

The microRNA Signature in Response to Insulin Reveals Its Implication in the Transcriptional Action of Insulin in Human Skeletal Muscle and the Role of a Sterol Regulatory Element–Binding Protein-1c/Myocyte Enhancer Factor 2C Pathway

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OBJECTIVE—Factors governing microRNA expressions in response to changes of cellular environment are still largely unknown. Our aim was to determine whether insulin, the major hormone controlling whole-body energy homeostasis, is involved in the regulation of microRNA expressions in human skeletal muscle.

RESEARCH DESIGN AND METHODS—We carried out comparative microRNA (miRNA) expression profiles in human skeletal muscle biopsies before and after a 3-h euglycemic-hyperinsulinemic clamp, with TaqMan low-density arrays. Then, using DNA microarrays, we determined the response to insulin of the miRNA putative target genes in order to determine their role in the transcriptional action of insulin. We further characterized the mechanism of action of insulin on two representative miRNAs, *miR-1* and *miR-133a*, in human muscle cells.

RESULTS—Insulin downregulated the expressions of 39 distinct miRNAs in human skeletal muscle. Their potential target mRNAs coded for proteins that were mainly involved in insulin signaling and ubiquitination-mediated proteolysis. Bioinformatic analysis suggested that combinations of different downregulated miRNAs worked in concert to regulate gene expressions in response to insulin. We further demonstrated that sterol regulatory element–binding protein (SREBP)-1c and myocyte enhancer factor 2C were involved in the effect of insulin on *miR-1* and *miR-133a* expression. Interestingly, we found an impaired regulation of miRNAs by insulin in the skeletal muscle of type 2 diabetic patients, likely as consequences of altered SREBP-1c activation.

CONCLUSIONS—This work demonstrates a new role of insulin in the regulation of miRNAs in human skeletal muscle and suggests a possible implication of these new modulators in insulin resistance. *Diabetes* 58:2555–2564, 2009

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Insulin is one of the major hormones involved in the control of energy expenditure and carbohydrate, lipid, and protein metabolism. It also regulates a variety of biological processes such as protein turnover, cell growth and differentiation, and DNA synthesis. To perform these actions in a concerted manner, insulin coordinates a complex program of transcriptional changes (1,2) in the human skeletal muscle.

Recently, our understanding of complex gene regulatory networks governing cell physiology has rapidly evolved with the discovery of microRNAs (miRNAs). They represent a large class of evolutionary conserved RNAs of 21–22 nt, which act as negative regulators of gene expression either by inhibiting mRNA translation or promoting mRNA degradation through base pairing to the 3' untranslated region (UTR) of target mRNAs (3). These small noncoding RNAs are transcribed by RNA polymerase II—producing long primary transcripts (pri-miRNAs), which are then processed by Drosha and Pasha (also DGCR8), which result in a stem-loop precursor called premiRNA. The pre-miRNA is subsequently transported from the nucleus to the cytoplasm by exportin-5 (XPO5). Then, DICER1 (RNase III endonuclease) processes the stem-loop to produce a 21-bp RNA duplex. One strand of the duplex, the miRNA, enters the RNA-induced silencing complex (RISC) and directs the complex to target mRNA (4). In agreement with their important regulatory functions, global effects of miRNAs upon the transcriptional profile and tissue specificity of mRNA expression have been reported in response to experimental manipulations of miRNA levels (5–7), and the latest estimate indicates that they may regulate up to one-third of the mammalian genome (8).

Important roles for these miRNAs have emerged in the control of metabolic pathways, as suggested by studies implicating miRNAs in the regulation of fat metabolism, adipocyte differentiation, energy homeostasis, and glucose-stimulated insulin secretion (9).

Skeletal muscle is one of the largest tissues in the human body and is the major site for insulin-dependent glucose disposal. Changes in mRNA and protein abundance in this tissue are central to a large number of metabolic and other disorders, including insulin resistance. The discovery that some miRNAs are expressed specifically in skeletal muscle raises the question of their

potential involvement in the transcriptional action of insulin in this tissue (10). Here, we report that insulin regulates miRNA expressions in the human skeletal muscle in vivo, which in return participate in insulin transcriptional action in this tissue. We also demonstrated that *miR-1* and *miR-133a*, specifically expressed in muscle tissues (11–13), are regulated by insulin via sterol regulatory element-binding protein (SREBP)-1c and myocyte enhancer factor 2C (MEF2C). Furthermore, we found that *miR-1* and *miR-133a* have impaired insulin response in the skeletal muscle of type 2 diabetic patients.

RESEARCH DESIGN AND METHODS

Euglycemic-hyperinsulinemic clamps. Volunteers provided written consent, and protocols were approved by an ethics committee (Hospices Civils, Lyon, France). Fifteen subjects without history of diabetes were submitted to a 3-h euglycemic-hyperinsulinemic clamp to achieve supraphysiological plasma insulin concentrations (insulin infusion rate of $2 \text{ mU} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$). Five subjects among 15 were selected based on their ages (51 ± 2 years). Their response to insulin infusion was compared with the response of five insulin-resistant type 2 diabetic patients (aged 50 ± 3 years) who had interrupted their usual treatment with oral antidiabetes agents at least 5 days before investigations. Metabolic parameters are presented in the online appendix in Table S1 (available at <http://diabetes.diabetesjournals.org/cgi/content/full/db09-0165/DC1>). Percutaneous biopsies of the vastus lateralis muscle were performed under basal conditions and after the clamp study.

Hyperglycemic-euinsulinemic clamp. Seven healthy men were submitted to a 3-h hyperglycemic-euinsulinemic clamp with infusion of somatostatin to inhibit endogenous insulin release. Muscle biopsies of vastus lateralis muscles were performed under basal conditions and after the clamp study as described previously (14).

Streptozotocin-induced diabetic mice. Three groups of 10-week-old C57Bl/6 males were used ($n = 6$). The control group was given daily intraperitoneal injections of sodium citrate buffer for 3 days. The remaining two groups were made diabetic by daily intraperitoneal injection of streptozotocin (STZ) for 3 consecutive days. Glucose levels were monitored daily, and mice were studied when they achieved fed blood sugars $>500 \text{ mg/dl}$ for at least 3 days. One group was further treated with two injections of insulin (3 mU Insulatard; Novo Nordisk). Twenty-four hours after the first injection of insulin, all animals were killed and gastrocnemius muscles were removed.

miRNA expression profiles in human muscle cells or skeletal muscle biopsies. Differentiated myotubes were prepared from three different skeletal muscle biopsies from three healthy volunteers (15). We quantified the expression of 365 human miRNAs in myoblasts and in differentiated myotubes, or in skeletal muscle biopsies, by using the TaqMan low-density arrays with the Applied Biosystems 7900HT fast real-time PCR system.

Quantification of mature miRNAs. Mature human or mouse miRNA expressions were quantified by using the TaqMan miRNA assays according to the manufacturer's instructions. To account for possible differences in the amount of starting RNA, all samples were normalized to RNU48.

Quantitative real-time PCR. Primers are given in supplementary Table S2. Quantitative RT-PCR data were computed as fold using geometric means and normalized to the mean value of the control sample in each paradigm, defined as one. All experiments were performed in triplicate, and comparisons were analyzed using Student's *t* test. Data are expressed as means \pm SE. Significance was defined as $P < 0.05$.

Microarray analysis. cDNA microarrays are from The Stanford Functional Genomics Facility (<http://www.microarray.org/sfgf/>). The dataset is available from GEO database (GSE 11868). The 6 insulin-sensitive subjects used for microarray analysis were included in the group of 15 (supplementary Table S1). Signal intensities were log transformed, and normalization was performed by Lowess method. Only spots with recorded data on the six slides were selected for analysis. With these criteria, 26,108 spots were retrieved. Among them, 11,864 had a gene symbol and were considered in this study as the list of genes expressed in human muscle. Genes with fold changes ≥ 1.19 were considered as significantly regulated (i.e., corresponding to the 95th percentile of genes based on the magnitude of the fold changes), which represented 1,681 genes (944 upregulated and 737 downregulated). Correction for multiple testing was performed using the Benjamini and Hochberg procedure.

Modulation of SREBP-1c mRNA levels in primary cultures of human muscle cells. Differentiated myotubes were infected for 48 h with adenoviruses expressing either green fluorescent protein (control) or nuclear SREBP-1c, as described previously (15). To knockdown SREBP-1, differentiated

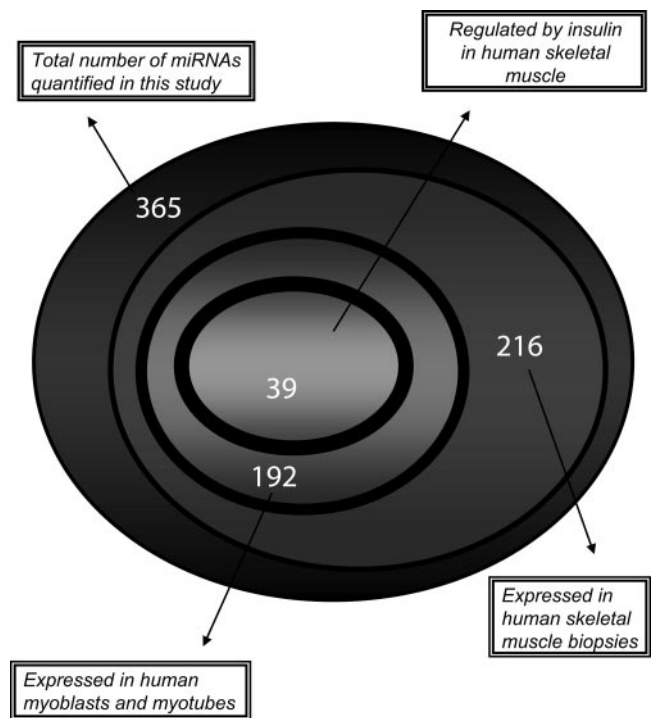


FIG. 1. Determination of miRNAs specifically expressed in muscle cells and regulated by insulin.

human myotubes were transfected with siRNA against SREBP-1 (Qiagen) for 36 h. Then, they were treated with 100 nmol/l insulin (Sigma Aldrich) for 5 h. **Chromatin immunoprecipitation.** Differentiated C2C12 cell lines were infected for 48 h with recombinant adenoviruses. Protein-DNA complexes were fixed for 5 min with 1% formaldehyde and proceed according to Active Motif's ChIP-IT Express Kit (Active Motif Europe) with MEF2C antibodies (E-17, sc-13266; Santa Cruz).

RESULTS

miRNAs are regulated by insulin in human skeletal muscle. To identify miRNAs regulated by insulin in human skeletal muscle, we carried out comparative miRNA expression profiling in biopsies from healthy subjects, before and after a 3-h euglycemic-hyperinsulinemic clamp, by using TaqMan low-density arrays combined with multiplex RT-PCR to quantify 365 mature human miRNAs from the miRBase database (16). A subset of 216 human miRNAs was found to be expressed in skeletal muscle biopsies (Fig. 1). Since miRNAs are expressed in a tissue-specific manner (10), we identified those specifically expressed in human muscle by using primary cultures of myoblasts and in vitro-differentiated myotubes prepared from human skeletal muscle biopsies. Among 216 miRNAs expressed in skeletal muscle, 192 were expressed in myoblasts and/or myotubes, confirming their presence in muscle cells (Fig. 1 and supplementary Table S3). Among them, 39 were found downregulated by insulin in skeletal muscle of healthy subjects (none were upregulated) (Fig. 1 and Table 1). They represent two different classes of miRNAs; 39% are synthesized from primary transcripts located in the noncoding regions (i.e., intergenic miRNAs) and 61% are expressed from introns of protein-coding transcripts (i.e., intronic miRNAs). Although they have two different modes of transcription (17,18), their coordinated downregulation during hyperinsulinemia indicated that they could share common features for regulation in response to insulin. Of note, seven other miRNAs were also regulated

TABLE 1

miRNAs, expressed in human muscle cells, regulated in vivo by insulin in human skeletal muscle, during a 3-h hyperinsulinemic-euglycemic clamp

miRNA names (miRBase version 9)	new miRNA names*	Subject 1	Subject 2	Subject 3	Means \pm SE	Host genes	Genome context
hsa-miR-95		0.75	0.81	0.6	0.72 \pm 0.06	ABLIM2	Intron Sense
hsa-miR-324-3p		0.54	0.61	0.91	0.69 \pm 0.11	ACADVL	Intron Antisens
hsa-miR-324-5p		0.19	0.44	0.41	0.35 \pm 0.08	ACADVL	Intron Antisens
hsa-miR-24		0.64	0.91	0.68	0.74 \pm 0.08	C9orf3 (24-1) and intergenic (24-2)	Intron Sense
hsa-miR-27b		0.28	0.69	0.55	0.51 \pm 0.12	C9orf3	Intron Sense
hsa-miR-30a-3p	hsa-miR-30a*	0.83	0.84	0.64	0.77 \pm 0.07	C6orf155	Intron Sense
hsa-miR-30a-5p		0.32	0.62	0.65	0.53 \pm 0.11	C6orf155	Intron Sense
hsa-miR-30c		0.6	0.84	0.66	0.7 \pm 0.07	NFYC (30c-1) and C6orf155 (30c-2)	Introns Sense
hsa-miR-423	hsa-miR-423-3p	0.62	0.84	0.78	0.75 \pm 0.07	CCDC55	Intron Sense
hsa-miR-532	hsa-miR-532-5p	0.66	0.91	0.8	0.79 \pm 0.07	CLCN5	Intron Sense
hsa-miR-660		0.5	0.49	0.83	0.61 \pm 0.11	CLCN5	Intron Sense
hsa-miR-152		0.5	0.68	0.7	0.63 \pm 0.06	COPZ2	Intron Sense
hsa-miR-26b		0.6	0.74	0.94	0.76 \pm 0.1	CTDSP1	Intron Sense
hsa-miR-26a		0.52	0.78	0.89	0.73 \pm 0.11	CTDSP1 (26a-1) and CTDSP2 (26a-2)	Introns Sense
hsa-miR-616	hsa-miR-616*	0.34	0.8	0.73	0.62 \pm 0.14	DDIT3	Intron Sense
hsa-miR-126		0.56	0.93	0.86	0.78 \pm 0.11	EGFL7	Intron Sense
hsa-miR-330	hsa-miR-330-3p	0.92	0.51	0.82	0.75 \pm 0.12	EML2	Intron Sense
hsa-miR-181b		0.59	0.89	0.96	0.81 \pm 0.11	novel transcripts	Introns Sense
hsa-miR-615	hsa-miR-615-3p	0.18	0.77	0.82	0.59 \pm 0.21	HOXC4	Intron Sense
hsa-miR-491	hsa-miR-491-5p	0.37	0.85	0.96	0.73 \pm 0.18	KIAA1797	Intron Sense
hsa-miR-1		0.59	0.89	0.53	0.67 \pm 0.11	MIB1 (1-2) and C20orf166 (1-1)	Introns Sense or antisens
hsa-miR-133a		0.27	0.75	0.59	0.54 \pm 0.14	MIB1 (133a-1) and C20orf166 (133a-2)	Introns Sense or antisens
hsa-miR-30e-3p	hsa-miR-30e*	0.73	0.81	0.58	0.71 \pm 0.07	NFYC	Intron Sense
hsa-miR-107		0.49	0.7	0.77	0.65 \pm 0.08	PANK1	Intron Sense
hsa-miR-29c		0.3	0.64	0.44	0.46 \pm 0.10	Intergenic	
hsa-miR-29a		0.28	0.65	0.38	0.44 \pm 0.11	Intergenic	
hsa-miR-30b		0.62	0.7	0.74	0.69 \pm 0.04	Intergenic	
hsa-miR-30d		0.54	0.74	0.66	0.64 \pm 0.06	Intergenic	
hsa-miR-130a		0.35	0.88	0.84	0.69 \pm 0.17	Intergenic	
hsa-miR-210		0.76	0.57	0.31	0.54 \pm 0.13	Intergenic	
hsa-miR-376a		0.11	0.1	0.22	0.14 \pm 0.04	Intergenic	
hsa-miR-432		0.91	0.45	0.72	0.69 \pm 0.13	Intergenic	
hsa-miR-125a	hsa-miR-125a-5p	0.64	0.89	0.92	0.82 \pm 0.09	Intergenic	
hsa-miR-181d		0.74	0.52	0.89	0.72 \pm 0.11	Intergenic	
hsa-miR-27a		0.28	0.4	0.75	0.48 \pm 0.14	Intergenic	
hsa-miR-296	hsa-miR-296-5p	0.44	0.9	0.4	0.58 \pm 0.16	Intergenic	
hsa-miR-206		0.8	0.63	0.34	0.59 \pm 0.13	Intergenic	
hsa-miR-125b		0.42	0.84	0.86	0.71 \pm 0.14	Intergenic	
hsa-miR-193a	hsa-miR-193a-3p	0.68	0.74	0.78	0.73 \pm 0.03	Intergenic	

*miRNA identifications are from <http://microrna.sanger.ac.uk/>.

by insulin in muscle samples (Table 2), but as they were not detected in muscle cells, we assumed that they arose from other cell types. The subset of miRNAs downregulated by insulin included muscle-specific miRNAs (i.e., *miR-1*, *miR-133a*, and *miR-206*) but also miRNAs broadly expressed in other tissues including insulin-sensitive tissues (i.e., adipose tissue and liver) (10).

To further characterize the mechanism involved in the downregulation of these miRNAs by insulin, we investigated the effects of insulin on the expression of key components of the miRNA synthesis machinery. We found a significant upregulation of the nuclear protein DROSHA mRNA ($P < 0.01$) and the cytoplasmic protein DICER1

($P < 0.05$) (Fig. 2), whereas expressions of DGCR8 and XPO5 were unchanged. Regarding the expression of the Argonaute proteins, components of the RISC complex, mRNA levels of all except one (EIF2C1) were significantly increased by insulin. Together, these data indicated that insulin positively modulated a number of specific actors of the miRNA synthesis machinery.

Insulin regulates *miR-1* and *miR-133a* at the transcriptional level. As we found that insulin induced a downregulation of 39 miRNAs, while at the same time expressions of genes involved in miRNA synthesis were upregulated, we postulated that insulin might directly affect miRNA levels either by regulating pri-*miR* transcrip-

TABLE 2

Other miRNAs regulated in vivo by insulin in human skeletal muscle, during a 3-h hyperinsulinemic-euglycemic clamp not expressed in human muscle cells

miRNA names (miRBase version 10)*	Subject 1	Subject 2	Subject 3	Means ± SE	Host genes	Genome context
hsa-miR-200c	8.85	1.33	1.37	3.85 ± 2.50	Intergenic	
hsa-miR-517c	0.34	0.48	0.18	0.33 ± 0.09	Intergenic	
hsa-miR-518b	0.39	0.41	0.76	0.52 ± 0.12	Intergenic	
hsa-miR-518c	0.93	0.45	0.27	0.55 ± 0.20	Intergenic	
hsa-miR-96	15.01	15.47	25.83	18.77 ± 3.53	Intergenic	
hsa-miR-575	7.50	15.87	9.11	10.82 ± 2.56	SCD5	Intron Sense
hsa-miR-646	3.00	3.67	2.82	3.16 ± 0.26	Novel transcript	Intron Sense

*miRNA identifications are from <http://microrna.sanger.ac.uk/>.

tion or by inducing their degradation. To clarify these points, we decided to focus on two representative muscle-specific miRNAs, *miR-1* and *miR-133a* (19). Their downregulation by insulin was confirmed by quantitative RT-PCR in muscle samples from a larger group of 15 healthy subjects (Fig. 3A). We also investigated *miR-1* and *miR-133a* regulations in an insulin-deficient mice model obtained by streptozotocin treatment (20). Compared with control mice, STZ-induced diabetic mice showed increased *miR-1* and *miR-133a* levels (Fig. 3B), whereas insulin treatment resulted in the decrease of both miRNAs (Fig. 3B). These data supported the downregulation of *miR-1* and *miR-133a* expression by insulin, observed in human skeletal muscle. However, glucose uptake by skeletal muscle is robustly increased during the hyperinsulinemic clamp, and STZ-induced diabetic mice are also markedly hyperglycemic (20). To verify whether glucose, a strong transcriptional regulator (14), played a role in the observed effects of insulin on miRNA levels, we measured *miR-1* and *miR-133a* expression in the skeletal muscle of seven healthy men during a 3-h hyperglycemic-euinsulinemic clamp, an experimental situation designed to distinguish between the transcriptional effects of glucose and those of insulin (14). Figure 3A showed that hyperglycemia alone, in absence of hyperinsulinemia, did not modify *miR-1* or *miR-133a* levels in human muscle.

Since *miR-1* and *miR-133a* are derived from introns of protein-coding transcripts (i.e., MIB1 and C20orf166), it could be conceivable that the observed downregulation of *miR-1* and *miR-133a* by insulin might be a consequence of the downregulation of their host genes. Figure 4 indicated

that two host genes were in fact upregulated during insulin infusion with a significant effect for MIB1 ($P < 0.05$) in muscle samples. Therefore, regulations of MIB1 and C20orf166 in response to insulin were unlikely to explain the decreased levels of *miR-1* and *miR-133a*.

Another potent mechanism controlling miRNA expression is the editing of their precursors by adenosine deaminases acting on RNAs, which can inhibit their processing and thus can decrease mature miRNA levels (21). This mechanism has been well described for *miR-376a* (21) and included in the list of 39 downregulated miRNAs by insulin (Table 1). We therefore verified whether primary transcripts of *miR-1* and *miR-133a* were edited in response to insulin by sequencing pri-*miRNA-1-1*, pri-*miRNA-1-2*, pri-*miRNA-133a-1*, and pri-*miRNA-133a-2* cDNAs in muscle samples from three healthy subjects before and after hyperinsulinemia. Sequences were identical before and after insulin infusion (data not shown), indicating that the decreased levels of mature *miR-1* and *miR-133a* were not related to RNA editing.

Decreased expressions of *miR-1/miR-133a* by insulin are correlated with MEF2C downregulation and require SREBP-1c. Previous studies have shown that the bicistronic transcript *miR-1/miR-133a* is regulated by MEF2C, myogenic differentiation 1 (MYOD1), and serum response factor (SRF), which regulate the activity of enhancers located either upstream of *miR-1/133a* locus or between the *miR-1* and *miR-133a* coding regions (11,13,22). To determine whether these transcription factors could be involved in the action of insulin, we quantified their expression in human muscle in response to insulin. Results indicated that MEF2C mRNA level was significantly reduced after insulin infusion ($P < 0.05$, $n = 15$) (Fig. 5A), whereas expressions of MYOD1 and SRF were slightly increased or unchanged. Thus, one could postulate that reduction of MEF2C expression could contribute to the downregulation of *miR-1* and *miR-133a*.

Recently, we have found that MEF2C was downregulated by SREBP-1c (15), a key mediator of insulin transcriptional action in human muscle (23,24). This observation suggested that insulin could downregulate MEF2C and subsequently *miR-1* and *miR-133a* expressions through activation of SREBP-1c. Figure 5B demonstrated that overexpression of SREBP-1c in human myotubes robustly downregulates MEF2C mRNA levels. Furthermore, overexpression of SREBP-1c was also associated with a significant reduction of *miR-1* and *miR-133a* expressions in myotubes (Fig. 5C). To determine whether this reduction was a consequence of the downregulation of MEF2C, we performed chromatin immunoprecipitation

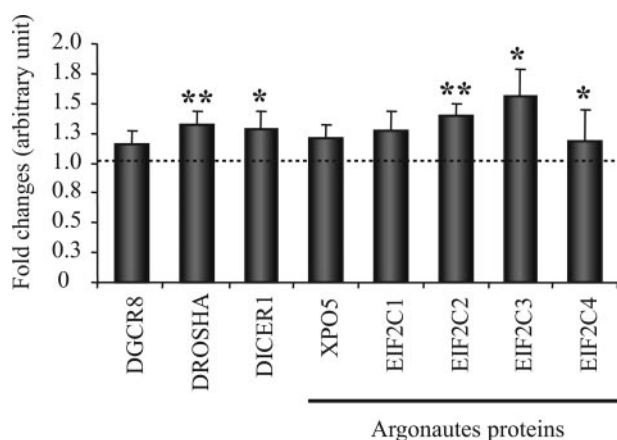


FIG. 2. Transcript levels of components of the miRNA biogenesis pathway before and after insulin infusion in 15 healthy subjects, determined by qRT-PCR. * $P < 0.05$ and ** $P < 0.01$ vs. control, respectively.

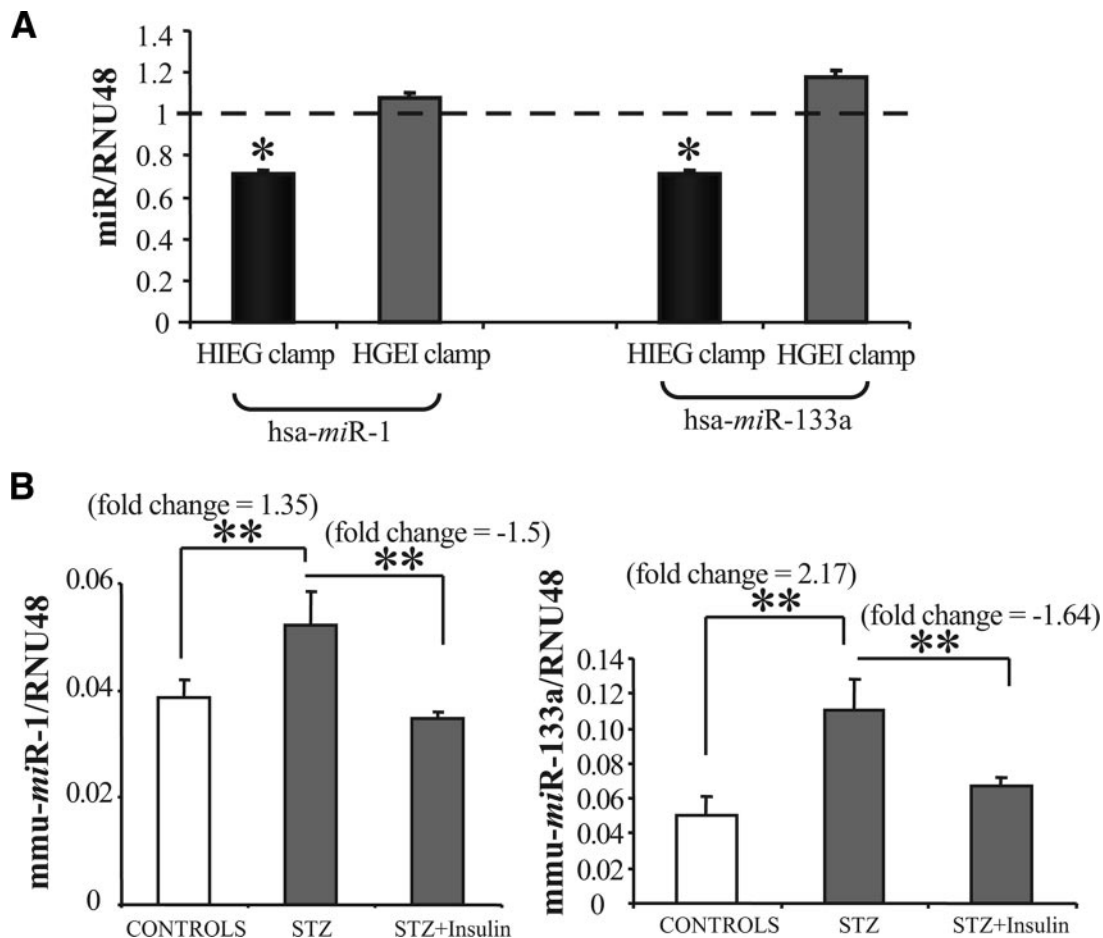


FIG. 3. A: Regulation of *miR-1* and *miR-133a* during a hyperinsulinemic-euglycemic clamp (HIEG, ■) or during a hyperglycemic-euinsulinemic clamp (HGEI, □) in human skeletal muscle. * $P < 0.05$ vs. before clamp. **B:** *miR-1* and *miR-133a* expressions in skeletal muscle of control, STZ-induced diabetic, and insulin-treated STZ mice. ** $P < 0.01$ vs. control mice.

assay by using specific antibodies against MEF2C combined with PCR amplification of the MEF2C-binding site in the enhancer region located between *pri-miRNA-1-2* and *pri-miRNA-133a-1* (22). Figure 5D shows that after 48 h of SREBP-1c overexpression in muscle cells, it was not possible to detect by PCR MEF2C binding on this enhancer. This result confirmed that SREBP-1c mediated *miR-1* and *miR-133a* downregulation through the downregulation of MEF2C in muscle cells. Finally, to demonstrate that downregulations of *miR-1* and *miR-133a* by insulin were mediated by SREBP-1c, we knocked down SREBP-1 expression in human myotubes before stimulation by insulin and quantified the expressions of *miR-1* and

miR-133a. Figure 5E showed that downregulations of *miR-1* and *miR-133a* by insulin observed in muscle cells transfected with green fluorescent protein siRNA (control) was not detectable in cells transfected with SREBP-1 siRNA, confirming that insulin regulation of these two miRNAs was mediated by SREBP-1.

To determine whether the mechanism involving SREBP-1c/MEF2C in the regulation of *miR-1*/*miR-133a* expressions could be generalized to the downregulatory effect of insulin on the set of miRNAs observed in the muscle of healthy individuals, we quantified the expression of five of them in muscle cells overexpressing SREBP-1c. As shown in the supplementary Fig. S4, *miR-107* and *miR-95* displayed increased expressions, which is the opposite of the marked downregulation observed during the clamp. These results indicated that other cellular pathways than SREBP-1c/MEF2C are likely involved in the regulation of some of the 39 miRNAs in response to insulin.

Regulations of *miR-1* and *miR-133a* are altered in skeletal muscle of type 2 diabetic patients. Because impaired insulin stimulation of SREBP-1c has been consistently observed in skeletal muscle of type 2 diabetic patients (25,26), we suspected that regulations of *miR-1* and *miR-133a* by insulin could be altered in the muscle of these patients. Figure 6A showed that basal expressions of *miR-133a* and *miR-1* were not significantly different in skeletal muscle from healthy individuals and insulin-resistant type 2 diabetic patients. However, the effect of insulin

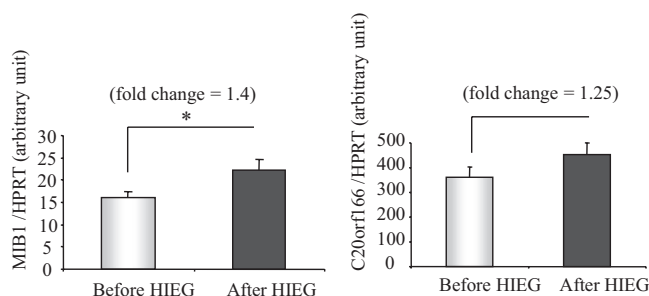


FIG. 4. Quantification by qRT-PCR of *miR-1* and *miR-133a* host genes, MIB1 and C20orf166, during insulin infusion in skeletal muscle ($n = 15$). * $P < 0.05$ vs. before clamp. HIEG, hyperinsulinemic-euglycemic clamp.

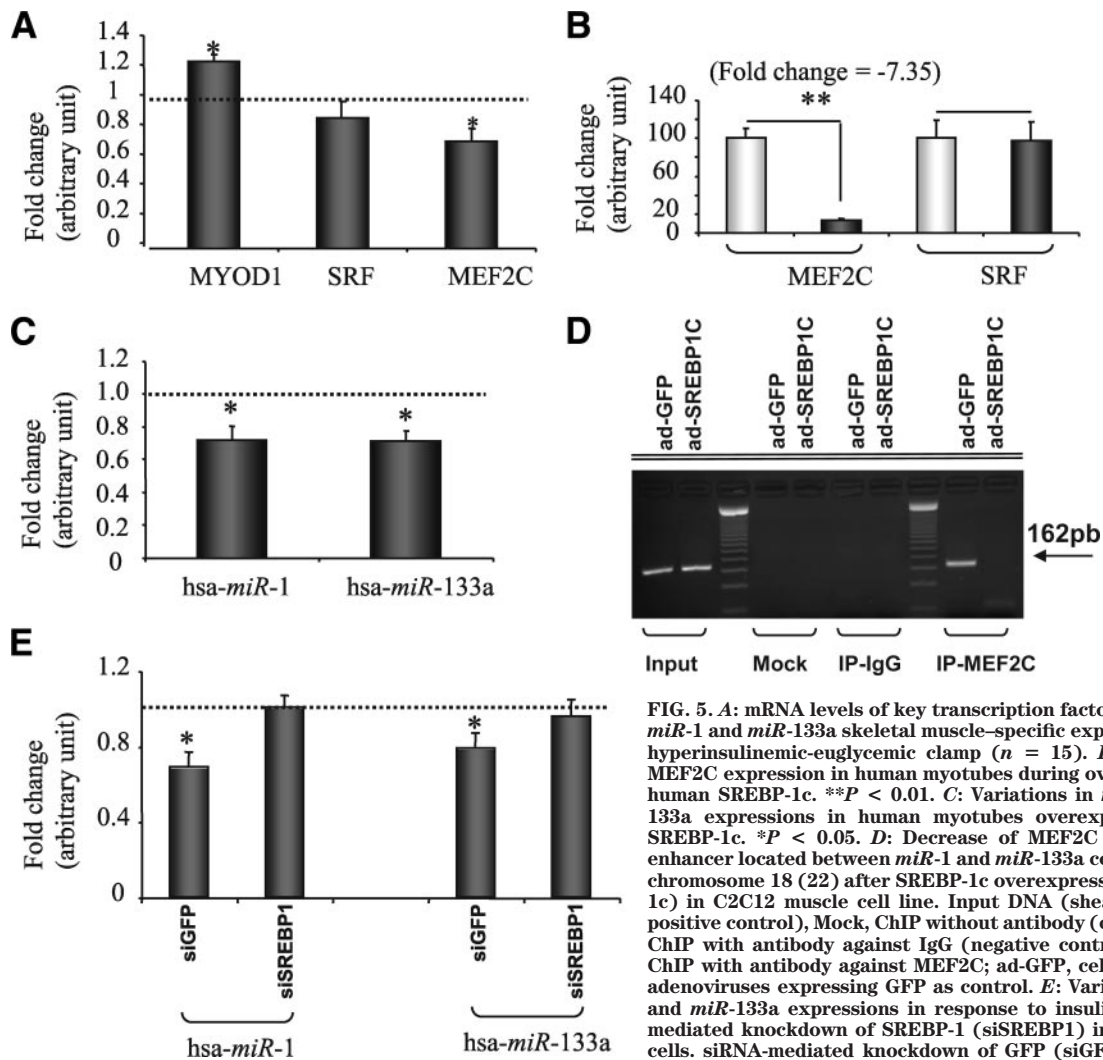


FIG. 5. A: mRNA levels of key transcription factors implicated in *miR-1* and *miR-133a* skeletal muscle-specific expressions, during hyperinsulinemic-euglycemic clamp ($n = 15$). **B:** Variations in MEF2C expression in human myotubes during overexpression of human SREBP-1c. $**P < 0.01$. **C:** Variations in *miR-1* and *miR-133a* expressions in human myotubes overexpressing human SREBP-1c. $*P < 0.05$. **D:** Decrease of MEF2C binding on the enhancer located between *miR-1* and *miR-133a* coding regions on chromosome 18 (22) after SREBP-1c overexpression (ad-SREBP-1c) in C2C12 muscle cell line. Input DNA (sheared chromatin, positive control), Mock, ChIP without antibody (control); IP-IgG, ChIP with antibody against IgG (negative control); IP-MEF2C, ChIP with antibody against MEF2C; ad-GFP, cells infected with adenoviruses expressing GFP as control. **E:** Variations in *miR-1* and *miR-133a* expressions in response to insulin, after siRNA-mediated knockdown of SREBP-1 (siSREBP1) in human muscle cells. siRNA-mediated knockdown of GFP (siGFP) was used as control.

was altered in the skeletal muscle of diabetic patients. The response to insulin was completely abolished for *miR-133a*, and the effect on *miR-1* expression was markedly reduced, showing only a modest decrease that did not reach significance ($P < 0.2$) (Fig. 6A). Concomitantly, the significant reduction in MEF2C expression observed during the clamp in insulin-sensitive individuals was not found in type 2 diabetic patients (Fig. 6B).

Detection of miRNA signals within the set of genes regulated by insulin in human skeletal muscle. Previous microarray analyses have shown that reduction of transcript levels can be observed following miRNA transfection (6,7,22,27). It has been demonstrated that insulin affects the expression level of ~1,000 genes in vivo in human skeletal muscle (1,2). It was therefore important to determine whether insulin-regulated miRNAs could contribute to the network of gene expression induced by insulin in skeletal muscle. To this aim, we first analyzed by cDNA microarrays the global changes in mRNA levels in the muscle of six healthy middle-aged subjects (including samples from the three subjects used for miRNA analysis) in response to insulin. Then we analyzed the 3' UTR region of the upregulated mRNAs, assuming that downregulated miRNAs by insulin would have their target genes upregulated during the clamp. Among 944 upregulated genes, 357 can be predicted as a target of at least one miRNA

downregulated by insulin (predictions using TargetScan version 4.2) (28). Since we observed that several binding sites for distinct miRNAs were present on the same transcript, we analyzed more in detail the distribution of the number of miRNA-binding sites in this set of genes. Figure 7 showed that the proportions of genes upregulated by insulin that can be a target of at least one (or more) distinct miRNA downregulated by insulin were significantly greater than the proportions of upregulated genes that were not targets. Moreover, these proportions increased with the number of distinct miRNA-binding sites in the 3' UTR (Fig. 7). This observation suggested that single miRNA induces moderate changes in expression of their targets in response to insulin, while combinations of different miRNAs can work in concert to exert significant effects on gene expression. This result is in agreement with a previous study (29) showing that the number and arrangement of miRNA recognition sites can influence the degree and specificity of miRNA-mediated gene repression.

To further explore the role of miRNAs in the insulin transcriptional network, we analyzed the functions of the predicted target genes for 39 downregulated miRNAs by using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (30). To take into account that miRNAs act as negative regulators of gene expression also by

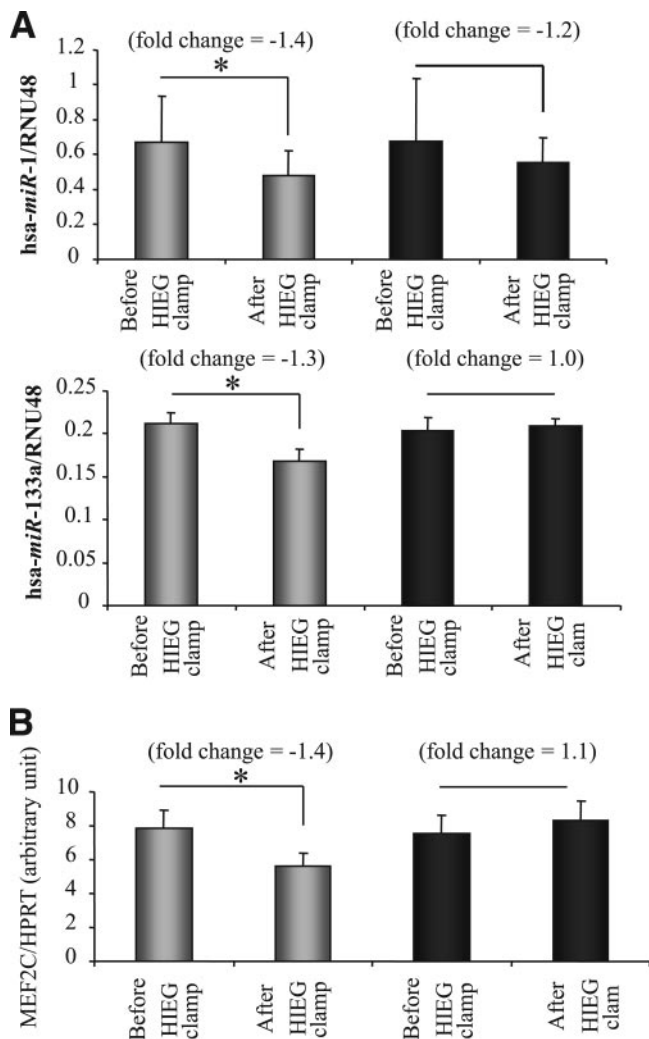


FIG. 6. Regulation of *miR-1*, *miR-133a* (A), and *MEF2C* (B) expression during a 3-h hyperinsulinemic-euglycemic clamp (HIEG) in skeletal muscle of healthy subjects (□) and type 2 diabetic patients (■). * $P < 0.05$ vs. before clamp.

inhibiting mRNA translation, we considered all potential targets including those that were not regulated at the mRNA level. Among 11,863 genes expressed in human skeletal muscle (see RESEARCH DESIGN AND METHODS), 4,481 were the target of at least one regulated miRNA. Among 4,481 potential target genes, 1,279 were recorded at least in one KEGG pathway. Table 3 shows KEGG pathways that are most significant in terms of containing more genes than expected ($P < 0.05$). This analysis revealed that several signaling pathways and ubiquitin-mediated proteolysis are the major pathways potentially affected by 39 insulin-regulated miRNAs.

DISCUSSION

miRNAs form a novel class of regulators that add a new level of regulation and fine-tuning in the control of gene expression. They are important for a wide range of cellular functions, and recent studies have provided evidence that miRNAs affect critical pathways of metabolic control, such as adipocyte and skeletal muscle differentiation, amino acid metabolism, lipid homeostasis, and insulin secretion from pancreatic β -cells (9). However, factors that govern miRNA synthesis and expression in response to changes in

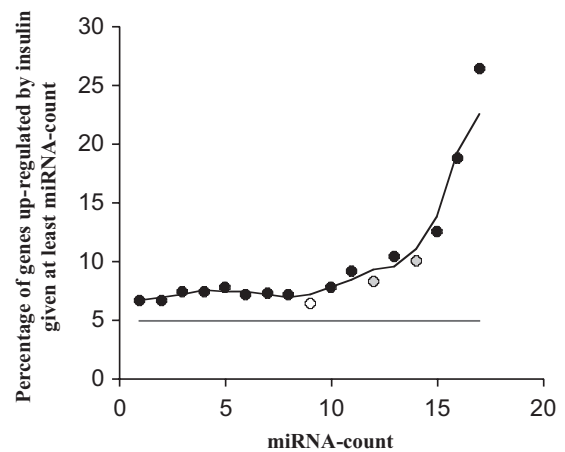


FIG. 7. Global analysis of the relationship between the fold changes of genes upregulated by insulin and the number of distinct miRNA-binding sites in their 3' UTR (predictions from TargetScan 4.2). Genes upregulated by insulin were divided into different subgroups according to the number of miRNA-binding sites in 3' UTR (i.e., 39 miRNAs downregulated by insulin). In each subgroup, we counted the number of upregulated genes to compute the proportion of such genes in the subgroup (e.g., miRNA count = 5, contains genes that are targeted by five or more distinct miRNAs). Subgroups in which the proportion of upregulated genes is significantly higher than when not the target are in black (adjusted $P < 0.05$) or in gray (adjusted $P < 0.10$). The absence of significant difference (white) is due to the small size of the subgroup for miRNA count = 9. The black curve was obtained from simple moving average over three data points, and the black line indicates the percentage of genes upregulated by insulin that are not targeted by miRNAs (~5%).

the cellular environment are still largely unknown. In this study, we demonstrate for the first time that insulin, the major hormone controlling whole-body energy homeostasis and metabolism, is involved in the regulation of miRNA expression in human skeletal muscle, providing evidence that regulation of miRNA expressions is a novel pathway of insulin action *in vivo*. The fact that this regulation is also observed in the murine model and that most of the regulated miRNAs are expressed in other insulin-sensitive tissues suggests that insulin action on miRNAs is not restricted to our experimental situation and occurs more generally in other tissues. Interestingly, 12 of 39 insulin downregulated miRNAs have been previously found downregulated in primary muscular disorders (31) (i.e., *miR-133a*, *miR-181d*, *miR-423*, *miR-30c*, *miR-95*, *miR-30b/d*, *miR-206*, *miR-30a-5p*, *miR-26a*, and *miR-29a/c*), suggesting an important role of these miRNAs in specific physiological pathways in skeletal muscle.

Although hundreds of miRNAs have been cloned in various species, molecular actors involved in *pri-miRNA* transcription are largely unknown. Recently, it has been demonstrated that hepatocyte nuclear factor-1 α induces *miR-194* expression during intestinal epithelial cell differentiation (32), that p53 transactivates *miR-34a* in pancreatic cells (33), that high mobility group at-hook 1 (HMGA1) downregulates five different miRNAs in mouse embryonic fibroblasts (34), and that two different MEF2-dependent enhancers regulated *miR-1* and *miR-133a* expressions during muscle differentiation (11,13,22). One important result of this study is the demonstration that SREBP-1c and MEF2C contribute to insulin action, at least on *miR-1* and *miR-133a*, which were studied as representative miRNAs. The transcription factor SREBP-1 is mainly involved in cholesterol and fatty acid metabolism (35). Recently, we have published a microarray analysis of human muscle

TABLE 3

KEGG pathways potentially affected by the 39 insulin-regulated miRNAs during a 3-h hyperinsulinemic-euglycemic clamp in human skeletal muscle

KEGG pathways	KEGG annotations	Number of genes annotated on microarray	Number of targeted gene (six or more miRNAs)*	P (Fisher test)†	Adjusted P (Benjamini)
MAPK signaling pathway	ko04010	352	42	0.000392	0.00503
VEGF signaling pathway	ko04370	93	16	0.000527	0.00503
Calcium signaling pathway	ko04020	187	26	0.000613	0.00503
Ubiquitin-mediated proteolysis	ko04120	206	27	0.001161	0.007934
mTOR signaling pathway	ko04150	90	13	0.009689	0.039725
Wnt signaling pathway	ko04310	255	28	0.01207	0.044987
Jak-STAT signaling pathway	ko04630	179	21	0.015888	0.054284

The 11,864 genes retrieved from microarray data analysis were classified into KEGG categories. We determined significant associations between the set of genes collectively targeted by the 39 insulin-downregulated miRNAs and specific KEGG pathways. *We considered genes targeted by at least six different miRNAs, which corresponded to percentile 95th of the distribution of predicted miRNA target sites in 3' UTR, for the 11,864 expressed in human muscle, when using TargetScan 4.2 (conserved site across human, mouse, rat, and dog). †Enrichment was significant for $P \leq 0.05$.

cells overexpressing SREBP-1c and revealed several hundred potential new target genes of this transcription factor, including MEF2C. Here, we found that both insulin in vivo in skeletal muscle and SREBP-1c overexpression in

muscle cells induced the coordinated downregulation of MEF2C, *miR-1*, and *miR-133a* expressions. Thus, these data led us to propose the following mechanism (Fig. 8): Insulin activates the translocation of the SREBP-1c active

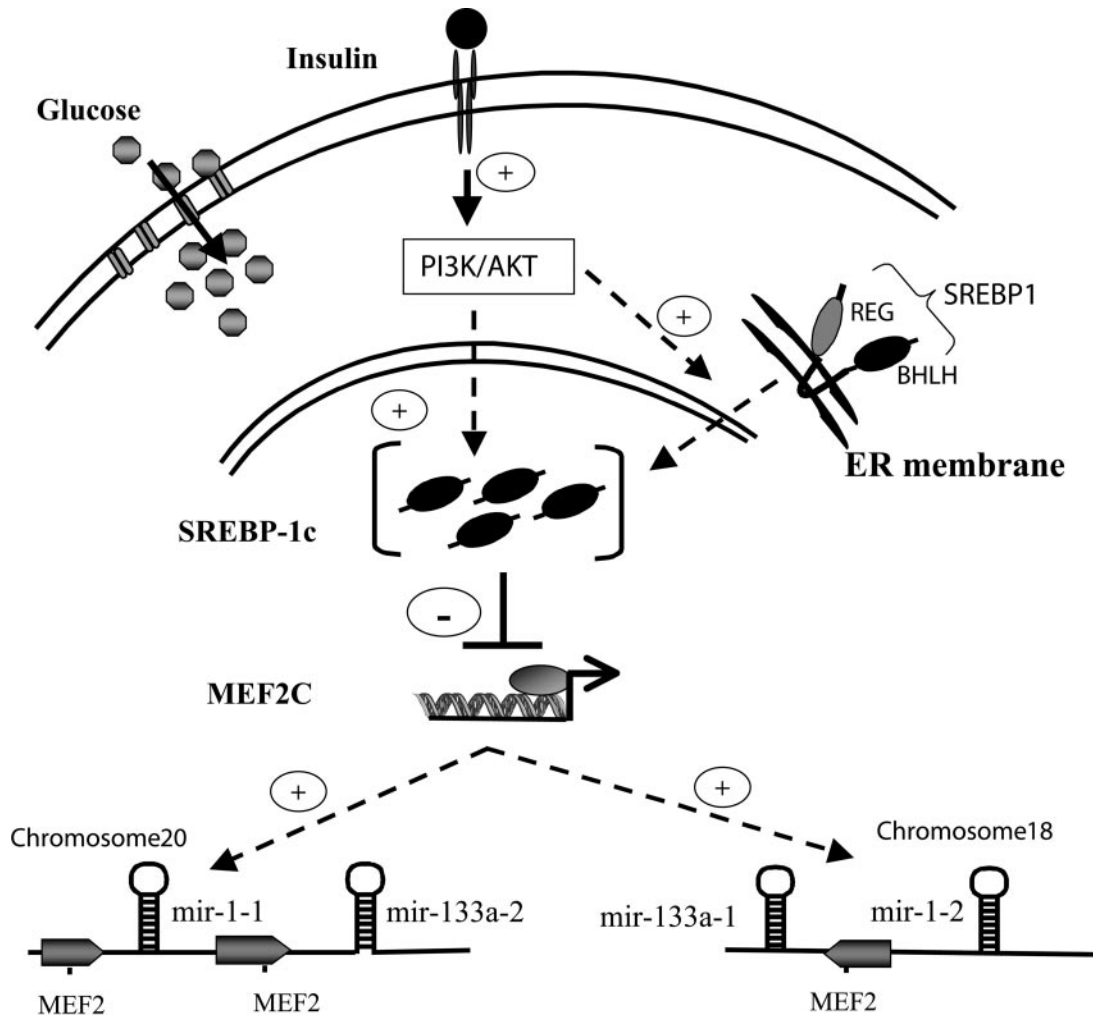


FIG. 8. Proposed mechanism to explain the downregulation of *miR-1* and *miR-133a* by insulin in human skeletal muscle. Insulin activates the translocation of SREBP-1c (BHLH) active form from the endoplasmic reticulum (ER) to the nucleus and, concomitantly, induces SREBP-1c expression via the phosphatidylinositol (PI) 3-kinase (PI3K) signaling pathway (36). SREBP-1c mediates MEF2C downregulation (15) through a mechanism that remains to be determined. As a consequence of lower MEF2C binding on their enhancer region, the transcription of *miR-1* and *miR-133a* is reduced, leading to decreased levels of their mature forms in muscle, after insulin treatment. Altered activation of PI 3-kinase and SREBP-1c may explain the defective regulation of *miR-1* and *miR-133a* expression in response to insulin in muscle of type 2 diabetic patients (25).

form to the nucleus and also the induction of its own expression via the phosphoinositide 3-kinase signaling pathway (36). Then SREBP-1c induces MEF2C downregulation and reduction of its binding to the pri-miRNA-1-2 and pri-miRNA-133a-1 enhancer region. This leads to the reduction of *miR-1* and *miR-133a* transcription and thus to the decreased levels of their mature forms. Testing this mechanism on five other miRNAs than *miR-1* and *miR-133a*, we found that two of them (*miR-107* and *miR-95*) were actually upregulated in muscle cells overexpressing SREBP-1c. This observation strongly suggests that other mechanisms than the pathway involving SREBP-1c/MEF2C are likely involved in the effects of insulin on miRNA levels. Particularly, we cannot rule out the fact that the decrease in miRNA concentrations may reflect possible regulations of the miRNA-processing machinery mediated by the miRNAs themselves. The insulin-induced decrease in miRNA levels should thus be followed by increased levels of target mRNAs, including those coding for the proteins involved in their processing. In addition, regulation of miRNA level during clamp could also be the results of changes in the protein level or activity of components of the miRNA processing machinery. Unfortunately, the small size of the muscle biopsies did not allow us to obtain enough material to verify these hypotheses in human samples. Further studies are now required to identify these alternative mechanisms.

One important result, which also supports the above-proposed mechanism of insulin action on miRNA expressions, is the observation that *miR-1/miR-133a* regulations are altered in the muscle of type 2 diabetic patients during the hyperinsulinemic clamp. Defective regulation of gene expressions in response to insulin in peripheral tissues of insulin-resistant type 2 diabetic patients has been reported, including a marked alteration in the induction of SREBP-1c expression (25,26). The present study demonstrates that one of the unexpected consequences of this defect is a marked alteration in the regulation of specific miRNAs. Therefore, our data could also support the implication of miRNAs in the pathophysiology of type 2 diabetes and insulin resistance in humans. Interestingly, in a murine model of diabetes, 15 miRNAs have been found differentially expressed in skeletal muscle when compared with control rats (37). Two of 15 are present in the list of insulin-regulated miRNAs (Table 1). Furthermore, overexpression of *miR-29* has been shown to reduce insulin action on protein kinase B/AKT activation in adipocytes (37). Interestingly, *miR-29a* and *miR-29c* are among the most regulated miRNAs in response to insulin in our study. Taken together, these data strongly suggest that some miRNAs regulated by insulin might be implicated in insulin resistance.

Bioinformatic analysis showed that target genes of 39 miRNAs downregulated by insulin are mainly involved in signaling pathways and in ubiquitination-mediated proteolysis. Previous data (1) have suggested that the ubiquitin-proteasome system is an important component of insulin action. Regarding intracellular signaling pathways, our observation confirmed previous *in silico* studies indicating that miRNAs significantly target proteins involved in signal transduction (38,39). In agreement, a role for specific miRNAs has been described in the regulation of insulin receptor substrate-1 (40) and phosphatase and tensin homolog (41) proteins. Here, our data suggested that p85 α phosphoinositide 3-kinase, a key mediator of insulin signaling is a potential target for six different miRNAs down-

regulated by insulin (i.e., *miR-376a*, *miR-107*, *miR-30a-3p*, *miR-30e-3p*, *miR-29a*, and *miR-29c*) (supplementary Table S5).

This study reveals the contribution of miRNAs in the transcriptional action of insulin in human skeletal muscle. Although further studies are now required to understand the biological impact of miRNAs in insulin action, the observed dysregulation in patients with type 2 diabetes may open potential perspectives for the understanding of insulin resistance and development of therapeutic strategies.

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No potential conflicts of interest relevant to this article were reported.

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