Blunted hypertrophic response in aged skeletal muscle is associated with decreased ribosome biogenesis

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Abstract

Background: The ability of skeletal muscle to hypertrophy in response to a growth stimulus is known to be compromised in older individuals. We hypothesized that a change in the expression of protein-encoding genes in response to a hypertrophic stimulus contributes to the blunted hypertrophy observed with aging. To test this hypothesis, we determined gene expression by microarray of plantaris muscle from 5- and 25-month old mice subjected to 1, 3, 5, 7, 10 and 14 days of synergist ablation to induce hypertrophy.

Results: Overall, 1607 genes were identified as being differentially expressed across the time course between young and old groups; however, the difference in gene expression was modest, with cluster analysis showing a similar pattern of expression between the two groups. Despite ribosomal protein gene expression being higher in the aged group, ribosome biogenesis was significantly blunted in aged compared with young skeletal muscle in response to the hypertrophic stimulus (50% versus 2.5-fold, respectively). The failure to up-regulate pre-47S ribosomal RNA (rRNA) expression in old muscle undergoing hypertrophy indicated ribosomal DNA transcription by RNA polymerase I was impaired.

Conclusions: Contrary to our hypothesis, the findings of the study suggest that impaired ribosome biogenesis was a primary factor underlying the blunted hypertrophic response observed in old skeletal muscle rather than dramatic differences in the expression of protein-encoding genes. The diminished increase in total RNA, pre-47S rRNA and 28S rRNA expression in aged muscle suggest that the primary dysfunction in ribosome biogenesis occurred at the level of rRNA transcription and processing.
Keywords: Hypertrophy, age, skeletal muscle, microarray, ribosomal biogenesis
**Background**

Human and rodent studies have reported that skeletal muscle hypertrophy is significantly diminished with old age (8, 14, 16, 26, 28, 30, 33). Although the underlying mechanism responsible for the blunted hypertrophic response in the elderly remains to be clearly defined, an alteration in protein metabolism is thought to be a primary factor (25). In particular, activation of protein synthesis by the mechanistic target of rapamycin (mTOR) signaling pathway has been shown to be attenuated in aged skeletal muscle subjected to a hypertrophic stimulus (9, 23, 31). The notion that blunted mTOR signaling contributes to a compromised hypertrophy with age is consistent with the pioneering work by Bodine and colleagues showing that mTOR activity is absolutely necessary to mount a full hypertrophic response (3).

In an effort to identify other genes and/or pathways that might contribute to the age-related difference in skeletal muscle hypertrophy, microarray analyses have been performed to identify changes in gene expression between young and old individuals in response to an acute bout of resistance exercise or following a training program (24, 27, 29). Although these studies were able to identify pathways that may have a role in the diminished hypertrophic response of the elderly, the power of these analyses was limited by the small number of time points, typically a pre- and post-measurement. Given the dynamic nature of gene expression during skeletal muscle hypertrophy, it is likely that this design limitation resulted in potentially important changes in gene expression being missed (5). This idea is supported by the finding that acute changes in gene expression are no longer observed following a resistance exercise training
program, and, therefore, may not truly reflect those biological processes governing hypertrophic growth (29).

The purpose of this study was to perform a comprehensive transcriptome analysis of old skeletal muscle undergoing hypertrophy in an effort to identify differentially expressed genes. Skeletal muscle hypertrophy was induced by synergist ablation with gene expression measured by microarray analysis after 1, 3, 5, 7, 10 and 14 days. To identify age-associated genes that were differentially expressed in response this hypertrophic stimulus, we compared this newly generated data set against our previously published transcriptome analysis in young skeletal muscle subjected to the identical perturbation (5). Despite observing only modest differences in gene expression between the two groups, pathways associated with the regulation of protein synthesis, metabolism and immune response were highly enriched in old skeletal muscle. Somewhat paradoxical though, we found that ribosomal biogenesis was significantly impaired at the level of rDNA transcription in aged skeletal muscle undergoing hypertrophy. These findings suggest that the blunted hypertrophic response in old skeletal muscle was primarily the result of a failure to sufficiently increase the translation capacity of the muscle more so than changes in gene expression.
Methods

Animal care and use

All experimental procedures performed in this study were approved by the University of Kentucky Institutional Animal Care and Use Committee. Male C57BL/6J mice, 5 months of age (The Jackson Laboratory, Bar Harbor, ME) and 25 months of age (National Institute on Aging, Bethesda, MD) were housed in a temperature- and humidity-controlled room and maintained on a 14:10 hour light: dark cycle with food and water ad libitum.

Young (5 months of age) and old (25 months of age) mice were subjected to bilateral synergist ablation surgery to induce hypertrophy of the plantaris muscle as previously described (18). Briefly, following anesthetization with a mixture of 95% oxygen and 5% isoflurane, the soleus and the majority of the gastrocnemius muscles were surgically excised via an incision on the dorsal aspect of the hind limb. Particular attention was made to ensure neural and vascular supply to the plantaris muscle remained intact and undamaged. Sham surgery controls involved the same procedure without gastrocnemius and soleus muscle excision. Following recovery from surgery, mice were anesthetized as described above at the designated time point and plantaris muscles were excised, weighed, placed in RNAlater (Ambion, Austin, TX) and stored at 4ºC. Plantaris muscle was collected at 1, 3, 5, 7, 10 and 14 days after the surgery (n = 6 per time point) during the same 4 hr time period (10am-2pm) in a rested, feed state, thus insuring a similar metabolic state between the groups. Control plantaris muscles (n=6) were collected from mice subjected to the sham synergist ablation surgery. Following
collection of the plantaris muscles, mice were euthanized by cervical dislocation under anesthesia.

**RNA isolation**

Total RNA was prepared from plantaris muscle using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's directions. RNA samples were treated with TURBO DNase (Ambion, Austin, TX) to remove genomic DNA contamination. Total RNA concentration and purity was assessed by measuring the optical density (230, 260 and 280 nm) with a Nanodrop 1000 Spectrophotometer (ThermoFisher Scientific, Wilmington, DE). RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA); the average RIN (RNA integrity number) value for all samples was 9.12 ± 0.17 (scale 1–10) indicating high-quality RNA with minimal degradation products.

**Microarray analysis**

The microarray analysis was performed at the University of Kentucky Microarray Core Facility according to the manufacturer’s protocol (Affymetrix, Santa Clara, CA). Gene expression was measured using the Mouse Gene 1.1 ST chip which provides coverage of 28,000 protein-coding transcripts and 7,000 non-coding transcripts of which approximately 2,000 are long intergenic non-coding transcripts. Microarray analysis of plantaris muscle of young mice undergoing hypertrophy has been previously published by us (5) and was used in the current study to compare to data generated from the plantaris muscle of old mice. As in the earlier study, two gene chips were processed at each time point from 250 ng of total RNA. The total RNA was derived from a pooled
sample of either the right or left plantaris muscle from six animals. We pooled RNA
samples based on the experimental results reported by Kendziorski et al., showing that
gene expression from a pooled RNA sample is similar to the average from the individual
samples comprising the pooled sample (15). To minimize variability due to systematic
biases (such as dye effects and/or hybridization artifacts) the chips for both the young
and old samples were hybridized at the same time with the resulting probe signal for
each transcript summarized using RMA and quantile normalized using the Affymetrix
Expression console software. Further, these normalized data sets were then all
uploaded to Partek Genomics Suite so that the young data set was re-analyzed with the
old data set. At this step, we did not set a lower cutoff for the signal intensity to avoid
excluding low expressing genes that might show a significant age-associated up-
regulation in response to synergist ablation. Data was log transformed and duplicate
probes sets for the same gene were removed, with the probe set demonstrating the
highest signal intensity being retained in the analysis. In order to facilitate downstream
pathway analysis, only the probes sets with annotation information were included.
Following processing, 21,735 genes were exported and used for further analysis. Gene
expression data has been made available at GEO (http://www.ncbi.nlm.nih.gov/geo) for
the young (GSE47098) and old (GSE67160) hypertrophy studies.

Identification of differentially expressed genes
Detection of differential gene expression profiles was performed using the R-based
Bioconductor statistical software, version 2.6 (11). In order to detect gene expression
differences between the young and old groups, data was analyzed using the maSigPro
package (7). This package is specifically designed to identify differential expression profiles across experimental groups in time course microarray data. It utilizes a regression-based analysis that allows for time to be maintained as an independent variable. First, genes exhibiting significantly different profiles across the time course were detected using least-squares technique to determine the parameters of the general regression model. Any gene with different profiles between the 5-month group and 25-month group will show some statistically significant coefficient, and its corresponding regression model will be statistically significant. The p-value associated to the F-statistic in the general regression model was used to select significant genes between age groups. Second, once gene models showing significant differences were identified, backwards stepwise regression was performed on this set of genes to identify the conditions for which genes shows statistically significant profile changes.

Cluster and pathway analysis

Ingenuity Pathway Analysis (IPA) was used to identify those pathways that were significantly enriched across all differentially expressed genes, restricting to those pathways associated with whole tissues (analysis conducted in June, 2014). In order to elucidate how these biological pathways were affected by age, genes were categorized based on their response to the synergist ablation (i.e., up- or down-regulated) and then mean fold-change at each time point was generated for both the up- and down-regulated genes. Hierarchical clustering was performed on all differentially expressed genes based on similar expression profiles across time.
RT-PCR analysis

cDNA was generated from 250 ng of total RNA using SuperScript® III First-Strand Synthesis System (Invitrogen, Life Technologies, Grand Island, NY) using a combination of random hexamers and oligoDT primers. qPCR for each candidate gene was performed using KiCqStart® SYBR® Green qPCR ReadyMix (Sigma-Aldrich, St. Louis, MO) with the following cycle conditions: 95°C for 3 min, 40 cycles of 95 °C for 30s, 60 °C for 60 s. Primer sequences available upon request. All transcripts were normalized to the geometric mean of Gapdh and Rn7sk. These genes were selected based on having the lowest variability (0.24% and 0.34%, respectively) across age and treatment as determined by the microarray analysis.

Statistical analysis

The muscle wet weight, total RNA concentration and qPCR data were analyzed by two-way analysis of variance followed by Tukey’s post hoc test with significance set at \( p \leq 0.05 \). Significant gene profiles were determined using polynomial regression (degree=3) using an FDR-corrected \( p \)-value of 0.01 to determine statistical significance. For stepwise regression analysis the \( p \)-value for the regression coefficients was set to \( \alpha = 0.01 \). Only genes that had a clear trend and fit the model with an R-squared value of > 0.7 were used for further analysis. A right-tailed Fisher’s exact test was used to determine the top statistically significant canonical pathways from IPA. For this analysis, a \( p \)-value < 0.0001 was considered statistically significant.
Results and Discussion

In response to 14 days of synergist ablation, plantaris muscle weight was significantly increased in both young and old mice; however, the increase in normalized plantaris muscle weight was significantly less (40% versus 71%, respectively) in old mice compared to young mice (Fig. 1). Importantly, there was no difference in the body weight of young and old sham mice and following 14 days of synergist ablation. These findings are consistent with previous observations in aged animals showing blunted hypertrophic growth following mechanical overload ranging from 7 days to 8-weeks (2, 4, 6, 8, 22, 31).

In an effort to identify differentially expressed genes that may contribute to the blunted hypertrophic response observed in old skeletal muscle, we performed a time course microarray analysis of gene expression following 1, 3, 5, 7, 10 and 14 days of synergist ablation in young and old mice. Regression analysis using the two data sets identified 1607 genes that were differentially expressed across the time course between the young and old groups (Supplement Table 1).

We next used cluster analysis to determine how age affected the temporal pattern of expression of differentially expressed genes. We found that twelve clusters provided a sufficient number of discrete clusters with enough genes in each cluster to allow for biologically meaningful pathway analysis (Fig. 2). Cluster analysis showed that the pattern of expression of differentially expressed genes was remarkably similar between young and old groups, with the one exception being cluster 12 (Fig. 2). This cluster
contained genes which were expressed at a much lower level in old muscle across the time course and showed a different response to synergist ablation compared to young muscle. Within this cluster, about a third of the genes were from either the major urinary protein family (Mup1-4, 7, 11, 19 and 20) or the serine (or cysteine) peptidase inhibitor family (Serpina1a, b, c and e) (Supplementary Table 1).

Although few studies have looked at the effect of the Mup genes on skeletal muscle function, increasing levels of circulating Mup1 have been reported to enhance whole-body energy expenditure that was associated with the activation of Akt signaling and mitochondrial biogenesis in skeletal muscle (13). Thus, elevated expression of these genes in young skeletal muscle suggests a possible role in regulating the metabolic adaptations that occur with synergist ablation; however, it should be noted that the human genome does not harbor any functional Mup genes, thus limiting the potential translational significance of the finding.

Pathway analysis of the differentially expressed gene set revealed the primary biological processes affected by age were the regulation of protein synthesis (EIF2 and mTOR signaling), metabolism (oxidative phosphorylation and mitochondrial dysfunction) and immune response (T cell signaling) (Table 1). These pathways have been implicated in the physiological adaptation to chronic mechanical overload (1), with “mTOR signaling” identified as the most enriched pathway in a human transcriptome analysis of skeletal muscle in response to resistance exercise (24), thus giving us confidence that the informatics approach we utilized effectively captured genes involved in the hypertrophic
response. We focused on the differentially expressed genes associated with EIF2 and mTOR signaling pathways given the findings from previous studies showing that the mTOR pathway was mis-regulated in aging muscle subjected to a hypertrophic stimulus (9, 10, 23, 31).

Of the differentially expressed genes associated with EIF2 and mTOR signaling, almost half (24/54) encoded ribosomal proteins (Table 1). The expression of these ribosomal protein genes increased in a similar manner until day 5 in both age groups, after which expression remained higher in the old group (Fig. 3A). The ribosomal protein genes showing the greatest age-depended differences were \textit{Rpl24}, \textit{Rps19}, \textit{Rpl10a}, \textit{Rpl13} and, in particular, \textit{Rpl11} (Fig. 3B and Supplement Table 1). RT-PCR confirmed that \textit{Rpl11} expression was significantly increased by 4.6-fold in old group at day 10 but was unchanged from baseline at this time point in the young group (Fig. 3C). These results are consistent with findings from a recent human study that found increases in lean mass were negatively correlated with expression of 30 ribosomal protein genes (9).

Given the higher expression of ribosomal protein genes in old skeletal muscle in response to synergist ablation, we were curious to see what affect this might have on ribosome biogenesis. We and others have reported that synergist ablation-induced muscle hypertrophy is associated with a significant increase in ribosome biogenesis (12, 19, 32). To determine if ribosome biogenesis was altered in response to synergist ablation in old mice, we measured total RNA per unit of muscle mass at each time point; given that 85% of total RNA is ribosomal RNA (rRNA), total RNA per unit of tissue is
considered a measurement of ribosome content (34). There was no difference in the ribosome concentration of young and old sham plantaris muscle as total RNA per unit of muscle was the same between these two groups (Fig. 4). In response to synergist ablation, total RNA concentration of plantaris muscle of young mice significantly increased from day 5 through day 14, with ~2.5-fold peak expression occurring at days 7 and 10 (Fig. 4). In contrast, total RNA concentration of the plantaris muscle of old mice showed a modest ~50% increase at days 10 and 14 that was significantly less compared to young mice (Fig. 4).

The lower ribosome content of old plantaris muscle in response to synergist ablation might be caused by a failure to fully activate ribosomal DNA (rDNA) transcription in response to synergist ablation. To determine if rDNA transcription was altered in old plantaris muscle, we measured pre-47S rRNA abundance in sham and after 3, 7 and 10 days of synergist ablation in both young and old mice. We chose to focus on these time points because day 3 was the last time point before total RNA concentration diverged between the two groups and days 7 and 10 because it was when total RNA concentration peaked in the young group. There was no difference in pre-47S rRNA expression between young and old sham groups suggesting that rDNA transcription at baseline was not affected by age (Fig. 5A). Following three days of synergist ablation, there was a significant increase in pre-47S rRNA expression in both young and old groups, though the increase in the young group was significantly greater than in old (3-fold vs. 1.7-fold, respectively). Of note, the increased expression of pre-47S rRNA observed at day 3 in both groups preceded the increase in ribosome content as
assessed by total RNA concentration (compare Fig.4 and 5A). Although the current study does not provide a mechanism for the delay between 47S pre-rRNA expression and total RNA accumulation, Nagatomo et al., reported an acute increase in 60S ribosome formation preceded an increase in total RNA at day 5 in response to cardiac pressure overload (21). The authors proposed this delay in total RNA accumulation was a reflection of ribosome half-life of 10-12 days. Given that this was observed in the heart, it is reasonable to speculate that a similar process may be occurring in skeletal muscle in response to mechanical overload induced by synergist ablation and might be affected by age. At day 7, pre-47S rRNA expression in the young group remained significantly elevated by 3-fold whereas pre-47S rRNA expression returned to baseline level in the old group (Fig. 5A).

Using the same model of hypertrophy as we did, Nader and colleagues also reported an increase in pre-47S expression after 3 days of synergist ablation which, in contrast to our findings, returned to baseline by day 7 (32). In both studies, however, pre-47S expression paralleled the change in total RNA, further lending support to the idea that the rate of rDNA transcription dictates ribosomal biogenesis. These results suggest that polymerase I (Pol I) activity is compromised in aged animals during hypertrophic growth, which may underlie an impaired ability in increase ribosomal biogenesis, potentially limiting growth.

To determine if processing of pre-47S rRNA was altered in old muscle undergoing hypertrophy we measured the 28S rRNA expression. There was no difference in 28S
rRNA expression in young and old sham groups indicating that pre-47S processing was unaffected by age in resting skeletal muscle (Fig. 5B). In good agreement with pre-47S rRNA expression, 28S rRNA expression was significantly increased in response to synergist ablation at day 3, 7 and 10 in the young group; in contrast, 28S rRNA expression remained unchanged in the old group until day 10 (Fig. 5B). These findings suggest that rDNA transcription and processing had become uncoupled in old muscle during hypertrophic growth.

Based on our findings, we present a novel notion that dysfunctional ribosomal biogenesis is one the primary factor inhibiting muscle hypertrophy in aged animals. We suggest that in young animals, the expression levels of the core components of mature ribosomes promotes a cellular environment which is permissive for maximizing ribosomal biogenesis during muscle hypertrophy, which does not occur in aged animals. Our findings showing impaired increase in total RNA, pre-47S levels and 28S rRNA content suggest that the primary dysfunction is occurring at the level of rRNA transcription and/or processing. Since the synergist ablation model of hypertrophy induces such a robust hypertrophic growth, it would be of interest to determine if ribosome biogenesis would be altered in a more physiological model that induced a more modest level of hypertrophy with age.

Results from the current study suggest that while age-dependent differences exist across in the transcriptome profile between young and old skeletal muscle during hypertrophy, the differences appear to be modest. Moreover, the response of individual
genes to the mechanical overload resulting from synergist ablation appear to be conserved across age groups, with little evidence to suggest that disparities in transcript levels or mis-expression are driving the impaired hypertrophic growth in aged animals. Furthermore, it is important to factor in acute gene expression changes versus chronic changes, as we saw most of the changes occur early in the time-course. Recently, Nader and colleagues challenged the idea that using acute changes in gene expression to better understand the mechanisms regulating muscle hypertrophy are of limited value because most of these changes are not observed with chronic overload (20). Thus, the findings from this study provide support for the hypothesis that attenuated translational capacity is one of the primary factors governing the growth of skeletal muscle with age. To this end, many studies have focused on examining the translational efficacy of muscle and how this process is affected by age, but the role of age on translational capacity has been largely unexplored. Moreover, studies indicate that even acute differences in protein synthesis, via increased translational efficacy, are not sufficient to predict the degree muscle hypertrophy (17). These findings further argue for an important role of ribosomal biogenesis in regulating chronic changes in muscle size. By utilizing multiple time points during hypertrophic growth, our data indicates that one key determinant of translational capacity (i.e. ribosome biogenesis) is severely impaired in aged skeletal muscle and this appears to be at the level on rDNA transcription.

CONCLUSION
Currently the regulation of rDNA transcription and ribosomal biogenesis in skeletal
muscle adaptation is poorly understood, with the majority of studies focusing on assessing the activation of downstream components of the mTORC1 pathway as a readout of ribosome biogenesis. We propose that the regulation of ribosome biogenesis, in particular, the regulation of rDNA transcription, may provide additional insights into the regulation of protein synthesis during skeletal muscle hypertrophy and its importance with aging.

**List of Abbreviations:** rRNA: ribosomal RNA, Pol I: Polymerase I

**Competing Interests:** The authors declare that they have no competing interests.

**Authors’ contributions:** TJK performed bioinformatic analyses, RT-PCR and drafted the manuscript; JDL and JHE performed the synergist ablation surgeries, collected muscle tissue, RNA isolation and initial bioinformatic analyses of microarray data; TC performed bioinformatic analyses, drafted the manuscript and prepared the figures; KAE and JJM designed the study, assisted in the interpretation of the data and edited the manuscript. All authors have read and approve of the final version of the manuscript.

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References


Figure Legend

Figure 1. Aged skeletal muscle demonstrates blunted hypertrophic growth following mechanical overload. Normalized (to body weight) plantaris muscle weight from 25 month old mice (Old) showed impaired hypertrophy compared to young mice (Young) following 14 days of synergist ablation (SA-14). † denotes a significant increase relative to sham; * denotes a significant age-effect (p<0.05).

Figure 2. Hierarchical clustering of differentially expressed genes in response to synergist ablation. The expression pattern of genes within Cluster 12 demonstrated the most significant effect of age during hypertrophic growth. The majority of genes within this cluster belonged to either the major urinary protein family or the serine (or cysteine) peptidase inhibitor family. The young group is represented by a solid line and the old group is represented by a dashed line.

Figure 3. Increased expression of ribosomal proteins during hypertrophy with old age. A) Geometric mean expression of all differentially expressed ribosomal protein genes following synergist ablation. B) Rpl11 increases earlier during mechanical overload in both young and old animals; however the aged animals maintain higher expression levels at the later time points following synergist ablation. C) RT-PCR confirmed a significant increase in Rpl11 expression in old animals following 10 days of synergist ablation (SA-10) which did not occur in young animals. † denotes a significant increase relative to Sham; * denotes a significant age-effect (p<0.05).

Figure 4. Increased ribosomal content during hypertrophy is blunted in aged animals. A) Total RNA content, of which > 85% is ribosomal rRNA, increased in
response to synergist ablation indicating increased ribosomal biogenesis. Conversely, old skeletal muscle demonstrated a comprised ability to increase total RNA content in response to synergist ablation. Old animals demonstrated a delayed increase in RNA content, not occurring until at SA10 and SA14. Furthermore, at SA5 through SA14, RNA content was significantly lower in the old animals relative to the young animals. † denotes a significant increase from Sham; * denotes a significant age-effect at that time-point (p<0.05).

**Figure 5. Increased pre-47S and 28S rRNA expression during muscle hypertrophy are attenuated in old animals.** A) Pre-47S rRNA expression, a readout of polymerase I activity, increased in response to synergist ablation in young animals after 3 and 7 days of synergist ablation with old animals only showing a modest increase at day 3. Furthermore, pre-47S expression was significantly higher in young animals compared to old animals at both day 3 and 7 of synergist ablation. B) 28S rRNA expression increased in response to synergist ablation at from day 3 through day 10 in young animals, while only increasing at day 10 in old animals. 28S expression was significantly attenuated at days 3 and 7 in old animals. † denotes a significant increase from Sham; * denotes a significant age-effect at that time-point (p<0.05).

**Supplemental Table**

**Supplemental Table 1.** All 1607 differential expressed genes including respective hierarchical cluster based on similarity in expression profile and log-2 expression value for each time point.
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Figure 3A.

Graph showing RP expression (AU) over time (days) with synergist ablation. The graph includes data for young and old groups.

Figure 3B.

Graph showing Rpl11 expression (AU) over time (days) with synergist ablation. The graph includes data for young and old groups.

Figure 3C.

Graph showing Rpl11 expression (FC) over time with synergist ablation. The graph includes data for young and old groups.
A

Pre-47S rRNA expression (FC)

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B

2.8S rRNA expression (FC)

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