

Adipose Tissue MicroRNAs as Regulators of CCL2 Production in Human Obesity

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In obesity, white adipose tissue (WAT) inflammation is linked to insulin resistance. Increased adipocyte chemokine (C-C motif) ligand 2 (CCL2) secretion may initiate adipose inflammation by attracting the migration of inflammatory cells into the tissue. Using an unbiased approach, we identified adipose microRNAs (miRNAs) that are dysregulated in human obesity and assessed their possible role in controlling CCL2 production. In subcutaneous WAT obtained from 56 subjects, 11 miRNAs were present in all subjects and downregulated in obesity. Of these, 10 affected adipocyte CCL2 secretion in vitro and for 2 miRNAs (miR-126 and miR-193b), regulatory circuits were defined. While miR-126 bound directly to the 3'-untranslated region of *CCL2* mRNA, miR-193b regulated CCL2 production indirectly through a network of transcription factors, many of which have been identified in other inflammatory conditions. In addition, overexpression of miR-193b and miR-126 in a human monocyte/macrophage cell line attenuated CCL2 production. The levels of the two miRNAs in subcutaneous WAT were significantly associated with CCL2 secretion (miR-193b) and expression of integrin, α -X, an inflammatory macrophage marker (miR-193b and miR-126). Taken together, our data suggest that miRNAs may be important regulators of adipose inflammation through their effects on CCL2 release from human adipocytes and macrophages.

Obesity is associated with a low-grade inflammatory state in white adipose tissue (WAT), which influences fat cell function and may promote insulin resistance and type 2 diabetes (1,2). Adipocytes and infiltrating inflammatory cells (primarily macrophages) present within the tissue secrete key inflammatory proteins, such as tumor necrosis factor- α , interleukin (IL)-6, and chemokine (C-C motif) ligand 2 (CCL2)/monocyte chemoattractant protein, and their gene expression and release are markedly increased in obesity (3,4). *CCL2* may be

particularly important since it has been proposed to initiate adipose inflammation by attracting inflammatory cells from the blood stream into WAT (5,6). Studies in mice show that CCL2 production and signaling are essential for the development of WAT inflammation (7). Although a number of different cell types in WAT may produce CCL2, the fat cells are of particular interest since adipocyte-derived CCL2 may promote local inflammation independent of the presence of macrophages/leukocytes in human WAT (8). However, the mechanisms controlling WAT CCL2 production in obesity are not clear.

The pathogenesis of obesity involves a complex interplay between various types of factors. At the molecular level, such interdependencies can be conceptualized as transcriptional regulatory networks with regulatory proteins and different classes of RNA molecules as nodes and their interactions as edges (9). Gene transcription is controlled at several different levels of which some have been elucidated only in recent years. MicroRNAs (miRNAs) have emerged as important factors regulating gene expression through binding to complementary sequences of target mRNAs resulting in decreased mRNA levels (10). Alterations in the levels of miRNAs have been shown to affect gene expression and thereby cell function in several pathophysiological conditions, including inflammation (11). The miRNAs may act directly on the target genes or indirectly by first regulating transcription factors (TFs), which, in turn, control the expression of genes (11). The role of miRNAs in adipose inflammation and obesity is, however, not known.

In this study, we aimed to define adipose miRNAs dysregulated in human obesity and their possible role in controlling CCL2 production. Through a systematic and unbiased approach, we were able to identify 10 obesity-regulated miRNAs that affected adipocyte CCL2 secretion in vitro. For two of these (miR-126 and miR-193b), we could define their mechanism of action, which involved direct or indirect (through TFs) regulation of CCL2 production in human adipocytes and macrophages.

RESEARCH DESIGN AND METHODS

Cohorts. Cohort 1 comprised 30 obese (BMI >30 kg/m²) otherwise healthy and 26 nonobese (BMI <30 kg/m²) healthy women. They were investigated in the morning after an overnight fast in approximately the midst of their menstrual cycle. All were premenopausal and free of continuous medication. Height, weight, and waist circumference were determined. A venous blood sample was obtained for measurements of glucose and insulin. The values were used to construct an index of overall insulin sensitivity (homeostasis model assessment of insulin resistance [HOMA_{IR}]) as described (12). Thereafter, an abdominal subcutaneous WAT biopsy (~1.5 g) was obtained by needle aspiration as described (13). One part (300 mg) of the tissue was used for measurement of release of CCL2 per number of fat cells, as described (14). Similar results were obtained when protein release was related to the WAT weight (values not shown). Methodological studies show that protein release

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was linear with time for at least 3 h, suggesting no important cell damage. Another part of the tissue (700 mg) was subjected to collagenase treatment, and mean adipocyte volume and weight were determined as described (15). Next, 200 μ L packed fat cells as well as 400 mg intact WAT were frozen at -70°C for future mRNA and miRNA measurements. The remaining isolated fat cells were incubated for 2 h at 37°C in an albumin concentration buffer without or with increasing concentration of insulin, and lipogenesis (incorporation of [^3H] glucose into lipids) was determined as described (15). From the insulin concentration-response curves, lipogenesis at maximum effective insulin concentration was determined and expressed per number of fat cells. Clinical characteristics of this cohort are given in Table 1. The obese subjects displayed in vivo (HOMA_{IR}) and in vitro (insulin-stimulated lipogenesis) insulin resistance as well as increased adipose tissue CCL2 secretion (in vitro). For experimental studies, subcutaneous WAT was obtained from healthy subjects undergoing cosmetic liposuction. In this group, there was no selection for age, sex, or BMI. In a subgroup of these tissue donors ($n = 12$), global gene expression profiling was determined during in vitro differentiation of primary human adipocytes. This study was approved by the ethical committee at the Karolinska University Hospital. All subjects were informed in detail about the studies and written informed consent was obtained.

Affymetrix GeneChip Human Gene 1.0 ST Array protocol. From total RNA, we prepared and hybridized biotinylated complementary RNA to GeneChip Human Gene 1.0 ST Array (Affymetrix, Inc., Santa Clara, CA) and then washed, stained, and scanned the slides using standardized protocols (Affymetrix, Inc.). Preprocessing was performed using the Affymetrix Expression Console version 1.1 and the following settings: summarization, PLIER; background correction, PM-GCBG; and normalization, Global Median. Gene and miRNA expression have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (<http://ncbi.nlm.nih.gov/geo>) and are accessible using Gene Expression Omnibus series accession number GSE25402.

Affymetrix GeneChip miRNA Array protocol. Total adipose RNA (1 μg) from each subject in cohort 1 was labeled using the FlashTag biotin HSR labeling kit (Genisphere, Hatfield, PA) according to the supplier's protocol. The labeled samples were placed in a hybridization cocktail mix containing 4% formamide and hybridized overnight to Affymetrix miRNA arrays following the indicated Genisphere protocol. The arrays were washed, stained, and scanned in an Affymetrix GCS 3000 scanner. Signal intensities and present calls were generated by using the miRNA QC tool by Affymetrix.

Cell culture. Isolation, culture, and in vitro differentiation of human adipocyte progenitor cells from subcutaneous WAT were performed exactly as described (16). 3T3-L1 and THP1 cells were handled as recommended in the protocols from American Type Culture Collection (Manassas, VA).

miRNA transfection. In vitro-differentiated adipocytes were treated with 40 nmol/L miRIDIAN miRNA Mimics or 100 nmol/L miRIDIAN miRNA Hairpin Inhibitors (Thermo Fisher Scientific, Lafayette, CO) and HiPerFect Transfection Reagent (QIAGEN, Hilden, Germany) according to the manufacturers' protocols. Optimal transfection conditions were determined in separate titration experiments, and transfection efficiency was assessed with quantitative real-time PCR (qRT-PCR) using miRNA probes and fluorescent transfection controls (Thermo Fisher Scientific). To rule out unspecific effects, control cells were transfected with miRIDIAN miRNA Mimic or Hairpin Inhibitor Negative Controls (Thermo Fisher Scientific). THP1 macrophages were transfected as described above for adipocytes.

RNA isolation, cDNA synthesis, and qRT-PCR. Total RNA was extracted from in vitro-differentiated adipocytes, 3T3-L1 cells, isolated adipocytes, intact

WAT, and macrophages. RNA concentration as well as purity was measured spectrophotometrically using a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific), and high quality (total and small) RNA was confirmed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

Total RNA was reverse transcribed using Omniscript First-strand cDNA synthesis kit (QIAGEN) and random hexamer primers (Invitrogen, Carlsbad, CA). For confirmation and further studies of microarray results, expression levels of selected target genes were measured in 384-well TaqMan Low Density Array plates (Applied Biosystems, Foster City, CA). Equal amounts of cDNA were dispersed on the plates together with a TaqMan gene expression master mix (Applied Biosystems). Amplification of the PCR products was followed and registered in a 7900HT Fast Real-Time PCR System (Applied Biosystems). The resulting data were analyzed with the RQ Manager and Data Assist software (Applied Biosystems). Other human transcripts were detected using TaqMan Universal PCR Master Mix (Applied Biosystems) and TaqMan primers and probes (Applied Biosystems). qRT-PCR was performed on an iCycler IQ (Bio-Rad, Hercules, CA) using the amplification protocol provided by Applied Biosystems. All samples were run in duplicates, and arbitrary units were calculated using a comparative cycle threshold (Ct) method (i.e., $2^{\Delta\text{Ct-target gene}/2^{\Delta\text{Ct-reference gene}}}$). 18S rRNA was used to normalize the expression of all analyzed human genes.

miRNA cDNA was synthesized using TaqMan miRNA Reverse Transcription Kit and human Megaplex primer pool A without preamplification (Applied Biosystems). For miRNAs not included in the primer pool, specific probes designed by Applied Biosystems were used. qRT-PCR was performed as described above on an iCycler IQ (Bio-Rad) using the amplification protocol provided by Applied Biosystems. Expression of miRNA was normalized to an internal reference gene *RNU48*, and arbitrary units were calculated as described above.

Western blot. Cells were lysed in radioimmunoprecipitation assay buffer as described previously (17), and 15–20 μg total protein was separated by SDS-PAGE. Western blot was performed according to standard procedures, and secondary antibodies mouse/rabbit IgG-horseradish peroxidase were from Sigma-Aldrich. β -Actin (Sigma-Aldrich) was used as a reference for all Western blots. Primary antibodies detecting MYC-associated factor X (MAX) were unspecific and, therefore, could not be used.

Enzyme-linked immunosorbent assay. Conditioned media from in vitro-differentiated adipocytes, macrophages, and subcutaneous WAT pieces (300 mg in 3 mL medium) was saved at -70°C for determination of CCL2 and IL-6 levels by ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Luciferase reporter assay. The luciferase reporter construct containing 3'-untranslated region (3'UTR) of v-ets erythroblastosis virus E26 oncogene homolog 1 (ETS1) (pGL3 ETS1 3'UTR/155up) was provided by Dr. Giovanna Marziali (Istituto Superiore di Sanità, Rome, Italy) and has been described previously (18). The empty luciferase reporter vector and vectors containing 3'UTR of *CCL2*, *ARNT*, *MAX*, and *RELB* were obtained from GeneCopia (Rockville, MD). 3T3-L1 cells were plated in 24-well plates and transfected 48 h postplating with 150 ng luciferase-3'UTR reporter constructs or with the empty expression vector 2 ng Renilla luciferase reporter pRL-SV40 (Promega, Madison, WI) (for ETS1 only) together with miRIDIAN miRNAs (miRNA mimic or negative controls) at final concentration of 40 nmol/L using Lipofectamine and Plus Reagent (both from Invitrogen) according to the manufacturer's protocol. After 4 h of incubation, the medium was refreshed with Dulbecco's modified Eagle's medium/F12 plus 10% FCS. The cells were incubated for 24 h and subsequently

TABLE 1
Clinical characteristics of cohort 1

Clinical parameter	Obese ($n = 30$)	Nonobese ($n = 26$)	T test P value
Age (years)	43 \pm 2	43 \pm 3	0.833
Weight (kg)	111.7 \pm 4.1	67.5 \pm 1.7	0.0001
BMI (kg/m^2)	40.9 \pm 1.3	24.1 \pm 1.8	0.0001
Waist circumference (cm)	121.7 \pm 13.9	85.2 \pm 9.2	0.0001
Body fat (%)	56.5 \pm 1.6	33.7 \pm 1.1	0.0001
Adipose CCL2 secretion*	32.5 \pm 3.1	15.4 \pm 3.0	0.0001
Fat cell volume (μL)	897.8 \pm 38.5	503.5 \pm 30.3	0.0001
Log_{10} HOMA_{IR}	0.458 \pm 0.060	-0.01 \pm 0.049	0.0001
Log_{10} insulin-stimulated adipocyte lipogenesis \dagger	0.346 \pm 0.077	0.763 \pm 0.089	0.0009

Cohort 1 comprised 30 obese otherwise healthy and 26 nonobese healthy women who were investigated in the morning after an overnight fast. Height, weight, waist circumference, fat cell volume, HOMA_{IR} (an index of overall insulin sensitivity), and insulin-stimulated glucose uptake (measured as incorporation of [^3H]glucose into lipids) were determined and evaluated. Results were analyzed using t test and are shown as mean \pm SEM. *Related to the number of fat cells incubated (i.e., ng per 10^7 cells). \dagger Expressed as nmol per 2 h and 10^7 cells.

lysed, and firefly as well as Renilla luciferase activities was determined using the Dual-Glo Luciferase Assay System (Promega). All experiments were performed in triplicate, and the values of firefly luciferase activity were normalized by Renilla luciferase activities and empty vector, which served as internal controls.

Analysis of expression arrays. Differences in WAT expression of miRNAs/miRNAs between obese/nonobese subjects and during adipocyte differentiation were analyzed using significance analysis of microarrays, false discovery rate 5%, as described previously (19).

Motif activity response analysis and network construction. Motif activity response analysis (MARA) is extensively described in the main text and supplement of our previous study (20). In brief, the method relies on the assumption that TFs regulate the expression of genes through binding to DNA sequence elements in promoter regions and that gene expression is a linear function of the number of TF binding sites in the promoter of the gene. Activities of known motifs recognized by human TFs were fitted to gene expression levels as determined by microarray. For interactions between TFs and their target genes, genes with a minimum number of the TF binding sites in their promoter region and with expression correlating with the corresponding motif activity over a threshold were identified as nodes in a transcriptional regulatory network together with the TFs. Finally, the subnetwork predicted to regulate CCL2 was extracted. Exact details on MARA and construction of networks used in this study are further described in RESULTS.

Reagents. Unless otherwise stated, details on the reagents used in this study, including relevant catalog numbers, are listed in Supplementary Table 1.

Statistical analyses. Data shown in the figures and tables are mean \pm SEM. When appropriate, the data were log transformed to become normally distributed. Results were analyzed with unpaired *t* test, linear regression (simple and multiple), sign test, or ANOVA (repeated-measures). For analysis of genome-wide data, see the respective subheading.

RESULTS

Mapping of miRNA expression in adipose tissue and adipocytes. Using global miRNA expression profiling of WAT, we identified 20 miRNAs that were expressed in all subjects (cohort 1) and concomitantly altered in obesity (2 up- and 18 downregulated) (Table 2). The results were validated by qRT-PCR in intact WAT showing that 14 miRNAs were regulated in accordance with the array data (70% concordance). Adipose tissue contains many cell types.

TABLE 2
Mapping of miRNA expression in adipose tissue and fat cells

Probeset name	Intact tissue, fold change obese/nonobese		T test <i>P</i> value	Isolated fat cells, fold change obese/nonobese	T test <i>P</i> value
	Microarray data	qRT-PCR data			
hsa-miR-193a-5p_st	0.81	0.65	0.0003	0.56	0.0002
hsa-miR-30c_st	0.85	0.67	0.0021	0.36	0.0002
hsa-miR-26a_st	0.76	0.81	0.0158	0.48	0.0003
hsa-miR-143_st	0.63	0.79	0.0276	0.43	0.0005
hsa-miR-92a_st	0.81	0.72	0.0295	0.64	0.0014
hsa-miR-145_st	0.86	0.65	0.0003	0.49	0.0036
hsa-miR-652_st	0.68	0.58	0.0004	0.45	0.0051
hsa-let-7d_st	0.75	0.79	0.0056	0.53	0.0113
hsa-miR-193b_st	0.90	0.72	0.0063	0.59	0.0151
hsa-miR-126_st	0.65	0.80	0.0472	0.67	0.0181
hsa-let-7a_st	0.70	0.77	0.0132	0.44	0.0244
hsa-miR-378_st	0.78	0.71	0.0008	0.57	0.0651
hsa-miR-197_st	0.86	0.79	0.0168	0.75	0.1233
hsa-miR-139-5p_st	0.86	0.84	0.0499	0.95	0.3072
hsa-let-7i_st	0.66	0.81	0.0559	0.37	0.0352
hsa-miR-486-5p_st	0.73	0.68	0.0562	0.58	0.0353
hsa-miR-151-5p_st	0.74	0.84	0.0621	0.73	0.0228
hsa-miR-222_st	1.27	1.19	0.0632	1.41	0.0136
hsa-miR-342-3p_st	1.15	0.85	0.1008	0.56	0.0030
hsa-miR-16_st	0.57	0.81	0.1423	0.41	0.0001

Global miRNA expression profiling comparing intact adipose tissue from obese ($n = 30$) and nonobese ($n = 26$) subjects identified 20 miRNAs regulated by obesity. For confirmation and further studies of microarray results, miRNA levels were measured with qRT-PCR in paired samples of human intact tissue and isolated adipocytes ($n = 44$). Results were analyzed using *t* test and are presented as fold change relative to the nonobese.

Since we were primarily interested in miRNAs expressed in human fat cells, we determined their expression in isolated adipocytes from cohort 1 and whether they were affected by obesity. All 20 miRNAs were expressed in mature fat cells. However, when overlapping the data from isolated fat cells and intact WAT, only 11 (miR-26a, -30c, -92a, -126, -143, -145, -193a, -193b, -652, let-7a, and let-7d) were found to be significantly altered (all downregulated) in obesity. These miRNAs were considered as candidate miRNAs.

miRNAs regulate adipocyte CCL2 secretion. To determine whether our candidate miRNAs could influence CCL2 secretion, we performed a transfection screen by overexpressing individual miRNAs in human in vitro-differentiated adipocytes. Nine miRNAs (miR-26a, -92a, -126, -143, -193a, -193b, -652, let-7a, and let-7d) significantly reduced CCL2 secretion, one had no effect (miR-30c), and one led to increased release (miR-145) (Fig. 1A). We chose to focus our functional studies on the nine miRNAs that upon overexpression, downregulated CCL2 production since their expression in obesity (Table 2) and effects on adipocyte secretion (Fig. 1A) were concordant.

To assess whether the miRNAs could directly interact with the 3'UTR of *CCL2*, we performed an in silico analysis using information available in public databases (see RESEARCH DESIGN AND METHODS). According to this search, only miR-126 was predicted to bind to the 3'UTR of *CCL2*, which was confirmed in a luciferase reporter assay in 3T3-L1 cells (Fig. 1B). The specificity of this interaction was determined by transfecting a random subset of the other candidate miRNAs (miR-26a, -92a, and let-7d) in parallel experiments showing no effect on luciferase activity (Fig. 1B). That miR-126 regulates CCL2 production at the transcriptional level was supported by the fact that miR-126 overexpression downregulated *CCL2* mRNA in human in vitro-differentiated adipocytes (Fig. 1C).

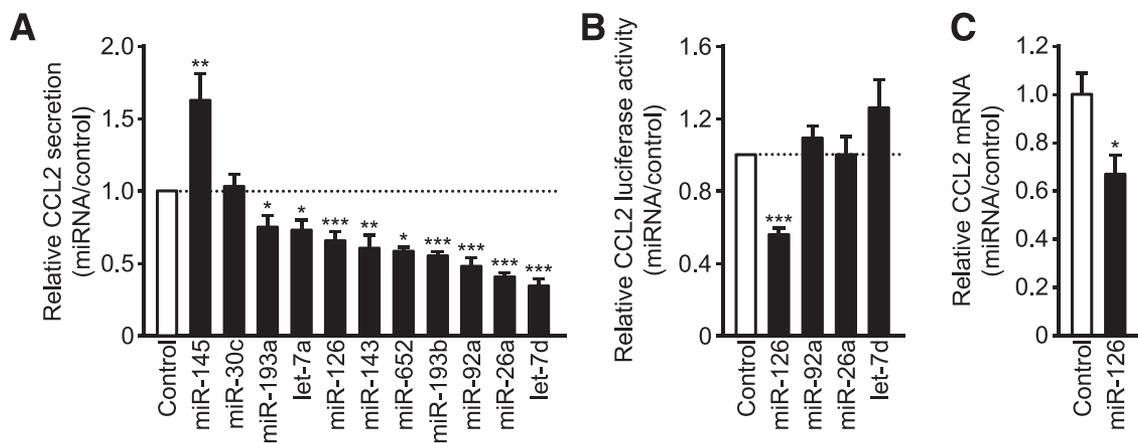


FIG. 1. Regulation of CCL2 by miRNAs. **A:** Each candidate miRNA was overexpressed in in vitro differentiated adipocytes, and secreted levels of CCL2 were assessed. **B:** In 3T3-L1 cells, a subset of the candidate miRNAs were individually transfected together with reporter constructs, and alterations of luciferase activity were monitored. **C:** CCL2 mRNA levels were determined after miR-126 overexpression in human adipocytes differentiated in vitro. Protein secretion, luciferase activity, and mRNA levels were measured 24/48 h after transfection. To rule out unspecific effects, control cells were transfected with a negative control miRNA. Results were analyzed using *t* test and are presented as fold change \pm SEM relative to the negative control. ****P* < 0.001, ***P* < 0.01, **P* < 0.05.

Identification of an adipocyte-specific transcriptional regulatory network controlling CCL2 expression in obesity.

Since only 1 out of 10 candidate miRNAs was predicted to directly interact with *CCL2*, we set out to construct a transcriptional regulatory network by which miRNAs could control CCL2 secretion. On the basis of global gene expression profiling in cohort 1, we performed MARA, a method previously developed by us to find key regulatory elements in monocyte differentiation (20,21). Using MARA, we characterized the activity of regulatory motifs by modeling gene expression patterns in terms of genome wide-predicted TF regulatory sites in proximal promoters (for details, see Supplementary Results). We studied 193 previously defined TF motifs, and a principal component analysis of the data revealed obesity as the second strongest factor affecting TF activity (Fig. 2A). While the first component showed no association with obesity-related anthropometric measures (data not shown), the second component correlated markedly and positively with BMI (Fig. 2B) and several other anthropometric measures, such as waist circumference and fat cell size (data not shown). Thus, there appears to be a clear association between the second component and obesity. Furthermore, we identified 96 TFs and corresponding

motifs with altered activity in obesity (Supplementary Table 2). To assess whether these TFs are present in adipocytes, 47 were randomly chosen for qRT-PCR on paired samples of intact WAT and isolated fat cells from cohort 1 (Supplementary Table 3). Of the TFs, 45 were expressed in both adipose preparations and 36 (80%) were significantly more highly expressed in the fat cells. Altogether, these results allowed us to identify motifs and their cognate TFs with altered activity in adipocytes of obese subjects.

Since our transfection screen was performed in human in vitro-differentiated adipocytes, we wanted to ensure that the motifs and cognate TFs identified above were also present in our cell model system. We therefore performed a MARA during in vitro differentiation of cultured human fat cells. The motifs identified in the two MARAs were overlapped, and 32 out of the 96 were regulated in both analyses (Supplementary Table 2). An adipocyte-specific transcriptional regulatory network controlling CCL2 production was constructed by linking TFs identified by MARA to CCL2 and our candidate miRNAs (for details, see Supplementary Results and Supplementary Table 4). Five (miR-92a, -126, -193b, -652, and let-7a) of the 10 candidate miRNAs were predicted to target TFs within the CCL2 network (Fig. 3A).

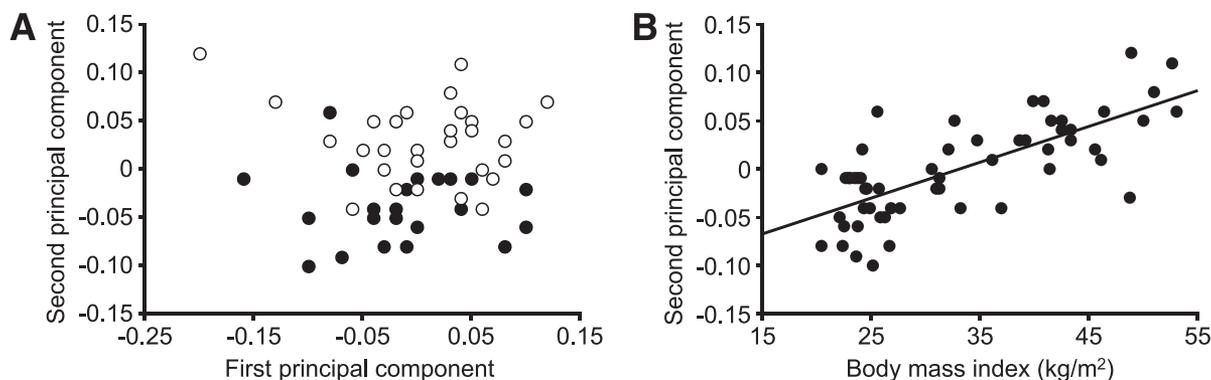


FIG. 2. Characterization of TF motif activities in obesity. **A:** Principal component analysis of TF motif activities in obese (○, *n* = 30) and nonobese (●, *n* = 26) subjects. **B:** Regression analysis comparing the second principal component with BMI (*n* = 56; *r* = 0.730; *P* < 0.0001).

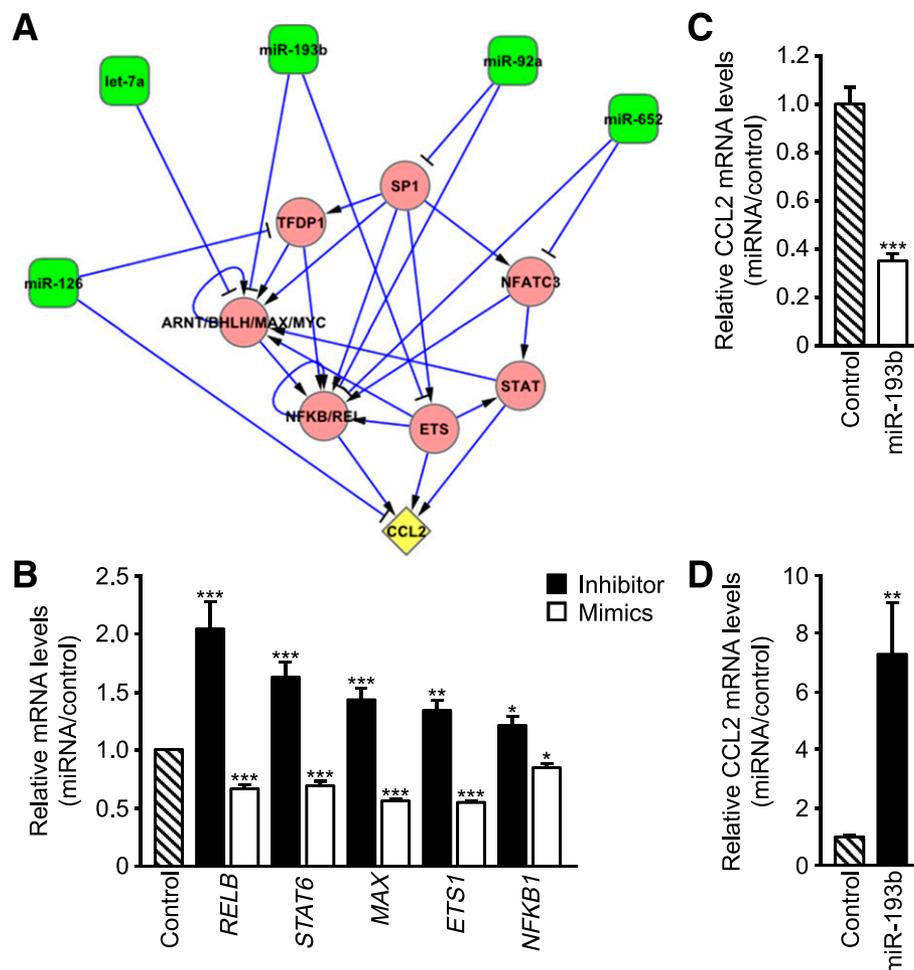


FIG. 3. Regulators of *CCL2* in human adipocytes and obesity. **A:** The proposed *CCL2* network depicting miRNAs (squares) as well as motifs and cognate TFs (circles) altered by obesity and potentially targeting *CCL2* (diamond). T-bars represent inhibition; arrows represent stimulation. **B–D:** miR-193b was overexpressed (white bar) or inhibited (black bar) in in vitro-differentiated adipocytes, and mRNA levels of direct (*MAX* and *ETS1*) and indirect targets (*RELEB*, *STAT6*, and *NFKB1*) (**B**) and *CCL2* (**C** and **D**) were determined. To rule out unspecific effects, control cells were transfected with a negative control miRNA (striped bar). mRNA levels were measured 48 h after transfection. Results were analyzed using *t* test and are presented as fold change \pm SEM relative to the negative control. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. SP1, Sp1 transcription factor; NFATC3, nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3; BHLH, basic helix-loop-helix; MYC, v-myc myelocytomatosis viral oncogene homolog; TFDP1, transcription factor Dp-1. (A high-quality color representation of this figure is available in the online issue.)

Validation of miRNA-regulated pathways affecting adipocyte and macrophage *CCL2* expression. The proposed connections between miRNAs and their predicted TF targets were challenged by overexpressing each miRNA and assessing the effect on TF mRNA expression in human adipocytes. Only miR-92a and miR-193b altered the expression of their predicted targets (Table 3). However, the average effects of miR-92a were small ($\sim 20\%$) and with some interexperimental variability. Furthermore, 3'UTR analyses (by reporter assays in 3T3-L1 cells) demonstrated that only miR-193b was able to interact with and directly regulate its proposed TFs (Table 3). We therefore focused further validation experiments on miR-193b and its role in the regulation of *CCL2*. As predicted, overexpression of miR-193b downregulated *RELEB*, *STAT6*, *MAX*, *ETS1*, nuclear factor of κ light polypeptide gene enhancer in B-cells 1 (*NFKB1*), and *CCL2* mRNA levels, while inhibition of miR-193b had reciprocal effects (Fig. 3B–D). The effects of miR-193b on *RELEB*, *STAT6*, and *ETS1* were confirmed at the protein level (Supplementary Fig. 1). The subnetwork comprising miRNAs, TFs, and *CCL2* validated by these experiments is shown in Fig. 4A. To assess the functionality of this regulatory circuit, we expanded our network and

included *IL-6* as a target. According to our bioinformatic analysis, miR-193b is a predicted regulator of *IL-6* via *NFKB1/RELEB*. This was confirmed by the observation that overexpression of miR-193b in human adipocytes attenuated *IL-6* secretion (Fig. 4B). In contrast, there was no predicted direct or indirect (through TFs) connection between miR-126 and *IL-6*. In accordance with this, miR-126 overexpression did not affect *IL-6* release (Fig. 4B).

Our results identified a transcriptional framework in human adipocytes controlling *CCL2* production. However, *CCL2* is secreted by several cell types present within the adipose tissue among which macrophages play an important role. Therefore, we assessed the role of miR-193b and miR-126 in controlling *CCL2* production in a human monocyte/macrophage cell line (THP1). Similar to our findings in adipocytes, overexpression of either miRNA decreased *CCL2* mRNA levels (Fig. 4C) and secretion (Fig. 4D).

Correlation between miR-193 and miR-126 expression, *CCL2* secretion, and an M1 macrophage marker in human adipose tissue. Associations between adipose expression of miR-193b, miR-126, *CCL2* secretion, and a marker of macrophage infiltration were determined by multiple regression analysis in cohort 1 using BMI as a covariate.

TABLE 3
Validation of predicted miRNA-TF interactions

miRNA	Predicted target	qRT-PCR		Luciferase activity	
		Fold change miRNA/control	T test <i>P</i> value	Fold change miRNA/control	T test <i>P</i> value
let-7a	<i>MYC</i>	0.914	0.2203		
miR-126	<i>TFDP1</i>	0.988	0.9095		
miR-193b	<i>ARNT</i>	0.862	0.0046	1.001	0.99108
miR-193b	<i>ETS1</i>	0.54	0.0001	0.493	0.00077
miR-193b	<i>MAX</i>	0.576	0.0001	0.482	0.00001
miR-652	<i>NFATC3</i>	1.098	0.5063		
miR-652	<i>NFKB1</i>	1.054	0.6819		
miR-92a	<i>RELB</i>	0.564	0.0001	0.917	0.11786
miR-92a	<i>SPI</i>	1.071	0.5431		

Predicted interactions between miRNAs and TFs were investigated by overexpressing each miRNA and assessing the effects on TF mRNA expression in human in vitro differentiated adipocytes. Overexpression of miR-92a and miR-193b altered the expression of TF levels, and to assess whether this interaction was direct, 3'UTR analyses (by reporter assays in 3T3-L1 cells) were performed. To rule out unspecific effects, control cells were transfected with a negative control miRNA. Luciferase activity and mRNA levels were measured 24/48 h after transfection. Results were analyzed using *t* test and are presented as fold change relative to the negative control. *MYC*, v-myc myelocytomatosis viral oncogene homolog; *TFDP1*, transcription factor Dp-1; *NFATC3*, nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 3; *SPI*, Sp1 transcription factor.

There was a significant relationship between miR-193b expression and CCL2 secretion independent of BMI (Table 4). Furthermore, the expression of both miR-193b and miR-126 correlated with integrin, α -X (ITGAX) mRNA levels (a gene specific for proinflammatory [M1] macrophages), regardless of body fat mass (Table 4).

DISCUSSION

In this study, we identified a set of miRNAs uniformly expressed in human WAT that are altered in obesity and that regulate CCL2 secretion from human fat cells and macrophages. *CCL2* is a key factor involved in the development of obesity-induced inflammation. Our findings show that miRNAs regulate CCL2 production via both direct and indirect mechanisms, the latter involving a network of distinct TFs.

miRNAs have emerged as important transcriptional regulators in both physiological and pathophysiological conditions. A number of studies identify several miRNAs to be dysregulated in obese WAT (22–24). However, there has been only a modest overlap concerning the specific miRNAs that have been described in each study. Our present results defining a set of 11 adipocyte-specific miRNAs downregulated in obesity were found in the largest cohort of subjects published so far ($n = 56$) and performed in both WAT and isolated adipocytes. Admittedly, our candidates only partially overlap with the previous studies, possibly explained by differences in study designs, for example, the use of only limited numbers of lean subjects (24), the inclusion of patients with diabetes (22), and/or the use of different technical methodologies (22–24).

By use of an unbiased approach involving several genome-wide and computational studies, we were able to dissect pathways linking miRNAs, TFs, and *CCL2*. By overexpressing each miRNA, we found that 10 out of 11 candidate miRNAs affected CCL2 secretion from fat cells. Nine of these miRNAs attenuated CCL2 release, which is in line with the observation that they are downregulated in obesity. In contrast, overexpression of miR-145 resulted in increased CCL2 secretion. However, since miR-145 is downregulated in obesity, the role of this miRNA remains to be defined.

MARA allowed us to construct a transcriptional regulatory network present in adipocytes and altered by obesity

that could mediate the effects of individual miRNAs on CCL2 secretion. The predicted network comprised several TFs with well-characterized roles in obesity-induced inflammation (1) and five miRNAs that were directly or indirectly linked to *CCL2*. For two of the miRNAs, we were able to confirm the regulatory pathways proposed by our model. In particular, we demonstrated a signal circuit from miR-126 directly to *CCL2* and from miR-193b to *CCL2* either via *ETS1* as a single TF step or via a series of other TFs (*MAX*, *NFKB1*, *RELB*, and *STAT6*). These findings were corroborated by several independent validation experiments and demonstrate a hitherto unknown role for miRNAs in the regulation of adipocyte CCL2 production. miR-193b may be of particular importance since its expression was associated with CCL2 secretion from subcutaneous WAT, independent of BMI. To our knowledge, several miRNAs identified in this study have not been previously associated with adipocyte function (miR-26a, -92a, -126, -145, -652, and let-7a/d) or controlling inflammatory pathways (miR-92a, -193a/b, and -652). Moreover, in recent murine studies, let-7 and miR-193b were implicated in controlling muscle glucose metabolism (25) and brown fat differentiation (26), respectively. This further supports a role for miRNAs in regulating different aspects of metabolism.

In this study, we used primary cultures of in vitro differentiated human adipocytes and the human monocyte/macrophage cell line (THP1) in our functional experiments. It could be argued that freshly isolated mature fat cells and adipose-derived macrophages should have been investigated. However, recent publications show that purified mature fat cells are not ideal for studies on inflammation since the isolation procedure per se affects inflammatory pathways (27). In addition, for both practical and ethical reasons, it is not possible to obtain sufficient amounts of adipose-derived macrophages from human biopsies.

Using the present approach, we were not able to identify how 8 out of 10 candidate miRNAs affect CCL2 secretion. There could be several explanations for this. Most important, the network constructed using the present approach is not complete. For instance, TFs without known DNA-binding motifs and other regulatory proteins (i.e., transcriptional cofactors) are not included in the current model.

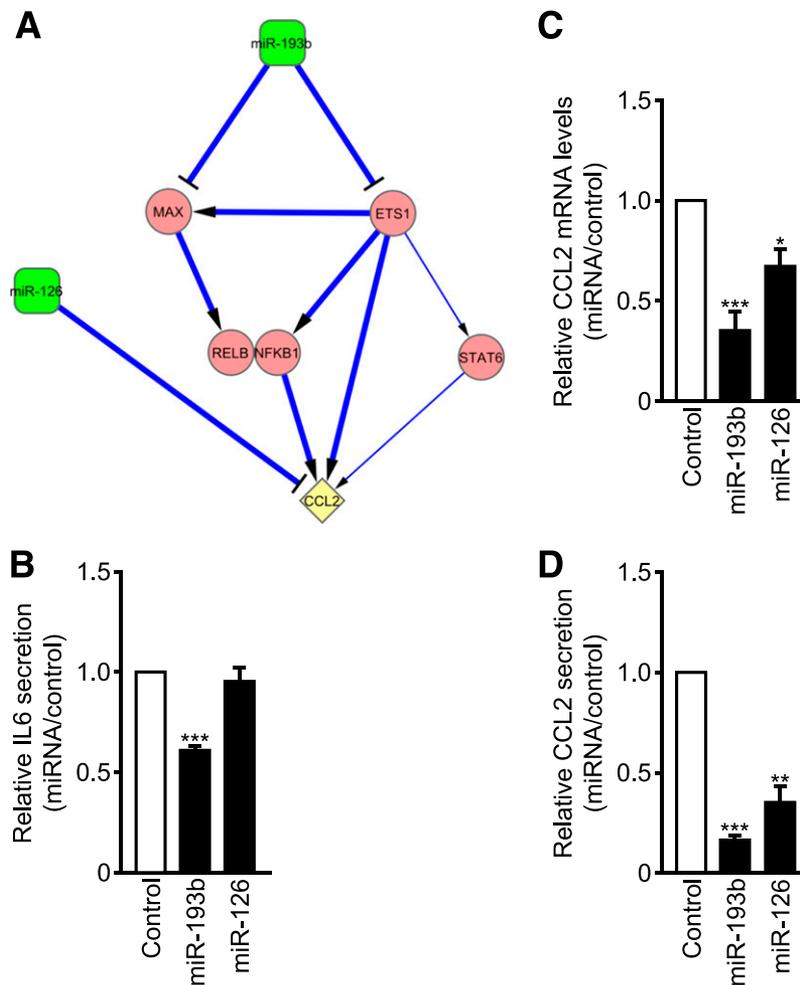


FIG. 4. Validated regulatory circuits controlling *CCL2* levels. **A:** The validated transcriptional regulatory network in fat cells included two miRNAs (miR-126 and miR193b; squares) and five TFs (*MAX*, *ETS1*, *RELB*, *NFKB1*, and *STAT6*; circles) all directly or indirectly regulating the production of *CCL2* (diamond) in human adipocytes. Bold edges represent physical interactions between nodes described previously or demonstrated in this study, and thin lines (*ETS1-STAT6-CCL2*) are predicted by network analyses. **B–D:** The effect of overexpression of miR-126 and miR-193 on IL-6 secretion from adipocytes (**B**) and *CCL2* expression and secretion in human macrophages (**C** and **D**) were examined. Results were analyzed using *t* test and are presented as fold change \pm SEM relative to the negative control. ****P* < 0.001, ***P* < 0.01, **P* < 0.05. (A high-quality color representation of this figure is available in the online issue.)

Therefore, more complex transcriptional regulatory networks, which include these and other factors, need to be constructed in future studies. Furthermore, we examined only subcutaneous WAT obtained from female donors and although unlikely, we cannot exclude sex- and region-specific differences in the regulation of *CCL2* production.

Although the focus of this study was on fat cells, our data in THP1 cells suggest that miR-193b and miR-126 may

TABLE 4
Association between miR-193b and miR-126 expression and adipose tissue inflammation

Parameter	<i>CCL2</i>	<i>ITGAX</i>
miR-193b	−0.286 (0.0248)	−0.303 (0.0174)
miR-126	−0.064 (0.6326)	−0.296 (0.0204)
<i>CCL2</i>		0.420 (0.0114)

Expression levels of miR-193b and miR-126 were correlated with adipose chemokine *CCL2* secretion and *ITGAX* mRNA levels in human adipose tissue obtained from 56 subjects. Results were evaluated using multiple regression analysis with BMI as covariate. Data are presented as partial *r* value (*P* value) for miRNAs, *ITGAX*, and *CCL2*.

also be important for the regulation of *CCL2* secretion in macrophages. However, whether the signaling circuit established in adipocytes is also present in macrophages remains to be determined. In any case, the clinical relevance of our findings is supported by the observation that there was a strong association between the expression of miR-193b or miR-126 and the mRNA levels of the M1 macrophage marker *ITGAX*, independent of BMI.

While *CCL2* may induce insulin resistance in skeletal muscle cells via direct mechanisms (28), there are no reports of such effects in fat cells. This suggests that the link between *CCL2*, adipose tissue inflammation, and local insulin resistance is indirect. Hence, *CCL2* may initiate and possibly sustain migration of proinflammatory cells into WAT, which in turn (together with the adipocytes) secrete a number of proteins (e.g., tumor necrosis factor- α and IL-6), which affect insulin signaling directly or indirectly, as discussed previously (29).

Taken together, our results suggest that specific miRNAs may be important regulators of inflammation in human adipose tissue through their effects on *CCL2* secretion from adipocytes and macrophages. This may be mediated by direct interactions with *CCL2* mRNA and/or via indirect

effects on TF circuits. miRNAs could therefore constitute novel potential targets for treating insulin resistance and type 2 diabetes in obesity. The approach presented herein may form the basis for future studies focusing on complex interactions regulating other aspects of human adipose tissue function.

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E.A. and C.O.D. designed the study; conducted microarray data, motif activity response, and network analyses; and wrote the manuscript. N.M. designed the study; conducted microarray data, motif activity response, and network analyses; collected tissue; performed the functional analyses; and wrote the manuscript. A.K., M.C., S.L.-C., A.E., J.L., P.H., and J.-F.T. collected tissue and performed the functional analyses. P.J.B., M.P., K.D.-W., Y.H., I.D., and E.v.N. conducted microarray data, motif activity response, and network analyses. M.R. collected tissue, performed the functional analyses, and wrote the manuscript. P.A. designed the study and wrote the manuscript. All authors contributed to data interpretation and reviewed and approved the final manuscript. C.O.D. and P.A. are the guarantors of this work and, as such, had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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