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Roles of the mitochondrial genetics in cancer metastasis: Not to be ignored any longer

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Abstract

Mitochondrial DNA (mtDNA) encodes for only a fraction of the proteins that are encoded within the nucleus, and therefore has typically been regarded as a lesser player in cancer biology and metastasis. Accumulating evidence, however, supports an increased role for mtDNA impacting tumor progression and metastatic susceptibility. Unfortunately, due to this delay, there is a dearth of data defining the relative contributions of specific mtDNA polymorphisms (SNP), which leads to an inability to effectively use these polymorphisms to guide and enhance therapeutic strategies and diagnosis. In addition, evidence also suggests that differences in mtDNA impact not only the cancer cells, but also the cells within the surrounding tumor microenvironment, suggesting a broad encompassing role for mtDNA polymorphisms in regulating the disease progression. mtDNA may have profound implications in the regulation of cancer biology and metastasis. However, there are still great lengths to go to understand fully its contributions. Thus, herein we discuss the recent advances in our understanding of mtDNA in cancer and metastasis, providing a framework for future functional validation and discovery.

Keywords

Mitochondrial genetics; polymorphism; metastasis; metabolism; tumor progression

1 Introduction

Cancer is understood to have a genetic origin, with a primary emphasis of research being placed upon nucleus-encoded genes. Metastasis accounts for the overwhelming majority of cancer-related deaths; therefore, a deeper understanding of the genetic mechanisms of this process is needed to enhance overall survival and quality of life. Recently, we began to consider mitochondrial encoded factors that play roles in tumorigenesis or metastasis. New ideas and questions regarding mitochondrial roles in cancer progression will likely result in exciting advances moving forward. Apart from acquired mutations, the mitochondrial genome also reflects maternal ancestry. The latter may further provide insight into germline susceptibility and possibly racial disparities in cancer susceptibility and progression,

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concepts rarely analyzed in the context of metastasis. Mitochondrial single nucleotide polymorphisms (SNP) are evolutionarily selected based on environmental pressures as human ancestors migrated to changing landscapes and corresponding differential energetics across populations based upon maternal lineages. Therefore, we hypothesize that, as a cancer cell disseminates from the primary tumor and spreads throughout the body, metabolic differences will lead to patient-to-patient variability in metastatic susceptibility and organotropism. This review explores the rapidly expanding literature connecting mitochondrial genomes, cancer progression, and metastasis. Our goal is to begin establishing a framework for the field to address the mitochondria's role in metastasis moving forward.

1.1 Mitochondria in evolution

Current models suggest that the mitochondrial organelle evolved through an endosymbiotic relationship between a bacterium from the phylum *Alphaproteobacteria* living inside a host cell [1]. Human mitochondria have since evolved to comprise numerous nuclear encoded proteins. Current theories surrounding SNP associated with human haplotypes suggest that as human populations migrated north out of Africa environmental factors selected for mitochondrial mutations that altered energetic capacities of the individual resulting in enhanced survival in the foreign environment [2–4,1,5–7]. During the process of ATP generation via the electron transport chain (ETC), variations in efficiency resulted in altered thermal output, with more efficient mitochondria consuming fewer calories and lower heat output, which is known as coupling efficiency. As humans migrated into colder climates, decreased coupling efficiency increased heat production thereby providing a selective advantage for early populations. Coupling efficiency, however, is likely not the only factor leading to enhanced selection, since changes in overall cell homeostasis might also have led to signaling that was more advantageous to thriving in varying regions [3]. For example, haplogroups N and M were the first to emigrate from Africa. Two of the identifying amino acid variants in MT-ND3 ((nt) 10398G>A) and MT-ATP6 ((nt) 8701G>A) alter mitochondrial membrane potential and calcium regulation [8] (Figure 1).

Today most of those early selective pressures have been mitigated by technological advancements, which has also been accompanied by enhanced longevity and increased incidence of age-associated pathologies, such as neurodegenerative disorders and cancers. Both SNP and somatic mutations in mitochondrial DNA (mtDNA) are associated with development of these disorders [9], leading to questions of whether mtDNA adaptations required for early survival of human ancestors may impact susceptibility to disease and/or disease progression.

1.2 Mitochondrial DNA

The human mitochondrial genome consists of 16,569 bp of circular DNA encoding 13 protein ETC subunits, 2 rRNA (16S and 12S), 22 tRNA regulated by a transcriptional control region termed the D-Loop (Table 1). The ETC subunits exist in four of the five ETC complexes: Complex I (NADH dehydrogenase complex (ND1, ND2, ND3, ND4, ND4L, ND5, ND6)); Complex III (CoQ-cytochrome c reductase complex (Cytb)); Complex IV (Cytochrome c oxidase complex MT-CO1, MTCO2, and MT-CO3); and Complex V (ATP

Synthase complex (ATP6 and ATP8)). All subunits for Complex II (Succinate dehydrogenase complex) are encoded in the nucleus [10]. The ETC is a critical regulator of energy production via reduction of oxygen and generation of an electrochemical gradient necessary to produce ATP. In addition to energy metabolism, mitochondria regulate reactive oxygen species (ROS) and other free radical levels, apoptosis, cellular pH, and calcium levels through interaction with the endoplasmic reticulum. Alterations in any of these cellular components can lead to signaling changes and modifications of the epigenome [4,6,11,12].

Otto Warburg and colleagues observed that cancer cells ingest large amounts of glucose and produce lactate via glycolysis despite the presence of oxygen, a process termed aerobic glycolysis or the so-called Warburg hypothesis [13–15]. This led Warburg to hypothesize that mitochondrial dysfunction was a primary cause of aerobic glycolysis and a driver of tumorigenesis [16]. Over time, this hypothesis has been modified since it has been confirmed that cancer cells maintain mitochondrial respiration [17,18]. Still, Warburg's observation remains a seminal hallmark of cancer biology since it ushered in the idea that mitochondria are pivotal to cancer biology.

In addition, we now appreciate that gain-of-function mutations in TCA cycle enzymes IDH1/2 can potentially be cancer drivers [19]. Yet, while some clear roles for nDNA-encoded genes regulating tumorigenesis have been identified [20,21], defining mtDNA SNP or mutations as drivers of cancer is generally not the case [22]. In part, this is due to insufficient baseline data to distinguish between driver and passenger mtDNA mutations associated with cancer progression. However, a consistent trend, correlates increased ROS with an increase in mtDNA mutational burden and tumor progression [23,24]. Also, previous studies have nicely laid the ground work that begins to define roles of mtDNA mutations within cancer. Unfortunately, interpretation remains challenging due to sample size and discrimination between tissue types. Nonetheless, two recent studies that analyzed TCGA datasets identified some intriguing correlations [25,26]. Details from those studies will be elaborated below.

While any such correlations are useful for hypothesis generation, limitations of experimental models and technology restrict functional testing. Additionally, existence of multiple copies of mtDNA with individual mitochondria and cells imposes challenges to experimental design and interpretation.

1.3 Mitochondrial DNA Mutations

The mutation rate of mtDNA is ~10x higher than in nDNA [27] and is thought to be due to mitochondria being the major producer of cellular ROS, which then cause DNA damage [28]. Some of the mtDNA SNP can be associated with potential evolutionary differences between closely related species and cancer cell subpopulations. Why would mtDNA be more susceptible to mutations? Besides increased levels of ROS in the local milieu, a lack of protective histones and reduced DNA repair are thought to contribute [29]. ROS cause single strand breaks in mtDNA which, in turn, leads to degradation and mitochondria turnover, thus highlighting mitochondria-specific mechanisms of DNA maintenance [30]. These data should not be misconstrued to suggest a lack of mtDNA repair mechanisms. Indeed,

mitochondria repair DNA, but their repair enzymes appear to be comparatively less effective than nuclear DNA polymerases [31].

The role of ROS in mtDNA mutations is a little less clear. Only 4% of mtDNA mutations result from guanine oxidation, which is typically associated with ROS induced damage. Still, ROS increase mutational burden of mtDNA in other contexts, which typically results in either thymine glycols for pyrimidines or 8-oxodG for purines [30,32]. 8-oxodG has low mutagenicity but prevents polymerase activity; whereas, thymine glycol allows polymerase activity but has high mutagenicity resulting in G:T transversions. Additionally, many mtDNA mutations arise from DNA replication errors with heavy and light chain strand biases [25,26]. mtDNA includes regions with abundant nucleotide repeats, which are another attribute of mtDNA that makes mutational analysis difficult. The nucleotide repeats may contribute to observations of increased mtDNA mutations, as DNA polymerases are more prone to slippage and introduction of run length variation at these sites [25,26,33].

2 Mitochondrial DNA and Cancer

Acquired mtDNA sequence differences can exist in a heteroplasmic state, in which a subset of mitochondria within a cell harbor one or more mutations while others do not. In contrast to homoplasmy, in which all mitochondria in a single cell are identical, heteroplasmy can impact disease severity and a disease subtype [12]. The existence of heteroplasmy, therefore, increases complexity regarding how mtDNA polymorphisms could regulate (cancer) cell functions. With the advent of next generation sequencing (NGS), the breadth of mtDNA mutations detected in neoplasms has greatly increased concomitant with greater depth of reads. With accumulating mutations associated with aging (and increased likelihood of heteroplasmy in cells [34,35]), discernment of driver *vs* passenger mutations becomes increasingly challenging. Nuclear mutations associated with cancer are more easily tracked as there are only two copies per cell. With the large variation in the number of mitochondria across tissues and cancers, mitochondrial mutations create a very different picture. The existence of heteroplasmy itself contributes to genetic instability and reductions in overall fitness [36]. There appears to be selection against heteroplasmy even within cancer cells [25,26], which suggests homoplasmy may be vital for maintenance of mitochondrial function. Some questions remain: Is it known how the stringency/preference for homoplasmy is enforced in the cell? Are constraints applied by homoplasmy enforcement? Does homoplasmy constrain dictate a preferential selection of particular mitochondria?

Despite high frequency mtDNA mutations in cancer specimens, not all cancers are created equal. For example, Ju *et al.* demonstrated that gastric, hepatocellular, prostate, and colorectal cancers had the highest number of mtDNA mutations, whereas hematological cancers had the fewest [26]. This observation raises several interesting questions: Do selective pressures drive acquisition of, or selection for, mtDNA mutations? Are there tissue specific variations in mtDNA roles in cancer or metastatic progression? Do ‘hotspots’ of mtDNA mutation occur concomitant with cancer progression? Are the somatic mtDNA mutations drivers, passengers or hitchhikers? Are there germline differences in mtDNA (i.e., SNP) that predispose people to cancer and/or metastasis? And, given recent data showing that mitochondrial subcellular localization changes during different cellular processes (such

as migration and invasion [37–41]), do mutations or SNP in mitochondria regulate cellular distribution?

Nonetheless, multiple studies have carefully analyzed mtDNA sequences in cancer and have identified correlations. To date, few driver mutations have been identified in cancer. While this review will focus on mtDNA mutations in metastasis, some of the mtDNA changes associated with carcinogenesis may also shed insight onto the metastasis problem.

We suggest reviews from Chatterjee *et al.* [42], Lu *et al.* [43], Hertweck *et al.* [44], as well as the MITOMAP database [45] for readers interested in potential mtDNA drivers of cancer. For example, some reports suggest that the mutational frequency was higher within the hypervariable D-Loop [25,46–48]. It is unclear whether D-loop mutations are simply correlative or drivers of tumorigenesis, however. A common deletion (in a D310 stretch of cytosines) is highly variable across many different cancers, but no clear correlation has yet been made with cancer progression.

The above mutations reflect somatic genetic changes in mtDNA. Despite the limitations in ascribing cause-effect relationships with mtDNA and cancer development, hints to associations have been made. As will be shown below, there are also some mtDNA SNP in the germline that may alter susceptibility to cancer development and progression. The relative abundance of germline changes associated with tumorigenicity or metastasis is still unknown.

3 Mitochondrial DNA and Metastasis

Just as humans underwent environmental selection during evolution, metastatic cells also undergo selection in different microenvironments that lead to organotropism. We reviewed the literature for cases of mtDNA alterations resulting in or associated with variations in metastatic colonization. There are enticing hints regarding mitochondrial genetics in metastasis, but the jury is still unclear which are the most critical changes. As one reads the literature, it is critical to remember that the mitochondrial genome changes are likely metastasis *modifiers* rather than drivers *per se*. In other words, mtDNA encodes quantitative trait loci (QTL) that combine with both nuclear and mitochondrially-encoded genes to regulate complex diseases like cancer and disease severity [49–51]. QTL are a group of alleles that influence a particular phenotype or trait [52]. Measurable trait differences can be influenced by the combined interactions of multiple different polymorphisms present within the genome, as well as environmental factors. It should therefore be noted that mitochondrial polymorphisms are likely not going to be the complete explanation for differences in disease progression, but may contribute and/or influence disease progression through complex interactions with other genes as well as altering responses to changes in environmental factors. We believe this is an important clarification, as mitochondrial polymorphisms would otherwise be expected to exhibit strong maternal inheritance patterns. Instead nuclear-mitochondrial crosstalk as well as individual responses to changing environmental factors are more likely influenced by mitochondrial polymorphisms. In addition, we hypothesize that changing a cancer cell’s environment through metastatic dissemination and colonization

may also play a role in regulating selective effects by mitochondrial polymorphisms that may alter the ability of these cells to differentially survive in relation to altered landscapes.

Consistent with this idea, Webb *et al.* detected no correlation between overall colorectal cancer risk and haplogroup association [53]. They did, however, find promising correlations between individual SNP including a polymorphism in the small non-coding region 4 between the mt-tRNA^{Ala} and mt-tRNA^{Asp} (A5657G) that had an increased association with colorectal cancer in comparison to rectal cancer, and a synonymous change in MT-ND2 (T4562C) that had a high association with the microsatellite instability subtype of colorectal cancer [53].

The pioneering work that established a genetic background component to metastasis came from Kent Hunter's group. They crossed FVB/NJ-TgN (MMTVPyMT)^{634nul} mice (PyMT) to dams from different mouse strains and found that F_1 progeny had varied metastatic efficacy [54–57]. While nuclear DNA-encoded metastasis efficiency modifiers have been mapped in breast and prostate cancers [56,58], an alternative interpretation was posited because of maternal mitochondrial inheritance [59].

To test this latter hypothesis without complexities of nDNA cross-over, we developed a mouse by transferring pronuclei from one strain into the enucleated embryonic cytoplast of another strain [60,61]. The resulting embryo was then transferred into a pseudopregnant nuclear-matched mouse and allowed to develop to term. We termed this mouse mitochondrial-nuclear exchange (MNX). Crossing nDNA-matched mice with female MNX mice allows segregation of mtDNA influence from nuclear genes. Using this model, we demonstrated that the mitochondrial genome alters tumor progression and metastasis [62,63,59] in the PyMT model nearly identical to what was observed in the wild-type mouse crosses. Furthermore, crossing MNX mice with mice over-expressing the HER2/Neu oncogene, we observed driver-dependent alterations in tumorigenesis and metastasis [62]. While not the focus of this review *per se*, MNX mice have uncovered existence of mtDNA modifier loci in cardiovascular disease [61], atherosclerosis [61,64], and obesity [63], strongly supporting a closer look at mitochondrial quantitative trait loci [65,66]. Early studies to dissect the molecular mechanisms identified significant and, importantly, selective changes in the methylation patterns and gene expression profiles of wild-type and MNX mice [63]. The latter point deserves emphasis since simply modifying metabolism (and, by inference, metabolite pools that affect DNA methylation or histone modification) would result in global, rather than site- or gene-specific, changes.

Results with MNX mice were not the first to implicate mitochondrial genetics in metastatic behavior. Kaori Ishikawa and colleagues were pioneers in this effort [67,23,68]. They demonstrated that mitochondrial dysfunction enhanced tumorigenicity and metastatic potential of lung and breast cancer cells [68,67,23]. Mutations that disrupted the function of complex I (e.g., insertion in the MT-ND6 gene (13885insC)) led to a reduction in complex I activity and elevated ROS, with a corresponding increase of metastatic propensity [23,69]. This impediment imparted by the mtDNA mutation also led to an increase in transcription of glycolytic- and metastasis-related genes [69]. The functional role of complex I mutations in metastasis was later highlighted in the MDA-MB-231 breast cancer cell line whose

metastatic potential appears to be linked to mutations in MT-ND6 (C12084T) and MT-ND5 (A13966G) [68].

In addition, missense and nonsense mutations in MT-ND6 result in an increase in invasion and migration in the A549 lung cancer cell line [70]. Furthermore, Koshikawa *et al.* uncovered multiple other mutations in mitochondria NADH dehydrogenase genes with a high predictability of altering complex I function that were associated with distant metastasis. Two of these mutations had previously been identified as germline SNP in the MT-ND1 gene (C3497T and T3394C). In addition, the authors found multiple mutations that changed evolutionarily conserved amino acids (T3398C, T12338C, G3709A, T10363C, C11409T, G13103A and T14138CC). Two mutations - T3398C and T12338C - had been previously reported to be associated with mitochondrial diseases [71,72]. The authors also found 3 polymorphisms in MT-ND1 (C3689G, G3709A and G3955A) that are likely to cause a conformational change. Lastly, three mutations cause a premature termination of either the MT-ND5 or MT-ND6 proteins (G12813A, G13366A and 14504delA) [69]. Whereas these data were unable to identify individual driver mutations, strong correlations between these mutations and metastatic lung and colon cancers provide strong rationale for the future analysis of these individual point mutations.

Comparing T3394C mutations in tumors with the adjacent mucosa identified T3394C as an acquired somatic mutation [69]. This observation further highlights important questions: While the MNX data suggest germline modifiers of metastatic efficiency, are there acquired somatic mutations that drive cells to a metastatic phenotype? Are the mtDNA mutations associated with metastases to select microenvironments? Do some mutations drive metastasis in general? Insufficient data exist to address these questions, but some anecdotal data suggest that there are context-dependent mtDNA mutation-selection events in cancer.

Kenny *et al.* compared non-invasive *vs* invasive cell lines and quantified heteroplasmy and mtDNA mutations, which were increased in the invasive cell lines [73]. Consistent with other findings, non-invasive cell lines predominantly utilized oxidative phosphorylation. However large inter-experimental variability in OCR or ECAR were observed. The authors attributed the variability to levels of mtDNA heteroplasmy, most notably in the MDA-MB-157 human breast carcinoma cell line. However, on average the invasive cell lines had an increase in a shift toward a more glycolytic phenotype [73]. Other papers report the ratio of oxidative phosphorylation to glycolysis to either increase [74] or decrease [75] as invasion and metastasis increases. Some of these differences could be associated with tumor cell origin, but experimental data to support this interpretation are lacking.

Other reports have also identified the importance of the mitochondria in mediating metastasis through the use and analysis of antioxidants. Goh *et al.* demonstrated that PyMT tumor metastatic potential increased inversely with mitochondrial catalase (mtCAT) expression. Consistent with their other findings, decreased metastatic potential corresponded to reduced ROS production. mtCAT expression in the microenvironment also induced an increased resistance to ROS, suggesting that mtCAT could neutralize the effects of ROS [76]. Furthermore, mtCAT reduced the presence of $F4/80^{+}$ macrophages within the PyMT primary tumors while also reducing expression of $CD34^{+}$ endothelial cells [77]. Thus, the

connections between metastasis and ROS could be more microenvironment-related rather than intrinsic to tumor cells themselves.

Blein *et al.* [78] examined patients with pathogenic BRCA1 or BRCA2 mutations and sequenced their mtDNA to determine whether mitochondrial SNP were associated with increased risk. As BRCA1 and BRCA2 are important for DNA repair the authors hypothesized that alterations in ROS production would differentially regulate susceptibility due to differences in acquisition of DNA damage. Of 7,432 breast cancer cases and 7,104 controls without BRCA1 mutations as well as 3,989 invasive breast cancers and 3,689 control unaffected BRCA2 mutation carriers, they found that the T haplogroup was associated with an increased risk of developing breast cancer in relation to BRCA1 and BRCA2 mutations [78]. In contrast, data from Ju *et al.* [26] found no correlation between oncogenic drivers that impact nuclear DNA (nDNA) mutation rates and mtDNA mutation rates, including BRCA1 and BRCA2 genes. Likewise, there were no correlations between tobacco use and lung cancer or UV and melanoma with regard to mtDNA mutations.

Oncogenes play a large role in the restructuring of tumor metabolism [79–81]. It stands to reason that mitochondrial SNP/mutations may also differentially regulate tumorigenesis in the context of altered oncogenic drivers. Combinatorial effects of mtDNA QTL and nDNA QTL (including oncogenes) is supported by data showing that mtDNA SNP cooperate differentially with different oncogenic drivers to alter metastasis efficiency [62]. However, as identified below the context specific nature and association of specific mutations/polymorphisms requires much more in-depth analysis and experimentation. Also, while data support roles for mtDNA metabolic dysfunction and increased ROS in promoting metastasis, cause-effect relationships are still lacking.

3.1 mtDNA Metastatic Correlations

With increasing use of NGS sequencing, an increasing number of analyses of patient data are being reported that attempt to associate mtDNA mutations with metastatic susceptibility. Unfortunately, most early GWAS or EWAS studies did not include mtDNA in genomic analyses. It should be stated, however, that issues exist in sequencing of the mtDNA, including a lack of definitive detection due to multiple copies and heteroplasmy, as well as possible contamination with normal tissue. In addition, mtDNA sequences have been found integrated into the nuclear genome of cancer cells, further complicating the analysis [82]. Despite these caveats, more inclusion of mtDNA in future genomic studies will hopefully improve understanding of the role of specific mtDNA in metastasis. The true value in these types of studies is that they look for common mutations across patients that are enriched at specific metastatic sites *vs* the site of the tumor origin. The premise is that non-random changes will be observed if there are specific mtDNA driver mutation(s) associated with metastasis. Below we summarize early studies that are charting the way for larger cohorts. A significant limitation is the paucity of matched primary and metastatic lesions for most tumor types. However, more investigators are recognizing this need and institutions are collecting the necessary samples. Thus, at this juncture, it is critical to recognize that cohorts are small and care must be taken not to over-interpret, nor over-extrapolate, the findings or

conclusions. Nonetheless, some intriguing insights are being garnered from these efforts (Figure 2).

McGeehan and colleagues [83] chose to sequence whole blood DNA from 13 patients with breast-to-brain metastasis. They observed that 45% of the variations between the patients occurred in the D loop, 5% simultaneous mutations in MT-ND6 and MT-ND5, and 6% mutations in mt-Cyb. Of 23 non-synonymous variants the authors hypothesized that 3 of these variants may lead to functional differences based on predicted structures. These variants included an insertion (T14819insTTCTATA) into MT-CYB altering amino acid S25, a mutation (A9664G) in MT-CO3 altering amino acid E153G, and a mutation (T8821C) in MT-ATP6 altering amino acid S99P. Readers are cautioned because structures of mitochondrial proteins are often inferred from different species. However, the systematic approach is likely to pay dividends eventually. Also, the cellular origin of cell-free DNA isolated in blood cannot yet be determined unequivocally. The cfDNA may be from inflammatory or other stromal cells, tumor cells or both. Additionally, there is accumulating evidence that mtDNA can be transferred between cells or species [84–90].

Arnold and colleagues [91] compared mtDNA sequences from 10 prostate tumors with distant metastases. The tissues analyzed included the primary prostate, soft tissue and bone metastases, and adjacent normal tissues. The authors identified an acquired A10398G mutation in 7/10 patients with bone metastases. Interestingly, this same mutation is a germline polymorphism associated with cancer susceptibility. Heteroplasmy/homoplasmy may have also been positively selected based upon selective pressures within the microenvironment, as they selected for mutations in all three cancer lesions tested, including G9820A in MT-CO3. The MT-CO3 mutation was heteroplasmic in both the primary tumor and soft tissue metastasis but was homoplasmic in the bone metastasis. Based upon this finding, the authors concluded that homoplasmy was critical for metastatic outgrowth in bone. However, asynchronous seeding and outgrowth with another mutation (either nuclear or mitochondrial) could not be excluded as an explanation. In addition, the authors also uncovered mutations that were negatively selected against within the soft tissue metastatic site including a mutation at nucleotide position 9377 in MT-CO3. Taken together these data highlight a tissue specific selection process for mtDNA mutations advantageous for cancer outgrowth. These results highlight important mtDNA-mediated differences in metastatic potential, and, whereas mutational burden was higher in the metastatic lesions, the conserved nature of some of these mutations suggest a potential selection pressure for their acquisition. Clinical associations of the A10398G SNP vary, depending upon the study, but an explanation for this variability is unclear. Impacts of SNP and mutations on phenotypic traits vary depending on SNP and mutations in the complementary genome (nDNA/mtDNA), although testing this hypothesis would be extremely difficult to address in highly genetically variable human populations. What is not always clear from many clinical studies, even using TCGA, is that the analyses occur with varying levels of stromal contamination (i.e., non-laser-captured tumor cells), meaning that ascribing germline or somatic origins of mutations are sometimes tenuous. Further, the authors also identified mutations in the mt-tRNA^{Arg} and mt-tRNA^{Thr} at nucleotide positions T10463C and G15928A, respectively. The latter findings illustrate that nonprotein-coding components of mtDNA could also impact metastatic efficiency.

Hopkins *et al.* [92] analyzed primary tumor and matched metastatic samples from 6 pancreatic ductal adenocarcinoma cancer patients. Four patients had similar mtDNA mutation profiles in both primary and metastatic tumors while one had a mtDNA mutation (G6021A) in the primary tumor that was not present in the metastases. However, another patient exhibited mtDNA G316A and G2976A mutations in all metastatic sites and G2145A mutations only in diaphragm and omentum metastases. Neither mutation was detectable in the primary tumor [92]. While these data suggest that some mtDNA mutations may be important for metastatic progression overall, other mutations may mediate organ specific outgrowth.

The D-loop contains heavy and light chain promoters (ITH1 and ITL) for mitochondrial DNA transcription [10], which can have huge impacts due to reduction/alteration in mitochondrial transcription. Van Trappen *et al.* [93] hypothesized that the D-loop mtDNA mutations may be altered in ovarian cancer metastases. Majority (13/17) of the primary and matched omentum metastases had similar D-Loop mtDNA mutation profiles. However, 4/17 had mutation differences compared to contralateral ovarian cancers with the metastases derived from the tumor containing the additional mutation.

Ebner *et al.* [94] compared the mtDNA control region in 351 malignant melanoma patients and 1598 healthy controls. They identified polymorphisms A16183C, T16189C, C16192T, C16270T and T195C to be significantly associated with development of melanoma. Interestingly, Breslow thickness was associated with A302CC-insertion and T310C-insertion polymorphisms and the T16519C polymorphism had about a 10% increase in association with metastasis.

Recent data also suggest a role for small non-coding RNAs in mediating the mitochondria to nuclear cross-talk [95]. Consistent with this a recent study analyzing the commonly associated MELAS mutation 3243A>G demonstrated that the mutation can promote increased transcription with development and EMT, through modulation of miRNA levels [96]. These results further support a mechanism by which alterations in the mtDNA can alter metastatic susceptibility and tumor progression.

There are many potential links between mitochondrial genetics and metastatic susceptibility or efficiency. Notwithstanding stochastic differences between patients, we and others hypothesize that specific combinations of nDNA and mtDNA SNP and oncogenic drivers will be predictive. Because of its pivotal role in regulating metabolism, most interpretations regarding the role of mitochondria in the process of metastasis focus on metabolism. However, as can be seen from the various examples above, there are myriad alternative explanations that will require significantly more extensive experimentation to sort out.

4 Mitochondrial Haplogroups and Cancer

As mentioned above, there are globally two roles for mitochondrial genetics in metastasis. Evidence for both somatic mutations during the process of tumor progression as well as germline polymorphisms that predispose/protect metastasis exists. However, determining cause-effect relationships in population-based studies is extremely difficult due to the

prevalence of confounding variables. The strongest evidence comes from experimental models, most commonly mice, since the variables can be controlled more easily. However, extrapolating murine data to humans is not a precise science at this time. Just as in humans, most studies focus on a limited number of haplogroups, making it challenging to separate nuclear and mitochondrial contributions. Therefore, review of mtDNA haplogroup association below is not complete, but rather provides examples of compelling mtDNA polymorphism correlations with cancer susceptibility. For a more thorough analysis of the strengths and weaknesses of animal models studying mtDNA polymorphisms in cancer risk association, please refer to a recent review from Bussard and Siracusa [97]. Following a brief discussion of the population-based studies, we will briefly summarize some of the newer experimental approaches to dissect the contributions of mitochondrial DNA in cancer progression and metastasis.

Li *et al.* examined colorectal cancer risk in a large cohort of men and women from Hawaii and California. The cohort was primarily comprised of Americans of Asian (28.69%), African (24.35%), European (21.42%), Latino (20.45%), or Native Hawaiian ancestry (4.90%) [98]. Of 2,453 CRC cases and 11,930 controls, the most significant mtDNA SNP was A4917G, which is associated with the T haplogroup. Overall, the T haplogroup was associated with increased risk of developing colorectal cancer. Interestingly, in the European-American group G4655A was highly associated with colorectal cancer risk, but not significantly across the entire population, suggesting that this SNP may pose an added risk depending upon the nuclear factors with which it interacts.

Canter *et al.* [99] demonstrate that the disease-associated G10398A polymorphism, which is associated with several neurodegenerative disorders is also associated with an increased risk of breast cancer in African-American women. This study was a part of the University of North Carolina Breast Cancer Study (654 cases and 605 controls). No difference was observed for Caucasian women harboring the allele.

4.1 Complex I

As noted above, a common SNP associated with migration of *H. sapiens* is the 10398 MT-ND3 polymorphism (haplogroup N). G10398A polymorphism has been analyzed in multiple different contexts and has been associated with several neurodegenerative disorders and is also associated with an increased risk of breast cancer in African-American women [99]. G10398A is accompanied with decreased NADH dehydrogenase activity, increased ROS, increased apoptotic resistance and increased tumorigenicity in cybrid models [100]. Since the G10398A SNP emerged early during human evolution (i.e., at the time of branching to haplogroups M and N), the population base for studying this cohort is large and could facilitate population studies. However, the context under which these SNP are studied may have important implications as additional polymorphisms or differential interaction with the nuclear genome may still alter these susceptibilities. Therefore, combining data from both population and experimental systems will probably be necessary to definitively understand mechanisms involved.

These issues are further elucidated as additional reports demonstrated a role for the alternative polymorphism A10398G in disease susceptibility. Czarnecka *et al.* [101] report

that the A10398G is highly enriched in Polish patients with breast cancer (23%) in comparison to controls (3%). The authors collected 44 breast tumors, 31 head and neck tumors and 100 specimens from healthy donors. Tengku Baharudin *et al.* [102] report that the MT-ND3 A10398G polymorphism is also associated with an increased risk of invasive breast cancer. The authors focused on Malay females. This SNP has also been demonstrated to alter mitochondrial pH and intracellular calcium, as well as the modulate ATP production and apoptosis. Additionally, the BAX to BCL-2 ratio was higher in breast cancer patients that harbored the 10398G SNP, suggesting increased apoptosis in the $10398G^{+}$ tumors. However, this only adds to the complexity of the 10398G paradigm as these tumors might be expected to have a reduced growth rate as well.

4.2 Complex IV

Mutations or SNP located near well-documented mutations may promote similar (albeit potentially reduced) impacts on mitochondrial function. A deeper understanding of these effects will likely accompany understanding of mitochondrial protein structure and folding. Petros *et al.* detected a mutation in MT-ATP6 C8932T (P136S) [103], which is located 20 amino acids away from the well characterized 8993G L156R mutation associated with Leigh syndrome [104], which itself leads to reduced lymphoblast respiration [105] and decreased ATP synthesis and elevated mitochondrial ROS [106]. Introduction of the T8993G MT-ATP6 mutant mtDNA into PC3 prostate cancer cells increases tumor growth rate and mitochondrial derived ROS [103]. Thus, these data suggests that the close proximity of the C8932T mutation may also promote tumor growth. Recent association of this mutation with neuromuscular disorders further supports this hypothesis along with predictions suggesting that this mutation would likely disrupt the protein structure [107]. This has important implications for patient populations as well, since the C8932T polymorphism was detected in two individuals during haplotype characterization [2].

Intriguingly, the T8993G mutation in prostate cancer further enhances prostate cancer growth through promotion of FGF1 and FAK in the context of the bone microenvironment [108], suggesting that specific alterations in the mitochondrial genome could act to promote site-specific metastatic outgrowth.

4.3 Complex III

After observing loss of cytochrome C oxidase I (MT-CO1) in a prostate cancer sample due to the insertion of a stop codon, Petros *et al.* [103] hypothesized that MT-CO1 mutations may be associated with prostate cancer. A large number of MT-CO1 mutations were found in prostate cancers. Four mutations were found in multiple tumor samples. Three patients in haplogroup H had tumors containing T6253C mutations, two patients in haplogroups H and N had C6340T mutations, six patients in haplogroups J, T, L1, and N had tumors with G6261A mutations and five patients in the L2 or an unclassified haplogroup had A6663G mutations. The authors did not analyze other mtDNA genes and therefore MTCO1 mutations could not be directly correlated with prostate cancer susceptibility. However, the high prevalence of the G6261A and A6663G mutations suggest a role for MT-CO1 mutations in promoting prostate cancer. The G6261A mutation has also been associated with a patient who exhibited LHON-like optic neuropathy [109], while T6253C and A6663G

have been associated with primary open-angle glaucoma [110], supporting a functional role in regulating mitochondrial function. Whether these diseases are linked is also an intriguing possibility.

4.4 MT-Control Region

The D-loop is a non-coding region that spans from nt 16,024–516, and acts as a promoter for both the heavy and light strands of mtDNA. This region harbors several important transcription and replication elements [111,112]. The D-loop region contains the origin of replication for the leading strand, thus mutations in this region may impact respiration downstream. Two common hotspots for mtDNA mutations are the (CA)n dinucleotide repeat polymorphism between nucleotide position 514 and 523 in the third hypervariable region, and the poly-C repeat between 303 and 315 nucleotides (D310). These regions are associated with mitochondrial genome instability [113,114]. Multiple other hotspots have been identified in the D-loop region including the T16189C polymorphism in endometrial cancer [115], rectal cancer [116], melanoma [94], and prostate cancer [117,118]. Interestingly, this SNP has been associated with metabolic disease and type II diabetes [119,120], further supporting a linkage between mitochondrial polymorphisms, metabolism and neoplastic development. A C150T polymorphism was found to be associated with an increased risk of HPV infection and cervical cancer progression [121]. Reports also suggest that the C150T polymorphism may also play a role in enhancing longevity [122], which, when coupled with the association with HPV, might suggest a role for C150T in the regulation of the immune system.

5 mtDNA and the Tumor Microenvironment

Included in the many examples above is evidence that mtDNA both influences and is influenced by local microenvironments. Since metastatic cells interact with myriad other cells, matrices and solutions during both primary tumor growth as well as in transit, a closer look at mitochondrial mutations and how they impact various microenvironments is warranted.

Mitochondria and immune activation are intricately linked due to the ability of mitochondria to regulate the immune system through the activation of innate immune cells through recognition of cellular damage and activation of the NLRP3 inflammasome [123], or through recognition of formylated mitochondrial peptides through formylated protein receptors [124]. Formylated peptides are also present in bacterial cells, but not present in nuclear encoded peptides. The mitochondria can also indirectly and directly regulate the immune system through differential production of ROS.

Cancer associated fibroblasts (CAF) can also utilize glycolysis and produce high energy fuels (such as pyruvate, lactate, ketone bodies, and fatty acids) that can be utilized by the cancer cells within the tumor microenvironment, through a mechanism termed the ‘Reverse Warburg effect’ [125–129]. In addition, altered metabolite levels in the tumor microenvironment can have drastic impacts on the ability of immune cells to carry out their functions [130]. Metabolic efficiency within immune cell populations is also tightly linked to immune cell differentiation and differences in glycolysis and oxidative phosphorylation

predict polarization potential [131]. Consistent with this, a disruption in mitochondrial proteins can lead to immunodeficiencies within patient populations [132].

mtDNA haplotypes have also been demonstrated to impact the microbiome [133]. The Human Microbiome Project Consortium found ethnicity/racial background to be one of the strongest associations between microbial communities and clinical metadata, which suggests genetic influence [134]. Whereas the taxonomic profiles remained similar based upon collection site, the relative abundance of microbial species varied across the differing haplogroups. In addition, they were able to correlate specific polymorphisms with distinct microbial population changes, which suggests that potential metabolic or oxidative differences imparted by changes in the mitochondrial genome may be giving rise to alterations in microbial populations [133]. Indeed, we observe similar changes in microbiota in the MNX mouse model (Manley and Welch, Manuscript in Preparation). When coupled with the newest findings linking microbiomes and immune function (as well as responses to immunotherapies) [135–139], the interrelationships between those phenotypes and metabolism are recognized as particularly important.

Lastly, as mitochondria are primary consumers of oxygen; so, changes in oxygen concentrations in hypoxic tumor microenvironments drastically changes metabolism [140]. Mitochondria derived ROS have also been demonstrated to induce the Hypoxia-inducible factor α (HIF-1α), which directly links mitochondria to the hypoxia response [141]. Thus, hypoxia will have overarching effects on many cells within the tumor microenvironment, which may be further impacted by differences in mtDNA.

6 What Are the Signals?

If readers will allow us to stipulate that mtDNA mutations and that SNP are modifiers of metastasis efficiency and/or organotropism, a key remaining question is what signal(s) come from the mitochondria to coordinately regulate the large number of genes that are involved in the metastatic cascade? The retrograde (from mitochondria to nucleus) signals will also be coupled with anterograde (from nucleus to mitochondria) signals that couple the transcriptome to cytoskeleton and other cytoplasmic functions such as secretion and proper protein placement in organelles. Already, metabolite changes are implicated as described above.

Taking cues from early mitochondrial evolution and cellular homeostasis may provide a framework for understanding mitochondria-nuclear crosstalk [1]. Initially, the two unicellular microorganisms communicated to eventually establish a symbiosis. With time, it is thought that duplicated proteins were eliminated in the mitochondrion. Coordination of nuclear and mitochondrial signals determine how cells respond to stress and other outside-in signals to regain and maintain homeostasis. Multiple different mechanisms used by mitochondria to signal to the nucleus include: ROS and TCA metabolite release, calcium homeostasis, changes in mitochondrial membrane potential, peptide production, and AMPK activation [142,143]. Which of these, or yet undiscovered, signals are critical in controlling metastasis?

Numerous studies report that mtDNA SNP/mutations that increase ROS are associated with increased metastatic potential [144–146], which highlights the need for better understanding the signaling mechanisms that are driving the enhanced metastatic propensity. Koshikawa *et al*. demonstrated that a disruption in ETC function and increased ROS results in a switch toward increased glycolytic metabolism and increased transcription of metastasis-associated genes [67,23,69]. Increased accumulation of ROS can also lead to protein oxidation and disruption of protein folding. Taking this into consideration Kenny *et al*. analyzed the role of the mitochondrial unfolded protein response (UPR^{MT}) [73] and found that activation of SOD2 through a SIRT3/FOXO3a/SOD2 axis downstream of the UPR^{MT} may increase mitochondrial biogenesis and the antioxidant response in aggressive disease. Furthermore, the authors demonstrated that SOD2 expression is an important component in the regulation of the invasion, but is not sufficient to drive the invasive phenotype. They also found a significant association between SOD2 expression and metastatic lesions, and by using cybrids were able to demonstrate that the expression of the SIRT3/FOXO3a/SOD2 signaling axis proteins were elevated in the presence of mtDNA from invasive cell lines [73]. However, SOD2 was not found to be a regulator of metastasis in other studies [147,148].

Differences in mitochondria-nuclear crosstalk also likely have important roles in racial disparities and racial differences in cancer aggressiveness and progression, as varying nuclear and mitochondrial ancestral backgrounds may result in differential crosstalk and regulation of disease. Interestingly, numerous studies have demonstrated that certain polymorphisms can be significantly associated with disease susceptibility in one ethnic group and not another, which supports a role for nuclear and mitochondrial crosstalk, examples of which are highlighted below.

7 Models to Study the role of the Mitochondrial DNA

Despite the tantalizing population-based and clinical data correlating some mitochondrial SNP and mutations with cancer metastasis, defining a cause-effect relationship will require complementary experimental evidence. Since metastasis involves so many steps and so many interactions with different cell types, tissues and fluids during the cascade, it is only studied *in vivo*. *In vitro* assays are perfectly appropriate for studying steps and some biochemical processes, but the gold standard for assessment of metastasis involves experimental animals [149].

Genetic crosses can be used to assess contributions of mtDNA to phenotypes; but, nuclear cross-over complicates interpretation. So, the most commonly utilized approaches to study the role of the mtDNA in shaping disease involve methods that combine wild-type or mutant mtDNA and a common nuclear genomic background. To achieve this several different approaches have been used.

Cybrids utilize methods to eliminate the mtDNA, which promotes the transfer and incorporation of a foreign mitochondrial genome [150]. The term cybrid is derived from the combination of nucleated cells with cytoblasts (nonnucleated cells), and they have previously been extensively reviewed [151]. The first step in this process is the generation of the rho-null cell, which is defined as a cell devoid of mtDNA (nuclear-encoded

mitochondrial proteins are still present). Typical methods for the elimination of mtDNA stemmed from early discoveries in yeast in which natural depletion of mtDNA occurred during conditions that favored glycolysis. Since then many labs utilized ethidium bromide (EtBr), a DNA intercalating agent that binds to the negatively charged mtDNA and prevents polymerase activity, to eliminate the mtDNA. The ETC-dependent dihydroorotate dehydrogenase step in pyrimidine synthesis is greatly impaired by EtBr [152] and results in depletion of uridine, which can be overcome by the replacement in cell culture media [153]. As a result, rho-null cells do not carry out oxidative phosphorylation; but, they do maintain a membrane potential [154]. Rho-null cells require glycolysis for energy production, which results in a NADH and NAD imbalance [155]. This imbalance can be overcome through the addition of pyruvate, as pyruvate can be generated into lactate to increase the levels of NAD. Thus, cybrid generation is reliant on the exogenous presence of pyruvate and uridine. Since mtDNA replication is blocked with EtBr, as cells divide, mitochondria progeny lack mtDNA. While a powerful technique [23], there is a caveat to their use: EtBr also intercalates nDNA and is a mutagen [156–158]. Unless deep sequencing of nDNA is performed, it is virtually impossible to assure that all effects are associated with mtDNA. Additional approaches have been aimed at eliminating mtDNA through inhibition of DNA polymerase gamma (Polγ), which can be accomplished through either the inhibition of Polγ using the Polγ inhibitor, ditercalinium, or through expression of a dominant negative Polγ [159]. In addition, dideoxynucleoside analogues that can interfere with mtDNA replication, and rhodamine 6-G inhibition of ETC activity, deplete mtDNA [160–164]. Additionally, mtDNA depletion and rho-null cells have also been generated through mitochondrial targeting of EcoR1, which leads to degradation of the mtDNA [165,166].

Mito-Mice were one of the first models developed to analyze roles of mtDNA mutations in mice. Using a cybrid cell line carrying the mtDNA mutation of interest, cells were enucleated and the resulting cytoplast was fused with a pronucleus stage embryo via electroporation. In one study [167], the authors successfully transferred mutated mtDNA into a recipient embryo and obtained heteroplasmy in the range of 40–80%. The deleterious effects of heteroplasmy were apparent since all mice died within 6-months due to kidney failure and did not live long enough to fully determine the extent of disease progression due to the mtDNA deletion. Due to the increased kidney failure observed in the Mito-Mice the authors next asked whether or not methods could be developed to alter these phenotypes. Also, using Mito-Mice, Inoue and colleagues asked whether bone marrow transplantation would impact severity of mitochondrial disease under the pretense that stem cells derived from the bone marrow may aid in the regeneration of tissues through transdifferentiation. While transdifferentiation was not observed, survival was prolonged and apoptosis was decreased in kidneys [168]. Unfortunately, interpretation of all of these results was complicated by heteroplasmy.

mtDNA mutator-mice represent a method of studying random mitochondrial mutagenesis, and can further be used to define mutations that regulate cancer progression. These mice contain a proof-reading deficient version of polymerase gamma, which results in an increase in mtDNA mutations [169]. Comparison of heterozygous and homozygous mutator-mice identified elevated levels of tumorigenesis in the heterozygous mice and a lack of tumor formation in the homozygous [170]. These results suggest that there is a threshold between

mitochondrial mutagenesis and functionality that is required for optimal tumor formation. A caveat exists however as the homozygous mice had a shortened lifespan and may not have had sufficient time for tumor formation. In order to address this question and more accurately determine the role of specific mtDNA mutations, Kaupila *et al.* outlined a strategy in which a heterozygous female mutator-mouse is crossed with a wild-type male mouse thus allowing for the generation of offspring that have a small number of novel mtDNA mutations derived from the maternal gamete and carry only wild-type Polγ alleles [171].

Conplastic strains take advantage of maternal inheritance of mtDNA and utilize backcrossing to ensure pure nuclear and cytoplasmic backgrounds of choice [172]. Over the course of ~10 generations the mitochondrial and nuclear components will be effectively pure. However, depending upon the nuclear backgrounds involved and relative crossover frequencies, there can be residual nuclear contamination.

We developed MNX mice to obviate these obvious technical limitations. We avoided use of mutagens and, because pronuclei were transferred mechanically, no backcrossing was required [97,63,61]. The mice have been stable for at least 10 generations and measurement of mtDNA contributions to disease progression can be segregated from nDNA contributions as long as the MNX cross utilized matched nuclear backgrounds and female mice.

A significant limitation to fulfilling all of Koch's postulates relating mtDNA to a phenotype is the challenge of altering mtDNA by site-directed mutagenesis in every mitochondrion *and* in every copy of mtDNA within a cell (10–100 copies per mitochondrion). Theoretically, selective targeting of the mtDNA is possible, especially in the era of CRISPR technologies. However, efficiency is far below 100%, resulting in a heteroplasmic cell.

Nonetheless, nucleases and mtDNA editing enzymes have some efficacy [173]. Briefly, mitochondria-targeted endonucleases can specifically cleave either the wild-type or mutant mtDNA, resulting in the elimination of one copy over the other. Unfortunately, there is limited specificity available for the selective targeting by endonucleases. Instead an alternative method has been developed that attaches non-specific nucleases to zinc finger DNA binding molecules to increase the specificity of cleavage, termed mitoZFN. Mitochondria targeted transcription activator-like effector nucleases (TALENs) provide another method for specific cleavage of mitochondria DNA. mitoZFNs and mitoTALENs typically are engineered to localize to the mitochondria by removal of the nuclear localization sequence and inclusion of a mitochondrial localization sequence from either the SOD2 gene or COX8A gene [174]. MitoZFNs and mitoTALENs have been effectively utilized to deplete mutated copies of mtDNA in heteroplasmic conditions and therefore restore a homoplasmic balance of the wild-type mtDNA. This method is effective, since mitochondria do not appear to activate a double strand break repair response, and rather instead promote degradation of the damaged mtDNA [175]. One disadvantage of the mitoTALENs is their relatively large size. So to improve on this, Pereira *et al.* [176] designed a smaller homing nuclease from the T4 phage (I-TevI) and demonstrated that the molecular hybrid mitoTEV-TALE is highly effective at eradicating mutated mtDNA and restoring oxidative phosphorylation.

It will take some time for these methods (alone and in combination) to mature, but improvements are occurring. Perhaps combining mitochondrial transfer (or mutagenic approaches) with selective elimination of copies not harboring the desired mutation may provide a mechanism by which mtDNA editing can occur.

8 Concluding Remarks

Accumulating evidence implicates mitochondrial functions and genetics in cancer as well as multiple steps in the process of metastasis. Historically, the possibility of mtQTL was largely ignored, probably because the contributions of a ~16 thousand nt circular DNA were predicted to be overwhelmed by ~3 billion bases of nuclear DNA. Despite a paucity of models that unequivocally segregate contributions of mtDNA QTL in cancer, the evidence is strong that the assumption was incorrect. We can no longer ignore contributions of mtDNA to complex human diseases, in this case cancer metastasis. The complexity of the genetics is daunting, especially when one considers both intrinsic (i.e., tumor cell) and extrinsic (i.e., stroma cell) effects. We must always be cognizant that mitochondria are conveyers of signals to and from the microenvironment. Clearly, some mtDNA polymorphisms will change signals being relayed both to and from the nucleus. The effects of mtDNA polymorphisms appear to be context-dependent and may regulate tumorigenesis differently in altered microenvironments as well as steps along the metastatic cascade.

Since mtDNA alters metastatic propensity based upon germline inheritance, the relatively small size of mtDNA may provide an opportunity to utilize sequence information to guide physicians in treatment planning, i.e., sparing low risk patients from unnecessary treatment. There would be both technical and economic advantages to sequencing mtDNA since results would be obtained more quickly and at lower cost. And, if the predictive value were realized, costs for treatment could be reduced. Of course, all of this discussion is mere speculation at this time.

Looking forward, the keys will be: to expand the analyses to multiple cancer types with paired primary tissues and metastases from different organs/tissues; to develop methodologies that allow site-directed mutagenesis of mtDNA; to reduce the time required to develop homoplasmic models; and to discern the signals responsible for both anterograde and retrograde signaling by mitochondria.

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Abbreviations

bp	Base pair
CAF	Cancer associated fibroblasts

cfDNA	cell-free DNA
CTC	circulating tumor cell
ROS	Reactive Oxygen Species
ETC	Electron transport chain
EWAS	epigenome-wide association study
EMT	epithelial-mesenchymal transition
ECAR	extracellular acidification ratio
GWAS	genome-wide association study
mtDNA	Mitochondrial DNA
MNX	Mitochondrial-nuclear exchange mouse
NGS	Next generation sequencing
nDNA	Nuclear DNA
nt	Nucleotide
OCR	oxygen consumption ratio
SNP	Single nucleotide polymorphism

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Haplogroup		Complex I	Complex II	Complex III	Complex IV	Complex V
L0	"African"	ND1-3516A ND2-5442C ND4L-10589A ND4-12007A ND5-12720G			CO1-6185C CO3-9347G, 97558A	ATP6-9042T
M	"Asian"	ND3-10400T		CytB – 14783C, 15043A		
N	"Eurasian"	ND3-10398A ND4 – 10873T		CytB-15301G	CO3-9540T	ATP6-8701A

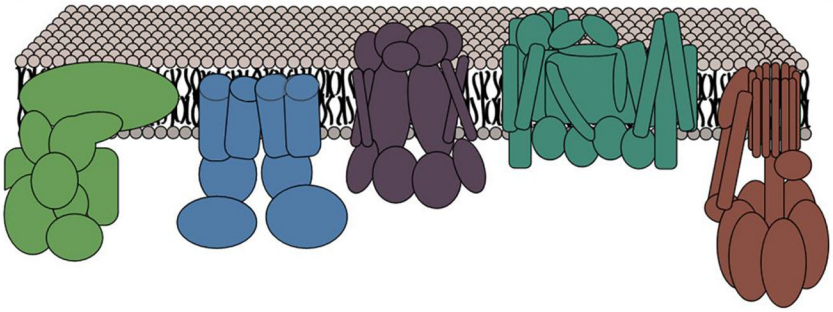

Figure 1:

Polymorphisms associated with major haplogroup lineages. Polymorphisms in genes coding electron transport chain proteins associated with major haplogroups. Nucleotide positions are followed by nucleotide changes that designate haplotype differences. Positions are based on the Revised Cambridge Reference Sequence ("rCRS"). GenBank's RefSeq database sequence number NC_012920.1. For a complete list of haplogroup markers reference https://www.mitomap.org/foswiki/bin/view/MITOMAP/HaplogroupMarkers

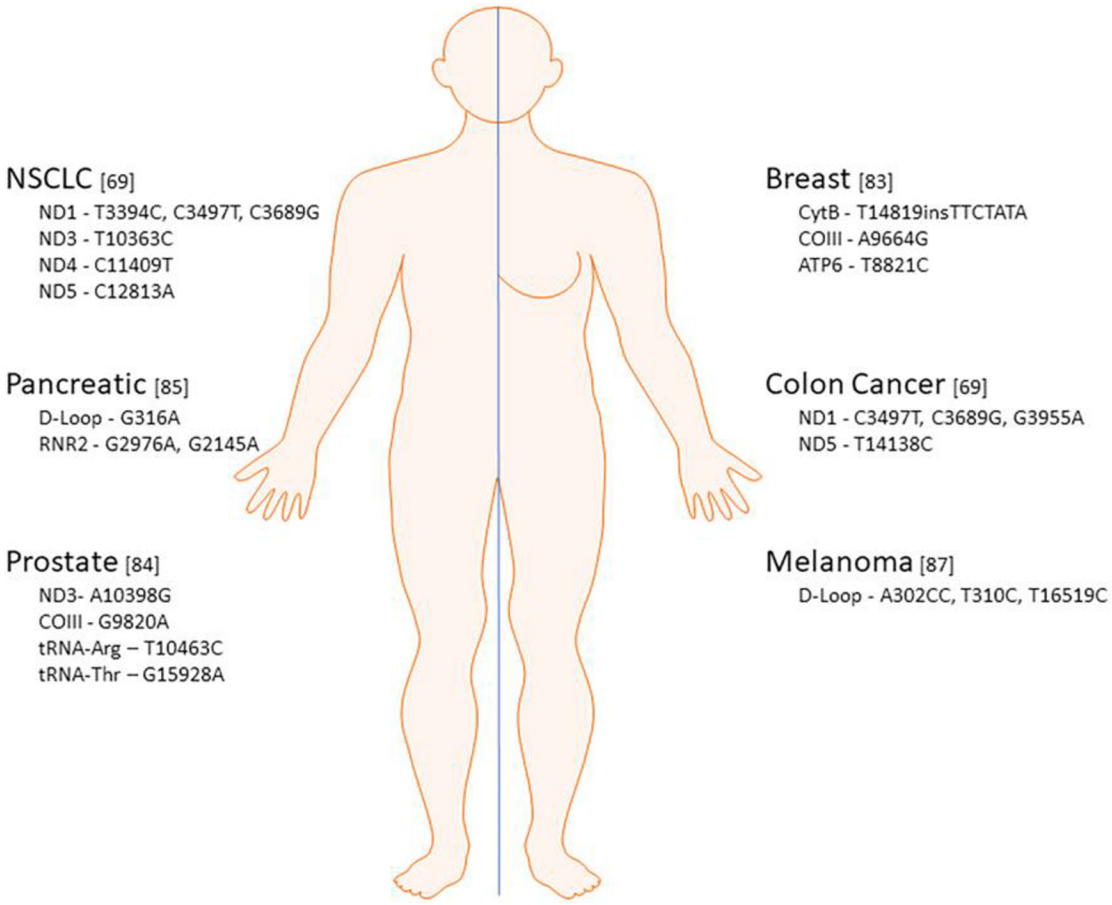

Figure 2:

Association of mtDNA polymorphisms with cancer metastasis. Summary of references associating mtDNA polymorphisms with metastatic dissemination across multiple different tumor types. References are designated by primary tumor type. Apart from gender specific cancers (prostate and breast), no other cancers exhibited gender bias.

Table 1:

mtDNA protein coding gene sequences. Gene positions are based on the Revised Cambridge Reference Sequence (“rCRS”). GenBank’s RefSeq database sequence number NC_012920.1.

		Complex I		
Gene	Starting	Ending	Shorthand	Description
MT-ND1	3307	4262	ND1	NADH Dehydrogenase subunit 1
MT-ND2	4470	5511	ND2	NADH dehydrogenase subunit 2
MT-ND3	10059	10404	ND3	NADH dehydrogenase subunit 3
MT-ND4L	10470	10766	ND4L	NADH dehydrogenase subunit 4L
MT-ND4	10760	12137	ND4	NADH dehydrogenase subunit 4
MT-ND5	12337	14148	ND5	NADH dehydrogenase subunit 5
MT-ND6	14149	14673	ND6	NADH dehydrogenase subunit 6
		Complex III		
Gene	Starting	Ending	Shorthand	Describtion
MT-CYB	14747	15887	Cytb	Cytochrome b
		Complex IV		
Gene	Starting	Endinig	Shorthand	Description
MT-CO1	5904	7445	COI	Cytochrome c oxidase subunit I
MT-CO2	7586	8269	coII	Cytochrome c oxidase subunit II
MT-CO3	9207	9990	coIII	Cytochrome c oxidase subunit III
		Complex V		
Gene	Starting	Ending	Shorthand	Description
MT-ATP8	8366	8572	ATPase8	ATPsynthase F0 subunit 8
MT-ATP6	8527	9207	ATPaseG	ATP synthase F0 subunit 6