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RESEARCH ARTICLE | *-Omic Approaches to Understanding Muscle Biology*

Identification of human skeletal muscle miRNA related to strength by high-throughput sequencing

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Mitchell CJ, D'Souza RF, Schierding W, Zeng N, Ramzan F, O'Sullivan JM, Poppitt SD, Cameron-Smith D. Identification of human skeletal muscle miRNA related to strength by high-throughput sequencing. *Physiol Genomics* 50: 416–424, 2018. First published March 30, 2018; doi:10.1152/physiolgenomics.00112.2017.—The loss of muscle size, strength, and quality with aging is a major determinant of morbidity and mortality in the elderly. The regulatory pathways that impact the muscle phenotype include the translational regulation maintained by microRNAs (miRNA). Yet the miRNAs that are expressed in human skeletal muscle and relationship to muscle size, strength, and quality are unknown. Using next-generation sequencing, we selected the 50 most abundantly expressed miRNAs and then analyzed them in vastus lateralis muscle, obtained by biopsy from middle-aged males ($n = 48$; 50.0 ± 4.3 yr). Isokinetic strength testing and midthigh computed tomography was undertaken for muscle phenotype analysis. Muscle attenuation was measured by computerized tomography and is inversely proportional to myofiber lipid content. miR-486-5p accounted for 21% of total miR sequence reads, with miR-10b-5p, miR-133a-3p, and miR-22-3p accounting for a further 15, 12, and 10%, respectively. Isokinetic knee extension strength and muscle cross-sectional area were positively correlated with miR-100-5p, miR-99b-5p, and miR-191-5p expression. Muscle attenuation was negatively correlated to let-7f-5p, miR-30d-5p, and miR-125b-5p expression. In silico analysis implicates miRNAs related to strength and muscle size in the regulation of mammalian target of rapamycin, while miRNAs related to muscle attenuation may have potential roles regulating the transforming growth factor-β/SMAD3 pathway.

atrophy; biomarker; muscle quality; sarcopenia; sequencing

INTRODUCTION

Skeletal muscle force production is the primary determinant of many measures of physical function (43) and has also been related to mortality risk (46), quality of life (50), and outcomes in acute illness (12). The principal factor regulating muscle strength is muscle size (11). However, there is a progressive loss of muscle size and strength that begins in midlife and accelerates with advancing age (17, 56). While muscle size is the predominant determinant of strength, there is evidence that alterations in muscle architecture (18) and quality (39) and loss of neural activation (47) also are important in the loss of physical function with aging. Currently, there remains a limited understanding of the mechanisms underpinning the loss of muscle size with aging. While muscle size is ultimately dictated by the dynamic balance of muscle protein synthesis (MPS) and muscle protein breakdown (MPB) (41), these pathways are regulated by muscle loading (4), nutrition (33), and health status. Subsequent impacts on either MPS or MPB are dictated by coordinated programs of transcriptional regulation, posttranslation regulation, and activation of protein kinases (7). There is increasing evidence for the importance of microRNAs (miRNAs) within these networks of regulation.

A subset of miRNAs have been termed "myomiRs" based on increased expression in skeletal muscle relative to other tissues and potential regulation of intrinsic muscular processes (28, 29). Currently there is no consensus on the precise definition of the term myomiRs because no unbiased identification of the relative abundances of specific miRNAs in healthy human skeletal muscle has been published. Furthermore, it is not known whether these muscle-specific miRNAs are predictive of skeletal muscle phenotypes. Of the available data, the relative expression of a subset of miRNAs, including some myomiRs, has been shown to correlate with lower leg muscle mass in comparative analysis of young and elderly males (45). Further evidence of the relationship between miRNA and muscle adaptation has been demonstrated in studies involving exercise training (30, 60). Resting skeletal muscle miRNA expression profiles are regulated differently in people who demonstrate the greatest responses to resistance training when compared with people with the lowest resistance training responses (9). Similarly powerlifters who have a radically different muscular phenotypes from other healthy adults also display a dramatically different muscular miRNA expression profile (6). Together these data suggest that intramuscular miRNA profile may be an important regulator of muscle phenotype and provide a potential biomarker of muscle morphology, composition and strength.

Therefore, the purpose of the present study was to undertake an untargeted and comprehensive analysis of the miRNA expressed in a skeletal muscle samples from healthy adult humans with next-generation sequencing (NGS). This analysis was conducted in a cohort of healthy middle-aged men, thus providing quantification of the expression profile of miRNA and the relationship to measured skeletal muscle phenotype, including strength, size, and lipid infiltration measured by computerized tomography (CT). Muscle radiographic density

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was measured by CT as the muscle attenuation coefficient, lower values of this coefficient reflect a higher muscle lipid content (15). The aims were then to identify the subset of miRNA, expressed within skeletal muscle, that correlate with measures of muscle size, strength, and composition. From the identified statistically correlated miRNA, in silico analysis was used to identify pathways potentially mediate the miRNA-dependent regulation of muscular phenotype.

METHODS AND MATERIALS

Participants. A total of 48 healthy middle-aged men (Table 1) were recruited. Participants underwent strength testing and muscle imaging before taking part in one of three previously published acute nutritional intervention studies (32–34). Participants were nonsmokers and free of neuromuscular and metabolic disease. They were sedentary to recreationally active and did not regularly perform resistance exercise. The body mass indexes of the participants ranged from 20 to 32 kg/m^2. All participants provided written and verbal informed consent before beginning the experiment. The study was approved by the Health and Disability Ethics Committee (New Zealand).

Blood biochemistry. Blood biochemistry analysis was performed with a Hitachi 902 autoanalyzer, (Hitachi High Technologies, Tokyo, Japan) by enzymatic colorimetric assay (Roche, Mannheim, Germany). The homeostatic model assessment insulin resistance was calculated based on the standard equation (27).

Strength testing. Participants were seated upright in a Kin Com isokinetic dynamotor (Chattanooga, TN) with the axis of rotation of the dynamotor aligned with the center of rotation of their right knee. The dynamotor was secured to the participant's leg with a Velcro strap 3 cm proximally of the malleolus. Five repetitions of knee extension were performed at a velocity of 60°/s with the participants instructed to give an 80% effort as a warm-up to enable familiarization with the movement. After a 2 min rest participants performed an additional five repetitions with a maximal effort. The machine was then repositioned, and the procedures were repeated for the left leg. The peak torque for each repetition was recorded, the three repetitions for each leg with the highest torque we recorded, and the torque measurements from the three best trials for each leg were then averaged to yield the strength value used in subsequent analysis. Participants were asked to refrain from vigorous activity for 3 days before strength testing. Muscle biopsies were performed at least 3 days after strength testing.

Muscle imaging. CT was conducted with a Lightspeed16 CT scanner (GE Medical Systems, Chicago, IL). An anterior-posterior scout scan was performed to measure femoral length. A single 10 mm thick axial slice at 50% of femoral length was completed. The voxel size was set at 0.7 mm^2. Cross-sectional area (CSA) of the thigh muscles was manually determined by a single investigator using ImageJ 1.46r (National Institutes of Health). Muscle attenuation (radiographic density), which is inversely related to lipid content, was expressed in Hounsfield units (HU) and averaged for the entire thigh muscle area. On this scale, distilled water is given a value of 0 HU. Adipose tissue typically has an attenuation value between −120 and −90 HU as it is less dense than water, whereas skeletal muscle typically has a value of ~50 HU as it is more dense than water. CT-derived muscle attenuation values are known to decrease by ~1 HU for every additional gram of lipid contained in 100 cm^3 of skeletal muscle (16). CT scans were performed on both legs, and the average CSA reported. CT scans were not conducted on three participants so muscle CSA and attenuation measurements are reported for 45 participants.

Muscle biopsy and miRNA isolation. Muscle biopsies were obtained, after an overnight fast, from the vastus lateralis muscle via a Bergström needle modified for manual suction under local anesthesia (1% xylocaine). Following collection, samples were immediately frozen in liquid nitrogen and stored at −80 until RNA extraction. Total RNA was extracted from ~20 mg of muscle tissue with the AllPrep DNA/RNA/miRNA Universal Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. RNA was quantified with a NanoDrop 1000 with quality determined by RNA 6000 NANO Chips (Agilent Technologies) on a 2100 Bioanalyzer (Agilent Technologies) to derive the RNA integrity number (RIN). Samples with an RIN ≥ 8.0 were utilized for sequencing. Whole body dual energy X-ray absorptiometry (Lunar Prodigy; GE, Waltham, MA) was used to quantify percentage body fat.

miRNA sequencing. Input RNA (3 μg) was freeze-dried on RNAstable (Biomatrica, San Diego, CA) in a 96-well plate and

Table 1. *Baseline characteristics of subjects*

	n = 48
Age, yr	50.0 ± 4.3
Body mass, kg	83.0 ± 10.1
Height, cm	176.6 ± 15.2
Body fat, %	22.3 ± 6.4
BMI, kg/m^2	26.0 ± 2.6
Glucose, mmol/l	5.47 ± 0.51
Insulin, μU/ml	7.47 ± 3.84
HOMA-IR	1.85 ± 0.74
Triglycerides, mmol/l	1.39 ± 0.55
LDL-C, mmol/l	3.25 ± 1.00
Cholesterol, mmol/l	5.39 ± 1.08
HDL-C, mmol/l	1.22 ± 0.21
Thigh muscle CSA, cm^2	158.9 ± 23.9
Knee extension torque are 60°/s, Nm	176.8 ± 34.3
Thigh muscle attenuation, HU	58.3 ± 2.7

Means ± SD. BMI, body mass index; CSA, cross-sectional area; HDL-C, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; LDL-C, low-density lipoprotein.

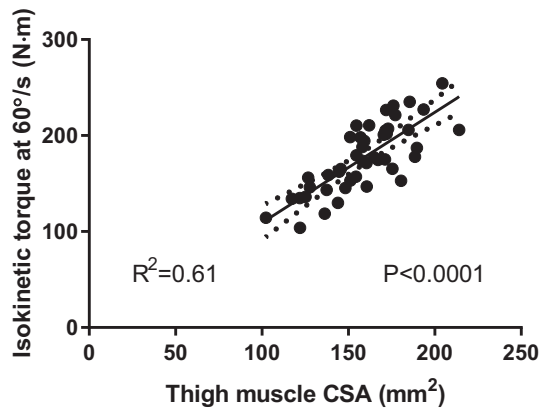

Fig. 1. Correlation between muscle and strength and size. Solid line shows relationship between size and strength and dotted lines show 95% confidence bands. CSA, cross-sectional area.

shipped for sequencing to Macrogen (Seoul, South Korea). Library preparation was performed with a Truseq small RNA kit (Illumina, San Diego, CA). Sequencing was conducted with Hiseq2500 50bpSR rapid mode.

Identification of known miRNAs. miRNA expression abundance was assessed with the mirna-soft package (54). In brief, reads were demultiplexed, the 3′ adapter sequences removed, and miRNA-seq reads >17 nucleotides in length retained. The trimmed and cleaned reads were aligned to known miRNAs by mirna-soft, matching the read sequences to the miRBase reference [version 18, http://www.mirbase.org (20)] with the reads tallied to generate total counts for each miRNA, generating an expression profile per sample. To maximize read counts and to incorporate reads representing putative isomiRs, we trimmed any unmatched, full length sequences 2 bp at both ends before performing another matching attempt. Finally, we normalized the read counts for each sample to counts per million by dividing the total read counts of a miRNA by the total read counts of the sample and multiplying this number by 1,000,000. The raw files and normalized counts of the NGS

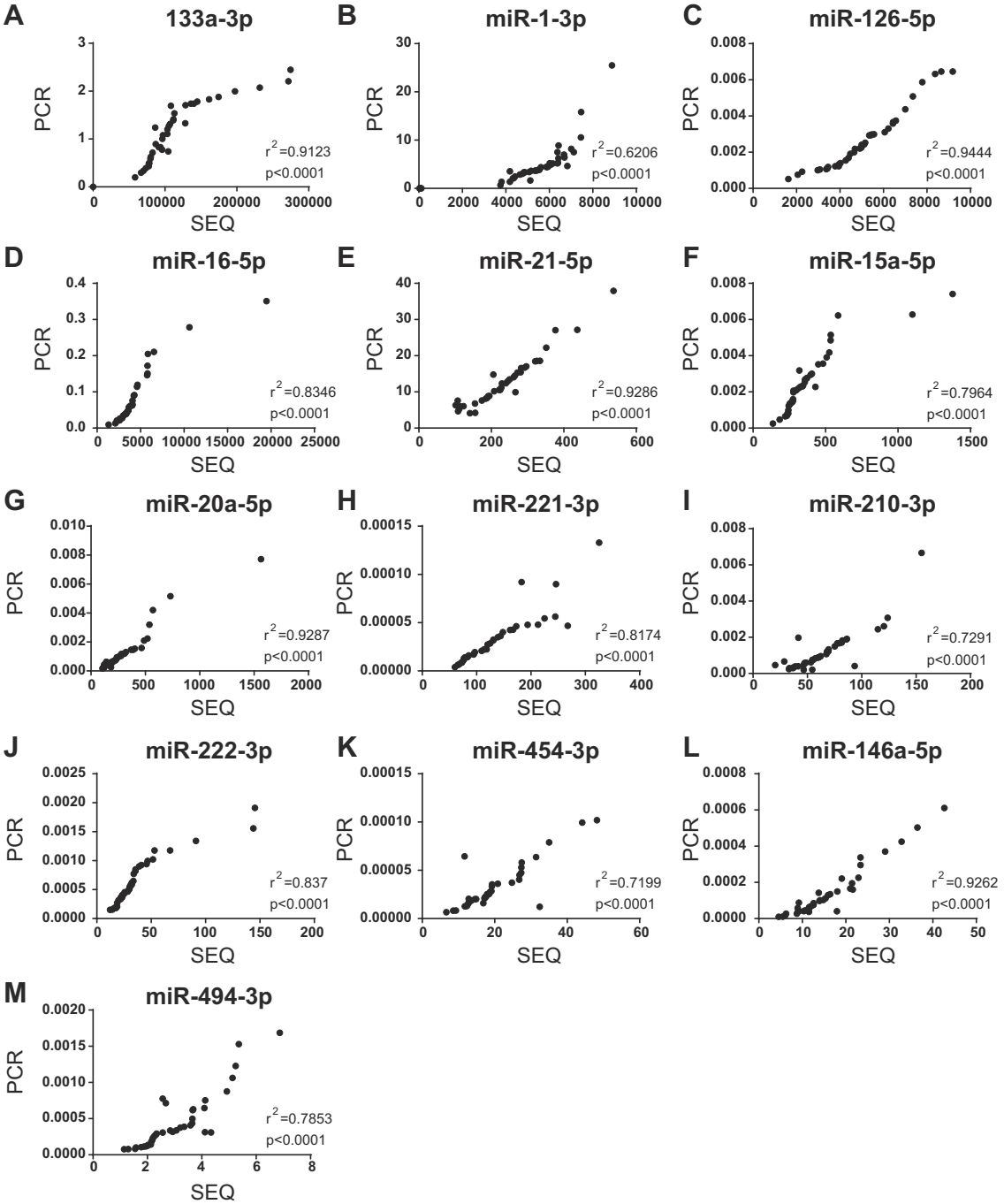

Fig. 2. Comparison of microRNA (miRNA) expressed determined with quantitative (q)PCR and next-generation sequencing (NGS). *A–M* compare the relative expressions of individual miRNAs between NGS and qPCR across a range of relative miRNA abundances.

data along with metadata are available at Gene Expression Omnibus (accession ID GSE103635).

RT-PCR. For validation of sequencing data, quantitative (q)RT-PCR of premultiplexed target miRNAs was undertaken. The multiplex technique is reported in detail (22). Briefly, 50 ng of stock total RNA was reverse-transcribed with the TaqMan microRNA RT kit (#4366596) and matching miRNA-specific stem-loop primers (TaqMan microRNA assay kit, #4427975) with some modifications. A custom RT primer pool was prepared by pooling miRNA-specific stem-loop primers of interest. Final concentrations of 0.05× of each primer was achieved by diluting in 1× Tris-EDTA (TE). We added 6 μl of the primer mixture, 0.3 μl 100 mM dNTP, 3 μl enzyme (50 U/μl), 1.5 μl 10 × RT buffer, and 0.19 μl RNase inhibitor (20 U/μl) to the RNA to achieve a final volume of 15 μl for reverse transcription (30 min at 16°C annealing, 30 min at 42°C extension, and 5 min at 85°C for denaturation). cDNA was then stored at −20°C.

To preamplify multiple cDNAs simultaneously, a new primer pool targeting the previously reverse-transcribed miRNAs consisting of 5 μl of each individual 20× TaqMan Small RNA Assay (#4427975) was diluted to 500 μl in 1× TE. The reaction mix was prepared by combining 3.75 μl of the primer mix with 2.5 μl of RT product, 12.5 μl of 1× TaqMan Universal PCR MasterMix (2×), no uracil-N-glycosylase (UNG) (#4440040), and 6.25 μl diethyl pyrocarbonate-treated (DEPC) water in a final volume of 25 μl.

All RT-qPCRs were carried out in duplicate 20 μl reactions containing 0.2 μl multiplexed muscle cDNA, 7.5 μl 2× TaqMan Universal PCR MasterMix, no UNG (#4440040), and 1 μl of 20× TaqMan Small RNA Assay (#4427975) in each well. Plates were analyzed on a HT7900 thermal cycler system (Thermo Fisher Scientific, Carlsbad, CA) by the procedure described in Le Carre et al. (22). Each primer was tested across a 1,000× concentration curve to determine linearity of primer efficiency as described in (8).

Total RNA, cDNA synthesis, and real-time PCR were conducted as previously reported (6). The geometric mean of three reference genes was used for normalization. Chromosome 1 open reading frame 43 (*C1orf43*), charged multivesicular body protein 2A (*CHMP2A*), and heat shock protein 90 (*HSP90*) were identified as the least variable and used as reference genes. Standard and melting curves were performed for every target to confirm primer efficiency and single-product amplification. The abundance of mRNAs were measured by $2^{(-\Delta CT)}$ method, and the abundance of Type 2a (*MYH2*) was compared with Type 1 (*MYH7*) fiber to determine the ratio of Type IIa/Type I fiber. Muscle myosin gene expression was available for 39 participants.

Pathway analysis. Ingenuity pathway analysis (IPA) was used to identify the mRNA targets of the miRNAs, related to either muscle CSA/strength or attenuation. Only targets with experimentally observed or highly predicted miRNA-mRNA interactions were included in the analysis. The resulting genes were then analyzed with the IPA core, which identified enriched pathways.

Statistical analysis. The 50 most highly expressed miRNAs, which account for 96.7% of the total sequencing reads, were included in the statistical analysis. Principal component analysis was used as a dimension reduction strategy, components with an Eigenvalue of >1 were retained, and varimax rotation was used. Pearson's product moment correlation was used to test the relationship between each of the identified principal components and muscle strength, CSA, muscle attenuation, and myosin heavy chain ratio. Significance was set at alpha ≤ 0.05. Where significant correlations were observed the five miRNAs with the largest absolute loading score for each principal component were identified and then Pearson's correlations were used to identify relationships between phenotype and candidate miRNAs. The Benjamini-Hochberg procedure was used to control for a false discovery rate of 5%; statistical significance was accepted when $P < Q$ (3). All statistical analyses were conducted with SPSS version 20.0 (IBM, Armonk, NY). Data are shown as means ± SE.

RESULTS

Muscular phenotype. Isokinetic knee extension strength at 60°/s averaged between legs was 176.8 ± 10.0 Nm and ranged from 103.9 to 254.3 Nm. The average thigh muscle CSA between the left and right leg was 158.9 ± 3.6 cm^2 and ranged from 102.3 to 214.2 cm^2. Muscle attenuation (a proxy of lipid content) was 58.3 ± 0.4 HU and ranged from 51.2 to 63.7 HU. Muscle strength was correlated ($P < 0.001$) with thigh muscle CSA and explained 60.9% of the variance (Fig. 1). Muscle attenuation was not correlated to muscle strength ($P = 0.658$). The ratio of type IIa:I myosin ranged from 0.05 to 0.66 with an mean value of 0.25 ± 0.16, with a higher ratio indicating a greater abundance of myosin type IIa mRNA.

miRNA abundance. The 50 most abundantly expressed miRNAs represent 96.7% of the total observed reads (supplemental material). (The online version of this article contains supple-

Table 2. *Principal components*

Principal Component #	Eigenvalues	Variance Explained, %	Cumulative Variance Explained, %	Correlation with Strength, r	Correlation with CSA, r	Correlation with Attenuation, r
1	8.08	16.84	16.84	−0.293*	−0.249	−0.219
2	7.95	16.56	33.40	−0.008	−0.028	−0.457*
3	5.75	11.99	45.39	−0.212	−0.161	−0.074
4	4.36	9.09	54.47	−0.032	−0.110	0.094
5	3.51	7.31	61.78	0.436*	0.345*	0.019
6	2.78	5.79	67.58	0.068	0.184	0.051
7	2.52	5.26	72.84	0.106	−0.019	−0.309*
8	2.43	5.05	77.89	−0.059	−0.092	0.082
9	2.41	5.03	82.91	−0.150	−0.235	−0.106
10	1.57	3.27	86.19	0.086	0.057	−0.200

*Significant correlation $P < 0.05$.

mental material.) The top five most highly expressed miRNAs were miR-486-5p, miR-10b-5p, miR-133a-3p, miR-22-3p, and miR-378a-3p, representing 21.19, 14.78, 11.83, 9.99, and 7.70% of total sequencing reads. With the exception of miR-499a-3p, 342 miRNAs were expressed in the vastus lateralis of all 48 study participants.

RT-PCR validation. Multiplex qPCR identified significant correlations (Fig. 2) between sequenced miRNA levels and PCR abundance for 13 miRNAs ranging from the third most abundant to the 377th most abundant miRNAs.

Principal components. A total of 10 principal components (PC) were extracted from the miRNA expression data, explaining 86.2% of the variance observed. PCs 1 and 5 correlated with muscle strength, while PC5 also correlated with muscle CSA. PC1 also showed a trend toward a correlation with muscle CSA ($P = 0.099$) (Table 2). PCs 2 and 7 correlated with muscle attenuation. The miRNAs with the highest loading scores in PC1 were miR-486-5p, miR-378a-3p, miR-191-5p, 133a-3p, miR-24-3p. The miRNAs with the highest loading scores in PC5 were miR-100-5p, miR-99b-5p, miR-99a-5p, miR-125b-5p, 30b-5p. The miRNAs with the highest loading scores in PC2 were miR-148a-3p, miR-26a-5p, miR-125b-5p, miR-30d-5p, let-7f-5p. The miRNAs with the highest loading scores in PC7 were miR-30e-3p, miR-30a-3p, miR-26a-5p, miR-148a-3p, miR-98-5p. PC2 and PC10 correlated with myosin isoform ratio.

Relation between miRNA expression and phenotype. The miRNAs identified as highly related to PCs of interest were then correlated with measures of muscular phenotype. miR-191-5p, miR-100-5p, and miR-99b-5p expression was correlated with strength (Table 3), and these were included in subsequent pathway analyses. The same pattern of correlations was observed between miRNAs and CSA; however, they did not reach statistical significance after correction for false discovery rate. The expression of miR-125b-5p, miR-30d-5p, and let-7f-5p was correlated with muscle attenuation (Table 4), and these were included in subsequent pathway analysis. miR-378a-5p and miR-125b-5p correlated with myosin isoform ratio, indicating a positive relationship with higher type IIa mRNA expression (Table 5).

Pathway analysis. Pathway analyses were conducted separately for miRNAs related to muscle CSA/strength (Table 6) and attenuation (Table 7).

Table 3. *Strength and CSA correlations*

	CSA			Strength		
microRNA	r	*P*	Q	r	*P*	Q
miR-100-5p	0.328	0.028	0.010	0.438*	0.002	0.005
miR-99b-5p	0.376*	0.011	0.015	0.436*	0.002	0.010
miR-191-5p	0.397	0.007	0.005	0.358*	0.013	0.015
miR-378a-3p	−0.204	0.178	0.030	−0.273	0.06	0.020
miR-30b-5p	−0.228	0.115	0.025	−0.245	0.093	0.025
miR-133a-3p	−0.265	0.078	0.020	−0.24	0.101	0.030
miR-99a-5p	0.191	0.212	0.035	0.233	0.119	0.035
miR-125b-5p	0.082	0.592	0.040	0.12	0.416	0.040
miR-24-3p	−0.078	0.612	0.045	−0.089	0.547	0.045
miR-486-5p	−0.02	0.894	0.050	0.022	0.88	0.050

*Significant correlation after 5% false discovery rate correction ($P < Q$).

Table 4. *Muscle attenuation correlations*

	Muscle Attenuation		
microRNA	r	*P*	Q
let-7f-5p	−0.465*	0.001	0.006
miR-30d-5p	−0.379*	0.01	0.013
miR-125b-5p	−0.374*	0.011	0.019
miR-26a-5p	−0.295	0.049	0.025
miR-30e-3p	−0.283	0.06	0.031
miR-148a-3p	−0.204	0.108	0.038
miR-30a-3p	0.236	0.119	0.044
miR-98-5p	−0.027	0.862	0.050

*Significant correlation after 5% false discovery rate correction ($P < Q$).

DISCUSSION

In the cohort of healthy middle-aged men, muscle strength exhibited a ~2.5-fold variation, while muscle CSA exhibited a ~2-fold variation, demonstrating the considerable diversity in muscle strength and size evident in the population. Next-generation miRNA sequencing was conducted, demonstrating the presence of 259 transcripts that were expressed in at least 80% of samples and averaged at least 15 reads per sample based on a sequencing depth of 1,000,000, reads per sample. From this, it was demonstrated that miR-486-5p, which has been predicted to target the anabolic PI3K/Akt signaling (48), was consistently the most highly expressed miRNA in human skeletal muscle. Furthermore, the top four most abundantly expressed miRNAs were demonstrated to account for more than 50% of the total recorded reads (miR-486-5p, miR-10b-5p, miR-133a-3p, and miR-22-3p). However, these abundantly expressed miRNAs were not related to muscular phenotype. When examining the association between sequencing reads and phenotype, we identified very few relationships. From these data, the transcript levels of just three miRNAs, miR-191, -100, and -99b, accounting for 1.1, 0.3, and 0.6%, respectively, of total sequencing reads, correlated with both muscle size and strength. Bioinformatic analysis indicates that these miRNAs target a number of genes implicated in the regulation of the mTOR and STAT3 pathways. Further analysis of the relationship to muscle phenotype identified transcript levels of let-7f-59, miR-30d-5p, and miR-125b-5p as being negatively correlated with muscle attenuation, which acts as an inverse biomarker of lipid content (16). These miRNA species were unrelated to strength in the present cohort of middle-aged men.

Middle age is a period of life generally agreed to encompass individuals aged 40 to 60 yr. In this cohort, both the average isokinetic torque and the variance are comparable to previously observations in cohorts of a similar age (10, 38), suggesting

Table 5. *Myosin heavy chain isoform ratio correlations*

microRNA	r	*P*	Q
miR-378a-5p	0.503*	0.001	0.006
miR-125b-5p	0.397*	0.012	0.013
miR-26a-5p	0.323	0.045	0.019
miR-148a-3p	0.217	0.184	0.025
miR-30d-5p	0.214	0.192	0.031
let-7f-5p	0.211	0.197	0.038
miR-24-3p	0.194	0.237	0.044
miR-206	−0.072	0.663	0.050

*Significant correlation after 5% false discovery rate correction ($P < Q$).

Table 6. *Pathway enrichment of miRNAs related to muscle strength*

Pathway	Genes, *n*	*P* Value	Regulated Genes
Glucocorticoid receptor signaling	7/287	5.62×10^{-4}	*CEBPB, FGFR3, FKBP5, IL6, PPP3CA, SMARCA4, TAF5*
JAK/Stat signaling	4/83	7.67×10^{-4}	*CEBPB, FGFR3, IL6, MTOR*
IL-17 signaling	4/85	8.39×10^{4}	*CEBPB, CRP, FGFR3, IL6*
Role of macrophages, fibroblasts, and endothelial cells in rheumatoid arthritis	7/309	8.67×10^{-4}	*CEBPB, FGFR3, FZD8, IL6, PLCD1, PPP3CA, TLR3*
Cardiac hypertrophy signaling	6/235	1.11×10^{-3}	*FGFR3, IGF1R, IL6, MTOR, PLCD1, PPP3CA*

Generated with Ingenuity Pathway Analysis software.

this population is typical of men in Western countries. The thigh muscle size and attenuation values were also analogous to what has been reported in healthy adults (49). Values for muscle strength, size, and density were much higher than what has been reported in a larger population of older men (average 74 yr), indicating that in the next 20–25 yr participants in the current study can expect ~18% decline in muscle size, ~25% decline in muscle strength, but a ~36% decline in muscle attenuation (15). In the present study muscle size was a strong predictor of strength, explaining 60% of the variance, whereas muscle composition did not explain variance in muscle strength. The current cohort of middle-aged men had relatively homogenous muscle compositions with little lipid infiltration (high muscle attenuation). It is likely that as this cohort ages the importance of muscle composition in predicting changes in strength and function will increase dramatically (55).

We identified miR-99b and -100 as being positively related to both muscle size and strength in middle-aged men. The expression of these miRNAs has previously been demonstrated to decline acutely following resistance exercise in young but not older men (59). Both miR-99b and -100 negatively regulate the anabolic mTOR pathway (57), which is stimulated by resistance exercise and protein feeding (36). It is possible that the maintenance of miR-99b and -100 levels after exercise in older adults might partially explain the age-related blunting of the anabolic response to resistance exercise (21, 35). miR-99b and -100 are known to suppress the mTOR pathway (57), yet their measured abundance in the current study was positively correlated with muscle size, suggesting a potential suppression of the anabolic mTOR pathway in the strongest individuals. Specifically, it is possible that greater baseline suppression of the mTOR pathway allows a higher magnitude of activation following acute anabolic stimuli such as resistance exercise or protein feeding (1). It is also possible that miR-99b and -100 act as a negative feedback mechanism to control upregulation of mTOR via another mechanism.

miR-191 was also positively correlated with both muscle strength and CSA in our study. It is predicted to target mitogen-activated protein kinases, interleukin-6 signaling, and serine/threonine-protein phosphatase PP1-beta catalytic subunit (PPP1CB). Skeletal muscle is enriched in PPP1CB (2), and it has potential functions in both insulin signaling (40) and myogenesis (61). All of these proteins have multiple cellular functions, including direct or indirect regulation of the mTOR pathway. Collectively, miR-191, -99b, and -100 also target the Stat3 pathway, which is essential for both myogenesis and muscle repair (52).

In older adults changes in muscle attenuation better explain declining muscle strength than changes in CSA (15). In the present cohort, muscle attenuation was not predictive of strength; however, it is possible a higher muscle lipid content in middle age might be related to later function decline. Three miRNAs (let-7a, miR-30c, and miR-125b) were identified as related to muscle attenuation. Pathway analysis predict these transcripts as potential regulators of the transforming growth factor(TGF)-β pathway and downstream SMAD signaling. This pathway is known to control adipogenesis (58), fibrosis (13, 23), and myogenesis (25), while TGF-β signaling is also known to inhibit myogenesis (25) and acts to stimulate both adipogenesis and myofiber lipid accumulation. As participants in the present study had high-quality muscle, with a low lipid content, the identified miRNAs should be tested longitudinally to determine if they are related to the degree of muscle quality (attenuation) loss associated with aging.

Multiple miRNAs including miR-208b and -499 are encoded within the genes that code for different myosin isoforms and participate in the regulation of muscle fiber type composition (53). It has recently been demonstrated that in animal models

Table 7. *Pathway enrichment of miRNAs related to muscle attenuation*

Pathway	Genes, *n*	*P* Value	Regulated Genes
Molecular mechanisms in cancer	34/374	5.88×10^{-11}	*BAK1, BID, BMPR1B, CASP3, CASP6, CCND1, CCND2, CCNE2, CDC25A, CDKN1A, CDKN2A, E2F5, E2F6, FANCD2, FAS, FASLG, GNA13, GNAI2, JUN, KRAS, MAPK12, MYC, NRAS, PRKAR2A, RASA1, RB1, RHOB, SMAD1, TGFBR1, TGFBR2, TP53, WNT1, WNT5A, WNT9A*
STAT3 pathway	15/73	2.24×10^{-10}	*BMPR1B, CDC25A, CDKN1A, IGF1R, KRAS, MAP3K10, MAPK12, MYC, NRAS, SOCS1, SOCS3, SOCS4, TGFBR1, TGFBR2, TGFBR3*
Estrogen-mediated S-phase entry	9/24	2.82×10^{-9}	*CCNA1, CCND1, CCNE2, CDC25A, CDKN1A, E2F5, E2F6, MYC, RB1*
Cardiac hypertrophy signaling	22/235	8.89×10^{-8}	*ADRA2A, ADRB2, ADRB3, GNA13, GNAI2, GNG10, GNG5, IGF1R, IL6R, JUN, KRAS, MAP2K7, MAP3K10, MAP3K13, MAP3K2, MAPK12, NRAS, PPP3CA, PRKAR2A, RHOB, TGFBR1, TGFBR2*
IGF-1 signaling	14/106	3.2×10^{-7}	*CSNK2A1, CTGF, GRB10, IGF1R, IGFBP3, JUN, KRAS, NEDD4, NRAS, PRKAR2A, RASA1, SOCS1, SOCS3, SOCS4*

Generated with Ingenuity Pathway Analysis software.

miRNA expression varies by fiber type (42). In the current study, the ratio of type IIa to type I myosin heavy chain mRNA was used as a transcriptional marker of fiber type composition. miR-378a-5p and miR-125b-5p were identified as correlating with the ratio of type IIa to type I myosin heavy chain mRNA expression. miR-378a-5p is transcribed within the peroxisome proliferator-activated receptor-γ coactivator-1β gene, which regulates mitochondrial biogenesis (5). miR-378 transcript knockout in mice resulted in an increased oxidative phenotype consistent with the present data demonstrating greater miR-378-5p expression in those men with greater relative type II myosin expression (5). More work is necessary to elucidate definitively the role of myosin heavy chain isoform composition in explaining differences in miRNA expression; however, it is likely the miRNAs play an important role promoting different gene expression patterns in each myofiber type (42).

Consistent with Git et al. (14) we show a variable relationship between NGS and a multiplex-based PCR comparator method. Interestingly, all measured miRNAs that underwent qPCR were correlated, including miR-15a-5p, which was previously shown not to correlate with NGS data (14). Most of the miRNAs profiled display linear correlations between PCR and sequencing data across the breadth of measurements. However, for some miRNA targets the relationships became sigmoidal at either very low or very high expression levels. Although the PCR primers were validated as linear over a 1,000-fold variation range, it is still unclear if the lack of agreement between sequencing and PCR at high expression levels is a function of the amplification of the RNA that takes place before either PCR or sequencing (51).

The experimental design employed in the current study intended to generate novel hypotheses concerning which miRNAs and key molecular pathways might regulate adult muscular phenotype. The study design did not allow for the identification of direct cause-and-effect relationships and did not measure putatively regulated pathways at the level of gene or protein expression. The in silico pathway analysis that was performed is limited by the experientially validated targets reported in the Ingenuity database and may have provided different results if a higher proportion of the entries were related to that of healthy muscle (19). The relative abundances of human muscle miRNAs we report differ dramatically from studies that have employed Nanostring (44) arrays or different NGS library preparation methods (31) used previously to characterize the human muscle miRNAome. However, our results are comparable to a recent study using similar sequencing methods (37), suggesting that methodological choices, particularly library preparation (24), are likely to be important in replication our findings.

In conclusion, our study indicates that middle-aged men show a large variation in both muscle size and strength while displaying a relatively homogenous and high muscle quality indicated by a low lipid content. miRNA transcript levels represent potential molecular biomarkers of variations in muscle phenotype. miR-486-5p is by far the most abundantly expressed miRNA in human skeletal muscle, while traditional myomiRs are among the top 50 most highly expressed miRNAs in human muscle. miR-22-3p, -378a-3p, and -101-3p were highly expressed in the present cohort relative to reports in other tissues (26) and may fit within the myomiR definition (29). let-7f-5p, miR-30d-5p, and miR-125b-5p were found to be related to muscle quality and have putative roles in SMAD3 signaling and lipid accumulation. miR-100-5p, miR-99b-5p, and miR-191-5p related to muscle strength and may regulate muscle size by acting on the mTOR pathway. Future research should investigate the roles these miRNAs play in regulating muscle size and quality as well as their potential utility as biomarkers of muscle phenotype in populations with declining muscle function.

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AUTHOR CONTRIBUTIONS

C.J.M., J.M.O., S.D.P., and D.C.-S. conceived and designed research; C.J.M., R.F.D., and N.Z. performed experiments; C.J.M., W.S., and F.R. analyzed data; C.J.M. and W.S. interpreted results of experiments; C.J.M. prepared figures; C.J.M. drafted manuscript; C.J.M., R.F.D., W.S., N.Z., F.R., J.M.O., S.D.P., and D.C.-S. approved final version of manuscript; R.F.D., W.S., N.Z., F.R., J.M.O., S.D.P., and D.C.-S. edited and revised manuscript.

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