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## HIGHLIGHTED TOPIC | Epigenetics in Health and Disease

# Evidence for microRNA involvement in exercise-associated neutrophil gene expression changes

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**Radom-Aizik S, Zaldivar F Jr, Oliver S, Galassetti P, Cooper DM.** Evidence for microRNA involvement in exercise-associated neutrophil gene expression changes. *J Appl Physiol* 109: 252–261, 2010. First published January 28, 2010; doi:10.1152/jappphysiol.01291.2009.—Exercise leads to a rapid change in the profile of gene expression in circulating neutrophils. MicroRNAs (miRNAs) have been discovered to play important roles in immune function and often act to attenuate or silence gene translation. We hypothesized that miRNA expression in circulating neutrophils would be affected by brief exercise. Eleven healthy men (19–30 yr old) performed 10, 2-min bouts of cycle ergometer exercise interspersed with 1-min rest at a constant work equivalent to ~76% of maximal oxygen uptake ( $\dot{V}O_{2max}$ ). We used the Agilent Human miRNA V2 Microarray. A conservative statistical approach was used to determine that exercise significantly altered 38 miRNAs (20 had lower expression). Using RT-PCR, we verified the expression level changes from before to after exercise of seven miRNAs. In silico analysis showed that collectively 36 miRNAs potentially targeted 4,724 genes (2 of the miRNAs had no apparent gene targets). Moreover, when we compared the gene expression changes ( $n = 458$ ) in neutrophils that have been altered by exercise, as previously reported, with the miRNAs altered by exercise, we identified three pathways, Ubiquitin-mediated proteolysis, Jak-STAT signaling pathway, and Hedgehog signaling pathway, in which an interaction of miRNA and gene expression was plausible. Each of these pathways is known to play a role in key mechanisms of inflammation. Brief exercise alters miRNA profile in circulating neutrophils in humans. These data support the hypothesis that exercise-associated changes in neutrophil miRNA expression play a role in neutrophil gene expression in response to physical activity.

leukocytes; granulocyte; epigenetic; immune system

A SINGLE BRIEF BOUT of exercise causes a profound perturbation of cellular homeostasis and leads to a transient activation of immune cells and inflammatory mediators even in healthy humans (39). We recently discovered that exercise leads not just to increased numbers of neutrophils in the circulation, but also to substantial expression changes in functional groups of genes and gene pathways (10, 34). It is becoming increasingly clear that when neutrophils are chronically activated they can contribute to disease (9). Consequently, we reasoned that exercise would not only stimulate gene expression in neutrophils, but also influence homeostatic processes, specifically microRNAs (miRNAs) that play a role in modulating protein translation and, ultimately, cell function.

miRNAs are a group of small noncoding RNA molecules ~22 nucleotides (nt) in length that are now known to regulate a variety of immune functions (1, 3, 24). In general, the miRNAs function to mitigate or silence protein translation (2). A growing number of animal-model and human studies point toward key regulatory roles for miRNAs in the neutrophil (1, 24). For example, miRNA-223 has been shown to influence granulocyte development in humans (14). Johnnidus and co-workers (21) found marked neutrophilia and abnormal nuclear morphology in miRNA-223-deficient transgenic mice. In cultured human neutrophils, Bazzoni et al. (5) found miRNA-9 was upregulated with exposure to proinflammatory mediators like LPS, IL-1 $\beta$ , and TNF- $\alpha$ , and may target the NF $\kappa$ B inflammatory regulatory pathway. To date, no studies have examined the effect of brief exercise on miRNA expression in human neutrophils in vivo.

Our exploration of miRNAs was facilitated by observations we made in a recent study, in which we found that brief exercise led to changes in the expression of 526 probe sets (458 annotated genes) in circulating neutrophils (34). We could, therefore, first test the hypothesis that brief exercise altered miRNAs in circulating neutrophils in humans. Simultaneously, as an initial step in determining whether there was a relationship between the miRNAs that were altered by exercise and the gene expression also altered by exercise, we utilized in silico analysis with neutrophil gene expression data obtained in our laboratory but from a different group of volunteers studied earlier who also performed aerobic exercise. These participants performed cycle ergometry at ~79% of each individual's maximal work rate for 30 min. The participants in the present study (from whom we extracted miRNA) performed 10, 2-min bouts of constant work rate cycled ergometry at ~76% maximal work rate. Each bout was separated by a 1-min period of rest. We reasoned that there would be a link between the particular miRNAs identified and the pattern of gene expression that had been altered by exercise.

## MATERIALS AND METHODS

**Participants.** Eleven healthy men (19–30 yr old) participated in this study (Table 1). Seven of the participants were Caucasian; one was Hispanic; one was Asian, and two participants were of mixed ethnicity. The decision to include only men in this investigation was made primarily because we wanted in these initial studies to minimize possible confounding effects related to sex [e.g., menstrual cycle hormones, often difficult to time precisely, that are known to influence stress and inflammatory responses (19)]. Elite athletes, individuals who participated vigorously in competitive sports, and anyone with a

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Table 1. *Anthropometric characteristics and exercise responses of the 11 subjects*

	Value
Age, yr	22.2 ± 1.0
Height, cm	173.1 ± 3.0
Body mass, kg	75.3 ± 3.5
BMI, kg/m <sup>2</sup>	25.1 ± 0.9
Peak $\dot{V}O_2$ , ml·kg <sup>-1</sup> ·min <sup>-1</sup>	42.0 ± 2.0
Average $\dot{V}O_2$ during constant work rate as percent of peak $\dot{V}O_2$	75.7 ± 2.2
Neutrophils, % change from before to after exercise ( <i>P</i> = 0.00005)	56 ± 8

Values are means ± SE. BMI, body mass index;  $\dot{V}O_2$ , oxygen uptake.

history of any chronic medical conditions or medication use were excluded from participation. The Institutional Review Board at the University of California Irvine approved the study, and written informed consent was obtained from all participants on enrollment.

**Anthropometric measurements.** Standard, calibrated scales and stadiometers were used to determine height and body mass.

**Measurement of fitness.** Each subject performed a ramp-type progressive cycle ergometer exercise test using the SensorMedics metabolic system (Ergoline 800S, Yorba Linda, CA). After sitting comfortably without pedaling ("resting") on the cycle ergometer for 3 min and 1 min of unloaded pedaling, the work rate (WR) was incremented at 20–30 W/min to the limit of the subject's tolerance. Subjects were vigorously encouraged during the high-intensity phases of the exercise protocol. Gas exchange was measured breath-by-breath and the anaerobic (lactate) threshold and peak  $\dot{V}O_2$  were calculated using standard methods (11).

**Exercise protocol.** At least 48 h, but not exceeding 7 days, following the completion of the ramp test, each subject performed 20 min of exercise consisting of 10, 2-min bouts of constant work rate cycle ergometry, with 1-min rest interval between each bout of exercise (i.e., a 30-min interval). The work rate was individualized for each subject and was calculated to be equivalent to the work rate corresponding roughly to 50% of the work rate between the anaerobic threshold and the peak oxygen uptake (as determined noninvasively from the ramp-type test). On average, this work rate was equivalent to 76% of the participants' peak  $\dot{V}O_2$ .

**Blood sampling and analysis.** An indwelling catheter was inserted into the antecubital vein. A baseline sample was taken 30 min after the placement of the catheter and before the onset of exercise. We waited 30 min to ensure that measurable physiological parameters of stress (e.g., heart rate and blood pressure) were at baseline levels. Subjects then completed the 30 min of intermittent exercise, and additional blood samples were obtained immediately after exercise. Complete blood counts for white blood cell analysis were obtained by standard methods from the clinical hematology laboratory.

**Neutrophil isolation.** Neutrophils were isolated from 30 ml EDTA-treated peripheral blood using OptiPrep Density Gradient Medium (SIGMA). The duration from blood draw to stabilization of RNA never exceeded 90 min. Using Wright-Giemsa stain, we determined that this approach to neutrophil isolation consistently yielded ≥98% purification.

**RNA extraction.** Total RNA was extracted using TRIzol (Gibco BRL Life Technologies, Rockville, MD). RNA pellets were resuspended in diethyl pyrocarbonate-treated water. RNA integrity was assessed (before beginning target processing) by running out a small amount of each sample (typically 25–250 ng/well) onto a RNA Lab-On-A-Chip (Caliper Technologies, Mountain View, CA) that was evaluated on an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA).

**miRNA microarrays.** To normalize the total RNA extracted between the before- and after-exercise conditions in which the neutrophil concen-

tration increased, we used a common amount of total RNA (100 ng) in both cases. This approach to normalizing samples in studies of gene expression in leukocytes is used by others (7); however, one cannot be absolutely certain that the 100 ng of total RNA reflected the same number of neutrophils under both conditions. The total RNA was labeled with the fluorescent dye Cyanine 3-pCp (Cy3) using the miRNA Labeling Reagent and Hybridization Kit (Agilent Technologies) following the manufacturer's protocol. Cy3-labeled RNA from each sample was hybridized to an Agilent Human miRNA Version 2 Microarray. The hybridized array was then washed and scanned according to Agilent specifications, and data were extracted from the scanned image using Feature Extraction version 10.2 (Agilent Technologies).

The results were analyzed using GeneSpring GX 10.0.2 Software (Agilent Technologies). All raw signal values lower than 1 were adjusted to 1 and normalized using percentile shift (90th percentile). Only entities that had a present or marginal flag in at least 50% of values in any one of the two conditions were selected for further analysis. Overall, 282 of 821 entities represented on the array met these criteria. The microarray raw data have been deposited in the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>; series accession no. GSE18999). Traditional Student's paired *t*-test was first applied to each probe set, and false discovery rate (FDR) (Benjamin-Hochberg) procedure was carried out.

The final list of significantly changed miRNAs was then additionally analyzed using TargetScan data base (provided by GeneSpring) (<http://www.targetscan.org/>).

We looked for the predicted target genes for each miRNA (context percentile = 50, conserved database). Briefly, TargetScan predicts gene targets of miRNAs by searching for the presence of conserved 8mer and 7mer sites that match the seed region (positions 2–7 of a mature miRNA) of each miRNA (18, 22, 23). We then submitted each list of target genes into DAVID, the Database for Annotation, Visualization, and Integrated Discovery (<http://david.abcc.ncifcrf.gov/>), to classify the genes into KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways. We present only pathways with EASE score < 0.05. EASE score is a modified Fisher exact *P* value in the DAVID system used for gene-enrichment analysis. EASE score *P* value = 0 represents perfect enrichment. *P* value ≤ 0.05 was considered as significant gene enrichment in a specific annotation category ([http://david.abcc.ncifcrf.gov/helps/functional\\_annotation.html#summary](http://david.abcc.ncifcrf.gov/helps/functional_annotation.html#summary)).

**mRNA microarray analysis.** The methods for neutrophil gene expression analysis are described in our previous publication (34).

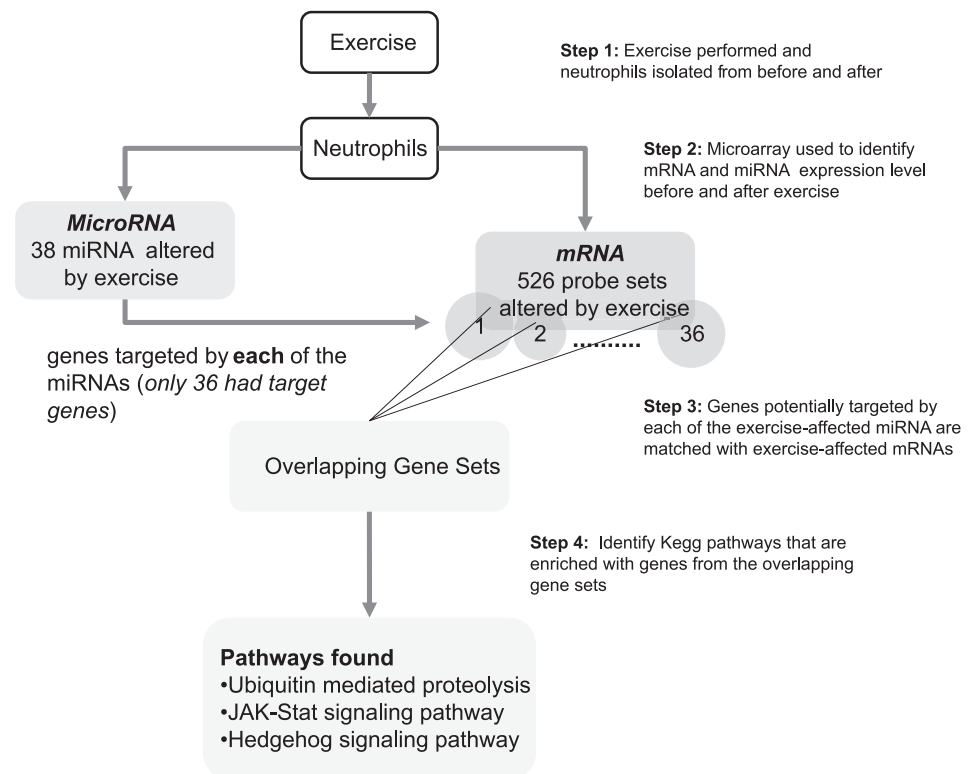
**An intersecting analysis of miRNA and gene expression level (Fig. 1).** We performed an additional analysis using miRNA target genes and the 458 genes whose expression was affected by exercise. We searched for genes and pathways that were potentially targeted specifically by those miRNAs that had also been altered by exercise (Table 2). We then performed pathway analysis in this overlapping gene set that included miRNAs and their gene targets that had both been altered by exercise. We present only pathways with EASE score ≤ 0.05.

As noted, there is experimental and theoretical evidence that, in general, downregulation of miRNA will lead to upregulated expression of targeted genes and, conversely, that miRNA upregulation will lead to downregulated target-gene expression. For each miRNA affected by exercise, we calculated the percent of target genes that were up- or downregulated (Fig. 2).

**Physiological data.** The physiological data are presented as mean and standard error (SE). The two-sided paired *t*-test was applied for testing changes from before to after the exercise, and the significance level was set at 0.05.

**Quantitative real-time polymerase chain reaction (RT-PCR).** For confirmation of miRNA microarray expression findings, TaqMan assays were carried out on seven miRNAs. Five were associated with gene expression of either the ubiquitin-mediated proteolysis or Jak-STAT signaling pathways (hsa-miR-106a, hsa-miR-17, hsa-miR-20a, hsa-miR-20b, and hsa-miR-16); and two miRNAs that are relevant to the immune system, hsa-miR-18a and hsa-miR-223 (24).

Fig. 1. An intersecting analysis of the specific neutrophil microRNAs (miRNAs) whose expression was significantly altered by exercise and genes whose expression was also significantly altered by exercise. This approach identified 3 significant pathways, providing direction for further investigations of how miRNAs altered by exercise regulate neutrophil function.



Reverse transcriptase (RT) reactions were carried out using the TaqMan miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. Briefly, each RT reaction contained 0.25 mM each dNTPs, 3.33 U/ $\mu$ l MultiScribe Reverse Transcriptase, 1 $\times$  Reverse Transcription Buffer, 0.25 U RNase Inhibitor, water, 10 ng total RNA and miRNA-specific RT primer. The 15- $\mu$ l reactions were incubated in a 96-well plate for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, and then held at 4°C in a Techne TC-512 thermal cycler (Burlington, NJ).

RT-PCR was performed using a standard TaqMan PCR protocol on an Applied Biosystems 7900HT Sequence Detection System. Each TaqMan reaction contained 1 $\times$  TaqMan miRNA assay (primers/probe) specific to the miRNA of interest, a 1:15 dilution of the RT product for that miRNA of interest, 1 $\times$  Universal MasterMix (no AmpErase UNG), and water to 20  $\mu$ l. The reactions were carried out in a 96-well plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. All reactions were run in duplicate. The cycle threshold (Ct) for each sample was determined using SDS software version 2.3 (Applied Biosystems) and was normalized to endogenous control RNU44.

## RESULTS

**Anthropometric and physiological characteristics.** The anthropometric and physiological characteristics of the 11 subjects appear in Table 1. The average body mass index (BMI) was within normal limits.

**Plasma lactate.** The exercise bout caused a mean increase of  $6.2 \pm 0.5$  mmol/l in lactate levels in the plasma [before ( $1.7 \pm 0.3$  mmol/l) vs. after ( $7.9 \pm 0.4$  mmol/l),  $P < 0.0000003$ ].

**Leukocyte response to exercise.** As shown in Table 1, the number of neutrophils was significantly elevated at peak exercise ( $P < 0.00005$ ).

**Effects of exercise on neutrophil miRNA expression (Table 2).** Thirty minutes of intermittent exercise (i.e., the 10 2-min bouts) altered the expression level of 38 miRNAs (20 had

lower expression). In silico analysis showed that 36 miRNAs potentially targeted 4,724 genes (2 miRNAs did not have any known target genes). Subsequent pathway analysis resulted in a total of 49 distinct pathways from the genes targeted by each of the miRNAs individually.

**RT-PCR verification of specific miRNA.** We verified the expression level changes from before to after exercise of seven miRNAs. The TaqMan results were significant for all,  $P < 0.05$ .

**Intersecting analysis of miRNA and gene expression level.** As shown in Fig. 1, the miRNA-gene expression overlapping data set (i.e., those genes from the 458 that were both affected by exercise and potentially targeted by one or more of the 36 miRNAs that had also been affected by exercise) consisted of 137 genes. When the pathway analysis was performed for the targeted genes of the individual miRNAs that had also been affected by exercise, three significant pathways were found: ubiquitin-mediated proteolysis, Jak-STAT signaling pathway, and Hedgehog signaling pathway. Intriguingly, the ubiquitin-mediated proteolysis pathway was found to be significant in 6 of the 36 miRNAs analyzed (Table 2).

## DISCUSSION

We have demonstrated that a relatively brief exercise session, one that mimics patterns of physical activity naturally found in humans, is sufficient to change the expression pattern of 38 miRNAs of 826 entities represented on the chip. There is great interest in the miRNAs as "fine tuners" of gene expression and protein translation in an ever-growing range of cell and physiological function (15, 41). This paradigm fits well with emerging concepts of the regulation of the acute inflammatory response to exercise, in which physical activity leads to an initial activation of neutrophils (29, 31, 34). This rapid

Table 2. Summary of key findings: association between miRNA and gene expression in response to brief exercise in human neutrophils and gene pathways that were significantly affected

miRNA and Fold Change	Target Genes (no.): Enrich in Kegg Pathways EASE < 0.05	Genes Affected by Exercise 1) Known To Be Targeted by the Specific miRNA; and 2) Whose Expression Level Was Altered by Exercise in Our Previous Study	Common Genes Enrich in Kegg Pathways EASE ≤ 0.05
hsa-miR-20a ↓ 1.24	870 Target genes: TGF-β signaling pathway, Prostate cancer, Renal cell carcinoma, Axon guidance, Glioma, Bladder cancer, Chronic myeloid leukemia, Melanoma, Pancreatic cancer, Non-small cell lung cancer, Colorectal cancer, ErbB signaling pathway, MAPK signaling pathway, Endometrial cancer, Regulation of actin cytoskeleton, mTOR signaling pathway, Acute myeloid leukemia, Small cell lung cancer, Focal adhesion, Circadian rhythm, Adherens junction	36 Genes: ANKRD12(↓), HBP1(↓), C10orf46(↓), DNAJB9(↓), PTEN(↓), SSH2(↓), DNAJB9(↓), UBE2J1(↓), STK38(↑), TAGAP(↑), USP24(↑), NPAT(↑), ZNF148(↑), SERTAD2(↑), FBXW11(↑), CNOT6L(↑), TRIM37(↑), ATP2B4(↑), CENTD1(↑), SYNE1(↑), PIK3R1(↑), SLC5A3(↑), TMEM50B(↑), MAP3K12(↑), ARHGEF3(↑), C1orf63(↑), PIK3R1(↑), AHNAK(↑), HSPA8(↑), SMAD7(↑), CCND2(↑), PAFAH2(↑), PTPN4(↑), RUNX3(↑), DUSP2(↑), LDLR(↑)	Ubiquitin-mediated proteolysis
hsa-miR-106a ↓ 1.39	880 Target genes: Axon guidance, TGF-β signaling pathway, Bladder cancer, Prostate cancer, Renal cell carcinoma, Glioma, Chronic myeloid leukemia, MAPK signaling pathway, Melanoma, Pancreatic cancer, Non-small cell lung cancer, Colorectal cancer, ErbB signaling pathway, Circadian rhythm, Endometrial cancer, mTOR signaling pathway, Regulation of actin cytoskeleton, Acute myeloid leukemia, Small cell lung cancer, Adherens junction	35 Genes: ANKRD12(↓), HBP1(↓), C10orf46(↓), SRGAP2(↓), PTEN(↓), SSH2(↓), DNAJB9(↓), UBE2J1(↓), STK38(↑), TAGAP(↑), USP24(↑), NPAT(↑), ZNF148(↑), SERTAD2(↑), FBXW11(↑), CNOT6L(↑), TRIM37(↑), ATP2B4(↑), CENTD1(↑), SYNE1(↑), SLC5A3(↑), TMEM50B(↑), MAP3K12(↑), ARHGEF3(↑), C1orf63(↑), KLF12(↑), AHNAK(↑), HSPA8(↑), SMAD7(↑), CCND2(↑), PAFAH2(↑), PTPN4(↑), RUNX3(↑), DUSP2(↑), LDLR(↑)	Ubiquitin-mediated proteolysis
hsa-miR-20b ↓ 1.29	871 Target genes: TGF-β signaling pathway, Axon guidance, Bladder cancer, Prostate cancer, Renal cell carcinoma, Glioma, Chronic myeloid leukemia, Melanoma, Pancreatic cancer, MAPK signaling pathway, Nonsmall cell lung cancer, Colorectal cancer, ErbB signaling pathway, Regulation of actin cytoskeleton, Circadian rhythm, Endometrial cancer, mTOR signaling pathway, Focal adhesion, Acute myeloid leukemia, Small cell lung cancer, Adherens junction	34 Genes: ANKRD12(↓), HBP1(↓), C10orf46(↓), PTEN(↓), SSH2(↓), DNAJB9(↓), UBE2J1(↓), STK38(↑), TAGAP(↑), USP24(↑), NPAT(↑), ZNF148(↑), SERTAD2(↑), FBXW11(↑), CNOT6L(↑), TRIM37(↑), ATP2B4(↑), CENTD1(↑), SYNE1(↑), SLC5A3(↑), TMEM50B(↑), MAP3K12(↑), ARHGEF3(↑), C1orf63(↑), KLF12(↑), AHNAK(↑), HSPA8(↑), SMAD7(↑), CCND2(↑), PAFAH2(↑), PTPN4(↑), RUNX3(↑), DUSP2(↑), LDLR(↑)	Ubiquitin-mediated proteolysis
hsa-miR-17 ↓ 1.40	875 Target genes: TGF-β signaling pathway, Axon guidance, Bladder cancer, Prostate cancer, Renal cell carcinoma, Glioma, Chronic myeloid leukemia, MAPK signaling pathway, Melanoma, Pancreatic cancer, Non-small cell lung cancer, Colorectal cancer, ErbB signaling pathway, Circadian rhythm, Endometrial cancer, mTOR signaling pathway, Regulation of actin cytoskeleton, Acute myeloid leukemia, Small cell lung cancer, Adherens junction	37 Genes: ANKRD12(↓), HBP1(↓), C10orf46(↓), PTEN(↓), SSH2(↓), DNAJB9(↓), UBE2J1(↓), STK38(↑), TAGAP(↑), TAGAP(↑), USP24(↑), NPAT(↑), ZNF148(↑), SERTAD2(↑), FBXW11(↑), CNOT6L(↑), TRIM37(↑), ATP2B4(↑), CENTD1(↑), TAGAP(↑), SYNE1(↑), SLC5A3(↑), TMEM50B(↑), MAP3K12(↑), TAGAP(↑), ARHGEF3(↑), C1orf63(↑), KLF12(↑), AHNAK(↑), HSPA8(↑), SMAD7(↑), CCND2(↑), PAFAH2(↑), PTPN4(↑), RUNX3(↑), DUSP2(↑), LDLR(↑)	Ubiquitin-mediated proteolysis
hsa-miR-93 ↓ 1.239805	871 Target genes: TGF-β signaling pathway, Axon guidance, Bladder cancer, Prostate cancer, Renal cell carcinoma, Glioma, Chronic myeloid leukemia, MAPK signaling pathway, Melanoma, Pancreatic cancer, Regulation of actin cytoskeleton, Nonsmall cell lung cancer, ErbB signaling pathway, Adherens junction, Circadian rhythm, Endometrial cancer, mTOR signaling pathway, Acute myeloid leukemia, Colorectal cancer, Small cell lung cancer	31 Genes: ANKRD12(↓), HBP1(↓), PTEN(↓), SSH2(↓), DNAJB9(↓), UBE2J1(↓), STK38(↑), TAGAP(↑), USP24(↑), NPAT(↑), ZNF148(↑), SERTAD2(↑), FBXW11(↑), CNOT6L(↑), TRIM37(↑), CENTD1(↑), SYNE1(↑), SLC5A3(↑), TMEM50B(↑), MAP3K12(↑), ARHGEF3(↑), C1orf63(↑), AHNAK(↑), HSPA8(↑), SMAD7(↑), CCND2(↑), PAFAH2(↑), PTPN4(↑), RUNX3(↑), DUSP2(↑), LDLR(↑)	Ubiquitin-mediated proteolysis

Continued

Table 2.—Continued

miRNA and Fold Change	Target Genes (no.): Enrich in Kegg Pathways EASE < 0.05	Genes Affected by Exercise 1) Known To Be Targeted by the Specific miRNA; and 2) Whose Expression Level Was Altered by Exercise in Our Previous Study	Common Genes Enrich in Kegg Pathways EASE $\leq$ 0.05
hsa-miR-520d-3p $\uparrow$ 2.82	515 Target genes: Colorectal cancer, mTOR signaling pathway, MAPK signaling pathway, ECM-receptor interaction, Focal adhesion, TGF- $\beta$ signaling pathway, Regulation of actin cytoskeleton, Pancreatic cancer, Endometrial cancer	19 Genes: TARDBP( $\downarrow$ ), UBE2B( $\downarrow$ ), UBE2J1( $\downarrow$ ), WDR68( $\uparrow$ ), TAGAP( $\uparrow$ ), USP24( $\uparrow$ ), ZNF148( $\uparrow$ ), ATP2B4( $\uparrow$ ), RALGDS( $\uparrow$ ), TIPARP( $\uparrow$ ), ARHGEF3( $\uparrow$ ), UBE3A( $\uparrow$ ), ASF1A( $\uparrow$ ), PRKACB( $\uparrow$ ), AHNAK( $\uparrow$ ), TOX( $\uparrow$ ), CCND2( $\uparrow$ ), DUSP2( $\uparrow$ ), MYBL1( $\uparrow$ )	Ubiquitin mediated proteolysis
hsa-miR-130b $\downarrow$ 1.41	599 Target genes: TGF- $\beta$ signaling pathway, mTOR signaling pathway, Colorectal cancer, Renal cell carcinoma, Prostate cancer, Pancreatic cancer, Chronic myeloid leukemia, Hedgehog signaling pathway, Glioma, mErbB signaling pathway, Wnt signaling pathway, Axon guidance, MelanoTarget genesis, Focal adhesion, Ubiquitin-mediated proteolysis	27 Genes: ANKRD12( $\downarrow$ ), TARDBP( $\downarrow$ ), HBP1( $\downarrow$ ), KIF13A( $\downarrow$ ), ARFGEF1( $\downarrow$ ), DICER1( $\downarrow$ ), SBF2( $\downarrow$ ), BAG5( $\downarrow$ ), NPAT( $\uparrow$ ), PHF20( $\uparrow$ ), P15RS( $\uparrow$ ), ZNF148( $\uparrow$ ), CFBF( $\uparrow$ ), FBXW11( $\uparrow$ ), TRIM37( $\uparrow$ ), CENTD1( $\uparrow$ ), TMEM50B( $\uparrow$ ), MAP3K12( $\uparrow$ ), PHACTR2( $\uparrow$ ), PRKACB( $\uparrow$ ), HSPA8( $\uparrow$ ), PTPN4( $\uparrow$ ), RUNX3( $\uparrow$ ), MAF( $\uparrow$ ), LDLR( $\uparrow$ ), ENPP4( $\uparrow$ ), MYBL1( $\uparrow$ )	Hedgehog signaling pathway
hsa-miR-16 $\downarrow$ 1.23	776 Target Genes: Acute myeloid leukemia, Insulin signaling pathway, Prostate cancer, Cell cycle, Chronic myeloid leukemia, Non-small cell lung cancer, Ubiquitin-mediated proteolysis, Endometrial cancer, Melanoma, Colorectal cancer, Pancreatic cancer, Glioma, Adherens junction, TGF- $\beta$ signaling pathway, mTOR signaling pathway, Renal cell carcinoma, Wnt signaling pathway, Thyroid cancer, Hedgehog signaling pathway, Type II diabetes mellitus, MAPK signaling pathway, Gap junction, O-glycan biosynthesis	30 Genes: ZCCHC2( $\downarrow$ ), C10orf46( $\downarrow$ ), DICER1( $\downarrow$ ), UBE2J1( $\downarrow$ ), BAG5( $\downarrow$ ), AP1GBP1( $\uparrow$ ), TUBB( $\uparrow$ ), WDR68( $\uparrow$ ), PHF20( $\uparrow$ ), OTUD4( $\uparrow$ ), SLC2A3( $\uparrow$ ), C13orf18( $\uparrow$ ), MGAT4A( $\uparrow$ ), CNOT6L( $\uparrow$ ), TRIM37( $\uparrow$ ), PURA( $\uparrow$ ), CDC42EP2( $\uparrow$ ), PIK3R1( $\uparrow$ ), SLC5A3( $\uparrow$ ), PHACTR2( $\uparrow$ ), IL10RA( $\uparrow$ ), SMAD3( $\uparrow$ ), PLEKHA1( $\uparrow$ ), SMAD7( $\uparrow$ ), CCND2( $\uparrow$ ), RNF125( $\uparrow$ ), SH2D1A( $\uparrow$ ), SYTL2( $\uparrow$ ), TGFBR3( $\uparrow$ ), MYBL1( $\uparrow$ )	Jak-STAT signaling pathway
hsa-let-7i $\downarrow$ 1.25	558 Target genes: p53 signaling pathway, ECM-receptor interaction, MAPK signaling pathway, Colorectal cancer, Pancreatic cancer, Focal adhesion, O-glycan biosynthesis, mTOR signaling pathway, Melanoma, TGF- $\beta$ signaling pathway, Chronic myeloid leukemia, Adherens junction	14 Genes: ZNF294( $\downarrow$ ), RIOK3( $\downarrow$ ), PMAIP1( $\downarrow$ ), KLHL6( $\downarrow$ ), USP24( $\uparrow$ ), MGAT4A( $\uparrow$ ), TMEM2( $\uparrow$ ), DNAAIC1( $\uparrow$ ), ZFP36L2( $\uparrow$ ), SLAMF6( $\uparrow$ ), PHACTR2( $\uparrow$ ), RASGRP1( $\uparrow$ ), GNPTAB( $\uparrow$ ), CREM( $\uparrow$ )	None
hsa-miR-107 $\downarrow$ 1.26	376 Target genes: Hedgehog signaling pathway, O-glycan biosynthesis, Ubiquitin-mediated proteolysis, Basal cell carcinoma, Circadian rhythm, Leukocyte transendothelial migration, Wnt signaling pathway, MelanoTarget genesis	17 Genes: ZCCHC2( $\downarrow$ ), EIF5( $\downarrow$ ), DICER1( $\downarrow$ ), UBE2J1( $\downarrow$ ), PHF20( $\uparrow$ ), OTUD4( $\uparrow$ ), SMEK2( $\uparrow$ ), CUL4A( $\uparrow$ ), ENSA( $\uparrow$ ), CNOT6L( $\uparrow$ ), PIK3R1( $\uparrow$ ), SLC5A3( $\uparrow$ ), PLEKHA1( $\uparrow$ ), RNF125( $\uparrow$ ), EOMES( $\uparrow$ ), TGFBR3( $\uparrow$ ), MYBL1( $\uparrow$ )	None
hsa-miR-126 $\downarrow$ 1.53	12 Target genes: NO Kegg pathway	None	None
hsa-miR-130a $\downarrow$ 1.61	601 Target genes: TGF- $\beta$ signaling pathway, mTOR signaling pathway, Renal cell carcinoma, Colorectal cancer, ErbB signaling pathway, Prostate cancer, Hedgehog signaling pathway, Glioma, Dorso-ventral axis formation, MelanoTarget genesis, Wnt signaling pathway, Axon guidance, Pancreatic cancer, Chronic myeloid leukemia, Focal adhesion	28 Genes: ANKRD12( $\downarrow$ ), TARDBP( $\downarrow$ ), HBP1( $\downarrow$ ), KIF13A( $\downarrow$ ), ARFGEF1( $\downarrow$ ), DICER1( $\downarrow$ ), SBF2( $\downarrow$ ), BAG5( $\downarrow$ ), NPAT( $\uparrow$ ), PHF20( $\uparrow$ ), P15RS( $\uparrow$ ), ZNF148( $\uparrow$ ), CFBF( $\uparrow$ ), FBXW11( $\uparrow$ ), TRIM37( $\uparrow$ ), ATP2B4( $\uparrow$ ), CENTD1( $\uparrow$ ), TMEM50B( $\uparrow$ ), MAP3K12( $\uparrow$ ), PHACTR2( $\uparrow$ ), PRKACB( $\uparrow$ ), HSPA8( $\uparrow$ ), PTPN4( $\uparrow$ ), RUNX3( $\uparrow$ ), MAF( $\uparrow$ ), LDLR( $\uparrow$ ), ENPP4( $\uparrow$ ), MYBL1( $\uparrow$ )	None
hsa-miR-151-5p $\downarrow$ 1.60	5 Target genes: NO Kegg pathway	None	None
hsa-miR-185 $\downarrow$ 1.28	134 Target genes: GnRH signaling pathway	1 Gene: MAP4( $\uparrow$ )	None
hsa-miR-18a $\downarrow$ 1.35	169 Target genes: Tight junction, p53 signaling pathway	7 Genes: DICER1( $\downarrow$ ), USP24( $\uparrow$ ), TMEM2( $\uparrow$ ), LMO4( $\uparrow$ ), CCND2( $\uparrow$ ), ENC1( $\uparrow$ ), PDE4D( $\uparrow$ )	None

Continued

Table 2.—Continued

miRNA and Fold Change	Target Genes (no.): Enrich in Kegg Pathways EASE < 0.05	Genes Affected by Exercise 1) Known To Be Targeted by the Specific miRNA; and 2) Whose Expression Level Was Altered by Exercise in Our Previous Study	Common Genes Enrich in Kegg Pathways EASE $\leq$ 0.05
hsa-miR-18b ↓ 1.29	169 Target genes: p53 signaling pathway, Tight junction	7 Genes: DICER1(↓), USP24(↑), TMEM2(↑), LMO4(↑), CCND2(↑), ENC1(↑), PDE4D(↑)	None
hsa-miR-194 ↓ 1.26	185 Target genes: TGF-β signaling pathway, ErbB signaling pathway	3 Genes: MGAT4A(↑), PDE4D(↑), SOCS2(↑)	None
hsa-miR-22 ↓ 1.28	276 Target genes: Chronic myeloid leukemia, Adherens junction, ErbB signaling pathway, Pancreatic cancer, Alkaloid biosynthesis II	8 Genes: H3F3B(↓), PTEN(↓), MAX(↓), CNOT6L(↑), TMEM50B(↑), PHACTR2(↑), DDIT4(↑), SOCS2(↑)	None
hsa-miR-363 ↓ 1.34	555 Target genes: Regulation of actin cytoskeleton, Calcium signaling pathway, Adherens junction, Long-term potentiation, Focal adhesion	21 Genes: H3F3B(↓), ING3(↓), PTEN(↓), PTEN(↓), HIVEP1(↓), DNAJB9(↓), NFYC(↓), C10orf118(↓), TAGAP(↑), OTUD4(↑), TMF1(↑), FOSL2(↑), ATP2B4(↑), PTGER4(↑), TMEM50B(↑), PLEKHA1(↑), DDIT4(↑), SMAD7(↑), NPC1(↑), NPC1(↑), EOMES(↑)	None
hsa-miR-660 ↓ 1.231584	67 Target genes: Regulation of actin cytoskeleton, GnRH signaling pathway	1 Gene: TOX(↑)	None
hsa-miR-96 ↓ 1.28	578 Target genes: Long-term potentiation, GnRH signaling pathway, Gap junction, ErbB signaling pathway, Long-term depression, Glioma, Tight junction, Focal adhesion, Prostate cancer, MAPK signaling pathway, Fc epsilon RI signaling pathway, Calcium signaling pathway, Glycosphingolipid biosynthesis - ganglioseries, Renal cell carcinoma, Natural killer cell-mediated cytotoxicity, Axon guidance, Insulin signaling pathway, Chronic myeloid leukemia, T cell receptor signaling pathway, Phosphatidylinositol signaling system, MelanoTarget genesis, Regulation of actin cytoskeleton	13 Genes: ELAVL1(↑), FBXW11(↑), CNOT6L(↑), FYN(↑), SMAD7(↑), ATP2B4(↑), ARHGFE3(↑), PIK3R1(↑), UBE2L3(↑), TMEM50B(↑), EOMES(↑), NCALD(↑), TOX(↑)	None
hsa-miR-1225-5p ↑ 1.55	41 Target genes: NO Kegg pathway	None	None
hsa-miR-1238 ↑ 1.58	61 Target genes: TGF-β signaling pathway	2 Genes: SLC8A1(↓), NCALD(↑)	None
hsa-miR-125a-5p ↑ 1.22	506 Target genes: TGF-β signaling pathway, Glycerolipid metabolism, O-glycan biosynthesis	13 Genes: KLHL6(↓), DICER1(↓), IER2(↓), UBE2J1(↓), PHF23(↑), PHF20(↑), ZNF148(↑), MGAT4A(↑), UBE2L3(↑), TSEN54(↑), MAPRE2(↑), SLC7A1(↑), MAF(↑)	None
hsa-miR-145 ↑ 1.22	439 Target genes: Axon guidance, TGF-β signaling pathway, Chronic myeloid leukemia, Wnt signaling pathway, Adherens junction	13 Genes: APIG1(↓), C7orf28B(↓), SRGAP2(↓), CCNL1(↓), SSH2(↓), CHP(↓), CENTD1(↑), H2AFX(↑), PHACTR2(↑), SMAD3(↑), SLC7A1(↑), RUNX3(↑), PDGFD(↑)	None
hsa-miR-181b ↑ 1.64	652 Target genes: Dorso-ventral axis formation, Axon guidance, Long-term potentiation, Renal cell carcinoma, TGF-β signaling pathway, T cell receptor signaling pathway, MAPK signaling pathway	24 Genes: APIG1(↓), TARDBP(↓), SF1(↓), BCLAF1(↓), SRGAP2(↓), UBE2B(↓), UBE2J1(↓), KLF6(↑), OTUD4(↑), SLC2A3(↑), TMF1(↑), HYOU1(↑), ATP2B4(↑), RALA(↑), PHACTR2(↑), ARHGFE3(↑), SLC38A2(↑), DMXL2(↑), DDIT4(↑), TOX(↑), SMAD7(↑), RNF125(↑), NCALD(↑), MYBL1(↑)	None
hsa-miR-193a-3p ↑ 1.58	120 Target genes: Acute myeloid leukemia, Dorso-ventral axis formation, Endometrial cancer, Renal cell carcinoma, Thyroid cancer, Colorectal cancer, Prostate cancer, Focal adhesion, Non-small cell lung cancer	2 Genes: DNAJB9(↓), WDR68(↑)	None
hsa-miR-197 ↑ 1.38	117 Target genes: Ubiquitin-mediated proteolysis	2 Genes: SSH2(↓), SMEK2(↑)	None

Continued

Table 2.—Continued

miRNA and Fold Change	Target Genes (no.): Enrich in Kegg Pathways EASE < 0.05	Genes Affected by Exercise 1) Known To Be Targeted by the Specific miRNA; and 2) Whose Expression Level Was Altered by Exercise in Our Previous Study	Common Genes Enrich in Kegg Pathways EASE $\leq$ 0.05
hsa-miR-212 $\uparrow$ 1.44	219 Target genes: Prostate cancer, TGF- $\beta$ signaling pathway, Axon guidance, Endometrial cancer, GnRH signaling pathway, Renal cell carcinoma, MelanoTarget genesis, Adherens junction, Non-small cell lung cancer, Colorectal cancer, ErbB signaling pathway, Thyroid cancer	8 Genes: EGR1( $\downarrow$ ), SRGAP2( $\downarrow$ ), H3F3B( $\downarrow$ ), SSH2( $\downarrow$ ), FBXW11( $\uparrow$ ), TMEM2( $\uparrow$ ), FLJ10357( $\uparrow$ ), NCALD( $\uparrow$ )	None
hsa-miR-223 $\uparrow$ 1.29	180 Target genes: NO Kegg pathway	8 Genes: AP1GBP1( $\uparrow$ ), RHOB( $\uparrow$ ), PDE4D( $\uparrow$ ), PRKACB( $\uparrow$ ), PURA( $\uparrow$ ), RGS1( $\uparrow$ ), SLC8A1( $\downarrow$ ), C13orf18( $\uparrow$ )	None
hsa-miR-340* $\uparrow$ 1.25	No target genes		None
hsa-miR-365 $\uparrow$ 1.37	139 Target genes: Wnt signaling pathway, MelanoTarget genesis, Apoptosis, Prostate cancer	6 Genes: CHP( $\downarrow$ ), SSH2( $\downarrow$ ), CNOT6L( $\uparrow$ ), HSPA8( $\uparrow$ ), ZNF148( $\uparrow$ ), SERTAD2( $\uparrow$ )	None
hsa-miR-485-3p $\uparrow$ 2.92	240 Target genes: Wnt signaling pathway, Adherens junction, MAPK signaling pathway, VEGF signaling pathway, Long-term potentiation, Pancreatic cancer, Chronic myeloid leukemia, Colorectal cancer	5 Genes: MGAT4A( $\uparrow$ ), WDR7( $\uparrow$ ), PDE4D( $\uparrow$ ), OTUD4( $\uparrow$ ), SLC5A3( $\uparrow$ )	None
hsa-miR-505 $\uparrow$ 1.24	130 Target genes: Tight junction, Cell cycle	2 Genes: H3F3B( $\downarrow$ ), SF1( $\downarrow$ )	None
hsa-miR-629* $\uparrow$ 1.44	No target genes		None
hsa-miR-638 $\uparrow$ 1.36	17 Target genes: NO Kegg pathway	2 Genes: PGK1( $\downarrow$ ), LMO4( $\uparrow$ )	None
hsa-miR-939 $\uparrow$ 1.40	75 Target genes: NO Kegg pathway	2 Genes: TSC22D3( $\uparrow$ ), LDOC1L( $\uparrow$ )	None
hsa-miR-940 $\uparrow$ 1.39	203 Target genes: T cell receptor signaling pathway, MAPK signaling pathway, Axon guidance, Focal adhesion	4 Genes: SSH2( $\downarrow$ ), CHP( $\downarrow$ ), CNOT6L( $\uparrow$ ), PDE4D( $\uparrow$ )	None

innate immune cellular activity appears to be an essential response to “danger” in which the immune cells are primed (i.e., achieve an initial response state) and positioned to act effectively in the event of an invading pathogen, an injury or wound, or the need to signal other components of the immune system (12, 26).

This may have been beneficial when our human progenitors were, for example, rapidly fleeing a predator or pursuing prey over rough terrain; however, any beneficial effects of immune cell activation would be lost if the proinflammatory response was unfettered and not balanced by equally robust compensatory mechanisms. Indeed, the clinical phenomena of exercise-induced asthma and even exercise-associated anaphylaxis attest to the seriousness of unregulated immune responses to exercise (12). Our results are the first positive steps in determining whether miRNAs play a role in neutrophil homeostasis in response to physical activity.

There is at present very little research on how exercise impacts epigenetic phenomena such as miRNAs. Safdar et al. (35) found that acute endurance exercise altered miRNA expression levels in muscle tissue of C57Bl/6J mice. Drummond et al. (13) showed that an anabolic stimulus of resistance exercise combined with the ingestion of essential amino acids altered the expression level of muscle miRNAs in young and elderly humans. They also noted that aging was associated with higher basal skeletal muscle primary miRNA expression and a dysregulated miRNA response.

To the best of our knowledge, ours is the first report demonstrating an acute effect of exercise on miRNA expression in circulating neutrophils. We used stringent statistical

approaches in analyzing the data to limit the possibility of false-positive results. We found that a different technique, TaqMan RT-PCR in a small group of miRNAs, corroborated the microarray-derived miRNA data. Twenty of the 38 differentially expressed miRNAs had lower expression level immediately after exercise, and in 18 the expression level increased.

Many of the affected miRNAs in neutrophils in our study are known to regulate genes that are involved in immune processes and apoptosis. For example, miR-17, miR-18a, and miR-20a are all part of the miR-17–92 cluster and had lower expression level after exercise. Moreover, a paralog [miRNAs that are similar to one another and possibly derived from gene duplication (40)] of the miR-17–92 cluster, miR-106a, also had lower expression after exercise and is a member of the miR-106a-363 cluster. Both clusters (miR-17–92 and miR-106a-363) are well conserved, having arisen from a series of ancient genomic duplications and deletions (3). In myeloid development, expression of miR-17, miR-20a, and miR-106a decreases during monocytogenesis (monocytic differentiation and maturation) *in vitro* (16). Finally, as noted above, myeloid-specific miR-223 negatively regulates progenitor proliferation and granulocyte differentiation and activation (21). In our study, we found an increase in miR-223 expression level immediately after exercise.

The predominance of research into the regulatory functions of miRNAs involves identifying, often *in silico*, the potential gene targets of the miRNAs that were changed or found to be expressed in the particular experiment. Currently, there are a number of approaches used to match miRNA regulation with gene expression, protein production, or cell function (4). It is known that in cell culture systems that are highly enriched with



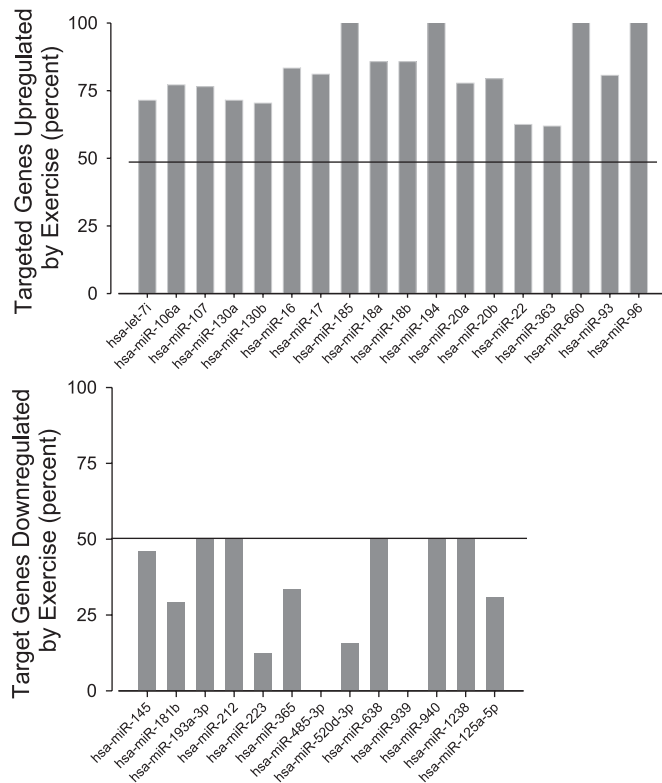


Fig. 2. Relationship between exercise-associated changes in miRNA expression and their targeted genes whose expression was also significantly altered by exercise. *Top*: individual miRNAs that were downregulated by exercise and the proportion of their targeted genes that were upregulated. Previous studies suggest that, in general, downregulated miRNAs will lead to upregulation of targeted genes. As seen in the figure, this phenomenon is corroborated by the data from neutrophils as ~80% of the targeted mRNAs were upregulated when their associated miRNA was downregulated. *Bottom*: individual miRNAs that were upregulated by exercise and the proportion of their targeted genes that were downregulated. In contrast to the downregulated miRNAs, upregulation of miRNAs did not lead to downregulation of their targeted genes.

miRNAs, the gene targets of the enriched miRNAs tend to have lower expression levels due most likely to degradation of mRNA once the miRNA is attached (27, 38). If degradation of mRNAs following attachment of miRNA were the predominant mechanism that was responsible for changes in neutrophil gene expression in our exercise studies, then one would expect first that a downregulation of miRNA would be accompanied by an upregulation of gene expression; and, conversely, an

upregulation of miRNA would lead to a downregulation of gene expression.

We therefore examined the relationship between the specific directional change in miRNA and gene expression. As shown in Fig. 2, for ~80% of the downregulated miRNAs, there was, indeed, an upregulation of their targeted genes. However, this relationship did not hold for the upregulated miRNAs in which we did not find a robust pattern of downregulation of the targeted genes. We now know that miRNAs are capable of silencing gene expression in the cytoplasm by mechanisms that can result in translational repression in which degradation of mRNA is not the primary mechanism (30). Some investigators suggest that degradation of mRNAs occurs only when there is a perfect match between the miRNA and its mRNA target (20). Why downregulation of miRNAs was associated with upregulation of gene expression in neutrophils while upregulation of miRNAs was not associated with downregulation of gene expression under in vivo conditions of exercise remains enigmatic.

As diagrammed in Fig. 1, in this initial exploration of gene pathways in neutrophils that were influenced by exercise we employed an intersecting analysis in which we focused solely on the 458 genes that were altered by exercise. This conservative analytic approach led to the identification of three major pathways involving both miRNAs' target genes and genes whose expression level had been influenced by exercise: the ubiquitin-mediated proteolysis pathway, Jak-STAT signaling pathway, and Hedgehog signaling pathway. Interestingly, in 2004 John et al. (20) using an in silico analysis of the then described miRNAs concluded that the most predominant pathway enriched by miRNA target genes was the ubiquitin-mediated proteolysis pathway. Exercise altered the expression level of nine genes that are linked to the ubiquitin-mediated proteolysis pathway (Ease score = 0.028) (Table 3). We further found that six different miRNAs that had been altered by exercise targeted specific genes involved in the ubiquitin-mediated proteolysis pathway (Table 3).

While we did not specifically examine the functional aspects of the ubiquitin-mediated proteolysis system in the neutrophils of our participants before and after exercise, there are mounting data that ubiquitin pathways play key regulatory roles in inflammatory function. For example, Skaug and coworkers (37) recently reviewed the multiple and complex ways in which ubiquitin pathway genes alter NF- $\kappa$ B regulatory pathways leading to control of key immune and inflammatory

Table 3. Genes associated with the ubiquitin-mediated proteolysis pathway that were altered by exercise

Gene Name	Gene Symbol	Fold Change
f-box and wd-40 domain protein 7 (archipelago homolog, <i>Drosophila</i> )	FBXW7	0.5
Ubiquitin-conjugating enzyme e2, j1 (ubc6 homolog, yeast)	UBE2J1	0.6
Ubiquitin-conjugating enzyme e2b (rad6 homolog)	UBE2B	0.7
Cullin 4a	CUL4A	1.3
Ubiquitin-conjugating enzyme e2l 3	UBE2L3	1.4
f-box and wd-40 domain protein 11	FBXW11	1.4
Tripartite motif-containing 37	TRIM37	1.4
Ubiquitin protein ligase e3a (human papilloma virus e6-associated protein, Angelman syndrome)	UBE3A	1.6
Suppressor of cytokine signaling 1	SOCS1	1.6

Fold change is defined as the geometric mean of expression levels of After/Before. A fold change of 0.6 indicates the expression level after exercise is 60% of the expression level before the exercise. A fold change of 1.3 indicates the expression level after the exercise is 130% of the expression level before the exercise.

Table 4. *Genes in the Jak-STAT signaling pathway that were altered by exercise*

Gene Name	Gene Symbol	Fold Change
Interleukin 2 receptor, beta	IL2RB	2.2
Signal transducer and activator of transcription 1, 91 kDa	STAT1	0.6
Cyclin d2	CCND2	1.8
Interferon (alpha, beta and omega) receptor 2	IFNAR2	1.3
Signal transducer and activator of transcription 4	STAT4	2.6
Suppressor of cytokine signaling 2	SOCS2	2
Phosphoinositide-3-kinase, regulatory subunit 1 (p85 alpha)	PIK3R1	1.7
Interleukin 10 receptor, alpha	IL10RA	1.6
Suppressor of cytokine signaling 1	SOCS1	1.6

Fold change is defined as the geometric mean of expression levels of After/Before. A fold change of 0.6 indicates the expression level after exercise is 60% of the expression level before the exercise. A fold change of 1.3 indicates the expression level after the exercise is 130% of the expression level before the exercise.

functions such as apoptosis. These workers noted that both ubiquitination and deubiquitination were prime regulators of immune pathways. There is relatively little research into the specific role of miRNA control over ubiquitin-mediated proteolysis pathway in neutrophils. In a recent study, Marois et al. (25) found that silencing of the expression of one of the ubiquitin ligases (E3a ubiquitin ligase)—perhaps by small interfering RNAs—led to decreased signaling and phagocytosis in neutrophils.

Exercise altered the expression level of nine genes that are linked to the Jak-STAT pathway, Ease score = 0.059 (Table 4). From our intersecting analysis, we further found that mir-16 had been significantly downregulated by exercise and targeted specific genes involved in the Jak-STAT pathway (Table 2). The Jak-STAT associated genes can modify granulopoiesis and neutrophil immune function through the SOCS (suppressors of cytokine signaling) and STAT (signal transducer and activator of transcription) (28). Moreover, activation of Jak-STAT pathways can rescue neutrophils from apoptosis under certain conditions (17), all functions that appear to be influenced by exercise. Finally, far less is known about the specific role that the Hedgehog signaling pathway plays in leukocytes; however, there is mounting evidence that this pathway does play a role in chronic inflammation (6).

Thus we have shown for the first time that a naturally occurring perturbation to the cellular milieu, physical exercise, altered the profile of neutrophil miRNA expression in the circulating blood. The specific exercise-affected miRNAs and their target genes identified three specific gene pathways: ubiquitin-mediated proteolysis, Jak-STAT signaling pathway, and Hedgehog signaling pathway, all of which reflect plausible biological mechanisms involved in neutrophil priming that occurs with exercise. The rapid miRNA response that we observed in vivo is consistent with similarly rapid miRNA responses observed in vitro—for example, Simone et al. (36) recently noted a robust miRNA response in cultured fibroblasts to oxidative stress occurred within an hour of the initial exposure.

There are a number of limitations in the design of the study that could influence its interpretation. We did not use a crossover protocol in which we could compare spontaneous changes in neutrophil miRNAs in resting individuals over a 30-min period as our goal was first to establish whether or not the added expense involved in crossover designs would be merited. To our knowledge, spontaneous changes in miRNAs over such short intervals have not been

studied even in cell culture in unperturbed cells. Previous studies of gene expression in leukocytes support the assumption that there is little change over very short time periods (8), but the possibility of circadian effects certainly cannot be ruled out.

Another possible confounding factor is that we used the results of two separate studies (namely, the gene expression data and the miRNA data) for part of our analysis. Both protocols consisted of aerobic exercise (cycle ergometry) at roughly the same relative work rate, but the data from the previous study involved 30 min of continuous exercise while in the present study we used a protocol made up of 10 2-min bouts separated by 1 min of rest. We increasingly use the latter protocol because we have found that while it is heavy exercise (note the increase in lactate), it is much more readily tolerated and completed by healthy people with average or below fitness. In fact, using the 10 2-min aerobic exercise protocol we have been able to identify gene expression changes in circulating leukocytes in early- and late-pubertal children (32, 33). Nonetheless, it is possible that gene expression changes in neutrophils might be somewhat different in the 10 2-min bout protocol compared with 30 min of continuous cycle ergometry.

Exercise certainly leads to both physical and chemical perturbations such as changes in pH, local temperature, and neutrophil shear stress, and systemic increases in cytokines and growth factors (e.g., IL-6, growth hormone), all of which possibly could alter genomic regulatory mechanisms and lead to changes in miRNA profiles. Alternatively, the miRNA changes we observed might have resulted from an equally elegant regulatory strategy in which neutrophils residing outside of the circulation (e.g., margined on the endothelium, bone marrow, or pulmonary circulation) express, through, perhaps, differences in maturation or other as yet unknown mechanisms, miRNAs differently than does the population of circulating cells. On exercise, neutrophils from the marginal pools move into the circulation and, in this way, the profile of the circulating neutrophils is changed. By whatever mechanism, our data now demonstrate that alterations in miRNAs may play a role in the effects of exercise on neutrophil function.

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## DISCLOSURES

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

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