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Changes in the leukocyte methylome and its effect on cardiovascular-related genes after exercise

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Denham J, O'Brien BJ, Marques FZ, Charchar FJ. Changes in the leukocyte methylome and its effect on cardiovascular-related genes after exercise. *J Appl Physiol* 118: 475–488, 2015. First published December 24, 2014; doi:10.1152/japplphysiol.00878.2014.—Physical exercise has proven cardiovascular benefits, yet there is no clear understanding of the related molecular mechanisms leading to this. Here we determined the beneficial epigenetic effects of exercise after sprint interval training, a form of exercise known to improve cardiometabolic health. We quantified genome-wide leukocyte DNA methylation of 12 healthy young (18–24 yr) men before and after 4 wk (thrice weekly) of sprint interval training using the 450K BeadChip (Illumina) and validated gene expression changes in an extra seven subjects. Exercise increased subjects' cardiorespiratory fitness and maximal running performance, and decreased low-density lipoprotein cholesterol concentration in conjunction with genome-wide DNA methylation changes. Notably, many CpG island and gene promoter regions were demethylated after exercise, indicating increased genome-wide transcriptional changes. Among genes with DNA methylation changes, epidermal growth factor (*EGF*), a ligand of the epidermal growth factor receptor known to be involved in cardiovascular disease, was demethylated and showed decreased mRNA expression. Additionally, we found that in microRNAs miR-21 and miR-210, gene DNA methylation was altered by exercise causing a cascade effect on the expression of the mature microRNA involved in cardiovascular function. Our findings demonstrate that exercise alters DNA methylation in circulating blood cells in microRNA and protein-coding genes associated with cardiovascular physiology.

epigenetics; sprint interval training; DNA methylation; cholesterol; microRNA

EXERCISE TRAINING CAN PREVENT and attenuate symptoms of cardiometabolic diseases such as insulin resistance, elevated blood pressure (BP), excess adiposity, and vascular impairment (18). The precise molecular mechanisms underpinning the health benefits gained from regular exercise training, however, are not known. DNA methylation affected by environmental factors modulates gene expression and may therefore mediate the health benefits gained from exercise training (19).

Recently, individuals involved in a 6-mo moderate-intensity exercise training program displayed genome-wide skeletal myocyte and adipocyte DNA methylation changes in genes associated with type 2 diabetes (49, 55). Additionally, older adult leukocyte DNA methylation increased in the proinflammatory *PYCARD* gene after 6 mo of aerobic exercise training to levels observed in their middle-aged peers (47). Although 6 mo of moderate-intensity aerobic exercise modulates the DNA methylome, it is unknown whether shorter training regimes affect DNA methylation. Moreover, although leukocytes are known to contribute to vascular disease (58), it is not known whether exercise is an environmental factor that regulates the leukocyte methylome and, in turn, contributes to improved vascular health.

Given that the effects of acute exercise on DNA methylation are intensity-dependent (5), we aimed to determine whether leukocyte DNA methylation changes occur after short-term (4 wk), yet intense exercise training. We used sprint interval training as a form of exercise training because it rapidly improves vascular functioning (53, 54), insulin sensitivity (3), cardiorespiratory function, and physical performance [reviewed in (25, 57)]. We aimed to *1*) identify the effects of 4 wk of thrice weekly exercise on genome-wide leukocyte DNA methylation in healthy young men; *2*) establish whether specific DNA methylation changes occurred reciprocally with altered gene expression; and *3*) identify the acute effect of maximal exercise on genes and microRNAs (miRNAs) related to cardiovascular disease. We hypothesized that 4 wk of exercise training would cause significant changes in DNA methylation in genes and miRNAs related to pathways involved in cardiovascular health.

MATERIALS AND METHODS

Participants. Twenty-six healthy young men not already engaged in intense exercise training were recruited for this study. Participants were initially screened for any chronic diseases by health and physical activity readiness questionnaires. During the initial assessment, participants' height, weight, and body mass index were recorded. Resting BP was measured with the subjects seated using an electronic BP monitor (3AQ1; Microlife, Clearwater, FL). Subjects' BP was measured after a 10-min rest and was the average of two measurements divided by a 1-min rest period. Body fat percentage was estimated by summing seven skinfolds as described previously (31).

All subjects gave written informed consent, and this study was approved by the Human Research Ethics Committee of Federation University Australia.

Cardiopulmonary exercise testing and training. To assess cardiorespiratory fitness, participants completed one maximal oxygen consumption ($\dot{V}O_{2max}$) test on the same day of the initial assessment and 48–96 h following their final exercise session. Participants began training 3 days but not longer than 1 wk after their $\dot{V}O_{2max}$ test and completed 12 training sessions (3 per week) over 4 wk. Participants completed two maximal treadmill exercise ($\dot{V}O_{2max}$) tests before and after exercise training. Before the $\dot{V}O_{2max}$ test participants were fitted with a two-way breathing valve (Hans Rudolph, Shawnee, KS) and expired air was collected into an online metabolic system (Moxus) for gas (O_2 and CO_2) analysis. The metabolic system was calibrated before each test using ambient air and gas of known composition. After a standardized 5-min warm-up, the treadmill speed was increased 1 km per hour every minute until the participant reached volitional exhaustion. The $\dot{V}O_{2max}$ of each subject was determined as the highest oxygen consumption over 1 min and was expressed as a relative value in $ml \cdot kg^{-1} \cdot min^{-1}$ by dividing the $\dot{V}O_{2max}$ by body wt.

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To restrict any potential DNA methylation changes to the exercise program, participants were requested not to deviate from their normal physical activity and recreational exercise training habits because physical activity and exercise training habits influence leukocyte DNA methylation (19). Participants were also requested not to make changes to their normal diet because dietary changes have been associated with DNA methylation alterations (46, 66).

Before each exercise session, participants were instructed to perform a warm-up involving a brief, low-intensity run (5 min), dynamic stretches, and some short (20 m) high-intensity runs. Table 1 outlines the training schedule performed. Briefly, participants were required to complete three sprints at maximal intensity. To prevent overtraining, a two-up one-back training model was used. For example, during *week 1* of training, participants completed three sprints in the first session, four sprints in the second session, and five in the third and final session of *week 1*. Participants then completed four sprints in the first session of *week 2* (Table 1). The training was progressively overloaded until participants performed eight sprints in the final exercise session. Participants had 48–72 h recovery before the subsequent training session to allow adequate recovery. Exercise sessions were monitored and supervised by an accredited exercise physiologist (with Exercise and Sports Science Australia) to ensure the safety of participants. Training was performed on a university athletics oval. Participants were requested to run at maximal intensity for each 30-s effort. To ensure participants were running at maximal intensity, participants received constant verbal motivation. Participants began each effort at one end of the oval and individually ran around the oval until a whistle was blown to indicate the 30 s had been completed. Participants were then given a 4-min rest, which involved a slow walk back to where they had started their effort, followed by passive rest.

Blood processing. Participants donated a resting blood sample before and after their initial and final (after 4 wk of exercise training) treadmill tests. Participants were asked to refrain from consuming alcohol or caffeinated beverages 24 h before blood draw and were seated for ~30 min prior to their donations before and after the exercise intervention. Circulating blood was drawn from the antecubital vein into a serum separating tube and an EDTA tube with the participant seated. All resting blood samples were collected from participants in the morning following an overnight fast. The final blood collection was obtained 48–96 h after the final exercise session. Blood was temporarily stored on ice before further processing. The serum separating tube was left to clot at room temperature before centrifugation (3,500 *g*) and was subsequently used to quantify blood lipid concentration using the CHOL2, TRIGL, and HDLC3 reagents that were run on the Roche c701 instrument at Melbourne Pathology (Melbourne, Australia). All DNA and RNA were extracted on the same day, within 3 h of blood draw to prevent possible de novo influences on DNA methylation and gene expression. DNA and RNA were extracted from whole blood stored in EDTA tubes using the PureLink Genomic DNA Mini Kit (Life Technologies, Grand Island, NY) and miRNeasy Mini Kit (Qiagen, Valencia, CA), respectively, following the manufacturers' recommendations. Whole blood leukocytes were washed twice with the erythrocyte lysis wash buffer (included in the miRNeasy Mini Kit) to isolate leukocytes from plasma, serum, platelets, and lysed erythrocytes. Therefore, only whole blood leukocyte miRNA and gene expression were analyzed in our study.

Table 1. *Description of the 4-wk exercise program*

Week	Session	Training Load*	Training Sprint Time, min	Total Session Time, min
1	1	3 Sprints	1.5	9.5
	2	4 Sprints	2	14
	3	5 Sprints	2.5	18.5
2	4	4 Sprints	2	14
	5	5 Sprints	2.5	18.5
	6	6 Sprints	3	23
3	7	5 Sprints	2.5	18.5
	8	6 Sprints	3	23
	9	7 Sprints	3.5	27.5
4	10	6 Sprints	3	23
	11	7 Sprints	3.5	27.5
	12	8 Sprints	4	32
	Total Time	66	33	249

*All sprints were completed at maximum intensity and separated by 4 min of passive recovery.

Genome-wide DNA methylation and EpiTYPER assay. Although 19 participants completed the exercise intervention, whole-genome DNA methylation analysis was performed only in 12 subjects at rest before and after the 4-wk exercise intervention. The 450k BeadChip uses two types of Illumina chemistry to provide a comprehensive DNA methylation status of more than 480,000 CpG sites spanning 99.9% of refseq genes. DNA methylation is represented as a β value, which corresponds to the percentage of methylation at a given CpG site. All samples quantified on the BeadChip passed the GenomeStudio Methylation Module's quality control procedure. To identify technical variation and increase the chances of detecting DNA methylation changes caused by exercise, raw β values underwent Subset-quantile Within Array Normalization (SWAN) (44). Data were subsequently imported into and analyzed using the bioinformatics software, Partek (Genomic Suite, version 6.6, Singapore). All β values were log-transformed into M values using the logit function $\{\log^2[\beta/(1-\beta)]\}$. M values are a more valid alternative for detecting DNA methylation changes because they eliminate the heteroscedasticity of higher- and lower-end β values (22). Whereas M values are more appropriate for analyzing DNA methylation, β values are more suitable for displaying biological relevance because they correspond to the DNA methylation percentage of an individual CpG site. For this reason, M values were converted back to β values for all graphs and tables. Hierarchical clustering and pathway analysis was analyzed using the Partek software. Due to the uneven CpG distributions between the two chemistries used on the Infinium HumanMethylation450 BeadChip (Illumina) - Infinium I (135,501) and Infinium II (350,076), we analyzed these separately. Whole-genome DNA methylation was quantified using an Infinium HumanMethylation450 BeadChip (Illumina) according to the manufacturer's guidelines and with the assistance of the Australian Genome Research Facility (Melbourne, Australia).

The EpiTYPER (Sequenom) was used to validate the DNA methylation change of the epidermal growth factor (*EGF*, cg12093976) and uracil-DNA glycosylase (*UNG*, cg20982606) genes, and these assays were performed by GeneWorks (Melbourne, Australia). Experiments were conducted according to the manufacturer's procedures. Briefly, 1 μg of DNA was bisulfite-converted and PCR-amplified using the following primer sets: cg12093976: sense, aggaagagagAGTTATAATTTTTGGATTGGGGTTG and antisense, cagtaatacgactcactatagggagaaggctTAATTTAATTTTATCTCCATCCTTCCAA; cg20982606: sense, aggaagagagGATTATTTTGGAGTTGAGGAGGTAG and antisense, cagtaatacgactcactatagggagaaggctCCTTAAAAACCTATCCAAAAAACAA. Base-specific (C and T) cleavage and in vitro transcription was performed, followed by mass spectrometry (MALDI-TOF). Data were analyzed using the EpiTYPER 1.2 software (Sequenom).

Gene and miRNA expression. The gene and miRNA expression of blood collected before and after initial and final treadmill exercise testing was assessed by quantitative PCR (qPCR) using TaqMan assays (Life Technologies). RNA samples were reverse transcribed using the High Capacity Reverse Transcription Kit and the TaqMan MicroRNA Reverse Transcription Kit for gene and miRNAs, respectively, following the manufacturer's procedures (Life Technologies, Australia). Experiments on 384-well plates consisting of samples, endogenous positive and negative controls, all in duplicate were run on the ViiA 7 Real-Time PCR System (Life Technologies, Australia).

Table 2. *TaqMan assay information*

Gene/MicroRNA	TaqMan ID
CBX7	Hs00545603_m1
CDC20	Hs00426680_mH
EGF	Hs01099999_m1
GAPDH (mRNA reference gene)	Hs02786624_g1
IGFL3	Hs00419511_g1
ISM1	Hs01382748_m1
SNCAIP	Hs00917423_m1
UNG	Hs01037093_m1
hsa-miR-21	000397
hsa-miR-210	000512
RNU44 (miRNA reference gene)	001094

Standard TaqMan assay procedures were followed, and TaqMan assays used in the experiments are outlined in Table 2. The cycle threshold (Ct) of genes and miRNAs were compared with glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and the small-nucleolar RNA, *RNU44*, respectively. The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (40) and the results were graphically represented as fold change. The cycling conditions were as follows: a hold at 50° for 2 min and at 95° for 20 s, followed by 40 cycles at 95° for 1 s and 60° for 20 s. As a quality control, duplicate samples greater than one Ct apart were re-run before being included in the analysis. All qPCR experiments were performed by the same researcher.

Statistical analyses. Participant phenotypes were assessed for normality using Kolmogorov-Smirnov and Shapiro-Wilks tests. Parametric data are expressed as means ± SD, and nonparametric data are expressed as median (interquartile range). Nonparametric data were log-transformed before further analyses. Although a paired *t*-test was used to identify statistically significant changes to phenotypes, repeated-measures ANOVA was used to show gene expression changes after 4 wk of exercise training using the statistical software IBM SPSS Statistics (version 21.0). Genome-wide DNA methylation changes in relation to CpG islands and gene regions were assessed using χ^2 and Wilcoxon matched-pair signed ranked tests. DNA methylation changes caused by exercise training were determined using two-way ANOVA after β values were log-transformed. To control for the discovery of false-positives, a false discovery rate (FDR) correction was applied to the whole-genome DNA methylation *P* values by converting *P* values to *q* values. Whole genome data were analyzed using the software Partek Genomic Suite (version 6.6). Gene and miRNA expression were analyzed using a two-way repeated-measures ANOVA. The miRNA-mRNA targets were predicted using the miRWalk website (23), which includes predictions from the most commonly used prediction databases, and pathway analysis was conducted using Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.7) (29, 30). Statistical significance was determined as $P < 0.05$.

RESULTS

Exercise adherence. Of the 26 participants recruited into the intervention, 19 successfully completed the 12 exercise sessions. One participant withdrew due to a quadriceps musculoskeletal injury during training, one had a previous injury that was exacerbated by the exercise, another experienced an unrelated health concern, and three stopped training for undisclosed reasons. The exercise adherence was ~73%.

Exercise improves cardiorespiratory health and fitness. Participants' phenotypes before and after exercise training are shown in Table 3. Exercise training increased cardiorespiratory fitness ($\dot{V}O_{2max}$) by 2.1 $ml \cdot kg^{-1} \cdot min^{-1}$ (4.7%, $P = 0.03$). Maximal running speed during $\dot{V}O_{2max}$ testing improved by 1

Table 3. *Participant phenotypes before and after exercise training*

Phenotype	Before Exercise Mean ± SD	After Exercise Mean ± SD	% Change	*P*
$n = 19$				
Age, yr	21.1 ± 2.7			
Height, cm	180.6 ± 7.4			
Anthropometric				
Weight (kg)	78.1 ± 9.4	78.1 ± 9.8	−0.03 ± 2.3	0.99
BMI, kg/m^2	23.9 ± 2.3	23.9 ± 2.4	−0.2 ± 2.4	0.68
Waist, cm	79.2 ± 5.3	78.9 ± 4.8	−0.4 ± 2.5	0.50
Hip, cm	82.8 ± 6.0	82.4 ± 5.4	−0.3 ± 1.8	0.37
WHR	0.96 ± 0.02	0.96 ± 0.02	−0.1 ± 2.3	0.94
∑ 7 skinfolds, cm	98.1 ± 38.3	91.3 ± 30.7	−4.5 ± 13.2	0.13
Body density	1.07 ± 0.01	1.07 ± 0.01	0.2 ± 0.5	0.13
Fat %	13.0 ± 4.9	12.2 ± 4.1	−4.2 ± 12.7	0.11
Cardiovascular				
RHR, beats/min	64.1 ± 10.8	59.5 ± 9.3	−4.0 ± 18.2	0.055
SBP, mmHg	124.0 ± 12.1	124.8 ± 7.8	1.1 ± 10.5	0.77
DBP, mmHg	72.0 ± 6.7	69.5 ± 7.0	−3.4 ± 7.9	0.06
MAP, mmHg	89.4 ± 7.9	87.9 ± 6.7	−1.3 ± 8.8	0.42
PP, mmHg	51.9 ± 8.4	55.4 ± 5.8	7.6 ± 16.8	0.07
Total CHOL, mmol/liter	4.42 ± 0.79	4.24 ± 0.67	−3.3 ± 9.3	0.06
Triglycerides, mmol/liter	0.97 ± 0.35	0.95 ± 0.48	−4.2 ± 22.4	0.70
HDL-C, mmol/liter	1.34 ± 0.24	1.31 ± 0.20	−0.9 ± 11.7	0.50
LDL-C, mmol/liter	2.64 ± 0.78	2.48 ± 0.61	−3.9 ± 11.2	**0.047**
LDL-C/HDL-C, mmol/liter	2.06 (1.5–2.8)	2.0 (1.5–2.5)	−1.6 ± 15.0	0.24
CHOL/HDL-C, mmol/liter	3.41 (2.7–4.2)	3.31 (2.7–4.2)	−2.0 ± 9.1	0.35
Cardiorespiratory fitness				
$\dot{V}O_{2max}$, $ml \cdot kg^{-1} \cdot min^{-1}$	49.5 ± 4.7	51.6 ± 3.9	4.7 ± 10.1	**0.03**
Final treadmill speed, km/h	19 (18–21)	20 (19–21)	4.0 ± 5.1	**0.001**

∑, sum of; % change, percentage change; BMI, body mass index; WHR, waist to hip ratio; RHR, resting heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PP, pulse pressure; CHOL, cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol. Data are expressed as means ± SD or as mean (interquartile range) from paired *t*-tests or related samples Wilcoxon signed rank test, respectively.

km/h (4%, $P = 0.001$) and LDL cholesterol was decreased after the intervention (−3.9%, $P = 0.047$). Resting heart rate (−4%, $P = 0.05$), diastolic BP (−4.3%, $P = 0.06$), and total cholesterol (−3.3%, $P = 0.06$) were reduced, whereas pulse pressure was increased (7.6%, $P = 0.07$), all with borderline statistical significance.

Whole-genome leukocyte DNA methylation changes after exercise training. We analyzed whole-genome DNA methylation using the 450 BeadChip (Illumina) to determine whether exercise altered the leukocyte methylome. The principal component analysis demonstrated separation between paired subject DNA methylation from samples taken before and after exercise training, indicating large changes to genome-wide DNA methylation (data not shown). DNA methylation changes occurred across the leukocyte genome in relation to gene regions and CpG islands (Fig. 1). To gather an overview of where these DNA methylation changes had occurred, we determined the average DNA methylation before and after exercise in relation to CpG islands and location in relation to the nearest gene. DNA methylation in relation to the nearest gene decreased in the promoter and intron regions for Infinium I and II assays, but increased in the 3′-untranslated regions (UTR) (Fig. 2*A*, $P < 0.0001$). There was a decrease in 5′ UTR and exon DNA methylation in Infinium II assay (Fig. 2*A*, $P < 0.0001$). CpG island DNA methylation decreased in both Infinium I and II assays (Fig. 2*B*, $P < 0.0001$). Whereas Infinium II-assessed northern shore (N shore) DNA methylation deceased, southern shore (S shore) and northern shelf (N shelf) DNA methylation increased ($P < 0.0001$, Fig. 2*B*). Although the effect of specific regional DNA methylation changes are not completely understood, the CpG island and promoter region demethylation indicate an increase in transcriptional activity or gene unsilencing. Therefore, DNA methylation changes occurred across the leukocyte methylome after exercise training.

CpG methylation after exercise training. Out of the 485,577 CpG sites quantified for DNA methylation status on the Infinium HumanMethylation450 BeadChip (Illumina), exercise induced DNA methylation changes, ranging from 0.1 to 62.8% at 205,987 sites relating to 32,445 transcripts after FDR ($q < 0.05$). Although 81,576 CpG sites relating to 16,256 transcripts became more methylated (Fig. 2*C*, $q < 0.05$), 124,411 CpG sites corresponding to 27,263 transcripts were demethylated after exercise (Fig. 2*D*, $q < 0.05$).

In the search for biologically relevant changes to CpG site methylation, we identified CpG sites with a change of ≥5% after exercise ($q \leq 0.05$) (Fig. 3). Due to the large number of CpG sites ($n = 2{,}909$) with a difference of ≥5%, we narrowed our focus to CpG sites ($n = 81$) with a change of ≥10% ($q < 0.005$, Table 4) and ≥20% after exercise ($q < 0.005$, Table 5). Of the CpG sites that had large changes (≥20%) in DNA methylation after exercise, 8 had increased methylation and 11 had less methylation after exercise. Next, after applying a stringent FDR ($q < 0.001$) we used gene ontology to identify DNA methylation changes in genes enriched for numerous cellular and molecular processes, including those involved in metabolic activity, biological adhesion, and antioxidant activity (Fig. 4). Using pathway analysis, we found that CpG sites altered by exercise ($q < 0.005$) were those of genes related to pathways important for cardiovascular physiology, including focal adhesion, calcium signaling, and mitogen-activated protein kinase (MAPK) signaling ($q < 0.05$, Table 6). Therefore, differentially methylated CpG sites after exercise are enriched for pathways crucial for cardiovascular health.

DNA methylation validation. DNA methylation within a gene promoter region is typically associated with decreased gene expression, thus we focused on CpG sites with the largest and most statistically significant DNA methylation changes within gene promoter regions (≥10% and $P < 1.0 \times 10^{-5}$). To that end, we analyzed CpG sites within the *EGF* (cg12093976) and uracil-DNA glycosylase (*UNG*) (cg20982606) promoter regions using the EpiTYPER (Sequenom). We were unable to obtain meaningful data due to the acquisition of peaks from additional fragments other than the fragment of interest.

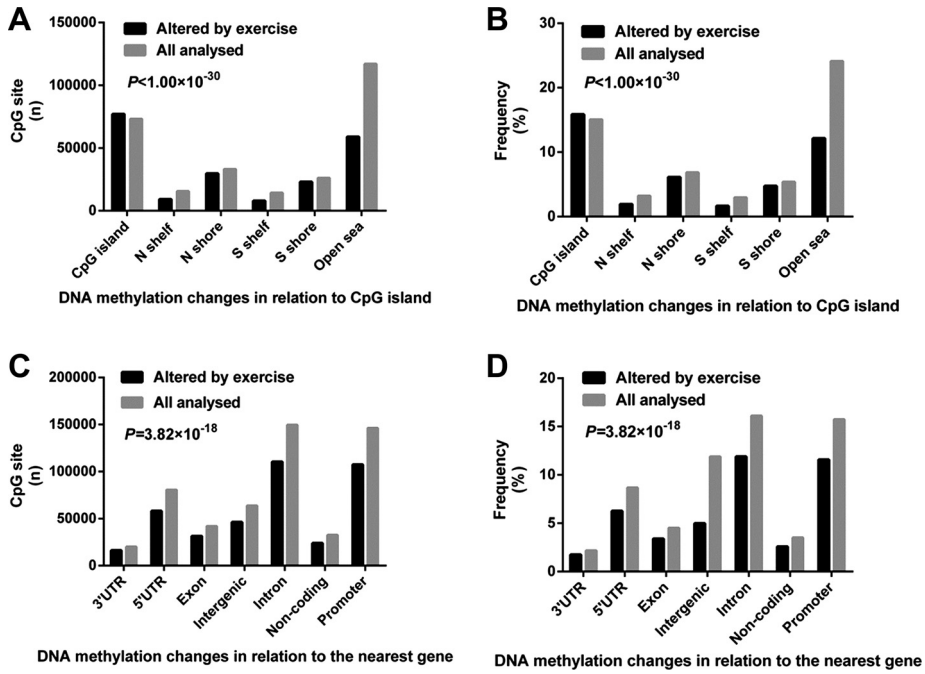

Fig. 1. DNA methylation changes in relation to CpG islands (*A* and *B*) and the nearest gene (*C* and *D*), respectively. Data are from χ^2 tests.

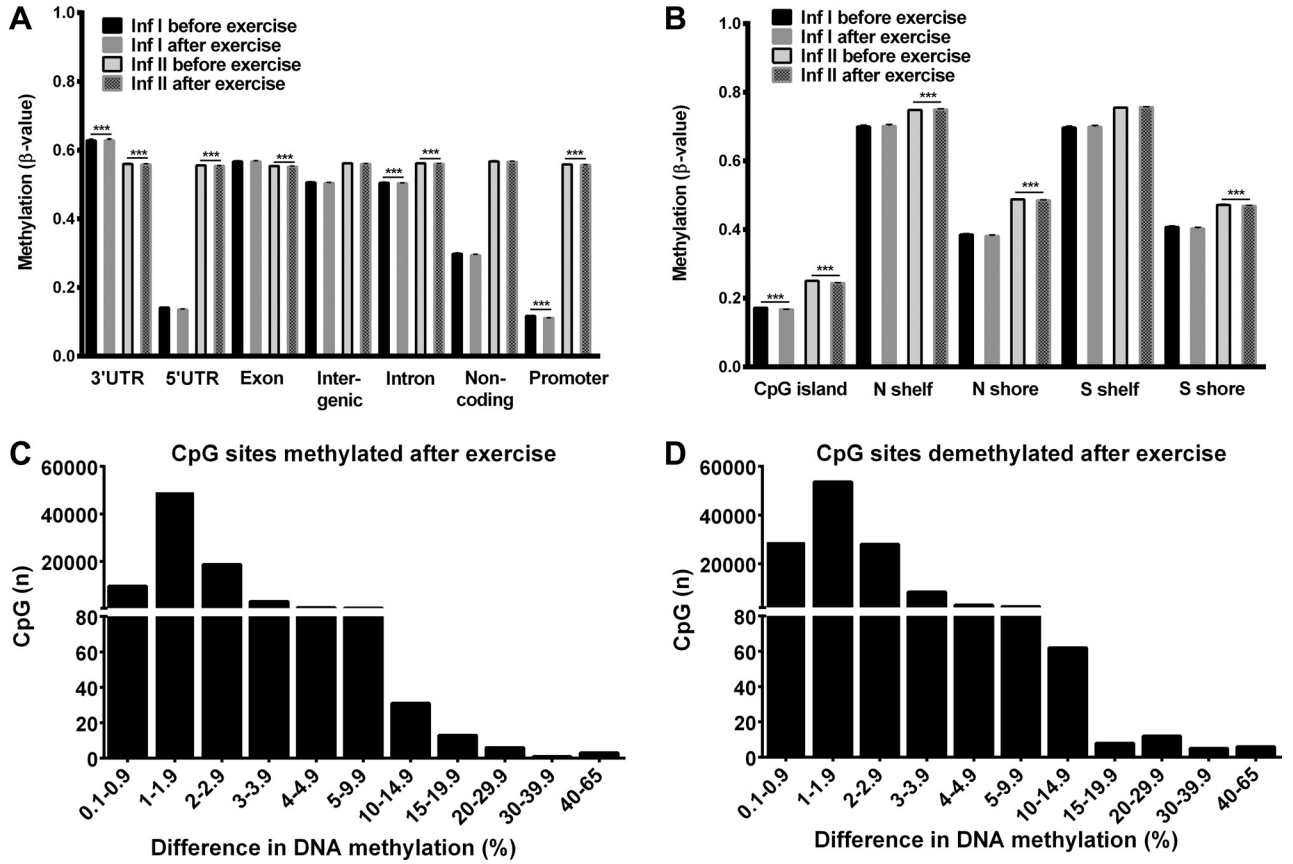

Fig. 2. Whole-genome DNA methylation changes caused by exercise. Genome-wide DNA methylation across all 485,577 CpG sites analyzed on the Infinium HumanMethylation450 BeadChip were compared before and after exercise in relation to the nearest gene (*A*) and CpG island (*B*). Data are from Wilcoxon matched-pair signed ranked test and are expressed as means ± SE. Number of CpG sites with increased methylation (*C*) and decreased methylation (*D*) with magnitude of DNA methylation change after exercise ($q < 0.05$). ***$P < 0.0001$. N, northern; S, southern; 3′UTR: 3′-untranslated region; 5′-UTR: 5′-untranslated region; promoter, gene promoter region (1–1,500 bases upstream of the transcription start site).

Exercise-induced gene and miRNA expression. We then aimed to identify whether a change in DNA methylation caused by exercise would alter gene mRNA expression. We quantified the expression of genes that had a DNA methylation change (>10%) that were most statistically significant ($q < 0.001$) within the promoter region or gene body (Fig. 5, *A–F*). We also quantified mature miR-21 and miR-210 because their genes had a modest (1.3–4.2%) change in DNA methylation at multiple regions across the gene ($q < 0.05$, Table 7). These miRNAs are inversely correlated to cardiorespiratory fitness and implicated in cardiovascular disease (11).

Gene expression changes after the initial $\dot{V}O_{2max}$ test at rest after exercise training and after the second $\dot{V}O_{2max}$ test (after exercise training) are displayed in Figure 5. We were unable to successfully amplify insulin-like family member 3 (*IGFL3*) mRNA, suggesting that this gene is lowly expressed in leukocytes. Genes fitted into one of five categories; specifically, *1*) genes that were responsive after acute exercise after exercise training only (cell division cycle 20, *CDC20*); *2*) genes that responded to acute exercise before and after exercise training (isthmin 1, *ISM1*); *3*) genes that responded to acute exercise before and after exercise training and that had altered gene expression at rest after exercise training (*EGF*, *UNG* and miR-210); *4*) those that were altered only at rest after exercise training (miR-21); and finally, *5*) those that were unchanged by acute or chronic exercise (chromobox homolog 7, *CBX7* and synuclein α interacting protein, *SNCAIP*, data not shown). Interestingly, the demethylated (11.6% and 12.9%, respectively) *EGF* and *UNG* promoter was accompanied by a significant decrease and increase in mRNA expression, respectively (relative expression ± SE, 10.75 ± 1.68 to 8.25 ± 0.95, $P < 0.05$ and 1.72 ± 0.14 to 2.12 ± 0.15, $P = 0.05$). These data suggest that exercise training-induced DNA methylation changes within genes (including miRNA genes) cause changes to mRNA and mature miRNA levels (Fig. 6).

MicroRNA-mRNA targets. Finally, we used miRWalk (23) to predict mRNA targets of miR-21 and miR-210. We then used DAVID (29, 30) to identify pathways and diseases associated with mRNA molecules targeted by miR-21 and miR-210. Notably, miR-21 mRNA targets were enriched for pathways including MAPK signaling, Toll-like receptor signaling, apoptosis, and fatty acid metabolism. Moreover, mRNA targets of miR-210 were enriched for genes relating to calcium signaling, B-cell receptor signaling, and transforming growth factor-beta signaling pathways. Although the mRNA targets of miR-21 were from genes associated with diseases such as ischemic heart disease ($n = 6$), nephropathy ($n = 5$), and coronary atherosclerosis ($n = 11$), miR-210 mRNA targets were from genes associated with focal dystonia ($n = 2$). Collectively, these data suggest a role for miR-21 and miR-210

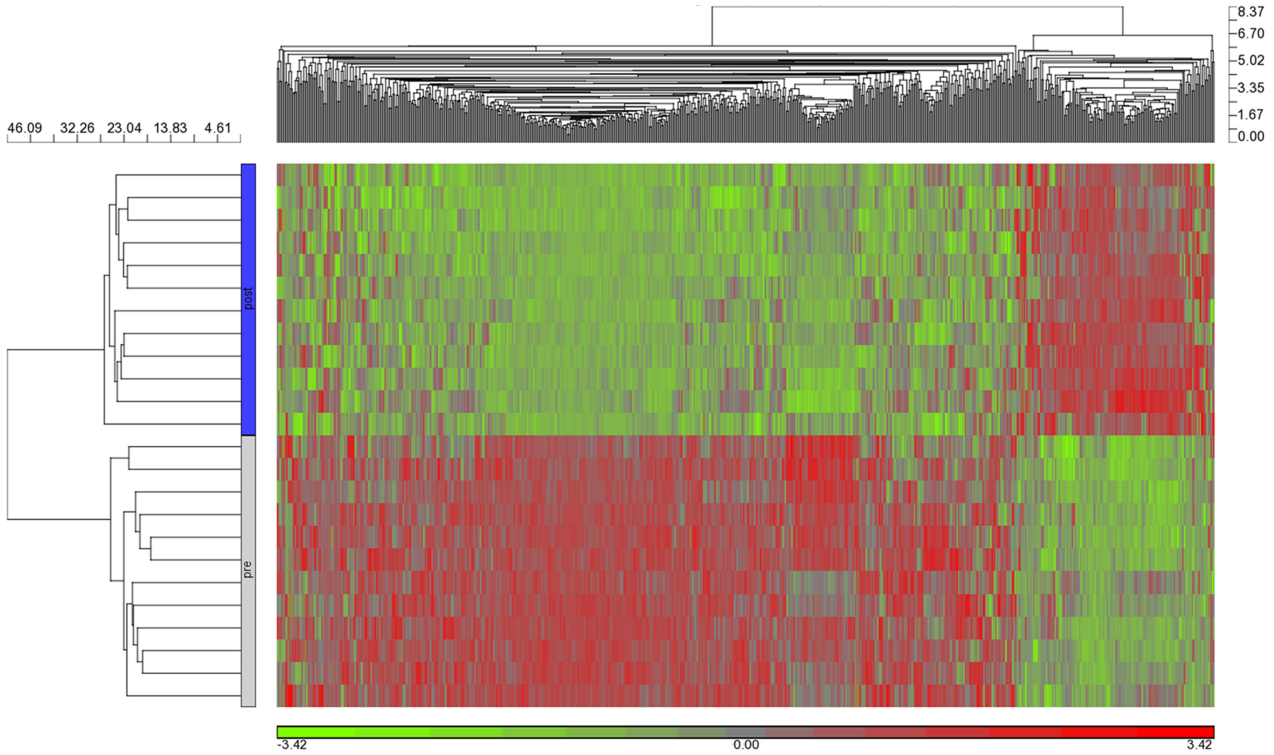

Fig. 3. Hierarchical clustering of CpG methylation before and after exercise training. The CpG methylation status is indicated by green (low methylation) and red (high methylation) in 12 subjects before (gray bar) and after (blue bar) exercise training. Each branch of the array tree on the left of the gray and blue bars are subjects and are separated before and after the exercise training. The array tree at the top of the hierarchical cluster indicates clusters of CpG sites modified by exercise. Data are from CpG sites with a fold-change of >5% ($q < 0.001$).

in the positive cardiovascular adaptations associated with short-term intense exercise training.

DISCUSSION

Our study is the first, to our knowledge, to show genome-wide leukocyte DNA methylation changes caused by short-term exercise in healthy young men. We found global and specific leukocyte DNA methylation changes with concomitant changes to mRNA and miRNA expression, in addition to favorable cardiovascular health adaptations after 4 wk of exercise training. Specifically, we observed DNA methylation changes to CpG islands and gene promoter and gene bodies. We showed exercise training-induced demethylation of cg12093976 and cg20982606, which decreased and increased the mRNA expression of the *EGF* and *UNG* genes, respectively. Furthermore, exercise training-induced leukocyte DNA methylation changes across miRNA genes (*MIR21* and *MIR210*) known to influence cardiovascular physiology (20, 60), subsequently influencing their mature miRNA expression.

Sprint interval training causes cardiopulmonary and metabolic adaptations similar to that of traditional long-duration aerobic training, but demands dramatically less time commitment (10). Given a common barrier to engagement in exercise is lack of time, sprint interval training may be an attractive alternative exercise training regime that improves health and fitness. We demonstrated that 4 wk of sprint interval training increased $\dot{V}O_{2max}$ and reduced total and low-density lipoprotein (LDL) cholesterol in healthy young men, thereby improving cardiorespiratory fitness and blood lipid profile. We are, to our knowledge, the first to demonstrate that 4 wk of sprint interval training can significantly decrease LDL cholesterol in young men. Although some have demonstrated 8 wk of sprint interval training in the form of running is effective at reducing LDL and total cholesterol (56), other shorter (6 wk) sprint interval training interventions have not reduced LDL cholesterol (27). Considering the role of LDL cholesterol in blood vessel health (13, 15), sprint interval training may be a cheap and effective short-term strategy to promote a favorable blood lipid profile.

Global leukocyte DNA methylation changes seem to be influenced by physical activity and exercise training, but data are equivocal. Although global leukocyte DNA methylation is increased in middle-aged individuals performing moderate (65) or high amounts (63) of physical activity in some studies, in others, global methylation is inversely (9, 41) related to physical activity or unrelated (67). The DNA methylation of exon 1 of the *PYCARD* gene was increased in older adults who engaged in 6 mo of moderate-intensity exercise to levels comparable to those of their younger peers (47). We are the first to analyze the exercise training-induced changes to genome-wide leukocyte DNA methylation in healthy subjects. Notably, we found a subtle decrease in leukocyte global methylation, consistent with previous findings (9, 41). These data may indicate widespread transcriptional changes to leukocytes. In particular, we found CpG island and gene body and promoter regions were, on average, demethylated after exercise training, indicating the modulation of transcriptional activity.

The exercise-induced changes to skeletal myocyte (49) and adipocyte (55) genome-wide DNA methylation have been analyzed previously. Strikingly, the exercise training-induced changes to DNA methylation found in our study included CpG

Table 4. *DNA methylation changes after 4 wk of exercise training (≥10%, q ≤ 0.005)*

Location in Relation to					DNA Methylation, %				
CpG	Closest Gene	Gene Region	CpG Island	Chr	Before Exercise	After Exercise	Diff	*P*	*q* Value, ≤ 0.005
cg19933985			S shelf	5	53.8 ± 4.4	70.8 ± 4.1	17	1.93×10^{-7}	1.49×10^{-5}
cg10633981	*C11orf58*	3′UTR	Open sea	11	49.5 ± 6.7	66.4 ± 5.1	16.9	2.13×10^{-8}	3.97×10^{-6}
cg02732134	*DNMBP*	5′UTR	Open sea	10	42.3 ± 2.8	57.9 ± 3.6	15.6	4.79×10^{-8}	6.26×10^{-6}
cg01287788	*SNORD94*	TSS200	Open sea	2	49.5 ± 4.2	65.0 ± 5.2	15.5	2.96×10^{-7}	1.96×10^{-5}
	PTCD3	Body							
cg27379715			Open sea	2	55.1 ± 3.3	70.0 ± 3.3	14.8	8.39×10^{-9}	2.39×10^{-6}
cg17393016	*C17orf55*	TSS1500	N shore	17	54.2 ± 13.2	68.7 ± 15.9	14.5	0.002	0.005
cg02308712			Open sea	12	47.5 ± 3.9	61.7 ± 4.3	14.1	9.38×10^{-8}	9.45×10^{-6}
cg15897635			N shelf	1	30.7 ± 6.6	44.4 ± 5.3	13.8	0.0003	0.001
cg21937244	*CDC42BPB*	Body	Island	14	64.4 ± 5.9	78.0 ± 3.4	13.7	3.10×10^{-8}	4.86×10^{-6}
cg12578536			S Shelf	5	48.44.0	61.8 ± 3.5	13.4	2.74×10^{-7}	1.86×10^{-5}
cg17931986	*COL11A2*	3′UTR	S shore	6	53.8 ± 3.1	67.2 ± 3.6	13.4	6.86×10^{-9}	2.16×10^{-6}
cg04205664	*CLEC2L*	Body	S shore	7	28.3 ± 1.9	41.5 ± 4.1	13.2	2.09×10^{-7}	1.57×10^{-5}
cg05239225	*AKAP13*	5′UTR	Open sea	15	46.6 ± 7.3	59.5 ± 4.3	12.9	0.0003	0.001
cg13810766	*PRKAG2*	Body	Open sea	7	33.6 ± 1.9	45.9 ± 3.1	12.3	2.72×10^{-9}	1.39×10^{-6}
cg12949141	*PCBD2*	Body	Open sea	5	35.1 ± 5.6	47.1 ± 4.9	12.1	8.56×10^{-5}	0.0007
cg02771392	*ZNF273*	TSS1500	Open sea	7	59.3 ± 3.1	71.3 ± 2.0	12	1.58×10^{-8}	3.35×10^{-6}
cg04250181	*SLCO1B3*	5′UTR	Open sea	12	65.0 ± 6.0	76.9 ± 3.5	11.9	1.90×10^{-6}	6.24×10^{-5}
cg01775802	*RGS6*	Body	Open sea	14	27.6 ± 5.1	39.3 ± 5.2	11.7	9.61×10^{-5}	0.0007
cg22870994			Open sea	11	44.3 ± 2.3	55.9 ± 2.4	11.6	1.06×10^{-7}	1.02×10^{-5}
cg14497545	*MAML3*	Body	Open sea	4	36.5 ± 2.3	48.1 ± 4.0	11.5	5.62×10^{-7}	2.95×10^{-5}
cg23817637	*CLRN3*	TSS1500	Open sea	10	60.3 ± 6.2	71.8 ± 2.6	11.5	0.0001	0.0009
cg18022036			Open sea	5	47.3 ± 3.9	37.4 ± 4.6	−10	0.0001	0.001
cg06611444			Open sea	4	70.1 ± 5.5	60.2 ± 5.4	−10	8.78×10^{-6}	0.0002
cg16859420			Open sea	8	31.6 ± 5.4	21.6 ± 3.9	−10	0.0002	0.001
cg20090957	*MAPKAPK5*	TSS1500	Island	12	34.1 ± 3.1	24.1 ± 2.4	−10	2.10×10^{-6}	6.63×10^{-5}
	C12orf47	Body							
cg17502267	*BNIP2*	Body	Island	15	39.4 ± 2.5	29.4 ± 4.0	−10	2.59×10^{-5}	0.0003
cg22876894			*N shore	20	31.5 ± 4.2	21.5 ± 2.6	−10	1.25×10^{-7}	1.13×10^{-5}
cg18825597			N shelf	8	61.2 ± 4.4	51.1 ± 6.4	−10.1	0.001	0.004
cg16146432	*C3orf50*	TSS200	N shore	3	26.1 ± 4.5	16.1 ± 3.1	−10.1	1.52×10^{-6}	5.43×10^{-5}
cg17479131	*LOC401431*	Body	N shelf	7	54.2 ± 2.3	44.1 ± 4.4	−10.1	5.14×10^{-5}	0.0005
cg06180061	*C16orf91*	Body	S shelf	16	62.2 ± 3.5	52.0 ± 4.6	10.1	1.98×10^{-8}	3.84×10^{-6}
cg08339189	*GGTA1*	Body	N shore	9	21.8 ± 1.7	11.6 ± 2.3	10.2	1.71×10^{-6}	5.83×10^{-5}
cg17932934	*CALN1*	5′UTR	Open sea	7	72.9 ± 2.1	62.7 ± 5.7	10.2	3.92×10^{-5}	0.0004
		1st Exon							
cg14366742			Open sea	4	43.4 ± 4.0	33.2 ± 4.6	10.2	5.62×10^{-8}	6.85×10^{-6}
cg11381792	*TRAPPC10*	Body	Island	21	31.9 ± 1.3	21.7 ± 1.8	10.2	1.64×10^{-9}	1.12×10^{-6}
cg04669668	*BANP*	Body	Island	16	57.2 ± 3.7	46.9 ± 4.8	10.3	0.0001	0.0008
cg09700085	*SLC6A20*	5′UTR	Island	3	26.0 ± 2.2	15.7 ± 1.9	10.3	2.08×10^{-8}	3.93×10^{-6}
		1st Exon							
cg08206623	*CDKN1C*	TSS1500	Island	11	45.8 ± 1.5	35.5 ± 1.8	10.3	2.61×10^{-8}	4.38×10^{-6}
cg20144008			Island	2	45.3 ± 2.1	35.0 ± 2.4	10.4	1.11×10^{-8}	9.05×10^{-7}
cg07894334	*SOCS5*	TSS1500	†N shore	2	59.2 ± 7.2	48.8 ± 5.8	10.5	2.78×10^{-5}	0.0003
cg27130012			S shore	6	74.6 ± 2.7	64.1 ± 4.6	10.5	1.02×10^{-5}	0.0002
cg15386880			Island	16	50.5 ± 4.8	40.0 ± 7.5	10.5	0.0001	0.0009
cg00001793	*ETV6*	Body	Open sea	12	59.2 ± 5.0	48.7 ± 7.1	10.5	0.0001	0.0008
cg21265548	*NSDHL*	5′UTR	‡S shore	X	46.3 ± 8.1	35.7 ± 7.1	10.6	0.001	0.004
	CETN2	TSS1500							
cg05280527	*NRXN3*	3′UTR	S shore	14	47.7 ± 3.0	37.1 ± 3.5	10.6	9.17×10^{-8}	9.35×10^{-6}
cg02678768	*EVPL*	3′UTR	N shore	17	67.1 ± 9.1	56.4 ± 8.7	10.7	3.77×10^{-6}	9.51×10^{-5}
cg23917513	*LMX1A*	5′UTR	Open sea	1	66.1 ± 4.1	55.4 ± 5.5	10.7	3.92×10^{-5}	0.0004
		Body							
cg13985765	*PCSK6*	Body	Open sea	15	68.7 ± 6.4	57.9 ± 6.4	10.8	0.0005	0.002
cg25152348	*NCAPH2*	5′UTR	Island	22	47.2 ± 2.1	36.3 ± 1.8	10.9	5.29×10^{-8}	6.62×10^{-6}
		1st Exon							
	LMF2	TSS1500							
cg16500605	*BAT3*	TSS1500	Island	6	31.9 ± 3.5	20.9 ± 1.4	11	1.05×10^{-6}	4.29×10^{-5}
cg01558909	*HBM*	TSS200	Island	16	14.8 ± 9.0	3.8 ± 0.5	11	0.0006	0.002
cg15694789	*STARD8*	5′UTR	S shore	X	38.3 ± 4.6	27.3 ± 3.3	11	2.69×10^{-6}	7.71×10^{-5}
cg05524038	*CSF1R*	TSS1500	Open sea	5	80.0 ± 2.9	68.9 ± 2.5	11.1	1.51×10^{-6}	5.42×10^{-5}
cg11123847	*ACTR10*	3′UTR	Open sea	14	80.9 ± 3.1	69.8 ± 4.3	11.2	1.14×10^{-5}	0.0002
cg14506366	*SLC6A3*	Body	Open sea	5	66.5 ± 7.0	55.3 ± 6.6	11.2	0.001	0.003
cg06082548	*NKX6-2*	Body	*Island	12	32.3 ± 2.1	21.0 ± 1.9	11.3	3.50×10^{-10}	5.28×10^{-7}
cg26979339	*RIC8B*	TSS1500	Island	12	35.7 ± 1.5	24.3 ± 2.2	11.4	1.45×10^{-8}	3.23×10^{-6}
cg25474648	*ZDHHC14*	3′UTR	S shore	6	74.2 ± 2.5	62.7 ± 4.8	11.5	2.62×10^{-6}	7.58×10^{-5}

Continued

Table 4.—*Continued*

Location in Relation to					DNA Methylation, %				
CpG	Closest Gene	Gene Region	CpG Island	Chr	Before Exercise	After Exercise	Diff	*P*	*q* Value, ≤ 0.005
cg01994308	*PLAG1* *CHCHD7*	5′UTR TSS1500	N shore	8	40.0 ± 6.7	28.5 ± 4.2	11.5	0.0005	0.002
cg12093976	*EGF*	TSS200	Open sea	4	34.3 ± 2.5	22.8 ± 3.0	11.6	7.18×10^{-9}	2.20×10^{-6}
cg12861974	*CXorf57*	TSS200	Island	X	33.4 ± 4.8	21.8 ± 2.9	11.6	3.04×10^{-6}	8.32×10^{-5}
cg20929922	*C5orf28*	TSS1500	S shore	5	37.7 ± 3.1	26.0 ± 3.4	11.7	3.23×10^{-5}	0.0004
cg19349861	*OPCML*	5′UTR 1st Exon	Open sea	11	34.7 ± 3.0	22.9 ± 2.3	11.7	1.62×10^{-8}	3.41×10^{-6}
cg03909902	*PRDM4*	5′UTR	Island	12	35.6 ± 2.8	23.8 ± 1.7	11.8	1.75×10^{-8}	3.54×10^{-6}
cg04981696	*ABCC1*	Body	S shore	16	41.5 ± 4.3	29.5 ± 4.0	12	3.30×10^{-6}	8.76×10^{-5}
cg05005382	*CHTF18*	Body	Island	16	68.5 ± 3.3	56.4 ± 5.0	12.1	0.0003	0.001
cg00556029	*MARCKSL1*	TSS200	Island	1	30.9 ± 4.8	18.7 ± 2.6	12.2	3.22×10^{-5}	0.0004
cg03252499			Open sea	11	63.3 ± 7.4	51.0 ± 5.3	12.3	0.001	0.004
cg25501666			N shelf	2	65.8 ± 4.5	53.1 ± 4.8	12.7	3.68×10^{-6}	9.35×10^{-5}
cg20982606	*UNG*	TSS1500 TSS200	Island	12	29.1 ± 3.0	16.2 ± 2.4	12.9	9.23×10^{-9}	2.52×10^{-6}
cg16379462	*SAMD4A*	Body	Open sea	14	64.6 ± 3.8	51.6 ± 6.4	12.9	0.0004	0.002
cg14266237	*KLF1*	3′UTR	N shore	19	49.8 ± 6.9	36.8 ± 8.3	13	3.93×10^{-8}	5.62×10^{-6}
cg21393587			N shore	X	74.4 ± 2.4	61.4 ± 4.0	13	5.53×10^{-8}	6.76×10^{-6}
cg06808467	*LOC339290* *C18orf18*	TSS1500 Body	Island	18	39.2 ± 2.4	25.6 ± 3.1	13.6	2.56×10^{-8}	4.36×10^{-6}
cg17092349	*DALRD3* *NDUFAF3* *MIR425* *MIR191*	5′UTR TSS1500 TSS1500 TSS200	N shore	3	47.5 ± 6.6	33.8 ± 5.8	13.7	5.10×10^{-5}	0.0005
cg26081875			Open sea	6	36.2 ± 7.9	22.3 ± 5.3	13.9	0.001	0.004
cg01525244	*CBX7*	TSS200	N shore	22	30.3 ± 1.2	16.3 ± 1.9	14	4.20×10^{-10}	5.96×10^{-7}
cg09844907	*MPV17L*	5′UTR 1st Exon	Island	16	34.0 ± 1.5	19.4 ± 2.6	14.6	1.87×10^{-8}	3.70×10^{-6}
cg27494111			N shore	19	67.6 ± 5.1	52.5 ± 10.4	15.1	3.25×10^{-6}	8.68×10^{-5}
cg26186549			S shore	6	68.7 ± 11.4	53.3 ± 11.9	15.4	2.77×10^{-5}	0.0003
cg16524049	*LMX1A*	Body	Island	1	43.1 ± 2.7	27.1 ± 4.6	16	4.54×10^{-6}	0.0001

Data are expressed are beta values ± SD. Chr, chromosome number; Diff, difference; 3′, three prime; 5′, five prime; UTR, untranslated region; TSS1500, 1,500 bases upstream of transcription start site; TSS200, 200 bases upstream of transcription start site. *DMR, differentially methylated region; †CDMR, cancer differentially methylated region; ‡RDMR, reprogrammed differentially methylated region.

sites in genes involved in pathways similar to those previously shown to be affected in skeletal myocytes after 6 mo of exercise training (49). For example, 6 mo of moderate-intensity exercise altered skeletal muscle DNA methylation in genes related to MAPK signaling, progesterone-mediated oocyte maturation, Wnt signaling, melanogenesis, hedgehog signaling, and calcium signaling pathway (49)—pathways that are also modulated in leukocytes of individuals from our study

Table 5. *DNA methylation changes after 4 wk of exercise training (≥20%,* q ≤ *0.005)*

Location in relation to					DNA methylation (%)				
CpG	Closest Gene	Gene Region	CpG Island	Chr	Before Exercise	After Exercise	Diff	*P*	*q* Value, ≤0.005
cg21036194	*SNCAIP*	Body	Open sea	5	24.1 ± 23.1	86.2 ± 1.8	62.1	5.05×10^{-7}	2.76×10^{-5}
cg22588144	*ISM1*	TSS1500	Island	20	4.9 ± 1.0	53.1 ± 8.3	48.2	1.69×10^{-12}	5.35×10^{-8}
cg01309395	*HLA-DPB2*	Body	Open sea	6	37.9 ± 34.7	82.1 ± 4.1	44.2	0.0004	0.002
cg07658590	*SLC19A1*	TSS1500	S shore	21	15.6 ± 18.2	47.6 ± 3.9	32.0	0.0004	0.002
cg18531559	*ULK4*	TSS200	S shore	3	8.4 ± 1.9	36.1 ± 0.6	27.7	1.70×10^{-11}	1.56×10^{-7}
cg11950805	*CDC20*	TSS1500	Island	1	1.7 ± 0.5	25.8 ± 15.0	24.1	5.47×10^{-5}	0.0005
cg26919805	*PPPDE2 XRCC6*	5′UTR 1st Exon TSS1500	Island	22	1.9 ± 0.5	25.2 ± 18.2	23.3	0.001	0.003
cg09459740			Open sea	11	74.1 ± 1.2	96.2 ± 5.2	22.1	9.12×10^{-8}	9.34×10^{-6}
cg07170824	*ACVRL1*	5′UTR 1st Exon	Island	12	52.7 ± 3.1	32.7 ± 5.3	−20.0	2.51×10^{-7}	1.75×10^{-5}
cg16700025	*CMIP*	Body	Open sea	16	62.2 ± 15.4	36.1 ± 0.6	−26.0	0.0001	0.001
cg18684755	*RYR1*	Body	S shelf	19	86.9 ± 1.6	56.7 ± 25.4	−30.2	0.002	0.005
cg06390613	*TMEM198*	3′UTR	N shelf	2	76.2 ± 2.0	43.9 ± 18.2	−32.3	0.0001	0.0009
cg03229033			Island	1	44.5 ± 5.8	12.0 ± 21.6	−32.4	0.0001	0.0009
cg15224432	*IGFL3*	TSS1500	Open sea	19	73.8 ± 1.8	36.1 ± 0.6	−37.7	7.17×10^{-16}	4.55×10^{-11}
cg04600795			Open sea	6	88.7 ± 1.2	45.1 ± 20.9	−43.7	1.50×10^{-5}	0.0002
cg10580110	*KRT6A*	TSS1500	Open sea	12	88.5 ± 1.7	44.8 ± 29.6	−43.7	1.40×10^{-5}	0.0002
cg08975528	*ZBTB12*	3′UTR	Island	6	74.6 ± 2.9	19.8 ± 34.1	−54.7	0.0002	0.001
cg17415355	*TOLLIP*	Body	S shelf	11	86.5 ± 1.5	24.9 ± 35.8	−61.6	8.60×10^{-5}	0.0007
cg24810735	*ANGPT1*	Body	Open sea	8	79.3 ± 3.5	16.5 ± 32.2	−62.8	1.95×10^{-5}	0.0003

Data are average beta-values ± SD.

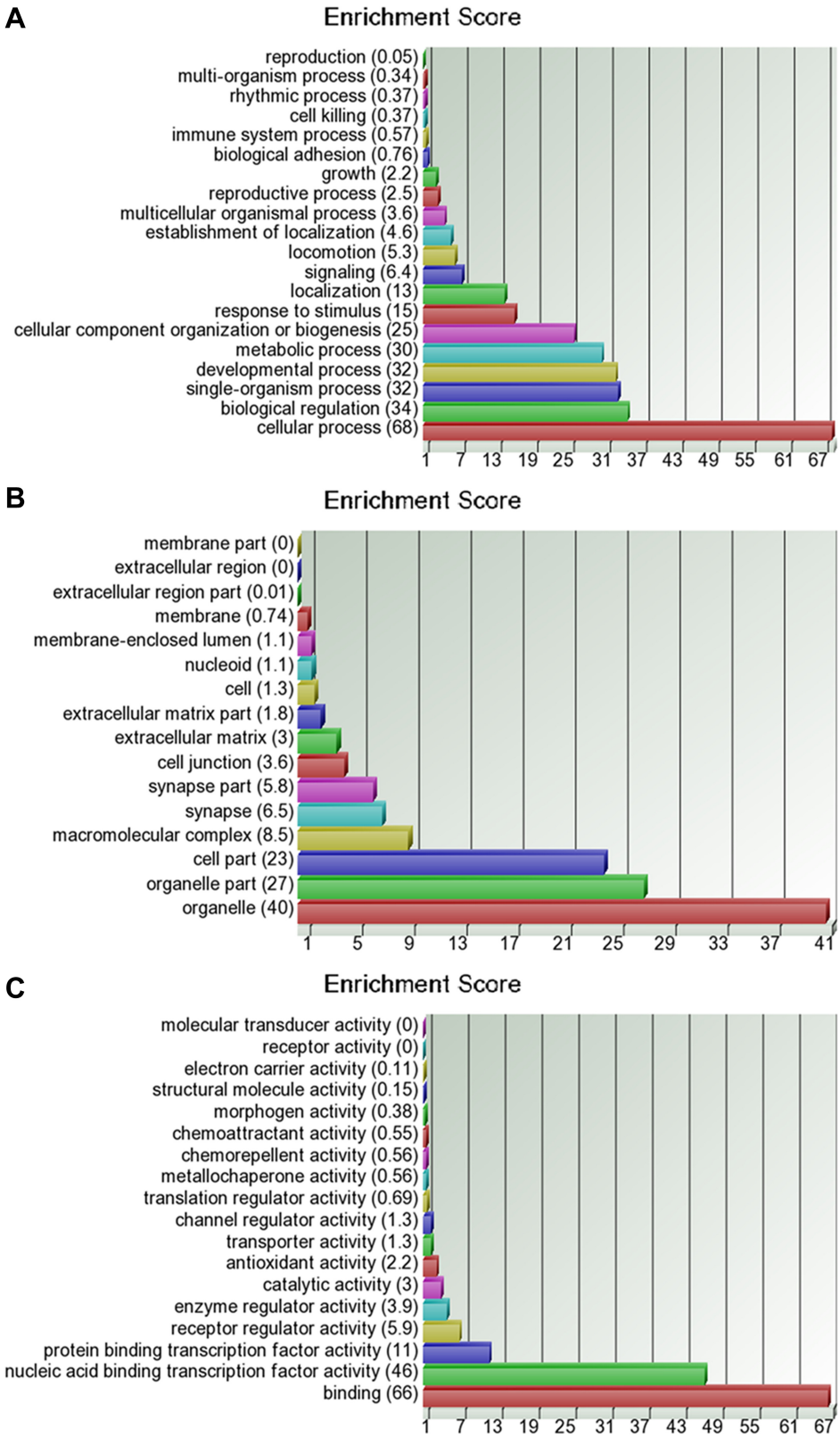

Fig. 4. Gene ontology for genes with CpG DNA methylation changes after exercise. Gene ontology is based on CpG sites with altered DNA methylation ($q < 0.001$) after exercise training. Gene ontology enrichment scores are given and organized by biological processes (*A*), cellular components (*B*), and molecular functions (*C*).

(Table 6). Furthermore, some of the mentioned pathways regulated by DNA methylation changes caused by exercise may also be governed by miRNA-mediated mRNA regulation, as indicated by our miRNA pathway analysis.

Although skeletal myocytes and adipocyte DNA methylation changes are predominantly indicative of metabolic transcriptional changes caused by exercise, leukocyte changes could be used as biomarkers of systemic changes to health and

Table 6. *Pathways modulated by exercise training (*$q \leq 0.05$*)*

Pathway Name	Enrichment Score	Enrichment *P*	Enrichment *q*	No. of Genes	Pathway ID
Endocytosis	16.44	7.23×10^{-8}	1.22×10^{-5}	115	kegg pathway 211
HTLV-I infection	13.52	1.35×10^{-6}	0.0001	154	kegg pathway 251
Wnt signaling pathway	11.93	6.61×10^{-6}	0.0003	88	kegg pathway 142
Proteoglycans in cancer	11.71	8.21×10^{-6}	0.0003	122	kegg pathway 164
Pathways in cancer	11.15	1.44×10^{-5}	0.0005	171	kegg pathway 128
Axon guidance	10.8	2.04×10^{-5}	0.0006	77	kegg pathway 176
Gap junction	10.44	2.92×10^{-5}	0.0007	58	kegg pathway 127
Regulation of actin cytoskeleton	9.97	4.66×10^{-5}	0.001	120	kegg pathway 179
Glutamatergic synapse	9.05	0.0001	0.002	67	kegg pathway 182
Calcium signaling pathway	9.03	0.0001	0.001	101	kegg pathway 263
Hippo signaling pathway	9.02	0.0001	0.002	92	kegg pathway 20
MAPK signaling pathway	8.93	0.0001	0.002	129	kegg pathway 119
p53 signaling pathway	8.6	0.0002	0.002	32	kegg pathway 149
Melanogenesis	8.28	0.00025	0.003	64	kegg pathway 206
Cholinergic synapse	7.94	0.00035	0.004	69	kegg pathway 237
Amphetamine addiction	7.53	0.0005	0.006	37	kegg pathway 50
Focal adhesion	7.13	0.0008	0.008	103	kegg pathway 5
Circadian entrainment	7.07	0.0008	0.008	56	kegg pathway 51
Basal cell carcinoma	7.02	0.0009	0.008	39	kegg pathway 35
Dopaminergic synapse	6.99	0.0009	0.008	68	kegg pathway 91
Dilated cardiomyopathy	6.34	0.002	0.01	53	kegg pathway 134
Epstein-Barr virus infection	6.05	0.002	0.02	113	kegg pathway 252
Cocaine addiction	5.92	0.003	0.02	28	kegg pathway 107
Long-term potentiation	5.88	0.003	0.02	35	kegg pathway 208
Hedgehog signaling pathway	5.32	0.005	0.03	33	kegg pathway 167
Chronic myeloid leukemia	5.17	0.006	0.03	42	kegg pathway 240
Melanoma	5.17	0.006	0.03	42	kegg pathway 249
Fc gamma R-mediated phagocytosis	5.16	0.006	0.03	45	kegg pathway 77
Glioma	4.95	0.007	0.04	38	kegg pathway 161
B cell receptor signaling pathway	4.95	0.007	0.04	38	kegg pathway 188
ECM-receptor interaction	4.94	0.007	0.04	47	kegg pathway 41
Glycosaminoglycan biosynthesis - chondroitin sulfate/dermatan sulfate	4.92	0.007	0.04	17	kegg pathway 248
Other types of O-glycan biosynthesis	4.92	0.007	0.04	17	kegg pathway 38
PI3K-Akt signaling pathway	4.74	0.009	0.04	166	kegg pathway 45
Oocyte meiosis	4.69	0.009	0.04	60	kegg pathway 22
T cell receptor signaling pathway	4.6	0.01	0.046	54	kegg pathway 70
Aminoacyl-tRNA biosynthesis	4.59	0.01	0.046	27	kegg pathway 116
Spliceosome	4.5	0.01	0.049	75	kegg pathway 131
Small cell lung cancer	4.48	0.01	0.049	42	kegg pathway 114
Progesterone-mediated oocyte maturation	4.36	0.01	0.05	50	kegg pathway 254

fitness. Leukocytes circulate through the body and their gene expression mimics that of their internal and external environments, making them useful biomarkers (37). Likewise, the epigenetic landscapes are malleable to the internal (biological) and external environments (such as physical exercise) (7, 28). Therefore, leukocyte changes to epigenetic modifications could be relevant biomarkers of phenotype changes, and in this case, biomarkers of the changes in cardiorespiratory fitness and accompanying health and performance adaptations (total and LDL cholesterol, and maximal running speed). Leukocytes are also implicated in the pathogenesis of atherosclerosis (58), and the DNA methylation changes observed in our study provide mechanistic insights into how exercise attenuates the risk of atherosclerosis.

Interestingly, exercise training caused DNA methylation changes with paralleled miRNA changes. DNA methylation can downregulate gene expression by working in concert with other epigenetic modifications (e.g., histone protein methylation and acetylation) and DNA binding proteins to compact chromatin and, in turn, inhibit transcription factor binding (17, 32, 33, 68). We identified that DNA methylation changes to *MIR21* and *MIR210* genes influenced mature miR-21 and miR-210 expression. The microRNAs miR-21 and miR-210 are ubiquitously expressed hypoxamirs involved in cardiovascular diseases, inflammation, and angiogenesis (14, 61). Indeed, miR-21 is upregulated in atherosclerotic plaques (52) and acute coronary syndromes (64). The increased miR-21 expression, however, may be a protective response to the disease and acute coronary damage. For example, upregulated miR-21 was protective against ischemia-induced cell apoptosis by downregulating programmed cell death 4 (*PDCD4*) mRNA after myocardial infarction in rats (21). Previous in vitro experiments showed that miR-21 attenuates vascular smooth muscle cell apoptosis caused by reactive oxygen species (ROS; hydrogen peroxide, H_2O_2) through downregulation of programmed cell death 4 (*PDCD4*) (38). Additionally, human endothelial cell miR-21 was augmented by shear stress and led to decreased phosphatase and tensin homolog (*PTEN*)-mediated apoptosis, facilitated endothelial nitric oxide synthase (eNOS) phosphorylation, and nitric oxide production (62); an effect exerted by exercise training (26, 36). MicroR-21 is particularly responsive to exercise training because it is upregulated after chronic exercise training in serum (4), spinal cord (39), cardiac (42), and skeletal myocytes (2) in mammals. Thus exercise

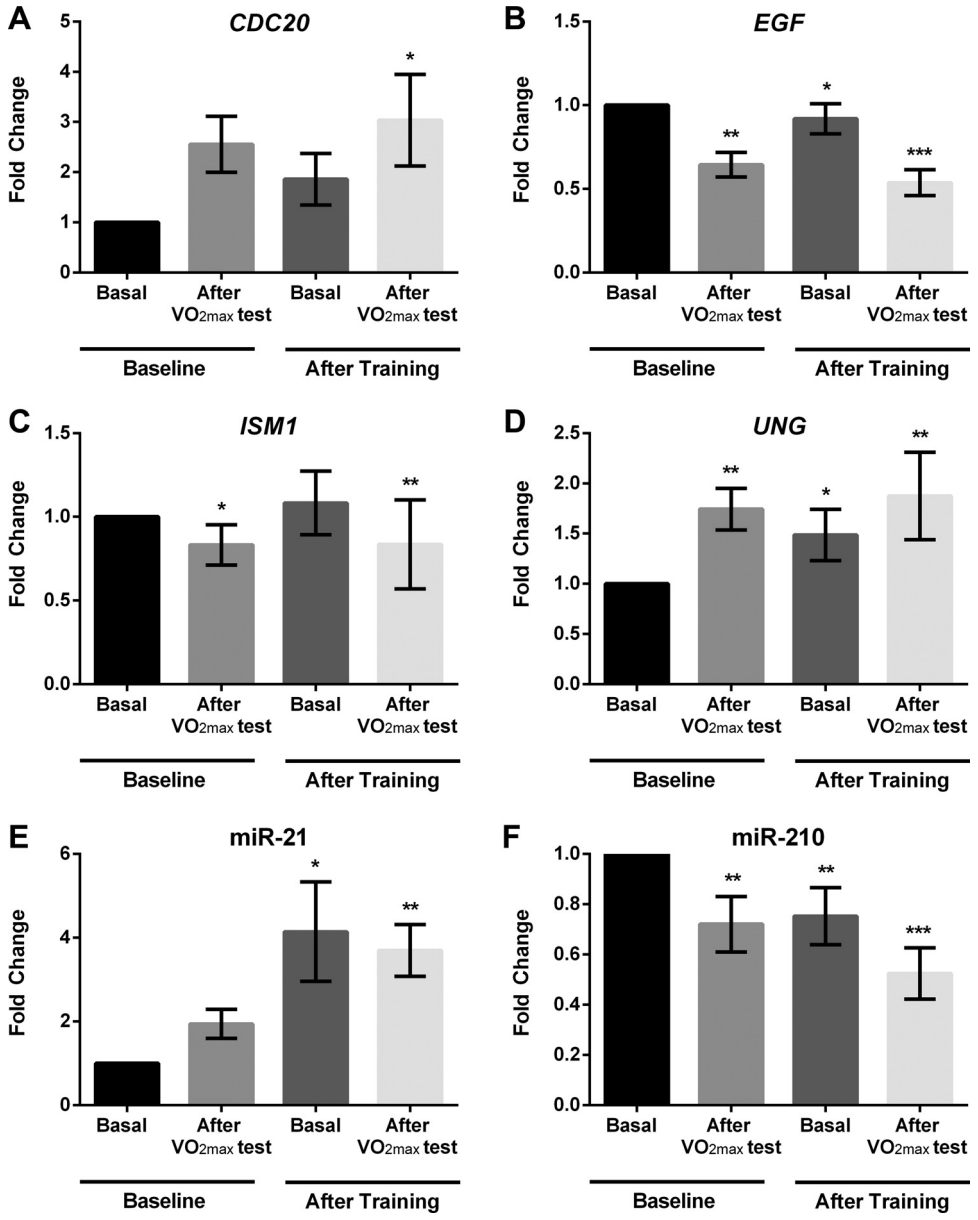

Fig. 5. Gene and microRNA (miRNA) expression changes caused by exercise. Data are from two-way repeated-measures ANOVA and are expressed as fold change of genes and mature miRNA expression (means ± SE). A significant difference between basal and other time points is indicated by asterisks. *CDC20*, cell division cycle 20 gene; *EGF*, epidermal growth factor gene; *ISM1*, isthmin 1 gene; *UNG*, uracil-DNA glycosylase gene; miR-21, microRNA-21; miR-210, microRNA-210. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ vs. basal.

may maintain vascular integrity and prevent vascular disease through upregulating miR-21 expression. This in turn contributes to modulation of miR-21 downstream mRNA targets involved in the eNOS pathway, oxidative stress, and apoptosis.

MicroR-210 is induced by hypoxia and is upregulated in numerous cardiovascular diseases, especially those associated with ischemia (20). Similar to miR-21, miR-210 is upregulated in atherosclerotic plaques (52) and myocardial infarction (8), possibly by oxidative damage and cellular senescence. ROS upregulate miR-210 through phosphorylation of AKT, ERK1/2, and platelet-derived growth factor receptor β (34). MicroR-210 is also known to facilitate ROS production and to induce double-stranded DNA breaks in human cells undergoing replicative senescence (24). Interestingly, serum miR-21 and miR-210 were shown to be inversely correlated to $\dot{V}O_{2max}$ in a cohort of 100 healthy subjects (11). Our data also suggest that miR-210 may be indicative of $\dot{V}O_{2max}$ because our participants exhibited elevated $\dot{V}O_{2max}$ with decreased leukocyte miR-210 expression after exercise training. We showed that predicted targets of miR-21 are particularly enriched for mRNA from genes associated with cardiovascular pathology (ischemic heart disease and coronary atherosclerosis) that supports the concept but warrants further study. The impact of these exercise-induced miRNA changes on genes and biological implications is also left for future research. Collectively, it is likely that miR-21 and miR-210 are exercise-responsive miRNAs that underpin the salubrious adaptations associated with exercise training.

Demethylation of *EGF* and *UNG* gene promoter regions attenuated and augmented the mRNA levels, respectively. Promoter demethylation is generally associated with gene activation (32), but not exclusively, because promoter demethylation does not always increase gene expression (12, 35, 45). Alternatively, the observed decrease to *EGF* mRNA expression could be due to other means of transcriptional regulation such as histone modifications, small noncoding RNA mole-

Table 7. *MIR21 and MIR210 DNA methylation after 4 wk of exercise training ($q < 0.05$)*

Location in Relation to					DNA Methylation, %				
CpG	Closest Gene	Gene Region	CpG Island	Chr	Before Exercise	After Exercise	Diff	*P*	*q* Value, ≤0.005
cg27023597	*MIR21*	TS1500	Open Sea	17	67.4 ± 3.6	64.4 ± 4.0	−3	0.007	0.01
cg04276626	*MIR21*	TSS200	Open Sea	17	79.3 ± 3.4	76	−3.3	0.008	0.01
cg02515217	*MIR21*	TSS200	Open Sea	17	80.0 ± 2.4	78.5 ± 2.1	−1.5	0.009	0.01
cg07181702	*MIR21*	Body	Open Sea	17	78.4 ± 3.0	74.6 ± 3.9	−3.8	0.02	0.03
cg02471760	*MIR210*	TSS1500	S Shore	11	54.6 ± 4.7	50.4 ± 4.6	−4.2	0.002	0.004
cg08200293	*MIR210*	TSS1500	Island	11	13.2 ± 1.2	11.8 ± 2.1	−1.4	0.01	0.02
cg05858042	*MIR210*	TSS1500	Island	11	24.5 ± 3.9	21.7 ± 5.1	−2.7	0.005	0.009
cg07410811	*MIR210*	TSS1500	Island	11	8.1 ± 1.2	6.7 ± 1.5	−1.4	0.003	0.007
cg15482500	*MIR210*	TSS200	Island	11	11.6 ± 0.7	9.3 ± 0.7	−2.3	9.39×10^{-5}	0.0007
cg01277369	*MIR210*	TSS200	Island	11	7.5 ± 1.1	6.2 ± 0.9	−1.3	0.007	0.01
cg03880841	*MIR210*	Body	Island	11	18.5 ± 0.01	15.9 ± 1.2	−2.6	6.2×10^{-5}	0.0005

Data are average beta values ± SD.

cules, or transcription factor activity. Importantly, epidermal growth factor (EGF) is a ligand for the EGF receptor (EGFR), which is hyperactive in atherosclerosis (43). The increased EGFR activity by elevated EGF may facilitate increased inflammation and blood vessel damage, leading to atherosclerosis (43). Acute exercise training increases peripheral but not central artery distensibility (53), which is an effect established after chronic exercise training (54). Moreover, exercise training increases and decreases muscle microvascular density and arterial stiffness, respectively (16). Therefore, the present study indicates that the attenuation of the EGFR-ligand, *EGF*, expression by promoter DNMT gene changes caused by exercise training could benefit vascular health. Similarly, the observed DNA methylation and gene expression change of *UNG* could serve as a mechanism for the demethylation of most genes analyzed in the present study. Active DNA demethylation occurs via the hydroxylation of 5-methylcytosine to 5-hydroxymethylcytosine by ten-eleven translocation (TET) proteins (1–3), followed by the active base-excision repair initiated by UNG (51, 59). Together, these data demonstrate the complex regulation of gene expression whereby DNA methylation can directly regulate mRNA levels or, indirectly modulate gene expression through the modulation of miRNA expression subsequently influencing gene expression and cardiovascular phenotypes.

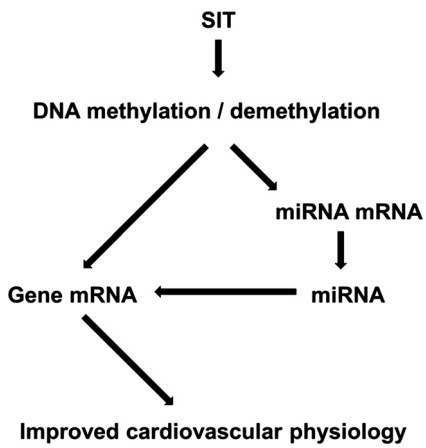

Fig. 6. Schematic of DNA methylation regulation on cardiovascular physiology. Exercise training in the form of sprint interval training (SIT) influences the leukocyte DNA methylome. This in turn modulates the expression (mRNA levels) of protein-coding genes (*EGF* and *UNG*) and also microRNAs (miR-21 and miR210). MicroRNAs miR-21 and miR-210 would subsequently regulate other protein-coding genes to inevitably influence cardiovascular physiology and enhance cardiovascular health and performance.

Although not measured, the numerous changes to DNA methylation observed in the present study could be a result of the altered DNA methyltransferase enzymes (DNMT1, 3A and 3B). Whereas DNMT1 is responsible for maintaining DNA methylation during mitosis, DNMT3A and DNMT3B are responsible for de novo methylation changes (6). It is possible that exercise training-induced changes to activity of DNMT enzymes could have affected the leukocyte methylome because expression was altered in the hippocampi of mice after 7 days of voluntary wheel running (1). Passive loss of DNA methylation during cell division is an alternate explanation for the DNA methylation changes observed in our study. This is, however, unlikely because of the short duration of study intervention period. The DNA methylation changes (up to 62.8%) caused by exercise training in our study could be explained by the intensity of exercise training. Previous work (5) revealed skeletal myocyte global and metabolic gene (*PPARGC1A*, *TFAM*, *PPARD*, *MEF2A*) DNA demethylation is intensity dependent, such that higher intensity exercise elicited greater DNA demethylation. Moreover, the acute increase in ROS and other metabolites may influence TET proteins and histone acetyltransferase and deacetylase activity to alter DNA methylation (50), but this remains to be experimentally demonstrated.

Our study has some limitations. Blood leukocyte counts were not quantified before or after exercise training, and it is possible that shifts in leukocyte subsets could be responsible for the change in leukocyte DNA methylation observed in our study. This is, however, unlikely because we quantified leukocyte DNA methylation from resting blood samples unaffected by the leukocytosis associated with acute exercise. Furthermore, there is no evidence to suggest that leukocyte subsets change with chronic exercise training and a similar high-intensity exercise training study did not alter leukocyte subsets (48). Although we did not include any experimental controls (i.e., subjects that did not participate in any exercise for 4 wk), data from our laboratory indicate that genome-wide leukocyte DNA methylation is stable over the course of 8 wk because no changes were observed in a cohort of young men not engaging in any high-intensity aerobic exercise (unpublished data). We could not successfully validate the 450K DNA methylation

results using EpiTYPER due to issues with the genomic regions being analyzed. Exercise training was not performed in a laboratory setting, and the exercise intensity of participants was not directly monitored during the course of the intervention. Lastly, we cannot rule out that potential changes to participants' diet caused DNA methylation changes.

In conclusion, we have demonstrated that 4 wk (249 min) of exercise training significantly alters the leukocyte methylome. These DNA methylation changes influenced miRNA and mRNA levels, improved cardiorespiratory fitness, and enhanced the blood lipid profile of healthy young men. These DNA methylation changes could be relevant biomarkers for monitoring adherence to exercise interventions and demonstrates a dynamic role for epigenetic regulation in the change to cardiovascular health and fitness caused by short-term exercise training.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

J.D., B.J.O., F.Z.M., and F.J.C. conception and design of research; J.D. performed experiments; J.D. analyzed data; J.D., B.J.O., F.Z.M., and F.J.C. interpreted results of experiments; J.D. prepared figures; J.D. drafted manuscript; J.D., B.J.O., F.Z.M., and F.J.C. edited and revised manuscript; J.D., B.J.O., F.Z.M., and F.J.C. approved final version of manuscript.

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