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# Biomarkers of Cardiac Health: Effects of Nutrition Interventions and Aging

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## Abstract

Wnt activity increased in aged tissues including skin, serum and skeletal muscle. A bioinformatics data mining approach was taken to study rodent cardiac aging gene expression data. The results suggested that the Wnt/β-catenin signaling was decreased in aged rodent heart. Our study also demonstrated that nutritional treatments opposed heart aging by upregulating Wnt/β-catenin signaling. In addition, the expression of β-catenin gene decreased up to threefold in aged heart but returned to its young level with nutritional interventions. Our study further suggested that β-catenin was an excellent candidate as a cardiac aging biomarker and nutritional target. Molecular mechanisms underlying such action were proposed.

**Glossary of Abbreviations**
**CPCs:** Cardiac Progenitor Cells
**CR:** Calorie Restriction
**DEGs:** Differentially Expressed Genes
**FDR:** False Discovery Rate
**PCA:** Principal Components Analysis
**RMA:** Robust Multichip Average
**SAM:** Significance Analysis of Microarray
**TGF-β2**: Transforming Growth Factor-β2

## Introduction

Cardiac disease is a common problem in pets. One study showed approximately 11% of dogs suffered cardiac disease,[1] most of which occurred in older animals. The aging heart undergoes a variety of structural, functional and physiological changes and becomes susceptible to disease.[2] Although much effort has been devoted to studying the aging heart, the molecular mechanisms underlying cardiac aging remain poorly understood.

Aging is characterized by decreased stem and progenitor cell functions and a decline in tissue regeneration.[3] Aged tissues exhibit reduced intrinsic resistance to injury or damage. Cardiac stem cells and cardiac progenitor cells (CPCs) are unspecialized cells that are capable of self-renewal. In response to tissue injury or damage, these cells can proliferate rapidly and develop into heart muscle or vascular cells.[4,5] However, these regenerative functions decline as tissue ages. In mice, the number of functionally competent CPCs peaks at 20 months of age and declines sharply by 28 months of age.[6] It is believed that the stem and progenitor cell dysfunction may contribute to aging.

Wnt signaling has been implicated in the control of stem cells' self-renewing state.[7] Three recent studies underlined a connection between the Wnt/β-catenin signaling pathway and stem-cell aging.[8-10] The Wnt/β-catenin signaling pathway describes a network of proteins best known for their roles in embryogenesis and cancer but also involved in normal physiological processes in adult animals. The Wnt/β-catenin pathway regulates cell fate decisions. The activated Wnt-ligand serves to inhibit degradation of β-catenin, an integral cell-cell adhesion adaptor protein as well as transcriptional co-regulator. In the absence of the Wnt-signal, β-catenin is targeted for degradation. The presence of Wnt binding allows for stabilization of β-catenin levels, supporting Rac1-dependent nuclear translocation and transcriptional activation.

**Key Words**
Cardiac Aging
Gene Expression
Nutrition Intervention
Wnt/β-Catenin

During development, the Wnt/β-catenin pathway integrates signals from many other pathways including retinoic acid, FGF, TGF-β and BMP in many different cell types and tissues. In heart tissues, Wnt not only controls heart development but also is thought to play a pivotal role in adult cardiac remodeling. Liu et al. discovered that Wnt signaling increased in various tissues and organs of an animal model of accelerated aging.[9] Continuous Wnt exposure caused cellular senescence. Brake and his colleagues showed increased Wnt signaling in aged skeletal muscle stem cells.[8] Wnt signaling promoted myogenic-to-fibrogenic conversion of muscle stem cells and inhibition of this signal preserved myogenic fate.

Contrary to the above findings, Ye et al. showed that

inhibition of Wnt activity induced premature cellular senescence, while its activation promoted cell proliferation and delayed senescence.[10] In addition, some studies also have shown that increased Wnt signaling may increase hair and bone regenerations in young animals.[11,12] The opposing effects of the Wnt signal are not unprecedented. There are ample examples for the pleiotropic effects of Wnt gene; these can be cell-, tissue- and stage-specific.[13,14] In the heart, it was hypothesized that Wnt signaling increased with aging and that this signaling drove cardiac stem and progenitor cells into fibrogenic lineage.[15,16] However, this hypothesis has not been supported with experimental data.

**Table 1: Description of the Three Studies**

| Name | Source | Organism | Groups | | | | Sample # |
|---|---|---|---|---|---|---|---|
| | | | Yng(mo) | Old(mo) | CR | Resv | |
| Study 1 | NCBI | Mouse | 5 | 30 | 30+CR | 30+Resv | 5 |
| Study 2 | NCBI | Rat | 4 | 28 | 28+CR | / | 6 |
| Study 3 | Proprietary | Mouse | 5 | 25 | / | / | 7 |

This paper presents a bioinformatics study to evaluate molecular mechanism underlying heart aging. Data from three rodent cardiac aging studies were identified. These studies included age effects and two nutritional treatments. The nutrition treatments included calorie restriction and resveratrol, an antioxidant bioflavonoid found in grapes and red wine, both previously confirmed to retard aging. Bioinformatics data mining and data integration approach were applied to the gene expression data from these studies.

## Materials and Methods

### Gene Expression Data

Study 1 data were obtained from the NCBI Gene Expression Omnibus (GEO) data repository (*http://www.ncbi.nlm.nih.gov/geo/*) with accession number GSE11291. As described in the original paper, mice had been fed a control diet from 6 weeks until 14 months of age. From this time point, mice were divided into three feeding groups (N=5) until 30 months of age: control diet, calorie restriction (CR) with 25% caloric reduction, or 50 mg/kg resveratrol supplement. In addition, a group of young mice fed the control diet until 5 months of age served as young controls.[17] Study 2 data were also obtained from the GEO with accession number GSE6718. Heart tissues were obtained from the National Institutes of Health-National Institute of Aging rodent tissue bank. There were three groups of rats: young (4 months), old (28 months) and CR (40% diet reduction).[18] Study 3 included two groups of mice: young (5 months) and old (25 months). The description of the three studies is shown in Table 1. Gene expression experiments were performed on the Affymetrix mouse genome 430 2.0 (Studies 1 and 3) and rat genome 230 2.0 (Study 2) gene chips that house over 45,000 and 31,000 probes, respectively.

### Statistics and Bioinformatics

Affymetrix CEL files were downloaded from the GEO database.[19] The Robust Multichip Average (RMA) algorithm was used for background adjustment, data normalization and data summarization.[20-22] Data quality was inspected with Bioconductor's Simpleaffy package[23] and Partek's PCA data visualization tool. Statistical analysis was performed using Significance Analysis of Microarray (SAM)[24] algorithm implemented as the "samr" package for R. Comparisons were made between young vs. old, CR vs. old, and resveratrol vs. old in each data set using SAM's two-class unpaired test with the permutation number set to 100. The false discovery rate (FDR) cutoff was set to 1%. Data analyses were performed using statistical software R[25] and BioConductor.[26]

Pathway analysis was performed using GenMAPP and MAPPFinder software.[27,28] All probes were used to create GenMAPP expression dataset, but color set of visualization was created with fold change of 1.2 or greater and FDR of 0.01 or less. Pathways and gene ontology with significant association with the data were identified and displayed using MAPPFinder. Pathways with adjusted p values of 0.05 or less were identified as significant.

In order to identify nutrients that modulate Wnt/β-catenin activity, Pathway Studio's ResNet database version 6.0 by Ariadne Genomics[29] were searched for small molecules that directly or indirectly interact with β-catenin. Among the small molecules, nutrients were identified and their roles in modulating Wnt activities were further investigated.

## Results

***Differential pathways in young vs. old heart.*** Differentially expressed genes (DEGs) were first identified in all three studies using SAM (Significance Analysis of Microarrays) software,[24] with FDR 0.01 or less than and fold change 1.2 or greater. We obtained 1,695, 2,537, and 2,142 DEGs in Studies 1 to 3, respectively. The same thresholds were used to search for significant pathways in the GenMAPP database using Studies 1 and 3. Simply, pathways with the most associated DEGs will have the smallest p values. Using an adjusted p value of 0.05, three pathways — TGF-β receptor pathway, Wnt pathway and Androgen receptor pathway — were significant in Studies 1 and 3.

***Downregulation of Wnt/β-catenin signaling pathway in the aged heart.*** As Wnt signaling pathway was identified as significant using two of the three studies, the expressional changes of genes in this pathway were further examined. Eleven of 14 genes in this pathway showed decreased expression in old vs. young heart in Study 1 (Figure 1, Column 2). These included both positive and negative regulators of the Wnt pathway. Most notably was Ctnnb1, the β-catenin gene. Ctnnb1 is one of the key components of the Wnt pathway as it relays extracellular Wnt signal to the nucleus via a cascade of molecular interactions in the cell.[30] In the microarray chip, there were three Ctnnb1 probes, two of which were determined to be DEGs and showed a twofold or threefold increase in expression. Akt1 is a protein kinase that phosphorylates and inactivates GSK3, which is part of the destruction complex that promotes β-catenin degradation. It showed a 1.8-fold decrease in expression. Rac1, a member of the Rho family of small GTPases, positively regulates β-catenin's nuclear localization. It showed a twofold downregulation. Together, our data suggested that the Wnt/β-catenin signaling pathway was downregulated in aged heart.

***CR and resveratrol reversed Wnt signal changes in the aged heart.*** The expressions of the 14 genes were further examined in CR vs. old and resveratrol vs. old hearts (Figure 1, Columns 3 and 4). Interestingly, the majority of these genes' expression changes were reversed to their young levels by either CR or resveratrol treatment. Remarkably, the β-catenin gene showed up to a threefold increase in expression CR- or resveratrol-treated hearts vs. old control heart. In one of the Ctnnb1 probes, its expression was increased 3.2-fold in CR-treated hearts and 2.8-fold in resveratrol-treated hearts. In another probe, its expression increases were 2.1- and 2.0-fold, respectively. Thus, CR and resveratrol supplement had changed β-catenin's expression to its young level.

Similar alterations by CR and resveratrol were observed in other genes. In addition, data visualization by Principal Components Analysis (PCA) provided an interesting observation. PCA is a data reduction method[31] that captures sample associations using the expression of all genes. Samples with similar expression profile are clustered together. Separation was visible between young and old samples, while data from the CR and resveratrol groups fell in the midline of young and old. Furthermore, data integration of the three studies showed similar expressional profile changes in three genes: Magi3, Ctnnb1 and Camk2d. These three genes were downregulated in old vs. young heart across all three studies (Table 2).

***Nutritional intervention of Wnt signal via β-catenin.*** Recent studies have shown that resveratrol and apegenin, both bioflavonoids, affected Wnt signaling by modulating β-catenin's nuclear location.[32,33] Although our data showed that resveratrol increased Wnt signaling, in their first report demonstrating a Wnt inhibitory activity of resveratrol, Hope et al. reported that low concentration of resveratrol inhibited Wnt signaling throughput in colon-derived cells.[33] The study also showed that this resveratrol inhibition of Wnt signaling was partially through modulating β-catenin's nuclear location. Shukla et al. showed that apigenin exerted its effect on Wnt signaling also by affecting β-catenin's nuclear location.[32] A search for β-catenin-interacting small molecules identified 80 small molecules, among which 21 were nutrients. There were five bioflavonoids including resveratrol and apigenin (Table 3).

***TGF-β signaling in cardiac aging.*** TGF-β signaling

| Symbols | Old vs. Yng | CR vs. Old | Resv. vs. Old |
|---|---|---|---|
| Dlgh1 | -1.528 | 1.154 | 1.150 |
| Magi3 | -1.366 | 1.195 | 1.244 |
| Akt1 | -1.814 | -1.119 | -1.315 |
| Dab2 | -1.296 | 1.201 | 1.227 |
| Rac1 | -2.004 | 1.712 | 1.509 |
| Ctnnb1 | -3.038 | 3.177 | 2.787 |
| Ctnnb1 | -1.904 | 2.143 | 2.017 |
| Camk2d | -1.933 | 1.541 | 1.235 |
| Mapk1 | -1.290 | -1.035 | -1.020 |
| Senp2 | -1.538 | 1.071 | 1.261 |
| Smad3 | -1.371 | 1.412 | 1.285 |
| Mark2 | 1.531 | -2.069 | -2.113 |
| Ccnd1 | 1.395 | -1.651 | -1.158 |
| Pias4 | 1.306 | -1.514 | -1.495 |

**Figure 1:** Expression changes (fold change vs control) of Wnt genes in Study 1. Data columns: Column 2: fold changes in old control vs. young control; Column 3: calorie restricted old vs control old; Column 4: Resveratrol old vs control old. Color indicates significance in false discovery rate (FDR): dark gray, expression decrease, FDR<=1%; medium gray, expression increase, FDR<=1%; light gray, expression increase, FDR<=5%; white: FDR>5%.

**Table 2: Selected Wnt signaling gene expression changes in old vs. young heart common to all three data sets, from Studies 1-3. See text for more detail. Light gray, downregulation; dark gray, upregulation; blank, not significant.**

| GeneName | Study 1 | Study 2 | Study 3 |
|---|---|---|---|
| Dlgh1 | -1.5 | -1.3 | |
| Magi3 | -1.4 | -1.4 | -1.6 |
| Ctnnb1 (probe 1) | -3.0 | -1.5 | -1.3 |
| Ctnnb1 (probe 2) | -1.9 | | |
| Camk2d | -1.9 | -1.2 | -2.1 |
| Mapk1 | -1.3 | -1.2 | |
| Senp2 | -1.5 | -1.3 | 1.5 |

**Table 3: Bioflavonoids that may modulate the activity of β-catenin. The results were obtained from the Pathway Studio's ResNet database. All have antioxidant activity.**

| Name | Description |
|---|---|
| Resveratrol | Bioflavonoid in grapes and red wine |
| Apigenin | Bioflavonoid in leafy plants and vegetables (parsley, artichoke, basil) |
| Genistein | Isoflavone in soybean |
| Naringenin | Bioflavonoid in grapefruit and other citrus fruits |
| Quercetin | Flavonoid in apples and red onions, and grains |

pathway also topped the chart of the most significant pathways, with adjusted p value of 0.006 for Study 1 and 0.011 for Study 3. To investigate the pathway further, we examined expressional changes of genes in the pathway. Twenty-four genes displayed expression changes in old vs. young hearts in Study 1. Among those, six genes showed increased expressions. Integration analysis was also performed to compare TGF-β pathway genes across the three studies. Remarkably, the transforming growth factor-β2 (TGF-β2) showed consistent change in all studies. Its expression increased 1.60-, 2,47-, and 1.65-fold in old vs. young heart in Studies 1, 2 and 3, respectively (data not shown). CR and resveratrol treatments decreased TGF-β2's expression to its young level.

## Discussion and Conclusion

In an effort to understand molecular mechanism underlying cardiac aging in pets, we undertook a bioinformatics data mining approach to examine gene-expression profiles in aged versus young hearts in rodent. The microarray gene expression data were selected from appropriate rodent studies evaluating both age and nutritional effects. Data integration analysis revealed a novel role for Wnt/β-catenin pathways in cardiac aging.

The Wnt/β-catenin signaling pathway has been known to play a critical role in early embryogenesis and later in various aspects of animal development.[30,34-38] However, scientists have only recently begun to discover that the Wnt/β-catenin signaling pathway also exerts its role in aging.[39] Three 2007 studies underscored the connection between Wnt signaling and aging. In one study, the authors found evidence showing that downregulation of Wnt2 gene triggered cellular senescence of a human diploid fibroblast cell line.[10] In the other two, the results were in contrast. Liu et al. reported that Wnt signaling increased in various tissues and organs of animal models of accelerated aging,[9] while Brake et al. showed that Wnt signaling increased in aged skeletal muscle stem cells.[8] The discrepancy may be partially due to the fact that the results from the study by Ye et al. were derived solely from cell cultures, while the other two studies were *in vivo* mouse studies. Additionally, the pleiotropic or even antagonistic effects of the Wnt signaling have been observed in multiple biological processes.[13,14]

In this study, we reported that Wnt signaling was decreased in aged rodent hearts. Some of the positive determinants of the Wnt pathway were downregulated in aged vs. young hearts. Remarkably, Ctnnb1, a key regulator of the Wnt signaling pathway, showed consistent downregulation with age across all three studies. Ashton et al. also suggested that Wnt signaling was repressed in aged heart under normoxic condition.[40] These results differ from the effect postulated by Deb et al., who suggested that Wnt signaling increased with aging and that this signaling drove cardiac stem and progenitor cells into fibrogenic lineage. According to that theory, interruption of Wnt signaling in heart would preserve cardiac progenitor cell fate.[15] However, to our knowledge, there is no direct experimental data in support of this hypothesis. On the contrary, our data showed that Wnt signaling was decreased with aging. More experiments are needed to address the discrepancies.

Nutritional intervention plays a major role in aging research. Both CR and resveratrol were previously shown to retard aging, although the mechanisms are not entirely clear. Here, we demonstrated that both CR and resveratrol upregulated Wnt signaling in aged hearts. In either CR- or resveratrol-treated hearts, the expressions of Wnt pathway genes were reversed to their young levels. This was most evident in Ctnnb1's expressional profile change. These effects are consistent with other studies demonstrating that several bioflavonoids, such as resveratrol, can affect Wnt signaling. Two recent studies showed that the inhibitory effects of either resveratrol or apigenin on Wnt were at least partially via modulating Ctnnb1's nuclear localization,[32,33] thus suggesting a role of bioflavonoid in controlling Wnt signaling.

Our study has shown that the Wnt/β-catenin signaling pathway was deregulated in the aged heart. Two nutrition treatments known for their ability to retard aging, calorie restriction and resveratrol supplement, were able to increase Wnt/β-catenin signaling in aged heart to the level of young heart. Most notably, the expression of β-catenin, a key component of Wnt signaling, was decreased up to threefold in aged heart across all three studies. CR and resveratrol supplement almost completely reversed the change. In addition, our study also has suggested a role of TGF-β signaling pathway in heart aging. Methods of nutrition interventions to heart aging via modulating Wnt/β-catenin signaling were also explored. Learning from this rodent cardiac aging study is being applied to investigate cardiac aging and disease in pets.

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