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High responders to resistance exercise training demonstrate differential regulation of skeletal muscle microRNA expression

Peter K. Davidsen,[1,2] Iain J. Gallagher,[2] Joseph W. Hartman,[3] Mark A. Tarnopolsky,[4] Flemming Dela,[1] Jørn W. Helge,[1] James A. Timmons,[1,2] and Stuart M. Phillips[3]

[1]*Centre for Healthy Ageing, Department of Biomedical Sciences, University of Copenhagen, Blegdamsvej, Denmark;* [2]*Lifestyle Research Group, The Royal Veterinary College, University of London, London, United Kingdom; and* [3]*Exercise Metabolism Research Group, Department of Kinesiology, and* [4]*Pediatrics and Medicine, McMaster University Medical Centre, Hamilton, Ontario, Canada*

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Davidsen PK, Gallagher IJ, Hartman JW, Tarnopolsky MA, Dela F, Helge JW, Timmons JA, Phillips SM. High responders to resistance exercise training demonstrate differential regulation of skeletal muscle microRNA expression. *J Appl Physiol* 110: 309–317, 2011. First published October 28, 2010; doi:10.1152/japplphysiol.00901.2010.—MicroRNAs (miRNA), small noncoding RNA molecules, may regulate protein synthesis, while resistance exercise training (RT) is an efficient strategy for stimulating muscle protein synthesis in vivo. However, RT increases muscle mass, with a very wide range of effectiveness in humans. We therefore determined the expression level of 21 abundant miRNAs to determine whether variation in these miRNAs was able to explain the variation in RT-induced gains in muscle mass. Vastus lateralis biopsies were obtained from the top and bottom ~20% of responders from 56 young men who undertook a 5 day/wk RT program for 12 wk. Training-induced muscle mass gain was determined by dual-energy X-ray absorptiometry, and fiber size was evaluated by histochemistry. The expression level of each miRNA was quantified using TaqMan-based quantitative PCR, with the analysis carried out in a blinded manner. Gene ontology and target gene profiling were used to predict the potential biological implications. Of the 21 mature miRNAs examined, 17 were stable during RT in both groups. However, miR-378, miR-29a, miR-26a, and miR-451 were differentially expressed between low and high responders. miR-378, miR-29a, and miR-26a were downregulated in low responders and unchanged in high responders, while miR-451 was upregulated only in low responders. Interestingly, the training-induced change in miR-378 abundance was positively correlated with muscle mass gains in vivo. Gene ontology analysis of the target gene list of miR-378, miR-29a, miR-26a, and miR-451, from the weighted cumulative context ranking methodology, indicated that miRNA changes in the low responders may be compensatory, reflecting a failure to "activate" growth and remodeling genes. We report, for the first time, that RT-induced hypertrophy in human skeletal muscle is associated with selected changes in miRNA abundance. Our analysis indicates that miRNAs may play a role in the phenotypic change and pronounced intergroup variation in the RT response.

hypertrophy; phenotypic change; microRNA abundance

MUSCLE ADAPTATION TO STRENGTH training represents one of the most dynamic in vivo situations, at least in adulthood, of net muscle protein accretion. The key variable affecting muscle accretion with resistance training (RT) appears to be muscle protein synthesis (7). Feeding- and exercise-induced changes in protein synthesis are four- to sixfold greater than changes in muscle protein breakdown and regulate the acute, and presumably chronic, changes in net muscle protein balance. However, RT-induced gains in muscle mass are highly variable between individuals (5, 24). A number of factors, including age, nutritional support, and, obviously, genetic predisposition, might affect the hypertrophic response. Individual genetic polymorphisms that can explain a small degree of variability in the RT hypertrophic phenotype have been identified (10, 34). In rodents (3) and humans (44), the phosphorylation (presumably reflecting activation) of the translation initiation protein p70 S6 kinase (S6K), which is involved in protein translation, correlates with the degree of hypertrophy occurring with RT. This implies that local factors regulating muscle protein synthesis, as opposed to systemic factors (51, 52), are of paramount importance in determining hypertrophy. In larger well-controlled RT studies where age, training status, exercise adherence, and nutritional support are controlled, large variation in the RT hypertrophy response, measured by whole body muscle mass gains or changes in muscle fiber cross-sectional area (CSA), still exists (22).

Noncoding RNA has emerged in recent years (45) as being important for skeletal muscle biology (18, 38). In particular, microRNAs (miRNAs) are accepted regulators of mammalian cell phenotype (4, 20, 39). miRNAs are ~22-nucleotide post-transcriptional regulators of gene product abundance that are able to block the translation of protein-coding genes (27). miRNAs regulate development and differentiation (15, 36), and brain and skeletal muscle tissue have the most tissue-specific miRNA species (26). miRNAs have also been implicated in the regulation of metabolism (14, 15), insulin secretion (30), and muscle disorders (13, 17), including type 2 diabetes (18). In vivo in humans, it would appear that miRNAs may affect protein synthesis, rather than mRNA stability (18), where they can be considered significant regulators of muscle protein expression. Several miRNAs are highly regulated in vivo and in vitro during muscle development and, in turn, regulate muscle differentiation (9). The most-studied miRNAs are miR-133a/b, miR-206, and miR-1, which are induced during differentiation of myoblasts into myotubes (36) and are collectively referred to as the "myomirs." The myomirs, which are also highly expressed in vivo, may also be regulated during changes in muscle phenotype. For example, miR-1 and miR-133a are downregulated in murine skeletal muscle during bilateral synergist ablation-induced hypertrophy of the plantaris muscle (32). An acute bout of resistance exercise results in a similar, ~50%, downregulation in miR-1, while miR-133a expression is unaltered (11). However, in humans, it is important to link acute changes in gene expression with the pheno-

Address for reprint requests and other correspondence: J. A. Timmons, The Royal Veterinary College, 4 Royal College St., Univ. of London, London NW1 0TU, UK (e-mail: Jamie.Timmons@gmail.com).

typic outcome relevant to the intervention, because not all subjects demonstrate a robust physiological adaptation or molecular response to exercise training (46, 47). While this has been clearly demonstrated for endurance training, it is also reasonable to suspect a similar scenario in RT; hence, in the absence of knowledge of how each subject would adapt to chronic training, the acute molecular responses to acute exercise may or may not be informative.

In the present study, we utilized the physiological heterogeneity of the hypertrophic response of a relatively large number of subjects ($n = 56$) that undertook 12 wk of intense RT. Although a number of salient factors that might affect hypertrophy [diet, training status, compliance, age, and initial lean body mass (LBM)] were controlled, substantial variation in the hypertrophic response was found (22). We, a priori, categorized the subjects as being low or higher responders, in terms of gain in lean muscle mass (whole body) and muscle fiber area, and determined the expression of a group ($n = 21$) of miRNAs deemed to be highly expressed according to published global miRNA profiling experiments (18, 33). Our results demonstrate that responder status is an important factor to consider, while only a discrete number of miRNAs appear to be regulated during skeletal muscle hypertrophy in humans, and these may influence subjects' "responder" status with RT.

METHODS

Subjects and sampling. Subjects have been described in a previous publication (22). Briefly, young (18–30 yr of age) men ($n = 56$) were recruited locally from the McMaster University campus or local Hamilton area by posters and advertisements. Subjects were screened before participation by questionnaire against standard criteria to ensure that they were free from any medical condition that would preclude their participation in the study. On the basis of the subjects' responses to the questionnaire, they were deemed healthy. None of the subjects were active weight lifters, and the only structured recreational activity they undertook in the study period was the study training. Therefore, subjects were not completely sedentary but participated in <2–3 h/wk of structured physically active or leisure-time activities. Percutaneous needle muscle biopsies were taken before and after (48 h after the final training session) the 12 wk of training from the vastus lateralis muscle under local anesthesia (2% xylocaine) with use of a 5-mm Bergstrom needle modified for manual suction, as described previously (40). Muscle samples (100–150 mg) were immediately dissected free of any visible fat or connective tissue and oriented under low-power (×20) magnification, so that the muscle could be embedded in optimal cutting medium and sectioned for histochemical analysis of fiber type, as previously described (25, 40, 43). The protocol was approved by the local ethics committee of McMaster University and Hamilton Health Sciences Research Ethics Board. The study and analysis protocols conformed to all local and international standards, including the revised 1983 Declaration of Helsinki, on research on human subjects.

Protocol. Each subject undertook a supervised 5 day/wk rotating split-body RT program for 12 wk. The training program is described in detail elsewhere (22). Briefly, the training program consisted of three groups of exercises: pushing, pulling, and leg exercises, with 60 weight-lifting sessions in total (20 workouts in each group). Dual-energy X-ray absorptiometry scans (model QDR-4500A, Hologic, Waltham, MA) before and after the exercise intervention were used to establish changes in body composition, as previously described (22).

Subject stratification. We initially selected the top and bottom 15% of subjects based on gain in whole body lean mass in response to training. We further examined these subjects to ensure that they fell into at least the top, or bottom, 15–20% for training-induced gain in type I and type II fiber cross-sectional area. We then examined the subjects that fell into these categories (8 high responders and 9 low responders) and used their strength gains in three leg exercises (leg press, knee extension, and hamstring curl) to arrive at two groups of subjects who were, collectively, in the top (or bottom) 15% for training-induced dual-energy X-ray absorptiometry-measured lean mass gain and 20% for training-induced fiber type (I and II) area increases and the top 15% or bottom 25% for strength gains assessed in three leg exercises. This resulted in two groups that were statistically significantly different with respect to lean mass gains (Fig. 1), fiber area gains, and strength gains and yet not different with respect to any other measured physical or dietary characteristic. It is notable that high responders were found in all three of the randomized groups from our original study ($n = 2$ control, $n = 2$ soy, $n = 4$ milk), and low responders were also found in all three groups ($n = 3$ control, $n = 5$ soy, $n = 1$ milk). All molecular analysis was carried out by an investigator who was blind to subject identity.

Total RNA extraction. Total RNA was isolated from vastus lateralis muscle biopsies (~20-mg samples that were flash-frozen at the time of sampling) by chloroform-phenol-based extraction. Briefly, ~20-mg paired tissue samples (obtained before and after training) were processed simultaneously in 1 ml of TRIzol (catalog no. 15596-018, Invitrogen) using a Mini-Beadbeater-8 (Biospec) for 15 s on the "homogenize" setting. After 5 min of incubation at room temperature, chloroform (200 μl; catalog no. C2432, Sigma-Aldrich) was added, and the samples were shaken vigorously by hand. Samples were briefly incubated on ice prior to 15 min of centrifugation at 12,000 *g*. The supernatant was removed and mixed with isopropanol (catalog no. I9516, Sigma-Aldrich) and spun once more at 12,000 *g* for 10 min after 10 min of incubation. After they were washed with 75% ethanol, RNA pellets were resuspended in 40 μl of diethylpyrocarbonate-treated water (catalog no. AM9906, Ambion) and quantified using a spectrophotometer (NanoDrop Technologies). RNA purity was assessed using the ratio of absorbance at 260 nm to absorbance at 280 nm and the ratio of absorbance at 260 nm to absorbance at 230 nm. We further evaluated the RNA quality using the Bioanalyzer 2100 (Agilent Technologies, Stockport, UK). An RNA integrity number <7 was predefined as the cut-off value for low-quality RNA (6), and no sample failed these quality assurance measures. RNA was stored at −80°C until further analysis.

Detection of mature microRNAs in skeletal muscle. We used the TaqMan miRNA reverse transcriptase kit and TaqMan miRNA assays (Applied Biosystems, Foster City, CA) to quantify mature miRNA expression levels. Each target miRNA was quantified according to the manufacturer's protocol with minor modifications. Briefly, reverse

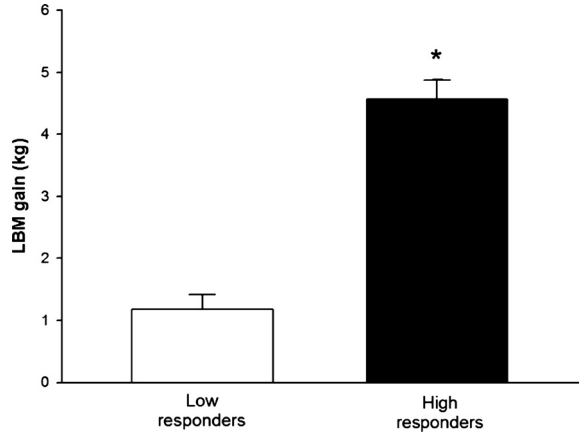

Fig. 1. Differential group change in lean body mass (LBM) in response to 12 wk of resistance training (RT). Values [means ± SE ($n = 8$–9 subjects/group)] are presented as training-induced changes in LBM relative to baseline plotted as a function of group. *Significantly different after 12 wk of RT ($P < 0.001$).

transcriptase reactions were performed with miRNA-specific reverse transcriptase primers and 5 ng of purified total RNA for 30 min at 16°C, 30 min at 42°C, and finally 5 min at 85°C to heat-inactivate the reverse transcriptase. All volumes suggested in the manufacturer's protocol were halved, as previously reported (18). Real-time PCRs for each miRNA (10 μl total volume) were performed in triplicate, and each 10-μl reaction mixture included 2.4 μl of 10×-diluted reverse transcriptase product. Reactions were run on a PRISM 7900HT Fast Real-Time PCR System (Applied Biosystems) at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Twofold dilution series were performed for all target miRNAs to verify the linearity of the assay. To account for possible differences in the amount of starting RNA, all samples were normalized to the small nuclear RNA RNU48 (catalog no. 4373383). Importantly, resistance exercise did not alter RNU48 quantities compared with preexercise values (tested against 18S rRNA). All reactions were run singleplex, in triplicate, and quantified using the cycle threshold ($\Delta\Delta C_t$) method (29).

miRNA target prediction and gene ontology analysis. We used gene ontology (GO) analysis (2) to obtain an overview of the functions of predicted target gene lists. The binding of miRNA to target mRNA occurs between the "seed" region of the miRNA (nucleotide 2–7 of the 5′ end of the mature miRNA) and the 3′-untranslated region of the mRNA. Gene lists of predicted targets for each modulated miRNA were obtained using TargetScan 4.2 (28). We used our previously published in vivo target analysis methodology (18) to generate lists of genes targeted by multiple regulated miRNAs. The rationale behind our methodology is that multiple changes in miRNA abundance can occur in vivo (13) and simultaneously be up- and downregulated. Furthermore, miRNAs can target the same gene, but with a range of predicted efficacies (20), such that it is important to calculate the net impact in the tissue of interest, rather than examine individual genes targeted by individual miRNA. For GO analysis, we used skeletal muscle-enriched gene expression profiles (derived from Affymetrix U133+ 2.0 chip data) and utilized topGO (1) to filter the predicted gene target lists to explore the biological processes most overrepresented. The topGO package allows genes contributing to significance at one level of the GO to be removed before testing higher levels. We utilized the muscle transcriptome as the "background" gene expression data set for GO analysis (18), as misleading ontological enrichment *P* values are yielded when a generic (genome-wide) reference data set is utilized, as all tissues have, by definition, an enriched and already biased GO profile.

Statistics. Values are means ± SE. Student's paired *t*-tests were used to assess changes in subject characteristics with training and to test for differences in miRNA level with training. Linear regression was carried out to define correlation between miRNA content and fiber type composition at baseline. Furthermore, linear regression was performed to test the linearity of any correlation between change in miRNA levels with training and gain in LBM. If normality or homogeneity of variances was not obtained, the data were logarithmically transformed. Differences are considered significant at $P < 0.05$. Statistical calculations were performed using statistical software (version 3.5, SigmaStat, Chicago, IL). Bioinformatic statistical procedures were carried out using the R language for statistical computing and the Bioconductor software suite, as previously described (18).

RESULTS

The general (and biochemical) characteristics of the subjects are listed in Table 1. Prior to training, there were no apparent differences between high and low responders in terms of weight, fat, and bone-free mass (i.e., LBM), or fiber areas. Training-induced changes in fat- and bone-free mass and type I and type II fiber area were, however, statistically greater in the high than low responders. We also analyzed diet records from these same subjects and observed no significant differences in total energy intake, protein intake, fat intake, or changes in these variables with training (data not shown). The LBM changes induced by 12 wk of RT were significantly different ($P < 0.001$) when group means were compared (Fig. 1). Indeed, the high responders gained almost fourfold more LBM than the low responders. Further physiological data, as well as the group average responses for comparison, can be found in Table 1.

Many "abundant" miRNAs are unaffected by resistance exercise in human skeletal muscle. Nineteen mature miRNAs, including the myomirs, were measured in skeletal muscle biopsies from low ($n = 9$) and high ($n = 8$) responders. The 21 candidate miRNAs were chosen on the basis of relative fluorescence signal in skeletal muscle from two gene-chip miRNA expression studies: one from human skeletal muscle and the other from rodent soleus muscle (18, 33). Although the two microarray studies were performed on different gene-chip platforms, Exiqon and mParaFlo chip (33), they showed reasonable agreement for the rank order of miRNA signal intensities. It is, however, clearly understood that signal intensities do not give an absolute indication of abundance because of different probe performance. Indeed, certain miRNAs that

Table 1. *Subject characteristics*

	Group Median ($n = 56$)	High Responders ($n = 8$)	Low Responders ($n = 9$)	*P* Value
Age, yr	22	22 ± 1	23 ± 1	0.78
BMI, kg/m^2	26.6	26.3 ± 1.7	27.2 ± 1.6	0.65
Pretraining FBFM, kg	61.3	62.6 ± 2.1	60.9 ± 2.9	0.49
Type I CSA, μm^2				
Pretraining	4,235	4,560 ± 347	4,125 ± 455	0.44
Δ	418	729 ± 195	240 ± 63*	0.021
Type II CSA, μm^2				
Pretraining	4,663	4,780 ± 584	4,438 ± 452	0.36
Δ	692	1,238 ± 132	362 ± 69*	<0.001
Leg press 1 RM, kg				
Pretraining	205	210 ± 10	202 ± 17	0.46
Δ	194	212 ± 16	180 ± 19	0.18
Leg extension 1 RM, kg				
Pretraining	129	136 ± 15	124 ± 19	0.47
Δ	93	117 ± 16	73 ± 12	0.075

Values are means ± SE. FBFM, fat- and bone-free mass; BMI, body mass index; CSA, cross-sectional area; RM, repetition maximum. *Significantly different from high responder.

Table 2. *Stable miRNA expression with exercise in human skeletal muscle*

	High Responders (n = 8)		Low Responders (n = 9)	
miRNA	Mean ± SE	P value	Mean ± SE	P value
miR-1	0.70 ± 0.15	0.20	0.96 ± 0.33	0.43
miR-16	0.77 ± 0.17	0.42	1.38 ± 0.41	0.50
miR-22	0.85 ± 0.35	0.51	0.85 ± 0.24	0.81
miR-23a	1.23 ± 0.48	0.20	1.35 ± 0.29	0.37
miR-23b	1.70 ± 1.13	0.30	1.02 ± 0.52	0.25
miR-24	0.98 ± 0.09	0.90	0.97 ± 0.13	0.86
miR-26b	0.90 ± 0.12	0.39	0.82 ± 0.27	0.50
miR-30a	1.17 ± 0.25	0.23	0.73 ± 0.15	0.19
miR-30c	0.67 ± 0.13	0.26	0.62 ± 0.14	0.18
miR-125b	1.14 ± 0.29	0.44	1.31 ± 0.45	0.50
miR-126	1.05 ± 0.15	0.79	1.39 ± 0.39	0.35
miR-133a	0.96 ± 0.19	0.86	0.72 ± 0.18	0.21
miR-133b	1.28 ± 0.43	0.26	1.04 ± 0.27	0.93
miR-206	1.40 ± 0.28	0.11	1.12 ± 0.24	0.68
miR-208b	0.78 ± 0.17	0.17	1.05 ± 0.29	0.81

Values are expressed as fold change relative to baseline (basal = 1.0).

yielded high array signals were modestly expressed according to the TaqMan real-time assay (see below).

Among the 21 "highly expressed" miRNAs, the expression levels of 15 were unaffected by 12 wk of RT (Table 2). Expression of miR-378 was significantly reduced (P = 0.008), whereas miR-451 expression was significantly increased (P = 0.025), in low responders with RT (Fig. 2). The expression levels of miR-26a and miR-29a demonstrated a clear tendency toward a decrease in low responders after 12 wk of RT ($0.05 < P < 0.10$; Fig. 2). We found that 18S rRNA and RNU48 abundance was stable with respect to training status (with average C_t values of ~22 and 25.7 arbitrary units at their respective cDNA dilutions). Furthermore, there were no significant differences in gene abundance prior to training between the groups.

Change in miR-378 level with resistance exercise is correlated with gain in LBM. Gene expression changes should be related to physiological status, whenever possible (46, 48). While we a priori defined the analysis strategy as a comparison of the average response in each of the training groups, we also examined the data for linear relationships between change in LBM and change in these four regulated miRNAs, since conceivably miRNA abundance could reflect protein accretion across a continuum of biological concentrations. Interestingly, we found that the intrasubject changes in miR-378 level after 12 wk of RT were significantly and strongly correlated (R = 0.71, P = 0.001) with gain in LBM, suggesting that maintenance of miR-378 could be an important determinant of a gain in LBM in vivo in humans (Fig. 3).

Basal expression level of miR-126 tends to correlate with muscle fiber type composition. Measurement of muscle fiber type composition is a time-consuming process and is subject to significant measure variation. miRNA expression has previously been associated with fiber type composition: mouse

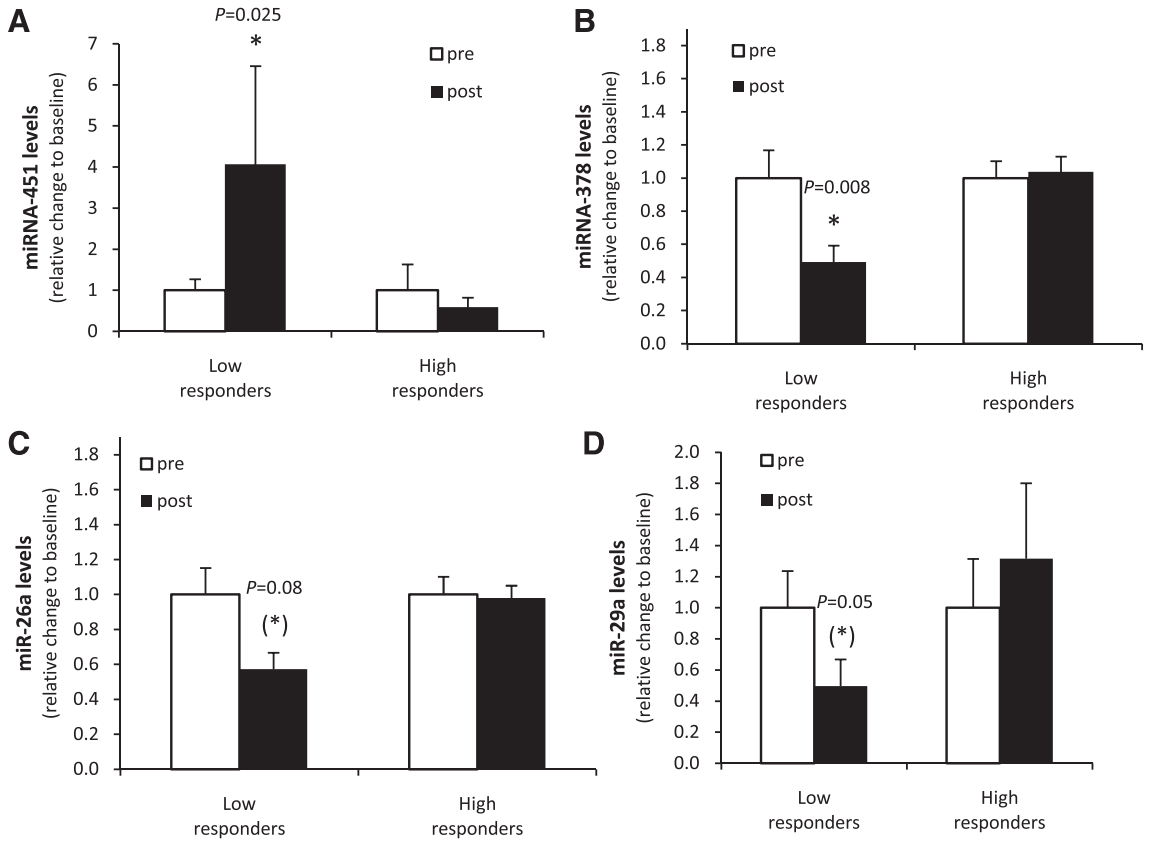

Fig. 2. Changes in microRNA (miRNA) content with exercise in human skeletal muscle. Mature miRNA levels in low and high responders, respectively, before (pre) and after (post) 12 wk of RT. Values [means ± SE (n = 8–9 subjects/group)] are presented as fold change relative to baseline (basal level = 1.0). 10×-Diluted cDNA yielded mean C_t values of 22 and 25, respectively. *Significantly different after 12 wk of RT. (*)Tends to be significantly different after 12 wk of RT.

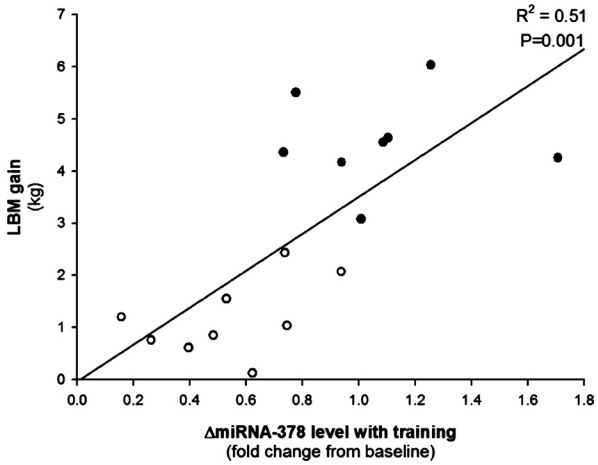

Fig. 3. Correlation plot for change in LBM with change in miR-378 level after 12 wk of RT. Change in miRNA level with training is shown as fold change relative to baseline (basal = 1.0). ●, high responders; ○, low responders. Regression line is shown, along with Pearson's product-moment correlation coefficient (R^2). Correlation coefficient was significant ($P = 0.0013$).

soleus had sevenfold more miR-206 than faster-twitch plantaris (32). Thus we tested the hypothesis that miRNA expression could be a useful biomarker for muscle fiber type composition also in humans. While we did not find any association between miR-206 and measured fiber type composition in humans, miR-126 content at baseline tended to positively correlate with a higher percentage of type I muscle fiber ($R^2 = 0.46$, $P = 0.06$). This association was relatively modest and is not robust enough for practical use as a biomarker of skeletal muscle fiber type in humans.

GO analysis of predicted target lists. We recently devised and validated a novel ranking system for examining multiple miRNA-induced changes in protein expression (18). Compared with previous approaches, our approach predicts which proteins should be altered on the basis of multiple changes in miRNA expression. The ranking procedure was evaluated by identification of statistically enriched and biologically validated GOs and canonical signaling pathways following adjustment for multiple-comparison testing. Each miRNA target site context score is adjusted by the training-induced changes in miRNA expression, giving rise to a weighted context score (wCS). Then each wCS per gene is summed to provide the weighted cumulative context score (wCCS) reflecting all miRNA activity on the target mRNA. This process yields a list of genes with a high statistical chance of being regulated in vivo by the change in muscle miRNA expression (18). This list was then subjected to GO analysis, a further stringent statistical hurdle, to determine which biological pathways were overrepresented. Ontological analysis is complex, and for analysis of these wCCS-adjusted target lists, we combined the two, nonoverlapping lists (mRNA targeted by up- and downregulated miRNAs) to explore the targeted biological processes. Highly significant enrichment was uniquely found within the first quartile of ranked genes, including processes involving cell interactions with the extracellular matrix (ECM; $P < 0.001$), ECM organization ($P = 0.001$), and angiogenesis ($P < 0.01$, all Benjamini-Hochberg-adjusted; Fig. 4). The fourth quartile of conserved wCCS targets did not demonstrate any such enrichment. (See Supplemental Data Sheet in Supplemental Material for this article, available online at the Journal website, for probable targets of the downregulated miRNAs, along with additional information on the GO analysis.)

Ontological enrichment of a target gene list provides statistical evidence of distinct biological processes being targeted by the miRNAs that change with RT, but it remains a further challenge to pinpoint the signaling pathways involved in the adaptive responses from these alone. Canonical pathway analysis was used to visualize whether first-quartile genes belong to known RT-related processes. The highest-ranked signaling pathway in the present analysis was mammalian target of rapamycin (mTOR) signaling ($P < 0.001$), which is extensively implicated in skeletal muscle hypertrophy (49). Furthermore, ECM-receptor interaction ($P < 0.001$) and focal adhesion kinase ($P < 0.001$) were significantly targeted by the modulated miRNAs, according to our analysis, and both are involved in mechanotransduction in striated muscle (12).

mRNA profiling of miRNA targets. Next we examined the mRNA expression changes of three genes that were predicted to be regulated at the protein level in skeletal muscle tissue: insulin-like growth factor I (IGF-I), VEGF-A, and eukaryotic translation initiation factor 4E type 2 (eIF4E2). These miRNA target genes were selected because they appear near the top of the wCCS-ranked gene list and because they have a plausible link to skeletal muscle remodeling. We have found that the miRNA changes do not directly relate to mRNA abundance in vivo in human skeletal muscle (18); rather, we profile these targets to look for a coherent transcriptional response between high and low responders. Interestingly, IGF-I mRNA levels increased significantly ($P = 0.016$) with training in high responders only (Fig. 5). This seems in accordance with numerous studies suggesting that muscle-produced IGF-I may be involved in muscle hypertrophy through activation of mTOR signaling (37, 41). Interestingly, IGF-I mRNA levels were unaltered with RT in the low-responder group. The mRNA levels of VEGF and eIF4E2 were not significantly regulated by RT in either group, although their profile was consistent with that of IGF-I. Thus the pattern of miRNA changes may be an attempt to compensate for a lack of change in selected mRNA abundance, or vice versa (see below).

DISCUSSION

In general, alterations in skeletal muscle phenotype following progressive RT include gains in strength, increases in LBM and muscle fiber hypertrophy, and enhanced insulin sensitivity. Hence, the significance of an individual's ability to adapt to RT may ultimately influence multiple risk factors for counteracting age-related sarcopenia and may also be important for long-term cardiovascular health by reducing insulin resistance (23). In the present study, we demonstrate, for the first time, that successful RT-induced hypertrophy in human skeletal muscle is associated with selected changes in miRNA abundance. The selective differences in miRNA expression in response to RT in high and low responders suggest that miRNAs may play a role in regulating the translation of key gene networks responsible for human skeletal muscle growth. Interestingly, the expression levels of miR-133a/b and miR-1, which have been previously linked to short-term murine muscle hypertrophy (32), were unaffected after a 12-wk RT program in humans.

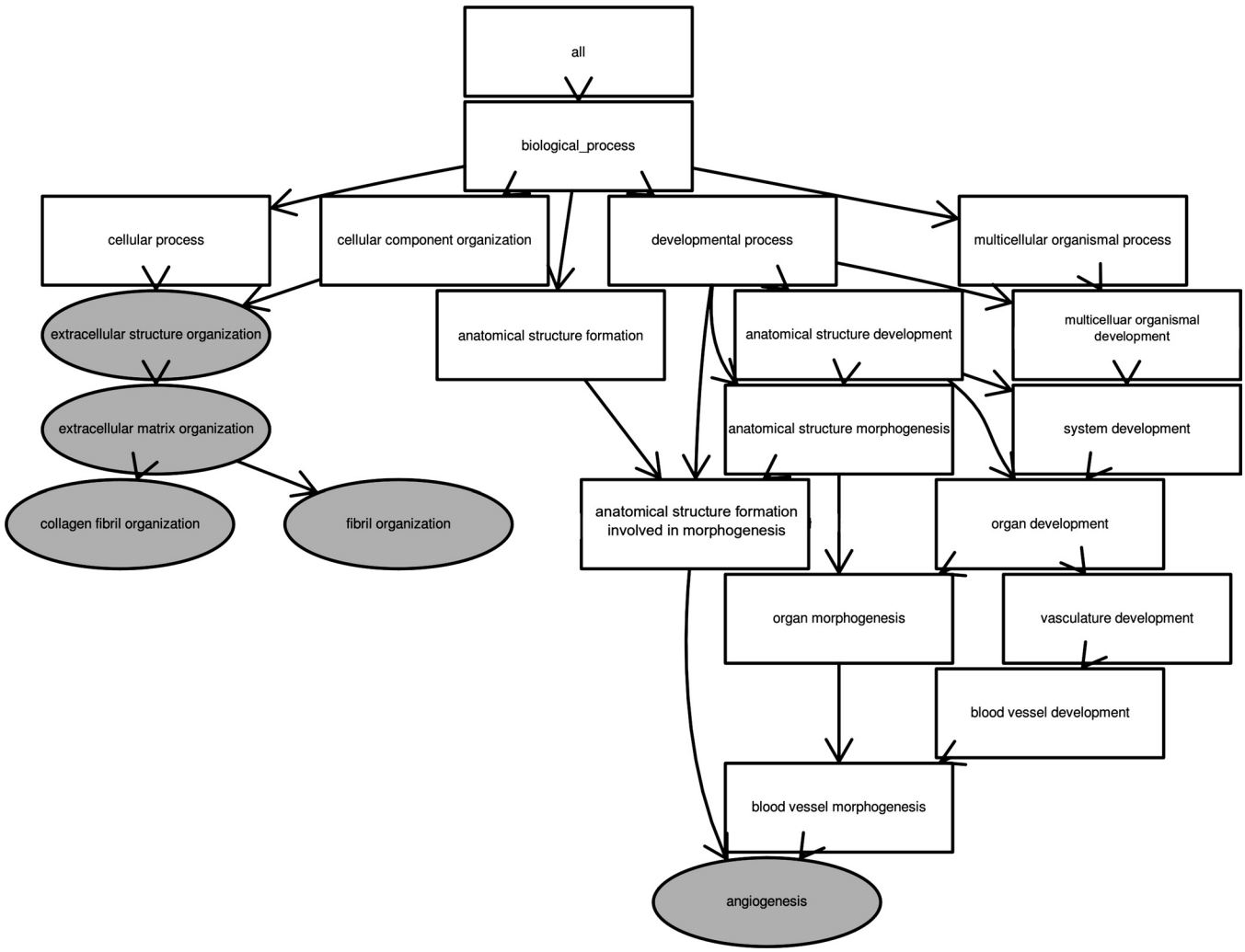

Fig. 4. Gene ontology (GO) analysis of the weighted cumulative context score 1st quartile-ranked genes. GO categories ancestral to "striated muscle development" were examined for enrichment in targets of the coregulated miRNAs. Enriched categories are shown as grey ovals ($P < 0.01$, Benjamini-Hochberg-adjusted).

Heterogeneous muscle hypertrophy response in humans. It is widely accepted that progressive RT causes gains in skeletal muscle mass. However, human subjects demonstrate large variability in their responsiveness to progressive RT. Factors such as sex, age, diet, physical activity level, and previous training status have been postulated to affect the degree of muscular adaptation (16, 50). Interestingly, previous studies (5, 24), as well as the original study on which this work is based, showed considerable variation in the extent of training adaptation, even when many of these variables are controlled (22). Hence, more information is needed to help define which factors significantly influence the intersubject variation in muscular adaptation to RT. At some point during RT, muscle hypertrophy can be limited by the availability of muscle satellite cells (35). A variable hypertrophy response in humans (5) allowed for the exploration of a causal association between acute mTORC1 signaling and the gains in muscle mass observed after 16 wk of RT (31). mTORC1-related signaling is elevated 24 h after acute resistance exercise, while regulation (autoinhibitory domain phosphorylation) of S6K1 was found to correlate positively with increased myofiber size after 16 wk of training (31). In the present study, we attempted to predict the relevance of the selected modulation of miRNA expression in the context of the degree of functional hypertrophy observed. We recently demonstrated that, in vivo in human skeletal muscle, miRNA changes appear to work in a combinatorial manner, such that valid protein targets can be identified by our recent bioinformatic analysis approaches (18). It is noteworthy that the selected panel of miRNAs, of which we had literature evidence for robust expression, belonged (in terms of seed sequence-based target analysis) to 14 independent "families" of miRNA. Thus it could be expected that they could coordinate a very large and diverse set of gene networks.

In the present study, the net effect of changes in miR-378, miR-29a, miR-26a, and miR-451 expression were statistically demonstrated to target mTOR pathway signaling ($P < 0.001$), an observation in strong agreement with the findings of Mayhew et al. (31). We also profiled some relevant members of the gene networks targeted by the cumulative impact of miR-378, miR-29a, miR-26a, and miR-451 modulation to further understand if these genes were over- or underregulated in relation to muscle hypertrophy. For example, loss of miRNA expression

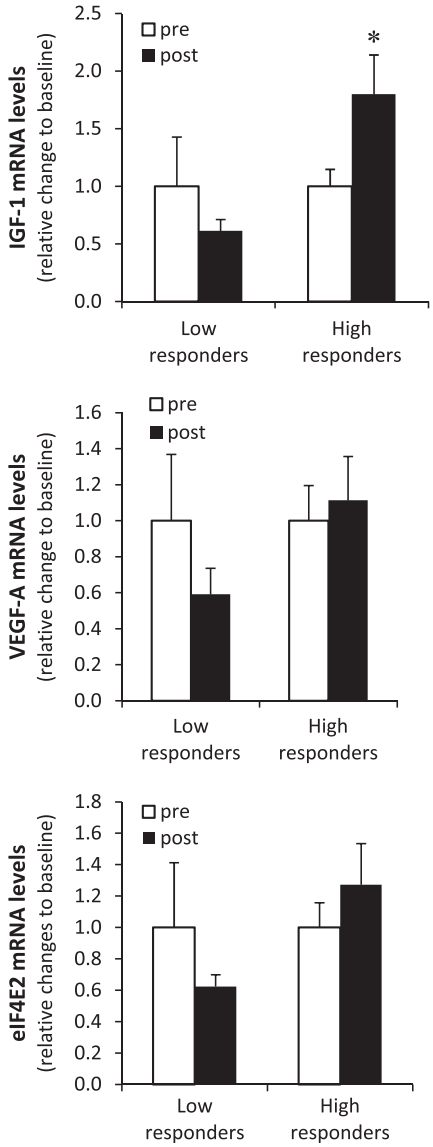

Fig. 5. mRNA levels in low and high responders, respectively, before and after 12 wk of RT. Values [means ± SE (n = 8–9 subjects/group)] are presented as fold change relative to baseline (basal level = 1.0). *Significantly different after 12 wk of RT (P = 0.016).

could compensate for lack of activation of transcription of an important target gene by allowing more efficient protein synthesis from the available mRNA substrate (recall that miRNAs inhibit protein production). Indeed, there was some support for our hypothesis, in that loss of miRNA repression (miR-378, miR-29a, and miR-26a) was associated with a reduced mRNA profile for IGF-I, VEGF-A, and eIF4E2, although only the change in IGF-I was significant (and, admittedly, we did not explore the relative importance of the known IGF-I splice variants). Ideally, sequence-based analysis of the "less-activated" mRNAs could be examined to explore the potential basis for their lack of modulation in vivo, thus supporting our interpretation that the altered miRNA profile may be compensatory. Alternatively, lower mRNA responses may be secondary to reduced miRNA expression, and neither may be causally related to the lower hypertrophy. A more detailed time-series analysis of changes in vivo would help address this point.

Notably, under the conditions encountered in vivo, we do not find evidence indicating that the miRNAs directly target the net breakdown of mRNA (18), but rather we believe that they regulate translational control. Recent claims that miRNAs largely modulate transcript abundance in mammals (21) are most likely an artifact of in vitro conditions and the ratio of nonlinear detection methodologies. The present analysis indicates that multiple levels of control attempt to mount a functional hypertrophy response in vivo and that transcription, providing mRNA "substrate" to protein synthesis machinery, may be partially "tuned" by the abundance of miRNA (regulating how efficiently the mRNA substrate is used as a template for net protein synthesis). Clearly, large-scale transcriptome and proteome analysis of tissue collected from in vivo experiments is needed for a robust test of such hypotheses, even though such ideas are supported by cell-based data (39). Nevertheless, the wCCS-based analysis of the four miRNAs altered with RT correctly identified signal transduction pathways for mechanotransduction and muscle hypertrophy.

miRNA changes and models of muscle hypertrophy. Very few studies have addressed miRNA expression in response to dynamic changes in muscle mass. In contrast to the present findings of an unchanged expression of miR-1 and miR-133a with 12 wk of RT, McCarthy and Esser (32) recently showed that the expression of these two myomirs was downregulated by ~50% following 7 days of functional overload. This disparity could suggest that these two muscle-specific miRNAs may be regulated only during the initial response of skeletal muscle to functional overload. Indeed, our findings could indicate that this initial response subsides when the RT period is prolonged and does not appear to be linked to the extent of physiological change. However, direct comparison of data is also made difficult by the different interventions and methods for normalizing gene expression; furthermore, tight correlation between animal and human data does not always exist (19, 42). More recently, McCarthy et al. (33) failed to find a link between the three classic myomirs, miR-1, miR-206, and miR-133a, and skeletal muscle atrophy over 28 days (nor were these myomirs acutely modulated at *day 2*). Their data, based on global chip normalization methods or normalization to small nucleolar RNU87 [in contrast to the earlier work (32)], and the present findings suggest that the myomirs are not centrally involved in long-term skeletal muscle hypertrophy or atrophy in vivo. This is somewhat in contrast to the apparent central role for miR-133a/b in adaptive cardiac hypertrophy (8), where loss of miR-133a/b facilitates hypertrophy.

McCarthy et al. (33) reported that miR-208b is upregulated during loss of muscle mass. In the present study, downregulation of miR-378, miR-29a, and miR-26a and upregulation of miR-451 were only observed in subjects who failed to demonstrate a large hypertrophy response, and no (converse) changes were reported for these miRNAs in the miRNA gene chip study of McCarthy et al. We also examined miR-208a and miR-208b expression in the present study (miR-208a has the same seed sequence as miR-208b). TaqMan-based analysis indicated that miR-208a and miR-208b are very poorly expressed in human skeletal muscle, and no reliable pattern of change was noted (data not shown). Finally, using a third housekeeping strategy (5S rRNA), it was recently claimed that miR-1 is downregulated by a combination of acute resistance exercise and essential amino acid ingestion in young, but not

older, men (11), while the other myomirs were unchanged and levels did not differ at baseline with age. Given the present data, we would suggest that miR-378, miR-29a, miR-26a, and miR-451 may be interesting to examine in the context of muscle aging or frailty. While we present novel miRNA associations with functional hypertrophy in vivo in humans and strong statistical evidence that such associations may target relevant signaling pathways, it must be acknowledged that, in this particular study (because of lack of biopsy material), we do not explore the net impact on protein abundance. We do, however, provide additional evidence for transcriptional differences between high and low responders (e.g., IGF-I mRNA), which, together with the miRNA data, demonstrates that subjects with a poor ability to accumulate LBM during RT have a unique molecular response to the intervention.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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