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Intracellular and microenvironmental regulation of mitochondrial membrane potential in cancer cells

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Abstract

Cancer cells have an abnormally high mitochondrial membrane potential (Ψ_m), which is associated with enhanced invasive properties *in vitro* and increased metastases *in vivo*. The mechanisms underlying the abnormal Ψ_m in cancer cells remain unclear. Research on different cell types has shown that Ψ_m is regulated by various intracellular mechanisms such as by mitochondrial inner and outer membrane ion transporters, cytoskeletal elements and biochemical signaling pathways. On the other hand, the role of extrinsic, tumor microenvironment (TME) derived cues in regulating Ψ_m is not well defined. In this review, we first summarize the existing literature on intercellular mechanisms of Ψ_m regulation, with a focus on cancer cells. We then offer our perspective on the different ways through which the microenvironmental cues such as hypoxia and mechanical stresses may regulate cancer cell Ψ_m.

Graphical Abstract

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Conflicts of Interest
The authors declare that they have no conflicts of interest.

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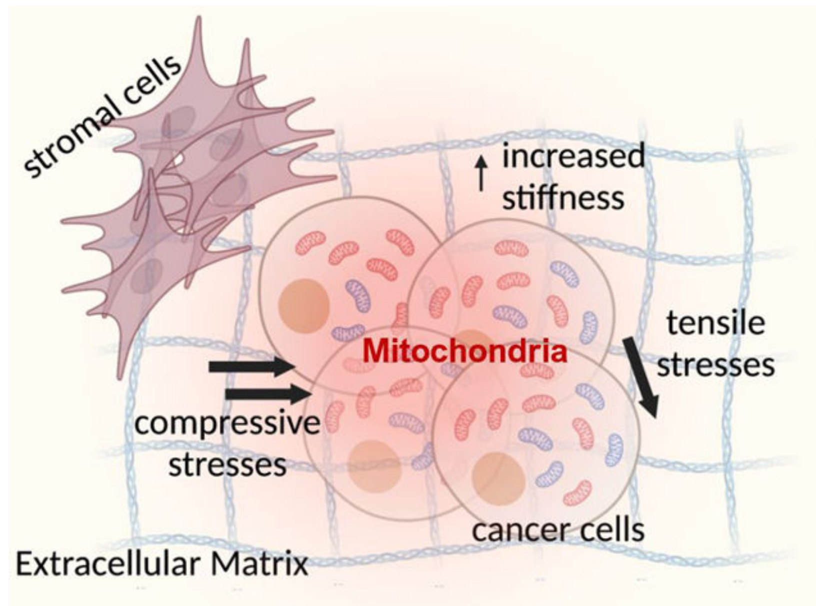

Keywords

Mitochondrial membrane potential (Ψ_{m}); intracellular signaling; tumor microenvironment; metastasis

1. Introduction

Nearly a century ago, Otto Warburg discovered that cancer cells have enhanced levels of glycolysis, and therefore abnormally high glucose uptake and lactate production even in the absence of hypoxic conditions (a phenomenon he called ‘aerobic glycolysis’) (Koppenol et al., 2011). It is now known that this phenomenon, i.e., the Warburg effect, is not a universal characteristic of all cancer cell types. Rather, tumors are highly heterogenous comprising of subpopulations of cancer cells displaying ‘Warburg-like’ metabolic characteristics (Hanahan & Weinberg, 2011). Nevertheless, this increased propensity for glucose consumption by some cancer cell subpopulations is routinely exploited for diagnostic fluorodeoxyglucose-positron emission tomography (FDG-PET) scans in many cancer types including breast and lung cancers (Zhu et al., 2011). Furthermore, such metabolic heterogeneity has emerged as an important cancer cell hallmark and a potential therapeutic target (Ashton et al., 2018; Ward & Thompson, 2012). Interestingly, studies investigating the mitochondrial activity of these metabolically heterogenous cancer cells have found an association between dysregulated mitochondrial activity and their invasiveness and metastatic potential (Dupuy et al., 2015; Porporato et al., 2014; Simoes et al., 2015; Whitaker-Menezes et al., 2011). A mechanistic understanding of the factors that lead to altered cancer cell mitochondrial activity is thus crucial to develop novel therapeutics, for instance, by targeting the mitochondrial support of metastatic behaviors.

A unique observation of cancer cell mitochondrial function is that many epithelial cancer cell types have an unusually high mitochondrial membrane potential (Ψ_{m}), compared to

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their normal counterparts (Chen, 1988; Summerhayes et al., 1982). Further studies showed an association of cancer cell Ψ_m with a range of cell behaviors including decreased susceptibility to apoptosis (Heerdt et al., 2003) and the acquisition of an invasive phenotype (Heerdt et al., 2005). In an *in vivo* mouse model of metastatic breast cancer, we found that mice injected with cancer cells of high Ψ_m have a greater lung metastatic burden than those with low Ψ_m cells, four weeks post tail-vein injection (Begum et al., 2019). The Ψ_m is thus associated with the metastatic development, and a greater understanding of the mechanisms by which it affects cell phenotypes will help in the discovery of novel therapeutic targets. However, whether the high Ψ_m is related to the 'Warburg-phenotype', and how it is differentially regulated in cancer cells, remains unclear.

One possibility is that the abnormally high Ψ_m in cancer cells is a result of genetic mutations that affect their intrinsic mitochondrial activity. Indeed, mutations in mitochondrial DNA (mtDNA) and variations in its copy number have been reported in several types of cancers (Ashton et al., 2018; Beadnell et al., 2018). Furthermore, it is possible that the altered Ψ_m results from the activity of oncogenes such as MYC, AKT, RAS, BRAF and reduction of tumor suppressors like p53, which were found to affect various metabolic pathways including those involving mitochondrial metabolism (Levine & Puzio-Kuter, 2010; Nagarajan et al., 2016; Ward & Thompson, 2012). While it is possible that the altered cancer cell Ψ_m is primarily a result of these cancer-specific mutations, there is also growing evidence supporting alternative mechanisms of physical interaction and biochemical signaling taking place within the tumor microenvironment (TME) (Pavlova & Thompson, 2016). In this review, we will briefly summarize how the Ψ_m is established and regulated, with a focus on TME-derived cues that have the capacity to regulate cancer cell Ψ_m. We will then discuss the current *in vitro* and *in vivo* techniques used to probe Ψ_m, and their potential applications in investigating Ψ_m-driven tumor progression.

2. How is the Ψ_m established?

Mitochondria are double membrane bound organelles. Transport of metabolites and ions across the outer mitochondrial membrane is regulated by large porin protein channels such as Voltage-Dependent Anion Channels (VDACs) that are permeable to solutes up to 5 kDa (Alberts et al., 2002). On the other hand, the mitochondrial inner membrane is largely impermeable, and transport of molecules across this membrane requires specialized membrane transport proteins (Alberts et al., 2002; Kühlbrandt, 2015). The inner membrane also forms deep invaginations into the mitochondrial matrix, called cristae (Fig. 1). The presence of cristae on the inner mitochondrial membrane greatly increases its effective surface area. It is also lined with respiratory complexes of the Electron Transport Chain (ETC) and is the site of mitochondrial adenosine triphosphate (ATP) synthesis. The mitochondrial matrix contains various enzymes that support the replication and transcription of mtDNA (Kühlbrandt, 2015). In addition, it the site of the tricarboxylic acid (TCA) cycle and contains TCA cycle associated enzymes.

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Tricarboxylic acid (TCA) cycle:

Pyruvate from the cytosol (one of the end products of glycolysis) is transported into the mitochondrial matrix through a pyruvate carrier present on the inner mitochondrial membrane and converted to acetyl coenzyme A (acetyl CoA) by the enzyme pyruvate dehydrogenase. Acetyl CoA enters the TCA cycle, where it is oxidized to produce carbon dioxide and water (Pelley, 2012). During this process, electron carrier molecules nicotinamide adenine dinucleotide (NAD) and flavine adenine dinucleotide (FAD) are reduced, forming NADH and $FADH_2$ from NAD^+ and FAD respectively. These reduced electron carriers then take part in the ETC in the inner mitochondrial membrane (Pelley, 2012) (Fig. 1).

Electron transport chain (ETC):

The ETC takes place in the inner mitochondrial membrane and oxidizes electron carriers from the TCA cycle. Briefly, electrons from NADH and $FADH_2$ are transferred to coenzyme Q by complexes I and II of the ETC respectively. Coenzyme Q carries these electrons to complex III, which donates them to cytochrome c (cyt c). Cyt c then diffuses along the membrane and transfers the electrons to complex IV, which then transfers these electrons to oxygen, eventually producing water (Pelley, 2012). In complexes I, III and IV, the activity of electron transfer is coupled to the pumping of protons into the mitochondrial intermembrane space (Pelley, 2012) (Fig. 1). This generates an electrochemical proton gradient across the mitochondrial inner membrane, which gives rise to the mitochondrial membrane potential (Ψ_m).

3. Functional significance of the Ψ_m

ATP synthesis:

Complex V of the ETC is an ATP synthase. Driven by the proton gradient generated by the ETC, it pumps protons back into the mitochondrial matrix and uses this energy to combine a molecule of adenosine diphosphate (ADP) with an inorganic phosphate to produce ATP (Pelley, 2012). This process of ATP production is termed oxidative phosphorylation (OXPHOS) since it requires the presence of oxygen. The ATP produced by the mitochondrial ATP synthase is transported out of the mitochondrial matrix in exchange for a molecule of cytosolic ADP by an adenine nucleotide transporter (ANT) (Fig. 1). Under some conditions of stress (such as ischemia), it has been reported that the ATP synthase and ANT reverse their activity – the ANT now transports ATP into the mitochondrial matrix in exchange for ADP, and the ATP synthase utilizes this ATP to form ADP and pump out protons into the mitochondrial intermembrane space, in an attempt to maintain the Ψ_m (Belous et al., 2003). In this state, mitochondria become major consumers of leftover cellular ATP, and try to maintain the Ψ_m in order to delay the onset of autophagic or apoptotic processes (which are associated with sustained Ψ_m depolarization). Zorova *et al.* suggest that the maintenance of Ψ_m at the expense of cellular ATP levels demonstrates how important it is for the cells to maintain stable Ψ_m levels (Zorova, Popkov, Plotnikov, Silachev, Pevzner, Jankauskas, Zorov, et al., 2018; Zorova, Popkov, Plotnikov, Silachev, Pevzner, Jankauskas, Babenko, et al., 2018).

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Reactive oxygen species (ROS) production:

As explained in the previous section, there is a transport or shuttling of electrons across the complexes of the ETC in the inner mitochondrial membrane. However, this electron transfer is not perfect, and there is electron leakage at Complex I (in the direction of the mitochondrial matrix) and at Complex III (in both directions of the mitochondrial matrix and the intermembrane space) (Li et al., 2013). This leads to the partial reduction of molecular oxygen (O_2) forming highly reactive free radicals called Reactive Oxygen Species (ROS) and include the superoxide anion, hydrogen peroxide and hydroxyl ions (Thannickal & Fanburg, 2000). In isolated rat heart mitochondria, ROS levels are low and unaffected by values of Ψ_m below 140 mV (Korshunov et al., 1997; Lee et al., 2001). Any increases in the Ψ_m beyond that threshold value leads to very high increases in ROS levels (Korshunov et al., 1997). Inhibiting the ATP synthase using oligomycin in cancer cells led to Ψ_m hyperpolarization along with increased ROS levels (Suski et al., 2012). These results suggest that a higher Ψ_m is associated with high ROS levels. However, in pathological conditions that affect the ETC complexes, low-Ψ_m levels have also been associated with high ROS levels (Lebiedzinska et al., 2010). Similarly, antimycin A (ETC Complex III inhibitor) treatment leads to a decrease in Ψ_m levels and increased mitochondrial ROS production (Suski et al., 2012). In summary, in mitochondrial disorders related to mitochondrial ATP synthase, increased Ψ_m is associated with high ROS levels; in disorders related to ETC subunits, decreased Ψ_m is associated with high ROS levels (Suski et al., 2012). To explain this apparent paradox, Li *et al.* propose an "ROS balance" hypothesis, suggesting that there is an optimal level of Ψ_m that is associated with physiological (low) levels of ROS (Li et al., 2013). Any extreme fluctuations in the Ψ_m levels (too low or too high) would lead to high ROS levels either by affecting the rate of electron transfer through the ETC or disrupting the balance of antioxidant enzymes (Li et al., 2013).

Mitochondrial quality control:

The mitochondrial network within cells consists of motile mitochondria (Hyde et al., 2010) that can fuse together when they encounter each other (also called mitochondrial fusion) or split into daughter mitochondria (a process called mitochondrial fission). Together with mitophagy (selective elimination of damaged mitochondria), these processes form the cells' quality control strategy for maintaining the bioenergetic efficiency and integrity of their mitochondrial networks (Twig, Hyde, et al., 2008). The role of Ψ_m in regulating the processes of mitochondrial fusion, fission and mitophagy is examined below.

Mitochondrial fusion: The process of mitochondrial fusion facilitates rapid diffusion of mitochondrial content, allowing exchange of nutrients and metabolites within the mitochondrial network (Chen & Chan, 2004). Long-term tracking of fusion events in individual mitochondria showed that fusion occurs as frequently as one event every 5–20 minutes per mitochondrion in cell lines derived from rodent kidney and pancreatic beta-cells (Twig, Elorza, et al., 2008; Twig, Hyde, et al., 2008). Mitofusins Mfn1 and Mfn2 mediate mitochondrial outer membrane fusion whereas Opa1 mediates fusion of inner mitochondrial membranes (van der Bliek et al., 2013). Fusion appears to be a selective event based on Ψ_m levels: mitochondria with high-Ψ_m have a greater probability to undergo fusion when compared to low-Ψ_m mitochondria (Twig, Elorza, et al., 2008); reduction of Ψ_m by

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uncoupling drugs hinders fusion and enhances fragmentation of the mitochondrial network (Legros et al., 2002; Meeusen et al., 2004; Twig, Elorza, et al., 2008). Depolarizing, i.e., a reduction in the Ψ_m is associated with proteolytic processing and therefore inactivation of OPA-1 (Duvezin-Caubet et al., 2006; Liu et al., 2009), and this could be a potential mechanism by which the Ψ_m regulates fusion. Ψ_m repolarization was found to restore fusion in breast cancer cells (Legros et al., 2002). Together, these studies suggest that the process of mitochondrial fusion requires an intact Ψ_m, and controlling the Ψ_m can regulate mitochondrial fusion events. Conversely, inhibiting the mitofusin proteins leads to a decrease in the overall Ψ_m of the cells, suggesting that the overall Ψ_m of the cells is maintained through fusion by allowing intermixing of mitochondrial matrix as well as membrane components (Chen & Chan, 2004).

Mitochondrial fission: Mitochondrial fission is the process by which an individual mitochondrion splits into two daughter mitochondria. Interestingly, this form of mitochondrial division appears to be asymmetrical – more than four-fifths of all tracked fission events led to the formation of a depolarized and a hyperpolarized daughter mitochondrion (Twig, Elorza, et al., 2008). The depolarized daughter mitochondrion is approximately six times less likely than the hyperpolarized daughter mitochondrion to undergo a subsequent fusion event (Twig, Elorza, et al., 2008). In this manner, fission generates a distinct pool of low- Ψ_m mitochondria that are now segregated from the overall mitochondrial network of the cell. Fission is thus a mechanism by which defective mitochondria can be compartmentalized and subsequently removed from the mitochondrial network (Chen & Chan, 2004). The process of mitochondrial fission is mediated by the protein Drp1. Under normal conditions, Drp1 is localized to the cytoplasm. During fission, it is recruited to the outer mitochondrial membrane, where it forms complexes that join and then separate the outer and inner membranes (Shirihai et al., 2015). In myoblast cultures, the presence of excess fatty acids like palmitate in the medium leads to enhanced mitochondrial fragmentation mediated by Drp1, and this is associated with a reduction in Ψ_m (Jheng et al., 2012). Inhibiting Drp1 in this model prevents the fission-associated loss of Ψ_m (Jheng et al., 2012). This suggests that the recruitment of Drp1 to the mitochondrial membranes could play a role in reducing Ψ_m.

Strikingly, Drp1 is markedly upregulated in invasive breast carcinoma and lymph node metastases when compared to normal breast tissue (Zhao et al., 2013). Inhibiting Drp1 in breast cancer cells is associated with a decrease in their *in vitro* migration and invasiveness (Zhao et al., 2013). Similar results are also obtained with hepatocellular carcinoma cells, where mitochondrial fission is associated with cell migration (Sun et al., 2018). These findings suggest that fission may play an important regulatory role in the development of metastases.

Mitophagy: In instances of premature electron leak from the ETC or dysregulation of certain mitochondrial enzymes, there is an accumulation of ROS (Shirihai et al., 2015), which are known to oxidize and damage mitochondrial DNA as well as mitochondrial proteins including the respiratory proteins themselves (Marchi et al., 2012). From a quality control perspective, it is important for cells to eliminate these mitochondria

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from the larger mitochondrial network to prevent propagation of harmful mitochondrial DNA mutations or damaged mitochondrial proteins. This process of selectively degrading defective mitochondria is known as mitophagy (Mijaljica et al., 2014).

Depolarized mitochondria (mostly arising from fission with asymmetric distribution of Ψ_m) are targeted for removal by mitophagy. Interestingly, depolarization of Ψ_m occurs long before these mitochondria are eliminated by mitophagy, and inhibiting mitophagy does not recover their Ψ_m (Twig, Elorza, et al., 2008). Instead, it leads to an accumulation of low- Ψ_m mitochondria (Twig, Elorza, et al., 2008). Mitophagy is thus important to clear away the pool of low- Ψ_m mitochondria and maintain the overall cellular Ψ_m. Studies have shown that the mechanism mediating mitophagy of low- Ψ_m specific mitochondria involves the Ψ_m-dependent activation of PINK1-Parkin proteins (Twig & Shirihai, 2011). Briefly, low- Ψ_m stabilizes PINK1 which is present in the outer mitochondrial membrane (Vives-Bauza et al., 2010). Activated PINK1 then recruits cytosolic Parkin (Twig & Shirihai, 2011; Vives-Bauza et al., 2010). The recruited Parkin then ubiquitinates and inactivates the mitofusin proteins, thus isolating the low- Ψ_m mitochondria by preventing the mitochondria from undergoing fusion and rejoining the overall mitochondrial network (Tanaka et al., 2010; Twig & Shirihai, 2011). Parkin activation also leads to recruitment of pro-mitophagy proteins such as p97 (Tanaka et al., 2010). Mitophagy is thus an important mitochondrial quality control mechanism, and inhibition of mitophagy leading to accumulation of damaged mitochondria has been implicated in promoting tumorigenesis (Chourasia et al., 2015; Mijaljica et al., 2014).

Mitochondrial and cellular motility:

For cells to be motile and migrate in the direction of certain cues, they need increased ATP production especially at the leading edges of the migrating cell layer. It has been reported that the cells at the leading edge of an epithelial migrating sheet have higher Ψ_m (Chen, 1988). A higher Ψ_m has also been associated with increased migration in cancer cells (Heerdt et al., 2005). Thus, the upregulation of Ψ_m may play an important role in mediating cell migration. Additionally, even within individual cells, high- Ψ_m mitochondria are transported selectively towards local regions of increased energy demand (Hyde et al., 2010). In neurons for example, one study reported that mitochondria that travel from the cell body (soma) to the synapse regions have high Ψ_m compared to the mitochondria that are transported from the synapse back to the cell body (Saxton & Hollenbeck, 2012). Together, these results suggest that the Ψ_m is an important regulator of both overall cell migration as well as individual mitochondrial motility within the cells.

4. Intrinsic regulation of the Ψ_m

Ψ_m regulates important mitochondrial as well as cellular processes including production of ROS (which can serve as important cell signaling molecules when at physiological levels) (Thannickal & Fanburg, 2000), mitochondrial dynamics and quality control mechanisms, as well as mitochondrial and cell motility. But what regulates the Ψ_m? In the following section we will examine different intracellular mechanisms controlling the Ψ_m, from ion

channels on the inner mitochondrial membrane to regulation by the cytoskeleton and cell signaling pathways (Fig. 2A).

Regulation of Ψ_m by inner membrane ion transporters:

To ensure that the electrochemical energy created by the proton gradient generated by the ETC is most effectively used for ATP production, the inner mitochondrial membrane (IMM) must be mostly impermeable to ions except through the ATP synthase. This would ensure that any Ψ_m dissipation is tightly coupled with ATP production. Conversely, the flux of various ions across the IMM through their respective ion channels (such as K^+, Ca^{2+}) can regulate Ψ_m and uncouple it from ATP production (O'Rourke, 2007).

Mitochondrial calcium uniporter (MCU): These channels are the major source of Ca^{2+} influx into the mitochondrial matrix (Giorgi et al., 2018). Ψ_m provides the driving force for Ca^{2+} flux through MCUs; dissipating the Ψ_m with chemical uncouplers such as FCCP reduces Ca^{2+} flux through these channels (Friel & Tsien, 1994; Giorgi et al., 2018; Jean-Quartier et al., 2012). Furthermore, Ψ_m itself is regulated by the MCU activity. Influx of Ca^{2+} ions leads to short-term Ψ_m depolarization (Gunter & Pfeiffer, 1990; Kamer et al., 2018). Sustained high levels of Ca^{2+} in the mitochondrial matrix could induce the mitochondrial permeability transition pore (MPTP) and permanent loss of Ψ_m (Granatiero et al., 2019). Thus, MCUs can regulate Ψ_m by determining matrix Ca^{2+} levels. Indeed, several studies have shown that manipulating MCU levels can affect Ψ_m. In Drosophila S2 cells, knockdown (k/d) of MCU rescued hydrogen peroxide induced Ψ_m-loss (Choi et al., 2017). Interestingly, in liver cancer cells it was found that k/d of MCUR1, a regulator of MCU that promotes mitochondrial Ca^{2+} uptake, is associated with a decrease in Ψ_m (Ren et al., 2018). One possible explanation could be MCUR1 k/d leads to an overall decrease in matrix Ca^{2+}, which could reduce the activity of Ca^{2+}-sensitive ETC enzymes (O'Rourke et al., 2005) hence negatively affecting the H^+ pump that gives rise to the Ψ_m.

K^+ channels: There are several types of K^+ channels on the IMM: mitoK_{ATP}, mitoBK_{Ca} and mitoKv1.3, which are regulated by ATP, Ca^{2+} and voltage respectively (Szewczyk et al., 2009). These mitochondrial K^+ channels are thought to be involved in the regulation of Ψ_m, ROS generation as well as mitochondrial matrix volume (Laskowski et al., 2016). When there is Ψ_m hyperpolarization or an increase in ATP concentration, the opening of these channels leads to the dissipation of the Ψ_m (Bednarczyk, 2009; Szabò et al., 2012) and an amelioration of the oxidative stress associated with enhanced ROS levels at high Ψ_m (Laskowski et al., 2016). The Ψ_m of cervical cancer cells overexpressing the pore-forming subunit of the mitoK_{ATP} channel is significantly lower than that of the control cells, highlighting the ability of mitochondrial K^+ flux in regulating Ψ_m (Paggio et al., 2019).

Uncoupling proteins (UCPs): The ETC Complexes I, III and IV and the major producers of proton gradient; they pump protons into the mitochondrial intermembrane space. One of the major consumers of this proton gradient is the ATP synthase (ETC Complex V) when it is operating in its normal ATP-producing state. Over the past two decades, studies have found the presence of yet another class of consumers of the proton

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gradient on the inner mitochondrial membrane – the uncoupling proteins (UCPs) (Rousset et al., 2004). Originally found in brown adipose tissue (BAT), UCP1 is activated by free fatty acids and transports protons into the mitochondrial matrix, thus dissipating the Ψ_m without any resultant ATP production (Ricquier & Bouillaud, 2000). UCP1 is associated with energy dissipation as heat, which is essential in non-shivering thermogenesis in BAT (Ricquier & Bouillaud, 2000). Later, other types of UCPs were found to be expressed in several different cell types and associated with regulating cell functions such as proton leak, Ψ_m, ROS levels, insulin secretion and resting metabolic rate (Rousset et al., 2004). Notably, overexpression of UCP5 in neuroblastoma cells was found to decrease their Ψ_m (Kwok et al., 2010). In aortic endothelial cells, manipulations that decreased or increased UCP levels led to an increase or decrease in Ψ_m respectively (Shimasaki et al., 2013). These results provide compelling evidence that the Ψ_m can be regulated by the level and activity of UCPs in cells.

Regulation of Ψ_m by outer membrane ion transporters:

As mentioned earlier, VDACs form channels that selectively allow passage of solutes through the outer mitochondrial membrane based on their open/closed configuration. VDACs control mitochondrial function by regulating the transport of important charged metabolites through the mitochondria (such as succinate^{2-} and ATP^{4-}) (Colombini, 2004). Low Ψ_m lead to an ‘open’ configuration of VDAC which favors the selective transport of anions over cations through these channels. Higher magnitudes of Ψ_m (> 40 mV) lead to a ‘closed’ configuration which favors the transport of cations (Maldonado & Lemasters, 2012). Meanwhile, VDAC closure could regulate Ψ_m by reducing the OMM permeability to adenine nucleotides (like ANT) (Szabo & Zoratti, 2014). This would decrease substrate availability for the Ψ_m-consuming ATP synthase activity, thus increasing the overall Ψ_m. Evidence for VDAC regulation of Ψ_m has been reported in several studies. In neuroblastoma cells, dopamine stimulation leads to a transient decrease in Ψ_m, which can be prevented by modulating the open/close configuration of the VDACs (Premkumar & Simantov, 2002). Moreover, the Ψ_m-regulation by free cellular tubulin is also reported to be dependent on VDAC phosphorylation; inhibiting VDAC phosphorylation by protein kinase A (PKA) abrogates the inhibitory effect of enhanced free tubulin level on Ψ_m (Sheldon et al., 2011). Further investigation on VDAC isoforms shows that, while inhibition of different VDACs (using siRNA) all leads to a decrease in Ψ_m, VDAC3 k/d causes the greatest Ψ_m reduction (Maldonado & Lemasters, 2012; Maldonado et al., 2013). Additionally, VDAC activity can also be regulated by binding to hexokinase, a cytoplasmic enzyme involved in glycolysis (Pastorino & Hoek, 2008). K/d of hexokinase using shRNA decreases the Ψ_m of cancer cells, an effect that can be reversed by the presence of VDAC inhibitors (Dubey et al., 2016).

Ψ_m regulation by cytoskeletal elements:

The cytoskeleton regulates the spatial organization of subcellular organelles and couples them biochemically and physically to the extracellular cues (Fletcher & Mullins, 2010). It is mainly comprised of three types of polymers: microtubules, microfilaments (or actin filaments), and intermediate filaments (Fletcher & Mullins, 2010). Studies have shown that the cytoskeletal filaments can control mitochondrial motility, morphology and subcellular

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location (Kuznetsov et al., 2020). In this section, we will examine the role of these cytoskeletal filaments in regulating the Ψ_m.

Microtubules: Microtubules are one of the main cytoskeletal elements associated with the transport of mitochondria within the cells (Kuznetsov et al., 2020). They are the stiffest cytoskeletal element (Fletcher & Mullins, 2010) and are made of tubulin heterodimers. Microtubule polymerization can be blocked by drugs that act as microtubule destabilizers such as nocodazole, rotenone and colchicine (Maldonado et al., 2010), which increase levels of free tubulin in cells (Maldonado et al., 2010). On the other hand, microtubule polymerization can be stabilized or enhanced by treatment with drugs such as paclitaxel, which decreases free tubulin levels as shown in hepatocellular carcinoma cells (Maldonado et al., 2010). Interestingly, microtubule depolymerization (increased free tubulin) decreases Ψ_m while its stabilization (decreased free tubulin) increases Ψ_m in cancer cells (Maldonado et al., 2010). This contrasts with non-cancer cells where microtubule stabilization does not affect Ψ_m (Maldonado et al., 2010). Other studies show that both depolymerizing and stabilizing microtubule decrease Ψ_m in myocytes (Kumazawa et al., 2014). In neurons, microtubule stabilization with paclitaxel leads to a significant decrease in the Ψ_m (Melli et al., 2008). In human dermal fibroblasts, depolymerizing microtubule with nocodazole does not change Ψ_m (Kandel et al., 2016). Together, these studies show that the microtubule control of Ψ_m is highly cell-type specific. A potential mechanism by which microtubule stability affects Ψ_m involves the binding of free tubulin to and closing of VDACs in the outer mitochondrial membrane (Rostovtseva et al., 2008), which reduces ADP transport into the mitochondria and overall mitochondrial activity (Maldonado et al., 2010; Rostovtseva et al., 2008).

Actin filaments (or microfilaments): In contrast to microtubules which mediate long-distance transport of mitochondria within the cells, actin filaments have been associated with short-range mitochondrial transport as well as mediating the anchorage of the mitochondria in regions of high energy demand (Boldogh & Pon, 2006). Monomeric actin (G-actin) can polymerize and form diverse structures of filamentous actin (F-actin) (Fletcher & Mullins, 2010). The activity of several types of actin regulatory proteins can dictate the overall structure of the cell's actin network (Fletcher & Mullins, 2010). For example, actin assembly in the presence of formins leads to the formation of liner actin filaments, whereas the activity of the Arp2/3 complex leads to branched actin networks (Davidson & Wood, 2016). In T-cells and neurons, gelsolin (an actin severing protein that caps actin filaments and prevents their elongation) is reported to prevent the loss of Ψ_m that is associated with the onset of apoptosis (Harms et al., 2004; Koya et al., 2000). Additionally, a small fraction of the overexpressed gelsolin in T-cells was found to colocalize with the mitochondria, suggesting that it may regulate Ψ_m by direct binding (Gourlay & Ayscough, 2005b; Koya et al., 2000). Interestingly, a more detailed study revealed that the mitochondrial fraction of the overexpressed gelsolin specifically bound to the VDACs, suggesting a regulatory mechanism of Ψ_m by gelsolin through VDACs (Kusano et al., 2000). Coronin, another actin binding protein that opposes the activity of Arp2/3 and thus inhibits F-actin formation is essential for maintaining Ψ_m in T-cells. Loss of Coronin-1 leads to a decrease in Ψ_m, which can be partially recovered by treatment with latrunculin A (an actin depolymerizing

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drug) (Foger et al., 2006). On the other hand, jasplakinolide treatment, which increases F-actin, leads to a decrease in Ψ_m (Dustin, 2006; Foger et al., 2006). These results suggest that the ratio of G-actin to F-actin in cells could potentially regulate their Ψ_m. A recent study in cancer cells reported that at any given time, about 20% of the mitochondria are associated with actin filaments (F-actin), and this association while regulating mitochondrial dynamics, had no effect on their Ψ_m (Moore et al., 2016). Regulation of Ψ_m by the actin cytoskeleton could therefore be highly context dependent and cell-type specific.

The relationship between actin dynamics and mitochondrial activity has also been explored in other model organisms such as yeast and plant cells. In yeast cells, mutations in actin regulatory proteins that promote the turnover of F-actin (Sla1p and End3p) lead to a loss of Ψ_m (Gourlay & Ayscough, 2005a). In plant root hair cells, both actin polymerizing and depolymerizing treatments (with jasplakinolide and latrunculin B respectively) reduce the Ψ_m (Wang et al., 2010). In agreement with these results, latrunculin treatment in mung bean plant cells also reduces the Ψ_m (Lo et al., 2011). Taken together, these studies provide compelling evidence that the dynamics of cellular actin cytoskeleton could regulate Ψ_m.

Intermediate Filaments (IFs): IFs have the least stiffness out of the three types of cytoskeletal elements and are thus better suited for receiving mechanical stimuli of tensile rather than compressive nature (Fletcher & Mullins, 2010). Mutations in IF genes are associated with altered mitochondrial distribution and morphology in several pathological conditions including Charcot-Marie-Tooth disease and epidermolysis bullosa simplex (Matveeva et al., 2015). In mouse fibroblasts, a portion of vimentin IFs colocalizes with mitochondria (Nekrasova et al., 2011). Further, disrupting the vimentin IFs leads to a redistribution of the mitochondrial network as well as increased mitochondrial motility, suggesting that vimentin IFs are involved in regulating the spatial organization and motility of mitochondria within these cells (Nekrasova et al., 2011). Interestingly, motile mitochondria were found to have a lower Ψ_m than their stationary counterparts (Chernoivanenko et al., 2011); restoring vimentin expression in the vimentin-null fibroblasts decreased mitochondrial motility (Nekrasova et al., 2011) while increasing their Ψ_m (Chernoivanenko et al., 2015; Chernoivanenko et al., 2011). Conversely, silencing vimentin using shRNA in wild-type cells (which express normal levels of vimentin) leads to a decrease in Ψ_m (Chernoivanenko et al., 2015). A more detailed study revealed that the vimentin IF-mitochondria association could be disrupted by the phosphorylation of vimentin at its Ser-55 site by Rac-1, and activated Rac-1 increases mitochondrial motility and reduces the Ψ_m (Matveeva et al., 2015). Together, these results strongly suggest that the association of mitochondria with vimentin IFs helps anchor the mitochondria at specific intracellular locations (thus reducing their motility) and increases their Ψ_m.

Regulation of Ψ_m by signaling pathways:

The activities of several mitochondrial proteins, including the ETC complexes, can also regulate the Ψ_m. These activities can be expression of tissue-type specific isoforms of ETC subunits, their allosteric inhibition from interactions with small molecules (such as ADP), and their altered phosphorylation state by cell signaling molecules (Hüttemann et al., 2008). For example, both ETC Complexes I and IV, which contribute to Ψ_m through proton

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pumping, are regulated via PKA-mediated phosphorylation induced by c-AMP (Hüttemann et al., 2007). Akt was found to localize to the mitochondria following PI3K signaling, to phosphorylate ATP synthase (ETC Complex V) (Bijur & Jope, 2003). Whether the phosphorylation of ETC Complexes serves to activate or inhibit them seems to be dependent on the subunit type and site of phosphorylation. Notably, several other signaling molecules, including tyrosine kinases such as Abl, Src, EGFR and ErbB2 and serine threonine kinases like JNK, GSK3β, were also found to localize to the mitochondria and modulate ETC Complex activity (Lim et al., 2016). As such, various upstream signaling pathways can potentially regulate the Ψ_m.

To summarize, several studies suggest that the regulation of Ψ_m can be associated with mitochondrial ion channels, by cytoskeletal elements and cellular signaling pathways, and some of these results are derived from cancer cells. However, carefully designed experiments are needed to investigate specifically whether and how the Ψ_m is differentially regulated in normal vs. cancer cells by each of these factors.

5. Ψ_m of normal vs. cancer cells

More than thirty years ago, Summerhayes et al. first reported that most *in vitro* cultures of epithelial cancer cells have a much higher Ψ_m (as assessed by uptake and retention of Ψ_m-sensitive dye rhodamine 123) when compared to non-transformed cells (Summerhayes et al., 1982). Apart from these cancer cells, they found cardiac muscle cells and myotubes to possess such “unusually” high levels of Ψ_m (Summerhayes et al., 1982). Other cancer cell types screened from blood cancers, connective tissue derived cancers (osteosarcoma) and neuroblastomas do not have the high Ψ_m that is characteristic of epithelial carcinomas (Chen, 1988). To find out whether intrinsic differences in the Ψ_m of cancer cells can affect tumor growth and progression, Heerdt et al. subcloned isogenic colonic carcinoma cells with stable differences in their Ψ_m (Heerdt et al., 2003). They found that the intrinsic Ψ_m differences of these cells are associated with differences in mitochondrial mRNA levels, butyrate-induced cell cycle arrest and susceptibility to apoptosis (Heerdt et al., 2003). Further studies with these cells showed that the high-Ψ_m subclones have higher vascular endothelial growth factor (VEGF) and matrix metalloproteinase 7 (MMP7) secretion, as well as higher invasiveness (Heerdt et al., 2005, 2006). Similarly, high-Ψ_m breast cancer (MCF-7) subclones are also shown to have greater VEGF secretion than the low-Ψ_m subclones, suggesting a correlation of Ψ_m with invasive properties in these cells (Houston et al., 2011).

Bonnet et al. attempted to selectively target the high-Ψ_m of cancer cells. They found that treatment with dichloroacetate (DCA), a pharmacological inhibitor of mitochondrial pyruvate dehydrogenase kinase (PDK), selectively lowers the Ψ_m of cancer cells (breast and lung carcinoma cells, as well as glioma cells) while having no effect on the Ψ_m of non-cancerous epithelial cells (Bonnet et al., 2007). Several types of cancer cells have re-wired metabolic pathways, most notably, increased glycolysis relative to oxidative metabolism (Warburg effect). By inhibiting PDK (which phosphorylates and inactivates pyruvate dehydrogenase, thus reducing mitochondrial pyruvate influx), DCA increases mitochondrial pyruvate influx and shifts the balance from glycolysis to oxidative

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metabolism in cancer cells. Further, DCA treatment also causes release of cytochrome c from the mitochondria, thus inducing apoptosis (Bonnet et al., 2007). In *in vivo* studies, it was found that DCA treatment can reduce tumor cell proliferation and increase their apoptosis, thus reducing the overall tumor size (Bonnet et al., 2007).

Most studies that reported observations of high Ψ_m of cancer cells were performed using *in vitro* cultures. Recently, Momcilovic et al. used a positron emission tomography (PET) imaging technique to investigate the Ψ_m of lung cancer cells *in vivo* (Momcilovic et al., 2019). They found a tumor-subtype dependent Ψ_m heterogeneity in these mouse models – adenocarcinomatous tumors have a higher Ψ_m whereas small squamous cell carcinoma tumors have lower Ψ_m (Momcilovic et al., 2019). In summary, cancer cells are found to have highly dysregulated mitochondrial functions (Vyas et al., 2016), which can manifest as differences in their Ψ_m (Chen, 1988).

6. How does the TME regulate Ψ_m of cancer cells?

The immediate environment surrounding tumors *in vivo,* commonly referred to as the tumor microenvironment (TME), is a rich source of biophysical and biochemical cues that directly influence cancer cell behaviors. Several different cell types apart from the primary tumor cells are found in the TME: immune cells, fibroblasts, adipocytes, mesenchymal stem cells, endothelial cells and pericytes (Balkwill et al., 2012). Biochemical cues derived from cell-type specific signaling are important in directing tumor progression. For example, the interaction of cancer cells and dendritic cells in the TME is thought to regulate T_{reg} expansion within the tumor and thereby mediate tumor escape from immune attacks (Whiteside, 2008). In addition, tumor progression in the TME has been associated with biophysical cues such as low oxygen concentrations (hypoxia) (Ando et al., 2017; Petrova et al., 2018), remodeling of the extracellular matrix (ECM) resulting in a change in both its composition and stiffness (Winkler et al., 2020), as well as compressive and shear stresses (Chaudhuri et al., 2018; Nia et al., 2020). These TME-derived cues are potential regulators of cancer cell mitochondrial activity and their roles in influencing the Ψ_m are explored below (Fig. 2B).

Hypoxia:

Exposure to hypoxia induces several changes in mitochondrial metabolism and the Ψ_m response to hypoxia appears to be highly cell-type specific and metabolic state-dependent. In myoblasts (Hawkins et al., 2010), infected macrophages (Wiese et al., 2012) and kidney proximal tubule cells (Weinberg et al., 2000), hypoxia has been reported to depolarize the Ψ_m (Khacho et al., 2014). However, in blastocysts (Ma et al., 2017) and hypoxia-sensitive glioblastoma cells (Turcotte et al., 2002), the Ψ_m increases in response to hypoxic stimuli. Furthermore, in hypoxia-resistant glioblastoma cells (Turcotte et al., 2002), uninfected macrophages (Wiese et al., 2012) and renal cell carcinoma cells (Papandreou et al., 2006), the Ψ_m remains unchanged under hypoxia. One potential explanation for these differences is in the distinct ways individual cell types adapt the efficiency and rate of mitochondrial respiration under hypoxia. In hypoxia-resistant glioblastoma cells, the Ψ_m remains constant under hypoxia, likely due to a compensatory decrease in overall

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oxygen consumption or stabilization of the ETC, leading to constant proton flux into the mitochondrial intermembrane space despite the reduced electron flux, which is not seen in the hypoxia-sensitive glioblastoma cells (Turcotte et al., 2002). Additionally, hypoxia has been associated with several other mechanisms that could determine the Ψ_m response. These include HIF-1α induced inhibition of pyruvate dehydrogenase, which decreases the overall mitochondrial respiration by reducing substrate availability, expression of hypoxia-specific isoforms of ETC complexes (cytochrome c oxidase), inhibition of ETC complex I, and the inhibition of ATP-synthase.

Substrate Stiffness and Composition:

Stromal secretion of lysyl oxidase (LOX) enzymes in the TME has been associated with increased ECM stiffness, promoting cancer cell migration and invasion (Winkler et al., 2020). Substrate stiffness has been shown to regulate the Ψ_m of several different cell types *in vitro*. Pulmonary arterial endothelial and smooth muscle cells (Bertero et al., 2016), and cardiomyocytes (Morishima et al., 2018) demonstrate enhanced Ψ_m when cultured on softer substrates. Another study on vascular smooth muscle cells found that their Ψ_m was the highest when cultured on a substrate with stiffness close to the *in vivo* stiffness of the tissue of origin (Bartolák-Suki et al., 2017), while both softer and stiffer substrates significantly reduced the Ψ_m of these cells. In addition to ECM stiffness, the overall ECM composition can also be modified in the TME which may in turn alter Ψ_m. In several tumor types, there is an increased deposition of fibrillar collagen, fibronectin and hyaluronan, leading to a desmoplastic phenotype that is linked to poor prognosis(Winkler et al., 2020). It was shown that pancreatic cancer cells cultured on substrates coated with fibronectin and laminin have a much higher Ψ_m than those on collagen coated or non-adherent substrates (Vaquero et al., 2003). Furthermore, altering the composition of anabolic vs. catabolic ECM proteins in nucleus pulposus cells was also found to change their Ψ_m (Wu et al., 2018). In vascular smooth muscle cells, silencing an ECM protein (cartilage oligomeric matrix protein) led to a decrease in their Ψ_m (Jia et al., 2018). Together, these studies suggest a possible role of ECM stiffness and composition in regulating cancer cell Ψ_m.

Compressive and tensile stresses:

As tumors grow, they push against the surrounding stromal tissue and accumulate solid stresses within the tumor. It was shown that there are compressive forces at the tumor core and tensile stresses at the tumor periphery (Jain et al., 2014). While the compressive stress has been reported to enhance the migration of breast cancer (Tse et al., 2012) and glioma (Kalli et al., 2019) cells, its effect on Ψ_m is still unclear and remains to be investigated. On the other hand, tensile stresses have found to regulate Ψ_m in various cell types *in vitro*. In human neuroblastoma cells, mild to moderate stretch was shown to depolarize the Ψ_m (Wang et al., 2014). Interestingly, sustained mechanical stretch reduces the Ψ_m of cardiomyocytes but has no effect on that of cardiac fibroblasts (Liao et al., 2004). Interestingly, bovine aortic smooth muscle cells have increased Ψ_m under both monotonous and variable stretching (Bartolak-Suki et al., 2015). These data demonstrate a potential role of static and dynamic tensile stresses in TME in altering the Ψ_m in cancer cells.

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7. Techniques to investigate Ψ_m

In vivo techniques:

Determining the Ψ_m of cells *in vivo* is not very straightforward and remains challenging through mitochondrial dyes and direct imaging. Some recent studies have reported the use of novel approaches to measure the Ψ_m of live cells *in vivo*. Lipophilic cations such as triphenylphosphonium (TPP) can readily accumulate in the mitochondria (owing to the Ψ_m), which can be conjugated with compounds for mitochondrial targeting (Zielonka et al., 2017). A novel TPP-based approach was reported by Logan et al. where two TPP-based probes with complementary click chemistry moieties are injected *in vivo* (in mouse models) that accumulate in the mitochondria in a Ψ_m (and plasma membrane potential) dependent manner, where they form a stable "MitoClick" compound. The amount of "MitoClick" accumulated in different cell types or tissues can be quantified by using liquid chromatography-tandem mass spectrometry (LC-MS/MS), and this approach was shown to sensitively report small changes in Ψ_m *in vivo* (Logan et al., 2016). The second TPP-based approach to measure Ψ_m *in vivo* uses a radiolabeled TPP tracer that accumulates in tissues in a Ψ_m-dependent manner and can then be imaged using positron emission tomography (PET). Using this approach in a mouse model of lung tumor, Momcilovic et al. report that the lung tumors could be segregated into distinct subtypes (adenocarcinoma vs. squamous cell carcinoma) based on their estimated *in vivo* Ψ_m (Momcilovic et al., 2019). These TPP-based approaches are promising new techniques for *in vivo* estimation of $\Psi_{m.}$

In vitro techniques:

The most widely used techniques to monitor the Ψ_m *in vitro* are based on lipophilic cationic fluorescent dyes that accumulate in the mitochondria in a Ψ_m-dependent manner (Perry et al., 2011). The Ψ_m can then be assessed by quantifying the fluorescence of these mitochondria-accumulated dyes using either fluorescence microscopy, flow cytometry or microplate reader-based approaches. Some of the commonly used Ψ_m fluorescent probes include tetramethylrhodamine methyl ester (TMRM), 5,5′,6,6'-tetrachloro-1,1',3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), 1,1′,3,3,3′,3′-hexamethylindodicarbo - cyanine iodide (DiIC1(5)), and rhodamine 123 (Rho123) (Martin et al., 2011; Perry et al., 2011; Walsh et al., 2017). Out of these dyes, TMRM is known to have the least inhibitory effect on mitochondrial activity and hence is the most preferred dye to use for *in vitro* Ψ_m measurements (Perry et al., 2011). Dye-based imaging of Ψ_m has several advantages such as real-time measurement of Ψ_m in response to administered cues (for reversible dyes), and spatial resolution allowing visualization of spatially resolved Ψ_m heterogeneity within a cell population. However, with many of these dyes, there is a concentration dependence that would determine the interpretation of the observed fluorescence intensity. As Perry et al. have reported, there is a 'non-quenching' mode where the dyes are used between 0.5 – 30 nM concentration, where Ψ_m hyperpolarization would lead to increased fluorescence intensity whereas Ψ_m depolarization would decrease observed fluorescence intensity. However, when these dyes are used at higher concentrations between 50 – 100 nM, increased aggregation of the dye in the mitochondria in response to Ψ_m hyperpolarization would result in 'quenching' of the dye fluorescence. Furthermore, in our own experience, the concentration of the Ψ_m-dyes as well as the staining time needs to be optimized for

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each cell line, such that any experimental treatments performed to investigate changes in the Ψ_m must be performed in the phase of dye-loading where the observed dye fluorescence plateaus. Another challenge with reversible dye-based approaches is the fact that the dye needs to remain in the imaging buffer, or it would lead to a steady loss of fluorescence over time and the samples that are imaged at the beginning would seem to have a higher- Ψ_m than the ones imaged at the end. However, having the dye in the imaging buffer would also interfere with imaging quality and resolution. There is hence a need for novel approaches to probe Ψ_m within cells. Recently, a new technique to probe Ψ_m has been reported that utilizes photoinduced electron transfer (PeT)-based Rhodamine Voltage Reporter (RhoVR) instead of the traditional lipophilic dyes that accumulate in the mitochondria in a Ψ_m-dependent manner (Klier et al., 2021) and has been shown to be sensitive to FCCP-induced Ψ_m-depolarization as well as allowing simultaneous probing of cells for cytosolic Ca^{2+} and plasma membrane potential with other probes. Additionally, Okkelman et al. report using TMRM for fluorescence lifetime imaging microscopy (FLIM), and shows promise for simultaneous probing of mitochondrial bioenergetics and Ψ_m (Okkelman et al., 2020).

In vitro models to study the microenvironmental regulation of Ψ_m:

As summarized in the previous sections, Ψ_m can be differentially regulated by cues that remodel the cellular cytoskeleton. The TME is a rich source of biophysical cues that play a role in remodeling cancer cell cytoskeleton to facilitate their invasive and migratory properties (Li & Wang, 2020). We have previously developed an *in vitro* micropatterning approach to recapitulate the confinement of tumor islands by stromal cells (Begum et al., 2019; Shen et al., 2014). A spatial distribution of Ψ_m was found within the micropatterned tumor islands, where the MCF-7 breast cancer cells at the edges of the micropattern had higher Ψ_m compared to those at the micropattern centers (Begum et al., 2019). Interestingly, altering the density of stromal cells surrounding the micropatterned tumor island can also regulate the spatial distribution of Ψ_m – a high degree of stromal confinement led to a very narrow region of cancer cells with high Ψ_m (Begum et al., 2019). To ensure that these results were not confounded by biochemical signals derived from the stromal cells, we mechanically confined the cancer cells in a micropattern surrounded by a thin layer of polydimethylsiloxane (PDMS). This led to a uniformly low- Ψ_m throughout the micropatterned tumor island, indicating that physical confinement as a key mechanism by which the surrounding stromal cells regulate the spatial Ψ_m distribution in the micropatterned tumor model.

Interestingly, RNA-sequencing of cancer cells from the centers and edges of the tumor micropatterns revealed that pathways related to adherens junctions (E-cadherin mediated intercellular adhesions) are upregulated at the micropattern centers (Begum et al., 2021). Through inhibition, overexpression, and knockdown of E-cadherin, we confirmed that the confinement cues from the TME regulate cancer cell Ψ_m through their effects on E-cadherin mediated intercellular adhesions. Thus, *in vitro* models recapitulative of the TME can be important tools in discovering novel mechanisms that regulate the Ψ_m in cancer cells.

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8. Conclusions

It has been known for decades that cancer cells have abnormally high Ψ_m. However, it remains unclear whether this phenotype is a result of cell-intrinsic mutations specific to and characteristic of cancer cells, or due to the activation of aberrant intracellular signaling pathways or from extrinsic cues in the TME. Given the crucial role of the TME in dictating cancer cell behavior, we propose that specific cues from the TME can alter the Ψ_m of cancer cells through intracellular physicochemical signaling. A better understanding of the source of altered cancer cell Ψ_m can lead to the discovery of new targets for attacking cancer cells, by reducing their metabolic plasticity and potentially their metastatic ability. Since the abnormally high Ψ_m has been observed in a wide variety of epithelial carcinoma cells, a detailed understanding of the underlying mechanisms giving rise to this phenotype will be beneficial for developing new therapeutic targets for a wide range of carcinomas.

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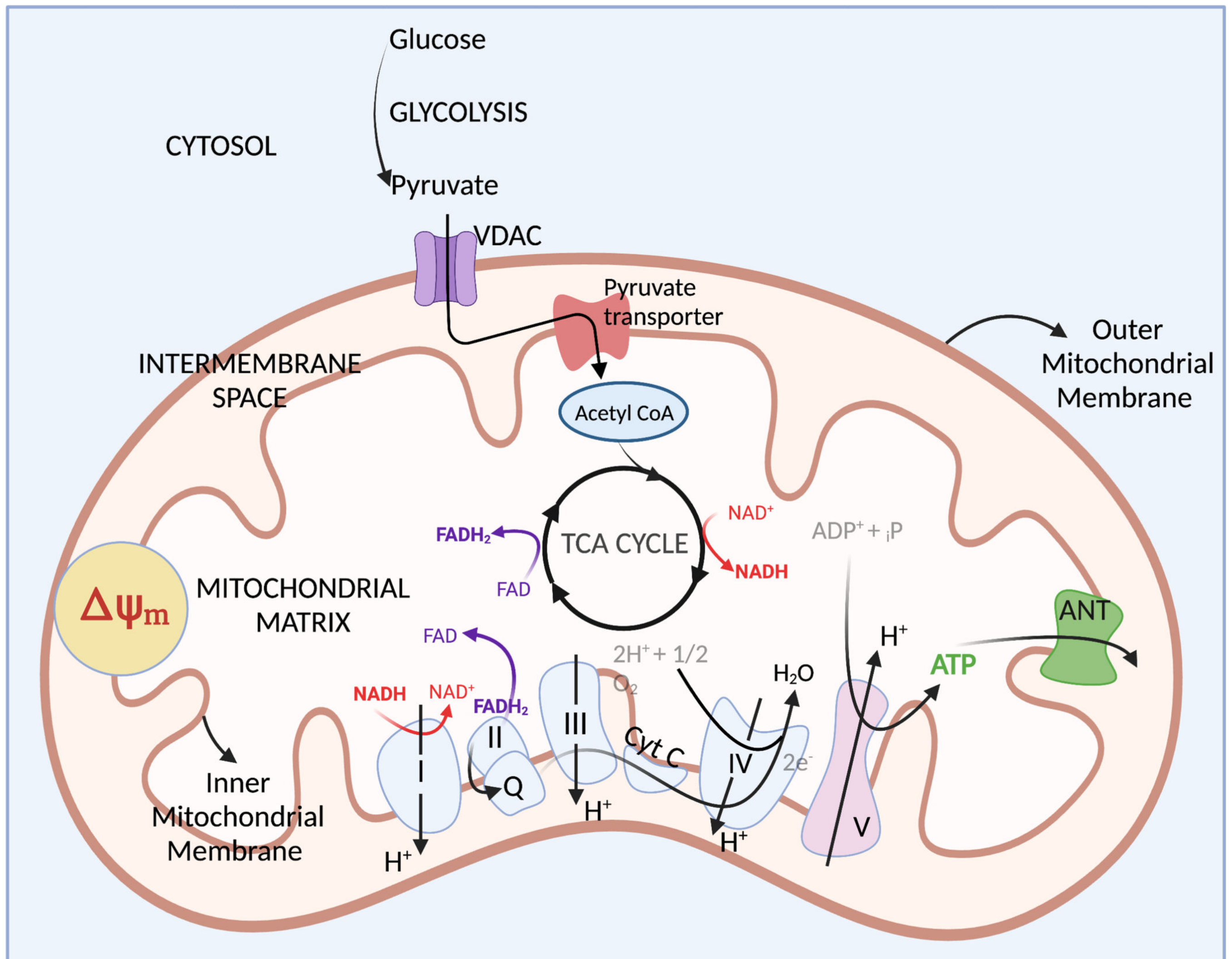

Figure 1:
Schematic showing the tricarboxylic acid (TCA) cycle and electron transport chain (ETC) within the mitochondria.

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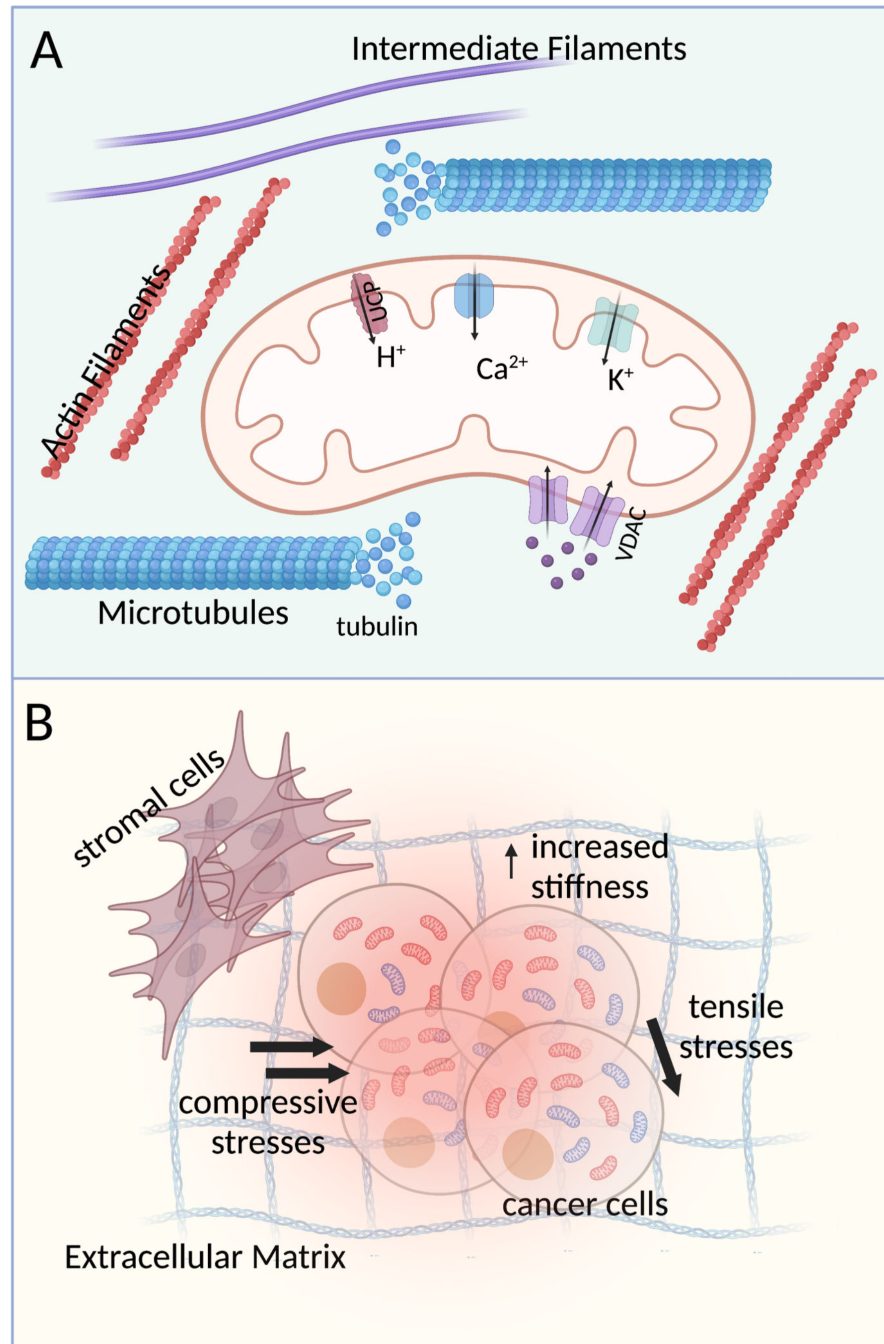

Figure 2:

Schematic showing intrinsic (A) and extrinsic (B) factors that may regulate cancer cell Ψ_m.