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Obesity Associated Modulation of miRNA and Co-Regulated Target Transcripts in Human Adipose Tissue of Non-Diabetic Subjects

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Abstract: *Objective*: Micro RNAs (miRNAs) are a class of non-coding regulatory RNAs. We performed a transcriptome-wide analysis of subcutaneous adipose tissue and *in vitro* studies to identify miRNAs and co-regulated target transcripts associated with insulin sensitivity (S_I) and obesity in human. *Methods*: We selected 20 insulin-resistant (IR, S_I=2.0±0.7) and 20 insulin-sensitive (IS, S_I=7.2±2.3) subjects from a cohort of 117 metabolically characterized non-diabetic Caucasians for comparison. *Results*: After global profiling, 3 miRNAs had marginally different expressions between IR and IS subjects. A total of 14 miRNAs were significantly correlated with %fat mass, body mass index (BMI), or S_I. The qRT-PCR validated the correlation of miR-148a-3p with BMI (r=-0.70, P=2.73X10^{-6}). MiRNA target filtering analysis identified DNA methyltransferase 1 (*DNMT1*) as one of the target genes of miR-148a-3p. *DNMT1* expression in adipose tissue was positively correlated with BMI (r=0.47, p=8.42X10^{-7}) and was inversely correlated with miR-148a-3p (r=-0.34). Differentiation of SGBS preadipocytes showed up-regulation of miR-148a-3p and down-regulation of *DNMT1* in differentiated adipocytes. After transfecting miR-148a-3p mimics into HeLa-S3 cells, *DNMT1* was down-regulated, while transfection of adipose stem cells with miR-148a-3p inhibitor up-regulated *DNMT1*. *Conclusions*: Our results indicate that miR-148a-3p-mediated regulation of *DNMT1* expression may play a mechanistic role in obesity.

Keywords: Adipose tissue, insulin resistance, obesity, miRNA, transcriptome.

INTRODUCTION

Studies by our laboratory and others have identified transcriptional dysregulation of genes in tissues involved in glucose homeostasis as a key molecular mechanism associated with insulin resistance and obesity [1-5]. Most of these studies have focused on protein-coding genes. However, emerging evidence suggests that there are more genes encoding non-coding RNAs than those encoding proteins in the human genome [6]. Many non-coding RNAs are involved in regulating genomic organization and contribute to control of gene expression through modulation of transcriptional or post-transcriptional processing of protein-coding genes [6]. Thus, similar to protein-coding RNAs, transcriptional dysregulation of non-coding RNAs may be important in the pathophysiology of insulin resistance and obesity [7].

MicroRNAs (miRNAs) are short (20-24 nt) non-coding RNAs that influence post-transcriptional regulation of gene expression by affecting both mRNA stability and translation [8]. Base-pairing interaction between miRNA and the target mRNAs silences the target gene by inducing translational repression or deadenylation and degradation [9]. Thus, interactions between miRNAs and target genes are a crucial regulatory layer overlaying transcription factor-mediated control mechanisms for coding transcripts. Recent animal studies have implicated several miRNAs in insulin secretion and action, and in glucose and cholesterol metabolism [10, 11]. Studies have also identified a role for miRNA in adipocyte differentiation [12, 13]. However, interactions between miRNAs and target genes, and the metabolic consequences of these interactions in humans, are not fully understood. In the current study, we profiled miRNA expression in subcutaneous adipose tissue of 40 metabolically well-characterized Caucasian subjects, to identify associations of these non-coding transcripts with insulin resistance and obesity. Genome-wide mRNA expression data of an expanded cohort of 99 Caucasian subjects were used to identify protein-coding target genes regulated by metabolic trait-associated miRNAs. We hypothesized that a subset of miRNAs would influence obesity and insulin sensitivity by modulating expression of their target genes in adipose tissue. We performed *in vitro* studies, including differentiation of preadipocytes and experimental manipulation of miRNA levels in cells, to evaluate miRNA-mRNA interactions and pathophysiology of obesity and insulin resistance.

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MATERIALS AND METHODS

Human Subjects

The participants in this study are a subset of 117 non-diabetic Caucasian subjects from a previous study [1]. Among these subjects, 99 had been characterized by insulin modified (0.03 U/kg) frequently sampled intravenous glucose tolerance tests (FSIVGT), and genome-wide mRNA expression data were available from subcutaneous adipose tissue biopsy samples. Methods for recruitment, clinical characterization, physiological assessments, and biopsy protocols have been published [1]. Subjects were men and women in general good health, between 19 and 60 years of age, with a body mass index (BMI) between 19 and 45 kg/m^2. Individuals were ranked by S_I (insulin sensitivity derived from MINMOD analysis [14] of FSIVGT data) after adjustment for age, gender, and natural logarithm of BMI. We selected 40 individuals from the leading and lagging tails of the distribution of the standardized residual of S_I (20 insulin-resistant and 20 insulin-sensitive). The individuals selected had adipose biopsy tissue available for extraction of RNA samples suitable for genome-wide miRNA expression analysis. Characteristics of the study sample are shown in Table **1**. This selection strategy is similar to our previous published study [2] and focuses on identification of gene expression changes associated with insulin sensitivity. However, since the selected subjects had a broad range of BMI and % fat mass (PFAT, measured by DEXA), our approach allowed identification of gene expression changes correlated with obesity/adiposity.

All biopsies were obtained from fasting volunteers. Adipose biopsies were obtained under local anesthesia from abdominal subcutaneous fat (near the umbilicus) by Bergstrom needle biopsy, rinsed in saline, frozen in liquid nitrogen, and stored at -80^0C for further use. All study participants provided written, informed consent under protocols originally approved by the University of Arkansas for Medical Sciences. Our current study was approved by the Institutional Review Board at Wake Forest School of Medicine.

Laboratory Measurements

Insulin was measured by the UAMS Clinical Research Center core laboratory using an immuno-chemiluminometric assay (Invitron Limited, Monmouth, UK). Plasma glucose was measured by the glucose oxidase method at LabCorp, Inc. (Burlington, NC). Plasma triglyceride, total cholesterol, and HDL cholesterol concentrations were measured directly (at LabCorp) by enzymatic colorimetric methods. LDL cholesterol and VLDL cholesterol concentrations were calculated indirectly by the Friedewald equation.

RNA Isolation

Total RNA was isolated from subcutaneous adipose tissue by using the mirVana miRNA isolation kit (Ambion, Austin, TX, cat # AM1561) following the manufacturer's instruction. This method retains small RNA (<200bp) species in the extracted RNA. The total RNA from adipocyte and stromal vascular fractions (AF and SVF) of adipose tissue was isolated by using the RNeasy Lipid Tissue Mini kit (Qiagen, Valencia, CA) and RNAqueous kit (Ambion), respectively, following the manufacturer's instructions. Fresh tissue for AF and SVF separation was available for a subset of 29 randomly selected Caucasian non-diabetic subjects. Total RNA from cells grown in standard conditions and from Simpson-Golabi-Behmel syndrome (SGBS) preadipocytes at different stages of differentiation was isolated with an miRNeasy Mini Kit; RNA from cell transfection experiments was isolated by an RNeasy Mini Kit (both from Qiagen) following the manufacturer's instructions. The quantity and quality of the isolated total RNA samples were determined by ultraviolet spectrophotometry (Nanodrop, Thermo Scientific, Pittsburgh, PA) and electrophoresis (Experion nucleic acid analyzer, BioRad Laboratories, Hercules, CA), respectively. RNA samples with RIN (RNA Integrity Number) >7 and A260/A280≥ 1.8 were used for all analysis.

Genome-Wide Analysis for miRNA

All experiments were conducted at Exiqon Services (Vedbaek, Denmark). High-quality total RNA re-verified by an Agilent 2100 Bioanalyzer profile at Exiqon was used for this experiment. A pool of all RNA samples in the study was used as the reference sample. 300 ng total RNA from both the sample and reference was labeled with Hy3™ and Hy5™ fluorescent label, respectively, using the miRCURY LNA™ microRNA Hi-Power Labeling Kit, Hy3™/Hy5™ (Exiqon). The Hy3™-labeled samples and a Hy5™-labeled reference RNA sample were mixed pair-wise and hybridized to the miRCURY LNA™ microRNA Array 7th Gen (Exiqon; Batch # 35005), which contains capture probes targeting all microRNAs for human, mouse or rat registered in the miRBASE 18.0. The hybridization was performed according to the miRCURY LNA™ microRNA Array Instruction manual using a Tecan HS4800™ hybridization station (Tecan, Austria). After hybridization the microarray slides were scanned and stored in an ozone-free environment (ozone level below 2.0 ppb) to prevent potential bleaching of the fluorescent dyes. The miRCURY LNA™ microRNA Array slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies, Inc., USA) and the image analysis was carried out using the ImaGene® 9 (miRCURY LNA™ microRNA Array Analysis Software, Exiqon). Sensitivity and reliability of this technology is validated by miRQC and other studies [15-17]. The quantified signals were background corrected (Normexp with offset value 10) [18] and normalized using the global Lowess (LOcally WEighted Scatterplot Smoothing) regression algorithm [19]. The background threshold was calculated for each individual microarray slide as 1.2 times the 25th percentile of the overall signal intensity of the slide. Using the probes expressed above background in ≥ 20% of samples, we identified expression of 495 human miRNA genes. However, to select expressed probes with greater confidence, we interrogated data from two published studies that used RNA seq to analyze miRNAs in adipose tissue [20, 21]. MiRNAs were selected if they were considered expressed in one of these published studies and if their expression was above background level in at least two IR or IS subjects in our study. We selected 231 miRNAs for further analysis. Considering 20 samples in each group (IR and IS), we had 85% and 73% power to detect 1.6 and 1.5 fold change, respectively, at a per-gene value of α <0.05.

Table 1. Anthropometric and metabolic phenotypes of study subjects.

Trait	All samples		IR-group		IS-group		P-value
	Mean	SD	Mean	SD	Mean	SD	
N (M/F)	40 (16/24)		20 (8/12)		20 (8/12)		
Age	40.1	10.5	40.1	10.9	40.2	10.3	0.99
WHR	0.93	0.28	0.98	0.38	0.87	0.1	0.22
BMI (kg/m^2)	26.7	4.3	26.7	4.2	26.8	4.4	0.92
%Fat mass	31.8	8.9	32.4	7.3	31.2	10.4	0.69
Systolic BP	121	11	126	11	116	8	0.004
Diastolic BP	75	9	78	11	72	6	0.08
Cholesterol, Total (mg/dL)	186	32	190	35	181	28	0.39
HDL Cholesterol (mg/dL)	57	23	55	25	59	20	0.54
LDL Cholesterol (mg/dL)	107	28	111	27	103	28	0.32
Triglycerides (mg/dL)	121	57	145	57	98	48	0.007
S_I [$x10^{-4}.(mU/L)^{-1}.min^{-1}$]*	4.6	3.1	2.0	0.7	7.2	2.3	1.31E-09
AIR_G [$mU.L^{-1}.min$]*	559.5	437.5	767.9	486.9	351.0	253.8	0.002
DI*	1872.4	1417.4	1375.8	796.6	2368.9	1723.4	0.027
Fasting Glucose (mg/dl) ¶	84.9	7.9	87.2	9.4	82.6	5.4	0.069
2hr Glucose (mg/dl) ¶	104.4	28.2	119.1	25.4	89.7	23.2	0.0005
Fasting Insulin (mu/L) ¶	6.4	6.0	7.6	4.3	5.2	7.3	0.21
2 hr Insulin (mu/L)* ¶	50.7	51.2	79.8	56.7	21.6	19.6	0.0002
Matsuda Index¶	8.6	6.8	4.5	2.1	12.7	7.4	0.0001
HOMA-IR¶	1.4	1.3	1.7	1.1	1.1	1.5	0.15
SR of SI			-1.0	0.3	1.5	1.0	2E-10
SR of Matsuda Index			-0.8	0.4	0.9	1.3	1.52E-05

Continuous variables are shown as mean ± SD. *, Metabolic traits from MINMOD analysis of FSIGT evaluation of non-diabetic individuals; ¶, Metabolic measurements from OGTT. BMI, Body mass index; WHR, waist to hip ratio; LDL, Low density lipoprotein; HDL, High Density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; S_I, insulin sensitivity index; AIR_G, Acute insulin response to glucose; DI, Disposition index; SR, Standardized Residual.

Genome-Wide Analysis for mRNA

Genome-wide transcriptome analysis and initial array processing were performed by the Center for Human Genomics Core Laboratory (Wake Forest School of Medicine) using HumanHT-12 v4 Expression BeadChip (Illumina, San Diego, CA) whole genome gene expression arrays according to the vendor-recommended standard protocol. In this study, we used results from analyses performed in the Caucasian subset (N=99) described above and published elsewhere [1].

Cell Culture

HeLa S3 cells (ATCC® CCL2.2™; American Type Culture Collection, Manassas, VA) were obtained from Cell and Viral Vector Core Laboratory at Wake Forest and cultured in Ham's F-12K (Kaighn's) Medium (GIBCO, Life Technologies, Grand Island, NY), supplemented with 10% fetal bovine serum (Bench Mark FBS, Gemini, West Sacramento, CA), L-glutamine, and antibiotics. Human adipose stroma-derived stem cells (ADSCs) were obtained from Coriell Cell Repositories (Camden, NJ). We used abdominal subcutaneous ADSCs from two different donors (AG17928, 53yrs/female and AG172929, 61yrs/female) to account for variability in genetic architecture. Cells were cultured in *Mesenchymal Stem Cell Basal Medium* (ATCC, PCS-500-030) supplemented with mesenchymal stem cell growth kit (ATCC, PCS-500-040) and antibiotics per the vendor's recommendation. Cells were cultured at 37°C in a humidified incubator maintaining a 5% CO_2 atmosphere.

Differentiation of Human Simpson-Golabi-Behmel Syndrome (SGBS) Preadipocytes

SGBS cells (provided by Martin Wabitsch) were cultured as described previously with slight modifications [22]. Briefly, SGBS preadipocytes were cultured at 37°C in a humidified incubator maintaining a 5% CO_2 atmosphere. The growth medium consisted of DMEM:F12 (1:1) (GIBCO), 33 mM biotin, and 17 mM pantothenate containing 10% fetal bovine serum (Hyclone, Logan, UT) and 1% penicillin-streptomycin. For this study, SGBS preadipocytes were plated at $1x10^5$ cells per well on a 6-well plate. The cells were cultured in 1.5 ml growth medium (described above), grown to confluence, and induced to differentiate into adipocytes one day post-confluence by addition of a serum-free differentiation medium. The differentiation medium consisted of DMEM:F12 (1:1) [prepared by mixing DMEM without glucose (Sigma) and Hams F12 nutrient mixture containing 10 mM glucose (Sigma) in a 1:1 ratio to obtain final glucose concentration of 5mM] supplemented with dexamethasone (25nM), 3-isobutyl-1-methylxanthine (IBMX, 500 µM), rosiglitazone (2 µM), human transferrin (0.01 mg/ml) , insulin (2×10^{-8} M), cortisol (10^{-7} M), T3 (0.2 nM), biotin (33 mM), and pantothenate (17 mM). The cells were maintained in differentiation medium for 4 days, after which the medium was changed to a serum-free adipogenic medium, which is essentially similar to the differentiation medium but without IBMX, dexamethasone and rosiglitazone. The medium was renewed every two days from the initiation of differentiation. Cells were harvested in triplicates for each specific time point including 0, 6, 12, 24, 48, 96 and 192 hours, following the initiation of differentiation.

Transfection

HeLa S3 cells and ADSCs were treated with miRNA mimic and miRNA inhibitor respectively using HiPerFect Reagent (Qiagen # 301705) according to the Fast-Forward transfection protocol optimized based on the manufacturer's instructions. In this procedure, cell monolayers with 80-90% confluence level were trypsinized to obtained uniform single cell suspension. Cells were counted (by Scepter Cell counter and 60mM sensor, Millipore, USA) and shortly before transfection, $1.2X10^5$ cells were seeded per well on a 12-well plate in 1100 µl of appropriate culture media containing serum and antibiotics. MiRNA mimic, miRNA inhibitor and appropriate control reagent were complexed with HiPerFect Transfection Reagent in serum-free media at room temperature for 10 minutes and then added directly to the cells in each well. The HeLa S3 cells were treated with Syn-hsa-miR-148a-3p (Qiagen, miScript miRNA Mimic #MSY0000243) or AllStars Negative Control siRNA (Qiagen # 1027280) at a final concentration of 5nM, while ADSCs were treated with Anti-hsa-miR-148a-3p (Qiagen, miScript miRNA inhibitor #MIN0000243) or miScript miRNA Inhibitor Negative Control (Qiagen # 1027271) at a final concentration of 50nM. These doses were selected, based on available literature, vendor recommendations, and initial dose response experiments (tested 1-5nM doses for mimics and 25-100nM doses for inhibitors). Cells were incubated with the transfection complexes under their normal growth conditions (37°C and 5% CO_2) and harvested at 48 hours after initiation of transfection.

cDNA Synthesis and qRT-PCR

Total RNA from adipose tissue and cells were reverse transcribed using miScript II RT kits (QIAGEN) following the manufacturer's instructions to prepare mi cDNA for mature miRNA quantification by real-time PCR. In this protocol we gently mixed total RNA template with miScript HiSpec Buffer, Nucleics Mix and mi Script Reverse Transcriptase. The mix was incubated for 60 min at 37 ^{0}C on a thermal cycler, followed by incubation for 5 min at 95 ^{0}C to inactivate miScript Reverse Transcriptase. This method selectively converts mature miRNAs and certain snoRNAs or snRNAs into cDNA. The oligo-dT primers used in this protocol have a 3' degenerate anchor and a universal tag sequence on the 5' end, allowing amplification of mature miRNAs in the real-time PCR step. We used diluted mi cDNA (1 ng / mL) and miScript Primer Assays (Qiagen) in combination with the miScript SYBR Green PCR Kit (QIAGEN) for quantification of mature miRNAs by real-time PCR. We mixed diluted mi cDNA (7ng/ reaction) with universal primer, assay specific primer and QuantiTect SYBR Green PCR Master Mix in a 25µl reaction volume and analyzed in a quantitative real time PCR instrument (ABI 7500 Fast, Applied Biosystems, Foster City, CA) using thermal cycling and data acquisition parameters recommended by the vendor. We have analyzed data by relative quantification method (DDCt method, as implemented in ABI SDS software v1.3.1) using Hs_SNORD68 as the endogenous control.

To analyze the target mRNA transcripts, total RNA from tissues and cells was reverse transcribed using QuantiTect reverse transcription kit (Qiagen) following the manufacturer's protocol, which includes a DNAse digestion step to remove genomic DNA contamination. Transcript-specific oligonucleotide primers for quantitative real-time PCR (qRT-PCR) were designed to capture most splice variants. Transcripts were measured by qRT-PCR using Power SYBR green chemistry (Applied Biosystems) as described previously and normalized to the expression of appropriate endogenous control genes (including 36B4/RPLP0 and Cyclophilin B/PPIB) depending on the tissue and cell type. Data was analyzed either by relative quantification (DDCt) or by absolute quantification method. The standard curves were generated for absolute quantification using pooled cDNA from the samples assayed. Details of the purchased or designed qRT-PCR assays are shown in Supplementary Table **1**. The qRT-PCR validation of adipose tissue target mRNA transcripts were performed in cDNA samples available for 101 non diabetic Caucasians (a subset of the 117 subjects described above).

Statistical and Bioinformatic Analysis

Lowess normalized genome-wide miRNA expression data was log2 transformed (log2 transformed median ratios) and was used in all analyses. The nonparametric Wilcoxon statistic implemented in Statistical Analysis for Microarray (SAM) software [23] was used to compare miRNA expression between the 20 IR and 20 IS subjects. We additionally compared the two groups by the Student's T-test. We considered results significant for a false discovery rate (q-value) < 5% based on 1000 permutations in SAM and unadjusted T-test $p \leq 0.05$. We then evaluated the correlation between the expression of each miRNA and metabolic phenotypes (BMI,

% fat mass, and S_I adjusted for age and gender) in the 40 subjects.

Selected miRNAs were validated by qRT-PCR. The expression data (after normalization with the appropriate endogenous control genes suggested by published literature) from qRT-PCR analysis were compared between IR and IS subjects by the T-test. A partial correlation coefficient (after adjustment for age and gender) was calculated to validate the relationship between natural log transformed metabolic phenotypes and miRNA expression values derived from qRT-PCR. Analyses were implemented in SPSS 13.0 (SPSS Inc., Chicago, IL).

We selected target genes for significant miRNAs from miRTarBase, an experimentally validated microRNA-target interactions (MTI) database [24]. Target genes with functional MTI status in the miRTarBase (downloaded September 2014) were considered. To identify miRNA-mediated regulation of target gene mRNA, we analyzed the correlation of selected target transcripts with metabolic phenotypes. As described above, data for probes of selected target mRNAs in 99 Caucasian subjects were mined from our published genome-wide expression analysis. Standard statistical methods used to analyze correlations between phenotypes and expression are published elsewhere [1]. In brief, gene expression data of all expressed probes in adipose were first inverse normal transformed. Then a nonparametric semi-partial correlation analysis was performed with log transformed values for S_I, PFAT, BMI, and WHR after adjusting for age and gender. We considered genes significantly correlated with the trait when at least one probe showed a Spearman's $r \geq 0.3$ with a p-value <0.01 and all probes were in same direction. All correlation analyses were done using SAS 9.2 software (SAS Institute Inc., Cary, NC). An inverse relationship between miRNA level and phenotype compared to mRNA target and phenotype was considered as evidence for putative co-regulation. Validation of the inverse relationship between a selected miRNA-mRNA target pair was performed by calculating the correlation between miRNA and mRNA expression levels.

Experimental conditions in *in vitro* experiments were compared using the Student's T-test.

RESULTS

miRNA Levels in Subcutaneous Adipose Tissue are Associated with Insulin Sensitivity and Obesity

Subjects had a wide range of BMI (19.6-37.4 kg/m^2), % fat mass (10-49%), and insulin sensitivity (S_I, 1.00-13.01). Consistent with the study design, insulin-resistant (IR, N=20, S_I=2.0±0.7) subjects had significantly lower S_I compared to the insulin-sensitive (IS, N=20, S_I=7.2±2.3) subjects ($p=1.31X10^{-9}$), but no significant differences in BMI or % fat mass (Table **1**). Comparison of subcutaneous adipose tissue miRNA profiles between IR and IS subjects identified significant ($p<0.05$ and $q<5\%$) up-regulation of let-7c ($p=3.19X10^{-3}$) and miR-222-3p (p=0.012) and down-regulation of miR-652-3p (p=0.04) in IR subjects compared to IS subjects. Real time qRT-PCR validated the significant up-regulation of let-7c (p=0.029, Supplementary Table **2**). However, none of these miRNAs was ≥1.5 fold differentially expressed (Table **2**).

We assessed the genome-wide miRNA expression data to identify correlations between their expression levels in adipose tissue with BMI, PFAT, and S_I in all 40 subjects (Table **2**). No miRNA was significantly correlated with these phenotypes after stringent correction for multiple statistical hypothesis testing. However, 14 miRNAs were nominally significantly correlated with at least one phenotype (uncorrected $p<0.01$). Real-time qPCR analysis of selected miRNAs (considering our results and published literature) further validated the significant positive and negative correlation of miR-146b-5p ($r=0.46$, $p=5.81X10^{-3}$) and miR-148a-3p ($r=-0.70$, $p=2.73X10^{-6}$; Fig. **1A**) with BMI (adjusted for age and gender, Supplementary Table **2**), respectively. These two miRNAs also showed strong correlation with PFAT.

Inverse Relationship of miRNA and Target Genes with Metabolic Phenotypes

To identify putative miRNA-mediated regulation of target gene mRNA we first searched mRNA target genes for metabolic phenotype-associated miRNAs in miRTarBase and then we analyzed the correlation of selected target transcripts with metabolic phenotypes. Mining of miRTarBase identified functional miRNA target interactions (MTIs) for nine phenotype-associated miRNAs from our study (Supplementary Table **3**). We analyzed genome-wide mRNA expression data from 99 Caucasian subjects to identify correlation of target genes with metabolic phenotypes. Pairing each miRNA with miRTarBase-indicated target genes showed evidence for inverse relationship of 6 miRNAs and 21 target genes with metabolic phenotypes. Specifically, miR-146a-5p and miR-21-5p were positively correlated with BMI, while their target genes *IRAK2* (interleukin-1 receptor-associated kinase 2, $r=-0.41$, $p<0.0001$) and *PPARA* (peroxisome proliferator-activated receptor α, $r=-0.58$, $p<0.0001$) were negatively correlated with BMI. Interestingly, miR-148a-3p was negatively correlated with BMI, and its target genes *DNMT1* (DNA methyltransferase 1; $r=0.35$, $p=0.0004$) and *DNMT3B* (DNA methyltransferase 3 beta; $r=0.34$, $p=0.0006$) were positively correlated with BMI (Supplementary Table **4**). DNA methyltransferases are involved in methylation of cytosine residues of DNA molecules. The *DNMT3b* enzyme is involved mainly in *de novo* DNA methylation, while *DNMT1* is considered a maintenance methyltransferase. This observation suggests a novel mechanistic link in the pathophysiology of human obesity. Thus, we selected miR-148a-3p and its putative target gene *DNMT1* and *DNMT3b* for further validation studies.

Real-time qPCR analysis in 101 Caucasian non-diabetic subjects validated a positive correlation of *DNMT1* with BMI ($r=0.47$, $p=8.42X10^{-7}$; Fig. **1B**) and PFAT ($r=0.53$, $p=2.17X10^{-8}$; adjusted for age and gender). Adipose tissue cDNA for real-time qPCR analysis of both mRNA and miRNA was available for a subset of 37 Caucasian non-diabetic subjects. We validated the inverse correlation between the *DNMT1* and miR-148a-3p expression level ($r=-0.34$, $p=0.04$, and $r=-0.48$, $p=0.0037$ after adjustment for age and gender; Fig. **1C**). We also tested the expression of the *DNMT3b* gene in adipose tissue by qRT-PCR using a total *DNMT3b* and *DNMT3b*-splice isoform 1-specific primer. However, *DNMT3b* expression was too low to be measured reliably.

Table 2. Genome wide analysis identified microRNAs correlated with metabolic phenotypes or differentially expressed in subcutaneous adipose tissue of insulin-resistant subjects.

miRNA	mirbase accession	%Fat Mass		BMI		S_I		Fold Change (IR/IS)	p-value** IR vs. IS
		r	P*	r	P*	r	P*		
hsa-miR-146b-5p	MIMAT0002809	0.55	**2.33E-04**	0.58	**9.58E-05**	-0.40	0.011		NS
hsa-miR-21-5p	MIMAT0000076	0.54	**3.11E-04**	0.58	**7.80E-05**	-0.35	0.03		NS
hsa-miR-146a-5p	MIMAT0000449	0.45	**0.004**	0.51	**7.50E-04**	-0.40	0.011		NS
hsa-miR-222-3p	MIMAT0000279		NS		NS	-0.43	**5.51E-03**	1.16	**0.012**
hsa-let-7c	MIMAT0000064		NS		NS	-0.35	0.03	1.16	**3.19E-03**
hsa-miR-652-3p	MIMAT0003322	-0.33	0.04		NS	0.42	**6.36E-03**	0.87	0.04
hsa-miR-378c	MIMAT0016847	-0.41	**0.009**	-0.35	0.03		NS		NS
hsa-miR-148a-3p	MIMAT0000243	-0.42	**0.007**	-0.32	0.05		NS		NS
hsa-miR-378d	MIMAT0018926	-0.43	**0.006**	-0.37	0.02		NS		NS
hsa-miR-376c-3p	MIMAT0000720	-0.43	**0.005**	-0.34	0.03		NS		NS
hsa-miR-320a	MIMAT0000510	-0.44	**0.005**	-0.37	0.02		NS		NS
hsa-miR-33b-5p	MIMAT0003301	-0.48	**0.002**	-0.36	0.02	0.37	0.02		NS
hsa-miR-320b	MIMAT0005792	-0.48	**0.002**	-0.41	**0.009**		NS		NS
hsa-miR-378a-3p	MIMAT0000732	-0.48	**0.002**	-0.43	**0.006**		NS		NS
hsa-miR-574-3p	MIMAT0003239	-0.49	**1.43E-03**	-0.44	**0.005**		NS		NS

*, p-value from correlation analysis (adjusted from age and gender); **, P-value from T-test; IR, insulin resistant subjects; IS, insulin sensitive subjects.

Although mature adipocytes are the predominant cells in adipose tissue, preadipocytes, macrophages, and other cell types are also present [25]. Thus, to define the contribution of adipose tissue cells to the observed relationship between *DNMT1* expression and obesity, we compared its expression in RNA from adipocytes and adipose stromal vascular cells. *DNMT1* expression was ~4 times higher in RNA isolated from the stromal vascular fraction (SVF) compared to the adipocyte fraction (AF) of adipose tissue (Fig. **1D**). As expected, expression of *ADIPOQ* (adiponectin) and *CD68* (tissue macrophage-specific marker) was very high in AF and SVF, respectively (Fig. **1D**), and indicates the quality of isolated SVF and AF. We identified a significant positive correlation between *DNMT1* expression and PFAT (Spearman's =0.38, p=0.043) in SVF RNA from a subset of 28 Caucasian non-diabetic subjects. After adjustment for age and gender, this relationship showed a trend toward significance (p=0.08) (Fig. **1E**). Correlations between SVF expression of *DNMT1* and BMI were not significant in this cohort. Appropriate SVF and AF RNA samples were unavailable to test miR-148a-3p expression.

Expression of miRNA and Target Gene Expression During Adipocyte Differentiation *In Vitro*

To understand the role of selected miRNAs associated with BMI or PFAT, we analyzed the *in vitro* expression at different stages of SGBS preadipocytes. In these cells, miR-148a-3p expression was high (p= 0.028; Fig. **2A**) and expression of its putative target gene DNMT1 was low (p=0.001) at day 8 of differentiation induction compared to undifferentiated cells (Fig. **2B**). Expression of two other obesity- associated miRNAs (miR-146a-5p and miR-146b-5p) was lower at day 8 of differentiation induction compared to undifferentiated cells (p=0.0015-0.0029; Fig. **3A**). Expectedly, adiponectin (*ADIPOQ)* expression was significantly higher ($p=1.7X10^{-5}$; Fig. **2C**) in differentiated SGBS cells. This pattern suggests that miR-148a-3p mediates regulation of *DNMT1* transcript expression.

miR-148a-3p Modulates DNMT1 Expression *In Vitro*

We measured miR-148a-3p and DNMT1 expression in HeLa cells, human preadipocytes, and human adipose-derived stem cells (ADSCs, primary preadipocytes). Expression of miR-148a-3p was almost absent in HeLa cells, and DNMT1 expression was highest in these cells. Conversely, ADSCs showed high expression of miR-148a-3p, but DNMT1 expression was significantly lower compared to HeLa cells (Figs. **3A** and **B**). This shows cell-specific expression of miR-14as-3p and also indicates a putative inverse relationship between miR-148a-3p and *DNMT1* expression.

Finally, we investigated whether experimental manipulation of miR-148a-3p expression modulates *DNMT1* expression in an *in vitro* cell culture system. As described above,

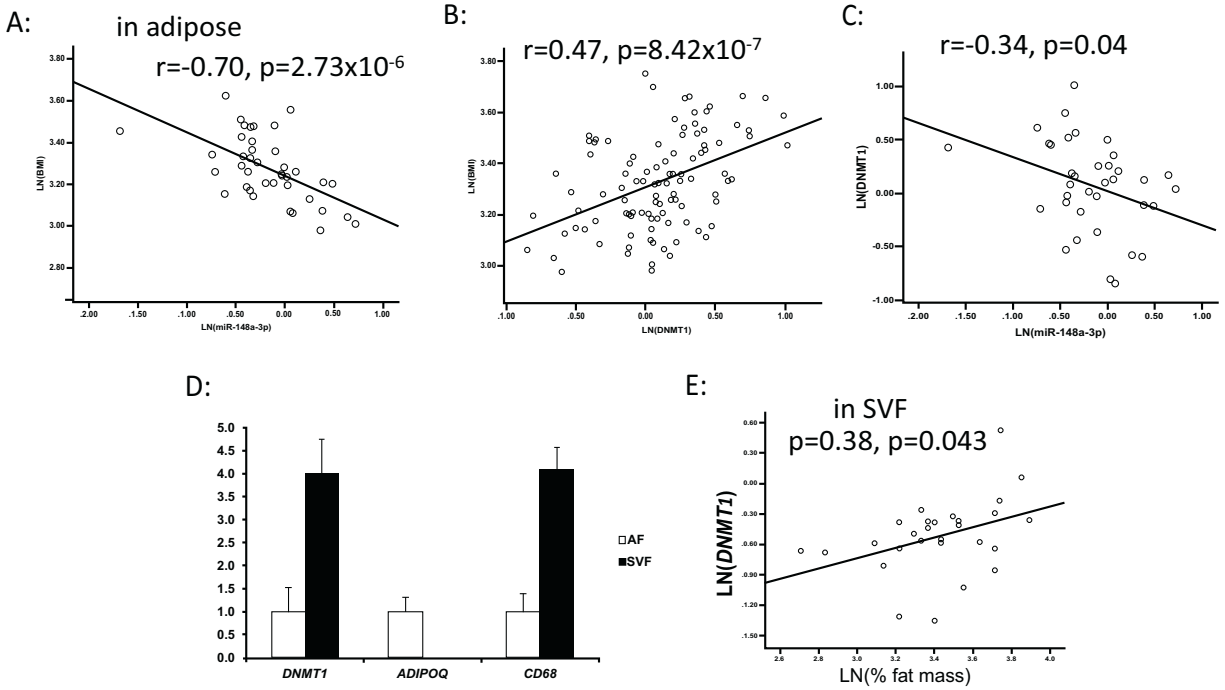

Fig. (1). Expression and phenotypic correlation of miR-148a-3p target gene DNMT1 in subcutaneous adipose tissue of Caucasian non-diabetic subjects. Real time–qPCR analysis indicated **A**) inverse correlation of BMI with miR-148a-3p (N=40 subjects), **B**) direct correlation of BMI with DNMT1 transcript expression (N=101 subjects), and **C**) inverse correlation of miR-148a-3p expression with DNMT1 expression (N=37 subjects). **D**) Relative expression of *DNMT1*, Adiponectin (*ADIPOQ*), and *CD68* transcript in pooled RNA samples from stromal vascular fractions (SVF) compared to adipocyte fractions (AF) of human subcutaneous adipose tissue. The SVF was separated from the AF after collagenase digestion of freshly collected adipose biopsy samples of non-diabetic subjects. Bar graph represents mean and SD of data from three replicated samples. **E**) *DNMT1* transcript expression levels in SVF were positively correlated with % fat mass in Caucasian non-diabetic subjects (N=28). Expression of DNMT1 was normalized with 36B4, and miR-148a-3p was normalized with SNORD68 expression. Natural log (LN) transformed data were used for the analysis.

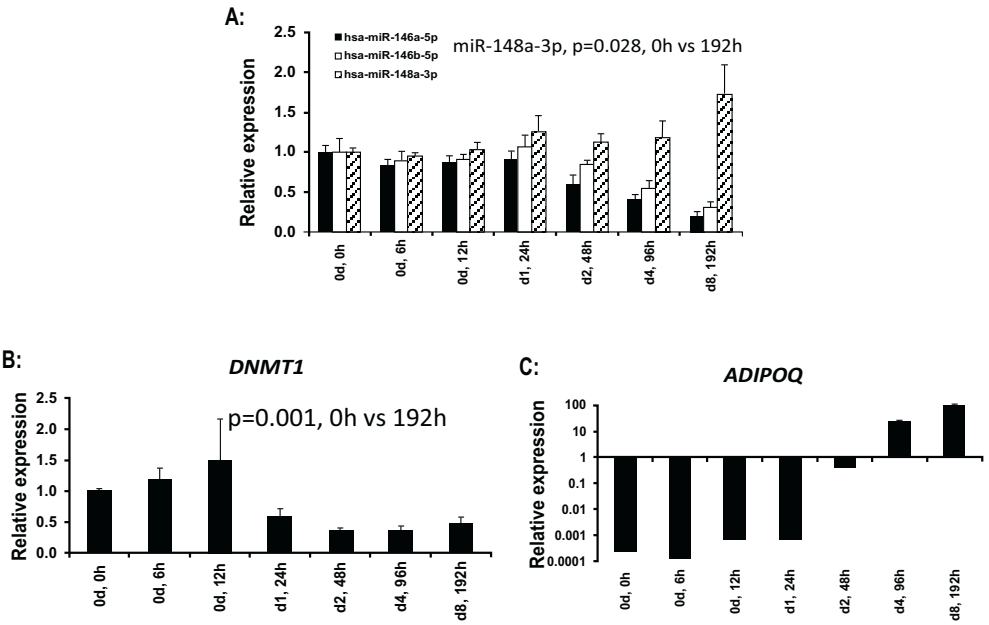

Fig. (2). Expression of miR-148a-3p and target gene DNMT1 at different stages of differentiation of Human Simpson-Golabi-Behmel syndrome (SGBS) preadipocytes *in vitro*. Relative expressions of **A**) selected obesity-associated miRNAs, **B**) *DNMT1*, a target gene for miR-148a-3p, and **C**) Adiponectin (*ADIPOQ*) at different days after induction of differentiation compared to undifferentiated (baseline, 0 hrs) cells are shown. Bar graph represents mean and SD of relative expression data from three biological replicate samples. Expression of *DNMT1* and *ADIPOQ* transcripts was normalized with 36B4 and expression of miRNAs was normalized with SNORD68 expression. Significant differences ($p<0.05$) in expression compared to 0 hrs are marked by * on the respective bar.

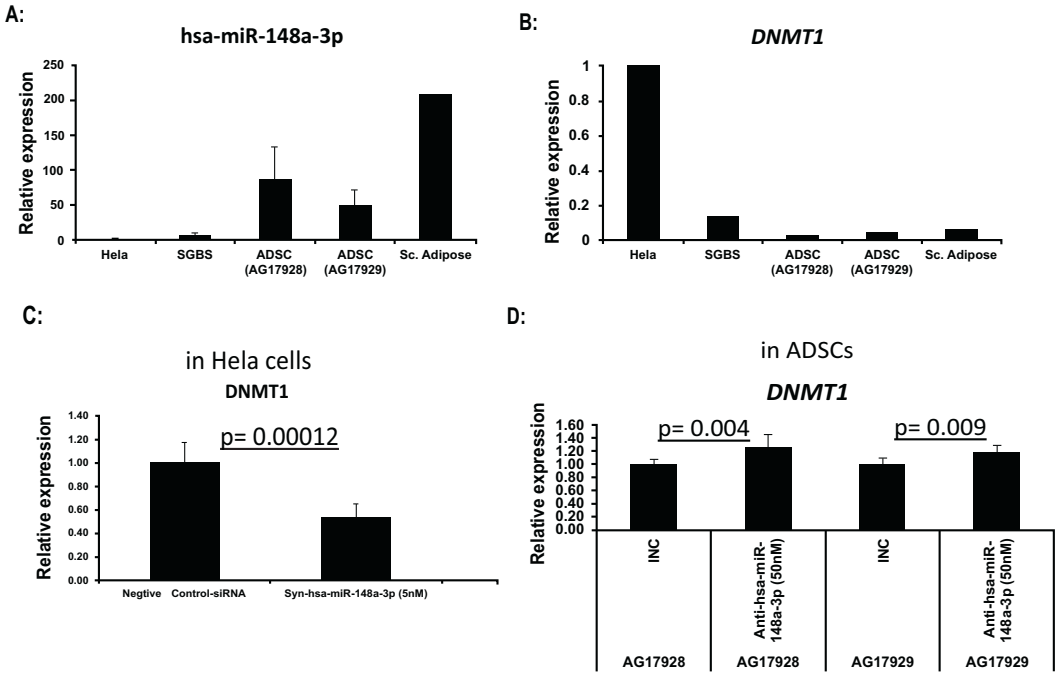

Fig. (3). Relative expression of miR-148a-3p target gene DNMT1 in different human cell types and effect of ectopic overexpression or inhibition of miR-148a-3p *in vitro* on expression of DNMT1. Relative expression of **A**) miR-148a-3p and **B**) DNMT1 transcripts in Simpson-Golabi-Behmel syndrome (SGBS) preadipocytes, human adipose-derived stem cells (ADSCs) from two donors and subcutaneous adipose tissue (pooled RNA from study subjects) compared to HeLa cells. Bar graph represents mean and SD of data from two experiments, each with 2-3 replicated samples. **C**) Expression of *DNMT1* in HeLa cells transfected with miR-148a-3p mimics (Syn-hsa-miR-148a-3p, 5nM) compared with cells transfected with appropriate negative control siRNA; **D**) Expression of *DNMT1* in ADSCs (from two donors AG17928 and AG17929) transfected with miR-148a-3p inhibitor (Anti-hsa-miR-148a-3p, 50nM) compared with cells transfected with appropriate control (miScript miRNA Inhibitor Negative Control, INC) transected cells. Bar graph represents mean and SD of relative expression of *DNMT1* at 48hrs post transfection. Data from two independent experiments, each with 2-3 biological replicates for each condition, are shown. DNMT1 expression in HeLa and ADSCs were normalized to 36B4 and Cyclophilin B, respectively.

miR-148a-3p expression is very low in HeLa cells and high in ADSCs. Thus, we transfected HeLa cells with miR-148a-3p mimics (Syn-hsa-miR-148a-3p) and ADSCs with miR-148a-3p inhibitor (Anti-hsa-miR-148a-3p). Transfection of HeLa cells with miR-148a-3p mimics significantly reduced the expression of *DNMT1* transcript (p=0.00012; Fig. **3C**), while transfection of ADSCs with miR-148a-3p inhibitor caused a small but significant increase (p= 0.004-0.009; Fig. **3D**) in the expression of *DNMT1* transcript 48 hr after transfection, compared to appropriate control transfected cells.

DISCUSSION

Insulin resistance is strongly associated with obesity, and both are key risk factors for type 2 diabetes (T2D) and cardiometabolic disorders [26, 27]. The association between obesity and insulin resistance is likely a cause-and-effect relationship. However, not all obese persons develop insulin resistance and some lean subjects may have insulin resistance similar to T2D subjects [27]. Investigators have used cross-sectional, longitudinal and interventional cohorts to elucidate the molecular mechanisms involved in obesity-dependent or -independent insulin resistance [1-5, 28]. Those studies by us and others have advanced our knowledge of the involvement of protein-coding transcripts in modulating insulin sensitivity and obesity. In the current study, integrating physiological, genomic, and cell biological data, we provide evidence that the expression of a small subset of miRNAs is modestly modulated in subcutaneous adipose tissue, and these non-coding regulatory RNAs subsequently modulate expression of target genes in insulin-resistant and/or obese subjects. Our results indicate that miR-148a-3p-mediated regulation of *DNMT1* expression (a gene involved in DNA methylation) plays a mechanistic role in obesity, possibly by affecting adipocyte differentiation.

Similar to other studies on mRNA expression, differences in miRNA levels between IR and IS subjects in this study were nominal. The expression of let-7c was higher in IR subjects compared to IS subjects and this was validated by real time-qPCR. This finding replicates previous results from transgenic animal studies [29, 30] suggesting that let-7 overexpression causes insulin resistance and impairs glucose tolerance.

We identified statistically significant correlations between miRNAs and obesity or adiposity in non-diabetic subjects. The strongest correlations were for miR-146b-5p, miR-21-5p, and miR-146a-5p. Previous cell and animal studies identified involvement of several miR-146 and miR-21 isoforms in immune and inflammatory responses and adipogenesis [31-33]. MiR-146a and miR-146b target interleukin-1 receptor-associated kinase-1 (*IRAK1*) and tumor necrosis factor receptor-associated factor-6 (*TRAF6*), both part of the NF-κB pathway, for repression [32]. Increased expression of

these miRNAs in subcutaneous adipose tissue of obese subjects validates results from two previous genome-wide studies [20, 21] that used small RNA sequencing methods for quantification (versus the Lock Nucleic Acid probe-based array used in this study). The role of these miRNAs is tissue-specific. In mice, high levels of miR-146a expression in insulin-secreting cells adversely affected insulin release and cell survival. On the other hand, depletion of miR-146a targeting genes like Med1 enhanced insulin sensitivity, improved glucose tolerance, and conferred resistance to high-fat diet-induced obesity [32]. Further investigation is required to determine the role of these up-regulated miRNAs in obesity and adipose tissue physiology. Civelek *et al* showed that the expression of miR-204-5p in adipose is most significantly positively correlated with BMI [20]. However, this correlation was not replicated in our study. Since we validated several other top signals from their study, it is likely that other factors contributed to this difference. For example, participants in their study were older than ours (average age 55.6±4.9 vs 40.1±10.5 years).

Global correlation analysis in two previous studies showed little or no evidence of negative correlation between mRNA and miRNA levels in human adipose tissue [20, 21]. Different technologies used for measurement of mRNA and miRNA species, and heterogeneity in the effect of mRNA and miRNA expression on phenotypes of individuals may account for this lack of significant findings. In the current study, we searched mRNA target genes for metabolic phenotype-associated miRNAs in miRTarBase, a carefully curated database of experimentally validated miRNA-target interaction [24]. Using this approach, we found evidence for inverse relationship of selected miRNAs and target genes with metabolic phenotypes. For example, we identified significant down-regulation of miR-148a-3p and up-regulation of its target gene DNA (cytosine-5)-methyltransferase-1 (*DNMT1*) with obesity (correlated with BMI and % fat mass). The miR-148a-3p was significantly correlated with obesity in RNA-seq-based genome-wide analysis by Civelek *et al* (Pearson correlation= -0.179, p= 0.011 and p-value of association= 0.009 with % fat mass) and Parts *et al* (Log2 likelihood ratio= -0.469, p= $4.13X10^{-5}$ between lean and obese subjects) [20, 21]. However, it was not among the top modulated miRNAs in those studies and was ignored. Replication by real time-qPCR showed a strong negative correlation between miR-148a-3p expression and obesity. Inverse regulation of miR-148a-3p and its target gene *DNMT1* reveals a novel mechanistic role of miRNA in obesity by modulating DNA methylation level in tissues.

Xie *et al* showed up-regulation of miR-148a during differentiation of mouse 3T3-L1 preadipocytes and down-regulation of its expression in adipose tissue of obese (ob/ob) versus wild-type lean mice [13]. They found an inverse correlation of miRNA expression during adipogenesis and obesity (miRNAs induced during adipogenesis were decreased in adipocytes from genetically mutated or diet-induced obese mice and vice versa) [13]. Similarly, we observed miR-148a-3p up-regulation during differentiation of SGBS cells (human preadipocytes), and miR-148a-3p down-regulation in obese subjects.

Londono *et al* (2013) showed accelerated adipocyte differentiation upon overexpression of miR-148a in mouse preadipocytes (3T3-L1) [34]. Similarly, a recent study by Shi *et al* (2015, published during preparation of this manuscript) also showed that overexpression of miR-148a enhances adipogenic differentiation of human primary preadipocytes (hMSCs-Ad), while knockdown of miR-148a inhibits adipogenic differentiation [35]. These studies support the functional correlation of miR-148a-3p expression and adipocyte differentiation. However, an *in vitro* study by Shi *et al* [35] suggests Wnt1 as the target of miR-148a. They showed positive correlation of miR-148a expression with BMI in visceral adipose tissue, but showed no data on Wnt1 expression in adipose tissue. Thus, further studies are required to resolve the connection between miR-148a-3p mediated modulation of adipocyte differentiation and its effect on BMI or % fat mass.

Several *in vitro* studies showed interactions between miR-148a-3p and its target genes *DNMT1* and *DNMT3b* [36-39]. A recent study showed inverse regulation of miR-148a and *DNMT1* expression during differentiation of mouse 3T3-L1 preadipocytes [34]. Our studies in SGBS cells replicated this finding. We also showed inverse and cell type-specific expression of miR-148a-3p and *DNMT1*. Experimental manipulation of miR-148a-3p levels in cells also modestly modulated *DNMT1* expression. Expression of *DNMT1* is likely to be modulated by many miRNA species. Indeed, miRTarBase provides evidence in support of six functional MTI (hsa-miR-148a-3p, hsa-miR-152-3p, hsa-miR-126-3p, hsa-miR-342-3p, hsa-miR-185-5p, and hsa-miR-140-5p) for *DNMT1*. Thus, manipulating expression of just one miRNA may only have a modest effect on overall *DNMT1* expression and cellular methylation levels [40]. However, miRNAs may regulate expression during translation, and lack of protein level expression data is a limitation of our study. Alteration of CpG site DNA methylation in regulatory regions of the genome was associated with obesity in humans [41]. Dietary or genetic perturbation may modulate miR-148a-3p expression and thereby cause obesity by modulating *DNMT1* levels and subsequently altering DNA methylation in adipose tissue. However, alternatively, the obesogenic factor may induce DNMT1 expression, which can hypermethylate the miR-148a-3p gene promoter and cause its down-regulation. A recent study showed that obesity induced induction of *DNMT1* selectively hypermethylates and stimulates compact chromatin structure in adiponectin (*ADIPOQ*) gene promoter, and impedes expression of adiponectin, a key adipokine involved in obesity mediated modulation of insulin sensitivity [42].

CONCLUSION

Obesity associated modulation of miR-148a-3p may also be indirectly involved in complex regulation of adiponectin level by altering DNMT1 level in adipose tissue. Further studies are needed to determine causality or timing of these events, and whether a feedback regulatory mechanism exists in cell type-specific regulation of miR-148a-3p and *DNMT1* levels in adipose tissue.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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DISCLOSER

The authors declare no conflict of interest. The views presented in this article are solely that of the authors and do not necessarily reflect those of the Food and Drug Administration

AUTHOR CONTRIBUTIONS

SKD designed the project, researched and analyzed data, and wrote, reviewed, and edited manuscript. NKS, VV and LM carried out experiments, researched and analyzed data and reviewed the manuscript. SJH analyzed data and reviewed the manuscript. All authors read and approved the final manuscript.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers web site along with the published article.

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