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Aging and microRNA expression in human skeletal muscle: a microarray and bioinformatics analysis

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Departments of 1 Physical Therapy, 2 Internal Medicine, 3 Biochemistry and Molecular Biology, and 4 Preventive Medicine and Community Health and 5 Sealy Center on Aging, University of Texas Medical Branch, Galveston, Texas; and 6 Department of Physiology, University of Kentucky, Lexington, Kentucky

Submitted 10 August 2010; accepted in final form 28 September 2010

Drummond MJ, McCarthy JJ, Sinha M, Spratt HM, Volpi E, Esser KA, Rasmussen BB. Aging and microRNA expression in human skeletal muscle: a microarray and bioinformatics analysis. Physiol Genomics 43: 595–603, 2011. First published September 28, 2010; doi:10.1152/physiolgenomics.00148.2010.—A common characteristic of aging is loss of skeletal muscle (sarcopenia), which can lead to falls and fractures. MicroRNAs (miRNAs) are novel posttranscriptional modulators of gene expression with potential roles as regulators of skeletal muscle mass and function. The purpose of this study was to profile miRNA expression patterns in aging human skeletal muscle with a miRNA array followed by in-depth functional and network analysis. Muscle biopsy samples from 36 men [young: 31 ± 2 (n = 19); older: 73 ± 3 (n = 17)] were analyzed for expression of miRNAs with a miRNA array, validated with TaqMan quantitative real-time PCR assays, and identified (and later validated) for potential gene targets with the bioinformatics knowledge base software Ingenuity Pathways Analysis. Eighteen miRNAs were differentially expressed in older humans (P < 0.05 and >500 expression level). Let-7 family members Let-7b and Let-7e were significantly elevated and further validated in older subjects (P < 0.05). Functional and network analysis from Ingenuity determined that gene targets of the Let-7s were associated with molecular networks involved in cell cycle control such as cellular proliferation and differentiation. We confirmed with real-time PCR that miRNA expression of cell cycle regulators CDK6, CDC25A, and CDC34 were downregulated in older compared with young subjects (P < 0.05). In addition, PAX7 mRNA expression was lower in older subjects (P < 0.05). These data suggest that aging is characterized by a higher expression of Let-7 family members that may downregulate genes related to cellular proliferation. We propose that higher Let-7 expression may be an indicator of impaired cell cycle function possibly contributing to reduced muscle cell renewal and regeneration in older human muscle.

Sarcopenia; Ingenuity; Let-7; cell cycle

Sarcopenia is characterized by a gradual, but progressive, loss in skeletal muscle mass and strength that typically does not become evident until the seventh or eighth decade of life. Since skeletal muscle contains the largest bulk of proteins in comparison to the existing cells in the human body (50–75%), significant reductions in skeletal muscle mass and strength can severely compromise muscle function, lead to muscle injury and immobilization, and eventually limit independence (13). As the aging population increases, age-related sarcopenia will become even more prominent. Therefore, it is imperative to further characterize the molecular mechanisms associated with age-related muscle loss.

An efficient method to identify genomewide transcript expression patterns and relate them to general gene function in older human skeletal muscle is performance of a gene microarray. This technique has helped identify several genes that mediate aging in human skeletal muscle (16, 45, 55, 58). However, no studies have provided a comprehensive evaluation of the role of microRNAs (miRNAs) in young and older adult skeletal muscle. miRNAs are ~20-nt noncoding RNA fragments that regulate mRNA transcripts through translational repression and/or degradation (42). It has been estimated that miRNAs regulate nearly two-thirds of the entire mammalian genome (14). Thus it is likely that miRNAs posttranscriptionally regulate a majority of skeletal muscle gene profiles expressed in young and older humans. Undoubtedly, sarcopenia is a result of changes in multiple cellular systems rather than a single dysfunctional gene, protein, or system. Therefore, miRNA research is appealing because miRNAs can regulate a variety of genes, inducing changes in multiple systems simultaneously.

Recent data have suggested that miRNAs may play a part in regulating the aging process (1, 3, 17, 19, 23, 24, 34, 39, 40, 57, 60). For example, Boehm and Slack (3) reported in Caenorhabditis elegans that miRNA lin-4 expression decreased with advancing age but when overexpressed tissue aging was reduced and life span increased. Age-related miRNA expression is also evident in higher eukaryotic cells. Nishino and colleagues (38) identified in aged mouse neural stem cells an increased Let-7 expression that was related to increasing age and reduced self-renewal. Other reports indicate altered miRNA expression patterns in aging human cells (1, 19, 30, 33, 39) that potentially regulate cellular pathways involved in inflammation (1) and stress (33). Together, these data suggest that miRNAs are dysregulated with aging over a span of cell types and are likely involved in many cellular processes characteristic of aging. However, a thorough miRNA examination is warranted in aging human skeletal muscle, as it would provide a new perspective underlying the mechanisms of sarcopenia.
Therefore, we chose to perform a miRNA microarray in a large pool of young and older human vastus lateralis skeletal muscle biopsy samples. Because miRNA gene regulation is very complex such that one miRNA can target hundreds of mRNAs and likewise one mRNA can be targeted by multiple miRNAs, we chose Ingenuity Pathways Analysis (IPA) to reveal molecular functions and networks and gene targets that are associated with muscle aging. We hypothesized that aging dysregulates miRNA expression in human skeletal muscle and that these miRNAs would be related to genes associated with cell cycle control, inflammation, and stress (16, 38, 58).

METHODS

Subjects. We analyzed skeletal muscle biopsy samples from 19 young and 17 older male subjects that have participated in our previous and present research experiments. Subject characteristics are found in Table 1. The subjects were not engaged in any regular exercise training at the time of the enrollment; however, they were physically independent and overall healthy. Screening of subjects was performed with clinical history, physical exam, and laboratory tests including complete blood count with differential, liver and kidney function tests, coagulation profile, fasting blood glucose and oral glucose tolerance test, hepatitis B and C screening, human immunodeficiency virus (HIV) test, TSH, urinalysis, and drug screening. All subjects gave informed written consent before participating in the study, which was approved by the Institutional Review Board of the University of Texas Medical Branch (which is in compliance with the Declaration of Helsinki). Once subjects were recruited, a dual-energy X-ray absorptiometry (DEXA) scan (Hologic QDR 4500W, Bedford, MA) was performed to measure body tissue composition and lean mass.

Study design. All subjects were admitted to the Clinical Research Center on the day before the experiment, were provided a standardized dinner, and were studied after an overnight fast under basal conditions. Subjects were studied at the same time of day to avoid potential circadian changes and refrained from exercise 48 h before study participation. On the morning of the study, polyethylene catheters were inserted into the antecubital vein for tracer infusion and in the contralateral antecubital vein for blood sampling. After a background blood sample was drawn, a primed continuous infusion of L-[ring-13C6] or L-[ring-2H6]phenylalanine was started and maintained at a constant rate until the end of the experiment. The priming dose for the labeled phenylalanine was 2 μmol/kg (0.3 mg/kg), and the infusion rate was 0.05 μmol·kg⁻¹·min⁻¹ (0.5 mg·kg⁻¹·h⁻¹). Two hours after the tracer infusion was started, the first muscle biopsy was obtained from the vastus lateralis of the leg. The biopsy was performed with a 5-mm Bergström biopsy needle under sterile procedure and local anesthesia (1% lidocaine). Two hours later, a second biopsy was obtained from the same incision but angled away from the previous biopsy (~5 cm). Muscle biopsy samples were immediately blotted and frozen in liquid nitrogen and stored at −80°C until analysis. The first muscle biopsy collected from each subject was used for miRNA and gene analysis, while both of the muscle biopsies were used to calculate a basal muscle protein synthesis rate.

Mature protein synthesis. Muscle tissue samples were grounded and intracellular free amino acids and muscle proteins were extracted as previously described (11). Blood and muscle intracellular free concentration and enrichment of phenylalanine were determined by gas chromatography-mass spectrometry (GC-MS; 6890 Plus GC, 5973N MSD, 7683 autosampler, Agilent Technologies, Palo Alto, CA) using appropriate internal standards (59). Mixed muscle protein-bound phenylalanine enrichment was analyzed by GC-MS, after protein hydrolysis and amino acid extraction (11), with the external standard curve approach (5). We calculated the fractional synthetic rate (FSR) of mixed muscle proteins by measuring the incorporation rate of the phenylalanine tracer into the proteins (ΔEi/Δt) and using the precursor-product model to calculate the synthesis rate: FSR = (ΔEi/Δt)(Ei(0) + Em(0)/2·60·100, where ΔEi is the increment in protein-bound phenylalanine enrichment between two sequential biopsies, t is the time between the two sequential biopsies, and Ei(0) + Em(0)/2 are the phenylalanine enrichments in the free intracellular pool in the two sequential biopsies. Data are expressed as percent per hour.

RNA isolation. Total RNA was isolated from skeletal muscle biopsy samples as conducted previously (12, 36). We used the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA) to assess RNA integrity; the average RNA integrity number (RIN) value for all samples was 8.31 (scale 1–10), and the 18S-to-28S ratio was 1.51, indicating high-quality RNA with minimal degradation.

miRNA array and data analysis. Total RNA was sent to LC Sciences (Houston, TX) for miRNA expression profiling using their proprietary μParaFlo microfluidic chip containing 837 human mature miRNA probes (Sanger miRBase 11.0). Expression values were normalized by removing system-related variation (sample amount variations, different labeling dyes, and signal gain differences of scanners) by a locally weighted regression method (cyclic LOWESS). Data adjustment included data filtering, log2 transformation, gene centering, and normalization. The data filtering removed miRNAs with normalized intensity values below a threshold value of 32 across all samples. The log2 transformation converted intensity values into log2 scale. Gene centering and normalization transformed the log2 values using the mean and the SD of individual genes across all samples with the following formula: Value = [(Value) − Mean(Gene)]/SD(Gene).

MicroRNA gene targets were determined with the online miRNA database TargetscanHuman (5.1). Gene data were uploaded into IPA (version 8.6, Ingenuity Systems, Redwood City, CA) to understand the interacting networks, molecular and cellular functions, and canonical pathways related to the progression of muscle loss with aging (sarcopenia).

TaqMan RT-PCR. To validate the array results the relative expression levels of Let-7a, b, and c and miR-98, miR-22, -24, -27a, -27b, -223, and -378 were determined using a TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA) according to the manufacturer’s directions. Relative fold changes were determined from the threshold cycle (Ct) values with the 2⁻ΔΔCt method (35). Data were normalized to RNU48 to account for possible differences in the amount of starting RNA.

RT-PCR analysis. First-strand cDNA synthesis from total RNA was performed with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time PCR (qQ5 Multicolor Real-Time PCR cycler, Bio-Rad) was performed on diluted cDNA with SYBR Green fluorescence on custom [β2-microglobulin (Sigma-Aldrich) and CMYC (Invitrogen)] and predesigned TaqMan (CDK6, CDC25A, CCND1, CDC34 and PAX7; Applied Biosystems) primers. Gene expression levels were normalized to β2-microglobulin.

Table 1. Baseline characteristics of young and older male subjects

<table>
<thead>
<tr>
<th></th>
<th>Young Adults (n = 19)</th>
<th>Older Adults (n = 17)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>30 ± 2</td>
<td>70 ± 1</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Height, cm</td>
<td>177 ± 2</td>
<td>173 ± 2</td>
<td>0.09</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>83 ± 2</td>
<td>81 ± 2</td>
<td>0.42</td>
</tr>
<tr>
<td>Lean mass, kg</td>
<td>63 ± 2</td>
<td>58 ± 2</td>
<td>0.06</td>
</tr>
<tr>
<td>Fat mass, kg</td>
<td>20 ± 2</td>
<td>22 ± 1</td>
<td>0.35</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>23 ± 1</td>
<td>27 ± 1</td>
<td>0.09</td>
</tr>
<tr>
<td>FSR, %/h</td>
<td>0.054 ± 0.004</td>
<td>0.058 ± 0.004</td>
<td>0.60</td>
</tr>
</tbody>
</table>

Values are means ± SE. FSR, fractional synthetic rate. Statistical significance was determined with an unpaired Student’s t-test.
Table 2. Basal microRNA expression level in older adult skeletal muscle

<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>Fold Change</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Upregulated microRNAs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa-Let-7a</td>
<td>1.2</td>
<td>0.04</td>
</tr>
<tr>
<td>hsa-Let-7b</td>
<td>1.2</td>
<td>0.02</td>
</tr>
<tr>
<td>hsa-Let-7e</td>
<td>1.5</td>
<td>0.04</td>
</tr>
<tr>
<td>hsa-Let-7f</td>
<td>1.2</td>
<td>0.04</td>
</tr>
<tr>
<td>hsa-miR-25</td>
<td>1.3</td>
<td>0.04</td>
</tr>
<tr>
<td>hsa-miR-98</td>
<td>2.3</td>
<td>0.03</td>
</tr>
<tr>
<td>hsa-miR-195</td>
<td>1.2</td>
<td>0.04</td>
</tr>
<tr>
<td>hsa-miR-1268</td>
<td>1.9</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Downregulated microRNAs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa-miR-22</td>
<td>0.7</td>
<td>0.02</td>
</tr>
<tr>
<td>hsa-miR-24</td>
<td>0.8</td>
<td>0.03</td>
</tr>
<tr>
<td>hsa-miR-27a</td>
<td>0.9</td>
<td>0.04</td>
</tr>
<tr>
<td>hsa-miR-27b</td>
<td>0.9</td>
<td>0.03</td>
</tr>
<tr>
<td>hsa-miR-30d</td>
<td>0.8</td>
<td>0.04</td>
</tr>
<tr>
<td>hsa-miR-133a</td>
<td>0.9</td>
<td>0.02</td>
</tr>
<tr>
<td>hsa-miR-133b</td>
<td>0.9</td>
<td>0.01</td>
</tr>
<tr>
<td>hsa-miR-223</td>
<td>0.3</td>
<td>0.04</td>
</tr>
<tr>
<td>hsa-miR-378</td>
<td>0.7</td>
<td>0.01</td>
</tr>
<tr>
<td>hsa-miR-378*</td>
<td>0.6</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Values are fold change from values in younger adults and are ordered alphanumerically. Statistical significance was determined with a Mann-Whitney test. 378* represents the mature miRNA strand that was identified second from the hairpin structure.

Statistical analysis. Noise reduction and background correction on miRNA array data were performed by LC Sciences. The Shapiro-Wilk test was run on the preprocessed data (background correction and scaling) to determine whether the reporters were normally distributed. We were unable to uniformly assume a normal distribution of the data, as many of the reporters from the miRNA array failed the Shapiro-Wilk test including the logarithm transformations of the data. As a result, nonparametric statistics (Mann-Whitney) were employed to test for significant differences between the young and older age groups. The significance level we chose to use was $\alpha = 0.05$. The Benjamini-Hochberg and Holm methods were used for multiple hypothesis corrections, resulting in no significant findings. As a result, we chose to only accept significant reporters with mean values $>500$ intensity as reported from the miRNA array. A value of $>500$ is considered biologically meaningful ($9)$. This method reduced the number of significant reporters by $\sim 75\%$. The statistical package R was used for microarray data calculations (v. 2.9.0, R Development Core Team, Vienna, Austria). The significant reporters were further analyzed for biological context by IPA. The right-tailed Fisher’s exact test was used to calculate a $P$ value to determine the statistical significance of association of our predicted reporters related to the molecular networks, biological functions, and top canonical pathways from IPA.

Data gathered from the miRNA array validation and gene expression experiments were analyzed with an unpaired, two-tailed Student’s t-test. Linear regression tests were carried out to determine correlations between the validated miRNAs and predicted target(s). A $P$ value $< 0.05$ was considered statistically significant. Data are reported as means $\pm SE$ except in the case of Fig. 5, in which individual values are reported.

RESULTS

Subject characteristics. As expected, young and older subjects differed from one another in age ($P < 0.05$) and tended to have differences in lean body mass ($P = 0.06$). There were no significant differences in regard to height, weight, fat mass, percent body fat, or FSR of mixed muscle proteins (Table 1).

MicroRNA array. There were variant expression levels across miRNAs, most of which were expressed at a signal intensity $<500$ (88%), whereas miRNAs that were expressed at the highest level ($>10,000$) represented a much smaller fraction of the total miRNAs examined (3%). Not surprisingly, some of the highly expressed miRNAs were muscle specific (i.e., miR-1, -133a/b, -206), with miR-1 being the highest expressed miRNA ($\sim 54,000$) in skeletal muscle, not too far off from the maximum detection intensity of the miRNA chips (65,000). Of the 875 miRNA probes examined in skeletal muscle samples of young and older men, 75 miRNAs were significantly different between the age groups (40 upregulated, 35 downregulated; $P < 0.05$). To eliminate false positives and identify miRNAs that have the greatest chance of being associated with age-related muscle sarcopenia, we narrowed the number of significant miRNAs by only accepting miRNAs with intensity levels $>500$, which are considered to be of biological significance ($9$). As a result, the total significant miRNA probes were reduced to 18. These miRNAs can be found in Table 2 and are ranked by those that were upregulated (Let-7a, -b, -e, -f, miR-25, -98, -195, -1268) and downregulated (miR-22, -24, -27a, -27b, -30d, -133a, -133b, -223, -378, -378*) relative to younger subjects ($P < 0.05$).

Real-time PCR miRNA validation. We next attempted to validate a subset of miRNAs (10 of 15) in order to pursue potential gene targets. We chose to validate four overexpressed miRNAs (Let-7a, -b, -e, miR-98) and six underexpressed miRNAs (miR-22, -24, -27a, -27b, -223, -378) in the majority of the young ($n = 18$) and older ($n = 16$) skeletal muscle biopsy samples that were used for the array. Unexpectedly, most of the miRNAs tested could not be validated (Let-7a, miR-98, -22, -24, -27a, -27b, -223, and -378). However, we found that Let-7b and -e were significantly upregulated ($\sim 40$ and 60%, respectively; Fig. 1) in the older subjects, which is in agreement with and of similar magnitude as those identified with the miRNA array analysis.

Recent reports indicate that Let-7s may be related to stem cell renewal (38), while aging is marked with an overall reduction in satellite cell number (27, 41, 44, 49, 56). Therefore, we measured in the whole skeletal muscle biopsy sample...
the mRNA expression of PAX7, a key marker of satellite cell abundance. We found that PAX7 expression was significantly lower in older versus younger adults (Fig. 2), providing evidence that satellite cell turnover may be impaired by aging.

MicroRNA database and Ingenuity analysis. Since we were successful in the validation of the differential Let-7b and -e expression, we determined putative downstream targets of the Let-7 family with the TargetScanHuman algorithm (5.1) as well as a subset of targets that has been validated in published reports. The TargetScan miRNA database ranks predicted gene targets based on context score—the more negative the value, the greater likelihood that the miRNA regulates the predicted gene. Predicted genes with context scores <0.30 from TargetScan were considered biologically relevant (18) and thus were considered acceptable for IPA. A predicted gene list was generated for the Let-7s and uploaded into the IPA for functional and network analysis with the appropriate filter settings.

To identify potential pathways and gene targets, we went through a series of steps using IPA to best determine the biological relevance of an upregulated Let-7 expression in older subjects. First, we determined the relationship between gene products by the merger of these products into networks. A network was defined as direct and indirect interaction between the uploaded predicted targets (called focus molecules) and other interacting molecules produced from the Ingenuity knowledge base. These were grouped into a maximum of 35 molecules/network. Ingenuity analysis identified two primary networks with functions related to 1) cellular development, cellular growth and proliferation, and connective tissue development and function (18/35; score 29) and 2) DNA replication, recombination, and repair, gene expression, and cell cycle (14/35; score 20). Table 3 identifies the top five networks, their functions, and the molecules that are associated with these networks.

Next, the top biological functions related to the products of the genes within these networks were identified. The ranking of these molecular function and canonical pathways are based on Fisher’s exact test; smaller P values suggest significant association of the molecules to the molecular functions in the network and higher rank for the network. Figure 3 shows these molecular and cellular functions. They were the following: 1) cell cycle (P value range: 2.66–0.75), 2) cellular growth and proliferation (P value range: 3.00–0.14), and other interacting molecules produced from the Ingenuity knowledge base. These were grouped into a maximum of 35 molecules/network. Ingenuity analysis identified two primary networks with functions related to 1) cellular development, cellular growth and proliferation, and connective tissue development and function (18/35; score 29) and 2) DNA replication, recombination, and repair, gene expression, and cell cycle (14/35; score 20). Table 3 identifies the top five networks, their functions, and the molecules that are associated with these networks.

Table 3. List of top network pathways generated from Ingenuity Pathway Analysis software identifying potential Let-7 targets and associated molecular products

<table>
<thead>
<tr>
<th>Network</th>
<th>Top Network Functions</th>
<th>Score</th>
<th>Focus Molecules</th>
<th>Molecules in Network</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cellular development, cellular growth and proliferation, connective tissue development and function</td>
<td>29</td>
<td>18</td>
<td>Akt, BTC, CASP3, CCND1, CCND2, CCR7, CDK6, CDKN1A, CX3CL1, CYP19A1, E2F5, EDN1, ERK1/2, ESR1, FAS, GJA1, Histone h3, IGFR1, IgG, IL6, IL1F6, IL1F8, IL1F9, Jnk, KRAS, KRT13, MYC, NAP1L1, NEU3, PDZK1, PTK2, POLR2D, RNA polymerase II, STAT5, VEGFB</td>
</tr>
<tr>
<td>2</td>
<td>DNA replication, recombination, and repair, gene expression, cell cycle</td>
<td>20</td>
<td>14</td>
<td>ACVR2A, ADRB2, BRCA1, CCL7, CDC25A, CDC34, COIL, DUSP5, E2F2, GEMIN7, GJC1, HAND1, HNF4A, ID1, Ikb, IL1B, MAP3K1, MIR146A, MIR146B, MYL2, MYOCBD, NFKBIA, NFKBIE, NR4A2, RB1, RRM2, Scf Trcp beta, SMN1, SMN2, Sod, TERT, TNF, TRP53, TP53I3, UBQLN2</td>
</tr>
<tr>
<td>3</td>
<td>Hematologic disease, respiratory disease, cardiac enlargement</td>
<td>2</td>
<td>1</td>
<td>HIF3A, VHL</td>
</tr>
<tr>
<td>4</td>
<td>Cancer, cell death, cellular development</td>
<td>2</td>
<td>1</td>
<td>CASP1, HMGA2</td>
</tr>
<tr>
<td>5</td>
<td>Cancer, drug metabolism, endocrine system development and function</td>
<td>2</td>
<td>1</td>
<td>APBB3, NCOA1</td>
</tr>
</tbody>
</table>

Molecules in bold indicate probable Let-7 gene targets (focus molecules).
3) cellular development (P value range: 2.45E-06–1.41E-02), 4) cell death (P value range: 1.65E-05–1.41E-02), and 5) post-translational modification (P value range: 4.65E-05–8.19E-03). Finally, the top canonical pathway that was identified by IPA was Cell Cycle: G1/S checkpoint regulation. The focus molecules that were associated with this pathway were c-Myc, CDK6, cyclin D1, cyclin D2, Cdc25A, p21 Cip1, E2F2, and E2F5.

Gene expression validation by real-time PCR. The information gained from IPA allowed us to identify a common biological theme (e.g., cell cycle control) and some candidate genes that could serve as possible targets of the Let-7 family of miRNAs. We chose to evaluate the expression of MYC, CDK6, CDC25A, CCND1, and CDC34 because 1) these genes have been identified as potential targets of the Let-7s (22, 26, 32, 50, 52), 2) Let-7s possess a conserved function as a tumor suppressor and therefore downregulate the expression of genes associated with cellular proliferation and promote differentiation (4, 26, 47), and 3) the focus molecules identified in the canonical pathway produced by IPA were mostly associated with cell cycle progression. We validated and confirmed that CDK6, CDC25A, and CDC34 mRNA expression were significantly reduced in older versus younger subjects (P < 0.05; Fig. 4), while expression of MYC and CCND1 were unchanged (data not shown) between the age groups.

We were successful in inversely relating Let-7 expression with that of three potential gene candidates. Therefore, we used linear regression tests to identify whether an upregulation of Let-7b or Let-7e is correlated to a downregulation of the mRNA expression of CDK6, CDC25A, and CDC34 in the older subjects. We were unable to statistically detect a miRNA-gene relationship (data not shown), therefore, we plotted the individual Let-7a–e and CDK6/CDC25A/CDC34 fold change values from 16 subjects (Fig. 5). These data provide a visual representation of a potential relationship between the miRNA and its predicted gene target.

DISCUSSION

We conducted a comprehensive miRNA analysis on skeletal muscle biopsies of 36 young and older adults, using a miRNA array followed by a bioinformatics analysis and real-time PCR to validate miRNA expression and candidate gene targets. In summary, we validated and confirmed that the expression of Let-7b and Let-7e was significantly higher in older versus younger subjects. Second, IPA identified that the Let-7 family predicted gene targets were related to pathways and biological functions associated with cell cycle control. To correlate Let-7 expression to cell cycle regulation, we validated that mRNA expression of CDK6, CDC25A, and CDC34, positive regulators of cell cycle progression, were downregulated in older subjects. This was further supported by a lower mRNA expression of PAX7 in older skeletal muscle. Together, our data indicate that older human skeletal muscle is characterized with a lower expression of genes related to cell cycle regulation and satellite cell abundance and may be governed by a Let-7 miRNA mechanism.

Let-7s were first discovered in *C. elegans* and were one of the first miRNAs identified (43) but later to be found conserved across species including humans (29). The primary finding of our study was that two members of the Let-7 family (Let-7b and Let-7e) were expressed at a higher level in older adults, which was validated with real-time PCR (Fig. 1). In humans, the Let-7s are composed of 10 family members (Let-7a, -b, -c, -d, -e, -f, -g, -i, miR-98, and -202), as recognized by an identical “seed” sequence (nucleotide 2 through 8) (47). It is interesting to point out that of the 10 Let-7 family members that were assessed by the miRNA array, all of the mean Let-7 values (although not significant) were greater in the older subjects (data not reported). Unfortunately, the data from the miRNA array only showed that five of these members were significantly elevated in the older subjects (Let-7a, -b, -e, -f, miR-98; Table 2) while even fewer were confirmed to be higher as detected with real-time PCR (Let-7b and -e; Fig. 1). The difficulty in confirming differences in the expression of Let-7 family members was probably due to relatively small differences between the age groups. However, the fact that all 10 Let-7 family members appeared to be elevated in older adults should not be ignored but instead raise the significance of our validated Let-7b and Let-7e data. The small but statistically undetectable age-related differences in the other Let-7 family members could suggest that the Let-7 family work together as a group to significantly alter gene expression or a common cellular function. Another possibility is that specific Let-7 family members may be in transition and require several more years of muscle aging before Let-7 expression reaches a level that can be detected with statistical significance. Nonetheless, our data indicate that Let-7 expression (Let-7b and Let-7e) was increased in older humans, which may be a novel biomarker of human skeletal muscle aging.

One of the major roles of the Let-7 miRNA family is to reduce cellular replication, therefore functioning as a tumor gene repressor. Loss-of-function studies indicate that overexpression of Let-7 suppresses proliferation while underexpression results in an accumulation of cells (7, 25, 26, 43). In fact, the downregulation of Let-7 expression is a common marker in lung cancer (54). However, inconclusive information is available describing the function of Let-7 expression in aging cells. For example, Let-7s induced cellular differentiation during maturation in *C. elegans* (43), but the expression decreased with advancing age, possibly serving as a regulator of longevity (24), whereas in mice Let-7 expression was upregulated in...
aging neural stem cells and associated with decreased self-renewal (38). Aging skeletal muscle is also characterized by a reduced capacity to regenerate (6, 48) and appears to be partly a function of the myogenic potential of satellite cells (2, 8, 53). Furthermore, some reports (27, 44, 49, 56), but not all (10, 41, 46), indicate that aging reduces the satellite cell pool in humans. In an attempt to provide evidence that an increased Let-7 expression may be associated with a reduced number of satellite cells in older humans, we measured the expression of PAX7 mRNA in human skeletal muscle biopsies. Interestingly, PAX7 mRNA expression was lower in older versus younger subjects (Fig. 2). Therefore, it is likely that an increased Let-7 expression in older humans may be linked with a lower abundance of satellite cells, including other supporting cells, which likely affects regenerative capacity of muscle tissue.

One of the major challenges of miRNA biology is the identification of specific gene targets. Since a single miRNA can posttranscriptionally regulate hundreds of genes, handpicking candidate targets out of a miRNA online prediction database can be quite unproductive. A powerful hypothesis-creating tool is the use of bioinformatics software such as IPA. Large gene lists can be uploaded into the server and used to identify molecular networks, key biological functions, and common cellular pathways. This approach can significantly improve the ability to identify relationships between miRNAs and their target genes. In this experiment, we generated a list of genes predicted to be targeted by the Let-7s and then uploaded them into the IPA application. In summary, we found that the gene targets of the Let-7 family were most closely related to functions associated with cell cycle control. We came to this conclusion by analyzing the expression of Let-7s and their predicted gene targets using microRNA profiling in older human skeletal muscle biopsies. The results are presented in Fig. 5, which shows individual line graphs from 16 older subjects plotted to identify individual fold change values for Let-7s and predicted gene targets. Each graph represents a specific pair of Let-7 and its target gene: A: Let-7b and CDK6, B: Let-7b and CDC25A, C: Let-7b and CDC34, D: Let-7e and CDK6, E: Let-7e and CDC25A, F: Let-7e and CDC34.
conclusion based upon the evidence of the top two molecular networks identified in Table 3 as well as the primary molecular and cellular functions indicated in Fig. 3.

The information generated from the Ingenuity knowledge base is consistent with the role of the Let-7s as a repressor of cellular replication (26, 47). Therefore, we focused on Let-7 targets that are positive regulators of cellular proliferation with the hypothesis that an increased Let-7 expression in older adults would be associated with decreased expression of these gene targets. We found that the mRNA expression of a cluster of cell cycle regulators, CDK6, CDC25A, and CDC34 (Fig. 4), were significantly downregulated in older adults, while MYC and CCND1 were unchanged. These data are in agreement with those of Johnson et al. (26), which indicate that overexpression of Let-7 expression in lung cancer cells reduced cell cycle progression in part by targeting the expression of many cell cycle genes including CDK6, CDC25A, and CDC34. Cyclin-dependent kinase (CDK) activity is tightly regulated by various interacting proteins (37). In particular, CDK6 activity is enhanced by the binding with cyclin Ds (D1, D2, D3). This complex then phosphorylates proteins that activate the entry into the cell cycle. However, the activity of CDKs can be inhibited by the CDK inhibitor CDKN2A (p16ink4a), a well-known tumor suppressor (21, 51). Interestingly, the upregulation of p16ink4a is a common feature of tissue aging (15, 28), and we have also found that INK4a gene expression is higher in older human skeletal muscle (Drummond MJ and McCarthy JJ, unpublished observations). To add further intricacy to miRNA gene regulation, high-mobility group A2 (HMGA2) inhibits the expression of p16ink4a and is also a purported Let-7 target (20, 38). Therefore the Let-7s could act directly or indirectly (i.e., HMGA2) on the expression of CDK6 to impact cell cycle control.

Our data indicate that CDC25A and CDC34 gene expression were downregulated and support reduced cell proliferation in older subjects. Cell division cycle 25A (CDC25A) codes for a protein that removes phosphates initiated by protein kinases such as Wee1, thereby increasing CDK activity (37). Indeed, the Let-7-CDC25A relationship has been recognized by others using sophisticated prediction-based software (22) and identified in Let-7 overexpression studies (26). CDC34, an E2 ubiquitin conjugating enzyme of the SCF (Skp1/cullin/F-box) complex, mediates protein kinases such as Wee1. Taking these findings together, we propose that the downregulation of CDC25A and CDC34 by Let-7 indirectly promotes Wee1 to inactivate CDKs, thereby reducing cellular proliferation, possibly affecting the turnover of satellite cells and overall slowing muscle regeneration (Fig. 6).

Limitations. Our miRNA array analysis initially identified 75 differentially expressed miRNAs between the age groups. This list was narrowed down to only 18 by eliminating those miRNAs that were expressed at low levels <500 (9). Although they are expressed at “low” levels, their individual or synergistic impact on gene expression may significantly alter system function. It is possible we may have eliminated some miRNAs that participate in other characteristics of muscle aging (e.g., inflammation and stress). However, since miRNA research has recently begun to be explored in human aging we wanted to eliminate some possible false positives by being conservative with our data analysis and interpret with certainty that our miRNAs identified in young and older persons were truly differentially expressed. Second, although the correlations between Let-7b/e and the candidate genes were not statistically significant, we do provide a visual representation in Fig. 5 showing nearly every older subject characterized with a higher Let-7 expression and lower CDK6, CDC25A, and CDC34 expression. Although there may not be a direct relationship between a single miRNA and its target, the correlation does not take into account that related miRNAs (i.e., Let-7 family) may act synergistically to posttranscriptionally regulate gene expression level. Finally, the limitations of human research put us into a position to collect and analyze data that are correlative in nature. However, our goal with this experiment was to spur further research interest by the use of a miRNA array as a first-line approach to identify miRNA candidates that may serve as potential biomarkers of muscle sarcopenia and use state-of-the-art bioinformatic software to most closely associate miRNAs to their downstream targets. Clearly, mechanistic data are needed to identify the direct targets of the Let-7 family in human skeletal muscle and to identify whether or not Let-7 expression and targets are localized to satellite cells.

In summary, we performed an in-depth miRNA analysis on a relatively large pool of young and older subjects, using a microarray followed by a thorough bioinformatics analysis using IPA. We later confirmed with real-time PCR that the Let-7s (Let-7b and Let-7e) were upregulated in older adults and that this miRNA family was associated with a downregulation of the cell cycle regulators CDK6, CDC25A, and...
CDC34. We conclude that aging is characterized by an elevated Let-7 expression that suggests a reduced cell proliferation potential and the daily renewal and regeneration of skeletal muscle cells. Additional research is required to determine the contribution of a higher Let-7 expression and downregulation of cell cycle regulators to the development of sarcopenia.

ACKNOWLEDGMENTS
We thank the nurses and staff at the Institute for Translational Sciences Clinical Research Center (ITS-CRC) and the Claude E. Pepper Older Americans Independence Center recruitment coordinators for their assistance in screening, admitting, and assisting with the subjects during data collection.

GRANTS
This study was supported in part by National Institutes of Health (NIH) Grants R01-AR-049877 (B. R. Basmann), R01-AR-45617 (K. A. Esser), 5R01-AG-018311 (E. Volpi), and P30-AG-024832. Additional support came from 1UL1-RR-029876-01 from the NIH/National Center for Research Resources.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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