Additive effects of miRNAs and transcription factors on CCL2 production in human white adipose tissue

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Short title: Additive regulation of CCL2 by miRNAs and TFs

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Abstract

Adipose tissue inflammation is present in insulin resistant conditions. We recently proposed a network of microRNAs (miRNAs) and transcription factors (TFs) regulating the production of the pro-inflammatory chemokine (C-C motif) ligand-2 (CCL2) in adipose tissue. We presently extended and further validated this network and investigated if the circuits controlling CCL2 can interact in human adipocytes and macrophages. The updated sub-network predicted that miR-126/-193b/-92a control CCL2 production via several TFs, including ETS1, MAX and SP1. This was confirmed in human adipocytes by the observation that gene silencing of ETS1, MAX or SP1 attenuated CCL2 production. Combined gene silencing of ETS1 and MAX resulted in an additive reduction in CCL2 production. Moreover, overexpression of miR-126/-193b/-92a in different pair-wise combinations reduced CCL2 secretion more efficiently than either miRNA alone. However, while effects on CCL2 secretion by co-overexpression of miR-92a/-193b and miR-92a/-126 were additive in adipocytes, the combination of miR-126/-193b was primarily additive in macrophages. For miR-92a and -193b, their signals converged on the NFκB pathway. In conclusion, TF and miRNA-mediated regulation of CCL2 production is additive and partly relayed via cell-specific networks in human adipose tissue which may be important for the development of insulin resistance/type 2 diabetes.

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Introduction

White adipose tissue (WAT) function plays an important role in the development of insulin resistance/type 2 diabetes (T2D). Fat cells present in WAT secrete a number of molecules, collectively termed adipokines, which affect insulin sensitivity via auto- and/or paracrine mechanisms (1, 2). In insulin resistant obese subjects, WAT displays a chronic low-grade inflammation, which is proposed to alter insulin signaling via increased fatty acid release and altered adipokine production (3).

Chemokine (C-C motif) ligand-2 (CCL2), also referred as monocyte chemoattractant protein-1 (MCP-1) is an early pro-inflammatory signal in WAT which acts by promoting adipose infiltration of macrophages (4, 5). Following diet-induced obesity, Ccl2−/− mice are less insulin resistant and display a less pronounced inflammatory reaction in WAT (6). Both adipocytes and macrophages contribute significantly to CCL2 secretion from WAT.

Little is known about the mechanisms regulating WAT inflammation. Results in recent years have demonstrated that microRNAs (miRNAs) play important roles in controlling inflammation in other conditions and non-adipose tissues as reviewed (7). In mammals, miRNAs act by inhibiting the expression of target genes following binding to defined complementary sites in target 3’-untranslated regions (UTRs) (8).

Cellular function is controlled by both transcriptional and posttranscriptional mechanisms (TFs and miRNAs, respectively). Using global expression profiling data and mathematical models, the transcriptome can be mapped into transcriptional regulatory networks (TRNs) with regulatory elements, e.g. TFs and miRNAs, as nodes and their interactions as edges (9,
10). We recently described and partly experimentally verified a miRNA-TF network in subcutaneous WAT of insulin-resistant obese women, which controls the expression of CCL2 (11). This TRN suggests that several miRNAs and TFs interact in different combinations to regulate CCL2 production. Although the reason for this complex regulation and redundancy is not entirely clear, it may allow for more exact fine-tuning of gene expression and/or signal enhancement in response to specific environmental cues. In line with this, recent studies in cancer cells have proposed that partly overlapping regulatory circuits allow signal amplification, in an additive or synergistic manner (12, 13). To the best of our knowledge, it is not known if these types of mechanisms are present in WAT or if they differ between specific cell types within the tissue.

Through combinatorial gain-/loss-of-function studies focusing on TFs and miRNAs, we investigated how transcriptional regulators (TFs and miRNAs) can integrate effects on the expression of inflammatory factors in WAT. First, we extended and further validated the proposed TRN (11). We subsequently silenced potentially important TFs in the network, alone or in combination, and investigated how this altered CCL2 production in human adipocytes. Finally, we determined whether concomitant overexpression of miRNAs in either human adipocytes or macrophages resulted in increased effects when compared with overexpressing each miRNA alone.

**Research Design and Methods**

**Clinical material**

Cohort 1 comprised 30 obese (body mass index >30 kg/m²) and 26 non-obese (BMI <30 kg/m²) women who had no chronic disease and were free of continuous medication. This
cohort has been described in detail previously (11). The subjects came to the laboratory in the morning after the overnight fast. Height, weight, and waist circumference were determined. An abdominal subcutaneous WAT biopsy (~1.0-1.5 g) was obtained by needle aspiration as described (14). One part (300 mg) of the tissue was used for measurement of CCL2 release and expressed per number of fat cells as described (15). Another part of the tissue (at least 500 mg) was subjected to collagenase treatment, and mean adipocyte volume and weight were determined as described (16). 200 µl of packed fat cells and 400 mg intact WAT were frozen at -70 °C for future mRNA and miRNA measurements as described (11). This study was approved by the ethical committee at the Karolinska University Hospital, Stockholm, Sweden. All subjects were informed in detail about the studies and written informed consent was obtained.

For in vitro studies, subcutaneous WAT was obtained from healthy men and women undergoing cosmetic liposuction. In this experimental group there was no selection for age, sex or body mass index. Isolation of human adipocyte progenitor cells from subcutaneous WAT was performed as described (17). The progenitor cells obtained from separate individuals were not mixed.

**Affymetrix GeneChip Human Gene 1.0 ST and microRNA array protocols**

Data obtained from gene and microRNA arrays from human subcutaneous WAT (GEO number GSE25402) and the protocols have been described (11).

**Cell culture**

Culture and in vitro differentiation of human adipocyte progenitor cells were performed as described (17). 3T3-L1 and THP1 cells were handled as recommended in the protocols from
ATCC (Manassas, VA). For the induction of monocyte–macrophage differentiation, THP1 cells were stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA).

**MicroRNA and siRNA transfection**

In vitro differentiated adipocytes (day 10-12 post-induction) were treated for 24-48 hours with various concentrations (5-40 nM) of miRIDIAN miRNA mimics (for overexpression of miRNA activity) or 60 nM of miRIDIAN miRNA Hairpin inhibitors (for native miRNA inhibition) (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. Transfection efficiency was assessed with real-time PCR (qRThPCR) using miRNA probes/assays (Applied Biosystems, Foster City, CA and Qiagen, respectively). To rule out unspecific effects, control cells were transfected with miRIDIAN miRNA Mimic or Hairpin Inhibitor Non-Targeting Negative Controls (Thermo Fisher Scientific).

In some experiments, *in vitro* differentiated adipocytes were co-transfected with miScript Target Protector (for the miR-92a binding site on SP1, 500 nM, Qiagen) and miR-92a mimics (40 nM) or appropriate control reagents (miScript Target Protector Negative Control (Qiagen) and miRIDIAN miRNA Mimic Non-Targeting Negative Control (Thermo Fisher Scientific). THP1 cells were transfected with miRNA mimics as described above for adipocytes 48 h post-incubation with PMA.

For RNAi experiments, adipocytes were treated with various concentrations (10-40 nM) as described above using ON-TARGETplus SMARTpool siRNA for ETS1, SP1 or MAX and
appropriate concentrations of siRNA Non-Targeting Negative Control (Thermo Fisher Scientific) instead of miRNA reagents.

**RNA isolation, cDNA synthesis and real-time PCR**

Total RNA was extracted from in vitro differentiated adipocytes, 3T3-L1 cells and THP1-macrophages and purity control performed as described (11). Synthesis of coding-gene cDNA and RT-qPCR using Taqman probes (Applied Biosystems) was performed as described (11). Synthesis of miRNA cDNA and following RT-qPCR was performed using Applied Biosystems reagents as described (11) or using miScript II RT Kit and miScript Primer Assays (Qiagen) according to the manufacturer’s instructions. Relative gene expression was calculated using the comparative Ct-method, i.e. $2^{\Delta \text{Ct-target gene}}/2^{\Delta \text{Ct-reference gene}}$ with 18S as internal control. Expression of miRNA was normalized to a reference gene RNU48 or SNORD68. Levels of 18S, RNU48 and SNORD68 did not differ between groups.

**Western blot**

Cells were lysed in RIPA buffer as described (18). 15-20 µg of total protein was separated by SDS-PAGE and Western blot was performed according to standard protocols. The membranes were blocked in 3 % ECL Advance™ Blocking Agent (GE Healthcare, Buckinghamshire, UK), primary antibodies against ETS1 were from Novocastra™ LeicaBiosystems AB (Wetzlar, Germany), SP1 were from Santa Cruz Biotechnology (Santa Cruz, CA). β-Actin (Sigma-Aldrich, St. Louis, MO) was used as a loading control. Secondary antibodies mouse/rabbit IgG-horseradish peroxidase were from Sigma-Aldrich. All tested primary antibodies detecting MYC-associated factor X (MAX) were unspecific and results therefore could not be demonstrated. Antibody-antigen complexes were detected by chemiluminescence using ECL™ Select Western Blotting Detection Kit (GE Healthcare).
ELISA

CCL2 levels in conditioned media from *in vitro* differentiated adipocytes and macrophages were analyzed using an ELISA assay from R&D systems (Minneapolis, MN).

Luciferase Reporter Assay

Empty luciferase reporter vector and vector containing a part of 3’UTR of SP1 (enclosing predicted binding site for hsa-miR-92a) were obtained from GeneCopoeia (Rockville, MD). The luciferase reporter assay in 3T3-L1 cells was performed as described (11).

Global gene expression analysis, motif activity response analysis and network construction

Motif activity response analysis (MARA) and construction of the adipocyte CCL2 transcriptional regulatory network used in this study has been described in detail (11, 19). Predicted targets of microRNAs were updated according to the latest release of TargetScan (March, 2013).

Statistical analyses

Data presented are mean ± standard error of the mean (SEM). When appropriate, the data was log-transformed to become normally distributed. Results were analyzed with unpaired t-test, linear regression (simple and multiple) or analysis of variance (repeated-measures).
Results

Strategy for investigating additive effects of TFs and miRNAs

In order to dissect the regulation of adipocyte CCL2 production by miRNAs and TFs, we extended and further validated a recently described transcriptional regulatory network (TRN) present in human fat cells and perturbed in obese insulin resistant subjects (11). In brief, this \textit{in silico} generated TRN links for several miRNAs (miR-92a/-126/-193b/-652 and let-7a) directly or indirectly, through one or two intermediate TF-steps, to CCL2 production. For the present study, we focused on a sub-part of the TRN comprising two partly validated circuits (from miR-126 and -193b to CCL2) and a third possible candidate miR-92a, which was not fully characterized in our previous study (Figure 1A).

\textbf{miR-92a regulates CCL2 production via SP1 in adipocytes}

Because the regulatory edges linking miR-92a to CCL2 have not been explored in detail, we first tested if miR-92a affected its predicted target genes \textit{SP1} and \textit{ETS1} in human adipocytes. We overexpressed or inhibited miR-92a using miRNA mimics/inhibitors and evaluated expression of SP1 as well as mRNA expression of \textit{ETS1}. Modulation of miR-92a expression levels affected the mRNA and protein of SP1, while \textit{ETS1} expression remained unaltered (Figure 1B-D and Table 1), suggesting that SP1, but not ETS1, is a direct target of miR-92a. The levels of CCL2 in conditioned media mirrored the changes in SP1 mRNA/protein expression (Figure 1B, 1D). Luciferase reporter assays confirmed that miR-92a interacted directly with the 3’-UTR region of SP1 (Figure 1E).

To verify that SP1 could regulate CCL2 production in human adipocytes, independently of miR-92a, the expression of \textit{SP1} was silenced using siRNA at two different concentrations (10
and 20 nM). *SP1* gene knockdown significantly attenuated CCL2 secretion (Figure 2A) and reduced SP1 mRNA/protein levels (Figure 2B-C). In addition, *SP1* silencing affected mRNA expression of two predicted direct targets in the TRN: *CCL2* and *REL* (a part of the NFκB network; Table 1). To further investigate the role of miR-92a in CCL2 production via *SP1*, we concomitantly inhibited expression of miR-92a and *SP1*. The combination of miR-92a inhibition and silencing of *SP1* abolished their individual effects on CCL2 production (Figure 2D). Attenuation of miR-92a levels increased CCL2 secretion to the same extend as previously shown in Figure 1D. Moreover, simultaneous transfection of miR-92a mimics and a target protector (corresponding to the miR-92a target sequence on CCL2 3’UTR) abolished the effect of miR-92a on CCL2 production (Figure 2E).

Previous validations of the interactions in the CCL2 TRN identified that miR-126 targeted CCL2 directly while miR-193b affected its production indirectly through several TFs including ETS1 and MAX (11). In the present study we identified two additional TFs as possible targets for miR-126 and miR-193b, REL and SP1 respectively (Figure 1A). However, overexpression of miR-126 or miR-193b did not alter *REL* and *SP1* mRNA levels suggesting that they are not true targets for these miRNAs in human adipocytes (Table 1).

**ETS1 and MAX regulate CCL2 production in human adipocytes via TRN in an additive fashion**

According to Figure 1A, ETS1 and MAX are entry-TFs for miR-193b. Moreover, they have a predicted interaction and have been validated as direct targets of miR-193b (11). To determine whether ETS1 and MAX could affect CCL2 secretion independently of miR-193b, we knocked down ETS1 and MAX in adipocytes using various concentrations of siRNAs followed by measurements of mRNA levels of their first predicted neighbors in the TRN
We could confirm that silencing of \textit{ETS1} gene decreased the mRNA levels of \textit{MAX}, \textit{STAT6}, \textit{NFKB1} and \textit{CCL2} while silencing of MAX decreased the mRNA levels of \textit{RELB} (Table 1).

Silencing of ETS1 and MAX with 10 nM of siRNA had modest effects on CCL2 secretion, but higher concentrations, i.e. 20 nM, resulted in significantly decreased CCL2 production (Figure 3A). As CCL2 protein levels were affected by both TFs and \textit{MAX} mRNA expression was affected by silencing of ETS1, we assessed whether ETS1 and MAX could interact with each other in controlling CCL2 secretion. Indeed, concomitant silencing of ETS1 and MAX using low concentrations (10 nM) of siRNA resulted in a more pronounced reduction of CCL2 secretion (10+10 nM of each siRNA) when compared to single knockdown of either TF (Figure 3A). Expression of CCL2 mRNA followed the same trend as CCL2 secretion (Supplemental Figure 1A).

Knock-down efficiency was determined by quantifying \textit{ETS1} and \textit{MAX} mRNA levels (Figure 3B-C). There was a marked decrease in mRNA levels of \textit{ETS1} and \textit{MAX} either when silenced alone at 10/20 nM or in combination (10+10 nM). ETS1-siRNA treatment reduced protein levels of ETS1 (Figure 3D). Due to the poor specificity of tested MAX antibodies, we were not able to determine protein levels of this TF.

As mentioned above we observed that 20 nM of siRNA (either ETS1 or MAX) caused more pronounced decrease in CCL2 production than 10 nM. Unfortunately, we did not detect significant quantitative differences in the mRNA levels of \textit{ETS1} and \textit{MAX} between 10 and 20 nM of siRNA at a given time point. To control for off-target effects, we treated \textit{in vitro} adipocytes with transfection agent alone or transfected with various concentrations of siRNA.
non-targeting negative control followed by mRNA measurements. Indeed, basal expression levels of \textit{CCL2}, \textit{ETS1} or \textit{MAX} remained stable (Supplemental Figure 2).

Taken together, experiments in Figures 1-3 demonstrate that miR-193b and -92a attenuate CCL2 production in human adipocytes independently via TFs nodes comprising ETS1/MAX and SP1, respectively.

**MicroRNA-193b, -126 and -92a cooperatively affect CCL2 production in human adipocytes**

Since our data presented above indicate that TFs are able to additively control CCL2 secretion, we assessed if this mode of action is also present when combining miRNAs. We have previously shown that single overexpression of either miR-193b, -126 or -92a at high concentrations, i.e. 40 nM, downregulate CCL2 production to a substantial degree (30 to 50 %) (11). Here we assessed how co-overexpression of miRNAs affected CCL2 secretion. A caveat is that overexpression of multiple miRNA mimics, particularly at high concentrations, increases the risk for off-target effects. Therefore, lower concentrations of miRNA mimics were used in the combined transfections. At 5 nM, single overexpression of miR-193b, -126, and -92a had significantly weaker effects on CCL2 secretion compared with concomitant overexpression of miR-92a with either miR-193b or -126 (Figure 4A). However, combining miR-193b and -126 did not cause a more pronounced effect than miR-126 alone. The effects on CCL2 secretion were paralleled by similar changes in mRNA expression (Supplemental Figure 1B).

To rule out the possibility that the additive effects on CCL2 production by combined overexpression of miRNAs were not due to alterations in transfection efficiency, we
quantified miRNA levels after transfection. Indeed, there were no differences in miRNA abundance irrespective of whether they were overexpressed alone or in pairs (Supplemental Figure 3).

As suggested in Figure 1A, the signaling circuits for miR-193b and -92a converge on TFs in the NFκB pathway. We therefore quantified mRNA levels of *NFKB1* and *RELB* in the miRNA co-overexpression experiments. While there were no changes in the expression levels of *NFKB1* (Figure 4B), *RELB* mRNA expression was attenuated by the combined overexpression of miR-92a with either miR-193b or miR-126 (Figure 4C).

**Co-operative effects of miRNA 193b, -126 and -92a on CCL2 production in human macrophages.**

It is well known that CCL2 is secreted by several other cell types present within WAT among which macrophages are probably the most important (20). Therefore, we assessed the effects of miR-193b, -126 and -92a on CCL2 secretion in a human monocyte/macrophage cell line (THP1). We have previously shown that miR-193b and -126 affected CCL2 production in these cells using high concentrations of mimic reagents (40 nM) (11). To observe possible additive effects of miRNAs on CCL2 production, we overexpressed miRNAs at lower concentrations (10 nM). In this experimental setting, overexpression of miR-92a alone significantly decreased CCL2 secretion, while overexpressing miR-193b caused somewhat increased (albeit not statistically significant) CCL2 secretion. In contrast to the results obtained in adipocytes, co-overexpression miR-193b and -126 decreased the secretion of CCL2 significantly while the combinations of miR-193b/-92a and miR-126/-92a showed less prominent effects (Figure 4D).
Similar to the experiments in adipocytes, we quantified the expression of miRNAs post-transfection in THP1-macrophages. There were no differences in the abundance of miR-126 or -92a irrespective of whether they were overexpressed alone or in pairs (Supplemental Figure 4). Due to technical problems of tested probes for miR-193b, we were not able to determine levels of this miRNA in THP1 cells.

**Association between expression of adipose tissue miR-193b, -126 and -92a and CCL2 secretion**

The possible *in vivo* relevance was assessed by correlating expression levels of miRNAs (miR-193b, -126 and -92a) with CCL2 secretion in adipose tissue of Cohort 1 (results in Table 2). In simple regression, only miR-193b correlated significantly (and negatively) with CCL2 secretion. However, when miRNA levels were combined in a stepwise regression, miR-193b/-92a and miR-193b/-126 interacted significantly in the negative correlation with CCL2. The combinations explained 21-24% of the inter-individual variations in CCL2 secretion (adjusted $r^2$). There was no significant correlation with CCL2 release when miR-92a and -126 were combined as regressors (values not shown). MicroRNA-193b always entered as first step in the models.

As shown in Figure 1A, miR-193b targets two TFs in the TRN: ETS1 and MAX. We applied regression analysis to further investigate if the activity of these TFs co-varied in their relationship with adipose CCL2 secretion (Table 2). In simple regression, the motif activity of either TF was significantly and positively correlated with adipose CCL2 secretion (Table 2). In a stepwise regression, their motif activities acted together in a significant manner with regard to their correlation with CCL2 secretion. Together they explained 40% of the CCL2 variation (i.e. adjusted $r^2$). ETS1 entered as first step in the relationship. We also investigated
by regression analysis if the activity of SP1 (target of miR-92a) co-varied with adipose CCL2 secretion. In a simple regression, the SP1 motif activity was significantly and positively correlated with adipose CCL2 secretion ($r=0.42$, $p=0.003$).
Discussion

In this study, we tested whether combined silencing of TFs (ETS1 and MAX) and overexpression of miRNAs (miR-193b/-126/-92a) resulted in augmented effects in CCL2 production. Our main conclusion is that the three investigated miRNAs (miR-193b, -126 and -92a) regulated CCL2 production via distinct pathways involving specific TFs. Concomitant overexpression of miRNA-pairs or paired silencing of their cognate target TFs (ETS1 and MAX) resulted in additive effects on CCL2 production. The enhanced effects of miRNA-pairs were qualitatively different in human adipocytes compared with human macrophages. This suggests that TRNs regulating CCL2 production are cell-specific and that the combined effects of miRNAs influence cellular phenotypes.

Several biological processes, including diseases such as cancer, have been causatively associated with disturbances in the interplay between miRNAs and TF both in vitro and in vivo as reviewed (21). MicroRNAs and TFs are trans-activating factors that interact with cis-regulatory elements potentially generating complex combinatorial effects. Here we extensively characterized the first downstream targets of miRh193b and h92a in the proposed CCL2 TRN. By knocking down ETS1, MAX and SP1, we demonstrate that these TFs down regulate CCL2 production in fat cells independently of the targeting miRNAs. This suggests that these three TFs are upstream regulators of CCL2 in human adipose tissue. The results with ETS1 and SP1 are in accordance with findings in non-adipose tissue demonstrating that both TFs control CCL2 (22, 23). Although the MYC/MAX/MAD-family are well-established regulators of key processes in basic cell physiology including cell metabolism (24), their involvement in the regulation of CCL2 has not been described before. Our study therefore suggests a novel role for MAX in the regulation of adipose inflammation. Since both ETS1
and MAX were direct targets for miR-193b, we explored the effects on CCL2 production of combined knockdown of these two genes. This resulted in more pronounced inhibition of CCL2 production compared to single knockdown of either TF. Thus, miR-193b may amplify its signal to CCL2 through interactions within the TF network.

To study the combined effects of miRNAs in human adipocytes, we overexpressed miR-193b/-126/-92a individually and in pairs. In fat cells the combination of miR-92a with either miR-193b or -126 caused a more marked down regulation of CCL2 production compared to either miRNA alone. This demonstrates two types of signal amplifications which are summarized in Figure 5. For miR-92a and -193b there are interactions through the TF network that in an additive manner amplify the effects on CCL2 secretion (interaction A1 and A3). For miR-92a and -126 the effects are most probably obtained due to a combination of the TF network (miR-92a acts through SP1) and the direct interaction of miR-126 with CCL2 (interaction A2).

We also investigated if combinations of miRNAs could alter signaling further downstream in the TF network. Co-overexpression of miR-92a with either miR-193b or miR-126 down regulated RELB mRNA expression. This suggests that in adipocytes an additive effect of miRNAs on CCL2 production may be due to signals converging onto the NFκB/REL pathway. These results are in concordance with previously published data on other cell types demonstrating that NFκB/REL are downstream of SP1, ETS1 and MYC (an interaction partner with MAX) (25-27).

To study cell-specific miRNA effects on CCL2 regulation, we performed similar experiments in the human macrophage THP1 cell line. In contrast to fat cells, only the combination of
miR-193b and miR-126 had an additive effect on CCL2 production (Figure 5; interaction M1). Whether the miRNA-TF regulatory circuits established in adipocytes is also acting in macrophages remains to be determined. Nevertheless, our data demonstrates that miRNAs regulate CCL2 differentially in human macrophages compared with fat cells.

Are additive effects of miRNA signaling of clinical relevance? Unfortunately, it is not possible to determine this in vivo. In order to shed some light on this relevant issue, we performed extensive correlation analyses between miRNA expression, TF activities and CCL2 secretion in human subcutaneous adipose tissue. The combined expression of miR-193b/-92a or miR-193b/-126 explained inter-individual variations in CCL2 secretion to a larger extent than either miRNA alone. The same was true for the combined expression of MAX and ETS1. This supports the hypothesis that additive effects of miRNA and TFs may be clinically relevant although, admittedly, caution should be taken when extrapolating these statistical correlations into an in vivo situation. It is also important to stress, that the TRN constructed using the present approach is not complete and most likely includes other factors not evaluated in the current and previous analysis (11).

The majority of work characterizing miRNAs associated with obesity and/or insulin resistance concerns single miRNAs rather than combinations/clusters of miRNAs. We propose the following model for how miRNAs affect CCL2 production in human adipose tissue where the signal may be relayed in at least three different ways (Figure 5): (1) as the combined effects of direct interactions with CCL2 and indirect interactions with specific TFs; (2) as the interaction of several miRNAs in a TF network; (3) as the interaction of a single miRNA with several TFs. These combined effects are specific for different cell types within adipose tissue (e.g. fat cells and macrophages). In addition, there are probably other signaling
pathways that could be important. For example, the expression of other cyto-chemokines is probably under the control of other TRNs and other WAT regions besides the subcutaneous fat could be controlled by miRNAs in a different way.

In summary, human adipose CCL2 production is regulated by a local miRNA-TF network which allows diverse signal amplification and is specific for different cell types present in the tissue. This may be an important factor linking adipose tissue inflammation, insulin resistance and type 2 diabetes.
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A.K. and Y.B. designed the study, performed the functional analyses and wrote the manuscript. S.L.C. and C.B. performed the functional analyses and wrote the manuscript. E.A. and C.O.D. conducted microarray data, motif activity response, and network analyses; and wrote the manuscript. P.H. collected tissue and wrote the manuscript. M.R. designed the study, collected tissue and wrote the manuscript. N.M. designed the study, conducted microarray data, motif activity response, and network analyses, 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.

P.A. and A.K. are the guarantors of this work and, as such, had full access to all 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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**Figure legends**

**Figure 1. MicroRNA-92a regulates SP1 and CCL2 in human adipocytes.**

**A:** The proposed detailed CCL2 regulatory sub-network in human adipocytes depicting miR-193b, -126 and -92a (squares) as well as motifs and cognate TFs (circles) altered by obesity and targeting CCL2 (diamond). T-bars represent inhibition; arrows represent stimulation. Bold edges represent physical interactions between nodes described previously in adipocytes and thin lines represent predictions by network analyses and/or interactions in other cell types. Under the “NFκB” complex following TFs are merged: NFκB1 (nuclear factor of kappa light polypeptide gene enhancer in B-cells 1), RELA (v-rel reticuloendotheliosis viral oncogene homolog A (avian), RELB (v-rel reticuloendotheliosis viral oncogene homolog B) and REL (v-rel reticuloendotheliosis viral oncogene homolog (avian). Abbreviations of the TFs: SP1 (Specificity Protein 12), ETS1 (v-ets erythroblastosis virus E26 oncogene homolog 1 (avian), MAX (MYC associated factor X), ETS2 (v-ets erythroblastosis virus E26 oncogene homolog 2 (avian), STAT6 (signal transducer and activator of transcription 6, interleukin-4 induced).

**B:** Predicted interaction between miR-92a and SP1 was investigated by overexpressing miR-92a and assessing the effects on *SP1* mRNA expression in human *in vitro* differentiated adipocytes. Changes of CCL2 secretion in the same experiments are shown in parallel (n=8).

**C:** Representative Western blots of SP1 protein after overexpression and inhibition of miR-92a.

**D:** Quantification of SP1 protein levels after overexpression and inhibition of miR-92a. Changes of CCL2 secretion are shown in parallel (n=6-9).

**E:** MicroRNA-92a was overexpressed together with SP1 3’-UTR luciferase reporter in 3T3-L1 cells and luciferase activity was measured (n=8).
Figure 2. SP1 regulates CCL2 in human adipocytes.

A: Transcription factor SP1 was silenced with siRNA at 10 and 20 nM final concentrations in vitro differentiated adipocytes and secreted levels of CCL2 were assessed by ELISA (n=12-18).

B: Transcription factor SP1 was silenced with siRNA at 10 and 20 nM final concentration in vitro differentiated adipocytes and mRNA levels of SP1 were assessed (n=6-9).

C: Protein levels of SP1 were measured after silencing of SP1 (n=6). Representative blot is shown.

D: Expression of SP1 and levels of miR-92a were attenuated using siRNA and inhibitor of miRNA-92 individually or in pair with each other and CCL2 secretion was assessed using ELISA (n=6-14).

E: Interaction of miR-92a-SP1 was studied by co-transfecting human in vitro differentiated adipocytes with miR-92a mimics and target protector (corresponds to the binding site of miR-92a on the 3’UTR of SP1) following evaluation of CCL2 secretion by ELISA (n=7).

In order to rule out unspecific effects, cells in parallel were transfected with appropriate negative control (non-targeting negative control for miRNA mimics or inhibitor, non-targeting negative control for siRNA or negative control for target protector). Levels of mRNA were calculated using a comparative Ct-method, i.e. $2^{\Delta\Delta Ct}$-target gene/$2^{\Delta\Delta Ct}$-reference gene with 18S as reference gene. SP1 protein levels were evaluated by Western blotting. Levels of β-actin were used to control loading. Data were analyzed using t-test and are presented as fold change ± SEM relative to the negative control. ***, P<0.001; **, P<0.01; *, P<0.05.
Figure 3. ETS1 and MAX regulate CCL2 production in human adipocytes.

A: Transcription factors ETS1 and MAX were silenced with siRNA alone at 10 or 20 nM final concentration in vitro differentiated adipocytes and secreted levels of CCL2 were assessed by ELISA. ETS1 and MAX were also co-silenced in pair with each other (10+10 nM of each miRNA) and secreted levels of CCL2 were assessed (n=11-17).

B: ETS1 and MAX were silenced alone (10 and 20 nM) or co-silenced in pair with each other (10+10 nM of each miRNA) and mRNA of ETS1 was assessed (n=11-17).

C: ETS1 and MAX were silenced alone (10 and 20 nM) or co-silenced in pair with each other (10+10 nM of each miRNA) and mRNA of MAX was assessed (n=11-17).

D: Protein levels of ETS1 were measured after silencing (n=6). Representative blot is shown. In order to rule out unspecific effects, cells in parallel were transfected with siRNA non-targeting negative control. Levels of mRNA were calculated using a comparative Ct-method, i.e. $2^{△Ct}$-target gene/$2^{△Ct}$-reference gene with 18S as reference gene. ETS1 protein levels were evaluated by Western blotting. Levels of β-actin was used to control loading. Data were analyzed using t-test and are presented as fold change ± SEM relative to the negative control.

***, P<0.001; **, P<0.01; *, P<0.05.

Figure 4. MicroRNAs regulate CCL2 in an additive manner in human adipose tissue.

A: miR-193b, -126 and -92a were overexpressed alone at 5 nM final concentration or in co-overexpressed in pairs (5+5 nM of each miRNA) in in vitro differentiated adipocytes and secreted levels of CCL2 were assessed by ELISA (n=16-24).

B: miR-193b, -126 and -92a were overexpressed alone at 5 nM final concentration or in co-overexpressed in pairs (5+5 nM of each miRNA) in in vitro differentiated adipocytes and mRNA levels of NFkB1 were assessed (n=14-19).
C: miR-193b, -126 and -92a were overexpressed alone at 5 or 10 nM final concentration or in co-overexpressed in pairs (5+5 nM of each miRNA) in in vitro differentiated adipocytes and mRNA levels of RELB were assessed (n=14-19).

D: miR-193b, -126 and 92a were overexpressed alone at 10 or 20 nM final concentration or co-overexpressed in pairs (10+10 nM of each miRNA) in in vitro differentiated THP1 cells and secreted levels of CCL2 were assessed by ELISA (n=9).

In order to rule out unspecific effects, cells in parallel were transfected with equal concentration of non-targeting negative control for miRNA mimics. Levels of mRNA were calculated using a comparative Ct-method, i.e. $2^{\Delta\Delta Ct}$-target gene/$2^{\Delta\Delta Ct}$-reference gene with 18S as reference gene. Results were analyzed using t-test and are presented as fold change ± SEM relative to the negative control. ***, P<0.001; **, P<0.01; *, P<0.05.

Figure 5. Suggested model of integrated regulation of CCL2 by miRNAs and transcription factors in human adipocytes and macrophages.

The validated transcriptional regulatory sub-network including three microRNAs (miR-126, -193b and -92a, squares), three transcription factors (MAX, ETS1 and SP1, circles) and an NFkB sub-network all directly or indirectly regulating the expression and secretion of CCL2 (diamond) from the adipose tissue in obesity (upper part). In the lower part of the model microRNAs (miR-126 and -193b, squares) and possible TF network (circle) regulating CCL2 production in macrophages are presented. T-bars represent inhibition; arrows represent stimulation. Bold edges represent interactions between nodes described in this and previous studies in adipocytes and thin lines are predicted by network analyses or shown in other cells types. Dashed edges/lines in the lower part represent possible interactions.
Supplemental figure legends

Supplemental Figure 1. ETS1, MAX and miRNAs regulate CCL2 expression in human adipocytes in additive manner.

A: Transcription factors ETS1 and MAX were silenced with siRNA alone at 10 or 20 nM final concentrations or co-silenced in pair with each other (10+10 nM of each miRNA) in \textit{in vitro} differentiated adipocytes and mRNA levels of CCL2 were assessed by qRT-qPCR (n=5).

B: miR-193b, -126 and -92a were overexpressed alone at 5 nM final concentration or co-overexpressed in pairs (5+5 nM of each miRNA) in \textit{in vitro} differentiated adipocytes and mRNA levels of CCL2 were assessed (n=14-22).

In order to rule out unspecific effects, cells in parallel were transfected with appropriate concentrations of siRNA/miRNA non-targeting negative control. Levels of mRNA were calculated using a comparative Ct-method, i.e. $2^{\Delta\Delta Ct}$-target gene/$2^{\Delta\Delta Ct}$-reference gene with 18S as reference gene. Data were analyzed using t-test and are presented as fold change ± SEM relative to the negative control. ***, P<0.001; **, P<0.01; *, P<0.05; t, P<0.1.

Supplemental Figure 2. Treatment with siRNA non-targeting negative control does not affect mRNA levels of CCL2, ETS1 and MAX.

\textit{In vitro} differentiated adipocytes were treated with transfection agent HiPerfect only (mock transfection) or transfected with 10 and 20 nM of siRNA non-targeting negative control. After 48 hours, mRNA of CCL2, ETS1 and MAX were assessed by qRT-qPCR (n=9). Levels of mRNA were calculated using a comparative Ct-method, i.e. $2^{\Delta Ct}$-target gene/$2^{\Delta Ct}$-reference gene with 18S as reference gene. Data were analyzed using t-test and are presented as fold change ± SEM relative to the mock transfection (HiPerfect only); ns, non-significant.
Supplemental Figure 3. Quantification of overexpression of miR-193b, -126 and -92a in adipocytes. MicroRNAs were expressed individually or co-overexpressed in in vitro differentiated adipocytes and expression of miRNAs was quantified using RT-qPCR (n=11-16).

Data was log10-transformed, analyzed using t-test and are presented as fold change ± SEM relative to the negative control. ***, P<0.001; **, P<0.01; *, P<0.05. Levels of miRNAs were calculated using a comparative $2^{\Delta Ct}$-target gene/$2^{\Delta Ct}$-reference gene with RNU48 as reference gene.

Supplemental Figure 4. Quantification of overexpression of miR-126 and -92a in in vitro differentiated THP1 cells. MicroRNAs were expressed individually or co-overexpressed in in vitro differentiated THP1 cells and expression of miRNAs was quantified using RT-qPCR (n=3).

Data was log10-transformed, analyzed using t-test and are presented as fold change ± SEM relative to the negative control. ***, P<0.001; **, P<0.01; *, P<0.05. Levels of miRNAs were calculated using a comparative $2^{\Delta Ct}$-target gene/$2^{\Delta Ct}$-reference gene with RNU48 or SNORD68 as reference gene.
References

1. Fain JN. Release of inflammatory mediators by human adipose tissue is enhanced in obesity and primarily by the nonfat cells: a review. Mediators of inflammation 2010;2010:513948.
20. Fain JN. Release of interleukins and other inflammatory cytokines by human adipose tissue is enhanced in obesity and primarily due to the nonfat cells. Vitam Horm 2006;74:443-77.


Table 1. Interactions between miRNAs or TFs and first predicted neighbors.

<table>
<thead>
<tr>
<th>miRNA/TF siRNA</th>
<th>Predicted first neighbor</th>
<th>Fold change treatment/NegC</th>
<th>t test P-value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-92a</td>
<td>SP1</td>
<td>0.782</td>
<td>0.031</td>
<td>this study (Figure 1B)</td>
</tr>
<tr>
<td></td>
<td>ETS1</td>
<td>1.09</td>
<td>0.3108</td>
<td>this study</td>
</tr>
<tr>
<td>miR-193b</td>
<td>ETS1</td>
<td>0.54</td>
<td>0.0001</td>
<td>(11)</td>
</tr>
<tr>
<td></td>
<td>MAX</td>
<td>0.58</td>
<td>0.0001</td>
<td>(11)</td>
</tr>
<tr>
<td>miR-126</td>
<td>CCL2</td>
<td>0.67</td>
<td>0.0112</td>
<td>(11)</td>
</tr>
<tr>
<td>siSP1</td>
<td>CCL2</td>
<td>0.76</td>
<td>0.007</td>
<td>this study</td>
</tr>
<tr>
<td></td>
<td>REL</td>
<td>0.77</td>
<td>0.0365</td>
<td>this study and (28) (not adipocytes)</td>
</tr>
<tr>
<td></td>
<td>NFκB1</td>
<td>0.85</td>
<td>0.3563</td>
<td>this study</td>
</tr>
<tr>
<td></td>
<td>ETS2</td>
<td>not evaluated due to low expression</td>
<td>-</td>
<td>this study and (29) (not adipocytes)</td>
</tr>
<tr>
<td>siETS1</td>
<td>MAX</td>
<td>0.82</td>
<td>0.0178</td>
<td>this study</td>
</tr>
<tr>
<td></td>
<td>NFκB1</td>
<td>0.80</td>
<td>0.0242</td>
<td>this study and (27) (not adipocytes)</td>
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<tr>
<td></td>
<td>STAT6</td>
<td>0.67</td>
<td>0.0023</td>
<td>this study</td>
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<tr>
<td></td>
<td>CCL2</td>
<td>0.56</td>
<td>0.0055</td>
<td>this study and (30) (not adipocytes)</td>
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<tr>
<td>siMAX</td>
<td>RELB</td>
<td>0.84</td>
<td>0.0106</td>
<td>this study</td>
</tr>
</tbody>
</table>
Predicted interactions between miRNAs and first neighbors were investigated by overexpression of each miRNA and assessing mRNA expression of TFs in human *in vitro* differentiated adipocytes. Predicted TF interactions with first neighbors were investigated by transfecting human *in vitro* differentiated adipocytes with siRNA and assessing the effects on mRNA expression of first neighbors.
Table 2. Correlation between CCL2 secretion, expression of miRNAs in adipose tissue and activity of ETS1 and MAX determined by MARA.

<table>
<thead>
<tr>
<th>Regressor</th>
<th>Single regression</th>
<th>Stepwise regression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r-value</td>
<td>p-value</td>
</tr>
<tr>
<td>miR-193b alone</td>
<td>-0.39</td>
<td>0.007</td>
</tr>
<tr>
<td>miR-126 alone</td>
<td>-0.21</td>
<td>0.15</td>
</tr>
<tr>
<td>miR-92a alone</td>
<td>-0.28</td>
<td>0.053</td>
</tr>
<tr>
<td>miR-193b + 92a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>For miR-193b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>For-92a</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>miR-193b + 126</td>
<td></td>
<td></td>
</tr>
<tr>
<td>For miR-193b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>For miR-126</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ETS1 alone</td>
<td>0.57</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>MAX alone</td>
<td>0.41</td>
<td>0.004</td>
</tr>
<tr>
<td>ETS1 + MAX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>For ETS1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>For MAX</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

F-value > 4 indicates that the regressors have significantly entered the model. CCL2 secretion was expressed per number of fat cells.
Figure 1

A) Schematic diagram of the signaling pathways involved in the regulation of CCL2 expression.

B) Bar graphs showing relative mRNA level of SP1 and relative CCL2 secretion. The x-axis represents different treatments: Mimics, NegC, and Mimics miR-92a. The y-axis represents the fold change in expression compared to NegC.

C) Western blots showing relative SP1 protein levels and relative CCL2 secretion. The blots compare Mimics and Inhibitor treatments with NegC.

D) Bar graphs showing relative SP1 protein levels and relative CCL2 secretion. The treatments include Appropriate, Mimics, and Inhibitor with NegC and miR-92a.

E) Bar graph showing relative luciferase activity for SP1 3'UTR.

Diabetes
**Figure 2**

(A) Graph showing relative CCL2 secretion (fold change siRNA/NegC) for appropriate, 10 nM, and 20 nM siSP1 treatments.

(B) Graph showing relative SP1 mRNA (fold change siRNA/NegC) for appropriate, 10 nM, and 20 nM siSP1 treatments.

(C) Graph showing relative CCL2 secretion (fold change treatment/NegC) for appropriate, siSP1, and siSP1 + Inhibitor miR-92a treatments.

(D) Graph showing relative CCL2 secretion (fold change treatment/NegC) for appropriate, siSP1, and siSP1 + Inhibitor miR-92a + SP1 target protector treatments.

(E) Graph showing relative CCL2 secretion (fold change treatment/NegC) for appropriate, Mimics miR-92a, and Mimics miR-92a + SP1 target protector treatments.
Figure 3

A

Relative CCL2 secretion (fold change siRNA/NegC)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>10 nM</th>
<th>20 nM</th>
<th>10+10 nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appropriate NegC</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>siETS1</td>
<td></td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>siMAX</td>
<td></td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>siETS1 + siMAX</td>
<td></td>
<td>0.7</td>
<td></td>
</tr>
</tbody>
</table>

B

Relative ETS1 mRNA (fold change siRNA/NegC)

C

Relative MAX mRNA (fold change siRNA/NegC)

D

Western Blot

siETS1 NegC (20 nM)

ETS1-Actin-50 kDa

ETS1-Actin-42 kDa
Figure 4

A (adipocytes)

B (NFkB1)

C (RELB)

D (macrophages)
Figure 5

Diabetes

Adipocytes

Macrophages
Supplemental figure 3

miR-193b

miR-126

miR-92a