Mitochondrial haplotype affects tumorigenesis and metastatic efficiency through cell-autonomous and non-cell autonomous mechanisms

By

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Abstract

Metastatic disease is responsible for the vast majority of cancer related deaths, yet remains therapeutically elusive. Recent evidence has shown there are genetic underpinnings to the cascade of events that result in fully formed metastases. We utilized Mitochondrial Nuclear Exchange (MNX) mice, a unique model created by transferring a nucleus isolated from a fertilized oocyte of strain x into an enucleated oocyte of strain y, to directly test mitochondrial haplotype's role in tumorigenesis and metastasis. Through a series of genetic crosses we show that mitochondrial haplotype alters tumor latency, total metastatic number, and metastatic size, but does not affect growth of primary mammary tumors. By comparison to previous work, we show that these mitochondrial effects in mammary cancer are oncogene dependent. We next paired metastatic mammary and melanoma cell lines with nuclear matched MNX mice. We show that pulmonary metastatic burden of both mammary and melanoma cells is altered by mitochondrial haplotype of the host environment. We found that scavenging of mitochondrial superoxide in highly metastatic backgrounds was able to decrease the total number of metastases to the same level as that in low metastatic backgrounds. We show that both mitochondrial haplotype as well as superoxide scavenging alters the expression of select nuclear encoded genes. Preliminary data shows that mitochondrial load, membrane potential, copy number, and metabolic and glycolytic flux profiles vary slightly between strains, but do not correlate with metastatic data. Taken together, we conclude that mitochondria from within tumor cells as well as mitochondria located in the secondary environment can alter metastasis. We posit that mitochondrial-nuclear crosstalk through reactive oxygen species signaling alter nuclear gene expression allowing permissive and restrictive metastatic environments.

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Dedication

I dedicate this work to Ann and Ron Brinker. There are no words that could convey the depth of my gratitude to you both. We are the three luckiest kids on the planet.

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2-DG	2-deoxyglucose
ADP	Adenine diphosphate
ATAD3A	ATPase family AAA domain containing 3A
ATP	Adenine triphosphate
BCL-2	B-cell lymphoma-2
BM	Bone marrow
BRMS-1	Breast cancer metastasis suppressor-1
CAM	Cellular adhesion molecules
COX2	Cytochrome c oxidase subunit 2
Cybrid	Cytoplasmic hybrid
CytC	Cytochrome C oxidase
D-Loop	mitochondrial displacement loop
DHU Loop	Dihydrouridine loop of tRNA
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
ECAR	Extracellular acidification rate
ECM	Extracellular matrix
ES cell	Embryonic stem cells
EtBr	Ethidium bromide
ETC	Electron transport chain
FFCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FAD	Flavin adenine dinucleotide
GPx	Glutathione peroxidase
H-Strand	Heavy Strand
H_2O_2	Hydrogen peroxide
Her2	Receptor tyrosine-protein kinase erbB-2 in FVB/N-Tg(MMTVneu)202Mul/J
HSP	Heavy strand Promoter
IACUC	Institutional animal care and use committee
IP	Intraperitoneal
IV	Intravascular
L-Strand	Light strand
LSP	Light Strand Promoter
MCL-1	Myeloid leukemia cell differentiation protein
MEF	Mouse embryonic fibroblasts
MGME1	Mitochondrial genome maintenance exonuclease 1
miR	Micro ribonucleic acid
MMTV	Mammary tumor virus
MNX	Mitochondrial nuclear exchange
MT-Cyb	Mitochondrial cytochrome b
mtDNA	Mitochondrial deoxyribonucleic acid
NAC	N-acetyl cysteine
NAD	Nicotinamide adenine dinucleotide
NCR	Mitochondrial non-coding region
ND3	NADH dehydrogenase subunit 3

ND4	NADH dehydrogenase subunit 4
ND5	NADH dehydrogenase subunit 5
nDNA	Nuclear deoxyribonucleic acid
neo	Neomycin
OCR	Oxygen consumption rate
O_H	Origin of replication for the heavy strand
O_L	Origin of replication for the light strand
P_i	Inorganic Phosphate
PyMT	Polyoma middle T antigen in FVB/N-Tg(MMTV-PyVT)634Mul/J
Q	Co-enzyme Q
QTL	Quantitative trail loci
qPCR	Quantitative polymerase chain reaction
RFLP	Restriction fragment length polymorphism
ROS	Reactive oxygen species
S-phase	Synthesis phase
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
TCA	Tri-carboxylic acid
TFAM	Mitochondrial transcription factor A
TFB1M	Dimethyladenosine transferase 1, mitochondrial
TFB2M	Dimethyladenosine transferase 2, mitochondrial
tRNA	Transfer ribonucleic acid
WASF3	Wiskott-Aldridge syndrome protein family 3

Chapter I: Introduction

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Cancer has become a global health problem. Despite great progress resulting in chemotherapy, radiation, and surgical treatments to ablate primary tumors; it was estimated that roughly 600,000 people would die of cancer in the United States alone in 2016 (Siegel, Miller, & Jemal, 2016). The vast majority of these cancer related deaths can be attributed to metastasis, the process of cancer spread (Liotta & Stetler-Stevenson, 1993). Metastasis is a complex series of events involving many different cellular and environmental factors, and its pathobiology is not yet well-understood.

The Metastatic Cascade

The process that a cancer cell must go through to break away from the primary tumor, invade through the microenvironment, disseminate, and eventually colonize secondary sites is termed the metastatic cascade. There are many routes for metastasis. Cells can metastasize across body cavities, a process known as transcoelomic metastasis, which is prevalent in ovarian cancer (Tan, Agarwal, & Kaye, 2006). Cells have also been seen to metastasize along nerve cells (Liebig, Ayala, Wilks, Berger, & Albo, 2009). We focus here on the hematogenous (Figure 1) and lymphatic routes. These routes are common in breast and melanoma metastasis (Lee, 1983; Meier et al., 2002) and are utilized in our experimental modelling of metastasis.

The process of metastasis actually begins during tumor formation. Tumor cells are inherently genetically unstable and give rise to mutations during proliferation which create subpopulations of cells with the ability to metastasize (Fidler & Kripke, 1977; Welch & Tomasovic, 1985). These mutations result in cells that can move through and remodel the extracellular matrix, are capable of anchorage independent growth and resistance to anoikis (Abercrombie & Heaysman, 1954). In addition metastatic cells need to be capable of surviving

Figure 1

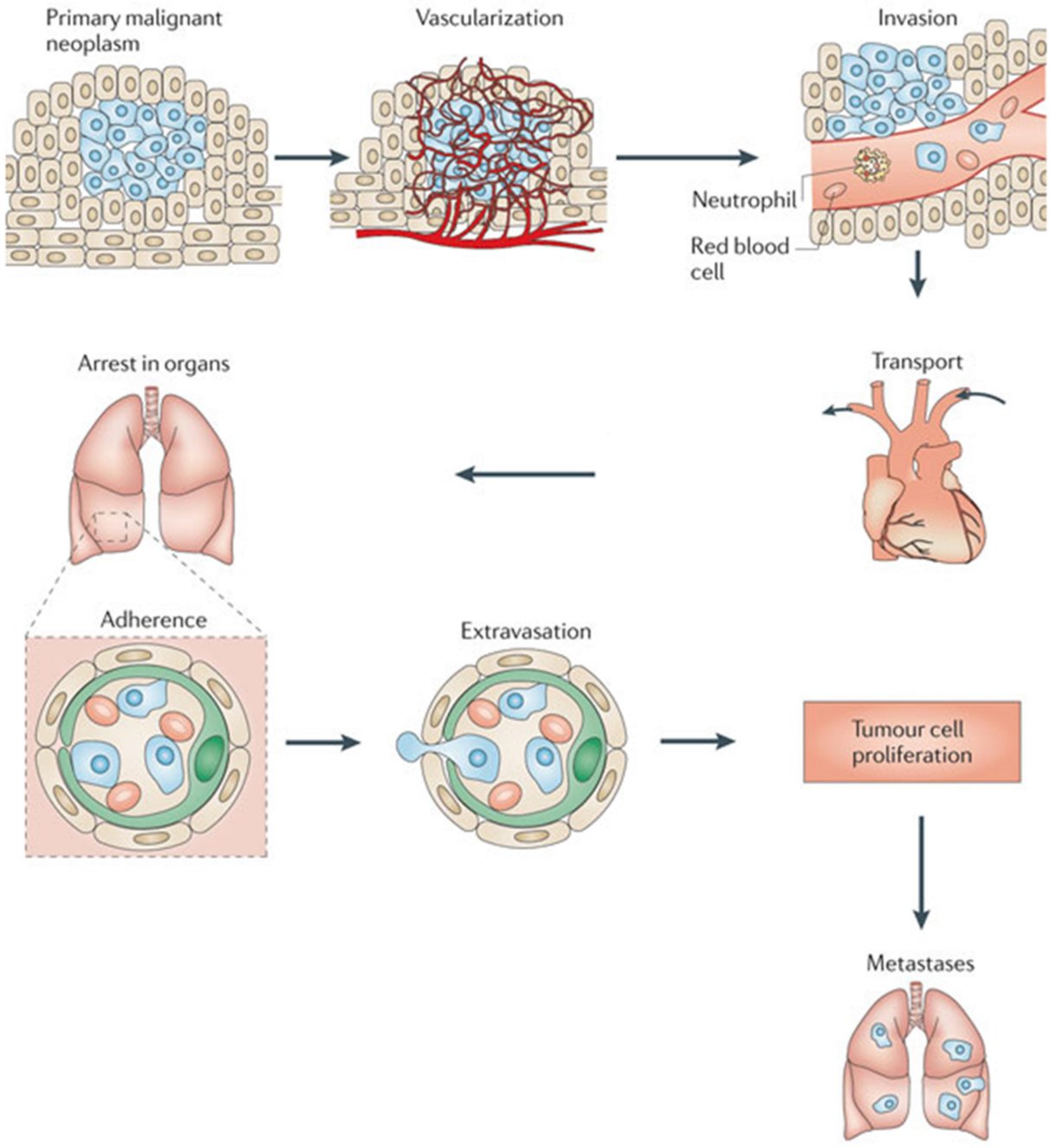

Primary malignant neoplasm
Vascularization
Invasion
Neutrophil
Red blood cell
Transport
Arrest in organs
Adherence
Extravasation
Tumour cell proliferation
Metastases

Figure 1: Representation of the hematogenous metastatic cascade. Primary tumors secrete factors to induce vascularization. Metastatic cells invade the surrounding tissue and push into the blood stream where they are then transported throughout the body. Metastatic cells arrest in capillary beds of organs where they adhere to vessels and extravasate into secondary tissues. Metastatic cells proliferate and colonize their secondary site. This figure was adapted and published here with full permission from the Nature Publishing Group, and originally appeared in (Francia, Cruz-Munoz, Man, Xu, & Kerbel, 2011).

shear forces in the blood stream while evading immune detection, and require the ability to enter and exit the vascular system (Vanharanta & Massague, 2013).

Emerging evidence suggests that during tumor growth, not only are metastatic variants being created and fostered, but the secondary microenvironment is as well. In 1889 Stephen Paget observed that cells of certain tumor types would seed specific secondary sites, or soil (Paget, 1989). Clinical observations such as breast cancer metastasizing to bone, brain, and lung while 70% of colon cancer metastasizes to the liver (Riihimaki, Hemminki, Sundquist, & Hemminki, 2016) supports the theory that cancer cells have a metastatic niche. David Lyden's group found that tumor derived exosomes were able educate bone marrow (BM) progenitor cells (Peinado et al., 2012). They found that these educated BM cells then traveled to secondary metastatic sites and initiated genetic changes of host tissue to form a pre-metastatic niche (Kaplan et al., 2005). Together this evidence suggests that pre-metastatic niches can be formed, although this theory is still in its infancy.

During tumorigenesis, vascularization and pseudo-vascularization occurs which aides in metastatic cell escape. Tumors and tumor associated fibroblasts release pro-angiogenic factors to recruit and generate new lymph and blood vasculature to and within the tumor (Folkman, Merler, Abernathy, & Williams, 1971; Orimo & Weinberg, 2006; Tannock, 1968; Watnick, 2012). Genetically deregulated tumor cells can mimic vascularization by forming micro-channels to aid in transport of nutritional building blocks and waste (Maniotis et al., 1999). In addition to allowing tumors to grow beyond the 2 mm passive diffusion limit (Folkman, 1971), this vascularization and pseudo-vascularization provide avenues for metastatic cells to exit the tumor and gain access to the blood and lymph systems (Juncker-Jensen et al., 2013; Wagenblast et al., 2015).

In order to break away from the primary tumor, metastatic cells need to be able to travel through their surrounding environment. Liotta proposed the three-step model of invasion: adhesion, dissolution, and motility (Liotta, 1986, 2016). In order to respond to cellular signals which aid in migration and differentiation, metastatic cells must be able to communicate through surface receptors with the extracellular matrix (ECM). In order to migrate, they must maintain a balance between no adherence, and too tight of an adherence to the ECM. They do this through modulation of interactions with integrins (Hynes, 1992), cadherins (Vleminckx, Vakaet, Mareel, Fiers, & van Roy, 1991), and cellular adhesion receptors called CAMs (Balzar et al., 2001). These metastatic cells then need to move through sometimes tightly woven ECM. They can achieve this through modulation of enzymes that aid in breakdown of proteins such as serine proteinases, cysteine proteinases, carboxyl proteinases, matrix metallo-proteinases, and endoglycosidases (Chambers & Matrisian, 1997; Nakajima, Welch, Belloni, & Nicolson, 1987; Ostrowski et al., 1988; Reich et al., 1988). They can also move through the ECM by passive invasion utilizing hydrostatic pressures to squeeze between ECM factors (Jain, Martin, & Stylianopoulos, 2014). Finally, metastatic cells have several ways in which they move. They can move individually as single cells through amoeboid like movements utilizing f-actin protrusions and integrin adhesions, and collectively by collective cell migration where lead cells carve paths and adhered cells follow and by multi-cellular streaming where individual cells control their interactions with the ECM but influence the cells around them (Tozluoglu et al., 2013; Wolf et al., 2003; Wolf et al., 2007). Through these mechanisms cancer cells are able to break away from tumors, move through the ECM, intravasate through basement membranes, and enter the blood or lymph circulation.

Metastatic cells need to survive in circulation and exit circulation at their secondary site. Once in circulation metastatic cells need the ability to overcome anoikis, a special type of apoptosis that occurs when cells are detached from ECM (Frisch & Francis, 1994; Frisch & Screaton, 2001). They must then stay intact during travel as the shear forces in the blood stream particularly are high, especially when travelling through the heart where pressures can be immense (Weinbaum, Cowin, & Zeng, 1994; Weiss, Orr, & Honn, 1989; Wirtz, Konstantopoulos, & Searson, 2011). Additionally cells need to evade detection by the immune system. Some cancer cells achieve this by aggregating with platelets so that interior metastatic cells are protected from both shear stress and immune detection and are able to survive (Jurasz, Alonso-Escolano, & Radomski, 2004). Some cancer cells are able to kill immune cells through counter attack (Dong et al., 2002; Ryan, Shanahan, O'Connell, & Houston, 2006). When arriving at their secondary site, cancer cells can become trapped in capillary beds or can adhere to vascular adhesion molecules (Pasqualini & Ruoslahti, 1996; Seguin, Desgrosellier, Weis, & Cheresh, 2015). Metastatic cells then crawl out of the blood stream in a process termed extravasation.

Even metastatic cells which reach their secondary site do not always grow and form metastases. Some cells will undergo apoptosis while others will stay as dormant single cells, neither proliferating nor dying. It is thought that interactions between the secondary microenvironment and disseminated cancer cells will influence whether these metastatic cells will proliferate and successfully colonize (Chambers, Groom, & MacDonald, 2002; Townson & Chambers, 2006). As discussed previously, factors from the primary tumor may induce remodeling of these secondary sites, turning growth signals on and restrictive signals off in order for metastases to successfully proliferate (Quail & Joyce, 2013).

This cascade of events is complex and inefficient. Many millions of cells can be shed per gram of tumor in a day (Butler & Gullino, 1975), yet relatively few metastases form. This was demonstrated when clinicians, in an attempt to alleviate pain, shunted malignant tumor cells in ascites directly into the blood, but very few patients developed metastases (Tarin et al., 1984). Although immense progress has been made in understanding this process, the molecular mechanisms behind each step have not yet been fully elucidated. This can be seen in the lack of therapies that target metastasis. The complexity of the process also makes experimental modelling in the laboratory challenging.

The Genetics of Metastasis

Individuals exposed to the same environmental factors and with similar lifestyles can still differ in both their susceptibility to cancer as well as to the ways in which the disease progresses. These observations coupled with familial tracking of cancer severity, indicate an underlying genetic component to metastatic disease. Indeed, cancer susceptibility and severity markers have been hypothesized since Broca's observations of familial breast cancer clustering in 1866 (Broca, 1866), and have since been mapped to the nuclear genome (Berwick & Vineis, 2000; Comino-Mendez et al., 2011; J. R. Smith et al., 1996; Xu et al., 1998; Zuo et al., 1996).

Metastasis suppressor genes, first discovered in 1988 (Steeg et al., 1988), are important genetic regulators of the metastatic cascade. These genes can function at any of the aforementioned steps of the metastatic cascade to block final metastasis formation. KISS1, first discovered in 1996, arrests metastatic cell growth at the secondary site, pushing already seeded metastatic cells into dormancy (Lee, 1983; Nash et al., 2007). Breast cancer metastasis suppressor-1 (BRMS1) functions at several points in the metastatic cascade to promote apoptosis and inhibit cell migration (Cook et al., 2012; Khotskaya et al., 2014; Phadke, Vaidya, Nash,

Hurst, & Welch, 2008). In addition to genes that regulate metastasis, recent research shows that genetic variation in chromosomal regions can affect metastasis.

Kent Hunter's recent research into variant modifiers of neoplastic disease utilized a transgenic mouse model of spontaneous tumorigenesis and metastasis. His group bred this transgenic male mouse to females of multiple different inbred mouse strains and observed the transgene positive F1 females for pulmonary metastatic burden (Lifsted et al., 1998). What his group found was that the incidence of metastasis varied greatly with the strain of inbred female mouse (Figure 2) (Hunter, 2006). His interpretation of the data was that there were inherited dominant genetic modifiers in the inbred mouse strains that helped determine metastatic susceptibility. He has gone on to validate multiple nuclear quantitative trait loci (QTL) which function in the process of metastasis (Hsieh, Look, Sieuwerts, Foekens, & Hunter, 2009; Y. Hu et al., 2012; Yang et al., 2005). The breeding scheme of his mice, with the male transgenic being mated to females of the inbred strains, means that the offspring inherit their mitochondria from the various inbred strains (strictly through the maternal lineage). This results in the offspring representing distinct mitochondrial genetic pools, and potentially indicates that mitochondrial DNA may play a role in susceptibility of metastasis.

Mitochondrial Genetics

Compared to the mammalian nuclear genome which is greater than 3 billion base pairs in size and codes for over 21,000 genes, the mammalian mitochondrial genome is extremely small and is passed exclusively through the female lineage (Giles, Blanc, Cann, & Wallace, 1980; Lander et al., 2001). The mitochondrial genome in humans was first sequenced in 1981 and is highly conserved from person to person at 16,569 bp (Anderson et al., 1981; Andrews et al., 1999). The size of the mitochondrial genome does differ from species to species. Interestingly

Figure 2

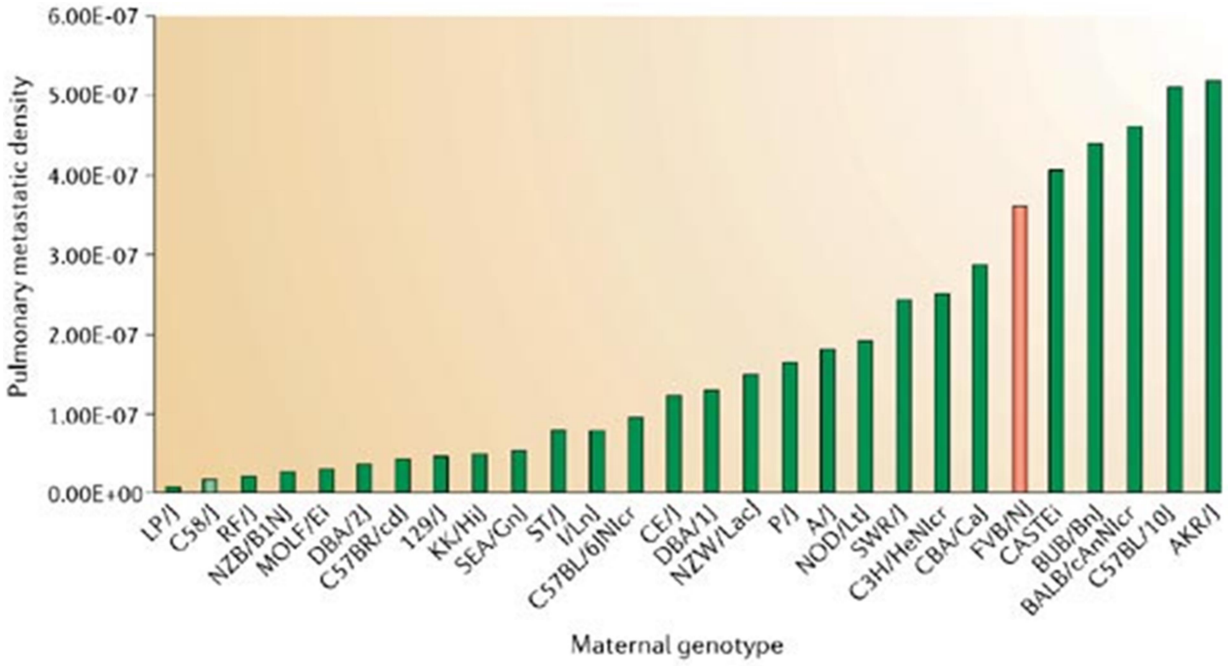


Figure 2: Offspring of inbred mouse strains show distinct differences in metastatic susceptibility. Female mice from multiple inbred strains were mated with a male mouse expressing the PyMT oncogene under the Mammary Tumor Virus (MMTV) promoter. Offspring were observed for tumor formation and upon necropsy lung metastases were quantified. The results show that maternal genotype is influencing metastatic propensity. This figure was published here with full permission from the Nature Publishing Group, and originally appeared in (Hunter, 2006).

the mouse mitochondrial genome is directly comparable to the human sequence with only 274 fewer nucleotides (Bibb, Van Etten, Wright, Walberg, & Clayton, 1981). This difference is almost entirely located within the displacement loop (D-loop) which is highly species specific and is currently designated as a non-coding region (Gillum & Clayton, 1978).

The mitochondrial genome is a double stranded circular DNA molecule (Figure 3). The two DNA strands are the heavy (H-strand) which is guanine rich, and the light strand (L-strand) which is cytosine rich (Anderson et al., 1981). The H-strand promoters, the L-strand promoter, as well as the H-strand origin of replication are located within the non-coding region (NCR) containing the D-Loop (Mercer et al., 2011). The D-Loop is a unique region within the NCR that incorporates a third strand of DNA called 7S which separates the H-strand and L-strand forming its namesake D-shaped structure (Kasamatsu, Robberson, & Vinograd, 1971). Interestingly, the D-Loop is not present in every mitochondrial DNA (mtDNA) copy (Nicholls & Minczuk, 2014). Even though mtDNA replication has historically been classified as independent of the cell cycle, levels of 7S do appear to correspond with nuclear S-phase (Antes et al., 2010). Although yet unproven, 7S levels may act as a gatekeeper to mtDNA replication much as checkpoint modifiers function during nuclear cell cycle (Nicholls & Minczuk, 2014). Much is still to be elucidated about the functions of the mitochondrial D-Loop.

The process for mtDNA replication is also still being debated. Multiple theories as to the exact stimuli, timing, and mechanisms involved in this process currently exist (Clayton, 1982; Yasukawa et al., 2006). Mitochondrial DNA, although lacking histones, is loosely organized into protein-DNA complexes called nucleoids which are located along the inner mitochondrial membrane (Bogenhagen, 2012). The machinery for mtDNA replication, called the replisome, is located within these nucleoids and consists entirely of nuclear encoded components

Figure 3

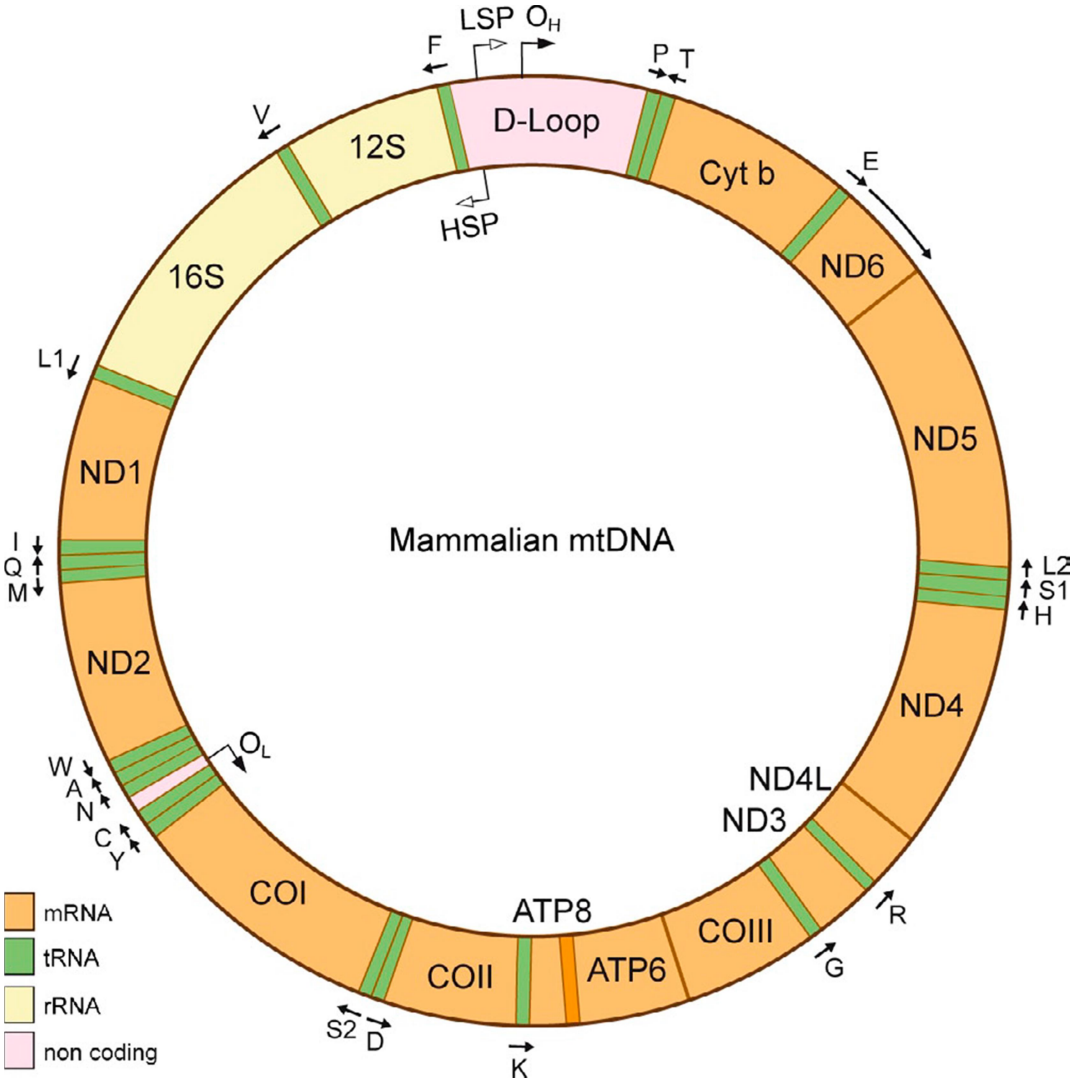

LSP
O_H
F
P
T
V
12S
D-Loop
Cyt b
E
HSP
16S
ND6
L1
ND5
ND1
Mammalian mtDNA
I
Q
M
L2
S1
H
ND2
ND4
O_L
W
A
N
C
Y
ND4L
ND3
COI
ATP8
COIII
R
COII
ATP6
G
S2
D
K
mRNA
tRNA
rRNA
non coding

Figure 3: Depiction of the double stranded mammalian mitochondrial DNA unit. Protein coding regions for electron transport subunit genes are in orange, while tRNA genes are in green and labeled with their one letter code, and rRNA subunit genes are in yellow. The NCR is marked here in pink. HSP (Heavy-Strand Promoter), LSP (Light-Strand Promoter), OH (Origin of replication for the H-Strand), and OL (Origin of replication for the L-Strand) are denoted along with their currently understood direction of replication. This figure was published here with full permission from the Wiley Publishing Group, and originally appeared in (Lagouge & Larsson, 2013)

(Chinnery & Hudson, 2013). The trimeric DNA polymerase γ functions with mitochondrial single stranded binding proteins, as well as mitochondrial helicases, Twinkle and C10Orf2, and the exonuclease MGME1 to complete replication (Copeland, 2014; Milenkovic et al., 2013; Uhler et al., 2016). Due in part to its polycistronic nature, mtDNA is incredibly efficient. Mitochondrial genes lack introns and are contiguous, with only one or two non-coding base pairs separating genes from each other (Anderson et al., 1981; Andrews et al., 1999; Eperon, Anderson, & Nierlich, 1980). Over 90% of the mitochondrial genome codes for protein (Chinnery & Hudson, 2013), with the NCR being the current exception.

The mitochondrial genome codes for thirty-seven genes: twenty-two mitochondrial specific tRNAs, two mitochondrial ribosomal RNAs (16s rRNA and 12s rRNA), and a mere thirteen proteins which comprise the subunits of the oxidative phosphorylation system (Chinnery & Hudson, 2013). Mitochondrial RNA is transcribed by a mitochondrial RNA polymerase called POLRMT in conjunction with mitochondrial transcription factors TFB1M and TFB2M and is regulated by mitochondrial transcription factor A (TFAM) (Gaspari, Larsson, & Gustafsson, 2004; Shi et al., 2012). Transcription initiates from the two H-strand and one L-strand promoters as single polycistronic precursor transcripts (Ojala, Montoya, & Attardi, 1981). These transcripts are subsequently cleaved to first release the tRNAs, then the rRNA and mRNA are released as a byproduct of the tRNA cleavage in a process termed the "tRNA punctuation model" (Ojala et al., 1981). New data suggest that additional cleavage outside of the tRNA products could occur, however this is still being debated (Sbisa, Tullo, Nardelli, Tanzariello, & Saccone, 1992; Slomovic, Laufer, Geiger, & Schuster, 2005). RNA products then undergo processing where tRNA have a CCA trinucleotide added to the 3' end and mRNA and rRNA are 3' polyadenylated

(Nagaike, Suzuki, Katoh, & Ueda, 2005). Unlike nuclear DNA, no 5' capping occurs. Having their own gene set yet relying on a vast array of nuclear proteins for DNA replication and for basic structure and function, belies the intimate relationship between mitochondria and the nucleus.

Despite the relatively small number of gene products, the mitochondrial genetic system is rather cumbersome. For each gene there can be between 100-10,000 copies, and each one of these copies has the potential to accumulate single nucleotide polymorphisms (Chatterjee, Mambo, & Sidransky, 2006). mtDNA accumulates mutations over time at a greater rate than nuclear DNA (Cormio et al., 2005; Haag-Liautard et al., 2008). Each potential polymorphism in oxidative subunit genes may subsequently shift the bioenergetics of the cell in a heritable manner. It is this concept that forms the basis for using mitochondrial single nucleotide polymorphisms (SNPs) to trace the evolution of human populations. The mitochondrial paradigm postulates that as populations moved out of central Africa into other climes, they accumulated mitochondrial mutations that shifted their metabolism in a way that favored survival in their new environment (Wallace, 2005), giving rise to distinct mitochondrial haplogroups.

Mitochondrial Genetics and Cancer

If mitochondria play a role in cancer and metastatic disease susceptibility, we would expect to see different rates and outcomes between patients of different mitochondrial haplogroups. Indeed, racial disparity exists in cancer. There have been many epidemiological studies, specifically in breast cancer, that note differences in both the rates of metastasis, and the overall outcome of patients that vary by race and subsequently mitochondrial SNPs (Chlebowski et al., 2005; Kulawiec, Owens, & Singh, 2009; Silber et al., 2013). Although African American women are less likely to develop breast cancer, they are much more likely to have more

aggressive cancers and a higher mortality rate than Non-Hispanic whites (Albain, Unger, Crowley, Coltman, & Hershman, 2009; Bain, Greenberg, & Whitaker, 1986; Chen et al., 1994; Miller, Hankey, & Thomas, 2002; Siegel et al., 2016). Although compelling, results from these studies are often confounded by factors influenced by socioeconomic status, availability of treatments, and time from diagnosis to treatment. Determining mitochondria's role in cancer is further complicated by the fact that most proteins that make up a mitochondrion are coded for in the nucleus.

Nuclear encoded mitochondrial genes have been implicated in many different oncogenic roles. The B-cell lymphoma-2 (BCL-2) family of genes is nuclear encoded but localize to the mitochondria where they regulate mitochondrial outer membrane permeability and as a consequence, apoptosis (Youle & Strasser, 2008). ATPase Family AAA domain containing 3A (ATAD3A) resides in the mitochondrial membrane and was found to stabilize Wiskott-Aldridge syndrome protein family 3 (WASF3) (Teng et al., 2016). The stabilization of WASF3 results in promotion of metastasis through actin polymerization and downregulation of the metastasis suppressor KISS1 (Teng et al., 2016). Despite the large number of nuclear encoded genes that make up a mitochondrion, there have been a variety of pathogenic polymorphisms found within mtDNA specifically.

Mitochondrial SNPs have been indicated in a variety of inherited clinical disorders (Taylor & Turnbull, 2005). The first disease found to be linked to a mitochondrial polymorphism is Leber's Hereditary Optic Neuropathy, caused by the maternally inherited mitochondrial G11778A SNP (Wallace et al., 1988). When mitochondrial DNA of patient tumors was sequenced, it was found that specific cancer types correlated with specific mitochondrial mutations. For instance, breast tumors commonly had mutations in mitochondrial NADH

dehydrogenase subunit 4 (ND4), while bladder tumors contained mutations in mitochondrial cytochrome b (MT-CYB), NADH dehydrogenase subunit 3 (ND3), and NADH dehydrogenase subunit 5 (ND5) (Chatterjee et al., 2006). While it is telling that these studies found that not all mitochondrial mutation was random, they were unable to determine if mtDNA mutations are driving these cancer types, or simply hot-spot by-products of the oncogenic process.

Some of the clearest evidence of mitochondria's involvement in the process of metastasis comes from a series of elegantly designed studies from Ishikawa and colleagues. Ishikawa utilized two mouse lung carcinoma cell lines, one with high metastatic potential, the A11 line which has a G13997A mutation in a mitochondrial-encoded subunit for Complex 1, and one with low metastatic potential, the P29 line which does not carry the mutation. They utilized the cytoplast fusion method (discussed later) to engineer four cytoplasmic hybrid (cybrid) lines that combine the nucleus of the highly metastatic A11 with the mitochondria of the low metastatic P29 (A11mtP29), the nucleus of the low metastatic P29 with the mitochondria of the high metastatic A11 (P29mtA11), and then to recombine the original strains, P29mtP29 and A11mtA11 as controls. They found that the P29mtA11 strain was highly metastatic *in-vivo* while the A11mtP29 was not (although it should be noted that this data is written about in (Ishikawa, Koshikawa, Takenaga, Nakada, & Hayashi, 2008), and highly cited throughout the field, the data is not shown in any of the references currently available). They went on to repeat this process with several additional cancer and human lines to find that when mitochondria from highly metastatic cells with mtDNA mutations are present, it resulted in a highly metastatic cell line (Imanishi et al., 2011; Ishikawa, Imanishi, Takenaga, & Hayashi, 2012; Ishikawa, Koshikawa, et al., 2008; Ishikawa, Takenaga, et al., 2008). Ishikawa's cybrid studies were the first experiments that attempted to directly test mtDNA's role in metastasis. Since mtDNA codes mainly for

critical subunits of the electron transport chain, we next analyze the effects of metabolism on the process of tumorigenesis and metastasis.

Metabolism

The Tri-Carboxylic Acid (TCA) Cycle as well as Oxidative Phosphorylation are processes located within the mitochondria. During oxygen rich periods, the process of glycolysis, which occurs in the cytosol, feeds metabolic components into the TCA cycle which in turn feeds products into the electron transport chain (ETC) (Figure 4). During glycolysis, one molecule of glucose is broken down in a series of enzymatic steps utilizing the reduction of NAD+ to NADH to form two pyruvates and a net of two adenosine tri-phosphates (ATP) (Cori, 1983). Under oxygen poor conditions, pyruvate is further broken down to lactate and the NADH is oxidized to regenerate NAD+ to be utilized again in glycolysis (Pietrocola, Galluzzi, Bravo-San Pedro, Madeo, & Kroemer, 2015). Under normal oxidative periods, pyruvate from glycolysis freely enters the mitochondrion where it is converted into acetyl-CoA and enters into the TCA Cycle (Herzig et al., 2012). During the TCA cycle, acetyl-CoA is further broken down through a series of enzymatic steps which generate ATP and reduce NAD+ and FAD to NADH and $FADH_2$ (Akram, 2014). These electron carriers pass their electrons to complex 1 and complex 2 (respectively) of the ETC, then re-enter the TCA cycle (Sazanov, 2015). The electrons from these carrier molecules are passed from complex to complex in order to drive the transport of hydrogens from the mitochondrial matrix to the inner mitochondrial membrane (Figure 5) (Rich, 2003). This proton gradient, known as mitochondrial membrane potential, then drives complex 5 (ATP synthase) which synthesizes ATP (Sazanov, 2015). From one molecule of glucose a net of thirty to thirty-six ATP molecules are formed from oxidative phosphorylation (Rich, 2003). In

Figure 4

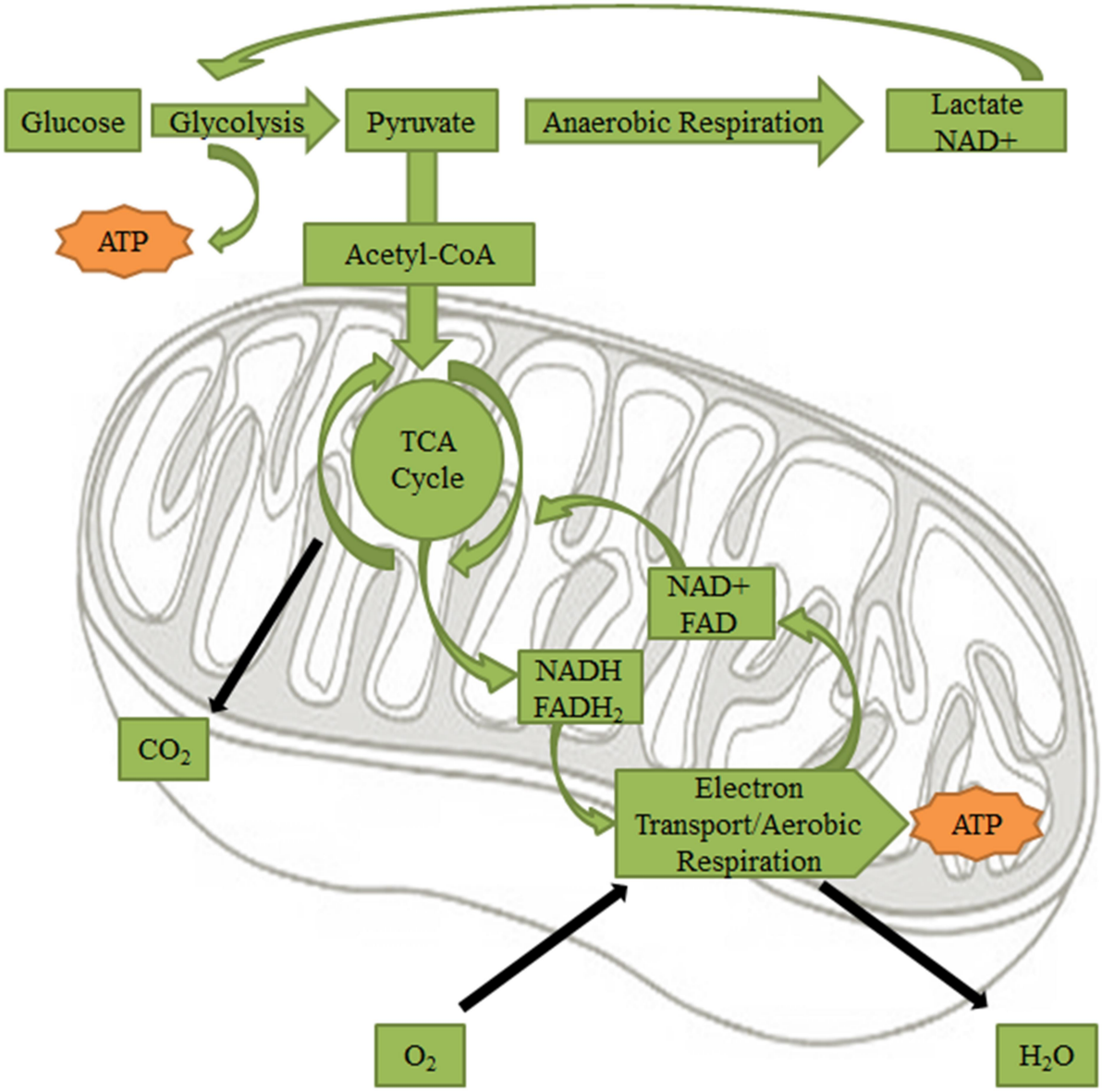

Glucose
Glycolysis
Pyruvate
Anaerobic Respiration
Lactate
NAD+
ATP
Acetyl-CoA
TCA
Cycle
NAD+
FAD
NADH
FADH2
CO2
Electron
Transport/Aerobic
Respiration
ATP
O2
H2O

Figure 4: Representation of the main pathways in mitochondrial metabolism. Glucose is first broken down to pyruvate during glycolysis with the aid of the electron transporter NAD+ to yield a small amount of ATP and NADH. Pyruvate under anaerobic respiration is broken down to lactate and NAD+ is regenerated from NADH to feedback to the glycolytic pathway. Under oxidative conditions pyruvate is converted to acetyl-CoA which is transported into the mitochondria and enters the TCA cycle. The TCA cycle reduces electron carriers NAD+ and FAD to NADH and $FADH_2$. These carriers shuttle electrons to the ETC which is the main site of ATP production. Cancer cells rely more heavily on glycolysis and anaerobic respiration even under oxidative conditions; however oxidative phosphorylation capabilities are not lost.

Figure 5

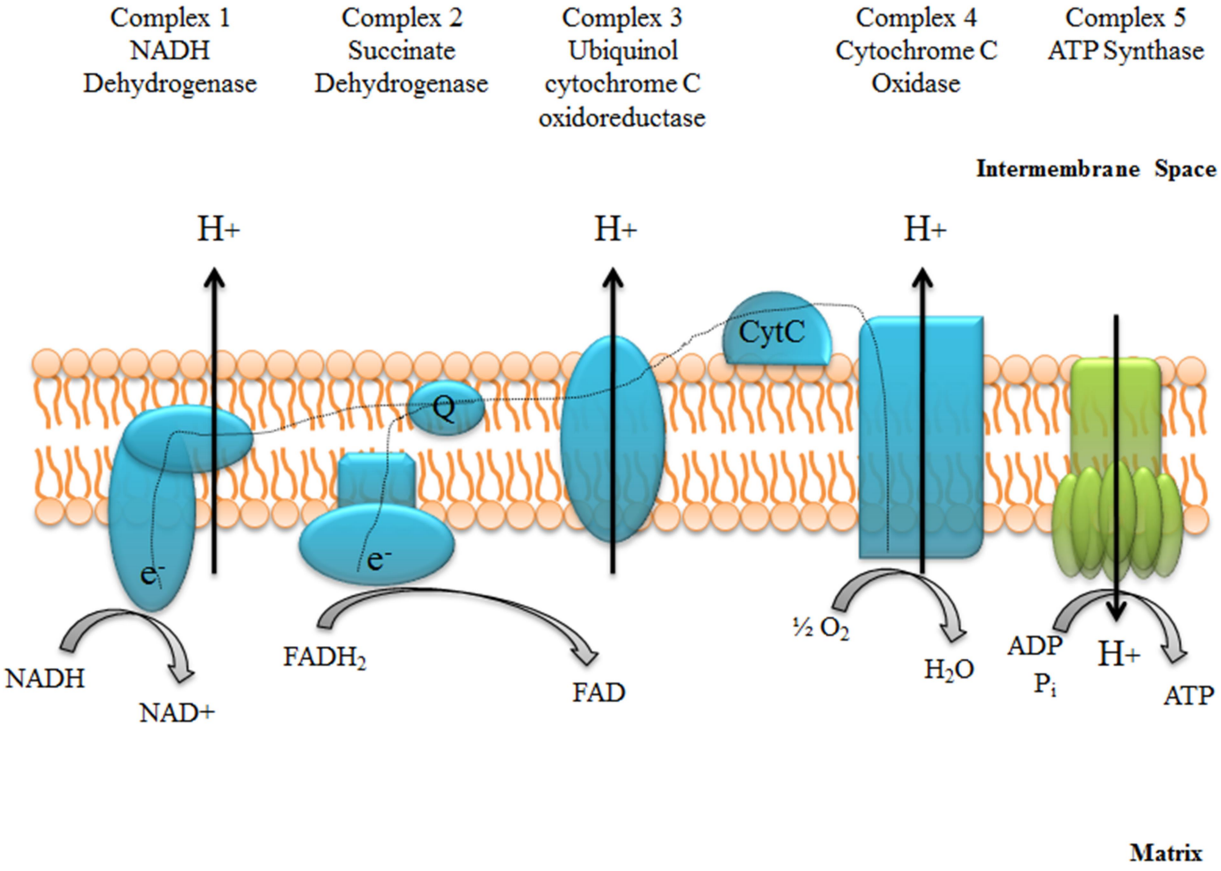

Complex 1
NADH
Dehydrogenase
Complex 2
Succinate
Dehydrogenase
Complex 3
Ubiquinol
cytochrome C
oxidoreductase
Complex 4
Cytochrome C
Oxidase
Complex 5
ATP Synthase
Intermembrane Space
H+
H+
H+
CytC
Q
e-
e-
NADH
NAD+
FADH2
FAD
½ O2
H2O
ADP
Pi
H+
ATP
Matrix

Figure 5: Portrayal of the electron transport chain located within the mitochondrial inner membrane. Electrons from NADH and $FADH_2$ enter the electron transport chain at Complex 1 and Complex 2 respectively. They are then transferred from subunit to subunit via redox reactions which are coupled to the transport of protons from the matrix into the intermembrane space. ATP Synthase utilizes this proton gradient to synthesize ATP from ADP and P_i. The path of the electrons is indicated with a dotted line. Coenzyme Q is denoted as Q, and Cytochrome C as CytC.

terms of energy production, oxidative phosphorylation provides a much higher output of ATP, yet tumor cells often rely on anaerobic respiration.

Metabolism and Cancer

Otto Warburg first noted that despite being in oxidative environments, tumor tissues (and subsequently, metastases) relied heavily on glycolysis and have increased glucose uptake compared to their normal counterparts (Warburg, Wind, & Negelein, 1927). Although the mechanisms behind this metabolic shift have still not been fully elucidated, it is thought that reliance on glycolytic cycling increases the bioavailability of substrates for rapidly proliferating cancer cells (Potter, 1958; Vander Heiden, Cantley, & Thompson, 2009). Since this observation, metabolic reprogramming has become a hallmark of cancer (Hanahan & Weinberg, 2011).

The metabolic shift can aid in transforming the cancer microenvironment. Increased uptake of glucose by tumor cells decreases local glucose availability. It was found that this decrease in available glucose renders T-cells in the microenvironment dysfunctional (Chang et al., 2015; Ho et al., 2015) which may aid in metastatic cell escape. Changes in metabolism have also been shown to alter nuclear gene expression through nutrient sensing histone modifications such as methylation, acetylation, and O-GlcNAcylation (Lu & Thompson, 2012; Wellen & Thompson, 2012). These metabolic alterations can arise from mutations in TCA cycle genes and ETC genes (Baysal et al., 2000; Mullen et al., 2012). Another facet of metabolism that affects tumorigenesis and metastasis is oxidative stress.

Reactive Oxygen Species

Mitochondrial reactive oxygen species (ROS) are mainly generated during electron transport between ETC subunits. The ETC is made up of five complexes, Complex 1 called

NADH dehydrogenase, Complex 2 called Succinate dehydrogenase, Complex 3 called Ubiquinol cytochrome C oxidoreductase, Complex 4 Cytochrome C oxidase, and Complex 5 ATP synthase (Figure 5) (Murray, Granner, Mayes, & Rodwell, 2003). With the exception of Complex 2 which is entirely nuclear-encoded, these complexes are made up of subunits that are both nuclear and mitochondrial-encoded (Rich, 2003). The first four complexes act as a coupled series of electron donors and acceptors which terminate in oxygen (Murray et al., 2003). During transfer between complexes, electrons can leak and partially reduce oxygen resulting in a species called superoxide. Although production of superoxide has been reported at each of the ETC complexes (Turrens, 2003), Complex 1, which releases superoxide to the matrix, and Complex 3, which releases superoxide to both the matrix and inner-membrane are the main sites of electron leak and subsequent production of superoxides (Figure 6) (St-Pierre, Buckingham, Roebuck, & Brand, 2002). These superoxides can dismutate to form a variety of reactive oxygen species.

Superoxide is the precursor to most mitochondrial ROS. The highly electrophilic superoxide is actually relatively long lived in the absence of scavengers and is capable of inactivating enzymes (Fridovich, 1983). However superoxide can dismutate to hydrogen peroxide (H_2O_2) spontaneously or through a reaction catalyzed by a superoxide dismutate (SOD) (Li et al., 2013). There are two types of SOD that function in the mitochondria. SOD1, which is characterized by a copper zinc binding site in each subunit, scavenges ROS in the intermembrane space while SOD2, which is characterized by a manganese binding site in each subunit, functions mainly in the mitochondrial matrix (Okado-Matsumoto & Fridovich, 2001). The resulting H_2O_2 can react with metals to produce the DNA damaging hydroxyl radical (Lloyd, Phillips, & Carmichael, 1997), or can be processed into water by Glutathione peroxidase (GPx) which is located both in the matrix and inner mitochondrial membrane, or by catalase in the cytosol

Figure 6

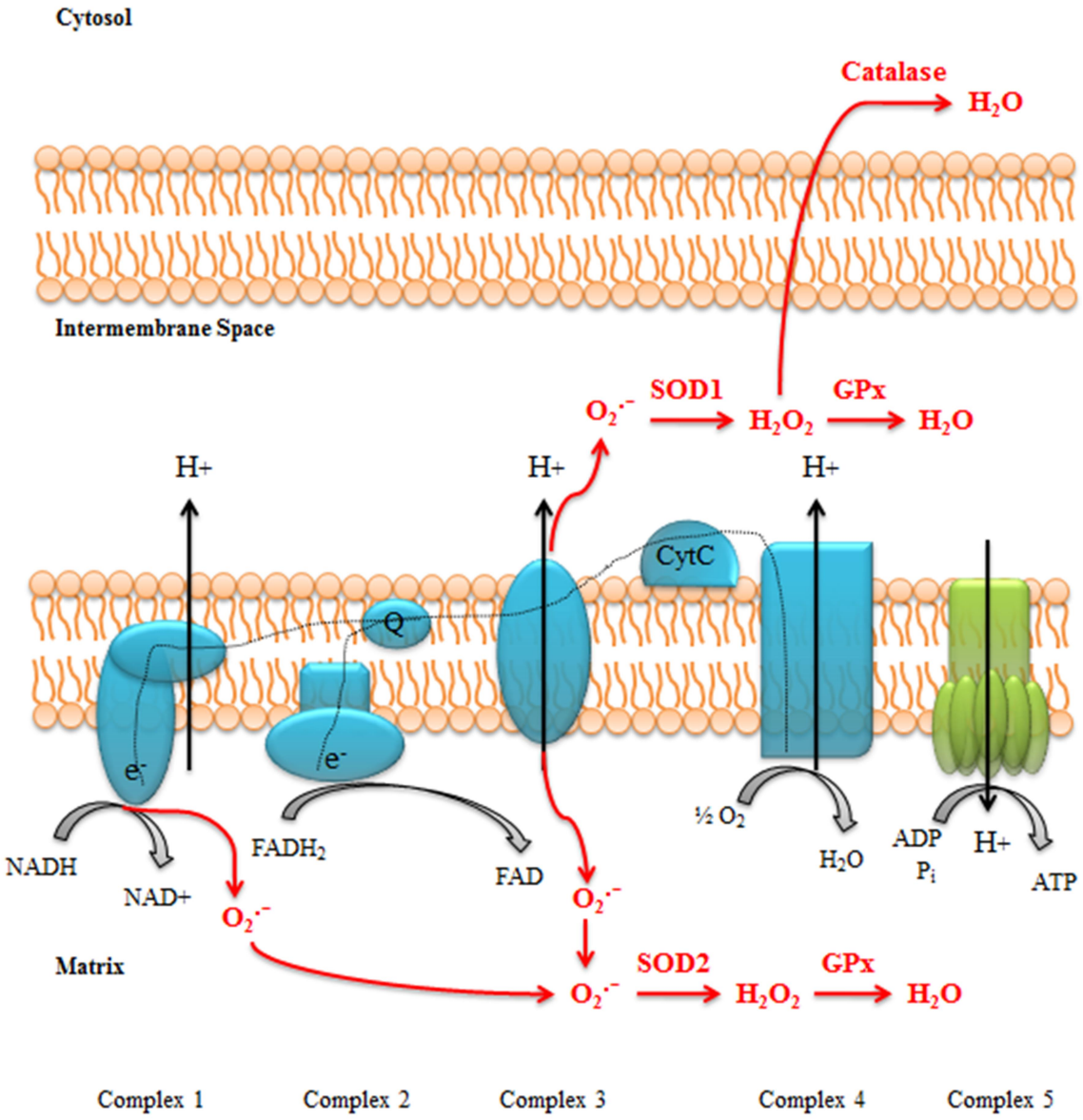

Cytosol
Catalase
H_2O
Intermembrane Space
$O_2^{\cdot -}$
SOD1
H_2O_2
GPx
H_2O
H+
H+
H+
CytC
Q
e-
e-
NADH
NAD+
$FADH_2$
FAD
$O_2^{\cdot -}$
$O_2^{\cdot -}$
½ O_2
H_2O
ADP
P_i
H+
ATP
Matrix
SOD2
GPx
$O_2^{\cdot -}$
H_2O_2
H_2O
Complex 1
Complex 2
Complex 3
Complex 4
Complex 5

Figure 6: Depiction of the electron transport chain focusing on the sites of ROS generation and scavenging. Superoxide is generated when electrons leak from the ETC. This mainly occurs as Complex 1, which leaks to the matrix, and Complex 3 which leaks to both the matrix and intermembrane space. Superoxide is converted to hydrogen peroxide by SOD2 in the matrix and SOD1 in the intermembrane space. This hydrogen peroxide is scavenged by glutathione peroxidase within the mitochondria, but is able to pass through lipid bilayers where it can travel to the nucleus or be dismuted by catalase to water.

(Drevet, 2006). H_2O_2 is relatively stable and if not quickly processed, can pass through membranes and act as a secondary messenger in processes as diverse as insulin signaling, growth factor signaling and redox signaling (Sies, 2014). Increases in ROS cause feedback through multiple mechanisms with in turn increase expression of antioxidant genes such as the aforementioned SODs, glutathione family proteins, and catalase (Sies, 2017). When antioxidant defense and ROS levels become unbalanced, it is termed oxidative stress.

Reactive Oxygen Species and Metastasis

Dysfunctional metabolism and hypoxia, such as that which occurs in cancer cells, as well as mtDNA mutations in ETC genes, often results in oxidative stress (Park et al., 2009). In normal cells, an excess of oxidative stress would trigger apoptosis; however, cancer cells are able to bypass these mechanisms by increasing antioxidant machinery giving them the ability to continue proliferating (Hanahan & Weinberg, 2011). A group of researchers recently found that when breast tumor cells were detached from the extracellular matrix, they upregulated SOD2 in order to overcome high amounts of ROS during anoikis. When they depleted SOD2, the cells were not able to metastasize *in-vivo* (Kamarajugadda et al., 2013). Although indirect, this study suggests that scavenging of ROS could lead to more aggressive cancers. Additionally, a group of researchers found that metastasis of subcutaneously implanted melanoma tumors was promoted when treated daily with the ROS scavenger N-Acetyl Cysteine (NAC) (Piskounova et al., 2015). Despite this evidence many studies suggest that scavenging of ROS could decrease a cancer's ability to metastasize.

Ishikawa's cybrid studies found that in multiple tumor cell types, mtDNA mutations which increased ROS resulted in highly metastatic phenotypes (Ishikawa, Koshikawa, et al., 2008; Ishikawa, Takenaga, et al., 2008). When they decreased ROS by using the scavengers

Ebselen (an H_2O_2 scavenger) or NAC (glutathione precursor that increases global scavenging of ROS), these cells became less metastatic (Ishikawa, Koshikawa, et al., 2008; Ishikawa, Takenaga, et al., 2008). Interestingly, they found that increased ROS triggered increased expression of the nuclear encoded anti-apoptotic gene Induced Myeloid Leukemia Cell Differentiation Protein (MCL-1) which could be reversed upon scavenging (Ishikawa, Koshikawa, et al., 2008). This further shows that ROS can act as messengers to communicate with the nuclear genome and alter oncogenic pathways. When testing these facets in cybrids from breast lines with mtDNA mutations in different ETC subunits, they found that again, metastatic potential followed the mitochondrial contributor, but that in this case it acted through ROS independent means (Imanishi et al., 2011). In addition to cancer type, metastatic location may also be a factor in the ROS and metastasis relationship. A microarray study utilizing breast metastases that were extracted from bone, lung, brain, and liver found that despite all originating from invasive ductal carcinoma, the expression level of ROS related proteins differed from site to site, with lung having the highest expression levels while bone had the lowest (Kim, Jung, & Koo, 2014). Taken together this data suggests that ROS are driving metastatic potential, but that it may do so dependent upon cancer type and metastatic location.

The opposing views of ROS scavenging in cancer may be due to a matter of balance. Several distinguished labs have proffered the opinion that moderate increases in mitochondrial ROS could feedback to the nucleus, affect oncogenic signaling, and subsequently increase metastasis. On the other hand, mitochondrial ROS above a certain threshold would activate apoptosis and lead to decreased metastasis (Porporato et al., 2014; Sena & Chandel, 2012; Weinberg et al., 2010). This model could also help to explain why antioxidants in cancer patients have had mixed results (Yasueda, Urushima, & Ito, 2016). Studies have shown that ROS can act

as potent retrograde signaling molecules to alter nuclear-expression of genes which drive metastatic progression or drive apoptotic cancer cell death. It is clear that ROS within the context of metastasis is a concept that warrants further exploration.

Mitochondrial Methods of Study

The small number of protein coding genes, the varied gene copy number, and the variation of mitochondrial number from cell to cell make mitochondria a difficult target for cancer research. Advances in recent years have led to a variety of new tools that have rekindled interest in the field such as the cybrid model. In this system one cell is depleted of its mitochondrial DNA through treatments of low level DNA intercalators such as ethidium bromide (EtBr) or ditercalinium; these cells are termed ρ^0 cells. Transfection of the ρ^0 cells with an antibiotic resistance cassette allows for future selection. These ρ^0 cells can then be either fused with platelets (which naturally do not contain a nucleus) (Figure 7 A), or a cytoplast which is a cell that has had its nucleus removed (Figure 7 B), generally through ultracentrifugation. Antibiotic is then applied, and only those cells which have successfully fused with the antibiotic resistant ρ^0 cell can be selected and further propagated (Coon, 1978; Yatscoff, Mason, Patel, & Freeman, 1981). By comparing behavior of cybrids which contain the same nucleus, but different mitochondria, we can start teasing out mitochondrial implications for metastatic disease.

Cybrids have been used to tremendous effect in some ground breaking studies as was previously noted. There are however some cautions. DNA intercalators, although they bind preferentially to mtDNA, can still bind to nuclear DNA (Coon, 1978; Yatscoff et al., 1981). The Ishikawa studies utilized ditercalinium, which interacts with the major groove of DNA resulting in an unwound conformation that can cause malfunction of the nuclear DNA excision repair system (Williams & Gao, 1992). Additionally, several antibiotics have been shown to affect the

Figure 7

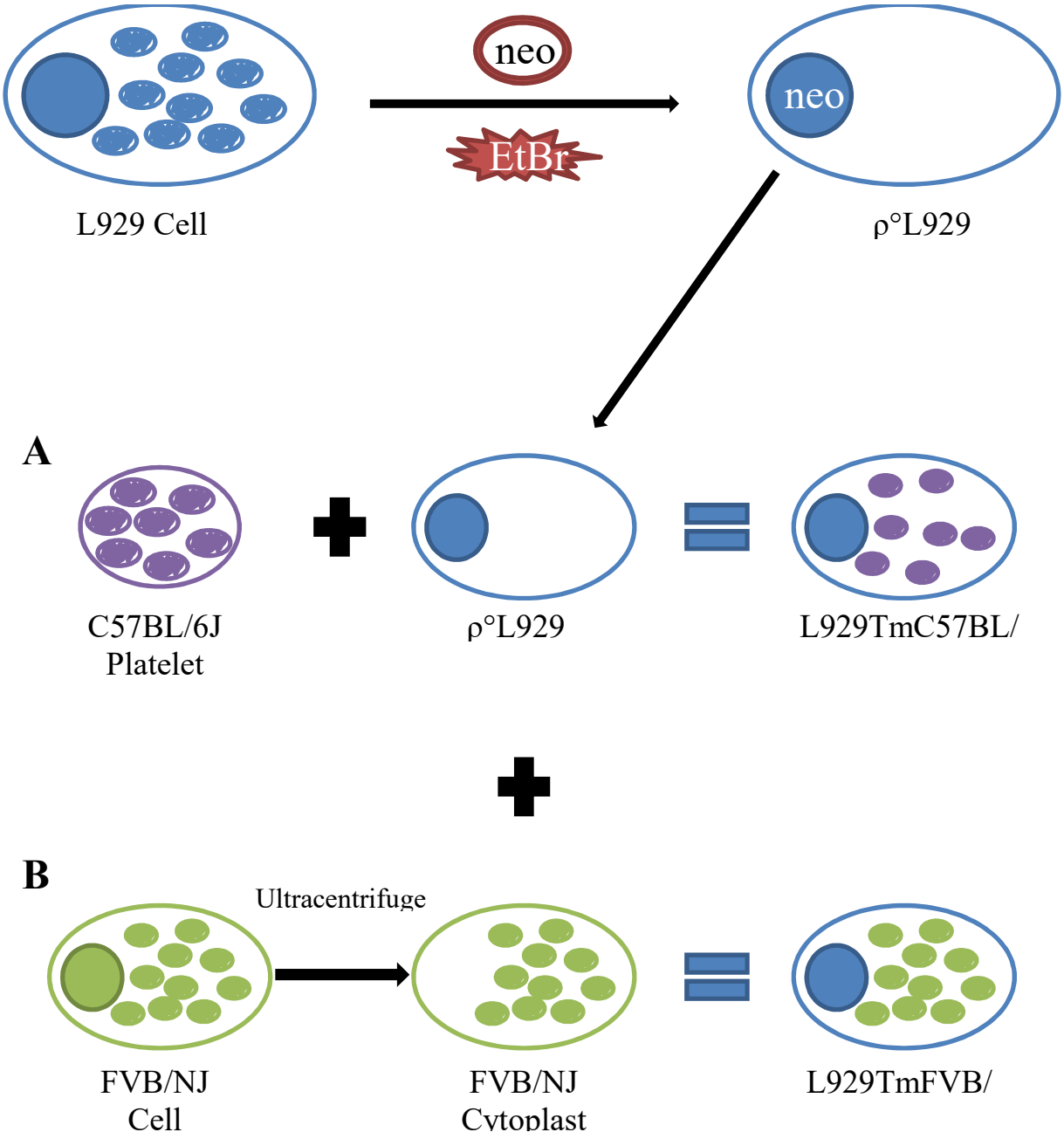

neo
EtBr
neo
L929 Cell
ρ°L929
A
C57BL/6J
Platelet
ρ°L929
L929TmC57BL/
B
Ultracentrifuge
FVB/NJ
Cell
FVB/NJ
Cytoplast
L929TmFVB/

Figure 7: Construction of a trans-mitochondrial cybrid. Rho null cells (ρ^0) are made by treating cells with ethidium bromide (EtBr) which preferentially binds to and degrades mtDNA as well as transfection with a neomycin resistance plasmid that allows for antibiotic cell selection. These ρ^0 cells can be **A:** fused with platelets which contain no nuclear DNA or **B:** fused with cytoplasts, a cell that has had its nucleus removed via differential ultracentrifugation. These new cells are denoted as nuclear contributor followed by Tm followed by mitochondrial contributor.

metastatic cascade (H. Hu et al., 2016; Lin et al., 2015; Qin et al., 2015; Zhou et al., 2012). Cybrid studies may still yield powerful preliminary data; however, with the potential for the nuclear background to be affected as well as the process of metastasis, there is need for a yet more stringent model.

Metastasis in particular is difficult to study in *in-vitro* systems. Many metastatic cell lines have been isolated and despite their *in-vivo* aggressiveness, they may not show the typical hallmarks of metastatic cells such as resistance to anoikis, invasiveness, and anchorage independent cell growth when cultured *in-vitro* (Hurst & Welch, 2011). It is for this reason that animal models are the gold standard for metastasis research, despite their relative scarcity.

Conplastic mice are one such model that has been developed to research mitochondrial effects *in-vivo*. Conplastic mice are made by taking a female mouse of the desired mitochondrial strain, breeding that mouse with a male of the desired nuclear strain, then backcrossing resulting litters multiple times (generally 10 generations) with male mice of the desired nuclear strain (Yu et al., 2009). These mice require a great amount of time to create, can only approximate a complete shift in the nuclear background, and due to combination effects, potential nuclear crossovers, and genetic bottlenecking during breeding, can yield ambiguous results.

To better differentiate contributions of the mitochondria to metastasis from that of the nucleus, several types of mitochondrial exchange mice have recently been created. One type is the mito-mice, which rely on chemical treatments for enucleation and mitochondria elimination in mouse embryonic stem (ES) cells or zygotes, similar to the creation of cybrids (Nakada & Hayashi, 2011). Yokota and colleagues were able to transfer mtDNA with the specific mutation G13997A, which Ishikawa identified in his cybrids as causing complex 1 defects, into mouse

cells. After fusion with donors, those ES cells were implanted back into pseudo-pregnant females resulting in chimeric mito-mice (Yokota et al., 2010). These mice showed complex 1 defects similar to their cybrid predecessors, but other metastatic phenotypes were missing. Unfortunately, the mito-mice model, with its reliance on chemical treatments, may have nuclear effects just as the cybrid model.

An alternative to mito-mice are the newly engineered mitochondrial nuclear exchange (MNX) mice. These mice are made by enucleating an oocyte of one strain by physical removal utilizing micro-pipette manipulation and exchanging that nucleus for a nucleus of a different mouse strain (Kesterson et al., 2016) (Figure 8). This process results in stable mouse lines that can be freely bred to transgenic models of metastasis. This approach eliminates the need for DNA intercalators, antibiotics, or the numerous backcrossing of traditional conplastic mice, and results in a 100% exchange of nuclear and mitochondrial DNA. This model has been used by Fetterman and colleagues to show that susceptibility of certain mouse strains to cardiovascular disease is directly related to their mitochondrial haplotype (Fetterman et al., 2013).

We propose the use of these MNX mice to further elucidate the effect of a cancer cell's mitochondrial haplotype on tumorigenesis and metastasis, to identify if mitochondrial haplotype at the secondary metastatic site can alter metastasis, and to determine if these effects are dependent upon nuclear oncogenic drivers. By studying if the inherited polymorphisms which define mitochondrial backgrounds affect tumor growth and metastatic spread, we hope to give new insights into potential clinical indicators of disease behavior.

Figure 8

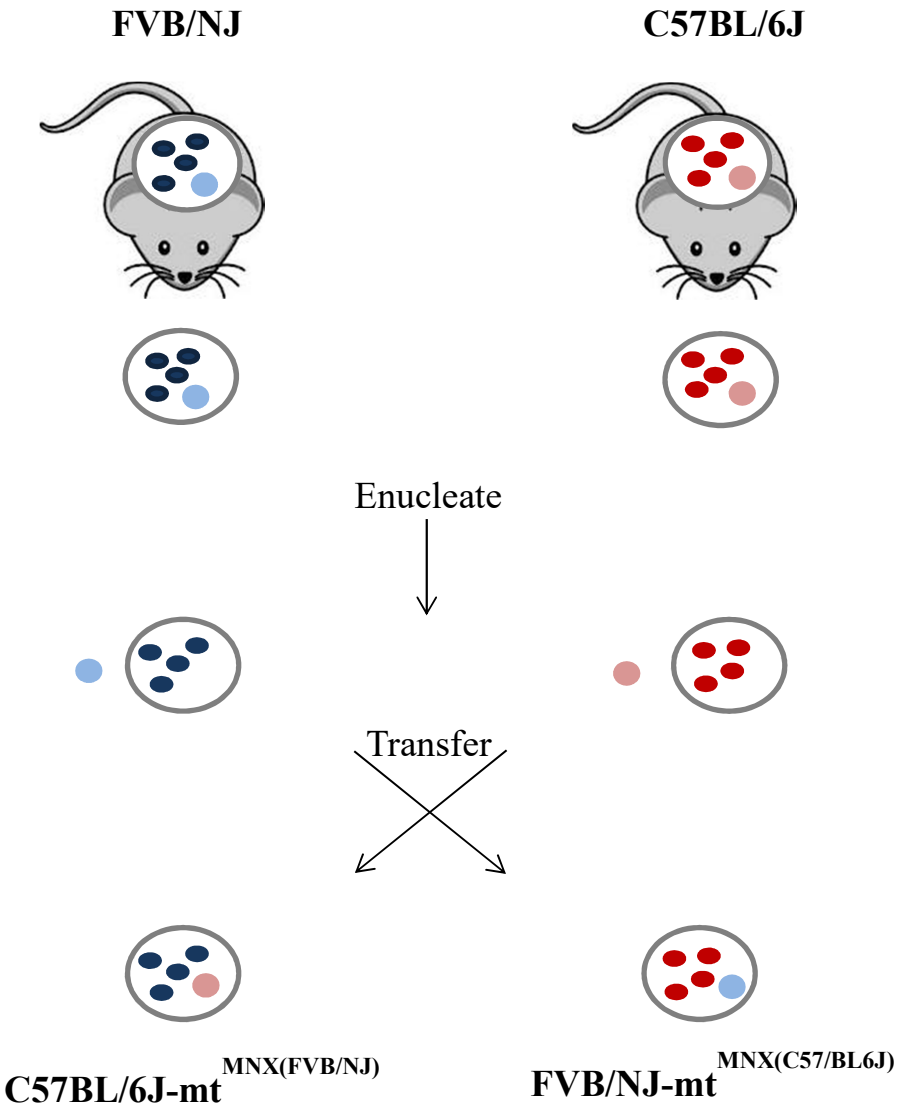

FVB/NJ
C57BL/6J
Enucleate
Transfer
C57BL/6J-mt MNX(FVB/NJ)
FVB/NJ-mt MNX(C57/BL6J)
Implant

Figure 8: Construction of the mitochondrial nuclear exchange mice. Fertilized oocytes are isolated from two different strains of mice. These oocytes are enucleated and the nucleus transferred from one strains oocyte to the other. These oocytes are the re-implanted into pseudo-pregnant female mice. These mice are denoted as: Nuclear Contributor-mt$^{MNX(Mitochondrial\ Contributor)}$

Chapter II: Mitochondrial haplotype alters breast cancer tumorigenicity and metastasis in a cell-autonomous, oncogene dependent manner

Abstract

Increasing data support roles for mitochondrial genomes contributing to complex diseases, including cancer. We hypothesized that differences in primary tumor formation and metastasis can arise from inherited mitochondrial haplotype. To directly test the role of mitochondrial DNA (mtDNA) in mammary cancer, we generated Mitochondrial Nuclear Exchange (MNX) mice. We utilized the FVB/N-Tg(MMTVneu)202Mul/J (Her2) transgenic mouse line crossed with FVB/NJ nuclear MNX mice having mtDNA from either C57BL/6J or BALB/cJ strains. To ensure that cytoplasmic factors would not confound tumor and metastasis studies, we first bred male MNX lines to female Her2 mice. This produced pups that had cytoplasm from both Her2 and MNX mice, but did not carry the MNX mitochondrial genome. We found that the offspring showed no differences in tumor latency, metastatic number, or metastasis size. We then bred male Her2 mice to female MNX mice and show that tumor latency is significantly ($p<0.001$) delayed in C57BL/6J (262 days) and BALB/cJ (293 days) mitochondrial mice compared to FVB/NJ (225 days). Metastatic number is decreased in both C57BL/6J and BALB/cJ mitochondrial mice compared to FVB/NJ (mean 5, 7, 15 respectively), while average metastatic size significantly varies (1.2, 1.4, 1.0 mm respectively). These data, taken together with previous PyMT studies, show that mtDNA background impacts tumor onset and metastasis in a cell autonomous, model dependent manner.

Introduction

It is estimated that 1,600 people per day died of cancer in the United States in 2016 (Siegel et al., 2016). Despite extensive progress, breast cancer remains among the most lethal

cancers and accounts for the greatest number of newly diagnosed cancer cases in American females of all ages (Siegel et al., 2016). Yet, it is the metastatic spread and not the primary tumor that is responsible for the vast majority of all cancer related morbidity and mortality (Liotta & Stetler-Stevenson, 1993). Unfortunately the pathobiology of metastatic disease remains relatively poorly understood. There are however, recent data which suggests an underlying genetic component to the process of cancer spread.

The inbred mouse has long been used to model human cancers. Just as humans have unique genetic populations with distinct cancer incidence, mice have unique strains which show differences in susceptibility to cancers (Bult et al., 2015; Heston & Vlahakis, 1971; Koch et al., 2007; Szymanska et al., 2014). For example, offspring of C57BL/6J and BALB/cJ female mice mated with male transgenic mice carrying the Polyoma Virus middle T antigen have altered tumor latency and decreased metastatic burden compared to offspring of similarly mated FVB/NJ mice (Lifsted et al., 1998). The authors show dominant nuclear quantitative trait loci which affect the process of metastasis (Hsieh et al., 2009; Y. Hu et al., 2012; Lancaster, Rouse, & Hunter, 2005; Yang et al., 2005). These studies have shown that an individual's underlying genetics can affect their susceptibility to metastatic disease. There is however, an additional genome present in every cell, the mitochondrial genome.

In addition to the nuclear contributors, the mitochondrial genome may be influencing the process of metastasis. The offspring of the previous Polyoma Virus middle T studies carried the mitochondria of the female, and in so doing represent distinct mitochondrial haplotypes. In this vein, Ishikawa and colleagues showed metastatic characteristics followed the mitochondria in cybrid cells constructed from combinations of high and low metastatic nuclei and mitochondria

(Ishikawa, Koshikawa, et al., 2008; Ishikawa, Takenaga, et al., 2008). Together this data suggests that mitochondria may influence the process of metastasis.

In order to determine if mitochondria could be playing a role in this process, Mitochondrial Nuclear Exchange (MNX) mice were selected which have the same nuclear backgrounds, but different mitochondrial backgrounds. As previously described, these were created by enucleating a fertilized oocyte of one strain of mouse, leaving the cytoplasm and mitochondria, then transferring in a nucleus from another mouse strain (Fetterman et al., 2013). These mouse strains are designated as: Nuclear Background-mt$^{\text{MNX (Mitochondrial Background)}}$. For example, the MNX line FVB/NJ-mt$^{\text{MNX(C57BL/6J)}}$ was made with a FVB/NJ nucleus and C57BL/6J mitochondria.

We previously utilized the FVB/NJ-mt$^{\text{MNX(C57BL/6J)}}$ and FVB/NJ-mt$^{\text{MNX(BALB/cJ)}}$ MNX strains crossed with the FVB/N-Tg(MMTV-PyVT)634Mul/J (hereafter PyMT) and showed tumor latency patterns and metastasis size also tracked with mtDNA (Feeley et al., 2015). These data suggest that in addition to the nuclear DNA, mtDNA plays a role in both tumor onset as well as metastasis. An additional study utilizing a pancreatic model system has shown that nuclear modifiers are driver dependent (Winter et al., 2017). No such study has yet been performed to determine whether mitochondrial DNA effects on metastasis are oncogene specific.

Given the abundance of research showing retrograde communication between the mitochondria and nucleus (Biswas et al., 1999; Biswas, Anandatheerthavarada, Zaidi, & Avadhani, 2003; Liao, Small, Srere, & Butow, 1991; Luo, Bond, & Ingram, 1997; Parikh, Morgan, Scott, Clements, & Butow, 1987), we hypothesized that the effects of mtDNA on tumor latency and metastasis would vary with the nuclear encoded oncogenic driver. Through a series

of genetic crosses we found that mitochondrial haplotype altered tumor latency and metastasis in a manner dependent on the nuclear oncogene. In addition we found that once seeded the rate of tumor growth did not vary with mitochondrial background. Using an inverse breeding schema we also show that tumor latency and metastasis are not affected by any potential long lived cytoplasmic factors, which further indicates this phenomenon is predicated on active mitochondria-nuclear crosstalk.

Materials and Methods

Mouse Lines

Mitochondrial Nuclear Exchange (MNX) stable mouse lines were created as previously reported (Fetterman et al., 2013). Briefly, pronuclei were isolated from fertilized oocytes of FVB/NJ mice and transferred into enucleated fertilized oocytes of either C57BL/6J or BALB/cJ origin. Wild-type FVB/NJ, C57BL/6J, BALB/cJ, and FVB/N-Tg(MMTVneu)202Mul/J (Her2) mice were purchased from Jackson labs. MNX colonies were maintained by breeding MNX females with nuclear matched male mice. All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Kansas Medical Center (IACUC 2014-2215)

Mouse Breeding for Determination of Cytoplasmic Effects on Tumorigenesis and Metastasis

Her2 female mice were bred with eighth generation FVB/NJ-mt$^{MNX(C57BL/6J)}$ or FVB/NJ-mt$^{MNX(BALB/cJ)}$ males. Offspring were genotyped to ensure mitochondrial homoplasmy and

presence of transgene. Subsequent males were bred back to parental Her2 female mice. Female second generation offspring were genotyped to ensure homoplasmy and presence of transgene.

Mouse Breeding for Determination of mtDNA Effects on Tumorigenesis and Metastasis

Her2 male mice were bred with eighth generation FVB/NJ-mt$^{MNX(C57BL/6J)}$ or FVB/NJ-mt$^{MNX(BALB/cJ)}$ females. Offspring were genotyped to ensure mitochondrial homoplasmy and presence of transgene. These females were bred back to parental Her2 male mice. Female second generation offspring were genotyped to ensure homoplasmy and presence of transgene.

Genotyping

Tail clips of no more than 3 mm in length were collected from all breeding and experimental mice at weaning. DNA extraction was performed using REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich: XNAT-100). Restriction Fragment Length Polymorphism (RFLP) was used to ensure homoplasmic mitochondrial background as described (Feeley et al., 2015; Fetterman et al., 2013). Briefly, primers were designed to span regions of single nucleotide polymorphisms (listed below) which distinguish C57BL/6J (C9461T) and BALB/cJ (A9348G) mtDNA from FVB/NJ (Bayona-Bafaluy et al., 2003). The mutation at 9461 present in C57BL/6J mtDNA will not allow a restriction digest site for *Bcl1* to incorporate. Upon incubation with *Bcl1* (New England Biolabs: R0160S) cleavage will only occur in animals with FVB/NJ mitochondria (Figure 1A). The mutation at 9348 present in BALB/cJ mtDNA results in the incorporation of a restriction digest site for *Pflf1* (New England Biolabs: R0595S). Upon digest with *Pflf1*, cleavage will occur in animals with BALB/cJ mtDNA but not in those with FVB/NJ mtDNA (Figure 1B). Presence of the transgene was determined using the strain specific standard PCR genotyping protocol from the Jackson Labs product page. Briefly, total DNA isolated from

Figure 1

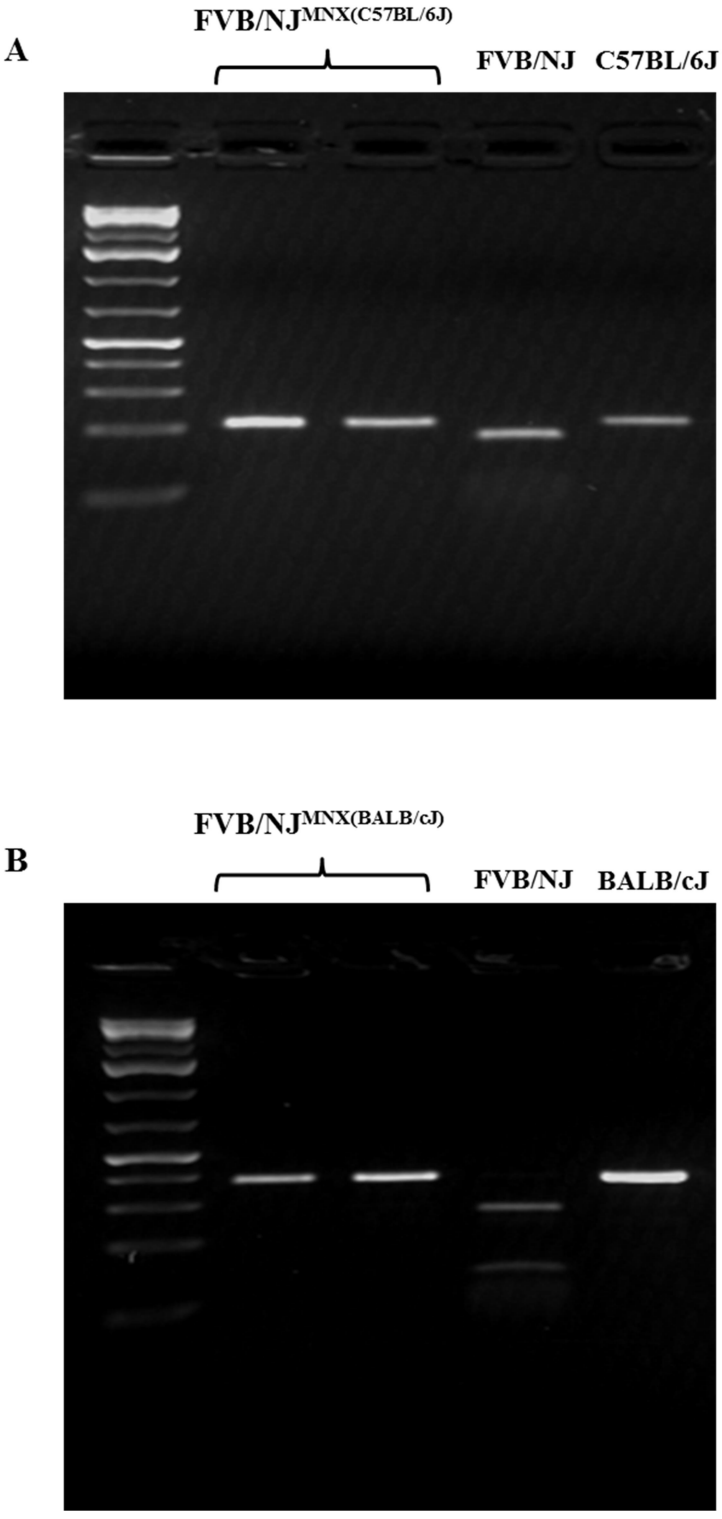

A
FVB/NJMNX(C57BL/6J)
FVB/NJ
C57BL/6J
B
FVB/NJMNX(BALB/cJ)
FVB/NJ
BALB/cJ

Figure 1: Genotyping of MNX strains**.** Representative gels showing genotyping of MNX and matching wild-type strains. Total DNA was isolated from Tail clips of weanling mice. DNA was amplified using primers that span **A:** 9461 C→T polymorphism in C57BL/6J mitochondria which does not allow the incorporation of a *Bcl1* restriction site, which is present in FVB/NJ or **B:** 9348 A→G polymorphism present in BALB/cJ but not FVB/NJ which does not allow the restriction site for *Pflf1* to incorporate, this DNA was then exposed to restriction enzymes, **A:** *Bcl1* and **B:** *Pflf1* and resolved on an agarose gel.

tail clips was utilized in a multiplexed PCR reaction using primers for the transgene as well as an internal reference site (Table 1).

Tumorigenicity

To determine tumor latency, beginning at 8 weeks of age mouse mammary glands were palpated daily and date of tumor onset was noted. Time from birth to tumor onset was recorded in days. Significance of tumor latency differences between groups was determined using the Kruskal-Wallis One Way Analysis of Variance on Ranks test followed by Dunn's Method, a pairwise multiple comparison procedure.

To determine tumor growth rates, the length (longest diameter) and width (diameter orthogonal to length) of the first tumor to arise was measured once per week for a period of 75 days. Tumor growth rate was determined as previously described (Hather et al., 2014). Briefly, tumor volumes were calculated using the modified formula for an ellipsoid:

$\frac{1}{2}$ (Length x width^2)

Since the rate based model assumes exponential growth, these measurements were log transformed, low volumes (<50 mm^3) were truncated, and log volume was plotted against time. Slopes were calculated and compared between groups using the Kruskal-Wallis test. Onset of additional tumors was noted and measured, but not utilized to determine growth rates.

Metastasis Measures

Mice were euthanized 75 days after tumor onset. Lungs were harvested, stained for one hour in Bouin's Solution (Sigma-Aldrich: HT10132), and rinsed twice in PBS. Since 90% of lung metastases are visible on the surface (Welch, Neri, & Nicolson, 1983) metastatic lesions were counted utilizing a dissecting scope. Due to the spherical nature of metastases

Table 1

Site	Direction	Sequence
9461	Forward	5' - TTC CAA TTA GTA GAT TCT GAA TAA ACC CAG AAG AGA GTG AT - 3'
	Reverse	5' - AAA TTT TAT TGA GAA TGG TAG ACG - 3'
9348	Forward	5' - CGA AAC CAC ATA AAT CAA GCC C - 3'
	Reverse	5' - CTC TCT TCT GGG TTT ATT CAG A - 3
MMTVneu Transgene	Forward	5’ – CGG AAC CCA CAT CAG GCC C – 3’
	Reverse	5’ – TTT CCT GCA GCA GCC TAC GC – 3’
MMTVneu Internal Reference	Forward	5’ – CAA ATG TTG CTT GCT TGG TG – 3’
	Reverse	5’ – GTC AGT CGA GTG CAC AGT TT – 3’

Table 1: Primer sequences for genotyping. Table showing sequences of primers used for RFLP analysis to distinguish between mitochondrial backgrounds and primers used for determination of presence or absence of Her2 transgene

(Welch et al., 1983) the diameter of each metastasis was measured using an ocular micrometer. A normal distribution was not assumed; therefor the Kruskal-Wallis test followed by Dunn's Method was applied to determine significance between all groups.

Results

During the process of MNX construction, oocytes are enucleated prior to transfer of a new nucleus from a donor strain. The result is that not only do the mitochondria of the previous strain remain, but the cytoplasm does so as well. In order to separate possible cytoplasmic effects on tumor latency and metastasis from mitochondrial effects, male FVB/NJ-mt$^{MNX(C57BL/6J)}$ and FVB/NJ-mt$^{MNX(BALB/cJ)}$ mice were bred to female Her2 mice as shown in Figure 2A and B. In this schema, male mice do not pass on the C57BL/6J or BALB/cJ mitochondria, but do pass on cytoplasm.

As seen in Figure 2C, there is no difference in tumor latency of second filial (F2) generation offspring from FVB/NJ-mt$^{MNX(C57BL/6J)}$ (n = 31) or FVB/NJ-mt$^{MNX(BALB/cJ)}$ (n = 31) founders compared to Her2 mice. Metastases from these mice were further quantified, and again, no significant difference between the F2 offspring and Her2 mice were observed in either number of metastases (Figure 3A; average number metastases FVB/NJ-mt$^{MNX(C57BL/6J)}$ 11 ± 3, FVB/NJ-mt$^{MNX(BALB/cJ)}$ 8 ± 3, Her2 15 ± 2) or size of metastases (Figure 3B; 0.9, 0.8, 0.8 respectively). These data indicate that residual cytoplasmic factors are not influencing the process of tumorigenesis or metastasis.

To determine if mtDNA effects are driver dependent, FVB/NJ-mt$^{MNX(C57BL/6J)}$ and FVB/NJ-mt$^{MNX(BALB/cJ)}$ MNX strains were chosen to mimic previous studies

Figure 9

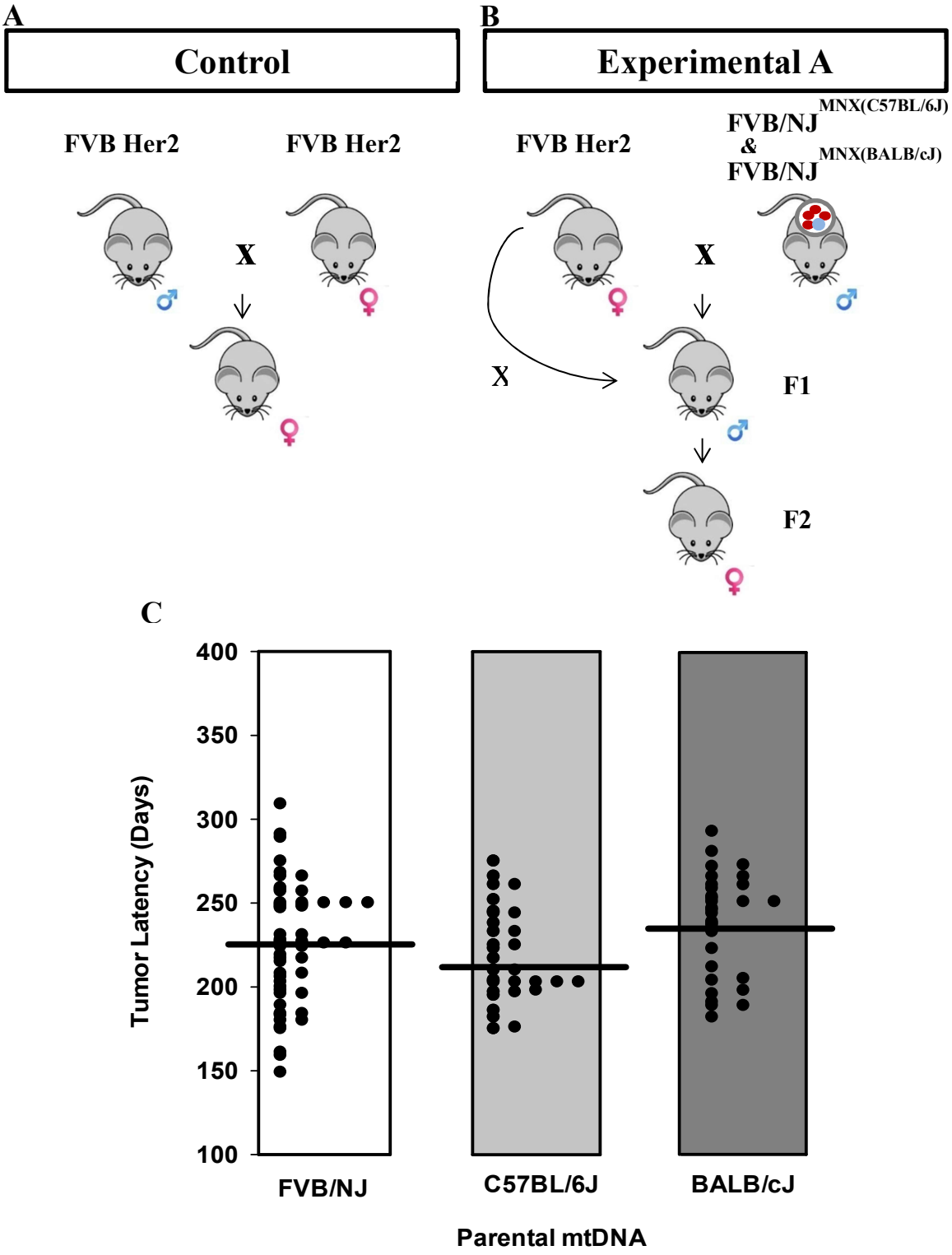

A
Control
FVB Her2
FVB Her2
X
B
Experimental A
FVB Her2
FVB/NJ MNX(C57BL/6J)
&
FVB/NJ MNX(BALB/cJ)
X
X
F1
F2
C
Tumor Latency (Days)
400
350
300
250
200
150
100
FVB/NJ
C57BL/6J
BALB/cJ
Parental mtDNA

Figure 2: Cytoplasmic factors from parental nuclear transfer do not alter tumor latency. **A:** To generate mice with cytoplasm but not mitochondria of MNX mice with desired nuclear oncogene, female Her2 mice were bred to male MNX with C57BL/6J or BALB/cJ mitochondria. Male offspring were bred back to female Her2 mice and second generation pups were utilized in further experiments. **B:** F2 offspring from mice mated as shown in A were observed for tumor latency. All mice carry FVB/NJ nuclear and mitochondrial DNA, but cytoplasm from parental C57BL/6J or BALB/cJ. Each dot is representative of a single animal while black bars indicate mean group tumor latency. FVB/NJ n=57, mean 225 days, SEM 5 days, C57BL/6J n=31, mean 219 days, SEM 5 days, BALB/cJ n=31, mean 238 days, SEM 6 days

Figure 3

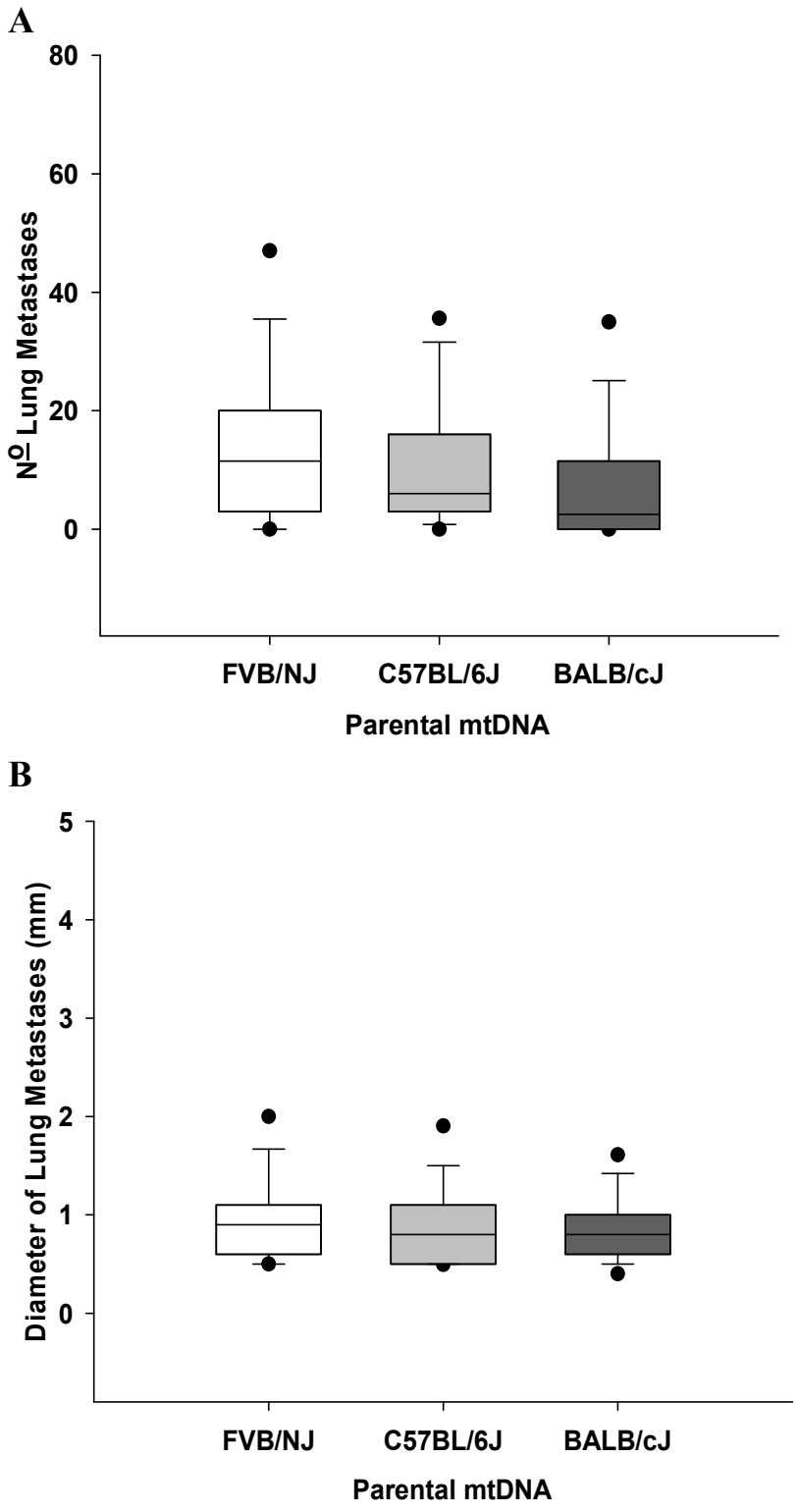

A
80
60
40
20
0
Nº Lung Metastases
FVB/NJ
C57BL/6J
BALB/cJ
Parental mtDNA
B
5
4
3
2
1
0
Diameter of Lung Metastases (mm)
FVB/NJ
C57BL/6J
BALB/cJ
Parental mtDNA

Figure 3: Cytoplasmic factors from parental nuclear transfer do not alter metastasis. Second generation pups were aged seventy-five days past tumor onset, **A:** gross metastases in lungs were counted (FVB/NJ n=54, mean 15, SEM 2, C57BL/6J n=23, mean 10, SEM 2, BALB/cJ n=18, mean 8, SEM 2) **B:** Diameter of gross metastases in lungs were measured using ocular micrometer (FVB/NJ mean 1.00 mm, SEM 0.02, C57BL/6J mean 0.90 mm, SEM 0.03, BALB/cJ mean 0.88 mm, SEM 0.03). Bottom of boxes are 25^{th} percentile, top of boxes are 75^{th} percentile, midline is median value, whiskers are maximum and minimum values of upper and lower fences respectively, dots represent statistically determined individual outliers.

(Feeley et al., 2015; Lifsted et al., 1998) and paired with the Her2 strain. Both MNX strains as well as the Her2 mice carry the same FVB/NJ nuclear background. The Her2 strain of mouse used here overexpresses the unactivated form of the proto-oncogene *neu* driven by the mammary tumor virus promoter and forms focal tumors in half of females with a relatively long latency. Of these tumor bearing females, 72% go on to form lung metastases (Guy, Webster, et al., 1992). To generate homozygosity of the transgene with selective mtDNA, a two-generation breeding protocol was required. Filial generation two female offspring were followed for tumor latency and growth rate, as well as metastasis (Figure 4A and B). Figure 4C shows that both FVB/NJ-$mt^{MNX(C57BL/6J)}$ and FVB/NJ-$mt^{MNX(BALB/cJ)}$ mice had statistically greater tumor latency than FVB/NJ mice. FVB/NJ-$mt^{MNX(BALB/cJ)}$ took significantly greater time to form tumors than even FVB/NJ-$mt^{MNX(C57BL/6J)}$ mice, with an average difference of 31 days (Table 2).

Several different endpoints could have been employed for this metastasis study. Since mitochondria altered the time to tumor onset, choosing to euthanize at a set age could have biased metastasis results by giving some tumors more or less time to metastasize. In addition, the effects of mitochondrial haplotype on tumor growth rate were not yet known, so euthanasia at a set tumor diameter was also eliminated. In order to limit confounding effects, mice were euthanized at a set number of days past first tumor onset. Sub-sets of mice were euthanized at 30, 60, 90, and 100 days past tumor onset. It was determined that 75 days gave large enough metastasis numbers to see variations, yet was a short enough period to minimize morbidity. Both age and tumor volumes were recorded at endpoint, however, upon review; neither factor altered the mitochondrial effects shown here.

To determine if mtDNA affected the growth rate of Her2 tumors, first primary tumors to arise in each mouse were measured weekly for the period of 75 days between tumor onset and

Figure 4

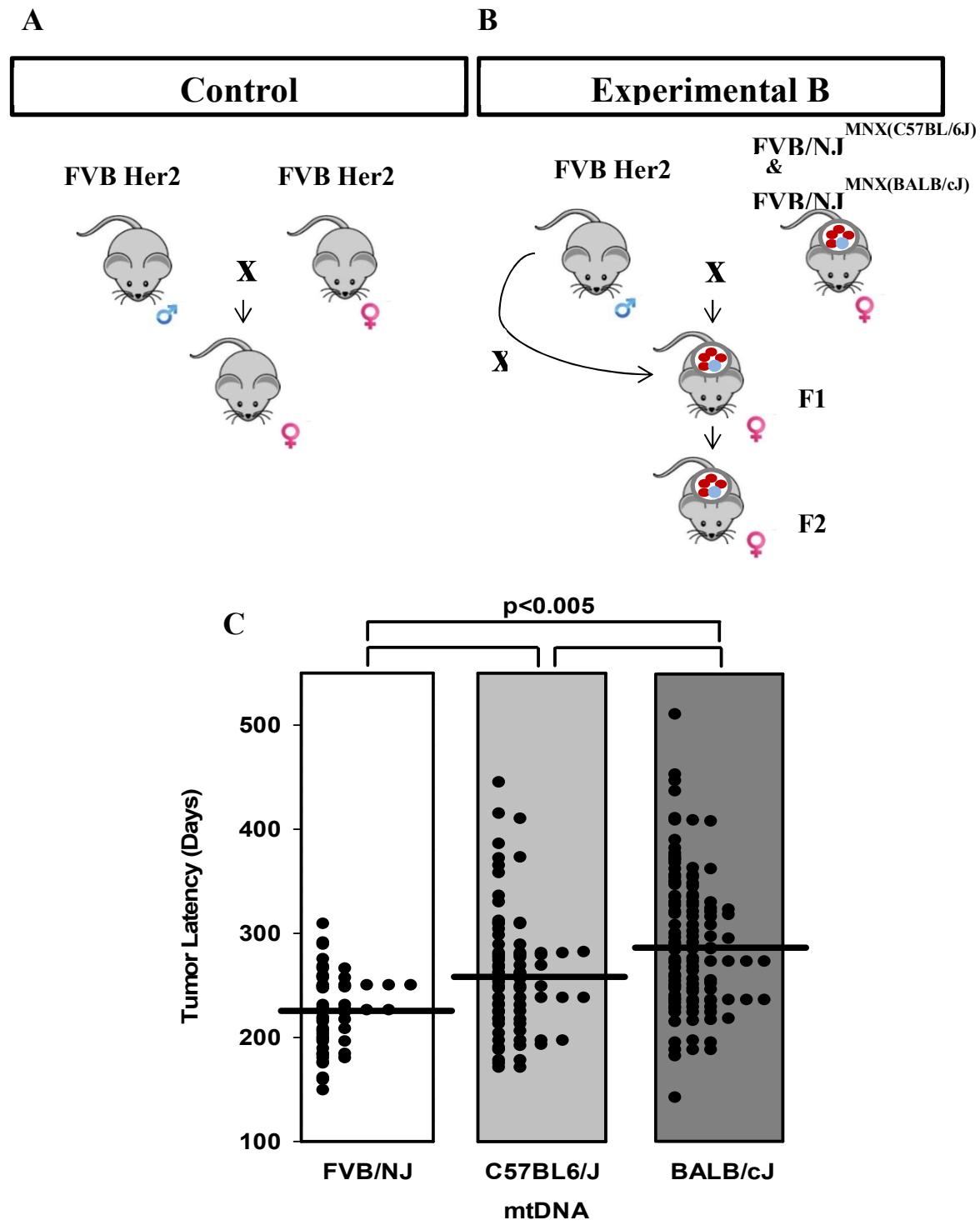

A
B
Control
Experimental B
FVB Her2
FVB Her2
X
FVB Her2
FVB/N.J MNX(C57BL/6J)
&
FVB/N.J MNX(BALB/cJ)
X
F1
F2
C
p<0.005
Tumor Latency (Days)
500
400
300
200
100
FVB/NJ
C57BL6/J
BALB/cJ
mtDNA

Figure 4: Mitochondrial haplotype alters tumor latency. **A:** Control mice were generated by breeding male and female Her2 mice. Female offspring were utilized in experiments **B:** To generate mice with desired nuclear oncogene and different mitochondrial background, male Her2 mice were bred to female MNX with C57BL/6J or BALB/cJ mitochondria. Female offspring were bred back to male Her2 mice and second generation pups were utilized in further experiments. **C:** Tumor latency of mice carrying FVB/NJ nuclear DNA with FVB/NJ (n=57, mean 225 days, SEM 5 days), C67BL/6J (n=78, mean 262 days, SEM 7 days) or BALB/cJ (n=119, mean 293 days, SEM 6 days) mitochondria. Each dot is representative of a single animal while wide black bars indicate mean group tumor latency.

Table 2

mtDNA	**N^{o} Mice**	**Latency ($\bar{x}$ ± SEM)**
FVB/NJ	57	225.175 +/- 4.61
C57BL/6J	78	262.167 +/- 6.73
BALB/cJ	119	293.487 +/- 6.00

Table 2: Tumor latency is increased by C57BL/6J and BALB/cJ mtDNA. Onset of first tumor was determined by palpation. The number of mice observed, mean tumor latency, and standard error of the mean for each mitochondrial group was recorded.

euthanasia. As shown in Figure 5, there is no statistical difference in rate of Her2 tumor growth in mice with different mtDNA haplotypes. Once the tumor has formed, it does not appear that these mitochondrial backgrounds play a role in tumor growth.

In order to explore whether mitochondrial haplotype affects metastasis, mice were euthanized 75 days post first tumor onset, then surface lung metastatic surface lesions were counted and measured. As Figure 6A shows, there are statistically fewer metastases ($p<0.001$) in both the C57BL6/J and BALB/cJ mitochondrial mice than the FVB/NJ mitochondrial mice, with the fewest (Table 3) in C57BL/6J. When diameters of metastatic lesions in Her2 mice were compared (Figure 6B), both the C57BL/6J and BALB/cJ mitochondrial mice had larger metastases (mean diameter 1.2 mm and 1.4 mm respectively, Table 3) than the FVB/NJ mitochondrial mice (mean diameter 1.0 mm). This data shows that both overall number as well as the size of metastatic lesions is affected by mitochondrial DNA.

Discussion

Previous studies implicate mitochondrial genetics as playing a role in tumorigenesis and metastasis, but have only utilized a single oncogenic driver (Feeley et al., 2015). The purpose of this study was to determine if breast tumorigenesis and metastasis are affected by mitochondrial haplotype in a driver dependent manner. By exchanging the nucleus of mouse strains promoting or inhibiting metastatic efficiency, mtDNA effects can be distinguished from phenotypes which would occur due to nuclear admixing. We show that mitochondrial haplotype causes differences in tumor onset and metastasis under the Her2 driver.

Figure 5

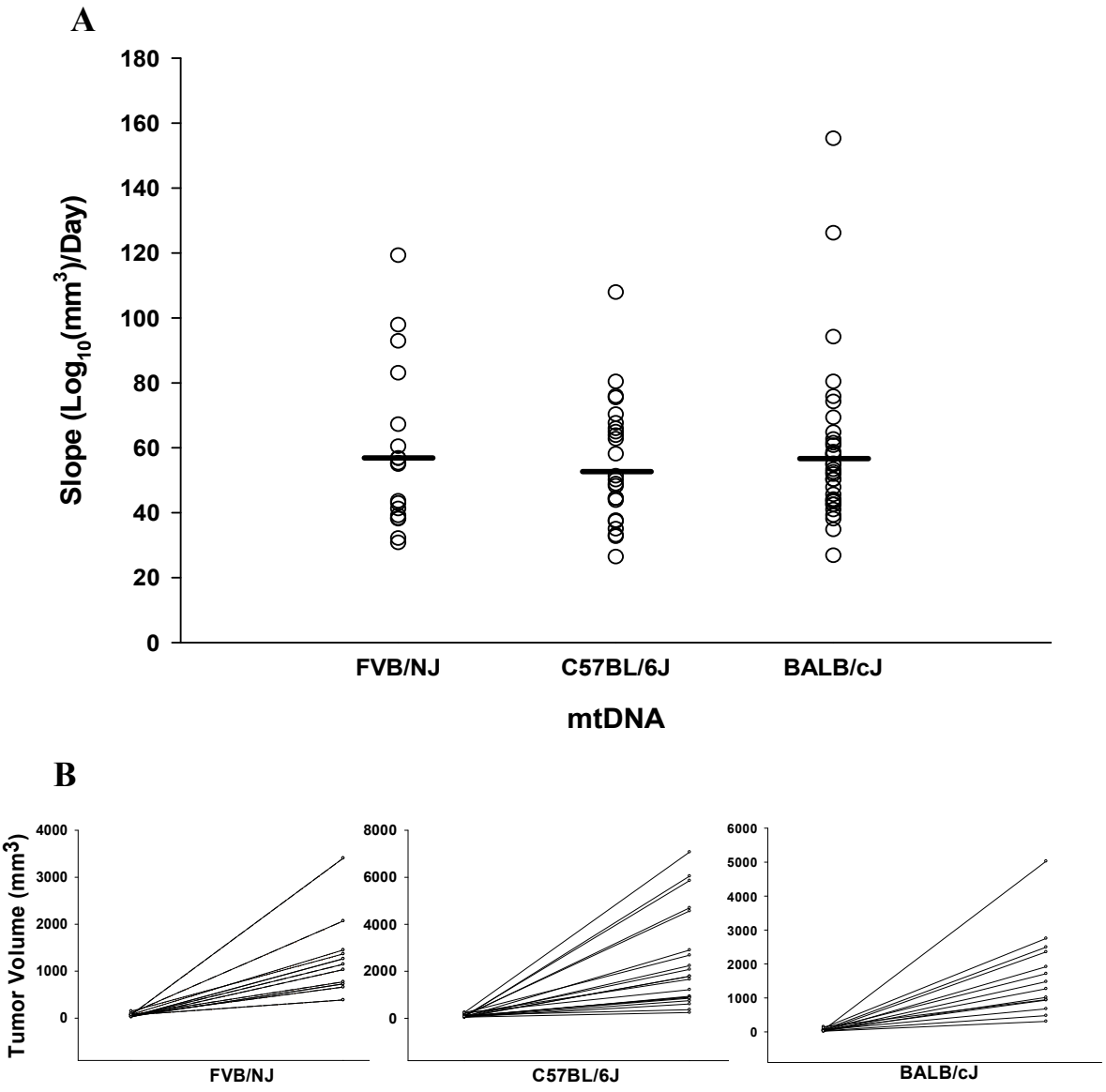

A
180
160
140
120
100
80
60
40
20
0
Slope (Log10(mm3)/Day)
FVB/NJ
C57BL/6J
BALB/cJ
mtDNA
B
Tumor Volume (mm3)
4000
3000
2000
1000
0
8000
6000
4000
2000
0
6000
5000
4000
3000
2000
1000
0
FVB/NJ
C57BL/6J
BALB/cJ

Figure 5: Mitochondrial DNA does not affect rate of primary tumor growth. First tumors to arise were measured once per week for seventy-five days, and tumor volumes were calculated. **A:** Each open circle represents the slope calculated from log transformed tumor volumes at each time point, wide black bars represent mean slope for each group (FVB/NJ mean 58, SEM 6, C57BL/6J mean 55, SEM 4, BALB/cJ mean 59, SEM 4). **B:** First and last tumor volumes for first tumors to arise were plotted.

Figure 6

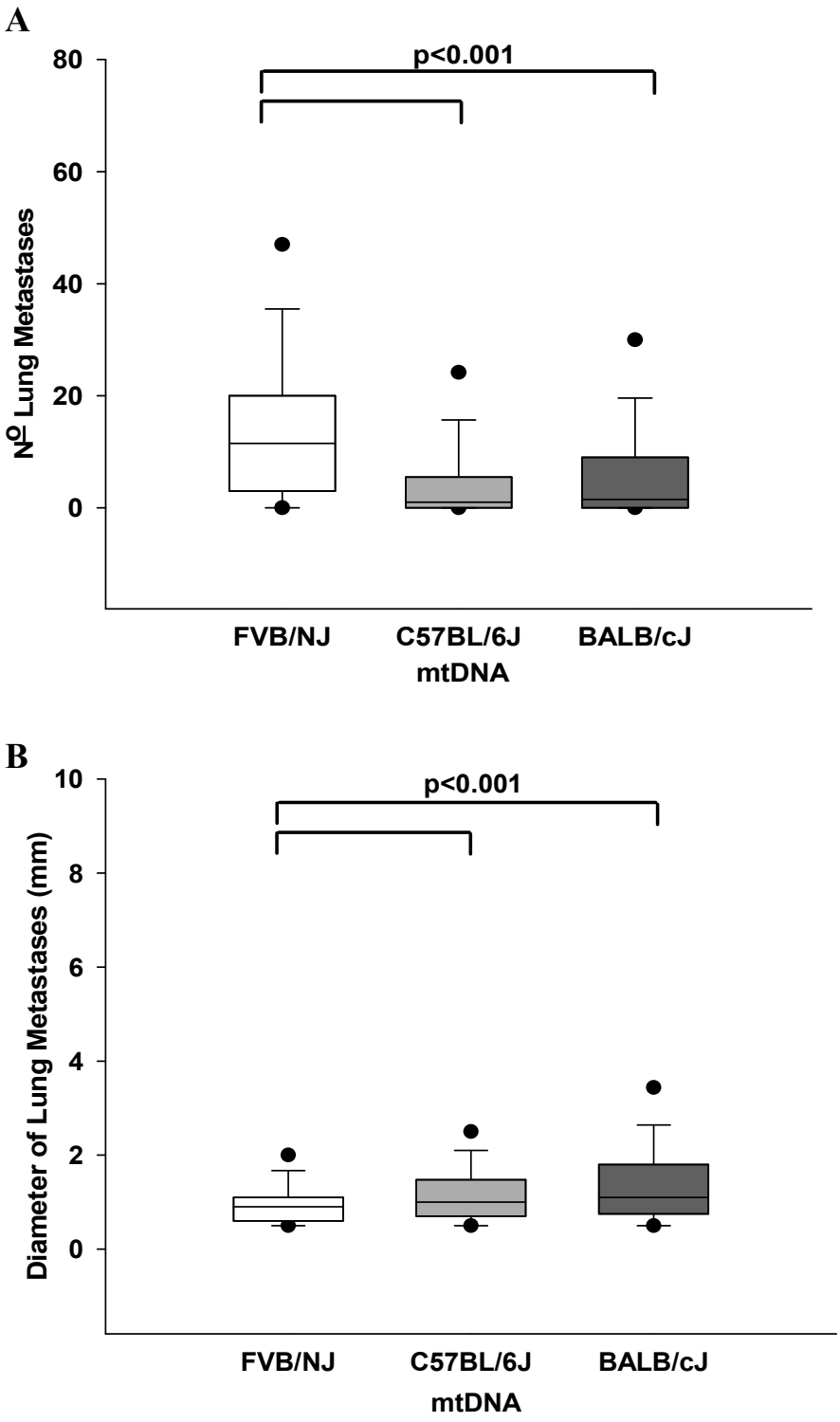

A
p<0.001
80
60
40
20
0
Nº Lung Metastases
FVB/NJ
C57BL/6J
BALB/cJ
mtDNA
B
p<0.001
10
8
6
4
2
0
Diameter of Lung Metastases (mm)
FVB/NJ
C57BL/6J
BALB/cJ
mtDNA

Figure 6: Metastasis is altered by mitochondrial DNA. Second generation pups were aged seventy-five days past tumor onset, **A:** gross metastases in lungs were counted (FVB/NJ n=54, mean 15, SEM 2, C57BL/6J n=52, mean 5, SEM 1, BALB/cJ n=76, mean 6, SEM 1). **B:** Diameter of gross metastases in lungs were measured using ocular micrometer (FVB/NJ mean 1.00 mm, SEM 0.02, C57BL/6J mean 1.23 mm, SEM 0.06, BALB/cJ mean 1.41 mm, SEM 0.04). Bottom of boxes are 25th percentile, top of boxes are 75th percentile, midline is median value, whiskers are maximum and minimum values of upper and lower fences respectively, dots represent statistically determined individual outliers.

Table 3

mtDNA	Nº Mice with Metastases	Total Metastases	Nº Metastases ($\bar{x}$ ± SEM)	Metastases Size ($\bar{x}$ ± SEM)
FVB/NJ	45	802	15 +/- 2.07	1.00 +/- 0.02
C57BL/6J	26	224	5 +/- 1.14	1.23 +/- 0.06
BALB/cJ	39	525	7 +/- 1.22	1.41 +/- 0.04

Table 3: Total metastatic burden and size are altered by mtDNA. Seventy-five days after day of first tumor onset, mice were euthanized and lungs were analyzed for number and size of metastases. The total number of mice observed, the total number of metastases in each group and the mean and standard error of the mean were recorded for each mitochondrial group.

In order to evaluate cytoplasmic factors present in our MNX models and determine if they are confounding the tumorigenesis and metastasis data presented here, we bred male MNX to our female Her2 parental line. The resulting second generation pups theoretically carry any interfering cytoplasmic factors in relatively similar amounts to the simultaneously bred experimental pups, but do not carry different mitochondrial backgrounds. We show that neither tumorigenesis, nor metastatic number or size differed among these groups. In addition this implies that the nuclear genomes were not permanently altered in the parental generation.

Surprisingly, relative tumor latency with the same mitochondrial backgrounds under Her2 did not always match those from PyMT studies (Feeley et al., 2015). Mice with C67BL/6J mtDNA had a mean tumor latency that was 16% greater than FVB/nJ under the Her2 driver, which is similar to those under the PyMT driver which showed a delay of 13%. In contrast, Her2 mice with BALB/cJ mtDNA took 30% more days to form tumors while the PyMT BALB/cJ mtDNA mice took 7% fewer days to form tumors than FVB mitochondrial mice (Feeley et al., 2015). This suggests that mtDNA is able to affect tumor latency, but it does so in an oncogene-dependent manner.

Once tumor initiated, no difference was found among the mitochondrial backgrounds under either the Her2 or PyMT oncogenes. This suggests that once formed, mitochondria are not influencing the rate of tumor growth under these drivers. This data together with the tumor latency data indicates that mitochondria's role in tumorigenesis may be limited to early stages. Further study into mitochondria's effects, specifically on transformation, is needed.

In addition to tumor latency, we explored mitochondrial DNA's effects on metastasis. We show that mice with mitochondria from C57BL/6J mice had the fewest overall number of metastases compared to both BALB/cJ and FVB/NJ mitochondrial mice, with BALB/cJ mice still having significantly fewer metastases than FVB/NJ mice. This is in striking contrast to the PyMT crosses which did not show a significant difference in number of metastases between any of the groups. With both Her2 and PyMT drivers, metastatic size differed between the mitochondrial groups. Under the Her2 oncogene the FVB/NJ mitochondrial group had the smallest metastases while under the PyMT oncogene the C57BL/6J mitochondrial group had the smallest metastases. Taken together this data shows that metastatic seeding and growth can be influenced by mitochondria and that process can be dependent upon the nuclear oncogenic driver.

The Her2 and PyMT oncogenes have very different defining characteristics. They differ greatly in time to tumor onset and time to metastatic spread. The Her2 oncogene has relatively long tumor latency with an average of four months and only 37% of females with metastasis at eight months of age (Guy, Webster, et al., 1992). In contrast, PyMT has rapid tumor onset at an average of 30 days with 94% of females presenting with metastasis at 3 months of age (Guy, Cardiff, & Muller, 1992). The lack of change in tumorigenesis and metastasis in the cytoplasmic control animals implies that the BALB/cJ and C57BL/6J haplotype effects in experimental animals may be predicated on active mitochondrial-nuclear crosstalk. Perhaps the mechanism behind BALB/cJ mitochondria's effect on tumorigenesis and metastasis requires a lengthier exposure to the oncogenic driver, while C57BL/6J's mechanism is highly penetrant and fast acting. With the vastly different oncogenic phenotypes of the Her2 and PyMT models, it seems

logical that the mitochondrial effects on tumorigenesis and metastasis that we have shown are dependent upon the drivers present.

Data presented here underscore the importance of the mitochondrial genome's interaction with nuclear oncogenic drivers. Although under development (Bacman, Williams, Pinto, Peralta, & Moraes, 2013; Gammage et al., 2016; Jo et al., 2015; Minczuk, 2010; Minczuk, Papworth, Miller, Murphy, & Klug, 2008) there are currently no genome editing tools that can completely knock out a single mitochondrial gene due to the complexity and redundancy of the mitochondria genome. This complicates our ability to define exactly which variants or groups of variants in each mitochondrial haplotype are responsible for the different susceptibilities to tumorigenesis and metastasis. However as these tools are refined, it is our hope that this research can pinpoint inherited mitochondrial variants which when combined with nuclear variations, would better predict patient outcomes and aid in treatment planning.

Chapter III: Mitochondrial haplotype of the host environment alters metastasis in a non-cell autonomous manner

Abstract

Mitochondrial Nuclear Exchange (MNX) mice, created by transferring the nucleus from an oocyte from strain x into an enucleated oocyte of strain y, showed that mammary tumor formation and metastasis can be regulated by inherited mitochondrial polymorphisms in an oncogene dependent manner. These studies utilized a model where both tumor and stromal cells have altered nuclear-mitochondrial composition. We now hypothesize that mitochondrial polymorphisms in non-cancer compartments could exert effects on tumor formation or metastasis. Tumor cells were injected into wild-type mice (control) and tumor growth and metastasis were compared to cells injected in syngeneic MNX mice sharing the same nuclear but different mtDNA backgrounds. Compared to intravenous injections in to wild-type mice, EO771 mammary carcinoma and B16-F10 melanoma cells (both syngeneic to C57BL/6J), formed significantly ($p<0.01$) more lung metastases in C57BL/6J-$mt^{MNX(C3H/HeN)}$ while K1735-M2 melanoma cells (syngeneic to C3H/HeN) formed significantly fewer lung metastases in C3H/HeN-$mt^{MNX(C57BL/6J)}$. These results have been replicated three times using ≥ 10 mice per experiment. Interestingly, C57BL/6J mitochondria confer resistance to metastasis in both cell autonomous and non-cell autonomous experiments. Initial *in-vivo* studies show mitochondrial superoxide scavenging of host prior to K1735-M2 or EO771 seeding decreases the incidence of metastasis in C3H/HeN mitochondrial mice to the same levels as C57BL/6J mitochondrial mice. *Ex-vivo* studies utilizing lung tissue from wild-type and MNX strains show differences in expression of nuclear encoded genes dependent upon the mitochondrial-nuclear pairings which are influenced by superoxide scavenging. These data suggest that mitochondrial haplotype can influence mitochondrial-nuclear communication. This modified cross-talk could result in alterations of nuclear encoded signaling pathways which mediate a tumor cell's ability to

metastasize in that environment. Together, our findings highlight the striking influence that mitochondrial-nuclear crosstalk can exert on tumorigenicity and metastasis via both intrinsic and extrinsic mechanisms.

Introduction

Otto Warburg's discovery that cancer cells utilize glycolysis followed by lactic acid fermentation over oxidative phosphorylation (Warburg et al., 1927) has helped fuel research into mitochondria's role in tumor formation and progression. It has since been shown that mitochondria contribute to tumor growth through multiple metabolic pathways, regulation of extracellular pH, calcium signaling, and apoptosis (Payen, Porporato, Baselet, & Sonveaux, 2016; Porporato, Payen, Baselet, & Sonveaux, 2016; Wallace, 2012; Wang & Youle, 2009; Weinberg & Chandel, 2009). Mitochondria have also been implicated in metastasis. Several studies showed cybrid cells containing mitochondria from aggressively metastatic cell lines but nuclei from non-metastatic cell lines were aggressively metastatic (Ishikawa, Koshikawa, et al., 2008; Ishikawa, Takenaga, et al., 2008; Kaipparettu et al., 2013). These data together with our previous research implicate mitochondria present within a cancer cell play a role in determining that cells tumorigenicity and metastatic propensity.

Stephen Paget proposed that in addition to the cancer cell itself which he termed the "seed", the secondary microenvironment, the "soil", also plays a role in determining whether cancer cells successfully metastasize to an area (Paget, 1989). Fidler and colleagues went on to show that although vascular arrest plays a role in metastatic distribution, which was espoused by Virchow (Virchow, 1989), without the proper microenvironment, metastatic cells will fail to

colonize (Hart & Fidler, 1980). We hypothesize that mitochondria contribute to the ability of the secondary site to support or undermine the growth of metastases.

The Mitochondrial Nuclear Exchange mice present a unique opportunity to study host mitochondrial effects. MNX mice are made by enucleating a fertilized oocyte of one mouse strain, then transferring in a nucleus from a donor oocyte of a different mouse strain (Feeley et al., 2015; Fetterman et al., 2013; Kesterson et al., 2016). For ease of reading, we have abbreviated the MNX strains as can be seen in Table 1. By pairing known mouse metastatic cell lines with MNX strains that match the nuclear component of the cells but have different mitochondria (Table 2), we can study mitochondrial host effects on metastasis *in-vivo*.

We have utilized four different MNX strains and their wild-type counterparts in a series of experimental and spontaneous metastasis assays. We show that mitochondria in non-cancer compartments can affect the process of metastasis in both mammary and melanoma models. In addition we utilize a series of *in-vitro* approaches to show variations in mitochondrial load, membrane potential, mitochondrial DNA copy number and metabolic profiles of mitochondria in the MNX and wild-type host tissue. We show that reactive oxygen species (ROS) scavenging of host tissue *in-vivo* alters the metastatic propensity of these tissues. Finally, we use *ex-vivo* approaches to analyze expression of metastatic genes with and without ROS scavenging to show that different mitochondrial backgrounds result in differential nuclear gene regulation. In turn we show that nuclear gene expression can be altered by mitochondrial ROS levels. Taken together this data shows for the first time that mitochondria in the metastatic secondary environment, affects the ability of metastatic cells to colonize. Although a defined mechanism for this observation has yet to solidify, our data indicate that ROS and mito-nuclear crosstalk play a role.

Table 1

Strain	Nuclear DNA	Mitochondrial DNA	Abbreviation
FVB/NJ-mt$^{MNX(FVB/NJ)}$	FVB/NJ	FVB/NJ	FF
FVB/NJ-mt$^{MNX(C57BL/6J)}$	FVB/NJ	C57BL/6J	FC
FVB/NJ-mt$^{MNX(BALB/cJ)}$	FVB/NJ	BALB/cJ	FB
BALB/cJ-mt$^{MNX(BALB/cJ)}$	BALB/cJ	BALB/cJ	BB
C57BL/6J-mt$^{MNX(C57BL/6J)}$	C57BL/6J	C57BL/6J	CC
C57BL/6J-mt$^{MNX(C3H/HeN)}$	C57BL/6J	C3H/HeN	CH
C3H/HeN-mt$^{MNX(C3H/HeN)}$	C3H/HeN	C3H/HeN	HH
C3H/HeN-mt$^{MNX(C57BL/6J)}$	C3H/HeN	C57BL/6J	HC

Table 1: Mouse strain nomenclature and abbreviations. Mitochondrial nuclear exchange mice were made by enucleating an oocyte of one mouse strain and transferring in a nucleus from a donor oocyte. Nuclear and mitochondrial contributors are listed next to their respective MNX strain. Abbreviations for each strain are defined.

Materials and Methods

Mouse Lines

Mitochondrial Nuclear Exchange (MNX) stable mouse lines were created as previously reported (Kesterson et al., 2016). Briefly, pronuclei were isolated from fertilized oocytes of FVB/NJ mice and transferred into enucleated fertilized oocytes of either C57BL/6J or BALB/cJ origin. Additional lines were created by exchanging pronuclei between oocytes of C57BL/6J origin and C3H/HeN origin. Wild-type FVB/NJ, C57BL/6J, BALB/cJ mice were purchased from Jackson labs, while C3H/HeN were purchased from Harlan laboratories. MNX colonies were maintained by breeding MNX females with nuclear matched male mice. All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Kansas Medical Center (IACUC 2014-2215)

Genotyping

Tail clips of no more than 3 mm in length were collected from all breeding and experimental mice at weaning. DNA extraction was performed using REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich: XNAT-100). Restriction Fragment Length Polymorphism was used to ensure homoplasmic mitochondrial background as described (Feeley et al., 2015; Fetterman et al., 2013). Briefly, primers were designed to span regions of single nucleotide polymorphisms (listed below) which distinguish C57BL/6J (C9461T) mtDNA from FVB/NJ and C3H/HeN, and BALB/cJ (A9348G) mtDNA from FVB/NJ (Bayona-Bafaluy et al., 2003). The mutation at 9461 present in C57BL/6J mtDNA will not allow a restriction digest site for *Bcl1* to incorporate. Upon incubation with *Bcl1* (New England Biolabs: R0160S) cleavage will only occur in animals with FVB/NJ or C3H/HeN mitochondria. The mutation at 9348 present in BALB/cJ mtDNA results in the incorporation of a restriction digest site for *Pflf1* (New England Biolabs: R0595S). Upon

digest with *Pflf1*, cleavage will occur in animals with BALB/cJ mtDNA but not in those with FVB/NJ mtDNA.

In-Vivo **Metastasis Assays**

For experimental metastasis assays, syngeneic mouse metastatic cancer lines were injected into the tail vein of MNX and nuclear matched wild-type mice at four weeks of age (Table 2). Following an incubation of 3 (Pei1) or 2 (EO771, B16-F10, K1735-M2) weeks, mice were euthanized and lungs were harvested upon necropsy.

For spontaneous metastasis assays, syngeneic mouse metastatic cancer lines were ectopically injected (mammary fat pad for Pei1 and EO771 cells, intradermal for B16-F10 and K1735-M2) into 6 week old MNX and nuclear matched wild-type mice. Tumors were measured with digital calipers every other day and mice were euthanized when tumors reached an average diameter of 15 mm. Lungs were harvested upon necropsy.

Metastasis Measures

For non-melanotic cell lines (Pei1, EO771, K1735-M2), lungs were stained for one hour in Bouin's Solution (Sigma-Aldrich: HT10132), and rinsed twice in PBS. Melanotic B16-F10 treated lungs were harvested then rinsed three times in PBS. Since 90% of lung metastases are visible on the surface (Welch et al., 1983) lungs were photographed and metastatic lesions were counted utilizing a dissecting scope. The Kruskal-Wallis one way analysis of variance on ranks followed by Dunn's Method of pairwise multiple comparison was utilized to determine significance between MNX and wild-type groups.

Table 2

Cell Line	Cancer Type	Origin	Mouse Strain	Cell Nº Experimental	Cell Nº Spontaneous	Incubation Period Experimental
Pei1	Mammary	FVB	FF, FB, FC	$5x10^4$	$5x10^4$	3 weeks
EO771	Mammary	C57BL/6	CC, CH	$5x10^4$	$1x10^5$	2 weeks
B16-F10	Melanoma	C57BL/6	CC, CH	$1x10^5$	$1x10^5$	2 weeks
K1735-M2	Melanoma	C3H	HH, HC	$2x10^4$	$2x10^5$	2 weeks

Table 2: Metastatic cell line origin, dosing, and incubation. Metastatic cell lines were paired with MNX mice which have matching nuclear backgrounds. These cells were injected into the tail vein (experimental) and ectopically (spontaneous) at the indicated dose. Time for metastatic formation used in experimental assays is listed.

Mitochondrial Load and Membrane Potential

In order to test mitochondrial load and membrane potential, mouse embryonic fibroblasts (MEF) were generated from all MNX and wild-type strains. MEF lines were passaged once in culture then harvested and stained individually and in combination with MitoTracker Red CMXRos (Molecular Probes by Invitrogen: M7512) and MitoTracker Green FM (Molecular Probes by Invitrogen: M7514) fluorescent probes followed by analysis of fluorescence using a flow cytometer as described previously (Poot, 2001). Briefly, 100,000 cells from each line were added to cytometer tubes, and then stained with 200 nM of each probe alone and in combination. Cells from each line were also left as unstained controls. Cells were protected from light and dye was allowed to incubate for fifteen minutes at thirty seven degrees Celsius. Cells were then placed on ice. Flow cytometry was performed with parameters from Molecular Probes website (MitoRed: Excitation-579 and Emission-599, MitoGreen: Excitation-490 and Emission-516). Fluorescence intensity at the appropriate emission wavelengths was captured.

Metabolic Profiling

Mitochondrial flux was determined using the XF Seahorse Bioanalyzer and XF Cell Mitochondrial Stress Test Kit (Seahorse Bioscience: 101706) as described in the provided kit user guide. Briefly, MEF lines were cultured and passaged once prior to plating 40,000 cells per well in the provided Seahorse Plate. Cells were allowed to attach overnight. Sensor cartridge was loaded with Seahorse calibrant and placed at thirty seven degrees Celsius without CO_2 overnight. Cells were checked for confluent seeding then media was replaced with warmed unbuffered DMEM at pH 7.35. Cell plate was then incubated at thirty seven degrees Celsius without CO_2 for one hour. Measurements of oxygen consumption were taken before and after injections of

oligomycin, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), antimycin A and rotenone (5 µM, 3 µM, 1.2 µM, and 1 µM respectively). After Seahorse analysis was completed, cells were lysed and total protein concentration was analyzed using the Bradford method. Area under the curve for all measurements was normalized to total protein content.

Glycolytic flux was also determined using the XF Seahorse Bioanalyzer with a subset of MNX MEF lines. The XF Glycolysis Stress Test Kit (Seahorse Bioscience: 103020) was utilized as described in the kit user guide. Briefly, MEF cells and sensor plate were loaded and incubated as listed above. Measurements of oxygen consumption and extracellular acidification were taken before and after injections of glucose, oligomycin, 2-deoxy glucose (2-DG), antimycin A, and rotenone (10 mM, 1 µM, 100 mM, 2 µM, 1 µM respectively). Cells were lysed and total protein concentration was used to normalize all oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) measurements.

Significance was determined by first determining equal variance through the Brown-Forsythe test followed by the Holm-Sidak pairwise multiple comparison test.

Total DNA Isolation

Total cellular DNA was isolated utilizing procedures that minimize loss of mitochondrial DNA as described previously (Guo, Jiang, Bhasin, Khan, & Swerdlow, 2009). Briefly, equal weights of lung tissue were homogenized in a Tris/EDTA/SDS lysis buffer while on ice. Proteinase K (NEB: P81025S) was added to homogenate, samples were vortexed then incubated at fifty five degrees Celsius for two hours. Samples were centrifuged at 16,000 x g for 15 minutes and supernatant was collected in new microcentrifuge tubes. Phenol:chloroform:isoamyl alchohol 25:24:1 solution (Sigma P3823) was added to supernatants and vortexed briefly.

Samples were centrifuged and supernatant was collected in a new tube. Chloroform was added to supernatant, gently mixed by inversion (20x) then centrifuged. Supernatants were transferred into new tubes and sodium acetate and isopropanol were added. Samples were mixed gently by inversion (10x) and incubated at negative twenty Celsius overnight to allow DNA precipitation. Samples were centrifuged and supernatant was discarded. DNA pellet was washed twice with seventy percent ethanol and air dried. DNA was suspended in nuclease-free water and DNA quantity and quality was analyzed via Nanodrop.

Mitochondrial DNA Content

In order to determine mitochondrial DNA copy number, lungs were harvested from untreated four week old MNX and wild-type mice and snap frozen. Total cellular DNA was isolated utilizing the procedure listed in the previous section. Five nanograms of DNA was utilized in each triplicate qPCR reaction with Taqman Fast Advanced MasterMix (Applied Biosystems: 4444557) and mouse specific Taqman probes. Expression levels of the nuclear encoded 18s ribosomal RNA (18s, ThermoFisher: Mm04277571), and both mitochondrial encoded genes NADH dehydrogenae subunit 1 (ND1, ThermoFisher: Mms04225274), and mouse cytochrome c oxidase subunit II (COX2, ThermoFisher: Mm03294838) were obtained using the Life Technologies ViiA 7 qPCR instrument. Fold change for the mitochondrial genes was calculated and normalized to fold change for nuclear 18s. To determine significance of mitochondrial content between mouse strains a Kruskal-Wallis One Way Analysis of Variance on Ranks was performed for both COX2 and ND1.

***In Vivo* Metastasis Assays with Reactive Oxygen Species Scavenging**

Four week old MNX mice were randomized into two groups, control and treatment. This process was repeated with four week old nuclear-matched wild-type mice. Treatment group

animals were intra-peritoneal (IP) injected with 0.7mg/kg of the mitochondrial specific superoxide scavenger MitoTEMPO (Enzo Life Sciences: ALX-430-150-M005) in 0.1% Dimethyl sulfoxide (DMSO) twenty four and again one hour prior to metastatic cell line injection. Control group animals received intra-peritoneal injections of 0.1% DMSO at twenty four and one hour prior to metastatic cell line injection. Metastatic cells (K1735-M2 cells into HH and HC mice, EO771 cells into CC and CH mice) were introduced via the tail vein and allowed to incubate as described in Table 2. Mice were euthanized and lungs were harvested, stained, and metastases counted as listed in previous section. Significance of metastasis number between all groups was determined using the The Kruskal-Wallis one way analysis of variance on ranks followed by Dunn's Method of pairwise multiple comparison.

Gene Expression Analysis of TEMPO Treated and Untreated Mouse Lung

Four week old MNX mice were randomized into two groups, control (receiving 0.1% DMSO) and treatment (receiving 0.7 mg/kg MitoTEMPO in 0.1% DMSO). This process was repeated with four week old nuclear-matched wild-type mice. Groups received vehicle or MitoTEMPO intra-peritoneal injections, twenty four hours later groups then received a second injection. One hour later mice were euthanized; lungs were harvested and then snap frozen. Lungs were homogenized using the Bead Bug Microtube Homogenizer. RNA was harvested using the Rneasy Microarray Tissue Kit (Qiagen: 73304) with Qiagen RNase-Free DNase set (Qiagen: 79254). cDNA was synthesized using the iScript cDNA Synthesis Kit (BioRad: 1708891). qPCR was performed in triplicate using the ViiA7 from Life Technologies, with ThermoFisher TaqMan Fast Advanced MasterMix and ThermoFisher mouse specific Taqman Primers for microRNA (miR) 199a (Mm04238139), miR125b (Mm04238249), fam120a

(Mm01327068), dnmt1 (Mm01151063), ago2 (Mm03053414), sgtb (Mm00522889), scai (Mm00618853), rab6b (Mm00620652), and sod2 (Mm01313000).

Results

In order to determine if metastasis is affected by the host mitochondria, metastatic cells were introduced into MNX mice that had the same nuclear genomic background, but different mitochondrial genomic background. As a control, the metastatic cells were injected back into wild-type mice from which the cell lines originated, and therefore have the same nuclear and mitochondrial genetic background as the cell line. These cells were introduced to the mice by two methods. The cells were directly injected into the vasculature via the tail vein in an experimental metastasis assay. The cells were also introduced in orthotopic injections in order to recapitulate the entire metastatic cascade from tumor formation through colonization in a spontaneous metastasis assay. The spontaneous method gives a picture of the entire metastatic cascade; however, if tumor formation is affected, then metastasis effects are hard to tease out. When paired with the experimental metastasis assay which bypasses tumor formation steps, a more complete picture of metastasis is achieved. Each experimental and spontaneous assay was repeated three independent times with a minimum of 10 mice per group. Representative experiments are shown.

Pei1 metastatic mammary cells form a greater number of lung metastases in control FVB/NJ animals than in BALB/cJ or C57BL/6J mitochondrial mice. As seen in Figure 1A, thirteen FF mice had an average of 33 metastases while eleven FB mice had an average of 11 metastases in the experimental assays. In spontaneous metastasis assays (Figure 1C)

Figure 1

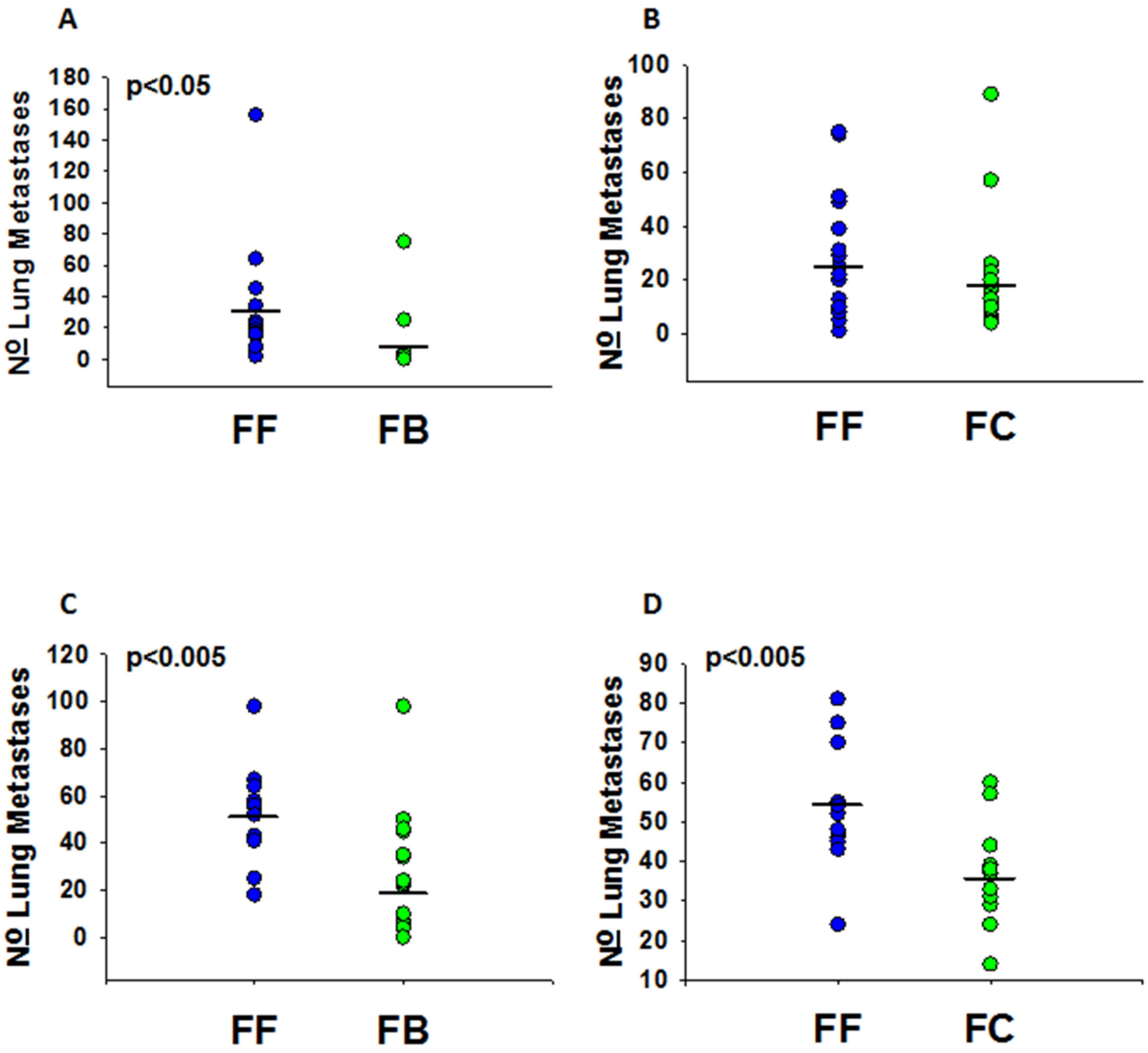

A
p<0.05
Nº Lung Metastases
180
160
140
120
100
80
60
40
20
0
FF
FB
B
Nº Lung Metastases
100
80
60
40
20
0
FF
FC
C
p<0.005
Nº Lung Metastases
120
100
80
60
40
20
0
FF
FB
D
p<0.005
Nº Lung Metastases
90
80
70
60
50
40
30
20
10
FF
FC

Figure 1: Mitochondrial haplotype alters total pulmonary metastases of mammary cells in spontaneous and experimental models. Pei1 metastatic mouse mammary cells were injected into the **A & B:** tail vein and mice were euthanized three weeks after injection. **C & D**: Pei1 cells were also injected into the mammary fat pad and lungs were harvested once primary tumors reached 15 mm in average diameter. Upon euthanasia lungs were harvested and gross metastases were counted. Individual dots represent total number of metastases in each individual animal, black bars represent mean for the mitochondrial group. **A:** Tail vein injected FF mice (n=13 mice) had more (mean 33, SEM 11) metastases than FB mice (n=11, mean 9, SEM 7). **B:** FF and FC tail vein injected mice did not differ in their total number of metastases (n=20, mean 25, SEM 5 and n=21, mean 18, SEM 4 respectively). **C:** Orthotopically injected FF mice (n=14) had more metastases (mean 52, SEM 5) than similarly injected FB mice (n= 20, mean 20, SEM 6). D: Orthotopically injected FF mice (n=13) had more metastases (mean 55, SEM 4) than similarly injected FC mice (n=14, mean 36, SEM 3).

the FF mice had an average of 52 metastases (n=14), while FB mice had an average of 20 metastases (n=20). Pei1 cells also show a greater number of metastases in the spontaneous assay (Figure 1D) for wild-type FF animals than C57BL/6J mitochondrial mice after injection of cells into the mammary fat pad (average lung metastases of 55 and 36 from n=13 and 14 animals respectively). Following tail vein injections, the FF and FC groups did not show a significant difference in number of metastases observed in the lung (Figure 1B) however the FF group had more metastases on average than the FC group, 25 to 18 from 20 and 21 animals respectively.

Mice with C3H/HeN mitochondria had more metastases than C57BL/6J mitochondrial mice when utilizing both mammary and melanoma cancer lines. In Figure 2A, the mouse metastatic mammary line, EO771, was introduced via the tail vein into both wild type C57BL/6J mice as well as MNX mice with C57BL/6J nuclear, but CH3/HeN mitochondria. CC mice had an average of 15 lung metastases while CH mice had an average of 45 lung metastases. When the B16-F10 mouse metastatic cell line was introduced to these same mouse strains, we show that CC mice again have fewer metastases (12) than CH mice (29) (Figure 2B). When switching the nuclear backgrounds, now to C3H/HeN, more metastases were still observed in the C3H/HeN mitochondrial mice than C57BL/6J mitochondrial mice utilizing the mouse melanoma cell line K1735-M2 (figure 2C).

The cell lines, EO771, B16-F10, and K1735-M2 were also utilized in spontaneous metastasis assays. EO771 were introduced to the mammary fat pad while B16-F10 and K1735-M2 were injected intradermally. These cell lines formed rapid, large tumors which quickly necrosed. As seen in Figure 2D – F, most mice were unable to reach metastasis and were instead euthanized in accordance with IACUC guidelines.

Figure 10

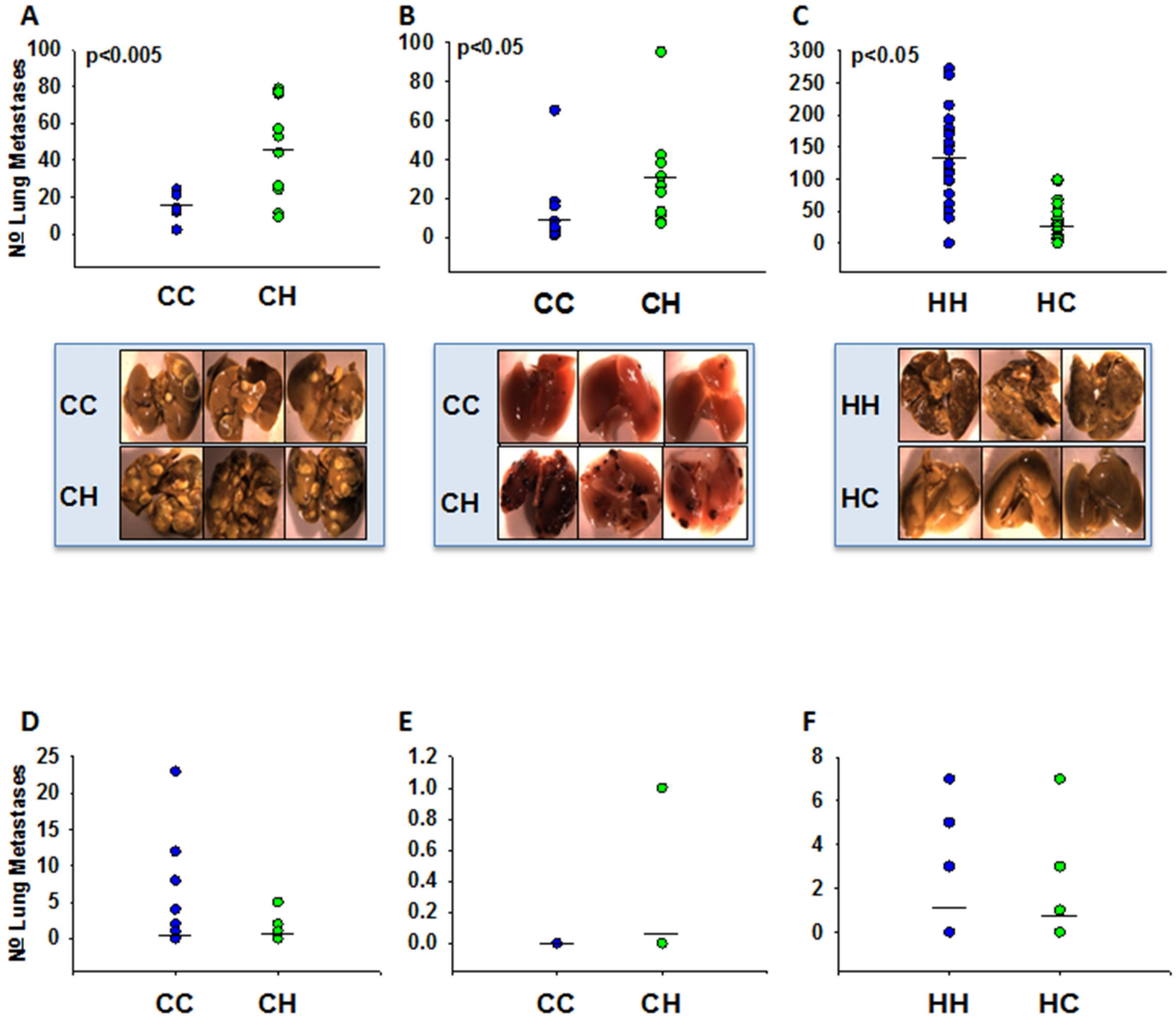

A
p<0.005
№ Lung Metastases
100
80
60
40
20
0
CC
CH
B
p<0.05
100
80
60
40
20
0
CC
CH
C
p<0.05
300
250
200
150
100
50
0
HH
HC
CC
CH
CC
CH
HH
HC
D
№ Lung Metastases
25
20
15
10
5
0
CC
CH
E
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CC
CH
F
8
6
4
2
0
HH
HC

Figure 2: Mitochondrial haplotype alters metastatic propensity in both mammary and melanoma models. Metastatic mouse cell lines were injected into the **A, B, C:** tail vein and **D, E, F:** orthotopic site of nuclear matched MNX mice. Tail vein injected mice were euthanized two weeks after injection while orthotopically injected mouse tumors were measured with digital Vernier calipers and mice euthanized when tumors reached 15 mm in average diameter. Circles represent total number of metastases in each animal; black bars represent average number of metastases for the mitochondrial group. **A:** EO771 metastatic mammary cells injected into the tail vein of CC mice (n=9) resulted in fewer (mean 15, SEM 2) metastases than CH mice (n=10, mean 45, SEM 9). **B:** B16-F10 metastatic melanoma cells injected into the tail vein of CC mice (n=10) showed fewer (mean 12, SEM 6) metastases than CH mice (n=10, mean 29, SEM 8). **C:** K1735-M2 cells injected in the tail vein of HH mice (n=20) resulted in more metastases (mean 134, SEM 16) than similarly injected HC mice (n=20, mean 31, SEM 7). **D:** EO771 cells injected into the mammary fat pad of CC (n=19) and CH (n=17) mice resulted in tumors which grew rapidly and had a low rate of metastases. No difference in number of metastases was observed (mean 2, SEM 1 and mean 1, SEM 0.3 respectively). **E:** B16-F10 cells injected into the dermis of CC (n=10) and CH (n=10) mice resulted in rapidly growing tumors with no difference in metastases (mean 0, SEM 0 and mean 0.1, SEM 0.1 respectively) **F:** K1735-M2 cells injected into the dermis did not show a difference in total number of metastases between HH (n=19, mean 1, SEM 0.5) and HC (n=20, mean 1, SEM 0.4) mice.

To begin exploring possible mechanisms contributing to the mitochondrial host effects on metastatic susceptibility, a series of experiments were performed to determine if any baseline mitochondrial differences occur between the MNX and wild-type mouse strains. These experiments utilized MEF cell lines created from a single embryo per line. Mitochondrial load as well as membrane potential was evaluated. In addition mtDNA copy number was analyzed, and basic metabolic profiles were explored.

The total number of mitochondria present as well as the membrane potential of those mitochondria did not differ between MNX and wild-type animals analyzed. A small initial study utilized fluorescent flow cytometry probes, MitoTracker Red, whose accumulation in mitochondria is dependent upon membrane potential, and MitoTracker Green, which accumulates in mitochondria independent of membrane potential. These probes were used in conjunction with MEF lines created from each MNX and wild-type strains. As seen in Figure 3A, there is no significant difference in overall number of mitochondria or in their membrane potential between the different wild-type and MNX strains. Even when membrane potential is normalized to the number of mitochondria (as is routine for this type of measurement), there is no difference between strains (Figure 3B).

Metabolic flux profiles of MNX and wild-type MEF lines show differences among the strains in initial experiments. MEF lines made from a single embryo per line were analyzed for oxygen consumption in quadruplicate (Figure 4A) or quintuplicate (Figure 4B) using the Seahorse instrument. As seen in Figure 5A, FC and FF cells had higher basal (mean 95.725, 95.63 pmol/min/µg respectively) and maximal (mean 175.604, 152.906 pmol/min/µg respectively) respiration than BB (basal mean 61.636 pmol/min/µg, maximal mean 81.228 pmol/min/µg) cells. The ATP-linked oxygen consumption rate does not appear to differ among

Figure 11

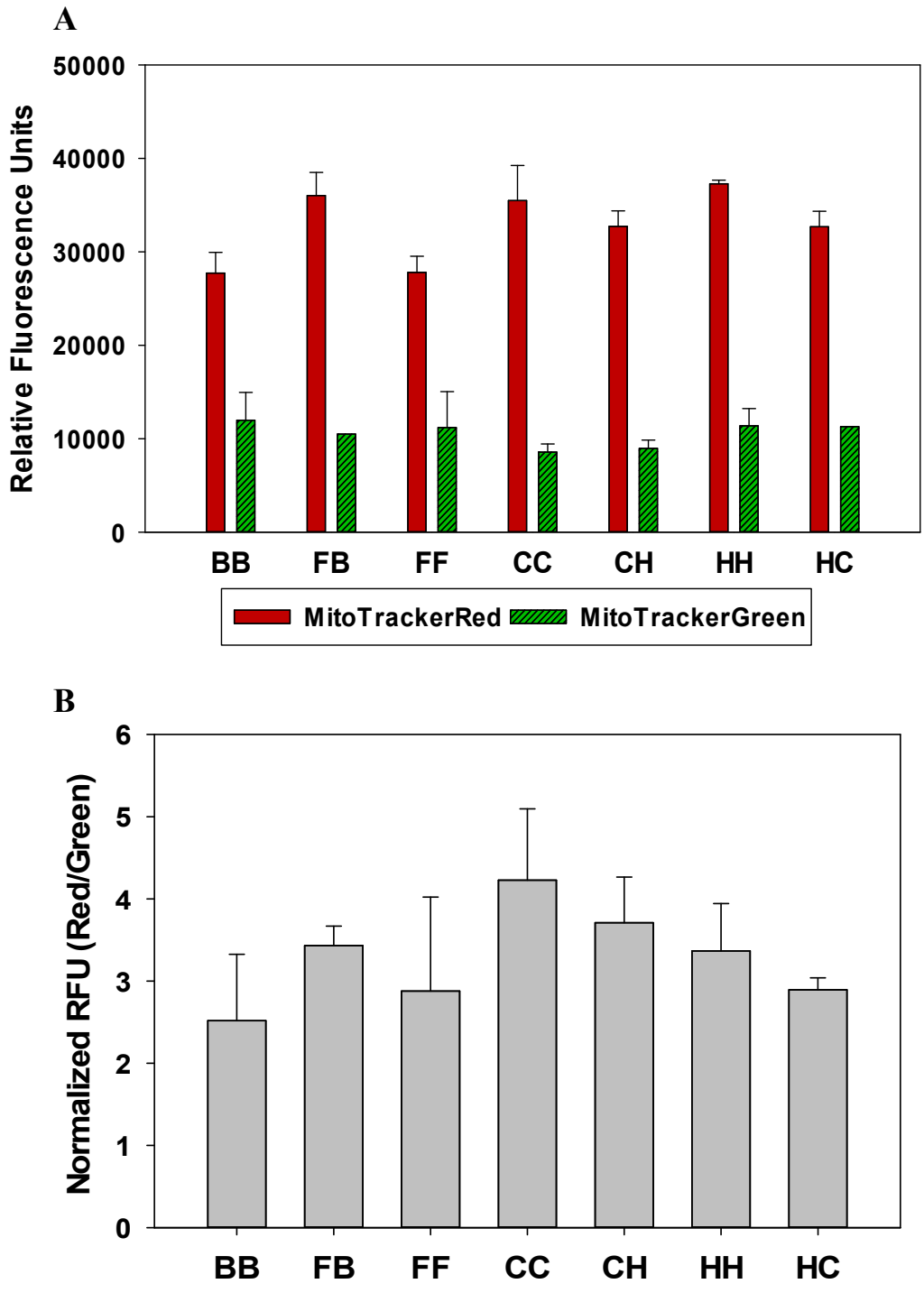
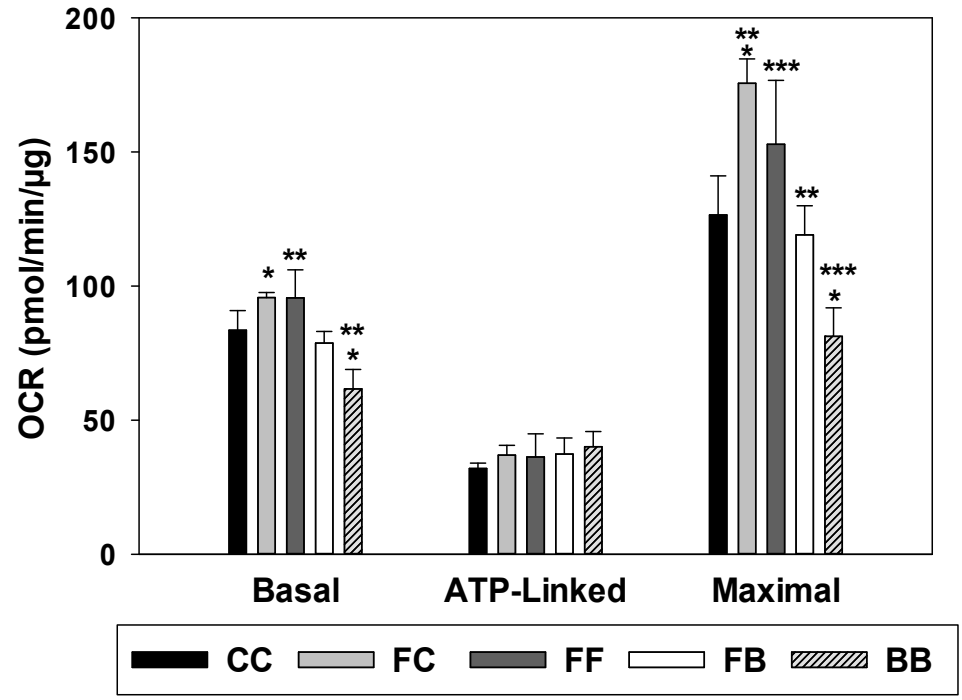

A
Relative Fluorescence Units
50000
40000
30000
20000
10000
0
BB
FB
FF
CC
CH
HH
HC
MitoTrackerRed
MitoTrackerGreen
B
Normalized RFU (Red/Green)
6
5
4
3
2
1
0
BB
FB
FF
CC
CH
HH
HC

Figure 3: Neither mitochondrial load nor membrane potential differ between MNX and wild-type strains. Mouse embryonic fibroblasts were isolated from each MNX and wild-type strain and stained with MitoTracker Green FM to identify overall mitochondrial load and with MitoTracker Red CMXRos to identify membrane potential. Probe fluorescence in each cell was analyzed by flow cytometry. **A:** Relative fluorescence for each probe showing mitochondrial load and raw membrane potential. **B:** MitoTracker Red is normalized to MitoTracker Green to show membrane potential per mitochondrion. Error bars represent standard error of the mean.

Figure 4

A


200
150
100
50
0
OCR (pmol/min/µg)
Basal
ATP-Linked
Maximal
CC
FC
FF
FB
BB

B

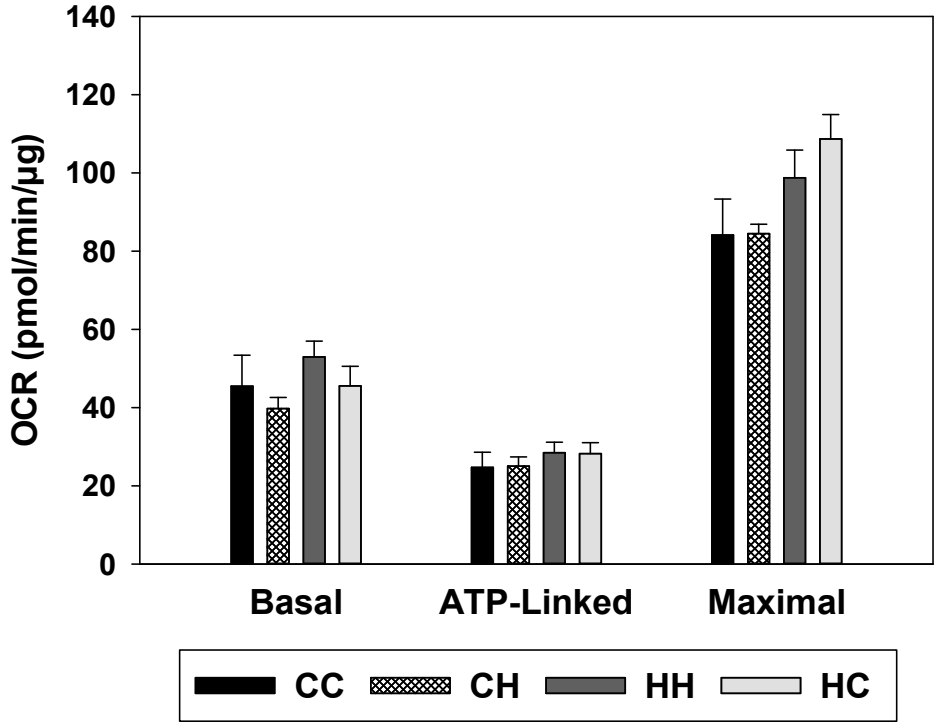

140
120
100
80
60
40
20
0
OCR (pmol/min/µg)
Basal
ATP-Linked
Maximal
CC
CH
HH
HC

Figure 4: Mitochondrial flux of MNX and wild-type mice is variable. MNX and wild-type mouse embryonic fibroblasts from each strain were plated and the Seahorse analyzer was used to measure oxygen consumption prior to compound addition (basal respiration), after oligomycin addition (ATP-linked respiration), and after addition of the uncoupler FCCP (maximal respiration). * denotes $p<0.05$ and error bars represent standard error of the mean. **A:** Basal respiration differs significantly between * FC and BB, and ** FF and BB. Maximal respiration differs significantly between * FC and BB, ** FC and FB, as well as ***FF and BB. **B:** Basal, ATP-linked, and maximal respiration does not differ significantly between CC, CH, HH, and HC strains

Figure 12

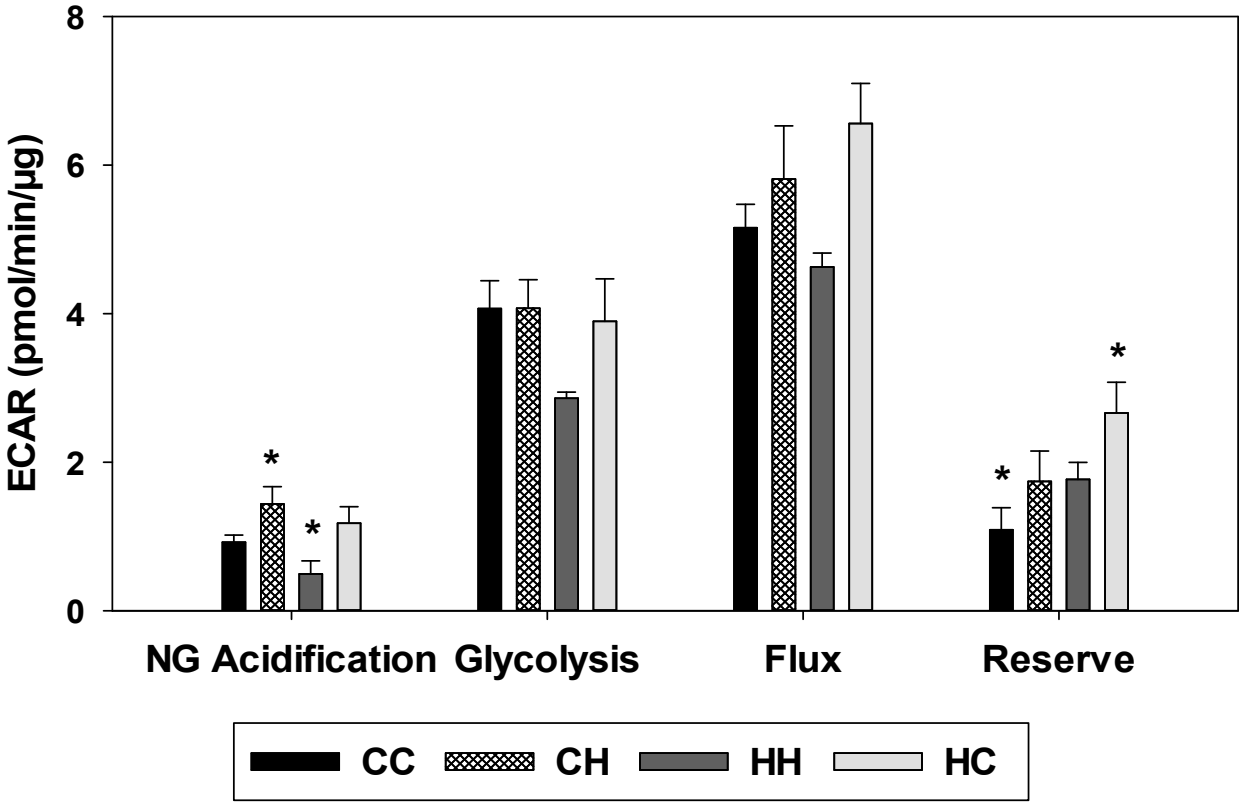

8
6
4
2
0
ECAR (pmol/min/µg)
*
*
*
*
NG Acidification
Glycolysis
Flux
Reserve
CC
CH
HH
HC

Figure 5: Glycolytic flux profiles vary between C57BL/6J and C3H/HeN MNX and wild-type strains. Mouse embryonic fibroblast from CC, CH, HH, and HC mouse strains were passaged once then analyzed for ECAR (ExtraCellular Acidification Rate) under serum starved conditions with no glucose to determine non-glycolytic acidification, after addition of glucose (glycolysis), after addition of oligomycin to determine glycolytic capacity (flux), and after addition of 2-DG (glycolytic reserve). * denotes $p<0.05$ and error bars represent standard error of the mean. CH and HH strains differ significantly in non-glycolytic acidification while CC and HC differ significantly in glycolytic reserve.

strains. No significant differences were seen between oxygen utilization profiles of CC, CH, HH, and HC mice (Figure 4B). Although differences in metabolic flux occur between the strains, these differences do not appear to mirror the metastatic phenotypes shown above.

Glycolytic flux profiles of MEF lines isolated from MNX and wild-type mice show differences between strains in initial experiments. Single embryo MEF lines from CC, CH, HH, and HC mice were analyzed for extracellular acidification rate in quintuplicate using the Seahorse instrument (Figure 5). Non-glycolytic acidification rates differed with CH having the highest (mean 1.437 mpH/min/µg) and HH having the lowest (mean 1.178 mpH/min/µg). Reserve capacity also differed between CC derived cells (mean 1.089 mpH/min/µg) and HC derived cells (mean 2.663 mpH/min/µg). Although some slight variations are present, taken as a whole, the glycolytic flux profiles between these four strains are fairly similar. The differences that are observed do not parallel the metastatic phenotypes shown above.

mtDNA copy number was evaluated to see if differences exist when dissimilar nuclear and mitochondrial genomes are combined. DNA from lung samples was isolated and expression of two mitochondrial specific genes (mtCO2, and ND1) was analyzed and normalized to expression of a nuclear specific gene (18s). As shown in Figure 6, HH and HC strains have a slightly higher (30%) mitochondrial copy number than CC and CH strains. Unfortunately, error for these groups is too high to tell if a significant difference is present (CC n=5, CH n=7, HH n=5, HC n=8). This study is being repeated to include a greater number of mice.

Mitochondrial reactive oxygen species have been indicated in metastatic disease (Ferraro et al., 2006; Ishikawa, Koshikawa, et al., 2008; Ishikawa, Takenaga, et al., 2008; Kaur et al., 2016; Laurila, Laatikainen, Castellone, & Laukkanen, 2009; Porporato et al., 2014). To

Figure 13

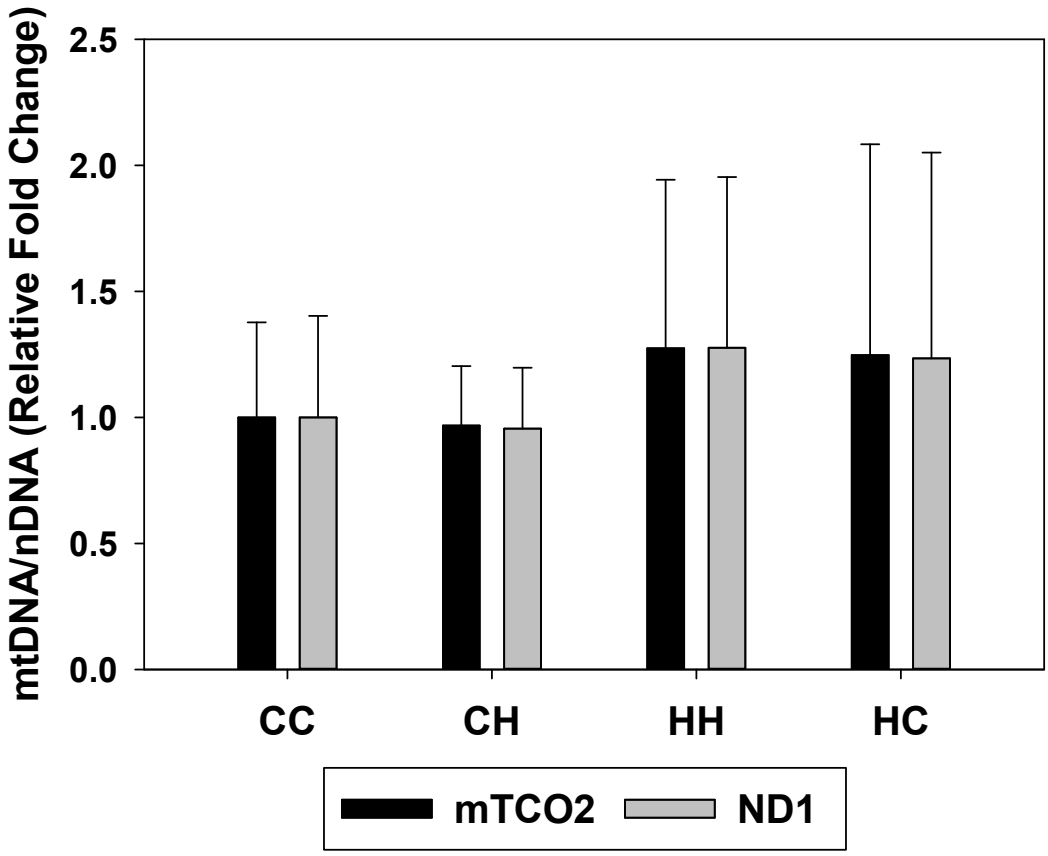

mtDNA/nDNA (Relative Fold Change)
2.5
2.0
1.5
1.0
0.5
0.0
CC
CH
HH
HC
mTCO2
ND1

Figure 6: Mitochondrial DNA content does not differ between C57BL/6J and C3H/HeN MNX and wild-type strains. Lungs were isolated from four week old CC (n=5), CH (n=7), HH (n=5), and HC (n=8) mice. Total DNA was isolated from each lung taking care to preserve mtDNA. qPCR utilizing Taqman primers for mitochondrial encoded mtCO2 and ND1 as well as nuclear encoded 18s was performed and fold change for mitochondrial genes was calculated relative to nuclear 18s. Error bars represent standard error of the mean.

determine if reactive oxygen species are playing a role in metastatic susceptibility seen with varying mitochondrial genomes, we utilized a mitochondrial superoxide scavenger, MitoTEMPO. MitoTEMPO acts as a superoxide dismutase 2 mimic and specifically scavenges superoxide in the mitochondria (Dikalova et al., 2010; Nazarewicz et al., 2013). Superoxide is produced in complex I and III from single-electron reduction of oxygen. Superoxide dismutates to longer lived hydrogen peroxide, which can partially reduce to the hydroxyl radical, or can react with nitrogen to form nitric oxide and peroxynitrite species (Turrens, 2003). By targeting superoxide, we are targeting the most upstream oxidant.

When MNX and wild-type mice were pre-treated with MitoTEMPO or vehicle twenty-four and again 1-hour previous to metastatic cell line tail vein injection, metastasis from C3H/HeN mitochondrial mice was suppressed. When K1735-M2 melanoma cells were used, metastasis numbers in the vehicle treated animals matched those of untreated animals in the previous studies with HH mice having an average of 94 metastases and HC an average of 16 metastases (Figure 7A). When treated with MitoTEMPO, HH mice now had average of 11 metastases while HC mice had an average of 18. As shown in Figure 7C, when the metastatic mammary line EO771 was utilized after superoxide scavenging in the host, CH mice had a reduced number of metastases (mean 11) compared to vehicle treated CH mice (mean 24) although significance was not reached. With both cell types and under both C3H/HeN and C57BL/6J nuclear genomes, the MitoTEMPO treatment did not eliminate metastases in the C3H/HeN mitochondrial mice, but rather reduced the number to that of the C57BL/6J mitochondrial mice. These data imply a role for differential ROS scavenging as a mechanism behind metastatic susceptibility differences.

Figure 7

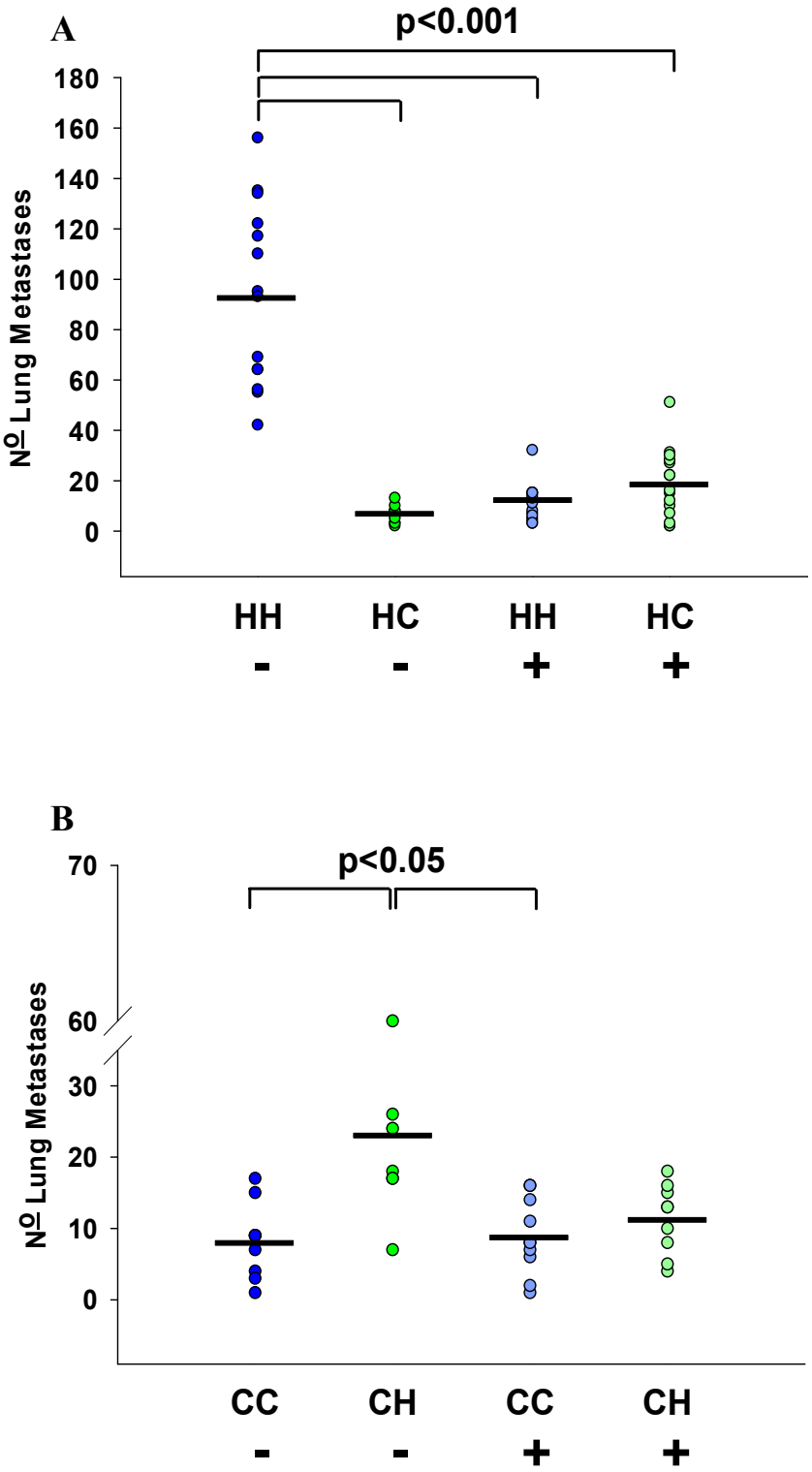

A
p<0.001
180
160
140
120
100
80
60
40
20
0
Nº Lung Metastases
HH
HC
HH
HC
-
-
+
+
B
p<0.05
70
60
30
20
10
0
Nº Lung Metastases
CC
CH
CC
CH
-
-
+
+

Figure 7: Mitochondrial superoxide scavenging selectively decreases metastasis in C3H/HeN mitochondrial mice. DMSO vehicle (-) or MitoTEMPO (+) was IP injected into **A:** 4 week old HH (- n=14, + n=15), and HC (- n=10, + n=15) mice 24 hours and again 1 hour prior to IV injection of K1735-M2 cells and **B:** 4 week old CC (- n= 9, + n=10), and CH (- n=8, + n=9) mice 24 hours and again 1 hour prior to IV injection of EO771 cells. Mice were euthanized two weeks post cell injection, lungs were harvested, and gross pulmonary metastases were quantified. **A:** K1735 injected HH vehicle mice had significantly more metastases (mean 94, SEM 10) than HC vehicle (mean 16, SEM 3), HH MitoTEMPO (mean 11, SEM 2), and HC MitoTEMPO (mean 18, SEM 4) treated mice. **B:** EO771 injected CH vehicle treated mice had more metastases (mean 24), than CC vehicle (mean 8), CC MitoTEMPO (mean 9), and CH MitoTEMPO (mean 11) treated mice.

In order to determine if ROS could be acting to alter nuclear genes, we first determined if expression levels of nuclear genes were altered between different mitochondrial haplotypes. RNA was isolated from lung tissue gathered from two different vehicle treated mice from CC, CH, HH, and HC strains. Initial expression levels for nine different metastasis related genes were analyzed following qPCR performed in triplicate for each sample. In CH MNX mice, several genes including *miR199a*, *miR125b*, *fam120a*, and *scai* appear to have expression levels similar to that of the mitochondrial wild-type, HH (Figure 8). In contrast, genes such as *dnmt1*, *ago2*, and *sgtb* appear to have expression levels in CH MNX mice that are in between the nuclear CC and mitochondrial HH wild-type strains. In contrast, the HC mice have expression levels similar to the nuclear wild-type, HH strain. There are also genes, such as *sod2* whose expression level does not change after mitochondrial exchange. Together these data indicate that nuclear gene expression can be altered by mitochondrial haplotype.

To determine if reactive oxygen species could be playing a role in the gene expression changes shown above, RNA was isolated from lungs of three different mice of CC, CH, HH, and HC strains after MitoTEMPO treatment. Gene expression was analyzed following qPCR performed in triplicate for each sample. Interestingly, MitoTEMPO treatment raised expression levels for *miR199a*, *miR125b*, *dnmt1*, *ago2*, *fam120a*, *rab6b*, *sgtb*, and *scai* for both CC and CH mice (Figure 9A-D, F-I). Expression levels in HH and HC mice appear unaffected by MitoTEMPO. Although expression patterns of these particular genes in treated mice do not match the metastatic scavenging experiments, it is clear that superoxide scavenging within the lung can affect nuclear gene expression. Further gene sets should be evaluated.

Figure 8

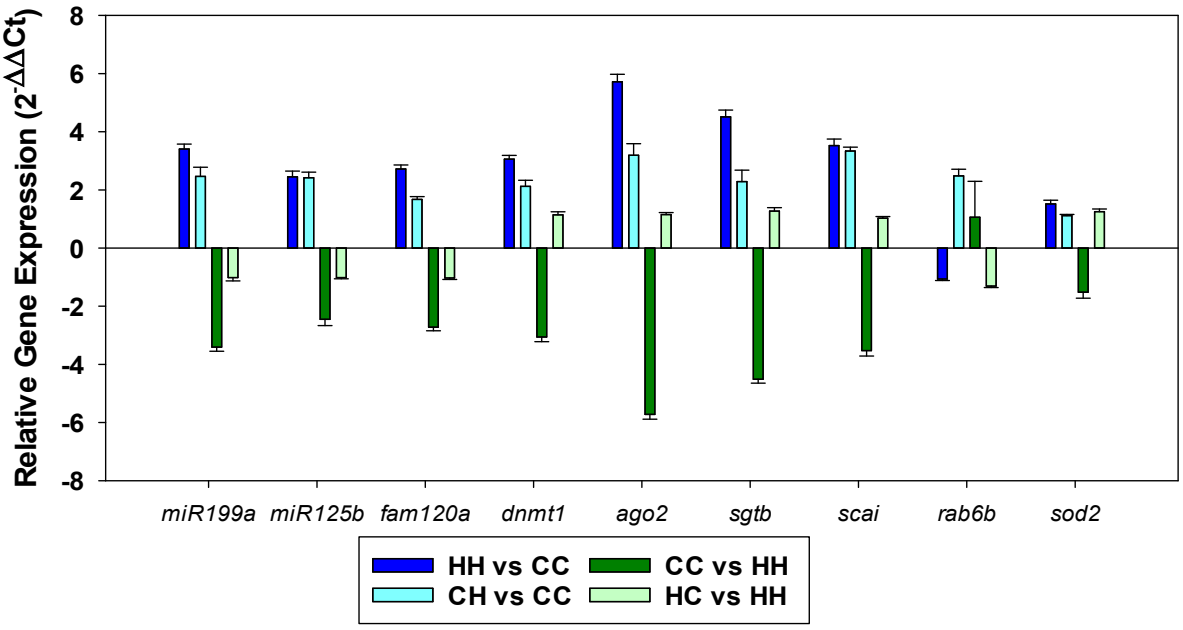

Relative Gene Expression ($2^{-\Delta\Delta Ct}$)
8
6
4
2
0
-2
-4
-6
-8
miR199a
miR125b
fam120a
dnmt1
ago2
sgtb
scai
rab6b
sod2
HH vs CC
CC vs HH
CH vs CC
HC vs HH

Figure 8: MtDNA affects expression of select nuclear genes. RNA was isolated from lungs of 4 week old CC, CH, HH, and HC mice. qPCR was performed with Taqman primers to determine expression of nuclear genes normalized to the nuclear 18s housekeeping gene. Error bars represent standard error of the mean. Blue bars represent gene expression levels of HH and CH mice relative to CC while green bars represent gene expression levels of CC and HC mice relative to HH mice.

Figure 9

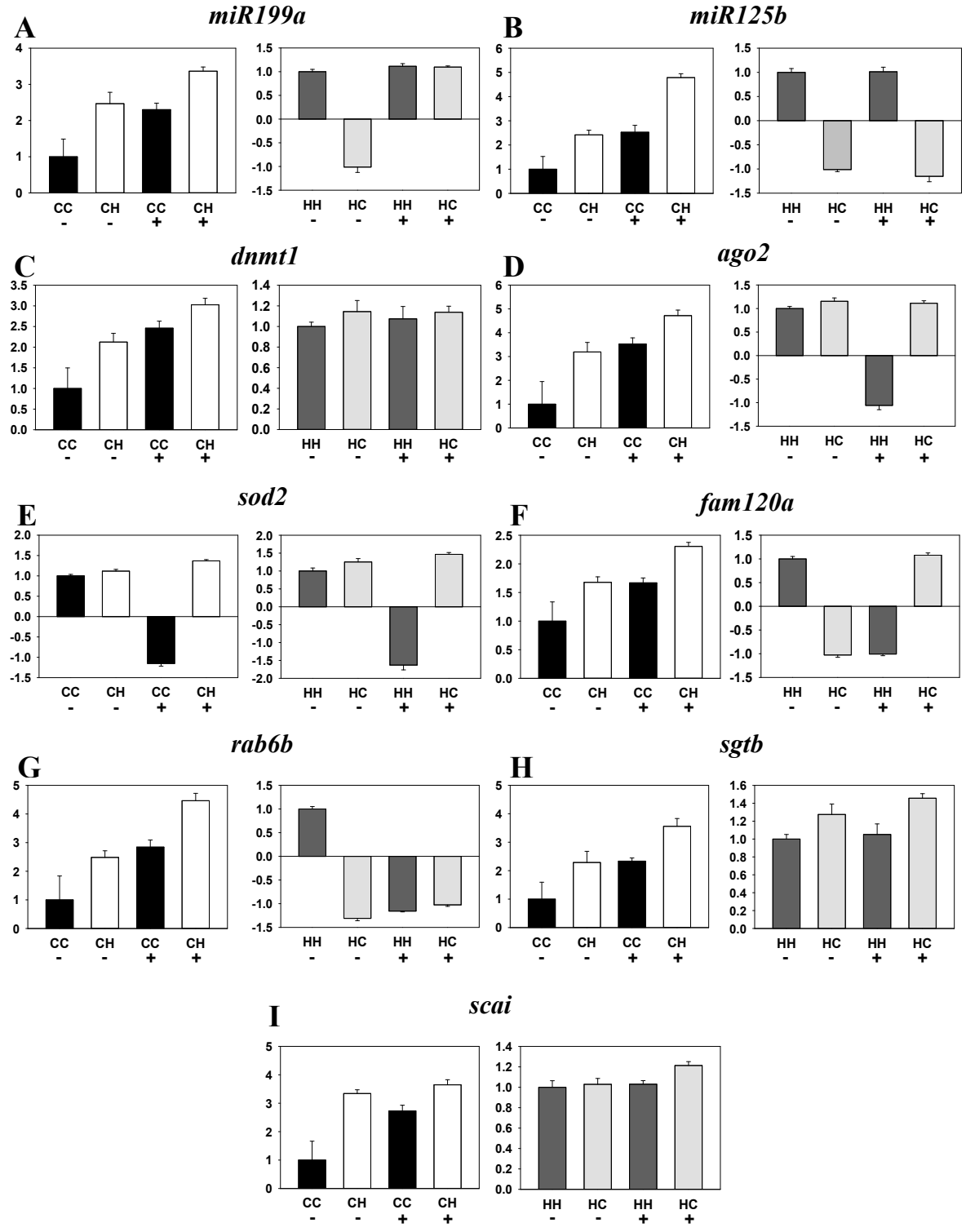

A
miR199a
B
miR125b
C
dnmt1
D
ago2
E
sod2
F
fam120a
G
rab6b
H
sgtb
I
scai
CC - CH - CC + CH +
HH - HC - HH + HC +

Figure 9: Mitochondrial superoxide scavenging selectively alters nuclear gene expression. RNA was isolated from lungs of 4 week old CC, CH, HH and HC mice treated with DMSO vehicle (-) or MitoTEMPO (+) 24 hours and 1 hour prior to euthanasia. qPCR was performed with Taqman primers to determine gene expression of **A:** *miR199a* **B:** *miR125b* **C:** *dnmt1* **D:** *ago2* **E:** s*od2* **F:** *fam120a* **G:** *rab6b* **H:** *sgtb* and **I:** *scai*. All expression was normalized to *18s*.

Discussion

The purpose of this study was to determine if mitochondria at the secondary site of metastasis would affect the ability of a metastatic cell to colonize this environment. We have shown through a series of *in-vivo* experiments that mitochondria of the host environment is able to affect metastasis in both mammary and melanoma models. Interestingly, orthotopic tumor growth does not appear to be affected by mitochondrial background. With orthotopic injections, a relatively large number of cells are implanted into an area. During the process of metastasis, these cancer cells and small groups of cancer cells must burrow through their microenvironment, squeeze into the lymph, or in this case the circulatory system, travel to the secondary site then extravasate to the secondary tissue where they again have to crawl through the secondary tissue to colonize (Hurst & Welch, 2011). This process involves countless interactions of cancer cells with host tissue cells, and therefore many opportunities for host mitochondria to impact metastatic capabilities of the cell. Since the experimental injections, which only recreate the second half of the metastatic cascade, also showed the alteration of metastatic capability, we focused our mechanistic studies on the secondary site tissue, the lung.

The mitochondrial DNA of the four inbred mouse strains utilized here are extremely similar. Utilizing the know mitochondrial strain sequences and alignment tools from NCBI as well as previous literature, it is known that there are only five single nucleotide polymorphisms (SNP) which define the mitochondria of these mouse strains (Bayona-Bafaluy et al., 2003). Most of these SNP occur in subunits of electron transport chain genes. FVB/NJ mitochondria have a G7777T SNP in ATPase subunit 8 which changes the aspartic acid residue to a tyrosine. Additionally, FVB/NJ as well as C57BL/6J mitochondria have a A9348G mutation in

cytochrome oxidase subunit 3, which is the main transmembrane subunit of Cytochrome C Oxidase (electron transport chain complex IV). Despite this mutation coding for valine instead of isoleucine, it is thought to be a neutral mutation (Bayona-Bafaluy et al., 2003; Moreno-Loshuertos et al., 2006). C57BL/6J have a C to T mutation at 9461 located at the start site for NADH Dehydrogenase subunit 3, part of the electron transport chain Complex I, which is non-synonymous. Interestingly, C57BL/6J and C3H/HeN have mutations in the mitochondrial transfer RNA (mt-tRNA) for arginine. C57BL/6J mitochondria have an adenine deletion at position 9821 while C3H/HeN mitochondria have an additional thymine located at 9820, both of which occur in the DHU loop (or D-arm).

Due to the mutations in electron transport gene subunits that differentiate these mitochondrial backgrounds, we first evaluated whether differences occurred in membrane potential, overall mitochondrial load, and metabolic and glycolytic flux. Our experiments, performed utilizing MEF cell lines, did not find differences in load, potential, nor metabolic and glycolytic flux that would help explain the metastatic phenotypes. Interestingly, when these backgrounds were probed for oxygen consumption previously using mammary tumor tissue (Feeley et al., 2015) and cardiomyocytes (Fetterman et al., 2013), C57BL/6J had significantly different oxygen consumption profiles from the other mitochondrial backgrounds (higher than FVB /NJ and BALB/cJ mitochondria in mammary tumor and lower than C3H/HeN mitochondria in cardiomyocytes). Metabolism and mitochondrial number can vary greatly from tissue to tissue (Kunz, 2003; Leary, Battersby, & Moyes, 1998; Rossignol, Malgat, Mazat, & Letellier, 1999). Although MEF were originally utilized because they represent an early stem-like phenotype, we cannot rule out differences that may occur in mitochondrial load and metabolic profiles in our

adult lungs. In order to fully evaluate these points, further studies utilizing full metabolomic profiling in mouse lung tissue are currently underway.

Since the DHU loop of tRNA is responsible for stabilizing the binding and localization of the ribosome and defects here can alter transcription (D. Smith & Yarus, 1989a, 1989b), we analyzed the mtDNA content by copy number analysis. While not significant, we see an almost thirty-percent greater amount of mtDNA in strains with the nuclear background originating from the C3H/HeN mouse, despite the mitochondrial background. This onservation suggests that nuclear factors are able to influence the amount of mitochondrial coded DNA. Due to the high variability of these measurements, a greater number of mice are now being evaluated.

While reconstructing the mutational history of the L929 cell lines, the Wallace laboratory found that mitochondrial mutations which increase ROS production, in turn increase cell proliferation (Fan, Lin, Potluri, Procaccio, & Wallace, 2012). They postulated that this may be a mechanism by which mitochondria contribute to cancerous phenotypes. Several labs have gone on to show in cybrid studies that increases in the number of adenines in the mt-tRNA coding for arginine's DHU-loop, increases the level or ROS which in turn increases not only proliferation, but also invasion (Jandova, Shi, Norman, Stricklin, & Sligh, 2012; Moreno-Loshuertos et al., 2006). With C3H/HeN having an additional adenine within the A-track as well as an additional thymine directly adjacent to the A-track when compared to C57BL/6J, we evaluated the number of metastases in mice with these mitochondrial backgrounds after ROS scavenging. We show that scavenging of ROS decreases the number of metastases in C3H/HeN mitochondrial mice to the same level as that of C57BL/6J mitochondrial mice. Studies to determine the level of ROS *in-situ* are currently underway. These data, taken together with the previous studies indicate that the A-track polymorphisms could be causing differences in ROS production between the

mitochondrial genotypes. These ROS differences could in turn result in differential signaling to growth and invasion factors in the nucleus.

Reactive oxygen species have recently been shown to act as secondary messengers to the nuclear genome where they alter expression levels of certain genes (Chae et al., 2013; Cook-Mills, Marchese, & Abdala-Valencia, 2011; Formentini, Sanchez-Arago, Sanchez-Cenizo, & Cuezva, 2012; He et al., 2012; Jajoo et al., 2013; Parikh et al., 1987; Raddant & Russo, 2014) and can cause changes to cell proliferation and growth (Burdon, 1995; Irani et al., 1997; Liu et al., 2002; Sauer, Wartenberg, & Hescheler, 2001; Sundaresan, Yu, Ferrans, Irani, & Finkel, 1995). We show that not only can gene expression differ when the mitochondrial background is changed, but that nuclear-mitochondrial combinations result in unique gene expression profiles. This indicates that active mitochondrial-nuclear crosstalk defines nuclear gene expression. To determine if ROS could be a messenger for this cross-talk, we also evaluated expression profiles of mice treated with a ROS scavenger. We found that changing ROS levels alters expression of specific genes from specific backgrounds.

Several nuclear encoded metastasis suppressors affect the microenvironment (Hurst & Welch, 2011). ROS alterations to expression of these suppressors could explain why host cell or "soil" mitochondria can alter the metastatic capacity of seeding cancer cells. Although we show that ROS can alter expression of nuclear genes which have been indicated in metastasis, the expression patterns of this particular gene set do not appear to follow the trends of our *in-vivo* ROS scavenging data. Future studies to evaluate nuclear gene expression profiles of metastasis suppressors are underway.

Chapter IV: Conclusions, future directions, and significance

Cancer is set to outpace heart disease as the number one killer of Americans by the year 2030 (Siegel et al., 2016). With metastatic spread being responsible for the majority of all cancer related deaths, it is imperative that we focus research efforts on further elucidating this multifaceted process. Metastatic spread occurs through a series of processes that take place in multiple tissues (hematologic or lymphatic), involving a myriad of factors. This makes metastasis a complex process to study, and its pathobiology is not completely understood. Previous studies have shown that there are genetic underpinnings to metastasis. The discovery of metastasis suppressor genes as well as quantitative trait loci underscores the importance of genetic influence on metastasis.

There is however, a second genome at work in every cell, the mitochondrial genome. From Warburg's early observations of metabolic switching (Warburg et al., 1927) to Ishikawa's cybrid studies showing metastatic characteristics in some cell lines followed the mitochondrial contributor(Ishikawa et al., 2012), mitochondria has become a target for cancer and specifically metastatic research. The redundancy and complexity of mitochondrial genetics has thus far limited research approaches. We proposed the use of a novel mouse model, the Mitochondrial Nuclear Exchange (MNX) mice, which are stable mouse lines that have 100% of the mitochondria of one mouse strain, and 100% of the nuclear background of a second strain. We utilized these unique mouse strains in a series of genetic and autochthonous approaches to elucidate cell-autonomous and host effects of mitochondrial haplotypes.

Mitochondrial haplotype alters tumor latency and metastasis in a cell-autonomous oncogene dependent manner

We utilized two strains of MNX mice which shared the same nuclear FVB/NJ background but had different (C57BL/6J and BALB/cJ) mitochondria, in a series of genetic crosses to the FVB/NJ mouse carrying the overexpressed Her2 mammary tumor oncogene. We then observed tumor latency, tumor growth rates, and metastatic outcomes. We show that tumor latency, metastatic size, and overall number of metastases is affected by mitochondrial haplotype, but not tumor growth rate. Additionally we demonstrate that these effects were not simply a byproduct of the MNX process by showing tumorigenesis and metastasis are not affected by residual cytoplasmic factors.

By comparing our Her2 outcomes to outcomes from identical crosses utilizing PyMT expressing mice, we show for the first time, that within breast cancer, these mitochondrial effects can vary from oncogene to oncogene. This underscores the importance of nuclear factors when considering mitochondrial genetic effects. Indeed anterograde and retrograde communication has been demonstrated between mitochondria and the nucleus (Kaipparettu et al., 2013; Ma, Bai, Trieu, & Wong, 2010; Rodley et al., 2012; Sripathi et al., 2011), which can result in differential expression of nuclear genes. When secondary crosses were performed with Her2 females to male MNX mice, eliminating the altered mitochondrial genome, we did not observe differences in tumorigenic or metastatic characteristics. This implies that the previous mitochondrial genome of the parental MNX mice did not result in heritable alterations of the nuclear genome. However, the altered mitochondrial genome (C57BL/6J or BALB/cJ) was never within the same cell as the nuclear genome which carried the Her2 overexpressed oncogene. In order to determine if mitochondrial effects on the nuclear oncogene could be truly reversible, we propose future studies which breed F2 generation male mice which contain both the Her2 nuclear oncogene and the altered mitochondria, back to female Her2 mice. The resulting offspring would no longer

contain the altered mitochondrial background. If these new mice no longer differed in metastatic and tumorigenic outcomes from Her2 parents, it would indicate that the mechanism for the cell-autonomous effects we demonstrate, are truly reversible and dependent upon active mitochondrial-nuclear cross-talk.

Metastasis is altered in a non-cell autonomous manner by mitochondrial haplotype of the host environment

The MNX mice presented the unique opportunity to study the effect of mitochondrial haplotype in the host environment on metastasis. By pairing metastatic cell lines with MNX strains which matched the nuclear but had different mitochondrial backgrounds from the cell lines, we demonstrated that metastatic burden varied with the host mitochondrial haplotype. This demonstrates a further mechanism by which mtDNA can heritably alter an individual's susceptibility to metastasis. We went on to show that non-cell autonomous mtDNA effects could be demonstrated in mammary cancer and melanoma models.

Reactive oxygen species have previously been shown to play a role in mtDNA's effects on the metastatic cascade (Ishikawa, Koshikawa, et al., 2008; Ishikawa, Takenaga, et al., 2008; Porporato et al., 2014). These previous studies focus on ROS generation and scavenging within the cancer cell. We show that specific scavenging of mitochondrial superoxide from the host tissue attenuates metastasis in mice with highly metastatic mitochondrial haplotypes. Even more interestingly, we show that mitochondrial ROS scavenging does not ablate all metastasis, but brings the number of metastases in highly metastatic haplotypes down to the same level as those

found in low metastatic haplotypes. It is our hypothesis that this may be due to ROS role as a signaling molecule and resultant changes to expression of nuclear DNA.

We show that differences in mtDNA resulted in differential expression of nuclear genes that are associated with metastasis within the secondary environment. Additionally we demonstrated that expression of a subset of these genes could be altered after superoxide scavenging. This could mean that mitochondrial haplotype helps determine secondary environmental characteristics not only through metabolic outputs, but additionally through modification of nuclear gene expression which may be driven by ROS messengers. Therefore, mitochondrial DNA may help determine pre-metastatic niche.

There is preliminary evidence that mtDNA or whole mitochondria can be transferred from healthy cells to damaged or dysfunctional cells (Acquistapace et al., 2011; Spees, Olson, Whitney, & Prockop, 2006). The mitochondrial host effects could potentially be explained by mitochondrial transfer from the secondary environment to the invading metastatic cells. This could reprogram them in a way that either inhibits or enhances colonization. We are currently proposing future studies that analyze laser capture micro-dissected metastases and surrounding secondary site tissue. Mitochondrial DNA sequencing of the metastases could be compared to the haplotype of the normal host tissue, the metastatic adjacent tissue, and the original cell lines. This could help determine if mitochondria or mtDNA are being transferred to the metastatic cells or from the metastatic cells.

We have presented evidence that mitochondrial haplotypes affect the process of metastasis. At this time, we are unable to determine which individual or combinations of SNP are responsible for these effects. Current tools to manipulate mitochondrial DNA are limited.

Mitochondria-targeted Zinc Finger Nucleases, TALENS, and CRISPR-Cas9 are able introduce site specific mutations to mtDNA, as well as suppress overall copy number (Bacman et al., 2013; Gammage et al., 2016; Jo et al., 2015; Minczuk, 2010; Minczuk et al., 2008). However, due to the complexity of mitochondrial genetics, these mutations have never been successfully homoplasmic. As these tools evolve, we plan to test the SNP from our highly metastatic and lowly metastatic mouse lines to better pinpoint the mechanism behind mtDNA effects on metastasis.

Significance

With the advent of personalized medicine, patients are now having both their normal nuclear and tumor DNA sequenced in order to inform treatment strategies and gauge possible outcomes. The discovery of the *BRCA1* and *BRCA2* genes has seen currently healthy people take preventative steps to minimize their risk of cancer. The data presented here highlights the importance of an additional genome, the mitochondrial genome. It may be that by considering both the nuclear and mitochondrial DNA as well as how they interact, we can drive progress for both preventative screening measures, as well as identify new targets for late stage patients.

We have shown that not only does mtDNA from within a cancer cell affect tumorigenesis and metastasis but it does so in a manner dependent upon the oncogenic driver. This serves to strengthen the connection between mitochondria-nuclear cross-talk and cancer and metastasis. We have also shown, for the first time, that mitochondrial haplotype of the host tissue can alter metastasis. Additionally, we show that ROS plays a role in the mtDNA mediated metastatic process, and that both mitochondrial haplotype and ROS level can affect nuclear gene expression

patterns. Together, this data underscores the importance of the mitochondrial genome in cancer and metastasis.

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