

## RESEARCH ARTICLE | *Novel Aspects of Adipocyte Biology*

# Comprehensive functional screening of miRNAs involved in fat cell insulin sensitivity among women

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**Dahlman I, Belarbi Y, Laurencikiene J, Pettersson AM, Arner P, Kulyt  A.** Comprehensive functional screening of miRNAs involved in fat cell insulin sensitivity among women. *Am J Physiol Endocrinol Metab* 312: E482–E494, 2017. First published March 7, 2017; doi:10.1152/ajpendo.00251.2016.—The key pathological link between obesity and type 2 diabetes is insulin resistance, but the molecular mechanisms are not entirely identified. micro-RNAs (miRNA) are dysregulated in obesity and may contribute to insulin resistance. Our objective was to detect and functionally investigate miRNAs linked to insulin sensitivity in human subcutaneous white adipose tissue (scWAT). Subjects were selected based on the insulin-stimulated lipogenesis response of subcutaneous adipocytes. Global miRNA profiling was performed in abdominal scWAT of 18 obese insulin-resistance (OIR), 21 obese insulin-sensitive (OIS), and 9 lean women. miRNAs demonstrating differential expression between OIR and OIS women were overexpressed in human in vitro-differentiated adipocytes followed by assessment of lipogenesis and identification of miRNA targets by measuring mRNA/protein expression and 3'-untranslated region analysis. Eleven miRNAs displayed differential expression between OIR and OIS states. Overexpression of miR-143-3p and miR-652-3p increased insulin-stimulated lipogenesis in human in vitro differentiated adipocytes and directly or indirectly affected several genes/proteins involved in insulin signaling at transcriptional or posttranscriptional levels. Adipose expression of miR-143-3p and miR-652-3p was positively associated with insulin-stimulated lipogenesis in scWAT independent of body mass index. In conclusion, miR-143-3p and miR-652-3p are linked to scWAT insulin resistance independent of obesity and influence insulin-stimulated lipogenesis by interacting at different steps with insulin-signaling pathways.

insulin resistance; white adipose tissue; microribonucleic acid

OBESITY IS A MAJOR contributor to development of insulin resistance (IR), e.g., impaired cellular response to insulin, which constitutes a key pathological link to type 2 diabetes (T2D). Insulin resistance is associated with metabolic disturbances in liver, skeletal muscle, and white adipose tissue (WAT) and is clinically characterized by hyperinsulinemia and dyslipidemia (36). WAT can expand by increasing the number and/or volume of adipocytes causing distinct adipose morphologies termed hyperplasia (many small adipocytes) or hypertrophy (few large adipocytes). Within adipose tissue, hypertrophic fat cells, increased spontaneous lipolysis, and blunted ability of insulin to stimulate fat synthesis through glucose conversion

into lipids (lipogenesis) lead to a flux of free fatty acids in the general circulation, resulting in systemic IR (20, 29).

The key genes in the insulin-signaling pathway are well described (56). However, the complex molecular signals causing obesity-associated IR are not fully understood. Only ~25% of obese women are markedly insulin resistant (24). One explanation is varying genetic predisposition (89). micro-RNAs (miRNAs) comprise an additional layer of gene regulation and play an important role in the regulation of protein-encoding genes. miRNAs are small noncoding RNA molecules that control gene function by binding to the 3'-untranslated region (UTR) of target mRNAs and blocking translation or causing mRNA degradation (8). miRNAs are known to have fine-tuning effects, and expression levels have been described to be dysregulated in various disorders related to the metabolic syndrome as recently reviewed (104). However, regarding a pathophysiological role, only a small number of miRNAs has been confirmed experimentally, even less in human fat cells (4).

Herein we aimed to identify miRNAs linked to IR and delineate their regulatory mechanisms for fat cell insulin sensitivity in obese humans. For this purpose, we applied global profiling of miRNA expression in subcutaneous white adipose tissue (scWAT) from obese insulin-resistance (OIR), obese insulin-sensitive (OIS), and lean women. We further performed a comprehensive functional screening of differentially expressed miRNAs for impact on insulin-stimulated lipogenesis and identified the affected responsive genes within the insulin-signaling and lipogenic pathways in human adipocytes. As far as we know, this is the first study of miRNAs in human adipose tissue in relation to IR of fat cells.

## MATERIALS AND METHODS

### Patients

The subjects were selected from an ongoing study on the genetics of insulin-stimulated lipogenesis in isolated human subcutaneous fat cells (5, 18). The subjects were recruited by local advertising, and all lived in Stockholm, Sweden. The regional board of ethics approved the study, and written informed consent was obtained from all participants. Data from this study are not related to results presented in the previous publications (5, 18). For the present investigation, 419 subjects were assessed [body mass index (BMI) range 18–62 kg/m<sup>2</sup> and age 20–64 yr]. To define insulin sensitivity, we used insulin-stimulated minus basal lipogenesis in isolated fat cells (see below) for all healthy lean subjects ( $n = 71$ ; BMI <25 kg/m<sup>2</sup>). Values above the threshold for the upper 10th percentile were defined as high insulin sensitivity, and values below the threshold for the lower 10th percent

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tile were defined as low sensitivity. To define the insulin resistance measure, we adopted the method of Ferrannini et al. (23). The 10th percentile of lean and healthy subjects was used to define insulin resistance to avoid the influence of confounding factors such as obesity, diabetes mellitus, hypertension, or other conditions or treatments that could by themselves influence insulin action (23).

For this study, we finally selected women from which we had stored frozen abdominal scWAT samples (see below) and were either lean regardless of level of insulin sensitivity ( $n = 9$ ) or obese (BMI 30 kg/m<sup>2</sup> or more) with either high (OIS,  $n = 21$ ) or low (OIR,  $n = 18$ ) insulin sensitivity according to the definition above. The subjects in all groups were matched for age. The two obese groups were matched for BMI, waist circumference, and fat cell size, all of which may influence insulin sensitivity independent of insulin-stimulated lipogenesis. No woman was diagnosed with diabetes. Clinical characteristics are shown in Table 1, which also contains information about medications.

Patients came to the laboratory for clinical examination following an overnight fast. A nurse determined height, weight, and waist circumference. A venous blood sample was obtained for measuring lipids and glucose at the routine clinical chemistry laboratory. Plasma insulin was measured by ELISA (Mercodia, Uppsala, Sweden). Systemic IR was estimated according to the homeostasis model adjustment (HOMA)-IR formula (9). Low-density lipoprotein cholesterol was calculated according to the formula of Friedewald et al. (26). The highest reported phosphotriglycerides were 3 mmol/l. Following the clinical examination, an abdominal subcutaneous fat biopsy was obtained by needle aspiration, as previously described (50). Tissue pieces were rapidly rinsed in saline, and a small part was immediately used for determination of mean fat cell volume and for *ex vivo* quantification of adipocyte lipogenesis (described below). The rest was frozen in liquid nitrogen and kept at  $-70^{\circ}\text{C}$ .

### Cell Models

**Cell isolation from scWAT biopsies.** Mature adipocytes from biopsies were prepared using the collagenase procedure as described (82, 98). Mean fat cell weight and volume were determined as described (65).

**Primary adipocyte cultures.** Primary adipocyte culture for *in vitro* studies was obtained from scWAT from healthy nonobese men and women (BMI <30) undergoing cosmetic liposuction. In this experimental group, there was no selection for age, sex, or BMI. The stroma vascular fraction (SVF) cells were isolated as described (78, 99). The precursor cells obtained from separate individuals were not mixed. Part of plastic-adherent SVF cells was directly plated (30,000–50,000 cells/cm<sup>2</sup>) and differentiated to adipocytes as described (99). The degree of differentiation was controlled under the microscope as accumulation of lipids, and response to insulin in lipogenesis assay was evaluated (see below). Cultures with a differentiation degree below ~80% were not used for the experiments. Remaining SVF cells were suspended in fetal calf serum supplemented with 10% DMSO and stored in liquid nitrogen until further usage. Upon the experiments, the SVF was thawed, washed with inoculation medium, plated, and differentiated as described above.

### Insulin-Stimulated Lipogenesis

***In ex vivo isolated mature adipocytes.*** Lipogenesis in mature adipocytes obtained from biopsies and isolated from adipose tissue specimen by collagenase procedure was conducted as described (65, 78). Data were expressed as amount of glucose incorporated in lipids (nmol glucose  $\cdot 2 \text{ h}^{-1} \cdot 10^{-7}$  fat cells). The basal lipogenesis (no insulin present) and lipogenesis at the maximum effective concentration for insulin were measured. The sensitivity of the adipocytes to insulin was expressed using the equation  $\text{pD}_2 = -\log(\text{EC}_{50})$ , where  $\text{EC}_{50}$  is the concentration (mol/l) of the hormone that produces a half-maximum effect and was calculated from logistic conversion of the dose-response curve as described (74).

***In vitro differentiated adipocytes.*** To measure lipogenesis in SVF-derived adipocytes differentiated *in vitro*, the cells were first washed one time with DMEM without glucose (Biochrom, Berlin, Germany) and incubated in insulin-free DMEM (Biochrom) supplemented with 1  $\mu\text{M}$  glucose for 3 h. Following the starvation, the cells were incubated for 2 h in the presence or absence of  $10^{-7}$  mol/l insulin and D-[3-<sup>3</sup>H]glucose (37 MBq/ml; Perkin Elmer-Cetus, Norwalk, CT) diluted 1:1,000. Subsequently, the cells were washed three

Table 1. Characteristics of study groups

	Lean ( $n = 9$ )	Obese IS ( $n = 21$ )	Obese IR ( $n = 18$ )	P Value (IR vs. IS)
Age, yr	37 $\pm$ 11	37 $\pm$ 8	40 $\pm$ 8	0.290
BMI, kg/m <sup>2</sup>	23 $\pm$ 1	41 $\pm$ 5	40 $\pm$ 6	0.475
Waist circumference, cm	80 $\pm$ 4	121 $\pm$ 11	123 $\pm$ 12	0.567
Diastolic blood pressure, mmHg	75 $\pm$ 9	75 $\pm$ 8	83 $\pm$ 9	0.008
Systolic blood pressure, mmHg	121 $\pm$ 20	122 $\pm$ 1.3	134 $\pm$ 18	0.026
Plasma glucose, mmol/l	4.7 $\pm$ 0.5	5.2 $\pm$ 0.6	5.3 $\pm$ 0.4	0.447
Plasma insulin, mU/l*	6.0 $\pm$ 4.3	11.4 $\pm$ 4.6	16.7 $\pm$ 9.5	0.024
log10-HOMA-IR, index	1.3 $\pm$ 1.1	2.6 $\pm$ 1.1	4.0 $\pm$ 2.3	0.020
P-cholesterol, mmol/l	4.4 $\pm$ 0.7	4.6 $\pm$ 0.7	5.1 $\pm$ 0.9	0.088
P-HDL cholesterol, mmol/l	1.6 $\pm$ 0.3	1.3 $\pm$ 0.3	1.1 $\pm$ 0.2	0.079
P-LDL cholesterol, mmol/l	2.46 $\pm$ 0.73	2.71 $\pm$ 0.70	3.11 $\pm$ 1.08	0.17
P-triglycerides, mmol/l	0.8 $\pm$ 0.3	1.4 $\pm$ 0.7	1.6 $\pm$ 0.7	0.256
Fat cell volume, pl	424 $\pm$ 174	864 $\pm$ 167	949 $\pm$ 124	0.104
Basal lipogenesis, nmol/10 <sup>7</sup> cells*	2.9 $\pm$ 2.5	2.8 $\pm$ 2.2	0.5 $\pm$ 0.3	$1.3 \times 10^{-13}$
Insulin-stimulated lipogenesis, nmol/10 <sup>7</sup> cells*	6.0 $\pm$ 4.7	7.1 $\pm$ 4.8	0.8 $\pm$ 0.4	$6.1 \times 10^{-9}$
Insulin-basal lipogenesis, nmol/10 <sup>7</sup> cells*	3.0 $\pm$ 2.3	4.3 $\pm$ 3.0	0.3 $\pm$ 0.2	$2.7 \times 10^{-12}$
Insulin pD <sub>2</sub> of lipogenesis	14.4 $\pm$ 1.6	13.7 $\pm$ 1.6	11.8 $\pm$ 2.4	0.008

Values are averages  $\pm$  SD;  $n$ , no. of subjects. IR, insulin resistant; IS, insulin sensitive; BMI, body mass index; HOMA, homeostasis model adjustment; P, phospho; HDL, high-density lipoprotein; LDL, low-density lipoprotein. IR and IS groups were compared with *t*-test. One lean woman took contraceptive pills. Two obese IS women took contraceptive pills, and 1 woman took a selective serotonin reuptake inhibitor (SSRI). Three obese IR women took SSRI and one woman another antidepressant drug (duloxetine). Two obese IR women were prescribed a combination of thiazide and amiloride diuretics. Finally, the following pharmaceuticals were each prescribed to one obese IR woman: angiotensin-converting enzyme inhibitor, statin, contraceptive pills, infliximab (tumor necrosis factor- $\alpha$  receptor antagonist), methotrexate nonsteroid anti-inflammatory drug, selective histamine H1-receptor antagonist, thyroid hormone, the antiepileptic drug topiramate, the antipsychotic drug ziprasidone, the opioid receptor modulator buprenorphine, and an inhalable corticosteroid and inhalable selective  $\beta_2$ -stimulatory agonist. \*Values were log10 transformed before analysis.

times with cold PBS and lysed in 0.1% SDS/H<sub>2</sub>O. Lysate (10  $\mu$ l) was saved for determination of protein concentration. The rest of the lysate was transferred to cuvettes containing scintillation fluid [toluene with 5 g/l 2,5-diphenyloxazol and 0.3 g/l 1,4-bis(4-methyl-5-phenyl-2-oxazolyl)-benzene; all from Sigma-Aldrich, St. Louis, MO], and counts per minute were recorded after overnight phase separation.

#### RNA Isolation, cDNA Synthesis, and Real-Time PCR

Total RNA was extracted from adipose tissue specimens (300 mg), mature adipocytes ex vivo, and cell culture samples using the miRNeasy kit (Qiagen, Hilden, Germany). RNA concentration and purity were measured using a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Lafayette, CO), and high-quality total and small RNA was confirmed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

For clinical samples, cDNA of miRNAs was synthesized using the TaqMan miRNA Reverse Transcription Kit and human Megaplex primer pools A/B without preamplification (Applied Biosystems, Foster City, CA). RT-qPCR was performed using the amplification protocol provided by Applied Biosystems. Expression of miRNAs was normalized to the internal reference gene *RNU48*.

cDNA synthesis of samples obtained in in vitro experiments was performed using the miScript II RT-Kit and miScript HiFlex Buffer (Qiagen) enabling detection of multiple miRNAs and mRNAs from a single cDNA preparation. RT-qPCR of coding genes or miRNAs was performed using commercial Taqman probes (Applied Biosystems) or miScript Primer Assays (Qiagen), respectively. Expression was normalized to the internal reference genes *LRP10* and/or *18s* (for coding genes) or *SNORD68* (for miRNAs) using the  $2^{-\Delta CT}$  method (63).

#### Affymetrix GeneChip miRNA Array

Total adipose RNA (1  $\mu$ g) was labeled using the FlashTag biotin HSR labeling kit (Genisphere, Hatfield, PA) according to the supplier's protocol. The labeled samples (400 ng) were placed in a hybridization cocktail mix containing 4% formamide and hybridized overnight to Affymetrix GeneChip miRNA 1.0 arrays (Affymetrix, Santa Clara, CA) following the indicated Genisphere protocol. The array included oligonucleotide probe sets for 847 human miRNAs from Sanger miRBase (v11). The arrays were washed, stained, and scanned in an Affymetrix GCS 3000 scanner. Instrument control and automatic interpretation of raw pixel intensity values were performed using Affymetrix GeneChip Operating Software. Signal intensities and present calls were generated in the miRNA QC tool (Affymetrix) using default settings, i.e., BC-CG background adjustment, quantile normalization, and median polish summarization. Sample quality was assessed using QC metrics, and figures were generated in the miRNA QC Tool. All samples passed quality control. Subsequent microarray analysis was limited to the 205 human miRNAs that were scored as present according to the miRNA QC tool in >40 samples. Array data have been submitted to GEO (<http://www.ncbi.nlm.nih.gov/geo/>; accession no. GSE87379).

#### Transfection of miRNA

For overexpression/inhibition of miRNA activity, in vitro differentiated adipocytes (day 10–12 postinduction) were transfected with miRIDIAN miRNA mimics/inhibitor (Dharmacon/Thermo Fisher Scientific or Qiagen, respectively) in 12-, 24-, or 48-well plates and HiPerfect Transfection Reagent (Qiagen), respectively, at 9, 4.5, or 2  $\mu$ l according to the manufacturer's protocol. Control cells were transfected with miRIDIAN miRNA Mimic Non-Targeting Negative Control or Inhibitor Negative Control (Qiagen). The cells were incubated for 48 h at which time in vitro lipogenesis was assessed (in cells plated on 48-well plates) and RNA or proteins were collected.

#### 3'-UTR Assay

Empty luciferase reporter vector and vector containing 3'-UTR of ectonucleotide pyrophosphatase/phosphodiesterase 1 (*ENPP1*) were obtained from GeneCopoeia (Rockville, MD). The luciferase reporter assay in 3T3-L1 cells was performed as described in detail (2). Because of sequence length, the 3'-UTR of *ENPP1* was divided into two constructs with 110-base pair (bp) overlap generating *fragment A* of the 3'-UTR (–25 to 2,511 bp) and *fragment B* (2,401–4,660 bp). miRNA-652-3p had predicted binding sites on both fragments that were used for transfections.

#### Analysis of Protein Expression

Approximately 250,000 of in vitro differentiated adipocytes were lysed in 150  $\mu$ l RIPA buffer as described (90). Total protein (20–30  $\mu$ g) was separated by SDS-PAGE, and Western blot was performed according to standard procedures. The membranes were blocked in 3% ECL Advance Blocking Agent (GE Healthcare, Buckinghamshire, UK). Primary antibodies against ENPP1 (Abcam, Cambridge, UK), AMP-activated protein kinase (AMPK)- $\alpha_2$  (Cell Signaling Technologies, Danvers, MA) and  $\beta$ -actin as a loading reference (Sigma-Aldrich) were used. Secondary rabbit IgG antibodies were conjugated to horseradish peroxidase (Sigma-Aldrich). Protein bands were detected by chemiluminescence using the ECL Select Western Blotting Detection Kit (GE Healthcare) in the Chemidoc XRS system (Bio-Rad, Hercules, CA), and quantified by Quantity One software (Bio-Rad).

#### ELISA

Cell lysates were prepared from SVF-derived adipocytes differentiated in vitro transfected with miRNA mimics. After 48 h, medium was changed to a 1:1 mix of DMEM without glucose (Biochrom) and Ham's F-12 (Life Technologies/Thermo Fisher Scientific) supplemented with 2.4 g/l NaHCO<sub>3</sub>, 16 mg/l biotin, 8 mg/l D-panthothenate, and 7.14 g/l HEPES (pH 7.4) and incubated for 3 h. Following starvation, the cells were incubated in the presence or absence of insulin ( $10^{-7}$  mol/l) for 15 min. Protein lysates and quantification of proteins were performed according to the PathScan Sandwich ELISA kit instructions (Cell Signaling Technology). We measured total protein kinase B (Akt) 2 (no. 7046C), phospho-Akt2 (Ser<sup>474</sup>, no. 7048C), total insulin receptor substrate (IRS)-1 (no. 7328C), phospho-IRS1 (panTyr, no. 7133C), insulin receptor- $\beta$  (no. 7069C), and phosphoinsulin receptor- $\beta$  (panTyr, no. 7082C) proteins.

#### miRNA Target Prediction Analysis

We used the miRWalk web tool to predict the targets of the selected miRNAs (21). A list of insulin-signaling related genes (92 genes) was imported in miRWalk, and predictions of miRNAs were performed using default settings. Five algorithms were used for filtering purposes, each one using different sets of properties for mRNA-miRNA targeting (TargetScan, miRanda, miRDB, DIANA-mT, and RNA22). The search was performed in December 2014.

#### Statistical Analysis

Data are shown as means  $\pm$  SD. Results were analyzed by paired or unpaired *t*-test. Relationships were determined by simple and multiple regression analysis. Microarray results were analyzed by the Bioconductor package significance analysis of microarrays using two-group unpaired comparison (97).

## RESULTS

#### Clinical Observations

Relevant clinical data of the individuals involved in the study are summarized in Table 1. As expected, the insulin-



stimulated lipogenesis was markedly lower in OIR compared with OIS women, whereas there was no difference in BMI between the two groups. Insulin-stimulated lipogenesis did not differ between OIS and lean women. Systemic IR was assessed by HOMA-IR and was more pronounced in OIR compared with OIS women. HOMA-IR was higher in OIS than in lean women.

#### Expression of miRNAs in scWAT from IRO and ISO Women

We first compared expression of the 205 human miRNAs detected in human scWAT between OIS and OIR women. Fifteen miRNAs were differentially expressed between these groups with false discovery rate <5% (Table 2). Five miRNAs were higher expressed, and 10 miRNAs were lower expressed in OIR compared with OIS women. Fold changes in expression between OIR and OIS women varied from 1.14 to 1.41 for upregulated miRNAs and 0.74 to 0.93 for downregulated miRNAs. In general, miRNA expression in lean was further distant to the expression in OIR than to the OIS, e.g., miR-149-star mean expression in lean = 745, in OIS = 853, and in OIR = 1,081. The only exception was let-7b where the miRNA expression in lean was closer to the expression in OIR than OIS women.

We further validated array results by RT-qPCR in intact scWAT, which revealed that 11 miRNAs were regulated directionally coherent with the array data (Table 2). These miRNAs

were considered as candidate miRNAs for further functional studies despite that RT-qPCR results of four miRNAs (miR-30d, miR-193b-star, miR-324-3p, and miR-26a) did not reach statistical significance.

#### Screening of Differentially Regulated miRNAs for Impact on Lipogenesis

To determine whether the 11 candidate miRNAs could influence adipocyte lipogenesis in vitro, the miRNAs were overexpressed using miRNA mimics in human adipocytes differentiated in vitro followed by measurements of basal and insulin-stimulated lipogenesis. This was achieved by measuring uptake of radioactive-labeled glucose in adipocyte lipids in basal and insulin-stimulated states. At micromolar glucose concentrations, glucose transport but not further metabolism of glucose to lipids is the rate-limiting step for lipogenesis in fat cells (3). Two miRNAs (miR-143-3p and miR-652-3p) significantly increased glucose incorporation into lipids by ~20% in response to insulin (Fig. 1A).

#### Relationship between Insulin-Stimulated Lipogenesis and Expression of Candidate miRNAs

The possible in vivo relevance of the candidate miRNAs was assessed by correlating expression values of the miRNAs in

Table 2. Differentially expressed miRNAs in scWAT from lean, insulin-resistant, and insulin-sensitive obese women

miR ID	Sequence	Microarray				RT-qPCR		
		Lean (n = 9)	Obese IS (n = 21)	Obese IR (n = 18)	IR/IS	P Value (IR vs. IS)	IR/IS	P value (IR vs. IS)
miR-149-star <sub>st</sub> (hsa-miR-149-3p)	AGGGAGGGACGGGGGCGUGGC	745 ± 137	853 ± 191	1,081 ± 245	1.27	0.0023	0.93	0.3216
miR-1228-star <sub>st</sub> (hsa-miR-1228-5p)	GUGGGCGGGGCGAGGUGUGUG	375 ± 78	460 ± 121	587 ± 164	1.28	0.0083	0.90	0.2645
miR-638 <sub>st</sub> (hsa-miR-638)	AGGGAUCGCGGGCGGGUGGCGGCCU	1,804 ± 455	2,274 ± 460	2,723 ± 625	1.20	0.0141	1.61*	0.0018*
miR-572 <sub>st</sub> (hsa-miR-522)	GUCGCGUCGGCGGUGGCCCA	55 ± 17	76 ± 21	107 ± 43	1.41	0.0053	0.71	0.0469
miR-1207-5p <sub>st</sub> (hsa-miR-1207-5p)	UGGCAGGGAGGCUGGGAGGGG	166 ± 44	228 ± 45	283 ± 83	1.24	0.0116	0.77	0.0027
miR-30d <sub>st</sub> (hsa-miR-30d-5p)	UGUAAACAUCCCGACUGGAAG	403 ± 34	369 ± 50	300 ± 56	0.81	0.0002	0.87*	0.0653*
miR-145 <sub>st</sub> (hsa-miR-145-5p)	GUCCAGUUUCCAGGAUCCCU	8,625 ± 618	7,557 ± 551	6,941 ± 637	0.92	0.0025	0.82*	0.0178*
miR-193b-star <sub>st</sub> (hsa-miR-193b-5p)	CGGGUUUUAGGGCGAGAUGA	159 ± 41	124 ± 37	91 ± 22	0.74	0.0021	0.90*	0.1539*
miR-324-3p <sub>st</sub> (hsa-miR-324-3p)	ACUGCCCCAGGUGCUGCUGG	124 ± 13	116 ± 13	98 ± 17	0.85	0.0008	0.90*	0.2146*
let-7b <sub>st</sub> (hsa-let-7b-5p)	UGAGGUAGUAGGUUGUGUGGUU	13,485 ± 1,384	14,029 ± 2,286	12,578 ± 863	0.90	0.0155	0.84*	0.0298*
miR-422a <sub>st</sub> (hsa-miR-422a)	ACUGGACUUAGGGUCAGAAGGC	623 ± 123	535 ± 148	437 ± 91	0.82	0.0202	0.74*	0.0064*
miR-378 <sub>st</sub> (hsa-miR-378a-3p)	ACUGGACUUGGAGUCAGAAGG	1,956 ± 400	1,907 ± 442	1,622 ± 290	0.85	0.0251	0.81*	0.0193*
miR-26a <sub>st</sub> (hsa-miR-26a-5p)	UUCAAGUAAUCCAGGAUAGGCU	6,493 ± 28 ± 5	6,290 ± 664	5,871 ± 461	0.93	0.0306	0.94*	0.2569*
miR-652 <sub>st</sub> (hsa-miR-652-3p)	AAUGGCGCCACUAGGGUUGUG	760 ± 110	642 ± 128	558 ± 90	0.87	0.0248	0.72*	0.0052*
miR-143-star <sub>st</sub> (hsa-miR-143-3p)	UGAGAUGAAGCACUGUAGCUC	5,379 ± 392	4,522 ± 547	4,139 ± 531	0.92	0.0336	0.71*	0.0078*

Values are averages ± SD miRNA, micro-RNA; scWAT, subcutaneous white adipose tissue; ID, identification. Significant according to significance analysis of microarrays (SAM) between IR and IS with a false discovery rate of 5%. Annotation of miRNAs according to miRBase release 21 is indicated in parentheses. \*miRNAs considered as candidate miRNAs for further functional studies.

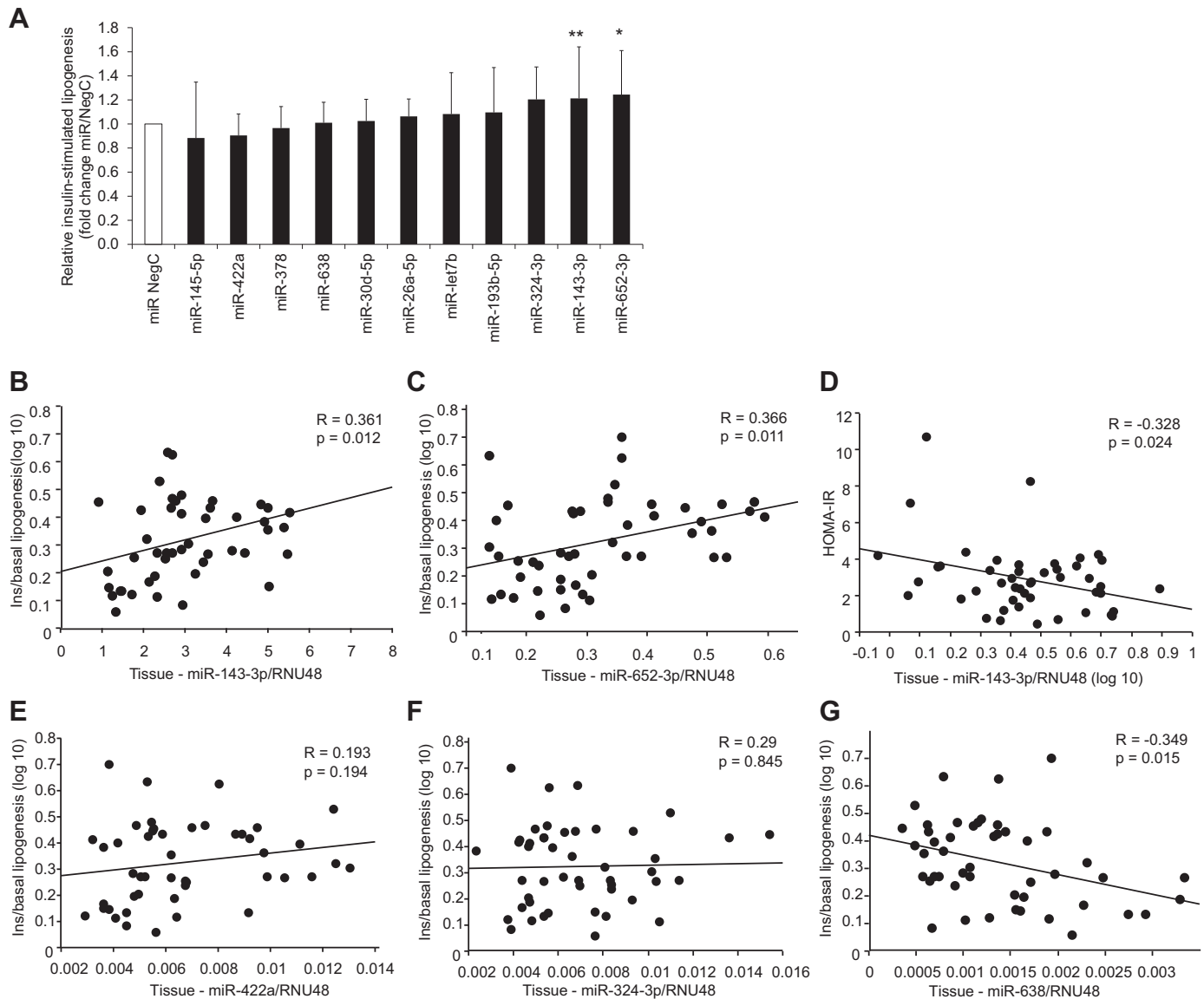


Fig. 1. Effects of differentially regulated micro-RNAs (miRNAs) in insulin-resistant (IR) and insulin-sensitive (IS) subjects on lipogenesis in vitro and their association with physiological parameters of IS. **A**: effects of miRNA overexpression on insulin-stimulated lipogenesis in human adipocytes. Stroma vascular fraction (SVF)-derived adipocytes differentiated in vitro were transfected with 40 nM of miRNA mimics for 48 h followed by evaluation of basal and insulin-stimulated lipogenesis. Relative insulin-stimulated lipogenesis was calculated against the miRNA mimics negative control (NegC) at an insulin-stimulated state. Maximal insulin response for NegC was minimum 3-fold in all experiments. Results are based on three to five biological/independent experiments.  $*P < 0.05$  and  $**P < 0.01$ . **B** and **C**: association between insulin-stimulated lipogenesis/basal lipogenesis and expression of miR-143-3p (**B**) and miR-652-3p (**C**) in sc white adipose tissue (scWAT) from 48 subjects. Expression of respective miRNA was assessed by RT-qPCR in intact WAT (48 subjects) and correlated using simple regression with lipogenesis values (expressed as insulin vs. basal) in the isolated adipocytes ex vivo (shown as relative units). **D**: association of homeostasis model adjustment (HOMA)-IR index and expression of miR-143-3p assessed with by RT-qPCR in intact WAT from 48 subjects using simple regression (shown as relative units). **E–G**: association between insulin-stimulated lipogenesis/basal lipogenesis and expression of miR-422a (**E**), miR-324-3p (**F**), and miR-638 (**G**) in scWAT from 47 subjects. Expression of respective miRNA was assessed with by RT-qPCR in intact WAT and correlated using simple regression with lipogenesis values (expressed as insulin vs. basal) in the isolated adipocytes ex vivo (shown as relative units). Expression of miRNAs was normalized to the reference gene *RNU48*. Values of lipogenesis were log10 transformed.

adipose tissue, according to RT-qPCR, with insulin-stimulated lipogenesis of fat cells in the original clinical cohort of 48 individuals. Specifically, expression of the miRNAs affecting glucose incorporation to lipids in vitro (miR-143-3p and miR-652-3p) was related positively to insulin-stimulated lipogenesis [for miR-143-3p  $R = 0.361$ ,  $P$  value = 0.012; for miR-652-3p  $R = 0.366$ ,  $P$  value = 0.011 (Fig. 1, **B** and **C**)] but not to BMI (Table 3). Expression of miR-143-3p, but not miR-652-3p, correlated negatively with the HOMA-IR index (Fig.

1D and Table 3). Additionally, we selected three other candidate miRNAs for similar correlation analysis: 1) miR-422a that was downregulated in IR patients to the same extent as miR-143-3p and miR-652-3p (Table 2); 2) miR-324-3p that demonstrated similar effect on lipogenesis in vitro (Fig. 1A); and 3) miR-638 that was instead upregulated in IR individuals contrary to miR-143-3p and miR-652-3p (Table 2). Only expression of miR-638 correlated with insulin-stimulated lipogenesis of fat cells ( $R = -0.349$ ,  $P$  value = 0.015), but the

Table 3. Relationship between insulin-stimulated lipogenesis and expression of miR-143-3p and -652-3p in adipose tissue

Regressor:	Lipogenesis (insulin/basal)		BMI		HOMA-IR		BMI	
	Partial <i>r</i>	<i>P</i> value	Partial <i>r</i>	<i>P</i> value	Partial <i>r</i>	<i>P</i> value	Partial <i>r</i>	<i>P</i> value
miR-143-3p-3p	0.424	0.007	0.155	0.306	-0.221	0.135	0.304	0.042
miR-652-3p-3p	0.372	0.011	0.04	0.774	-0.139	0.324	0.364	0.012

Expression of respective miRNA was assessed by quantitative RT-qPCR in intact white adipose tissue (WAT) (48 subjects), correlated using simple regression with lipogenesis values (expressed as insulin vs. basal) in the isolated adipocytes ex vivo or HOMA-IR (47 subjects), and shown as relative units. Expressions of miRNAs were normalized to the reference gene *RNU48*. Values of lipogenesis and miRNA expression levels in HOMA-IR analysis were log10 transformed.

overexpression of this miRNA did not affect lipogenesis in vitro (Fig. 1, A and E–G).

#### Identification of miRNA-143-3p and miR-652-3p Direct and Indirect Targets in Lipogenic Pathways

To delineate genes directly or indirectly targeted by miR-143-3p and miR-652-3p, we applied the following strategy. First, we searched for predicted direct targets of the miRNAs among a set of genes known to be involved in insulin signaling/insulin resistance/insulin sensitivity/lipogenesis pathways (92 genes; Table 4). This allowed us to identify 41 and 11 target genes for miR-143-3p and miR-652-3p, respectively (partially shown in Table 5 and further explained below).

Second, the proposed connections between miRNAs and their predicted targets were challenged by overexpressing each miRNA and assessing the effect on gene expression in human adipocytes. We choose to measure expression of the genes that displayed the highest target-prediction score or were well-established players in insulin signaling (35) (13 and 8 genes for miR-143-3p and miR-652-3p, respectively). Finally, we manually searched for genes that are known to inhibit insulin signaling; given that overexpression of miR-143-3p and miR-652-3p increases insulin-stimulated glucose uptake into lipids, a direct target of these miRNAs should have an inhibitory effect on insulin signaling (Table 5). Very low expressed genes were excluded from further analysis.

#### Validation of Genes Regulated by miRNA-143-3p and miR-652-3p in Human Adipocytes Differentiated In Vitro

Consequently, we could detect that overexpression of miR-143-3p significantly increased the expression of protein kinase, AMP-activated,  $\alpha_2$ -catalytic subunit (*PRKAA2*) by 35%, whereas inhibition of miRNA decreased the expression of *PRKAA2* by 27% (Fig. 2A). Overexpression of miR-652-3p reduced the expression of *ENPP1* by 30%, whereas inhibition of the miRNA increased *ENPP1* expression with 20% (Fig. 2B). Findings of miRNA overexpression were confirmed at the protein level (Fig. 2, A and B). The possible in vivo relevance of miRNAs on gene expression was assessed by overexpressing miR-652-3p and miR-143-3p using varying concentrations of miRNA mimics (10, 20, 40, and 60 nM) in human adipocytes and correlating expression levels of these miRNAs, according to RT-qPCR, with the expression levels of *ENPP1* and *PRKAA2*, respectively. Expression of *ENPP1* was related negatively to the expression of miR-652-3p [ $R = -0.523$ ,  $P$  value = 0.0005 (Fig. 2C)]. Expression of miR-143-3p demonstrated a trend of positive relation to the expression of *PRKAA2* but did not reach statistical significance (data not shown). We have observed higher variation in *PRKAA2* expression most likely because *PRKAA2* is not a direct target of miR-143-3p.

However, concentration of miRNA mimics (10–60 nM) applied for overexpression in vitro correlated directly with the expression levels of miR-143-3p (Fig. 2D), supporting the functionality of the miR-143-3p mimics. Overexpression of miR-652-3p using different concentrations of miRNA mimics (10–60 nM) leads to very stable increase of the miRNA levels

Table 4. List of genes reported to be involved in insulin resistance mechanisms

No.	Gene ID	Ref. No.	No.	Gene ID	Ref. No.
1	<i>ACAP1</i>	57	47	<i>PIK3R1</i>	73
2	<i>AKT1</i>	43	48	<i>PIK3R2</i>	73
3	<i>AKT2</i>	43	49	<i>PIK3R3</i>	73
4	<i>AKT3</i>	56	50	<i>PIK3R4</i>	73
5	<i>APPL1</i>	85	51	<i>PIK3R5</i>	73
6	<i>ARF6</i>	109	52	<i>PIK3R6</i>	73
7	<i>ARHGAP4</i>	109	53	<i>PLCG1</i>	40
8	<i>ASPSR1</i>	108	54	<i>PREX1</i>	6
9	<i>CAV1</i>	32	55	<i>PRKAA2</i>	35
10	<i>CBL</i>	60	56	<i>PRKCA</i>	14
11	<i>CBLB</i>	60	57	<i>PRKCB</i>	56
12	<i>CD36</i>	34	58	<i>PRKCD</i>	11
13	<i>CHUK/IKK1</i>	110	59	<i>PRKCG</i>	56
14	<i>CLASP2</i>	54	60	<i>PRKCI</i>	56
15	<i>CLIP3</i>	81	61	<i>PRKCZ</i>	70
16	<i>CRK</i>	17	62	<i>PTEN</i>	95
17	<i>DEPTOR</i>	55	63	<i>PTRF</i>	61
18	<i>DOC2B</i>	17	64	<i>RAB10</i>	86
19	<i>ENPP1</i>	68	65	<i>RAB14</i>	80
20	<i>EXOC1</i>	56	66	<i>RAB31</i>	64
21	<i>EXOC2</i>	56	67	<i>RAB5A</i>	92
22	<i>EXOC4</i>	56	68	<i>RALA</i>	16
23	<i>EXOC7</i>	41	69	<i>RHOQ</i>	17
24	<i>EXOC8</i>	56	70	<i>RICTOR</i>	53
25	<i>FASN</i>	93	71	<i>RPTOR</i>	77
26	<i>GGA1</i>	58	72	<i>RUVBL2</i>	105
27	<i>GGA2</i>	58	73	<i>SGK3</i>	111
28	<i>GGA3</i>	58	74	<i>SH2B2</i>	1
29	<i>HMGA1</i>	25	75	<i>SLC2A1</i>	59
30	<i>IKK complex</i>	84	76	<i>SLC2A4</i>	59
31	<i>INPPL1</i>	101	77	<i>SLC2A4RG</i>	49
32	<i>INSR</i>	33	78	<i>SNAP23</i>	46
33	<i>IRS1</i>	79	79	<i>Snappin</i>	7
34	<i>IRS2</i>	88	80	<i>SORBS1</i>	48
35	<i>KIAA0528</i>	106	81	<i>SORT1</i>	56
36	<i>KIF5B</i>	106	82	<i>SQSTM1</i>	30
37	<i>LNPEP</i>	69	83	<i>SREBF1</i>	35
38	<i>LRP1</i>	42	84	<i>STX4</i>	46
39	<i>MLST8</i>	77	85	<i>STXBP3</i>	45
40	<i>MTOR</i>	77	86	<i>STXBP4</i>	72
41	<i>MYO1C</i>	10	87	<i>TBC1D1</i>	76
42	<i>PDPK1</i>	71	88	<i>TBC1D13</i>	19
43	<i>PIK3CA</i>	73	89	<i>TBC1D4</i>	91
44	<i>PIK3CB</i>	73	90	<i>TNFA</i>	62
45	<i>PIK3CD</i>	73	91	<i>TRIP10</i>	15
46	<i>PIK3CG</i>	73	92	<i>VAMP2</i>	102

Table 5. Analysis of known genes involved in insulin signaling that are predicted targets of miR-143-3p and miR-652-3p

Gene	miR-143-3p			miR-652-3p		
	Predicted to bind by databases (no.)	Reason for selection	Fold change (miR/NegC) $\pm$ SD	Predicted to bind by database (no.)	Reason for selection	Fold change (miR/NegC) $\pm$ SD
<i>PIK3R1</i>	5	High prediction score	1.01 $\pm$ 0.50	5	High prediction score	1.18 $\pm$ 0.67
<i>AKT1</i>	4	High prediction score	0.95 $\pm$ 0.48			
<i>AKT2</i>	4	High prediction score	1.08 $\pm$ 0.19		Well-known player in insulin signaling (35)	0.84 $\pm$ 0.15
<i>PIK3R3</i>	4	High prediction score	0.92 $\pm$ 0.42			
<i>SORBS1</i>	4	High prediction score	1.14 $\pm$ 0.33			
<i>PIK3R4</i>	3	High prediction score	1.08 $\pm$ 0.24			
<i>HMGA1</i>	4	High prediction score	0.92 $\pm$ 0.33			
<i>IRS1</i>	1	Well-known player in insulin signaling (35) and predicted target	1.02 $\pm$ 0.32			
<i>PRKAA2</i>	1	Well-known player in insulin signaling (35) and predicted target	1.35 $\pm$ 0.43**	1	Well-known player in insulin signaling (35) and predicted target	0.85 $\pm$ 0.28
<i>SREBF1</i>		Well-known player in insulin signaling (35)	1.07 $\pm$ 0.16	1	Well-known player in insulin signaling (35) and predicted target	1.09 $\pm$ 0.30
<i>IRS1</i>		Well-known player in insulin signaling (35)	1.02 $\pm$ 0.32	1	Well-known player in insulin signaling (35) and predicted target	0.73 $\pm$ 0.15***
<i>INSR</i>		Well-known player in insulin signaling (35)	1.06 $\pm$ 0.21		Well-known player in insulin signaling (35)	0.80 $\pm$ 0.18**
<i>IRS2</i>		Well-known player in insulin signaling (35)	0.98 $\pm$ 0.13		Well-known player in insulin signaling (35)	0.76 $\pm$ 0.15***
<i>ENPP1</i>		Negative regulator of IR	1.10 $\pm$ 0.41	2	Negative regulator of IR and predicted target	0.78 $\pm$ 0.14***
<i>SLC2A4</i>				3	High prediction score	0.91 $\pm$ 0.28

Predicted interaction between miRNAs and its targets was investigated by overexpressing each miRNA and assessing effects on the mRNA level by RT-qPCR in human in vitro differentiated adipocytes. NegC, negative control. \*\* $P < 0.01$ . \*\*\* $P < 0.001$ .

precluding from obtaining statistical significance between these two parameters (Fig. 2E).

Because miR-652-3p reliably downregulated ENPP1 expression, we forecasted that it might directly interact with the 3'-UTR of ENPP1. Indeed, luciferase reporter assay confirmed that miR-652-3p binds directly to the 3'-UTR region of ENPP1 (Fig. 2F). Finally, expression of other predicted targets of the miRNAs was not affected at all or not affected in concordance with the miRNA-stimulating effects on lipogenesis in vitro (data not shown).

#### Identification of miRNA-143-3p and miR-652-3p Indirect Targets Regulated at the Posttranslational Level

PRKAA2 is a catalytic subunit of AMPK, an energy sensor protein kinase that plays a key role in regulating cellular energy metabolism. Because AMPK acts via direct phosphorylation of metabolic enzymes involved in insulin signaling, we hypothesized that miR-143-3p and possibly miR-652-3p can indirectly affect lipogenic pathways also at the posttranslational level. To investigate this, we overexpressed both miRNAs in adipocytes differentiated in vitro, stimulated the cells with insulin, and using ELISA measured total and phosphoproteins of the main insulin signaling players. Indeed, overexpression of miR-143-3p affected phosphorylation of AKT2 (Ser<sup>474</sup>), whereas overexpression of miR-652-3p affected phosphorylation of AMPK $\alpha$  at activating residue Thr<sup>172</sup> as well as total phosphorylation of IRS1 (Fig. 3A). Total AMPK levels were not affected by overexpression of miR-143-3p (data not shown).

#### DISCUSSION

By using comprehensive miRNA transcriptome profiling of human WAT and functional in vitro studies, we have found that miR-143-3p and miR-652-3p enhance insulin-stimulated glucose incorporation into lipids in human fat cells. Adipose expression of these miRNAs in clinical samples is positively associated with insulin-stimulated lipogenesis, consistent with

a role for miR-143-3p and miR-652-3p in development of fat cell IR in humans. We identified that miR-652-3p binds to ENPP1 and thereby downregulated its expression. In addition, we demonstrate that overexpression of miR-652-3p affects activating phosphorylation of AMPK and total phosphorylation of IRS1 proteins. For miR-143-3p, we discovered that the miRNA is involved in insulin signaling through enhancing phosphorylation of AKT2 and affecting expression of AMPK $\alpha_2$ .

Insulin resistance has a multifactorial etiology where each underlying event has a limited impact. In agreement with such a limited impact of individual factors, the fold change in miRNAs differential expression in WAT between OIR and OIS women was modest in our study. However, even small differences in adipose miRNA expression have been described to be of clinical importance (2, 51, 52). In this study, only 2 of 11 differentially expressed miRNAs displayed a coherent impact on insulin-stimulated lipogenesis in vitro. However, the significant correlation between adipose miR-143-3p and miR-652-3p expression and insulin-stimulated lipogenesis in the clinical cohort supports the notion that these miRNAs are relevant for insulin sensitivity in women. Importantly, both miR-143-3p and miR-652-3p were expressed at the same level in adipose tissue and isolated adipocytes as evaluated by qPCR (data not shown), which supports that scWAT reflects adipocyte expression of these miRNAs. Circulating levels of miR-143-3p are lower in obese compared with lean subjects (47). Similarly, circulating miR-652-3p are decreased in obesity (13). It is possible that such circulating miRNAs act locally in adipose tissue and thus that the impact of miR-652-3p and miR-143-3p on adipose tissue could go beyond locally produced miRNAs.

miRNA-652 has previously been shown to be dysregulated in obese human WAT, and to have effects on lipolysis (66) and CCL2 production (2), implying pleiotropic effects of this miRNA on fat cells. Here we identified that miR-652-3p



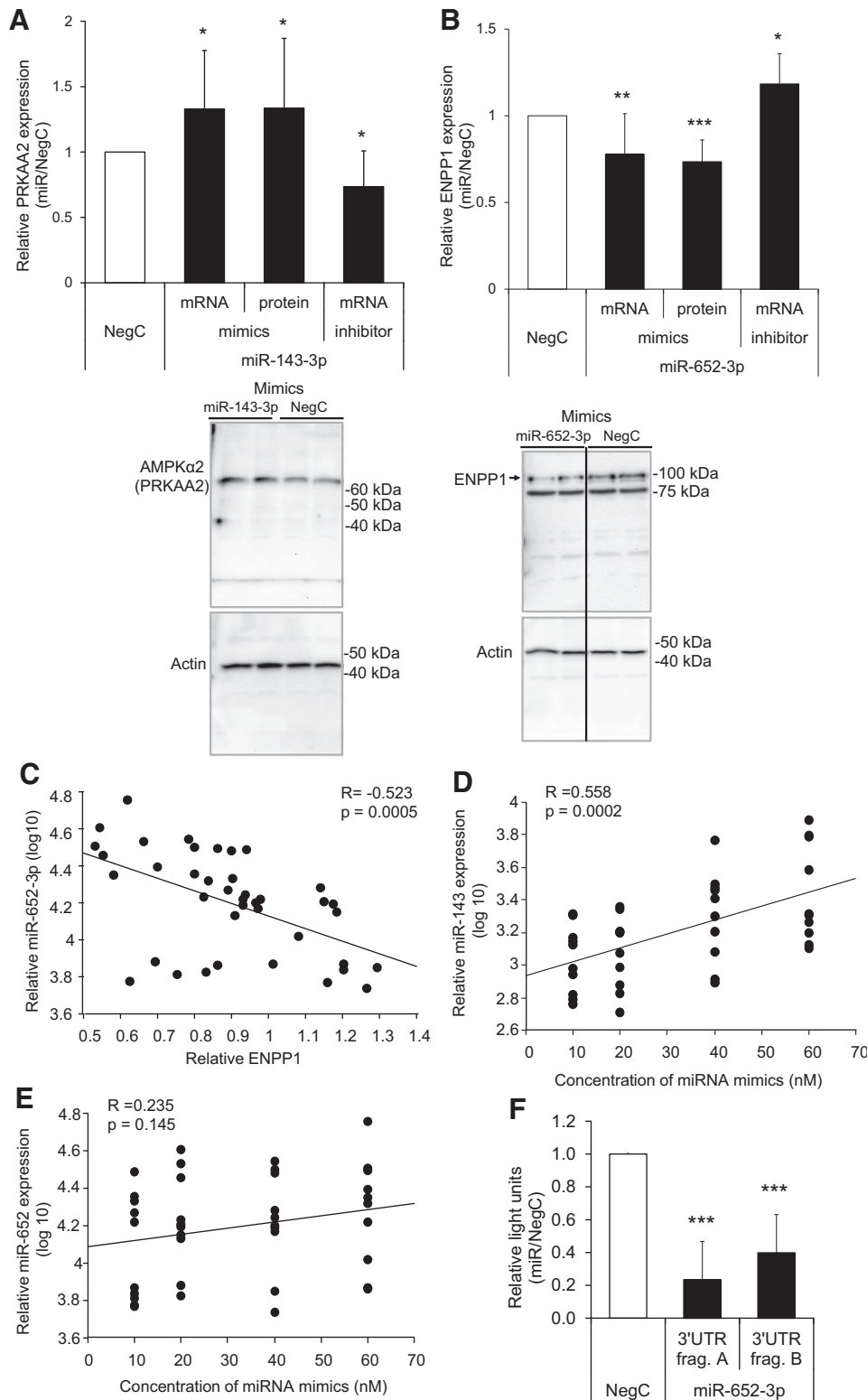


Fig. 2. Effects of miRNA on their predicted targets in human adipocytes. **A**: expression levels of protein kinase, AMP-activated,  $\alpha_2$ -catalytic subunit (PRKAA2) gene and protein were determined after miR-143-3p overexpression (40 nM of miRNA mimic) or inhibition (25 nM of miRNA inhibitor) using RT-qPCR and Western blot. Representative Western blots are shown. **B**: expression levels of ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) gene and protein were determined after miR-652-3p overexpression (40 nM of miRNA mimic) or inhibition (60 nM of miRNA inhibitor) in human adipocytes differentiated in vitro using RT-qPCR and Western blot. **A** and **B**: results are based on three (miRNA inhibition), four (miRNA overexpression) or five (for protein analysis) biological/independent experiments. Representative Western blots are shown. The Western blots are derived from the same gel/membrane run at the same time. The miRNA or NegC samples were loaded in quadruplicates, and the line in the middle indicates discontinuous image panels due to removal of duplicate lanes. **C**: association between overexpression of miR-652-3p and ENPP1 mRNA expression in human adipocytes differentiated in vitro. miR-652-3p was overexpressed using different concentrations (10, 20, 40, or 60 nM) of miRNA mimic. Expression of miRNA and ENPP1 was assessed by RT-qPCR, correlated using simple regression, and shown as relative units. Expression of miRNAs or ENPP1 was normalized to the reference genes *RNU48* and *Irf10*, respectively. Values of miRNA expression were log10 transformed. Results are based on technical replicates from four biological/independent experiments. **D** and **E**: association between overexpression of miR-143-3p (**D**) and miR-652-3p (**E**) and concentration of miRNA mimics used for the overexpression of the miRNAs in human adipocytes differentiated in vitro. Expression of the miRNAs was assessed by RT-qPCR and correlated with amounts of miRNA mimics using simple regression (shown as relative units). Expression of miRNAs was normalized to the reference gene *RNU48* and log10 transformed. **F**: mimics of miR-652-3p-3p (40 nM) were cotransfected with luciferase reporter constructs containing 3'-UTR (fragments **A** and **B**) of ENPP1 or empty vector in 3T3-L1 cells, and changes of luciferase activity were measured. Results are based on four biological/independent experiments. Results were analyzed using *t*-test and are presented as relative fold change  $\pm$  SD vs. NegC. To rule out unspecific effects, control cells were transfected with NegC miRNA for mimics. \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , and \* $P < 0.05$ .

directly regulates ENPP1, which is a member of the ENPP family. It has been shown to negatively inhibit insulin receptor signaling and induce cellular IR when overexpressed in various cell types (31). Obese patients with IR have increased ENPP1 expression in adipose tissue (12). In the presence of a high-fat diet, ENPP1 overexpression in adipocytes induces a pernicious

metabolic profile with fatty liver, hyperlipidemia, and dysglycemia, thus recapitulating key manifestations of the metabolic syndrome (75). These findings are in line with ours that overexpression of miR-652-3p inhibits ENPP1 and increases insulin-stimulated lipogenesis. So far, only two studies describe regulation of ENPP1 by miRNA, both in other cells



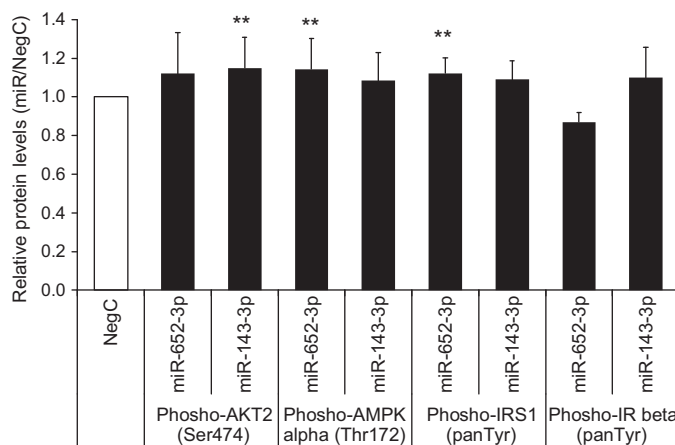


Fig. 3. miR-143-3p and miR-652-3p posttranslationally affect insulin-signaling pathways. A: miR-143-3p and miR-652-3p (40 nM) were overexpressed followed by insulin stimulation ( $10^{-7}$  mol/l) for 15 min. Protein extracts were prepared, and total and phosphoproteins were measured using ELISA. Values of phosphoproteins were normalized to the amount of total protein and expressed as fold change miRNA vs. NegC at the insulin-stimulated state. Results are based on 4 biological/independent experiments. Results were analyzed using *t*-test and are presented as relative fold change  $\pm$  SD vs. NegC. To rule out unspecific effects, control cells were transfected with NegC miRNA for mimics. Coefficient of variation for protein kinase B (AKT), AMP-activated protein kinase (AMPK), and insulin receptor substrate (IRS)-1 ELISAs was 8% and for IR $\beta$  was 14%. \*\**P* < 0.01.

types than adipocytes (67, 94). Thus, our results add novel data on the regulation of ENPP1 in obesity-associated IR.

miRNA-143 is one of few adipose miRNAs with established multiple functions, including regulation of adipogenesis (22, 103). We performed overexpression of its activity late in differentiation and therefore are confident that observed effects on lipogenesis are not secondary to an impact on differentiation. Other reported findings pointing to an important role for miR-143 in metabolic disease are that this miRNA is dysregulated in obesity, associated with WAT inflammation (2, 66, 103), and may control IR in the liver of mice (44). We demonstrate that miR-143-3p indirectly targeted and upregulated PRKAA2 encoding a catalytic subunit of AMPK. miRNAs usually downregulate their direct targets, but indirect targets can be either downregulated or upregulated. PRKAA2 was predicted as a target of miR-143-3 by one of five databases only, supporting that the gene is not a direct target of miR-143-3p. However, because miR-143-3p is downregulated in IR subjects, effects of overexpression of miRNA on PRKAA2 expression are in concordance with the clinical data. PRKAA2 is a subunit of the AMPK complex, which is an energy sensor activated by energy depletion and switching on catabolic processes (38). In adipose tissue, AMPK might have beneficial effects. Thus, deficiency of the AMPK  $\alpha_2$ -subunit results in adipose hypertrophy (100), and *Prkaa2*<sup>-/-</sup> mice exhibit decreased insulin sensitivity of peripheral tissues. In accordance with our findings, AMPK has been found to be less active in adipose tissue of obese IR subjects than in weight-matched insulin-sensitive subjects (107).

We hypothesized that miRNAs could regulate insulin sensitivity by indirectly affecting protein phosphorylation. Indeed, both miR-143-3p and miR-652-3p affected phosphorylation of key proteins in the insulin-signaling pathway, namely AKT2, AMPK $\alpha$ , and IRS1, hereby providing novel information on the

regulation of these genes. The target of miR-143-3p (AKT2) is an important molecule in the insulin-signaling pathway that can be phosphorylated and activated by, e.g., phosphatidylinositol 3-kinase, 3-phosphoinositide-dependent protein kinase-1, and mammalian target of rapamycin complex-2/PDK2 (87, 96). None of these kinases is predicted to be a direct target of miR-143, emphasizing that the link between miR-143 and AKT2 phosphorylation is complex and involves multiple steps. There is some evidence for a functional link between the miR-143-3p target PRKAA2/AMPK and AKT. Whereas AKT2 has been reported to inhibit AMPK (37), AMPK activity and AKT2 levels are strongly correlated in subcutaneous adipose tissue, but no mechanistic link has been reported (27). Thus, we conclude that miR-143 might impact PRKAA2 expression and AKT2 phosphorylation via independent pathways.

One should note that one miRNA targets multiple mRNAs, and we cannot rule out that there are additional targets of either miRNA in the very complex insulin-signaling pathways. The majority of previous studies attempting to identify miRNAs involved in insulin-resistance pathways were performed in rodents or other cells types than adipocytes, as reviewed (39, 83). Interestingly, there is almost no overlap between miRNAs in adipose tissue and muscle linked to IR/T2DM, supporting an organ-specific function (28). The present study was performed in women. It is well known that, in general, obese men have a more adverse metabolic profile than obese women, and consequently gender differences in adipocyte physiology may exist. Therefore, at the moment, it is not clear whether our findings are equally relevant for men.

The results presented herein add essential pieces of information to our understanding of the multifactorial etiology of insulin sensitivity. We demonstrate that, in fat cells, miR-652-3p and miR-143-3p increase insulin-stimulated lipogenesis, and directly, as well as indirectly through phosphorylation, regulate key genes in the insulin-signaling pathway. The positive association with insulin-stimulated lipogenesis in a clinical cohort strengthens the clinical importance of these miRNAs. These miRNAs may constitute important mediators of obesity-related IR and become targets for antidiabetic therapy.

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#### DISCLOSURES

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

#### AUTHOR CONTRIBUTIONS

ID, AK and PA designed the study and all co-authors researched and analyzed data. ID and PA selected and characterized the clinical cohort. YB,

AK and JL performed in vitro experiments. AP, JL, YB and AK performed literature search for the identification of insulin signaling genes. AK, YB and ID wrote the manuscript. All co-authors contributed to the discussion, revised the manuscript critically and approved a final version. AK and ID are the guarantors of this work and take full responsibility for the full content.

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