic Cre-GFP construct lentivirus vector and sorted to make clones of B16F10 that stably maintain Cre-GFP expression. tdTomato^{+} clonal cell lines were generated by co-culturing B16 Cre-GFP^{+} with various cells derived from ROSA26-LsL-tdTomato reporter mice for 2 days, sorted, and cloned by limited dilution cloning. Bone marrow-derived macrophages (BM-derived MΦ/BMDM) were isolated from bone marrow cells of reporter mice and cultured *in vitro* in the presence of M-CSF for 6 days. Adult fibroblasts (ADF) were generated from dermis of the tail of reporter mice.

Mice

ROSA26-LsL-tdTomato (C57BL/6 background) and immune deficient RAG1$^{-/-}$ (C57BL/6 background) mice were purchased from the Jackson Laboratory (Sacramento, CA, USA). All experiments were conducted in accordance with Institutional Animal Care and Use Program (IACUC) at University of California, San Diego.

Injection of tumor cells

In all experiments, tumor cells were trypsinized, washed, and resuspended in 1X HBSS w/ Ca^{2+} Mg^{2+} (Corning). $1x10^5$ B16 Cre-GFP^- or B16 Cre-GFP^+ cells were injected subcutaneously into the flank of 8-12 weeks C57BL/6 mice or ROSA26-LsL-tdTomato mice. $1x10^6$ B16 Cre-GFP^+ and $tdTomato^+$ clonal cell lines were injected into $RAG1^{-/-}$ to assess tumorigenicity of $tdTomato^+$ clonal cell lines. For all experiments, tumor growth was monitored every 2-3 days and all experiments were terminated when tumor size reaches 10x10 mm^2.

Flow cytometry analyses and cell sorting

For all *in vitro* experiments, cells were trypsinized, washed, and resuspended in FACS staining buffer (1X PBS w/ 1% BSA). For immunostaining of co-culture, cells were harvested and incubated with 1:100 anti-CD24 (eBioscience, Clone M1/69) and 1:100 anti-Sca-1 (Biolegend, Clone D7) for 15-20 minutes, and washed with FACS staining buffer. 1 μg/ml of 7-Aminoactinomycin D (7-AAD) was used to stain and exclude dead cells from analysis. For cell sorting, cells were sorted using BD FACS Aria II Cell Sorter performed by UCSD Human Embryonic Stem Cell Core Facility.

Co-culture, transwell, and exosome experiments

All *in vitro* co-culture experiments were done in complete RPMI1640 previously mentioned. B16 Cre-GFP^+ cells were co-cultured with mouse

embryonic fibroblasts (MEFs), bone marrow-derived macrophages (BM-derived MΦ/BMDM), adult dermal fibroblasts (ADFs), peritoneal cavity macrophages (Peritoneal MΦ), keratinocytes, and splenocytes at different ratios and analyzed at different time points by flow cytometry and immunofluorescence microscopy for GFP and tdTomato expressions. For transwell experiment, 0.4 μm pore-size transwell was used. Briefly, MEFs reporter cells were placed in the bottom chamber of cell culture dish and B16 Cre-GFP$^+$ melanoma cells were cultured in the upper chamber for 2 days. On day 3, MEFs reporter cells were trypsinized and analyzed by flow cytometry for tdTomato expression. For exosome experiments, B16 Cre-GFP$^+$ cells were cultured in exosome-free complete RPMI1640 media. To isolate exosomes, cells were cultured in exosome-free complete RPMI1640. Supernatant was collected after several days of culture, centrifuged at 20,000 xg for 20 minutes at 4 ^{o}C using Beckman Avanti J-30I. After centrifugation, supernatant was then collected and subjected to two sets of centrifugation at 100,000 xg for 70 minutes each at 4 ^{o}C. Reporter cells were then treated with isolated exosomes derived from B16 Cre-GFP$^+$ for 2 days.

Immunofluorescence of tissue sections

B16 Cre-GFP$^+$ tumors were harvested from reporter mice at day 20 post transplantation. Tumors were flash frozen, sectioned, and imaged using confocal microscopy (Nikon Eclipse TE 2000-E). For time-lapse imaging, B16 Cre-GFP$^+$ cells were co-cultured with reporter cells for 24 hours.

DNA content analysis by flow cytometry and karyotyping

To assess DNA content by flow cytometry, cells were harvested and fixed in 70% EtOH for an hour at 4^{o}C and stained with 2 μg/ml 7-AAD. Ploidy was calculated based on mean fluorescent intensity (MFI) of 7-AAD staining, and MFI of MEFs was set as reference. For karyotyping experiments, cells in culture were treated with 0.1 μg/ml of KaryoMAX Colcemid™ Solution (Life Technologies) for 3-4 hours. Cells were then harvested and treated with hypotonic solution (0.8% sodium citrate) at RT for 10 mins, and centrifuged for 10 mins at 4^{o}C. Supernatant was aspirated and cells were treated with Carnoy's fixative (75% MeOH, 25% glacial acetic acid) for 10 mins at RT and repeated two additional times. To prepare the slide, a drop of cells in fixative was released onto the slide and let sit until dry. Cells were then stained with Giemsa for 20 minutes and mounted in mounting medium for analysis. At least 15 cells karyotype were counted for each cell line.

Cell proliferation assay

Cell proliferation was assessed by flow cytometry. At day 0, 2,000 cells were seeded in 24-well plate, and cell number was counted by flow cytometry at day 1, 2, and 3. Dying cells were stained with 1 μg/ml 7-AAD and excluded from analysis. Each experiment was done in parallel with B16 Cre-GFP^{+} and repeated at least two times. Data is presented as average relative cell number compared to day 0.

RNA isolation, cDNA synthesis, and RT-qPCR

RNA was extracted using TRIzol® reagent (Ambion®). RNA was then subjected to cDNA synthesis using High Capacity cDNA Reverse Transcription Kit (Applied Biosystem) according to manufacturer's protocol. Real-time quantitative polymerase chain reaction was done using SYBR® Green PCR Master Mix (Applied Biosystem) and carried out in CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Gene expression was analyzed using $2^{-\Delta\Delta Ct}$ method. Real-time primers used are: mHPRT forward 5'-GCTTGCTGGTGAAAAGGACCTCTCGAAG-3', mHPRT reverse 5'-CCCTGAAGTACTCATTATAGTCAAGGGCAT-3', mTrpm1 forward 5'-GAGCTGAAGGAGGCTAGGCTG-3', mTrpm1 reverse 5'-CTTGGTGTCCTCTCCTGTTGT-3', mTyrosinase forward 5'-CCTCCTGGCAGATCATTTGT-3', mTyrosinase reverse 5'-GGCAAATCCTTCCAGTGTGT-3', mMITF forward 5'-GCCTTGTTTATGGTGCCTTC-3', mMITF reverse 5'-GTCCTCCTCCCTCTACTTTCTGT-3', c-MET forward 5'-GCATGTCAGCATCGCTCAA-3', c-MET reverse 5'-TGCAGGCCCAGCTGTTTC-3', mSca1 forward 5'-CTCTGAGGATGGACACTTCT-3', mSca1 reverse 5'-GGTCTGCAGGAGGACTGAGC-3', mCd24a forward 5'-CTGCTGGCACTGCTCCTAC-3', mCd24a reverse 5'-GGTGGTGGCATTAGTTGGAT-3'.

***In vitro* and *in vivo* chemoresistance assay**

To assess chemoresistance of tdTomato$^+$ clonal cell lines *in vitro*, 25,000 cells of B16 Cre-GFP$^+$ and tdTomato$^+$ clonal cell lines were seeded into 24-well plate and were incubated at 37 ^oC with 5% CO_2 tissue culture incubator overnight. Cells were then treated with Doxorubicin (Bedford Laboratories™, Bedford, OH, USA) or Paclitaxel (TEVA Pharmaceuticals, Sellersville PA, USA) at two different doses, as indicated in Figure 6A, for 24 hours. Cell number was counted by flow cytometry. Each data point represents average percent survival of three independent experiments of each cell line compared to untreated controls. To determine chemoresistance of tdTomato$^+$ cells from *in vitro* direct co-culture, MEFs, bone marrow-derived macrophages (BMDM), and adult fibroblasts (ADF) derived from ROSA26-LsL-tdTomato reporter mice were co-cultured with B16 Cre-GFP$^+$ for 2 days before treated with Paclitaxel (0.1 uM and 10 uM) for 24 hours. Frequency of tdTomato$^+$ cells was assessed by flow cytometry. Data is representative of at least three independent experiments. For *in vivo* chemoresistance experiments, $1x10^5$ B16 Cre-GFP$^+$ cells were injected subcutaneously into the flank of ROSA26-LsL-tdTomato reporter mice. On day 6, mice were intraperitoneally treated with either HBSS (Corning) or 15 mg/kg of Paclitaxel every two days for six times. Tumors were harvested when the size reaches >10x10 mm^2 and frequency of tdTomato$^+$ of total tumor homogenate was determined using flow cytometry.

Tumor sphere assay

Cells were plated on ultra-low attachment plate at three different cell density: 50,000 cells/ml, 10,000 cells/ml, were grown 7-14 days in serum free sphere media, which consists of 1% methylcellulose in 1X DMEM/F12 media pH 7.5 supplemented with 50 IU/ml and 50 µg/ml of Penicillin/Streptomycin (Life Technologies), 1X N-2 supplement (GIBCO, Life Technologies), 100 ng/ml human epidermal growth factor (hEGF) (Peprotech), and 10 ng/ml basic fibroblast growth factor (bFGF) (Peprotech).

Migration assay

Cells were trypsinize, washed with 1X DPBS, and resuspended in serum-free RPMI1640. $1x10^4$ cells were plated on top of the transwell insert and complete RPMI1640 media was added to the bottom chamber enough to submerge the transwell membrane. After 48 hours, media and non-migrated cells were removed from the top of transwell membrane. Cells in the membrane were then fixed in 70% EtOH for 10 minutes and were allowed to dry for 15 minutes. 0.2% crystal violet was then used to stain cells. Membrane was cut and placed onto a slide and stained with DAPI for analysis.

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