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Quality Control Systems in Cardiac Aging

Ellen K Quarles[1], **Dao-Fu Dai**[1], **Autumn Tocchi**[1], **Nathan Basisty**[1], **Lemuel Gitari**[1], and **Peter S Rabinovitch**[1]

Ellen K Quarles: equarles@gmail.com; Dao-Fu Dai: ddaofu@u.washington.edu; Autumn Tocchi: uw.tocchi@gmail.com; Nathan Basisty: nbasisty@uw.edu; Lemuel Gitari: mlemuel@uw.edu; Peter S Rabinovitch: petersr@u.washington.edu

[1]University of Washington School of Medicine, Department of Pathology Box 357470 Seattle, WA 98195-7470 United States of America

Abstract

Cardiac aging is an intrinsic process that results in impaired cardiac function, along with cellular and molecular changes. These degenerative changes are intimately associated with quality control mechanisms. This review provides a general overview of the clinical and cellular changes which manifest in cardiac aging, and the quality control mechanisms involved in maintaining homeostasis and retarding aging. These mechanisms include autophagy, ubiquitin-mediated turnover, apoptosis, mitochondrial quality control and cardiac matrix homeostasis. Finally, we discuss aging interventions that have been observed to impact cardiac health outcomes. These include caloric restriction, rapamycin, resveratrol, GDF11, mitochondrial antioxidants and cardiolipin-targeted therapeutics. A greater understanding of the quality control mechanisms that promote cardiac homeostasis will help to understand the benefits of these interventions, and hopefully lead to further improved therapeutic modalities.

Keywords

Quality control; aging; heart; cardiac function; proteostasis

1. Introduction

Cardiac aging is an intrinsic process that results in impaired cardiac function, along with cellular and molecular changes. These degenerative changes are intimately associated with quality control mechanisms. This review provides a general overview of the clinical and cellular changes which manifest in cardiac aging, and the quality control mechanisms involved in maintaining homeostasis and retarding aging. Finally, we discuss aging interventions that have been observed to impact cardiac health outcomes.

Corresponding Author: Peter S Rabinovitch, K081 HSB, University of Washington School of Medicine, Department of Pathology, Box 357470, Seattle, WA 98195-7470, United States of America, petersr@u.washington.edu, 206-685-3761.

2. Overview of Cardiac Aging

2.1 Human Cardiac Aging

A growing body of studies examining human aging and centenarians are beginning to address what healthy aging means for the CV system (Galioto *et al.* 2008). Centenarians have lower prevalence of CV diseases, hypertension, myocardial infarction, angina, and diabetes than younger persons (ages 70 to 99 years) (Selim *et al.* 2005; Galioto *et al.* 2008). This trend toward protection from CV-related causes of death (hypertension, heart disease, diabetes) is also present in their descendants, pointing to a genetic or epigenetic healthy aging profile (Perls & Terry 2003). Multiple studies have followed CV risks factors and CV health in long-lived populations and while some aspects of disease incidence and primary risk factors differ between groups, the recurring conclusion is that a boost to cardiac health occurring early in life (either through genetics or lifestyle) and maintained through life (also by some combination of genetics and lifestyle) is a common piece of the longevity puzzle (Curb *et al.* 1990; Yashin *et al.* 2006). As more studies (and the cohorts within them) mature, there will be more data available on why some humans succumb to aging-related disease early, while others last into their 10^{th} decade.

The Framingham Heart Study and the Baltimore Longitudinal Study on Aging demonstrated that in apparently healthy adults, aging is associated with increase in left ventricular wall thickness measured by echocardiography. The Doppler measurement of the E/A ratio, the ratio between early (E) and late (A) diastolic LV filling, declines dramatically with age (Dai & Rabinovitch 2009; Dai *et al.* 2009). This decline in the E/A ratio suggests that a greater portion of blood filling in the LV results from late diastolic filling as opposed to early diastolic filling, which is clinically defined as diastolic dysfunction or heart failure with preserved ejection fraction (HFpEF). The prevalence of LV hypertrophy and diastolic dysfunction significantly increased in the elderly (Bursi *et al.* 2006), even in an apparently healthy elderly population without hypertension, suggesting that intrinsic cardiac aging may manifest as the above changes.

Although systolic function determined from ejection fraction is relatively preserved at rest in the elderly, exercise capacity and cardiovascular reserve after prolonged exercise significantly declines with age (Correia *et al.* 2002). Aging also contributes to the decline of the maximal heart rate during strenuous exercise, but does not affect the resting heart rate when lying face up (Fleg *et al.* 1995). The decrease in exercise capacity in the elderly is attributed to a modest decrease in ejection fraction after maximal exercise and a prominent decline in maximal heart rate at peak exercise. Likewise, there is age-dependent decline in maximal cardiac index, another measure of systolic function calculated as the cardiac output normalized to the body surface area, which is mostly due to a decline in maximal heart rate after strenuous exercise.

The increased fraction of LV filling performed by atrial contraction in diastolic dysfunction also increases atrial pressure, adversely contributing to atrial hypertrophy and dilatation and subsequently increasing the risk of atrial fibrillation, consistent with the significant age-dependent increase in the prevalence of atrial fibrillation (Lakatta 2003; Lakatta & Levy 2003b; Lakatta & Levy 2003a). Atrial fibrillation adversely affects exercise capacity in the

geriatric population. It also predisposes to the development of HFpEF. Indeed, HFpEF accounts for more than half of all heart failure cases in patients older than 75 years old, especially in those without structural or ischemic heart diseases.

Valvular changes in old age include myxomatous degeneration, deposition of collagen and calcium leading to sclerosis of the valves. Aortic valve sclerosis is present in 30–80% of the elderly (Stewart *et al.* 1997; Nassimiha *et al.* 2001; Karavidas *et al.* 2010), which is detected by echocardiography as calcification of aortic valve leaflets and aortic annulus (Otto *et al.* 1999; Freeman & Otto 2005). Age-related aortic valve sclerosis predisposes to the development of aortic stenosis and increased leaflet calcification and decreased leaflet mobility may predict the progression to aortic stenosis. Hypertension, LV hypertrophy, hyperlipidemia, smoking, end-stage renal disease and congenital bicuspid aortic valves are important risk factors for the progression to aortic valve stenosis (Olsen *et al.* 2005).

In the elderly, fibrosis and valvular calcification are the most common factors contributing to the development of aortic stenosis, which occurs when the aortic valve opening narrows due to the stiffening and calcification of the aortic valve leaflets (Olsen *et al.* 2005). This narrowing prevents effective blood pumping through aortic valve, generating a pressure gradient between the aorta and the left ventricle. To compensate for this obstruction, the walls of the left ventricle thicken with myocardial hypertrophy to maintain sufficient systolic function. Later in the progression, increased wall stress due to pressure overload causes the left ventricle to dilate, leading to deterioration of systolic function. In addition, aortic regurgitation, also related to the calcification of the aortic cusps and annulus, increases with age, and is present in 13–16% of the elderly population (Nassimiha *et al.* 2001). The presence of aortic regurgitation results in ineffective work of the left ventricle and volume overload that may lead to LV dilatation and systolic heart failure.

The above ventricular and valvular changes in cardiac aging compromise the cardiac functional reserve capacity as well as lower the threshold for development of heart failure (Correia *et al.* 2002). This makes the aged heart more susceptible to stress and disease-related challenges, leading to increased prevalence of heart failure and CV mortality in the geriatric population.

2.2 Large mammal models of cardiac aging

Canine hearts develop several aging changes, including myocardial hypertrophy, accumulation of lipofuscin and amyloid which cause increased myocardial stiffness. Degenerative valvular heart diseases are also common in dogs older than 16 years, the prevalence of which approaches 75% in some breeds (Kwart & Haggstrom 2000). The dog model has been widely used for electrophysiological studies since the distribution of cardiac conduction system and activation sequence (electrophysiological properties) in dogs closely resembles that of the human heart (Hamlin & Smith 1960). Aged dog hearts demonstrated prolonged action potential duration and decreased responsiveness to adrenergic stimulation as well as increased risk of developing sick sinus syndrome and atrial fibrillation (Anyukhovsky *et al.* 2005).

Aged rhesus monkey demonstrate several age-related cardiac pathologies, including aortic and mitral valve degenerative calcifications, loss or degeneration of myocardial fibers with hypertrophy of remaining cardiomyocytes, lipofuscin accumulation and variable degrees of myocarditis, multifocal interstitial fibrosis, myocardial infarction, and congestive heart failure (Lane *et al.* 1999; Lane *et al.* 2002; Mattison *et al.* 2003; Roth *et al.* 2004). As shown by the National Institute of Aging's longitudinal study of aging in rhesus monkeys (*Macaca mulatta*), monkeys fed with normal diets develop many of the above cardiac pathologies but did not develop spontaneous atherosclerotic plaques unless they were fed high fat diets.

2.3 Rodent models of cardiac aging

Cardiac aging in the mouse model closely recapitulates the age-related changes found in human hearts (Dai *et al.* 2009; Boyle *et al.* 2011). Using echocardiography to examine the age-related changes in cardiac structure and function in a mouse longevity cohort, we found a significant age-dependent increase in LV mass index (LVMI) and left atrial dimension (Dai *et al.* 2009). Systolic function measured by fractional shortening showed only a modest decline with age. Diastolic function, measured by tissue Doppler imaging of Ea/Aa, significantly declined with age, with substantial fraction of mice older than 24 months with diastolic dysfunction (defined by Ea/Aa < 1). Morphometric analysis indicated an increased myocardial fiber size, increased fibrosis and amyloid deposition with age, especially in the subendocardial areas (Dai *et al.* 2009). Myocardial performance index (MPI), an indicator of global systolic and diastolic function, was also significantly impaired with age. All of the above aging phenotypes are also found in middle age mitochondrial mutator ($Polg^{D257A/D257A}$) mice, a model of "premature aging" (Dai *et al.* 2010).

Previous studies in Fischer rat heart aging using a pressure-volume catheter and echocardiography consistently revealed age-dependent left ventricular hypertrophy, impairment of systolic and diastolic function, as well as increased prevalence of mitral regurgitation (Anversa *et al.* 1989; Forman *et al.* 1997; Boluyt *et al.* 2004). Histopathology of aged rat hearts demonstrated cardiomyocyte hypertrophy and increased LV fibrosis (Forman *et al.* 1997), which reduced LV elasticity and led to diastolic dysfunction. Aging rat hearts also showed decreased responsiveness to sympathetic and dobutamine stimulation (Ahmet *et al.* 2011).

3. Mechanisms of Cardiac Aging

The mechanism of age-dependent LV hypertrophy in mice includes activation of the calcineurin-NFAT pathway, which is well known to mediate pathological hypertrophy (Dai *et al.* 2009). Calcineurin is a phosphatase that dephosphorylates and activates the transcription factor NFAT, which then translocates into nucleus and interacts with several other transcription factors (e.g., GATA4) to initiate transcription of hypertrophic genes, such as atrial natriuretic peptides and brain natriuretic peptides. The mechanisms of diastolic dysfunction in aged heart include fibrosis and subsequent reduced elasticity of the ventricles. In addition to increased interstitial collagen, there is increased matrix metalloproteinase (MMP) and decreased tissue inhibitor of metalloproteinase (TIMP) abundance in fibrotic aged heart (Lindsey *et al.* 2005). Delay in active ventricular relaxation in aged heart is attributable to a reduced abundance of sarco(endo)plasmic reticulum ca ATP-ase

(SERCA2), and to an oxidative modification of SERCA2, both of which affect the rate of diastolic calcium reuptake (Adachi *et al.* 2004; Dai *et al.* 2009).

3.1 Metabolic Changes in Cardiac Aging

Metabolic dysfunction is associated with aging and many age-related diseases (Hu & Liu 2014). In the heart, aging changes in metabolism include reduced fatty acid (FA) metabolism and oxidative phosphorylation (OSPHOS), and compensatory increases in glucose uptake and glycolysis in mice (Stanley *et al.* 2005). These changes in FA metabolism and OXPHOS recapitulated those seen in humans (Kates *et al.* 2003). Despite intense research in this area, there is little consensus on the causes of this "metabolic substrate shift" or whether the shift in substrate utilization in failing and aging myocardium is detrimental or compensatory (van Bilsen *et al.* 2009). The possibility of the latter is raised by observations that increasing glucose as a substrate, rather than FAs, increased contractility in the LV in humans, pigs, and dogs (Stanley *et al.* 2005). One explanation for this is that FA oxidation both leads to excessive loss of ATP through UCP3, and that it carbohydrate utilization is more efficient than FA oxidation in terms of ATP synthesis (Lardy & Pressman 1956; Himms-Hagen & Harper 2001; Stanley & Chandler 2002). Consequently, the power of the LV contraction seems to be improved when the primary metabolic substrate is glucose rather than fatty acids in adult Sprague-Dawley rats (Burkhoff *et al.* 1991). It could be that the substrate shift is therefore a compensatory mechanism to improve contractility in the aging heart.

Sirtuins may play an important role in cardiovascular aging. Sirtuins are NAD^+-dependent class III histone deacetylases involved in the post-translational modification (PTM) of numerous targets (Longo & Kennedy 2006). They can catalyze succinylation, malonylation, and lysine deacetylation ((Du *et al.* 2011; Rardin *et al.* 2013) and reviewed in (Nakagawa & Guarente 2011)). Sirtuins are highly conserved, being found in organisms from bacteria to humans (Brachmann *et al.* 1995). The yeast Sir2 (silent information regular 2) was the first to be well characterized and shown to effect lifespan and stress response (Kaeberlein *et al.* 1999). Seven mammalian sirtuins (Sirt1-Sirt7) have been described (Dali-Youcef *et al.* 2007), and Sirt3, -4, and -5 are present in the mitochondria. Of these, Sirt3 has the strongest activity in the heart. Sirt1, -6, and -7 are largely nuclear, and Sirt2 is located in the cytoplasm (Houtkooper *et al.* 2012).

There are many examples in the literature suggesting connections between sirtuin enzyme activity, metabolism, and aging. (For recent review of this topic see (Pillai *et al.* 2014; Rehan *et al.* 2014)). It is thought that sirtuins contribute to the regulation of metabolism by modifying mitochondrial enzymes and by acting as a sensor of energy status through their dependence on NAD^+ concentration (Wu *et al.* 2014). Due to this important connection to energy metabolism and the broad range of downstream targets, sirtuins have been proposed to function as "watchdogs" for energy dysregulation (Choudhary *et al.* 2009; Ozden *et al.* 2011). SIRT3 in particular may act as a regulator of mitochondrial metabolism and fatty-acid oxidation (Hirschey *et al.* 2010).

3.2 Age-related contractility changes

There are many potential causes of the contractility changes in aging noted above, including alterations in autophagy, proteostasis, inter-/intracellular signaling, mitochondrial lipid composition, and circulating factors.

In a study of 20-week calorie restriction (CR) treatment on aged C57BL/6 mice, changes in indicators of autophagy (LC3B-II to LC3B-I ratio, Beclin-1 expression) following CR were associated with preservation of cardiac geometry and contractile function, as determined by echocardiography (Han *et al.* 2012). Cardiomyocyte cell area was reduced by CR as was phosphorylation of mTOR. Interestingly, a decrease of phosphorylation of Akt/glycogen synthase kinase-3β (GSK-3β) due to CR treatment aligns this study with others that suggest that Akt regulation of autophagy in the heart is disrupted by aging. Hua and colleagues investigated the role of Akt on cardiac aging through the use of Akt over activation transgenic mice (Hua *et al.* 2011). They found that both wild-type Akt and especially overactive Akt were associated with decreased autophagic flux, Ca^{2+} dysregulation, and cardiac hypertrophy in aged (24–26 month old) mice. The authors suggest that autophagic dysregulation may play an important role in cardiac aging phenotypes such as contractile defects and hypertrophy (Hua *et al.* 2011).

The insulin/insulin-like growth factor (IGF) signaling cascade contains a multitude of participating enzymes and co-factors and affects many other signaling cascades (Corpas *et al.* 1993; Abbas *et al.* 2008). Perturbations in the IGF signaling have been implicated in alterations in body composition and neuroendocrine signaling along with cardiac functional declines (Yakar *et al.* 2005). IGF plays a significant role in somatic growth and regulation of apoptosis. Due to the varied functions of IGF, combined with its reduced secretion with aging, researchers have looked to IGF signaling as a critical link in the biology of aging (Corpas *et al.* 1993). Cardiomyocyte mechanical function may be detrimentally affected by circulating insulin-like growth factor-1 (IGF-1) levels in aging mammals. A 2008 study found that liver IGF-1 deficient mice had improved cardiomyocyte function compared to aged controls. It also discussed that down-regulation of Akt, Klotho and phosphorylated-AMPK (adenosine monophosphate-activated protein kinase) due to aging was abrogated by IGF-1 deficiency, and this might play a role in protection from aging-induced cardiac functional decline (Li *et al.* 2008).

Regulation of endothelin-1 (ET-1) appears to be associated with contractile function. ET-1, which is secreted by endothelium, binds to membrane-bound receptor ETA on cardiomyocytes (Takayanagi *et al.* 1991; Yamamoto *et al.* 2005). It is involved in cell hypertrophy and vasoconstriction and it may be up-regulated with aging (Ito *et al.* 1993; Pieske *et al.* 1999). Ceylan-Isik and colleagues found that short-term treatment of 26–28 month old C57BL/6 mice with ETA receptor antagonist, and knock-out of the ETA receptor, partially abrogated aging-associated decline in contractile function and cardiac hypertrophy (Ceylan-Isik *et al.* 2013). This effect was dependent on autophagy and also resulted in a reduction of ROS generation and protection from protein damage (Ceylan-Isik *et al.* 2013).

Many studies have pointed to the regulation of autophagy in aging hearts as a lynchpin for the preservation or loss of cardiac function/geometry with interventions or aging,

respectively. Promotion of autophagy by various mechanisms is associated with decreased hypertrophy, improved contractile function, reduced protein damage, and intracellular Ca2+ regulation (Goswami & Das 2006; Gurusamy & Das 2009; Taneike *et al.* 2010; Hua *et al.* 2011; Han *et al.* 2012; Kobayashi & Liang 2014; Mei *et al.* 2014). See section 4.3 below.

Alterations in mitochondrial cardiolipin (CL) composition in aging heart mitochondria have been associated with cardiac functional impairment and mitochondrial respiratory dysfunction (Lee *et al.* 2006; Chicco & Sparagna 2007). Interestingly, a recent study by Mulligan and colleagues found that inhibition of certain types of CL remodeling could improve cardiac contractile function, along with hypertrophy and dilation, in 25 month old mice without altering age-associated disruption of mitochondrial function and ROS production (Mulligan *et al.* 2014). Delta-6 desaturase inhibition was used to prevent the age-related reallocation of poly-unsaturated fatty acids (PUFAs) on CL, in particular to prevent the switch between of linoleic acid (in young animals) to long-chain PUFAs (found in older animals). Contractility was much improved in the treated cohort, despite an apparent lack of change in mitochondrial function or measures of ROS production such as H2O2 production or lipid peroxidation (Mulligan *et al.* 2014). Studies of a novel CL-targeted therapeutic, SS-31, are discussed in section 5.4 below.

Nitric oxide (NO), produced by three nitric oxide synthases (NOS) in myocardium, effects many aspects of cardiac maintenance and function including endothelial vasorelaxation, gene expression, contractility, oxygen consumption, apoptosis, remodeling during hypertrophy, and regeneration (Massion *et al.* 2005; Sverdlov *et al.* 2014). Negative CV outcomes associated with aging also appear to be associated with a proportionate decrease in measurable NO availability in the myocardium either by decreased NO production or increased scavenging (Paulus 2001; Massion *et al.* 2005). An exception to this is neuronal NO synthase (nNOS). By measuring RNA and protein levels, nNOS has been shown to be up-regulated, in humans with congestive heart failure (Damy *et al.* 2004). Whether the increased expression of nNOS with heart failure is beneficial or detrimental is as yet unknown.

4. Quality Control Mechanisms in Cardiac Aging

4.1 mTOR Signaling in regulation of protein homeostasis

The TOR (and in mammals, mTOR) signaling pathway is a mechanism of transmitting a wide variety of extracellular environmental cues (nutrient availability, amino acids, hormonal signals, mitogens) and producing adaptive responses within the cell. These adaptive responses are important throughout the body, and the heart is no exception. By regulating apoptosis, mitochondrial biogenesis, transcription, translation, lipid metabolism, glycolysis and inflammation, the mTOR pathway plays a critical role in cardiomyocyte growth, function, and structure in aging. Numerous reviews (Balasubramanian *et al.* 2009; Evans *et al.* 2011; C 2013; Johnson *et al.* 2015) are available regarding this pathway and its implications in growth, disease, and aging.

mTOR, the mechanistic target of rapamycin, is a serine/threonine kinase in the PI3K family. It is the catalytic subunit of two distinct complexes – mTORC1 and mTORC2. mTORC1 is

downstream of the AKT and PI3K pathways, and mTORC2 is activated by the RAS and RAF signaling cascade (Dobashi *et al.* 2011). mTORC1 has been the more thoroughly studied complex in mammalian aging, largely due to its inhibition by rapamycin. This pharmacological inhibition is mediated through the rapamycin-FKBP12 complex (Evans *et al.* 2011). Important pathways downstream of TORC1 include regulation of cap-dependent initiation of translation *via* 4EBP1, control of ribosomal protein biosynthesis via S6K, and regulation of autophagy via Ulk1 (Johnson et al., 2014). However, chronic treatment with rapamycin can also inhibit mTORC2 in a cell-specific manner (Lamming *et al.* 2012). mTOR complex 2 (mTORC2) includes Rictor. This complex may also regulate some aspects of cardiac homeostasis in aging through stimulating autophagy and the clearance of pro-apoptotic factors, and removal of Akt from FOXO3 (Gurusamy & Das 2009; Jung *et al.* 2010; Kurdi & Booz 2011).

4.1.1 mTORC1—mTORC1 is an important regulator of cell growth and size, and signaling of mTORC1 is depressed in stress conditions such as low ATP concentration and low nutrient availability (Dobashi *et al.* 2011). Modulation of mTORC1 has been shown to improve cardiac geometry and function (Balasubramanian *et al.* 2009). It has many downstream functions critical to proteostasis including stimulating protein synthesis, inhibiting autophagy, ribosomal biogenesis, and translation initiation. 4E-BP1, and eukaryotic elongation factor 2 (eEF2), and ribosomal protein S6 kinase (S6K) are downstream effectors of mTORC1 and are largely responsible for mTORC1's control over protein synthesis. A study by Wessells and colleagues provided evidence that in *Drosophila melanogaster*, upregulation of d4eBP was sufficient to mitigate the age-related decline in fly cardiac function (Wessells *et al.* 2009). 4eBP binds eIF4E, inhibiting cap-dependent initiation of translation (Sonenberg & Hinnebusch 2009).

4.2 Proteostasis

Protein homeostasis (proteostasis) is the equilibrium between protein synthesis, maintenance, and degradation. Maintenance of proper protein homeostasis is essential to cellular and organismal health – as illustrated by many studies indicating that age-related diseases and conditions are associated with the inability of the cell to maintain healthy proteins or get rid of defective proteins (Bedford *et al.* 2008). These conditions include neurodegenerative disease (Douglas & Dillin 2010), cardiac dysfunction (Hedhli *et al.* 2005; Christians & Benjamin 2012), cataracts (Surguchev & Surguchov 2010), and sarcopenia (Vinciguerra *et al.* 2010; Marzetti *et al.* 2012). Similar dysfunctions in proteostasis have been observed in "normally" aging cells which are free of disease (de Magalhães 2004), indicating a potentially important role for protein regulation in both health and aging.

Aside from the correlative association between aging, health, and protein quality control, direct interventions to modulate quality control mechanisms may potentially increase lifespan and improve health. Several such examples can be seen among interventions that inhibit mTOR, including rapamycin and calorie restriction, discussed further below. A number of other protein quality control interventions have been shown to improve health and aging in both invertebrate and mammalian models as well (Morimoto & Cuervo 2009; Douglas & Dillin 2010; Madeo *et al.* 2010; Koga *et al.* 2011).

Collectively, these studies may suggest that dysfunctional proteostasis has some causative role in aging or, alternatively, restoration of protein homeostasis machinery is protective against some other driving force in aging and age-related disease. In either scenario, the major mechanistic question of *how* these processes extend lifespan and healthspan remains as yet poorly answered, as an incomplete understanding of the various interactions, specificity, and targets of quality control pathways currently limits the ability of researchers to close this gap. Fortunately, several quality control pathways, such as autophagy and ubiquitin-mediated degradation, are receiving increased attention from several areas of biomedical research as their roles are recognized in a number of diseases (Madeo *et al.* 2010). In addition, the emergence of sophisticated tools in genomics and proteomics has provided powerful resources in cellular biology, allowing researchers to acquire and analyze an unprecedented depth and volume of data.

The aging cardiac proteome recapitulates most hallmarks of the aged cellular proteome including the appearance of protein aggregates and lipofuscin, increased protein oxidation and damage, increased ubiquitination, and declines in autophagy and the ubiquitin proteasome system (Morimoto & Cuervo 2009; Johnson *et al.* 2013). All of these changes have an impact on global levels of proteostasis to some degree, consistent with a notion of proteome remodeling during aging. It is unlikely, however, that all protein changes are equally or significantly contributing to the aging phenotype – presenting a challenge for researchers to identify the most phenotypically relevant downstream targets and their changes during aging.

The majority of studies in mice have reported a decline in the efficiency of protein degradation machinery with advanced age, contributing to a popular notion that aging is associated with a decrease in overall protein turnover. However, using a sensitive method of heavy label proteomics, our group has consistently observed that proteome turnover is either unchanged or modestly increased in the various mouse tissues examined to date, including mouse heart (Dai *et al.* 2014a) as well as skeletal muscle (submitted), brain (unpublished), and liver (submitted). Unlike earlier studies, these finding were based on direct measurements of individual protein turnover rates *in vivo* (Hsieh *et al.* 2012), rather than using bulk protein synthesis measurements or cellular markers of degradation as a proxy. Additionally, other recent studies utilizing a similar metabolic labeling-based MS approach to assess in vivo protein turnover have observed turnover rates consistent with our observations in aging mice (Price *et al.* 2010; Miller *et al.* 2012).

In mice, we have shown that the age related functional declines discussed previously are accompanied by proteomic remodeling of both energetic and structural pathways (Dai *et al.* 2014a). Levels of mitochondrial respiratory proteins, key for the production of most of the cardiac ATP, declined in the old heart, with concurrent reductions in metabolic proteins involved in fatty acid beta oxidation, amino acid metabolism, ketogenesis, and the TCA cycle, together likely contributing to an overall energy deficiency in aging hearts (Dai *et al.* 2014a). Conversely, glycolytic metabolic pathways as well as extracellular structural proteins were significantly *increased* in protein abundance with age (Dai *et al.* 2014a). The metabolic shift from TCA to glycolysis/gluconeogenesis was confirmed by metabolomics. This remodeling of the cardiac proteome with age is consistent with a number of proteomic

studies focused on cardiac aging and disease. These changes may be the result of an underlying decline in protein quality control systems, which in turn leads to accumulation of damaged proteins. Two processes are known to turn over the majority of cellular proteins: autophagy and the ubiquitin proteasome system.

4.3 Autophagy

Autophagy is one of two primary cellular systems which degrade the vast majority of proteins in the cell (its counterpart, the ubiquitin proteasome, is discussed below). Any cellular degradation involving lysosomes, single membrane vesicles containing various enzymes for the digestion of macromolecules, is generally categorized under the umbrella term “autophagy” (Morimoto & Cuervo 2009). There are three major ways by which proteins can be delivered to a lysosome for degradation, which define three primary categories of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. For brevity these will not be covered in detail, however, readers are referred to a number of comprehensive reviews on each topic (Morimoto & Cuervo 2009; Madeo *et al.* 2010; Koga *et al.* 2011).

There are four general physiological roles of autophagic degradation: cell and protein quality control, conserving cellular resources, cellular remodeling, and cell defense (Madeo *et al.* 2010). In context of quality control, autophagy is responsible for the clearance of damaged proteins, insoluble protein inclusions, and abnormal organelles, all of which are hallmarks of aged and dysfunctional tissues. Knocking down components of autophagy leaves cells unable to remove damaged organelles and proteins (Ravikumar *et al.* 2002; Madeo *et al.* 2010; Wong & Cuervo 2010), demonstrating that autophagy plays a key role in protein homeostasis.

Numerous lines of evidence suggest that autophagy is likely to have an important role in organismal aging, however, there has as yet been no “smoking gun”. Many studies, mostly in *C. elegans*, have demonstrated that autophagy components are required for lifespan extension by CR, mTOR inhibition, IGF-1 inhibition, and a few other longevity pathways (Ryazanov & Nefsky 2002; Douglas & Dillin 2010; Koga *et al.* 2011), although these have not yet been confirmed in other model systems. Unfortunately, there is no genetic or pharmacological intervention known to specifically increase autophagy without targeting other processes.

A recent report has found that genetic over-expression of ATG5, a vital autophagy protein involved in autophagosome formation, extends lifespan in mice (Pyo *et al.* 2013). ATG5 has an important pro-apoptotic function, which cannot be excluded as the longevity-promoting factor. In particular clearance of defective mitochondria by autophagy (mitophagy) is known or be essential in cardiac development and response to stress or injury. Thus, cardiac-specific knockdown of ATG5 in mice has been shown to accelerate aspects of aging in the heart, including accelerated left ventricular hypertrophy, decreased fractional shortening, and premature death (Taneike *et al.* 2010). In addition, the accumulation of ubiquitinated proteins and p62 in ATG5 mutant mice suggests that removal of damaged or aggregating proteins is a protective mechanism (Taneike *et al.* 2010; Wohlgemuth *et al.* 2014b). In agreement with this, a study performed using cardiomyocyte cell lines found that induction

of autophagy was protective against oxidative stress-induced protein aggregation and reduced levels of protein ubiquitination (Dutta *et al.* 2013). Further, this study found that induction of autophagy improved mitochondrial function and reduced cell death, confirming that autophagy has an important role in maintaining mitochondrial quality.

Sirt1 expression stimulates basal levels of autophagy, likely through deacetylation of autophagy genes (Atg)5, -7, and -8,, and Sirt1 overexpression increases autophagic flux in cultured mouse embryonic fibroblasts (Lee *et al.* 2008). The reduction in Sirt1 expression with age may contribute to susceptibility of the heart to injury, as it is no longer able to promote autophagy through deacetylation of FoxO1, leaving cardiomyocytes with a lessened ability to respond to ischemia (Hariharan *et al.* 2010). The induction of autophagy mediated by CR and nutrient deprivation, but not autophagy stimulated by rapamycin treatment, appear to be dependent on functioning Sirt1 (Morselli *et al.* 2010).

The mTOR pathway, when inhibited, is well known to increase autophagy and extend lifespan. In fact rapamycin (discussed below), by inhibiting mTOR, is one of the few drugs available which can be used to pharmacologically increase autophagy. Longevity studies with rapamycin, and other forms of mTOR inhibition, have reported increased autophagy in animals across many studies (Harrison *et al.* 2009; Morimoto & Cuervo 2009; Stanfel *et al.* 2009; Johnson *et al.* 2013), and offer further evidence that autophagy may play a central role in aging. Even so, due to the inability to specifically over-express autophagy components without targeting non-specific processes, it is still not certain whether augmenting autophagy can in itself extend lifespan or slow aging.

In the future, to better understand the mechanisms underlying improved cardiac function following induction of autophagy, it will be critical to better understand the detailed roles of autophagic pathways with oxidative stress, mitochondrial quality control, and clearance of unwanted proteins in the heart.

4.4 Ubiquitin-mediated turnover

The ubiquitin-proteasome system (UPS) is the primary non-lysosomal protein degradation pathway. In contrast to autophagy, its action is limited specifically to individual proteins, and cannot degrade other macromolecules, organelles, or groups of proteins. Where autophagy often degrades its targets in bulk, the UPS very specifically targets thousands of proteins and utilizes a sophisticated array of mechanisms to do so with spatial and temporal precision. The UPS is also active in all regions of the cell, and targets proteins localized in organelles. For most proteins, degradation through this pathway is characterized by 2 major steps: first the recognition and “tagging” of a protein for elimination with a poly-ubiquitin modification, followed by translocation to the proteasome for degradation (Morimoto & Cuervo 2009; Wong & Cuervo 2010; Koga *et al.* 2011). This extraordinarily complex process has been extensively studied and described in very great detail in literature, and readers are referred to several detailed reviews of UPS functions (Ryazanov & Nefsky 2002; Morimoto & Cuervo 2009; Douglas & Dillin 2010; Wong & Cuervo 2010; Koga *et al.* 2011; Jana 2012).

Similar to autophagy, the UPS is essential for maintaining overall cellular homeostasis. Inhibiting or deleting its components often leads to severe cellular phenotypes, toxicity, and cellular death. Almost immediately after inhibition, an accumulation of protein inclusions can be observed in cells. Interestingly these resemble the inclusions described in a number of neurodegenerative diseases (Ryazanov & Nefsky 2002; Keck *et al.* 2003; Bedford *et al.* 2008; Robinson 2008). Genetic depletion of proteasome subunits in the brains of mice has been shown to induce a neurodegenerative phenotype, suggesting a role in neurodegenerative diseases characterized by protein inclusions (Bedford *et al.* 2008). In the heart, the role of the UPS is less well known. Some evidence exists of proteasomal degradation of various cardiac proteins including myofibrillar proteins, connexins, actin, and myosin (Pagan *et al.* 2013). These mechanisms, for the most part, have not been well-characterized in heart. Pharmacological and genetic intervention of the UPS with proteasome inhibitors has, however, made it evident that the proteasome can have powerful effects on the heart. In models of ischemia-reperfusion injury, for instance, proteasome inhibitors decrease infarct size – sometimes by over 50% (Pagan *et al.* 2013). Little has been reported in the literature about the role of the ubiquitin proteasome in the context of cardiac aging specifically.

Most of the evidence linking the UPS-intervention to longevity comes from *C. elegans* studies (Li *et al.* 2007; Koga *et al.* 2011; Jana 2012). Generally, these can be explained by the specific action of UPS on longevity pathways, rather than a global change in the proteolytic system. The ubiquitin ligase RLE-1, for example, selectively targets and poly-ubiquitinates a key component in the homologous insulin/IGF pathway in worms, daf-16, and leads to its degradation by the proteasome (Li *et al.* 2007). As a result, inhibition of RLE-1 extends lifespan in *C. elegans*. In flies it has been shown that overexpression of parkin-1, a ubiquitin ligase involved in familial Parkinson's disease, extends lifespan (Rana *et al.* 2013).

On a broad scale, it is not known if general protein maintenance by the UPS is intimately involved in aging. Correlatively, proteasomal activity becomes less functional with age and is restored in long-lived animals under CR (Koga *et al.* 2011; Jana 2012). It is also important to note that autophagy and the UPS must work in synchrony to direct protein homeostasis and an intervention in either process is likely to cause changes in both. Interestingly, it has been shown that poly-ubiquitination can promote the clearance of proteins through autophagy (Tan *et al.* 2008).

4.5 Apoptosis

There is increasing evidence of significant age-related loss of cardiac myocytes (Kajstura *et al.* 1996a; Kajstura *et al.* 1996b; Li *et al.* 1997; Liu *et al.* 1998; Lee *et al.* 1999) which contributes to the increased susceptibility of the aged heart in models of myocardial infraction (MI), ischemic heart attack, and congestive heart failure (Liu *et al.* 1998; Azhar *et al.* 1999; Narula *et al.* 1999; Crow *et al.* 2004; Lehrke *et al.* 2006). Studies on the interrelation between age and apoptotic cell loss have been contradictory, with some apoptotic markers decreasing while others increase with age and in pathologies generally associated with aging (Maury & Teppo 1989; Levine *et al.* 1990; Lane *et al.* 1993; Torre-

Amione *et al.* 1996; Kavathia *et al.* 2009). However, there is consensus that apoptosis plays a significant role in deteriorating function of senescent hearts. Several cellular processes have been hypothesized to contribute to this. A significant increase in oxidative stress may precede cardiomyocyte apoptosis (Kajstura *et al.* 1996b; Nitahara *et al.* 1998; Mather & Rottenberg 2000; Phaneuf & Leeuwenburgh 2002; Crow *et al.* 2004). Similarly, the reduction of SIRT1 deacetylase activity of and the increased acetylation of the Foxo1 transcription factor in senescent hearts has been shown to lead to activation of pro-apoptotic Bim signaling (Sin *et al.* 2014). Sirt1 and Sirt7, both localized to the nucleus, modulate p53 activity to act in a protective manner against apoptosis (Alcendor *et al.* 2004; Vakhrusheva *et al.* 2008). Sirt3 can also influence the path to autophagy by targeting the mitochondrial permeability transition pore (MPTP). Cyclophilin D is a component of the MPTP and Sirt3 maintains cyclophilin D in its deacetylated form, preventing the release of pro-apoptotic factors (Hafner *et al.* 2010). Age-related decline in the Bcl2 anti-apoptotic marker and significant elevation of cytosolic cytochrome c in aged hearts can also trigger apoptosis (Narula *et al.* 1999; Phaneuf & Leeuwenburgh 2002). Furthermore, cytochrome c-dependent activation of cysteine proteases and caspase 3 is known to mediate myopathic apoptosis in human cardiomyopathy (Beltrami *et al.* 1994; Narula *et al.* 1999).

4.6 Mitochondrial Quality Control

Mitochondrial dysfunction and dysregulation are well documented in old age. Mitochondrial dysfunction in old age is associated with abnormal mitochondrial ROS production and detoxification (reviewed in (Terzioglu & Larsson 2007; Trifunovic & Larsson 2008; Mammucari & Rizzuto 2010)). Mitochondrial oxidative phosphorylation declined with age, as evident by the decline in mitochondrial state 3 respiration (maximal stimulated respiration), related to diminished activity of electron transport complexes I and IV in old age. The function of complexes II, III and V are relatively unaffected in old age (see review (Navarro & Boveris 2007)).

As the heart is a highly metabolic active organ and rich in mitochondria, it is particularly susceptible to mitochondrial oxidative damage. Several studies have demonstrated the deficiency of mitochondrial energetics in human and experimental animals with heart failure (Ventura-Clapier *et al.* 2008). The mechanisms by which mitochondrial dysfunction lead to heart failure may include mitochondrial biogenesis that does not keep up with the increasing demand in cardiac hypertrophy (see review (Goffart *et al.* 2004), mitochondrial uncoupling and decreased substrate availability (Murray *et al.* 2004), and increased mitochondrial DNA deletions (Dai *et al.* 2011b) and altered energetics (see section 3.1, above).

We have previously shown that mitochondrial protein carbonylation, indicative of oxidative damage to mitochondrial proteins, significantly increased in aged mouse hearts (Dai *et al.* 2009; Dai *et al.* 2010). This suggests that damaged mitochondria in aged mouse hearts produce more ROS than healthy mitochondria in young hearts. Furthermore, aged mouse hearts had a 3–4 fold increase in mitochondrial DNA point mutations and deletions (Dai *et al.* 2009). Defective mtDNA produce defective subunits of mitochondrial electron transfer complexes, especially complexes I and IV, leading to increased ROS production. This may lead to vicious cycle of ROS amplification within mitochondria (Dai *et al.* 2012a; Dai *et al.*

2012b). The most direct evidence for the critical role of mitochondrial ROS in cardiac aging was shown by mice overexpressing catalase targeted to the mitochondria (mCAT). The mCAT mice were significantly protected from the age-dependent increase in LVMI, decline in diastolic function and impairment of myocardial performance through better preservation of SERCA2, as well as amelioration of cardiac fibrosis and cardiomyocytes hypertrophy (Dai *et al.* 2009). Consistent with this, mCAT attenuates mitochondrial oxidative damage, as displayed by significant reductions of mitochondrial protein carbonyls and mtDNA deletion frequencies in aged mCAT hearts (Dai *et al.* 2009).

Another line of evidence for the critical role of mitochondria in aging is demonstrated by mice with proofreading-deficient homozygous mutation of mitochondrial polymerase gamma (Polg$^{D257A/D257A}$ designated as Polg$^{m/m}$), which induces a substantial increase in mtDNA point mutations and deletions (Trifunovic *et al.* 2004; Kujoth *et al.* 2005). The accumulation of mitochondrial DNA mutations has been shown to increase apoptosis (Kujoth *et al.* 2005). These mice were shown to have shortened lifespan and an “accelerated aging-like” phenotypes, such as kyphosis, graying and loss of hair, anemia, osteoporosis and age-dependent cardiomyopathy (Trifunovic *et al.* 2004; Dai *et al.* 2010), which include marked LV hypertrophy, systolic and diastolic dysfunction, impairment of myocardial performance, increased cardiac fibrosis, apoptosis and hypertrophy of remaining cardiomyocytes. The observations that mitochondrial damage and cardiomyopathy in these mice can be partially rescued by mCAT suggests that mitochondrial ROS and mitochondrial DNA damage are part of a vicious cycle of ROS-induced ROS release (Dai *et al.* 2010).

A recent paper reports the striking result that endurance exercise in Polg$^{m/m}$ mutant mice can prevent progeroid phenotypes in both skeletal and cardiac muscles (Safdar *et al.* 2011). It is proposed that the augmented mitochondrial biogenesis induced by endurance exercise in these mice is a critical factor in maintaining mitochondrial function in these muscles. Age-associated accumulation of mtDNA deletions have been documented in human hearts (Corral-Debrinski *et al.* 1991; Zhang *et al.* 1997). The beneficial effects of endurance exercise seen in Polg$^{m/m}$ mutant mice reinforce the well-known benefit of regular aerobic exercise for human hearts.

Sirtuins are believed to influence cardiac aging through modulation of mitochondrial stress responses. Sirt3 has been shown to contribute to this by up-regulating mitochondrial antioxidant defenses, leading to lower levels of ROS (Wu *et al.* 2014). Indeed, mice without Sirt3 have a compromised ability to benefit from caloric restriction in the face of oxidative stress (Tao *et al.* 2010) and develop cardiac hypertrophy and fibrosis very early in life (Sundaresan *et al.* 2009). Age-related loss of Sirt3 has been associated with cardiac aging phenotypes, including hypertrophy (Pillai *et al.* 2010; Pillai *et al.* 2014). Sirt3 modulates the Foxo3a and catalase to reduce ROS *in vitro*, along with decreasing signaling from Ras to the MAP/ERK and P13K/Akt pathways, preventing cardiomyocyte hypertrophy (Sundaresan *et al.* 2009). Sirt3 also deacetylates the mitochondrial antioxidant MnSOD *in vitro*, contributing to its improved antioxidant effects (Qiu *et al.* 2010).

4.6.1 Mitochondria fusion and fission—Mitochondria fusion/fission is a highly conserved quality control process in which a balance between fusion and fission is vital for

normal functioning of the mitochondria and overall cellular homeostasis. These processes regulate mitochondria number, morphology, and function (Bereiter-Hahn & Voth 1994) and their role in maintenance of mitochondrial quality control is likely necessary to retard the detrimental effects of aging. As noted above, mitochondrial dysfunction is a hallmark of aging and this is manifested by impairment of OXPHOS bioenergetics, and accumulation of ROS. Furthermore, cardiomyocyte mitochondrial morphological changes have been reported in aging and heart disease (Hom & Sheu 2009; Ong & Hausenloy 2010). This suggests that there are diminished mitochondrial quality control mechanisms with age and a greater understanding may offer some insight into the function decline in cardiac function with aging.

In recent years, it has become clear that mitochondria exist as a dynamic network within cells, in which the active processes of mitochondrial fission and fusion are a balanced process by which mitochondrial quality control is maintained (Hom & Sheu 2009). A key mechanism by which this is accomplished is that mitochondrial fission fragments that have low membrane potential (indicative of poor OSPHOS activity) are targeted for degradation by ubiquitination via the activity of Pink and Parkin (Matsuda *et al.* 2010). Key proteins that regulate mitochondrial fusion and fission are mitofusin1/2 and OPA1, and their dysregulation impairs mitochondrial structure and function, with loss of efficiency of cellular respiration in many tissues (Frieden *et al.* 2004; Szabadkai *et al.* 2004; Chen *et al.* 2005; Westermann 2010), including the ageing heart (Bossy-Wetzel *et al.* 2003; Papanicolaou *et al.* 2011). Complete genetic ablation of mfn1/2 is embryonic lethal. Deletion of mfn1 results in mitochondrial fragmentation, although mfn1-KO murine hearts have normal left ventricular function and their mitochondria exhibited normal respiratory function (Papanicolaou *et al.* 2012). However, other studies have found that Mfn1 partial deletion resulted in mild respiration deficiency, cardiac hypertrophy, and impaired contractile reserve (Papanicolaou *et al.* 2011).

Under normal conditions mitofusins 2 (Mfn2) is highly expressed in adult hearts and its deficiency in cardiomyocytes is associated with disruption of cell cycle progression, cardiac hypertrophy, reduced oxidative metabolism and altered mitochondrial permeability transition and systolic dysfunction (Papanicolaou *et al.* 2011). Piquereau *et al.* also found that partial down-regulation of Mfn2 and optic atrophy-1 (OPA1) in cardiac tissue resulted in altered mitochondrial morphology in which large pleomorphic mitochondria with disorganized cristae were arranged in irregular patterns (Papanicolaou *et al.* 2011; Piquereau *et al.* 2012). Suppression of mfn2 expression has also been reported in cardiac diseases such as SHR, murine pressure-overload hypertrophy and in β_2-TG mice with cardiomyopathy (Fang *et al.* 2007). Partial knockout of both Mfn1 and 2 in murine models results in mitochondrial fragmentation, impaired mitochondrial respiration, and fatal cardiomyopathy (Chen *et al.* 2011). Overexpression of Mfn2 may also promote apoptosis, however (Shen *et al.* 2007; Ikeda *et al.* 2014)

It is thus clear that mitochondrial dynamics, including fission and fusion, is a necessary component of cardiomyocyte homeostasis and maintenance of cardiac function. The decline in efficiency of these functions is likely implicated in cardiac aging, however, this is clearly a subject that warrants further study.

4.7 Cardiac matrix homeostasis: Matrix metalloproteases

Matrix metalloproteases (MMPs) are known to degrade the extracellular matrix (ECM) and play a role in tissue homeostasis (Parks *et al.* 2004). As organisms age, this homeostatic role can be thrown off balance with overexpression of MMPs. With increased age, MMP2, MMP9, and MMP28 have all been shown to play a role in the cardiac tissue (Chiao *et al.* 2012; Horn *et al.* 2012; Ma *et al.* 2013; Yabluchanskiy *et al.* 2014).

MMP2 is a gelatinase that is up-regulated in multiple aged organisms (Horn *et al.* 2012). A study using sheep demonstrated that MMP2 expression is increased in old animals, and this expression shows the same trend in old mice (Horn *et al.* 2012). When angiotensin-converting enzyme 2 (ACE2) is knocked out in mice, there is a spike in MMP2 expression that is not seen in wild type controls, suggesting an increase in matrix degradation when angiotensin cannot be properly regulated (Patel *et al.* 2014).

MMP9, a collagenase, is the most commonly upregulated MMP in aging heart (Parks *et al.* 2004; Chiao *et al.* 2012). Chiao *et al.* showed an increase in MMP9 expression and protein levels in both the left ventricle and plasma in senescent mice, which was tied to decreased collagen deposits and TGFβ activation. In the same study, when MMP9 was knocked out, MMP8, which has a high affinity for collagen I and III, was upregulated. Similar to MMP2, when ACE2 was knocked out, MMP9 was increased (Chiao *et al.* 2012). Despite MMP9 being a collagenase, with its increased expression collagen accumulates and there is increased angiogenic signaling (Yabluchanskiy *et al.* 2014). However, there is no increase in vessel numbers or prevention of vascular leakage unless MMP9 expression is attenuated (Yabluchanskiy *et al.* 2014).

MMP28, an epilysin and the most recently cloned MMP, has been shown to be dysregulated in cardiac aging (Ma *et al.* 2013). When MMP28 is knocked out, the aging heart has an increased inflammatory response (Ma *et al.* 2013). MMP28 will be a protease to investigate in the future, since many of its interactions and roles are still being elucidated, especially in cardiac aging.

Besides the MMPs, the role of collagen and fibrinogen in the ECM composition is important. If there is too much extracellular matrix there is an increase in cardiac stiffness and diastolic dysfunction in aged organisms (Ma *et al.* 2013). Cardiac stiffness increased with age due to the increased amount of collagen content and fibrinogen deposit in old animals (Horn *et al.* 2012; Rodriguez-Menocal *et al.* 2014; Yabluchanskiy *et al.* 2014).

5. Cardiac Aging Interventions

Interventions to prevent or delay CV disease have been widely publicized and often successful, however these have focused on cardiac-extrinsic risk factors, such as hypertension, cholesterol, smoking, diabetes and exercise (http://www.cdc.gov/heartdisease/risk_factors.htm). Age-related factors (see section 1) are generally considered immutable, even though aging is by far the largest risk factor for heart disease and failure. There are however, several recognized methods of delaying the negative outcomes of aging, the best characterized of these being restriction of caloric intake. More recently, there has been some

progress in defining caloric restriction (CR) mimetics that may recapitulate positive changes due to CR, without the necessary reduction of food intake. These largely function by modulating the activity of mTOR. Candidates for CR mimetics presently including rapamycin and resveratrol (Wohlgemuth *et al.* 2014a). Other interventions include antioxidants targeted to the mitochondria, circulating factors, and cardiolipin-targeted pharmaceutics. Below are summaries of some of the important cardiac aging interventions that have been pursued to date.

5.1 Caloric/dietary restriction and mimetics

5.1.1 CR—CR, also called dietary restriction (DR), is a powerful and reproducible technique to improve both healthspan and lifespan in many model organisms from yeast to mice (reviewed in (Speakman & Mitchell 2011). CR is sustained calorie restriction without restriction of vitamins and micronutrients. CR's cardioprotective activity includes reduced cardiac hypertrophy as measured by left ventricular mass and wall thickness, improved cardiomyocyte contractile function and reduced cardiomyocyte size (Han *et al.* 2012). While both CR and rapamycin affect metabolism through decreased mechanistic target of rapamycin (mTOR) activity, there is evidence that DR works though other pathways as well in a complex net of changes resulting in a whole-body stress response and adjustment in tissue maintenance (reviewed extensively in (Speakman & Mitchell 2011)). CR can delay the onset of cardiac aging and ameliorate the effects of cardiovascular disease. In humans, rodents and monkeys, chronic dietary restriction reduces the aging-associated decline in cardiac function, ameliorates cardiac hypertrophy, and reduces signs of cardiomyopathy (Maeda *et al.* 1985; Taffet *et al.* 1997; Colman *et al.* 2009; Niemann *et al.* 2010; Shinmura *et al.* 2011; Dai *et al.* 2014a).

CR protects against cardiomyopathy, in part by reducing age-associated apoptosis through protection from DNA damage, enhanced DNA repair, and alterations in apoptosis-related gene expression (Maeda *et al.* 1985; Dhahbi *et al.* 2006). It can also modulate expression of genes involved in fibrosis, extracellular matrix maintenance, inflammation, and fatty acid metabolism (Dhahbi *et al.* 2006). Many other processes associated with cardiac aging are modulated by DR, including reduced perivascular collagen deposition, reduced left ventricular cardiac hypertrophy, protective effects against ischemia, and a reduction of chronic vascular inflammation (Spaulding *et al.* 1997; Broderick *et al.* 2001; Dhahbi *et al.* 2006).

The mechanisms by which CR modulates cardiac aging are still a matter of intense research. One hypothesis is that limited nutrient/energy availability drives tissues from a proliferative and energetic state to a somatic maintenance state to allow the best use of limited resources. Indeed, Drake and colleagues (2013) found that DNA synthesis, a measure of proliferation, was reduced while measures of mitochondrial biogenesis were maintained during life-long CR (Miller *et al.* 2012; Drake *et al.* 2013). Many of these effects may be mediated through the mTOR pathway, particularly mTORC1, which regulates protein synthesis and autophagy (Dobashi *et al.* 2011). Aged rats that are subjected to CR over their lifespan show increased autophagy in conjunction with improved LV diastolic function (Shinmura *et al.* 2011). Conversely, when autophagy is reduced in the heart in Atg5 knockout mice, after three

months they exhibit a reduced lifespan, LV hypertrophy, decreased fractional shortening, and defective structure and function of cardiac mitochondria compared to controls (Taneike *et al.* 2010). Cardiovascular disease and cardiac aging have long thought to be influenced by oxidative stress. Since CR has been shown to reduce mitochondrial reactive oxygen species (ROS) production in the heart and other tissues, and decrease NAD(P)H oxidase activity, it may abrogate the effects of aging through a modulation of the redox environment (Gredilla *et al.* 2001; Csiszar *et al.* 2010; Csiszar & Ungvari 2010). The most direct evidence of this is derived from study of mice overexpressing catalase targeted to the mitochondria. These mice have longer mean and maximal lifespans (Schriner *et al.* 2005), and moreover, show functional and biochemical evidence of reduced cardiac aging (Dai *et al.* 2009). Notably, diastolic failure (HFpEF), which is common in aged mice, as well as in man, was substantially attenuated in mCAT mice.

As life-long CR is unlikely to be translatable as a human therapeutic regime, there has recently been greater attention to the potential benefits of short term CR initiated later in life. We have examined cardiac function and molecular alterations following 10 week CR given to mice at 24 months of age (Dai *et al.* 2014a). We found that both CR and rapamycin reversed age-related diastolic dysfunction and cardiac hypertrophy. Also, by using deuterated-leucine protein labeling, we observed protein turnover differences between CR-treated and old control animals.

5.1.2 Rapamycin—CR in humans is an unappealing regimen, and thus, the search continues for CR mimetics that reproduce beneficial effects of CR without necessitating drastic changes in diet.

Rapamycin is by far the best documented agent that is believed to function as a CR mimetic. By inhibiting the target of rapamycin, mTOR, several important growth and cellular quality control mechanisms are modulated including ribosomal biogenesis, autophagy, lipid synthesis, and translation (reviewed in (Johnson *et al.* 2013)). These effects have been long- and well-documented in invertebrate models of aging, including flies (Kapahi *et al.* 2004), nematodes (Vellai *et al.* 2003; Jia *et al.* 2004), and yeast (Kaeberlein *et al.* 2005; Powers *et al.* 2006). Interest in rapamycin in mammalian systems greatly increased following reports from the National Institute on Aging Intervention Testing Program that long-term treatment of mice with rapamycin improves healthspan measures and extends lifespan (Harrison *et al.* 2009; Anisimov *et al.* 2010; Miller *et al.* 2011). Aging tissues throughout the body are affected by rapamycin, including the liver, adrenal glands, tendons, bone marrow, and heart (Chen *et al.* 2009; Wilkinson *et al.* 2012). Benefits of rapamycin on the aging heart included a reduced incidence of nuclear atypia in cardiomyocytes (Wilkinson *et al.* 2012).

Since the longevity effect of rapamycin was very similar after the drug was administered from 9 months of age or beginning at 20 months of age, it has been suggested that the cardiac benefits of rapamycin might be delivered to even older mice after briefer treatment. Flynn *et al.* demonstrated an improvement in the age-related loss of contractile function and a reduction in evidence of age-related sterile inflammation after rapamycin was administered to 24 month old female mice for 3 months (Flynn *et al.* 2013). In our laboratory, we observed that 10 week rapamycin treatment of 25 month-old mice conferred a substantial

reversal of diastolic dysfunction and cardiac hypertrophy, as well as an attenuation of age-related cardiac proteomic changes (Dai *et al.* 2014a). While the total proteome turnover of the aging mouse heart was not significantly different from young controls, the cardiac proteome had a significantly increased half-life after both CR and rapamycin treatment, concurrent with a reduction of detectable protein oxidation and ubiquitination. These results may point to proteome remodeling as a mechanism behind the cardiac functional benefits granted by rapamycin. We also found an age-dependent abundance decrease in proteins associated with young mitochondrial functional profile (electron transport chain, TCA cycle, fatty acid metabolism) and an increase in proteins that transition the mitochondria to a more glycolytic program. Short-term rapamycin and CR both reversed this phenotypic age-related change (Dai *et al.* 2014a).

5.1.3 Resveratrol—Resveratrol, a potential CR mimetic initially studied for its anticancer benefits, has enjoyed some popularity as an aging intervention. Resveratrol (3, 4′, 5-trihydroxy-*trans*-stilbène) is a phytoalex that is produced by plants as a reaction to stresses such as infection or injury (Baur 2010). Many studies point to resveratrol as an activator of sirtuins, modulating protein acetylation states (Wohlgemuth *et al.* 2014a). Sirt1 and Sirt3 and cause the nuclear translocation of phosphorylated FoxOs (Mukherjee *et al.* 2010), and this mechanism may underlie resveratrol's hormetic action in preconditioning that increases stress response pathway activation and autophagy (Petrovski *et al.* 2011). Other possible mechanisms of action include activation of AMPK and inhibition of cAMP phosphodiesterases (PDEs) (Chung 2012). In an ischemia/reperfusion model of Sprague-Dawley rats, a formulation of resveratrol with 5% quercetin plus 5% rice bran phytate was shown to protect cardiac performance and minimize infarct area, while also increasing autophagy. It has also been suggested that resveratrol can be combined with short term CR to potentiate autophagy in the hearts of 26-month-old rats and provide protection against doxorubicin-mediated cardiac toxicity (Dutta et al. 2014). However, a recent meta-study found that the published work on resveratrol has sufficient study variability, including dose ranges and methodological variability, to reduce the confidence in conclusions drawn from the clinical literature (Pollack & Crandall 2013).

5.2 GDF11

There is recent evidence that the circulating growth differentiation factor 11 (GDF11, also known as bone morphogenetic protein 11, BMP11) may contribute to myocardial aging (Loffredo *et al.* 2013). Using heterochronic parabiosis, a technique that joins the circulatory systems of two mice, in this case old to young, Loffredo and colleagues determined that age-related cardiac hypertrophy was abrogated in old mice due to a circulating factor originating from the young mice. They concluded that GDF11 was the factor responsible for the age-related hypertrophy reversal. Indeed, old mice given GDF11 had similar cardiac benefits (Loffredo *et al.* 2013). However, the mechanism by which GDF11 may be working remains unclear. GDF11 shares much homology with myostatin, and myostatin is a negative regulator of muscle mass (McPherron 2010). Also the homologous gene myoglianin has been shown to preserve muscle function in fly aging models, together suggesting that GDF11 may preserve cardiac homeostasis and other tissues (Patel & Demontis 2014).

5.3 Mitochondrial antioxidants

Mitochondria are critical for maintaining protein, lipid, and overall cellular quality control, and mitochondrial dysfunction is associated with aging tissue dysfunction. Moreover, as noted above, mCAT mice show considerable protection from the functional and biochemical effects of aging (Dai et al 2009). Consequently several pharmaceutical therapies have been proposed to target and improve mitochondrial function.

Triphenylphosphonium ion (TPP^+) conjugation is an effective method of targeting the mitochondria by using the potential gradient across the inner membrane to trap the molecules there at up to 100- to 1000-fold higher concentration than in the cytosol (Murphy & Smith 2007). An example is TTP^+ conjugated to coenzyme Q, a compound termed MitoQ (Smith *et al.* 2012). MitoQ and other TPP^+ conjugates have been shown to reduce systolic blood pressure and cardiac hypertrophy in rats (Graham *et al.* 2009; Dikalova *et al.* 2010), prolong lifespan in SOD-/- flies (Magwere *et al.* 2006), and be protective against AD and PD in rodent models (Ghosh *et al.* 2010; Manczak *et al.* 2010).

Plastoquinone conjugated to TPP+ (SkQ1) (Skulachev *et al.* 2009), is another strategy employed to reduce intracellular ROS and improve lifespan (Skulachev 2013). Both MitoQ and SkQ1 can reduce IR injury, but they may also inhibit oxidative phosphorylation and ATP production (Szeto 2014).

5.4 Cardiolipin-targeted therapeutics

SS-31 (Szeto-Schiller compound 31, H-D-Arg-Dmt-Lys-Phe-NH_2) is a tetrapeptide which preferentially targets and concentrates in the inner mitochondrial membrane (Szeto 2014). SS-31 has been the focus of several recent Phase I and Phase II clinical trials under the name Bendavia (Chakrabarti *et al.* 2013; Szeto 2014). Though it can scavenge free radicals such as H_2O_2 hydroxyl radical and peroxynitrite, the *in vivo* effects seem to be primarily due to its interaction with cardiolipin. Cardiolipin is an inner mitochondrial membrane phospholipid critical for maintenance of cristae structure and the formation of electron transport chain super complexes (Zhang *et al.* 2002; Pfeiffer *et al.* 2003), as well as anchoring cytochrome c at the inner membrane (Rytomaa & Kinnunen 1994; Rytomaa & Kinnunen 1995). This interaction is important for cytochrome c function, but cardiolipin also causes cytochrome c to unfold through hydrophobic interactions. Peroxidase activity of cytochrome c is then greatly increased, which can lead to cardiolipin peroxidation and subsequent loss of proper cristae structure and super complex stability (Basova *et al.* 2007; Wiswedel *et al.* 2010). Quality control mechanisms of the mitochondria are dependent on homeostatic ROS, and this disruption of electron transport function and organization leads to increased ROS formation by complex 1 (Maranzana *et al.* 2013). SS-31 binding to cardiolipin blocks the peroxidase activity of cytochrome c (Birk *et al.* 2014).

After a coronary artery ligation method of myocardial infarction, rats chronically receiving Bendavia were found to have reduced LV volume, scar area, and ROS production, and improved LV fractional shortening and ejection fraction (Dai *et al.* 2014b). Interestingly, this was accompanied by reduced apoptosis in the infarct border zone and maintenance of mitochondrial function and gene expression.

Our laboratory has shown that SS-31 attenuates Gαq overexpression-induced heart failure and reduced angiotensin-II induced LV hypertrophy and diastolic dysfunction (Dai *et al.* 2011a). It may also protect cardiac mitochondrial ultrastructure in a pressure-overload model of transverse aortic constriction by preserving most of the mitochondrial and non-mitochondrial cardiac proteome in the pre-overload state (Dai *et al.* 2013).

6. Conclusion

Quality control of genetic material, proteins, and cellular processes degrades with aging. In the heart, this progressive loss of maintenance mechanisms leads to clinically relevant cardiac dysfunction and a susceptibility to age-associated diseases. Many aspects of cardiac dysfunction manifest similarly in humans and other mammals, allowing the use of genetically altered and pharmacologically treated model organisms to dissect mechanisms of cardiac aging. We now know that molecular pathways that affect longevity also tend to affect cardiac healthspan. Pathways that respond to and modulate proteostasis, nutrient signaling, autophagy and mitochondrial maintenance are clearly important for CV health, and interventions that directly interact with these pathways are promising avenues for preserving optimal cardiac function. Through powerful new methods of investigating quality control mechanisms and cardiovascular dysfunction, in tandem with progress in interventions that modulate them, we can look forward to more therapies that directly influence cellular maintenance to improve cardiovascular health.

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