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ARTICLE

Human adipose microRNA-221 is upregulated in obesity and affects fat metabolism downstream of leptin and TNF-α

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Abstract

Aims/hypothesis MicroRNAs (miRNAs) are short endogenous RNAs that regulate multiple biological processes including adipogenesis and fat metabolism. We sought to identify miRNAs that correlate with BMI and to elucidate their upstream regulation and downstream targets.

Methods Microarray-based expression profiling of 233 miRNAs was performed on subcutaneous abdominal adipose tissue biopsies from 29 non-diabetic Pima Indian participants. Correlation of the expression levels of eight miRNAs with BMI was assessed by quantitative reverse transcription (QRT) PCR in adipose samples from 80 non-diabetic Pima Indians with a BMI of 21.6–54.0 kg/m^2. The upstream regulation of one of these miRNAs, miR-221, was tested by treating cultured human pre-adipocytes with leptin, TNF-α and insulin. Predicted targets of miR-221 were validated using QRT-PCR, immunoblots and luciferase assays. The downstream effects of miR-221 overexpression were assayed by proteomic analysis.

Results Expression levels of miR-221 were positively correlated with BMI (particularly in women) and fasting insulin concentrations, while the levels of miR-193a-3p and miR-193b-5p were negatively correlated with BMI; other miRNAs did not show significant associations in the 80 samples. miR-221 was downregulated by leptin and TNF-α treatment in cultured human pre-adipocytes. Conversely, miR-221 overexpression upregulated several proteins involved in fat metabolism, mimicking peroxisome proliferator-activated receptor (PPAR) activation. Furthermore, miR-221 directly downregulated the adiponectin receptor 1 (ADIPOR1) and the transcription factor v-ets erythroblastosis virus E26 oncogene homolog 1 (ETS1). Adiponectin signalling is known to promote insulin sensitivity, and ETS1 is crucial for angiogenesis.

Conclusions/interpretation Our data suggest that miR-221 may contribute to the development of the insulin resistance that typically accompanies obesity, by affecting PPAR signalling pathways and by directly downregulating ADIPOR1 and ETS1.

Keywords ADIPOR1 · ETS1 · Insulin resistance · Leptin · MicroRNA · miR-221 · Obesity · Pima · Tumour necrosis factor

Abbreviations

ADIPOR1	Adiponectin receptor 1
AKR	Aldoketoreductase
ETS1	v-ets Erythroblastosis virus E26 oncogene homologue 1
FASN	Fatty acid synthase
HOMA1-IR	Homeostasis model assessment 1 of insulin resistance
HRP	Horseradish peroxidase
miRNA	MicroRNA
MMP	Matrix metalloproteinase
NIDDK	National Institute of Diabetes and Digestive and Kidney Diseases
NIH	National Institutes of Health
PPAR	Peroxisome proliferator-activated receptor
pri-miRNA	Primary miRNA
QRT	Quantitative reverse transcription
SAT	Subcutaneous adipose tissue
UTR	Untranslated region
VEGF	Vascular endothelial growth factor

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Introduction

Recent studies report the functional involvement of microRNAs (miRNAs) in metabolic and endocrine pathways [1–3] and adipogenesis [4–8]. Differential expression of miRNAs has been reported in the tissues of obese vs non-obese human individuals [2, 6, 9, 10] and in humans and animals with diabetes [11, 12]. However, the mechanisms of action of most miRNAs that are deregulated in obesity are unknown.

We used a hypothesis-free approach to identify miRNAs in adipose tissue with expression levels that correlated with BMI in Pima Indian individuals. We identified miR-221 as a highly expressed candidate miRNA that was upregulated in individuals with the highest BMIs, in agreement with previous studies [6, 13]. We then investigated the cause of miR-221 upregulation in obesity and assessed possible molecular mechanisms mediated by downstream targets of miR-221.

Methods

Participants and biopsy collection Participants were predominately of Pima Indian heritage and all were free of diabetes. Biopsies and clinical data were available for 80 healthy individuals who were not taking any medication (see electronic supplementary material [ESM] Table 1). Participants were admitted to the Clinical Research Unit, NIDDK, Phoenix, AZ, USA, where they consumed a weight-maintaining diet (containing 50% of calories as carbohydrates, 30% as fat and 20% as protein) for 2–3 days prior to clinical testing. A 3 h 75 g OGTT and abdominal subcutaneous adipose tissue (SAT) needle biopsies under local anaesthesia with 1% lidocaine were performed after a 12 h overnight fast. Variants in the miR-221 promoter were analysed for association with BMI by genotyping in a population-based sample of 3,500 Pima Indians (including 80 who underwent adipose tissue biopsies). Informed consent was obtained for all participants, and the study was approved by the institutional review board of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of Health (NIH).

RNA extraction, microRNA expression profiling and validation Total RNA was extracted from 80 frozen SAT biopsies using the miRNeasy kit (Qiagen, Venlo, the Netherlands), quantified with a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and its integrity assayed on an Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). Samples (*n*=29) showing the highest quality RNA were labelled with the 3DNA FlashTag Biotin RNA Labeling kit (Genisphere, Hatfield, PA, USA) and hybridised with GeneChip miRNA arrays (Affymetrix, Santa Clara, CA, USA) for expression profiling. Three random samples were rehybridised to new arrays to ascertain reproducibility. The array cartridges were processed on an Affymetrix Fluidics Station 450 and scanned on an Affymetrix GeneChip 3000 7G with AutoLoader. The results were normalised using the Affymetrix miRNA QC tool and analysed with MeV software v4.3 [14] (Dana Farber Cancer Institute, Boston, MA, USA) and Excel 2003 (Microsoft, Redmond, WA, USA).

Expression levels of specific miRNAs were validated in all 80 RNA samples by quantitative reverse transcription (QRT) PCR using TaqMan assays (Life Technologies, Carlsbad, CA, USA) on an ABI 7900HT Fast QPCR system (Life Technologies). U6 and U44 small nuclear RNAs were used for normalisation. The differential expression of specific miRNAs was correlated to individuals' BMI and insulin and glucose levels during the OGTT. The homeostasis model assessment 1 of insulin resistance (HOMA1-IR) was calculated as previously described [15]. miRNA target prediction used the TargetScan algorithm (for a list of predicted miR-221 targets, see www.targetscan.org/cgi-bin/targetscan/vert_61/targetscan.cgi?species=Human&gid=&mir_sc=miR-221/222/222ab/1928&mir_c=&mir_nc=&mirg=&sortType=cs&allTxs=&incl_nc=All) [16, 17].

Cell culture, treatment and transfection Primary human pre-adipocytes (Zen-Bio, Research Triangle, NC, USA) and SH-SY5Y human neuroblastoma-derived cells (American Type Culture Collection (ATCC), Manassas, VA, USA) were cultured in MEM/F-12 1:1 containing 10% FBS (ATCC). Cells were serum-starved for 24 h and then treated with recombinant human leptin (L4146; Sigma, St Louis, MO, USA) at concentrations of 0, 50, 200 and 500 ng/ml, or TNF-α (H8916; Sigma) at concentrations of 5–50 ng/ml, for 24 h prior to harvesting. Pre-adipocyte differentiation was induced in DM-2 medium (Zen-Bio) for more than 10 days. TNF-α treatment of differentiated adipocytes was for 10 or 16 days, at 10 or 100 ng/ml [18]. Insulin treatment was at 100 nmol/l for 1 h and 24 h. Total RNA was extracted using the miRNeasy kit (Qiagen).

For 3′ untranslated (UTR) reporter assays, JetPrime (Polyplus-transfection SA, Illkirch, France) was used to co-transfect HEK 293 cells (ATCC) with miRNA 3′ UTR target expression vectors for human *ETS1*, *ADIPOR1*, *ADIPOR2* or control (Genecopoeia, Germantown, MD, USA), and human miR-221 mimic (Dharmacon, Lafayette, CO, USA) or scrambled control oligonucleotide, in 24-well plates. Cells were lysed 48 h post-transfection, and dual-luciferase readings were performed using the Genecopoeia Luc-Pair miR Luciferase Assay Kit on a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA, USA). Transfection of pre-adipocytes for immunoblotting and proteomic analysis was in six-well plates and 75 cm^2 flasks, respectively.

Immunoblots We used Bio-Rad (Hercules, CA, USA) equipment and reagents for immunoblots, and the following antibodies: adiponectin receptor 1 (ADIPOR1; sc-99183; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and from Novus Bio (Littleton, CO, USA), v-ets erythroblastosis virus E26 oncogene homolog1 (ETS1) (NBP1-47474), β-actin (NB600-532), anti-mouse horseradish peroxidase (HRP;NB7539) and anti-rabbit HRP (NB730-H).

Proteomic analysis Relative quantification of digested peptides was performed by stable isotope labelling of primary amines as previously described [19]. Dimethylated peptides were separated by nanoflow liquid chromatography using a Waters (Milford, MA, USA) nanoAcquity LC system. Data dependent acquisition was used in the mass spectrometry analysis on Thermo LTQ-Orbitrap Velos (Waltham, MA, USA). Proteins were identified using Proteome Discoverer 1.3 (Thermo Scientific). Quantification was performed by integrating the extracted ion chromatogram of the differentially labelled peptides.

QRT-PCR for mRNAs This was performed on an ABI 7900HT Fast QPCR system using PerfeCTa FastMix (Quanta BioSciences, Gaithersburg, MD, USA). Primer sequences are given in ESM Table 2. *GAPDH* expression was used for normalisation.

Statistical analysis The statistical significance between groups was determined using SigmaStat (SPSS, Chicago, IL, USA) using two-way ANOVA, the Wilcoxon rank-sum test for two independent samples, the Mann–Whitney *U* test or the *t* test as appropriate.

Results

To identify miRNAs that might be relevant to studies of obesity, miRNA array analyses were performed using the GeneChip platform (Affymetrix) on RNA isolated from 29 abdominal SAT biopsies (ESM Fig. 1a). A comparison of miRNA expression levels between individuals in the highest tertile vs the lowest tertile of BMI (ten vs nine individuals, respectively) identified several miRNAs that were differentially expressed between the two groups (Fig. 1a). QRT-PCR was used to validate both the mature and primary (pri)-miRNA for these eight miRNAs in a larger sample of 80 individuals.

Three of the miRNAs, miR-221, miR-193a-3p and miR193b-5p, were significantly correlated with BMI after adjusting for age and sex (Fig. 1b–g, Table 1), at both the mature and primary transcript levels; the other miRNAs assayed in the larger sample did not show significant correlations with BMI that were consistent between the mature and the primary transcript forms. Sex differences were also analysed.

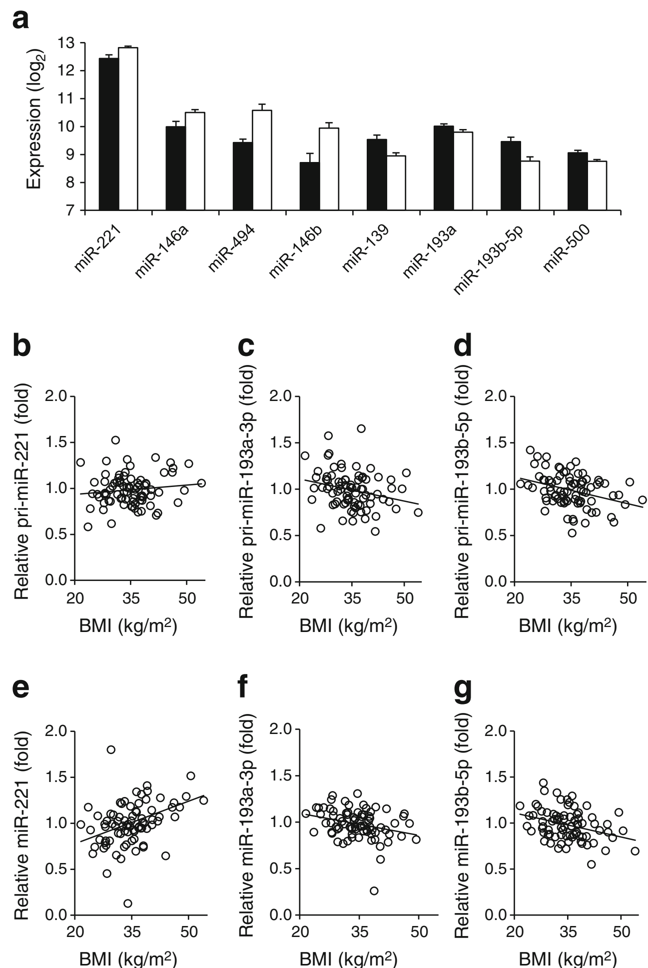

Fig. 1 Profiling and validation of miRNAs associated with obesity in a Pima Indian population. (**a**) miRNAs showing significantly different (p<0.05, Wilcoxon rank-sum test for two independent samples) expression levels in adipose tissue from nine individuals with a BMI below 30 kg/m^2 (black bars; mean BMI 27.5 kg/m^2; six men, three women) vs 10 individuals with a BMI of 37.0 kg/m^2 or more (white bars; mean BMI 40.3 kg/m^2; six men, four women). Error bars represent SEM. (**b–g**) QRT-PCR validation of BMI-associated miRNAs, miR-221, miR-193a-3p and miR-193b-5p, in 80 non-diabetic Pima Indians: (**b–d**) primary transcripts; (**e-g**) mature miRNAs. A comprehensive list of correlations and accompanying p values is given in Table 1

For the mature miR-221, the correlation with BMI was stronger when the analysis was restricted to women (p<0.0001). Although miR-221 maps to the X chromosome, there was no significant difference in miR-221 expression between males and females (data not shown). Expression levels for miR-193a-3p, miR-193b-5p and miR-221 also showed nominal correlations with plasma glucose levels or insulin responses to a 75 g OGTT (Table 1). Since miR-221 displayed the highest expression levels in adipose tissue, we focused on this miRNA and examined potential causes for its upregulation in obesity and the effects of miR-221 on predicted downstream targets.

The upregulation of miR-221 in obesity could either be caused by a *cis*-acting variation at the miR-221 locus or by a

Table 1 Summary of associations between BMI, glucose and insulin responses to an OGTT (all traits adjusted for age and sex) and the expression of mature and primary transcript (pri-) forms of miR-193a, miR-193b-5p and miR-221 in 80 SAT biopsies

miRNA type		BMI (n=80)	2 h glucose (n=80)	Fasting insulin (n=64)	2 h insulin (n=64)	HOMA1-IR (n=68)
miR-193a-3p	R	−0.004			−0.323	
	p	0.060	NS	NS	0.009	NS
pri-miR-193a	R	−0.009		−0.440		
	p	0.010	NS	0.018	NS	NS
miR-193b-5p	R	−0.006	−0.269	−0.267	−0.355	0.077
	p	0.047	0.016	0.053	0.004	0.056
pri-miR-193b	R	−0.008	−0.273	−0.383	−0.37	
	p	0.015	0.014	0.014	0.0026	NS
miR-221	R	0.015		0.302		0.135
	p	<0.0001	NS	0.005	NS	0.066
miR-221 (women only, n=35)	R	0.644		0.736		0.506
	p	<0.0001	NS	0.042	NS	0.004
pri-miR-221	R	0.007				
	p	0.035	NS	NS	NS	NS

The HOMA1-IR and insulin correlations were no longer significant when adjusted for BMI. R and p represent linear regression R and p values

secondary effect. To determine whether a variation in genomic sequence was the primary cause, the promoter region of *miR-221* was sequenced using DNA from 24 unrelated Pima Indian individuals. Two variants, rs2858060 and rs2745709, were identified, and subsequent genotyping in a population-based sample of Pima Indians (n=3,500) revealed no significant association between either variant and BMI or miR-221 expression levels in the 80 biopsies (data not shown). This suggests that the positive correlation observed between miR-221 expression and BMI is probably a consequence of obesity.

It is well known that obesity is associated with higher levels of leptin and TNF-α [20, 21], and also with a decrease in insulin sensitivity accompanied by hyperinsulinaemia [21]. Therefore, we examined whether leptin, TNF-α or insulin could potentially affect the expression of miR-221. Cultured human pre-adipocytes were treated with increasing concentrations of leptin (50, 200 and 500 ng/ml) for 24 h. Expression levels of miR-221 showed a dose-dependent downregulation, the largest decrease occurring with 500 ng/ml of leptin (Fig. 2a). Leptin signalling affects the brain as well as peripheral tissues; therefore, we tested whether leptin could regulate miR-221 levels in a neuronal cell model as well as in adipocytes. Treating SH-SY5Y human neuroblastoma-derived cells with leptin (100 and 500 ng/ml) caused a trend of downregulation of miR-221 (Fig. 2b).

To assess the effects of TNF-α on miR-221 expression, human pre-adipocytes were treated with 5, 10, 25 or 50 ng/ml of TNF-α for 24 h. Exposure to TNF-α for 24 h resulted in a decrease in miR-221 expression levels at all the concentrations tested (Fig. 2c). To evaluate the effects of chronic exposure to TNF-α on miR-221 expression in

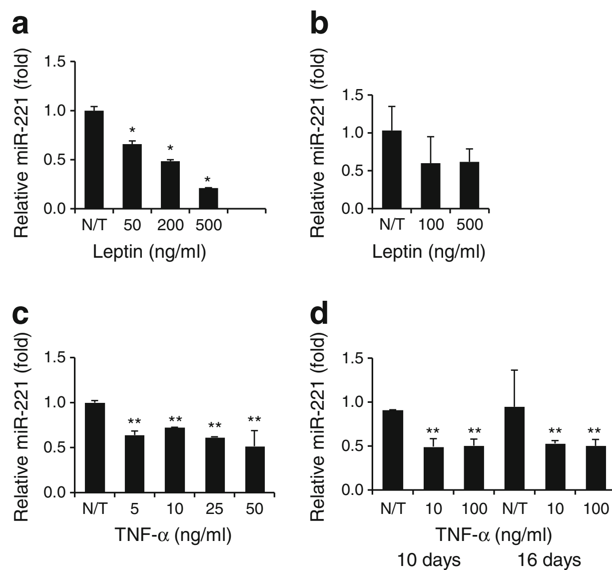

Fig. 2 miR-221 is downregulated by leptin and TNF-α in culture. (**a**) QRT-PCR for miR-221 in primary human pre-adipocytes cultured with or without 50–500 ng/ml leptin for 24 h. Error bars indicate SD (n=4). *p=0.02 compared with non-treated (N/T) cells (two-tailed Mann–Whitney U test). (**b**) QRT-PCR for miR-221 in the human neuronal cell line SH-SY5Y cultured with or without 100 and 500 ng/ml leptin for 24 h. Error bars indicate SD (n=3). (**c**) QRT-PCR for miR-221 in primary human pre-adipocytes cultured with or without 5–50 ng/ml TNF-α for 24 h. Error bars indicate SD (n=4). **p<0.01 compared with N/T cells (two-tailed Mann–Whitney U test). (**d**) QRT-PCR for miR-221 in differentiated human adipocytes cultured with or without 10 or 100 ng/ml TNF-α for 10 or 16 days. Error bars indicate SD (n=4). **p<0.01 compared with N/T cells (two-tailed Mann–Whitney U test)

differentiated adipocytes, human pre-adipocytes were treated with standard differentiation media and allowed to differentiate for over 10 days. The adipocytes were then treated with 10 or 100 ng/ml of TNF-α for 10 or 16 days. miR-221 expression levels were similarly decreased (Fig. 2d). In contrast to leptin and TNF-α, no significant change in miR-221 expression was observed following treatment with 100 nmol/l insulin (data not shown).

To find direct downstream targets of miR-221, the TargetScan prediction program was used to identify genes containing binding sites for the miRNA paralogs miR-221 and miR-222 (see Methods for the link containing the list of predicted gene targets). Two of the predicted target genes, *ADIPOR1* (Fig. 3a) and *ETS1* (Fig. 3b), could have a potential role in causing insulin resistance as a consequence of obesity and were therefore selected for further study.

To test whether miR-221 could downregulate *ADOPOR1* and/or *ETS1* expression, luciferase assays were performed using *ADOPOR1* and *ETS1* 3′ UTR reporter plasmids. To assess binding in this artificial system, HEK 293 cells (selected for rapid growth and ease of transfection) were co-transfected with a miR-221 mimic or scrambled non-specific oligonucleotide (negative control) along with an *ADIPOR1* 3′ UTR reporter plasmid, or with one of two *ETS1* 3′ UTR reporter plasmids. The 3′ UTR of *ETS1* is 3.6 kb in size and was therefore cloned into two separate constructs with overlapping sequence. A reporter plasmid containing the 3′ UTR of *ADIPOR2*, which also functions as an adiponectin receptor but has no predicted binding site for miR-221, was also tested. The miR-221 mimic caused a significant reduction in luciferase activity for the *ADIPOR1* and both *ETS1* 3′ UTR reporter plasmids, but not for the *ADIPOR2* 3′ UTR plasmid (Fig. 3c,d). In human pre-adipocytes, transfection with the miR-221 mimic reduced the levels of endogenous *ETS1* mRNA levels (Fig. 3e) but not *ADIPOR1* mRNA (data not shown). In contrast, both ETS1 and ADIPOR1 were reduced at the protein level (Fig. 3f,g).

Since we had demonstrated that both leptin and TNF-α could downregulate the expression of miRNA-221 and that miRNA-221 could regulate *ETS1* expression, we next determined whether leptin and/or TNF-α treatment would result in a downstream effect on *ETS1* expression. Human pre-adipocytes were treated with either leptin (50, 200 and 500 ng/ml) or TNF-α (10 ng/ml) for 24 h. *ETS1* mRNA expression levels were increased during treatment with both leptin (200 and 500 ng/ml) and TNF-α (Fig. 4a,b). To

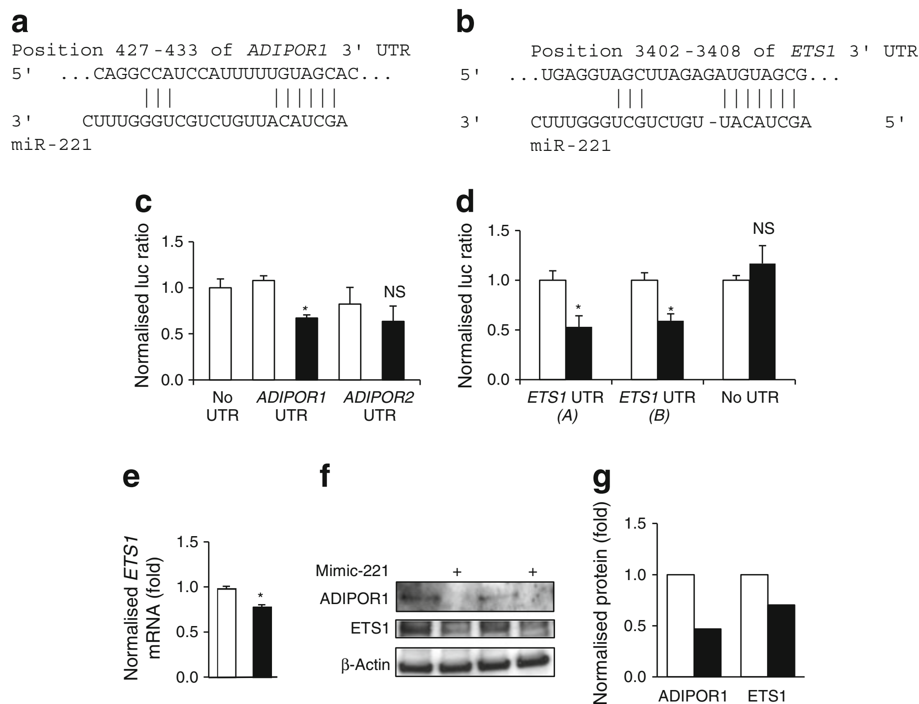

Fig. 3 miR-221 targets the 3′ UTRs of *ADIPOR1* and *ETS1* and decreases their protein levels. (**a,b**) TargetScan conserved predicted miR-221 binding site on the 3′ UTRs of *ADIPOR1* and *ETS1*. (**c,d**) Quantification of dual-luciferase assay in HEK 293 cells co-transfected with miR-221 mimic (black bars) or control oligonucleotide (white bars) and *ADIPOR1/2* (**c**) or *ETS1* (**d**) 3′ UTR reporter plasmids. (**d**) Two overlapping fragments of the *ETS1* 3′-UTR were subcloned into separate vectors, indicated (A) and (B). Firefly/renilla luciferase ratio (luc ratio) was normalised to no-UTR controls. Error bars indicate SD (n=5). *p<0.05 (two-tailed Mann–Whitney U test). (**e**) Quantification of QRT-PCR for *ETS1* mRNA in human pre-adipocytes transfected with miR-221 mimic (black bars) or control oligonucleotide (white bars). Error bars indicate SD (n=3). (**f**) Immunoblots for ADIPOR1, ETS1 and β-actin in human pre-adipocytes transfected with miR-221 mimic (+) or control oligonucleotide. (**g**) Quantification of the immunoblots shown in (**f**). Background-subtracted mean signal (n=2) for ADIPOR1 and ETS1, normalised to the loading control (β-actin). Black bars, miR-221 mimic; white bars, control oligonucleotide

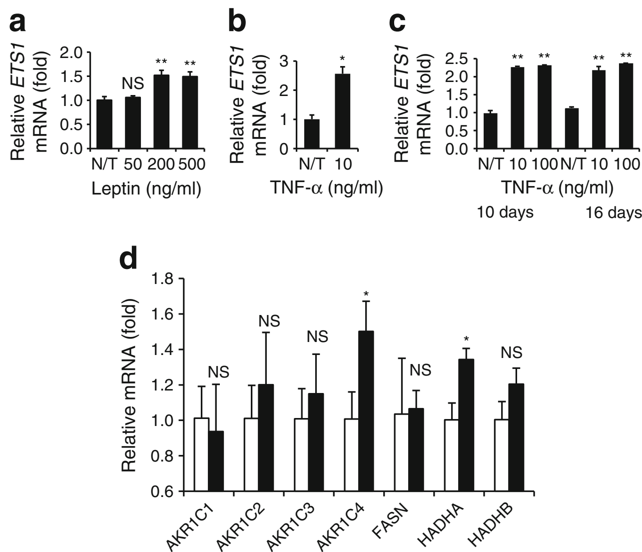

Fig. 4 ETS1 is upregulated by leptin and TNF-α in culture. (**a**) QRT-PCR for *ETS1* mRNA in primary human pre-adipocytes cultured with or without 50–500 ng/ml leptin for 24 h. Error bars indicate SD (n=4). **$p<0.01$ (two-tailed Mann–Whitney U test). (**b**) QRT-PCR for *ETS1* mRNA in primary human pre-adipocytes cultured with or without 10 ng/ml TNF-α for 24 h. Error bars indicate SD (n=3). *$p<0.05$ (two-tailed Mann–Whitney U test). (**c**) QRT-PCR for *ETS1* mRNA in differentiated human adipocytes cultured with or without 10 or 100 ng/ml TNF-α for 10 or 16 days. Error bars indicate SD (n=3). **$p<0.01$ (two-tailed Mann–Whitney U test). (**d**) Quantification of QRT-PCR in human pre-adipocytes transfected with miR-221 mimic (black bars) or control oligonucleotide (white bars). HADHA/B, hydroxyacyl-CoA dehydrogenase, α or β subunit, respectively. Error bars indicate SD (n=3). *$p<0.05$ (two-tailed Mann–Whitney U test)

examine chronic exposure to TNF-α in adipocytes, differentiated human adipocytes were treated with 10 and 100 ng/ml of TNF-α for 10 or 16 days. As with the pre-adipocytes, ETS1 mRNA levels were increased in adipocytes treated with TNF-α (Fig. 4c).

Specific analysis of ETS1 and ADIPOR1 as targets of miR-221 was hypothesis-driven. To examine the effects of miR-221 on protein expression with a hypothesis-free approach, mass spectrometry was used to perform a proteomic analysis on human adipocytes transfected with miR-221. Human pre-adipocytes were transfected with either a miR-221 mimic or a scrambled non-specific siRNA control and then treated with differentiation media for 10 days, before total protein was harvested. Out of approximately 2,200 detectable proteins, 251 (115 known and 136 uncharacterised) were significantly ($p<0.05$) upregulated or downregulated in the miR-221 mimic transfected cells compared with controls (ESM Table 3). Only five of the 251 differentially regulated proteins (upregulated: eukaryotic translation initiation factor 5 [EIF5]; member of the RAS oncogene family [RAB1A]; downregulated: guanine nucleotide binding protein alpha inhibiting 2 [GNAI2]; thrombospondin 1 [THBS1]; tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide [YWHAG]) were predicted miR-221 targets, suggesting that most observed changes in protein levels were a downstream effect or that some miR-221 target genes were missed by the TargetScan prediction program.

Ingenuity software was used to place the 115 known differentially expressed proteins into physiological interaction networks. The most significantly upregulated interaction network (with a score of 43 and 20 focus molecules) was that of energy production and fatty acid metabolism (ESM Fig. 1b). This network included fatty acid synthase (FASN), which was upregulated about fivefold in the miR-221-transfected cells, and members of the aldoketoreductase (AKR) family (AKR1C1–AKR1C4). The most significantly downregulated network (with a score of 44 and 22 focus molecules) was that of cellular assembly and maintenance and tissue development (ESM Fig. 1c).

Table 2 Top common regulators predicted to effect the proteomic changes observed in cultured adipocytes overexpressing miR-221, using Ingenuity pathway analysis software

Regulator	Molecule type	Predicted state	z score	p values of overlap
MYCN	Transcription regulator		1.913	1.05×10^{-10}
INSR	Kinase		1.782	6.24×10^{-10}
Pirinixic acid	Chemical toxicant	Activated	3.268	2.57×10^{-9}
Bezafibrate	Chemical drug	Activated	2.184	1.19×10^{-8}
TP53	Transcription regulator		0.692	4.05×10^{-8}
PPARα	Ligand-dependent nuclear receptor	Activated	3.108	6.44×10^{-8}
Dinoprost	Chemical – endogenous		0.254	7.10×10^{-8}
PPARγ	Ligand-dependent nuclear receptor	Activated	2.601	1.62×10^{-7}
Mannose	Chemical – endogenous		1.000	2.52×10^{-7}
ACOX1	Enzyme		0.113	4.57×10^{-7}

MYCN, v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian); INSR, insulin receptor; ACOX1, acyl-coenzyme A oxidase 1, palmitoyl

To determine whether proteins upregulated by miR-221 in the proteomic analysis were also upregulated at the mRNA level, QRT-PCR was used to measure the mRNA levels of seven of them (*AKR1C1–AKR1C4*, *FASN*, *HADHA and HADHB*) following miR-221 overexpression. Among these targets, *AKR1C4* and *HADHA* were significantly upregulated by miR-221, while several others showed a non-significant trend (Fig. 4d). Other affected proteins may be regulated post-transcriptionally by downstream effectors of miR-221.

Prediction of upstream regulators that could potentially control several of the differentially expressed proteins yielded four molecules with high activation scores (Table 2). Two of these predicted common regulators (peroxisome proliferator-activated receptor (PPAR)α and PPARγ) belong to the PPAR family, while the other two (pirinixic acid and bezafibrate) are PPAR agonists. Therefore, overexpression of miR-221 in cultured adipocytes appears to affect the proteome in a manner that mimics PPAR activation.

Discussion

Our results show that adipose miR-221 expression is positively correlated with increasing BMI in the Pima Indian population. This correlation is in agreement with previous reports also showing that adipose miR-221 is upregulated in obesity [6, 13]; however, the mechanism(s) for the upregulation in our study are unclear. Both leptin and TNF-α, whose levels are known to be increased in the obese state, were found to downregulate miR-221 in cultured cells. This suggests a more complex mechanism in vivo, which may include desensitisation to the effects of leptin and TNF-α in the chronically obese state [22–25].

In our proteomic analysis, we sought to identify the downstream consequences of miR-221 upregulation. The most significantly upregulated physiological network in adipocytes transfected with miR-221 consists of proteins involved in fatty acid metabolism, including FASN. It is thought that increased levels of fatty acids and increased expression of FASN may play a role in the development of insulin resistance [26–29]. Prediction of upstream regulators that can activate several of the differentially expressed proteins yielded PPARα, PPARγ and two PPAR agonists, which may again indicate that upregulation of miR-221 in the obese state activates pathways involved in insulin sensitivity [30, 31].

We have further shown that miR-221 directly downregulates *ADIPOR1* and *ETS1* expression; therefore, it may be possible that obesity-associated upregulation of miR-221 can result in a decrease in ADIPOR1 and/or ETS1, which may in turn lead to the development of obesity-related metabolic consequences such as insulin resistance or type 2 diabetes. ADIPOR1 functions as a receptor for adiponectin and mediates a signalling pathway that promotes insulin sensitivity and is suppressed in insulin resistance and type 2 diabetes caused by obesity [32]. ETS1 is a transcription factor that regulates the expression of a wide spectrum of genes including cytokines and chemokines [33], and it has been proposed that obesity-associated inflammation may also play a significant role in the development of type 2 diabetes [34].

In addition, the previously described role of ETS1 in vascular endothelial growth factor (VEGF)-induced angiogenesis through its regulation of matrix metalloproteinases (MMPs) [35] that is subject to regulation by other miRNAs, e.g. miR-199a-5p [36], and the importance of VEGF signalling for adipose function [37] suggest that higher miR-221 expression in obesity may lead to decreased vascularisation and increased hypoxia and inflammation in adipose tissue. Supporting this notion is our group's previous report that MMP3, a target of ETS1 [38], is downregulated in preadipocytes and stromal vascular cells from obese individuals [39], and that genetic variation in the MMP3 locus is only partially responsible for this downregulation. Additionally, downregulation of ETS1 may be essential to PPAR signalling [40], in line with the observed proteomic effects in which miR-221 overexpression mimicked PPAR activation. ETS1 and ADIPOR1 were absent from the list of proteins detected in our mass spectrometry analysis, possibly due to an overall lower expression level of these proteins, as well as a bias against membrane (such as ADIPOR1) and nuclear (such as ETS1) proteins inherent in the purification method employed.

miR-221 is a known player in oncogenesis, affecting the proliferation and behaviour of various cell types [41–47]. Deregulation of ETS1 in a variety of cancers suggests that ETS1 could mediate the effects of miR-221 in oncogenesis. Recent studies have shown that an ETS1-mediated transcription of members of the MMP family is responsible for remodelling of the extracellular matrix in both angiogenesis and invasion [48]. Resistance to TNF-α promotes tumourigenesis [49, 50], and elevated miR-221 may have a role in mediating these effects via its suppression of ETS1 [51]. Thus, chronic upregulation of miR-221 in obesity may potentially increase cancer risk. However, additional studies are required to demonstrate that miR-221 is a valid link between obesity and obesity-associated diseases such as the metabolic syndrome, type 2 diabetes and cancers.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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