Review

Aging-associated changes in cardiac gene expression

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Abstract

Cardiovascular diseases (e.g., vascular diseases, strokes, heart failure) reach epidemic proportions in the elderly and are the primary limits to survival in man. Age-associated changes in heart structure and function represent the major risk factors in heart failure (HF) syndromes and are associated with altered patterns of gene expression that can generally be seen as relative changes in the abundance of gene transcripts. An understanding of the molecular mechanisms underlying these changes should be tantamount to defining a genetic basis for aging; however, the analysis of processes as complicated as aging requires an accounting of biological diversity. Until recently, most of the changes in transcript abundance were identified one at a time, but the advent of gene expression arrays has permitted rapid, large-scale expression profiling. This has provided information about the dynamics of total gene expression, which can be used to identify pathways and elucidate regulatory events that may be affected during senescence or in response to disease. Importantly, very large sample sizes or meta-analyses of studies of smaller sample sizes should be sufficient to account for the diversity of altered gene expression that directs alterations in specific molecular pathways, which underlie changes in cardiac structure and function in senescence and disease.

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1. Introduction

Aging is associated with a random, passive decline in function that decreases an organism’s viability and ability to reproduce. Cellular aging is commonly associated with the instability of the mitochondrial and nuclear genomes and oxidative protein damage in response to environmental and genetic stress [1]. Several theories [2–5] have been proposed to account for the deleterious effects of aging (i.e., senescence), including the Free Radical Theory, which argues that aging results from DNA or protein damage generated by reactive oxygen species (ROS), and the Mutation Accumulation Theory, which suggests that aging is a cumulative result of random, late acting, adverse mutations. None of the major theories of aging (see Table 1), however, adequately explains the process of getting older or can fully account for senescence-associated changes that occur in humans.

The more comprehensive “Evolutionary Biologic Theory of Aging” proposes that senescence is non-adaptive and results as a consequence of a variety of gene actions that escape natural selection [6]. The theory neither supports the view that a “single” gene has evolved to cause aging nor that sequential gene actions are responsible for aging [7]; however, the use of forward genetics in invertebrate model systems (i.e., worms and flies) have led scientists to discover a set of longevity promoting genes that profoundly influence lifespan. These genetic alleles increase lifespan at the expense of reproductive capacity and implicate distinct genetic components in the process of aging [8–10]. C. elegans and D. melanogaster, however, are mostly composed of post-mitotic cells that do not develop cancers, a situation drastically removed from that of humans.

Experiments designed to study basic molecular mechanisms of aging in humans often proves impractical. In
Several other theories of aging have also been described (e.g., Error-Catastrophe, Immunological Basis of Aging, Neuroendocrine Theory, Cellular Senescence) which have been reviewed elsewhere [76].

man, the process of aging takes decades and is complicated by an increased incidence of diverse pathologic disorders like diabetes, Alzheimer’s, cancers and cardiovascular diseases [11]. These age-associated disorders may be due to polymorphisms, epigenetic changes (e.g., methylation, decreased telomere length), metabolic dysfunction (e.g., loss of mitochondria or mitochondrial genome disturbances), hormonal changes (e.g., decrease in melatonin or estrogen production), physical de-conditioning or loss of immunological responsiveness (e.g., thymus involution). Cardiovascular diseases (e.g., vascular diseases, strokes, HF) and cancers, in particular, reach epidemic proportions in the elderly and are the primary causes that limit survival and complicate the study of aging in humans. For these reasons and others, rodent model systems have often been used as a surrogate for human aging [12–14].

Until recently, gerontologists had only identified a finite number of quantifiable changes in mRNA abundance related to aging-associated risk factors in heart. Microarrays have emerged as the method of choice to study gene expression on a genomic scale, and transcriptome-based analyses are now widely employed to study human HF syndromes [15–20], but only a few have been used to study aging heart [21,22]. One of the ultimate goals of gene expression arrays, which can systematically assay the cardiac transcriptome (the identity and quantity of all cardiac mRNAs), is to foster our understanding of the dynamics of gene regulation and how diverse biological processes, like development, lifestyle, gender, disease and genetic background, interact to affect heart function with aging or senescence. It is therefore our aim in this review to illustrate how gene expression profiling techniques coupled with statistical analysis and publicly available bioinformatic tools can be employed to identify pathways, regulatory sequences and candidate genes implicated in aging, and theoretically, how this approach can be used to identify genetic causes of aging in humans.

2. Transcriptomic analyses

Gene expression arrays (i.e., microarrays) have emerged as the method of choice for studying gene transcripts on a genomic scale and are usually composed of either cDNA clones or oligonucleotides spotted at high density on a membrane, appropriately treated glass slide or other appropriate material [11]. Microarrays specifically assay small amounts of DNA or RNA, and are generally used to analyze a cellular transcriptome after hybridization with labelled probes generated from total RNA or mRNA transcripts. Differences in expression profiles are quantified, and changes in abundance are generally believed to reflect the cellular response to an environmental or genetic factor. [A discussion of data acquisition and analysis of micro-
arrays is beyond the scope of this review, but these issues have been described elsewhere [23–26], and the reader is referred to TIGR Microarray Resources (http://www.tigr.org/tdb/microarray/) for an overview of microarray-based techniques, specific protocols, software and gene indices.

When employed systematically to address biological processes, transcriptome analyses are capable of addressing questions of tremendous complexity and scope (e.g., aging); however, scientists seeking to harness the potential of these techniques are often challenged by the large amount of data. Computational genomics has therefore evolved to promote and further interactions between the fields of computer science and molecular biology in support of genomic-based techniques. The goal has been to design user-friendly software that can be used to track, integrate, qualify, and ultimately foster scientific insight from experimental results. Specifically, software is available for statistical analyses and modeling, pathway discovery, network elucidation and genomic correlates, only a few of which are mentioned here. TIGR (http://www.tigr.org/software/tm4/) has developed software tools to facilitate microarray data entry and analysis; while Robert Tibshirani (http://www-stat.stanford.edu/~tibs/) and Trevor Hastie (http://www-stat.stanford.edu/~hastie/) provide a number of statistical and modeling programs (supervised and unsupervised) to analyze microarray data sets. Ingenuity Pathways Analysis (http://www.ingenuity.com/) enables biologists to explore, understand, and discover therapeutically relevant networks by generating biological networks from large-scale gene expression and proteomics data sets [27]. Genomatix (www.genomatix.de) utilizes input sequences identified from microarrays to retrieve and analyze corresponding promoter regions to identify cis-elements and potential trans-binding factors [28,29].

3. Cardiac gene expression with aging

3.1. Aging-associated changes in rodents

Age-associated changes in both heart structure and function represent major risk factors in human HF syndromes [30]. While molecular and genetic studies have identified a finite number of genes directly responsible for a few cardiovascular diseases in humans [31,32], multiple genetic pathways are believed to be responsible for transducing stress-related, mechanical and neurohormonal stimuli into changes in gene expression. The balance between these pathophysiologic stresses and the ability of the senescent myocardium to adapt ultimately determine the threshold for clinical manifestation, its severity and the prognosis for patients who develop age-related, cardiac diseases. This has led to the hypothesis that identification of age- or disease-specific patterns of gene expression at a molecular level could assist in the diagnosis and ultimately the prognosis (i.e., morbidity and mortality) of disease syndromes. The structural and functional adaptations that occur to maintain cardiac function in response to aging, however, involve complex, multipart events that are influenced, not only by genetics, but also by life-style, gender and disease [30].

Adult cardiomyocytes are terminally differentiated cells that are largely unable to divide, and it has been reported that myocyte loss occurs with aging in both humans and rodents [30]. Consequently, the myocardium and, specifically, cardiomyocytes must adapt to meet the continued functional demands of the organism. Because of the difficulty in studying human aging in heart, simple model systems have been utilized to gain molecular insights into aging and aging-associated events. Mice and rats, for example, have a much shorter lifespan than humans and, experimentally, are amenable to pressure and volume overload, environmental stress and genetic manipulation. Except for some transgenic models, rodents are largely free from cardiovascular diseases that commonly affect man. While this may call into question the relevance of some findings with respect to humans, rodent models have been useful in defining many of the age-associated changes in gene and protein expression described to date.

It is well accepted that aging in rodents is associated with an isoform shift in myosin heavy chain (MHC) transcript/protein abundance concomitant with a decrease in myosin ATPase activity [33]. Both sarcomeric α-actin isoforms (cardiac and skeletal) are decreased with aging [34]. Changes in the relative amount of the Na+/Ca2+ exchanger and sarco(endo)-plasmic reticulum Ca2+-ATPase type 2 [35,36] may contribute to an improvement in the energy-efficiency or regulation of cardiac contraction [30], but the latter is associated with impaired calcium uptake by the sarcoplasmic reticulum in humans [37]. Cardiac expression of such functionally distinct genes as atrial natriuretic factor [38], angiotensin II receptor subtypes AT1 and AT2 [39], inositol 1,4,5-triphosphate receptor (IP3R) [40], cyclooxygenase 2 [41] and transcription factor NF-κB [42] increase with age, while cardiac expression of other transcripts, like those for collagen isoforms [43,44], alpha 1, beta 1 (and possibly alpha 2) thyroid hormone receptor levels, RXR gamma receptors [45], M2-cholinergic receptor [46] and the estrogen receptor [47], decrease with age. Although initially beneficial at the cellular level, long-term changes in gene expression may be detrimental for the organism.

As proposed by Harman [48], aging-associated changes are due to molecular damage caused by free radicals, and the production of free radicals in mammalian hearts is inversely proportional to the maximum lifespan [2]. Since mitochondria are the main source of ROS, which is produced naturally as a by-product of the mitochondrial electron transport, then this organelle would be expected to be a possible target for free radical damage. Consistent with this notion, a general reduction in mitochondrial-encoded gene expression, followed by impaired mitochondrial function
with age, has been observed [49,50]. This reduction in mitochondrial function is attributed to accumulated deletions within mitochondrial genomic DNA [51] and mitochondrial loss [52]. Age-associated reductions in mitochondrial gene expression, like cytochrome c oxidase subunit 1, specifically lead to significant (~35%) decreases in protein translation, with an accompanying fall (30%) in enzymatic activity [53]. Since mitochondrial activity (i.e., energy production) is critical for the normal function of heart, all of these adaptations explain, at least partially, some of the functional changes seen with aging [54]. While other examples exist [55,56], only a limited number of aging-associated alterations in gene expression had actually been described by traditional molecular techniques.

Changes in mRNA abundance with aging have recently been examined by gene expression arrays. Bodicky et al. [21] compared ventricular cardiomyocytes isolated from 4- and 20-month-old male C57Bl/6 mice to identify 43 gene transcripts that accumulated at significantly different levels with age. These included the well-established changes in transcript abundance for α-MHC, SERCA2 and the α-actins, and the data implicated a reduction in the activity of the mitochondrial electron transport system. Uniquely, they found reduced mRNA levels of several transcription factors (e.g., Nkx2.5, GATA-4, JunB) that might be implicated in aging. In contrast to the limited changes in transcript abundance found in isolated cardiomyocytes, Lee et al. showed that ~10% of the transcripts in whole heart from B6C3F1 mice demonstrated significant changes in abundance with aging [22]. A majority of age-associated changes in transcript abundance may therefore be associated with non-cardiomyocytes, strain differences or altered transcript abundances associated with isolation procedures and not aging. It is therefore critical to take into account biological diversity when performing studies of aging.

Some of “transcriptional alterations” associated with aging were consistent with a metabolic shift from fatty acid to carbohydrate metabolism (e.g., reduced carnitine palmitoyltransferases 1 and 2, carnitine acetyltransferase, and triacylglycerol hydrolase, and induced phosphofructokinase and pyruvate dehydrogenase kinase isoenzyme 4). Transcripts associated with protein synthesis (e.g., elongation factor 2 and numerous ribosomal-associated proteins) were also significantly reduced. These results support the notion of impaired mitochondrial function, and it was postulated that the reduction in transcripts for ribosomal proteins might reflect an adaptation to reduced ATP synthesis. Additionally, there was an increase in the number of transcripts encoding molecules associated with the extracellular matrix, collagen deposition, cell adhesion and cell growth, consistent with previous findings that the aging heart undergoes ECM protein deposition, fibrosis and cardiomyocyte hypertrophy.

Lee et al. [22] examined a second cohort of animals (30 months of age) that were subjected to caloric restriction, which increases the maximum longevity of many mammalian species [2,22]. When compared to ad libitum fed 30-month-old mice, the authors suggested that the altered diet preserved fatty acid metabolism and led to the accumulation of transcripts thought to reduce endogenous DNA damage and innate immune reactivity. Changes consistent with altered cytoskeletal structure and modulation of apoptosis were reported in response to caloric restriction. Because the authors did not take into account the relative changes in lifespan induced by the caloric-restricted animals, it is difficult to interpret these data. It is possible that the “preserved fatty acid metabolism” was only delayed in caloric restricted animals, and if the data had been compared at the same relative lifespan (as a percentage of maximum lifespan), then these differences may not have been detected. Absolute versus relative lifespan must be considered when studying models of aging.

Finally, when studying altered gene transcript abundance in heart, numerous points, which may complicate the interpretation of results, should be considered. First investigation of heart tissue is complicated by factors such as tissue heterogeneity, genetic variability, disease state and pharmacological intervention. Second, gene expression can be rapidly altered in response to cell cycle progression (i.e., non-cardiomyocytes) or events associated with cell death. Third, stochastic mechanisms are ubiquitous in biological systems, and may involve circadian clocks or a decrease in the precision of cellular signals. Mammalian circadian clocks, in particular, consist of complex integrated feedback loops, which are believed to be important for the daily timing of physiological processes. Recent reports have shown that circadian gene regulation is extensive (>8–10% of the genes expressed in heart), and that the specificity of circadian regulation cannot be accounted for by tissue-specific gene expression [57]. Importantly, these circadian-associated changes in transcript abundance involve diverse processes, and different circuits and effector genes; however, the importance of circadian clocks in aging has not yet been addressed experimentally.

3.2. Aging-associated changes in humans

It is critical to account for biological variability when performing microarray analyses; however, most human heart samples are acquired as biopsies (fresh, frozen or fixed), as hearts rejected for transplantation, or as explants of diseased myocardium [11]. In addition, these samples, which contain mixed cell populations, are obtained from patients of different ages and gender, making it exceedingly difficult to determine by microarrays to what extent gene products are responsive to age and not to other sources of biological variability.

To illustrate this point, we recently employed gene expression arrays as a screening tool to quantify the expression of gene products in failing and non-failing human myocardium [15]. We identified 162 candidate “HF-responsive” gene products (p<0.05), including many that
were not identified using a ratio-based approach to determine fold-changes in abundance. We then performed qRT-PCR analyses on a larger and more diverse sample population (n = 34, including 15 non-failing heart samples) to validate and examine the role of contributing biological variables (i.e., age and gender). The statistical analyses (multiple linear regression modeling with backward elimination procedure) demonstrated that the majority of changes in abundance were subject to diverse biological inputs. We recently expanded these analyses to include 16 new gene products. Of the 47 candidate genes examined by qRT-PCR in this combined set, 66% (31 out of 47) demonstrated significant and verifiable changes in abundance, but only when modeled to take into account the three variables of HF, gender and age. Only five of these transcripts were linked to HF irrespective of sex or age (e.g., lumican and CCAAT-enhancer binding protein). Fifteen transcripts demonstrated HF-associated changes in expression, which varied by sex (e.g., calponin 1 and natriuretic peptide precursor A), and some of these involved only one sex (e.g., Cd163), while others like those for Gpr58 demonstrated opposite effects in men and women. Ten transcripts demonstrated complex interactions, involving HF, sex and age (Fig. 1a and b), but only one of the transcripts, methionine tRNA synthetase (Mars), demonstrated a HF response that varied only as a function age (Fig. 1c). These data clearly illustrate the absolute requirement to account for biological variability when analyzing samples from human hearts.

Because of the number of human heart samples available to us, we have gone back to examine the response of these transcripts as a function of age in non-failing myocardium. The hypertensive status of patients was however considered in this analysis. Among the 47 HF candidate gene products examined, two transcripts were age-responsive in non-failing myocardium. A good example is shown in Fig. 1d for metallothionein 1L (Mt1l), a protein implicated in cell defense [58]. Its abundance increases with age regardless of normalization (Gapd or Casq2) and independent of the hypertensive status. Because Mt1l is involved in the protection of tissues against various forms of oxidative injury [59], its increased abundance may be reflective of oxidative repair mechanisms activated with aging in humans [60]. These findings suggest that the analysis of large sample sets or perhaps meta-analyses of non-failing samples from smaller studies will facilitate the identification of aging-responsive transcripts in man.

Fig. 1. Statistical analysis of qRT-PCR data on a subset of candidate genes showing age-responsive expression profiles, some of which vary by HF and gender. The threshold cycle (Ct) of each sample was log-transformed, and normalized data are presented as a \( -\Delta \text{Ct (Ct-C}\text{reference gene} \) where Calsequestrin (Casq2) and glyceraldehydes-3-phosphate dehydrogenase (Gapd) were used as reference genes. (A) Metallothionein 1L (Mt1l), an age- and HF-responsive gene product (solid line—control samples; dashed line—for HF samples); (B) peptidyl-prolyl cis/trans isomerase (Pin1), an age-responsive gene product affected primarily in females (solid line—males; dashed—for females); (C) methionyl-tRNA synthetase (Mars), an age-responsive gene product that affected only HF patients (solid line—control samples; dashed line—for HF samples); (D) metallothionein 1L (Mt1l), a pure age-responsive gene product in non-failing myocardium of male patients (some data are adapted from Ref. [15]). Symbols: ●, non-failing male; ○, HF male; ■, non-failing female; □, HF female. For 1D, symbols: ●, non-hypertensive male; ○, hypertensive male.
4. Towards a meta-analysis of expression profiles

Several transcriptome analyses of the failing human myocardium have been published [15–20]; however, the results do not always agree. For example, when Tan et al. [18] compared expression profiles between patients with failing and compensated forms of ischemic and dilated cardiomyopathy, they found calponin transcripts to be decreased by ~2.7-fold. In contrast, Steenman et al. [19] showed in a similar patient cohort that calponin transcripts increased in abundance with HF. Our data however offered a partial explanation to this discrepancy when we demonstrated that calponin transcript abundance in human cardiac samples depended primarily on sex or HF × Sex interactions [15]. Gene products identified by these studies therefore do not change with age or to the same extent in men and women or with HF.

In our study, we found that age and sex accounted for only 71% of the statistical variance [15]. The additional variance suggested overlapping sources of biological variability like diabetes, hypertension, smoking, diet, obesity, medications, HF etiology and duration. Furthermore, the array technology itself introduces other sources of variance including microarray batch variation, hybridization platforms, gene sequence variance and sample preparation. Thus, to have sufficient accounting of the diversity of the altered gene expression to understand the molecular pathways leading to changes in cardiac structure and function in various types of human HF or with aging, a very large sample size or a meta-analysis of studies of smaller sample size is necessary [61] (Fig. 2a).

As a proof of concept, we have imported data from three studies where we were able to obtain raw data from individual failing and non-failing heart patients [15,18,19]. Altogether, we combined 18 expression profiles from patients with dilated cardiomyopathy and 16 from non-failing hearts of various ages (Boheler and Garg, unpublished data). Because the microarrays were performed on different platforms (cDNA vs. oligonucleotide), we employed a variety of techniques to match expression data for individual gene transcripts (cross-referencing gene identifiers with accession numbers via DAVID [62] or LocusLink http://www.ncbi.nlm.nih.gov/LocusLink/list.cgi). Following normalization and statistical tests (ANOVA) for each gene transcript, the \( p \)-value for each group was used to determine statistical significance in mean expression between the diseased and non-diseased hearts, but not as a function of age. Because many tests were performed (large number of target cDNAs), we took care with the interpretation of the \( p \)-values, because, by chance, one would expect 5% of the results to be statistically significant even when there are no differences between the two groups. Consequently, both the Bonferroni and FDR (false discovery rate) approaches were applied to adjust the \( p \)-values for multiple testing.

From this meta-analysis, thrombospondin-4 (Thbs4) was identified as a putative HF-responsive gene product. Thrombospondins are composed of a family of extracel-

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**Fig. 2.** (A) Meta-analysis algorithm used to identify, validate and statistically analyze putative candidate genes involved in the pathogenesis of dilated cardiomyopathy. Data were combined from three studies [15,18,19]. In the first two studies, both males and females, ranging in age from 26 to 69, were employed. In the data set used from Steenman et al., RNA from two male patients with dilated cardiomyopathy of ages 45 and 58 were combined, and a non-failing sample obtained from a 15-year-old male with cystic fibrosis was used as a control. From the meta-analysis, thrombospondin-4 (Thbs4) was identified as a putative HF-responsive gene product. (B) Statistical analysis of qRT-PCR data for Thbs4, showing that it decreases with age in non-failing hearts. Importantly, this decrease does not occur in samples from failing heart. (There was also a strong relationship of Thbs4 with sex, but this data is not shown.)
lular calcium binding proteins involved in cell proliferation, adhesion and migration. This gene family, consisting of at least five glycoproteins, is composed of structurally related, multidomain ECM proteins. THBS-4 appears to act as an adaptor protein in ECM assembly as it binds specifically to both collagenous and non-collagenous ECM proteins [63]. Recent findings suggest that the A387P (alanine to proline) variant of thbs4 may be an important determinant in the development of myocardial infarctions at any age [64]. Thbs4 is expressed in vascular cells, and it is thought to influence the vessel wall by modulating the proliferation of endothelial and smooth muscle cells. Moreover, the A387P substitution is a “gain-of-function” mutation, favoring a form of TSP4 that interferes with extracellular adhesion and proliferation and may thereby be proatherogenic [65].

To determine if Thbs4 changes as a function of age or age-associated interactions and validate this meta-analysis approach, real-time PCR was performed on 41 heart samples with unique primers. Eighteen samples were obtained from non-failing hearts, while the remaining had HF. The ages varied from 15 to 75, and included samples from 31 men and 10 women. QRT-PCR reaction protocols and statistical analyses were as described [15], and we modeled the relationship between gene values (ΔCt) and age, sex, hypertension, and HF. This enabled us to verify that Thbs4 was significantly different between diseased (dilated cardiomyopathy) and non-failing samples (p<0.0005), and that it was significantly altered with age (p=0.021) and with sex (p=0.003). Moreover, the analyses indicated a combined effect of sex and age (p=0.017) as well as that of HF and age (p=0.001) (Fig. 2b). Thus, the expression of Thbs4 in diseased and control samples is highly dependent on the age of the subject.

These data lead us to conclude that the combination of data sets from microarrays, followed by appropriate normalization and statistical analysis can be used to perform a meta-analysis of HF and aging. Based on this proof of concept, it should now be possible to discriminate among other aspects of biological diversity like smoking, diet, obesity, medications and HF etiology; however, to adequately address aging, much larger sample sizes of combined data sets will be required. Only through a sufficient accounting of biological diversity in humans will it be possible to distinguish age-associated changes in gene expression from those due to other causes.

5. Pathway analysis: cysteine metabolism

5.1. Transcripts altered with age, hypertension and heart failure

Cysteine is a naturally occurring non-essential amino acid that contains a thiol functional group. This moiety features prominently in the maintenance of the tertiary and quaternary structural integrity of many proteins through formation of disulfide bridges and in chemical processes that occur at the active sites of many enzymes. Cysteine is stored and transported in the configuration of the tripeptide glutathione. Catabolism of cysteine occurs in both aerobic and anaerobic environments, which have important biological functions related to the regulation of protein activity and antioxidant properties [66].

In our previous study on human HF and aging, only MARS demonstrated a purely age-dependent response among samples obtained from patients with failing myocardium. Mars proteins (MARS) are known to participate in methionine-tRNA synthesis; however, it is also catalytic for homocysteine (HCY)-thiolactone formation [67], which may enhance the immune response to proteins that have homocysteine moieties incorporated into the primary amino acid structure. Among the group of 31 genes analyzed, one other gene transcript, Tst, encoding Rhodenese was associated with cysteine metabolism. Although Tst was elevated in males and reduced in females with HF, the prevalence of changes in transcripts associated with cysteine metabolism (and defined by Ingenuity Networks) suggested a possible aging or aging-associated processes in heart. We therefore hypothesized that changes in pathways involving cysteine metabolism could contribute to the pathological conditions of aging and HF.

To test this hypothesis, we determined the transcript abundance of multiple enzymes that participate in cysteine metabolism, particularly those that could affect antioxidant status of cells or influence homocysteine levels. We examined heart samples obtained exclusively from male donors (to limit gender-dependent biological variability) and normalized qRT-PCR data to Gapd. We then modeled the relationship between expression (ΔCt) and three-way interactions, using a multiple linear regression model with backward elimination (SPSS statistical package) of age, HF, hypertension (Htn). We found that 6 of 15 cysteine metabolism associated transcripts were influenced (i.e., displayed interactions) by these independent variables (Table 2). Age was therefore a major contributor to the observed changes; however, the direction of transcript abundance differed among the groups. For example, Cth, Cbs and Gss demonstrated a decrease in transcript abundance with age in non-hypertensive males, but showed a significant increase (p<0.05) in hypertensive males. If these changes in transcript abundance equate with protein function, then one would predict altered cysteine availability, reduced Hcy removal through trans-sulfuration and a decrease in the amount of newly synthesized glutathione in elderly hypertensive males, which could adversely affect the antioxidative status. While these findings are speculative, the data demonstrate how transcriptome analyses promote the generation of new hypotheses involving biochemical pathways that can
be tested to address age- and disease-associated events in humans.

5.2. Putative regulatory elements and genetic correlates

Because transcriptome analyses potentially reflect changes both at the protein level and at the level of the gene, we have chosen to illustrate how some of the recent developments in computational genomics can be employed to address potential genetic correlates associated with age and HF. Specifically, we will describe how a set of gene products that shows common changes in expression can be used to putatively identify a common set of cis-elements that may contribute to their genetic regulation.

To study the potential regulation of cysteine-metabolism associated transcripts at the genomic level, we employed Genomatix (http://www.genomatix.de) software to identify common cis-elements for the three groups of genes. Statistically, the cysteine metabolism associated transcripts can be divided into three groups: HF- and HF×Age-dependent—Cbs and Mars; HF×Htn-dependent—Iars, Cth and Gss; and Htn×Age-dependent—Cth, Gss and Cbs. We have not included Tst from the Htn×Age-dependent group, because its expression with age was opposite to that seen for the other gene transcripts in this group. By inputting the accession numbers of the transcripts within each group, Genomatix rapidly retrieved verified promoter regions consisting of 500 bp upstream and 100 bp downstream of the start site of transcription. Because each group of transcripts depended on two independent variables, we used the task “Search for common framework” to identify multiple cis-elements that were located in a conserved order and separated by spacers. For HF×Age-dependent genes, the software identified a common framework of four elements: hypoxia inducible factor, bHLH/PAS protein (HIF), Nuclear Factor 1 (NF1F), E-box binding factor without transcription Activation (HEN1) and cAMP-Responsive Element Binding proteins (CREB) families. For the HF×Htn and Htn×Age groups, the frameworks each consisted of the same three elements (Fig. 3): CREB, CLOX and CLOX homology (CDP) factors (CLOX), GC-Box factors SP1/GC (SP1) and PAX-5/PAX-9 B-cell-specific activating protein (PAX5) families.

From these genomic analyses, a total of 37 potential transcription factors were predicted that could bind to these cis-elements. Because cis-regulatory elements potentially bind trans-regulatory factors that are organ-specific, it was necessary to identify those found in heart. For this, we employed the results from our meta-analysis described above. This enabled us to identify which transcripts, corresponding to the putative transcription factors, were present in human heart, but more importantly, which transcripts showed potentially significant changes in expression among the samples. Our rationale was based on the assumption that altered transcript abundance should be regulated by transcription factors that either had altered abundance or altered function. The meta-analysis thus facilitated our identification of cardiac transcription factors that were present in heart and which specifically showed altered transcript abundance in failing or non-failing hearts. Ten transcripts were statistically implicated in HF-related syndromes (not shown). Each of these transcripts was then analyzed by qRT-PCR analysis and subjected to multiple linear regression modeling. Eight transcription factors

![Fig. 3. Schematic indicating the conserved framework of cis-elements present in the promoters of the Htn×Age-responsive gene set identified through pathway analysis of cysteine metabolism related genes. The figure shows three genes that contained three cis-elements (CREB—cAMP-responsive element binding protein; SP1F—Transcription Factor Sp1-family; and PAX5—Paired box gene 5) in a defined order, including genes encoding glutathione synthetase (gss), cystathionine beta synthase (cbs) and cystathionase (cth). These conserved cis-elements were identified with the assistance of Genomatix, which identifies promoter modules composed of two or more transcription factor binding sites within a defined distance range. Note that PAX5 is not generally expressed in heart; however, this conserved cis-element serves as the model binding site for at least eight paired box genes.](image-url)
showed significant changes in abundance (Table 3), and almost all of these were Htn×HF-dependent. Additionally, five of eight transcription factors showed Htn×Age-dependent interactions.

When we compared the transcript profile of Htn×HF- and Htn×Age-dependent gene transcripts with the corresponding trans-regulatory factors, we found that several of the expression profiles were highly similar. For example, the CREB-family member transcription factor Atf-4 and SP1-family member Sp4 demonstrated increased transcript abundance with age in non-hypertensive males and decreased abundance with age in hypertensive males. Similarly, Cbs, which was potentially regulated by CREB- and SP1-family transcription factors, demonstrated an expression profile almost identical to that seen for Atf-4 and Sp4 (Fig. 4). Two other gene transcripts for Cth and Gss, which are putatively regulated by the same transcription factors, have similar expression profiles. Meanwhile in the Htn×HF-dependent groups, Atf-2 and Sp-1 showed expression profiles similar to that of Gss and Iars. Altogether, these data suggest that different members of CREB- and SP1-families of transcription factors may regulate the transcriptional activity of Htn×Age- and Htn×HF-dependent genes, respectively. Finally, cut-like (Drosophila-CLOX)- and PAX5-family members appear to be implicated in Htn×HF-mediated and Htn×Age-mediated gene expression profiles.

While these findings do not demonstrate cause and effect, the data illustrate how transcriptome analyses coupled with bioinformatics tools can be employed to predict outcomes related to genetic regulation. By applying the same techniques to specific questions of aging, including longevity and disease, we suggest that it will be possible to identify survival-associated transcripts and conserved cis-elements that effectively predict age-associated trans-regulatory factors that are linked to survival or aging in humans—a major goal of cardiac gerontology.

### 6. Summary

In conclusion, cardiac transcriptome analysis is a powerful approach to analyze gene function. We have specifically illustrated how gene expression profiling techniques coupled with statistical analysis and publicly available bioinformatic tools can be employed to identify pathways,
regulatory sequences and candidate genes implicated in aging in humans. We have also provided examples of how these data sets can be combined to perform a meta-analysis of human HF and aging. When coupled with data from the human genome, these approaches will be useful in the identification of putative genetic correlates of aging, and we predict that the same approaches will be applicable to the diagnosis of senescence-related diseases in humans. More comprehensive applications of expression profiling to cardiovascular medicine will eventually lead to new molecular correlates of cardiac disease and aging, and when coupled with genetic models, our understanding of the processes involved in human aging will rapidly improve, foretelling the development of rational strategies for cardiovascular therapy during senescence.

References


