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MicroRNAs in Muscle: Characterizing the Powerlifter Phenotype

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Powerlifters are the epitome of muscular adaptation and are able to generate extreme forces. The molecular mechanisms underpinning the significant capacity for force generation and hypertrophy are not fully elucidated. MicroRNAs (miRs) are short non-coding RNA sequences that control gene expression via promotion of transcript breakdown and/or translational inhibition. Differences in basal miR expression may partially account for phenotypic differences in muscle mass and function between powerlifters and untrained age-matched controls. Muscle biopsies were obtained from m. vastus lateralis of 15 national level powerlifters (25.1 ± 5.8 years) and 13 untrained controls (24.1 ± 2.0 years). The powerlifters were stronger than the controls (isokinetic knee extension at $60°$/s: 307.8 ± 51.6 Nm vs. 211.9 ± 41.9 Nm, respectively $P < 0.001$), and also had larger muscle fibers (type I CSA $9,122 \pm 1,238$ vs. $4,511 \pm 798$ μm^2 $p < 0.001$ and type II CSA $11,100 \pm 1,656$ vs. $5,468 \pm 1,477$ μm^2 $p < 0.001$). Of the 17 miRs species analyzed, 12 were differently expressed ($p < 0.05$) between groups with 7 being more abundant in powerlifters and five having lower expression. Established transcriptionally regulated miR downstream gene targets involved in muscle mass regulation, including myostatin and MyoD, were also differentially expressed between groups. Correlation analysis demonstrates the abundance of eight miRs was correlated to phenotype including peak strength, fiber size, satellite cell abundance, and fiber type regardless of grouping. The unique miR expression profiles between groups allow for categorization of individuals as either powerlifter or healthy controls based on a five miR signature (miR-126, -23b, -16, -23a, -15a) with considerable accuracy (100%). Thus, this unique miR expression may be important to the characterization of the powerlifter phenotype.

Keywords: microRNA, resistance training, gene expression, skeletal muscle, mRNA

INTRODUCTION

Powerlifting is a competitive sport which requires maximal lifts for three multi-joint exercises. Powerlifters display an extreme capacity to generate muscle force relative to their body size. Self-selection and genetic predisposition undoubtedly play a part in becoming an elite powerlifter. However, extensive resistance training is required to make a competitive powerlifter (Ostrander et al., 2009; Pitsiladis et al., 2013). Regardless of the relative importance of genetics and training, powerlifters embody a unique phenotype, which can aid in understanding the molecular mechanisms which regulate muscle mass and strength.

Powerlifters are stronger relative to body size in comparison to untrained individuals. Theoretically, this greater strength is due to a combination of increased fiber area, altered muscular architecture and an improved ability to activate the muscle (Kawakami et al., 1993; Brechue and Abe, 2002; Matta et al., 2011). However, the relative importance of each of these factors is not fully elucidated (Edman, 1979; Narici et al., 1989).

Muscle hypertrophy occurs through a net accretion of muscle contractile proteins. Chronic resistance training promotes muscle anabolism via a complex interaction of multiple competing pathways (Marcotte et al., 2015). A number of myogenic regulatory factors including MyoD and MyoG are upregulated in individuals after chronic resistance exercise (Kosek et al., 2006) and are involved in myogenic remodeling and programming following exercise (Yablonka-Reuveni et al., 1999). Conversely, resistance exercise reduces expression of some genes which promote muscle degradation such as myostatin and atrogin1, while increasing the expression others such as MuRF1 (Zanchi et al., 2009; Fernandez-Gonzalo et al., 2013). Satellite cells, which are PAX7+ multipotent cells resident in the stem cell niche between mature muscle fibers and the basal lamina (Yin et al., 2013), are involved in the maintenance and repair of damaged fibers, while also being capable of fusing, to increase fiber number or supporting hypertrophy via nuclear addition (Petrella et al., 2008; McCarthy et al., 2011; Roberts et al., 2015). PAX7 mRNA and positive cells expression have been shown to increase with both acute and chronic resistance exercise (Nielsen et al., 2012; Bellamy et al., 2014; Nederveen et al., 2017).

Central in the coordinated regulation of gene expression are microRNAs (miRs), small non-coding RNAs, that selectively bind, inhibiting translation or promote degradation of targeted mRNAs (Ambros et al., 2003; Mathonnet et al., 2007; Townley-Tilson et al., 2010). Hundreds of different miRs are expressed in human tissue, with many found to be highly enriched in specific tissue types (Landgraf et al., 2007; Walden et al., 2009). To date only a few studies have examined the effect of anabolic stimuli such as resistance exercise or feeding on muscle miR profiles (Drummond et al., 2008; Davidsen et al., 2011; Tonevitsky et al., 2013; Zacharewicz et al., 2013). Of these analyses, 8 miRs (miR-1, -133a, -133b, -206, -208a, -208b, -486, and -499a) have been consistently identified as myogenic miRNAs (myomiRs) that are vastly more abundant within skeletal muscle compared to other tissues (McCarthy and Esser, 2007; van Rooij et al., 2009; Small et al., 2010). These myomiRs have been implicated in a variety of roles within muscle. For example, miR-1, -133a, and -206, are all involved in the regulation of Pax7 and are themselves regulated by downstream genes such as MyoD and MyoG (Chen et al., 2006; Ikeda et al., 2009; Braun and Gautel, 2011). miR-208a, -208b, and 499a inhibit myostatin and thus are important in muscle catabolism (Drummond et al., 2009; Hitachi and Tsuchida, 2014). In addition, miR-499a inhibits Sox-6 expression which plays a role in the conversion between muscle fiber types (McCarthy, 2011).

Further to these established myomiRs, there is evidence from either myogenic cell lines, animal and human studies, of other miRs that may exert crucial roles in control of muscle hypertrophy, atrophy, myogenesis, and apoptosis via cell cycle regulation (Muscat and Dressel, 2000; Naguibneva et al., 2006; Fish et al., 2008; Wang et al., 2008; Williams et al., 2009; Aqeilan et al., 2010; Nakasa et al., 2010; Nan et al., 2010; Dey et al., 2011; Sun et al., 2012). From this increasing literature, only those miRNAs that have been demonstrated from both mechanistic analysis in cell lines and in-vivo were selected as being key candidates for analysis. This included an additional subset of 9 miRs [miR-30b (Naguibneva et al., 2006; Nielsen et al., 2010), -148b (Li and Xi, 2011; Gastebois et al., 2016), -145 (Cordes et al., 2009; Sachdeva et al., 2009), -23a (McCarthy, 2014b; Kapchinsky et al., 2015), -23b (Wang, 2013; Režen et al., 2014), -126 (Fish et al., 2008; Wang et al., 2008), -15a, -16 (Bandi and Vassella, 2011; Musumeci et al., 2011; Sun et al., 2012; Yin et al., 2012), and -451a (Mercken et al., 2013; Zang et al., 2015)].

Powerlifters are capable of generating very high relative muscular forces due to greater muscle size and quality than the general population (MacDougall et al., 1982). It is unknown if the basal expression of miRs differs between powerlifters and healthy controls. Further, it is unclear if miR expression may partially regulate the expression of genes related to the regulation of muscle mass. Hence, the aim of this study was to quantitatively analyse using real-time PCR, the regulation of the extended list of key myomiRs and key myogenic regulatory mRNA species in both elite powerlifters and healthy age matched controls. It was hypothesized that muscle specific miRs with defined roles in muscle mass regulation are differentially expressed between these distinctive muscular phenotypes, and that these differences will relate to the expression of downstream mRNAs.

METHODS

Participants

Thirteen recreationally active young students 24.3 ± 1.8 years and 15 elite Norwegian Powerlifters aged 23.5 ± 3.1 years were recruited (**Table 1**). Exclusion criteria were any injuries of the musculo-skeletal system that could prevent the participant from exerting maximal force, use of medication and the use of anabolic steroids. Subjects were asked to refrain from any strenuous exercise for 72 h prior to the study day. The study was complied with the standards set by the Declaration of Helsinki and was reviewed by the Regional Committee for Medical and Health Research Ethics (REC South-East). The nature and goals of the study were thoroughly explained, and all subjects provided a written informed consent.

TABLE 1 | Participant Characteristics.

	Healthy controls	Power lifters
Age (years)	24.3 ± 1.8	23.5 ± 3.1
Height (m)	178.6 ± 7.4	176.4 ± 7.1
Weight (kg)	77.4 ± 12.1	94.8 ± 16.7
BMI (kg/m^2)	24.1 ± 2.3	30.4 ± 4.3

Values presented as means $\pm$ SD, n = 13 controls and n = 15 power lifters.

Muscle Strength

Isokinetic torque of the knee extensor was measured at 60° per second over a range of 70° (from 20° to 90° when 0° is fully extended) using a dynamometer (HUMAC 2009NOMR CSMi. Testing and Rehabilitation System, USA). Participants were strapped to the dynamometer chair with two belts crossing over their chest. Hands were placed on these belts to ensure isolation of knee extensor muscles. Participants repeated the test three times after four warm up attempts with strong verbal motivation from the same individual with the best value being recorded. To correct for variance between body size between groups, peak torque was normalized to height as a form of allometric scaling of strength as proposed by Jaric (2003).

Muscle Biopsy Sampling

Muscle biopsies (200–300 mg) were obtained from m. vastus lateralis using a 6 mm sterile Bergström needle under local anesthesia (Xylocain-adrenaline, 10 mg/ml +5 mcg/mL, AstraZeneca, Södertälje, Sweden). Connective tissue and fat were dissected away before a bundle of fibers identified for later immunohistochemical analyses was mounted in OCT Embedding Matrix (Tissue-tek, O.C.T. compound, Sakura, USA) and immediately frozen in isopentane, which was pre-cooled ($\sim -140°C$) with liquid nitrogen and stored at $-80°C$ for later analysis. A ~ 20 mg piece was snap frozen in liquid nitrogen for RNA analysis.

miR/mRNA Isolation

Total RNA was extracted from ~ 20 mg of muscle tissue using the AllPrep® DNA/RNA/miRNA Universal Kit (QIAGEN GmbH, Hilden, Germany) following the manufacturer's instructions as described by Figueiredo et al. (2016).

miR cDNA/RT-PCR

10 ng of input RNA was used for cDNA synthesis using TaqMan™ Advanced miRNA cDNA Synthesis Kit (Thermo Fisher Scientific, Carlsbad, CA, USA), miR abundance were measured by RT-PCR on a QuantStudio 6 (Thermo Fisher Scientific, Carlsbad, CA, USA) using Applied Biosystems Fast Advanced Master Mix (Thermo Fisher Scientific, Carlsbad, CA, USA).

Target miRNAs were miR-15a-5p, -23a-5p, -23b-5p, -499a-3p, -206, -208a-3p, 208b-3p, -451a, -486-5p, -126-3p, -1-3p, -133a-3p, -133b, -148b-3p, -30b-3p, -145-5p, and -16-5p Thermo Fisher Scientific, Cat# A25576, Carlsbad, CA, USA) (Supplementary Table 1). The geometric mean of three reference miRNAs (miR-361-5p, -320a, -186-5p; Vandesompele et al., 2002) was used for normalization based on miRNAs that showed the least variation amongst the participants between and within groups. The abundance of miRs were measured using the $2^{(-\Delta CT)}$ method (Schmittgen and Livak, 2008).

mRNA cDNA /RT-PCR

1500 ng of input RNA was used for cDNA synthesis using the High–Capacity RNA-to-cDNA™ kit (Life Technologies, Carlsbad, CA). Messenger RNA (mRNA) was measured by RT-PCR on a LightCycler 480 II (Roche Applied Science, Penzberg, Germany) using SYBR Green I Master Mix (Roche Applied Science). Target mRNAs were decided based on previously published pubmed literature that associated targeted miRs with muscle regulatory genes. These included, cMYC, Myogenic Differentiation 1 (MyoD), Forkhead Box O3 (FOXO3), PAX7, Cyclin D1 (CCND1), Myogenin (MyoG), Neural Cell Adhesion Molecule (NCAM), Atrogin-1, Muscle-Specific RING Finger Protein 1 (MuRF1), Vascular Endothelial Growth Factor (VEGF), Myostatin, Histone Deacetylase 4 (HDAC4), Bone Morphogenetic Protein 2 (BMP2), SRY (Sex Determining Region Y)-Box 6 (SOX6), Phosphatase and tensin homolog (PTEN), Serum response factor (SRF), SPRED1, PAX3, and Forkhead Box 01 (FOXO1). Primers were designed using BLAST software (sequences in Supplementary Table 2). The geometric mean of four reference genes was used for normalization (Vandesompele et al., 2002). The recently proposed human reference genes (Eisenberg and Levanon, 2013), endoplasmic reticulum membrane protein complex subunit 7 (*EMC7*), valosin-containing protein (VCP), charged multivesicular body protein 2A (*CHMP2A*), and chromosome 1 open reading frame 43 (*C1orf43*) were identified as the least variable and therefore, used as reference genes (Supplementary Table 3). Standard and melting curves were performed for every target to confirm primer efficiency and single-product amplification. The abundance of mRNAs were measured using the $2^{(-\Delta CT)}$ method (Schmittgen and Livak, 2008).

Immunohistochemical Staining

Muscle biopsies were cut to 8 μm thick cross sections at $-20°C$ using a cryostat (CM 1950, Leica Biosystems GmbH, Nussloch, Germany) and mounted on microscope slides (Superfrost Plus, Menzel-Glaser, Brouschweig, Germany). Glass slides from the freezer were air dried at room temperature for 10 min and a PAP-pen (OmmEdge PEN. Vevtor Laboratories, Inc) was used to draw a lipid ring around sections. Next, primary antibodies and stains were applied for 45 min incubation in 1% Bovine Serum Albumin (BSA) (Dako, 10082504) and PBS-t solution (QC213624, Thermo Fisher Scientific, Carlsbad, CA, USA). BSA was removed and the slide dried using lint-free paper towel (Kimtect Science, Precision Wipes Tissue Wipers) to prepare for secondary antibody application. Specific secondary antibodies [Alexa-488 goat anti-mouse (Biotium, Inc, Hayward, CA, USA, 1:200) and CF-594 goat anti-rabbit (Biotium, 1:200) were applied after each primary antibody. Sections were mounted with a fluorescent anti-fade containing DAPI (for nuclear staining) (Invitrogen, 1266174, Life Technologies, Denmark, Naerum, Denmark) and coverslip were pasted together with slides and stored protected from light in a fridge (5°C). Satellite cells were visualized with an antibody against PAX7 (DSHB, 1:100) and laminin (Dako, 20025756, 1:400) as well as DAPI-stains (for nuclear staining) (Invitrogen, 1266174, Life Technologies, Denmark, Naerum, Denmark), while serial sections for MCH-II (DSHB, 1:1,000) and dystrophin (ABCam, ab15277), 1:500] and were added onto a separate slide for distinction of the myofiber border and myofibers type II.

Stained biopsies were visualized on a computer screen using a light microscope (Olympus BX61, Japan) connected to a

fluorescent light (EXFO, Xl120PC-Q, Canada) and was used to quantify the sections. The microscope was also connected to a digital camera (Olympus DP72, Japan) that took pictures at 20x zoom of the sections. All morphometric analysis was performed in Cell-F (Olympus, Japan), TEMA (ChekVision, Hadsund, Denmark) and ImageJ (version 2.0.0-rc-41/1.5 d, National Institutes of Health, Bethseda, MD, USA). Type I (unstained) and type II (stained) myofibers were differentiated, and myofiber area was determined. On average 543 ± 241 myofibers were analyzed per biopsy sample for the assessment of muscle fiber area. Satellite cells and myonuclei were identified using the following criteria: SC had to stain positive for PAX7 and be placed within the basal lamina; nuclei with a subordinate placement were considered myonuclei. The number of PAX7 positive satellite cells and myonuclei are presented relative to the number of type I and II myofibers. Myonuclear domain is expressed as the area of each fiber type supported by a single myonucleus. A total of 50 myofibers for each fiber type were analyzed for quantification of myonuclei, in accordance with previous methods (Mackey et al., 2009). The same investigator performed all analyses manually.
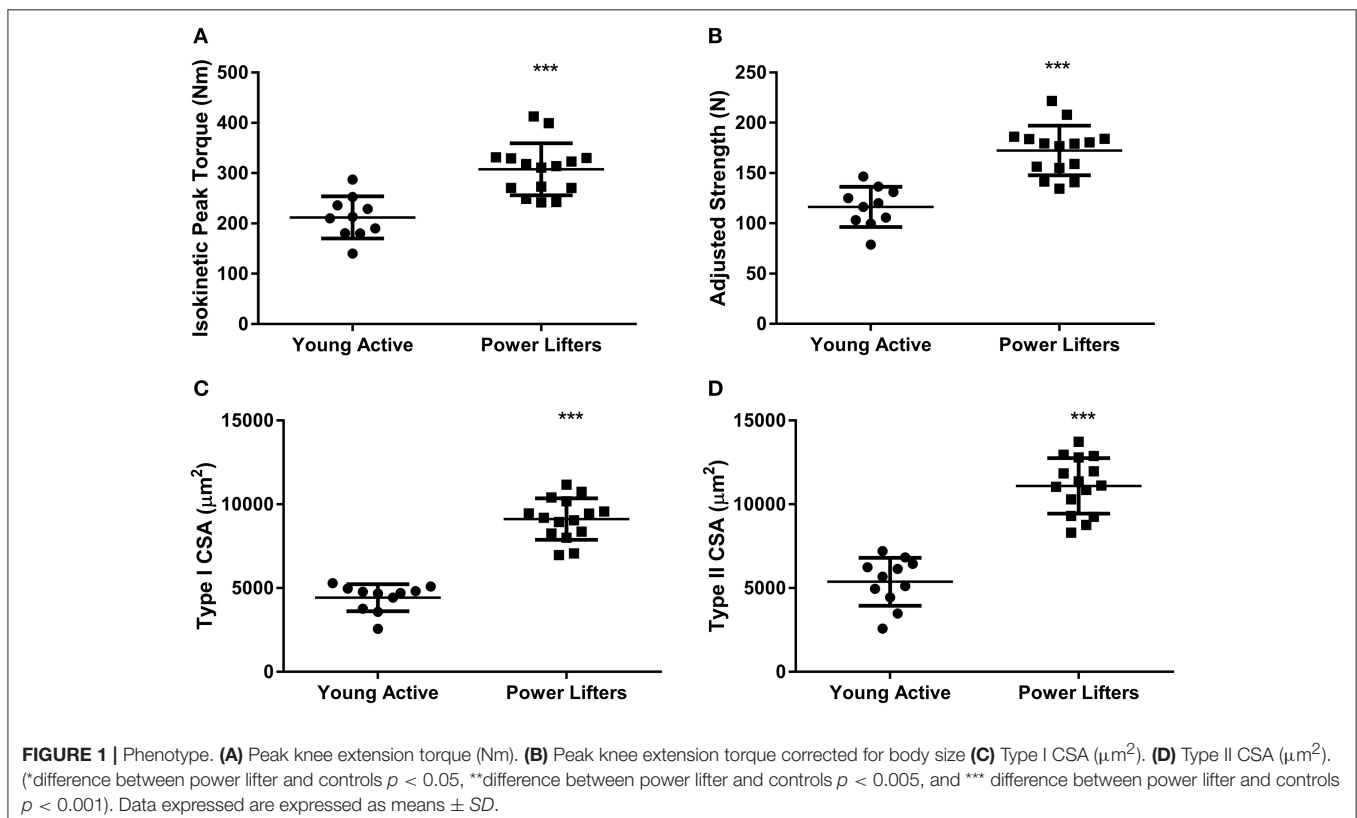
Statistical Analysis

Statistical analysis was performed using Graph Pad Prism Software (GraphPad Software Inc., La Jolla, CA). Differences between controls and powerlifters were determined using Student's t-test. Multiple comparison corrections were undertaken using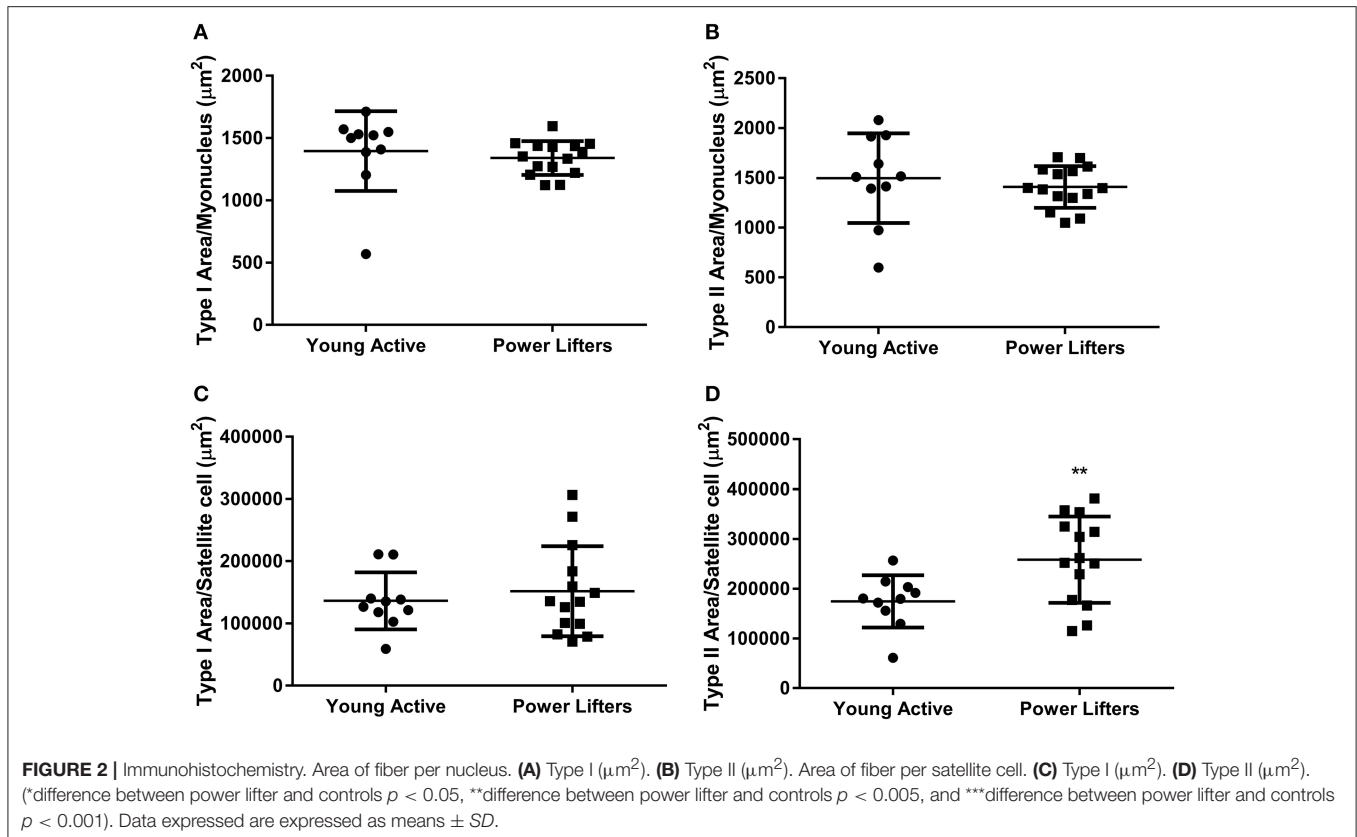 false discovery rates where $p < q$ was determined as significant with alpha set at $p < 0.05$ (Supplementary Table 4). Linear regression was performed using measures of phenotype including peak strength, fiber CSA, fiber area per satellite cell and fiber type as dependant variables with miRs as independent variables. Only miRs which independently correlated with the dependant variable and did not correlate with expression of any miRs already in the model were included in the linear regression models. Step wise discriminant analyses was performed using IBM SPSS for Windows Version 23 (IBM Corp. USA) to determine the ability of miRs to distinguish between powerlifters and controls, as per (Margolis et al., 2016). Receiver operating characteristic curves (Prism software) were then used to determine the area under the curve for each of the potential of miR-126, -23a, -16, -23a, and -15a in order to correctly identify the powerlifter phenotype. Prism software was also used to generate graphs. Data are shown as means ± SD. Statistical significance was accepted at $p < 0.05$.

RESULTS

Skeletal Muscle Strength and Histology

The powerlifters were stronger ($p < 0.001$) than the healthy control group. This difference was maintained when corrected for body size ($p < 0.001$; **Figures 1A,B**). Powerlifters also had larger muscle fibers ($p < 0.001$) of both fiber types (**Figures 1C,D**). The area per myonucleus (myonuclear domain) was not different between groups irrespective of fiber type

FIGURE 1 | Phenotype. **(A)** Peak knee extension torque (Nm). **(B)** Peak knee extension torque corrected for body size **(C)** Type I CSA (μm^2). **(D)** Type II CSA (μm^2). (*difference between power lifter and controls $p < 0.05$, **difference between power lifter and controls $p < 0.005$, and *** difference between power lifter and controls $p < 0.001$). Data expressed are expressed as means ± SD.

FIGURE 2 | Immunohistochemistry. Area of fiber per nucleus. **(A)** Type I (μm^2). **(B)** Type II (μm^2). Area of fiber per satellite cell. **(C)** Type I (μm^2). **(D)** Type II (μm^2). (*difference between power lifter and controls $p < 0.05$, **difference between power lifter and controls $p < 0.005$, and ***difference between power lifter and controls $p < 0.001$). Data expressed are expressed as means $\pm$ SD.

(type I $p = 0.555$ and type II $p = 0.515$; **Figures 2A,B**). No difference between area of type I fibers per satellite cell was seen between groups ($p = 0.560$) however, the powerlifters had increased type II area per satellite cell ($p = 0.007$; **Figures 2C,D**; **Table 2**).

miR and Gene Expression

Five of the seven myomiRs measured were differentially abundant between groups. Four were elevated (miR-486 $p = 0.003$, -499a $p = 0.012$, -133a $p < 0.001$ and -1 $p = 0.008$), one lower (miR-206 $p = 0.009$) in the healthy controls compared to the powerlifters, two were similarly expressed (miR-208a $p = 0.71$ and -208b $p = 0.496$) between groups. MyomiRs have putative roles in the control of known muscle mass regulators such as PAX7 ($p < 0.001$), PAX3 ($p = 0.008$), MyoD ($p = 0.011$), Myostatin ($p < 0.001$), MyoG ($p = 0.002$), HDAC4 ($p = 0.001$), SRF ($p < 0.001$), and SOX6 ($p = 0.008$) which were all more abundant in the powerlifters while PTEN ($p = 0.302$) transcript abundance was not different between groups (**Figure 3**).

miR-15a ($p < 0.001$), -16 ($p = 0.016$), and -451a ($p = 0.017$) were elevated in the powerlifters compared to the healthy controls (**Figures 4A–C**). Downstream target cyclin D1 ($p = 0.198$) was not different between groups while D2 ($p = 0.019$) (**Figure 4H**) was less abundant in the powerlifter group. Futhermore, miR-15a and -16 have reported roles in inhibiting angiogenesis via action on VEGF which trended toward being

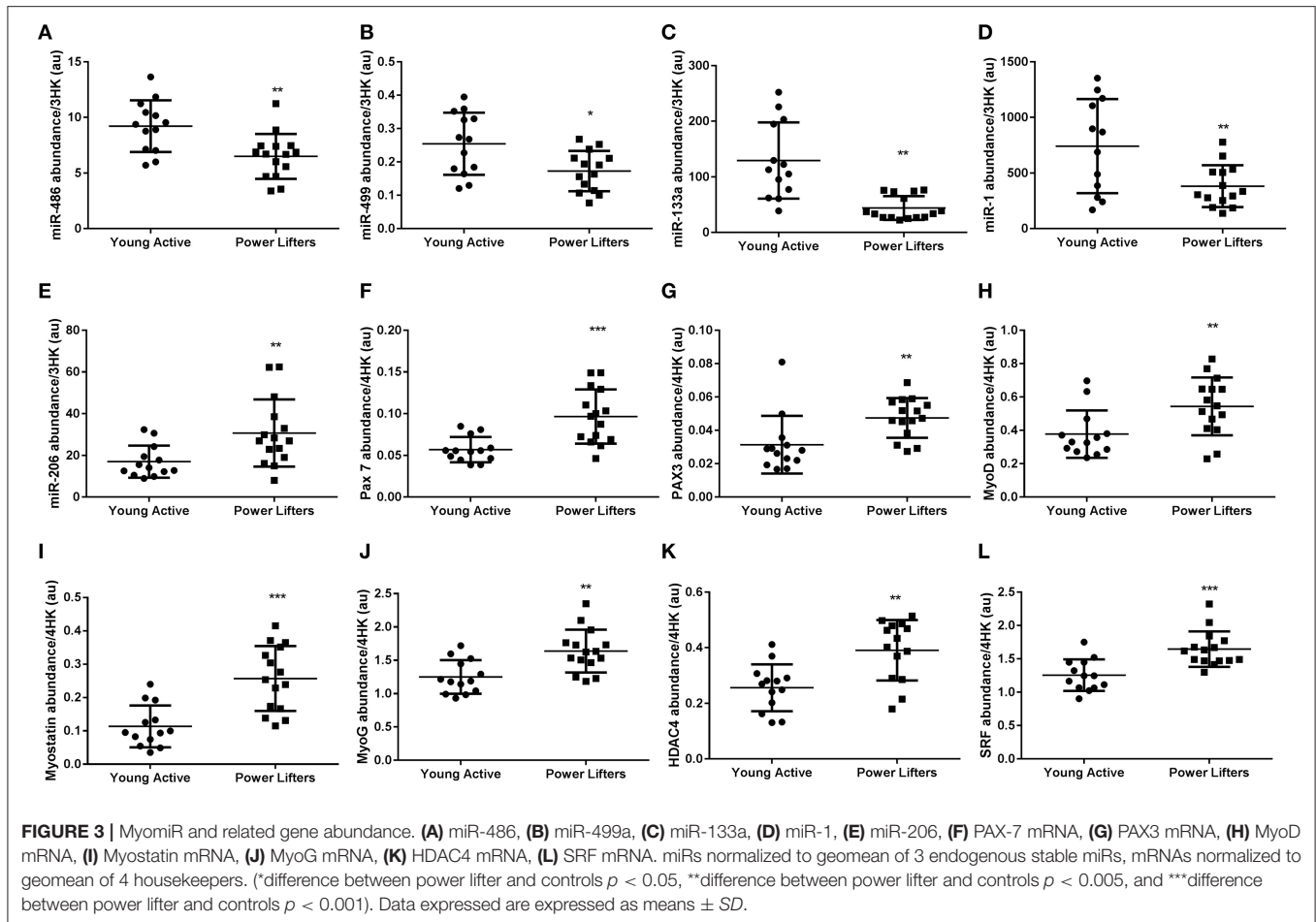**TABLE 2 |** Myonuclei and satellite cells.

	Healthy controls	Power lifters	p-values
Fiber ratio (Type I : II)	1.1 ± 0.4	0.8 ± 0.3	0.022*
Myonuclei/Type I Fiber	3.3 ± 0.4	6.8 ± 1.3	<0.001*
Myonuclei/Type II Fiber	3.6 ± 0.4	7.8 ± 0.9	<0.001*
Satellite cell/Type I Fiber	0.036 ± 0.007	0.071 ± 0.033	0.003*
Satellite cell/Type II Fiber	0.036 ± 0.014	0.049 ± 0.021	0.111

Values presented as means $\pm$ SD, n = 13 controls and n = 15 power lifters. *difference power lifters and controls $p < 0.05$.

more abundant in powerlifters ($p = 0.064$; **Figure 4I**). miR-145 showed no difference between groups ($p = 0.730$).

miR-23a ($p < 0.001$) and -23b ($P < 0.001$) are both inhibitors of catabolic gene expression (Wada et al., 2011) and were found to be elevated in the powerlifter group (**Figures 4D,E**). Catabolic genes FOXO1/3 ($p = 0.244$, $p = 0.213$) were unchanged between groups while MuRF1 ($p = 0.013$) and Atrogin1 ($p = 0.004$) all showed increased expression in powerlifters compared to controls (**Figures 4J,K**). The upstream regulator c-MYC was expressed similarly between groups ($p = 0.693$).

miR-30b expression ($p = 0.013$) was higher in powerlifters compared to controls (**Figure 4F**). miR-126 ($p < 0.001$) had lower expression in powerlifters compared to controls (**Figure 4G**). SPRED-1 ($p = 0.083$), an inhibitory target of miR-126 upstream of VEGF showed no difference between groups.

FIGURE 3 | MyomiR and related gene abundance. (A) miR-486, **(B)** miR-499a, **(C)** miR-133a, **(D)** miR-1, **(E)** miR-206, **(F)** PAX-7 mRNA, **(G)** PAX3 mRNA, **(H)** MyoD mRNA, **(I)** Myostatin mRNA, **(J)** MyoG mRNA, **(K)** HDAC4 mRNA, **(L)** SRF mRNA. miRs normalized to geomean of 3 endogenous stable miRs, mRNAs normalized to geomean of 4 housekeepers. (*difference between power lifter and controls $p < 0.05$, **difference between power lifter and controls $p < 0.005$, and ***difference between power lifter and controls $p < 0.001$). Data expressed are expressed as means ± SD.

Correlation Analysis

miRNAs were correlated against phenotype with both groups combined. With multiple regression analyses, we see a range of significant in relationships as described in **Table 3**.
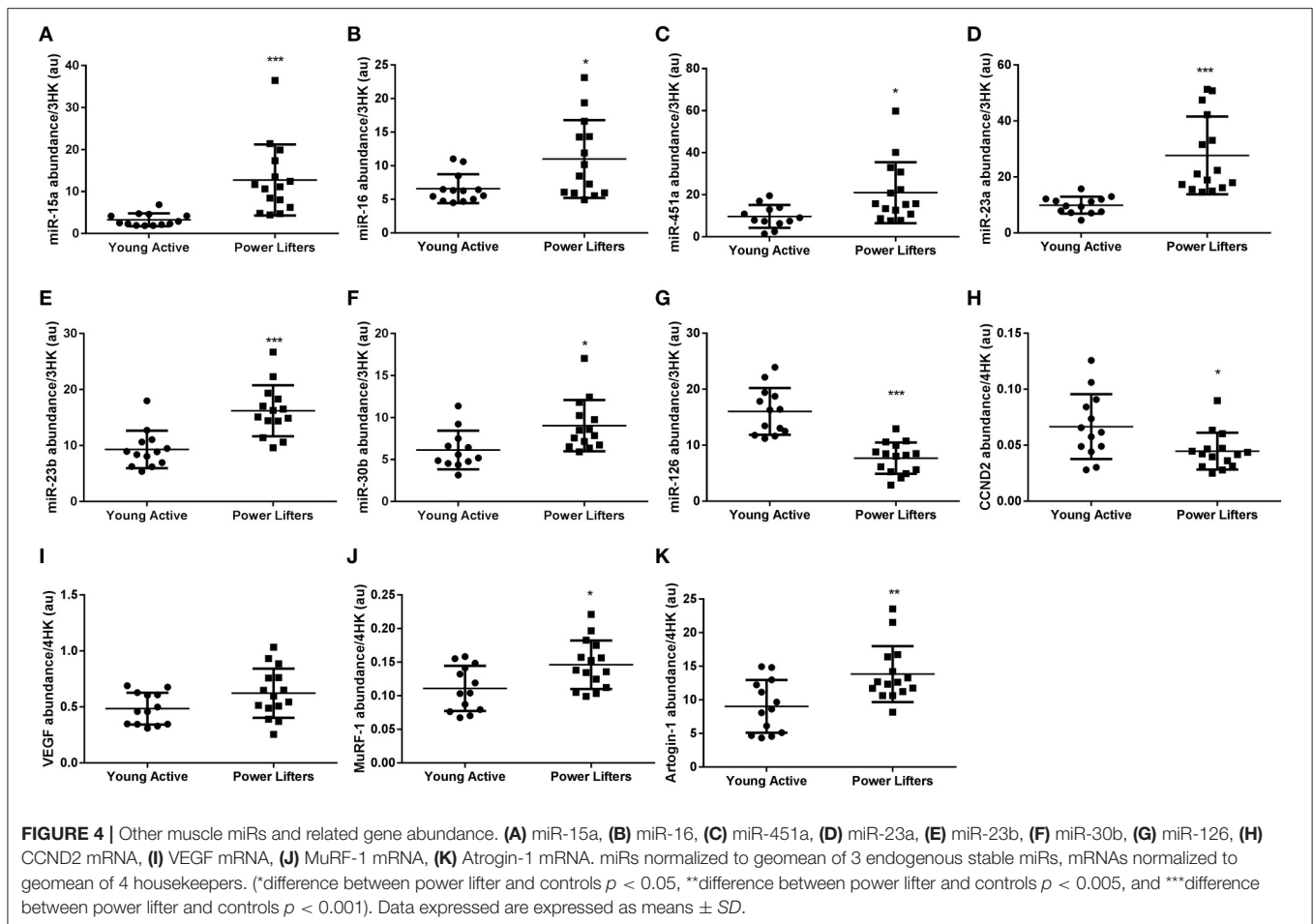
Discriminant Analysis

Of the 17 miRs analyzed, stepwise discriminant analyses revealed that the combination of five miRs (miR-126, -23b, -16, -23a, and 15a) correctly classified 100% of participants as powerlifters or controls. Receiver operator characteristic curves were then used to measure the sensitivity and specificity of these miRs (**Figure 5**). miR-126 differentiated powerlifters with an area under curve of 0.98, sensitivity 93% and specificity 100%. miR-23b distinguished powerlifters with an area under curve of 0.91, sensitivity 85.7% and specificity 84.6% whereas miR-16 had an area under curve of 0.74 with sensitivity 85.7% and specificity 46.2%. miR-23a had an area under curve of 0.98 with sensitivity 100% and specificity 92.3% while miR-15a had an area under the curve of 0.96 with a sensitivity of 93.3% and specificity of 84.6%.

DISCUSSION

Powerlifters represent an extreme muscular phenotype with muscle fibers double the size of controls and the ability to exert ~45% more torque. These athletes provide a unique opportunity to study how differences in muscle miR and gene expression may regulate the maintenance of the extreme increases in muscle size and strength. A targeted approach was used to analyse miRNA species that have previously been shown to be both differentially regulated within skeletal muscle, corresponding to a change in phenotype, and further have been established to be involved in the mechanistic control of muscle-regulatory genes. Of the 17 miRs analyzed, a total of 12 miRs were differently expressed in the biopsied skeletal muscle samples between powerlifters and healthy controls with miR-126, -23b, -16, -23a and -15a showing the greatest separation between groups. The different miR expression patterns in powerlifters have putative roles in the control of fiber type, protein turnover, muscle remodeling, and angiogenesis.

Chronic resistance training results in increases in fiber area, alteration in muscle architecture and improvements in neural drive which are associated improvements in peak torque (Kawakami et al., 1993; Brechue and Abe, 2002; Matta et al., 2011). The present study demonstrated that both muscle fiber types in powerlifters are approximately two-fold larger than those in healthy controls. This is in contrast to previous analyses indicating a 10 week period of resistance training produces ~12.2% increases in fiber CSA (Häkkinen et al., 1998). The

FIGURE 4 | Other muscle miRs and related gene abundance. **(A)** miR-15a, **(B)** miR-16, **(C)** miR-451a, **(D)** miR-23a, **(E)** miR-23b, **(F)** miR-30b, **(G)** miR-126, **(H)** CCND2 mRNA, **(I)** VEGF mRNA, **(J)** MuRF-1 mRNA, **(K)** Atrogin-1 mRNA. miRs normalized to geomean of 3 endogenous stable miRs, mRNAs normalized to geomean of 4 housekeepers. (*difference between power lifter and controls $p < 0.05$, **difference between power lifter and controls $p < 0.005$, and ***difference between power lifter and controls $p < 0.001$). Data expressed are expressed as means ± SD.

increased myonuclear content in powerlifters were offset by increases in fiber size showing support for myonuclear domain theory in both fiber types (Hawke, 2005; Petrella et al., 2006; Jackson et al., 2012). Whilst area per satellite cell for type I fibers was not different between groups, area per satellite cell in type II fibers was significantly higher (∼1.6-fold) in the powerlifters. In studies of untrained individuals who undertook 2–4 months of resistance training resulting in muscle hypertrophy, satellite cells per fiber area were seen to increase (Kadi and Thornell, 2000; Olsen et al., 2006). This finding is inconsistent with the powerlifters in the present study. The greater type II fiber area per satellite cell in the powerlifters may represent an adaptive ceiling not observed in shorter duration training studies.

The human genome has been shown to encode at least 1,881 non-coding miRNAs (miRbase.org Version 21). Amongst these, the importance of a subset have been identified as playing crucial roles in the myogenesis, hypertrophy, and atrophy of skeletal muscle (Winbanks et al., 2013; Hitachi and Tsuchida, 2014; Soriano-Arroquia et al., 2016). Most of these appear to exert conserved roles across species and have functions from the early phases of myogenesis, from stem cell differentiation through to myofiber atrophy. Within these a subset commonly identified as myomiRs are highly expressed within skeletal muscle (Kovanda et al., 2014) and have reported

roles in skeletal muscle maintenance processes (McCarthy, 2014a). We have identified a number of differences in miR expression between powerlifters and controls which through transcriptional regulation (**Figure 6**) may partially explain the divergent powerlifter phenotype. Several miRNAs were found to correlate with strength, fiber size, type II fiber area per satellite cell and type I:II fiber ratios and area. miR 133a and -486 were significantly correlated against strength and fiber size irrespective of fiber size. miR-206, -1, and 16 were correlated to fiber size alone.

For example, miR-499a has been shown to repress SOX6, a key inhibitor of type II to type I fiber conversion (McCarthy et al., 2009; Quiat et al., 2011; Yeung et al., 2012). Powerlifters showed a greater proportion of type II fibers, reduced miR-499a expression and an increased abundance of SOX6. It is likely that the reduced miR-499a expression allows for a greater SOX6 expression that may support the maintenance of a larger proportion of type II fibers. Since the powerlifter group expressed lower type I/II fiber ratios (0.8 vs. 1.1) they have an abundance of type II fibers compared to controls. This is consistent with other studies that have analyzed the fiber shifts associated with consistent resistance training (Tesch and Karlsson, 1985; Staron et al., 1990) but could also be the result of self-selection to the sport of powerlifting by individuals with a pre-existing

TABLE 3 | Phenotype vs. miR expression correlations.

	R^2	β	P-value
Strength (miR-133a)	0.368	−0.606	0.001
Corrected strength	0.47	–	0.001
miR-133a	–	−0.568	0.001
miR-486	–	−0.273	0.043
Type I CSA	0.63	–	<0.001
miR-206	–	0.446	0.002
miR-16	–	0.269	<0.001
miR-133a	–	−0.398	0.011
Type II CSA	0.668	–	<0.001
miR-206	–	0.351	0.007
miR-133a	–	0.446	0.018
miR-486	–	−0.277	<0.001
miR-1	–	−0.11	0.034
Type II Area/SC (miR-23b)	0.354	0.595	0.003
Fiber type ratio (miR-126)	0.324	0.569	0.002
Fiber type area ratio	0.337	–	0.011
miR-126	–	0.385	0.009
miR-145	–	0.399	0.029

abundance of type II fibers. We also find miR-126 that was less abundant in the powerlifters correlated to fiber ratios for cell population and for area representation. Previously it was reported that miR-126 may not be specific to type I fibers (Davidsen et al., 2011). As mentioned earlier since powerlifters have lower type fiber ratios, the idea that miR-126 is type I specific may warrant further analyses. miR-499a is also known to inhibit myostatin, which is a negative regulator of muscle mass (Lee and McPherron, 2001; Whittemore et al., 2003; Drummond et al., 2009). Powerlifters showed more abundant expression of this gene. Myostatin is an upstream regulator of mass accretion that through SMAD3 signaling, can directly inhibit muscle growth and differentiation pathways governed by PAX3/7, MyoD, MyoG, and SRF (Sakuma and Yamaguchi, 2012). The expression of these anabolic factors was significantly higher in powerlifters. Previous training studies indicate a down regulation of myostatin while positive regulators of mass, like MyoD and MyoG, are increased (Kosek et al., 2006; Zanchi et al., 2009; Fernandez-Gonzalo et al., 2013). However, the powerlifters in the present study have undergone years of rigorous training and may not be directly comparable to individuals who have undergone only 2–4 months of resistance training. The expression of other muscle catabolism markers MuRF1 and Atrogin1 showed greater abundance in the powerlifters despite upregulation of their direct inhibitors miR-23a/b. Together these data may be explained by an increased rate of muscle protein turnover in powerlifters compared to controls. It has previously been demonstrated that resistance training increases rates of muscle protein synthesis (Phillips et al., 2002), it is thus conceivable that increase in expression of genes related to both anabolism and catabolism would underpin a greater resting muscle protein turnover in powerlifters as well. It is also possible that the greater expression of catabolic genes such as myostatin, MuRF1 and Atrogin1 in the powerlifters represents a limit to

hypertrophic adaptation following long term training which is not seen individuals with lower training ages (Roth et al., 2003).
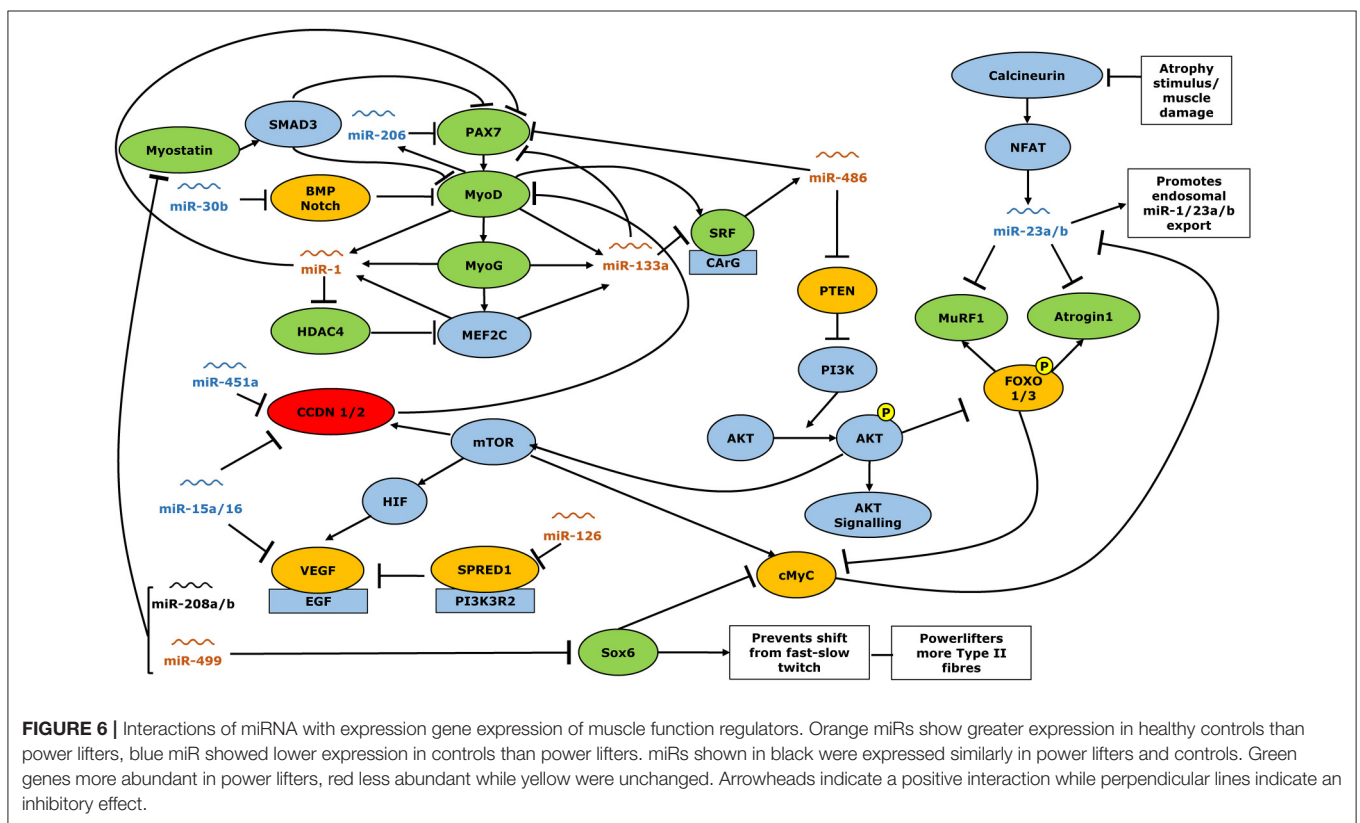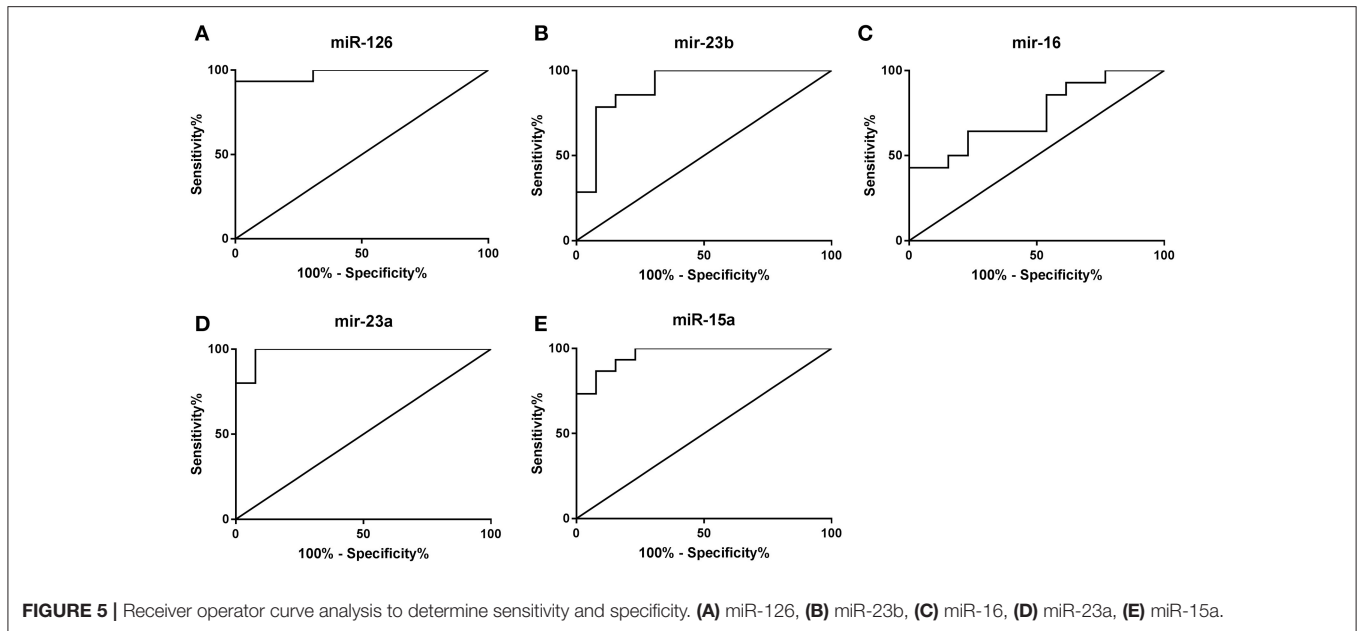
PAX3/7, MyoD, and MyoG are key components of the myogenesis pathway (Parker et al., 2003; Buckingham and Rigby, 2014) and are more abundantly expressed in powerlifters than the healthy controls. They have been shown to be involved in muscle repair and regeneration in various injury models as well as following resistance exercise (Chakkalakal et al., 2012; Urciuolo et al., 2013). PAX7 protein concentrations are downregulated by miR-1, -133a, -206, and -486 (Chen et al., 2006; Braun and Gautel, 2011). All except miR-486 are transcribed in response to increased MyoD and MyoG expression (Rosenberg et al., 2006; Braun and Gautel, 2011) and act to provide a negative feedback mechanism. miR-1 and -133a were found to be less abundant in powerlifters. The reduced abundance of miR-1 and miR-133a in powerlifters may explain higher HDAC4 and SRF expression, respectively. SRF is expected to promote miR-486 activity via MyoD (Chen et al., 2006; Dey et al., 2011) instead, we observed a lower expression of miR-486 despite elevated SRF expression. miRs -1, -133a, and -486 all directly or indirectly proved negative feedback to the processes of muscle repair and regeneration, despite MyoD and MyoG expression. miRs -1, -133a, and -486 remained down regulated in powerlifters compared to controls. This suggests a complex interaction between multiple miRs and genes related to muscle regeneration.

The cell cycle regulators Cyclin D1 and D2 directly inhibit MyoD and are themselves directly inhibited by miR-451a, -15a, and -16 (Muscat and Dressel, 2000; Aqeilan et al., 2010; Nan et al., 2010). These miRs were more abundant in the powerlifters, perhaps acting to support increased myogenesis and muscle remodeling. CCND2 gene had lower expression in powerlifters likely resulting from the increased expression of its inhibitors miR-451a, -15a, and -16.

Powerlifters exhibited no difference in expression of the proangiogenic gene VEGF which is partly regulated via the mammalian target of rapamycin (mTOR) pathway (Wan et al., 2006). Resistance training induces angiogenesis to support hypertrophy but on its own does not normally increase capillary density (Nederveen et al., 2017). The direct inhibitors of VEGF, miR-15a/16 were however, upregulated in the powerlifters, which may limit angiogenesis in powerlifters. miR-126 which increases VEGF expression via Sprouty-related protein (SPRED1) inhibition (Fish et al., 2008) was lower in powerlifters with no differences in SPRED1 expression between group. The direct regulation of angiogenesis by resistance training via miRs requires further elucidation.

The discriminant analysis identified miR-126, -23b, -16, -23a, and -15a as strong determinants of the powerlifter phenotype, correctly categorizing participants with 100% accuracy. Each of these five miRs independently, correctly group individuals by expression with at least 74% accuracy. The five identified miRs could be investigated further as markers of training adaptation.

The powerlifter phenotype is characterized by dramatic differences in muscle fiber size and force generation capacity when compared to age matched untrained controls. Regulation of mRNA via miR provides a likely mechanism which may

FIGURE 5 | Receiver operator curve analysis to determine sensitivity and specificity. **(A)** miR-126, **(B)** miR-23b, **(C)** miR-16, **(D)** miR-23a, **(E)** miR-15a.

FIGURE 6 | Interactions of miRNA with expression gene expression of muscle function regulators. Orange miRs show greater expression in healthy controls than power lifters, blue miR showed lower expression in controls than power lifters. miRs shown in black were expressed similarly in power lifters and controls. Green genes more abundant in power lifters, red less abundant while yellow were unchanged. Arrowheads indicate a positive interaction while perpendicular lines indicate an inhibitory effect.

underpin these phenotypic differences. However, because protein expression was not measured it is not possible to draw definite conclusions about how miRs might alter phenotype. The design of the study did not allow for the delineation of specific effects of long term resistance training and genetic endowment. Undoubtedly both play a role but it is probable that the majority

of the observed effects are due to a long history of high intensity resistance training.

The present study indicates maintenance of the distinct powerlifter phenotype may be modulated by robust differences in miR expression profiles at rest when compared to healthy controls. Specifically, miR-126, -23b, -16, -23a, and -15a

discriminate accurately between powerlifters and controls. Differences in miR expression are involved in the regulation of downstream mRNA but can also themselves be regulated by mRNA making understanding the system a complex undertaking. miRs identified from the rare elite powerlifter phenotype can now be investigated in other populations with disparities in muscle strength and size such as young and older adults and those with myopathies in order to validate their importance as biomarkers of muscle function.

AUTHOR CONTRIBUTIONS

Performed experiments: RD, TB, KA, NZ. Analyzed data: RD, TB. Primer design and pathway illustration: NZ. Drafted manuscript: RD and CM. Critically evaluated and contributed to the manuscript: RD, TB, CM, TR, and DC. CM is responsible for the final content of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2017.00383/full#supplementary-material

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