LINE-1 RETROTRANSPOSITION AND THE IMPACT ON AGING RODENT SKELETAL MUSCLE TISSUE

By

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ABSTRACT

Purpose: Long INterspersed Element 1 (LINE-1) is a class 1 transposable element known as a retrotransposon. LINE-1 is thought of as a genomic parasite due to its reverse transcription machinery, and ability to randomly copy and paste itself back into the genome. Studies have shown that there are an estimated 500,000 copies of LINE-1 accounting for roughly 17-18% of the total human genome. However, only around 100 of the 500,000 copies are functionally-active. Additional studies have shown that markers of tissue LINE-1 activity increase with age, and due to the ability of LINE-1 to randomly insert itself into the genome, this may negatively impact overall health. Currently, there is no research regarding the effects of aging on LINE-1 activity in rat skeletal muscle tissue. Therefore, the purpose of this study was to identify the effects of aging on LINE-1 activity markers in rat skeletal muscle tissue. **Methods**: In the current study mixed gastrocnemius muscle from male Fischer 344 rats that were 3, 12, and 24 months (mo) of age (n=9 per age group) were analyzed for LINE-1 mRNA expression, DNA expression, promoter methylation and euchromatin content. Primer sets for qPCR were designed for the youngest and most active form of LINE-1 (L1.3), older LINE-1 elements (L1.Tot), and ORF1. **Results**: L1.3, L1.Tot and ORF1 mRNA expression was higher in 12 and 24 mo versus 3 mo rats ($p<0.05$). L1.3 and ORF1 DNA expression was higher in the 24 mo versus other groups ($p<0.05$). ORF1 protein expression was higher in 12 and 24 mo versus 3 mo rats ($p<0.05$). L1.3 promoter methylation was numerically lower (but was not significantly different) in 24 mo versus 3 mo rats. Nuclear DNA methyltransferase (DNMT) activity was lower in the 12 and 24

mo versus 3 mo rats ($p<0.05$). Due to the lower nuclear DMNT activity within skeletal muscle of older rats we aimed to inhibit DNMT activity in L6-derived myotubes with 5-Azacytidine (5-AC) to determine its effects on LINE-1 mRNA expression. However, 5-AC treatments at 3 h and 24 h did not alter L1.3 or L1.Tot mRNA levels relative to vehicle-treated myotubes.

Conclusions: Markers of LINE-1 activity increase in rat skeletal muscle across the age spectrum, and this may be related to age-related alterations in LINE-1 methylation and chromatin changes. However, more research is needed to determine factors that alter LINE-1 promoter methylation and chromatin content with aging.

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CHAPTER I

INTRODUCTION

Long INterspersed Element 1 (LINE-1) is a class I transposable element better known as a retrotransposon. LINE-1 is considered a "jumping gene" due to its ability to randomly copy and paste itself back into the genome. LINE-1 accounts for roughly 17% of human DNA (75). In other mammalian species LINE-1 accounts for roughly 18% of mice DNA (141) and 23 % of rat DNA (43). Roughly 3,000 copies in mice, and 500 copies in rats are active LINE-1 copies (48, 106). While a majority of genomic LINE-1 copies are considered inactive due to either rearrangement, point mutations, or truncations, the human genome possesses around 100 active LINE-1 copies which are retrotransposition-competent (10).

A full length retrotransposition-competent LINE-1 is ~ 6 to 6.5 kilobases long and consists of a 5' untranslated region (UTR), two open reading frames (ORF1, ORF2) followed by a 3' UTR (110). ORF1 encodes a 40 kDa RNA binding protein that preferentially binds to LINE-1 in trimers to aid in translocation of the LINE-1 mRNA back into the nucleus. ORF2 encodes a 150 kDa protein that possesses endonuclease (37) and reverse transcriptase domains (91). LINE-1 retrotransposition begins with transcriptional machinery (e.g. RNA pol II) transcribing LINE-1 mRNA within the nucleus. LINE-1 mRNA is then transported out of the nucleus into the cytoplasm to ribosomes where it is translated into ORF1 and ORF2 proteins. Thereafter, within the nucleus both ORF1 and ORF2 proteins exhibit strong *cis*-preference and preferentially bind to an accessible LINE-1 mRNA forming a ribonucleoprotein particle (RNP) (35, 56, 88, 142). Once in the nucleus, the ORF2 protein on the RNP nicks genomic DNA and uses a free 3'

hydroxyl group to prime reverse transcription (29). This process is deemed target-primed reverse transcription (TPRT) (81), which is random, meaning that LINE-1 insertions can occur sporadically within the genome such as promoter / regulatory regions, or introns and exon sequences in order to affect gene expression (11). TPRT is not completely efficient given that not all LINE-1 copies are fully reversed transcribed back into the genome. This process occurs through premature termination of the TPRT process by inhibitory mechanisms, which results in truncated LINE-1 gene fragments within the genome.

Aging is defined as a failure or functional decline in multiple cells, tissues and organs in their ability to maintain homeostatic mechanisms to cope with stresses (80). In particular, skeletal muscle aging has been commonly synonymized with sarcopenia which is defined as a decline in skeletal muscle mass along with decreases in strength or function with age (90). In general, skeletal muscle aging is associated with genomic instability, epigenetic alterations, mitochondrial dysfunction, cellular senesce and atrophy (65, 76, 80). Skeletal muscle aging is also associated with fiber type conversion (54, 63, 77, 97, 117), a decline in myosin heavy chain mRNA (7, 8, 19, 89, 124) and a decline in myofibrillar protein content (49). Failure to maintain chromatin structure or methylation during aging can especially be problematic given that: a) there are numerous copies of genomic LINE-1, and b) LINE-1 5' UTR hypomethylation has been associated with over 100 different human diseases such as Duchenne muscular dystrophy, hemophilia A and B, and cancer (51). However, the functional effects of LINE-1 pathway activity in skeletal muscle remain unclear.

Interestingly, only one paper published to date has demonstrated LINE-1 mRNA expression and DNA copy number increases with age in both liver and skeletal muscle tissues of mice (32). However, this increase in skeletal muscle LINE-1 warrants further investigation to determine if LINE-1 is contributing to the aging skeletal muscle phenotype (76). Therefore, the primary purpose of this investigation was to determine if aging affects both LINE-1 mRNA expression and genomic DNA LINE-1 copy number in rat skeletal muscle tissue. Furthermore, we sought to examine how LINE-1 5'UTR promoter methylation and LINE-1 DNA content in euchromatin are impacted by aging in rat skeletal muscle tissue.

CHAPTER II

REVIEW OF LITERATURE

Transposable Elements

Transposable elements (TEs) or "jumping genes" were first discovered by Barbara McClintock at the Cold Spring Harbor Laboratory in the 1950's (92). Specifically, she observed in maize that two interacting genetic loci, activator (Ac) and dissociation (Ds) could transpose and or change positions on chromosomes (92). This led her to develop a theory, whereby these mobile elements could regulate genes through either inhibition or modulation. However, her findings and theories were heavily criticized until the late 1960's and early 1970's when other researchers discovered transposition in bacteria and yeast models, which validated her findings and led her to receive a Nobel Prize for Physiology or Medicine in 1983. Importantly, Dr. McClintock's discover of TEs proved that the genome is not a static collection of genes but is instead dynamic and ever changing.

Historically within mammalian genomes, TEs have been characterized as "junk DNA" (104). However, DNA sequencing along with molecular genetics and genomic studies have revealed that TEs are not simply "junk DNA", but these elements can play important biological roles for mammalian genomes (110). TEs were originally theorized to be in all eukaryotic species (39), and this has been found to be true for all currently investigated eukaryotic species (1, 3, 75). In this regard, this literature review will primarily focus on mammalian TEs, which account for ~37% of mouse, ~31% of canine and ~46% of human genomic DNA (33, 75, 79).

However, higher percentages have been suggested in human genomic DNA by computer algorithms (33).

TEs are commonly referred to as "jumping genes" or transposons due to their ability to mobilize and invade new chromosomal locations. Transposition events have been theorized to be important for specific biological components (58, 110), although others have speculated that TEs can be dangerous genome invaders (28, 44, 47). How TEs can transpose or mobilize is used to classify the different TEs that exist. The first classification system for TEs was proposed in 1989 by David J. Finnegan who defined two classes of TEs: class I or retrotransposons, and class II DNA transposons (38). Class I elements operate through a copy and paste mechanism which act via an RNA intermediate, whereas class II elements act via a cut and paste mechanism while utilizing a DNA intermediate (38). More recently, a newer classification system has been accepted to account for the abundance and diversity of TEs. The new TEs classification system was proposed in 2007 and maintains the previous two class system, but implements enzymatic and mechanistic criteria, and introduces levels of class, subclass, order, superfamilies, families and subfamilies (143). The focus herein will be on an order of class I TEs or retrotransposons termed Long INterspersed Element 1 (LINE-1). LINE-1 belongs to the order of long interspersed nuclear element, which consists of five superfamilies: R2, RTE, Jockey, LINE-1 and I (143).

LINE-1

LINE-1 was first discovered in 1980 by several different groups of investigators researching the same family of long interspersed nuclear elements (2, 69, 83, 85, 118, 126). However, LINE-1

has been in our genome for quite some time as it is proposed to be active within mammalian genomes for over 160 million years (21, 128, 147). There are sixteen primate specific subfamilies (PA1-PA16) of LINE-1 (72, 128). Analysis of these LINE-1 subfamilies have revealed that only LINE-1 elements from the PA1 subfamily are retrotransposition competent (10, 20, 98, 116, 128), and a majority of these PA1 LINE-1 elements belong to a smaller population termed transcribed-active subset (Ta-subset). These LINE-1 Ta-subsets are responsible for a majority of the retrotransposition activity within the human genome (11). LINE-1 Ta-subsets have been further categorized into (pre-Ta, Ta-0 and Ta-1) (14) and of these, Ta-1 has more active LINE-1 copies compared to both pre-Ta and Ta-0 (14).

Large scale sequencing projects of the human genome have determined that LINE-1 accounts for roughly 17% of human DNA (75). This equates to roughly 500,000 copies of LINE-1 within the human genome. However, a majority of LINE-1 is considered inactive from either rearrangement, point mutations or truncations, and therefore the human genome has around 100 active LINE-1 genes from the Ta-subset (10). In other mammalian species, such as rodents, similar large-scale sequencing projects have found that approximately 18% of mice DNA (141) and approximately 23 % of rat DNA is composed of LINE-1 (43). Studies have shown in mice that approximately 3,000 LINE-1 copies are full length and retrotransposition competent, whereas in rats, approximately 500 LINE-1 copies are full length and retrotransposition competent (48, 106). LINE-1 has been identified in other mammalian genomes and is reviewed in greater detail elsewhere (106).

Full length retrotransposition competent LINE-1s are ~ 6 to 6.5 kilobases long and consists of a 5' untranslated region (UTR), two open reading frames (ORF1, ORF2) and is followed by 3' UTR (110). ORF1 encodes for a 40 kDa RNA binding protein that preferentially binds to LINE-1 in trimers to aid in translocation of the LINE-1 mRNA back into the nucleus. ORF2 encodes for a 150 kDa protein that possesses endonuclease (37) and a reverse transcriptase activity (91). ORF2 is essential for LINE-1 reintegration via endonuclease nicking genomic DNA and catalyzing the reverse transcription of LINE-1 mRNA back into the genome.

LINE-1 retrotransposition begins with transcriptional machinery (e.g. RNA pol II) transcribing the LINE-1 mRNA within the nucleus. The LINE-1 mRNA is then transported out of the nucleus into the cytoplasm to ribosomes to be translated into ORF1 and ORF2 proteins. Thereafter, both ORF1 and ORF2 proteins exhibit strong *cis*-preference and preferentially bind to an accessible LINE-1 mRNA forming a ribonucleoprotein particle (RNP) (35, 56, 88, 142). The ORF1 protein then binds in trimers to LINE-1 mRNA and serves to act as a nuclear chaperone to transport the LINE-1 RNP back into the nucleus. The ORF2 protein uses its endonuclease activity to make a single stranded nick of genomic DNA and then uses the free 3' hydroxyl group to prime reverse transcription (29). This process is deemed target-primed reverse transcription (TPRT) (81). TPRT is not one hundred percent efficient as not all LINE-1 copies are fully reverse transcribed back into the genome, and thus creating truncated LINE-1 fragments or "genomic fossils". Specifically, these LINE-1 genomic fossils can be created through premature termination of the TPRT process by inhibitory mechanisms, which results in truncated LINE-1 gene fragments within the genome. LINE-1 TPRT insertion is random, meaning that

LINE-1 insertions can occur sporadically within the genome such as promoter / regulatory regions, or introns and exon sequences in order to affect gene expression (11). However, there are specific locations within the genome that allow for easier LINE-1 TPRT such as that of adenine and thymine rich locations within the genome where hydrogen bonds are weaker (37).

Regulation of LINE-1 activity

Due to the ability of LINE-1 to negatively impact the genome, multiple cellular mechanisms have developed to control or restrict LINE-1 activity. These cellular mechanisms include chromatin changes, DNA methylation, P-element induced wimpy testes (PIWI), piwi-interacting RNA (piRNAs), non-coding RNAs, ABOBEC, 3' repair exonuclease (Trex1), Moloney leukemia virus 10 (MOV10), Sirtuin 6 (SIRT6), and environmental stressors (32, 114). However, among all of these regulatory mechanisms of LINE-1 it appears that DNA methylation is the best-established mechanism (22).

DNA can be tightly compacted and packaged into chromatin, however, DNA must be accessible to allow for gene expression. Thus, the chromatin structure is dynamic in its ability to be compacted (heterochromatin) or loose (euchromatin) to allow for gene expression to occur at specific DNA locations. This process is controlled by chromatin regulating proteins such as enzymes which can modify histones by methylation and other processes, or by remodeling DNA histone structure (reviewed in (152)). DNA methylation is the process by which the nucleotide cytosine can be methylated to form 5-methylcytosine. DNA methylation typically occurs at CpG islands located within gene promoter regions, which causes a repression in gene expression. This

methylation of CpG islands can lead to altered chromatin structure through the formation of tightly packed heterochromatic regions either locally (148) or on the whole chromosome (6). The 5'UTR of LINE-1 is made up of approximately 13% of CpG islands (113), and measurements of the CpG island methylation within the LINE-1 promoter has ranged from 20-100% methylated (108). Typically, a greater methylation state of CpG islands within the LINE-1 5' UTR and has been associated with repressed LINE-1 gene expression (148).

In general, DNA methyltransferases such as Dnmt3a, Dnmt3b and Dnmt3L are responsible for *de novo* DNA methylation (103). In fact, studies using mice deficient in Dnmt3L have shown that LINE-1 mRNA and the expression of other transposon sequences is up-regulated (17). Additional mechanisms exist to regulate *de novo* LINE-1 methylation. The PIWI proteins are a class of the conserved Argonaute protein family, which interact with piRNAs to repress retrotransposons (5). This has been shown to be an effective defense mechanism against transposons by piRNAs being able to direct PIWI proteins to active transposon transcripts for cleavage in drosophila germlines (18, 84). Furthermore, in mice it has been shown that a deficiency in two murine PIWI clade proteins murine piwi (MIWI2) or miwi-like (MILI) leads to decreased methylation of retrotransposons leading to increased retrotransposon expression levels (109).

As previously noted, RNA binding proteins have been shown to modulate LINE-1 retrotransposition. The protein heterogeneous nuclear ribonucleoprotein L (hnRNPL), which is involved in alternate splicing, has been associated with LINE-1 in both mouse and human models (45, 105). In mice, the knockdown of hnRNPL caused an increase in both LINE-1

mRNA and ORF1 protein expression (105). In cell culture models another RNA binding protein named RNase L has been shown to restrict LINE-1 in human cell lines (150). Lastly, the poly A binding protein C1 (PABPC1) has also been associated with LINE-1 retrotransposition, and knockdown of poly A binding protein interacting protein 2 (PAIP2) a PABPC1 inhibitor caused an increase in LINE-1 retrotransposition (30). APOBEC3 is a gene family encoding for seven proteins which catalyze the process of deamination of cytidine to uridine (25). Studies have shown that APOBEC3A and APOBEC3B can robustly inhibit LINE-1 retrotransposition in cell culture (13), (23, 100, 119, 146). APOBEC3A has been shown to inhibit LINE-1 by deaminase pathways that target single stranded DNA such as that of LINE-1 during TPRT (111). Additionally, it has been speculated that APOBEC3B inhibits LINE-1 by deamination of cytidine to uridine (13), but the exact mechanism is still unknown. LINE-1 activity can also be inhibited through the Trex1, SAM domain and HD domain-containing protein 1 (SAMHD1), and Mov10 proteins. Trex1 is a 3' to 5' DNA exonuclease and has been shown to restrict LINE-1 retrotransposition in cell culture (130). SAMHD1 is a triphosphohydrolase which can reduce the level of intracellular deoxyribonucleotide triphosphates. In this regard, SAMHD1 has been shown in cell culture models to reduce ORF2 protein expression and suppress TPRT (153). Mov10 is an RNA helicase and part of the RNA induced silencing complex (RISC). Mov10 has been shown to directly bind with LINE-1 mRNA and co-localize with the ORF1 protein and LINE-1 ribonucleoprotein particles which inhibits the TPRT process (46). Additionally, SIRT6 has been proposed as a LINE-1 regulator. SIRT6 is a member of the sirtuin family, which are NAD+ dependent enzymes that play a role in chromatin signaling and maintenance of the

genome (133). SIRT6 has been shown to regulate LINE-1 elements by packing LINE-1 into heterochromatin regions by mono-ADP ribosylating KRAB-associated protein 1 (KAP1) a nuclear corepressor protein (140).

Furthermore, environmental factors such as calorie restriction and exercise have been shown to affect LINE-1. Specifically, in a lifelong mouse model it was found that calorie restriction reduced skeletal muscle and liver LINE-1 mRNA and DNA copy number (32). Additionally, in a human model our laboratory reported that both acute and chronic resistance training reduced skeletal muscle LINE-1 retrotransposon activity in college-aged males (114). However, it is currently unknown how both of these environmental factors mechanistically reduce LINE-1 activity.

Lastly, it is also noteworthy to mention that Jef Boeke's laboratory recently performed transcription factor profiling of the 5' UTR with the intent of identifying transcription factors and binding elements that affect LINE-1 mRNA expression in human cell types (131). Notably, these authors: a) identified 138 binding elements in the LINE-1 promoter, b) noted that there were multiple binding sites for the CCCTC-binding factor (CTCF), c-myc, and RNA pol II, and c) elegantly demonstrated that c-myc acts to repress LINE-1 transcription, whereas CTCF acts to increase LINE-1 transcription. Thus, alterations in LINE-1 5' UTR methylation presumably alters the ability of CTCF, c-myc, or other transcription factors to bind to and alter LINE-1 promoter activity.

LINE-1 and Diseases

In the 1980's it became appreciated that LINE-1 may directly cause certain diseases. For instance, Kazazin and colleagues examined over 200 boys with hemophilia A and discovered that two of the boys had mutagenic truncated LINE-1 insertions within the gene coding region of coagulation factor VIII (71). Upon examining the mother of one of the boys it was discovered that a full-length LINE-1 element was present at chromosome 22, which a portion was identical to her son's truncated LINE-1 insertion (34). Thus, this study was one of the first reports showing the deleterious effects that LINE-1 retrotransposition can have on the genome. Numerous subsequent studies have reported that LINE-1 retrotransposition has been associated with over one hundred different diseases ranging from Duchenne muscular dystrophy, hemophilia A and B, neurofibromatosis and cancer (51). In fact it has been estimated that LINE-1 retrotransposition events account for 1 in every 250 human pathogenic mutations (70, 145). These LINE-1 pathogenic insertions can cause genomic deletions at sequences that flank insertions sites such as with the PDHX, which causes pyruvate dehydrogenase deficiency (70, 96). LINE-1 transcripts have also been shown to be expressed in rheumatoid arthritis (101), and a rodent study has reported that increased LINE-1 mRNA expression manifests in cardiac tissue following ischemia-reperfusion (82).

LINE-1 retrotransposition may also contribute to tumorigenesis in certain tissues by creating genetic lesions through its endonuclease activity or by inserting new LINE-1 copies which affect gene expression (112). This was first shown in 1992 in a colorectal cancer patient where a LINE-1 insertion was found to disrupt the coding exon of the tumor suppressor gene

adenomatous polyposis coli (APC) (94). Additional studies using advanced sequencing techniques have also shown that LINE-1 insertions affect gene expression in lung cancer (62), colorectal cancer (78, 129), hepatocellular carcinomas (125), and ovarian as well as pancreatic cancers (78). The overexpression of the LINE-1 ORF1 protein is evident in multiple human cancers. Specifically, a study by Rodic and colleagues demonstrated that LINE-1 ORF1p detected through immunohistochemistry is present in a wide range of human neoplasms (e.g., detected in 90% in breast and ovarian cancers, 90% pancreatic cancers, 50-60% in gastrointestinal tract cancers, and 40% in prostate cancers) (112). Additionally, these authors suggest that as many as half of human cancers express ORF1 protein (112). More recently LINE-1 hypomethylation has been recognized as a promising prognostic cancer marker in patients that possess breast cancer (138), stage I non-small cell lung cancer (61), gastric cancer (122), rectal cancer (52), colorectal cancer (95) and hepatocellular carcinoma (52).

Aging and LINE-1

Aging is defined as a failure or functional decline in multiple cells, tissues and organs in their ability to maintain homeostatic mechanisms to cope with stresses (80). Generally speaking, skeletal muscle aging is associated with genomic instability, epigenetic alterations, mitochondrial dysfunction, cellular senesce and atrophy (other mechanisms of aging are reviewed here (65, 76, 80). The loss of skeletal muscle with age involves both quantitative and qualitative changes to the skeletal muscle structure (76). This decline in skeletal muscle mass along with decreases in strength or function is referred to as sarcopenia (90). However, this process is typically slow and

varies between individuals, but inevitably occurs in all humans irrespective of physical activity or health (24). Skeletal muscle also exhibits a fast to slow myosin isoform conversion with age, which has been repeatedly documented (54, 63, 77, 97, 117). This fiber type conversion is also associated with decreases in fast twitch myosin heavy chain mRNA expression, although slow twitch myosin heavy chain mRNA expression has been shown to be unaffected with age (7, 8, 19, 89, 124). Myofibrillar protein (e.g. actin and myosin) has additionally been shown to decrease with age (49).

The process of mitochondrial aging has been extensively studied for over four decades (53). In humans, mitochondrial content in skeletal muscle has been shown to decline with age by examination of mitochondrial DNA copy number, citrate synthase, and electron microscopy (9, 65, 115, 123, 137). Studies have also shown a progressive 5% reduction per decade of mitochondrial DNA, which may be driving the skeletal muscle aging process (65, 123). Interestingly, the sirtuin enzymes have been shown to be associated with aging. In this regard, SIRT1 overexpression in mice has been shown to attenuate DNA damage and better overall health and less carcinomas (55). Additional studies have shown that SIRT6 can promote longevity through DNA double-strand break repair (66, 86), reducing tumor formation (120, 139) and regulating telomere stability (93). Additional studies using a SIRT6 knockout mouse model reported that animals developed premature aging symptoms such as genomic instability, decreased bone mineral density and had shortened lifespans (99). However, SIRT6 overexpression mice exhibited longer lifespans, demonstrated an improvement in metabolic

function (68), and exhibited cancer resistance (67). Thus, sirtuins may play an important role in regard to the aging process.

The alteration of chromatin structure is a critical aspect of aging (102). The aging process can alter chromatin states, which can lead to altered expression of genes that are normally in a repressed state (149). In this regard, certain histone marks (e.g. H3K9me3 and H4K20me2) can act to repress gene expression through heterochromatin formation (16). Interestingly, these histone marks can change with age leading to altered chromatin states (12). This has led to a hypothesis by Sedivy and colleagues where chromatin alterations during aging leads to altered epigenetic control of retrotransposition (121). In support of this hypothesis, a study by De Cecco and colleagues demonstrated chromatin alterations in senescent human diploid fibroblasts led to an increase in transcription and transposition of LINE-1 (31).

Additionally, it has been well established *in vitro* and *in vivo* that global CpG methylation decreases with age (36, 42, 127, 134, 144). This change in genome wide DNA methylation has been used as a biomarker to determine chronological age in humans, and therefore DNA methylation can act as an "epigenetic clock" (57). Furthermore, this hypomethylation occurs at CpG islands within promoter regions of specific genes (4) such as those of TEs. In this regard, LINE-1 promoter methylation has been examined in DNA obtained from blood samples across the age span, and some studies report no change with age (15, 64, 151) whereas others report decreases (26, 40, 132).

Failure to maintain chromatin structure or methylation during aging can especially be problematic for LINE-1 due to its pathogenic potential. In mice LINE-1 mRNA expression has

been shown to increase with age in both liver and skeletal muscle tissues (32). In this study, De Cecco and colleagues observed an increase in LINE-1 DNA copy number within both liver and skeletal muscle tissue in 36-month mice compared to 5- and 24-month old mice (32). Additionally, these authors reported increases in heterochromatin structure in both mouse tissues (32), which could be contributing to the increased LINE-1 expression but was only speculated by the authors. Lastly, De Cecco and colleagues suggested that retrotransposition is an important aging process that can impact many tissues (32). These research findings warrant consideration as to how the increase in LINE-1 retrotransposition during aging affects skeletal muscle function.

Purpose statement and hypotheses

Given the limited amount of literature regarding LINE-1 and skeletal muscle tissue, the primary purpose of this investigation is presented below. Notably, experimental hypotheses are presented following the purpose statement.

Purpose: to determine if skeletal muscle LINE-1 mRNA and DNA expression differ between rats that are 3 months, 12 months and 24 months old. Furthermore, we aim to determine how skeletal muscle LINE-1 5'UTR promoter methylation and heterochromatin LINE-1 genomic DNA content are affected between these age groups.

Hypothesis: Given the prior findings in mice skeletal muscle by De Cecco and colleagues (32) and the plethora of data suggesting LINE-1 methylation is affected by aging we hypothesized that both LINE-1 mRNA and DNA expression would increase across the age spectrum, and

skeletal muscle LINE-1 5’UTR promoter methylation levels would decrease across the age spectrum.

CHAPTER III

METHODS

Animals

All animal experimental procedures were approved by Auburn University's Institutional Animal Care and Use Committee. Fischer 344 male rats aged 3, 12, and 24 months (n=9 per age group) were purchased via Envigo Laboratories (Indianapolis, IN, USA). Rats were housed two per cage at the Auburn University Biological Research Facility in quarters with constant 12 h light and 12 h dark cycles at ambient room temperature. Standard chow (24% protein, 58% carbohydrate, 18% fat; Teklad Global #2018 Diet, Envigo Laboratories) and tap water were provided to animals *ad libitum*.

The day prior to the necropsies, rotarod performance was assessed via a single-lane device (Product #: ENV-571R; Med Associates Inc., Saint Albans City, VT, USA). Specifically, rotarod tests occurred during the start of the light cycle, and involved placing the rat on the device and the motorized rotor was initiated and progressed from a speed of 4.0 to 40.0 revolutions/min. The automated timer tracked each rats time spent on the rod and once the rat dismounted from the rod, a laser beam break stopped the timer. Rotarod performance has been used to assess rodent balance, grip strength, motor coordination as well as muscular endurance (50).

The morning of the necropsies, rodents were removed from the Biological Research Facility, transported to the Molecular and Applied Sciences Laboratory in the School of Kinesiology, and were then acclimated for approximately 3-4 hours with *ad libitum* access to

water only. After acclimation, rodents were then euthanized under CO_2 gas induction in a 2 L chamber (VetEquip, Inc., Pleasanton, CA, USA). Thereafter, total body mass was recorded, and right-leg gastrocnemius muscle was dissected out and weighed using an analytical scale with a sensitivity of 0.0001 g (Mettler-Toledo; Columbus, OH, USA). Dissections on the right-leg gastrocnemius muscle were performed close to the origin and insertion sites, and any visible connective or fat tissue was removed. Tissues were then flash frozen in liquid nitrogen and stored at -80°C until analyses described below.

Tissue preparation for protein analyses

Mixed gastrocnemius tissues were removed from -80ºC storage, tissue was crushed on a liquid nitrogen-cooled mortar and pestle, and approximately 50 mg of tissue from each rodent were placed in 500 µL of ice-cold general cell lysis buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na-EDTA, 1 mM EGTA, 1% Triton, 20 mM sodium pyrophosphate, 25 mM sodium fluoride, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 and 1 µg/mL leupeptin] (Cell Signaling; Danvers, MA, USA). Tissues were homogenized via micro pestles and homogenates were centrifuged at 500 *g* for 5 min. After centrifugation insoluble proteins were removed and supernatants were stored at -80ºC prior to Western blotting.

Western blotting

Total protein determination on supernatants were performed following the dye-based bicinchoninic acid (BCA) colorimetric assay (Thermo Fischer Scientific, Waltham, MA, USA).

Supernatants were subsequently prepared for SDS-PAGE using 4x Laemmli buffer at 2 µg/µL, and 25 µL were loaded onto 4-15% SDS-polyacrylamide pre-casted gels (Bio-Rad Laboratories; Hercules, CA, USA). 1x SDS-PAGE run buffer (Ameresco; Framingham, MA, USA) was used for electrophoresis at 180 V for 60 min. Thereafter, proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories) via constant amperage (200 mA) for 120 min. Membranes were then stained with Ponceau S and digital images were captured using a gel documentation system (UVP, Upland, CA, USA) to ensure equal loading of samples between lanes. Membranes were then blocked at room temperature with 5% nonfat milk powder in Tris-buffered saline with 0.1% Tween-20 (TBST) for one hour. All of the following primary antibodies were incubated overnight at 4ºC in a solution of TBST containing 5% bovine serum albumin (BSA; Ameresco): a) Mouse anti-ORF1 (1:1,000, Abcam, Cambridge, MA, USA; catalog #: ab76726), b) Rabbit-anti DNMT3A (1:1,000, Cell Signaling; catalog #: 2160S), and c) Rabbit anti-TET1 (1:1,000, Abcam; catalog #: ab191698). The following day, membranes were incubated with HRP-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (1:2,000; Cell Signaling) in a solution of TBST containing 5% BSA at room temperature for one hour. Thereafter, membranes were developed using an enhanced chemiluminescent reagent (Luminata Forte HRP substrate; EMD Millipore, Billerica, MA, USA) where band densitometry was assessed by use of a digital gel documentation system and associated densitometry software (UVP). Densitometric values of white bands for each target were normalized to dark band Ponceau densitometry values. These values were then normalized to the 3-month group average to yield relative expression units (REUs).

RNA isolation, cDNA synthesis and real-time polymerase chain reaction (RT-PCR)

Approximately 20 mg of frozen mixed gastrocnemius muscle from each rodent were placed in 500 µL of Ribozol (Ameresco, Solon, OH, USA) per the manufacturer's recommendations. Thereafter, phase separation was achieved according to manufacturer's instructions for RNA isolation. Following RNA precipitation and pelleting, pellets were resuspended in 30 µl of RNase-free water, and RNA concentrations were determined in duplicate at an absorbance of 260 nm by using a NanoDrop Lite (Thermo Fisher Scientific). cDNA (2 µg) was synthesized using a commercial qScript™ cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA) per the manufacturer's recommendations. RT-PCR was performed with gene-specific primers and SYBR-green-based methods in a RT-PCR thermal cycler (Bio-Rad, Hercules, CA, USA). Primers were designed with primer designer software (Primer3Plus, Cambridge, MA, USA), and melt curve analyses demonstrated that one PCR product was amplified per reaction. Additionally, PCR products were resolved on 1% agarose gels in order to verify that a product produced at the anticipated molecular weight was obtained. Three primer sets were designed to interrogate LINE-1 mRNA and DNA expression. The first primer set (L1.3) amplified a portion of the 5'-UTR region and was designed to probe for the most active LINE-1 element based upon the findings of Kirilyuk et al. (74). The second primer set (L1.Tot) also amplified a portion of the 5'-UTR region and was designed to encompass full-length LINE-1 elements that contained a 5' promoter, but did not have the ability to retrotranspose based on mutations in the protein coding regions (74). We also designed a primer set to amplify a portion

of the ORF1 region. The forward and reverse primer sequences for all genes are listed in Table 1. Fold-change values from 3 month old rats were performed using the $2^{\Delta\Delta Cq}$ method where $2^{\Delta Cq}$ = 2^[housekeeping gene (HKG) Cq – gene of interest Cq] and $2^{\Delta\Delta Cq}$(or fold-change) = [$2^{\Delta Cq}$ value/$2^{\Delta Cq}$ average of 3 month age group]. The geometric mean of fibrillarin (FBL), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), cyclophilin (CYCLOPH), and hypoxanthine phosphoribosyltransferase (HPRT) was used as the HKG to normalize mRNA expression results. While data are represented as fold-change from the 3-month group, they are presented as REUs. The overall coefficient of variation values for Cq duplicates of the assayed genes were as follows: HKG = 0.99%, L1.3 = 0.47%, L1.Tot = 0.53%, ORF1 = 0.43%.

DNA isolation and RT-PCR for gDNA LINE-1

Mixed gastrocnemius were removed from -80ºC, and approximately 25 mg of each tissue from each rodent were processed using the commercially available DNA isolation kit DNeasy Blood & Tissue Kit (Qiagen, Venlo, Netherlands) per the manufacturer's recommendations. Following DNA precipitation and pelleting, pellets were resuspended in 200 µl of elution buffer from the kit, and DNA concentrations were determined in duplicate at an absorbance of 260 nm using a NanoDrop Lite (Thermo Fisher Scientific). DNA (25 ng) was subjected to RT-PCR analysis using the aforementioned methods. The geometric mean of Ribosomal Protein S16 (RPS16), Histone Deacetylase 1 (HDAC1) and Beta-2-Microglobulin (B2M) was used as a HKG to normalize DNA expression results. Fold-change values from 3-month rats were performed using the aforementioned $2^{\Delta\Delta Cq}$ method, and data are presented as REUs. Overall coefficient of

variation values for Cq duplicates of the assayed genes were as follows: L1.3 = 0.51% and L1.Tot = 0.62%, ORF1 = 0.31%, HKG = 0.24%.

LINE-1 promoter methylation analysis

LINE-1 promoter methylation analysis was performed on isolated gastrocnemius DNA (described above) from n=8 rats per age group using a commercially available methylated DNA immunoprecipitation (MeDIP) kit (product #: ab117133; Abcam). Prior to performing the assay, 1.5 µg of gastrocnemius DNA was digested using MseI (New England BioLabs, Ipswich, MA, USA). Following digestion reactions, total methylated DNA from a total of 1 µg input was immunoprecipitated using an anti-5-methylcytosine antibody provided within the kit. RT-PCR was then performed on 25 ng of the methylated DNA using the L1.3 primers described above to decipher fold-change in methylated LINE-1 5' UTR. Residual input DNA from each sample (25 ng) was used as a control to normalize RT-PCR results. Fold-change in promoter methylation was calculated using the $2^{\Delta\Delta Cq}$ method where $2^{\Delta Cq}$ = 2^[input L1.3 DNA Cq – methylated L1.3 DNA Cq] and $2^{\Delta\Delta Cq}$(or fold-change) = [$2^{\Delta Cq}$value/$2^{\Delta Cq}$ average of 3 month age group]. Fold-change values from 3-month rats were performed using the aforementioned $2^{\Delta\Delta Cq}$ method, and data are presented as REUs. Overall coefficient of variation values for Cq triplicates of the assayed genes were as follows: input L1.3 = 0.44% and methylated L1.3 = 0.35%.

LINE-1 chromatin accessibility analysis

LINE-1 chromatin accessibility was assessed from n=8 rats per age group using a commercially available kit (Chromatin Accessibility Assay Kit, product #: ab185901; Abcam) per the manufacturer's recommendations. Briefly, methods involved obtaining DNA, digesting the DNA using a proprietary nuclear digestion buffer, and performing RT-PCR on digested versus undigested samples. The premise of the assay operates through a gene of interest localized to euchromatin regions being more susceptible to digestion and, thus, possessing a lower RT-PCR amplification signal relative to genes in heterochromatin regions. RT-PCR was performed on 25 ng of digested DNA using the L1.3 primers described above to decipher fold-change in genomic LINE-1 residing in euchromatin. Undigested DNA from each sample (25 ng) was used as a control to normalize RT-PCR results. Fold-change in L1.3 euchromatin DNA was calculated using the $2^{\Delta\Delta Cq}$ method where $2^{\Delta Cq}$ = 2^[digested L1.3 DNA Cq – undigested L1.3 DNA Cq] and $2^{\Delta\Delta Cq}$(or fold-change) = [$2^{\Delta Cq}$value/$2^{\Delta Cq}$ average of 3 month age group]. Fold-change values from 3-month rats were performed using the aforementioned $2^{\Delta\Delta Cq}$ method, and data are presented as REUs. Overall coefficient of variation values for Cq triplicates of the assayed genes were as follows: undigested L1.3 DNA = 1.64% and digested L1.3 DNA = 0.36%.

DNA Methyltransferase Activity Assay

Prior to assaying DNA Methyltransferase (DNMT) activity, nuclear protein extraction was performed on frozen gastrocnemius muscle (~25 mg) using a commercially-available kit (Nuclear Extraction Kit; Abcam) per the manufacturer's recommendations. Global DNMT activity of nuclear isolates (10 µL) was assessed using a commercially available kit (DNMT

Activity Assay Kit; Abcam) per the manufacturer's recommendations. DNMT activity was expressed as relative expression units (REU), which were then normalized to input muscle weights. Overall coefficient of variation values for duplicate readings were 33.2%.

Histone Deacetylase Activity Assay

Global Histone Deacetylase (HDAC) activity on nuclear isolates (10 µL) was assessed using a commercially available fluorometric kit (Histone Deacetylase Activity Assay Kit; Abcam) per the manufacturer's recommendations. HDAC activity was expressed as relative fluorescence units (RFU), which were then normalized to input muscle weights. Overall coefficient of variation values for duplicates readings were 5.3%.

RNA-sequencing

RNA-sequencing was performed only on a subset of 3-month old (n=8) and 24-month old rats (n=8) with the intent of identifying how aging affected the mRNA expression of genes related to LINE-1 regulation. Gastrocnemius tissues were processed for RNA isolation using methods described above. Thereafter, RNA was shipped to LC Sciences (Houston, TX, USA) for RNA-sequencing. The steps described below were completed for this analysis.

Library Preparation and Sequencing. Total RNA quality and quantity were assessed using Bioanalyzer 2100 and RNA 6000 Nano LabChip Kits (Agilent, CA, USA), and all samples yielded RIN numbers >7.0. Total RNA was subjected to poly(A) mRNA enrichment with poly-T oligo-attached magnetic beads (Invitrogen). Following purification, the poly(A) mRNA fractions

were fragmented into small pieces using divalent cations under elevated temperature. The cleaved RNA fragments were subsequently reverse-transcribed to create final cDNA libraries in accordance with strand-specific library preparation by dUTP method. The average insert size for the paired-end libraries was 300±50 bp. Paired-end 2×150 base pair sequencing was performed on an Illumina Hiseq 4000 platform following the manufacturer's recommended protocol.

Transcripts Assembly. Cutadapt (87) and perl scripts were used to remove the reads that contained adaptor contamination, low quality bases, and undetermined bases. Sequence quality was verified using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). HISAT2 (73) was used to map reads to the genome of Rattus norvegicus (Version: v88). The mapped reads of each sample were assembled using StringTie (107). Thereafter, all transcriptomes from the 16 samples were merged to reconstruct a comprehensive transcriptome using perl scripts and gffcompare (https://github.com/gpertea/gffcompare/). After the final transcriptome was generated, StringTie (107) and Ballgown (41) were used to estimate the expression levels of all transcripts.

Differential expression analysis of mRNAs. StringTie (107) was used to measure expression levels for mRNAs by calculating FPKM values (FPKM=[total_exon_fragments/mapped_reads(millions)×exon_length(kB)]). Differentially expressed transcripts related to LINE-1 regulation between the young and old groups were determined by comparing FPKM values. Given that RNA-sequencing was used as a screening tool herein, differentially expressed transcripts related to LINE-1 regulation were considered meaningful if the un-adjusted p-value of a given mRNA between groups was $p<0.05$.

Age-related skeletal muscle phenotyping

Beyond examining skeletal muscle LINE-1 pathway markers in the three cohorts, we also sought to analyze muscle for age-related phenotypes. These methods are described below.

Total myofibrillar protein assessment. Myofibrillar protein isolations were performed based on the methods of Goldberg's laboratory (27). Briefly, frozen powdered muscle (8–13 mg) was weighed using an analytical scale sensitive to 0.0001 g (Mettler-Toledo, Columbus, OH, USA), and immediately placed in 1.7 mL polypropylene tubes containing 190 μL of ice cold homogenizing buffer (20 mM Tris–HCl, pH 7.2, 5 mM EGTA, 100 mM KCl, 1% Triton-X100) and 6.4 M spermidine (Alfa Aesar, Haverhill, MA USA) as previously reported by our laboratory (Roberts et al. 2019). Samples were homogenized on ice using tight-fitting pestles, and centrifuged at 3,000g for 30 min at 4 °C. The resultant pellet was resuspended in homogenizing buffer, and samples were centrifuged at 3000g for 10 min at 4 °C. Resultant supernatants from this step were discarded, resultant pellets were resuspended in 190 μL of ice cold wash buffer (20 mM Tris–HCl, pH 7.2, 100 mM KCl, 1 mM DTT), and samples were centrifuged at 3000g for 10 min at 4 °C; this specific process was performed twice. Final myofibril pellets were resuspended in 200 μL of ice-cold storage buffer (20 mM Tris–HCl, pH 7.2, 100 mM KCl, 20% glycerol, 1 mM DTT) and frozen at −80 °C until concentration determination. Myofibrillar protein concentrations were determined from a BCA assay, which were then normalized to input muscle weights.

Determination of myosin heavy chain and actin content. SDS-PAGE preps from resuspended myofibrils were performed using: (a) 10 μL resuspended myofibrils, (b) 65 μL

distilled water (diH2O), and (c) 25 µL 4x Laemmli buffer. Samples (5 µL) were then loaded on pre-casted gradient (4–15%) SDS-polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA, USA) and subjected to electrophoresis (200 V for 45 min) using pre-made 1x SDS-PAGE running buffer (Ameresco). Following electrophoresis gels were rinsed in diH2O for 15 min, and immersed in Coomassie stain (LabSafe GEL Blue; G-Biosciences, St. Louis, MO, USA) for 45 min. Thereafter, gels were de-stained in diH2O for 60 min, bright field imaged using a gel documentation system (UVP), and band densities were determined using associated software. Myosin and actin concentrations were expressed as arbitrary density units (ADU) per mg muscle.

Citrate synthase activity assay. Mixed gastrocnemius foils were removed from -80ºC storage, tissue was crushed on a liquid nitrogen-cooled mortar and pestle, and approximately 30 mg of tissue from each rodent were placed in 500 µL of ice-cold cell lysis buffer (recipe described above) (Cell Signaling). Tissues were homogenized via micro pestles and homogenates were centrifuged at 500 *g* for 5 min. After centrifugation insoluble proteins were removed and supernatants were stored at -80ºC prior to citrate synthase activity assessments. Citrate synthase activity as previously described by our laboratory (59). The assay principle is based upon the reduction of 5,50-dithiobis (2- nitrobenzoic acid) (DTNB) at 412 nm (extinction coefficient 13.6 mmol/L/cm) coupled to the reduction of acetyl-CoA by the citrate synthase reaction in the presence of oxaloacetate. Briefly, 2 µg of skeletal muscle protein was added to a mixture composed of 0.125 mol/L Tris–HCl (pH 8.0), 0.03 mmol/L acetyl-CoA, and 0.1 mmol/L DTNB. The reaction was initiated by the addition of 5 µL of 50 mmol/L oxaloacetate and the

absorbance change was recorded for 1 min. Overall coefficient of variation values for duplicate readings were 4.2%.

Muscle Triglyceride Assay. Gastrocnemius skeletal muscle tissue (~20 mg) was weighed using an analytical scale sensitive to 0.0001 g (Mettler-Toledo, Columbus, OH, USA), and immediately placed in 1.7 mL polypropylene tubes. Thereafter, triglyceride analysis was performed using a commercially available triglyceride colorimetric kit (Cayman Chemical, Ann Arbor, MI, USA), according to manufacturer's instructions. Gastrocnemius skeletal muscle triglyceride content was expressed μmol per gram wet muscle. Overall coefficient of variation values for duplicate readings were 1.2%.

In vitro experiments for 5-Azacytidine treatments

Passage 2, L6 myoblasts (Kerafast Inc. Boston, MA, USA), were grown in growth medium (DMEM, 10% FBS, 1% penicillin/streptomycin, and 0.1% gentamycin; Corning Inc., Corning, NY, USA) on 12-well plates at a seeding density of 1 x 10^5 under standard culture conditions (37°C in a 5% CO_2 atmosphere). Once myoblast growth reached 80–90% confluency 5 days after seeding, differentiation was induced by removing growth medium and replacing it with differentiation medium [DM; DMEM, 2% (vol/vol) horse serum, 1% penicillin/streptomycin, and 0.1% gentamycin; Corning]. DM was then replaced every 24 h for 6 d to allow for myotube growth.

5-Azacytidine (5-AC) (TCI America, Portland, OR, USA) was administered to myotubes during differentiation on day 5 at final concentration of 10 mM in DM (solubilized in <0.1%

dimethyl sulfoxide (DMSO); Corning) (n=4 wells per treatment) for 3 h and 24 h, and doses were based on Ikeda et al. (25) who used similar doses *in vitro*. Control (CTL) wells received DMSO administration during day 5 of differentiation in DM (n=4 wells per treatment), at a similar dose used to solubilize 5-AC. Following treatments, RNA was isolated using the aforementioned methods. Total RNA concentrations were analyzed using a Nanodrop Lite spectrophotometer (Thermo Fisher Scientific), and 1 µg of cDNA were synthesized using a commercial qScript cDNA SuperMix (Quanta Biosciences) per the manufacturer's recommendations. Real-time PCR using SYBR green chemistry was performed using the three LINE-1 primers (Table 1). LINE-1 mRNA at 3 h, 5-AC treatments were normalized to GAPDH, and LINE-1 mRNA at 24 h were normalized to B2M. Fold-change values from CTL treatments were performed using the $2^{\Delta\Delta Cq}$ as previously described. Overall coefficient of variation values for 3-h treatment Cq duplicates of the assayed genes were as follows: GAPDH = 0.31%, L1.3 = 0.18%, L1.Tot = 0.20%, and ORF1 = 0.12%. Overall coefficient of variation values for 24-h treatment Cq duplicates of the assayed genes were as follows: B2M = 0.11%, L1.3 = 0.30%, L1.Tot = 0.17%, and ORF1 = 0.21%.

Statistical Analyses

Statistics were performed using the open-source software R (54), R Studio (53) and SPSS v 23.0 (IBM, Armonk, NY, USA). Prior to statistical analysis assumption testing was performed on all dependent variables. For non-normally distributed data, values were log-transformed and re-analyzed for normality. Dependent variables for the rodent experiments were analyzed using

one-way ANOVAs with Fisher LSD *post hoc* tests or Welch's t-tests (when assumptions were violated) to assess differences in dependent variables between age groups. Dependent variables from *in vitro* treatments were compared using independent samples t-tests. Magnitude of effects are also expressed in the results using partial eta squared (η_p^2) effect size, and effect sizes of 0.01, 0.06 and > 0.14 were considered small, moderate and large. Statistical significance for all null hypothesis testing was set at $p<0.05$. All data is herein is presented as mean ± standard deviation (SD).

CHAPTER IV

MANUSCRIPT (to be submitted to AJP Cell Physiol in April, 2019)

LINE-1 retrotransposon activity is up-regulated in aged rat skeletal muscle

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Running head: LINE-1 Retrotransposon Activity in Muscle of Aging Rats

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ABSTRACT

The Long INterspersed Element 1 (LINE-1) gene is a retrotransposon capable of replicating and inserting newly-transcribed copies into the nuclear genome. While aging in mice increases markers of skeletal muscle LINE-1 activity, data is currently lacking in other mammalian species. Herein, mixed gastrocnemius muscle from male Fischer 344 rats that were 3, 12, and 24 months (mo) of age (n=9 per age group) were analyzed for LINE-1 mRNA expression, DNA expression, promoter methylation and euchromatin content. Primer sets for qPCR were designed for the youngest and most active form of LINE-1 (L1.3), older LINE-1 elements (L1.Tot), and a portion of the ORF1 sequence. L1.3, L1.Tot and ORF1 mRNA expression were higher in 12 and 24 mo versus 3 mo rats ($p<0.05$). L1.3 and ORF1 DNA expression were higher in the 24 mo rats versus other groups ($p<0.05$). ORF1 protein expression was higher in 12 and 24 mo versus 3 mo rats ($p<0.05$). L1.3 promoter methylation was numerically (but not significantly) lower in 24 mo versus 3 mo rats. RNA-seq screening indicated that DNA methylation may be lower in 24 mo versus 3 mo rats. Thus, we examined markers related to this pathway across age groups. Nuclear DNA methyltransferase (DNMT) activity was lower in the 12 and 24 mo versus 3 mo rats ($p<0.05$). Due to the lower nuclear DMNT activity within skeletal muscle of older rats we aimed to inhibit DNMT activity in L6 myotubes with 5-Azacytidine (5-AC) to potentially

recapitulate the aging effects of increased LINE-1 mRNA expression. Interestingly, 5-AC treatments at 3 h and 24 h did not alter L1.3, L1.Tot, or ORF1 mRNA levels relative to vehicle-treated myotubes. To summarize, skeletal muscle markers of LINE-1 activity increase across the age spectrum in rats, and this may be related to age-related alterations in LINE-1 methylation status and chromatin alterations. However, our *in vitro* results suggest this may not be directly related to DNMT activity and more research is needed to determine factors that alter LINE-1 promoter methylation and chromatin content with aging.

KEY WORDS: LINE-1, retrotransposons, skeletal muscle, aging

LIST OF ABBREVIATIONS

HKG, housekeeping gene

L1.3, younger, active LINE-1

L1.Tot, total LINE-1

LINE-1, Long INterspersed Element-1

MeDIP, methylated DNA immunoprecipitation

ORF, open reading frame

RNP, ribonucleoprotein

TE, transposable element

TPRT, target-primed reverse transcription

UTR, untranslated region

INTRODUCTION

The Long INterspersed Element 1 (LINE-1) is a class I transposable element. LINE-1 is considered to be a "jumping gene" due to its ability to "copy and paste" itself back into the genome. LINE-1 repeats account for roughly 17% of human DNA (32), 18% of mice DNA (57), and 23% of rat DNA (19). While a majority of genomic LINE-1 copies are considered inactive due to either rearrangement, point mutations, or truncations, there are roughly 3,000 active LINE-1 copies in mice, and 500 active LINE-1 copies in rats (20, 45), and 100 active LINE-1 copies in humans which are retrotransposition-competent (3).

A full-length retrotransposition-competent LINE-1 is ~ 6 to 6.5 kilobases long and consists of a 5' untranslated region (UTR), two open reading frames (ORF1, ORF2) followed by a 3' UTR (47). ORF1 encodes a 40 kDa RNA binding protein that preferentially binds to LINE-1 in trimers to aid in translocation of the LINE-1 mRNA back into the nucleus. ORF2 encodes a 150 kDa protein that possesses endonuclease (15) and reverse transcriptase domains (41). LINE-1 retrotransposition begins with transcriptional machinery (e.g. RNA pol II) transcribing LINE-1 mRNA within the nucleus. LINE-1 mRNA is then transported out of the nucleus into the cytoplasm to ribosomes where it is translated into ORF1 and ORF2 proteins. Both ORF1 and ORF2 proteins exhibit strong cis-preference and preferentially bind to an accessible LINE-1 mRNA forming a ribonucleoprotein particle (RNP) (13, 24, 38, 58). Once in the nucleus, the ORF2 protein on the RNP nicks genomic DNA and uses a free 3' hydroxyl group to prime reverse transcription (10). This process is deemed target-primed reverse transcription (TPRT)

(36), which is random, meaning that LINE-1 insertions can occur sporadically within the genome such as promoter/regulatory regions, or introns and exon sequences in order to affect gene expression (4). TPRT is not completely efficient, and the premature termination of the TPRT process can occur through inhibitory mechanisms resulting in truncated LINE-1 gene fragments within the genome.

Aging is defined as a failure or functional decline in multiple cells, tissues and organs in their ability to maintain homeostatic mechanisms to cope with stresses (35). In particular, skeletal muscle aging has been commonly synonymized with sarcopenia which is defined as a decline in skeletal muscle mass along with decreases in strength or function with age (40). In general, skeletal muscle aging is associated with genomic instability, epigenetic alterations, mitochondrial dysfunction, cellular senesce and atrophy (29, 33, 35). Skeletal muscle aging is also associated with fiber type conversion (23, 27, 34, 43, 49), a decline in myosin heavy chain mRNA (1, 2, 7, 39, 50) and a decline in myofibrillar protein content (21). Failure to maintain chromatin structure or methylation during aging can especially be problematic with regard to promiscuous LINE-1 mRNA expression given that: a) there are numerous copies of genomic LINE-1, and b) LINE-1 5' UTR methylation is thought to be the prominent mechanism to reduce LINE-1 pathway activity (60).

Interestingly, only one paper published to date has demonstrated LINE-1 mRNA expression and DNA copy number increases with age in both liver and skeletal muscle tissues of mice (12). However, the cause of increased LINE-1 mRNA and DNA content with aging was

not well-elucidated. Moreover, given that mice contain several-fold more active copies of LINE-1 that other mammals, it remains to be determined if this phenomena is observed across species. Therefore, the primary purpose of this investigation was to determine if aging affects both LINE-1 mRNA expression and genomic DNA LINE-1 copy number in rat skeletal muscle tissue. Furthermore, we sought to examine how LINE-1 5'UTR promoter methylation and LINE-1 DNA content in euchromatin regions are impacted by aging in rat skeletal muscle tissue.

MATERIALS AND METHODS

Animals

All animal experimental procedures were approved by Auburn University's Institutional Animal Care and Use Committee. Fischer 344 male rats aged 3, 12, and 24 months (n=9 per age group) were purchased via Envigo Laboratories (Indianapolis, IN, USA). Rats were housed two per cage at the Auburn University Biological Research Facility in quarters with constant 12 h light and 12 h dark cycles at ambient room temperature. Standard chow (24% protein, 58% carbohydrate, 18% fat; Teklad Global #2018 Diet, Envigo Laboratories) and tap water were provided to animals *ad libitum*.

The day prior to the necropsies, rotarod performance was assessed via a single-lane device (Product #: ENV-571R; Med Associates Inc., Saint Albans City, VT, USA). Specifically, rotarod tests occurred during the start of the light cycle, and involved placing the rat on the

device and the motorized rotor was initiated and progressed from a speed of 4.0 to 40.0 revolutions/min. The automated timer tracked each rats time spent on the rod and once the rat dismounted from the rod, a laser beam break stopped the timer. Rotarod performance has been used to assess rodent balance, grip strength, motor coordination as well as muscular endurance (22).

The morning of the necropsies, rodents were removed from the Biological Research Facility, transported to the Molecular and Applied Sciences Laboratory in the School of Kinesiology, and were then acclimated for approximately 3-4 hours with *ad libitum* access to water only. After acclimation, rodents were then euthanized under CO_2 gas induction in a 2 L chamber (VetEquip, Inc., Pleasanton, CA, USA). Thereafter, total body mass was recorded, and right-leg gastrocnemius muscle was dissected out and weighed using an analytical scale with a sensitivity of 0.0001 g (Mettler-Toledo; Columbus, OH, USA). Dissections on the right-leg gastrocnemius muscle were performed close to the origin and insertion sites, and any visible connective or fat tissue was removed. Tissues were then flash frozen in liquid nitrogen and stored at -80°C until analyses described below.

Tissue preparation for protein analyses

Mixed gastrocnemius tissues were removed from -80ºC storage, tissue was crushed on a liquid nitrogen-cooled mortar and pestle, and approximately 50 mg of tissue from each rodent were placed in 500 µL of ice-cold general cell lysis buffer [20 mM Tris–HCl (pH 7.5), 150 mM

NaCl, 1 mM Na-EDTA, 1 mM EGTA, 1% Triton, 20 mM sodium pyrophosphate, 25 mM sodium fluoride, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 and 1 µg/mL leupeptin] (Cell Signaling; Danvers, MA, USA). Tissues were homogenized via micro pestles and homogenates were centrifuged at 500 *g* for 5 min. After centrifugation insoluble proteins were removed and supernatants were stored at -80ºC prior to Western blotting.

Western blotting

Total protein determination on supernatants were performed following the dye-based bicinchoninic acid (BCA) colorimetric assay (Thermo Fischer Scientific, Waltham, MA, USA). Supernatants were subsequently prepared for SDS-PAGE using 4x Laemmli buffer at 2 µg/µL, and 25 µL were loaded onto 4-15% SDS-polyacrylamide pre-casted gels (Bio-Rad Laboratories; Hercules, CA, USA). 1x SDS-PAGE run buffer (Ameresco; Framingham, MA, USA) was used for electrophoresis at 180 V for 60 min. Thereafter, proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories) via constant amperage (200 mA) for 120 min. Membranes were then stained with Ponceau S and digital images were captured using a gel documentation system (UVP, Upland, CA, USA) to ensure equal loading of samples between lanes. Membranes were then blocked at room temperature with 5% nonfat milk powder in Tris-buffered saline with 0.1% Tween-20 (TBST) for one hour. All of the following primary antibodies were incubated overnight at 4ºC in a solution of TBST containing 5% bovine serum albumin (BSA; Ameresco): a) Mouse anti-ORF1 (1:1,000, Abcam, Cambridge, MA, USA;

catalog #: ab76726), b) Rabbit-anti DNMT3A (1:1,000, Cell Signaling; catalog #: 2160S), and c) Rabbit anti-TET1 (1:1,000, Abcam; catalog #: ab191698). The following day, membranes were incubated with HRP-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (1:2,000; Cell Signaling) in a solution of TBST containing 5% BSA at room temperature for one hour. Thereafter, membranes were developed using an enhanced chemiluminescent reagent (Luminata Forte HRP substrate; EMD Millipore, Billerica, MA, USA) where band densitometry was assessed by use of a digital gel documentation system and associated densitometry software (UVP). Densitometric values of white bands for each target were normalized to dark band Ponceau densitometry values. These values were then normalized to the 3-month group average to yield relative expression units (REUs).

RNA isolation, cDNA synthesis and real-time polymerase chain reaction (RT-PCR)

Approximately 20 mg of frozen mixed gastrocnemius muscle from each rodent were placed in 500 µL of Ribozol (Ameresco, Solon, OH, USA) per the manufacturer's recommendations. Thereafter, phase separation was achieved according to manufacturer's instructions for RNA isolation. Following RNA precipitation and pelleting, pellets were resuspended in 30 µl of RNase-free water, and RNA concentrations were determined in duplicate at an absorbance of 260 nm by using a NanoDrop Lite (Thermo Fisher Scientific). cDNA (2 µg) was synthesized using a commercial qScript™ cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA) per the manufacturer's recommendations. RT-PCR was performed

with gene-specific primers and SYBR-green-based methods in a RT-PCR thermal cycler (Bio-Rad, Hercules, CA, USA). Primers were designed with primer designer software (Primer3Plus, Cambridge, MA, USA), and melt curve analyses demonstrated that one PCR product was amplified per reaction. Additionally, PCR products were resolved on 1% agarose gels in order to verify that a product produced at the anticipated molecular weight was obtained. Three primer sets were designed to interrogate LINE-1 mRNA and DNA expression. The first primer set (L1.3) amplified a portion of the 5'-UTR region and was designed to probe for the most active LINE-1 element based upon the findings of Kirilyuk et al. (31). The second primer set (L1.Tot) also amplified a portion of the 5'-UTR region and was designed to encompass full-length LINE-1 elements that contained a 5' promoter, but did not have the ability to retrotranspose based on mutations in the protein coding regions (31). We also designed a primer set to amplify a portion of the ORF1 region. The forward and reverse primer sequences for all genes are listed in Table 1. Fold-change values from 3 month old rats were performed using the $2^{\Delta\Delta Cq}$ method where $2^{\Delta Cq}$ = 2^[housekeeping gene (HKG) Cq – gene of interest Cq] and $2^{\Delta\Delta Cq}$ (or fold-change) = [$2^{\Delta Cq}$ value/$2^{\Delta Cq}$ average of 3 month age group]. The geometric mean of fibrillarin (FBL), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), cyclophilin (CYCLOPH), and hypoxanthine phosphoribosyltransferase (HPRT) was used as the HKG to normalize mRNA expression results. While data are represented as fold-change from the 3-month group, they are presented as REUs. The overall coefficient of variation values for Cq duplicates of the assayed genes were as follows: HKG = 0.99%, L1.3 = 0.47%, L1.Tot = 0.53%, ORF1 = 0.43%.

Table 1. Rat primer sequences used for real-time PCR

Gene	Accession number	Amplicon length
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; HKG) FP (5' → 3'): TGATGCCCCCATGTTTGTGA RP (5' → 3'): GGCATGGACTGTGGTCATGA	NM_017008.4	163 bp
Fibrillarin (FBL; HKG) FP (5' → 3'): CTGCGGAATGGAGGACACTT RP (5' → 3'): GATGCAAACACAGCCTCTGC	NM_001025643.1	83 bp
Cyclophilin (CYCLO; HKG) FP (5' → 3'): GCATACAGGTCCTGGCATCT RP (5' → 3'): AGCCACTCAGTCTTGGCAGT	NM_017101.1	93 bp
Hypoxanthine phosphoribosyltransferse 1 (HPRT; HKG) FP (5' → 3'): AAGACAGCGGCAAGTTGAAT RP (5' → 3'): GGGCCTGTGTCTTGAGTTCA	NM_012583.2	192 bp
Ribosomal Protein S16 (RPS16; HKG) FP (5' → 3'): TCGCTGCGAATCCAAGAAGT RP (5' → 3'): CCCTGATCCTTGAGACTGGC	NM_001169146.1	87 bp
Histone Deacetylase 1 (HDAC1; HKG) FP (5' → 3'): GAGCGGTGATGAGGATGAGG RP (5' → 3'): CACAGGCAATGCGTTTGTCA	NM_001025409.1	74 bp
Beta-2-Microglobulin (β2M; HKG) FP (5' → 3'): GGAAACTGAGGGGAGTAGGG RP (5' → 3'): CCTGGGCTTTCATCCTAACA	NM_012512.2	143 bp
LINE-1 (L1.3) FP (5' → 3'): GACCATCTGGAACCCTGGTG RP (5' → 3'): GGGCCTGTGTCTTGAGTTCA	DQ100473.1	181 bp
LINE-1 (L1.Tot) FP (5' → 3'): GGAAGAGACCACCAACACTG RP (5' → 3'): GAAGGTTTAGCTCTCCCTCC	DQ100473.1 DQ100475.1 DQ100476.1 DQ100477.1 DQ100474.1 DQ100482.1	200 bp
Open Reading Frame 1 (ORF1) FP (5' → 3'): AAGAAACACCTCCCGTCACA RP (5' → 3'): CCTCCTTATGTTGGGCTTTACC	N/A	N/A

Legend: HKG, housekeeping gene; bp, base pairs.

DNA isolation and RT-PCR for gDNA LINE-1

Mixed gastrocnemius were removed from -80ºC, and approximately 25 mg of each tissue from each rodent were processed using the commercially available DNA isolation kit DNeasy Blood & Tissue Kit (Qiagen, Venlo, Netherlands) per the manufacturer's recommendations. Following DNA precipitation and pelleting, pellets were resuspended in 200 µl of elution buffer from the kit, and DNA concentrations were determined in duplicate at an absorbance of 260 nm using a NanoDrop Lite (Thermo Fisher Scientific). DNA (25 ng) was subjected to RT-PCR analysis using the aforementioned methods. The geometric mean of Ribosomal Protein S16 (RPS16), Histone Deacetylase 1 (HDAC1) and Beta-2-Microglobulin (B2M) was used as a HKG to normalize DNA expression results. Fold-change values from 3-month rats were performed using the aforementioned $2^{\Delta\Delta Cq}$ method, and data are presented as REUs. Overall coefficient of variation values for Cq duplicates of the assayed genes were as follows: L1.3 = 0.51% and L1.Tot = 0.62%, ORF1 = 0.31%, HKG = 0.24%.

LINE-1 promoter methylation analysis

LINE-1 promoter methylation analysis was performed on isolated gastrocnemius DNA (described above) from n=8 rats per age group using a commercially available methylated DNA immunoprecipitation (MeDIP) kit (product #: ab117133; Abcam). Prior to performing the assay, 1.5 µg of gastrocnemius DNA was digested using MseI (New England BioLabs, Ipswich, MA, USA). Following digestion reactions, total methylated DNA from a total of 1 µg input was

immunoprecipitated using an anti-5-methylcytosine antibody provided within the kit. RT-PCR was then performed on 25 ng of the methylated DNA using the L1.3 primers described above to decipher fold-change in methylated LINE-1 5' UTR. Residual input DNA from each sample (25 ng) was used as a control to normalize RT-PCR results. Fold-change in promoter methylation was calculated using the $2^{\Delta\Delta Cq}$ method where $2^{\Delta Cq}$ = 2^[input L1.3 DNA Cq – methylated L1.3 DNA Cq] and $2^{\Delta\Delta Cq}$ (or fold-change) = [$2^{\Delta Cq}$ value/$2^{\Delta Cq}$ average of 3 month age group]. Fold-change values from 3-month rats were performed using the aforementioned $2^{\Delta\Delta Cq}$ method, and data are presented as REUs. Overall coefficient of variation values for Cq triplicates of the assayed genes were as follows: input L1.3 = 0.44% and methylated L1.3 = 0.35%.

LINE-1 chromatin accessibility analysis

LINE-1 chromatin accessibility was assessed from n=8 rats per age group using a commercially available kit (Chromatin Accessibility Assay Kit, product #: ab185901; Abcam) per the manufacturer's recommendations. Briefly, methods involved obtaining DNA, digesting the DNA using a proprietary nuclear digestion buffer, and performing RT-PCR on digested versus undigested samples. The premise of the assay operates through a gene of interest localized to euchromatin regions being more susceptible to digestion and, thus, possessing a lower RT-PCR amplification signal relative to genes in heterochromatin regions. RT-PCR was performed on 25 ng of digested DNA using the L1.3 primers described above to decipher fold-change in genomic LINE-1 residing in euchromatin. Undigested DNA from each sample (25 ng) was used

as a control to normalize RT-PCR results. Fold-change in L1.3 euchromatin DNA was calculated using the $2^{\Delta\Delta Cq}$ method where $2^{\Delta Cq}$ = 2^[digested L1.3 DNA Cq – undigested L1.3 DNA Cq] and $2^{\Delta\Delta Cq}$ (or fold-change) = [$2^{\Delta Cq}$ value/$2^{\Delta Cq}$ average of 3 month age group]. Fold-change values from 3-month rats were performed using the aforementioned $2^{\Delta\Delta Cq}$ method, and data are presented as REUs. Overall coefficient of variation values for Cq triplicates of the assayed genes were as follows: undigested L1.3 DNA = 1.64% and digested L1.3 DNA = 0.36%.

DNA Methyltransferase Activity Assay

Prior to assaying DNA Methyltransferase (DNMT) activity, nuclear protein extraction was performed on frozen gastrocnemius muscle (~25 mg) using a commercially-available kit (Nuclear Extraction Kit; Abcam) per the manufacturer's recommendations. Global DNMT activity of nuclear isolates (10 µL) was assessed using a commercially available kit (DNMT Activity Assay Kit; Abcam) per the manufacturer's recommendations. DNMT activity was expressed as relative expression units (REU), which were then normalized to input muscle weights. Overall coefficient of variation values for duplicate readings were 33.2%.

Histone Deacetylase Activity Assay

Global Histone Deacetylase (HDAC) activity on nuclear isolates (10 µL) was assessed using a commercially available fluorometric kit (Histone Deacetylase Activity Assay Kit;

Abcam) per the manufacturer's recommendations. HDAC activity was expressed as relative fluorescence units (RFU), which were then normalized to input muscle weights. Overall coefficient of variation values for duplicates readings were 5.3%.

RNA-sequencing

RNA-sequencing was performed only on a subset of 3-month old (n=8) and 24-month old rats (n=8) with the intent of identifying how aging affected the mRNA expression of genes related to LINE-1 regulation. Gastrocnemius tissues were processed for RNA isolation using methods described above. Thereafter, RNA was shipped to LC Sciences (Houston, TX, USA) for RNA-sequencing. The steps described below were completed for this analysis.

Library Preparation and Sequencing. Total RNA quality and quantity were assessed using Bioanalyzer 2100 and RNA 6000 Nano LabChip Kits (Agilent, CA, USA), and all samples yielded RIN numbers >7.0. Total RNA was subjected to poly(A) mRNA enrichment with poly-T oligo-attached magnetic beads (Invitrogen). Following purification, the poly(A) mRNA fractions were fragmented into small pieces using divalent cations under elevated temperature. The cleaved RNA fragments were subsequently reverse-transcribed to create final cDNA libraries in accordance with strand-specific library preparation by dUTP method. The average insert size for the paired-end libraries was 300±50 bp. Paired-end 2×150 base pair sequencing was performed on an Illumina Hiseq 4000 platform following the manufacturer's recommended protocol.

Transcripts Assembly. Cutadapt (37) and perl scripts were used to remove the reads that contained adaptor contamination, low quality bases, and undetermined bases. Sequence quality was verified using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). HISAT2 (30) was used to map reads to the genome of Rattus norvegicus (Version: v88). The mapped reads of each sample were assembled using StringTie (46). Thereafter, all transcriptomes from the 16 samples were merged to reconstruct a comprehensive transcriptome using perl scripts and gffcompare (https://github.com/gpertea/gffcompare/). After the final transcriptome was generated, StringTie (46) and Ballgown (17) were used to estimate the expression levels of all transcripts.

Differential expression analysis of mRNAs. StringTie (46) was used to measure expression levels for mRNAs by calculating FPKM values (FPKM=[total_exon_fragments/mapped_reads(millions)×exon_length(kB)]). Differentially expressed transcripts related to LINE-1 regulation between the young and old groups were determined by comparing FPKM values. Given that RNA-sequencing was used as a screening tool herein, differentially expressed transcripts related to LINE-1 regulation were considered meaningful if the un-adjusted p-value of a given mRNA between groups was $p<0.05$.

Age-related skeletal muscle phenotyping

Beyond examining skeletal muscle LINE-1 pathway markers in the three cohorts, we also sought to analyze muscle for age-related phenotypes. These methods are described below.

Total myofibrillar protein assessment. Myofibrillar protein isolations were performed based on the methods of Goldberg's laboratory (9) Briefly, mixed gastrocnemius foils were removed from -80ºC storage, tissue was crushed on a liquid nitrogen-cooled mortar and pestle, muscle (~20 mg) was weighed using an analytical scale sensitive to 0.0001 g (Mettler-Toledo, Columbus, OH, USA), and immediately placed in 1.7 mL polypropylene tubes containing 190 µL of ice cold homogenizing buffer (20 mM Tris–HCl, pH 7.2, 5 mM EGTA, 100 mM KCl, 1% Triton-X100) and 6.4 M spermidine (Alfa Aesar, Haverhill, MA USA). Samples were homogenized on ice using tight-fitting pestles, and centrifuged at 3,000 g for 30 min at 4 °C. The resultant pellet was re-suspended in homogenizing buffer, and samples were centrifuged at 3000 g for 10 min at 4 °C. Resultant supernatants from this step were discarded, resultant pellets were re-suspended in 190 µL of ice cold wash buffer (20 mM Tris–HCl, pH 7.2, 100 mM KCl, 1 mM DTT), and samples were centrifuged at 3000g for 10 min at 4 °C; this specific process was performed twice. Final myofibril pellets were re-suspended in 200 µL of ice-cold storage buffer (20 mM Tris–HCl, pH 7.2, 100 mM KCl, 20% glycerol, 1 mM DTT) and frozen at −80 °C until protein concentration determination. Myofibrillar protein concentrations were determined using a BCA assay, which were then normalized to input muscle weights. Overall coefficient of variation values for triplicate readings were 2.04%.

Determination of myosin heavy chain and actin content. SDS-PAGE preps from resuspended myofibrils were performed using: (a) 10 µL resuspended myofibrils, (b) 65 µL distilled water (diH2O), and (c) 25 µL 4x Laemmli buffer. Samples (5 µL) were then loaded on pre-casted gradient (4–15%) SDS-polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA,

USA) and subjected to electrophoresis (200 V for 45 min) using pre-made 1x SDS-PAGE running buffer (Ameresco). Following electrophoresis gels were rinsed in diH2O for 15 min, and immersed in Coomassie stain (LabSafe GEL Blue; G-Biosciences, St. Louis, MO, USA) for 45 min. Thereafter, gels were de-stained in diH2O for 60 min, bright field imaged using a gel documentation system (UVP), and band densities were determined using associated software. Myosin and actin concentrations were expressed as arbitrary density units (ADU) per mg muscle, and this assay was performed in singlets due to its robust sensitivity (48).

Citrate synthase activity assay. Mixed gastrocnemius foils were removed from -80ºC storage, tissue was crushed on a liquid nitrogen-cooled mortar and pestle, and approximately 30 mg of tissue from each rodent were placed in 500 µL of ice-cold cell lysis buffer (recipe described above) (Cell Signaling). Tissues were homogenized via micro pestles and homogenates were centrifuged at 500 *g* for 5 min. After centrifugation insoluble proteins were removed and supernatants were stored at -80ºC prior to citrate synthase activity assessments. Citrate synthase activity as previously described by our laboratory (25). The assay principle is based upon the reduction of 5,50-dithiobis (2- nitrobenzoic acid) (DTNB) at 412 nm (extinction coefficient 13.6 mmol/L/cm) coupled to the reduction of acetyl-CoA by the citrate synthase reaction in the presence of oxaloacetate. Briefly, 2 µg of skeletal muscle protein was added to a mixture composed of 0.125 mol/L Tris–HCl (pH 8.0), 0.03 mmol/L acetyl-CoA, and 0.1 mmol/L DTNB. The reaction was initiated by the addition of 5 µL of 50 mmol/L oxaloacetate and the absorbance change was recorded for 1 min. Overall coefficient of variation values for duplicate readings were 4.2%.

Muscle Triglyceride Assay. Gastrocnemius skeletal muscle tissue (~20 mg) was weighed using an analytical scale sensitive to 0.0001 g (Mettler-Toledo, Columbus, OH, USA), and immediately placed in 1.7 mL polypropylene tubes. Thereafter, triglyceride analysis was performed using a commercially available triglyceride colorimetric kit (Cayman Chemical, Ann Arbor, MI, USA), according to manufacturer's instructions. Gastrocnemius skeletal muscle triglyceride content was expressed μmol per gram wet muscle. Overall coefficient of variation values for duplicate readings were 1.2%.

In vitro experiments for 5-Azacytidine treatments

Passage 2, L6 myoblasts (Kerafast Inc. Boston, MA, USA), were grown in growth medium (DMEM, 10% FBS, 1% penicillin/streptomycin, and 0.1% gentamycin; Corning Inc., Corning, NY, USA) on 12-well plates at a seeding density of 1 x 10^5 under standard culture conditions (37°C in a 5% CO_2 atmosphere). Once myoblast growth reached 80–90% confluency 5 days after seeding, differentiation was induced by removing growth medium and replacing it with differentiation medium [DM; DMEM, 2% (vol/vol) horse serum, 1% penicillin/streptomycin, and 0.1% gentamycin; Corning]. DM was then replaced every 24 h for 6 d to allow for myotube growth.

5-Azacytidine (5-AC) (TCI America, Portland, OR, USA) was administered to myotubes during differentiation on day 5 at final concentration of 10 mM in DM (solubilized in <0.1% dimethyl sulfoxide (DMSO); Corning) (n=4 wells per treatment) for 3 h and 24 h, and doses were based on Ikeda et al. (26) who used similar doses *in vitro*. Control (CTL) wells received

DMSO administration during day 5 of differentiation in DM (n=4 wells per treatment), at a similar dose used to solubilize 5-AC. Following treatments, RNA was isolated using the aforementioned methods. Total RNA concentrations were analyzed using a Nanodrop Lite spectrophotometer (Thermo Fisher Scientific), and 1 µg of cDNA were synthesized using a commercial qScript cDNA SuperMix (Quanta Biosciences) per the manufacturer's recommendations. Real-time PCR using SYBR green chemistry was performed using the three LINE-1 primers (Table 1). LINE-1 mRNA at 3 h, 5-AC treatments were normalized to GAPDH, and LINE-1 mRNA at 24 h were normalized to B2M. Fold-change values from CTL treatments were performed using the $2^{\Delta\Delta Cq}$ as previously described. Overall coefficient of variation values for 3-h treatment Cq duplicates of the assayed genes were as follows: GAPDH = 0.31%, L1.3 = 0.18%, L1.Tot = 0.20%, and ORF1 = 0.12%. Overall coefficient of variation values for 24-h treatment Cq duplicates of the assayed genes were as follows: B2M = 0.11%, L1.3 = 0.30%, L1.Tot = 0.17%, and ORF1 = 0.21%.

Statistical Analyses

Statistics were performed using the open-source software R (56), R Studio (55) and SPSS v 23.0 (IBM, Armonk, NY, USA). Prior to statistical analysis assumption testing was performed on all dependent variables. For non-normally distributed data, values were log-transformed and re-analyzed for normality. Dependent variables for the rodent experiments were analyzed using one-way ANOVAs with Fisher LSD *post hoc* tests or Welch's t-tests (when assumptions were

violated) to assess differences in dependent variables between age groups. Dependent variables from *in vitro* treatments were compared using independent samples t-tests. Magnitude of effects are also expressed in the results using partial eta squared (η_p^2) effect size, and effect sizes of 0.01, 0.06 and > 0.14 were considered small, moderate and large. Statistical significance for all null hypothesis testing was set at $p<0.05$. All data is herein is presented as mean ± standard deviation (SD).

RESULTS

Skeletal muscle mass, myofibrillar protein, citrate synthase, collagen and triglyceride levels

Table 2 contains data related to skeletal muscle aging phenotype. Notably, body mass, gastrocnemius mass and rotarod performance data from these rats have been previously reported (42, 44). There were no significant between-group differences for myofibrillar protein concentrations, myosin protein concentrations, actin protein concentrations, citrate synthase activity or muscle triglyceride concentrations.

Table 2. Effects of age group on skeletal muscle characteristics

	Age Group (months old)			ANOVA		
	3	12	24	F	p	η_p^2
Body mass (g)*	285 ± 29[b]	429 ± 20[a]	439 ± 42[a]	73.73	<0.001	0.86
Gastroc. mass (g) †	1.21 ± 0.11[c]	1.57 ± 0.08[a]	1.45 ± 0.08[b]	35.51	<0.001	0.75
Relative Gastroc. mass (g) †	4.31 ± 0.28[a]	3.67 ± 0.18[b]	3.32 ± 0.19[c]	52.04	<0.001	0.81
Rotarod Performance (sec) *	186.44 ± 44.31[a]	126.00 ±38.60 [b]	38.89 ± 12.63 [c]	41.12	<0.001	0.77
Myofibrillar Protein (µg/mg wet muscle)	78.34 ± 7.08	76.63 ± 7.21	78.12 ± 5.14	0.20	=0.818	0.02
Myosin Protein (ADU/mg wet muscle)	1.71 ± 0.28	1.67 ± 0.30	1.60 ± 0.34	0.35	=0.711	0.03
Actin Protein (ADU/mg wet muscle)	1.07 ± 0.16	1.17 ± 0.14	1.18 ± 0.15	1.33	=0.284	0.10
CS Activity (mM/min/mg protein)	0.23 ± 0.03	0.24 ± 0.05	0.21 ± 0.03	1.04	=0.369	0.08
Muscle Triglycerides (µmol/g wet muscle)	1.62 ± 0.48	1.57 ± 0.32	1.61 ± 0.44	0.05	=0.955	0.01

Legend: Data are expressed as means ± standard deviation. The F and p values are reported from one-way ANOVAs including age as the between-subjects factor. Superscript letters indicate significant between age group differences ($p<0.05$). *, indicates that these data were published by Mobley et al. (42) and † indicates these data were published by Mumford et al. (44) and are re-presented for the convenience of the reader. Abbreviations: ADU, arbitrary density units; CS, citrate synthase.

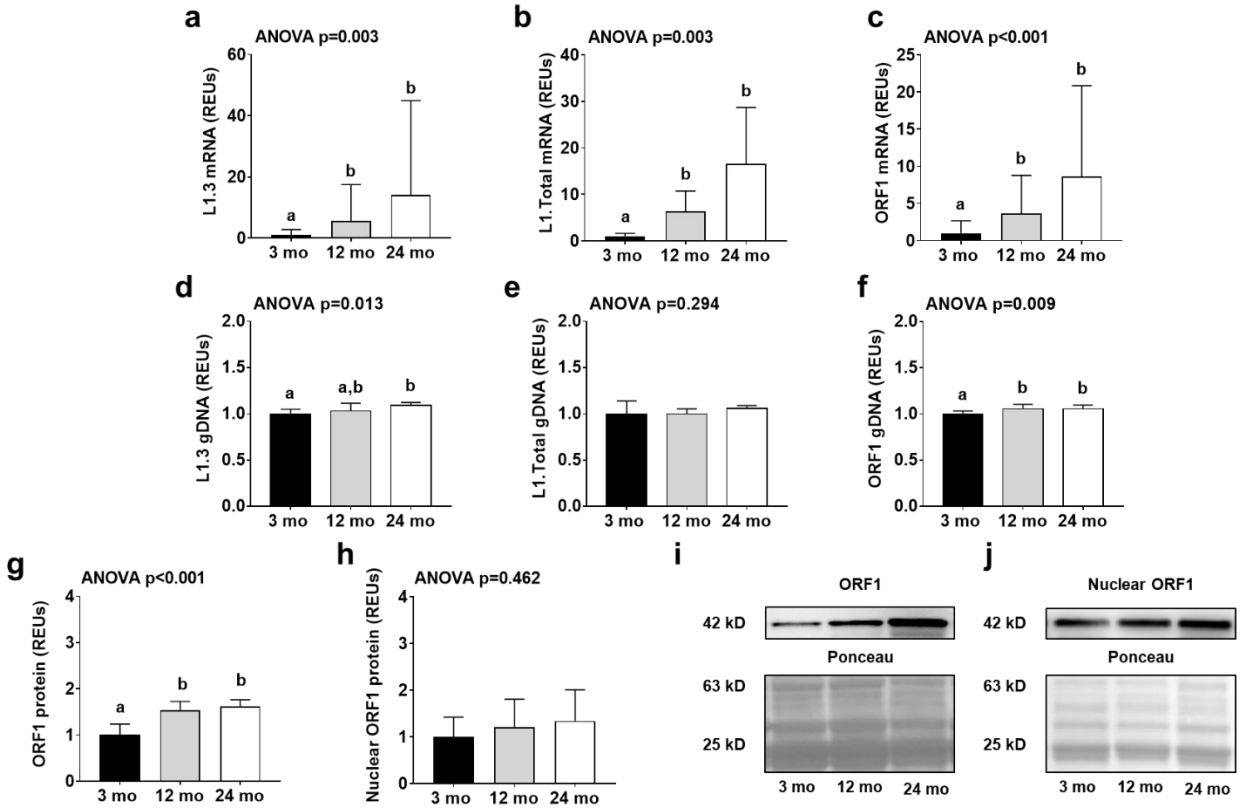
Gastrocnemius LINE-1 activity markers

L1.3, L1.Tot and ORF1 mRNA expression were not normally distributed and these variables were log-transformed prior to statistical analysis. L1.3 mRNA expression was significantly different between age groups ($p=0.003$, $\eta_p^2 = 0.38$; Fig 1a), and post hoc analysis revealed that 12- and 24-month rats had higher L1.3 mRNA expression compared to 3-month rats ($p<0.05$). L1.Tot mRNA expression was significantly different between age groups ($p=0.001$, $\eta_p^2 = 0.42$; Fig 1b), and post hoc analysis revealed that 12- and 24-month rats had

higher L1.3 RNA expression compared to 3-month rats ($p<0.05$). ORF1 mRNA expression was significantly different between age groups ($p<0.001$, $\eta_p^2 = 0.47$; Fig 1c), and post hoc analysis revealed that 12- and 24-month rats had higher ORF1 RNA expression compared to 3-month rats ($p<0.05$).

L1.3 gDNA expression was significantly different between age groups ($p=0.013$, $\eta_p^2 =0.31$; Fig. 1e), and post hoc analysis revealed that 24-month rodents had higher expression levels compared to 3-month rats ($p<0.05$). ORF1 gDNA expression was significantly different between age groups ($p=0.009$, $\eta_p^2 =0.33$; Fig. 1g), and post hoc analysis revealed that both 12- and 24-month rats had higher expression compared to 3-month rats ($p<0.05$). Total ORF1 protein expression was significantly different between age groups ($p<0.001$, $\eta_p^2 =0.69$; Fig. 1h), and post hoc analysis revealed that 12- and 24-month rats had higher expression levels compared to 3-month rats ($p<0.05$). There were no between group differences for L1.Tot gDNA (Fig. 1f) or nuclear ORF1 protein expression (Fig. 1i). Figure 1j/k illustrate representative Western blots from each age group.

Figure 1. Effect of aging on skeletal muscle LINE-1 mRNA, DNA and protein expression

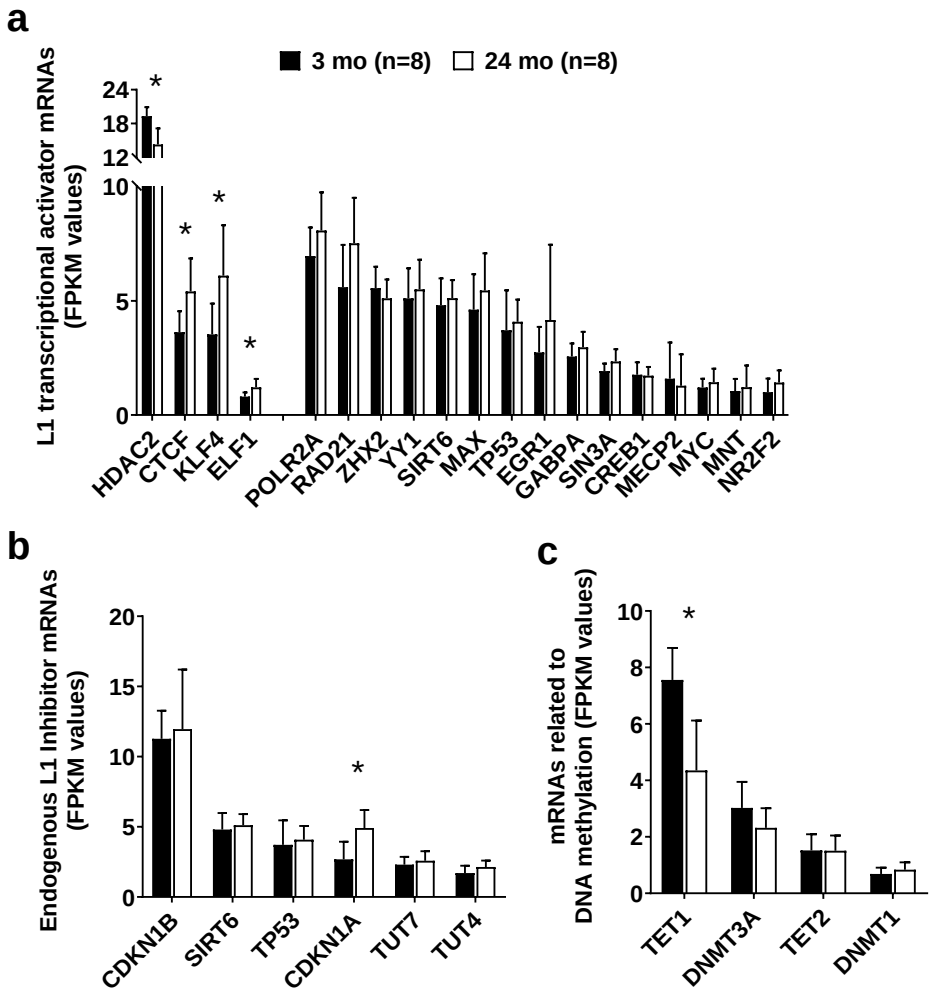


Legend: Age group differences in L1.3 mRNA (panel a), L1.Tot mRNA (panel b), ORF1 mRNA (panel c), L1.3 genomic DNA (gDNA) (panel e), L1.Tot gDNA (panel f), ORF1 gDNA (panel g), total ORF1 protein expression (panel h), and nuclear ORF1 protein expression (panel i). Panel d contains L1.3 primer validation through agarose gel electrophoresis, and panels j and k contain total and nuclear ORF1 representative Western blot images (panel j & k). Data are presented as mean ± SD and different superscript letters indicate significant between-group age differences ($p<0.05$).

RNA-sequencing results between 3-month and 24-month rats for generating downstream assays

Upon receiving RNA-sequencing results, we aimed to examine the mRNA expression patterns of genes related to LINE-1 regulation from pervious literature (52). In the first cluster of genes, LINE-1 transcriptional activators (Fig 2a), HDAC2 was significantly lower in 24- compared to 3-month rats ($p<0.05$). Additionally, CTCF, KLF4 and ELF1 were significantly higher in 24- compared to 3-month rodents ($p<0.05$). In the second cluster of genes, endogenous LINE-1 inhibitors (Fig 2b), CDKN1A was significantly higher in 24- compared to 3-month rodents ($p<0.05$). In the third cluster of genes, or genes related to DNA methylation (Fig 2c), TET1 was significantly lower in 24- compared to 3-month rodents ($p<0.05$). Based upon these findings, we surmised that the following molecular phenomena may be driving an increase in skeletal muscle LINE-1 mRNA with aging: a) an increase in nuclear CTCF protein given that there are numerous consensus binding sequences for this transcription factor in the 5' UTR region (52), b) a decrease in nuclear HDAC2 and/or a decrease in global nuclear HDAC activity due to significantly lower HDAC2 mRNA expression in older versus younger rats, and c) an alteration in global DNA methylation patterns due to significantly lower expression TET1 mRNA and numerically lower expression of DNMT3A mRNA ($p=0.105$) in older versus younger rats.

Figure 2. Muscle RNA sequencing results for 3 month and 24 month rats

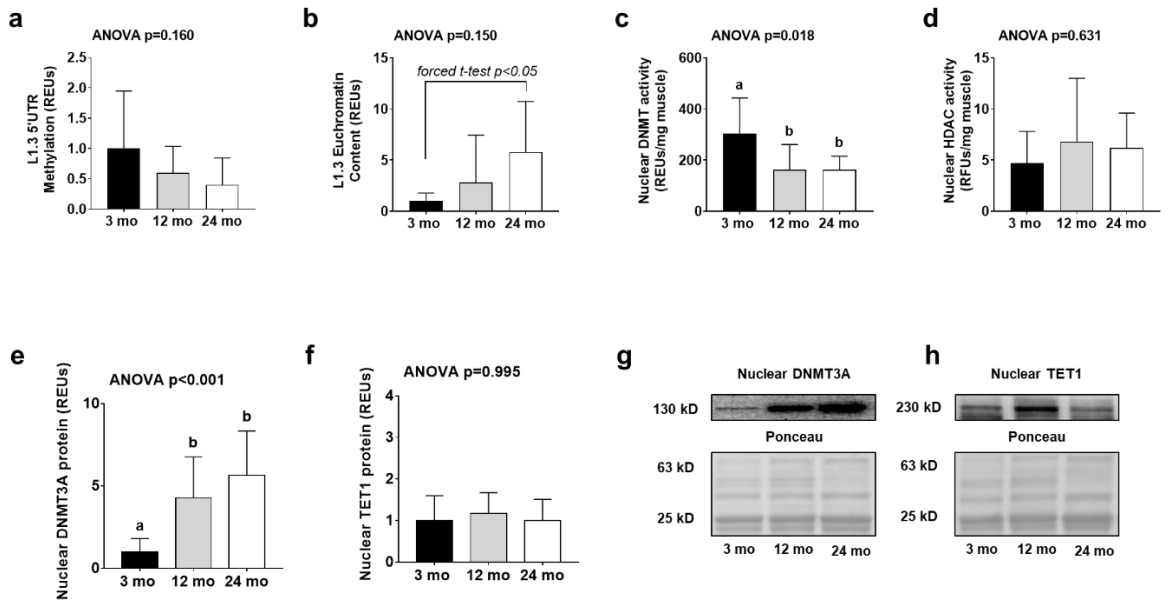


Legend: Age group differences in LINE-1 transcriptional activators (panel a), LINE-1 endogenous inhibitors (panel b) and genes related to DNA methylation (panel c). Data are presented as mean ± SD and different, and asterisks indicate significant between-group age differences ($p<0.05$).

Effect of aging on skeletal muscle L1.3 DNA methylation, L1.3 chromatin state, global DNMT activity, and global HDAC activity

L1.3 DNA methylation and expression values of L1.3 DNA in the euchromatin state were not normally distributed and these variables were log-transformed prior to statistical analysis. There were no between-group differences for L1.3 DNA methylation ($p=0.16$, $\eta_p^2 =0.16$; Fig. 3a) or L1.3 DNA in the euchromatin state ($p=0.15$, $\eta_p^2 =0.17$; Fig. 3b). However, nuclear DNMT activity was statistically significant different between age groups ($p=0.018$, $\eta_p^2 =0.32$; Fig. 3c), and post hoc analysis revealed that both 12- and 24-months rats had lower nuclear DNMT activity compared to 3-month rats ($p<0.05$). Nuclear DNMT3a protein expression was statistically significant ($p<0.001$, $\eta_p^2 =0.48$; Fig. 3e), and post hoc analysis revealed that both 12- and 24-months rats had paradoxically higher nuclear DNMT3a protein expression compared to 3-month rats ($p<0.05$). Notably, we attempted to assay nuclear CTCF protein per the RNA-sequencing results above and found that this protein was enriched in the total cell lysate, but not the nuclear fraction (*data not shown*).

Figure 3. Effect of aging on skeletal muscle LINE-1 promoter methylation and chromatin state

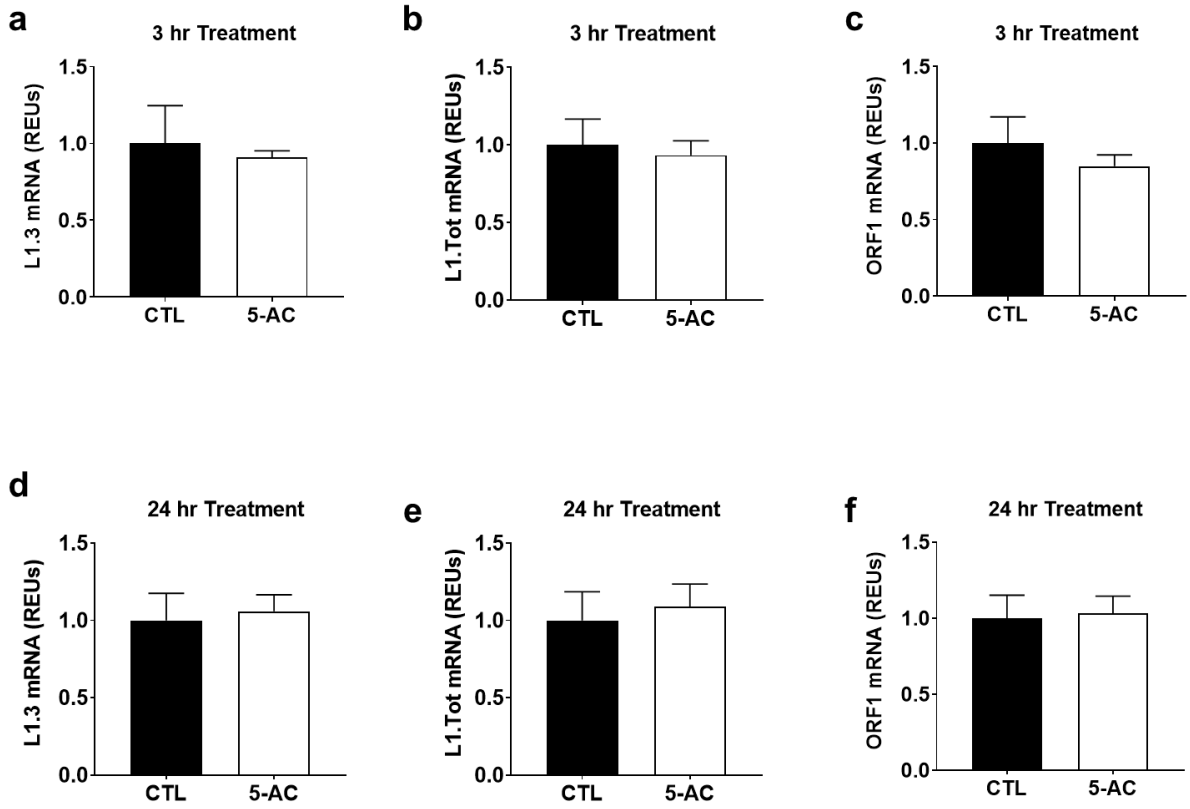


Legend: Age group differences in L1.3 promotor methylation (panel a), L1.3 euchromatin content (panel b), nuclear DNMT activity (panel c), nuclear HDAC activity (panel d), nuclear DNMT3a protein expression (panel e), and nuclear TET1 protein expression (panel f). Panels g and h contain DNMT3a and TET1 representative Western blot images. Data are presented as mean ± SD and different superscript letters indicate significant between-group age differences ($p<0.05$).

Effects of 5-AC treatment on LINE-1 mRNA expression in vitro

Given that nuclear DNMT activity decreased with aging, L1.3 DNA methylation trended downward with aging, and L1.3 DNA in the euchromatin state trended upward with aging, we developed the hypothesis that a decrease in skeletal muscle nuclear DNMT activity with aging may lead to decrements in L1.3 methylation which, in turn, increases L1.3 DNA in the euchromatin state, L1.3 mRNA expression, and perhaps L1.3 DNA integration. Thus, we reasoned that a well-known DNMT inhibitor (5-AC) may recapitulate aging effects in regard to increasing LINE-1 mRNA expression *in vitro*. Contrary to this hypothesis, there were no between-treatment differences for L1.3, L1.Tot or ORF1 mRNA at 3 h or 24 h when comparing 5-AC versus CTL treatments.

Figure 4. Effects of 5-AC on LINE-1 mRNA expression L6 myotubes



Legend: 5-Azacytidine (5-AC) and DMSO-only control (CTL) treatments were 3 h and 24 h. Data for 3-h treatments are presented in panels a-c for L1.3 mRNA, L1Tot mRNA, and ORF1 mRNA. Data for 24-h 5-AC treatments are presented in panels d-f for on L1.3 mRNA, L1Tot mRNA, and ORF1 mRNA. Data are presented as mean ± SD.

DISCUSSION

This is the first investigation examining skeletal muscle LINE-1 mRNA and DNA expression across the age spectrum in rats. Our observations of higher LINE-1 mRNA and genomic DNA expression in older versus younger rats is in agreement with De Cecco et al. (12) who previously reported skeletal muscle LINE-1 mRNA genomic DNA copy number was higher in 36-month old versus 5-month old mice. Similar findings between studies are quite interesting due to repetitive LINE-1 elements accounting for differing amounts of rat DNA (23%) (19) compared to mice DNA (18%) (57). Furthermore, rats possess less functionally-active copies of LINE-1 in their genome compared to mice as mentioned prior (20, 45). Notwithstanding, our data suggest that aging increases skeletal muscle LINE-1 mRNA and DNA expression in mice and, in lieu of the report from De Cecco et al., this phenomena appears to be conserved across rodent species.

While our findings as well as those from De Cecco et al. are insightful, we attempted to elucidate the primary mechanism through which aging increases skeletal muscle LINE-1 mRNA expression. In this regard, the RNA-sequencing data between younger and older rats was as a screening tool to determine if previously identified transcriptional activators and inhibitors of LINE-1 (52) were differentially expressed between the younger and older cohorts. Additionally, it has been well established *in vitro* and *in vivo* that global methylation decreases with age (14, 18, 51, 54, 59). Thus, we used the RNA-sequencing data to also screen how genes related to LINE-1 regulation and DNA methylation were differentially expressed between age cohorts.

mRNAs for HDAC2, which is a histone deacetylase, and TET1, which plays a role in DNA methylation, were both lower in the older cohort. LINE-1 methylation has been examined in DNA obtained from blood samples across the age span, and some studies report no change with age (5, 28, 61) whereas others report decreases (8, 16, 53). In regard to skeletal muscle, however, there is currently no information to our knowledge which has delineated LINE-1 methylation patterns with aging. Therefore, given this lack of information along with TET1 mRNA being lower in older versus younger rats, we were interested in examining the effects of aging on skeletal muscle LINE-1 methylation as well as DNMT activity and the protein expression of DNMT3A and TET1. We observed that nuclear DNMT activity was lower in the 12- and 24-month versus 3-month rats. Furthermore, we observed a step-wise decrease in L1.3 DNA methylation and a step-wise increase in L1.3 DNA in the euchromatin state across the age spectrum, albeit neither analysis yielded statistical significance. Collectively, we interpreted these data to suggest that age-related decrements in DNMT activity alters global methylation and chromatin structures to "expose" more active LINE-1-containing DNA regions. These phenomena, in turn, may lead to the up-regulation in LINE-1 mRNA expression and eventual increase in *de novo* LINE-1 integration. Notably, this working model agrees with De Cecco et al. (12) who posited that there is a "loosening" of specific LINE-1 genomic regions in postmitotic skeletal muscle fibers with aging, and this phenomenon leads to increases in LINE-1 mRNA expression and LINE-1 genomic insertions.

To test the aforementioned hypothesis we examined if reducing skeletal muscle DNMT activity via 5-AC *in vitro* increased LINE-1 mRNA expression. While our RNA-sequencing

screen and subsequent molecular analysis drove this experiment, it is notable that others have found that 5-AC affects LINE-1 methylation status in other cell lines (11) which strengthened the rationale. Contrary to our hypothesis, no differences in LINE-1 mRNA expression existed between 5-AC and CTL conditions following shorter-term (3 h) or longer-term (24 h) treatments. These findings suggest either: a) the age-related alterations in skeletal muscle DNMT activity noted herein are independent of LINE-1 pathway activity, or b) the relatively low passage number of the L6 myotubes examined may have exerted a protective effect from 5-AC-induced alterations in LINE-1 methylation and subsequent mRNA increases. The latter speculation is indeed plausible given that others have found that long-term sub-culturing in mesenchymal stromal cells elicits cellular senescence through substantial alterations in DNA methylation and chromatin arrangement (6). Thus, future experiments should attempt to age myoblasts through a series of passaging, and then observe if 5-AC is capable of increasing LINE-1 mRNA expression.

Based upon our current findings we, as well as DeCecco et al. (12), hypothesize that age-related increases in LINE-1 activity is likely a contributor to the progressive dysfunction of aging through nuclear genome destabilization. In order to address this potential relationship we sought to identify aged-related skeletal muscle phenotype differences between cohorts. Beyond skeletal muscle mass differences, however, the age-related skeletal muscle phenotype measures we assayed where not altered between cohorts (i.e., decrements in gastrocnemius contractile protein, decreases in mitochondrial volume, or higher levels of fat infiltration). These data indicate the 24-month rats are likely not old enough to present overt signs of muscle aging. Indeed, this is a

limitation, although our current findings do not exclude the potential for other measures to be associated with age-related alterations in LINE-1 markers (e.g., muscle fiber cross-sectional area, satellite cell number, myonuclear number, and skeletal muscle fibrosis). However, we did not preserve gastrocnemius sample for histological analysis. Therefore, future research in this area should more comprehensively phenotype the skeletal muscle from younger versus older rodents to determine if LINE-1 pathway markers are associated with age-related phenotypes. Further, the development of a mouse model which permits the muscle-specific overexpression of LINE-1 during adulthood would provide insight into whether increases in skeletal muscle LINE-1 gene expression drives an aging phenotype.

Conclusions

This is the first observation of higher skeletal muscle LINE-1 mRNA and DNA expression in older versus younger rats. We posit that this age-related increase is potentially due to alterations in LINE-1 methylation and chromatin state across the age span. Increased LINE-1 activity with aging is generally viewed as negative. Therefore, more research is needed to investigate how LINE-1 is affecting skeletal muscle, and if this pathway appreciably contributes to the skeletal muscle aging process.

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DISCLOSURES

The authors have no conflicts of interest to disclose regarding the publication of these data.

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