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TITLE: Effect of Ketone Bodies on Mitochondrial Cardiomyopathy and Heart Aging

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14. ABSTRACT
In this study, we used a novel mouse model of mitochondrial cardiomyopathy (transgenic mice overexpressing TMEM135; Tg-TMEM135) to test whether the heart disease can be prevented by supplementation of ketone body, which is a metabolite of one of the branched chain amino acids (BCAAs) whose metabolism is decreased in the heart of these mice. We also tested whether ketone body supplementation prevents pathologies associated with aging of the heart. Our results indicate that ketone body supplementation improved left ventricle hypertrophy of the heart, vacuole formation in the heart muscle, and the survival of Tg-TMEM135 mice. In aged mice, the left ventricle volume and mass showed increase compared to young mice. These measurements were significantly reduced in aged mice with ketone body supplementation, suggesting that ketone body supplementation may improve aging-associated hypertrophy of the heart. These findings suggest the possibility that ketone body supplementation may be effective in improving mitochondrial cardiomyopathy and heart abnormalities associated with aging in humans. Based on our finding that ketone body supplementation can substitute for impaired BCAA catabolism in Tg-TMEM135 mice, ketone body supplementation may be also effective in preventing cardiovascular disease in patients with impaired BCAA metabolism, which is being suggested as a risk factor/biomarker for cardiovascular disease.

15. SUBJECT TERMS
Mitochondria, mitochondrial dynamics, mitochondrial disease, cardiomyopathy, mouse model, transmembrane protein 135 (TMEM135), aging, ketone body, supplementation

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1. Introduction

In this research, we use a novel mouse model of mitochondrial cardiomyopathy to test whether the heart disease can be prevented by supplementation of a ketone body, which is a metabolite of a specific amino acid whose metabolism is decreased in the heart of these mice. Since this mouse model shows similar heart conditions and gene expression profiles as aged mice, we further hypothesize that the ketone body supplementation may also prevent aging of the heart, which we will test in aging mice. If successful, we will lay a foundation for an innovative strategy to prevent mitochondrial cardiomyopathy and heart aging. Knowledge gained from this study can be applied to develop preventative approaches for heart symptoms in patients with mitochondrial diseases as well as for heart aging in the general population.

2. Keywords

Mitochondria, mitochondrial dynamics, mitochondrial disease, cardiomyopathy, mouse model, transmembrane protein 135 (TMEM135), aging, ketone body, supplementation

3. Accomplishments

-What were the major goals of the project?

Aim 1: To test the effect of ketone bodies on the heart defects and sudden death of Tmem135 transgenic Tg-TMEM135) mice, a model for mitochondrial cardiomyopathy.

Aim 2: To test the long-term effect of ketone bodies on the age-associated abnormalities of the heart

-What was accomplished under these goals?

Aim 1: To test the effect of ketone bodies on the heart defects and sudden death of Tmem135 transgenic Tg-TMEM135) mice, a model for mitochondrial cardiomyopathy.

(1) Test the effect of ketone bodies on heart abnormalities and sudden death of Tg-TMEM135 mice at the weaning age

During the course of the study, we observed great variability in the severity of heart phenotypes in Tg-TMEM135, which is likely due to the mixed genetic background of mice (in which preliminary findings were made). Therefore, we have decided to use Tg-TMEM135 that are congenic on FVB background (backcross generation >N4; theoretically >~94% is FVB background; we will refer to these mice as FVB.Tg-TMEM135 mice) in this Aim. In a separate study, we found that the incidence of weaning age death increases as we backcross Tg-TMEM135 to the FVB strain while it decreases as we backcross them to the B6 strain, indicating the clear effect of the background. Therefore, we have decided to use FVB.Tg-TMEM135 mice that show severer phenotypes.

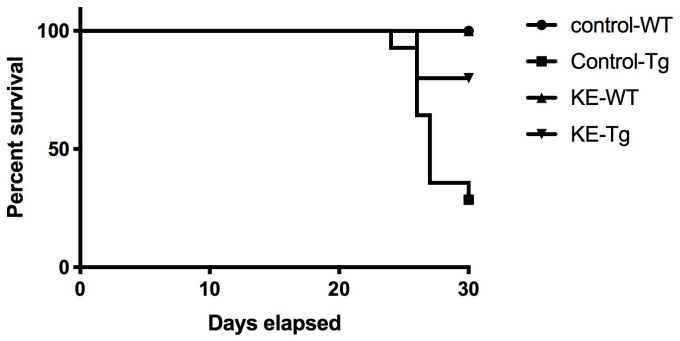

Fig. 1 Survival of FVB.Tg-TMEM135 and WT mice on KE or control diet around the weaning age.

To test whether ketone bodies improve the heart conditions and survival rate of FVB.Tg-TMEM135 mice at

weaning, we added ketone ester (KE), R,S-1,3-butanediol acetoacetate diester into the standard chow diet at 10% of volume. Saccharin was added at 1% for palatability. KE-diet was also supplied to wild-type (WT) littermate mice. To FVB.Tg-TMEM135 and WT mice in the control group, the standard chow diet mixed with water was supplied. The KE or control treatment was carried out from postnatal day (P)15 (pups start eating solid food around this age) to examine the mortality around weaning (P21- P30) (Fig. 1). We compared the survival curves of these four groups by Log-rank test and found that they are statistically different ($P<0.0001$). Notably, ten out of 14 FVB.TMEM135-Tg mice that were fed control diet died around the weaning age, whereas two out of 10 FVB.TMEM135-Tg mice that were fed KE diet died around the weaning age. Comparison of the survival curves of these two groups alone also showed statistically significant difference ($P=0.0238$).

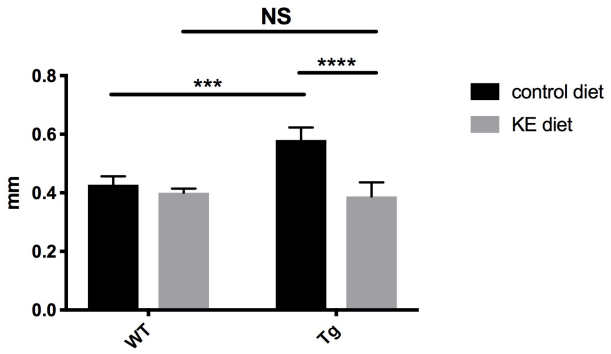

Fig. 2 Left ventricle (LV) posterior wall thickness at end-diastole of WT and FVB.Tg-TMEM135 mice on control or KE diet at P21.

We measured the heart functions of KE-treated and non-treated FVB.TMEM135-Tg mice as well as KE-treated and non-treated wild-type littermate control mice by echocardiograms at P21 using the VisualSonics Vevo770 system. We observed that the left ventricle (LV) posterior and anterior wall thickness at end-diastole are significantly increased in FVB.TMEM135-Tg mice on the control diet compared to those of WT control mice as well as those in KE-treated FVB.TMEM135-Tg and WT control mice (Fig. 2; $p=0.0001$, 2-way ANOVA with Bonferroni's multiple comparison test). These results show that the left ventricle wall thickness is increased in FVB.TMEM135-Tg mice compared to WT mice by P21 on control diet, while this increase is not observed in FVB.TMEM135-Tg mice that were fed KE-diet (P15-P21), suggesting that KE diet may prevent the development of LV hypertrophy in FVB.TMEM135-Tg mice.

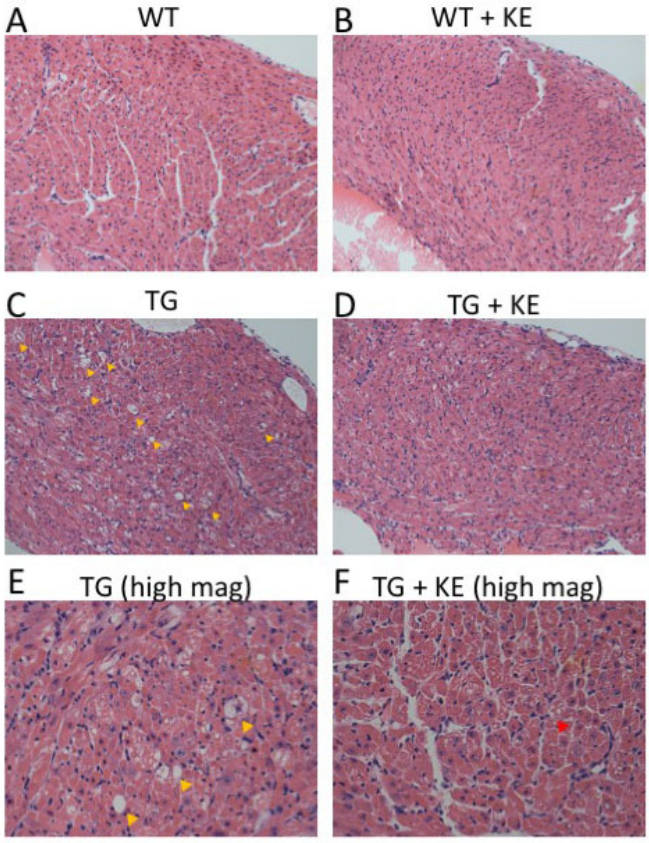

Fig. 3 H&E stained heart sections of WT mice on control (A) and KE diet (B) and FVB.Tg-TMEM135 mice on control (C) and KE diet (D) at P21. (E, F) Higher magnification views of hearts of FVB.Tg-TMEM135 mice on control (E) and KE diet (F).

We then performed histological analysis of the heart of these mice that were collected at P22. Hematoxylin and eosin (H&E) stained heart sections of FVB.TMEM135-Tg on control diet showed numerous vacuoles (Fig. 3C, E, yellow arrowheads) that are not observed in WT mice on control diet as well as KE diet (Fig. 3A-B). FVB.TMEM135-Tg mice on KE diet showed improved heart morphology with fewer and smaller vacuoles (Fig 3D, F, red arrowhead) compared to FVB.TMEM135-Tg mice on regular diet (Fig. 3C, E). These results indicate that vacuole formation in the heart of FVB.TMEM135-Tg mice was improved by ketone body supplementation.

We examined fibrosis in the heart of these mice by Masson's trichrome staining which visualizes collagen as blue. We quantified the percentage of blue-stained area using ImageJ. This analysis did not show any statistical difference between any of the genotype/treatment

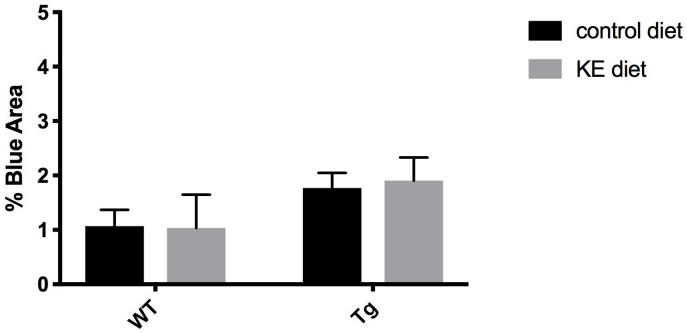

Fig. 4 Quantification of blue-stained area (% Blue) by Masson's trichrome-staining in hearts of WT and FVB.Tg-TMEM135 mice on control or KE diet at P22.

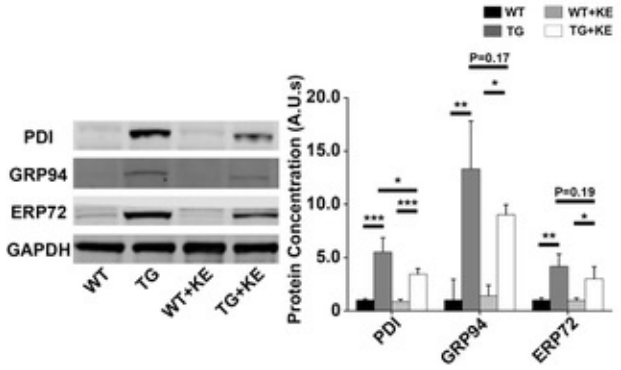

Fig. 5 Western blot analysis of ER stress markers in hearts of WT and FVB.Tg-TMEM135 mice on control or KE diet at P22.

groups by two-way ANOVA with Bonferroni's multiple comparison test (Fig. 4). This result indicates that fibrosis is not significantly increased in the FVB.TMEM135-Tg heart by the weaning age.

Heart samples were also collected at P22 to test the level of ER stress markers by western blot analysis. Signals for ER stress markers, PDI, GRP94, and ERP72 are all upregulated in FVB.Tg-TMEM135 mice compared to WT mice on control diet (Fig. 5). While signals for these ER stress markers are still higher in FVB.Tg-TMEM135 mice compared to WT mice on KE diet, comparison between FVB.Tg-TMEM135 mice on KE diet with FVB.Tg-TMEM135 mice on control diet shows significantly lower level of PDI and lower trends for GRP94 and ERP72 (although they did not reach statistical significance) in FVB.Tg-TMEM135 mice on KE diet (Fig. 5). These results show that ER stress is upregulated by overexpression of TMEM135 by the weaning age, which can be reduced by supplementation of ketone body.

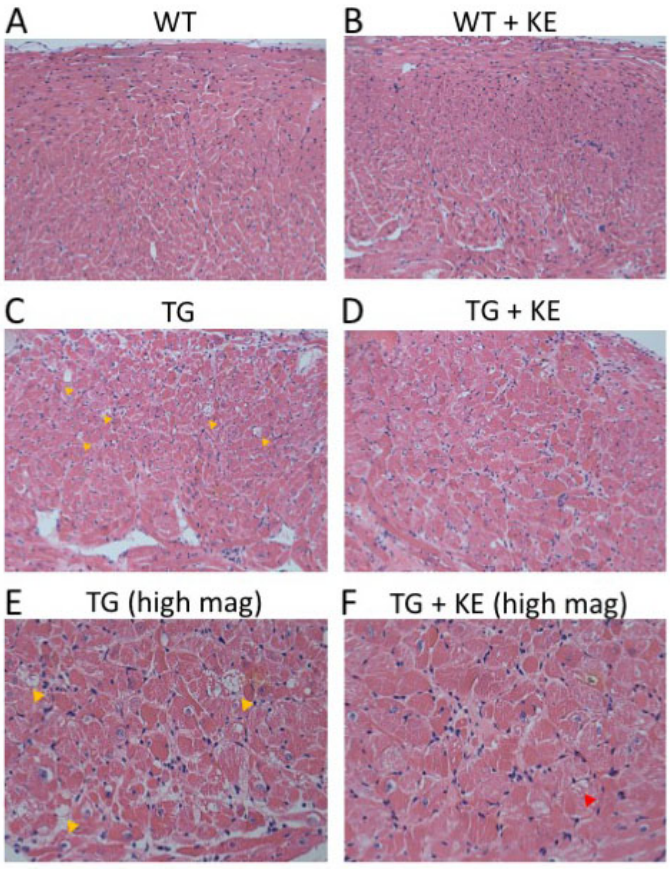

Fig. 6 H&E stained heart sections of WT mice on control (A) and KE diet (B) and FVB.Tg-TMEM135 mice on control (C) and KE diet (D) at 8 months of age. (E, F) Higher magnification views of hearts of FVB.Tg-TMEM135 mice on control (E) and KE diet (F).

(2) Test the long-term effect of ketone bodies on the Tg-TMEM135 heart

In order to test the long-term effect of ketone bodies on the FVB.Tg-TMEM135 mouse heart, we fed KE diet to FVB.Tg-TMEM135 mice along with WT littermate control mice, and control diet to FVB.Tg-TMEM135 mice and WT mice from P15 to 8 months.

At 8 months of age, heart functions of these mice were examined by echocardiography. Surprisingly, comparison between FVB.Tg-TMEM135 and WT mice on regular diet did not show significant difference in any of the measurements. However, it is interesting to note that several measurements including left ventricle volume and mass are significantly lower in FVB.Tg-TMEM135 mice on KE diet compared to the other 3 groups (WT mice on KE diet, WT mice on control diet, FVB.Tg-TMEM135 mice on control diet). This observation suggests that KE diet may have an effect to decrease the left ventricle volume/mass specifically in mice

overexpressing TMEM135.

H&E staining of the heart sections at 8-month-old FVB.Tg-TMEM135 mice on control diet (Fig. 6C, E) showed a disorganized structure with vacuoles (yellow arrowheads), albeit not as severe as those observed at P22 (Fig. 3). In FVB.Tg-TMEM135 mice on KE diet (Fig. 6D, F), the heart structure is less dis-organized with fewer/smaller vacuoles (red arrowhead) compared to FVB.Tg-TMEM135 mice on control diet (Fig. 6C, E).

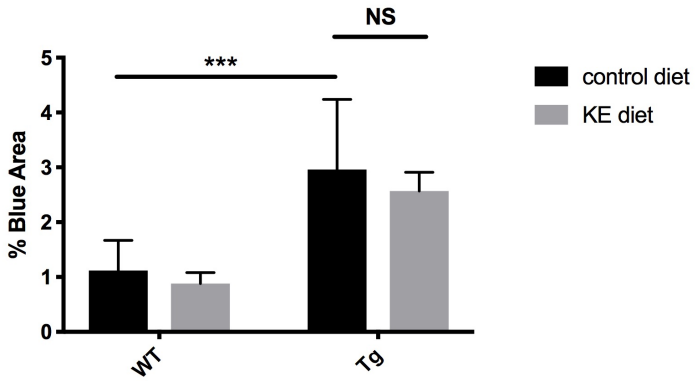

Fig. 7 Quantification of blue-stained area (% Blue) by Masson's trichrome-staining in hearts of WT and FVB.Tg-TMEM135 mice on control or KE diet at 8 months of age.

Masson's trichrome staining showed significantly increased collagen in FVB.Tg-TMEM135 mice compared to WT littermates on control diet (Fig. 7, P=0.0319 by two-way ANOVA with Bonferroni's multiple comparison test). Comparison between FVB.Tg-TMEM135 mice on control diet and FVB.Tg-TMEM135 mice on KE diet did not show significant difference (Fig. 7).

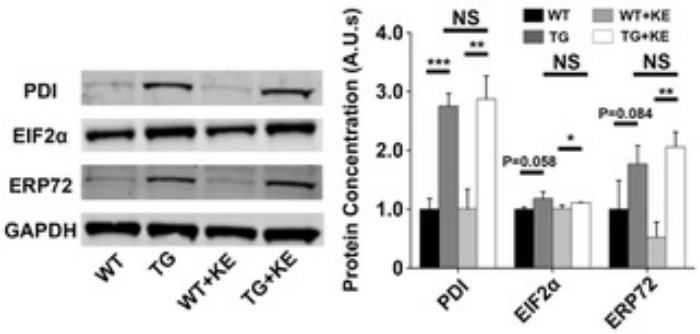

Fig. 8 Western blot analysis of ER stress markers in hearts of WT and FVB.Tg-TMEM135 mice on control or KE diet at 8 months of age.

Western blot analysis showed that the level of an ER stress marker, PDI, is significantly higher while the other 2 markers, EIF2alpha and ERP72, showed higher trends in FVB.Tg-TMEM135 mice compared to WT mice on control diet (Fig. 8, two-way ANOVA). KE diet did not affect this upregulation of ER stress markers in FVB.Tg-TMEM135 mice at 8 months of age (Fig. 8).

Aim 2: Test the long-term effect of ketone bodies on the age-associated abnormalities of the heart

In order to test the effect of ketone bodies on pathological changes of the heart associated with aging, we fed KE diet to C57BL/6J (B6) WT mice (n=10) from P21 (post weaning) to 16 months of age. B6 WT mice in the control group were fed non-treated control diet (n=10).

Echocardiography showed that LV mass and volume were both increased in 16 month old B6 WT mice on control diet compared to 2 month old B6 WT mice on control diet. These measurements were significantly reduced in 16 month old B6 WT mice on KE diet compared to 16 month old B6 WT mice on control diet (Fig. 9). This observation suggests that KE diet may improve the increase in LV volume and mass as the mice age.

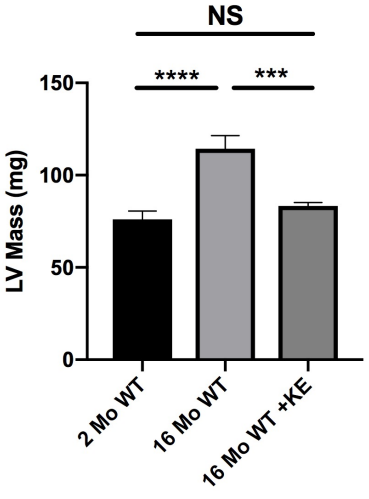

Fig. 9 Left ventricle (LV) posterior mass of 2 month old and 16 month old B6 WT mice on control diet and 16 month old B6 WT mice on KE diet.

In H&E stained sections of the heart of these mice, we did not observe any major differences between 2 month old B6 WT mice on control diet, 16 month old B6 WT mice on control diet, and 16 month old B6 WT mice on KE diet. Quantification of fibrosis by Masson's trichrome staining also did not show any significant differences between the three groups (Fig. 10, P=0.9982 by one-way ANOVA).

Western blot analysis also did not show difference in the levels of any of the ER stress markers tested between 2 month old B6 WT mice on

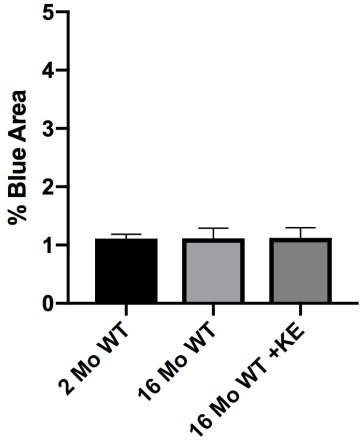

Fig. 10 Quantification of blue-stained area (% Blue) by Masson's trichrome-staining in hearts of 2 month old and 16 month old B6 WT mice on control diet and 16 month old B6 WT mice on KE diet.

control diet, 16 month old B6 WT mice on control diet, and 16 month old B6 WT mice on KE diet.

These results indicate that B6 WT mice do not develop histological abnormalities nor upregulation of ER stress by 16 months of age. It is possible that different inbred mouse strains show varied progression and severity of age-related changes in the heart. We had an opportunity to examine the heart of 20 months old B6 and A/J mice by Masson's trichrome staining. Interestingly, A/J mice show much higher level of fibrosis compared to B6 mice at 20 months of age, suggesting that the genetic background affects age-related heart abnormalities. It would be meaningful to test the effect of ketone body supplementation on mice with genetic background that is more susceptible to age-related heart abnormalities such as A/J mice in the future.

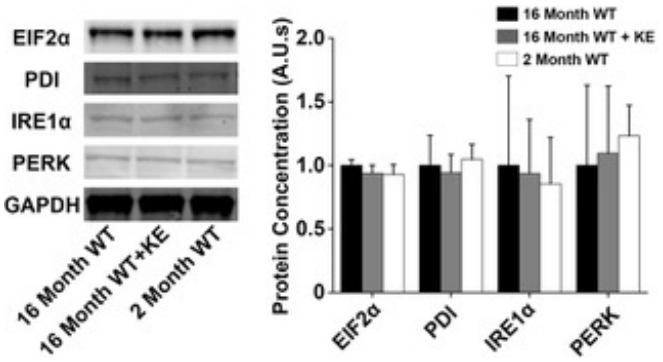

Fig. 11 Western blot analysis of ER stress markers in hearts of 2 month old and 16 month old B6 WT mice on control diet and 16 month old B6 WT mice on KE diet.

-What opportunities for training and professional development has the project provided?

The project has provided opportunities for Assistant Scientist, Wei-hua Lee to be trained to develop as an independent researcher. She learned how to manage and carry out the project.

-How were the results disseminated to communities of interest?

Nothing to report

-What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report

4. Impact

-What was the impact on the development of the principal discipline of the project?

The results obtained in this study showed that ketone body supplementation improved the survival and heart abnormalities of Tg-TMEM135 mice, a mouse model of mitochondrial cardiomyopathy. It also appears to improve aging-associated hypertrophy of the heart. These findings suggest the possibility that ketone body supplementation may be effective in improving mitochondrial cardiomyopathy and heart abnormalities associated with aging in humans. Recently, studies have found intriguing associations between increased concentrations of circulating branched-chain amino acids (BCAAs) and cardiovascular disease, suggesting that impaired BCAA metabolism may increase the risk for cardiovascular disease and could be its biomarker. Based on our findings that ketone body supplementation can substitute for impaired BCAA catabolism in Tg-TMEM135 mice, ketone body supplementation may be also effective in preventing cardiovascular disease in patients with impaired BCAA metabolism.

-What was the impact on other disciplines?
Nothing to report

-What was the impact on technology transfer?
Nothing to report

-What was the impact on society beyond science and technology?
Nothing to report

5. Changes/Problems

-Changes in approach and reasons for change
Nothing to report

-Actual or anticipated problems or delays and actions or plans to resolve them

Since our institutional animal protocol that covers the work for this award was up for renewal and renewal was approved by IACUC on 11/16/17, we needed to submit the documents to ACURO for review (11/29/17). The renewed protocol was approved by ACURO on 2/7/18. This delayed the project by approximately 2 months, and no-cost extension of the project was granted.

Earlier in the study, we noted that some of the Tg-Tmem135 mice experienced death upon cage transportation between laboratories, suggesting that they are generally sensitive to stress. Therefore, we reduced the number of cage changes and avoided unnecessary transportation of cages during the study. In addition, in order to avoid causing additional stress, we have decided not to collect blood from live mice (which is one of the laboratory procedures that have been shown to cause stress [Balcombe et al. 2004, PMID: 15669134]) during the course of the study for testing myocardial infarction markers (Aim 1 [2] and Aim 2). Heart functions of live mice were directly tested by echocardiography before collection of heart samples.

-Changes that had a significant impact on expeditures
Nothing to report

-Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report

6. Products, Inventions, Patent Applications, and/or Licenses
Nothing to report.

7. Participants & Other Collaborating Organizations
-What individuals have worked on the project?
Name: Akihiro Ikeda, Ph.D., D.V.M.
Project Role: PI
Researcher Identifier: ORCID ID: http://orcid.org/0000-0001-8440-3891
Nearest person months worked: 1.8
Contribution to Project: Dr. Ikeda was responsible for the experimental design, carrying out the experiments outlined, interpretation of data, supervision and coordination of the project.

Name: Wei-hua Lee, Ph.D.
Project Role: Assistant Scientist
Nearest person months worked: 6
Contribution to Project: Dr. Lee was responsible for carrying out the molecular biological experiments outlined, managing the mouse colony, phenotyping of mice and interpretation of data under the supervision of Dr. Ikeda.

-Has there been a change in the active other support of the PD/PI or senior / key personnel since the last reporting period?
Akihiro Ikeda (PI) has acquired the following funding during the reporting period:

Title: Role of chondroitin sulfate proteoglycans in photoreceptor - RPE interaction (R21 EY029067)
Time commitment: 1.2 calendar months
Supporting Agency: NIH/NEI
Period: 4/1/18-3/30/20
Major goal: To understand the roles chondroitin sulfate synthese 1 plays in the retina, and how a defect in this molecule causes accelerated aging phenotypes.
Role: PI
Overlap: None

Title: Molecular Genetics of Age-Dependent Retinal Degeneration (R01 EY022086)
Time commitment: 4.2 calendar months
Supporting Agency: NIH/NEI
Period: 9/30/18-7/31/23
Major goal: to understand the molecular mechanisms of age-dependent retinal degeneration using mouse molecular genetic approaches.
Role: PI
Overlap: None

-What other organizations were involved as partners?
Nothing to report.

8. Special Reporting requirements
None

9. Appendices
None