Published in final edited form as:
Aging Cell. 2013 October ; 12(5): 851–862. doi:10.1111/acel.12109.

Late-life rapamycin treatment reverses age-related heart dysfunction

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Summary

Rapamycin has been shown to extend lifespan in numerous model organisms including mice, with the most dramatic longevity effects reported in females. However, little is known about the functional ramifications of this longevity-enhancing paradigm in mammalian tissues. We treated 24-month-old female C57BL/6J mice with rapamycin for 3 months and determined health outcomes via a variety of noninvasive measures of cardiovascular, skeletal, and metabolic health for individual mice. We determined that while rapamycin has mild transient metabolic effects, there are significant benefits to late-life cardiovascular function with a reversal or attenuation of age-related changes in the heart. RNA-seq analysis of cardiac tissue after treatment indicated inflammatory, metabolic, and antihypertrophic expression changes in cardiac tissue as potential mechanisms mediating the functional improvement. Rapamycin treatment also resulted in beneficial behavioral, skeletal, and motor changes in these mice compared with those fed a control diet. From these findings, we propose that late-life rapamycin therapy not only extends the lifespan of mammals, but also confers functional benefits to a number of tissues and

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Competing interests
The authors declare that they have no competing interests.

Author contributions
SM conceived of the overall project with input from all authors and supervised the study. RS and PK provided assistance in a portion of the experimental design. BJG prepared samples for RNA-seq, and SM performed subsequent statistical analysis. AZ carried out additional bioinformatic analysis with support from SDM. Expert assistance in data analysis and interpretation was provided by CJR and MDN. JMF, BKK, and MNO participated in the design, execution, and data analysis of all experiments with assistance from CAZ, MPP, and ECA. JMF primarily wrote the manuscript, with contributions from all authors.

Supporting Information
Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

mechanistically implicates an improvement in contractile function and antihypertrophic signaling in the aged heart with a reduction in age-related inflammation.

Keywords

aging; hypertrophy; strain echocardiography; rapamycin; RAD; mTOR; RNA-seq

Introduction

Aging causes a progressive decline in function of all tissues. For decades, the reasons for such a decline have remained largely speculative. Interventions targeting one specific age-related disease often achieve only a mild extension of lifespan, frequently accompanied by comorbidities. Recently, there has been increasing enthusiasm for the development of treatments that target the underlying molecular mechanisms of aging rather than focusing on specific diseases that arise as a function of increasing age. The National Institute on Aging Intervention Testing Program has developed a program to identify candidate compounds that are safe, noninvasive, and effective in extending the lifespan of model organisms (Nadon *et al.*, 2008), and the most successful agent identified to date in the program is the FDA-approved drug rapamycin. In this study, we investigate potential mechanisms for the efficacy of rapamycin in the extension of lifespan of aging mice, concomitant with evaluating the functional benefits to the aging heart.

Inhibition of TOR (target of rapamycin) signaling has been the focal point of numerous studies because of robust effects in extending lifespan in multiple model organisms ranging from worms to mice (Vellai *et al.*, 2003; Kapahi *et al.*, 2004; Harrison *et al.*, 2009). In contrast, we sought to critically test the *in vivo* benefits of this ‘anti-aging’ compound, particularly in the context of an intervention in older animals with preexisting pathologies, such as age-related cardiac dysfunction. This is a critical point, because it is not optimal to simply extend lifespan without an accompanying improvement in the health and quality of life. To accomplish this overall goal, we employed a number of noninvasive measures to survey the skeletal structure, cardiovascular function, and metabolic health of individual C57BL/6J mice at 24 months of age. These animals were divided into two groups ($N = 18$ for each group, treated with rapamycin vs. nontreated) and aged for an additional 3 months, facilitating comparison of multiple metrics of health within individual subjects longitudinally over the 3-month interval. The late-life examination of each subject’s health status in multiple organ systems over a 3-month period allows us to determine whether rapamycin treatment could be beneficial not just to survival, but in reducing the functional decline of an individual’s tissues during late life.

From a broad series of *in vivo* measures, we observed the greatest benefit of rapamycin treatment in improving age-related cardiac health, with lesser effects on other tissues. The underlying molecular changes within cardiac tissue were examined using RNA-seq for whole-transcriptome analysis, cytokine profiling of sera and cardiac tissue, and additional validation at the protein level to confirm the RNA-seq results. This study provides the first evidence that late-life intervention with the lifespan-extending drug rapamycin has

beneficial functional effects on the cardiovascular system, demonstrating either a reversal or attenuation of age-related cardiac decline. Furthermore, we have utilized bioinformatic analysis to uncover suites of related genes which rapamycin modulates in the aged heart, finding that it affects the expression of genes involved in calcium regulation, mitochondrial metabolism, hypertrophy, and inflammation. These data led to a novel link between rapamycin treatment and increased levels of the cardiac signaling regulatory protein, ras-related associated with diabetes (RAD) (Wang *et al.*, 2010). Overall, these findings suggest that rapamycin improves the aging heart's function through a reduction in age-related sterile inflammation while also promoting RAD expression, which has been shown to promote antihypertrophic signaling and enhance cardiomyocyte excitation–contraction coupling (Wang *et al.*, 2010).

Results

Rapamycin treatment

The metrics in this study were chosen to closely parallel those that are commonly measured in clinical situations in human aging. This study centers on age-related heart disease, which remains the leading cause of death in developed nations. The body mass, blood glucose levels, and respirometry were also examined to assess the metabolic health status of these animals. Echocardiography was used to examine the heart with functional metrics similar to those used in the clinical setting. Behavioral analysis was used as a surrogate for the mobility and activity of aged individuals. MicroCT was also employed to measure skeletal changes related to kyphosis and osteoporosis that are major problems in the aged population.

In this study, we used a well-established oral delivery method of microencapsulated rapamycin diet at 14 parts per million (Harrison *et al.*, 2009). This dosage is effective at delivering the drug into the blood and tissues of mice at pharmacologically relevant dosages [Fig. S1A, (Harrison *et al.*, 2009)]. Female mice were employed in this study for two primary reasons: (i) to avoid the confound of differences in lifespan between genders making it improbable to select equivalent biological age for the treatment time frame in a longitudinal study and (ii) the efficacy of rapamycin and mTOR inhibition as a lifespan-extending strategy has been clearly demonstrated to be stronger in females than males (Harrison *et al.*, 2009), as well as in multiple genetic backgrounds. To verify that this regimen was working in our hands, we evaluated the level of inhibition of mTOR in tissue extracts of the heart and demonstrated that mTOR is specifically inhibited *in vivo* without significantly affecting mTORC2 signaling activity (Fig. S1B,C), consistent with prior results using this diet (Harrison *et al.*, 2009; Wilkinson *et al.*, 2012).

Metabolic effects of rapamycin

In previous studies of mice treated with rapamycin, and in clinical use, it has been noted that chronic treatment with rapamycin has the potential to cause deleterious metabolic side effects including weight gain and glucose insensitivity (Yang *et al.*, 2012). To determine whether this was also true in the context of our experimental design, we measured body mass and fasted glucose levels weekly throughout the duration of our 3-month study (Fig. 1A,B). The mice treated with rapamycin showed a small weight gain over their baseline

measurements, which peaked at 6 weeks of treatment before declining back to baseline levels, indicating weight gain is a transient phenomenon with rapamycin treatment in this study. The total body weights between the control group and the rapamycin-fed group were not significantly different at any point during the 12-week observation period (Fig. 1A).

The control mice showed a stable, but slightly declining glucose level over the course of 3-month evaluation (Fig. 1B). These data are consistent with the known decline in glucose levels in >24-month-old C57BL/6 mice. Rapamycin-fed mice showed an initial increase in fasted blood glucose levels that was significantly higher than the control diet at 7-, 8-, and 10-week treatment. However, by 12 weeks, this effect was not significantly different from the mice fed the control diet, suggesting that the modulation of glucose levels by rapamycin treatment is a transient variable in C57BL6/J mice. The transient nature of rapamycin's metabolic side effects was also noted in a recent study by Fang *et al.* in young male mice treated with rapamycin between 3 and 8 months of age (Fang *et al.*, 2013). However, where this study reported a prevention of weight gain in the control mice over the rapamycin treated, our treated aged mice showed the converse result, where rapamycin-treated mice maintain a higher body weight than the control mice before returning to baseline.

Because mTOR signaling is intricately tied to nutritional status and energy metabolism, we carried out whole-body metabolic measures in both groups of mice at the end of our 3-month study. The mice were tested in a metabolic cage system for 48 h to examine metabolic profiles during their diurnal and nocturnal cycles (Fig. 1C–E, $n = 8$ per group). Mice treated with rapamycin showed slightly lower mean energy expenditure (EE) than control mice. This phenomenon was further enhanced after normalization to the animal's body mass (Fig. 1C,D). These data were not consistent with the dimensionless respiratory quotient (RQ) we measured (Fig. 1E). To calculate the EE, there are assumed values involved for the amounts of carbohydrate, fat, and protein. This under- or overestimation of body composition in EE calculation of animal models is a known pitfall of indirect calorimetry (Tschöp *et al.*, 2012). Because RQ is not dependent on such assumptions, it provides a more accurate depiction of the metabolic status of the animal. Therefore, we conclude that rapamycin does not have a significant effect on the whole-animal metabolic rate in aged animals with 3-month treatment.

Effects on the skeleton of 3-month rapamycin treatment in old mice

It is well known that mice exhibit both muscle and bone loss with age, similar to that seen in human aging (Syed & Melim, 2011; Sheard & Anderson, 2012). To measure changes in bone with age over the 3-month rapamycin treatment, mice in both cohorts were scanned using microCT at 24 (baseline) and 27 months of age (after 3 months of rapamycin treatment). The resultant microCT images were then used to construct 3D whole-body models at 35 μM resolution. To examine the progression of age-related spinal deformation in these mice, the kyphosis index (KI) was calculated by determining the distance from the body of the 7th cervical vertebrae to the 1st sacral vertebrae and the perpendicular distance to the maximal curvature (Fig. S2A). Mice showed significant reduction in KI at 27 months, indicating increasing curvature within the 3-month window between baseline and the 2nd

time point. This deformation of the spine was not ameliorated with the administration of rapamycin (Fig. S2B).

Although rapamycin was unable to prevent/attenuate the gross changes in the spine over this 3-month interval, there was a slight trend toward improvement in the cortical bone volume of the femurs. The femurs of mice from both cohorts were scanned *ex vivo* at 9 μM resolution at the end of the study comparing rapamycin-treated animals vs. untreated controls. The mid-shaft bone volume of rapamycin-fed mice was slightly greater (although not significantly) than that of mice fed the control diet (Fig. S2C, $n = 8$, $P = 0.313$). Similarly, the endosteal and periosteal thicknesses were improved in these bones as well. The trabecular bone of the spine was also analyzed from the L1 to L3 vertebra. The trabecular bone volume and trabecular thickness showed a trend toward improvement (Fig. S2C, $n = 14$, $P = 0.125$ and $P = 0.097$, respectively).

Beneficial behavioral changes induced by rapamycin

While measured for respiration rate in metabolic cages at the end of the 3-month therapy, control and rapamycin-treated mice were simultaneously monitored for activity and behavioral changes throughout a 48-h measurement period. Mice fed a rapamycin diet displayed some distinct beneficial behavioral changes relative to controls (Table S1). Analysis of the behavioral time budget for the rapamycin-treated mice revealed that they spend significantly more time performing voluntary wheel running than the control group ($n = 8$, $P = 0.01$). Access to a running wheel was not present in their normal housing environment during the 3-month treatment period. This finding is similar to that of previous reports in which chronic rapamycin treatment (beginning at 7 or 9 months of age) showed a significant increase in activity later in life when compared with controls (Miller *et al.*, 2011). We further analyzed the data on voluntary activity by determining the speed and frequency of running wheel motion for each event (Fig. S3A). These data showed that the rapamycin-treated mice do not exercise more intensely, as the mean speed is the same between groups, but they do exercise more frequently compared with control mice. This translates to short rest periods and an increase in total distance traveled during their nocturnal cycle (Fig. S3B). Therefore, rapamycin treatment increases spontaneous mobility when given the opportunity to exercise on a running wheel, which suggests that inhibition of mTOR may confer a resistance to muscle fatigue and greater endurance in addition to other health benefits.

Reduction in cardiac tissue inflammation

One of aging's molecular hallmarks is the presence of a sterile inflammation and cytokine production (Freund *et al.*, 2010). Sterile inflammation is defined as the presence of an immune response as a result of cellular damage from noninfectious sources. We sought to critically test whether rapamycin treatment reduces inflammation systemically in the sera and within aged heart tissue. To examine the total circulating cytokine levels in sera before and after the treatment period, we employed a multiplex bead-based system for the measurement of cytokine levels before and after rapamycin treatment. We evaluated 32 cytokines in sera and identified 4 that were significantly altered from baseline to post-treatment between the control and rapamycin-treated mice (Fig. 2A, $n = 10$ per group, $P < 0.05$). We identified a reduction in granulocyte colony-stimulating factor (G-CSF), LPS-

induced CXC chemokine (LIX), interleukin-17 (IL-17), and interleukin- 7 (IL-7). These data reveal that there is a significant systemic reduction in cytokines linked to inflammation (both pro-inflammatory and anti-inflammatory).

To further investigate potential inflammation within the heart at the protein level, we employed an antibody array system to simultaneously test the levels of 145 cytokines within cardiac tissue lysates from hearts collected from both groups at the end of the study (Fig. 2B, $n = 4$, $P < 0.05$). Forty-five cytokines were significantly different between the groups, and all of these differentially expressed cytokines were reduced in tissues from rapamycin-treated animals. The four cytokines modulated by rapamycin in the sera of these mice were also significantly reduced in cardiac tissue. Each cardiac tissue sample independently clustered based upon the levels of these differentially expressed cytokines (2B, control = green, rapamycin = blue). These data suggest that rapamycin treatment in the aged heart results in a suppression of the pro-inflammatory signaling which arises with age (Freund *et al.*, 2010). Overall, our data also suggest that while potentially related to each other, the anti-inflammatory effects of rapamycin are more potent within cardiac tissue than systemically in the sera.

Cardiovascular improvements and prevention of age-related hypertrophy

To determine the potential beneficial effects of rapamycin treatment on cardiovascular function in aged mice, high-frequency ultrasound imaging was performed before and after drug treatment in both the control and rapamycin-treated mice. Hearts were imaged using a number of anatomical views and imaging modes via echocardiography to determine the physical and functional parameters of the myocardium in systole and diastole (Table 1). The baseline heart function metrics of the mice in this study are similar to that of previous echocardiography studies on mice of an advanced age (>20 months of age) (Dai *et al.*, 2009). To gauge individual improvements to the heart from this late-life intervention, the net effect from baseline to the post-treatment time point was calculated for each mouse.

Remarkably, rapamycin-treated mice showed a significant improvement in the ejection fraction (%EF), the fraction of blood ejected from the ventricle after each heart cycle, after 3 months treatment (Table 1). Mice fed a control diet on average decreased their %EF by 7.2% from 24 to 27 months of age, while rapamycin-fed mice increased by 8.5% ($n = 17$, $P = 0.03$), demonstrating an improvement over baseline and a reversal of age-related cardiac dysfunction. This indicates that within individual animals, it is possible to achieve significant left ventricular functional improvement with a late-life pharmacological intervention with 3 months of effective treatment.

It is well established that C57Bl/6J mice develop age-related heart hypertrophy and impaired cardiovascular function as they age, similar to human beings (Dai *et al.*, 2009). We determined the change in left ventricular mass for each mouse via echocardiography, normalized to tibial length as previously described (Buyse *et al.*, 2009; Table 1). We observed that in both groups, a majority of the mice had increased their left ventricular mass over the 3-month evaluation period. However, the mice fed rapamycin showed significantly less hypertrophy than the control group ($n = 17$, $P = 0.0003$). These data were also confirmed by measuring the normalized wet weight of whole hearts after euthanizing the

mice (n = 10 per group, P = 0.004, Table 1). Overall, we report that rapamycin treatment significantly slows or reverses the progression of age-related hypertrophy and improves ventricular function of the aging heart at a late-life point of intervention.

Doppler-derived measurements were also taken to interrogate transmitral and transaortic blood flow in these mice. The Myocardial Performance Index (MPI) provides a Doppler-derived measure of global left ventricular function (Tei *et al.*, 1997), using the time intervals of mitral valve inflow and aortic valve outflow. Rapamycin treatment did not improve MPI, as compared to controls. The ratio between early (passive) and late (active) mitral inflow velocities (E/A) was also calculated as an indicator of overall diastolic function. No differences were found between the rapamycin-treated mice vs. controls.

To further investigate the improvement in heart function, we carried out speckle-tracking strain analysis of the echocardiography data to identify specific improvements in the deformation of the heart during the cardiac cycle. Strain analysis is recognized as a useful determinant of global and regional ventricular function in mice (Bauer *et al.*, 2011). This analysis uses motion tracking to observe discrete changes in length of the ventricular wall throughout systole and diastole. Strain and its time derivative, strain rate, can therefore be calculated in three primary orientations, corresponding to ventricular deformation: longitudinal, radial, and circumferential (Figs 3 and 4). On average, peak global strain declined or remained unchanged in the control mice, in all three orientations (longitudinal, radial, and circumferential; Fig. 3). In contrast, only circumferential strain was significantly improved with rapamycin treatment (Fig. 3C,F, n = 17, P = 0.028). Similarly, systolic circumferential strain rate was also significantly improved (Fig. 4C,F, n = 17, P = 0.030). No differences in longitudinal or radial systolic strain rates were observed following rapamycin treatment. Interestingly, we also observed significant improvements in global diastolic radial (Fig. 4B,E) and circumferential (Fig. 4C,F) strain rates, despite there not being significant changes in other diastolic function metrics observed via standard pulse wave Doppler echocardiography (n = 17, P = 0.049 and P = 0.009, respectively). This implies that the global peak strain rate may be a more sensitive indicator of left ventricular diastolic function than the Doppler-derived metrics. All strain and strain rate measures are summarized in Table S2 (Supporting information).

Rapamycin-induced differential gene expression

Because of the striking functional improvement in the heart compared with the other tissues examined, we performed detailed analysis on the molecular changes within cardiac tissue that arise due to rapamycin treatment in late life. Total mRNA was extracted from whole heart tissue from the two groups (rapamycin-treated vs. controls) and sequenced via paired-end Illumina HiSeq (n = 10 per treatment) at a depth of approximately 10 million reads per sample, according to standard protocols on a HiSeq 2000. After mapping the resultant reads to the mouse genome using CLCbio, we carried out differential gene expression using the edgeR package in bioconductor (bioconductor.org). Following correction for multiple testing ($P < 0.05$), we determined that there were 700 genes (Table S3) differentially expressed in response to rapamycin treatment, with the majority of these gene transcripts being downregulated (521 genes) in the hearts of rapamycin-treated mice (Table S3). Within

these differentially expressed genes, the rapamycin-treated animals have a consistent and distinct transcriptional profile, suggesting that the transcriptional profile across the 10 animals per group is distinctive and robust in affecting the heart's molecular transcriptional machinery. The differentially expressed gene list was analyzed using both ingenuity pathway analysis (IPA) and DAVID bioinformatics databases (david.abcc.ncifcrf.gov) to determine the over-represented biological functions and canonical pathways in the differentially expressed genes. The majority of upregulated genes upon rapamycin treatment are related to pathways involved in metabolic function and energy metabolism, consistent with related studies showing rapamycin treatment enhances mitochondrial function (Schieke, 2006). The downregulated genes show a number of gene categories related to regulation of the immune system and inflammation. These transcriptome data suggest that with age, there is increased representation of inflammatory processes, which are known to be associated with aging (Puntmann *et al.*, 2011). Furthermore, rapamycin attenuates the enhanced cardiac inflammatory response we observed in untreated controls. This hypothesis is supported by a promoter motif analysis we carried out on the differentially expressed genes. We divided the differentially expressed genes (700 total) into two groups: the downregulated and upregulated genes. These gene lists were then searched for a motif within promoter regions (±1000 base pairs from transcription start site) for each gene group using MEME motif-finding software. From all derived motifs, we chose only those that are significantly overrepresented (hypergeometric probability < 0.05) in the upregulated or downregulated groups of genes compared with all other genes in mouse genome (Table S4). We also scanned the TRANSFAC database with each of the selected motifs and determined the best match. For six of eleven significantly overrepresented motifs, we identified a known motif (Fig. S4). For the upregulated gene set, we identified motifs that are similar to Zfp281, Sp4, PXR, and Sp1. The downregulated gene promoter motifs resemble two canonical sequences for Zfp281 and Pax-6. These data provide a basis for the anti-inflammatory effects of rapamycin. Specificity protein 1 (Sp1) is known to inhibit inflammatory signaling and has an age-dependent decline in expression during cellular senescence (Oh *et al.*, 2007). Additionally, the activation of pregnane X receptor (PXR) signaling represses the inflammatory response through inhibition of the NF-KB pathway (Shah *et al.*, 2007). The differential upregulation of genes under the control of these two anti-inflammatory transcriptional regulators provides a partial mechanism for the cardiac-specific effects of rapamycin, in the suppression of inflammatory cytokines we observed by both RNA-Seq and protein array data. Similar rapamycin-induced signaling mechanisms have also been identified in association with mTOR in other paradigms (Astrinidis *et al.*, 2010; Ng *et al.*, 2011).

Improvement in cardiac tissue function

The RNA-seq data and bioinformatics analysis of promoter motifs generated some intriguing inferences about the anti-inflammatory effects of rapamycin treatment of the aging heart. Our analysis suggested alterations in Sp1- and PXR-related gene expression, which are both implicated in modulating inflammation. However, reduced inflammation alone does not provide a mechanistic explanation for the improved contractile function within the heart we observed via echocardiography. To further investigate the mechanisms for improved tissue function due to rapamycin treatment, we examined a number of classical

markers within heart tissue that could reasonably explain the improved outcomes. Hypertrophic response of the myocardium can be mediated by physical stretch of the cardiomyocyte as a result of distention during the cardiac cycle. During such stress, there can be an increase in the release of the cardiac hormones ANP and BNP as a means of modulating the hypertrophic response (Nishikimi *et al.*, 2011). Therefore, we measured both proteins and determined that although the level of ANP is significantly reduced in response to 3 months of rapamycin treatment (Fig. 5A, $n = 9$, $P = 0.006$), BNP levels were not significantly affected (Fig. 5B). Hence, rapamycin reduces the ANP-induced stress response feedback in the heart, which may prevent or dampen hypertrophic signaling either directly or indirectly. It is currently unclear why the effects of rapamycin do not alter the levels of both ANP and BNP consistently. Whether the alteration of these hormones is a direct action of rapamycin or a subsequent effect of improved physical dimensions of the heart remains an area of open investigation. The increase in the peak diastolic strain rates within the hearts of the rapamycin-treated mice suggests an increase in elasticity of the heart wall and a potential reduction in cardiac fibrosis. To determine whether rapamycin treatment was altering age-related fibrosis; we assayed the amount of collagen III to collagen I as an indication of fibrosis content. Neither the ratio of collagen III/collagen I nor the total collagen levels were altered by 3 months of rapamycin treatment (Fig. 5). This indicates that a late-life rapamycin treatment has no effect on the age-related increase in cardiac fibrosis.

The RNA-seq analysis provided a potential list of cardiac-related gene pathways that may be altered by rapamycin including cardiac hypertrophy and calcium signaling (Fig. 6). Analysis of the differentially expressed gene list indicated upregulation of the heart disease-related protein RAD, a potential modulator of cardiac function capable of both inhibiting hypertrophy and enhancing cardiomyocyte function by modulating calcium channels, tropomyosin, and calmodulin signaling (Fig. S5; Chang *et al.*, 2007; Yada *et al.*, 2007; Wang *et al.*, 2010). The inactivation of RAD in young transgenic models has been shown to cause ventricular arrhythmia (Yada *et al.*, 2007). Additionally, RAD mRNA is suppressed in the left ventricle of patients with end-stage heart failure and is linked to hypertrophy via physical interaction with the calmodulin-dependent kinase II (CaMkII), an essential protein involved in calcium homeostasis (Chang *et al.*, 2007). To assess the level of RAD in rapamycin-treated and control hearts of old mice, we quantitated the levels of RAD by Western blot and observed a significant increase in the levels of RAD from heart tissue lysates of mice treated with rapamycin (Figs 5D and S6, $n = 8$, $P = 0.024$). These data provide a potential linkage between rapamycin treatment and suppression of hypertrophy via enhanced levels of RAD and potentially help explain the improved diastolic function observed with speckle-tracking strain imaging.

Discussion

Rapamycin has been well characterized with regard to mechanism of action and physiological effects under particular clinical indications, such as organ transplant (McMahon *et al.*, 2011) and particular forms of cancer (Witzig *et al.*, 2011). The use of rapamycin as an inhibitor of mTOR signaling may have beneficial properties that go beyond its antiproliferative effects. Recent studies examining rapamycin as a lifespan-extending compound of model organisms including mice have shown rapamycin to be the most

effective candidate yet evaluated (Miller *et al.*, 2011), as well as demonstrating some benefits in terms of retarding age-related pathology (Wilkinson *et al.*, 2012). This is of obvious importance to researchers interested in the biology of aging and discovery of agents that slow or prevent postdevelopment senescence leading to the age-related decline of multiple organ systems (Blagosklonny, 2006).

However, one major concern in the development of a potential life-extending treatment is the long-term safety of the pharmaceutical. Such an agent should first improve the health of the individual and the lifespan second. One notable concern with regard to rapamycin in this context has been the publication of reports indicating potential metabolic side effects, which includes weight gain and glucose intolerance (Yang *et al.*, 2012). This specific study examined the metabolic side effects for a period of less than 6 weeks. Additional studies have found that rapamycin did not improve insulin sensitivity in ob/ob mice (Miller *et al.*, 2008) or enhanced progression of type II diabetes (Fraenkel *et al.*, 2008). One potential limitation to these studies has been the time frame in which these effects were evaluated. As we observed in our study, rapamycin treatment in old-aged mice results in elevated fasted blood glucose levels and an increase in body weight. However, this metabolic effect is transient and dissipated through 12 weeks of treatment despite the continued inhibition of mTOR signaling. Our findings on these transient effects on glucose homeostasis are consistent with a recent report of rapamycin's metabolic effects in young mice (Fang *et al.*, 2013). We observed a weight gain in older mice treated with rapamycin, rather than decreased body mass seen in younger mice treated with rapamycin. The enhanced body weight may be unrelated to a metabolic disorder and could simply be a retardation of normal age-related weight loss seen in C57BL6 mice. Interestingly, a recent study by Lamming *et al.* has reported that rapamycin modulation of mTORC1 signaling is responsible for the extension of lifespan, while mTORC2 is associated with the metabolic effects of rapamycin treatment (Lamming *et al.*, 2012). The examination of mTORC1 and mTORC2 signaling components revealed that at the terminus of the study mTORC1 alone is affected by continuous drug treatment with this late-life intervention. Overall, we conclude that these metabolic side effects are dependent upon specific signaling events that dissipate with longterm treatment. This interplay between mTORC1 and mTORC2 signaling should be examined in more detail to determine whether alternative mTOR inhibitors could minimize the initial mild side effects while retaining efficacy with long-term treatment.

Rapamycin intervention in aged mice significantly improved the behavior and spontaneous activity of the mice in this study. This finding agrees with previous reports of a trend toward increased activity in multiple lifespan extension paradigms including rapamycin treatment (Miller *et al.*, 2011; Wilkinson *et al.*, 2012) and caloric restriction in mice. If increased activity in model organisms via pharmacological intervention translates to increased movement in humans, it could represent a significant reduction in mortality (de Groot *et al.*, 2004). Similarly, a study of middle-aged runners discovered significant cardiovascular and other additional benefits as well as an overall improvement in the quality of life (Chakravarty *et al.*, 2008). From our data, the mice did not exercise significantly harder than the control mice, yet they did exercise more frequently. This indicates that muscle function is not drastically enhanced, but the mice may recover from fatigue more rapidly and are

more energetic than untreated control mice. These data are somewhat counterintuitive as reduced mTOR signaling normally counteracts skeletal muscle growth and repair (Goodman *et al.*, 2011). However, in the context of aging, rapamycin may rejuvenate or help maintain the signaling events controlling cellular metabolism and thereby improve muscle function.

The effects of aging on the skeletal system are well described in the progression of osteoporosis. In this study, we chose to examine the effects of rapamycin on cortical bone maintenance and spinal posture using microCT. We found that there was no significant prevention in the progression of increased spinal curvature over a 3-month interval late in life. In humans, there are few treatment options for kyphosis, and it usually involves corrective surgical procedures. While rapamycin treatment did not delay or attenuate spinal changes, there was a suggestion of improvement in the cortical bone volume scanned *ex vivo* at high resolution. Analysis of the femur mid-shaft revealed that the rapamycin-treated mice trended toward a higher cortical bone volume. This trend was also seen in the trabecular bone of the lumbar vertebra where a slightly higher bone volume and trabecular thickness were observed. It has been suggested that the modulation of mTOR may activate signaling events which would prevent the progression of lytic bone disease (Smink & Leutz, 2010) and can enhance the proliferation and differentiation of osteoblasts (Darcy *et al.*, 2012). To fully determine whether rapamycin may attenuate bone aging, further studies with chronic rapamycin treatment beginning prior to any significant loss of bone due to aging (~3 months of age for C57BL/6 mice) are warranted.

Surprisingly, we determined that rapamycin was shown to significantly reduce the presence of specific inflammatory cytokines within the blood and more dramatically within the cardiac tissue. We report for the first time a genome-wide expression profile of the aging heart by RNA-seq that demonstrates a significant downregulation of inflammatory response genes relative to untreated controls. This indicates that one potential mechanism for the beneficial effects of rapamycin in mice may be the suppression of age-related inflammation, or ‘inflammaging’ (Freund *et al.*, 2010), independent of inflammatory responses seen as a consequence of organ transplant in other modalities of rapamycin therapies. The release of pro-inflammatory cytokines from cardiac cells has been demonstrated to promote fibrotic changes and hypertrophy, and therefore, a reduction in pro-inflammatory cytokines may prevent tissue damage both locally in the heart and systemically (Aoyagi & Matsui, 2011).

Our investigations of cardiac function in aged mice treated with rapamycin demonstrate that age-related heart dysfunction can be improved even in late life via appropriate pharmacological intervention. Our results strikingly show a functional improvement in ejection fraction and multiple other functional metrics with a reduction in hypertrophy of the heart over baseline levels prior to rapamycin treatment. These data showed a reversal of the typical progressive age-related decline seen in C57BL/6J mice (Dai *et al.*, 2009). This result is in direct contrast to recent studies examining acute models of heart disease, which determined that mTOR activation was protective in the context of ischemia–reperfusion injury or transaortic banding in young mice (Song *et al.*, 2010; Aoyagi *et al.*, 2012). Our results are more consistent with studies reporting the cardioprotective effects of mTOR inhibition (McMullen *et al.*, 2004; Gao *et al.*, 2006). Our data are further supported by clinical findings on organ transplant patients treated with an mTOR inhibitor, who

subsequently exhibited reduced risk of cardiac disease (Paoletti & Cannella, 2009). Additionally, we observed an improvement in the global peak strain and peak strain rates, a sensitive measure of myocardial systolic and diastolic function (Bauer *et al.*, 2011), and these measures are the first reports using strain analysis in the aged mouse heart. Due to the helical orientation of ventricular muscle fibers, deformation of the left ventricle can be observed in three different orientations: longitudinal, circumferential, and radial. Here, we observed significant improvements in the circumferential orientation, which appears directly related to the net improvement in ejection fraction. Importantly, these same measures are used clinically and significantly decline with age or hypertrophy in patients (Mizuguchi *et al.*, 2010). This result demonstrates for the first time the rapamycin's ability to prevent loss of heart function in the context of the aging mouse with an improvement in global peak strain and peak strain rates.

RNA-seq on RNA from heart tissue of treated vs. untreated mice identified a suite of genes that are differentially expressed. Of the 700 statistically significantly differentially expressed genes, a significant majority of these are downregulated by rapamycin treatment. Pathway analysis of these genes showed that a large number of these downregulated gene expression changes are related to a suppression of inflammation. These data were supported by additional promoter motif analysis of this data set which indicated an overrepresentation of genes that are modulated by anti-inflammatory transcriptional regulators, SP1 and PXR. We then determined gene expression patterns that were specifically related to heart function. This analysis led to the identification of overlapping gene sets that were involved in mitochondrial fatty acid metabolism, cell adhesion, cardiac hypertrophy, and calcium handling. The mitochondrial fatty acid metabolism genes are consistent with other reports on mTOR modulation of metabolism and increasing mitochondrial function (Schieke, 2006). Although we did not detect whole-animal changes in metabolic rate using respirometry, rapamycin has been shown to modulate mitochondrial function *in vitro* and may have tissue-specific effects (Ramanathan & Schreiber, 2009). The analysis of the remaining cardiac-related differentially expressed genes identified RAD as a potential modulator of cardiac function in the aged heart, which is a key protein in calcium signaling (Yada *et al.*, 2007), and has been linked to cardiac hypertrophy and heart failure (Chang *et al.*, 2007; Wang *et al.*, 2010). We therefore asked whether or not the protein levels of RAD in cardiac tissue from rapamycin-treated mice vs. controls reflected our transcriptomic results. We determined that RAD is indeed significantly upregulated with rapamycin treatment, which could be mechanistically linked with the antihypertrophic and improvement in cardiac contractility (Chang *et al.*, 2007; Wang *et al.*, 2010). RAD functions in the cardiomyocyte in part by direct binding to the $Ca_v\beta$ subunit of voltage-gated L-type calcium channels (Wang *et al.*, 2010). Through this interaction, the activity of L-type calcium channels is modulated, resulting in the suppression of calcium currents (I_{Ca}) and a reduction in action potential duration (Yada *et al.*, 2007; Wang *et al.*, 2010). The action of RAD therefore suggests a counteraction of age-related changes in cardiomyocyte excitation–contraction coupling, which can lead to heart failure. Overall, these data support the hypothesis that rapamycin treatment improves excitation–contraction coupling in cells while simultaneously reducing endogenous stress signaling in the heart, resulting in preserved if not improved cardiac

function with aging. Further studies are warranted to examine the specific effects of rapamycin on individual cardiomyocyte function in the context of aging.

Rapamycin's efficacy as a functional intervention against all age-related pathologies remains to be seen, and there are some recent data suggesting negative outcomes from long-term treatment in specific tissues (Wilkinson *et al*., 2012). There are also some concerns about the side effects with relation to dyslipidemia and diabetic complications. However, our study demonstrates a reversal or attenuation of age-related specific cardiovascular and behavioral benefits, which could impact the overall 'healthspan' and lifespan of an individual. We provide new evidence demonstrating a suppression of age-related inflammation in the heart, an upregulation of antihypertrophic signaling, and a physiological improvement of heart function over the course of 3 months late-life intervention. We show for the first time an efficacious pharmacological intervention for the age-related loss of heart function using an agent that has been demonstrated to extend lifespan in multiple species. Further studies on the metabolic and biochemical changes occurring within the aging murine heart are needed to fully appreciate how rapamycin's mechanism of action modulates its beneficial effects at the cellular level. The continued exploration of mTOR inhibitors will also provide a greater understanding of the optimal timing for intervening in aging and age-related pathologies. Therefore, the data presented here open new avenues of research to develop life-extending compounds that minimize adverse reactions while providing the maximal health benefits.

Experimental procedures

Full detailed methods and experimental procedures are available in online supplement. All Sequencing Data deposited in the GEO database under the accession GSE48043.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding support for this research was generously provided by the following grants; PO1 AG025901(SM), RO1 AG033373 (BKK), 1U01-AG022307 (RS), T32AG000266 (Buck Institute), and partial support from the Larry L. Hillblom Foundation Network grant (2007 2/F), the Ellison Medical Foundation(BKK), and the Glenn Foundation for Medical Research(SMand JF). This research was also supported in part by grants from Merck to SM (Investigator Initiated Studies Program, # 39326, and #50196).

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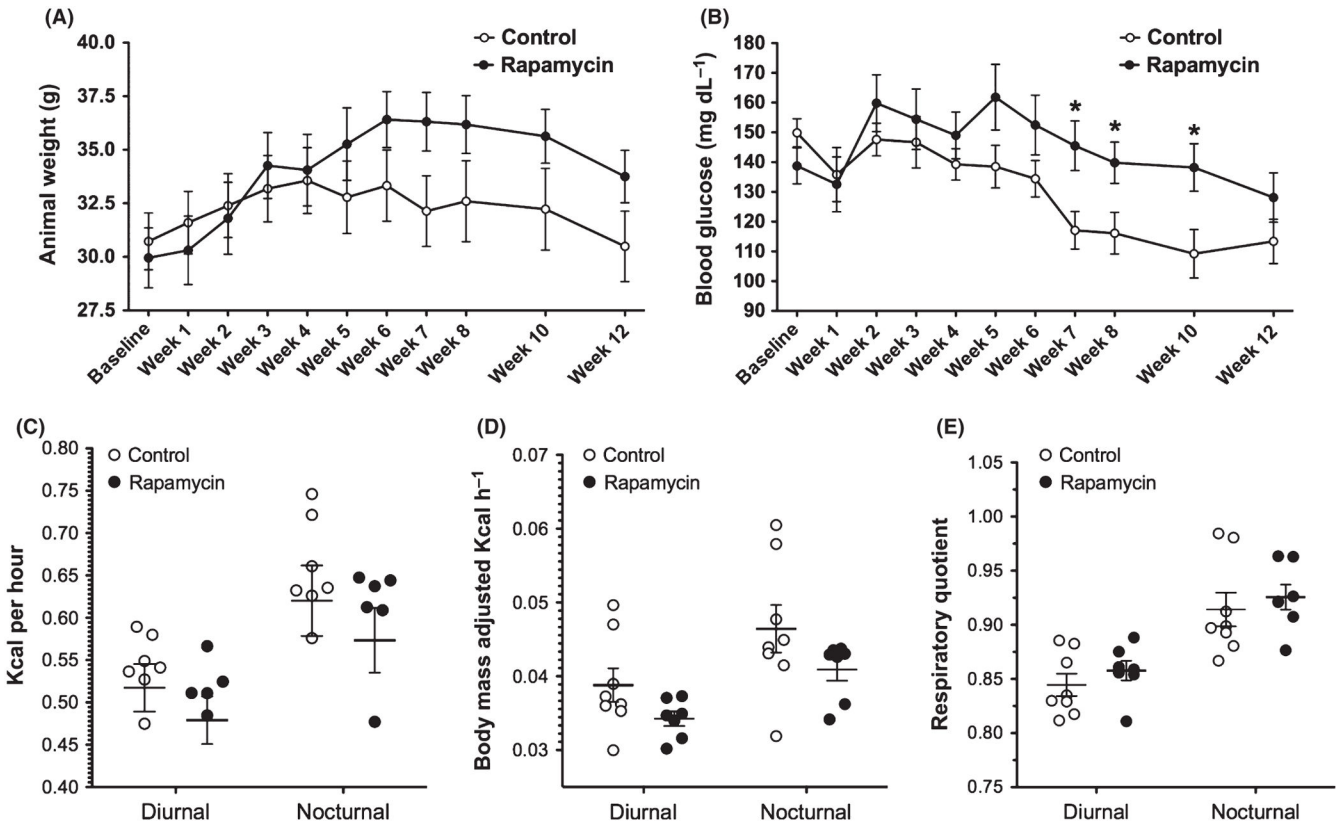

Fig. 1.

Body composition and metabolism. Metabolic profile of the mice determined by weekly whole-body mass (A) and fasted blood glucose (B) measurements. Rapamycin increased body mass from the baseline and peaked at 6 weeks, yet was not significantly different by 12 weeks of treatment. The fasted blood glucose levels were significantly different from mice on the control diet at 7, 8, and 10 weeks, but was not significantly different at 12 weeks (n = 11, $P < 0.05$). After 3 months of treatment, mice were tested in a metabolic cage system. The mean diurnal and nocturnal values for Kcal per hour, body mass–adjusted Kcal per hour, and the respiratory quotient (RQ) are shown for each mouse. The rapamycin-fed mice trended toward lower average energy expenditure (EE) (C) enhanced by normalizing the data to the body weight (D). Conversely, the RQ values (E) that are unbiased by estimation of fuel source or body mass are equal in both groups, indicating no significant enhancement of metabolism with rapamycin treatment.

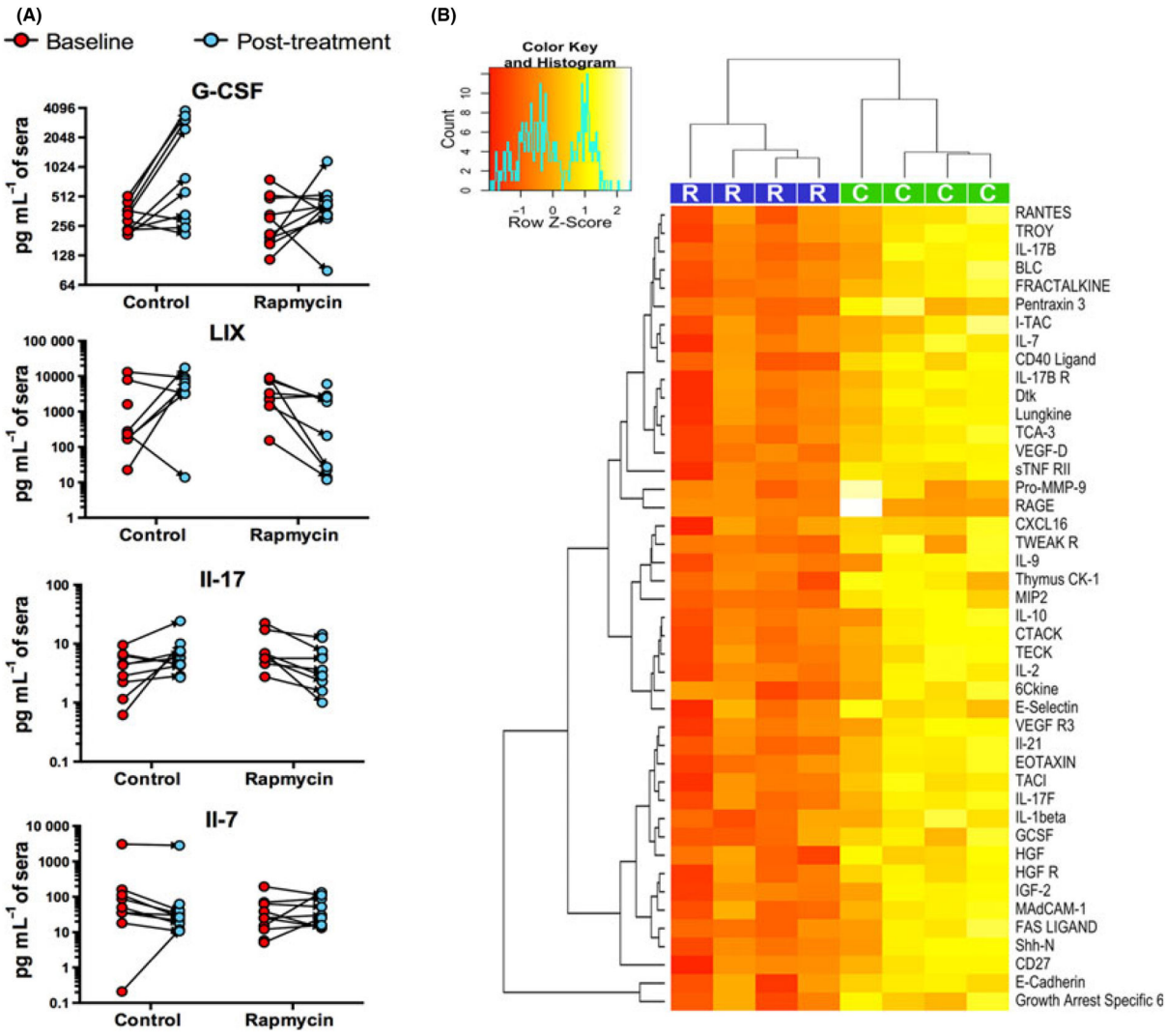

Fig. 2.

Inflammatory cytokine profiling of sera and cardiac tissue. (A) 32 cytokines were examined in the sera and are shown as log pg mL^{-1} from baseline to post-treatment. Only 4 of the cytokines tested were significantly altered by the treatment ($n = 10$, $P < 0.05$) G-CSF, LIX, IL-17, and IL-7. (B) Cardiac tissue inflammation was assayed by antibody array against 144 cytokines within heart tissue lysate. We discovered 45 differentially expressed cytokines between the rapamycin and control treatments ($n = 4$, $P < 0.05$). These cytokines are displayed as a heatmap showing that inflammation is uniformly downregulated within heart tissue of rapamycin (blue squares) that independently clusters from the control mice (green squares).

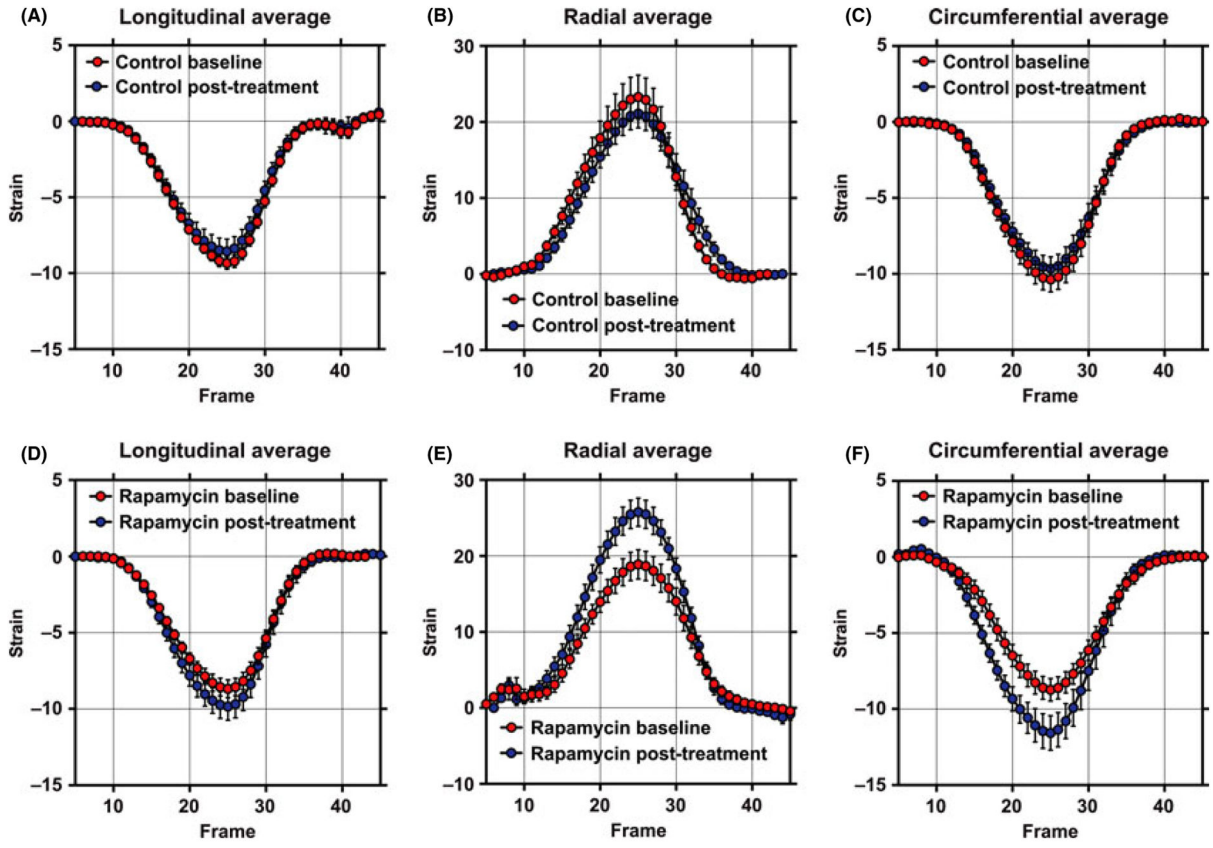

Fig. 3.

Speckle-tracking strain image analysis of peak strain. Echocardiography data were processed and analyzed with speckle-tracking software to identify the deformation of the whole heart wall throughout the cardiac cycle. This analysis resulted in peak strain measurement in multiple orientations. Control mice demonstrate a slight loss in the global peak strain from baseline (red) and post-treatment (blue) in the longitudinal (A), radial (B), and circumferential (C) views of the heart. Rapamycin-treated mice trend toward some improvement in the longitudinal (D) and radial (E) peak strain rates. The circumferential global peak strain (F) is significantly improved in these mice ($n = 17$ $P = 0.024$). All data are summarized in Table S2.

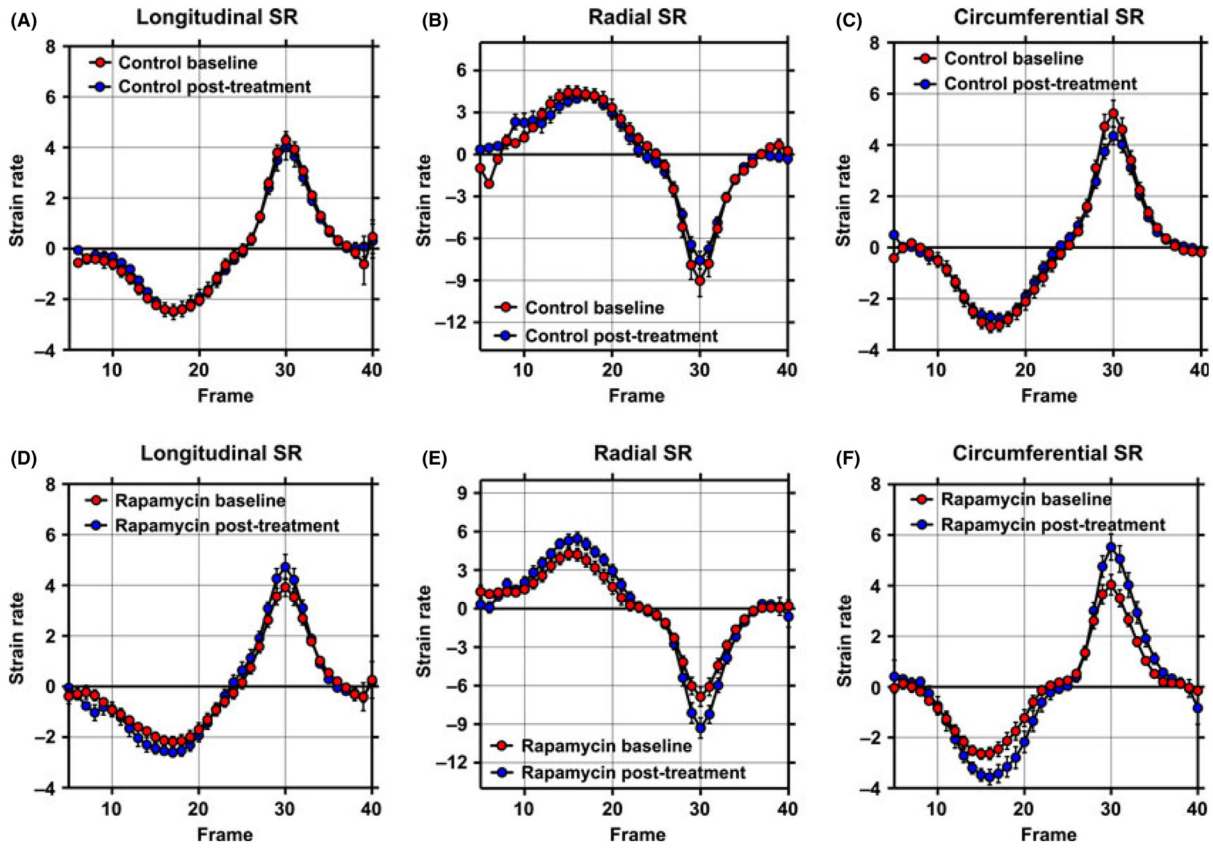

Fig. 4.

Speckle-tracking image analysis of systolic and early diastolic strain rate. Using the same analysis software as the strain calculation, the strain rate was also calculated for multiple orientations. This measurement results in the systolic peak rate (1st peak) and the early diastolic peak strain rate (2nd peak). The control mice demonstrate a very slight loss in the global peak strain rates from baseline (red) and post-treatment (blue) in all anatomical views of the heart (A–C). This same analysis of the rapamycin-treated mice (D–F) showed that the radial orientation (E) shows significant improvement in the early peak diastolic strain rate (n = 17, P = 0.49), but not in the systolic peak strain rate. The circumferential global peak strain rate (F) is significantly improved in both the systolic and early diastolic phases of the cardiac cycle (n = 17, P = 0.030 and P = 0.009, respectively). All data are summarized in Table S2.

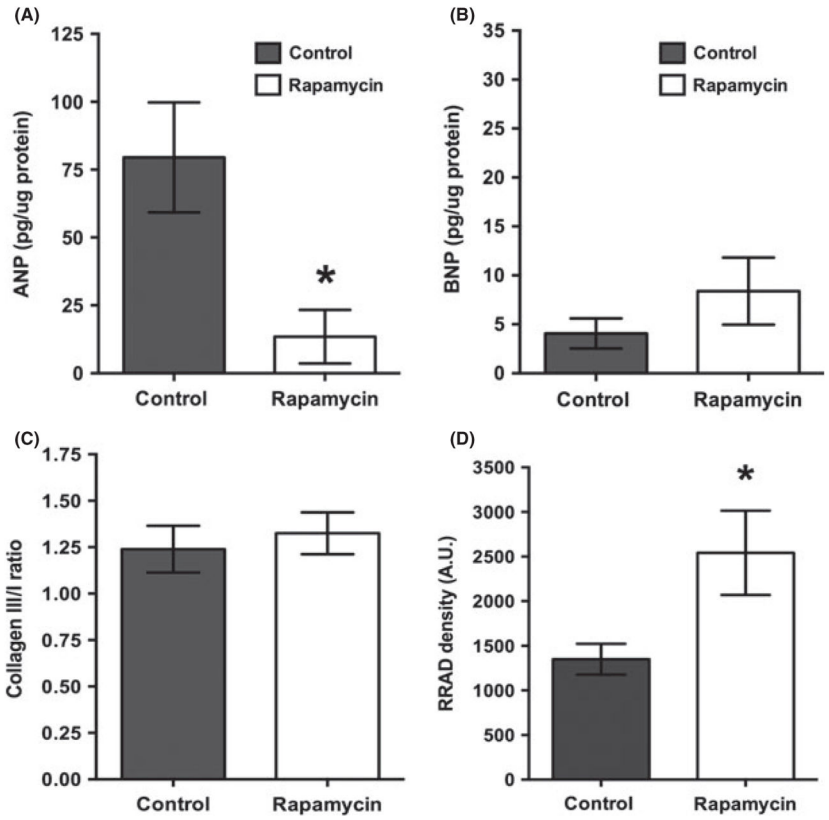

Fig. 5.

Cardiac signaling and fibrosis. The level of the cardiac hormones ANP (A) and BNP (B) was determined by ELISA. ANP was significantly reduced in the rapamycin-treated mice ($n = 9$, $P = 0.006$) as compared to controls (A). The level of brain BNP did not show a difference between treatments (B). The level of fibrosis determined by Western blot of collagen I and collagen III. The ratio or total level of collagen isoforms was not significantly altered by rapamycin ($n = 6$) (C). Western blot densitometry of hypertrophy modulator RAD is significantly upregulated by rapamycin treatment as compared to the control mice ($n = 8$, $P = 0.024$) (D).

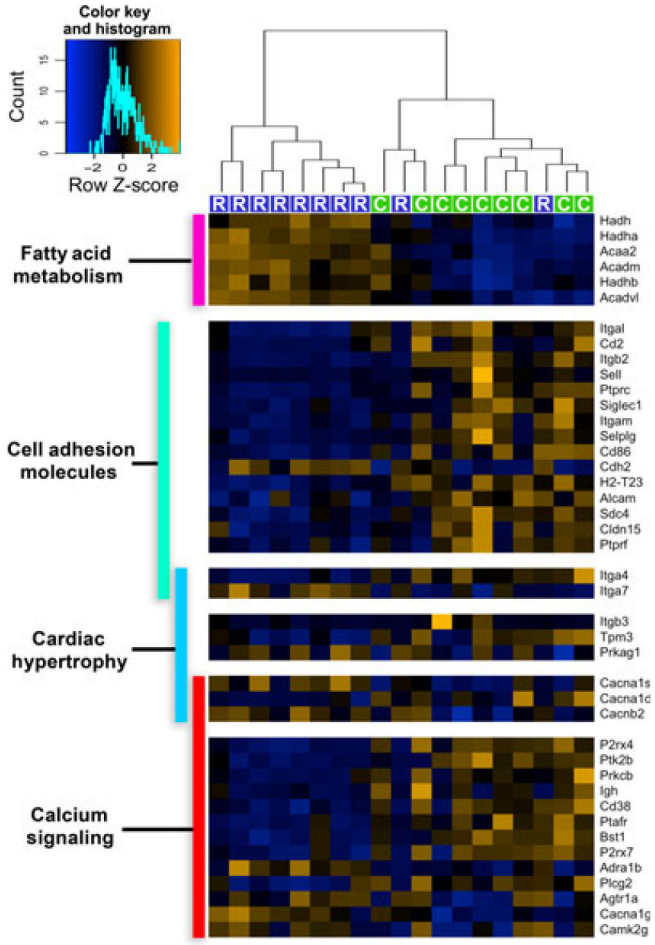

Fig. 6.

RNA-seq analysis of rapamycin-treated heart. Differential gene expression analysis revealed 700 genes that were differentially expressed between treatment groups (n = 10 tissues per group). This suite of genes was examined for overrepresented pathways using KEGG pathway analysis. Pathways most relevant to heart function were selected to identify genes of interest shown as a heatmap with the pathway identified on the left and the gene on the right. Expression data were independently clustered demonstrating the differential expression between the control mice (green box) and the rapamycin-treated mice (blue box) for each genetic pathway.

Table 1

Heart function and physical parameters. This table displays the physical and functional parameters of the heart at an advanced age between 24 (baseline) and 27 months (post-treatment). These parameters convey the changes observed with and without the intervention of rapamycin. Rapamycin significantly improves a number of parameters including the prevention of hypertrophy and improved ejection fraction

	Control				Rapamycin				Permutation test		
Metric	**Baseline**	**SD**	**Post-treatment**	**SD**	**Baseline**	**SD**	**Post-treatment**	**SD**	**N per Group**	***P*-value**	**Significant difference**
Hemodynamics											
Heart rate, bpm	484	46	482	37	470	30	468	41	17	0.81	n.s.
Doppler metrics											
E, cm s^{-1}	467.6	117.7	539.8	155.9	506.7	104.0	531.2	116.6	10	0.34	n.s.
A, cm s^{-1}	362.4	119.0	461.6	121.5	376.7	105.8	446.7	82.4	10	0.49	n.s.
E/A	1.3	0.3	1.2	0.3	1.4	0.2	1.2	0.1	10	0.07	n.s.
MPI	0.88	0.19	0.88	0.14	0.93	0.16	0.87	0.24	10	0.72	n.s.
LV structure											
LVSd	1.10	0.3	1.10	0.3	0.98	0.20	1.24	0.40	17	0.04	***
LVIDd	3.54	0.52	3.96	0.56	3.70	0.41	3.66	0.43	17	0.01	***
LVPWd	1.17	0.38	1.29	0.38	1.09	0.45	1.14	0.32	17	0.54	n.s.
LVSs	1.60	0.36	1.56	0.26	1.49	0.25	1.70	0.34	17	0.09	n.s.
LVIDs	2.43	0.77	2.86	0.74	2.63	0.53	2.41	0.48	17	0.02	***
LVPWs	1.55	0.52	1.69	0.41	1.52	0.46	1.55	0.33	17	0.54	n.s.
LVESV, μL	22.8	14.7	33.5	22.1	25.4	11.8	22.2	9.7	17	0.02	***
LVEDV, μL	62.0	18.1	79.3	24.2	66.2	16.4	68.4	16.9	17	0.02	***
LVSV, μL	39.2	6.2	45.8	11.4	40.8	9.3	46.3	10.6	17	0.49	n.s.
CO, mL min^{-1}	18.1	2.8	21.0	5.8	18.1	4.1	20.8	5.0	17	0.78	n.s.
LV Mass (mg)	113.2	27.2	171.1	45.2	133.3	30.8	138.9	19.9	17	0.0008	***
Heart mass											
ECHO-derived LV mass to tibial length (mg mm^{-1})	6.3	1.5	9.5	2.3	7.4	1.8	7.7	1.1	17	0.0003	***
Whole-heart weight to tibial length (mg mm^{-1})			13.9	2.8			10.7	1.5	10	0.004	***
LV function											
% Ejection fraction	67	15	61	16	63	11	69	9	17	0.03	***

	Control				Rapamycin				Permutation test		
Metric	**Baseline**	**SD**	**Post-treatment**	**SD**	**Baseline**	**SD**	**Post-treatment**	**SD**	**N per Group**	***P*-value**	**Significant difference**
% Fractional shortening	38	13	34	11	34	8	38	8	17	0.03	***

*** $P < 0.05$.