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Morphological Dynamics of Mitochondria – A Special Emphasis on Cardiac Muscle Cells

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Abstract

Mitochondria play a critical role in cellular energy metabolism, Ca^{2+} homeostasis, reactive oxygen species generation, apoptosis, aging, and development. Many recent publications have shown that a continuous balance of fusion and fission of these organelles is important in maintaining their proper function. Therefore, there is a steep correlation between the form and function of mitochondria. Many major proteins involved in mitochondrial fusion and fission have been identified in different cell types, including heart. However, the functional role of mitochondrial dynamics in the heart remains, for the most part, unexplored. In this review we will cover the recent field of mitochondrial dynamics and its physiological and pathological implications, with a particular emphasis on the experimental and theoretical basis of mitochondrial dynamics in the heart.

Keywords

mitochondria; mitochondrial fission and fusion; mitochondrial dynamics; cardiac muscle; ventricular myocytes

1. Introduction

The name, “mitochondrion,” derived from the Greek words “mitos” (thread), and “chondros” (grain), describes two shapes of mitochondria that were observed by light microscopy around 150 years ago [1]. Recent advancements in imaging techniques have revealed that the shape of mitochondria in living cells is quite dynamic, constantly interchanging between thread-like and grain-like morphology through what we know now as the fusion and fission processes. These fusion and fission processes together with the mitochondrial movement have been termed “mitochondrial dynamics” [2–5].

The general hypothesis of the origin of mitochondria, that they evolved from the endosymbiosis of an aerobic bacterium with a protoeukaryotic cell, is based in part on the organelle’s double membrane structure, and on similarities of both mitochondrial DNA (mtDNA) and mitochondrial protein synthesis machinery to those of bacteria [1]. Mammalian mtDNA is 16-kb and circular, encoding 22 mitochondrial rRNAs, two mitochondrial tRNAs, and 13 proteins that make up parts of the oxidative phosphorylation complexes I, III, IV, and V. The structure of the mitochondrion is comprised of an outer and inner membrane, a narrow intermembrane space, and a large matrix. The outer membrane contains well-regulated channels that are relatively permeable to many ions and metabolic

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compounds [6,7]. The inner mitochondrial membrane is invaginated, creating cristae structures that form microdomains of ions, nutrient molecules, ATP, ADP, and small soluble proteins [6]. The inner membrane contains the respiratory chain proteins for the generation of ATP, and many transporters that are important for the exchange of molecules in and out of the matrix. Contact sites, where the inner and outer mitochondrial membranes meet, is where complexes such as the porin/voltage dependent anion channel (VDAC), mitochondrial creatine kinase, and adenine nucleotide translocase (ANT) can be found [8]. These contact sites are also proposed to be the sites of mitochondrial fusion and fission [9].

Unlike the typical static kidney-bean shape shown in many textbooks, mitochondria are actually dynamic organelles which can move across great distances in the cell on microtubule networks, and undergo fusion or fission on average every two minutes in several cell types [10–12]. The fusion and fission processes occur in a careful balance in order to maintain proper mitochondrial dynamics. An increase in fusion or a decrease in fission can lead to elongated, interconnected mitochondria, whereas a decrease in fusion or an increase in fission can lead to punctate, fragmented mitochondria (Figure 1). Mitochondria display a wide range of shapes, ranging from small vesicles to rods to tubules to branched reticular networks, within individual cells and in different cell types [13]. The mitochondria from many mammalian cell lines also generally cluster around the perinuclear area [13]. Additionally, giant mitochondria have been found in normal cells, aging cells, and cells with metabolic injuries [14,15].

Mitochondria cannot be generated de novo, but rather derive from existing mitochondria through division and synthesis and import of proteins and lipids. Every mitochondrion contains several copies of the mtDNA genome, which is maternally inherited. The mtDNA genome can be replicated multiple times during each cell cycle, and cells that have high levels of mutations in mtDNA (60–90%) can have dysfunctional oxidative phosphorylation and respiration [16–18]. Defects in mitochondrial fusion can lead to accumulations of mtDNA mutations and, consequently, accumulations of dysfunctional mitochondria, apparently due to lack of complementation of mtDNA genomes and mixing of gene products. MtDNA mutations can result in many different diseases, one of which predominantly affects skeletal muscle, called myoclonic epilepsy associated with ragged-red fibers (MERRF). This disease is characterized by muscle weakness, increase in subsarcolemmal mitochondria, and lactic acidosis [19].

Fusion and fission processes require GTP hydrolysis and this expenditure of energy serves a purpose. Fusion between two or more mitochondria is important for the mixing of mitochondrial matrix contents [20]. Fused networks of connected mitochondria may facilitate the transmission of Ca^{2+} signals and membrane potential across distances within the cell [21,22]. A decrease in mitochondrial fusion activity results in decreased mitochondrial oxygen consumption and membrane potential, as well as a decrease in mtDNA [23]. Fragmented mitochondria are thought to be easily transportable and allow for rapid mitochondrial trafficking to energy-demanding regions of the cell [21]. Disruption of mitochondrial fission results in disruption of mitochondrial recruitment and location [22,24]. Mitochondrial fragmentation is also shown to be a necessary component for high glucose-induced respiration increase and reactive oxygen species (ROS) overproduction [25].

In this review we will describe the mammalian mitochondrial fission and fusion proteins, the regulation of these proteins, and their functional roles in health and disease. A special focus will be on the experimental and theoretical basis of mitochondrial dynamics in the heart.

2. The proteins involved in mitochondrial fusion and fission

Mitochondrial fusion is a complex process that involves the fusing together of four lipid bilayers. Mitochondrial fusion in mammals requires the two 85kD-GTPase isoforms mitofusin1 and mitofusin2 (Mfn1, Mfn2, Fzo1p in yeast) [26], which are outer mitochondrial membrane anchored proteins that contain two transmembrane domains connected by a small intermembrane-space loop, a cytosolic N-terminal GTPase domain and two cytosolic hydrophobic heptad-repeat coiled-coil domains (Figure 2) [26–28]. Mfn1 and Mfn2 can form homo- and hetero-oligomers, or the coiled-coil domains of Mfn1 can interact *in trans* with Mfn1 on another mitochondrion which serves to tether adjacent mitochondria together [23,29]. For a model of the fusion, see Figure 1. GTP hydrolysis is important in the fusion process. In cells where Mfn2 has a GTPase mutation, tethered mitochondria are found rather than fused mitochondria [29]. GTP hydrolysis may cause a conformation change in Mfn, but this process is not fully understood.

Mfn1 and Mfn2 share 77% homology and have overlapping functions, as they can replace each other in Mfn-null cells [27]. However, there are functions that are unique to each Mfn. In embryo development, Mfn1−/− and Mfn2−/− mice die in midgestation and Mfn2−/− mice have defective placentation [30]. During mitochondrial fusion, Mfn1 and Mfn2 have different affinities to GTP and different GTPase activity [31,32]. Mfn2 may also regulate mitochondrial metabolism. Mfn2 gene expression is reduced in type 2 diabetes and obesity, and expression is increased with exercise and weight loss [33–35]. Another study suggests that insulin stimulates the expression of Mfn2 protein and inhibits the downstream MEK-dependent signaling pathway [36].

Mitochondrial fusion requires another dynamin family 100kD-GTPase, optic atrophy 1 protein (OPA1, Mgm1p in yeast, Figure 2), which is found in the intermembrane space or bound to the surface of the inner mitochondrial membrane, and is required to tether and fuse mitochondrial inner membranes [37,38]. During mitochondrial fusion, after the outer membranes fuse, Mgm1p from both mitochondria interact in trans to tether and fuse the inner membranes [37]. In yeast, mitochondrial fusion requires a linker protein between outer and inner mitochondrial membrane proteins for proper fusion. Ugo1p found in the outer mitochondrial membrane connects Fzo1p to Mgm1p [39]. However, no homolog of this protein has been found in mammals.

Splicing of the mammalian OPA1 gene yields at least eight mRNA variants of OPA1 [40]. Post-translational proteolytic processing of OPA1 by mitochondrial processing peptidase (MPP) produces long isoforms that are anchored to the inner mitochondrial membrane, and cleavages at the S1 and S2 protease sites can yield additional short isoforms that are found in the intermembrane space [40,41]. The following proteins are associated with proteolytic processing of OPA1: presenilin-associated rhomboid-like protein (PARL) at S2 [42,43], the m-AAA proteases AFG3L2 [44] and paraplegin [45], and i-AAA protease Yme1 at S2 [41,46,47]. Loss of mitochondrial membrane potential regulates OPA1 cleavage by destabilizing the long OPA1 isoform and enhancing cleavage at S1 [41,44,45].

Loss of OPA1/Mgm1p results in mitochondrial fragmentation due to loss of mitochondrial fusion, and also leads to aberrations in cristae structure, suggesting that OPA1/Mgm1p plays a role in cristae maintenance [38,43,48–51]. OPA1 controls the shape of cristae by forming self-oligomers comprised of both the long and short isoforms [42]. The tightness of cristae junctions correlates with OPA1 oligomerization, while BID disrupts OPA1 oligomers [42]. Mitochondria with Mgm1p GED mutants display a decrease in normal cristae structures [37]. In addition to its fusion and cristae remodeling roles, OPA1 is also important in the resistance to free radicals. Loss of function of the C. elegans OPA1-homologue, eat-3, leads

to increased ROS sensitivity for injury either by paraquat, which produces superoxide radicals, or by the loss of mitochondrial superoxide dismutase sod-2 [52]. Moreover, these eat-3 mutants also overexpress sod-2 to prevent free radical damage [52].

Mammalian mitochondria undergo fission by the interaction of two proteins: dynamin-like protein 1 or dynamin-related protein 1 (DLP1/Drp1, Dnm1 in yeast), an 80–85-kD cytosolic GTPase, and human fission protein 1 (hFis1, Fis1p in yeast), a 17-kD outer mitochondrial membrane anchored protein. Overexpression of dominant negative DLP1 or dominant negative hFis1, or RNAi knockdown DLP1 or hFis1 induces mitochondrial elongation, which demonstrates that DLP1 and hFis1 are the required fission proteins [25,53]. hFis1 is anchored in the membrane by a single C-terminal transmembrane domain, while the N-terminal cytosolic region consists of six α-helices that contain two tetratricopeptide repeat-like folds that are involved in protein interactions (Figure 2). Cross-linking and fluorescence resonance energy transfer (FRET) indicates that DLP1 and hFis1 bind together [54]. hFis1 transiently interacts with a DLP1-fission complex via the hFis1 TPR, and specifically the α1-helix plays a role in this interaction, perhaps serving as a regulator for DLP1-hFis1 interactions [25]. There is evidence that oligomerization of hFis1 may also play a role in mitochondrial fission, as overexpression of hFis1 oligomerization-defective mutants display reduced ability to induce mitochondrial fission [55]. In yeast, Fis1p requires the WD40-motif cytosolic adaptor proteins, Mitochondrial division 1 (Mdv1p) or Caffeine-resistant 4 (Caf4), that bind Dnm1p to Fis1p [56–58]. There is no homolog of these proteins in mammals, yet other proteins are found, such as Bax and Endophilin B1, which will be discussed later. DLP1 is mainly localized in the cytosol and is recruited to the mitochondrial membrane by hFis1 to tips and constriction sites of mitochondria that are future sites of fission [59,60]. DLP1 translocation depends on actin and microtubules, as disruption of F-actin or retrograde motor protein complex, dynein/dynactin, can attenuate fission and DLP1 recruitment to mitochondria [61,62]. As studied in yeast with Dnm1, once at the mitochondria, DLP1 homo-oligomerizes into a ring formation around the mitochondrion [63]. DLP1 does not require hFis1 to assemble on mitochondria, as there was no difference in DLP1 distribution on mitochondria upon RNAi knockdown of hFis1 [53]. It is thought that because DLP1 and dynamin share a highly conserved GTPase domain and coiled-coil domain, that DLP1 also acts as a membrane-pinching mechanoenzyme. The coiled-coil regions of different DLP1 proteins can interact with each other and with the GTPase domain [64]. The self-assembly can stimulate the final step of fission, the hydrolysis of GTP, required for DLP1 constriction and disassembly [65–67]. A model of this fission process is described in Figure 1. In addition, DLP1 and hFis1 are found on peroxisome organelles that catabolize fatty acids and H_2O_2. DLP1 also localizes to undefined cytoplasmic vesicles and the endoplasmic reticulum (ER) as well [68,69].

Human membrane-associated RING-CH (MARCH)-5, also known as MITOL, is an E3 ubiquitin ligase that is found in the outer mitochondrial membrane [70–72]. MARCH5 interacts with and ubiquitinates hFis1 and DLP1, as well interacts with Mfn2. RNAi of MARCH5 leads to mitochondrial elongation which indicates inhibition of mitochondrial fission. MARCH5 may also regulate trafficking of DLP1, as there is abnormal clustering of DLP1 in cells that express MARCH5 RING mutants [70].

Mitochondria protein (MTP18, Mdm33 in yeast), is a protein that faces the intermembrane space and is most likely anchored in the inner mitochondrial membrane [73]. MTP18 expression is activated by phosphatidylinositol (PI) 3-kinase signaling [74]. Overexpression of MTP18 leads to mitochondrial fragmentation, whereas RNAi of MTP18 leads to elongated mitochondria. Overexpression of hFis1 leads to mitochondrial fragmentation via the known DLP1-mediated pathway [25]. However, this hFis1-induced fragmentation was suppressed by MTP18 knockdown, suggesting that hFis1 may depend on MTP18 [73]. The

mechanism of how MTP18 participates in fission of the inner mitochondrial membrane still remains unknown.

3. Post-translational regulation of mitochondrial fusion and fission

There are two types of mitochondrial fission, one being physiological, and the other pathological (which will be described in the next section). Mitochondrial fission can occur rapidly and reversibly by an increase in cytosolic Ca^{2+}, and requires mitochondrial Ca^{2+} uptake, suggesting an intramitochondrial Ca^{2+} component to regulating mitochondrial fission machinery [75]. DLP1 is regulated covalently by ubiquitination and sumoylation [71,76,77], or phosphorylation. DLP1 phosphorylation and dephosphorylation is controlled by cytosolic Ca^{2+} signaling pathways. DLP1 can be phosphorylated by cyclin B1, cAMP, or CaM kinase 1α, and dephosphorylated by calcineurin pathways [78–81]. Phosphorylation in the DLP1 GTPase effector domain (GED) at Ser600 by activation of Ca^{2+}/calmodulin-dependent protein kinase 1α (CaMK1 α) has been associated with increase in DLP1 translocation to mitochondria and increase in DLP1 affinity for hFis1 [78]. Phosphorylation in the DLP1 GED at Ser585 by activation of Cdk1/cyclin B protein kinase complex has been shown to induce mitotic mitochondrial fragmentation. Phosphorylation by PKA in the DLP1 GED at Ser637 decreases GTPase activity and prevents mitochondrial fragmentation [79]. Similarly, DLP1 translocates to mitochondria in a calcineurin-dependent manner in dysfunction-induced fragmentation, which requires DLP1 Ser637 [81]. Additionally, phosphorylation by cyclic AMP-dependent protein kinase at DLP1 GED at Ser656 and dephosphorylation by calcineurin is regulated by sympathetic tone, Ca^{2+} levels, and cell viability [80]. In a study of patients with chronic heart failure an association was found between calcineurin activation and an increase in peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α), an upstream regulator of lipid and glucose oxidative metabolism, and mRNA levels of proteins involved in degradation, fusion, and fission of mitochondria [82].

4. Mitochondrial dynamics in cell death

Although still controversial, there is evidence suggesting that mitochondrial morphology proteins are important in cell death pathways. Preventing mitochondrial fission through inhibition of DLP1 or hFis1 can delay or inhibit the markers of apoptosis such as cytochrome *c* release induced by staurosporin, actinomycin D, etoposide, or ER signals [25,53,83,84]. Conversely, excessive mitochondrial fragmentation caused by the overexpression of hFis1 leads to apoptosis [25,85].

In neonatal rat ventricular myocytes, H9c2 cells, bovine aortic endothelial cells, and mouse aortic smooth muscle cells, mitochondrial fragmentation occurs in the early stages of apoptosis [86]. Hyperglycemic conditions cause mitochondrial fragmentation, ROS increase, mitochondrial permeability transition (MPT), and cytochrome *c* release in these cardiovascular cells. Blocking mitochondrial fission can block ROS, MPT and cytochrome *c* release. Also bongkrekic acid, the adenine nucleotide translocase inhibitor, can prevent MPT in these conditions [86]. In another study, it was shown that C_2-ceramide in neonatal rat cardiomyocytes were able to cause mitochondria to fragment and apoptosis to be activated. Also, cardiomyopathy was induced by doxorubicin treatment, which caused mitochondrial fragmentation and apoptosis [87]. These findings indicate a link between mitochondrial fragmentation and apoptosis in heart.

The link between mitochondrial morphology and apoptosis has been mainly explored in non-cardiovascular cells. Ca^{2+} is an important regulator of apoptosis, and prolonged ER Ca^{2+} depletion due to thapsigargin can lead to mitochondrial fragmentation and apoptosis

[88,89]. ER Ca^{2+} depletion causes an accumulation of unfolded or misfolded proteins in the ER, leading to ER stress pathways. If protein folding cannot be restored, apoptosis is triggered and can be mediated by the mitochondria and/or activation of proapoptotic kinases [90]. However, this apoptosis can be blocked by expression of mutant DLP1-K38A, a dominant negative DLP1 [75]. Mitochondrial Ca^{2+} uptake is important in BAP31-induced apoptosis or BH3-only BIK activation, as mitochondrial Ca^{2+} uptake increases DLP1 recruitment to the mitochondria, mitochondrial fragmentation, and apoptosis [84,91].

Mitochondrial fission proteins also associate with proapoptotic proteins. It was demonstrated that endophilin B1/Bif1 triggers Bax and Bak (proapoptotic Bcl2 family proteins) activation upstream of mitochondrial fragmentation [92]. Subsequently, Bax and DLP1 colocalize together at scission sites on mitochondria [93]. After Bax translocation to the mitochondria, DLP1 becomes stabilized on the mitochondria, which correlates with a Bax/Bak-dependent sumoylation of DLP1 [94].

During apoptosis, mitochondrial morphology proteins, such as DLP1 and OPA1 have a role in addition to fission. DLP1 has a role in remodeling and opening cristae, which requires a functional permeability transition pore [91]. OPA1 also serves a function in apoptosis, and has been shown to be released during apoptosis with cytochrome *c* [95]. Downregulation of OPA1 causes cells to undergo apoptosis [50]. DLP1 and OPA1 may be involved in the release of cytochrome *c*. DLP1 is thought to facilitate mitochondrial outer membrane permeabilization (MOMP) through mitochondrial fragmentation. However, DLP1 may also have a role in MOMP. Upon thapsigargin treatment, cytochrome *c* was released without mitochondrial fragmentation, yet, cytochrome *c* release was inhibited when DLP1 was inhibited [75]. In another study, a chemical inhibitor of DLP1, mdivi-1, was used and partially blocked apoptosis by inhibiting MOMP in cells. In vitro, mdivi-1 blocked Bid-activated Bax/Bak dependent cytochrome c release. Taken together this suggests that DLP1 can regulate MOMP independent of the mitochondrial fission pathway, perhaps by directly modulating MOMP [96]. It is important to note, though, that apoptosis can occur without the fusion or fission proteins [83,94,97–100].

5. Mitochondrial morphology and human disease

The importance of functional mitochondrial morphology is apparent as morphological defects are related to many human diseases. These include myopathies, diabetes mellitus, liver diseases, neurodegeneration, aging, and cancer [101]. Mutations in mitochondrial fission can be lethal. A female infant with a dominant-negative mutation in DLP1 only lived for 37 days, and had defects in fission of mitochondria and peroxisomes. The patient had microcephaly, abnormal brain development, optic atrophy and hypoplasia, and metabolic abnormalities [102]. RNAi against DLP1 is lethal in *C. Elegans* embryos before 100 days [59]. Mutations in mitochondrial fusion can also be lethal. Mice with mutations in Mfn1, Mfn2, or OPA1 cannot survive past midgestation [27]. Downregulation of OPA1, Mfn1, or Mfn2 results in mitochondrial fragmentation, a reduction in oxygen consumption, and a decrease in mitochondrial membrane potential [23]. Mfn2 mutations cause peripheral neuropathy-Charcot-Marie-Tooth subtype 2A, characterized by defects in axonal neurons [103,104]. In addition, OPA1 is mutated in dominant optic atrophy, the defect in mitochondrial distribution and loss of retinal ganglion cells resulting in optic nerve atrophy [105]. Therefore, along with mtDNA mutations, mutations affecting mitochondrial fusion and fission proteins are a major source of human disease.

6. Mitochondrial dynamics in the heart

The great majority of work done so far on mitochondrial dynamics has utilized cultured cells because of the ease in performing live-cell imaging in these preparations. The mitochondria

in these cultured cells show dramatic motility and morphologic changes. This robust mitochondrial dynamism could be a result of cell culture, which promotes microtubule-directed mitochondrial trafficking and thus mitochondrial fission and fusion [106]. This high degree of mitochondrial dynamics is considered not applicable to native tissues like adult heart, liver, kidney, etc. Electron micrographs of adult cardiac muscle cells, especially ventricular myocytes, show that mitochondria are numerous, making up about 35% of the cell volume, and that mitochondria are highly organized and compacted between contractile filaments and next to T-tubules. This crystal-like pattern of mitochondria raises an interesting question: do the mitochondria in adult ventricular myocytes also undergo physiological fission, fusion, and movement just like other cell types? This important question has just begun to draw some research attention very recently and thus the definitive answers are still lacking. Intuitively, one could imagine that there is no need for mitochondria in adult ventricular myocytes to go through fission, fusion, and movement because they are being anchored by cytoskeleton, crowded among themselves in a space that is surrounded by contractile proteins and Z-lines. In dichotomy, however, all mitochondria have a limited life span and their biogenesis, turnover, autophagy, and maintenance of genomic integrity are dependent critically on fission, fusion, and motility [107,108]. Intriguingly, there are several studies indicated that all major proteins involved in mitochondrial fission and fusion do exist in adult hearts. Using Northern and Western blot to screen the expression of Mfn1 and Mfn2 in various adult human tissues, including heart, pancreas, skeletal muscle, brain, liver, placenta, lung, and kidney, one research group has found two Mfn1 transcripts in these human tissues, interestingly, with the highest amount in heart [109]. The group also found that while Mfn2 mRNA was abundantly expressed in heart and muscle tissue but expressed only at low levels in other tissues. The expression of Mfn1 and Mfn2 in rat and mouse heart has also been detected by RT-PCR [110,111]. The fission protein hFis1 has been shown to be ubiquitously expressed in isolated rat mitochondria from many tissues, including heart [112]. The identification of DLP1 mRNA in adult human tissues was detected by northern blot analysis that showed high levels in skeletal muscle, heart, kidney and brain [113]. Using RT-PCR, four mRNA transcripts for OPA1 were identified in adult mouse hearts [114]. These four OPA1 splice variants produced six distinctive protein bands in hearts suggested that OPA1 isoforms undergo posttranslational modification. Although all these studies convincingly show that the mitochondrial fission and fusion proteins exist in heart, it should be noted that heart consists numerous cell types such as fibroblasts, endothelial cells, vascular smooth muscle cells, and cardiac muscles. To be sure, these experiments should be repeated by using purified isolated cardiac myocytes.

The identification of these fission and fusion proteins in adult heart, however, has not yet been implicated in their functional role of dynamically controlling mitochondrial morphology in adult cardiac muscle cells. The reasons for the lack of this information could be in part due to the technical difficulties in imaging mitochondrial morphology in living adult ventricular myocytes. The morphology of normal mitochondria tends to be big and cylindrical- or ovular shaped, averaging around 0.5–1 μm in width and 1–2 μm in length [1]. The dimension of an adult ventricular myocyte is approximately 120 μm × 20 μm × 10 μm. There are several thousands of mitochondria situated by-and-large orderly in a single cell. This is the major reason why the confocal images taken from myocytes loaded with mitochondria-targeted fluorescent probes show a very regular pattern of mitochondrial distribution (Fig. 3A). Due to the limitation in the spatial resolution (~200 nm for x–y axis and ~ 500 nm for z axis), confocal microscopy, unlike electron microscopy, may not be able to distinguish more subtle differences between mitochondria when they are packed near each other. It has long been known that the electron micrograph of adult ventricular myocytes shows that the mitochondria located around the nucleus, between the myofibrils, and beneath the sarcolemma, appear to have different morphology and distribution, as well

as different oxidative metabolic activities [115,116]. Figure 4A shows such an image that we have obtained from longitudinal sectioning of an adult rat ventricular myocyte. The mitochondria clustered near the poles of the nucleus (N) are generally more irregularly distributed with a globular shape and a higher degree of variation in size. Interfibrillar (I) mitochondria are usually aligned in longitudinal rows between the myofibrils. They are elongated and usually about the same length as a sarcomere. However, some mitochondria are clearly fragmented and are in contact with neighboring mitochondria (Fig. 4B). These more subtle variations among the interfibrillar mitochondria can be manifested more clearly in the electron micrography taken from transversely sectioned cardiac myocytes (Fig. 4C). In this image, mitochondria are “wrapped” partially around the myofilaments with certain degrees of variations. This close apposition of mitochondria to the contractile machinery strategically allows mitochondria to deliver ATP more efficiently to the sites where energy demands are high. Subsarcolemmal (S) mitochondria are also varied in size and shape, being rod-like, globular, or horseshoe-like. These distinct patterns of mitochondrial distribution in different regions of adult ventricular myocytes may suggest a spatial and temporal heterogeneity of mitochondrial dynamics in adult ventricular myocytes. It can be hypothesized that fission and fusion could occur in a timely manner for the perinuclear and subsarcolemmal mitochondria but much rarely for the interfibrillar mitochondria. For comparison, Figure 3B shows that both thread-like and grain-like morphology of mitochondria co-exist in the cultured neonatal ventricular myocyte. This appearance of mitochondrial distribution is similar to other types of culture cells for which the mitochondria are usually going through fission, fusion, and movement.

Another important feature about the distribution of mitochondria in the adult ventricular myocyte is their proximity to the sarcoplasmic reticulum (SR), some of them are located as close as 37 nm to the foot of the SR ryanodine receptor (RyR) foot [117]. Thereby, mitochondria are located within microdomains of high micromolar range Ca^{2+} during excitation-contraction coupling and are poised for both regulating localized cellular Ca^{2+} homeostasis and ATP generation, linking energy metabolism to excitation-contraction coupling (Fig. 5). The heart beats perpetually during their life span with a resting heart rate varies significantly among different animal species (e.g. 70 beats per minute in human and 450 beats per minute in mouse). In addition, the heart rate could change quickly under sympathetic and parasympathetic innervations. Therefore, the generation of ATP by mitochondria must be regulated effectively to meet the challenges in balancing energy demand and supply. It has become more evident that mitochondria use Ca^{2+} as a key regulator for controlling their metabolic activities [118]. During the excitation-contraction cycles, mitochondria sequester a small amount of Ca^{2+} that activates several enzymes involving in the ATP generating machineries. This signal cascade process, which ensures the homeostasis of bioenergetics in beating heart, has been termed excitation-contraction-metabolism coupling [119,120]. It should be noted that this Ca^{2+} coupling between mitochondria and SR/endoplasmic reticulum (ER) for modulating oxidative metabolism occurs in numerous cell types [121]. One intriguing question is: do the mitochondrial dynamics play any role in excitation-contraction-metabolism coupling? Very interestingly, recent studies have shown that Ca^{2+} can regulate mitochondrial fission, fusion and motility [75,122–124]. Moreover, it has been reported that a new function of Mfn2 is to tether the endoplasmic reticulum and mitochondria to control the efficiency of mitochondrial uptake of Ca^{2+}[125]. Although it is still not known whether this Mfn2 tethering mechanism also exists in heart, this new discovery exemplifies again the multiple roles of mitochondrial dynamics in regulating cell function.

Taken together the above-mentioned experimental and theoretical evidence, several distinct aspects about mitochondrial dynamics can be raised in adult ventricular muscle cells: 1) the normal fusion/fission machinery (proteins) appears to exists, 2) the requirement of

mitochondria regeneration, turnover, autophagy, genomic protection suggests the need for fission and fusion, 3) the motility is minimal and could vary among different mitochondrial populations, 4) a four-dimensional (x, y, z axis and time) live-cell imaging is needed to detect possible movements like mitochondria winding slowly through the myofibrils in the third dimension, and 5) the possible tethering of mitochondria with SR implicates that the Ca^{2+} concentrations in microdomains may play a crucial role in regulating mitochondrial fission, fusion, and motility.

In other type of cardiac muscle cells, some recent reports demonstrated the physiological and pathological implications of mitochondrial dynamics. In embryonic stems cells, it has been demonstrated that anaerobic glycolytic metabolism has to be transformed into the more efficient mitochondrial oxidative metabolism for cardiac differentiation [126]. This energetic switch might be manipulated by modifying the copy number of regulators of mitochondrial fusion and fission. Therefore, mitochondria dynamics could be critically in regulating the differentiation of stem cells into a functional cardiac phenotype. Interestingly, in day 12 rat embryo, the mitochondria morphology in heart muscle cells are randomly distributed with both rod-like and spherical shape [127]. This may suggest that myocytes are undergoing a transition from a glycolytic state with more fragmented mitochondria to an oxidative state with more fused mitochondria. Therefore, mitochondrial dynamics may be more pronounced during the early development of cardiac muscle cells.

In cultured neonatal ventricular myocytes, we have demonstrated that inhibition of mitochondrial fission by overexpressing cells with a dominant-negative mutant form of DLP1, DLP1-K38A prevented the overproduction of reactive oxygen species (ROS), mitochondrial permeability transition, and subsequent cell death under sustained high glucose condition, suggesting that mitochondrial fission is an upstream factor for these events [86]. Similarly, treating cultured neonatal ventricular myocytes with ceramides affects mitochondrial dynamics and promotes mitochondrial fission that leads to apoptosis [87]. These studies suggest that mitochondrial fission may be involved in ROS homeostasis and oxidative stress-mediated cell injury.

In contrast to normal cardiac mitochondria, there are cases of cardiac disease, including end-stage dilated cardiomyopathy, myocardial hibernation, cardiac rhabdomyoma, and ventricular-associated congenital heart diseases, where mitochondria were found to be disorganized and abnormally small [128–133]. In one case of Tetralogy of Fallot, electron micrographs show disorganized clusters of fragmented mitochondria located away from contractile filaments, and the diameter of one mitochondrion was measured to be as small as 0.1 μm [133]. On the other hand, in senescent cardiomyocytes, accumulations of big and defective mitochondria correlate with age [134]. Using long-term culture of neonatal rat ventricular myocytes as an experimental model for studying aging, inhibition of autophagy with 3-methyladenine caused only moderate accumulation of “giant” mitochondria but drastically increased numbers of small mitochondria [134]. The authors conclude that the interplay between mitochondrial fission and autophagy control the rate of mitochondrial turnover and this balance may be disturbed in aging heart cells resulting in more giant mitochondria that were seen in aging heart. All together this suggests that there is a relationship between mitochondrial morphology and pathogenesis of cardiac disease, though this has not yet been explored in greater details.

7. Perspectives

Mitochondrial dynamics has drawn much research attention, especially during the last ten years. This is mostly due to the recent accumulation of vast knowledge indicating the biological significance of mitochondrial fission, fusion, and movement. One can appreciate

that, in numerous cell types, the separation between mitochondria-mediated life versus death resides in the finest balance between fission and fusion. In cardiac muscle cells, this important aspect of mitochondrial research is mostly missing. This could be in part due to the intuition that there is no need for the mitochondria in adult ventricular myocytes to have fission and fusion processes due to their tight organization. Intriguingly, the heart appears to have higher levels of proteins involved in mitochondria dynamics than other tissue studied. It is plausible that these proteins may be involved in cellular signaling processes such as the regulation of electron transport chain activity or the regulation of the permeability transition pore in addition to, though still lacking experimental evidence of, fission and fusion. As described earlier, translocation of DLP1 may involve the steps of phosphorylation/dephosphorylation by signaling proteins such as cAMP dependent protein kinase and calcineurin. Moreover, activation of the fission process does not necessarily always end up with mitochondrial fragmentation, especially during the early and intermediate stages of activation [135]. We have recently obtained data showing that translocation of DLP1 to mitochondria is an earlier event for Ca^{2+} mediated ROS generation in adult rat ventricular myocytes (Hom and Sheu, unpublished results). Finally, the mitochondria dynamics could be an integral part of the regulation of cellular bioenergetics, redox signaling, Ca^{2+} homeostasis, differentiation, and aging for heart muscle cells. Hopefully, this review may catalyze additional activities in this important area of heart research.

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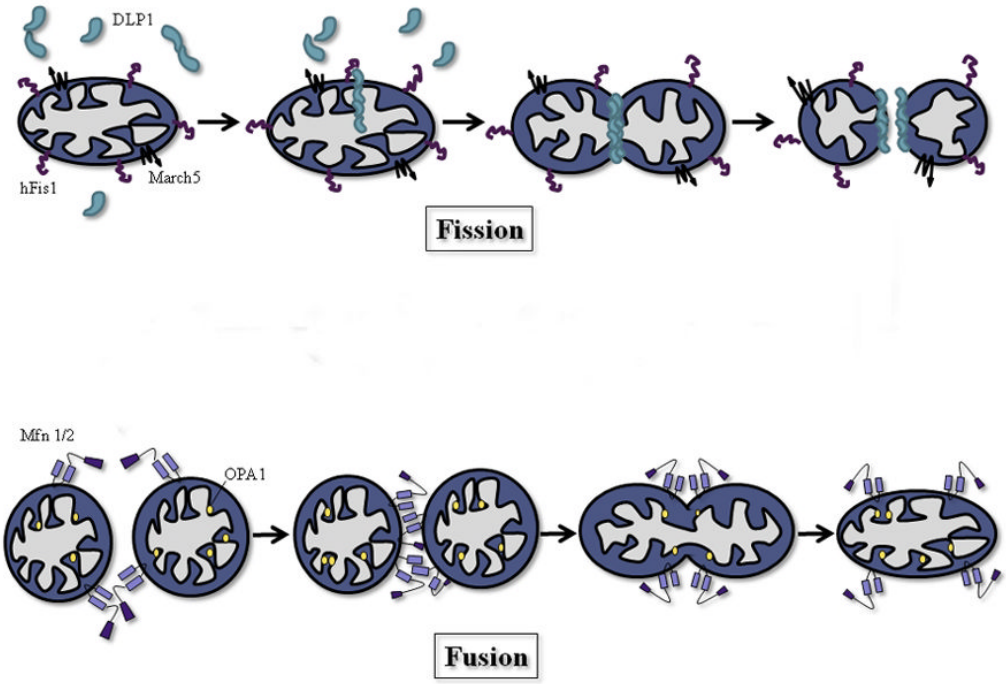

Figure 1. Model of the mechanism of mitochondrial fission and fusion in mammalian cells

Mitochondrial fission starts with DLP1 recruitment to the mitochondrial membrane. DLP1 can self-assemble in the cytosol. hFis1 and DLP1 are able to form a complex together, and it is thought that hFis1 serves as a transient receptor to recruit DLP1 to the mitochondria. After DLP1 is targeted to mitochondria, GTP-bound DLP1 forms a spiral completely around the mitochondrion. The constriction of the mitochondrial membrane may be driven by the assembly of DLP1 and/or a DLP1 conformational change driven by the hydrolysis of GTP into GDP + P_i. GTP hydrolysis allows the complete scission and disassembly of the DLP1 complex, thereby completing mitochondrial fission. Mitochondrial fusion requires opposing mitofusins to tether adjacent mitochondria together in a *trans* complex. GTP hydrolysis is essential for mitochondrial fusion. OPA1 is involved in inner mitochondrial membrane fusion and cristae remodeling. The mechanism by which the membranes come close enough for fusion, the actual fusion, or how mitofusins disassemble is not yet known.

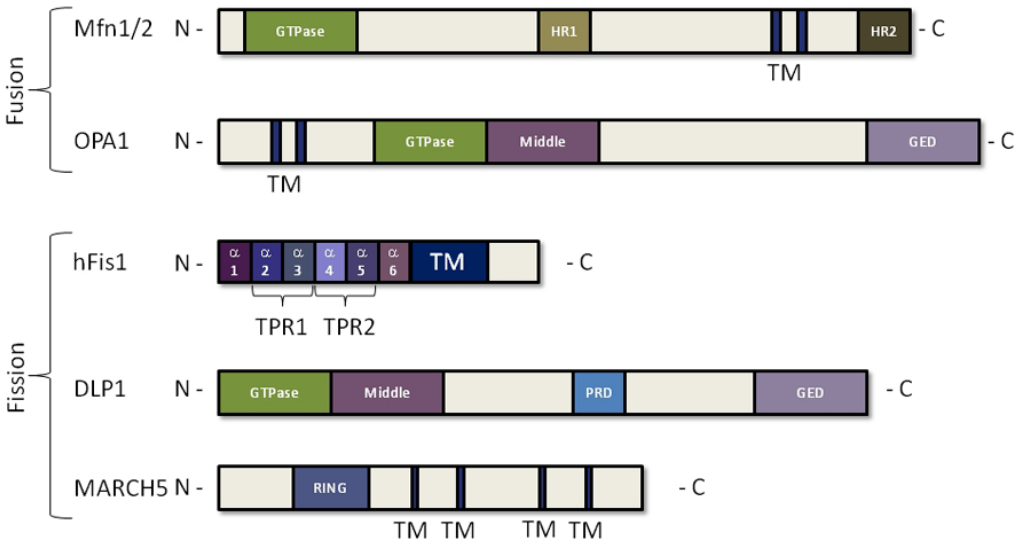

Figure 2. Structure of proteins involved in mammalian mitochondrial fusion and fission

Abbreviations: GTP hydrolysis domain (GTPase), hydrophobic heptad repeats (HR), transmembrane segment (TM), dynamin-homology middle domain (Middle), GTPase effector domain (GED), tetratricopeptide repeats (TPR), proline-rich domain (PRD), really interesting new gene (RING). Mfn1/2 and OPA1 are involved in mitochondrial fusion. hFis1, DLP1, MARCH5, and MTP18 are involved in mitochondrial fission.

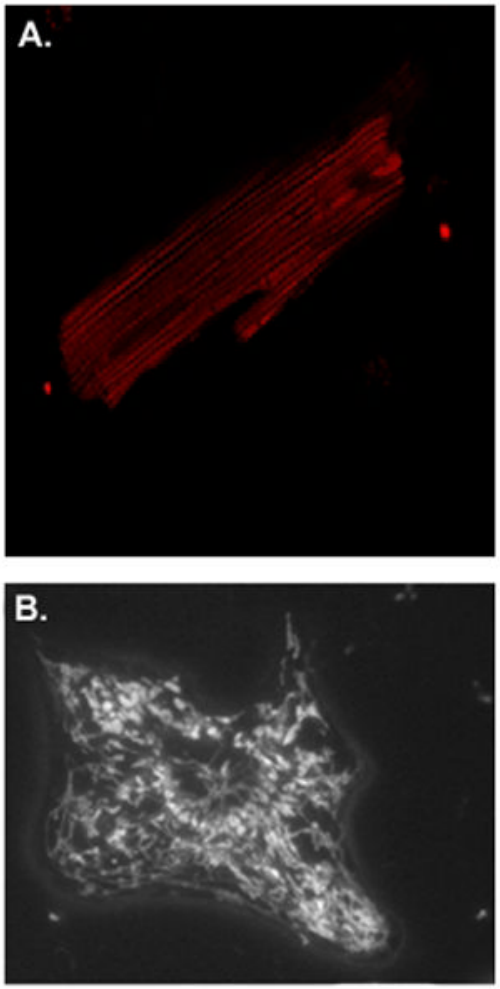

Figure 3. Mitochondrial morphology in adult and neonatal rat ventricular myocytes

A. One plane of mitochondria from an adult rat ventricular myocyte, visualized using TMRE under confocal microscope. Mitochondria within the cell are highly organized following patterns of the contractile apparatus. The mitochondria appear as uniform box-like shapes.

B. Mitochondrial morphology of normal neonatal rat ventricular myocytes (Day 3 in culture) visualized by a mitochondrial-targeted red fluorescent protein (mRFP). There are two populations of mitochondria: thin filamentous or large globular. Unlike the adult myocytes, these mitochondria are disorganized and do not appear to be poised for excitation-contraction-metabolism coupling at this stage in development. The mitochondrial morphology may change in development to accommodate increased energy demands of the cell.

C. Mitochondria from adult rat ventricular myocytes that are viewed from a longitudinal slice (left) appear as cylindrical or ovular shaped, whereas mitochondria that are viewed from a transverse slice (right are partially wrapped around myofilaments to different degrees.

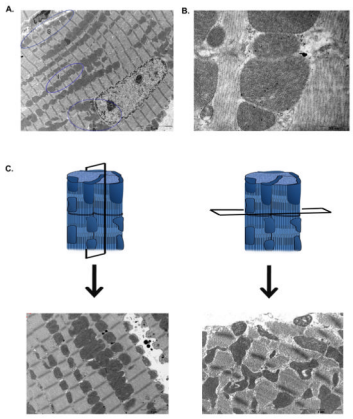

Figure 4. Different populations of mitochondria in the adult ventricular cardiomyocyte

A. Electron micrograph of adult rat ventricular myocyte. Abbreviations: S (Subsarcolemmal), I (Interfibrillar), N (Nuclear). Mitochondria directly under the sarcolemma vary in size, shape and organization. There is a mix of small and big mitochondria, with no distinct morphology and are not confined by contractile filaments. Interfibrillar mitochondria are constrained within contractile filaments, causing these mitochondria to be highly organized in a line with about one mitochondrion per sarcomere. These mitochondria tend to be big and ovular. Mitochondria around the nucleus tend to be highly disorganized and the size and shape vary greatly, ranging from small, spherical mitochondria to large globular mitochondria.

B. Increased magnification of mitochondria viewed under the electron microscope. The filled arrow shows an example of soft membranes that can be found between adjacent cardiac mitochondria. The border between these mitochondria appears blurry and might indicate the early or intermediate stages of mitochondrial fusion or fission. The open arrow shows an example of defined membranes. Each mitochondrion has its own distinct outer membranes that are not in contact with other membranes. The borders and clearly defined with a visible space in between the outer membranes.

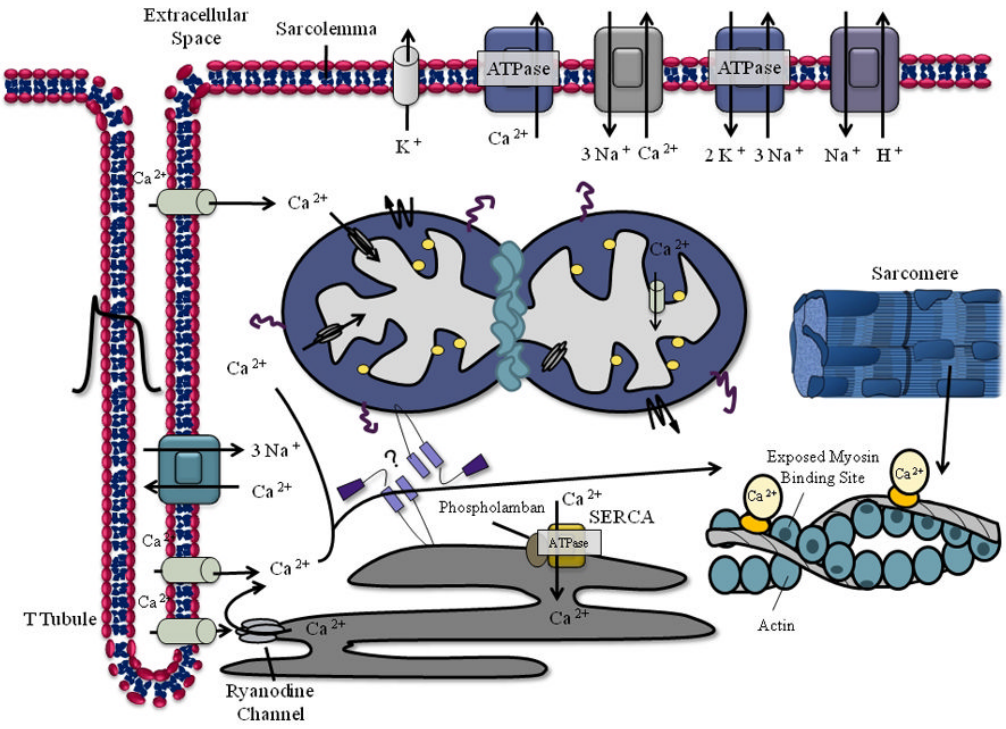

Figure 5. The role of mitochondria in excitation-contraction-metabolism coupling in the cardiac myocyte

During an action potential induced by pacemaker cells, L-type calcium channels open and allow Ca^{2+} to enter the cardiac myocyte. This Ca^{2+} influx activates ryanodine receptors on the SR to release calcium into the cytosol, causing a global increase in cytosolic Ca^{2+} concentration. This process is known as Ca^{2+}-induced Ca^{2+} release. The cytosolic Ca^{2+} binds to Troponin C, which shifts the tropomyosin complex off of the actin binding site, exposing the site for the myosin head to bind to the actin filament. This is known as excitation-contraction coupling. Mitochondria are situated close to high Ca^{2+} microdomains. Ca^{2+} enters mitochondria down its electrochemical gradient due to the highly negative mitochondrial membrane potential that is maintained by the electron chain complexes. Ca^{2+} activates ATP production via Ca^{2+}-activated dehydrogenases in the citric acid cycle and Ca^{2+}-activated ATP synthase [136,137]. ATP hydrolysis is needed for the myosin head to pull the actin filament to the center of the sarcomere. This unites metabolism to excitation-contraction coupling. For muscle relaxation to occur, intracellular Ca^{2+} is taken up by the SERCA pump or removed from the cell by the sodium-calcium exchanger or plasma membrane Ca^{2+} ATPase. This allows the tropomyosin complex to shift back over the active sites on the actin filaments.