EXERCISE TO IMPROVE AGE-RELATED LOSS OF FUNCTION AND CORRESPONDING

ALTERATIONS IN GENE EXPRESSION

By

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Abstract

The "gray wave," the increasing population aged 65 and older, will double over the course of the next 30 years. This is a concern as it results in an increased prevalence of age-associated diseases such as diabetes, cardiovascular disease, frailty (inability of the body to maintain homeostasis), and sarcopenia (age-related loss of muscle mass and strength).[1] Frailty and sarcopenia add to the progressive loss of functional ability and independence among older adults, resulting in a lower quality of life.[2] Exercise can reduce the risk of these diseases, but it is not a cure nor is it physically feasible for every individual[4]. Therefore, it is essential to investigate the latent molecular mechanisms of aging and exercise to develop future therapeutic targets[9]. Previously, we discovered that as aging occurs, calcium handling in skeletal muscle is changed. The sarcoplasmic reticulum calcium transport ATPase (SERCA), a pump that transports calcium to the lumen of the sarcoplasmic reticulum, stimulates relaxation of muscle[11]. Sarcolipin (SLN), an uncoupler of the SERCA pump, decreases the reaction rate of calcium reuptake by preventing the pumping of calcium into the lumen without negatively regulating ATP hydrolysis[12]. After monitoring four months of endurance training (two modes: voluntary wheel running, n=8 per groups; high intensity interval training, n=10 per group) in adult (10-month-old) and older (26-month-old) mice, we attempted to establish the skeletal muscle gene expression of SLN along with the similarly functioning proteins, myoregulin and phospholamban. Using gastrocnemius muscle, we isolated RNA, measured mRNA expressing with q-rt-PCR, and determined SLN content with Western Immunoblotting. We hypothesized that increased levels of SLN contributes to age-associated muscle dysfunction, but that endurance training might restore muscle health in older mice by lessening SLN expression. We

conclude that, compared to controls, significant improvement was observed in the physical function and body composition of both exercise groups.

Introduction

The "gray wave," the increasing population aged 65 and older, will double over the course of the next 30 years. This is a concern as it results in an increased prevalence of age-associated diseases such as obesity, diabetes, cardiovascular disease, frailty, and sarcopenia (age-related loss of muscle mass and strength)[1]. Frailty and Sarcopenia add to the progressive loss of functional ability and independence among older adults, resulting in a lower quality of life[2]. Frailty is a pre-disability syndrome that creates increased susceptibility to physical stress and physical deterioration due to the body's inability to maintain homeostasis, often leading to mortality[3]. Sarcopenia, the loss of muscle mass and strength, is believed to be a major contributor to frailty[4].

Aging and loss of physical function also contribute to the risk of hyperinsulinemia and insulin resistance due to increased body weight and fat mass[5]. Due to the limited programs and ineffective policies in place that promote healthy aging, diseases such as diabetes and cardiovascular, raise public health concerns[6]. According to the World Health Organization, epidemiological data has shown a rise in multimorbidity of age-associated diseases among older individuals, negatively impacting society by increasing health-care facility use and cost of treatment[7]. Negative impacts are also felt in the limitations of societal engagement, including participating in the paid labor force, volunteering in community events, and providing for the family[7].

Although exercise is not a cure and is not physically feasible for every individual, it can act as therapy for age-associated diseases in adult and older mice by improving functional

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performance and altering morphology[4,2]. Additionally, exercise training is suggested to be optimal treatments for frailty, insulin resistance, and cardiovascular disease due to its capability to alter body composition and fat mass[8,5].

It is essential to investigate the latent molecular mechanisms of aging and exercise to develop future therapeutic targets, which is what we aim to accomplish through animal models of exercise[9]. Mouse models are useful when investigating pharmacological treatments and other potential frailty interventions that mitigate age-associated diseases as they permit in-depth examination on exercise-stimulated gene expression changes and metabolic pathway alterations[10]. They also allow investigations into lifespan health through longitudinal studies, which are crucial when discussing age-related physical function, and offer a high control of variables, such as similar living environments and genetic homogeneity, which improves accuracy of results.

This study involved comparing exercise-stimulated gene expression in mice to established literature that details human exercise-induced gene expression. We hypothesized that the gene expression observed in our mouse models of exercise will be similar to known, exercise-stimulated gene expression in humans. Accordingly, this would validate our mouse models as accurate mimetics of human exercise training. We also hypothesized that compared to sedentary controls, adult mice would have greater improvements in functional markers and greater differences in gene expression after exercise than older mice.

Previously, we discovered that as aging occurs, calcium handling in skeletal muscle is changed. The sarcoplasmic reticulum calcium transport ATPase (SERCA), a pump that transports calcium to the lumen of the sarcoplasmic reticulum, stimulates relaxation of muscle[11]. Sarcolipin (SLN), a regulator of the SERCA pump, decreasing the reaction rate of calcium

reuptake and preventing the pumping of calcium into the lumen without negatively regulating ATP hydrolysis[12]. After monitoring four months of endurance training (two modes: voluntary wheel running, n=8 per groups; high intensity interval training, n=10 per group) in adult (10-month-old) and older (26-month-old) mice, we attempted to establish the skeletal muscle gene expression of SLN along with the similarly functioning proteins, myoregulin and phospholamban. We hypothesized that increased levels of SLN contributes to age-associated muscle dysfunction, but that endurance training might restore muscle health in older mice by decreasing SLN expression.

Methods

This study used 18 older (26-month-old) and 24 adult (10-month-old) C57BL/6 mice. It consisted of three groups of mice, with eight mice per group. The three groups included sedentary control (no exercise), voluntary wheel running (VWR), and high intensity interval training (HIIT).

Study 1 Aerobic Exercise in Adult Mice as a Mimetic of Human Training

Pre- and Post-Exercise Functional Testing:

<u>CFAB (Comprehensive Functional Assessment Battery)</u>

To assess the functional ability before the mice underwent exercise training, five tests were conducted: rotarod, grip tests, treadmill test, inverted cling, and voluntary wheel running. Standard deviations and means from 6-month-old mice were taken to determine each mouse's z-scores, which were then added to determine a composite functional assessment battery (CFAB) score.[9]

<u>Rotarod</u>

Balance, coordination, stamina, and power were tested using a Panlab LE820 to assess overall motor function of each mice. To get the mice acclimated to the rotarod, two practice sessions were conducted with three trials in each session. Testing day consisted of one 5-minute interval with three trials in which acceleration increased from 4 rpm to 40 rpm. The best time out of the three trails was reported as the outcome measure.

Grip Test

A Bioseb GT3 model grip strength tester was used to measure forelimb strength. Needing no acclimation period, the grip test consisted of five trials. Each mouse was held by the tail and their forepaws were gently placed on the bar until they gripped it. Each mouse was pulled back until they released their grip and the best of the trials was reported, in Newtons (N).

Treadmill

A Columbus Instruments Exer 3/6 treadmill was used to measure volitional endurance and fatigue. To get the mice acclimated, two practice sessions were conducted in which each mouse learned to walk and eventually run on the treadmill. A mild shock plate, which was not painful, was present at the bottom of the treadmill so the mice can be startled and motivated to keep running. Test day consisted of a 6 cm/s start with an acceleration of 1 cm/s every 20 seconds. Each mouse ran until they touched the shock grid three times for two seconds or until they refused to run even after being manually encouraged.

Inverted Cling

An Inverted Cling, consisting of an inverted grid and a padded floor, was used to measure overall strength and endurance. Needing no acclimation period, each mouse clings to the grid until they fall onto the padded floor. We measured the amount of time, in seconds, the mice hung

on before falling. The best of the two trials were reported. The test was repeated if the mice held on for less than 10 seconds.

Voluntary Wheel Running

Voluntary Wheel Running was used to measure activity and volitional exercise rate by individually placing the mice in cages with a running wheel. The running wheel contained a computerized magnetic revolution that recorded and reported the number of revolutions as kilometers per day (in km/day).

Other Outcome Measures:

EchoMri

To measure fat and lean content in the mice, magnetic resonance imaging was used. This tracked changes in body composition and fat percentage pre- to post-training.

Quantitative Real Time Polymerase Chain Reaction (q-rt-PCR)

To find targets that are altered with exercise and are associated with functional ability, the data from the Next Generation Sequencing (NGS) was analyzed. Q-rt-PCR will be used to test the isolated RNA and compare the changes in pre- to post-training gene expression between younger and older mice.

NGS RNAseq Transcriptomics

Using the samples of isolated RNA, NGS was done at the University of Texas Medical Branch (Galveston) Genomic Core, and analyzed by the University of North Carolina – Chapel Hill BARC (Bioinformatics and Analytics Research Collaborative) core facility.

Exercise for 12-14 weeks

This study involved two modes of exercise training, voluntary and involuntary. The voluntary mode was with the mice's own volition and at their own pace. The involuntary mode

involved specific rates, levels, and time lengths for the exercise training. To determine which mode resulted in a more favorable treatment, the two modes were compared.

A) *Activity Wheel (Voluntary wheel running).* The voluntary mode involved individually placing each mouse in a cage with a running wheel and a magnetic revolution counter that was attached to a computer to measure activity (km/day). When not testing voluntary aerobic conditioning training, the mice were returned to their original cages with other mice to retain social interaction. Before the next mice were housed in the cages, the cages and the detachable running wheels were cleaned with 70% ethanol.

B) *Treadmill (involuntary running).* The involuntary mode involved controlled treadmill running with a mild shock grid that motivated the mice to keep running. To measure endurance and fatigue, the mice were placed on a treadmill three times a week at an intensity specific to each mouse. The mice walked or ran until they touched the shock grid at the bottom three separate times for two or more seconds each. The amount of time and the speed each mouse ran was recorded between trials and the treadmill was cleaned with 70% ethanol.

Study 2 Gene Expression After Exercise Training in Adult and Older Mice

RNA was isolated from the gastrocnemius muscle in older (26-month-old) and adult (10-month-old) mice using the TRiReagent methodology and DNA analysis was conducted after NGS RNAseq transcriptomics. Q-rt-PCR was performed to measure mRNA and compare specific changes in gene expression among both older and adult mice. Western Immunoblotting was conducted to determine sarcolipin protein content.

Results
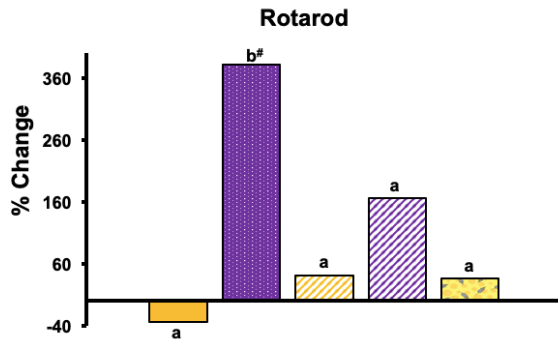
Study 1

CFAB: Functional Status and Exercise Capacity
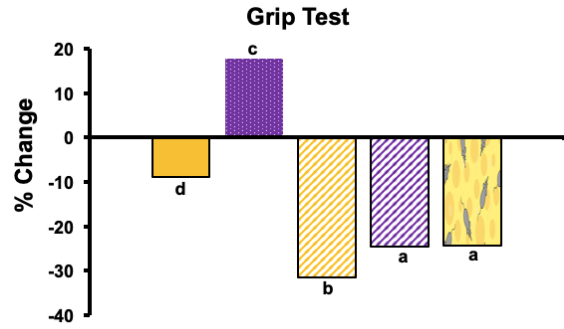
Figure 1. Rotarod vs. % Change

Figure 2. Grip Test vs. % Change
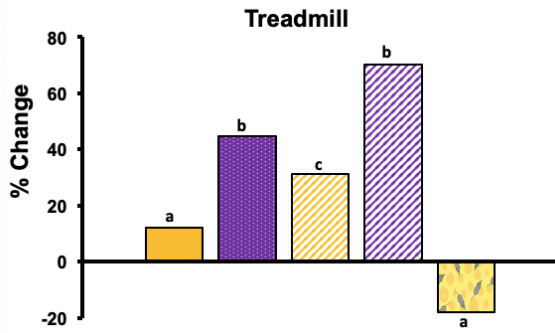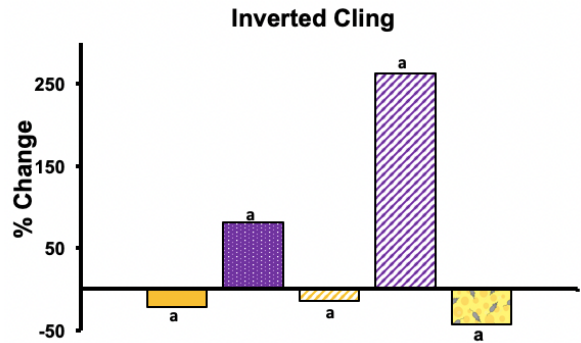
Figure 3. Treadmill vs. % Change

Figure 4. Inverted Cling vs. % Change
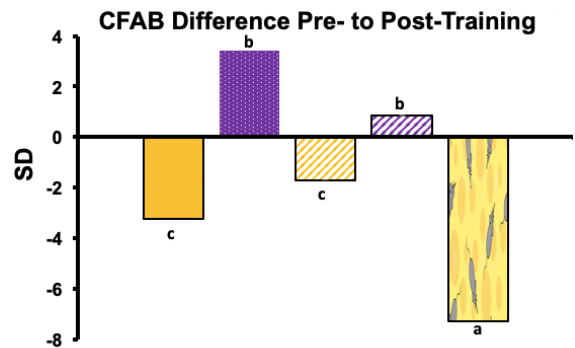
Figure 5. Voluntary Wheel Running vs. % Change
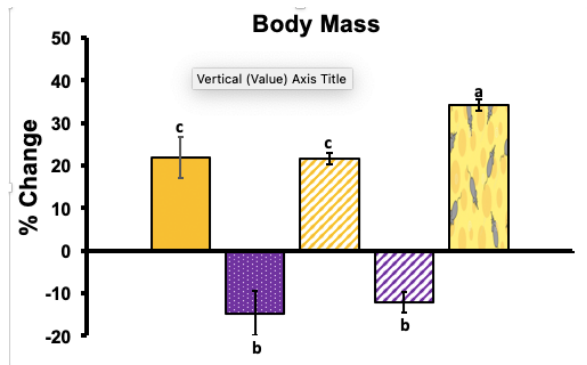
Figure 6. CFAB Difference Pre- to Post-Training vs. SD
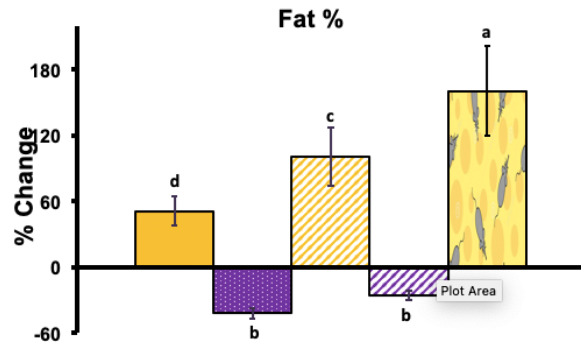
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Figure 7. Body Mass vs. % Change

Figure 8. Fat % vs % Change

🟧 VWR 10m 🟪 VWR 26m ▨ HIIT 10m ▨ HIIT 26m 🟨 10m Sed.

Key: VWR = Voluntary Wheel Running group. HIIT = High Intensity Interval Training group. Sed. = Sedentary Control. 10m = 10 months old at post-training. 26m = 26 months old at post-training. % Change = change from pre- to post-training.

Statistics: SPSS v27 (IBM) was used for statistical analysis. ANCOVA adjusted for body mass at pre- and post-training or ANOVA. Different letters = statistically significant (p<0.05), # = a trend (0.05<p<0.10). See table 1 for standard errors and exact means.

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Exercise Program:	VWR		HIIT		Sedentary Control
Test	26m	10m	26m	10m	10m
Rotarod (Seconds)	382% ± 324.2	-33% ± 4.9	165% ± 130.2	41% ± 25.4	-37% ± 5.2
Grip Test (Newtons)	18% ± 8.2	-9% ± 6.2	-25% ± 7.14	-32% ± 3.18	-24% ± 4.3
Treadmill (Seconds)	45% ± 20.4	12% ± 11.9	70% ± 18.9	31% ± 12.2	-18% ± 11.9
Voluntary Wheel Running (km/Day)	54% ± 101.1	15% ± 41.9	7% ± 41.3	5675% ± 4809.8	1097% ± 801.4
Inverted Cling (Seconds)	81% ± 44.8	-22% ± 14.6	263% ± 270	-15% ± 11.1	-43% ± 24.5
Body Mass (grams)	-15% ± 5.2	22% ± 4.8	-12% ± 1.9	22% ± 2.1	34% ± 3.5
Fat %	-42% ± 4.9	52% ± 13.2	-26% ±	101% ± 26.3	101% ± 40.5
CFAB Scores (SD)	3.4 ± 0.96	-3.3 ± 1.4	0.8 ± 1.8	-1.7 ± 0.6	-7.3 ± 1.4

Table 1. Exercise vs. Function Status Pre- and Post-Training. The graphs are representative of the information in this table. Key: numbers = mean % change + SE (standard error of the mean). SD = Standard Deviation, 26m = 26-month-old mice (older adults equivalent to humans in the mid-70's), 10m = 10-month-old mice (adults, equivalent to humans in the mid-30's).

Study 2
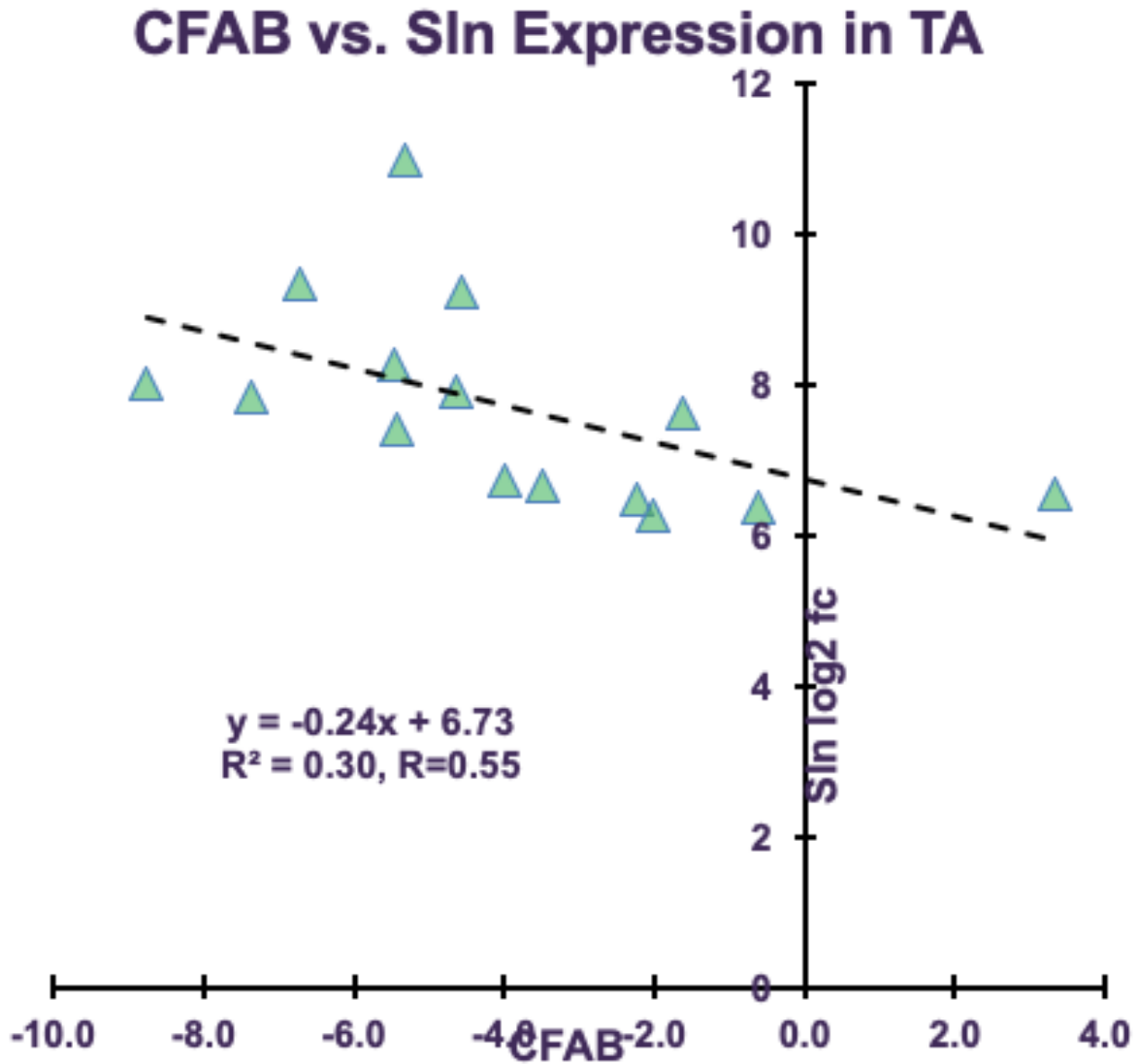
Next Generation Sequencing and Western Immunoblots

Figure 9. CFAB vs Sln Expression in TA. Mean log2 fold change (log2fc) = 4.33 (adj. p-val 1.08*10-6) between 6 and 28 months. Each symbol equals 1 mouse, Sln = sarcolipin, Equation is simple linear regression, TA = tibialis anterior muscle.
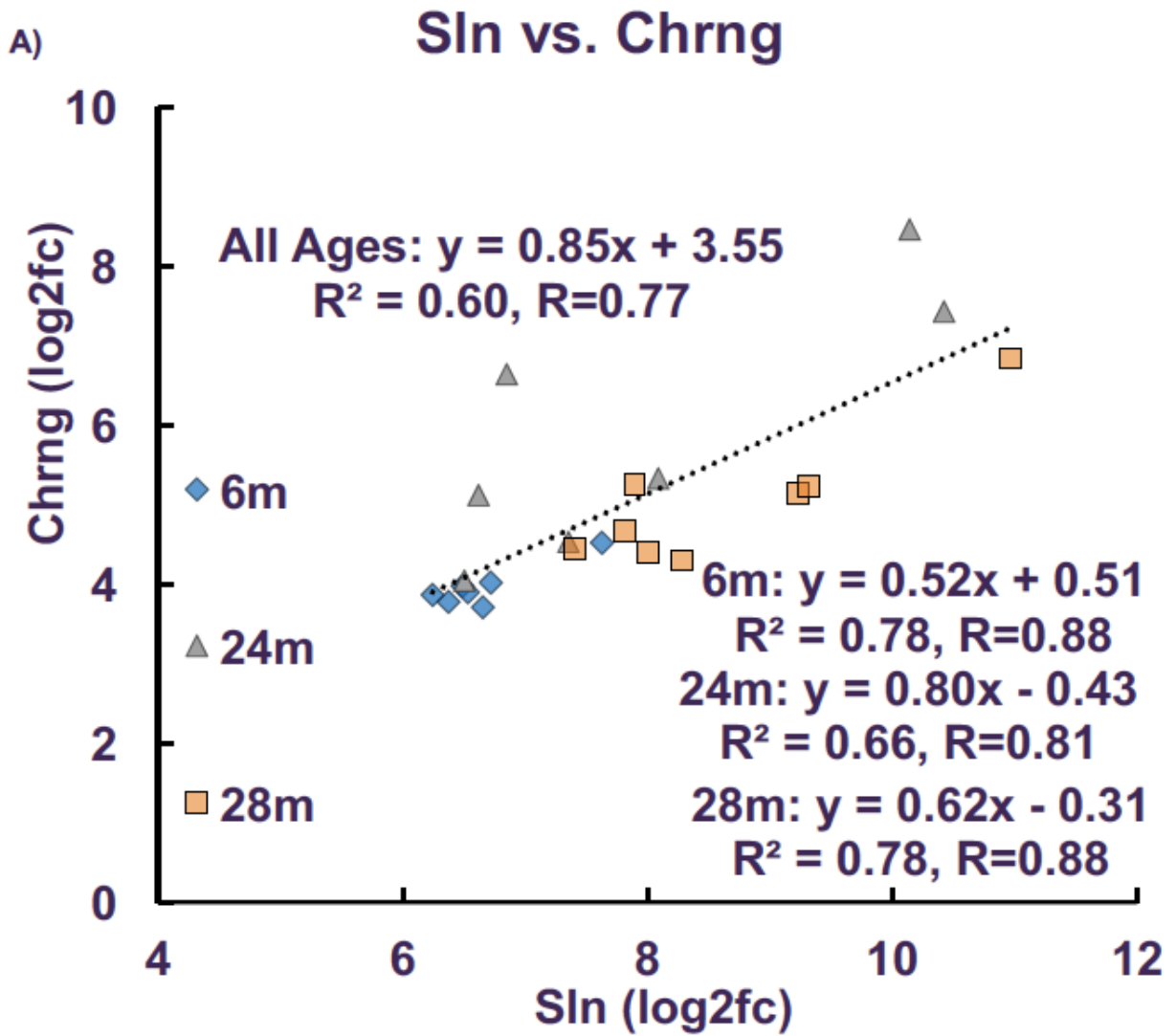
Figure 10. Sln vs. Chrng Gene Expression in TA: Sarcolipin (Sln) and acetyl choline receptor gamma (Chrng, present in denervated muscle cells), TA = tibialis anterior muscle.
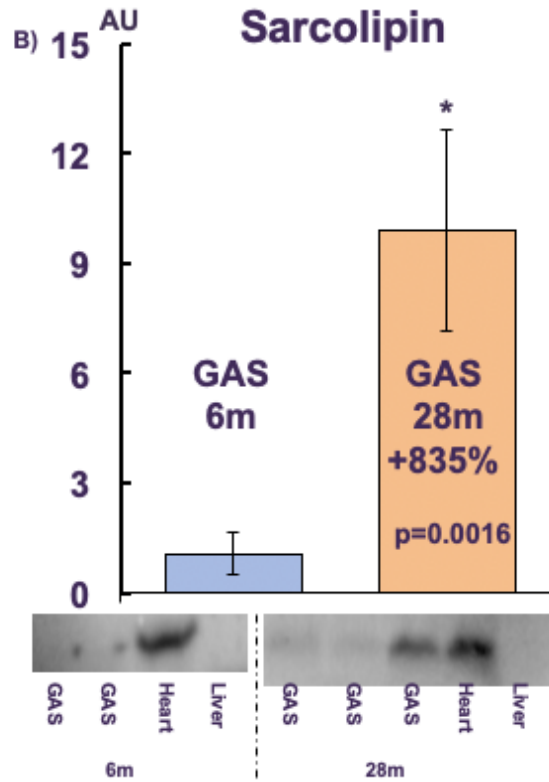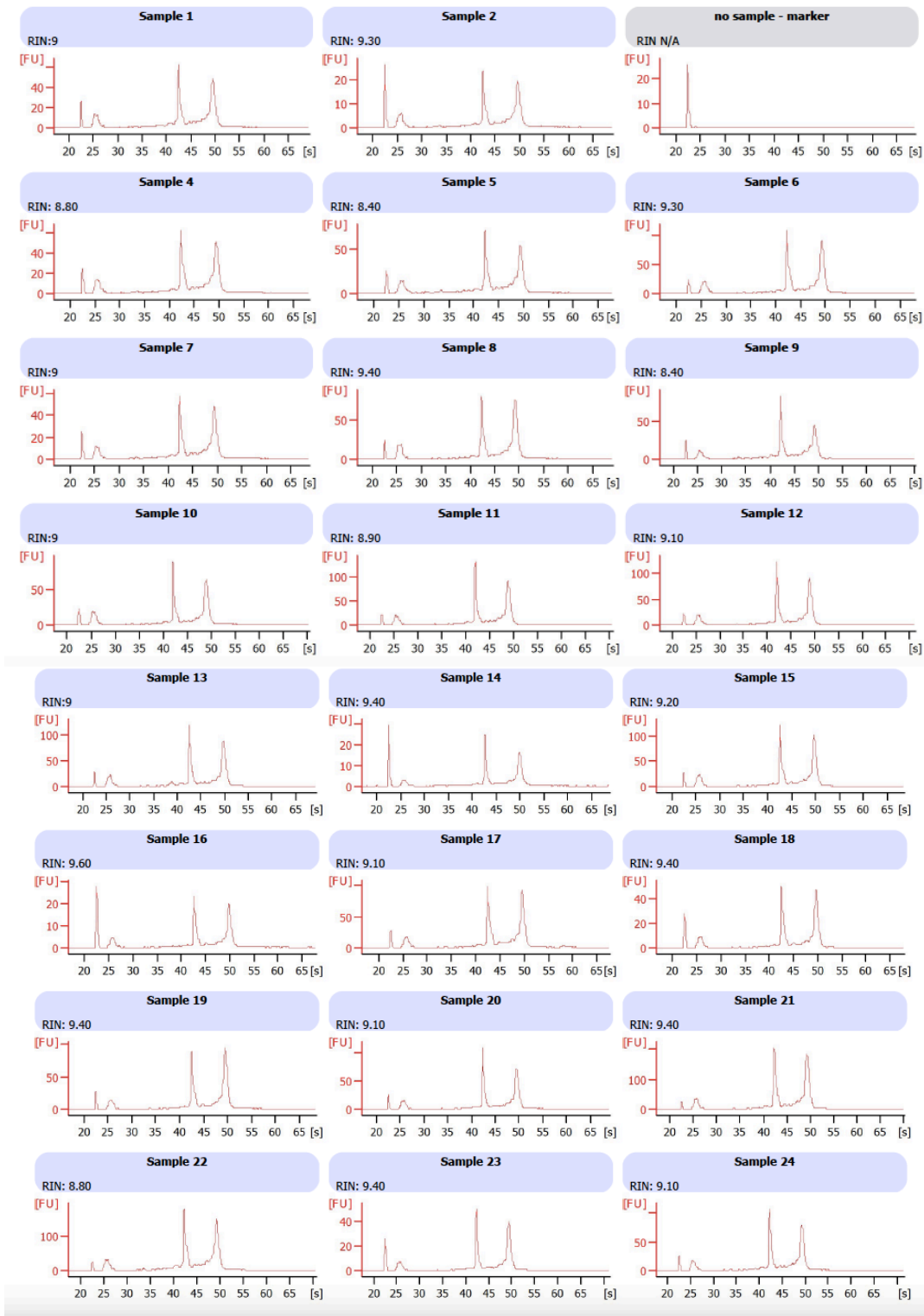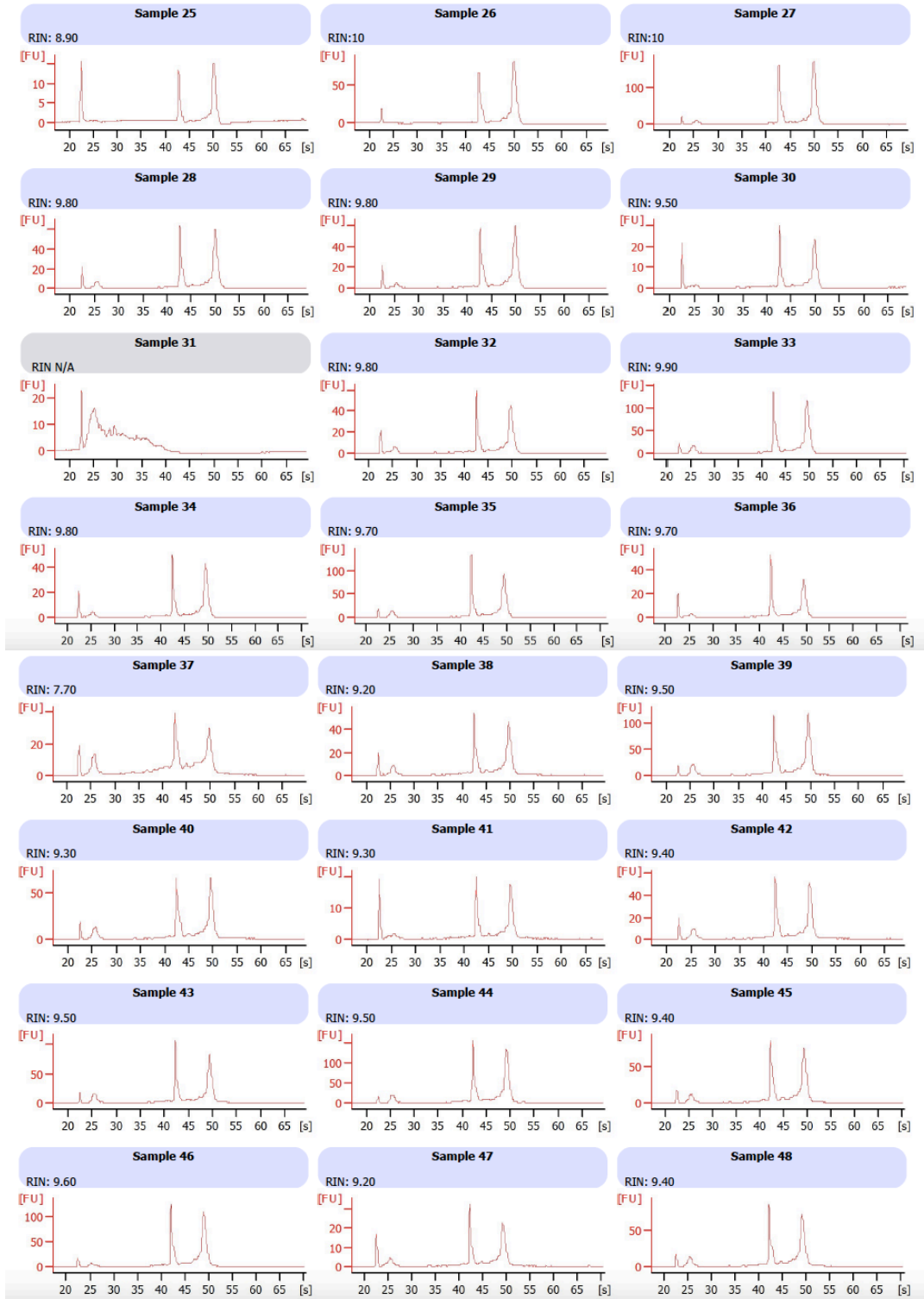
Figure 11. Sarcolipin Protein Expression: Heart muscle is a positive control and liver is the negative control, AU= arbitrary units of relative optical density. 6m = 6 months, 24m = 24 months, 28m = 28 months old, log2fc = log2 fold change, each symbol = 1 mouse, * = statistically significant (t-test).

RNA Isolations and Agilent BioAnalyzer

RNA (ng/µL)	A260/A280	A260/A230	RIN
1039.4 ± 823.4	1.9 ± 0.05	2.1 ± 0.2	9.1 ± 0.5

Table 2. Total RNA Extraction and Agilent BioAnalyzer. Numbers= mean of 60 RNA samples + SD (Standard Deviation). RNA = RNA concentration from Agilent BioAnalyzer. A260/A280 = ratio of RNA purity at an absorbance of 230 nm. Pure RNA is expected to have a ratio between 2.0-2.2. A260/A230 = ratio of RNA at an absorbance of 280 nm. Pure RNA is expected to have a ratio between 1.8-2.1. RIN = RNA Integrity Number. Range 1-10. RIN over 8 is significant. Samples 3, 31, 51 and 57 excluded from RNA and RIN (n/a).

Sample 25 — RIN: 8.90
Sample 26 — RIN:10
Sample 27 — RIN:10
Sample 28 — RIN: 9.80
Sample 29 — RIN: 9.80
Sample 30 — RIN: 9.50
Sample 31 — RIN N/A
Sample 32 — RIN: 9.80
Sample 33 — RIN: 9.90
Sample 34 — RIN: 9.80
Sample 35 — RIN: 9.70
Sample 36 — RIN: 9.70
Sample 37 — RIN: 7.70
Sample 38 — RIN: 9.20
Sample 39 — RIN: 9.50
Sample 40 — RIN: 9.30
Sample 41 — RIN: 9.30
Sample 42 — RIN: 9.40
Sample 43 — RIN: 9.50
Sample 44 — RIN: 9.50
Sample 45 — RIN: 9.40
Sample 46 — RIN: 9.60
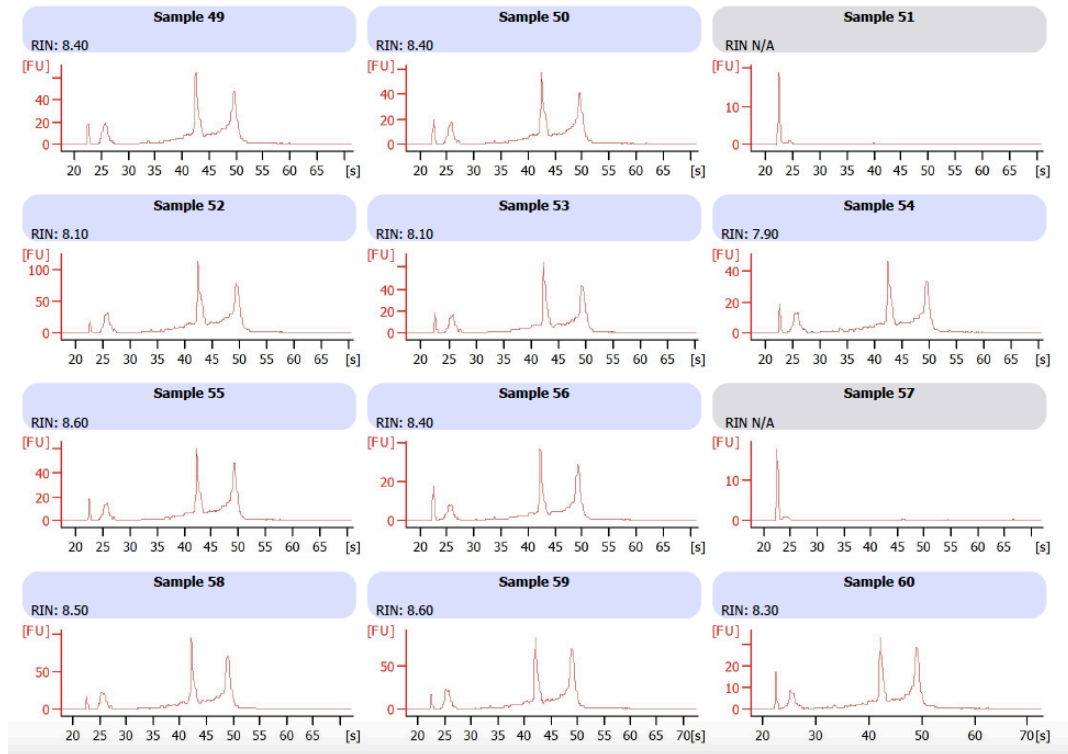Sample 47 — RIN: 9.20
Sample 48 — RIN: 9.40

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Figure 13. RNA BioAnalyzer Electropherogram for Sample 1-60. First peak: Standard. Second peak: 18S (Active center for protein synthesis in small subunit of rRNA). Third Peak: 28S (Structural rRNA for the large subunit). rRNA Ratio (28s/18s) is used to indicate the quality of total purified RNA. High quality ratios generally above 2.0 signify that the RNA has not degraded. Samples 3, 31, 51, 57 contained no RNA sample.

Discussion

Study 1

Comparing CFAB scores before and after both modes of exercise training (VWR and HIIT) showed that physical function was improved or preserved in both groups of mice. This proved exercise as a promising anti-aging therapeutic for age-associated physical decline. The older mice expressed a greater percent change in CFAB scores between pre-testing and post-testing than the adult mice, most notably in the rotarod, treadmill, and inverted cling scores. A reason for this could be that older mice may have lacked more muscle initially compared to the adult mice, which could have given them additional room for development. A similar trend was seen in the mice's morphology, in which both groups significantly improved in body

composition by the end of exercise training but only the older adults improved in fat percentage. This may be because older adults had a higher percentage of fat originally due to age-associated physical deterioration, giving them more room for development. When comparing the VWR mice and the HIIT mice on the grip test, the VWR had an increased performance, which could be due to the mice gaining practice by grabbing the ridges on the running wheel during the VWR exercise training. A cause of error could stem from using ANCOVA to adjust for body mass as insignificant increases in percent changes depend on a lean-to-fat ratio and overall mass. A limitation of this study was not having an older sedentary group so that percent changes could be compared to the same extent as adult mice. We were also extremely careful when accelerating and progressing the mice in the HIIT group, so they may not have benefited from maximal stimulation. We conclude that our exercise protocol, both VWR and HIIT, were validated as effective mimetics of human models as they demonstrated similar improvements in physical function.

 Study 2

Both VWR and HIIT preserved function but as can be seen in the results, sarcolipin is negatively correlated (R=-0.55) with CFAB scores. This means that as levels of sarcolipin expression increased in the mice, the functional status of the mice decreased. This gave evidence that overexpression of sarcolipin may reduce physical performance. We also found that sarcolipin expression in gastrocnemius muscle was upregulated 835% in older mice (28-month-old, n=10) compared to adult mice (6-month-old, n=9). This indicates that sarcolipin gene and protein expressions increased with age. When looking at our RNA samples, we reported the means of our 60 samples. The purity of our samples, given by the A260/A280 and the A260/A230 ratio, was high with the mean RNA Integrity Number being 9.1, which gave promise

for successful cDNA synthesis and RT-PCR. We noted that sample 26 and 27 had an RIN of 10, which is the highest RIN value given for electropherograms and indicated that the sample is extremely intact. Our next step is to quantify mRNA gene expression of sarcolipin and myoregulin in gastrocnemius muscle using rt-qPCR.

mRNA	Sequence (5' → 3')		Nucleotide #	Tm(°C)	Product (Bases)
		Primers:			
Sarcolipin (Sln)	Forward:	GCCTGACACACCGCTGCACTA	21	65.2	94
	Reverse:	AGCTAAGGCTCACTGGCTGGC	21	64.8	
Myoregulin (Mrln)	Forward:	GCGGCCATTCCCAGGACTTT	20	63.1	106
	Reverse:	CCTGCCCTTGCTGGACCAAA	20	63.0	
Gapdh[1]*	Forward:	ACCCTTAAGAGGGATGCTGCC	21	61.9	114
	Reverse:	CCGTTCACACCGACCTTCACC	21	63.5	
B2m[2]*	Forward:	CAGCATGGCTCGCTCGGTGA	20	65.3	114
	Reverse:	CATTCTCCGGTGGGTGGCGT	20	64.9	

Figure 14. Primers for Sarcolipin, Myoregulin, Gapdh, and B2m, mRNA that will be analyzed with rt-qPCR using QuantStudio 6 Flex Real-Time PCR System. [1]glyceraldehyde-3-phosphatedehydrogenase. [2]beta-2 macroglobulin. * Housekeeping Genes.

We conclude that mice and humans' physical function deteriorates as they age and this may be in part due to age-associated sarcolipin overexpression. Our current and future work involves investigating if exercise affects sarcolipin expression throughout the aging process. It also involves investigation into whether increased sarcolipin expression in adult muscle from an adeno-associated virus serum type 6 (AAV-6) vector with a desmin promoter will cause deterioration similar to expression during normal aging.

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