Review

George H. Kunkel, Pankaj Chaturvedi* and Suresh C. Tyagi

Epigenetic revival of a dead cardiomyocyte through mitochondrial interventions

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Abstract: Mitochondrial dysfunction has been reported to underline heart failure, and our earlier report suggests that mitochondrial fusion and fission contributes significantly to volume overload heart failure. Although ample studies highlight mitochondrial dysfunction to be a major cause, studies are lacking to uncover the role of mitochondrial epigenetics, i.e. epigenetic modifications of mtDNA in cardiomyocyte function. Additionally, mitochondrial proteases like calpain and Lon proteases are underexplored. Cardiomyopathies are correlated to mitochondrial damage via increased reactive oxygen species production and free calcium within cardiomyocytes. These abnormalities drive increased proteolytic activity from matrix metalloproteinases and calpains, respectively. These proteases degrade the cytoskeleton of the cardiomyocyte and lead to myocyte death. mtDNA methylation is another factor that can lead to myocyte death by silencing several genes of mitochondria or upregulating the expression of mitochondrial proteases by hypomethylation. Cardiomyocyte resuscitation can occur through mitochondrial interventions by decreasing the proteolytic activity and reverting back the epigenetic changes in the mtDNA which lead to myocyte dysfunction. Epigenetic changes in the mtDNA are triggered by environmental factors like pollution and eating habits with cigarette smoking. An analysis of mitochondrial epigenetics in cigarette-smoking mothers will reveal an underlying novel mechanism leading to mitochondrial dysfunction and eventually heart failure. This review is focused on the mitochondrial dysfunction mechanisms that can be reverted back to resuscitate cardiomyocytes.

***Corresponding author: Pankaj Chaturvedi,** Department of Physiology and Biophysics, School of Medicine, Health Sciences Centre, A-1216, 500 South Preston Street, Louisville, KY 40202, USA, e-mail: p0chat03@louisville.edu
George H. Kunkel and Suresh C. Tyagi: Department of Physiology and Biophysics, School of Medicine, Health Sciences Centre, A-1216, 500 South Preston Street, Louisville, KY 40202, USA

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Introduction

Heart failure (HF) is a multifaceted disease state encompassing a variety of factors including mitochondrial degradation and proteases leading to cardiomyopathies (1). Proteases such as calpain and matrix metalloproteinases molecularly degrade the cellular components of cardiomyocytes (2, 3). This review is focused on the mitochondrial aspects of cardiac disease and the proteolytic effects involved in heart failure (HF) which comprise proteases affecting cytoskeletal/contractile proteins, mitochondrial involvement, homocysteine activity and ultimately the effect of mitochondrial epigenetics leading to HF. Current work in the field of HF and its future direction is considered from various angles. All aspects center around the mitochondrial aspect of heart disease. The novel outlook on the epigenetic basis of mitochondrial damage leading to HF is expressed within this review.

The mitochondria are the ATP generators producing the energy needed for cardiac bioenergetics. An individual's cardiac health is directly related to their mitochondrial status since damaged mitochondria alter calcium maintenance and increase reactive oxygen species (ROS) within the myocardium. This imbalance activates proteases such as calpain and matrix metalloproteinases that are detrimental to cardiomyocyte stability (4). Though proteolytic activity is noted for its maladaptive cardiac remodeling, the Lon protease maintains the stability of the mitochondria by regulating oxidative protein aggregation (5). We propose that the Lon protease reduces ROS production, therefore acting as a benefactor to cardiac health. Coupling of Lon protease activity and human mitochondrial transcription factor A (TFAM) was seen in *Drosophila* Schneider cells (6). TFAM is responsible for mitochondrial DNA maintenance and repair. Since TFAM regulates *SERCA2a* gene promoter (7) and Lon proteases affect TFAM, we can speculate that the Lon protease has an indirect

regulation over mitochondrial calcium handling. The significance of TFAM was observed by Palacin and coworkers in a single-nucleotide polymorphism (SNP) within the *TFAM* gene of male smokers H haplogroup leading to early-onset myocardial infarction (MI) (8). The Lon protease may regulate ROS production (9). This speculation is based on the fact that ROS production is created by leaks in the electron transport chain, and preventing that leak via degradation of cytochrome c oxidase (COX) by Lon protease may prevent ROS upregulation.

Epigenetic analysis of mitochondrial DNA is of increasing awareness within the scientific community. Mutation within the mitochondrial DNA may cause mitochondrial damage leading to increased calcium and ROS production, which has been shown to result in cardiomyopathies. Hypermethylation of the mitochondrial gene PGC-α leads to cardiomyopathy in male rats (10). The depletion of mitochondrial DNA from human cardiac left ventricle promotes cardiac dysfunction in human dilated cardiomyopathy (11). The mitochondrial genes which are encoded by the nucleus play important roles, and methylation of these genes affects mitochondrial function (12). There is a profound effect of diet on mitochondrial DNA and proteins. Methionine-restricted diet lowers complex I ROS generation, DNA methylation, and oxidative damage to mtDNA and proteins in rat heart (13). Cigarette smoking alters DNA methylation allowing for chromatin exposure and mutation of mitochondrial DNA. The effects of cigarette smoking on methylation of mitochondrial genes could impact cardiac health through mitochondrial degradation (14). The aim of this review is to explore the mitochondrial and proteolytic mechanisms of myocyte dysfunction and how these mechanisms can be reverted back to resuscitate myocytes.

Heart failure epigenetics

Epigenetics is the environmental control of gene expression based on genetic methylation markers influencing genes. Universally, studies are showing that alterations in DNA methylation and histone modifications are responsible for 'malignant' gene expression. DNA methylation occurs at cytosine-guanine base pairing sites known as CpG sites. 5-Methylcytosine is the methylated mammalian form of cytosine. It is constituted by the addition of a methyl group to the fifth carbon of a cytosine ring of a CpG dinucleotide. Enzymes like DNA methyltransferases (DNMTs) mediate the addition of the methyl group. Notably, this action is vital since the addition of a methyl group can unequivocally turn the genes on and off. This may result in malignant expression or essential expression of a particular gene.

In the literature several articles can be found describing the alterations of DNA methylation found with failing heart cardiomyocytes. Haas et al. examined a genome-wide cardiac DNA methylation in dilated cardiomyopathic (DCM) patients. The group noted that abnormal DNA methylation changes lead to lymphocyte antigen 75 (*LY75*) and adenosine receptor A2A (*ADORA2A*) alterations in the mRNA expression. Of the 20 genes assessed, the results are differential to the specific gene LY75 which was hypermethylated in the diseased state, while *ADORA2A* was hypomethylated on their respective CpG sites. This suggests the possibility that genes associated with apoptosis may be differentially methylated leading to increased mRNA expression and cardiomyocyte death (15). Movassagh and colleagues analyzed DNA methylation and histone-3 lysine-36 trimethylation (H3K3Me3) in cardiomyopathic hearts. Analysis revealed an epigenomic pattern among the diseased hearts, including differences in the DNA methylation of promoter CpG island regions and H3K3Me3-enriched regions, specifically the *DUX4* locus. It was noted that DNA methylation differences were observed in the promoters of upregulated genes but not downregulated genes (16).

Kao et al. performed multiple studies regarding DNA methylation of CpG islands in the promoter region of the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (*SERCA2a*). In their first study on the epigenetic aspect of tumor necrosis factor alpha (TNFα, known to reduce SERCA2a expression) the group presented increased DNMT levels due to TNFα increase. The TNFα-induced DNMT-1 activity on *SERCA2a* promoter regions resulted in hypermethylation and reduced expression of *SERCA2a* (17). Further analysis was performed using a known HF drug, hydralazine. Interestingly, Kao and coworkers found that hydralazine increased calcium handling via upregulation of SERCA2a and decreased expression of DNMT-1. Therefore, the drug decreased *SERCA2a* promoter region methylation (18). Overall, Kao et al. expressed the notion of therapeutic actions towards ameliorating HF through a novel strategy of DNA methylation inhibition.

Koczor and colleagues utilized computational analysis to detect differential methylation of gene promoters in the failing left ventricle. Analysis via expression microarrays and gene promoter microarrays revealed 393 overexpressed and 349 underexpressed genes in DCM samples. Also, of the 158 gene promoters acquiring DNA methylation changes which correlated with change in gene expression, 51 were hypermethylated and six were hypomethylated.

Four genes (*AURKB*, *BTNL9*, *CLDN5* and *TK1*) were identified as significant due to differential DNA methylation and altered gene expression. Future studies may reveal a deeper understanding to this epigenetic alteration, but this experimental analysis is important because standardization is the key to therapeutic discovery (19). Xiao et al. presented a compelling study showing the reverse of norepinephrine-induced rat cardiac hypertrophy via inhibition of DNA methylation. The hypertrophic mice had increased blood pressure and DNA methylation, dynamic left ventricular protein expression and ventricular hypertrophy. The addition of 5-aza-2′-deoxycitidine (Aza: a DNA methylation inhibitor) to a portion of the affected rats alleviated hypertrophy and HF and corrected protein expression and DNA methylation patterns (20). Inhibiting DNA methylation can have astonishing results.

Other major players for histone modifications are histone acetyl transferases, which increase transcription, and histone deacetylases (HDACs), which decrease transcription (21). Histones can also be modified by acetylation and methylation of lysine. Methylation of arginine and phosphorylation of serine also act to modify core histones. Awad and associates expressed that phosphorylation of cardiac CaMKII enhances histone modification by phosphorylation of histone monomers. Phosphorylation of histones at the serine residue on histone 3 (H3) remodels chromatin during cardiac hypertrophy. As discovered in primary neonatal rat cardiomyocytes, nuclear CaMKII binds chromatin and regulates H3 phosphorylation at serine residues. CaMKIIδB, an isoform of camKII found in the nucleus of cardiomyocytes, is a key factor in calcium-mediated transcriptional gene regulation. Increased serine-10 phosphorylation coincides with increased CaMKIIδ activity and cellular hypertrophy (22).

During diastole, Ca^{2+} reabsorption into the SR takes place with the help of sodium-calcium exchanger (NCX1). Lu and colleagues demonstrated the effects of CaMKIIδ on NCX1, using a plasmid (T287D) encoding for active CaMKIIδB. The study states that MEF2 (myocyte enhancer factor-2)/HDAC function is regulated by the calcium-bound calmodulin. Blocking calmodulin in trans-aortic constricted (TAC) animals prevented the translocation of HDAC (class IIa histone) and CaMKIIδB. The study resulted in decreased left ventricular shortening by 40% and SERCA2a levels while increasing NCX1 protein expression (23). Angrisano et al. analyzed an epigenetic switch following a pressure-induced HF, in SERCA2a and beta-myosin heavy chain (*β-MHC*) gene promoters (*Atp2a2* and *Myh7*, respectively). The histone modifications observed post TAC were as follows: dimethylation of H3 at lysine (K) 4 (H3K4), 9 (H3K9) and 36 (H3K36) and trimethylation at lysine 27 (H3K27). Due to these epigenetic changes, the mRNA expression levels of SERCA2a dropped significantly, while β-MHC levels increased (24). The study demonstrated that epigenetic changes occurring in minor cardiomyopathies may cause increased progression towards HF.

Epigenetics plays a vital role in the progression of disease, and unanimously, studies have shown that genetic modifications can cause detrimental effects, factoring into the rate of cardiomyopathy development. Epigenetic gene silencing is the process of regulating gene expression through DNA methylation and histone tail modification. Ultimately, manipulative measures are key to advances in experimental technique and therapeutics.

Epigenetics of the mitochondrial genome

The mitochondrion is an integral organelle found ubiquitously within cells and it establishes the metabolic processes that enable and sustain life. It is the site for oxidative phosphorylation via the electron transport chain and ATP production through complexes I-V which are located in the intermembrane space. Mitochondria have small circular DNA known as mtDNA that contains 16 569 base pairs (16 kb) and encompasses 37 genes (25). mtDNA has fewer CpG dinucleotides (435) than the nuclear DNA and is free of introns, histones and retrotransposons (Line-1 elements). It actively contains two rRNAs and 22 tRNAs that assist in translation. Epigenetic modifications through mtDNA methylation is a naturally occurring process adding a methyl group to 5-methylcytosine or 5-hydroxymethylcytosine. mtDNA methylation plays a vital role in mtDNA stability and regulation. A multitude of studies report diseases associated with mtDNA methylation (26). The functional analysis of mitochondrial stability when altering mitochondrial DNA methylation is explained by Sanchez-Roman et al. They placed Wistar rats on a methionine restriction diet resulting in longevity of rat life span. According to the authors, this longevity is tied to decreased cardiac mitochondrial ROS production and lowered oxidative damage to the mitochondrial DNA (13). Based on additional studies, we can conclude that ROS affects mitochondrial transcription factor A, which is a required mitochondrial gene expression (27). Yue and coworkers described the protective role of lycopene (an antioxidant) in ischemia/reperfusion (I/R) injury caused by oxidative damage to mtDNA. The authors revealed that I/R injury increased 8-hydroxyguanine and decreased

mtDNA content and transcription levels resulting in cardiomyocyte mitochondrial dysfunction. Lycopene-ameliorated ROS production and Tfam protein decline, resulting in a cardioprotective role. Tfam/mtTFA/TFAM is widely studied and seems to be an important aspect of mtDNA activation. Therefore, transcription factor A may have a vital role in mtDNA transcription (27).

Interestingly, Rebelo et al. found that alterations in the *in vivo* mtDNA methylation were increased when mtDNA replication was stimulated. Also, upregulation in TFAM expression resulted in decreased methylation of CpG sites. Decreased methylation was mainly found in the mtDNA promoter region where TFAM binds with high affinity. siRNA knockdown of *TFAM* revealed a 35% depletion of mtDNA levels (28). Suarez et al. have noted a decrease in TFAM found in diabetic cardiomyopathy and the protective role of TFAM overexpression under hyperglycemic conditions. High glucose directly stimulated TFAM overexpression while prolonging calcium transients by 70%, which decreased SR Ca^{2+}ATPase 2a and cytochrome-oxidase subunit 1 (29). This inhibition resulted in increased calcium uptake and potential cytochrome c expression. Therefore, one of the mitochondrial regulatory roles is to decrease hyperglycemic ROS production and increase free calcium. Free calcium increase would induce protease (calpain) activity. We are curious to see if in future studies we can link TFAM binding to *COX*. Mechanistically, ROS may alter TFAM binding to *COX*, manipulating oxidative phosphorylation and resulting in the release of cytochrome c, inducing the apoptotic cascade. Current literature is lacking this study.

Mitochondrial epigenetics involved in cardiac diseases

Epigenetic analysis via mitochondrial DNA methylation is scarce in the literature and unseen in cardiac-specific studies. DNA methylation is observed as a potential factor and marker of disease. Here we unravel our novel hypothesis to the study of cardiovascular diseases (CVDs) due to alterations in methylation patterns and its effects on mitochondrial damage (Figure 1). Baccarelli et al. have reported that patients with CVD have significantly higher methylation of the mtDNA than healthy controls (30). The mtDNA methylation was measured in platelets, and the authors speculate that it could serve as a non-invasive marker for the etiology of CVD. Methylation of the mitochondrial gene *PGC-1α* contributes to cardiomyopathy in male rats (10). Potentially speaking, since the mitochondria are inherited maternally, changes in the mitochondrial DNA methylation markers during the mother's lifetime (pre-conception) could make a direct influence on the child's health and susceptibility to disease. Specifically in cardiac diseases, mitochondria play a vital role in cardiac bioenergetics, maintenance of calcium and ROS production. Obesity, insulin resistance and diabetes, which pose cardiovascular risk, are marked by abnormalities in epigenetic regulation. HDACs, for example, affect mitochondrial energy metabolism and, thus, function (31). Inhibition of class I and II HDACs improved energy metabolism in high-fat diet fed mice (31). Sirtuins, which behave as histone deacetylases, are induced in the heart (SIRT3) by exercise and protect against adverse cardiac

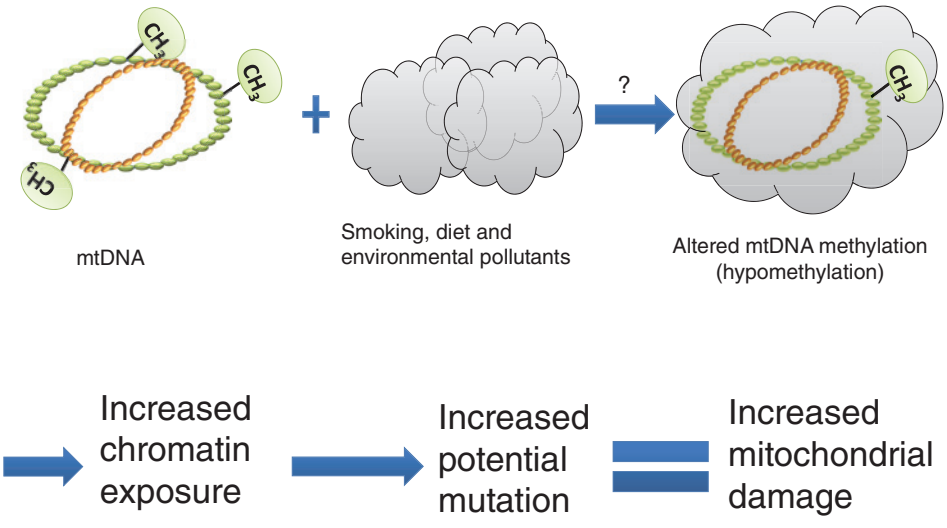

Figure 1: The effect of environmental factors like smoking.
Environmental factors like smoking, diet and pollutants alter the mtDNA methylation pattern leading to increased chromatin exposure, increase potential mutation and eventually mitochondrial damage.

remodeling following pressure overload (32). Hypoxia-inducible factors (HIFs), which are regulated by mitochondrial complex 3, are methylated at the CpG islands in diabetes and obesity (33, 34). For the mtDNA expression and maintenance, there is high crosstalk between nucleus and the mitochondria. The precise molecular mechanisms underlying these processes and the ones that affect cardiac function are so far to be deciphered.

Hyperhomocysteinemia affecting mitochondrial epigenetics

High levels of homocysteine or hyperhomocysteinemia (HHcy) has been established as an independent factor for cardiovascular risk. In our laboratory we have shown that high level of homocysteine affects DNA methylation (35). Our lab has recently reported a novel link between mitochondrial epigenetics, HHcy and bone remodeling. We showed that HHcy alters bone mitochondrial epigenetic remodeling through N-Hcy-collagen 1. HHcy mitochondrial epigenetic remodeling affects the MMP/TIMP (matrix metalloproteinase/tissue inhibitors of metalloproteinase) and elastin/collagen ratios causing bone impairment (36). Furthermore, another study within our laboratory established an epigenetic role of HHcy-induced skeletal muscle weakness through mitochondrial dysfunction. An increase was observed in DNMT3a and DNMT3b proteins and global DNA methylation in the HHcy treated C2C12 cell line. This evidence directly supports the epigenetic role that HHcy plays. Within this study a functional analysis was also performed aiding in the micro-RNA (mir) analysis of the enhancement of mir-494 and decrease in transcriptional regulator NRF-1 causing mitochondrial transcription factor A (mtTFA) protein quantity decline (37).

Our laboratory has earlier performed an epigenetic mechanistic analysis of cardiomyocytes during HHcy. Interestingly, HHcy causes increased expression of N-methyl-d-aspartate receptor 1 (NMDAR1), DNMT1, MMP9 and H3K9 acetylation along with decreased expression of HDAC1, mir-133a and mir-499. The results showed that HHcy causes chromatin remodeling, which is correlated with cardiac remodeling (35). Therefore, we can speculate that HHcy directly affects cardiac health via epigenetic modifications. We have performed another work in mice and human aortic samples and explored the epigenetic mechanistic role of HHcy during an aortic aneurism. Through this study we observed a possible role of histone remodeling and DNA methylation in the regulation of genes involved in matrix remodeling (*MMPs*, *TIMPs*, collagen I and collagen IV) and homocysteine metabolism (methylenetetrahydrofolate reductase and S-adenosyl-L-homocysteine hydrolase) (38). Further, we analyzed DNA methylation in HHcy aortic remodeling with the use of an epigenetic inhibitor (5′-azacytidine(Aza); a DNA methylation inhibitor). This experimental analysis showed that induction of Aza alleviates hypertension by reducing DNA methylation, which regulates extracellular matrix (ECM) remodeling and differential gene regulation (39).

Mitochondria and heart failure

As stated above, the mitochondria is a vital component of metabolic function. Impeding its action through damage from various sources leads to mitochondrial and cardiomyocyte decline. The basic regulatory function of the mitochondria relies on transporters such as Na^+/K^+-ATPase, Na^+/Ca^{2+} exchanger, and MCU (mitochondrial calcium uniport). Mitochondrial consumption of calcium is a factor in the heart muscle relaxation, and when damaged, the mitochondria inefficiently take up calcium, causing cytosolic calcium increase and increased protease activity (40). The Na^+/K^+-ATPase is an active transport mechanism that pumps three sodium ions out of the mitochondria while pumping two potassium ions in. This action maintains the mitochondrial membrane potential and ratio of potassium/calcium in the mitochondria (41).

Li and colleagues analyzed the function of sodium/potassium pump (NKA) by inhibiting it with cardiac glycosides, leading to proarrhythmic behavior due to impaired mitochondrial bioenergetics. Cardiac glycosides block sodium extrusion causing calcium increase via reversing the Na^+/Ca^{2+} exchanger (NCX). It was revealed that inhibiting the NKA caused decreased mitochondrial calcium uptake and increased oxidative phosphorylation activity resulting in increased ROS production (40). In the preservation of cardiomyocytes a channel known as Trpm2 mitigates ROS production, acting to stabilize the mitochondria. Hoffman et al. experimented with Trpm2 knockout (KO) mice and revealed that after ischemic-reperfusion the Trpm2 Ca^{2+} influx channel reduces mitochondrial ROS production. Also, the Trpm2 channel protects against doxorubicin cardiomyopathy, since there is a decrease in the survival rate of *Trpm2* KO mice (42). Yancey et al. analyzed the effects of a mitochondrial-targeted antioxidant known as mitoubiqnone (MitoQ) on Sprague-Dawley rat cardiomyocytes. Eight weeks post aortocaval fistula (ACF), mitochondrial ROS production increased and mitochondrial membrane potential decreased. MitoQ substantially improved the effects of

ACF in these rats but did not attenuate left ventricular dilation or fractional shortening (43).

Bondarenko et al. revealed the role of the mitochondrial calcium uniporter in mice lacking MCU expression. MCU formerly known as protein CCDC109A is an essential transporter focused on calcium uptake into the mitochondria (44). $MCU^{-/-}$ mice had significant alterations, such as the functional impairment of the ability to perform strenuous exercise and minor decreases in strength. All apoptotic markers and metabolic changes were the same among the mutant and wild type (WT) mice. $MCU^{-/-}$ mice had a 25% decrease in matrix calcium compared to the WT. Therefore, the MCU uniporter has an essential role in mitochondrial calcium uptake. Bondarenko et al. presented a link between the permeability transition pore (PTP) and the MCU transporter. MCU is the mediator of PTP opening, and when open, PTP depolarizes the membrane and inhibits ATP production. Michels et al. has noted the deficiency of two Ca^{2+} selective channels (mCa1 and mCa2) in the failing heart. These channels assist in calcium uptake into the mitochondria (45).

Bondarenko et al. revealed the coexistence of MCU-dependent and MCU-independent Ca^{2+} channels in the inner mitochondrial membrane. Mitochondrial calcium currents were assessed in MCU knockdown HeLa cells. Interestingly, the extralarge conductance of mitochondrial (Ca^{2+}) current (xl-MCC) was upregulated 2.3-fold, and the bursting currents remained unchanged. Therefore, these currents are not MCU dependent. The literature is lacking a follow-up article on the actions of the xl-MCC in HF patients. We suspect that the channel sustaining this current has met its maximum calcium uptake in the HF patient, leading to a decrease in the patient's status (44). Essentially, mitochondrial calcium transport channels sustain the stability of the mitochondria and cardiomyocytes. The cardiac degradative process within a HF patient is dependent upon increased calcium and ROS production by the unstable mitochondria. The distortion of channels and currents as mentioned above affects protease activity by ROS formation and decreased calcium handling. ROS products such as superoxide (O_2) and hydrogen peroxide (H_2O_2) are produced within the mitochondria under two conditions: when mitochondria are not producing enough ATP resulting in a high proton motive force and reduced coenzyme Q and when there is a high $NADH/NAD^+$ ratio in the mitochondrial matrix (46). The literature has noted that the deficiencies producing ROS lie within the supercomplexes of the electron transport chain. The leakage of electrons in the electron transport chain causes the superoxide (ROS) production. Maranzana et al. focused their experimentation on complex I by disrupting the association of complexes I-III. The investigation resulted in a substantial increase in ROS from complex I (47). Interestingly, the mental health drug haloperidol, a psychopharmacology drug, inhibits mitochondrial complex I in the brain tissue. The effects of this drug on cardiomyocytes are limited, but further investigation would be compelling (48).

Huang and coworkers presented a novel haloperidol derivative called N-n butyl haloperidol iodide (F2) that was shown to inhibit H_2O_2-induced sodium/calcium exchanger activation in rat cardiomyocytes. F2 protects against cardiac I/R injury by directly inhibiting the MEK (extracellular signal regulated kinase-kinase)/ERK (extracellular signal regulated kinase) pathway and Na^+/H^+ exchanger (NHE) activation (49). The cardiomyocyte NCX exchanger was shown by Eigel and colleagues to induce cardiomyocyte apoptosis post hypoxia in guinea-pig ventricular cardiomyocytes (50). Hinata et al. examined the mechanism by which H_2O_2 activates NCX in guinea-pig ventricular cardiomyocytes using an NHE inhibitor. They found an increase in the NCX current via H_2O_2 activation by two pathways, one of which activates NHE by a PI3K-dependent mechanism and the other by the activation of a tyrosine kinase (Src family) (51). NCLX, the sodium/calcium exchanger respective to the mitochondria, maintains the same antiport function within the mitochondria. The mitochondrial H^+/Ca^{2+} exchanger (Letm1) and NCLX are both factors in the mitochondrial membrane potential and act as apoptotic regulators. Although it is believed that Letm1 releases Ca^{2+} from the mitochondria when NCLX activity is low, the topic is controversial (52). De Marchi et al. clashes with the above statement. This group presents that NCLX is the mediator of calcium extrusion from the mitochondria, not Letm1, and support the idea that NCLX has a protective function by standardizing NAD(P)H production and regulating ROS. Increased $mtCa^{2+}$ showed increased auto-fluorescence of NAD(P)H, which was drastically diminished by NCLX overexpression (53).

Cardiac arrhythmias are a major factor represented in cardiomyopathies such as HF. Dysregulation of calcium is a direct cause of myocardial arrhythmogenic activity. Takeuchi et al. present NCLX as a regulator of automaticity in HL-1 cardiomyocytes. They knocked down NCLX with siRNA, and this knockdown resulted in prolonged action potential generation due to decreased SR calcium leak (54). Therefore, overall observation of calcium extrusion via MCU, NCLX and potentially Letm1 demonstrates that it maintains the normal cardiac physiological state. When there is dysregulation of calcium such as in HF, the transport systems decline in function. As a result, apoptotic signals are triggered resulting in degradation and decline.

Mitophagy and heart failure

Hemodynamic stress induces mitochondria to act as 'angels of death' promoting apoptosis by producing excess ROS with pro-death proteins and inhibiting ATP synthesis. Autophagy is the catabolic cellular digestion eradicating damaged cellular components using the lysosome. Mitophagy is mitochondrial-driven autophagy, which is a cardioprotective response acting as a beneficial adaptation to early induced stress. In the healthy myocardium a balance between mitophagy and mitochondrial biogenesis is present (55). The myocardium is composed of a variety of accumulated 'old' post-mitotic cells including defective mitochondria and aggregated irregular proteins known as lipofuscin. The catabolic nature of autophagocytosis and cellular digestion by proteases seems to be lacking in the myocardium. Lipofuscin proteins act to restrict the release of ROS from deficient mitochondria due to a lysosomal compartment. With the progression of age, lipofuscin-filled cardiomyocytes become overloaded with mitochondria causing increased oxidative stress and HF development. Dysregulation of deficient mitochondria is present in various cardiomyopathies, and some of them are caused by genetic deficiencies in mitochondrial DNA. Hemochromatosis, a genetic iron overload disease causing degradation of iron-saturated ferritin in lysosomes, leads to lysosome sensitivity by oxidative stress causing increased redox-active iron. Lysosomal activity is pertinent in the HF model, and up-regulation of lysosomal activity may degrade damaged mitochondria and lead to prolonged cardiomyopathy and congestive heart failure (CHF) development. Potentially, this is a target for future therapeutics and may increase the life span of CHF patients (56).

Cardiac health is dependent on increased mitophagy during stress. There seems to be a deficiency in research focused on the beneficial aspects of exercise-induced mitophagy. The cardioprotective nature of mitophagy would lead to the assumption that increased mitophagy would occur within the myocardium during exercise-induced stress. Regular physical training could lead to 'mitophagy exercise' within the myocardium and would increase mitophagy activity in daily life, preventing cardiomyopathies by degrading deficient mitochondria and limiting ROS production, especially in an older population. Selective mitophagy occurs within the diabetic heart, specifically in insulin-resistant and insulin-deficient diabetes. Both conditions are accompanied by hyperglycemia. As noted by Kobayashi and colleagues, high levels of glucose and high fat diets directly inhibit cardiomyocyte autophagy. Cardiomyocyte autophagy tends to express inverse roles in type 1 and 2 diabetes. Diminished autophagy is proposed to be cardioprotective in type-1 diabetic model, whereas decreased autophagy in type 2 diabetes contributes to cardiac injury (57). In type 1 diabetic model, attenuation of cardiac injury and decreased ROS production was observed in beclin-1 and Atg 16 deficient mice. Beclin-1 is a protein that is essential in the autophagy cascade. Xu et al. crossed beclin knockouts with OVE26 diabetic mice, and in this diabetic model beclin-1 deficiency attenuated cardiac injury (58). Therefore, mitochondrial clearance via mitophagy is beclin-1 dependent (59).

Increasing the rate of mitochondrial consumption by mitophagy causes an increase in efficient mitochondria to be present in the sarcoplasm, allowing for increased calcium uptake by mitochondria and decreasing the detrimental effects of high calcium. Therefore, we can assume that mitophagy is tied to the protease calpain and other calcium-dependent proteases. Efficient mitophaghy within the sarcoplasm would degrade malignant mitochondria allowing for increased 'good' mitochondria leading to decreased calcium levels and calpain activity. Calpain activity may be tied to mitophagy, which is not a readily studied area. Mitochondrial impairment results in a loss of ATP production; thus, the vast bioenergetic needs of the heart may not be met. Mitochondrial fission is a beneficial factor segregating (non-repairable) impaired mitochondria to be lysed. Do impaired mitochondria induce a secondary messenger, signaling impaired properties to healthy mitochondria, thereby increasing the number of impaired mitochondria? If so, this could be a factor in the degradative effects seen in HF. Another conjecture is the possibility that increased mitophagy could alleviate arrythmogenic responses in the myocardium by clearing the mitochondria which are not impaired.

Studies by Xue and coworkers revealed that calpain activity is induced during atrial fibrillation in the dog heart model, resulting in atrial remodeling. Though the focus of their study was not mitochondrial dynamics, it is stated that the cardiomyocyte mitochondria were misshaped and differentially sized (60). We assume that this change in shape may lead to mitochondrial impairment. Also, use of calpain inhibitors led to decreased calpain activity and reversed structural remodeling. A hunch may be proposed that mitochondrial degradative activity is linked to calpain and therefore HF.

Hyperhomocysteinemia and mitophagy

In our laboratory we have found that within the HHcy mouse model the cardiac-specific knockout of NMDAR1 resulted in decreased levels of mitochondrial MMP-9.

Mitochondrial MMP-9 within the HHcy mouse model has been found to induce mitophagy. Deletion of the NMDAR1 receptor prevents Hcy from activating MMP-9 and translocation of connexin-43. Translocation of the gap junction protein connexin-43 leads to a mitophagic response (61). In addition, we found that there is a decrease in the mitochondrial fusion/fission ratio, i.e. there is more fission than fusion, and it promotes mitophagy (see Figure 2) (62, 63). There is an increase in calcium uptake, increased ROS production and increased cytochrome c leakage which is detrimental to the cell. The use of mitochondrial division inhibitor was able to ameliorate the damage done by pressure overload-induced HF (63, 64). Mitophagy is related to increased calcium uptake and intracellular calcium concentration. We have reviewed that homocysteine induces intracellular calcium by (1) agonizing the NMDAR1 receptor which draws more calcium; (2) decreasing expression of peroxisome proliferator activator receptor (PPAR) which impairs NCX1 and hence calcium handling; and (3) impairing the SR Ca^{2+} ATPase (SERCA2a) (65).

Cardiomyocyte and mitochondrial proteases

Intra-mitochondrial Lon protease

Lon protease is an ATP-dependent protease and a key enzyme involved in the stabilization of the eukaryotic mitochondrial matrix. The mitochondrial Lon protease acts to eliminate oxidatively modified proteins and is seen to be upregulated in various cancers but is relatively unexplored in CVDs. It is noted for its actions as a chaperone, due to its ability to repair misfolded proteins within the mitochondria. Pinti et al. confirmed the Lon protease as a stress regulatory protein. Upregulation of D-deoxyribose and stavudine (d4T) activates the release of Lon protease in the presence of ROS. Mechanistic analysis concluded that variation in Lon transcriptional promoter regions allows for increased or decreased expression, and d4T upregulates Lon promoter activity in all cell lines (66). Kao and colleagues found that the Lon protease is a regulator of apoptosis through its association with heat shock proteins (HSP); HSP60-mtHSP70 complex (67). This interaction was confirmed by co-immunoprecipitation and immunofluorescence co-localization assay. Their findings included the identification of 76 Lon-associated proteins participating in the mitochondrial chaperone system. Also, since HSP60 binds to p53 (known as a significant factor in apoptosis), it inhibits apoptosis. This action is Lon dependent because the Lon protease acts to degrade proteins, maintaining the stability of the HSP60-mtHSP70 complex (67). Decreased Lon protease activity leads to increased apoptotic signaling and mitochondrial instability within the cardiomyocyte. This is detrimental, and embryonic death occurs in animal models of Lon protease knockouts as observed by Quiros et al. (68).

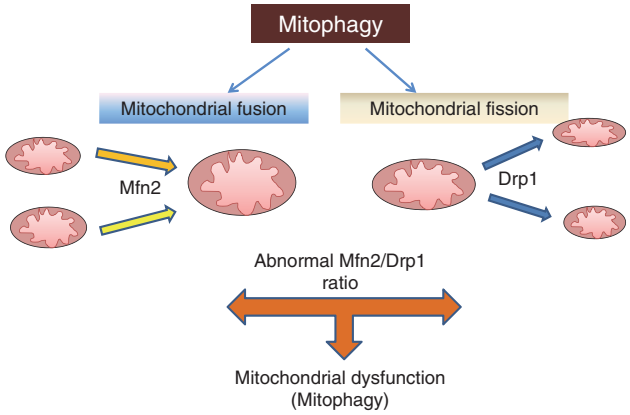

Figure 2: The role of mitochondrial fusion and fission in mitophagy. Mitochondrial fusion is promoted by Mfn2 gene, while Drp1 promotes mitochondrial fission. If the ratio of Mfn2 and Drp1 is altered, there is abnormal increase in the fission which leads to mitophagy. We have reported that mitochondrial division inhibitor has beneficial effects in pressure overload induced heart failure (63) and abnormal Mfn2/Drp1 ratio can lead to endothelial dysfunction (62).

Lon protease and TFAM

Interestingly, a link between the Lon protease and mtDNA has been established in the literature. Matsushima et al. have demonstrated a role of the Lon protease in mtDNA metabolism in *Drosophila* Schneider cells. This group showed that RNAi knockdown of Lon increases TFAM (human mitochondrial transcription factor A) expression and mtDNA copy number. Upregulation of Lon causes the reverse reaction. Lu et al. has also described a correlation of TFAM and the Lon protease via phosphorylation. More specifically, phosphorylation of TFAM within the high-mobility group box 1 inactivates transcription. Lu et al. also showed that unbound TFAM is degraded by the Lon protease. Interestingly, the anticancer drug bortezomib decreases Lon protease activity (69). Potentially, use of this drug could allow for increased intracellular ROS production due to Lon protease inhibition, potentially resulting in mitochondrial and cardiomyocyte decline. Additionally, Watanabe and coworkers found that TFAM and TFB2M regulate *SERCA2a* gene transcription. This

group established that mtDNA-specific transcription factors have the potential to regulate nuclear DNA. This was shown through TFAM and TFB2M binding to the *SERCA2a* gene promoter of rat neonatal cardiomyocytes. Watanabe et al. also stated that TFAM and TFB2M levels correlated with *SERCA2a* levels in the rat MI model (7). Knowing this correlation between TFAM and *SERCA2a* transcription, we can speculate that the Lon protease which regulates TFAM will indirectly regulate *SERCA2a*. Knowing that activation of *SERCA2a* would cause increased calcium uptake from the sarcoplasm, we can propose that the mitochondrial Lon protease is a major player in maintaining cardiac health (see Figure 3). Potentially, if maternal cigarette smoking alters mitochondrial *TFAM* methylation, this may correlate to the transcriptional activation of TFAM causing alterations in Lon protease and SERCA2a activity.

In a clinical study performed by Jerkins et al., it was revealed that bortezomib induces congestive HF (70). Could the underlying molecular mechanism be tied to decreased Lon protease activity and dysregulation of TFAM/mtDNA ratio and SERCA2a. Lu et al. showed that Lon depletion increased TFAM levels and mtDNA content.

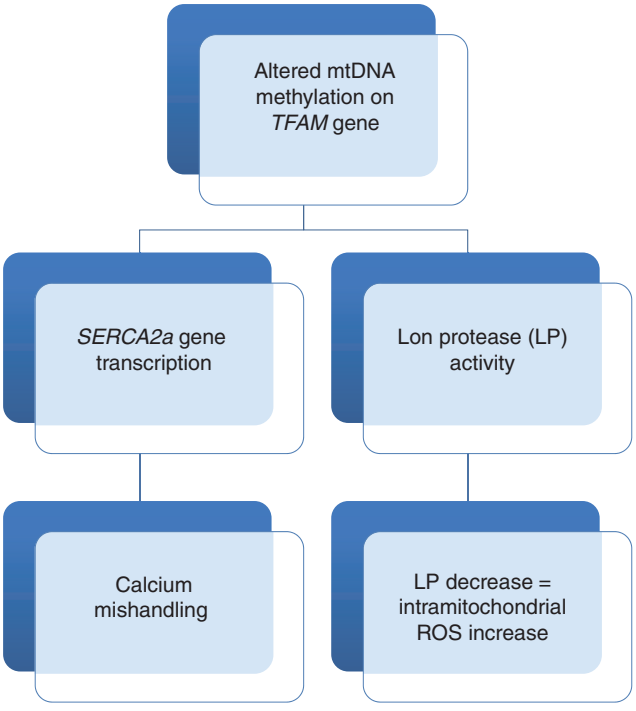

Figure 3: The role of altered mtDNA methylation.
mtDNA methylation can shut down certain beneficial genes like Lon protease which can lead to intramitochondrial ROS increase. On the other hand, hypomethylation of *TFAM* can lead to its increased expression and binding to *SERCA2a* gene, which subsequently leads to calcium mishandling. All these events together lead to mitochondrial dysfunction.

Therefore, this group presents a critical regulation activity performed by the Lon protease (69). Palacin et al. has performed a study analyzing the incidence of early onset of MI and its relation to SNPs within the *TFAM* gene. This study analyzed the H haplogroup and found four polymorphisms. Overall, this study concluded that H-haplogroup male tobacco smokers had a higher incidence of early-onset MI (8). This haplogroup type may be more susceptible to tobacco smoke intake and its effects on mitochondrial DNA. Smoking may play a vital role in *TFAM* gene regulation among all populations.

Lon protease and COX

Cytochrome c activation is associated with apoptotic signal pathways and is released from the mitochondria as leakage. Under hypoxic conditions the Lon protease is characterized as the protease that degrades COX 4 in the electron transport chain (71). Cytochrome c release may be indirectly linked to cytochrome oxidase 4 degradation. In relation to cardiac disease, cardiac hypoxia may be a factor in HF. A link is established between cardiac ischemia and cytochrome c release as performed by Borutaite et al. (9). The activation of the Lon protease is driven by increased intramitochondrial ROS production. Yang et al. showed that CSE (cigarette smoke extract) treatment decreased COX subunit II mRNA and protein levels in vascular endothelial cells (72). In an analysis by Fukuda and colleagues it was found that under hypoxic conditions HIF-1 regulates COX IV subunit expression. This is performed via transcriptional activation of the Lon gene, which degrades COX4-1 via protease release (73). Pawlak et al. also noted the decreased COX IV expression in a steroidogenesis study involving cigarette smoking (74). Kawashima et al. observed the effects of maternal cigarette smoking on placental gene expression. The study showed that the placental gene expression of placental growth factor (PGF) and HIF1A was significantly higher and the BAX/BCL2 mRNA ratio was significantly higher than in the non-smoker group (75). As we know BCL2 is a major factor in cytochrome c release.

Is it possible that decreased methylation markers seen on the COX subunit due to smoking allow for decreased Lon activity driving cardiomyocyte apoptosis via upregulated intracellular ROS? If a compromised mitophagic response and increased mitochondrial decline occurs, this may drive the significant increase in protease activity seen in HF patients, due to the vast ROS production and calcium mishandling. Although performed in WI-38 VA-13 human lung fibroblasts, Bota and coworkers showed

that Lon downregulation results in significant caspase 3 activation and apoptotic death due to mitochondrial decline (76). In the earlier studies of Lon protease function Bota et al. described the degradative abilities of Lon protease protein. Focusing on the mitochondrial oxidized aconitase, decreased presence of Lon protease results in increased oxidative aconitase, consequently resulting in protein aggregation (77). Therefore, Lon protease activity to degrade ROS production is essential because increased ROS drives increased oxidation of proteins leading to aggregation. ROS mainly caused by electron leak from the mitochondrial complexes of the electron transport chain can further oxidize proteins resulting in aggregation. As we know that Lon protease reduces oxidative stress and clears oxidized proteins, Ngo et al. proposed that Lon protease activation is biphasic and therefore upregulated in transient stress and downregulated in chronic stress (78). Although this may be true, it is likely that mtDNA transcription factors and signaling are interrupted causing the Lon protease decreased expression. Hart et al. showed that in high-capacity runner rats, antioxidant resveratrol increased aerobic performance and upper limb strength. We speculate that molecularly this antioxidant assisted in the degradation of ROS production allowing for increased healthy mitochondria. The study also showed that resveratrol activated AMP-activated protein kinase, SIRT-1 and TFAM. The authors propose that this increase in aerobic performance is due to activation of the AMPK-SIRT-1-PGC-1alpha pathway (79). The Lon protease plays a significant mitochondrial role, and we speculate that cigarette smoking will compromise the Lon protease at the mtDNA level.

Calpains and matrix metalloproteinases

Calpains are a family of intracellular cytosolic cysteine proteases activated by calcium and localized within the cytosol and mitochondria. Calpain 1 in particular has been implemented as a major degradative protease and factor in cardiomyopathies. Secondarily, MMPs are zinc-dependent endopeptidases, capable of degrading the ECM causing cardiac remodeling. This family of proteases has two well-studied members within the cardiac system: MMP2 and MMP9, which play a role in HF. MMPs are regulated by TIMPs. This family of proteases is noted for their involvement in cardiac remodeling and atherosclerotic plaque formation. Both proteolytic families play major roles in the degradative process of HF.

Interestingly, Vindis et al. analyzed an oxidized low-density lipoprotein apoptotic pathway driven by calpain activation. Upregulation of calpain caused Bid cleavage allowing for cytochrome c release through the mitochondrial transition pore. The release of cytochrome c activates caspase 3 (80). Elaboration on the previous discussion of Lon protease about indirect release of cytochrome c, this route may be driven by calpain. It is known that Bax proapoptotic protein is translocated from the cytosol to the mitochondria, which stimulates cytochrome c release. Gao et al. found that mitochondrial calpain cleaves the N-terminus of Bax creating the Bax/p18 fragment, which is stated to be a potent proapoptotic fragment that stimulates cytochrome c release independent of Bcl-2 (81). Therefore, mitochondrial calpain is a mediator of cytochrome c release.

Arrington and colleagues found that Calpain 10, the intra-mitochondrial protease, resides in the mitochondrial compartments. This group observed the cleavage of electron transport chain complex I subunits ND6 and NDUFV2 by calpain 10. Arrington et al. proposed that increased cytosolic calcium causes increased mitochondrial transport via the ruthenium red sensitive calcium channel uniporter. The increased mitochondrial matrix calcium activates calpain 10 causing cleavage of complex I (82). Although Arrington et al. have proposed this as a beneficial factor, Maranzana et al. noted that disrupting the association of complexes I-III resulted in a substantial increase in ROS from complex I (47). There may be a causative relationship between calpain 10 cleavage of the electron transport chain and decline of the mitochondria. We are considering the possibility that in pathological situations, Calpain 10 may cleave additional sections of the electron transport chain. This speculation is based on ROS production, when breaks in the complexes I-III occur leading to mtDNA modifications, as previously discussed. Our lab has previously established a link between HHcy and mitochondrial damage via activation of MMP-9. We showed that Hcy activates calpain-1 and induces its translocation from the cytosol to within the mitochondria, leading to MMP-9 activation through intra-mitochondrial oxidative bursts (83). It is possible that the mitochondrial calpain discussed by Gao et al. may be calpain 1 and is found within the mitochondria due to Hcy translocation.

Smoking and cardiomyopathies

A grand old American past time is associated with today's number 1 killer. Cigarettes are major factors in expediting a multitude of diseases and is a cofactor in the spike of CVD seen in recent years. According to the center for disease control, 600 000 people annually die from

cardiovascular-related diseases. The epigenetic generational effect, this addiction has had on our society, may be a detrimental factor in today's increased rate of CVD. We are proposing a mechanistic analysis to assess the role of mitochondrial epigenetics in cardiac health. Being that we receive our mitochondria from our mothers, a direct link between our mitochondrial health and the behavioral aspects of our mothers' preconception can be analyzed. It is quite interesting to think that if your mother smoked years before you were born, it may have affected your health today. The specifics to your mother's smoking habits before you were born and how it may relate to your cardiovascular health will be further discussed. Therefore, to assist the future of the human race, we need to establish this link because providing knowledge to the general population especially young adults may decrease the projectional increased rate of CVD caused by smoking. The young adult population is critical since according to the CDC 9, out of 10 smokers had their first cigarette before the age of 18 years.

The cigarette-smoking pandemic associated with lung pathology has also incorporated a multitude of effects on the myocardium. Hamed et al. performed a clinical study among patients with acute coronary syndrome showing that all of them had increased levels of MMP9 and 45% of them were smokers (84). Akbarzadeh and colleagues studied the effects of acute smoking on cardiac electrophysiology. Noting the variation in QT interval and QT dispersion (QTD), QTD is associated with cardiac arrythmias. Post smoking a single cigarette, the group observed that participants (smokers and non-smokers) had increased heart rate and mean QTD indicating that chronic exposure would result in arrythmogenic activity (85). A growing area of study includes the detrimental effects of smoking on the cardiomyocytes. An uninvestigated area of cardiac research involving cigarette smoke inhalation is the mitochondrial basis to this disease. Mitochondria are the ATP generators of the cell, and energy by ATP is required for normal function in cardiomyocytes. Research-based evidence from the Gvodzak group broke light on this subject in the 1980s, but since then, minimal research is focused on this area of CVD.

Gvodzak and coworkers analyzed passive cigarette smoking and its effects on the oxidative metabolism of the mitochondria within cardiomyocytes. Rabbit groups were subjected to varieties of exposure from a single 30-min smoke to twice a day over 2 weeks, and the last group smoked twice a day for 8 weeks. Findings in this study included a respiratory decline, respective to increased exposure as well as a decreased rate of cardiomyocyte mitochondrial oxidative phosphorylation. The study showed that the morphological changes within the myocardium are due to damaging mitochondria (86). Further analysis revealed that cardiomyocyte mitochondria contained decreased respiration, oxidative phosphorylation and cytochrome oxidase activity (87). The decrease in COX is tied to the upregulation of cytochrome c observed in mitochondrial damage. COX is the last enzyme in the mitochondrial electron transport chain. It allows for increased cytochrome c production, which is known to lead to apoptosis via amplification of ROS and caspases. Thus, decrease in COX decreases the efficiency of energy production within the mitochondria. Gvodzak et al. have further studied the effects of smoking and alcohol on the mitochondria of the myocardium in rabbits. Analysis of isolated cardiomyocytes showed that endogenous respiration was unaffected but stimulated respiration decreased (88).

Mansoor and colleagues described the mitochondrial damage that occurs via a single toxic component 2-ethylpyridine (2-EP) of cigarette smoke. This *in vitro* study of retinal epithelial cells (ARPE-19) concludes that 2-EP exposure decreases cell viability and increases caspase 3/7 and 9 activity as well as ROS. Increased caspase activity induces an apoptotic drive (89). Recently in the literature Gannon et al. showed the effects of smoking on murine granulosa cells stating that mice subjected to cigarette smoke have increased mitochondrial damage. The Gannon group observed upregulation of autophagy cascade proteins. The study resulted in a loss of follicles via autophagy-mediated granulosa cell death, caused by cigarette smoke inducing mitochondrial dysfunction (90). Naserzadeh et al. performed mitochondrial damage based cigarette smoking studies on isolated mitochondria from rat liver and skin. This group analyzed the effects of cigarette smoking by using a CSE and found a significant increase in mitochondrial ROS formation, lipid peroxidation and mitochondrial membrane collapse and swelling. This resulted in a CSE toxic disruption of the mitochondrial respiratory chain leading to cytochrome c release that increased apoptosis (91). Mitochondrial stability is a focal point of CVD, and therefore all aspects including epigenetics require further investigation.

Smoking and epigenetics: the mitochondrial outlook

DNA/mtDNA methylation and cigarette smoking

In recent years, cardiovascular epigenetics is a rising topic for discussion and a new area of unventured discovery.

The growing public health concern centered around cigarette smoking raises the epigenetic conversation of how this self-induced pathogen affects our cardiovascular health. The literature has a focus on how maternal smoking changes nuclear DNA methylation patterns in infants and newborns. Ivorra et al. has identified 185 CpG islands with altered methylation patterns in the infants of mothers who smoked during pregnancy. These changes correspond to 110 gene regions, of which 10 are newly identified regions including *FRMD4A*, *ATP9A*, *GALNT2* and *MEG3* and are significant in embryonic development. Ivorra et al. observed DNA methylation patterns in umbilical cord blood as compared to maternal peripheral venous blood. Findings of statistically significant differences were noted on 31 CpG islands that were associated with 25 genes. Analysis was performed using an Illumina Infinium Human Methylation 450Beadchip. Ivorra et al. observed that CpG islands of the exposed group had 90.3% higher methylation, with three hypomethylated sites (92).

Alterations in methylation pattern of nuclear DNA have also been noted in a massive study by Richmond and coworkers. This group observed 800 mother-offspring pairs involved in prenatal smoking. DNA methylation was observed at three time points, at birth, age 7 years and 17 years. Of the 7 gene regions and 15 CpG sites analyzed, 4 genes (*AHRR, MYO1G, CYP1A1, CNTNAP2*) maintained abnormal DNA methylation patterns throughout the time points. There was strong maternal association between maternal and offspring DNA methylation alterations as compared to paternal smoking, primarily due to the *in utero* cigarette smoke exposure (93). Intriguingly, this study links the *in utero* DNA methylation alterations to a potentially long-term effect of *in utero* cigarette exposure. Nielsen et al. analyzed maternal smoking during pregnancy and observed three DNA methylation time points: (1) during pregnancy in placenta, (2) at birth in cord blood and (3) buccal epithelium tissue. It is noted that an altered DNA methylation pattern occurred at specific genes: *CYP1A1, AhRR, FOXP3, TSLP, IGF2, AXL, PTPRO, C11orf52, FRMD4A* and *BDNF* (94). Zhang et al. observed a protective effect of demethylation treatment on CSE-induced mouse emphysema model. Analyzing mitochondrial transcription factor A (mtTFA) in various tested groups showed a 4-fold increased methylation in the CSE group compared to the control. The use of DNA methylation inhibitor (Aza) prevented this hypermethylation. Increased methylation correlated with a 3-fold decrease in mtTFA and COX subunit II mRNA and protein levels. Treatment with Aza decreased methylation and improved emphysema and mitochondrial COX activity (95).

mtDNA methylation is correlated to the exposure of chromatin and mutation in the genome. Mutation, although not always directly related to disease, may result in genetic modifications. Some mutations in the mitochondrial DNA may be associated with mitochondrial apoptosis creating increased ROS. This is an unfound area of study, because if the mother's mitochondrial epigenetic methylation markers are changed before giving birth to her offspring, then those specific markers may be passed to their young ones. This could potentially increase the rate of mutation within the mitochondria leading to the damage of mtDNA and mitochondrial proteins resulting in increased proteolytic activity. The same can be said for the healthy female who exercises regularly thereby passing 'good mitochondria' to her young. Therefore, does the brunt of cardiac disease stem from the stability of the mitochondria? Could methylation marker changes lead to increased mitochondrial fission and fusion? A recently published public health review article supports the potential detrimental effects that maternal pre-conception cigarette smoking may have on epigenetic changes (96). Pre-conception alterations in mitochondrial DNA methylation may play a greater role in today's cardiomyopathies than is readily studied.

Smoking: a link between cardiomyopathies, epigenetics and mitochondria

Smoking causes mitochondrial DNA damage and consequently leads to CVD (97). Cigarette smoke promotes atherogenic factors such as hypercholesterolemia and high oxidative stress, which cause DNA damage (98–100). The high oxidative stress is due to high ROS and reactive nitrogen species (RNS). *In vitro* studies have shown that ROS and RNS induce sustained mtDNA damage, altered mitochondrial transcript levels and decreased mitochondrial protein synthesis (98). *In vivo* studies on animal models of CVD have shown increased mtDNA damage (99). In humans, CVD patients have increased mtDNA damage as compared to healthy controls in both heart (101) and aorta (98). Tippetts et al. have shown that cigarette smoking inhibits mitochondrial respiration and increases cardiomyocyte ceramide accumulation (102). Hu et al. have shown that cigarette smoking exposure induces myocardial contractile and mitochondrial damage which can be rescued by metallothionein (103).

Recent studies by Byun exposed the effect of airborne pollutants on mitochondrial DNA methylation. Bisulfite-pyrosequencing analyzed samples from 40 males facing various pollutants such as metal-rich particle matter, air benzene and elemental carbon. Modifications due to their vulnerability were observed in specific mt-DNA regions. Those subjected to high metal-rich particle matter had higher transfers of RNA phenyalanine and higher methylation on the 12S ribosomal RNA gene. It also showed a strong correlation between methylation of the 12S ribosomal RNA gene and the mtDNA copy number. Though these modifications are non-smoking related, it is clear that outside exposure to toxic elements may affect mtDNA. If airborne elements faced on a daily basis affect mtDNA, then how might cigarette smoking toxicity affect it? (104).

Outlook

Reviving a dead myocyte: the mitochondrial puzzle pieces of heart failure

Whether a dead myocyte can be revived is a big question. We have highlighted two aspects that underline myocyte death: mitochondrial dysfunction and proteases. Mitochondrial dysfunction comprises epigenetic

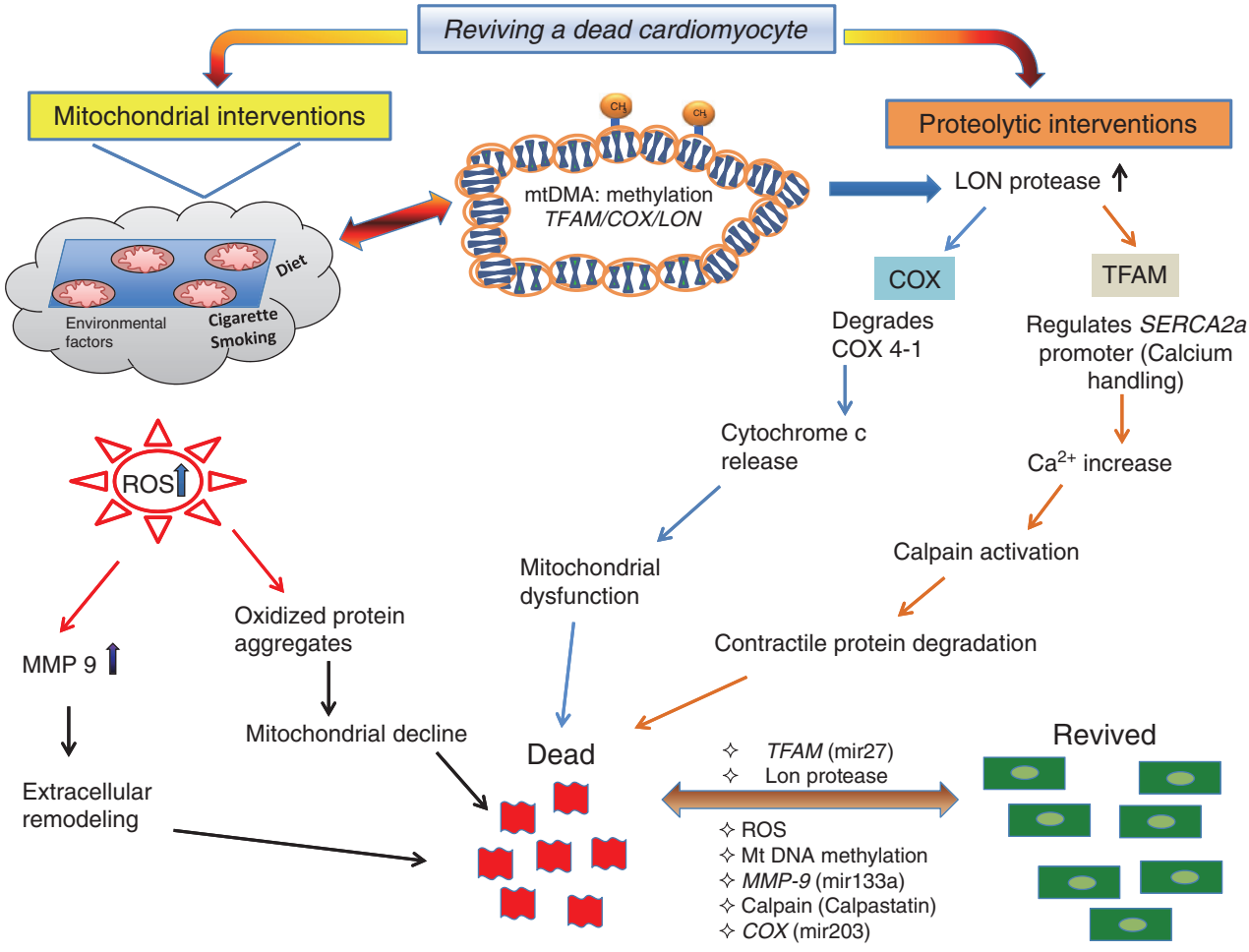

Figure 4: Proposed mechanisms for reviving a dead myocyte.
Myocytes can be revived by two proposed mechanisms: (1) mtDNA alterations and (2) proteolytic interventions. mtDNA methylation is altered by smoking, diet and environmental factors that lead to altered expression of some genes like *TFAM*, which transcriptionally regulates Lon protease and SERCA2a. Lon protease degrades COX to release cytochrome c and causes mitochondrial dysfunction. Lon protease can also lead to increase in the expression of SERCA2a and eventually calcium mishandling. Calcium increase induces calpain activation that can degrade contractile proteins. All these events can lead to myocyte death. Diet, smoking and environmental pollutants can cause altered DNA methylation and increase in ROS production. It can activate MMPs and cause cardiac remodeling. Strategies to revive a dead myocyte can involve managing *TFAM* (mir27), *COX* (mir203), ROS, Lon protease, *MMP9* (mir133a), calpain and mtDNA methylations.

modifications of the mtDNA and abnormalities in the calcium transport system, the electron transport chain and the ATP synthesis machinery. The epigenetic modifications of the mtDNA which includes DNA methylation at CpG islands affects genes like *TFAM*, Lon protease and *COX*. These genes can induce the expression of calpains and MMPs, which degrade the intracellular machinery of the myocytes and lead to myocyte death. We speculate that a dead myocyte can be revived if protected from the adverse effect of mitochondrial and cytosolic proteases like calpain and MMPs (Figure 4). We have published that mir133a regulates the differential methylation of DNA and affects the expression of *MMP9* gene (35). Mir133a is one of the potential candidates to revive impaired myocytes. Use of calpain inhibitors like calpastatin which are endogenous can reduce mitochondrial calpain levels and prolong myocyte death. Mitochondrial MMP9 can also be inhibited by the use of endogenous inhibitors like TIMPs. Mir27a and Mir27b can target *TFAM* and regulate its levels, hence regulating the levels of SERCA2a and calcium balance. Similarly, mir203 can be employed to regulate the levels of COX in the mitochondria. Finally, since dietary habits like methionine intake and smoking along with environmental factors like pollutants affect mtDNA methylation, these factors should be managed that can help resuscitate myocyte health.

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