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MicroRNA-192* impairs adipocyte triglyceride storage

Raghavendra Mysore [a], You Zhou [a], Sanja Sädevirta [a,b], Hanna Savolainen-Peltonen [c,d], P.A. Nidhina Haridas [a], Jarkko Soronen [a,e], Marja Leivonen [f], Antti-Pekka Sarin [e,g], Pamela Fischer-Posovszky [h], Martin Wabitsch [h], Hannele Yki-Järvinen [a,b], Vesa M. Olkkonen [a,i,*]

[a] Minerva Foundation Institute for Medical Research, Biomedicum 2U, FI-00290 Helsinki, Finland
[b] Department of Medicine, University of Helsinki, FI-00014 Finland
[c] University of Helsinki and Helsinki University Hospital, Obstetrics and Gynecology, FI-00029 HUS, Helsinki, Finland
[d] Folkhälsan Research Center, FI-00290 Helsinki, Finland
[e] National Institute for Health and Welfare/Public Health Genomics Unit, Biomedicum, FI-00290 Helsinki, Finland
[f] Department of Surgery, Helsinki University Central Hospital, FI-000290 HUS, Helsinki, Finland
[g] Institute for Molecular Medicine Finland FIMM, University of Helsinki, FI-00014 Helsinki, Finland
[h] Division of Pediatric Endocrinology and Diabetes, Department of Pediatrics and Adolescent Medicine, Ulm University Medical Center, D-89075 Ulm, Germany
[i] Department of Anatomy, Faculty of Medicine, University of Helsinki, FI-00014 Helsinki, Finland

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ABSTRACT

We investigated the expression of miR-192* (miR-192-3p) in the visceral adipose tissue (VAT) of obese subjects and its function in cultured human adipocytes. This miRNA is a 3′ arm derived from the same pre-miRNA as miR-192 (miR-192-5p) implicated in type 2 diabetes, liver disease and cancers, and is predicted to target key genes in lipid metabolism. In morbidly obese subjects undergoing bariatric surgery preceded by a very low calorie diet, miR-192* in VAT correlated negatively ($r = -0.387$; $p = 0.046$) with serum triglyceride (TG) and positively with high-density lipoprotein (HDL) concentration ($r = 0.396$; $p = 0.041$). In a less obese patient cohort, the miRNA correlated negatively with the body mass index ($r = -0.537$; $p = 0.026$). To characterize the function of miR-192*, we overexpressed it in cultured adipocytes and analyzed the expression of adipogenic differentiation markers as well as cellular TG content. Reduced TG and expression of the adipocyte marker proteins aP2 (adipocyte protein 2) and perilipin 1 were observed. The function of miR-192* was further investigated by transcriptomic profiling of adipocytes expressing this miRNA, revealing impacts on key lipogenic genes. A number of the mRNA alterations were validated by qPCR. Western analysis confirmed a marked reduction of the lipogenic enzyme SCD (stearoyl coenzyme A desaturase-1), the fatty aldehyde dehydrogenase ALDH3A2 (aldehyde dehydrogenase 3 family member A2) and the high-density lipoprotein receptor SCARB1 (scavenger receptor B, type I). SCD and ALDH3A2 were demonstrated to be direct targets of miR-192*. To conclude, the present data identify miR-192* as a novel controller of adipocyte differentiation and lipid homeostasis.

1. Introduction

MicroRNAs (miRNAs) are short (19–23 nt) non-coding single-stranded RNA molecules that contribute to regulation of gene expression via mRNA deadenylation or destabilization and translational repression or activation [1]. Bioinformatic analyses have suggested that more than 50% of all genes are subject to regulation by miRNAs [2]. Pri-miRNA transcripts typically contain several miRNA precursors [3]. During the process of maturation, generally one arm of each processed double-stranded miRNA precursor becomes functional. The other, 'passenger' strand denoted with an asterisk (*) is normally degraded. However, in some cases both strands of the duplex become functional miRNAs that target different mRNA populations [3].

Visceral or abdominal adipose tissue represents a fat depot located within the abdominal cavity and accumulates in subjects with abdominal-type obesity. Visceral adipocytes have a higher rate of lipolysis, secrete pro-inflammatory cytokines, and are more insulin-resistant than subcutaneous adipocytes [4]. VAT drains directly through the portal circulation to the liver [5], and it has been hypothesized that non-esterified fatty acids as well as adipocytokines released by VAT may contribute to hepatic triglyceride storage, insulin resistance and elevated serum triglyceride (TG) concentration [6].

Human VAT miRNAs have been investigated in several studies, focusing on miRNA expression patterns in type 2 diabetes [7], obese

Abbreviations: ALDH3A2, aldehyde dehydrogenase 3 family member A2; aP2, adipocyte protein 2; BMI, body mass index; GLUT4, glucose transporter type 4; miRNA, microRNA; NT, non-targeting; PLIN, perilipin 1; qPCR, quantitative reverse transcriptase PCR; SCARB1, scavenger receptor B, type I; SCD, stearoyl coenzyme A desaturase-1; SGBS, Simpson–Golabi–Behmel syndrome; TG, triglyceride; VAT, visceral adipose tissue.

* Corresponding author at: Minerva Foundation Institute for Medical Research, Biomedicum 2U, Tukholmankatu 8, FI-00290 Helsinki, Finland.
E-mail address: vesa.olkkonen@helsinki.fi (V.M. Olkkonen).

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subjects [8–10], or subjects with non-alcoholic fatty liver disease/steatohepatitis [11,12]. In several cases, there is a negative correlation between the expression of miRNAs and their predicted protein targets in the VAT of obese subjects, consistent with physiologically relevant gene regulation by the miRNAs [15]. Yu et al. [13] demonstrated that peroxisome proliferator activated receptor-γ (PPAR-γ) agonist stimulation has in the subcutaneous and visceral fat depots distinct but overlapping effects on the expression of miRNAs that target the transforming growth factor-β (TGF-β), insulin and Wnt/β-catenin signaling pathways with key roles in the control of adipogenesis. Moreover, in vitro experiments with preadipocyte/adipocyte cultures have provided evidence for functional roles of a number of miRNAs in adipogenesis: miR-103, miR-320, and miR-1908 enhance adipogenesis while miR-146 and miR-194 decrease adipocyte differentiation [9,14–18]. Of note, correlation was shown between dysregulation of miRNAs targeting white adipose tissue adipocytokine expression and non-alcoholic steatohepatitis [11], consistent with the hypothesis that adipocytokines regulated by miRNAs and secreted by adipose tissue impact the pathogenesis of non-alcoholic fatty liver disease.

Dysregulation of miR-192 has been associated with a number of pathologic conditions including type 2 diabetes, liver diseases, and cancers [19–21]. Our preliminary analyses indicated that both miR-192 and its 'passenger' arm miR-192* are expressed at substantial and highly similar levels in the VAT of obese human subjects. However, the function of miR-192* has not been investigated, although target predictions suggest that this species regulates the expression of a number of key genes in lipid metabolism. We therefore surmised that miR-192* could serve as a regulator of adipocyte differentiation and/or lipid metabolism, and set out to investigate its function in a cultured human adipocyte model. The study identifies miR-192* as a novel controller of adipogenesis and adipocyte lipid homeostasis.

2. Materials and methods

2.1. Study subjects

The morbidly obese subjects (BMI 46.8 ± 6.3) were recruited amongst patients undergoing laparoscopic bariatric surgery at the Peijas Hospital of the Hospital District of Helsinki and Uusimaa. The following inclusion criteria were applied: (i) age 18–65 years; (ii) no known acute or chronic disease except for obesity or obesity related diseases such as type 2 diabetes, non-alcoholic fatty liver disease, cardiovascular diseases and/or hyperlipidemia; based on history, physical examination, standard laboratory tests (complete blood count, liver functions tests, creatinine, TSH, electrolyte and lipid concentrations as well as glucose, C-peptide, and insulin) and electrocardiogram. Exclusion criteria were: (i) excessive use of alcohol (over 20 g/day), (ii) use of hepatotoxic medications or herbal products, and (iii) pregnancy or lactation. The subjects were studied in the morning after an overnight (10–12 h) fast one to two weeks prior to surgery. Weight, height, waist and hip circumferences were recorded as previously described [22]. Blood samples were taken for measurement of concentrations of high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol, triglyceride, glucose, C-peptide, and insulin, as well as of alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) activities as described [22]. Before the operation, the patients adhered to a 6–12 week very low calory diet (<800 kcal/day).

In the less obese (BMI 31.2 ± 2.5) female cohort, 17 women undergoing elective gynecological surgery for non-malignant reasons in the department of Obstetrics and Gynecology, Helsinki University Hospital, were recruited. Patients were scheduled for abdominal or laparoscopic hysterectomy, salpingo-oophorectomy, diagnostic laparoscopy, enucleation of uterine myoma, enucleation of ovarian cyst, or laparoscopic colpo-sacro-rectopexy. Reasons for surgery included uterine myomas accompanied with menorrhagia and/or pain, benign ovarian cysts, infertility and dysmenorrhea, or rectocele. Blood samples were drawn before the operation. Serum was isolated by centrifugation and stored at −20 °C until the analyses (see above). The characteristics of the subjects are shown in Table 1.

2.2. VAT biopsies

At the time of surgery, biopsies of omental VAT were taken and snap-frozen in liquid N_2. The adipose tissue samples were stored at −80 °C until further processing.

2.3. Ethics

The nature and potential risks of the study were explained to all subjects prior to obtaining their written informed consent. The protocol was approved by the Medicinal Ethics Committee of the Helsinki and Uusimaa Hospital District. The Ethics committee operates according to the principles of good clinical research practice (ICH-GCP-E6) and in accordance with the international obligations concerning the status of research subjects and the rules and guidelines that govern research [Medical Research Act 488/1999, chapter 2a (23.4.2004/295), section 5 and 10a].

2.4. Isolation of total RNA from VAT

Frozen visceral fat (approximately 200 mg) was homogenized and total RNA was isolated and purified by using the Lipid Tissue miRNeasy Mini® Kit (Qiagen, Valencia, CA). The quality of the RNA was verified by Agilent 2100 Bioanalyzer® (Agilent, Santa Clara, CA). The isolated RNA was stored at −80 °C.

Table 1
Characteristics of the study subjects.

Subjects undergoing bariatric surgery.	
Gender (females/males)	18/9
Age (yrs)	48.0 ± 9.7
Body composition	
Body weight (kg)	133.5 ± 25.9
Body mass index (kg/m^2)	46.8 ± 6.3
Biochemical parameters	
fP-glucose (mmol/L)	5.8 ± 0.9
fS-insulin (mU/L)	11.9 (7.3–20.0)
fS-C-peptide (nmol/L)	1.1 ± 0.40
fS-LDL cholesterol (mmol/L)	2.8 ± 0.9
fS-HDL cholesterol (mmol/L)	1.2 ± 0.3
fS-TG (mmol/L)	1.3 ± 0.5
fS-ALT (U/L)	34 (22.0–51.5)
fS-AST (U/L)	30 (24.0–39.5)
fS-ALP (U/L)	69 (56.0–82.5)
Subjects undergoing gynecologic surgery	
Gender (females/males)	17/0
Age (yrs)	46.0 ± 9.5
Body composition	
Body weight (kg)	85.0 ± 9.0
Body mass index (kg/m^2)	31.2 ± 2.5
Biochemical parameters	
fS-LDL cholesterol (mmol/L)	3.4 ± 0.8
fS-HDL cholesterol (mmol/L)	1.4 ± 0.4
fS-TG (mmol/L)	1.6 ± 2.0
fS-AST (U/L)	33 (21–145)

Abbreviations: f, fasting; P, plasma; S, serum; LDL low-density lipoprotein; HDL, high-density lipoprotein; TG, triglycerides; ALT alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase. Data are shown as mean ± SD or median (25%–75% percentile), as appropriate.

Table 2
The oligonucleotide primers used.

Gene	Primer sequence (5′–3′)
36B4	Forward (F): CATGCTCAACATCTCCCCCTTCTCC
	Reverse (R): GGGAAGGTGTAATCCGTCTCCACAG
SDHA	F: CATGCTGCCGTGTTCCGTGTGGG
	R: GGACAGGGTGTGCTTCCTCCAGTGCTCC
Adiponectin	F: GGCCGTGATGGCAGAGAT
	R: CCTTCAGCCCGGGTACT
PPAR-γ	F: GATCCAGTGGTTGCAGATTACAA
	R: GAGGGAGTTGGAAGGCTCTTC
AP2	F: GCTTTTGTAGGTACCTGGAAACTT
	R: ACACTGATGATCATGTTAGGTTTGG
GLUT4	F: TGGGCGGCATGATTTCCTC
	R: GCCAGGACATTGTTGACCAC
SCARB1	F: CCTATCCCCTTCTATCTCTCCG
	R: GGATGTTGGGCATGACGATGT
FABP7	F: GGCTTTGCCACTAGGCAGG
	R: TGACCACTTTGTCTCCTTCTTGA
ALDH3A2	F: TGGGTATTGTAGCCGCTGTG
	R: ACCAACGCAGTCTGTGAACT
ALDH3B2	F: ATGAAGGATGAACCACGGTCC
	R: GTTCCAGGGTGCGATGATGA
CYP3A5	F: ATCGAAGGTCTTTAGGCCCAG
	R: CTTCCCGCCTCAAGTTTCTC
SCD	F: TGGAGAAGCGGTGGATAAC
	R: AAAAATCCCACCCAATCACA
MSR1	F: GCAGTGGGATCACTTTCACAA
	R: AGCTGTCATTGAGCGAGCATC
APOH	F: CCCAAGCCAGATGATTTACCAT
	R: ACAGTCCTGTGAGAGGGCA

2.5. Cell culture

Simpson–Golabi–Behmel syndrome (SGBS) cells represent a human adipocyte model that responds to insulin (glucose uptake) and beta-adrenergic stimuli (lipolysis) and thus resembles human primary adipocytes [23]. SGBS pre-adipocytes were cultured at 37 °C in 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) F12 (Sigma-Aldrich, St. Louis, MO) supplemented with 33 μM biotin, 17 μM pantothenate, 10% foetal bovine serum (Gibco/Life Technologies, Grand Island, NY), 100 I.U./ml penicillin, and 100 μg/ml streptomycin. Differentiation into adipocytes was carried out under controlled serum-free conditions by employing a previously established 14-day protocol [23,24]. HuH7 cells were cultured in AQMEM medium (Sigma-Aldrich), 10% fetal bovine serum, and the above antibiotics.

2.6. Generation of SGBS cells overexpressing miR-192*

SGBS preadipocytes were transduced with shMIMIC® miR-192* (VHS5841-101207549, Thermo Scientific, Waltham, MA) or a non-targeting control miRNA (HRM5872, Thermo Scientific) encoding lentiviral particles at a multiplicity of three I.U./cell in the above DMEM medium without serum, with 8.0 μg/ml hexa dimethrine bromide (Sigma-Aldrich). Growth medium was added after 4 h of incubation. The cells were grown in standard medium for 48 h without selection, after which the growth medium was replaced with a medium containing 0.5 μg/ml puromycin (Sigma-Aldrich). The cells were cultured in the presence of puromycin until the adipogenic differentiation process, during which no antibiotic selection was applied.

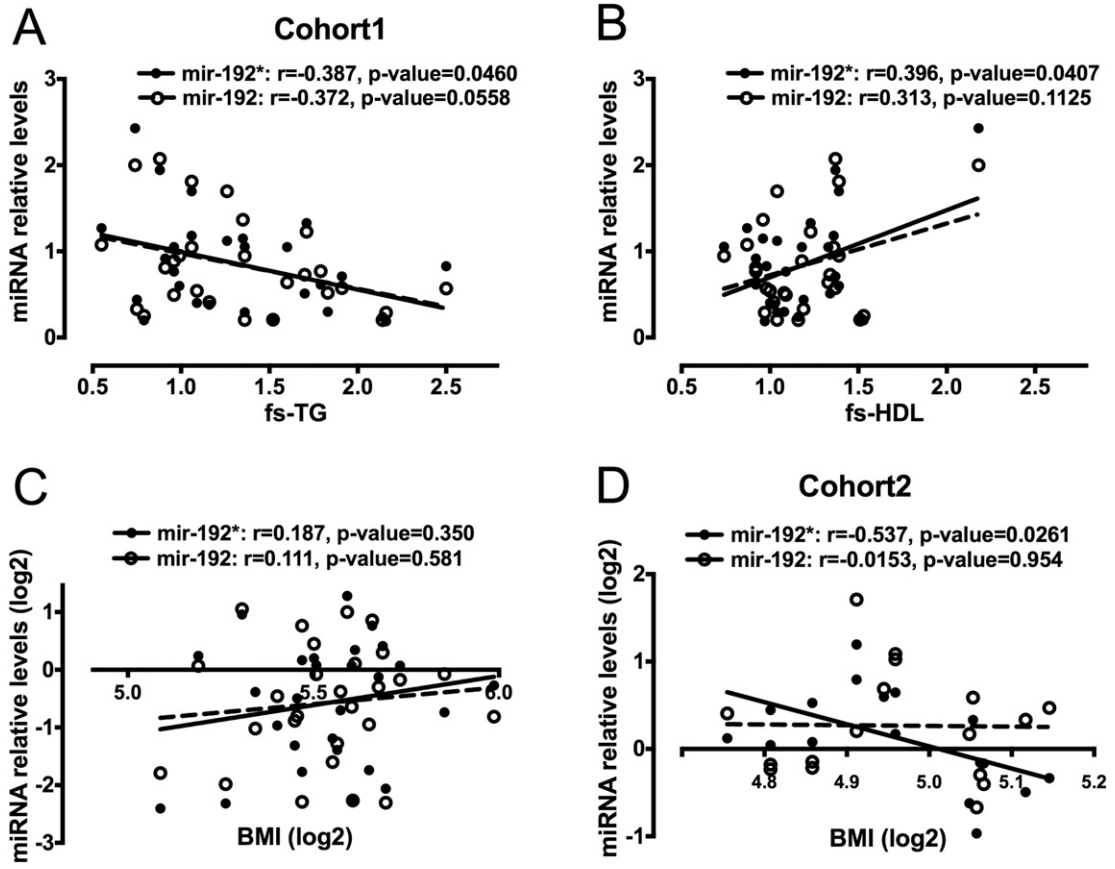

Fig. 1. Correlations of VAT miR-192* and miR-192 with serum TG, HDL-cholesterol and BMI. The two miRNA species were quantified by TaqMan qPCR by using RNU44 as a reference. A. Correlation of VAT miR-192* and miR-192 expression with fasting serum triglycerides (fS-TG) in cohort 1. B. Correlation of VAT miR-192* and miR-192 expression with fasting serum high-density-lipoprotein cholesterol (fS-HDL) in cohort 1. C. Correlation of VAT miR-192* expression with BMI in cohort 1. D. Correlation of VAT miR-192* expression with BMI in cohort 2. The r and p values are indicated. Cohort 1 = The morbidly obese subjects (N = 27); Cohort 2 = The less obese female cohort (N = 17).

2.7. Triglyceride determination of SGBS cells

For measurement of triglyceride (TG) content cells were lysed in 50 mM Tris–HCl (pH 7,4), 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1% SDS and Complete Mini® protease inhibitor cocktail (Roche Diagnostics, Basel Switzerland). Total protein content was determined with the DC assay (BioRad, Hercules, CA), and TGs were analyzed by using the GPO-PAP Triglyceride kit (Cobas, Roche/Hitachi, Tokyo, Japan).

2.8. RNA isolation and transcriptome profiling

SGBS adipocytes overexpressing miR-192* (designated SGBS-miR-192*) or a non-targeting control miRNA (SGBS-NT) were lysed in miRNeasy® lysis buffer, and total RNA was isolated and purified by using the miRNeasy Mini® Kit (Qiagen, Valencia, CA). The quality of RNA was verified by 2100 Bioanalyzer (Agilent). A modified standard Agilent 4x44K microarray (014850) containing 205 free positions (Agilent) was used for gene expression analysis. To these positions the EU FP7 consortium 'LipidomicNet' had added 201 probes corresponding to 119 lipid-related genes not previously present on the array. Total RNA (300 ng) was labeled with Cy3 using the Agilent Quick-Amp® Labeling Kit-1 color according to the manufacturer's instructions. cRNA was purified with the RNeasy Mini® Kit (Qiagen). The concentration and labeling efficiency of cRNA was measured with NanoDrop ND-1000 (PeqLab, Erlangen, Germany). The Agilent Gene Expression Hybridization Kit® was used for the hybridization and the arrays were incubated in Agilent SureHyb® chambers for 17 h in a hybridization oven at 65 °C while rotating. Washing was performed according to the manufacturer's instructions. Scanning was done with the Agilent G2565CA Microarray Scanner System®. The resulting TIFF files were processed with Agilent Feature Extraction® software 10.7. Generated raw data were analyzed using ChipInspector® software (Genomatix, Munich, Germany).

2.9. SGBS transcriptome data analysis

Background noise within arrays was corrected by using a normal-exponential convolution model [25]. Data were normalized by quantile method to ensure that intensities have the same empirical distribution across arrays. Box plot and density plot of data were used for quality control. Data were log2 transformed. Differential expression between SGBS-miR192* and SGBS-NT adipocytes was compared by empirical Bayes statistics. P-values were corrected for multiple testing by the Benjamini–Hochberg method. The data is deposited at http://www.ncbi.nlm.nih.gov/geo/ with access code GSE62951.

2.10. Bioinformatic analyses

Genes which differed significantly between SGBS-miR192* and SGBS-NT adipocytes were selected for Ingenuity Pathways Analysis® tool (IPA, Ingenuity Systems, Redwood City, CA, www.ingenuity.com). Briefly, enrichment of the genes in the pathways was assessed in comparison with a reference set in the whole Ingenuity pathway knowledge base. Fisher's exact test was used to determine the significance of each pathway and the Benjamini–Hochberg method was used to control for false discovery rate. Nine algorithms (RNA22, miRanda, miRDB, TargetScan, RNAhybrid, PITA, PICTAR, Diana-microT and miRWalk) were used to predict miRNA targets. miRNA binding sites were restricted to the 3′ UTR region. The minimum seed length for miRNA binding was 7 nucleotides. The other settings were default.

2.11. Quantitative real time reverse transcription PCR

Total RNA from SGBS cells (1.0 μg) was reverse transcribed using the Exiqon Universal® cDNA Synthesis Kit. Real-time qPCR was performed on LightCycler®480 (Roche Applied Science, Penzberg, Germany) using miRNA-specific primer sets (Exiqon) and the Roche SYBR-Green® master mix. Messenger RNAs were similarly quantified from cDNA preparations generated from the above total RNAs by using the VILO® reverse transcriptase kit (Invitrogen, Carlsbad, CA), and the gene-specific primer pairs specified in Table 2, by using 36B4 (acidic ribosomal phosphoprotein) and SDHA (succinate dehydrogenase complex, subunit A) as housekeeping references. Quantity of miR-192 and miR-192* in total RNA isolated from VAT biopsies and reverse transcribed with the Life Technologies/Applied Bioscience (Foster City, CA) TaqMan® microRNA Reverse Transcription kit was analyzed with miR-192 and miR-192*-specific TaqMan® MicroRNA assays by using RNU44 as a reference.

Table 3
Correlations of VAT miR-192* and miR-192 quantity with anthropometric and clinical parameters in morbidly obese subjects undergoing bariatric surgery.

	miRNA192*		miRNA192	
	r	p	r	p
Age	0.252	0.205	0.337	0.085
Weight	0.073	0.717	0.051	0.799
BMI	0.187	0.350	0.111	0.581
SBP	0.295	0.172	0.356	0.096
DIABP	0.281	0.193	0.292	0.176
fP-glucose	−0.047	0.820	0.128	0.533
fS-insulin	−0.081	0.687	−0.037	0.854
fS-C-peptide	−0.177	0.376	−0.114	0.572
fS-HbA1c	−0.260	0.199	−0.209	0.108
fS-TG	−0.387	0.046	−0.372	0.056
fS-HDL	0.396	0.041	0.313	0.112
fS-LDL	−0.054	0.789	−0.022	0.915
fS-AST	0.452	0.018	0.474	0.013
fS-ALT	0.330	0.092	0.353	0.071
fS-GGT	0.238	0.241	0.207	0.728
fS-ALP	0.013	0.948	0.010	0.962

Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DIABP, diastolic blood pressure; f, fasting; P, plasma; S, serum; HbA1c, glycated hemoglobin; TG, triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, γ-glutamyl transferase; ALP, alkaline phosphatase.

2.12. Validation of direct miRNA targets

SCD (HmiT016583-MT06) and ALDH3A2 (HmiT005407-MT06) 3'UTR luciferase constructs were obtained from GeneCopoeia (Rockville, MD). The predicted miR-192* target sequences cggtgtTTGGCA (SCD) and tcataaAATTGGCA (ALDH3A2) were mutagenized to cggtgtAAAAAT and tcataaCCCCCCCC, respectively. The constructs (0.5 μg DNA/well, SCD and ALDH3A) were transfected into HuH7 cells in 24-wells together with 20 nM Syn-hsa-miR-192-3p (miR-192*) miScript® miRNA mimics (Qiagen) or a non-targeting control by using Lipofectamine 2000® (Invitrogen). After 24 h firefly and Renilla luciferase activities were

Table 4
Correlations of VAT miR-192* and miR-192 quantity with anthropometric and clinical parameters in less obese/overweight female subjects undergoing gynecologic surgery.

	miRNA192*		miRNA192	
	r	p	*r*	p
Age	0.247	0.339	0.068	0.797
Weight	−0.393	0.118	0.0814	0.756
BMI	−0.537	0.026	−0.015	0.954
fS-TG	0.223	0.390	0.105	0.689
fS-HDL	−0.334	0.190	−0.469	0.057
fS-LDL	−0.282	0.291	−0.134	0.620
fS-AST	0.252	0.329	0.284	0.269

Abbreviations: BMI, body mass index; f, fasting; P, plasma; S, serum; TG, triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein; AST, aspartate aminotransferase.

Table 5
Comparison of relative VAT miR-192* and miR-192 expression levels between the morbidly obese Cohort 1 and the less obese/overweight Cohort 2, as well as between BMI < 30 and >30 subjects within Cohort 2.

	miR-192*	miR-192
Cohort 1 (N = 27)	0.94 ± 0.7[§]	0.97 ± 0.8
Cohort 2 (N = 17)	1.17 ± 0.4; p = 0.27	1.32 ± 0.6; p = 0.16
Cohort 2; BMI < 30 (N = 8)	1.43 ± 0.4	1.35 ± 0.8
Cohort 2; BMI > 30 (N = 9)	0.93 ± 0.3; p = 0.01	1.29 ± 0.5; p = 0.84

[§] Arbitrary units; the data represents mean ± SD; relative miRNA quantities were determined by using RNU44 as a reference. The p-values indicate the significance of differences between Cohorts 1 and 2 (the 3rd row) or between the subgroups of Cohort 2 (the bottom row).

determined by using the Dual-Luciferase Reporter Assay system and a Glomax 96 Microplate Luminometer (Promega, Madison, WI). The firefly luciferase activity values were normalized using the Renilla values.

2.13. Antibodies and Western blotting

Rabbit ALDH3A2 (HPA014769), aP2 (HPA002188) and β-actin (A2066) antibodies were from Sigma-Aldrich (St. Louis, MO), anti-PLIN (PAI-46,158) from Thermo Scientific, anti-SR-B1 (SCARB1; NB400-104) from Novus Biologicals (Littleton, CO), and anti-SCD (#2438) from Cell Signaling Technologies (Danvers, MA). Total protein extracts of SGBS adipocytes were separated on 12% Laemmli gels and transferred onto Protran® nitrocellulose membranes (Fisher Scientific, Waltham, MA). Bound primary antibodies were detected with secondary antibody-HRP conjugates (Jackson ImmunoResearch, West Grove,

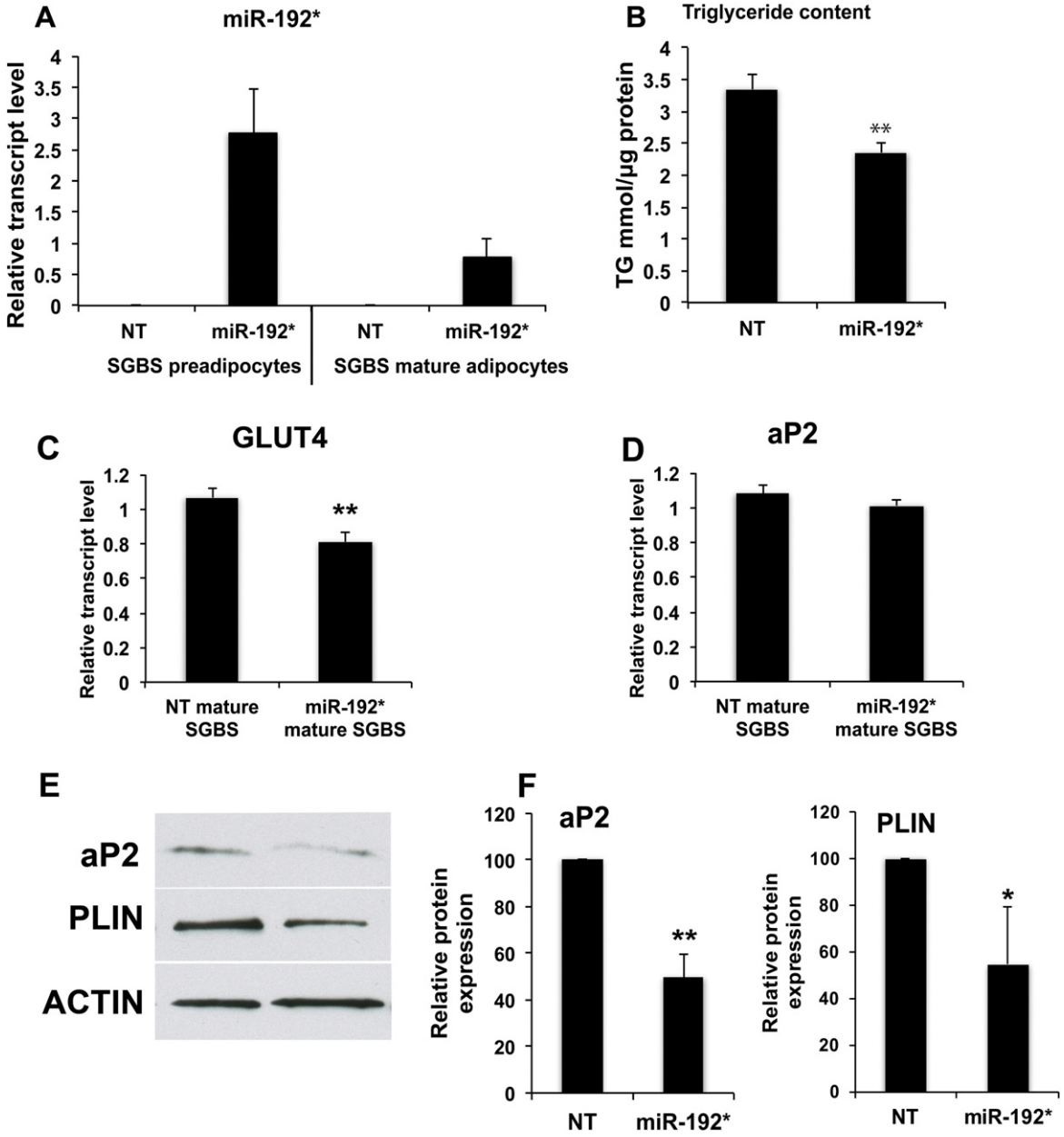

Fig. 2. SGBS adipocytes overexpressing miR-192* display defects in adipogenic differentiation and TG storage. A. qPCR analysis of miR-192* expression in the non-targeting miRNA (NT) or miR-192* (miR-192*) expressing preadipocytes and mature adipocytes. The data represents mean ± SD (N = 3). B. The TG content of mature SGBS-NT and SGBS-miR-192* adipocytes (N = 6, from two independently transduced cell pools, **p < 0.01). C-D. mRNA expression of the adipocytic differentiation markers GLUT4 (C) and aP2 (D) in mature NT and miR-192* expressing adipocytes (N = 3). E–F. Western analysis of aP2 and perilipin 1 (PLIN) expression in mature NT and miR-192* adipocytes; representative blots (E) and quantification (F) are shown. The data was normalized for the β-actin (ACTIN) signal (N = 3–4; *p < 0.05, **p < 0.01).

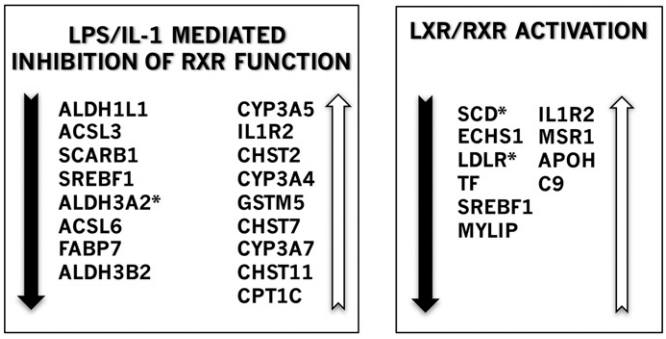

Fig. 3. The canonical pathways and their constituent genes significantly affected by miR-192* overexpression in SGBS adipocytes. Down- and upregulated mRNAs are indicated with arrows. mRNAs predicted by a minimum of two algorithms to be direct targets of miR-192* are marked with an asterisk.

PA) and visualized by enhanced chemiluminescence (ECL, Thermo Scientific). The signals were quantified by densitometry and normalized for β-actin.

2.14. Statistical analyses

Clinical parameters were tested for normality of distribution using a Kolmogorov–Smirnov test. Normally distributed data are shown as means ± standard error of mean (SEM) and non-normally distributed data are shown as median (25%–75% percentile). Correlations of VAT miR-192* and miR-192 expression levels with each other and with clinical-biochemical parameters were analyzed with Spearman's test. The significance of qPCR and Western findings was evaluated with Student's T-test (unpaired, two-tailed).

3. Results

3.1. miR-192* correlates negatively with serum triglycerides and positively with HDL in morbidly obese subjects.

Our analyses of visceral adipose tissue miRNAs in obese patients undergoing bariatric surgery revealed a negative correlation of miR-192* expression in VAT with serum TG concentration ($r = -0.387$; $p = 0.046$) (Fig. 1A), and a positive one with serum HDL cholesterol ($r = 0.396$, $p = 0.041$) (Fig. 1B) and aspartate aminotransferase (AST; $r = 0.452$, $p = 0.018$) (Table 3). A similar tendency of correlation with TG and HDL was observed for miR-192; however, it did not reach statistical significance (Fig. 1A, B), while miR-192 did correlate significantly with AST (Table 3). The abundances of miR-192 and miR-192* processed from the same pre-miRNA showed a strong positive correlation ($r = 0.919$; $p < 0.0001$). Inclusion of gender, age or BMI as covariates in analysis of variance did not significantly change the correlation between miR-192* and HDL/TG (data not shown). No correlation of miR-192* with BMI was observed in the morbidly obese cohort (Fig. 1C).

3.2. miR-192* correlates negatively with body mass index in less obese/overweight subjects

Further correlation analyses were carried out using VAT biopsies from female patients with a lower BMI (31.2 ± 2.5). Interestingly, no significant correlation of miR-192* with serum HDL, TG or AST was observed here (Table 4). However, the VAT miR-192* expression in this cohort showed a significant negative correlation ($r = -0.537$; $p = 0.026$) with BMI (Fig. 1D). Comparison of the relative VAT miR-192* and miR-192 quantities between the morbidly obese cohort and the less obese/overweight gynecological surgery patients revealed no significant difference. However, when the female cohort with a lower BMI was divided into two subgroups consisting of subjects with BMI < 30 or >30, significantly lower miR-192* quantity was observed in the BMI > 30 subgroup (Table 5).

Prompted by the above observations and results of target predictions, further studies were focused on the 3′ arm species miR-192*. Target predictions for miR-192* suggested that it regulates the expression of several key genes of lipid metabolism such as low-density lipoprotein receptor (LDLR), peroxisome proliferator activate receptor α (PPARA), patatin-like phospholipase domain containing 3 (PNPLA3), caveolins 1 and 2 (CAV1, CAV2), Krüppel-like factor 6 (KLF6), aldehyde dehydrogenase 3 family member A2 (ALDH3A2), and stearoyl coenzyme A desaturase 1 (SCD).

3.3. Characterization of human adipocytes overexpressing miR-192*

To assess the functional role of miR-192* in human adipocytes, its effects on adipocyte differentiation and triglyceride content were studied. We stably overexpressed this miRNA or a non-targeting control miRNA with a lentiviral vector in SGBS preadipocytes (designated SGBS-miR-192* and SGBS-NT, respectively), and induced their adipocytic differentiation. qPCR analysis verified overexpression of miR-192* in both SGBS-miR-192* preadipocytes and mature adipocytes (Fig. 2A). miR-192* expression impaired the adipocyte differentiation as evidenced by a 25% reduction in the cellular TG content in SGBS-miR-192* relative to the control cells (Fig. 2B). This effect was further confirmed by analyzing the expression of adipocyte markers. A significant reduction of GLUT4 mRNA and a similar tendency for the aP2 message, which did not quite reach statistical significance, were observed in the SGBS-miR-192* adipocytes (Fig. 2C, D). Western blot analysis demonstrated a marked reduction of the aP2 protein and of the lipid droplet surface protein perilipin 1 (Fig. 2E, F), the mRNA of which was not affected (data not shown). Collectively, these results suggested a moderate but clear defect in the adipocytic differentiation and TG storage of SGBS-miR-192*.

3.4. Alterations in the transcriptome of SGBS adipocytes overexpressing miR-192*

To identify the targets of miR-192* by which it regulates adipogenesis, a transcriptome profiling of SGBS-miR-192* ($n = 4$) and SGBS-NT ($n = 4$) adipocytes was carried out by using Agilent microarrays. A total of 637 mRNAs were found differentially expressed (Benjamini-Hochberg-corrected $p < 0.1$) between the SGBS-miR-192* and control cells. Of the 167 mRNAs down-regulated in SGBS-miR-192*, 31.1% overlapped with the predicted targets of miR-192*. The differentially expressed mRNAs were subjected to Ingenuity pathway analysis. The canonical pathways significantly affected by miR-192* overexpression were 'LPS/IL-1 mediated inhibition of RXR function' and 'LXR/RXR activation' (Fig. 3). In these pathways, a total of 14 mRNAs were significantly suppressed and 13 upregulated in SGBS-miR-192* as compared to SGBS-NT. Of note, the mRNAs suppressed in SGBS-miR-192* included a number of key lipogenic genes, such as ACSL3 (acyl-coenzyme A synthetase long chain-3), SREBF1 (sterol regulatory element binding protein-1), ACSL6, FABP7 (fatty acyl binding protein-7), and SCD (stearoyl coenzyme A desaturase-1). A subset of the downregulated mRNAs in the affected pathways [ALDH3A2, SCD, and LDLR (low-density lipoprotein receptor)] were predicted by a minimum of two different algorithms to be direct targets of miR-192* (Fig. 3). Besides the mRNAs suppressed in SGBS-miR-192*, a number of mRNAs in the above pathways were significantly upregulated, including CYP3A5 (cytochrome P450 3A5), IL1R2 (interleukin-1 receptor type II), MSR1 (macrophage scavenger receptor 1/scavenger receptor A), APOH (apolipoprotein H) and C9 (complement component 9).

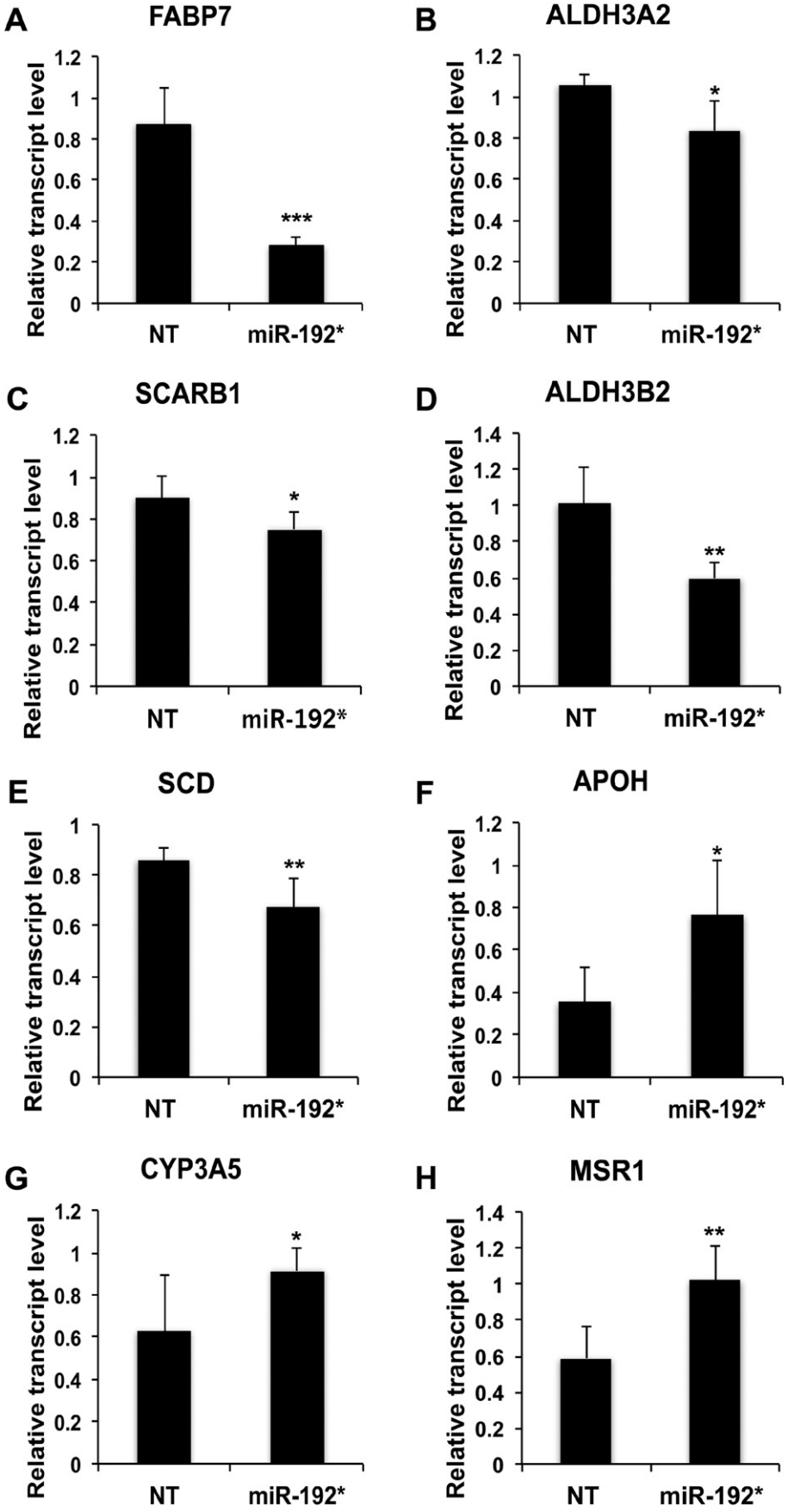

Fig. 4. Validation of changes in expression of key genes in the affected pathways by qPCR. Total RNA from SGBS-NT (NT) and SGBS-miR-192* (miR-192*) adipocytes was reverse transcribed and subjected to qPCR analysis. The genes are identified at the top. The data represents mean ± SD (N = 6 from two independently transduced cell pools). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

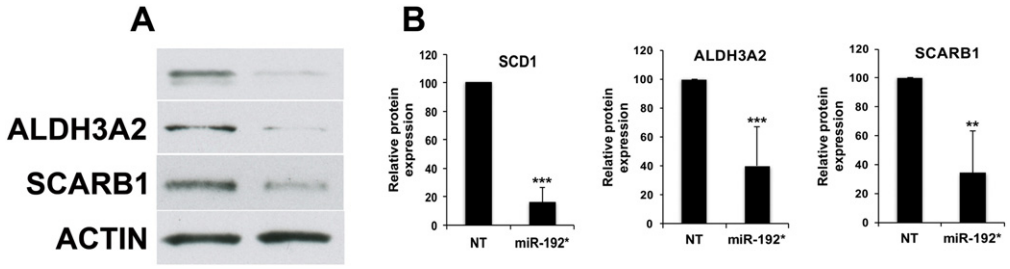

Fig. 5. Validation of miR-192* effects in SGBS adipocytes at the protein level. A. Total protein extracts (15 μg protein/lane) of mature SGBS-NT and SGBS-miR-192* (identified at the bottom) adipocytes were Western blotted with the indicated antibodies (SCD, ALDH3A2, SCARB1) and anti-β-actin. B. Quantification of the Western findings. The signals were analyzed by densitometry and normalized for β-actin. The data represents mean ± SD (N = 3–4); $^{**}p < 0.01$, $^{***}p < 0.001$.

3.5. miR-192 modulates the expression of genes associated with lipid metabolism.*

To validate gene expression changes observed in the microarray transcriptome profiling and in predicted targets of miR-192*, mRNA and protein expression in SGBS-miR-192* and SGBS-NT adipocytes were assessed by qPCR and Western blotting, respectively. Significant reduction of the FABP7, ALDH3A2, SCARB1, ALDH3B2, SCD and increase of APOH, CYP3A5, and MRS1 mRNAs was detected in the SGBS-miR-192* adipocytes (Fig. 4). Western blotting revealed significant decreases of the SCD, ALDH3A2, SCARB1 (SR-B1) proteins in SGBS-miR-192* as compared to the SGBS-NT adipocytes (Fig. 5).

To confirm that SCD and ALDH3A2 indeed represent direct targets of miR-192*, we obtained luciferase-3′-untranslated region (3'UTR) reporter constructs for these mRNAs, and transfected them into HuH7 cells together with miR-192* mimics or a non-targeting control RNA, followed by luminescence measurements by employing a dual luciferase assay system. For both SCD and ALDH3A2 3'UTR reporter constructs a significant reduction of firefly luciferase activity by the corresponding miRNA mimics was observed (Fig. 6). Of note, in both 3'UTRs mutagenesis of sequence complementary to the miR-192* seed region resulted in a significant attenuation of the miRNA effect, providing evidence that SCD and ALDH3A2 are subject to direct regulation by miR-192*.

4. Discussion

In the present study we investigated the expression of miR-192* (miR-192-3p) in the visceral adipose tissue (VAT) of obese human subjects and its function in cultured human adipocytes. This species is the 3′ arm produced from the same pre-miRNA as miR-192 implicated type 2 diabetes, liver disease and cancers. The quantity of miR-192* in VAT correlated in morbidly obese subjects negatively with serum triglyceride content and positively with HDL-cholesterol and AST. Of note, these correlations were absent in a female patient cohort with a lower BMI. In this cohort, however, the quantity of VAT miR-192* showed a negative correlation with BMI. Although the expression and function of miR-192 in type 2 diabetes and cancers have been extensively characterized, the functions of the 3′ arm, miR-192*, are poorly known. The only published study on this miRNA species reported its down-regulation in duodenal biopsies of coeliac patients and modulation of the levels of the miRNA in duodenal fibroblasts by gliadin peptides [26]. We demonstrated that stable overexpression of miR-192* in cultured human SGBS adipocytes impaired their adipocytic differentiation as evidenced by a decreased TG content and reduced expression of the adipocyte marker proteins aP2 and perilipin 1. aP2, also known as FABP4 (adipocyte fatty acid binding protein), plays a central role in fatty acid incorporation into TGs and is a widely used marker of adipocytes [27], while PLIN is a lipid droplet surface component induced during adipogenesis

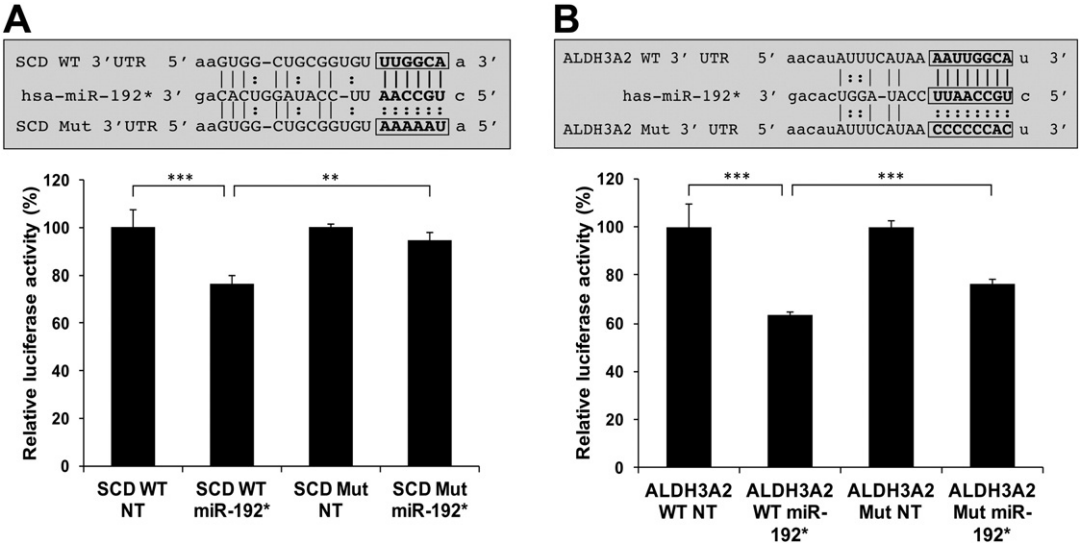

Fig. 6. SCD and ALDH3A2 are direct targets of miR-192*. A. SCD Top: Schematic presentation of the luciferase-SCD 3′UTR construct, the sequence predicted to be targeted by miR-192*, and the mutagenized nucleotide residues. Bottom: Relative reporter luciferase activity (%). B. ALDH3A2 Top: Schematic presentation of the luciferase-ALDH3A2 3′UTR construct, the sequence predicted to be targeted by miR-192*, and the mutagenized nucleotide residues. Bottom: Relative reporter luciferase activity. WT, wild-type 3′UTR; Mut, mutagenized 3'UTR; NT, non-targeting RNA; miR-192*, miR-192* mimic. The data represents mean ± SD (SCD, N = 7; ALDH3A, N = 6); $^{**}p < 0.01$, $^{***}p < 0.001$.

[28]. Individuals that carry the T87C polymorphism in the aP2 locus leading to reduced gene expression have lower serum TG levels and significantly reduced risk for coronary heart disease and type 2 diabetes as compared with subjects homozygous for the wild-type allele [29]. Albeit aP2 appears not to be a direct target of miR-192*, indirect regulation of adipocyte aP2 expression by this miRNA may be relevant for human cardiometabolic disease.

Transcriptome profiling and pathway analysis of differentially expressed genes in miR-192* overexpressing adipocytes and those expressing a non-targeting control miRNA revealed two significantly altered canonical lipid regulatory pathways,'LXR/RXR activation' and 'LPS/IL-1 mediated inhibition of RXR function'.'LXR/RXR activation' is a lipogenic pathway that enhances adipose tissue differentiation and lipid storage via stimulation of PPARγ [30]. Also the 'LPS/IL-1 mediated inhibition of RXR function' pathway contains a number of key lipogenic genes, including ACSL3, SREBF1, ACSL6, and FABP7, which were downregulated in the miR-192* expressing cells. Of the genes affected by miR-192*, down-regulation of SCD, ALDH3A2 and SCARB1 (SR-B1) was verified at the protein level. Moreover, SCD and ALDH3A2 were demonstrated to be direct targets of miR-192*. Of these, SCD is a central lipogenic enzyme that catalyzes the synthesis of monounsaturated fatty acids [31]. Mice with targeted disruption of SCD display reduced adiposity and insulin sensitivity and are resistant to diet-induced weight gain [32]. Moreover, a lipogenic diet in these animals failed to induce TG synthesis in the liver [33] and liver-specific SCD knock-out mice were protected from diet-induced obesity and hepatic steatosis [34], revealing a stringent requirement of endogenous SCD activity and monounsaturated fatty acids for TG synthesis. Adipose-specific deletion of SCD in mouse resulted in enhanced basal GLUT1-mediated glucose uptake into AT and reduction of adiponectin [35], an adipokine that promotes hepatic insulin sensitivity and fatty acid oxidation, and dampens inflammatory signaling [36]. Inhibition of SCD in 3 T3-L1 adipocytes resulted in a downregulation of several lipogenic genes and a 33% reduction in cellular TG content [37]. Interestingly, SCD was recently also shown to regulate inflammatory gene expression by changing DNA methylation in cultured adipocytes [38]. Expression of SCD was decreased in the VAT of morbidly obese insulin resistant human subjects as compared to overweight controls [39]. One can thus envision that suppression of SCD by miR-192* in the VAT could dampen the synthesis of TGs and enhance the flux of non-esterified fatty acids and inflammatory signals towards the liver.

ALDH3A2 is a fatty aldehyde dehydrogenase that converts long-chain aliphatic aldehydes to fatty acids [40]. This enzyme detoxifies oxidized lipid species, and its expression is decreased in murine models of insulin resistance [41]. Its suppression may thus promote lipotoxicity, which could result in reduced insulin sensitivity and elevated pro-inflammatory signaling in VAT.

SCARB1 (SR-B1) is a receptor for high-density lipoproteins (HDL) in several cell types [42], the function of which in adipocytes is not well studied. However, there is evidence that SCARB1 mediates the anti-inflammatory action and enhancement of glucose uptake by HDL in adipocytes [43,44]. Suppression of SCARB1 could thus interfere with the anti-inflammatory and pro-lipogenic actions of HDL in VAT.

In addition to the gene products suppressed by miR-192*, a number of mRNAs including CYP3A5, APOH and MSR1 (validated by qPCR), were found up-regulated in the miR-192* expressing adipocytes. This is consistent with the view that alterations in cellular miRNAs result in a multitude of complex, both direct and indirect effects on gene expression [45].

Serum TG concentration is determined by the rate of very-low-density lipoprotein (VLDL) production by the liver and clearance of chylomicron and VLDL TGs upon lipolysis of the TG-rich lipoproteins [46,47], and a strong inverse correlation exists between serum TG and HDL cholesterol levels due to intimate metabolic links between the two lipoprotein classes [48]. Elevated TGs associated with a low HDL-cholesterol concentration form a cardiovascular risk factor associated with obesity and the metabolic syndrome [49,50], Interestingly, a negative correlation between VAT miR-192* quantity and serum TG concentration was observed in the morbidly obese subjects while a positive correlation was evident with HDL cholesterol, whereas no correlation with LDL cholesterol was observed. One can envision that VAT miR-192* could via its metabolic effects either modulate VLDL and/or HDL production by the liver, which is closely connected with VAT via the portal circulation [5,6], or affect the metabolism of VLDL in a manner resulting in a sequential impact on HDL-cholesterol [49,50]. Interestingly, both miR-192* and miR-192 showed in the morbidly obese subjects a positive correlation with AST, a liver enzyme acting as a biomarker for hepatic damage. This is consistent with the notion that elevated expression of miR-192* may result in a defect in VAT fat storage and increased flux of lipid precursors towards the liver, leading to a metabolic status that involves liver dysfunction [51,52]. However, one cannot exclude the possibility that the miRNAs and serum AST are independent of each other but affected in the same direction by a third physiologic factor.

In a female cohort with a lower BMI, the correlations of miR-192* with HDL, TG and AST were absent, suggesting that these correlations are associated with the metabolic status of the morbidly obese bariatric surgery patients. We also cannot exclude the possibility that they could be affected by the very low calorie diet the subjects were advised to adhere to before surgery. Similar to the morbidly obese cohort, no correlation with LDL cholesterol was observed. Interestingly, VAT miR-192* quantity correlated negatively with BMI in the less obese female cohort. This observation is consistent with the cultured adipocyte data suggesting that miR-192* negatively regulates adipogenesis. No such tendency of correlation with BMI was seen in the subjects undergoing bariatric surgery; we find it likely that morbid obesity represents an altered metabolic status in which this type of subtle regulatory connections are no longer detectable. Comparison of miR-192* expression levels between the two cohorts revealed no significant difference. However, when the female cohort with a lower BMI was divided into two subgroups with BMI < 30 or >30, lower miR-192* expression was observed in the BMI > 30 subgroup, further supporting the notion that this miRNA species dampens lipid storage in adipocytes.

In order to assess a putative role of miR-192* in metabolic disease, it would be highly interesting to compare its abundance and the expression levels of its targets between VAT from lean and obese subjects. However, due to ethical limitations we were unable to perform this analysis in the present study.

In conclusion, we investigated the expression of miR-192* and miR-192 in human VAT biopsies and characterized the function of miR-192* in a cultured adipocyte model. VAT miR-192* expression correlated in morbidly obese human subjects negatively with serum TGs and positively with HDL, and displayed a negative correlation with BMI in a less obese patient cohort. Stable overexpression of this miRNA in cultured human adipocytes decreased the adipocyte TG content and expression of adipocyte markers as well as of distinct lipogenic genes. These findings identify miR-192* as a novel controller of adipocyte differentiation and lipid homeostasis.

Conflict of interest

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

Transparency document

The Transparency document associated with this article can be found, in the online version.

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