Epithelial-To-Mesenchymal Transition in Breast Cancer Metastasis: Mitochondria Take the Center Stage

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Abstract

Epithelial to Mesenchymal Transition (EMT), a process involved in organogenesis and wound healing, is now at the center stage of cancer metastasis. While most of the current research is focused on defects arising in the nuclear genome, the contribution of defects in mitochondria has remained understudied. In the past decade, the association between mitochondrial genomic (mtDNA) and functional defects resulting in altered metabolism with tumor progression and poor outcome has become more evident. Here we review the contribution of mitochondria in epithelial-to-mesenchymal transition, particularly focusing on breast cancer. Our goal is to highlight the critical role that mitochondrial genome defects induced retrograde signaling play in driving cellular plasticity. We will further discuss the molecular intermediates of the retrograde signaling pathway, which can potentially be novel therapeutic targets in mitochondrial stress induced EMT.

Keywords: Breast cancer metastasis; Epithelial-to-Mesenchymal Transition (EMT); Mitochondria; mtDNA; Mitochondrial Retrograde Signaling (MRS)

Abbreviations

brCSC : breast Cancer Stem Cells
Ca2+ : Calcium
C/EBPδ : CCAAT/Enhancer Binding Protein delta
Cn : Calcineurin
CREB : Camp Response Elements Binding Protein
ECM : Extra Cellular Matrix
EMT : Epithelial To Mesenchymal Transition
ER : Estrogen Receptor
ESRP1 : (Epithelial Splicing Regulatory Protein 1)
FOXC1 : Forkhead Box C1
HIF : hypoxia inducible factor
hnRNA2 : heterogeneous nuclear Ribonucleoprotein A2
mtDNA : mitochondrial DNA
NFAc : Nuclear Factor of Activated T-cells, Cytoplasmic
Calcineurin-Dependent 1
NFκB : Nuclear Factor kappa-light-chain-enhancer of activated B cells
PR : Progesterone Receptor
rRNA : ribosomal RNA
TAFAM : Transcription Factor A, Mitochondrial
TGFβ1 : Transforming Growth Factor beta
tRNA : transfer RNA

Epithelial-to-Mesenchymal Transition (EMT) in Cancer Cell Metastasis

Metastasis accounts for over 90% of cancer associated mortalities, despite recent advances in detection and treatment options of cancer patients [1,2]. Metastasis involves a series of processes by which a primary tumor spreads from its initial site to secondary tissues/organs. Over 80% of all carcinomas are epithelial in origin. In order to invade, disseminate to distant tissues and subsequently form metastatic colonies, neoplastic epithelial cells have to acquire, at least transiently, a more mesenchymal phenotype [3,4]. Cellular plasticity during this transition between epithelial and mesenchymal phenotypes is achieved by the activation of a cascade of transcriptional and cell biological events termed the Epithelial-to-Mesenchymal Transition (EMT) [5-9]. During an EMT, tumor epithelial cells lose their epithelial characteristics, including cell-cell adhesion, baso-apical polarity and motility, and acquire mesenchymal traits, including motility, invasiveness and, importantly, many of the attributes of stem cells. The involvement of cellular alterations resembling an EMT in tumor cell metastasis have been well documented in many types of carcinomas, including breast, prostate, colon, head and neck, ovary and lung cancers.

The process of tumor metastasis involves a number of sequential cellular events. The tumor cells disseminate from the primary mass, invade into the surrounding cellular matrix, intravasate into the lymphatic and blood vessels, enter and survive in the circulation, extravasate out from the capillary, home-in at a distant organ and proliferate to form secondary tumors in distant organs [10,11]. Acquired migratory capacity is a prerequisite for the epithelial carcinoma cell within the primary tumor mass to escape from the primary tumor and enter the circulation. Cell migration is achieved by multistep interrelated processes involving the formation of lamellipodia/membrane protrusions at the front edge, cycles of adhesion and detachment, cell body contraction, and tail retraction [12]. Most cells have the intrinsic potential for directional movement involving extension of the leading edge during normal cellular processes such as development, tissue remodeling and regeneration. The migratory pattern of tumor cells is random and involves front-rear polarity. The turnover of focal adhesion factors plays a pivotal role in tumor cell migration. Focal adhesions at the leading edge provide traction points moving the cell body forward while...
disassembly of focal adhesions at the rear allows the retraction of the rear and results in translocation of the cell in the direction of movement [13]. This migratory pattern is one of the hallmarks of aggressive tumors indicative of the transition from a benign to a malignant phenotype. To extravasate through capillary endothelium, cancer cells need to adhere to endothelial cells. The invasion into the surrounding matrix, requires the disassembly of the adhesions between cancer cells and the extracellular matrix [13]. Infact as high as 20% of the candidate genes involved in breast and colorectal carcinogenesis are adhesion-related genes, implying the significance of cell adhesion in cancer progression [14].

Signaling Factors Involved in Epithelial-to-Mesenchymal Transition

A number of signaling factors have been shown to drive EMT both under physiologic and pathologic conditions and these have been discussed in many previous reviews. Therefore in this review, we will briefly discuss the role of three major factors which are “drivers” of several downstream pathways involved in driving EMT: (1) hypoxia, (2) cellular Calcium (Ca\(^{2+}\)) levels and (3) metabolic reprogramming.

The hypoxic environment within the core of the solid tumor has been established as one of the crucial cellular factors driving EMT [15-19]. Uncontrolled cell proliferation of tumor cells leads to larger tumor mass. This results in limited availability of nutrients and oxygen in the microenvironment exposing tumor cells to intermittent hypoxic conditions. Higgins et al., demonstrated that hypoxia-induced EMT in renal epithelial cells depends on Hypoxia-Inducible Factors (HIF)-dependent signaling [18]. Studies by Coppole et al., also show that HIF-1α is important to induce EMT in hypoxic hepatocytes [20]. Luo et al., provided a strong evidence for the involvement of HIF-1α in inducing EMT by silencing HIF at 2% oxygen and over-expression of an oxygen-insensitive HIF mutant at 21% oxygen. Interestingly, expression of the oxygen-insensitive HIF mutant abrogated HIF mediated Snail activation and subsequent cell migration [21]. Their report identified Snail as a HIF target gene and provides novel insights into the regulation of Snail during hypoxia-induced EMT. In addition to HIF signaling, tumor cells activate transforming growth factor, TGFβ1 in response to hypoxia. The role of TGFβ in EMT has been demonstrated in numerous studies. A more direct evidence comes from a report showing Erbin, a Smad/Erk pathway inhibitor inhibits TGFβ induced EMT in renal tubular epithelial cells [22,23].

Another important factor which has emerged as a critical regulator of cell migration is Ca\(^{2+}\) which is the ubiquitous second messenger [24]. Ca\(^{2+}\) flux has been shown to be important for the migration of various cell types, including tumor cells [25-29]. Store-operated calcium release is the predominant Ca\(^{2+}\) entry mechanism particularly in non-excitible cells [30,31]. As mentioned in the earlier section, focal adhesion turnover is critical for tumor cell migration during metastasis. Blocking store-operated Ca\(^{2+}\) influx slows down focal adhesion turnover, resulting in larger focal adhesions and stronger adhesion that would impede the fast migration of metastatic tumor cells. Reduction of calcium channel proteins Orai1 or STIM1 either by genetic targeting in highly metastatic human breast cancer cells or treatment with a pharmacological inhibitor of store-operated calcium channels was reported to reduce tumor metastasis in animal models. Therefore, agents that block store-operated Ca\(^{2+}\) channels, such as SKF96365, genetically silencing Orai1 and STIM1, or antibodies that specifically block the channel activity of store-operated calcium could be potential therapeutics for tumor metastasis [32].

A number of reports provide evidence that metabolic reprogramming in tumor cells is a driver of tumor metastasis. The metabolic plasticity of tumor cells has gained renewed interest with the goal to defining novel pathways based on metabolic parameters involved in tumor initiation and progression [33]. Using a high-throughput metabolic phenotyping platform, it has been recently demonstrated that breast cancer stem cells, which are formed during an EMT, have a higher ability to utilize additional catabolic fuels perhaps as an adaptive survival mechanism [34]. This study showed that cancer stem cells acquired nutrients from glycolysis end products (pyruvate and lactate) and ketone bodies (β hydroxybutyrate) from extracellular microenvironment to support their mitochondrial functions. We have earlier reported that a metabolic switch to glycolysis and activation of Insulin-like Growth Factor-1 Receptor (IGF1R)/Akt1 kinase pathway and activation of glucose transporter-4 all of which were critical to the survival of tumorigenic transformation in our cellular model [35-37]. We observed a glycolytic switch accompanied by activation of the Akt1 kinase pathway in breast cancer cells during an EMT induction [38]. Another study reported in two different breast cancer cell lines, HER2-positive BT-474 and MCF-7, EMT induction was accompanied by increased aerobic glycolysis, over expression of glucose transporters, lactate dehydrogenase isoforms, monocarboxylate transporters and glycogen phosphorylase isofrom. Interestingly enzymes involved in anabolic pathways and gluconeogenesis were suppressed during EMT. Their observation that the metabolic changes are similar in different breast cancer cells during EMT induction, suggests the generality of the metabolic transition [39]. These differences in the metabolic properties between the cancer stem cells and the primary tumor cells can therefore be harnessed in designing therapeutic strategies targeting cells with metastatic potential.

Involvement of EMT in Breast Cancer Metastasis

In normal and neoplastic epithelial cells in vitro, EMT has been induced by various growth factors, including TGFβ1, hepatocyte growth factor and PDGF. These growth factors and their cognate receptors subsequently activate an array of transcription factors, which have the potential of inducing an EMT [40]. The Notch and β catenin signaling pathway has been established as a major driver of EMT by activating downstream transcription factors in various cancers including breast cancers [41-43]. These factors include the homeobox protein Gsc (Gsc), the zinc-finger proteins Snail (Snai1 and Snai2 (Slug), the basic helix-loop-helix protein Twist1 (Twist), the forkhead box proteins FOXC1 and FOXC2, and the zinc-finger, E-box-binding proteins Zeb1 and Sip1 (Zeb2) [6,44-49]. In addition to activation of transcription factors, members of the miR-200 family of micro-RNAs are down-regulated during an EMT [50-52]. Downregulation of these miRNAs subsequently results in the up-regulated expression of several critical target genes, notably Zeb1 and Zeb2. Therefore expression or activation of one of these transcription factors or down-regulation of the miR-200 family in neoplastic epithelial cell is sufficient to reprogram these cells into an EMT. Moreover, many of these factors are expressed concomitantly in the mesenchymal cells that have passed through an EMT. A number of comprehensive reviews on the contributions of each inducer to the EMT program have already been published before.

It is becoming evident that understanding the mechanisms and cellular pathways in breast cancer holds the key to improving therapeutic response in clinics. Therefore, studies have focused on identifying the molecular signatures of breast cancer types and signaling pathways that govern prognosis. Based on microarray
analyses clinical breast cancers have been classified into a number of distinct subtypes, such as luminal A and B, triple (ER-, PR- and Her2-) negative (TNBC), and HER2-expressing tumors. Significantly high numbers of TNBC tumors are basal-like in origin [53-60]. These subtypes have been useful in designing therapeutic approach, predicting metastasis and survival. In recent years, additional molecular subtypes of tumors have been characterized based on high throughput genomic and proteomic analyses [55,61]. A recent study proposed a computational method of Weighted Similarity Network Fusion (WSNF) to identify the microRNA-Transcription Factors - messenger RNA regulatory networks [62].

Developing novel computational approaches will be useful to characterize the functional relevance of the genomic drivers of breast tumor metastasis [63]. Several studies have identified individual factors that induce EMT in breast cancer. Two independent studies aimed to identify an EMT core gene signature either by ectopically expressing Gsc, Snail, Twist, or TGFβ1; or by silencing E-cadherin. These studies demonstrated that gene expression changes associated with the above mentioned EMT-inducing transcription factors correlated most closely with the claudin-low and metaplastic breast cancers [46,64]. Analysis of mRNA expression levels in cells over expressing EMT-inducers revealed that Twist, Snail, and TGFβ1 upregulate expression of Foxc2, Zeb1, and Zeb2. In a study designed to identify which of the established EMT inducers had the strongest correlation with poor prognostic outcome, it was shown that FOXC1 expression most closely correlated with a poorer survival of the breast cancer patients in the NKI and UNC datasets [46]. Therefore, FOXC1 is a potential strong drug target in metastatic breast cancer. Notably FOXC1 is highly expressed in metaplastic and basal-like breast cancer subtypes for which highly effective treatments are not currently available.

Involvement of Mitochondrial Defects in Cancer and EMT

Mitochondria, the cellular energy house which generates ATP, have emerged in the past decade as major cell signaling hubs regulating cell death and cell proliferation. Mitochondrial biogenesis and functions are controlled by both the nuclear and mitochondrial genome. Each cell contains 100-1000s of mitochondria depending on the energy requirement of the tissue and each mitochondrion contains several copies of mitochondrial genome (mtDNA). Human mtDNA encodes 13 essential subunits of the Oxidative Phosphorylation (OXPHOS) system as well as 2 rRNAs and 22 tRNAs used in mitochondrial translation. MtDNA is highly susceptible to damage from numerous cellular factors including reactive free radicals produced by electron transport chain, the hypoxic environment within solid tumors, defects in mtDNA transcriptional machinery and environmental toxins. Ironically many chemotherapeutic drugs targeting primary tumors damage mtDNA and impair mitochondrial functions. Furthermore, reduction in functional mtDNA copy increases in ratios of mutant: wild-type mtDNA owing to time-dependent accumulation of mutant DNA or reduction of mtDNA copy number resulting from defective replication are hallmarks of many pathological conditions. Loss of functional mtDNA resulting from mutations reported in nuclear encoded factors which control mtDNA transcription and translation such as polymerase gamma, the mtDNA helicase TWINKLE and defective TFAM [65,66]. Normal mitochondrial functions can be adversely affected by both mutations and mtDNA polymorphisms.

Impaired oxidative phosphorylation, a consequence of mitochondrial dysfunction, has been shown to be involved in tumorigenesis. One of the early theories about the involvement of mitochondrial dysfunction in tumors was derived from Warburg's hypothesis. Warburg observed that cancer cells were high in fermentation and low in respiration which led to his hypothesis that tumor cells originated from non-neoplastic cells which adopted anaerobic metabolism as an adaptive mechanism after defects in its respiratory system [67]. Even though Warburg's hypothesis remains contentious, changes in the number, shape, and impaired mitochondrial functions have been reported in various cancers in agreement with Warburg’s hypothesis [68,69]. Interestingly, abnormal mtDNA was observed in leukemic myeloid cells by electron microscope [70,71]. In the past decades, mutations in both the non-coding and coding regions of the mtDNA have been identified in various types of cancer.

Defects in mitochondrial enzymes, Succinate Dehydrogenase (SDH) [72-84] fumarate hydratase (fumarase) [85-91] (IDH) [92-108] account for deregulated bioenergetics and tumor cells' mitochondrial dysfunction favoring tumor progression. Moreover accumulation of these metabolites has been shown to cause epigenetic alterations by influencing the activities of histone and DNA methylases [109-111]. Nuclear-encoded mitochondrial deacetylase SIRT3 has also been shown to play a role as a tumor suppressor and loss of SIRT3 has been shown to increase tumorigenicity in various cancers [112-117]. Altered epigenetic status is associated with acquired stemness in cancer cells therefore it is of importance that changes in metabolism and mitochondrial functions can affect epigenetics driving an EMT phenotype.

A number of studies have shown that defects in nuclear DNA encoding mitochondrial proteins have been reported to be involved in tumorigenesis via either increasing production of Reactive Oxygen Species (ROS) or activation of Ca2+ dependent signaling [37,118-122]. As mentioned earlier, the elevated levels of reactive free radicals in the hypoxic core of the solid tumors impairs mitochondrial functions. Dysfunctional electron transport chain in turn results in increased production of mitochondrial ROS and the ROS dependent signaling which determine tumor cell fate [123]. Moreover ROS or Nuclear Factor kappa B (NFkB) have been reported to facilitate EMT in certain cell types and in a TNFα- dependent manner [124]. Interestingly, it has been demonstrated that H2O2 alone can promote EMT, mediated by NFkB, independent from TNFα-induced signaling. In numerous cell lines and animal models, EMT has been reported to be induced by various signaling pathways and numerous transcription factors [125-127] and mitochondrial ROS induced signaling pathway may be one of the leading factors of EMT. The contribution of mitochondrial stress induced Ca2+ signaling is discussed in a later section.

Mitochondrial Defects in Breast Cancer

Significant progress has been made in understanding the contribution of defects in the nuclear genome towards breast cancer metastasis. However, the mitochondrial heterogeneity among breast cancer patients has remained a neglected area of breast cancer research. Defects in mitochondrial functions, mitochondrial genome defects including reduced copy numbers, germline or somatic mtDNA mutations and Microsatellite Instability (MSI) have been reported in a high percentage of breast cancer patients [128-134]. Clinical reports from various cohorts and The Cancer Genome Atlas (TCGA) show a significant percentage of hormone receptor (Estrogen and Progesterone receptor) and Herceptin (HER2) Triple Negative Breast Tumors.
(TNBC) are highly aggressive and have poor prognostic outcomes. Tumors with reduced mtDNA copy numbers correlate with high metastasis and poor 5 year disease free survival rates in the absence of neoadjuvant chemotherapy [135]. A number of studies have identified the D-Loop of the mtDNA as the “hot spot” for mutations associated with over 42% breast cancers [136,137]. The GI0398A mtDNA polymorphism was the one of the early reported associations with an increased risk of breast cancer in African-American women [138]. One of the most frequently observed mtDNA defect in breast tumors in the ΔmtDNA4977 deletion [133,139-142]. Other frequently reported mtDNA mutations associated with breast cancer is at the NADH dehydrogenase subunits 1,2,4,5 ATP synthase F0 subunit 8, cytochrome c oxidase subunit III. Mutations in 12s and 16s rRNA have also been reported in breast cancer patients [132,143]. Another study identified novel tRNA mutations in two breast cancer cell lines [144]. It has been shown that introducing mitochondria from highly invasive breast cancer cell lines into noninvasive cells imparted increased tumorigenicity in the recipient cell [144,145]. It is now broadly accepted that mitochondria are significant contributors towards the cellular tumorigenic transformation.

Mitochondrial Retrograde Signaling in EMT

In the last decade, cellular signaling originating from dysfunctional mitochondria has been implicated in tumorigenic transformation in various cancers. Based on studies from various independent laboratories, broadly, these can be categorized into three different pathways depending on the stress inducer and/or propagator of the signaling: (1) Ca\(^{2+}\) activated calcineurin mediated signaling; (2) Mitochondrial reactive oxygen species dependent signaling; (3) Mitochondrial unfolded protein (UPR\(_{mt}\)) induced signaling.

The observation that dysfunctional mitochondria activate a cytosolic Ca\(^{2+}\) / calcineurin dependent signaling was initially made by the Avadhani laboratory in 1999 and is one of the most well-defined mitochondrial stress signaling pathways. Mitochondrial stress, owing to either reduction in mtDNA copy number (70-80% depletion) or defects in electron transport chain complexes initiates a mitochondrial retrograde signaling pathway (Figure 1) [35-37,145-155]. A consequence of impaired electron chain complex is the disruption of mitochondrial membrane potential. Perturbation of mitochondrial membrane potential (ΔΨ\(_{m}\)) affects the mitochondrial calcium uptake leading to increased cytosolic calcium. It is interesting that the onset of mitochondrial stress signaling involves calcium, which as mentioned earlier is a regulator of cell motility suggesting that mitochondrial retrograde signaling confers migratory capacity. This activates a Ca\(^{2+}\) dependent phosphatase, Calcineurin (Cn) and a set of nuclear transcription factors, such as CREB, C/EBPσ, Nfatc, Nfkb (cRelp50) and heterogeneous Ribonucleoprotein A2 (hnRNP2). The read out of this signaling is an alteration in the expression profile of nuclear genes involved in various oncogenic pathways [152-154].

While reduction in mtDNA has been reported in 63-80% of breast tumors, neither the functional relevance of this correlation nor the underlying mechanisms by which mtDNA reduction induces EMT was investigated until recently. We reported that 70-80% reduction in mtDNA copy number and impaired cellular respiration in both breast cancer MCF7 and normal MCF10A cells results in an epithelial-to-mesenchymal transition (Figure 1) characterized by loss of cell polarity and cell-ECM adhesions with an increase in migration, 3D tumoursphere-formation (Figures 2 and 3) and acquired invasive phenotype [38]. Interestingly, we observed the activation of calcineurin which is a propagator of the mitochondrial retrograde signaling. Downstream of activation of calcineurin, mesenchymal genes were induced along with a loss of epithelial markers conforming with the morphological transition from epithelial to mesenchymal state. The contribution of reduced mtDNA induced signaling in driving this cellular plasticity was evident by restoring the mtDNA contents which reversed the transition to epithelial state. A recent proteomic analyses aimed at identifying the altered proteins involved in metastasis of TNBCs showed that Single-Strand Binding Protein (SSBP-1) was downregulated in metastatic cells [156]. Decreased SSBP-1 expression resulted in loss of mtDNA copy number [156] and triggered a calcineurin mediated mitochondrial retrograde signaling similar to that previously defined from our group [35-37,148,149,152-154,157].
Even though the cell lineage and markers of “cancer stem cells” remains contentious, it is widely accepted that in many cancers, metastatic tumors are formed by a small population of cells during an EMT, which have the tumor initiating potential. These cells are characterized and isolated by their cell surface markers which vary depending on the tumor of origin. Interestingly, mtDNA loss generates brCSC-like cells with self-renewal capacity suggesting a link between low mtDNA copy number and cellular reprogramming to “stemness” [38]. Our study made a seminal contribution in delineating a defined role for mitochondrial retrograde signaling in driving mammary tumorigenesis, from initiation to progression through EMT [38].

As described in an earlier section, EMT involves a multitude of well-coordinated and tightly regulated steps. One of the mechanisms by which the requirement of the metastasizing cell is met is by increasing the protein diversity by alternative splicing. It is therefore significant that mitochondrial retrograde signaling modulates the expression of the Epithelial Splicing Regulatory Protein (ESRP)-1, which regulates the alternative splicing of a wide array of gene sets involved in inducing EMT [38,158-166]. Another key signaling protein of mitochondrial retrograde signaling pathway, Akt kinase is induced in mtDNA depleted human mammary epithelial cells. The EMT specific transcription factor, Twist has been shown to upregulate Akt resulting in inducing cellular invasion and acquired cell motility [143]. This is crucial because Akt kinase controls EMT-specific alternative splicing events by phosphorylating splicing factors such as serine/arginine-rich splicing regulator SRSF1 [167]. Therefore we postulate that mtDNA defects induced-mitochondrial retrograde signaling drives breast tumor EMT via modulating global changes in the patterns of alternative splicing and thereby increasing proteomic diversity.

Future Directions for Therapeutic Benefit

It seems plausible that dysfunctional mitochondria in tumor cells provide adaptive and survival advantage to metastasizing cells. Mitochondrial retrograde signaling is an upstream effector of EMT because it regulates the expression of nuclear genes involved in cellular reprogramming. Based on our studies and reports showing clinical correlations between mtDNA defects and poor prognostic outcome, we suggest that mtDNA copy number could be a useful prognostic marker.

Our evidence that mitochondrial stress signaling induces metastatic progression in an hnRNPA2-and ESRP1-dependent manner implies changes in alternative splicing events play a crucial role in this signaling driven EMT during breast cancer metastasis. Therefore gene isoforms that are altered specifically by low mtDNA during metastatic transition of primary tumors will suggest that therapeutics which target splicing machinery currently under clinical trials could be more beneficial to patients with primary breast tumors containing low mtDNA. In future studies, it needs to be explored whether reduction in mtDNA copy numbers in some primary tumors is due to defects in mitochondrial DNA transcription or translation and identify the drug-targetable factors to correct such defects. Reports that breast cancer cell lines lacking mtDNA (rho zero) showed decreased sensitivity to chemotherapeutic drugs such as doxorubicin, vincristine and paclitaxel [168], suggest that patients with low mtDNA content tumors are resistant to chemotherapeutic agents and accounts for the poor prognosis of breast cancer patients.

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Conflict of Interest

The authors declare no conflict of interest.

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