Altered miR-29 Expression in Type 2 Diabetes Influences Glucose and Lipid Metabolism in Skeletal Muscle

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MicroRNAs have emerged as important regulators of glucose and lipid metabolism in several tissues; however, their role in skeletal muscle remains poorly characterized. We determined the effects of the miR-29 family on glucose metabolism, lipid metabolism, and insulin responsiveness in skeletal muscle. We provide evidence that miR-29a and miR-29c are increased in skeletal muscle from patients with type 2 diabetes and are decreased following endurance training in healthy young men and in rats. In primary human skeletal muscle cells, inhibition and overexpression strategies demonstrate that miR-29a and miR-29c regulate glucose uptake and insulin-stimulated glucose metabolism. We identified that miR-29 overexpression attenuates insulin signaling and expression of insulin receptor substrate 1 and phosphoinositide 3-kinase. Moreover, miR-29 overexpression reduces hexokinase 2 expression and activity. Conversely, overexpression of miR-29 by electroporation of mouse tibialis anterior muscle decreased glucose uptake and glycogen content in vivo, concomitant with decreased abundance of GLUT4. We also provide evidence that fatty acid oxidation is negatively regulated by miR-29 overexpression, potentially through the regulation of peroxisome proliferator–activated receptor γ coactivator-1α expression. Collectively, we reveal that miR-29 acts as an important regulator of insulin-stimulated glucose metabolism and lipid oxidation, with relevance to human physiology and type 2 diabetes.

MicroRNAs (miRNAs) are short, noncoding RNA molecules of 18–24 nucleotides that regulate gene expression through posttranscriptional modification of target mRNA through binding at the 3′ untranslated region. The miRNA interaction with mRNA can destabilize mRNA or repress protein translation; a single miRNA is capable of altering the expression of hundreds of proteins (1,2). At least 1,800 human miRNAs have been identified (miRBase21), and while the total number is still unclear, miRNAs may account for 2–3% of all genes in the human genome, exerting posttranscriptional control over 30% of all genes. miRNAs regulate metabolism in most mammalian tissues, including liver, adipose tissue, and skeletal muscle (3–5). miRNAs also play important roles in skeletal muscle development and hypertrophy (6). The majority of evidence from skeletal muscle suggests that miRNAs regulate gene expression during development; miR-1, -133, and -206 have been implicated as regulators of myogenesis, each by distinct mechanisms. However, the role for miRNAs in the regulation of gene expression and metabolism in skeletal muscle is incompletely resolved.

The miR-29 family comprises three mature members, miR-29a, miR-29b, and miR-29c, which are encoded by two gene clusters. These miRNAs are highly expressed in insulin-sensitive tissues and are upregulated in rodent models of obesity or diabetes (4,7). A recent meta-analysis of miRNA expression profiles of patients with type 2 diabetes or rodent models of diabetes identified miR-29a as the most upregulated miRNA across different insulin-sensitive tissues (8). Overexpression of miR-29a in adipocytes inhibits insulin-stimulated glucose uptake and negatively regulates gluconeogenesis and insulin signaling in hepatocytes (4,9,10). These findings underscore the miR-29 family members as regulators of glucose homeostasis.

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Expression levels and function of miR-29 in human skeletal muscle remain poorly characterized. Here, we hypothesized that the miR-29 family regulates glucose metabolism and insulin sensitivity in skeletal muscle. Using gain- or loss-of-function approaches in vivo or in primary human skeletal muscle cells, we identified miR-29 as an important regulator of glucose uptake, insulin action, and lipid oxidation. Collectively, we reveal that miR-29 acts as an important regulator of skeletal muscle metabolism.

RESEARCH DESIGN AND METHODS

Human Subjects

Male volunteers with type 2 diabetes or normal glucose tolerance (NGT) were matched for age, weight, and BMI. Clinical characteristics of the participants are presented in Table 1. Patients with type 2 diabetes were treated with metformin, statins, thiazolidinedione, or sulfonylureas, and insulin-treated patients were excluded. Subjects with type 2 diabetes had increased fasting and 2-h glucose values, as well as increased HbA_{1c}. Cholesterol was reduced in subjects with type 2 diabetes, probably reflecting statin use (Table 1). Skeletal muscle biopsies were obtained from the vastus lateralis muscle under local anesthesia after an overnight fast, as described previously (11). The human exercise cohort has been previously described (12). Eight healthy, sedentary male volunteers performed short-term endurance exercise training by cycling for 60 min at 80% of $V_{O_{2peak}}$ for 14 consecutive days, as described elsewhere (12). Biopsies were taken from fasted volunteers before the first training session and again 16 h following the 14th training session. All participants provided written informed consent, and protocols were approved by the Karolinska Institutet and the Dublin City University Research Ethics Committees, in accordance with the Declaration of Helsinki.

Table 1—Subjects' characteristics

	NGT (n = 10)	Type 2 diabetes (n = 12)
Age (years)	59 ± 1.5	62 ± 1
Height (cm)	178.7 ± 2.3	175.4 ± 1.1
Weight (kg)	92.4 ± 2.2	97.3 ± 3.4
BMI (kg/m^2)	29.0 ± 0.5	31.6 ± 1.0
Waist (cm)	102.0 ± 1.7	105.8 ± 2.6
SBP (mmHg)	132.0 ± 3.1	139.2 ± 3.3
DBP (mmHg)	83.5 ± 2.5	83.3 ± 2.4
Fasting glucose (mmol/L)	5.4 ± 0.1	8.6 ± 0.5*
2-h glucose (mmol/L)	6.7 ± 0.9	16.3 ± 0.9*
HbA_{1c} (%)	4.6 ± 0.1	6.0 ± 0.2*
HbA_{1c} (mmol/mol)	27.3 ± 0.6	42.3 ± 2.5*
Insulin (pmol/L)	61.2 ± 5.9	76.8 ± 9.0
Cholesterol (mmol/L)	5.85 ± 0.21	4.19 ± 0.16*
HDL (mmol/L)	1.30 ± 0.13	1.30 ± 0.11
LDL (mmol/L)	3.82 ± 0.20	2.28 ± 0.16*
Triglycerides (mmol/L)	1.57 ± 0.16	1.38 ± 0.17

Data are mean ± SEM. DBP, diastolic blood pressure; SBP, systolic blood pressure. *$P < 0.05$ NGT vs. type 2 diabetes.

Animal Studies

Experiments were approved by the Regional Animal Ethical Committee (Stockholm, Sweden). Male C57BL/6J and C57BL/6.Cg-Lepob/J mice were purchased from Charles River Laboratories (Sulzfeld, Germany), housed under a 12-h light/12-h dark cycle, and received ad libitum access to water and standard rodent chow (Lantmännen, Sweden). After 1 week of acclimatization, tibialis anterior muscles of 12-week-old C57BL/6J mice were transfected by electroporation with either a control plasmid or a plasmid encoding for pri-miR-29a or pri-miR-29c (Origene, Rockville, MD), as previously described (13). One week after electroporation, mice were fasted for 4 h and in vivo glucose uptake was assessed by a modified oral glucose tolerance test, as described elsewhere (13). Mice were anesthetized with Avertin (2,2,2-tribromoethanol and tertiary amyl alcohol), and electroporated muscles were removed and immediately frozen. Glycogen content was determined using a glycogen assay kit (ab65620; Abcam), following the manufacturer's protocol.

Female Wistar rats were purchased from B&K Universal (Sollentuna, Sweden), fed a normal chow diet, and randomized to either an exercise group or a sedentary control group, as described previously (14). The exercise group was trained by means of a swimming program consisting of two 3-h bouts of swimming per day for 5 consecutive days. Rats were sacrificed 16 h after the last training session, and gastrocnemius muscle was used to measure miRNA expression.

Primary Human Skeletal Muscle Cell Culture

Primary cells were isolated from vastus lateralis skeletal muscle biopsies derived from healthy volunteers, as described elsewhere (15). Myoblasts were propagated in growth medium (F12/DMEM, 20% FBS, 1% penicillin-streptomycin [Invitrogen; Thermo Fisher Scientific, Stockholm, Sweden]) and differentiated according to the protocol for the LHCN-M2 cell line (16), with slight modifications. Cells were differentiated for 4 days with fusion media containing DMEM/M199, HEPES (0.02 M; Invitrogen), zinc sulfate (0.03 μg/mL), vitamin B_{12} (1.4 μg/mL; Sigma-Aldrich), insulin (10 μg/mL; Actrapid; Novo Nordisk), and apotransferrin (100 μg/mL; BBI Solutions). Cells were then cultured with postfusion media containing DMEM/M199, HEPES, zinc sulfate, vitamin B_{12}, and 0.5% FBS. Six days after inducing differentiation, cells were transfected with 20 nmol/L of miR-29a or miR-29c Ambion Pre-miRNA Precursors, or with negative control miRNA (Life Technologies). A second transfection was performed after 48 h. Each transfection was performed for 6 h in OptiMEM reduced serum media with Lipofectamine RNAiMAX transfection reagent (Invitrogen). The same double-transfection protocol was used for miRNA inhibition using 20 nmol/L of mirVana miRNA Inhibitors for hsa-miR-29a or -29c, or a negative control inhibitor (Life Technologies). Overexpression of miR-29a and miR-29c in primary human cells was determined

using quantitative PCR (qPCR) (Supplementary Fig. 1*A* and *B*). Transfection efficiency of miR-29a and miR-29c inhibitors was estimated using qPCR, reflecting inhibition activity (Supplementary Fig. 1*C* and *D*). Metabolic studies were conducted 48 h after the second transfection.

Glucose Uptake, Glycogen Synthesis, and Fatty Acid Oxidation in Cells

Glucose uptake, glucose incorporation into glycogen, and palmitate oxidation were determined in primary human skeletal muscle cells, as previously described (15,17). Glucose uptake was measured in myotubes incubated in the absence or presence of insulin (120 nmol/L) in glucose- and serum-free DMEM before the addition of 2-[1,2-^{3}H]deoxy-D-glucose and 10 μmol/L unlabeled 2-deoxy-D-glucose. Glycogen synthesis was determined based on D-[U-^{14}C]glucose incorporation into glycogen and assessed after preincubation in the presence or absence of insulin (120 nmol/L) in serum-free DMEM. Cells were starved of serum for 4 h before treatment with insulin. Lipid oxidation was estimated by incubating myotubes in serum-free DMEM supplemented with 25 μmol/L cold palmitate and [9,10-^{3}H]palmitic acid, then incubated in the absence or presence of 2 mmol/L AICAR for 6 h. Tritiated water released in culture media was measured.

Results were normalized by protein content (BCA Protein Assay Kit; Thermo Fisher Scientific, Rockford, IL). Data are the average of five or six independent experiments performed in duplicate using cells obtained from three different donors.

RNA Extraction and Gene Expression Analysis

Total RNA from mouse skeletal muscle and human cells was isolated with Trizol (Life Technologies), according to the manufacturer's recommendations. Total RNA concentration was quantified spectrophotometrically (NanoDrop ND-1000 Spectrophotometer; Thermo Fisher Scientific, Waltham, MA). RNA was reverse-transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit or a custom primer pool in order to quantify miRNA (Life Technologies). Gene expression was determined by real-time PCR using SYBR Green reagent (Life Technologies). miRNA expression was determined using the TaqMan reagent and primers (assay identifiers [IDs] 002112, 000413, 000587; Thermo Fisher Scientific). Reference genes were selected using the NormFinder algorithm. miRNA expression was normalized to U48 for human studies (assay ID 001006), and the geometric means of sno202 and sno234 for rodent studies (IDs 001232 and 001234; Thermo Fisher Scientific). Gene expression in human skeletal muscle was normalized by the geometric means of *TBP* and *RPLP0*, and in mouse skeletal muscle, to the geometric means of *Tbp* and *Rplp0*. Putative targets of miR-29 were determined for humans and mice using the TargetScan algorithm (version 6.7), and conserved predicted targets in the insulin signaling pathway were measured. SYBR primer sequences are reported in Supplementary Table 1.

Protein Abundance and Hexokinase Activity Analysis

Western blotting was performed as described elsewhere (13). Ponceau staining was used to confirm equal protein loading. The antibodies used are reported in Supplementary Table 2. Protein abundance was determined by densitometry using Quantity One software (Bio-Rad). Hexokinase activity was determined using the Hexokinase Colorimetric Assay Kit (Sigma-Aldrich), following the manufacturer's protocol.

Statistical Analysis

All data are presented as mean ± SEM. Differences in miRNA expression between subjects with NGT and patients with type 2 diabetes were analyzed using an independent Student *t* test. Differences in parameters for the human exercise cohort and electroporated mouse skeletal muscle were measured using a paired Student *t* test. Data from cells were analyzed using repeated-measures ANOVA followed by a Bonferroni post hoc test. Comparisons were considered statistically significant at $P < 0.05$. Analyses were performed using GraphPad Prism 7 software (GraphPad Software Inc.).

RESULTS

miR-29 Expression Profiling in Skeletal Muscle From Patients With Type 2 Diabetes and Primary Human Skeletal Muscle Cells Rendered Resistant to Insulin

We determined the expression of miR-29 family members in skeletal muscle biopsies obtained from people with NGT or type 2 diabetes. Expression of miR-29a and miR-29c was increased in skeletal muscle from patients with type 2 diabetes compared with the NGT control subjects, whereas miR-29b expression was unchanged (Fig. 1*A*–*C*). miR-29 expression was increased in skeletal muscle from *ob/ob* mice (Fig. 1*D*). The relative expression of the miR-29 family members was comparable between human skeletal muscle biopsies and primary human myotubes: miR-29a was sevenfold higher than miR-29c, whereas miR-29b had the lowest expression level (Supplementary Fig. 2*A* and *B*). In mouse skeletal muscle, expression of miR-29a was 200-fold higher than that of miR-29c (Supplementary Fig. 2*C*). To test the effects of systemic factors associated with insulin resistance on miR-29 expression, we studied primary human skeletal muscle cells exposed to tumor necrosis factor-α (TNF-α) or palmitate for 24 or 96 h. Exposure to either TNF-α or palmitate reduced insulin-stimulated glucose incorporation into glycogen (Supplementary Fig. 3*A* and *B*). Treatment with TNF-α or palmitate for 24 h did not affect the expression of the miR-29 family members (Fig. 1*E* and *F*). A 96-h treatment with palmitate, but not TNF-α, increased miR-29a and miR-29c expression (Fig. 1*E* and *F*), whereas miR-29b expression remained unaffected (data not shown).

miR-29 Overexpression Attenuates Glucose Metabolism in Primary Human Skeletal Muscle Cells

We next examined the effects of miR-29a and miR-29c on glucose metabolism in primary human myotubes. These family members were studied because they were differentially expressed in skeletal muscle from patients with type 2 diabetes. Glucose uptake was reduced by ~20% upon miR-29a and miR-29c overexpression under both basal

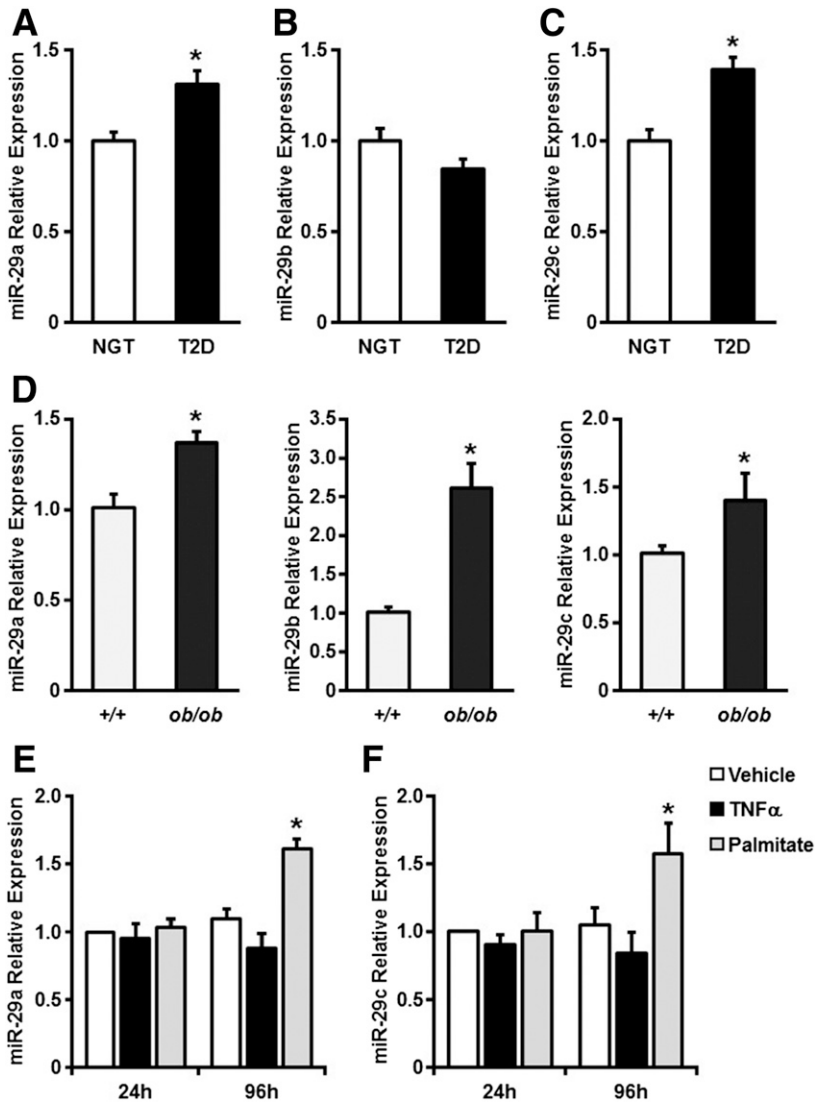

Figure 1—Expression of miR-29 in skeletal muscle. miR-29a (*A*), miR-29b (*B*), and miR-29c (*C*) expression was determined in skeletal muscle biopsies obtained from individuals with NGT (n = 10) and patients with type 2 diabetes (T2D) (n = 12). *D*: Expression of miR-29a, miR-29b, and miR-29c was determined in gastrocnemius muscles of wild-type mice and *ob/ob* littermates (n = 5). Expression of miR-29a (*E*) and miR-29c (*F*) was determined in primary human skeletal muscle cells treated with TNF-α or palmitate for 24 h (40 ng/mL TNF-α; 0.2 mmol/L palmitate) or 96 h (20 ng/mL TNF-α; 0.1 mmol/L palmitate) (n = 4). Data are presented as mean ± SEM. *$P < 0.05$.

and insulin-stimulated conditions (Fig. 2*A*). Basal glycogen synthesis was unaffected, whereas insulin-stimulated glycogen synthesis was reduced 31% and 23% by miR-29a and miR-29c overexpression, respectively (Fig. 2*B*). In addition, insulin-stimulated glucose oxidation was reduced following miR-29c overexpression (Fig. 2*C*).

miR-29 Overexpression Modulates Insulin Signaling in Primary Human Skeletal Muscle Cells

To determine the mechanism by which miR-29 overexpression attenuates glucose metabolism, we measured mRNA levels of genes involved in this process in primary human myotubes. miR-29c overexpression reduced expression of *GLUT1*. miR-29a and miR-29c overexpression reduced expression of hexokinase 2 (HK2), a rate-limiting enzyme of glycolysis (Fig. 2*D*). Using miRNA target prediction algorithms, we identified several miR-29 putative target genes involved in insulin signal transduction. Specifically, we identified insulin receptor substrate 1 (*IRS1*), phosphoinositide 3-kinase (PI3K) regulatory subunit 1 (*PIK3R1*), PI3K regulatory subunit 3 (*PIK3R3*), and *AKT2* as predicted targets of miR-29. Among these, miR-29 overexpression reduced *IRS1*, *PIK3R3*, and *AKT2* mRNA expression, further validating a role for miR-29a and miR-29c as modulators of insulin signaling and glucose metabolism (Fig. 2*D*). Western blotting of the insulin signaling pathway revealed that miR-29 overexpression decreased IRS1 protein abundance, as well as insulin-stimulated phosphorylation of Akt^{Ser473}, without altering total Akt abundance (Fig. 2*E* and Supplementary

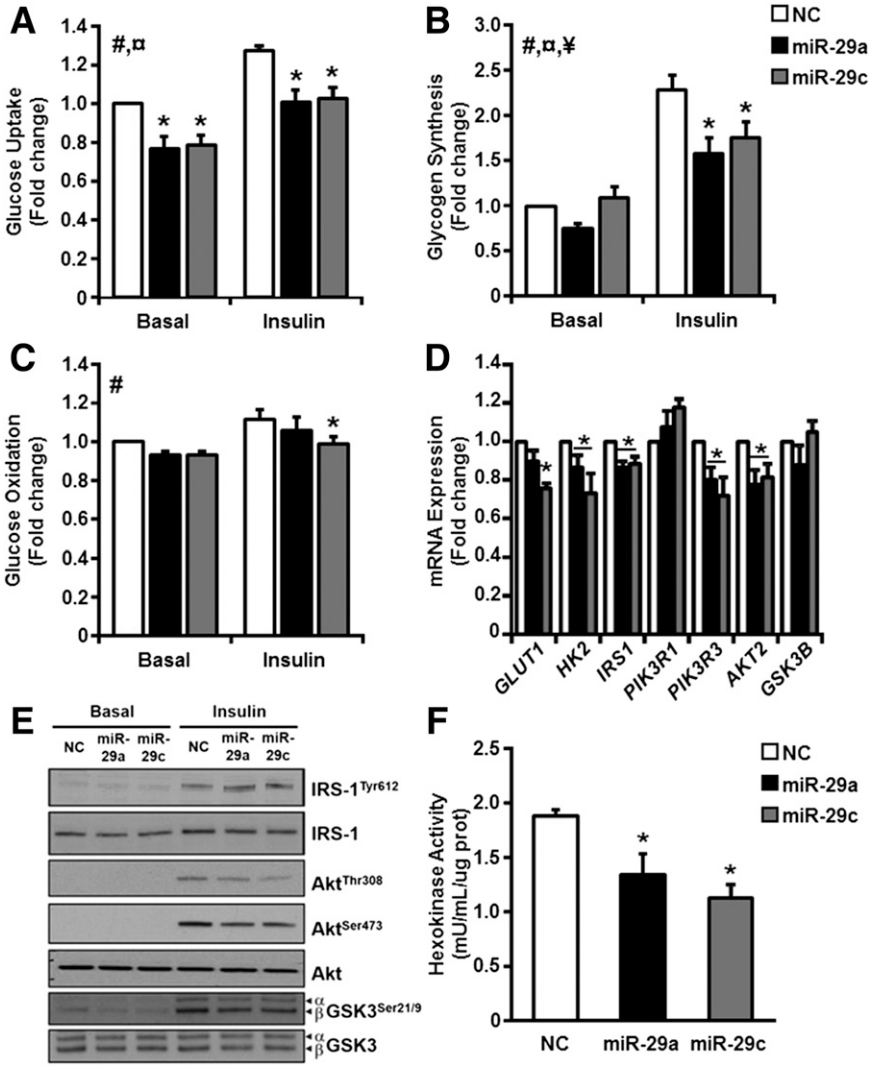

Figure 2—miR-29a and miR-29c overexpression modulates glucose metabolism in primary human skeletal muscle cells. Myotubes were transfected with 20 nmol/L of miR-29a or miR-29c Pre-miRNA Precursors or with negative control (NC) miRNA and subsequently incubated in the absence (basal) or presence of insulin (120 nmol/L) in order to assess glucose uptake and metabolism. ^{3}H-deoxyglucose uptake (*A*), ^{14}C-glucose incorporation into glycogen (*B*), and ^{14}C-glucose oxidation (*C*) were assessed. *D*: Gene expression was determined by qPCR. *E*: Representative immunoblots for pIRS1^{Tyr612}, total IRS1, pAkt^{Thr308} and pAkt^{Ser473}, total Akt, pGSK3α/β$^{Ser21/9}$, and total GSK3α/β. *F*: The effect of miR-29a and miR-29c overexpression on hexokinase activity was determined. Data are presented as mean ± SEM (n = 5–6). *P < 0.05; #transfection effect; ¤insulin effect; ¥interaction.

Tables 3 and 4). Overexpression of miR-29a and miR-29c also decreased glycogen synthase kinase (GSK) 3β protein abundance, concomitant with reduced insulin-stimulated phosphorylation of GSK3α/β$^{Ser21/Ser9}$ (Fig. 2*E* and Supplementary Tables 3 and 4). Consistent with the reduction in *HK2* mRNA, overexpression of either miR-29a or miR-29c robustly decreased hexokinase activity (Fig. 2*F*). These results provide mechanistic insight into the role of miR-29 in modulating glucose uptake and insulin-mediated glucose metabolism.

miR-29 Overexpression Alters Glucose Metabolism in Intact Mouse Tibialis Anterior Muscle

Mouse tibialis anterior muscle was electroporated with vectors expressing either pri-miR-29a or pri-miR-29c (a control vector was used in the contralateral leg) to assess the effects on glucose metabolism in vivo. Seven days after electroporation, mature miRNA levels were increased 14- and 1.6-fold for miR-29a and miR-29c, respectively, compared with the control leg (Fig. 3*A*). Consistent with the data obtained from cell cultures, in vivo glucose uptake assessed during a modified oral glucose tolerance test was reduced 15% and 13% in skeletal muscle transfected with vectors expressing either miR-29a or miR-29c, respectively (Fig. 3*B*). Furthermore, total intramuscular glycogen content was also reduced in skeletal muscle following 7 days of either miR-29a or miR-29c overexpression (Fig. 3*C*). Expression of *Glut4* was decreased in skeletal muscle overexpressing either miR-29a or miR-29c, whereas *Glut1* mRNA level was

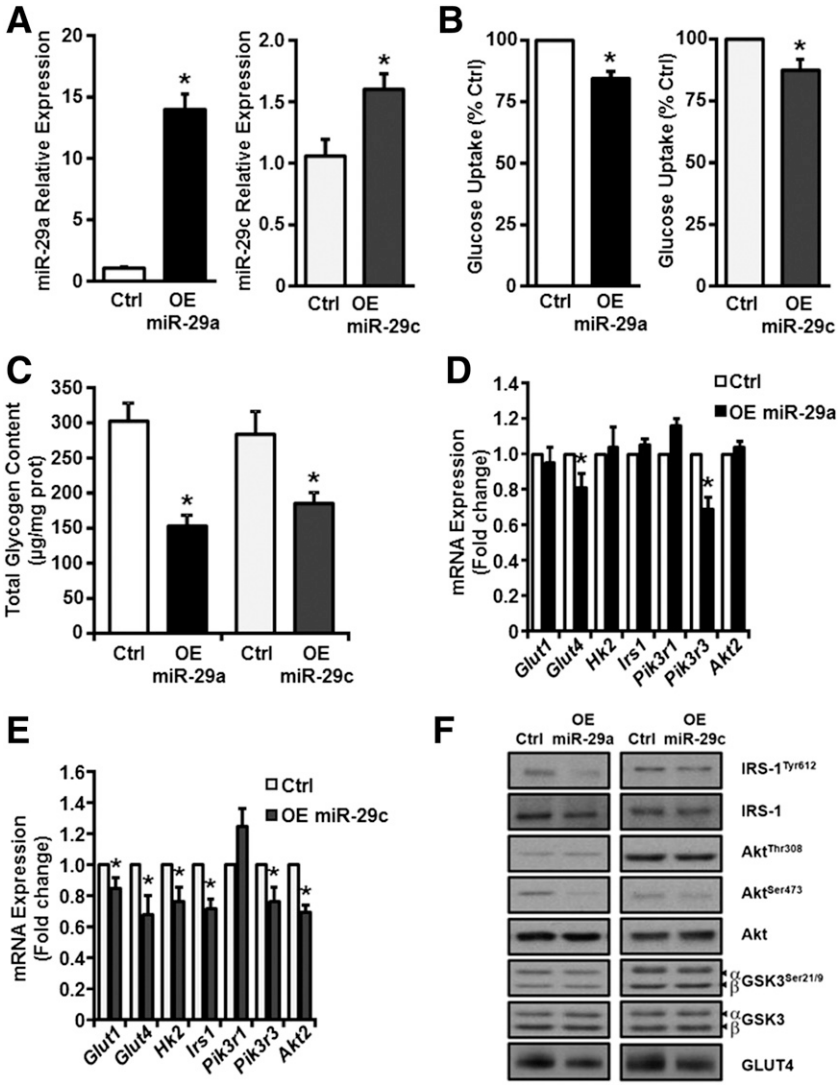

Figure 3— Overexpression (OE) of miR-29 attenuates glucose uptake in vivo in tibialis anterior muscle. *A*: Quantification of miR-29a and miR-29c overexpression in tibialis anterior muscle 7 days after electroporation by qPCR. *B*: In vivo ^{14}C-deoxyglucose uptake during a modified oral glucose tolerance test, reported as a percentage of the contralateral control (Ctrl) leg. *C*: Intramuscular glycogen content following miR-29 overexpression. *D* and *E*: Gene expression in mouse muscle following overexpression of miR-29a (*D*) or miR-29c (*E*). *F*: Representative immunoblots of pIRS1^{Tyr612}, total IRS1, pAkt^{Thr308} and pAkt^{Ser473}, total Akt, pGSK3α/β$^{Ser21/9}$, total GSK3α/β, and GLUT4. n = 10 mice for all data presented. Data are presented as mean ± SEM. *P < 0.05.

decreased in skeletal muscle transfected with miR-29c (Fig. 3*D* and *E*). We next assessed gene expression of predicted targets of miR-29. While miR-29a overexpression reduced expression of *Pik3r3*, miR-29c overexpression reduced mRNA levels of *Irs1*, *Pik3r3*, and *Akt2* (Fig. 3*D* and *E*). *Hk2* mRNA was decreased by miR-29c overexpression; however, HK2 enzyme activity was not affected by miR-29a or miR-29c overexpression (Supplementary Fig. 4). Western blotting revealed that phosphorylation of Akt^{Ser473} was reduced and total Akt protein abundance was unaltered in skeletal muscle overexpressing miR-29 following a 2-h oral glucose challenge (Fig. 3*F* and Supplementary Table 5). Total IRS1 abundance and IRS1^{Tyr612} phosphorylation were reduced in skeletal muscle overexpressing miR-29a or miR-29c (Fig. 3*F* and Supplementary Table 5).

miR-29 Inhibition Increases Glucose Metabolism in Human Primary Myotubes

To determine the effects of endogenous miR-29 on glucose metabolism, miR-29a and miR-29c functions were downregulated in human primary skeletal muscle cells using inhibitors that specifically bind to targeted miRNAs. Inhibition of miR-29c increased both basal and insulin-stimulated glucose uptake in human myotubes (Fig. 4*A*). A trend toward increased basal glucose uptake was observed after inhibition of miR-29a (P = 0.06) (Fig. 4*A*). Basal glycogen synthesis was increased after inhibition of miR-29c, but not miR-29a (Fig. 4*B*). Insulin-stimulated glycogen synthesis was not affected by inhibition of either miR-29a or miR-29c (Fig. 4*B*). The mRNA levels of *HK2* and *IRS1* were increased by miR-29a and miR-29c inhibition (Fig. 4*C*). In

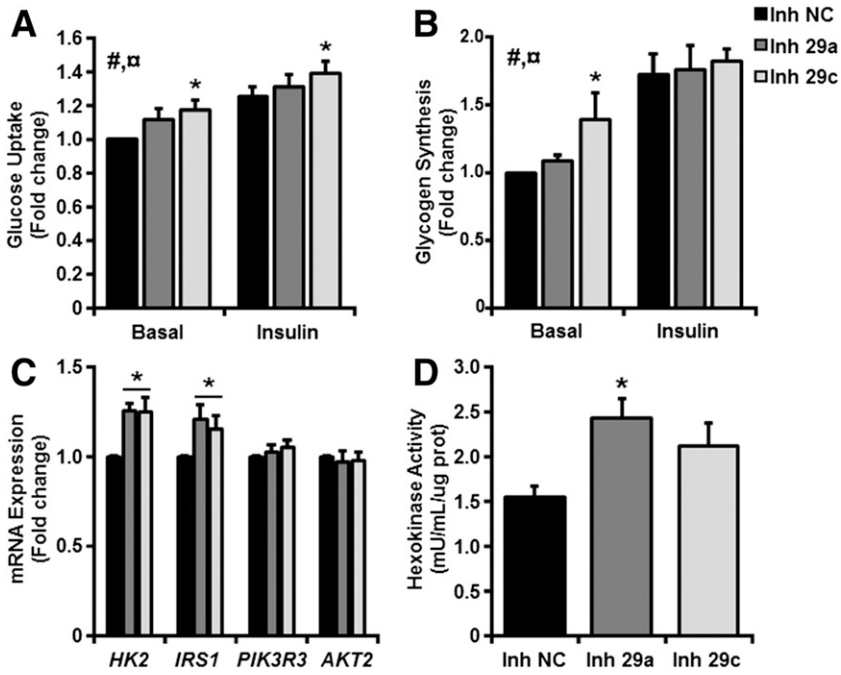

Figure 4—Effect of endogenous miR-29a and miR-29c inhibition on glucose metabolism in primary human skeletal muscle cells. Glucose metabolism was determined after repressing miR-29a and miR-29c in myotubes using specific inhibitors. ^{3}H-deoxyglucose uptake (*A*) and ^{14}C-glucose incorporation into glycogen (*B*) were assessed in the absence (basal) or presence of insulin (120 nmol/L). *C*: Gene expression was determined by qPCR. *D*: Hexokinase activity was determined biochemically. Data are presented as mean ± SEM ($n = 6$). *$P < 0.05$; #transfection effect; ¤insulin effect. Inh, inhibition; NC, negative control.

addition, hexokinase activity was increased by miR-29a inhibition (Fig. 4*D*).

Effect of miR-29 on Fatty Acid Metabolism in Primary Human Skeletal Muscle Cells

Overexpression of miR-29a and miR-29c decreased both basal and AMPK-activated (AICAR-stimulated) palmitate oxidation (Fig. 5*A*). Conversely, inhibition of miR-29a and miR-29c increased palmitate oxidation (Fig. 5*B*). Triglyceride synthesis was not affected by miR-29 overexpression (data not shown). *CD36* mRNA was unaffected by miR-29 expression modulation, whereas *PDK4* and *PGC1A* were decreased by miR-29 overexpression and increased after inhibition (Fig. 5*C* and *D*). In mouse tibialis anterior muscle, *Pgc1a* mRNA was decreased by overexpression of either miR-29a or miR-29c, whereas overexpression of miR-29c also reduced the mRNA level of *Cd36* and *Pdk4* (Fig. 5*E* and *F*). Abundance of mitochondrial complex proteins and the activity of citrate synthase were unaltered by modification of miR-29 expression (data not shown), suggesting that the effect of miR-29 on fatty acid oxidation is not related to alterations in mitochondrial content.

Effect of Endurance Exercise on miR-29 Expression in Skeletal Muscle

Given that miR-29 expression was increased in insulin-resistant skeletal muscle, we tested the hypothesis that modalities that enhance insulin sensitivity would decrease miR-29 expression. Thus, we determined the effect of exercise training on miR-29 family expression in skeletal muscle. In rodents, 5 days of swim training led to a reduction of miR-29a and miR-29c in gastrocnemius muscle (Fig. 6*A* and *B*). These effects seem to be specific for miR29a and miR-29c, since miR-29b was undetectable in rat gastrocnemius muscle. In humans, 14 consecutive days of endurance exercise training reduced miR-29c abundance in vastus lateralis skeletal muscle, whereas only a trend toward reduced abundance was observed for miR-29a (Fig. 6*C*–*E*).

DISCUSSION

Glucose metabolism and insulin action are regulated by miRNAs in several tissues, including liver and adipose (18). The role of miRNAs in the regulation of insulin action in human skeletal muscle is currently unknown. In this study, we determined the effects of the miR-29 family on glucose and lipid metabolism and insulin action in skeletal muscle. We show that miR-29a and miR-29c expression increased in skeletal muscle from patients with type 2 diabetes, and decreased in muscle from healthy young men following exercise training. Inhibition and overexpression approaches in primary human skeletal muscle cells reveal that miR-29 regulates lipid oxidation and insulin's action on glucose metabolism. Similarly, in vivo miR-29 overexpression decreases glucose uptake and subsequently glycogen content. At the molecular level, miR-29 alters insulin signaling and *PGC1a* and *HK2* mRNA levels. Our work uncovers a critical role for

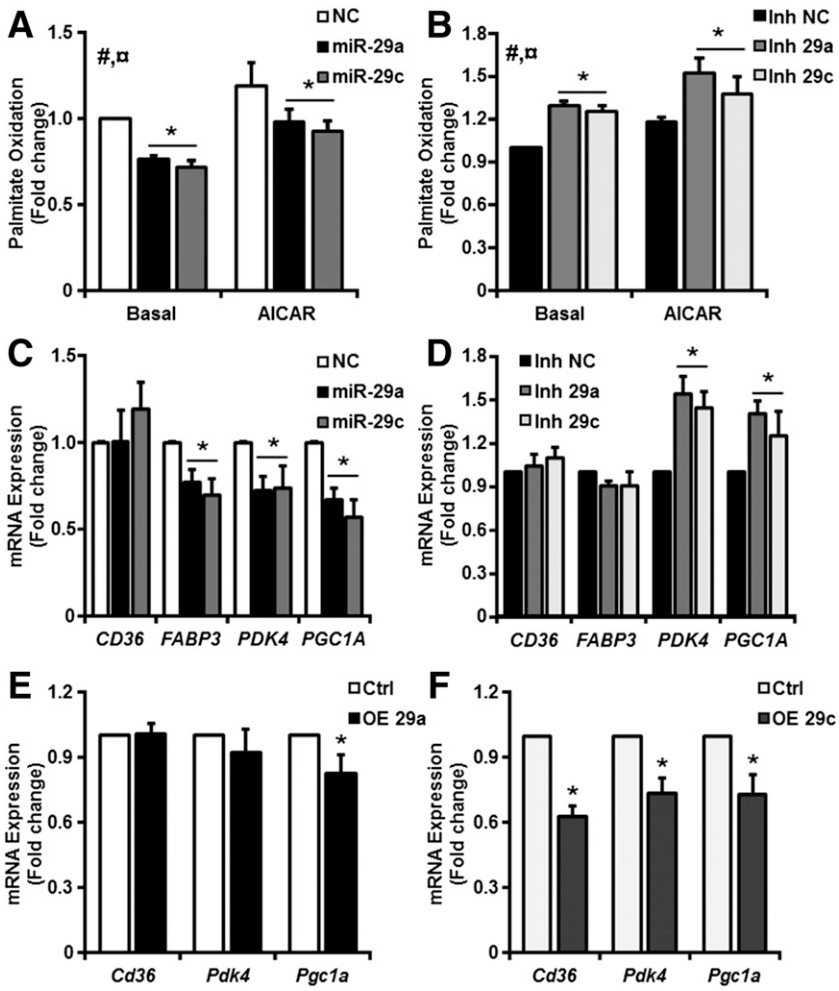

Figure 5—Effect of miR-29 on fatty acid oxidation and lipid-handling genes. Lipid oxidation was determined in primary human skeletal muscle cells incubated in the absence or presence of 2 mmol/L AICAR for 6 h (n = 6). ^{3}H-palmitate oxidation was assessed following either miR-29 overexpression (OE) (*A*) or inhibition (*B*). Gene expression was determined following either miR-29 overexpression (*C*) or inhibition (*D*) (n = 6). *P < 0.05; #transfection effect; ¤insulin effect. Gene expression was determined in mouse tibialis anterior muscle following overexpression either miR-29a (*E*) or miR-29c (*F*) (n = 10). Data are presented as mean ± SEM. *P < 0.05. Ctrl, control; Inh, inhibition; NC, negative control.

miR-29 in skeletal muscle metabolism, with relevance to insulin resistance in type 2 diabetes.

Meta-analysis of miRNA expression in insulin-responsive tissues highlighted miR-29 as a dysregulated miRNA in insulin-resistant conditions such as type 2 diabetes (8). miR-29 members are upregulated in liver and skeletal muscle of mice fed a high-fat diet and in obese animal models of diabetes, such as *db/db* mice and Zucker rats (4,7,10,19). In this study we provide evidence that miR-29a and miR-29c are increased in skeletal muscle from patients with type 2 diabetes. Moreover, we extend previous observations showing miR-29a and miR-29c are increased in skeletal muscle of *ob/ob* mice (4,20). Thus, miR-29 expression is robustly dysregulated in insulin-resistant tissues in obese rodent models of diabetes and in skeletal muscle from patients with type 2 diabetes. Circulating levels of fatty acids are increased in both patients with type 2 diabetes and obese rodent models of diabetes, and can thereby influence gene expression in peripheral tissues such as skeletal muscle (20). Consistent with previous findings in L6 rat myotubes, palmitate increased miR-29a expression in primary human skeletal muscle cells (7). Thus chronic high levels of circulating fatty acids might contribute to the increased abundance of miR-29 in insulin resistance. The mechanism by which palmitate increases miR-29 expression warrants further study. Overall, our data provide evidence that dysregulated expression of miR-29 family members are a common hallmark of insulin-resistant skeletal muscle.

Skeletal muscle is a major site of glucose disposal, and in patients with type 2 diabetes it is characterized by diminished insulin-mediated glucose transport and metabolism (21). We determined the effects of the type 2 diabetes–associated changes in miR-29a and miR-29c on glucose metabolism and insulin responsiveness by overexpressing these miRNAs in vitro in human skeletal muscle cells and in vivo in intact mouse skeletal muscle. Overexpression of

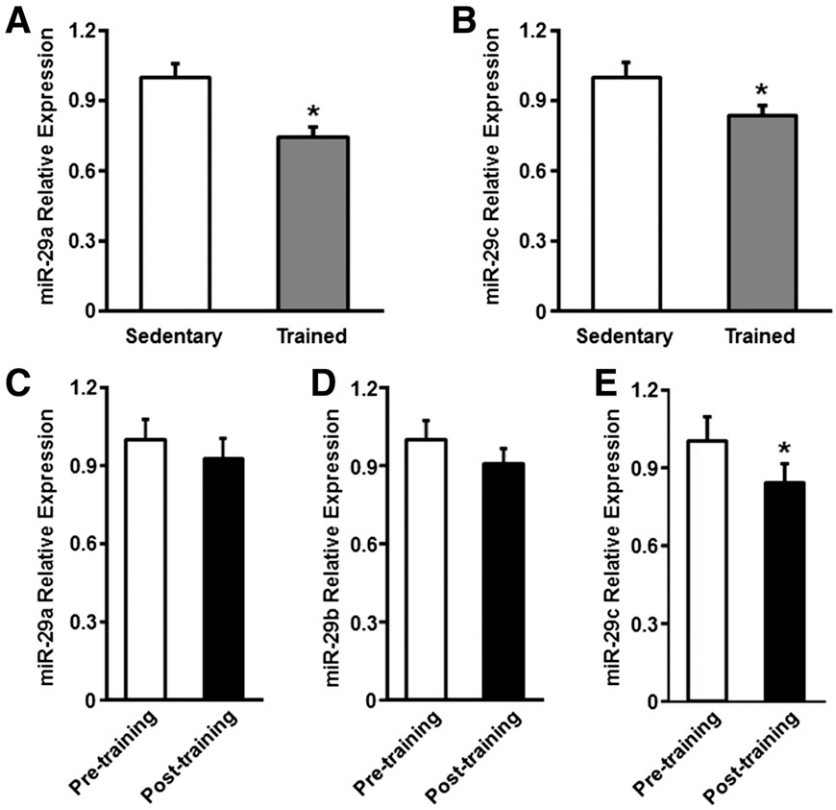

Figure 6—Effect of endurance training on miR-29 expression in rat and human skeletal muscle. miR-29a (*A*) and miR-29c (*B*) expression was determined in gastrocnemius muscle from sedentary ($n = 6$) or endurance exercise–trained rats ($n = 7$). miR-29a (*C*), miR-29b (*D*), and miR-29c (*E*) expression was determined in skeletal muscle from healthy young men ($n = 8$) before and after 14 consecutive days of endurance training. Data are presented as mean ± SEM. $^*P < 0.05$.

miR-29 reduced glucose uptake both in cultured cells and in vivo during an oral glucose challenge. Our data support findings of decreased glucose uptake upon miR-29 overexpression in rodent cell lines (4,7,22). In addition to reduced glucose uptake, we observed that miR-29 overexpression decreased insulin-stimulated glycogen synthesis in human primary skeletal muscle cells and reduced glycogen content in mouse skeletal muscle in vivo, strengthening our finding of a role for miR-29 in the regulation of glucose metabolism. While other studies determined a role for miR-29 in glucose metabolism solely based on supraphysiological overexpression, we also determined the functional role of endogenous miR-29 using miRNA inhibitors. Thus we provide new evidence that miR-29a and mir-29c regulate glucose uptake and insulin-stimulated glucose metabolism in skeletal muscle, both in vitro in human primary cells and in vivo in mature skeletal muscle.

Efficient glucose disposal in skeletal muscle is dependent on insulin-regulated processes, including GLUT4 trafficking, glucose phosphorylation by hexokinase, and subsequent glycogen storage. miR-29-induced alterations in glucose metabolism were associated with reduced expression of *IRS1*, *PIK3R3*, and *AKT2*, suggesting that miR-29 overexpression modulates insulin action by downregulating the expression of canonical mediators of insulin signaling in skeletal muscle. In addition to reduced IRS1 protein abundance, miR-29a and miR-29c decreased insulin signaling downstream of PI3K, at the level of Akt and GSK3 phosphorylation in human skeletal muscle cells. While the low abundance of GLUT4 in human primary cells precludes its role in glucose metabolism in this system, aberrant glucose metabolism following miR-29 overexpression, including diminished insulin-stimulated glucose transport in mouse skeletal muscle, was observed concomitantly with reduced GLUT4 protein abundance.

Following transport into a cell, glucose is rapidly phosphorylated by hexokinases into glucose-6-phosphate before undergoing glycolysis or storage as glycogen. Hexokinase activity was decreased in human muscle cells following overexpression of either miR-29a or miR-29c. Thus the loss of hexokinase activity in skeletal muscle cells overexpressing miR-29 is likely related to the reductions in *HK2* mRNA, as this gene encodes the main isoform of hexokinase in skeletal muscle (23). However, no binding site exists for miR-29 in the 3′ untranslated region of the *HK2* gene, suggesting that the effects are secondary. In skeletal muscle, insulin induces HK2 expression in a PI3K-dependent manner (24). In our study, overexpression of miR-29 reduced PI3K subunit expression,

which has been previously validated as a direct target of miR-29 (25). Collectively, this suggests that the miR-29-induced decrease in hexokinase activity may be related to attenuated signals emanating from PI3K. Defects in insulin signal transduction due to reduced IRS1/PI3K phosphorylation are associated with reduced glucose transport in skeletal muscle from severely obese people (26,27). Moreover, hexokinase expression and activity are reduced in patients with type 2 diabetes (28,29). Therefore, miR-29a and miR-29c may contribute to insulin resistance in skeletal muscle in type 2 diabetes by regulating glucose metabolism at multiple levels.

A well-characterized feature of skeletal muscle from patients with type 2 diabetes is diminished oxidative capacity and lower mitochondrial abundance (30,31). Because miR-29 was increased in skeletal muscle from patients with type 2 diabetes, we determined the effects of miR-29 family members on palmitate oxidation. We found that endogenous miR-29 negatively regulates fatty acid oxidation in skeletal muscle. Peroxisome proliferator–activated receptor γ coactivator-1α (PGC1α) is an important transcriptional coactivator that regulates glucose and lipid metabolism, and promotes mitochondrial biogenesis (32). PGC1α expression is induced by exercise, concomitant with increased lipid oxidation (33,34). Moreover, PGC1α expression is reduced in skeletal muscle from patients with type 2 diabetes, coincident with decreased oxidative capacity (35). Here we found that overexpression of miR-29a and miR-29c both in vitro and in vivo downregulates *PGC1A* expression. Conversely, miR-29 inhibition increased *PGC1A* expression. In C2C12 skeletal muscle cells, miR-29a directly targets *PPARD*, leading to reduced *PGC1A* expression (22). In cultured human myotubes or adult mouse skeletal muscle subjected to miR-29 overexpression, however, *PPARD* expression was unaltered, suggesting that the regulation of PGC1α by miR-29 occurs by direct targeting, as previously shown (10). Taken together, this evidence suggests that miR-29 contributes to a decreased capacity of skeletal muscle to oxidize fat, associated with a reduced capacity to oxidize glucose in response to insulin, as observed in type 2 diabetic muscle.

Exercise training can increase the sensitivity of skeletal muscle to insulin and prevent the progression of type 2 diabetes (36). Repeated bouts of physical activity improve glucose and lipid metabolism in skeletal muscle, concomitant with increased mitochondrial capacity (36). Here we measured miR-29 expression in skeletal muscle following endurance training in rats and humans. In young healthy men, miR-29c was downregulated after 14 consecutive days of endurance exercise training, whereas miR-29a expression tended to decrease. The effect of exercise training on miR-29 expression in humans was mimicked in rodents: both miR-29a and miR-29c were decreased by 5 days of endurance training (swimming). This reduction of miR-29 expression was accompanied by increased IRS1-associated PI3K activity (14). Our finding of exercise-induced reductions in miR-29 expression is consistent with previous findings in humans, whereby a 12-week resistance training program was associated with a reduction in miR-29a compared with baseline only in "low responders" (i.e., individuals who failed to demonstrate a hypertrophic response to exercise training) (37). Nevertheless, the mechanism by which exercise training decreases miR-29 abundance remains unknown.

In summary, expression of miR-29a and miR-29c in skeletal muscle is altered in patients with type 2 diabetes and animal models (Fig. 7). Molecular studies reveal that miR-29a and miR-29c modulate glucose and lipid metabolism in skeletal muscle by fine-tuning the expression of genes involved in the canonical insulin-signaling cascade

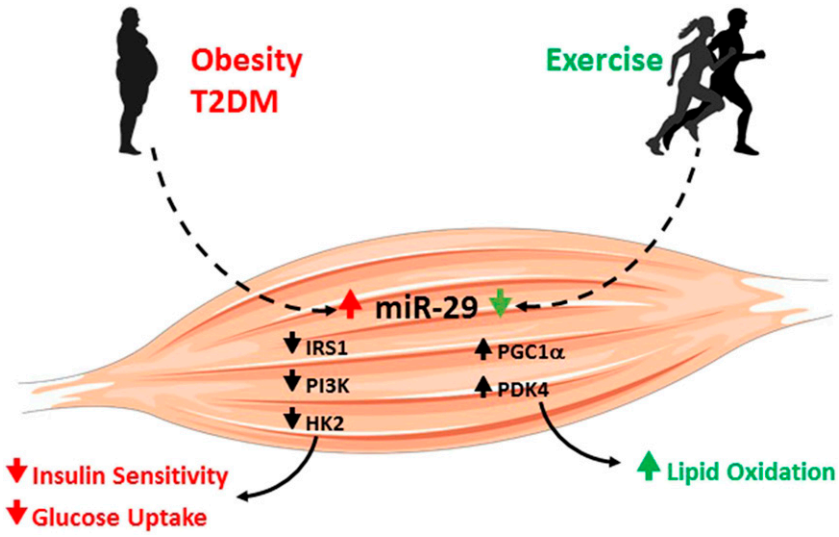

Figure 7—miR-29 expression modulates glucose and lipid metabolism in skeletal muscle. T2DM, type 2 diabetes mellitus.

and PGC1α. Upregulation of miR-29 leads to metabolic defects associated with type 2 diabetes, including insulin resistance, decreased glucose uptake, and impaired fatty acid oxidation. Conversely, downregulation of miR-29 following exercise may promote oxidative phosphorylation. In conclusion, miR-29a and miR-29c are important modulators of insulin action and oxidative capacity in skeletal muscle.

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Author Contributions. J.M. and R.J.O.S. analyzed and interpreted data. J.M., J.R.Z., and A.K. designed the study and wrote the manuscript. J.M., R.J.O.S., L.S.L., J.M.M., and N.F. performed experiments. D.J.O. and B.E. enrolled patients and collected data. All authors approved the manuscript. J.M., J.R.Z., and A.K. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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