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Molecular mechanisms of cardiomyocyte aging

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Abstract

Western societies are rapidly aging, and cardiovascular diseases are the leading cause of death. In fact, age and cardiovascular diseases are positively correlated, and disease syndromes affecting the heart reach epidemic proportions in the very old. Genetic variations and molecular adaptations are the primary contributors to the onset of cardiovascular disease; however, molecular links between age and heart syndromes are complex and involve much more than the passage of time. Changes in CM (cardiomyocyte) structure and function occur with age and precede anatomical and functional changes in the heart. Concomitant with or preceding some of these cellular changes are alterations in gene expression often linked to signalling cascades that may lead to a loss of CMs or reduced function. An understanding of the intrinsic molecular mechanisms underlying these cascading events has been instrumental in forming our current understanding of how CMs adapt with age. In the present review, we describe the molecular mechanisms underlying CM aging and how these changes may contribute to the development of cardiovascular diseases.

Keywords

aging; apoptosis; cardiomyocyte; cardiovascular disease

INTRODUCTION

Aging is often thought of as a progressive disorder that decreases an organism's ability to maintain 'normostasis' and reproductive capacity, but the functional consequences of aging tend to be cumulative, organ-specific and species-dependent. Age is strongly correlated with a higher incidence of disease disorders such as cancers, diabetes, Parkinson's disease, Alzheimer's disease and dementia. Moreover, age is a major independent risk factor for cardiovascular-related morbidity and mortality [1,2]. In fact, heart and blood vessel diseases remain the greatest threat to health in the U.S.A. and are common disorders in the elderly that require long-term medical attention. According to the CDC (Centers for Disease Control and Prevention), 12% of adults (18 years of age) have some form of cardiovascular disease, including hypertension, stroke, cardiomyopathies and HF (heart failure), and there is a positive relationship between age and the presence of heart disease (including coronary heart disease), hypertension and stroke. In individuals <65 years of age, ~1 % suffer from HF [3]; however, heart-related disease syndromes reach epidemic proportions in the very old (80

years of age). In fact, more than 10% of the very old will be afflicted with some form of heart disease [3,4].

A number of functional and anatomic changes occur in the heart with age [1,5–8]. An increase in LV (left ventricular) size (hypertrophy) is not uncommon and, although the heart wall may not always thicken, the ventricular chamber diameter often differs from that found in young individuals, and the shape of the heart may change from ellipsoid to globular [9]. Although species-dependent, CMs (cardiomyocytes) decrease as a percentage of total cell number, but are often enlarged [10,11]. The volume of blood present in the heart chambers at end-diastole either increases or is unchanged, and LV end-systolic pressure may be decreased relative to those in ‘young’ hearts. Valves inside the heart often thicken and become stiffer, and there is usually an accumulation of ECM (extracellular matrix) proteins). The SA (sinoatrial) node may lose pacemaker cells, and the pacemaker/conduction system may become fibrotic and accumulate fat deposits. These maladaptive changes may lead to a slightly slower heart rate without an increase in stroke volume or overall cardiac output, which may, in fact, decrease. Moreover, heart function in older persons tends to be slightly different from that of younger adults, and arrhythmias occur at a higher frequency in the elderly [8]. More specifically, systolic function is generally maintained, whereas diastolic function exhibits a number of changes, largely as a result of slowed diastolic relaxation [9]. Despite these age-related changes, the aged heart in the absence of disease is generally able to adequately supply blood to all parts of the body. However, in response to stress (infection, emotions, physical exertion and some medications), the aged heart is less able to tolerate increased workloads and $\dot{V}_{O_{2max}}$ (maximum oxygen consumption) is reduced, both of which can lead to shortness of breath and a general decrease in the quality of life [8].

Contributing directly to these changes in heart function are CMs, which display a number of physiological and morphological features that are affected by age (Table 1). Altered CM function is thought to contribute to reduced heart function, reduced stress tolerance and possibly to the manifestation of a disease state. Included among these changes are a decrease in the total number of CMs due to necrosis and apoptosis [12–14], alterations in myocyte activation, contraction and relaxation, hypertrophy, and an inability to repair or replace lost cells in sufficient quantities to meet added functional demands [15]. The underlying cause for many of these phenotypes is molecular in nature and is often the result of cell stresses that occur during aging. With that said, it is important to distinguish between ‘normal or healthy’ aging and ‘abnormal or pathological’ aging of heart cells. The former relates to damage that accumulates with age, whereas the latter is generally due to genetic anomalies or secondary to vascular problems, which in the heart may result in cardiomyopathies, failing myocardium or other disease syndromes. Although these disease states may be pathological in nature, their manifestation often has an age-related component, which can be gender-dependent. Understanding the mechanisms underlying normal CM aging and the consequences that this has on heart pathologies are therefore essential to our understanding of the aging heart and, ultimately, to the prevention and treatment of age-associated disease syndromes. Finally, there is a question regarding when aging begins. Some would argue that it begins from conception or from birth, when the heart cells undergo profound structural and functional changes due to increased workloads. This is particularly true with regards to pathological aging due to genetic anomalies or due to malnutrition during early development

[16,17]. The aim of the present review is, however, to discuss changes that occur in heart cells during the process of normal aging from ages 20 to 80, as well as to describe some CM pathologies that are affected by an aging component. CM cell division, which has been proposed as an adaptive response with age, will, however, be discussed from the early stages of heart development, because much of what we know on this subject is derived from early cells.

EARLY MOLECULAR STUDIES AND THE GENE-CENTRIC APPROACH

Some of the earliest changes in cardiac gene expression associated with age involved proteins implicated in cardiac contraction and relaxation [1,18,19]. Many initial studies were based on whole heart samples obtained from animal models or from diseased human myocardium that often lacked normal non-affected human heart controls. In simpler model systems, data were often compared with developmental studies of the heart, which led to the hypothesis of a fetal-type gene programme that was re-activated in the hypertrophied or aged myocardium. The initial reports of altered expression invariably highlighted potential functional consequences, but years of continued investigation, including genetic analyses, have demonstrated that some of the original conclusions and conjectures were at times too conservative and at other times misleading. For example, the ‘fetal-type gene programme’ is not a ubiquitous feature of cardiac aging; consequently, the presence of these markers does not support the idea of a re-activated fetal-type gene programme. Still other findings have led to recent clinical trials that may help reverse some of the functional defects found in aged and failing human heart.

The earliest gene-centric analyses focused on MHC (myosin heavy chain) proteins, which provide structural integrity to CMs and are a major component of the thick filaments of the sarcomere. Cardiac MHC isoforms can exist as either homodimers or heterodimers composed of α - and β -subunits, encoded by separate genes located on chromosome 14 in mice and humans. More specifically, an isoform shift in MHC transcript/protein abundance and altered myosin ATPase activities were observed in aging myocardium in rodents (Figure 1A) and in the atria of humans [20–22]. Although the human heart contains primarily the β -MHC isoform, a small but significant amount of α -MHC is present. With rodent development, β -MHC gene function is transcriptionally down-regulated [23], but, with aging and in disease, gene activity is up-regulated. This activation is thought to be metabolically advantageous, due to decreased ATPase activity [24], and this finding formed the basis for the original ‘fetal-type gene programme’ hypothesis. Perhaps more importantly, mutations (e.g. R403Q) in the β -MHC gene were found to be responsible for ~45% of FHCs (familial hypertrophic cardiomyopathies) in humans [25]; however, patients with FHC demonstrate a high degree of clinical variability and age-dependency. Some individuals aged <14 years of age die suddenly, whereas others show progressive cardiac insufficiency. Some women, in particular, remain clinically stable and relatively asymptomatic up until their sixth to seventh decades of life, indicating that age-associated factors are implicated in its manifestation.

Sarcomeric actin isoforms (α -cardiac and α -skeletal), critical components of the thin filaments, also undergo developmental changes in the human heart, and the transcripts encoding these proteins show significant decreases with aging [26]. In fact, α -skeletal actin

mRNA increases in the human heart during development and it is the major transcript present in control and failing adult hearts. Studies made possible by the development of isoform-specific antibodies have, however, revealed that α -cardiac is the predominant sarcomeric isoform in human donor hearts [27]. Actin transcription may therefore be implicated in the aging process and disease states, but, on the basis of protein data, any possible aging-associated consequences of actin isoform switches remain unclear.

Myocardial relaxation is slowed with age. This is in part due to functional changes in the SERCA2 [SR (sarcoplasmic reticulum)/ER (endoplasmic reticulum) Ca^{2+} -ATPase 2] pump, the protein responsible for pumping cytosolic $[\text{Ca}^{2+}]$ into the SR. In its unphosphorylated form, the regulatory protein PLB (phospholamban) represses SERCA2 activity, but, following phosphorylation by either PKA (protein kinase A) or CaMKII (Ca^{2+} /calmodulin-dependent protein kinase II) at Ser¹⁶ and Thr¹⁷ respectively, this inhibition is relieved. The ability of PLB to undergo cAMP-mediated phosphorylation and the relative responsiveness of SERCA2 to PLB phosphorylation does not appear to be diminished in the aged heart; however, SERCA2 ATPase activity, protein content and transcript abundance are reduced [19,28–31]. Consequently, the ratio of SERCA2a to PLB is decreased in senescent human and mouse myocardium [31,32]. Expression changes in NCX1 ($\text{Na}^+/\text{Ca}^{2+}$ exchanger) that may affect relaxation also occur with aging. More specifically, we have reported a significant increase in transcripts at 24 months when compared with 6- and 18-month-old animals [35]. Age-related changes in forward NCX1 protein exchange activity have, however, been inconsistent [19,33,34], and several groups have been unable to demonstrate altered transcript abundance as a function of age [29]. Using a Tg (transgenic) mouse model containing the rat *Ncx1* promoter upstream of a *LacZ* (β -galactosidase) gene cassette ([35], and K.R. Boheler and D.R. Riordon, unpublished work), we have observed an increase in both the amounts and tissue staining of β -galactosidase in ‘old’ Tg mice (19–24 months of age) (Figure 1B and 1C). These findings suggest that the promoter can be transactivated in response to aging, thus supporting a molecular basis to biological aging of heart.

Following these focused studies on contractile and lusitropic-associated proteins, additional cardiac transcripts and proteins were found to be altered with aging or disease [36]. In rats, aging was found to be accompanied by significant increases in the mRNA levels of IP₃R (inositol 1,4,5-trisphosphate receptor) and TRPC (transient receptor potential canonical) channels in both ventricles and atria, but mRNA levels of the type 2 SR calcium release channel (ryanodine receptor-2) were unchanged [37]. Numerous transcripts encoding factors associated with signalling pathways are also altered [38]. AT₁R and AT₂R [AngII (angiotensin II) receptor subtypes 1 and 2 respectively] [39], COX-2 (cyclooxygenase 2), β -arrestin and the transcription factor NF- κ B (nuclear factor κ B) increase with age, whereas cardiac transcripts/proteins to the M₂-cholinoreceptor, ET_A receptor [ET (endothelin) receptor subtype A] [40], β_1 -adrenergic receptor, the α -subunit of G_s proteins, adenylate cyclases and the oestrogen receptor diminish with age [4,41–43]. Transcripts to collagens type I and III decrease, but some changes are chamber-specific [44,45]. ANP (atrial natriuretic peptide) and BNP (brain natriuretic peptide) [19] have also been identified as excellent markers of aging-associated hypertrophy [46], and both plasma levels and the amplitude of cardiac secretion are increased with aging [47]. Many mitochondrial-encoded transcripts are also reduced, followed by a decrease in mitochondrial function with age. Both

of these latter changes can be attributed to accumulated deletions within mitochondrial genomic DNA and an increased rate of mitochondrial loss that varies among tissues, which may also have important implications for longevity (see below) [48,49].

TRANSCRIPTOME ANALYSES

Although targeted analysis of individual genes has been informative, molecular mechanisms underlying cardiac aging, using genome-wide transcriptome analyses, have yielded unique insights [50]. Most microarray studies have relied on samples from whole heart or ventricles, whereas a few have compared RNA profiles of CMs isolated from young and old animals. Using isolated CMs from 4- and 20-month-old male C57B1/6 mice, Bodyak et al. [51] identified 43 gene transcripts that were quantitatively and significantly altered as a function with age. Examples of quantitative differences included transcripts for α -MHC, SERCA2 and α -actins, and several transcripts implicated in the mitochondrial electron transport system. Reduced mRNA levels for several transcription factors (e.g. Nkx2.5 (NK2 transcription factor related locus 5), GATA4 and JunB) implicated in aging were also reported. In contrast, Lee et al. [52] found that $\approx 10\%$ of all transcripts in whole hearts from B6C3F1 mice showed significant changes with aging. The difference between the two studies suggests that some age-associated changes in transcripts seen in whole hearts may be non-CM in origin. Consistent with this conclusion, we have shown in our AGEMAP (Atlas of Gene Expression in Mouse Aging Project) study that the aging heart demonstrates distinct gene clustering patterns similar to that seen in highly vascularized tissues, such as the lung and spleen [53], suggesting that many of the reported inflammatory changes are more closely associated with tissue inflammation than with CMs themselves.

Despite the question of cell origin, four major findings have been generated from genome-wide transcriptome analyses. First, and as expected, cardiac transcript abundance varies significantly with aging. Although it is generally presumed to be due to altered gene expression/function, the data with sarcomeric actins (see above) show that such an assumption is often inappropriate (i.e. RNA changes are not always associated with similar protein changes). Moreover, altered transcript abundance is not necessarily a function of changed gene activity. Instead, it can be due to altered transcript stability or degradation. Secondly, changes in transcript abundance are species-specific and strain-dependent (see below). In AGEMAP, for example, 23 genes had age-sensitive expression in the heart, but only one gene group, the electron transport chain genes, showed commonalities in gene expression with studies from worms, flies, mice and humans [53]. Moreover, organ-specific differences have been observed [53–55]. Thirdly, a majority of cardiac gene transcripts significantly altered with aging appear to be increased ($\sim 83\%$) [54]; however, microarray analyses often fail to detect changes in RNA abundance of less than 2-fold. Consequently, this sort of analysis is biased towards increased expression. Fourthly, decreases in transcript abundance often reflect changes in either cell type and cell function or represent an adaptive response designed to preserve function or energy (e.g. reduced protein biosynthesis and proteasomal genes) [52,54,56].

Species- and strain-dependent differences in transcript abundance are now well established, but the interpretations are still the subject of vigorous debate. Park et al. [57], for example,

compared seven mouse strains (129sv, BALB/c, CBA, DBA, B6, C3H and B6C3F1) aged 5 and 25 months using an array that assayed 22626 gene transcripts. They found 15% of transcripts were altered in the 129sv strain, but only 6% in the C3H and CBA mouse strains, with aging. Remarkably, only one gene product (CoA hydratase 1) decreased among the seven mouse strains, whereas five other gene products [complement component 4, Cxcl14 (CXC chemokine ligand 14), Skap2 (Scr family associated phospho-protein 2), phenylalanine hydrolase and Sp100-rs] consistently increased. The majority of significant changes were not conserved across these strains, perhaps due to lifespan variations, which are well-documented between genetically homogeneous groups of mice. The 129 strain, for example, has an average lifespan of 850 days, whereas DBA mice only live on average 560 days [58]. As the age groups were not normalized to expected lifespan, the examination of animals at only one age (25 months) may therefore be misleading. To overcome this limitation, we have normalized transcriptome data as a function of average lifespan in three strains of rats, but surprisingly we only found one insulin-responsive transcript [Insig2 (insulin-induced gene 2)] implicated in fatty acid synthesis, one ubiquitin-associated transcript implicated in the degradation of p53 [Ube3a (ubiquitin protein ligase E3A)] and two heat-shock proteins [Hspa9a, which binds and sequesters p53, and Hspd1 (chaperonin)], which appear to be informative of cardiac aging independent of strain (K.R. Boheler and A. Sheydina, unpublished work). Strain-dependent changes normalized to absolute lifespan may not therefore fully account for the changes observed to date. Alternatively, variables such as sex also influence large-scale gene expression array analyses. For example, we have demonstrated that many putative HF-responsive genes in humans are in fact highly dependent on variables such as age and sex. Some, such as Pin1 [protein (peptidyl-prolyl *cis/trans* isomerase) NIMA-interacting 1], are age- and sex-dependent, but HF-independent (Pin1), whereas others [Ngp1 (guanine nucleotide-binding protein-like 2), Tgm2 (transglutaminase 2), Cdl63 and Map2k3 [MAPK (mitogen-activated protein kinase) kinase 3] are principally sex-responsive [59].

Overall, transcriptome analyses have strongly implicated genes associated with inflammatory responses, mitochondrial death signalling and decreased mitochondrial function (see below), cytoskeletal and structural proteins involved in CM hypertrophy, survival and growth (e.g. insulin signalling) [55,60]. Metabolic shifts from fatty acid toward carbohydrate metabolism (e.g. reduced carnitine palmitoyl transferases 1 and 2, carnitine acetyltransferase and triacylglycerol hydrolase, and induced phosphofructokinase and pyruvate dehydrogenase kinase isoenzyme 4) and decreased protein biosynthesis (e.g. elongation factor 2 and numerous ribosomal-associated proteins) have been reported [50–52,55,61]. Glutathione metabolism and insulin signalling appear to be ‘up-regulated’, whereas oxidative phosphorylation was generally ‘down-regulated’ [4,55]. Gene transcripts encoding Hsps also consistently show increased expression in heart. Many of these conserved changes between both the individual gene- and whole-genome-based studies are associated with cell stress and cell death, suggesting that these processes may be a major factor underlying the molecular mechanisms of CM aging and organ dysfunction. Of particular interest are mitochondrial defects and separately those genes that regulate cell proliferation, senescence and death.

MOLECULAR MECHANISMS UNDERLYING CM ADAPTATIONS WITH AGE (see Figure 2)

Proliferation

During mammalian heart development, increases in cardiac mass are due initially to differentiation and subsequent proliferation of CMs. During the fetal period, the rate of mitosis decreases and, in some animal models (e.g. sheep), hypertrophy becomes a major contributor to cardiac mass [62,63]. Perinatally, CMs undergo significant reductions in their proliferative capacity and undergo substantial hypertrophy, in part due to the increased functional demands placed on the heart. In neonatal mice, DNA synthesis can be attributed primarily to karyokinesis without cytokinesis (i.e. bi-nucleation), supporting the idea that CMs essentially withdraw from the cell cycle [64]; however, ^{14}C dating in humans has suggested a very low, but continuous, generation of CMs in the adult [65]. The intrinsic proliferative capacity of terminally differentiated adult CMs thus appears to be limited to less than 50% turnover in humans to non-existent in some animal models. Given the proliferative capacity of non-myocytes, the numbers of CMs decrease as a percentage of total heart cell number from ~40% to 20–30% in the adult rodent [64,66,67].

The molecular basis responsible for the transition from proliferating to quiescent cells is poorly understood. Functionally, we have shown, using an *in vitro* differentiation model, that early mouse CMs are initially highly proliferative, and in a pRb (retinoblastoma protein)-dependent manner, the cells withdrew from the cell cycle, underwent bi-nucleation and exhibited molecular traits consistent with contact inhibition [68,69]. Knockdown of pRb restored proliferation in early post-proliferative cells. In agreement, but in adult animals, the deletion of pRb and the related family member p130 led to a >100-fold increase in DNA-synthesizing cells as compared with control animals [70]. Similarly, adult mice expressing cyclin D proteins demonstrated an ~200-fold increase in DNA synthesis in CMs when compared with non-Tg control animals [71,72]. Moreover, overexpression of CDK (cyclin-dependent kinase) 2, which acts downstream of CDK4 and CDK6, promoted CM DNA synthesis by ~100-fold relative to littermate controls [73]. These experiments show that molecules that regulate the G₁ restriction point in the cell cycle are sufficient to drive CM DNA synthesis in adult hearts. Problematically, and as described above, most adult CMs from humans and mice do not proliferate, and any mitotic potential is insufficient to repair myocardial tissue following injury or with aging.

More recently, several studies have suggested that a fraction of endogenous cardiomyogenic stem or progenitor cells undergo proliferation and differentiation. Consequently, not all CMs in the adult heart may originate from the perinatal period, but, as described above, there may be species differences. Cardiomyogenic stem and progenitor cells have been described elsewhere, and the reader is encouraged to see, for example, [74] for further details. Although these cells may serve as a replacement pool for cardiac repair after myocardial injury and potentially with aging, the origin of the cells and their contribution to total cell numbers is still a matter of substantial debate and the mechanisms of activation are wholly unclear. Again, any contribution to myocardial repair following injury is insufficient to fully repair damaged myocardium.

Hypertrophy

In the absence of cell proliferation, cellular hypertrophy is the response of choice to meet increased functional demands and workload during the perinatal period and separately in the adult [15,75]. Moreover, CM hypertrophy appears to be the adaptive mechanism of choice and the direct result of CM loss with aging. Multiple activators have been implicated in cardiac hypertrophy, including neurohumoral factors, peroxisome proliferator activation in response to fatty acid oxidation and biomechanical stress, which results from increased workload. Neurohumoral factors known to induce CH include catecholamines, AngII, ET-1 and IGF-1 (insulin-like growth factor-1) [76], whereas biomechanical stress is sensed by the cell through a variety of mechanisms that involve stretch-sensitive ion channels, integrins and cytoskeletal proteins. Several neurohumoral factors mediate hypertrophic responses via receptor tyrosine kinases, but a majority activate G-protein-coupled receptors such as G_{aq} . Many of these receptor signals converge on calcium signalling. Changes in intracellular calcium promote activation of calcium-dependent protein kinases, phosphatases or MAPKs. These in turn activate the ERK1/2 (extracellular-signal-regulated kinase 1/2), the calcineurin/NFAT (nuclear factor of activated T-cells) pathway and the CaMKII/HDAC (histone deacetylase) pathway [76,77]. The molecular consequences of altered calcium signalling are profound. Transcription factors such as NFAT, GATA4, and MEF2 (myocyte enhancer factor 2) are up-regulated in response to calcium or hypertrophy [78–80], and these regulate the expression of several cardiac genes encoding α -MHC, ANP, troponins, SERCA2, Mlc2v (myosin light chain-2V), desmin and calcineurin. Activation of the calcineurin/NFAT pathway also leads to increased abundance of calcium-handling proteins (SERCA2, NCX1 and PLB) [81]. An apparently calcium-independent pathway of hypertrophy induction up-regulates Myc, Fos and JunB transcription factors, which are involved in 5S rRNA, U6 snRNA (small nuclear RNA) and precursors for tRNAs. Both ERK and c-Myc activate polymerase III transcription in CMs, as well as ribosomal protein S6 kinases, eukaryotic initiation factor 4E-binding protein 1 and the translation elongation factor eEF2 proteins [82–84]. A complete discussion of the molecular responses to altered intracellular calcium is beyond the scope of the present review, but additional information is available (for example, see [85]). It is generally believed that the same mechanisms that cause hypertrophy in normal young animals are utilized in the aged; however, the ability to hypertrophy is lost in senescent CMs [86,87].

Senescence

Cellular senescence is characterized by loss of replicative capacity in primary somatic cell cultures or *in vivo*, and it has been linked to the shortening of telomeres [88–91]. Telomeres are tandem repeats (TTAGGG in humans) located at the end of chromosomes that are shortened with each cell division. Telomerase, in turn, adds sequence repeats ('TTAGGG' in all vertebrates) to the ends of the telomeres to prevent shortening; however, this enzyme is poorly expressed in most adult somatic cells. Up until recently, cellular senescence was not considered a normal fate for CMs because these cells were believed to withdraw from the cell cycle early in life. The finding that CMs, particularly in humans, may be able to proliferate has, however, called this assumption into question. Moreover, telomerase activity in rat CMs is age- and gender-dependent [92].

Data in humans and animals suggest that myocyte maturation and aging are in fact characterized by loss of replicative potential, telomere shortening and the expression of the age-associated proteins such as p16^{INK4a} [86]. Surprisingly, both senescent (p16^{INK4a}-positive shortened telomeres) and non-senescent myocytes are present in the mouse heart at 3 months of age [93], thus underscoring the heterogeneity in CM populations. From *in vitro* studies, fetal human CMs undergo cellular senescence in approximately 20–25 passages, but this senescence is independent of telomere shortening [94]. Telomere shortening in mice deficient for telomerase is, however, associated with attenuated CM proliferation, cardiac remodelling, and increased apoptosis and CM hypertrophy, traits generally seen during aging. Eventually LV failure, analogous to end-stage dilated cardiomyopathy in humans, develops in these Tg mice, suggesting that telomere shortening with increasing age in human hearts could be a critical biological determinant of HF in the elderly [95]. In mice, forced expression of TERT (telomerase reverse transcriptase) in heart prevents telomere shortening. Overexpression of TERT also increases CM density and DNA synthesis through to 12 weeks of age, followed by cellular hypertrophy without fibrosis or impaired function [96]. Overexpression of telomerase thus delays cell-cycle withdrawal, induces hypertrophy in postmitotic cells and promotes CM survival. Moreover, short telomeres and decreased telomerase activity have been implicated in the activation of cellular damage pathways and, ultimately, in cellular dysfunction, senescence and apoptosis [97]. CM senescence and loss of telomerase activity may therefore be an important molecular factor contributing to the aging heart.

Caspase-dependent apoptosis

Apoptosis is an evolutionarily conserved, energy-dependent and highly regulated mechanism that is essential to normal development, maintaining the correct balance of cells and organ function. Morphologically, apoptosis is characterized by cellular shrinkage, chromatin condensation, DNA fragmentation and endocytosis of the dead cell by neighbouring cells. It is initiated by intrinsic or extrinsic mechanisms that trigger caspases and lead to apoptosome formation. The intrinsic pathway is turned on by mitochondria and ER through release of peptide factors such as AIF (apoptosis-inducing factor), Endo G (endonuclease G), Smac (second mitochondrial-derived activator of caspase)/Diablo [direct LAP (inhibitor of apoptosis)-binding protein with low pI] and cytochrome *c*. This pathway is regulated by the Bcl-2 (B-cell leukaemia/lymphoma-2) family of proteins, which may be anti-apoptotic (Bcl-2 and Bcl-xL) or pro-apoptotic (Bax and Bid) [98,99]. The extrinsic pathway is activated via binding of death receptors, such as FasL (Fas ligand), TNF (tumour necrosis factor)- α or TRAIL (TNF-related apoptosis-inducing ligand), with activation caspases 8/10 and usually requires signal amplification at the mitochondria by cleavage of BH3 (Bcl-2 homology domain 3) [100–102].

In the aged heart, CMs are susceptible to apoptosis [103]. Under normal conditions, as many as 30% of CMs are lost as a function of aging [10]. The molecular mechanisms responsible for programmed CM cell death are diverse, and the precise cause of CM apoptosis in healthy aging heart is not fully understood. It may result from altered gene expression in response to cellular stresses that up-regulate some pro-apoptotic genes. Alternatively, the stochastic accumulation of DNA mutations, and protein errors and misfoldings elicit ER stress that

activates the caspase cascade. Results supporting the first possibility are available from Tg and disease models. Cardiac-specific TNF- α overexpression, for example, activates both pro- and anti-apoptosis pathways in mouse myocardium, primarily in non-CMs [104], but overexpression of secreted TNF in mice increased CM apoptosis and led to a progressive loss of Bcl-2 [105]. Low serum levels of IGF-1 are also associated with an increased risk of HF in healthy aged individuals [106]. In contrast, overexpression of IGF-1 restores calcium dynamics and is anti-apoptotic [107]. Moreover, IGF-1 signalling has been implicated in the general aging response and is known to regulate the expression of numerous gene targets that mediate stress resistance, immunity, metabolic processes and toxin degradation [108].

Mechanistically, a variety of genotoxic stress pathways (including telomere- and p53-mediated DNA damage signalling) contribute to the aging process through their regulation of mitochondrial function and gene expression [109,110]. ROS (reactive oxygen species)-related stress in particular accelerates the senescence of CMs, and oxidative stress damage to mtDNA (mitochondrial DNA) in the heart has been shown to be inversely related to maximum lifespan [48,111–113]. Long-term accumulation of oxidative stress damage, independent of pathological aging, results in mitochondria that are enlarged, structurally disorganized, energy-production-deficient and prone to ROS leakage. Mutations to mtDNA, perhaps due to enhanced superoxide generation by Complexes I and III, disrupt mitochondrial protein biosynthesis and electron transport chain function [114]. These mutations also lead to altered gene expression, consistent with the transcriptome analyses described above. Moreover, the presence of a mixture of more than one type of mitochondrial DNA in a cell (i.e. heteroplasmy) resulting from these mutations can compromise mitochondrial energy generation. Symptoms of severe heteroplasmic mitochondrial disorders generally do not appear until adulthood because many cell divisions with concomitant mutant allele amplification are required to cause symptoms. If severe, as in advanced aging, energy generation may be reduced below threshold levels necessary to meet the demands of physiological myocardial functions, thus contributing to the development of cardiomyopathies [115–117]. Separately, a ROS-mediated pattern of mitochondrial dysfunction has been observed in adult CMs that involves the induction of the mPTP (mitochondrial permeability transition pore), which is a central regulator of cell dysfunction and death [118]. mPTP is the main target of ischaemia/reperfusion-related ROS injury, and its activation rapidly leads to programmed cell death in CMs. Moreover, the oxidant stress threshold for induction of the mPTP is substantially reduced in CMs from aged compared with young adult rats [113,119]. Mitochondrial dysfunction due to mutations, altered gene expression and reduced thresholds is therefore a leading cause of CM apoptosis, which ultimately contributes to the aging heart phenotype.

Caspase-independent apoptosis

Caspase-independent apoptosis represents an alternative pathway for cell death in heart. Autophagy, or self-eating, is essential for the functional integrity of CMs [120,121]. It is best known as a survival response to starvation that liberates amino acids and non-esterified 'free' fatty acids, but in non-starved cells it is also a mechanism for the degradation of damaged long-lived proteins and organelles. Because CMs are long-lived and have high energy requirements (oxidative stresses), mitochondria are a major target of autophagy. To

induce caspase-independent programmed cell death, AIF and Endo G or HtrA2/Omi (high temperature requirement protein A2) must be released from the inner membrane of mitochondria and translocate to the nucleus to induce DNA fragmentation. Although poorly studied in the heart, this mechanism may be very important during the late remodelling stage of pathological HF. AIF is of particular interest to the aging heart, because it is activated in response to oxidative stress, ischaemia/reperfusion and HR [121–124], and the Hq (harlequin) mouse strain, which contains a proviral insertion in the AIF gene, is a model of premature aging. In these mice, AIF is reduced by 80–90% in all tissues, including the heart [125]. Phenotypically, Hq mice are characterized by mitochondrial dysfunction and an increased risk of oxidative-stress-induced heart disease. Elevated ROS levels have also been reported, but no significant increase in the frequency of point mutations in nuclear DNA due to elevated ROS has been observed in the hearts from these mice [126,127]. In contrast, elevated mutations have been documented in the brains and skin of these animals. This would suggest that either ROS-mediated DNA damage is effectively repaired in the heart or that apoptosis or autophagy effectively removes cells or organelles that harbour DNA mutations throughout the accelerated period of aging observed in these animals.

INCREASED LONGEVITY

The critical importance of mitochondria and oxidative stress to normal CM aging and death has been demonstrated molecularly through the use of Tg animals. Tg mice with cardiac-restricted overexpression of catalase, an enzyme that metabolizes H_2O_2 into water and oxygen, decrease CM protein damage and reduced contractile dysfunction relative to controls. Perhaps more importantly, these animals have an extended lifespan (3 months longer) relative to control littermates [110,128]. Using a different Tg model system, Schriener and co-workers [129,130] targeted catalase overexpression to peroxisome, nuclei and mitochondria. Importantly, mitochondrial localization of catalase led to a pronounced increase in lifespan (20 % relative to controls), a strong cardioprotective effect and a reduction in the severity of common pathologies usually associated with aging myocardium [130]. These findings support the theory that ROS generation and mechanisms designed to reduce ROS in CMs play an essential role in cardiac aging [129,131].

Insulin resistance promotes ROS formation and is generally considered to be pro-inflammatory. Over-expression of IGF-1 in Tg mice, however, significantly prolongs lifespan and protects CMs from protein damage and apoptosis [107]. Altered cardiac expression of PI3K (phosphoinositide 3-kinase), a downstream effector of insulin receptor signalling transduction [132], also influences lifespan. In heart, enhanced PI3K expression significantly delays the onset of aging symptoms and prolongs survival, whereas reduced PI3K dramatically accelerates end-of-life cardiac phenotype and shortens the lifespan in mice [133]. Activation of this pathway may therefore counteract some of the deleterious effects associated with defective mitochondria.

Finally, cardiac-specific changes in two NAD^+ -dependent protein deacetylase proteins, SIRT (sirtuin) 1 and SIRT7, dramatically affect the cardiac aging phenotype. Although these proteins are not directly associated with ROS generation, the NAD^+ /NADH ratio mediates the SIRT1/PGC1 α (peroxisome-proliferator-activated receptor γ co-activator 1 α) pathway

for mitochondrial biogenesis and function. Moreover, cardiac-specific overexpression of SIRT1 delays aging of the heart in a dose-dependent manner [134,135]. Low (2.5-fold) to moderate (7.5-fold) overexpression attenuates age-dependent increases in cardiac hypertrophy, apoptosis and fibrosis, cardiac dysfunction, and expression of senescence markers. Moderate overexpression of SIRT1 also protects the heart from oxidative stress and increased expression of antioxidants, such as catalase, and retarded aging of the heart. In contrast, a high level (12.5-fold) of SIRT1 increased hypertrophy, enhanced apoptosis and augmented oxidative stress in the heart at baseline. Ultimately, cardiac function was decreased and a cardiomyopathy developed. Inactivation of SIRT7 reduces Tg mouse mean lifespan by approximately 50%, and the animals develop hypertrophy and inflammatory cardiomyopathy. SIRT7-mutant hearts are also characterized by increased collagen III deposition and extensive fibrosis. Sirt7-deficient primary CMs show an approximately 200% increase in basal apoptosis and significantly diminished resistance to oxidative and genotoxic stress [136]. Thus NAD⁺-dependent protein deacetylase proteins play a critical role in the regulation of cardiac stress responses and cell death.

RECENT AND EVOLVING INSIGHTS INTO CM AGING: EPIGENETIC REGULATION

The progressive impairment of cardiac function with time is thought to have primarily a genetic or an environmental component. Other molecular modifiers are, however, likely to play an essential role in determining heart function with aging. Epigenetics, for example, is the study of heritable changes in phenotype and gene expression due to mechanisms that do not involve changes to the underlying DNA sequence. Epigenetic modifications involve chemical and structural modifications of DNA or histones, and miR (microRNA) regulation of transcription or RNA stability. Recently, several of these pathways have been implicated in cardiac aging, but, generally speaking, this line of investigation is in its infancy.

One known epigenetic modification involves DNA methylation, i.e. the enzymatic addition of methyl groups to cytosine residues within palindromic CpG dinucleotide sequences or CGIs (CpG islands) [137,138]. Methylation can affect gene transcription by altering the binding affinity of transcription factors and other binding factors to DNA sequences. Sequence specific MBPs (methyl-CpG-binding proteins) can also act as transcriptional repressors, and sequence-independent MBPs may recruit protein complexes to sites of methylation to promote chromatin remodelling [139]. High levels of methylation are generally associated with repressed gene function and these patterns of DNA methylation are tissue-specific [138]. Overall DNA methylation may decline with aging, whereas CGI methylation may increase [138,140–142]. The number of studies implicating DNA methylation in heart aging is currently limited; however, available evidence suggests that DNA methylation regulates gene expression in the heart. For example, CpG-rich sequences appear to be methylated at a relatively young age in the heart [140]. Individual genes, however, show differential methylation with aging. An age-related increase in ER α gene methylation occurs in human right atrium [143], and DNA methylation of 5-LOX (5-lipoxygenase) has been reported in mice. Methylation of the oestrogen receptor gene generally leads to inactivation, whereas DNA methylation of 5-LOX is specifically increased

in the mouse heart with aging. This latter increase is correlated with decreased 5-LOX mRNA content [141], suggesting that increased DNA methylation directly leads to reduced levels of 5-LOX expression.

Another form of epigenetic regulation involves miRs [144,145]. miRs are short RNA molecules, averaging 22 nt in length, found in all eukaryotic cells. The mouse and human genomes are thought to contain 500 and 800 miRs respectively (<http://microrna.sanger.ac.uk>), and many of these function as post-transcriptional regulators of gene expression. To date, most studies of miR function in the heart have focused on development or have relied on targeted loss of the miR that occurs embryonically or in stem cells [145,146]. A few studies have, however, demonstrated that miRs are critical to normal cardiac function in the adult. Using an inducible system to knock out *Dgcr8* (DiGeorge syndrome critical region gene 8) in adult animals, Rao et al. [147] have shown that *Dgcr8*-deficient mice develop a dilated cardiomyopathy phenotype, which pathologically is very similar to that of surviving *miR133a-1/miR133aa-2*-knockout animals. Mice with these mutations that also survive to 2 or 4 months of age develop fibrosis and have thinned ventricles. The dilated cardiomyopathy observed in *Dgcr8*-knockout mice was also similar to the one reported in cardiac-specific *Dicer*-deficient mice [148]. *Dicer* is an RNase III endonuclease required for processing of pre-miRs into mature 22-nt miRs, and its loss should have affected all miR processing in the heart. Loss of *Dicer* in young animals induced biventricular enlargement, myocyte hypertrophy, myofibre disarray, ventricular fibrosis and the induction of some gene transcripts normally seen in fetal hearts. Lethality also occurred within 1 week of induced loss of *Dicer*, and this lethality was much faster than that observed in the *Dgcr8*-knockout mice. Dysregulation of miR expression or function thus can contribute to cardiac functional deterioration and, consequently, any dysregulation that may occur during aging is likely to affect the myocardial phenotype.

CONCLUSIONS

The pioneering molecular studies and subsequent genome-wide analyses of CMs have been invaluable to our understanding of the molecular mechanisms of cardiac aging. Although transcriptome analyses, in particular, led to a broader view of cardiac aging, many of the reported changes in transcript abundance are not conserved among all species and strains. The findings do, however, directly implicate and confirm previous molecular and genetic links to mitochondria and inflammation. These changes are conserved among mammalian species and provide a molecular basis for CM senescence and apoptosis in aging. Since CMs are poorly regenerative, surviving CMs receive signals that cascade until the cell has hypertrophied. As very few cells are replaced, more and more adaptations take place to meet the mechanical demands placed on the heart. The result is a positive-feedback loop that can lead to additional cell death and inflammation. This self-sustaining cycle ultimately leads to functional deficits that are unable to deal with additional stresses in advanced aging (Figure 2).

Importantly, the findings that we have described do not fully explain the molecular basis of aging myocardium. Clearly epigenetic regulation is going to be involved, and this avenue of research is likely to be very important to future analyses of the aging myocardium. Increased

CGI methylation will reduce the transcriptional activity of some genes, and miRs can have wide-ranging effects that are only beginning to be elucidated. Genome-wide association studies are also going to play a significant role in the identification of genes that are genetically implicated in heart dysfunction and aging phenotypes. Some of the findings will lead to the discovery of mutations (e.g. β -MHC) that contribute to cardiomyopathies and aging. Others will lead to the discovery of gene interactions that will affect function and survival of CMs. Moreover, specific protein adaptations, glycosylation events, the accumulation of protein aggregates [RAGE (receptors for advanced glycated end products) and lipofuscin], inflammation and fibrosis also contribute significantly to the aging heart phenotype [2,44,149–151]. Although we have focused on CMs, an understanding of molecular dynamics responsible for these adaptations is essential if we are to fully understand the molecular basis of myocardial aging.

In conclusion, significant insight into the molecular basis of cardiac aging has been made over the past 30 years. Early studies focused primarily on cataloguing changes in gene transcript abundance, and genetic studies have provided tremendous insight into the cause of some aging processes. The use of Tg models has recently opened the door for potential therapeutic interventions that may preserve CM cell number and function, whereas others have shown that improved function (SERCA activity) may be therapeutically applicable to aging and diseased myocardium. As impaired heart function strongly influences mortality and quality of life, it is likely that continued molecular advancements in the field of cardiac aging will lead to improved therapies that will reduce morbidity and improve the quality of life of a rapidly aging population.

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Abbreviations

AGEMAP	Atlas of Gene Expression in Mouse Aging Project
AIF	apoptosis-inducing factor
AngII	angiotensin II
ANP	atrial natriuretic peptide
BNP	brain natriuretic peptide
CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase II
CDK	cyclin-dependent kinase
CGI	CpG island

CM	cardiomyocyte
Dgcr8	DiGeorge syndrome critical region gene 8
ECM	extracellular matrix
Endo G	endonuclease G
ER	endoplasmic reticulum
ERK	extracellular-signal-regulated kinase
ET	endothelin
FHC	familial hypertrophic cardiomyopathy
HF	heart failure
Hq	harlequin
Hsp	heat-shock protein
IGF-1	insulin-growth factor 1
5-LOX	5-lipoxygenase
LV	left ventricular
MAPK	mitogen-activated protein kinase
miR	microRNA
MBP	methyl-CpG-binding protein
MHC	myosin heavy chain
mPTP	mitochondrial permeability transition pore
mtDNA	mitochondrial DNA
NCX1	Na ⁺ /Ca ²⁺ exchanger
NFAT	nuclear factor of activated T-cells
PI3K	phosphoinositide 3-kinase
Pin1	protein (peptidyl-prolyl <i>cis/trans</i> isomerase) NIMA-interacting 1
PLB	phospholamban
pRb	retinoblastoma protein
ROS	reactive oxygen species
SR	sarcoplasmic reticulum
SERCA2	SR/ER Ca ²⁺ -ATPase 2

SIRT	sirtuin
TERT	telomerase reverse transcriptase
Tg	transgenic
TNF	tumour necrosis factor
$\dot{V}O_{2\max}$	maximum oxygen consumption

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Figure I. Transcriptional changes in cardiac gene expression with age

(A) Time course of α - and β -MHC mRNA expression in left ventricles of aging Wistar rats ($n = 4-6$ per time point). (B) Expression of the *ncx1*-promoter-driven gene reporter protein, β -galactosidase (Beta-Gal), in aging Tg mouse hearts. Quantitative Western blot data showing a significant ($P = 0.012$) 6-fold increase in β -galactosidase protein in old hearts (19–20 months) compared with adult hearts sampled at 12 months of age. Inset, representative Western blots for β -galactosidase (25 μ g of total heart homogenate). Ctr, control. (C) Heart sections (20 μ m thick) showing the localization of β -galactosidase staining (blue) in portions of the right atria (RA) and ventricle (RV), as well as the

ventricular septum (VS) in adult (10 months) compared with old (24 months) NB mice. The thin arrows indicate regions of heart conduction tissue which maintain high levels of β -galactosidase-positive staining throughout adult life, whereas the thick arrows indicate regions with enhanced transgene expression in the aged myocardium, mo, months.

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Figure 2. Schematic diagram showing CM growth characteristics and how the loss of proliferation or survival promote an aging phenotype

Early CMs readily proliferate, but, during the peri-natal period, the cells withdraw from the cell cycle and enlarge primarily by hypertrophy. In the adult, the cell size has stabilized and proliferation is either absent or at very low levels. Because CMs are very long-lived, the cells accumulate damage (e.g. ROS, lipofuscin and mitochondrial heteroplasmy) and undergo adaptations with age. In the case of severe damage, cells may senesce or undergo cell death (apoptosis or autophagy). Owing to the loss of cells, the remaining cells undergo molecular adaptations that promote hypertrophy to meet the functional demands placed on the intact organ. In the short-term, many of these adaptations are beneficial, but, over time, a ‘vicious cycle’ is established, such that CM cell numbers continue to decrease and many of the remaining cells exhibit some dysfunction (e.g. decreased SR calcium uptake and insufficient energy generation). At the organ level, the loss of cells and functional anomalies lead to a heart that is insufficient in advanced age to meet additional functional demands. Blue lettering, CMs from embryonic and fetal stages of development; red lettering, cumulative effects that affect CMs during aging in adults.

Table I
General effects of aging on CM

This Table was adapted from J. Am. Coll. Cardiol., vol. 57 (I), H. Shih, B. Lee, R. J. Lee and A. J. Boyle, The aging heart and post-infarction left ventricular remodeling, pp. 9–17 Copyright, 2011, with permission from Elsevier.

Molecular changes	Cellular changes	Organ adaptations	Clinical effects in aged adults
Cyclin D1, cyclin D2, cyclin D3, pRb, p130 and CDK2	CM proliferation (reduced or absent proliferation; increased numbers of senescent cells)	Decreased CM/non-CM ratio	Impaired systolic and diastolic function
TERT, IGF-I, PI3K, ET-I, SIRT1, SIRT7, caspases, AIF and survivin	CM survival (reduced survival with increased cell death)	Decreased CM/non-CM ratio	Impaired systolic and diastolic function, and fibrosis
ANP, BNP, ERK1/2, NFAT, calcineurin and S6 kinases	CM diameter/hypertrophy (increased but augmented further when other cells undergo cell death)	Altered heart shape and possible increase in blood volume at end diastole	LV hypertrophy and fibrosis
MHC, SERCA2, NCX1, mitochondrial proteins and heteroplasmy, and Cx43	CM function (variable changes, but generally reduced contractile and lusitropic function in response to stress; mitochondrial function is significantly reduced)	Inability to tolerate increased workloads and reduced $\dot{V}_{O_2 \max}$	Impaired systolic and diastolic function, and arrhythmias