Research Article **Open Access**

Supplementation of selenium-enriched yeast attenuates age-dependent transcriptional changes of heart in mitochondrial DNA mutator mice

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Submission date: October 31, 2013; Acceptance date: March 24, 2014; Publication date: March 31, 2014

ABSTRACT

Background: Age is a major risk factor in developing heart diseases and has been associated with profound transcriptional changes in mammalian tissues. Low tissue selenium has recently been linked to several age-related diseases, including cardiovascular disease. This study investigated the global effects of age and dietary supplementation of selenium on heart transcriptional profiles in POLG mutator mice.

Methods: Heart transcription profiles from young (2-month-old) and old (13-month-old) animals fed either a control diet or a diet supplemented with 1.0 mg selenium from selenium-enriched yeast (SP)/kg diet were obtained and validated using microarray and real-time RT-PCR techniques.

Results: Aging led to significant transcriptional changes, where the expression of 1942 genes in old animals was changed by a fold change larger than 2.0, when compared to young animals. Age-regulated genes are associated with cardiovascular system development, immune and inflammatory response, and cellular oxidative stress response. Multiple genes linked with cardiomyocyte apoptosis, hypertrophy, and cardiac fibrosis, such as Myh7, Lcn2, Spp1, and Serpine1, were significantly up-regulated in old animals. SP supplementation also caused significant transcriptional changes in the heart, especially in old mice where many age-dependent transcriptional changes were totally or partially reversed by SP. Upstream regulator analysis further indicated that genes for Foxo1 and Foxo3, two transcriptional regulators involved in the regulation of cardiac muscle remodeling, were significantly activated by SP, suggesting that Foxo-mediated transcriptional activities play important roles in the anti-aging properties of SP.

Conclusions: Results of this study indicate that SP supplementation attenuated age-related transcriptional changes in the heart of old POLG mice, which implies a potential clinical application of dietary selenium in preventing decline of cardiac function in old animals.

Key words: Aging, heart, gene expression, selenium

BACKGROUND:

In humans and rodents, heart aging is characterized by a loss of myocytes (cardiomyopathy), enlargement of the remaining myocytes (hypertrophy), and cardiac fibrosis [1-3]. At the molecular level, aging has been associated with widespread transcriptional changes in the heart, where transcriptional profiles associated with aging have been characterized by a metabolic shift toward carbohydrate metabolism, induction of genes having structural roles, and reduced protein synthesis [4, 5]. It has also been associated with the impairment of the transcriptional responses to oxidative stress, as indicated by diminished induction of stress response genes and lower constitutive expression levels of antioxidant genes [5, 6]. Aging of the heart has also been related to down-regulation of genes involved in energy metabolism, particularly mitochondrial genes [7].

Aging related changes are modifiable. Recent studies comparing 30-month-old control and calorie-restricted (CR) mice showed that age-related changes in gene expression could be remarkably prevented, and that CR mice appeared to be biologically younger than animals receiving the control diet [5, 8]. Studies have also demonstrated that moderate CR (65% feed intake of *ad libitum*) in rats reduced the progression of cardiomyopathy [9], while lifelong CR prevented age-related impairments of late diastolic function in B6D2F1 mice [10]. The mechanisms leading to age-related alterations, however, are not well understood, although it is extensively accepted that increased oxidative stress contributes to myocardial dysfunction with advancing age [11, 12]. Previous studies indicated that several natural and synthetic compounds with antioxidant activity, including α-lipoic acid (LA), resveratrol (RE), coenzyme Q10 (CQ) and vitamin E (VE), contain the potential to slow down certain specific aspects related with aging [5]. For example, it has been shown that dietary supplementation with LA or CQ inhibited gene expression changes which are linked with the extracellular matrix, cellular structure, and protein turnover in aged mice [13]; while RE, a polyphenol compound found in red wine, has been reported to delay cardiac aging in mice [14, 15] and increase the survival of mice fed a high fat diet [16]. VE supplementation was reportedly effective in inhibiting the expression of genes associated with cardiomyocyte hypertrophy and increased innate immunity [17].

Selenium (Se) has long been associated with defense against oxidative stress. Lower tissue Se status has been linked to an increased risk of several aging-related diseases, including cardiovascular diseases [18, 19]. Several animal studies have also demonstrated the cardiovascular benefits of Se, whereby dietary Se supplementation prior to ischemia-reperfusion injury resulted in improved cardiac functional recovery, reduced incidence of reperfusion arrhythmias, and preservation of ventricular ultra-structure [20-22]. At a transcriptional level, the effect of Se supplementation on heart aging has not been reported.

In this study, we investigated the effects of dietary supplementation of Sel-Plex® (Alltech, Nicholasville, KY), a natural organic Se source derived from selenium-enriched yeast, on the cardiac transcriptional profiles of young and old animals, and evaluated its potential interventive effects on heart aging. POLG (proof reading-deficient mutation of polymerase gamma) mutator mice, an animal model of aging, were used in this study [23, 24]. POLG mice start to show significant premature aging phenotypes such as greying, osteoporosis, sarcopenia, and enlarged hearts as early as 7 - 9 months of age [25], and demonstrate marked cardiac hypertrophy and dilatation, impairment of systolic and diastolic function, and increased cardiac fibrosis [25]. We found age-dependent cardiac transcriptional changes such as increased expression of genes related to cellular immune and inflammatory response, cell death, as well as genes related with development of cardiomyocyte hypertrophy and fibrosis were partially reversed by SP. This suggests a potential clinical application of Se in preventing or delaying the decline of cardiac function in the aging population.

MATERIALS AND METHODS:

Animals and diets. The POLG ($PolgA^{D257A\ /\ D257A}$) mice were provided by Dr.Tomas A. Prolla (University of Wisconsin, Madison). Immediately after weaning at 21 days (PND21), male mice were randomly assigned to a selenium-deficient diet (SD) or a diet supplemented with 1 mg selenium/kg diet (SP) using selenium-enriched yeast (Sel-Plex®, Alltech Inc., Nicholasville, KY). Experimental diets were prepared by Harlan-Teklad (Madison, WI) and have been described in detail elsewhere [26]. To control the effects of non-selenium-related yeast components, the SD diet was supplemented with an equal amount of non-selenium enriched yeast (selenium < 0.5 ppm on a product basis). Selenium levels in dietary premixes were evaluated by atomic absorption spectroscopy [27]. The selenium concentration in the SD diet was confirmed to be < 0.03 ppm, whereas in the supplemented diet it was 1.05 ppm. Mice were housed two to three per cage, and food and water were provided *ad libitum*. At 60 days of age, a total of 20 mice, 10 each from SD and SP were euthanized by cervical dislocation, and the hearts were rapidly dissected, flash-frozen in liquid nitrogen, and stored at -80ºC. At 400 days of age, another set of 20 animals (10 animals from each diet group) were euthanized for sample collection. All procedures were approved by the Animal Care Committee at the William S. Middleton Veterans Administration Hospital.

Tissue selenium quantification. To evaluate the effects of selenium supplementation on the tissue mineral contents, liver selenium was measured using the method adapted from Gerber et al [28], with slight modifications. Weighted liver samples (25 to 45 mg) were placed in MARSXpress digestion vessels containing 2ml of 69% OPTIMA grade nitric acid (Fisher Scientific, NJ, USA) and 2ml of 30% (w/w) hydrogen peroxide solution (Sigma Aldrich, St. Louis, MO). The samples were then digested using the MARS 5 microwave digestion system (CEM Corporation, Matthews, NC) under the following conditions: ramp to 200°C for 20 min at 1200W, then held at this temperature for another 20 min before cooling down for 15 min. Digested samples were diluted to 50ml using 18.2MΩ/cm deionized water and then used for selenium analysis on Agilent 7700 series ICP-MS instrument (Agilent Technologies, Inc., Japan).

Total RNA extraction. Approximately 30 mg of left ventricle tissue was homogenized with a Qiagen TissueRuptor (Qiagen, Valencia, CA) and total RNA was extracted using an RNeasy Mini kit (Qiagen), following the protocol recommended by the company. To remove contaminating DNA, on-column DNA digestion with RNase-Free DNase (Qiagen) was performed. Integrity and purity of isolated RNA was assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE) and further confirmed with an Agilent 2100 Bioanalyzer System (Agilent Technologies, Santa Clara, CA).

Microarray procedures. cRNA preparation, hybridization, and scanning were performed following the standard protocols recommended by Affymetrix (Santa Clara, CA). Briefly, purified RNA was used for biotin-labeled cRNA synthesis using the Affymetrix GeneChip Expression 3'-Amplification One-Cycle Target Labeling Kit (Affymetrix), according to the manufacturer's recommended procedures. Labeled cRNA was hybridized to mouse genome MG-430_2.0 GeneChip arrays (Affymetrix) for 16 h at 45ºC, followed by washing, streptavidin-phycoerythrin (SAPE) staining, and finally scanning in an GeneChip Scanner 3000 7G (Affymetrix). Probe signal intensities were analyzed using an Affymetrix MAS5 algorithm scaled to the default trimmed mean signal intensity (SI) of 500. A total of 24 gene expression profiles, six each from SD-young, SD-old, SP-young, and SP-old animals were obtained.

Microarray data analysis. GeneSpring GX 12.5 (Agilent) was used to validate and normalize microarray data and to perform statistical and gene expression pattern analyses. Briefly, normalization was done by first scaling the intensity of probesets of the arrays to a mean target intensity of 500, followed by baseline transformation to median of all samples. Background corrections were done by MAS5 based on the Perfect Match (PM) and Mis-Match (MM) probe design of the microarray. To minimize the possibility of misleading findings, probe sets with low signal intensity and those which were labeled as 'Absent' by the MAS5 algorithm across samples were excluded from further analysis. The differentially expressed genes by age or diets were filtered using the volcano plot method where genes with $P < 0.05$ and corresponding signal intensity fold change (FC) > 1.2 or FC < -1.2 were deemed to be significantly different.

Pathway analysis of microarray data. To dissect the biological themes represented by altered transcription profiles by diet and age, two independent pathway analyses were applied. First, we performed parametric analysis of gene set enrichment (PAGE), a computational method that allows determination of significant changes in a defined gene set [29]. To identify functional clusters that characterize the transcriptional alterations associated with aging or dietary Se status, significantly changed genes by aging or diet were further grouped into networks, functions and canonical pathways using Ingenuity Pathways Analysis software (IPA, Ingenuity Systems, Redwood City, CA). Fischer's exact test was used to determine the significance of the association between the genes and the given network, biological function or canonical pathway.

Real-time quantitative-PCR confirmation. For validation of the effects of diet and age on gene expression, a subset of differentially regulated genes were selected for further confirmation based on the function of interest using real-time quantitative PCR (RT-qPCR) analysis. The same RNA used for microarray analysis was also employed for RT-qPCR analysis. Each sample of total RNA (0.5 μg) was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. RT-qPCR was performed in triplicate using Taqman® probes and Taqman® fast advanced master mix (Applied Biosystems), and the 7500 Real-time PCR FAST system (Applied Biosystems). Taqman® probes (Table 1) were pre-designed by Life Technologies. The relative quantification (RQ) was calculated as a ratio of the target gene to control gene using the delta-delta Ct (ΔΔCt) method. Conditions for RT-qPCR were as follows: 95°C for 20s, followed by 40 cycles of 95°C for 3s and 60°C for 30s. RT-qPCR results were analyzed using a Student's t-test. Values are presented as means ± SEM, and differences between treatment means were considered significant at $P < 0.05$.

Table 1: Primers used in RT-qPCR

Taqman® Probe ID*	Gene Symbol	Gene Title
Mm01335356_g1	Selh	Selenoprotein H
Mm00447333_m1	Snca	Synuclein, alpha
Mm00656767_g1	Gpx1	Glutathione peroxidase 1
Mm00435860_m1	Serpine1	Serine (or cysteine) peptidase inhibitor, clade E, member 1
Mm00809552_s1	Lcn2	Lipocalin 2
Mm00600555_m1	Myh7	Myosin, heavy polypeptide 7, cardiac muscle, beta
Mm01611440_m1	Spp1	Secreted phosphoprotein 1
Mm01341361_m1	Timp1	Tissue Inhibitor of metalloproteinase 1
Mm00491305_m1	Trim54	Tripartite motif-containing 54
Mm00477771_m1	Nf2^	Neurofibromatosis 2

* Primers were pre-designed by Life Technologies (Carlsbad, CA).

^ Nf2 was used as reference gene to account for non-biological variations.

Western blot analysis. Ventricular tissue samples from the same source material used in the microarray analysis were homogenized in cold lysis buffer (1.5 mM KCl, 50 mM Tris HCl, 0.125% sodium deoxycholate, 0.375% Triton X 100, 0.15% NP40, 3 mM EDTA) containing one tablet of Pierce protease and phosphatase inhibitor mini tablets on ice. The supernatant was subsequently collected and protein concentration was determined using a BCA assay (Pierce BCA Protein Assay Kit; Thermo Scientific, Rockford, IL). Total protein (25μg) was separated on a 12% Agarose gel and transferred to PVDF 2μm membrane (BIO-RAD). Membranes were incubated with primary antibodies for 2 h at room temperature. The primary antibody of MYH7 was purchased from Santa Cruz, while ACTB (LI-COR, Lincoln, NE) was used as an internal control for quantification. Positive signals on the membrane blots were detected using Amersham's enhanced chemiluminescence Western Blotting

Detection reagents (GE Healthcare Life Sciences) or fluorescent-labeled secondary antibodies (LI-COR, Lincoln, NE).

RESULTS:

Dietary effects on the liver Se concentration. To confirm the effects of Se supplementation, liver Se content was measured. As expected, liver Se concentration was significantly increased (P<0.01) in animals fed the SP supplemented diet when compared with animals fed the SD diet in both young (1771.6 ± 260.3 ppb vs. 318.1 ±26.1 ppb) and old animals (1778.5 ± 48.7 ppb vs. 139.23 ± 47.1 ppb).

Overview of aging-regulated gene expression patterns of POLG mutator mice. Clustering of gene expression profiles of animals fed with control diet indicated a dramatic difference on overall cardiac transcription profiles between young and old animals, as shown in Figure 1A, where expression profiles of old animals were clearly separated from those of young animals. Further comparison indicated that expression of 6830 genes in the heart were significantly different (P<0.05, FC >1.2 or FC <-1.2) between young and old mice. Among these, 1942 genes had an increased FC >2.0 or a decreased FC < -2.0 (Figure 1B). Details of these highly age-regulated genes are shown in supplemental table 1.

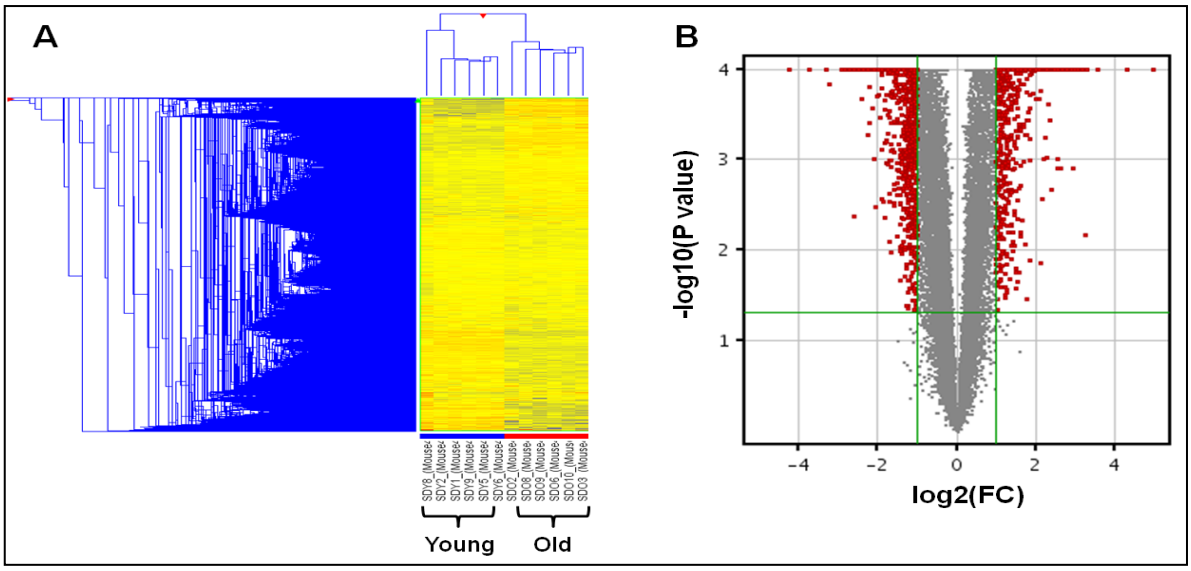

Figure 1. Effects of age on cardiac transcription profiles. A) Unsupervised hierarchical clustering of the expression profiles from old and young animals fed the control diet. In the heatmap, normalized gene expression values are shown in colors that reflect the expression changes compared to the mean value of each gene, where blue, red or yellow colors represent decreased, increased or no change in the level of expression intensity, respectively. The dendrogram on the top reflects the extent of similarity of expression profiles between arrays, while the dendrogram on the left side represents the changes of expression patterns of individual genes across the array. B) Volcano plot of age-regulated genes. 1943 genes that significantly altered by age (P<0.05, FC >2.0 or FC <-2.0) shown as red dots in the picture.

To minimize the noise associated with the large dataset, as well as to focus on the biological processes represented by the most significantly altered genes, functional analysis was based on the above1942 highly age-regulated genes. Results indicated that functional

classes associated with age-regulated genes are primarily involved in development of the cardiovascular system, immune and inflammatory response, tissue development and morphology, lipid metabolism and apoptosis (Figure 2A, supplemental table 2). Heart aging is generally related to the activation of these functions, which are indicated by the red squares in the green frames (Figure 2A). Canonical pathway analysis also suggested a strong connection between aging and pathways important in immune and inflammatory response, such as IL-8 signaling, clathrin-mediated endocytosis signaling, acute phase response signaling, Fcγ receptor-mediated phagocytosis in macrophages and monocytes, NF-κB activation, and complement system (Figure 2B).

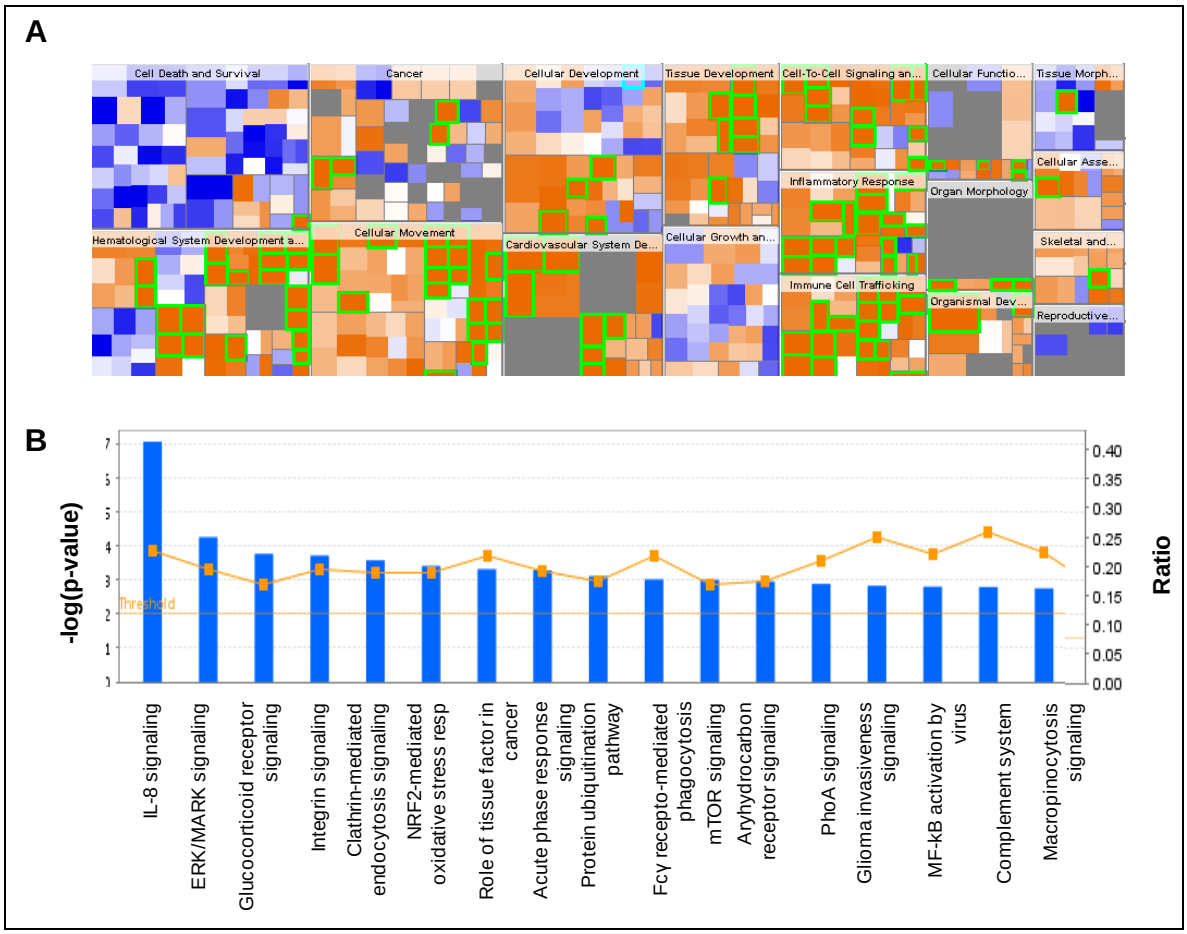

Figure 2. Aging associated biological functions and canonical pathways in the hearts. A) Biological functions associated to aging-regulated transcriptional changes. The significance of the biological functions to age-regulated genes was predicted based on Z-score (A positive score indicates an increase (red) in function while a negative z-score represent a suppressed (blue) function. Z-scores > 2 or < -2 were deemed to be significant and those functions are shown in green frames. B) Canonical pathways associated with age-regulated genes. The bar graph shows the significance of each age-associated pathway; line-connected dots represent the ratios of aging regulated genes to the total number of genes that make up that pathway. Significance was calculated based on Fischer's exact test.

This is consistent with previous reports that aging and age-related diseases are linked to a chronic inflammatory state, including local infiltration of inflammatory cells and higher circulatory levels of pro-inflammatory cytokines, complement components, and cell adhesion

molecules [30, 31] . Aging is also related to pathways involved in oxidative response, cellular maintenance, cellular morphology, DNA replication, apoptosis, protein degradation as well as others (Figure 2B). Since aging of the heart is frequently associated with cardiomyopathy, hypertrophy, and cardiac fibrosis [2], we examined the influence of aging on genes that had functionally been related to development of cardiomyocyte apoptosis, heart hypertrophy, and fibrosis. As shown in table 2, aging has a dramatic impact on expression of these genes as judged by the number of gene expression changes and the large FCs. For example, lipocalin 2 (Lcn2) is an adipose-derived cytokine that plays an important role in cardiomyocyte apoptosis. Its expression level was increased more than 30 times (FC = 31.8) in old animals compared with young animals. Similarly, several other well known gene biomarkers that have been related to cardiovascular diseases, such as Spp1, Myh7, Mmp3, and Serpine1, were also significantly up-regulated by aging (Table 2).

Table 2. Aging regulated genes that are functionally related to cardiomyocyte apoptosis, hypertrophy and fibrosis [*]

Gene	FC _Age [a]	Function associations [a]	Gene name
TXNIP	2.425	AP	Thioredoxin interacting protein
MMP2	2.487	AP	Matrix metallopeptidase 2
MTOR	2.227	AP	Mechanistic target of rapamycin
PLA2G5	-2.543	AP	Phospholipase A2, group V
BCL2L1	2.001	AP	BCL2-like 1
NFKBIB	3.584	AP	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta
BNIP3	2.033	AP	BCL2/adenovirus E1B 19kDa interacting protein 3
CAMK2D	-2.384	AP	Calcium/calmodulin-dependent protein kinase II delta
ADRB1	-3.072	AP	Adrenoceptor beta 1
LCN2	31.838	AP	Lipocalin 2
DICER1	2.271	AP	Dicer 1, ribonuclease type III
SLC8A1	-2.772	AP, FB	Solute carrier family 8 (sodium/calcium exchanger), member 1
TRIM54	3.982	AP, HT	Tripartite motif containing 54
AKT1	3.209	AP, HT	v-akt murine thymoma viral oncogene homolog 1
BCL2	-2.321	AP, HT	B-cell CLL/lymphoma 2
MAPK8	-2.031	AP, HT, FB	Mitogen-activated protein kinase 8
HMOX1	6.726	AP, HT, FB	Heme oxygenase (decycling) 1
SOD2	-2.168	AP, HT, FB	Superoxide dismutase 2, mitochondrial
SERPINE1	2.987	FB	Serpin peptidase inhibitor, clade E, member 1
LMNA	2.426	FB	Lamin A/C
BCL6	-4.261	FB	B-cell CLL/lymphoma 6
TRDN	-2.075	FB	Triadin
FN1	3.058	FB	Fibronectin 1
SGCB	-2.442	FB	Sarcoglycan, beta (43kDa dystrophin-associated glycoprotein)
AR	-2.026	FB	androgen receptor
RRM2B	-3.411	FB	Ribonucleotide reductase M2 B (TP53 inducible)
NFATC1	2.015	HT	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1
TGFB1	2.1	HT	Transforming growth factor, beta 1

CDKN1A	8.625	HT	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
ACE	2.242	HT	Angiotensin I converting enzyme (peptidyl-dipeptidase A) 1
MAPK3	2.748	HT	Mitogen-activated protein kinase 3
PPP3CB	-2.307	HT	Protein phosphatase 3, catalytic subunit, beta isozyme
MYH14	2.712	HT	Myosin, heavy chain 14, non-muscle
MYH7	4.368	HT	Myosin, heavy chain 7, cardiac muscle, beta
EGFR	-2.504	HT	Epidermal growth factor receptor
MEF2D	2.588	HT	Myocyte enhancer factor 2D
TTN	-2.72	HT	Titin
SMTN	2.389	HT	Smoothelin
NCF1	2.031	HT	Neutrophil cytosolic factor 1
TIMP1	7.788	HT, FB	TIMP metallopeptidase inhibitor 1
DES	2.286	HT, FB	Desmin
APOE	6.703	HT, FB	Apolipoprotein E
ATP2A2	-3.987	HT, FB	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2
CREB1	-2.851	HT, FB	cAMP responsive element binding protein 1

[*] Only a portion of gene identified was listed in this table. Function associations are based on Ingenuity Knowledge Base (Ingenuity Systems, Redwood City, CA).

[a] AP, apoptosis; FB, fibrosis; HT, hypertrophy.

Effects of SP supplementation on heart gene expression profiles of old and young mice. To determine the effects of dietary Se status on the cardiac gene expression of animals at different ages, we compared transcription profiles of 2-month-old mice fed SP (SPY) with 2-month-old control (SDY) mice, 13-month-old mice fed with SP (SPO) with 13-month-old control (SDO) mice, respectively. Relative to aging effects, dietary effects on the transcriptional profiles of the heart were moderate based on the number of changed genes alongside the magnitude of the changes shown.

In old animals, SP supplementation significantly ($P < 0.05$, $FC > 1.2$) changed the expression of 1685 cardiac genes (Up, 921; Down, 764), in which 88 genes had a $FC > 2.0$ or $FC < -2.0$ (Supplemental table 3). As expected, several selenoprotein encoding genes including Sepw1, Gpx1, and Gpx3, which are functionally important in cellular oxidation-reduction reactions, were significantly induced by SP (Supplement table 3). PAGE analysis also indicated that oxidation reduction was the most enhanced biological process in SP-fed animals (Supplemental figure 1). Notably, multiple biomarker genes that were significantly induced in the aged hearts, including Lcn2, Timp1, Mhy7, and Spp1, were among those found most significantly down-regulated by SP (Supplemental table 3, Figure 5). This prompted further comparative analysis on the effects of SP supplementation and aging on cardiac transcriptional profiles. Pathway analysis indicated a significant association between SP-regulated genes and increased carbohydrate metabolism and muscular cell development, while functions involved in immune cell trafficking, cell death, cardiovascular system development, and lipid metabolism were suppressed (Table 3).

Upstream regulator analysis (URA) further predicted that activation of Foxo1 and Foxo3, and the inhibition of Akt1, play important roles in gene expression changes related to SP supplementation (Figure 3). As shown in the figure, expression of Foxo1 and Foxo3, as well as many of its target genes, including biomarkers important in cardiovascular functions,

were significantly altered by SP, indicating that transcriptional regulations by SP could be important in prevention of cardiac hypertrophy in old mice [32, 33].

Table 3. Biological functions related to genes differentially expressed by SP supplementation in old POLG mutator mice

Functions Annotation [a]	P-Value	Molecules	Category	Activation [b]
Chemotaxis of cells	3.33E-05	46	Cellular movement	-5.0
Contractility of cardiac muscle	5.82E-04	15	Cardiovascular system development and function	-3.568
Synthesis of lipid	2.72E-03	53	Lipid metabolism	-3.326
Quantity of lymphocytes	6.85E-04	58	Hematological system development and function	-3.085
Quantity of leukocytes	1.35E-04	75	Tissue morphology	-3.023
Quantity of b lymphocytes	2.63E-03	30	Humoral immune response	-2.919
Proliferation of immune cells	7.30E-04	60	Cellular growth and proliferation	-2.891
Adhesion of immune cells	5.26E-03	28	Immune cell trafficking	-2.785
Mobilization of cells	1.20E-03	10	Cellular movement	-2.774
Inflammatory response	7.58E-04	54	Inflammatory response	-2.65
Migration of phagocytes	1.67E-03	24	Immune cell trafficking	-2.474
Migration of phagocytes	1.67E-03	24	Inflammatory response	-2.474
Cell movement of phagocytes	8.02E-04	43	Inflammatory response	-2.449
Cell viability	9.18E-03	91	Cell death and survival	6.415
Transactivation	1.02E-03	56	Gene expression	3.571
Phosphorylation of l-amino acid	1.35E-02	17	Post-translational modification	3.56
Differentiation of embryonic cells	6.48E-04	21	Cellular development	3.441
Mitogenesis	8.47E-03	18	Cell cycle	2.813
Expansion of cells	1.29E-02	24	Cellular growth and proliferation	2.704
Antiapoptosis	1.75E-04	26	Cell death and survival	2.646

[a] Functions analysis by IPA (Ingenuity Systems, Redwood City , CA).

[b] Predicted activation based on Z-score calculated by IPA, where a positive score predicts an increased activity while a negative score indicate a suppressive effect.

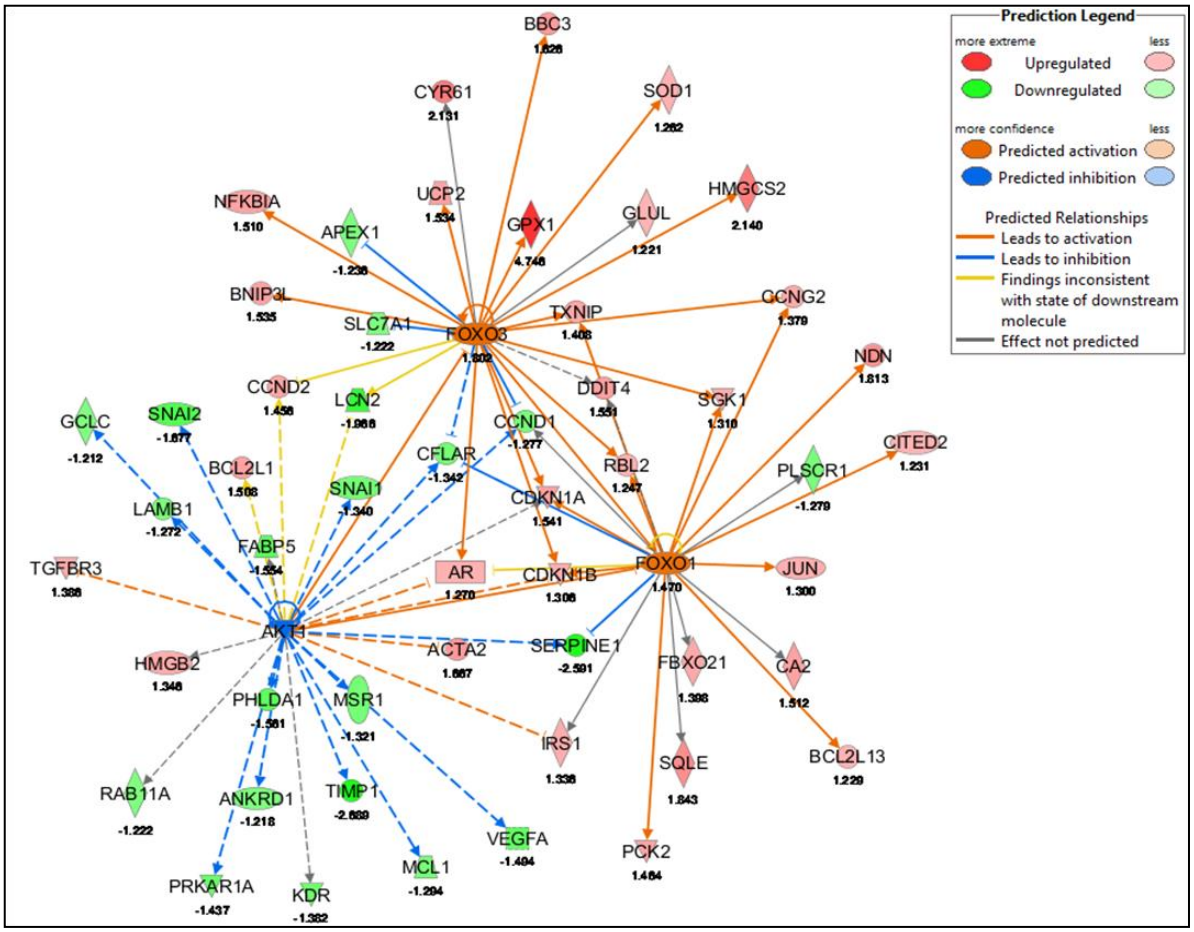

Figure 3. Transcription factors and targets genes regulated by SP in old mice. Upstream regulator analysis (URA) is based on expected causal effects between upstream regulators and targets; the expected causal effects are derived from the literature compiled in the IKB. The direction of change is the gene expression in the experimental samples relative to control. If the direction of change is consistent with the literature, URA predicts that the upstream regulator is more active in the experimental sample than in the control (Orange); if mostly inconsistent with the literature (anti-correlated with the literature), IPA predicts that the upstream regulator is suppressed (Blue). The color of target genes (nodes) in the pictures shows the expression changes (Red, Up; Green, Down) detected in this study.

In young mice, 660 heart genes were identified as significantly changed (Up, 273; Down, 387) by SP, but only 8 genes had a FC > 2.0 or FC < -2.0, including Sepw1 (Supplemental table 4). The small number of changed genes and moderate FCs indicate the effects of dietary Se status on cardiac transcription profiles of young mice is relatively small compared to those in old mice. Transcription factor analysis, however, also indicated Foxo3 was activated by SP supplementation as in old animals (Supplemental figure 2). Biological functions significantly associated with SP-regulated genes in young animals are shown in (Table 4), where several functions related to cell survival and cancer were decreased.

SP supplementation mitigates age-related cardiac transcriptional changes. To directly evaluate the effects of SP on aging-regulated genes, we compared the expression changes of

these genes in response to aging and SP supplementation. Among 1943 genes that were significantly changed by age, 1356 (~70%) were regulated in the opposite direction by SP (Figure 4A). Of these, 262 genes were significantly ($P < 0.05$, FC >1.2) changed by diet in the reverse direction to the effects of aging (Figure 4B, details in Supplemental table 5). The counteractive effects of SP on aging-related transcriptional changes are also apparent when comparing the biological functions that are significantly induced by aging with those suppressed by SP where many overlaps between age-increased functions and SP-decreased functions including inflammatory response, heart contraction, cell viability, and lipid metabolism were observed (Table 5).

Table 4. Biological functions related to genes differentially expressed by SP supplementation in young POLG mutator mice

Functions Annotation [a]	P-Value	Molecules	Category	Activation [b]
Cell viability of tumor cell lines	1.39E-02	40	Cell death and survival	-2.315
Tumorigenesis of cells	1.51E-02	15	Cancer	-2.299
Hyperplasia of epidermis	1.45E-02	5	Cancer	-2.219
Cell viability of blood cells	1.46E-02	19	Cell death and survival	-2.195
Transformation of blood cells	3.29E-03	5	Cancer	-2.156
Size of embryo	1.39E-02	23	Embryonic development	-2.101
Transactivation	4.48E-04	44	Gene expression	-2.092
Cell transformation	3.62E-03	34	Cancer	-2.086
Transmigration of antigen presenting cells	3.45E-03	4	Immune cell trafficking	-2.0
Processing of RNA	2.83E-04	24	RNA post-transcriptional modification	2.0

[a] Functions analysis using IPA (Ingenuity Systems, Redwood City , CA).

[b] Predicted activation based on Z-score calculated by IPA, where a positive score predicts an increased activity while a negative score indicate a suppressive effect.

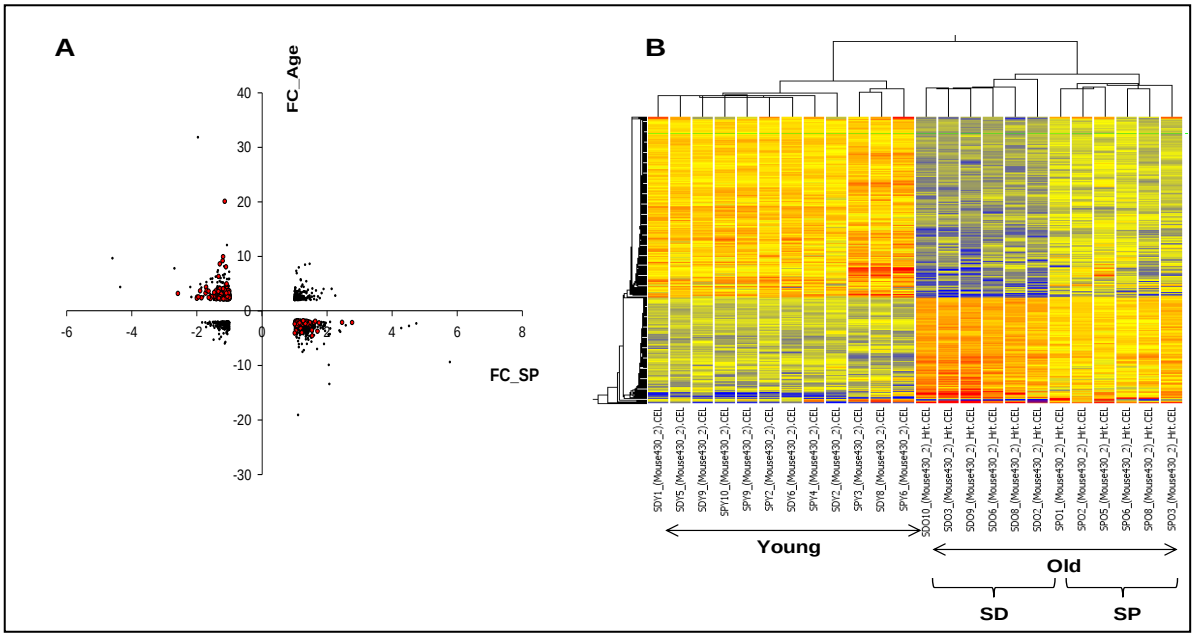

Figure 4. Effects of SP supplementation on the expression of age-regulated genes in old POLG mice: A) 1943 genes that changed by aging were displayed. The fold change of age FC_Age and diet FC_SP are shown in y-axis and x-axis, respectively. Red dots represent genes significantly different between the SP-supplemented group and old control group (P<0.05, FC >1.2). B) Unsupervised hierarchical clustering of the expression profiles from old and young animals. Age-regulated genes were significantly and oppositely altered by SP in old POLG mice. In the heatmap, normalized gene expressions were shown in colors that reflect the expression changes compared with mean intensity of each gene transcript, where blue, red or yellow colors represent decreased, increased or no change in the level of expression intensity, respectively. The dendrogram on the top reflects the extent of similarity of expression profiles between arrays, while the dendrogram on the left side represents the changes of expression patterns of individual genes across the array.

Table 5. Top biological functions significantly associated with genes induced by age or genes suppressed by SP *

Function annotations	Molecules	p-Value	Category	Z-score
Functions related to age-induced genes				
Viral Infection	159	7.33E-06	Infectious disease	7.202
Chemotaxis of phagocytes	42	2.00E-07	Inflammatory response	4.799
Phagocytosis	40	1.37E-07	Inflammatory response	4.195
Organization of cytoplasm	142	1.25E-07	Cellular assembly and organization	3.956
Inflammatory response	92	1.03E-10	Inflammatory response	3.83
Fibrogenesis	57	1.28E-08	Tissue development	3.082
Contraction of heart	26	6.00E-06	Cardiovascular system development and function	2.897
Release of fatty acid	27	3.10E-06	Lipid metabolism	2.752
Growth of tumor	49	2.50E-06	Cancer	2.716

Antiapoptosis	29	1.36E-04	Cell death and survival	2.621
Metabolism of protein	86	3.08E-05	Protein synthesis	2.596
Functions related to SP-suppressed genes				
Cell viability	89	2.55E-04	Cell death and survival	-5.862
Cell movement of tumor cell lines	57	1.02E-04	Cellular movement	-4.823
Proliferation of tumor cell lines	89	5.92E-03	Cellular development	-4.415
Leukocyte migration	65	8.16E-04	Cellular movement	-3.587
Contractility of cardiac muscle	15	5.82E-04	Cardiovascular system development and function	-3.568
Synthesis of lipid	53	2.72E-03	Lipid metabolism	-3.326
Transcription	141	1.86E-05	Gene expression	-3.005
Inflammatory response	54	7.58E-04	Inflammatory response	-2.65
Chemotaxis of mononuclear leukocytes	16	4.19E-03	Inflammatory response	-2.645
Contraction of heart	17	1.29E-03	Organ morphology	-2.425

*Predicted activation based on Z-score calculated by IPA, where a positive score predicts an increased activity while a negative score indicate a suppressive effect.

Validation of the microarray results. To perform an independent validation of the microarray data, we used quantitative RT-PCR and Western blot methods for a subset of genes that were significantly changed by age as well as by SP supplementation in old animals. These genes were selected based on their biological connections to the development of cardiovascular diseases and their significance in the expression change on microarray results. Similar FCs were obtained for the effects of SP and aging, with RT-PCR and microarray analysis on the selected genes as shown in Figure 5. Age-related increases in expression of Serpine1 (serine protease inhibitor 1), Myh7, Spp1, Timp1 and Trim54 (tripartite motif-containing 54), which were significantly suppressed by SP in old POLG mice as determined by microarray analysis, were confirmed by RT-PCR (Figure 5A). Age-related decrease of Gpx1 and Selh, two genes important in cellular oxidation and reduction process, were induced by SP in the heart (Figure 5A). The effects of aging and SP on the expression of Lcn2 and Snca (synuclein, alpha), both involved in cardiomyocyte apoptosis were also confirmed by RT-PCR (Figure 5). The increased expression of Foxo1 and Foxo3, two transcription regulators important in cardiac and skeletal muscle remodeling, was also confirmed in old animals fed with SP (Figure 5B). Western analysis indicated that the protein level of MYH7 (MHC-β), a molecular biomarker of cardiac hypertrophy [34] , was significantly decreased by SP in old POLG mice (Figure 5C).

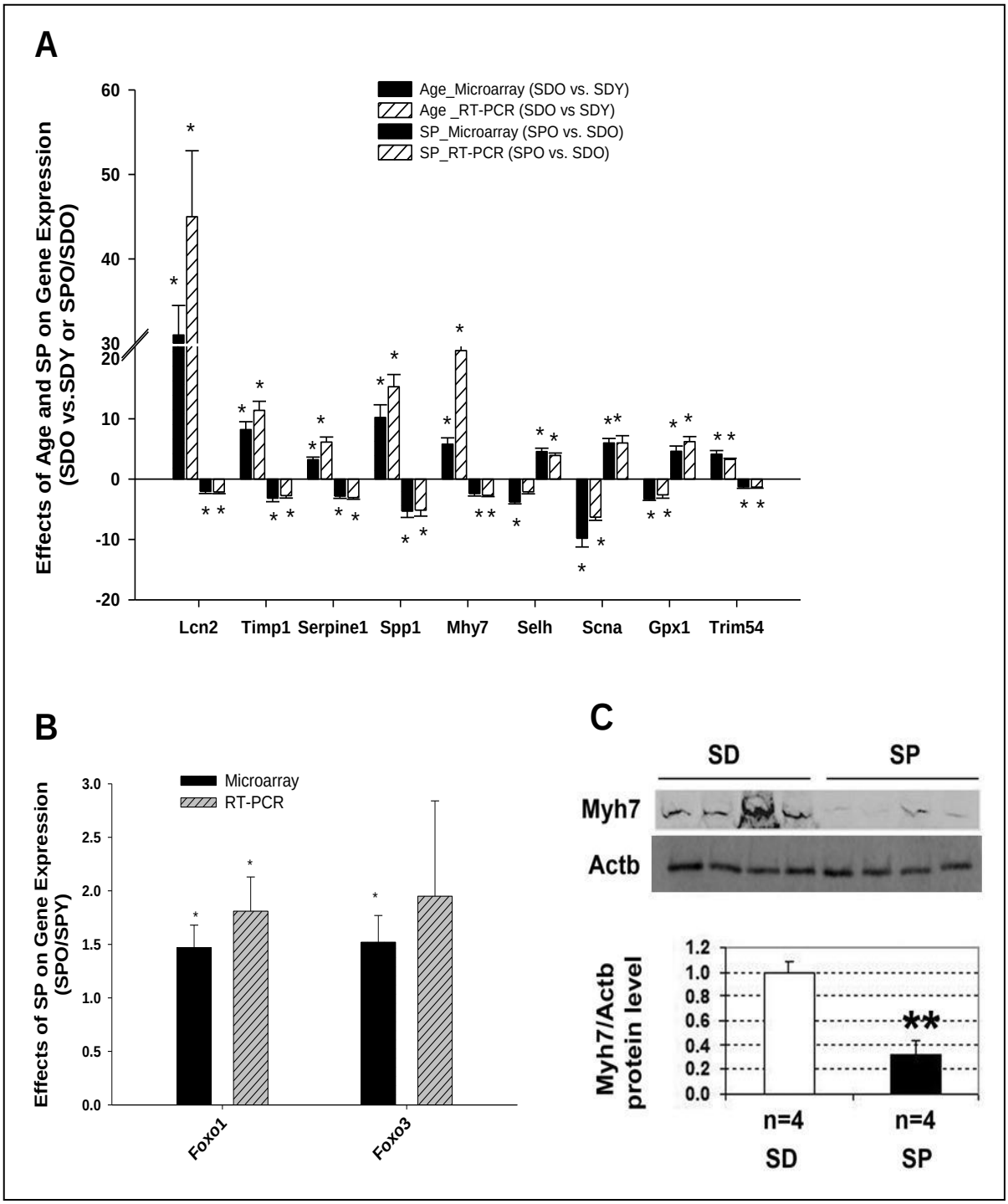

Figure 5. Gene expression change validation. A) Comparison of microarray and RT-qPCR results on the effects of aging and SP on select genes. Values represented are least square means ± SEM; n = 7. Selected genes were: Lcn2 = lipocalin 2; Selh = sSelenoprotein H; Snca = synuclein, alpha; Gpx1 = glutathione peroxidase 1; Serpine1 = serine (or cysteine) peptidase inhibitor, clade E, member 1; Myh7 = myosin, heavy polypeptide 7, cardiac muscle, beta; Spp1 = secreted phosphoprotein 1; Timp1 = tissue Inhibitor of metalloproteinase 1; Trim54 = tripartite motif-containing 54. B) Effects of SP on the expression of Foxo1 and Foxo3 in old animals. C) Effects of SP on the protein level of MYH7 in the heart of old POLG mice. * indicates $P < 0.05$, ** indicates $P < 0.01$. Student's t-test.

DISCUSSION:

The mechanisms that connect aging and cardiovascular disease are complicated, but accumulating evidence indicates that oxidative stress and activation of inflammatory pathways during aging play a central role [35, 36]. By comparing the genome-wide transcriptional profiles, we investigated the effects of aging on cardiac gene expression profiles as well as the preventive potential of dietary supplementation of selenium-enriched yeast (SP), a popular supplement for antioxidant effects, on age-related transcriptional alterations in POLG mutator mice.

The oxidative stress theory of aging postulates that reactive oxygen species (ROS) induce a variety of macromolecular oxidative modifications, and accumulation of such oxidative damage is a primary causal factor in the aging process [37]. Previous studies have indicated the associations between heart aging and impaired resistance to oxidative stress [5, 6]. Induction of stress response genes such as GADD45α and β was found decreased in aged hearts, where the expression of several antioxidant genes including Gpx4, Peroxiredoxin 1, Peroxiredoxin 2, Sod1 and Sod2 was also significantly lower than that of young mice [6].

In the present study, age-related expression changes on antioxidant genes were confirmed, as indicated by the decreased expression of Gpx 1 (FC = -2.3) and Sod2 (FC= -2.2) in old heart (Supplemental table 1). These findings indicate that the potential response to stress-induced injury is compromised in aged hearts. Given that many selenoproteins perform key antioxidatnt functions, it was not surprising to note increased expression of genes encoding SepW1, Gpx1, and Gpx3 in response to SP-supplemented diet (Supplemental table 3). The increased expression of genes encoding other proteins with antioxidant activity, namely Sod1 and Peroxiredoxin 6, may be explained by the fact that thioredoxin reductase (TxR), a selenium-dependent enzyme, is known to regulate Sod1 activity, whereas thioredoxin, in its reduced form, regulates the expression of peroxiredoxins [38, 39] .

Another hallmark of aging is the increase in inflammatory responses with age [30]. Given the systemic nature of the immune system, it is reasonable to suggest that changes with age in inflammatory and/or immune response would have an effect on different tissues that could be detected as common molecular signatures of aging. Inflammatory processes have been associated with various age-related diseases, such as Alzheimer's disease, diabetes, cancer, and sarcopenia [40]. Consistent with this hypothesis, the results of the current study indicate that age-regulated genes were significantly associated with induced biological pathways that are important in cellular immune and inflammatory responses, such as IL-8 signaling, acute phase response signaling, NF-κβ activation, and the complement system (Figure 2). On the other hand, function-association analysis suggested a suppressive effect of SP supplementation on genes involved in multiple immune and inflammatory responses in old hearts (Table 3), while this effect was not apparent in the young animals.

Along with the partial counteractive effects of SP supplementation on age-associated gene expression patterns shown in the pathway analysis, this study also compared the effects of aging and SP on the expression of biomarker genes that are important in cardiomyocyte apoptosis, myocyte hypertrophy and cardiac fibrosis and which are frequently associated with functional changes in old hearts [2]. Cardiomyocyte apoptosis plays a critical role in the progression of heart failure. For example, in patients with end-stage cardiomyopathy in

dilated and ischemic heart disease, loss of cardiomyocytes due to apoptosis is directly related to the progression of cardiac dysfunction [41-43]. In this study, the expression of many genes that are functionally involved in myocyte apoptosis was significantly altered by age, including Lipocalin 2 (Lcn2) (Table 2). Lcn2 is an adipose-derived proinflammatory marker that has been associated with insulin resistance and obesity-related metabolic disorders [44, 45]. Lcn2 may mediate the innate immune responses in the pathogenesis of heart failure [46]. Compared with young heart, Lcn2 expression was increased more than thirty times in old POLG mice, while SP significantly suppressed its expression in the heart of old POLG mice (Figure 5). Similarly, several well known biomarkers related to cardiomyocyte hypertrophy, such as Myh7, were significantly up-regulated in old animals (Table 2). Myh7 encodes the beta heavy chain subunit of cardiac myosin and is expressed predominantly in the heart. Changes in the relative abundance of this protein and the alpha heavy subunit of cardiac myosin correlate with the contractile velocity of cardiac muscle. Mutations in this gene are associated with moderate to severe hypertrophy [34, 47]. Decreased expression of Myh7 in old animals fed SP (Figure 5) suggests optimized dietary Se may be beneficial in preventing age-related cardiac hypertrophy.

Progressive fibrosis is another hallmark of aging in various organs. Increased fibrosis is a major determinant of increased myocardial stiffness, which, together with impaired relaxation, creates the basis for the development of diastolic dysfunction [48]. Many genes related to heart fibrotic remodeling were regulated in old POLG mice (Table 2), in which serpin peptidase inhibitor, clade E, member 1(Serpine1) and TIMP metallopeptidase inhibitor 1 (Timp1) are two extensively studied biomarkers for cardiac fibrosis. Serpine 1, also known as plasminogen activator inhibitor type 1 (Pai-1), encodes a member of the serine proteinase inhibitor (serpin), which plays significant roles in regulation of fibrosis by inhibiting the tissue collagenolytic activities and by protecting matrix proteins from proteolytic degradation [49]. The increased expression of this potent inhibitor *in vivo* suppresses the normal fibrinolytic system and creates a prothrombotic state, resulting in pathological fibrin deposition followed by tissue damage [50]. The significantly higher expression of Serpine1 in old mice, where expression of this gene was significantly decreased by SP, probably indicates a beneficial effect of SP supplementation in maintaining heart function under stress conditions related with aging and POLG mutation. Timp1 is a natural inhibitor of the matrix metalloproteinases (MMPs), which are involved in degradation of the extracellular matrix. Dysregulation of this gene has been related to dysfunction of many organs including heart, liver and kidney [51]. Aging caused significantly increased expression of the Timp1 gene in POLG mice, while its expression was significantly decreased by SP in old animals.

Upstream regulator analysis in this study indicated that transcriptional activity of Foxo1 and Foxo3, two members of the O subfamily of Forkhead/winged helix transcription factors (Foxo) were activated in old POLG mice (Figure 3). Similar activation effect on Foxo3 was also predicted in young animals fed the SP-supplemented diet (Supplemental figure 3). Foxo transcription factors regulate key physiological functions including response to stress, cell differentiation, protein degradation and apoptosis [52, 53], and it plays important roles in regulating cardiac and skeletal muscle remodeling [54, 55]. Expression of either Foxo1 or

Foxo3 in cardiomyocytes can inhibit agonist-induced hypertrophy growth through suppressing the calcineurin/NFAT signaling cascade, while cardiac hypertrophy observed in Foxo3 null mutant mice [32]. These results suggest that modulation of transcription activity of Foxos play important roles in the effects related to SP supplementation.

This study focused on the transcriptional profiling of young and old cardiac muscle from POLG mice fed either control or SP-supplemented diets over their lifetimes. Because the pathophysiology of aging is complex, it is likely that a detailed analysis of gene expression profiles in multiple tissues will reveal both tissue-specific and general patterns. One limitation of this study is that POLG mutator mice, a premature aging model, display aging phenotypes much earlier than normal mice. As a result, its transcriptional response to SP supplementation could be different from what may occur in normal mice over a normal lifespan. A similar experiment using wild-type animals would be of interest to establish whether these observations hold true against a normal genetic background.

CONCLUSIONS:

In summary, the results of this study indicate that SP supplementation can partially attenuate the effects of aging on cardiac transcriptional profiles in POLG mutator mice, and this protective effect may be mediated through several mechanisms. Firstly, through its involvement in antioxidant systems, SP supplementation induces the expression of genes important in stress resistance, a function usually compromised in old animals. Secondly, SP supplementation suppressed the expression of genes that are involved in age-dependent inflammatory and immune responses, which may prevent tissue damage caused by chronic inflammatory states in aged animals. More specifically, SP supplementation significantly suppressed the expression of age-induced genes that are functionally related to cardiomyocyte apoptosis, hypertrophy, and fibrosis, probably by regulating the transcriptional activity mediated by Foxo transcription factors. Taken as a whole, the data from the present study demonstrate that dietary supplementation with selenium-enriched yeast may ameliorate, at a transcriptional level, the effects of aging on cardiac function in old POLG mice. If similar effects hold true in relation to human heart, the health implications could be of major benefit from the perspective of geriatric health and well-being.

Competing Interests: The authors have no financial interests or conflicts of interest.

Authors' Contributions:

All authors contributed to this study.

Acknowledgements and Funding:

The authors would like to thank LifeGen Technologies (Madison, WI) for the help with the animal experiment. We also thank Dr. Kate Jacques and Judith Hower (Alltech, Nicholasville, KY) for helpful discussions and editing of the manuscript.

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