Impact of endurance exercise training on adipocyte microRNA expression in overweight men

Thomas Tsioulis,*† Joshua Pike,*† David Powell,‡ Fernando J. Rossello,§∥ Benedict J. Canny,*† Ruth C. R. Meex,*† and Matthew J. Watt*†,

*Monash Biomedicine Discovery Institute, †Department of Physiology, ‡Monash Bioinformatics Platform, Faculty of Biomedical and Psychological Sciences, §Australian Regenerative Medicine Institute, and ∥Department of Anatomy and Developmental Biology, Monash University, Clayton, Victoria, Australia; and ||School of Medicine, Faculty of Health, University of Tasmania, Hobart, Tasmania, Australia

ABSTRACT: Adipocytes are major regulators of metabolism and endurance exercise training improves adipocyte function; however, the molecular mechanisms that regulate chronic adaptive responses remain unresolved. microRNAs (miRNAs) influence adipocyte differentiation and metabolism. Accordingly, we aimed to determine whether adipocyte miRNA expression is responsive to exercise training and to identify exercise-responsive miRNAs that influence adipocyte metabolism. Next generation sequencing was used to profile miRNA expression of adipocytes that were isolated from subcutaneous abdominal (ABD) and gluteofemoral (GF) adipose tissue of overweight men before and after 6 wk of endurance exercise training. Differentially expressed miRNAs were overexpressed or silenced in 3T3-L1 adipocytes and lipid metabolism was examined. Next generation sequencing identified 526 miRNAs in adipocytes, and there were no statistical differences in miRNA expression when comparing pre- and post-training samples for ABD and GF adipocytes. miR-10b expression was increased in ABD compared with GF, whereas miR-204, miR-3613, and miR-4532 were more highly expressed in GF compared with ABD adipocytes. Blocking miR-10b in adipocytes suppressed β-adrenergic lipolysis but generally had a minor effect on lipid metabolism. Thus, unlike their critical role in adipogenesis, stable changes in miRNA expression do not play a prominent role in the regulation of adipocyte function in response to endurance exercise training. — Tsioulis, T., Pike, J., Powell, D., Rossello, F. J., Canny, B. J., Meex, R. C. R., Watt, M. J. Impact of endurance exercise training on adipocyte microRNA expression in overweight men. FASEB J. 31, 000–000 (2017). www.fasebj.org

KEY WORDS: Lipolysis · adipose tissue · gluteofemoral · metabolism · obesity

Adipose tissue is a major regulator of systemic energy balance via its role in lipid metabolism, hormone production and secretion, and immunity. Defects in these processes occur with obesity (1) and contribute to the pathogenesis of comorbidities, including type 2 diabetes mellitus, cardiovascular disease, nonalcoholic steatohepatitis, and some cancers (2). Lifestyle modification, including weight loss and exercise, are effective treatments for obesity and its comorbidities (3). Thus, understanding the cellular and molecular pathways that promote beneficial effects of diet and exercise may have important clinical implications.

Acute exercise invokes a myriad of changes in metabolic, neural, and hormonal stimuli that activate signaling cascades to regulate transcriptional and epigenetic changes in cells—events that form the basis for the adaptive response to exercise (4). Exercise training plays an important role in regulating many functions, including lipolysis (5, 6); mitochondrial biogenesis and the capacity for oxidative phosphorylation (7, 8); reprogramming of white adipocytes to a beige phenotype (9); altered expression and secretion of adipokines, including leptin, adiponectin, and resistin (10, 11); and reduced inflammation (10–12). Despite extensive investigation, the functional adaptations of adipocytes to exercise training (5, 9) and, in particular, the molecular mechanisms that mediate these chronic adaptive responses, remain incompletely described.

MicroRNAs (miRs/miRNAs) are small noncoding RNAs that regulate gene expression networks (13) to modify biologic functions (14–17). miRNAs play a prominent role in adipose tissue by regulating adipocyte differentiation, which is achieved by regulating preadipocyte clonal expansion and targeting essential transcription factors that induce adipocyte terminal differentiation (18, 19). Recent studies also demonstrate an important role for miRNAs, mir-196a and mir-30, in the recruitment of brown-like adipocytes to white

ABBREVIATIONS: ABD, abdominal subcutaneous; GF, gluteofemoral; HR max, maximal heart rate; miR/miRNA, microRNA; piRNA, piwi-interacting RNA

1 Correspondence: Department of Physiology, Monash University, 26 Innovation Walk, Clayton, VIC, 3800, Australia. E-mail: matthew.watt@monash.edu

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was estimated by using a validated equation on the basis of the

\[ \text{VO}_2 \text{max} = (0.01141 \times \text{Wpeak}) + 0.435 \]

where \( \text{Wpeak} \) is the participant’s maximum power output (25). Participants with an estimated \( \text{VO}_2 \text{ max} > 45 \text{ ml/min/kg} \) were excluded from the study.

Exercise training protocol

At least 3 d later, participants commenced 6 wk of aerobic exercise training, which consisted of 4 supervised sessions per wk. Three sessions consisted of 30 min of continuous exercise at an intensity of 75% HR max. Exercise was performed at Monash Sport and participants self-selected the mode of exercise, which included treadmill running (TRM833; Precor, Braeside, VIC, Australia), cycling (UBK833; Precor), cross-training (EFX835; Precor), or rowing (Concept 2 Model D; Precor). Exercise duration was increased by 5 min every 2 wk such that participants were performing 40 min of exercise per session by the conclusion of the training program. The fourth weekly session consisted of an interval session that was performed on a cycle ergometer, which consisted of 3-min intervals at 85% HR max followed by 3 min at 65% HR max. This was repeated 5 times and progressed to 7 intervals by the end of the training program. Heart rates were measured by using a Polar FT1 heart rate monitor (Polar Electro Oy, Kempele, Finland) throughout the exercise training program to ensure participants exercised at their target heart rate. All training sessions included a 5-min warm-up and cool-down period. Food intake was recorded over a 48-h period between wk 2 and 4 of the training program.

Blood sample analysis

Blood was spun at 8000 \( \times g \) for 3 min in a benchtop centrifuge (MiniSpin Plus; Eppendorf, Macquarie Park, NSW, Australia) and plasma was frozen at \(-80^{\circ} \text{C}\) until analysis. Free fatty acid concentration was determined by using the nonesterified fatty acids C analysis kit (Wako Pure Chemicals, Tokyo, Japan), triacylglyceride concentration was determined by using the Roche Diagnostics TG analysis kit (Roche, Basel, Switzerland), and plasma glucose was determined by using a handheld glucometer (Accu-Chek Performa; Roche).

Adipocyte isolation

Adipocytes were isolated in Krebs buffer (5 mM glucose, 5% bovine serum albumin, pH 7.4) by collagenase digestion (1 mg/ml collagenase type 2; Sigma-Aldrich, St. Louis, MO, USA) for 30 min

MATERIALS AND METHODS

Participants and experimental design

The Monash University Human Ethics Committee approved all experimental protocols (CF12/3804, 2012001746). Eleven overweight males volunteered to participate in the study by providing written informed consent after a detailed explanation of the experimental procedures and associated risks were outlined verbally and in writing. Participants were sedentary as defined by participating in <2 h of exercise per week, were not taking medications, were weight stable, and reported no history of cardiovascular disease, impaired glucose tolerance, or dyslipidemia.

Pre-experimental protocol

Participants visited the laboratory on 3 occasions. On the first visit, body mass and waist and hip circumferences were measured and aerobic capacity was assessed. Participants performed a graded cycling test to volitional exhaustion that involved cycling on a Lode cycle ergometer (Lode, Groningen, The Netherlands). Participants maximal heart rate (HR max) was recorded and \( \text{VO}_2 \text{max} \) was estimated by using a validated equation on the basis of the maximal power output attained at volitional exhaustion (\( \text{VO}_2 \text{ max} = (0.01141 \times \text{Wpeak}) + 0.435 \)), where \( \text{Wpeak} \) is the participant’s maximum power output (25). Participants with an estimated \( \text{VO}_2 \text{ max} > 45 \text{ ml/min/kg} \) were excluded from the study.

Pretraining protocol

Food intake was recorded in the 48 h before pretraining tissue sampling, and total energy intake and macronutrient breakdown was assessed (FoodWorks 7 Professional Edition, v. 7.0.3016; Xyris Software (Monash, VIC, Australia). Participants arrived at the laboratory after an 8- to 10-h fast, voided, and lay supine for 15 min. A blood sample was taken from an antecubital vein, transferred to an EDTA-coated tube, and placed on ice. Participants were then prepared for adipose tissue sampling. Local anesthesia (2% xylocaine, no epinephrine) was administered subcutaneously at 2 sites, 3-cm lateral from the umbilicus (ABD) and to the upper portion of the buttocks, lateral to the gluteal crease (GF). A small incision was made at each site and adipose tissue was obtained by using a 14 G, 3-1/8” needle (Braun Sterican; B Braun Medical, Bethlehem, PA, USA) attached to a Hepafix Syringe with Male Luer Lock (Qosina, Ronkonkoma, NY, USA) from a depth of ~50 mm under the skin. On average, 716 ± 25 mg of tissue was excised from each depot.
at 37°C in a rocking water bath. Collagenase buffer also contained 500 nM adenosine (Sigma-Aldrich) to simulate in vivo environments and inhibit unrestricted lipolysis (26).

**Metabolic assessment in isolated adipocytes**

**Lipolysis**

Isolated adipocytes were incubated in Krebs buffer for 2 h (pH 7.4) without and with the addition of isoproterenol (10 nM and 1 μM) or forskolin (20 μM), and glycerol release was measured (free glycerol reagent; Sigma-Aldrich).

**Lipogenesis**

Adipocytes were incubated for 2 h in Krebs buffer that contained D-[U-14C]-glucose (PerkinElmer, Boston, MA, USA). At the conclusion of incubation, triglycerides were extracted by using chloroform:methanol (2:1 v:v) and were isolated from other lipids by thin-layer chromatography. Radioactivity in the triglyceride was determined by liquid scintillation counting (Tri-Card 2810TR; PerkinElmer).

**Fatty acid metabolism**

Isolated adipocytes were incubated for 4 h with [1-14C]-oleate (0.5 μCi/ml; PerkinElmer) and cold oleate (0.5 mM), with 2% fatty acid–free bovine serum albumin in Krebs buffer. Adipocytes were extracted in chloroform:methanol (2:1 v:v). Fatty acid oxidation rates were calculated as the sum of 14CO2 and acid-soluble metabolite production. Fatty acid esterification into triglycerides was quantified by measuring radioactivity in triglycerides after separation of the organic phase by thin-layer chromatography. Results for all experiments were corrected for total cell count.

**Total RNA extraction**

Total RNA was isolated from adipocytes by using the mirVana miRNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer instructions. To assess the integrity and quantity of total RNA, samples were plated onto an Agilent RNA 6000 Nano Chip (Agilent Technologies, Santa Clara, CA, USA) and assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies). An RNA integrity number that ranged from 1 (completely degraded RNA) to 10 (completely intact RNA) was provided during the analysis and only samples with an RNA integrity number >8.0 were included in the subsequent sequencing and analysis. Whereas RNA was extracted from the adipocytes of 8 complete data sets (i.e., ABD and GF pre- and postexercise training), only 5 complete data sets passed the strict quality control measures and were used for next generation sequencing. Whereas this small sample size is likely to reduce the capacity to detect small differences between treatments, power analysis of the 30 highest-expressing miRNAs (Supplemental Table 1) indicated that, on average, 118 participants would be required to detect statistically meaningful differences between treatment groups (α = 0.05; β = 0.8).

**Small RNA preparation and next generation sequencing**

TruSeq Small RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) was used to generate small RNA libraries directly from the total RNA. In brief, 3' and 5' RNA adapters were specifically ligated to mature miRNAs. A reverse-transcription reaction was used to create single-stranded cDNA. cDNA was then PCR amplified by using a common primer and a primer that contained one of 48 index sequences, which was read on a high-sensitivity DNA chip. Finally, the amplified cDNA construct was gel purified in preparation for subsequent cluster generation.

**miRNA analysis**

Next generation sequencing reads were quantified by using mirDeep2 (Thermo Fisher Scientific) (27) against precursor and mature miRNAs from miRBase. Total mapped reads per sample averaged 2.45 × 106 reads (SEM = 0.50 × 106; n = 20). The resulting read counts for each miRNA were then log-transformed and samples were visualized by using MD5 plots from the limma and gplots toolsets (28, 29). Visualization showed a strong per-patient effect, which was corrected for by including a covariate for the patient in the linear model. Differential miRNA expression testing was performed by using voom/limma (30), and multiple testing correction by using Benjamini and Hochberg correction to control the false discovery rate.

Further investigation was then performed to explicitly look for piwi-interacting RNA (piRNA) in the small read data. Two approaches were taken. After testing several short read aligners, the best aligner for these very short reads was found to be the bwa aln method from the BWA tool (31), which was then used for the rest of this analysis. Reads were aligned against a database of known piRNA (32), which produced few uniquely mapping reads (<1%). The second approach was to align the reads to the human reference genome, allowing for multimapping reads, then to construct an annotation of piRNA clusters from the piRNA database to quantify against. piRNA clusters that were found to have a significant number of aligned reads were then inspected manually by using a genome annotation viewer. The reads mapping in these clusters were in small regions and those reads typically also mapped to other known noncoding RNA, such as transfer RNA. These inspections led us to believe there is little piRNA expression in this next generation sequencing read data.

**Cell culture**

3T3-L1 adipocytes were cultured in high-glucose DMEM (Thermo Fisher Scientific) with 10% fetal bovine serum and 1% penicillin-streptomycin (Thermo Fisher Scientific). To initiate differentiation, cells were incubated in high-glucose DMEM that contained isobutylmethylxanthine (500 μM), insulin (0.4 μM), dexamethasone (0.25 mM), and transferrin (0.1 μg/ml) for 3 d, which was then replaced with media that contained only insulin (0.4 μM) for another 3 d. Upon differentiation, cells were returned to high-glucose DMEM medium with 10% fetal bovine serum and 1% penicillin-streptomycin and incubated for a further 2 d before transfection. 3T3 adipocytes were transfected with miR mimic oligonucleotides (miRs-10b, -204, -4532) for overexpression studies, miR inhibitor oligonucleotides (miR-10b) for knockdown studies, and negative controls miR-NC or small inhibiting RNA. miR oligonucleotides (TaqMan; Thermo Fisher Scientific) were mixed with high-glucose DMEM (without fetal bovine serum) and HiPerfect (Qiagen, Valencia, CA, USA) transfection reagent. miRs were added to a final concentration of 10 pmol and cells were incubated for 48 h.

To confirm miRNA overexpression and inhibition, cells were lysed and total RNA was extracted by using the mirVana miRNA Isolation Kit. RNA was reverse transcribed by using the TaqMan small RNA Assays kit (Thermo Fisher Scientific),
and quantitative PCR was performed on a Bio-Rad T100 Thermal cycler (Bio-Rad, Hercules, CA, USA) with commercially available TaqMan probes using the following parameters: 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. RNAU6 was used as a reference gene, and relative abundances were calculated by using the ΔΔCt method.

Lipolysis and fatty acid metabolism experiments were performed 48 h after transfection. To measure lipolysis, 3T3-L1 adipocytes were incubated in DMEM high-glucose medium for 2 h without and with the addition of isoproterenol (10 nM and 1 μM) or forskolin (20 μM), and glycerol release was measured (free glycerol reagent). Fatty acid metabolism was assessed as described above for isolated adipocytes. Results for cell culture studies were corrected for protein content after bicinchoninic acid assay (BCA Protein Assay Kit; Pierce, Rockford, IL, USA) per manufacturer instructions.

Statistical analysis

All data are presented as means ± SEM, unless otherwise specified. Statistical analysis for cell culture studies was performed by using paired Student’s t tests or 1- or 2-way ANOVA with Bonferroni multiple comparisons tests where appropriate. Statistical significance was set a priori at P ≤ 0.05.

RESULTS

Participants’ response to exercise training

Six weeks of exercise training had no significant effect on body weight or body mass index, but we observed both reduced waist and hip circumferences (Table 1). Aerobic capacity was improved by training as evidenced by an increased peak power output during the graded exercise test and increased estimated VO2 max in post- vs. pretesting. Plasma free fatty acid concentration was decreased after exercise training, whereas plasma triglyceride, glucose, and insulin did not change (Table 1). There was no difference in energy intake or macronutrient composition during the training program (data not shown).

Metabolism in isolated adipocytes: evidence of adaptations to exercise training

Lipid metabolism was assessed in adipocytes that were isolated from ABD and GF adipose tissue before and after exercise training. Basal/unstimulated lipolysis was not different between ABD and GF adipocytes either before or after endurance exercise training (Fig. 1A). Lipolysis in ABD and GF adipocytes was increased from basal by isoproterenol and forskolin (main effect; P < 0.0001). Stimulated lipolysis was increased in ABD and GF adipocytes after exercise training, with no significant differences between the response of ABD and GF adipocytes (Fig. 1A). Lipogenesis was not different between ABD and GF adipocytes and was not affected by exercise training (Fig. 1B). Free fatty acid esterification into triglycerides was increased after vs. before exercise training (main effect; P = 0.006), and this response was not different between adipose tissue depots (Fig. 1C). Thus, adipocytes exhibited prominent metabolic adaptations to exercise training.

miRNA expression profile in human adipocytes

By using the bioinformatics tool miRDeep2 (27), RNA reads were assigned to novel and/or discovered miRNAs and their expression was quantified. Of miRNAs, 526 were identified when data were aligned to the database of miRNAs (Supplemental Table S1), and there was at least 1 read assigned to the miRNA across all samples. Heat maps of miRNAs were generated to visualize adipocyte miRNAs before and after exercise training (Fig. 2). There was no obvious clustering of miRNAs for adipocyte location (Fig. 2A, B; i.e., ABD and GF), and principal component analysis showed that miRNA expression profiles were not influenced by anatomic location or training status (Fig. 2C). Most of the variance was explained by the first 2 components with little contribution from component 3 onwards (data not shown). Of interest, the miRNA profile of individual participants did not cluster between trials, which indicated marked daily heterogeneity in adipocyte miRNA expression (Fig. 2D). Together, these data indicate that miRNA expression in ABD and GF adipocytes are similar, and that miRNA expression profiles of human adipocytes are not impacted in response to endurance exercise training.

The 20 most highly expressed miRs are shown in Table 2, several of which were previously reported to be involved in adipocyte function or metabolism. The
most highly expressed miRNA in both ABD and GF adipocytes was miR-143, which was previously identified as a regulator of adipogenesis (33, 34). miR-26a was the fifth highest expressing miRNA in adipocytes and is reported to regulate human white and beige adipocyte differentiation (35).

Exercise training had no impact on the miRNA expression profile of adipocytes, with no differentially expressed miRNAs in ABD or GF adipocytes when comparing pre- and postexercise samples. There was a tendency for the highly abundant miR-10b to be reduced in GF adipocytes after training (42%; not significant). Only 4 miRNAs were differentially expressed between GF and ABD adipocytes before exercise. miR-204 (3.2-fold; \( P = 5.73 \times 10^{-05}; \) Fig. 3A), miR-3613 (3.4-fold; \( P = 0.0005; \) Fig. 3B), and miR-4532 (3.7-fold; \( P = 0.0005; \) Fig. 3C) were more highly expressed in GF compared with ABD adipocytes. Of these, only miR-204 (1.7-fold; \( P = 0.02 \) was different between these adipose depots after exercise training. miR-10b is highly expressed in adipocytes (Table 2; ranked second most abundant) and was more abundant in ABD vs. GF adipocytes before and after exercise training (Fig. 3D, E).

**Effects of miR-10b, -204, and -4532 on adipocyte metabolism**

Functional effects of the differentially expressed miRNAs were examined in fully differentiated (mature) 3T3-L1 adipocytes by using miRs-10b, -204, and -4532 mimics. We did not examine the role of miR-3613 because of its very low endogenous expression (Supplemental Table S1). Baseline expression in 3T3-L1 adipocytes was highest for miR-4532 (C_{T}: 25.7), followed by miR-10a (C_{T}: 31.1) and miR-204 (C_{T}: 35.1). By comparison, the C_{T} of house-keeping genes was 26.2 for U6 and 14.3 for 18S. Transfection with miRNA mimics increased the expression of miR-10b, -204, and -4532 relative to controls as confirmed by quantitative real-time PCR (Fig. 4A). There was heterogeneity in the efficacy of miRNA mimics to increase miRNA levels despite identical transfection protocols; however, regardless of the differences in level of overexpression, effects on metabolism were uniformly marginal. Neither spontaneous (basal) nor \( \beta \)-adrenergic–stimulated (isoproterenol) lipolysis were different in adipocytes that were transfected with miR mimics compared with controls (Fig. 4B). There were no differences in fatty acid uptake between treatment conditions (Fig. 4C). miR-10b and miR-4532 mimics increased fatty acid oxidation (Fig. 4D; \( P < 0.05 \) compared with controls, but this increase accounted for only \( \sim 5\% \) of the total fatty acid uptake and is unlikely to be biologically important for adipocyte lipid balance. The rate of fatty acid incorporation into triglyceride was not different when comparing miR mimic–treated adipocytes with controls (Fig. 4E).
training (Supplemental Table S1), we next tested the effects of miR-10b knockdown on lipid metabolism in 3T3-L1 adipocytes. The inhibitor was a single-stranded oligonucleotide with chemical modifications that irreversibly binds and inactivates miR-10b. miR-10b silencing was first confirmed by quantitative real-time PCR (Fig. 5A). Spontaneous lipolysis was not different between adipocytes that were treated with control or miR-10b inhibitor (Fig. 5B). Stimulation of adipocytes with the β-adrenergic agonist, isoproterenol, increased lipolysis by 85% in control-treated cells, whereas no increase in lipolysis was observed under the same conditions in cells treated with miR-10b inhibitor (Fig. 5B). There was no difference in lipolysis when adipocytes were stimulated with forskolin, an agent that stimulates adenylate cyclase activity to activate PKA activity (i.e., distal to the β-adrenoceptors), which suggested potential effects of miR-10b in regulating β-adrenoceptors (Fig. 5B). However, direct analysis of Adrb2 and Adrb3 in 3T3-L1 adipocytes that were treated with miR-10b inhibitor or mimic revealed no difference compared with control-treated cells (data not shown). Similarly, Prkaca (protein kinase cAMP-activated catalytic subunit α), which is a predicted target of miR-10b, was not differentially expressed between treatments (control: 1.0 ± 0.23 vs. miR-10b mimic: 1.9 ± 0.36 vs. miR-10b inhibitor: 1.7 ± 1.0

Figure 2. miRNA expression profiles of human ABD and GF adipocytes. A, B) Heat maps showing miRNA expression profiles before (A) and after (B) 6 wk of endurance exercise training in both ABD and GF adipocytes. C) Principal component analysis comparing miRNA expression between ABD and GF adipocytes before and after exercise training. Note the absence of clustering for either adipocyte location or training status. D) Neighbor net showing the relationship between ABD and GF adipocyte miRNA expression levels before and after exercise training. Note the close clustering within each patient for each trial day; n = 5 individual miRNA sequences were used to generate expression profiles. PC, principal component.
Further studies demonstrated that miR-10b knockdown did not affect fatty acid uptake (Fig. 5C), fatty acid oxidation (Fig. 5D), or fatty acid esterification (Fig. 5E).

**DISCUSSION**

Exercise training causes remarkable changes in the adipose tissue transcriptional network, altering the expression of ~1500–2500 genes (8, 9), which likely underpins the beneficial effects of exercise in adipose tissue function. Herein, we extended these observations and report on the adipocyte miRNA profile in humans before and after endurance exercise training. The major findings of the present study were that the miRNA expression profile in adipocytes is unresponsive to endurance exercise training, which suggests that stable changes in miRNA expression are unlikely to be important molecular mediators of adipocyte adaptation to endurance exercise training.

Exercise training did not alter miRNA expression profiles of adipocytes, which contrasts with previous studies that reported stable exercise-mediated changes in miRNA expression in tissues other than adipose, most notably skeletal muscle (36), heart (37), and blood (17, 38, 39). Of 562 miRNAs that were identified in mature adipocytes, no single miRNA was significantly altered after exercise training. Only miR-10b tended to decrease after exercise training, and this was evident only in GF adipocytes. Exercise induces transient changes in mRNA transcription in skeletal muscle (40, 41) and adipose tissue (42), and exercise rapidly and transiently regulates several miRNAs in skeletal muscle (43). A caveat to the interpretation of the present study is the possibility that potential changes in miRNA expression in adipocytes may have been restored to baseline values in the timeframe between the cessation

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Numbers were calculated as the sum of raw reads from 20 samples (i.e., ABD and GF, pre- and post-training; n = 5 per condition).

Figure 3. Differentially expressed miRNAs in human ABD and GF adipocytes. A–C) miR-204 (A), miR-3613 (B), and miR-4532 (C) expression was significantly greater in GF compared with ABD adipocytes before exercise training. Within each panel, fold-change between tissues is shown to the left and mean expression levels from all samples to the right. D, E) miR-10b expression is significantly higher in ABD vs. GF adipocytes before (D) and after (E) exercise training. All data are shown as fold-change between tissues with 95% confidence intervals. Data assessed by paired Student’s t test; n = 5 for ABD and GF adipocytes. *P < 0.05.
of exercise training and adipose tissue sampling (i.e., 48 h). If adipocyte miRNA expression profiles are impacted in the hours immediately after exercise, this would be consistent with the notion that miRNAs are more fine-tuners, rather than important long-term regulators of the exercise response in adipocytes. To our knowledge, no study has examined the time course of miRNA expression changes after acute exercise, if indeed such changes do occur in adipocytes.

miRNAs play a major role in adipogenesis by regulating cell fate determination, clonal expansion, and terminal differentiation (19). A major advance of the present study was the demonstration that many miRNAs that were previously shown to be involved in adipogenesis were also highly expressed in mature adipocytes. For example, there are 9 miRNAs known to regulate cell fate determination toward an adipocyte lineage, and of these miRNAs, miR-22 and miR-30 were highly expressed in mature adipocytes. Similarly, let-7 and miR-92 are 2 of 5 known modulators of clonal expansion, and these miRNAs were also highly expressed in mature human adipocytes. There are 39 known miRNAs that act as regulators of terminal differentiation, and we show that 9 positive regulators (miRs-21, -26b, -30, -103, -143, -148, -181a, -199a, and -378) and 5 negative regulators of terminal differentiation (let-7, miRs-22, -125a, -193a/b, and -224) are expressed in mature adipocytes. Of these miRNAs, the positive adipogenic regulators are more abundant in mature adipocytes compared with negative regulators. Taken together, these data indicate that miRNAs that initiate early-stage adipogenesis are mostly suppressed in mature adipocytes, but that many miRNAs that are involved in terminal differentiation remain highly abundant, which suggests that this miRNA cluster regulates varied cellular functions via distinct stages of the cell cycle. Such regulation is consistent with differentiation in other tissues, such as vascular smooth muscle cells, which undergo phenotypic switching that is mediated by several miRNA species that remain up-regulated after differentiation (44).

Aside from their prominent role in adipogenesis, there is some evidence that miRNAs impact metabolism in mature adipocytes (18). Although much of this work has been performed in immortalized murine adipocytes, our examination of the highest expressing miRNAs in mature human adipocytes may provide clues as to the biologically relevant regulators of adipocyte function.
For example, we reported high expression of miRNAs that influence insulin action, including miR-143, which is the most highly expressed miRNA in human adipocytes, and suppresses insulin signaling (45) and miR-103, which impairs insulin-stimulated glucose uptake (46). The major metabolic function of adipocytes is to facilitate efficient storage and release of fatty acids. The present study shows that miRNAs that were previously implicated in triglyceride storage (i.e., via de novo lipogenesis or fatty acid esterification) are highly expressed in human adipocytes and include miR-224 (47) and miR-125b (48). Furthermore, several miRNAs that stimulate lipolysis were highly expressed in human adipocytes (Supplemental Table S1) and include miR-30c, -193b, and -378 (49), whereas known inhibitors of lipolysis were also prominently expressed. For example, miR-124a attenuates triglyceride lipolysis by targeting adipose tri-glyceride lipase (50), whereas miR-145 suppresses lipolysis by targeting the coactivator of adipose tri-glyceride lipase, comparative gene identification 58 (51). Factors that regulate expression of these miRNAs and the understanding of how these miRNAs coordinate lipolysis remains unsolved.

We directly assessed the functional relevance of several miRNAs on miR-10b because it is the second most abundant miRNA in adipocytes. A notable finding of this work was that inhibiting miR-10b reduced β-adrenergic, but not basal or forskolin-stimulated lipolysis in adipocytes. miR-10b is predicted to target β3-adrenergic receptors (miRanda, miRWalk, Targetscan), and further studies are required to precisely delineate the molecular interactions of miR-10b in lipolysis. Our studies in murine adipocytes demonstrate no effect of miR-10b on β-adrenergic receptor expression. Whereas the role of miRNAs in adipocyte metabolism is still being investigated, we have provided a comprehensive list of the most abundant miRNAs in mature human adipocytes—critical information that provides a platform for further examination of miRNAs in adipocyte biology.

Epidemiologic data suggests that body fat distribution is an important determinant of metabolic and cardiovascular risk, with increased GF adipose deposition (i.e., a pear shape) linked to decreased risk of developing type 2 diabetes and cardiovascular disease (52), and increased abdominal fat (i.e., an apple shape) associated with increased cardiometabolic risk (52, 53). We asked whether the miRNA expression profile of adipocytes contributes to the heterogeneity within adipose tissues. Our results showed that miRNA profiles of adipocytes that were isolated from ABD and GF regions are strikingly similar,
which suggests that miRNA regulation is unlikely to explain the apparent functional differences between ABD and GF adipose tissue.

In summary, whereas miRNA responses to exercise training are functionally important in skeletal muscle, the evidence provided herein demonstrates that stable changes in miRNA expression do not seem to be important for regulating adipocyte function in response to exercise training. Thus, whereas miRNAs are critical in adipocyte development by directing clonal expansion and adipogenesis (19), stable changes in miRNA expression do not seem to be obligatory for the metabolic adaptations observed with exercise training. Nevertheless, the description and quantification of miRNAs in mature human adipocytes will provide an important resource for further studies that examine miRNA function in adipocyte biology.

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AUTHOR CONTRIBUTIONS

R. C. R. Meex and M. J. Watt designed research; T. Tsiloulis, J. Pike, B. J. Canny, R. C. R. Meex, and M. J. Watt performed research and analyzed data; D. Powell and F. J. Rosello performed bioinformatics analysis; T. Tsiloulis and M. J. Watt wrote the paper; and B. Canny, D. Powell, F. J. Rosello, and R. C. R. Meex edited the paper.

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Thomas Tsiloulis, Joshua Pike, David Powell, et al.

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