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Suppression of lipin-1 expression increases monocyte chemoattractant protein-1 expression in 3T3-L1 adipocytes

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Abstract

Lipin-1 plays a crucial role in the regulation of lipid metabolism and cell differentiation in adipocytes. Expression of adipose lipin-1 is reduced in obesity, and metabolic syndrome. However, the significance of this reduction remains unclear. This study investigated if and how reduced lipin-1 expression affected metabolism. We assessed mRNA expression levels of various genes related to adipocyte metabolism in lipin-1-depleted 3T3-L1 adipocytes by introducing its specific small interfering RNA. In lipin-1-depleted adipocytes, mRNA and protein expression levels of monocyte chemoattractant protein-1 (MCP-1) were significantly increased, although the other genes tested were not altered. The conditioned media from the cells promoted monocyte chemotaxis. The increase in MCP-1 expression was prevented by treatment with quinazoline or salicylate, inhibitors of nuclear factor-κB activation. Because MCP-1 is related to adipose inflammation and systemic insulin resistance, these results suggest that a reduction in adipose lipin-1 in obesity may exacerbate adipose inflammation and metabolism.

Keywords: Lipin-1; Adipocyte; MCP-1; Obesity; Adipose inflammation

1. Introduction

Lipin-1 was cloned as the causative gene of fatty liver dystrophy (*fld*) mice, which is a spontaneous mutant strain characterized by lipodystrophy, neonatal fatty liver, and peripheral neuropathy [1]. Using database searches, lipin-2 and lipin-3 were also found. These three mammalian lipin family genes exhibit distinct patterns of tissue-specific expression, with lipin-1 predominantly expressed in adipocytes and skeletal muscle cells [2]. Lipin-1 has been involved in multiple pathways regulating metabolism. First, lipin-1 operates as an enzyme, phosphatidic acid phosphatase, which catalyzes the formation of diacylglycerol from phosphatidic acid in the penultimate step of triglyceride synthesis [3]. Second, lipin-1 is involved in adipogenesis and maintenance of adipocyte function to facilitate peroxisome proliferator-activated receptor (PPAR)-γ expression [4, 5]. Third, lipin-1 regulates lipid metabolism at the transcriptional level by interacting with transcription factors (PPAR-α) or coactivator protein (PPAR-γ coactivator-1α [6]. Collectively, these findings place lipin-1 as a multiple regulator of metabolism.

Adipose lipin-1 mRNA expression has been strongly correlated with insulin sensitivity in obese subjects, impaired glucose tolerance, metabolic syndrome, or in young healthy people [7-13]. Decreased lipin-1 expression in morbidly obese patients has been shown to recover after weight reduction surgery [8, 9]. A strong negative correlation was observed between adipose lipin-1 mRNA levels and fasting glucose and insulin levels, as well as the insulin resistance marker, HOMA-IR, in both mice and humans [10]. On the contrary, adipocyte-specific lipin-1 transgenic mice fed a high-fat diet developed obesity but unexpectedly showed an amelioration

in insulin resistance [14]. These *in vivo* studies confirmed that higher adipose lipin-1 expression levels are favorable for systemic insulin sensitivity. Therefore, understanding the mechanism of reduced adipose lipin-1 expression in obesity is important in the treatment of metabolic diseases, including obesity, metabolic syndrome and diabetes. Here, we investigated the effects of lipin-1 depletion in adipocytes on reduced systemic insulin sensitivity.

2. Materials and Methods

2.1. Cell culture and adipocyte differentiation

3T3-L1 fibroblasts (ATCC, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) containing 4.5 g/L glucose supplemented with 10% fetal bovine serum (FBS, BioWest, Nuaillé, France). Adipocyte differentiation using insulin (2 μM), 3-isobutyl-methyl-xanthine (0.5 mM), and dexamethasone (1 μM) and subsequent culture was performed in a 6-well plate as described previously [15]. Adipocytes were used 12 days after induction of differentiation. THP-1 monocytes (ATCC) were maintained in RPMI 1640 medium (Sigma) supplemented with 10% FBS.

2.2. Lipin-1 gene silencing by small interfering RNA (siRNA)

Predesigned siRNA against lipin-1 (siLip1-1 and siLip1-2, corresponding to the catalog numbers MSS204345 and MSS204346, respectively) and negative control (NC) siRNA (Stealth™ RNAi Negative Control Low GC Duplex #2) were purchased from Invitrogen (Carlsbad, CA, USA). Transfection into 3T3-L1 adipocytes was performed by lipofection using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instruction with each siRNA at a final concentration of 50 nM. The cells were examined 48 h after transfection.

2.3. Quantitative real-time PCR analysis

RNA extraction and quantitative real-time PCR analysis were performed with an Applied Biosystems 7500 Sequence Detection System using TaqMan Gene Expression master mix, according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA), as described previously [15]. Validated TaqMan Gene Expression Assays containing gene specific TaqMan probes and primers (Applied Biosystems) for lipin-1, lipin-2, lipin-1α, lipin-1β, monocyte chemoattractant protein-1 (MCP-1), PPAR-α, glucose transporter 4 (GLUT4), adiponectin, retinol binding protein 4 (RBP4), uncoupling protein-1 (UCP1), and toll-like receptor 4 (TLR4) were used for assay-on-demand gene expression products (Supplementary Table 1). The eukaryotic 18S rRNA gene was used as an endogenous control to normalize the relative expression of these genes. All experiments were performed at least in triplicate. Amplification was determined using the comparative threshold cycle (CT) method and Sequence Detection Software version 1.4 (Applied Biosystems). The 2-$\Delta\Delta$CT method was used to

calculate the relative expression of mRNA [15].

2.4. Protein extraction and Western blotting

Protein extraction and Western blotting were performed as described previously [16]. In brief, equal amounts of extracted protein (40 μg per lane) were resolved on 5–15% gradient SDS-PAGE for Western blotting using antibodies against mouse lipin-1 (Cell Signaling technologies, Danvers, MA, USA) or β-actin (Santa Cruz Biotechnology). For evaluating MCP-1 secretion, conditioned medium was obtained from 1.5-mL of fresh medium after 6 h incubation of 3T3-L1 adipocytes, transfected 42 h previously with lipin-1 specific (siLip1-1, siLip1-2) or NC siRNA. A 15-μL aliquot of the conditioned medium was then subjected to SDS-PAGE, followed by immunoblotting with MCP-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The bands were visualized with a Light Capture II system (ATTO Co., Tokyo, Japan).

2.5. Mouse MCP-1 ELISA

3T3-L1 adipocytes were cultured in 6-well plates and transfected with lipin-1 specific (siLip1-1) or NC siRNA. After 36 h of transfection, the culture medium was replaced with 1.5 mL of fresh medium, followed by further 12 h of incubation. A 50-uL aliquot of the medium was used for the MCP-1 assay, according to the manufacturer's instruction (mouse MCP-1 ELISA kit, Thermo Scientific, Rockford, IL, USA). The remaining medium was stored at −80°C for the monocyte

chemotaxis assay.

2.6. Monocyte chemotaxis assay

The chemotactic activity of the conditioned media from the 3T3-L1 adipocytes was investigated using a 24-well Transwell system with a 8-μm pore size membrane (Corning, Corning, NY, USA), with modifications of the method described by Youn et al. [17]. The conditioned medium (siLip1-1, NC) from the MCP-1 assay or control medium (DMEM with 10% FBS) was placed in the lower wells of a Transwell. THP-1 monocytes (2 x 10^5 cells per well) were incubated in the upper wells. After 1.5 h of incubation at 37°C in 5% CO_2 atmosphere, the number of migrated cells in the lower wells was enumerated using a hemocytometer under a light microscope. Cell viability was determined by trypan blue staining. The results were expressed as the number of cells per well after subtraction of the value obtained from the control medium that represented non-specific migration.

2.7. Treatment of cells with nuclear factor-κB (NF-κB) inhibitors

Cells were treated with a NF-κB inhibitor, sodium salicylate (Sigma) or 6-Amino-4-(4-phenoxyphenylethylamino) quinazoline (QNZ) (Santa Cruz Biotechnology), 0.5 h before initiating lipin-1 gene silencing [18,19]. The sodium salicylate was dissolved in water, whereas QNZ was dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 0.1% DMSO in the medium.

2.8. Statistical Analyses

The results are expressed as mean ± S.E. For comparison of more than two groups, ANOVA was performed followed by Turkey's multiple comparison tests using GraphPad PRISM software version 5 (GraphPad Software Inc., San Diego, CA, USA). Two-tailed unpaired Student's *t*-tests were used for comparison of the two groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Lipin-1 mRNA and protein expression were decreased by the specific siRNA in 3T3-L1 adipocytes

We first made a lipin-1 depletion model in adipocytes using two different lipin-1 specific siRNAs to eliminate the non-specific effects of a single use of siRNA. We also tested whether lipin-1 suppression may affect lipin-2 expression, because continuous lipin-1 suppression initiated before adipocytic differentiation increases lipin-2 expression in 3T3-L1 adipocytes [20]. As demonstrated in Fig. 1A, treatment with lipin-1 specific siRNAs (siLip1-1 and siLip1-2) resulted in suppression of lipin-1 mRNA expression by approximately 40% when compared to the vehicle (NC). Lipin-2 expression levels were considerably lower compared to lipin-1

expression levels and were not affected by lipin-1 depletion. Lipin-1α and lipin-1β, lipin-1 protein isoforms, derived from the Lpin1 gene by alternative mRNA splicing, have been shown to be expressed in adipocytes [21]. The expression of both lipin-1 isoforms was significantly reduced by lipin-1 suppression. The expression of both lipin-1 isoforms was significantly reduced by lipin-1 suppression, as expected by the design of siRNAs targeting lipin-1 (Fig. 1B). We also confirmed that protein expression was decreased by the suppression of the lipin-1 gene (Fig. 1C). Therefore, these findings established the model of lipin-1-depleted adipocytes that were used in subsequent experiments.

3.2. MCP-1 expression was increased in lipin-1 suppressed 3T3-L1 adipocytes

We next investigated the effects of lipin-1 depletion on expression of genes related to metabolism using quantitative PCR. We tested the expression of PPAR-α, GLUT4, and adiponectin, which have been shown to have a correlation with adipose lipin-1 expression levels in human studies [11-13]. We also examined the expression of other genes related to adipocyte function including adipokines (MCP-1, RBP4), metabolism (UCP1), and inflammation (TLR4). As shown in Figs. 2A–G, expression of MCP-1 was increased significantly by 1.8–2.0–fold in both lipin-1-depleted adipocytes (siLip1-1 and siLip1-2), whereas expression of the other genes tested (PPAR-α, GLUT4, adiponectin, RBP4, UCP1, and TLR4) remained unaltered.

3.3. MCP-1 secretion and chemotactic activity was increased in lipin-1-depleted adipocytes

We attempted to confirm whether increased MCP-1 mRNA expression induced by lipin-1 depletion was associated with its secretion and biological activity. Western blot (Fig. 3A) and ELISA assay (Fig. 3B) demonstrated that MCP-1 secretion in conditioned medium obtained from lipin-1-depleted 3T3-L1 adipocytes was significantly increased. Furthermore, the conditioned medium was able to significantly attract THP-1 monocytes (Fig. 3C). These results indicate that lipin-1 depletion in adipocytes promotes secretion of biologically active MCP-1.

3.4. Treatment of NF-κB inhibitor blocked the increase of MCP-1 mRNA expression induced by lipin-1 depletion in 3T3-L1 adipocytes

To investigate the mechanism by which lipin-1 depletion increased MCP-1 expression, we examined the possible involvement of NF-κB signaling [22]. We employed two inhibitors of NF-κB activation, quinazoline and sodium salicylate. siRNA-induced lipin-1 suppression (siLip1-1 and siLip1-2) was not altered by treatment with either inhibitor when compared to the vehicle (NC) (Figs. 4A-B), whereas the increase in MCP-1 expression was prevented (Figs. 4C-D). These results suggest that NF-κB signaling may be involved in lipin-1 depletion-induced MCP-1 expression in 3T3-L1 adipocytes.

4. Discussion

Our major findings are that lipin-1 depletion by RNA interference resulted in increased expression and chemotactic activity of MCP-1 in 3T3-L1 adipocytes. Secretion of MCP-1 from adipose tissue is involved in monocyte/macrophage chemotaxis and adipose inflammation in obesity [23-26]. Adipose MCP-1 is derived from stromal-vascular cells and adipocytes. Although the expression level of MCP-1 is lower in adipocytes compared with than in stromal-vascular cells [26], MCP-1 from adipocytes still has a crucial role in adipose inflammation and systemic insulin resistance. These effects have been demonstrated by adipose MCP-1 overexpression and deletion studies [27,28]. Studies on genetic deficiency or pharmacological inhibition of the MCP-1 receptor also provided evidence for the functions of MCP-1 [29]. Secretion of MCP-1 from human adipocytes correlates positively with adipocyte size [30]. Mice fed a high-fat diet were shown to have increased MCP-1 mRNA expression in adipose tissue at an early stage of obesity, when macrophages had not infiltrated [31]. Considering these implications of MCP-1 functions, adipose lipin-1 can be a crucial regulator for adipose inflammation and systemic insulin resistance by increasing MCP-1.

Kim et al. showed recently that lipin-1 represses transcriptional activity of a non-nuclear transcription factor, nuclear factor of activated T cells c4 (NFATc4), which has an important role in activation of cytokine expression including tumor necrosis factor (TNF)-α, interleukin-6, and resistin [32]. They also showed that lipin-1 suppression by small hairpin RNA (shRNA) induced TNF-α expression in 3T3-L1 adipocytes through an interaction between lipin-1 and NFATc4. We also

measured TNF-α expression in our model, although its expression was too low to calculate meaningful levels (data not shown). This discrepancy may be explained partly by the fact that the suppression levels of lipin-1 in the study by Kim et al were considerably lower (over 90% reduction) compared with that in our study (approximately 40% reduction). This greater suppression of lipin-1 expression may have resulted in increased expression of TNF-α. The degree of reduction of lipin-1 expression in our model was similar to that observed in obesity or metabolic syndrome in previous clinical investigations [7-13]. In terms of increased expression of inflammatory cytokines induced by in adipose lipin-1 depletion, our findings are consistent with theirs.

In view of the relationship between lipin and inflammation, lipin-2 may have an important role in regulation of inflammatory responses. For example, lipin-2 mutations are known to be involved in Majeed syndrome, an autoinflammatory disorder with a cluster of symptoms including recurrent fever, sterile osteomyelitis, cutaneous inflammation, and anemia [33,34]. Although the mechanism by which lipin-2 mutations cause these symptoms is not known, each lipin isoform may have a role in causing inflammation in different organs.

The mechanism by which lipin-1 depletion increases MCP-1 expression is at least partly through the NF-κB pathway. Sodium salicylate inhibits IκB kinase-β activity upstream of NF-κB activation signaling [18], whereas quinazoline inhibits the transcriptional activity of NF-κB [19]. Although we tested two different points of action in NF-κB signaling, a potential weakness of this study is the little information we obtained on the direct activation of NF-κB induced by lipin-1 depletion. Because lipin-1 modulates cellular lipid signaling by acting as a phosphatidic acid

phosphatase, depletion of lipin-1 expression increases phosphatidic acid and its derivatives. Increased lysophosphatidic acid levels in muscle have also been documented in a patient with lipin-1 deficiency [35]. This lipid signaling may affect the activity of NF-κB, as there is evidence lysophosphatidic acid activates NF-κB [36]. Further studies are needed to clarify the precise mechanism by which lipin-1 depletion induces NF-κB activation.

The mechanism of lipin-1 suppression is also an important issue in the reduction of adipose lipin-1 expression in obesity. We had previously demonstrated that TNF-α reduces lipin-1 expression in 3T3-L1 adipocytes through Jak2 signaling [15]. Adipose inflammation is now recognized as a crucial pathological feature in obesity with infiltrated macrophages in obese adipose tissue secreting TNF-α, thereby worsening adipose inflammation. The mutual interactions between secreted MCP-1 from adipocytes and secreted TNF-α from infiltrated monocyte/macrophage may contribute to exacerbation of adipose inflammation. Our findings support the concept that adipose lipin-1 has a crucial role in the "vicious cycle" of adipose inflammation.

In conclusion, these results indicate the potential importance of reducing adipose lipin-1 levels as a therapeutic molecular target in patients with obesity.

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Figure legends

Fig. 1. Lipin-1 specific siRNA downregulates lipin-1 mRNA and protein expression in 3T3-L1 adipocytes. The siRNA against mouse lipin-1 (siLip1-1 or siLip1-2), or negative control (NC) siRNA were transfected for 48 h by lipofection into 3T3-L1 adipocytes on day 12 at a final concentration of 50 nM. (A) Lipin-1 and lipin-2 mRNA expression were quantified by real-time PCR. (B) Expression of lipin-1α and lipin-1β mRNA was also quantified. The results were normalized relative to mRNA levels of 18S rRNA and calculated as change relative to the vehicle (NC). The results of at least three independent experiments produced similar results. Data are expressed as mean ± S.E. (n = 3 or 4). *$P < 0.05$ vs. vehicle (NC). (C) Lipin-1 protein expression was analyzed by Western blotting with a β-actin probe was used as the loading control.

Fig. 2. Effects of lipin-1 downregulation on gene expression related to adipocyte function. Expression of mRNA for monocyte chemoattaractant protein-1 (MCP-1, A), peroxisome proliferator-activated receptor-α (PPAR-α, B), glucose transporter 4 (GLUT4, C), adiponectin (D), retinol binding protein 4 (RBP4, E), uncoupling protein-1 (UCP1, F), and toll-like receptor 4 (TLR4, G) were measured by quantitative real-time PCR in artificially lipin-1 suppressed adipocytes. The results were normalized relative to mRNA levels of 18S rRNA and expressed as changes relative to the vehicle (NC). Similar results were obtained in at least two separate experiments. Data are expressed as mean ± S.E. (n = 4). *$P < 0.05$ vs. vehicle (NC).

Fig. 3. MCP-1 secretion and chemotactic activity in lipin-1-depleted 3T3-L1 adipocytes. 3T3-L1 adipocytes were transfected using lipofection with either siRNA against mouse lipin-1 (siLip1-1 or siLip1-2) or negative control (NC) siRNA at a final concentration of 50 nM. The cells were incubated with fresh medium for the last 6 h (A) or 12 h (B, C) of a 48 h culture following initiation of lipofection. Secretion of MCP-1 in the medium was detected by Western blotting (A) or measured by ELISA (B). (C) Monocyte chemotaxis induced by the conditioned medium was evaluated using a Transwell system with a 8-μm pore size membrane. The conditioned medium (NC or siLip1-1) or control medium (DMEM with 10% FBS) was placed in the lower wells. THP-1 monocytes (2×10^5 cells per well) were incubated in the upper wells. After 1.5 h of incubation, the number of migrated cells in the lower wells was counted by a hemocytometer under a light microscope. The results are expressed as number of migrated cells per well after subtraction of the value of non-specific migration (control medium). Data are expressed as mean ± S.E. (n = 4). $*P < 0.05$ vs. vehicle (NC).

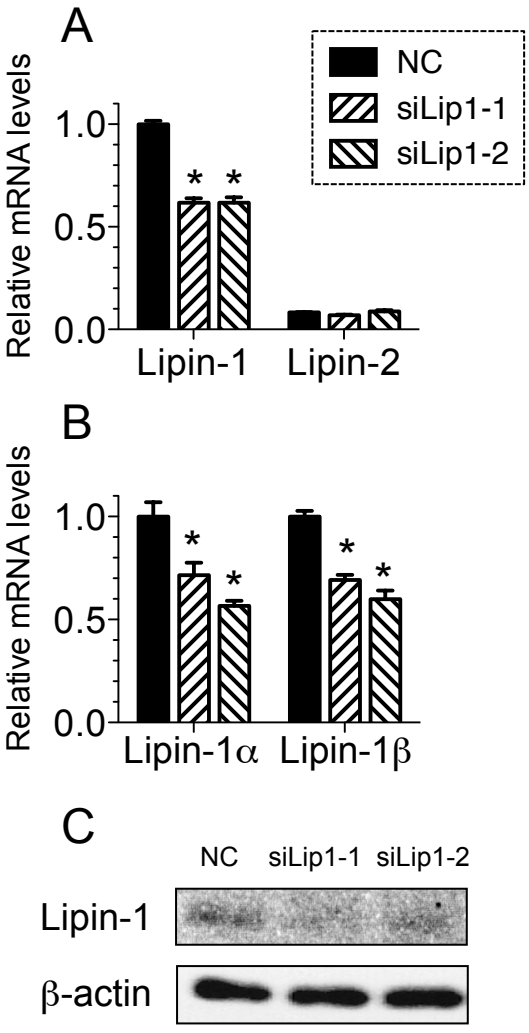
Fig. 4. The effects of NF-κB inhibition on increased MCP-1 expression induced by lipin-1 suppression. 3T3-L1 adipocytes (Day 12) were cultured in the presence of sodium salicylate (salicylate, 18 μM) (A, C), or quinazoline (QNZ, 20 μM) (B, D) for 0.5 h before lipofection of lipin-1 specific siRNA (siLip1-1 or siLip1-2) or negative control siRNA (NC). Sterilized water (A, C) or dimethyl sulfoxide (DMSO) (B, D) were used as the vehicle. After 48 h of transfection, lipin-1 (A, B) and MCP-1 (C, D) mRNA expression was determined by quantitative real-time PCR. The results were normalized against mRNA levels of 18S rRNA and expressed as changes

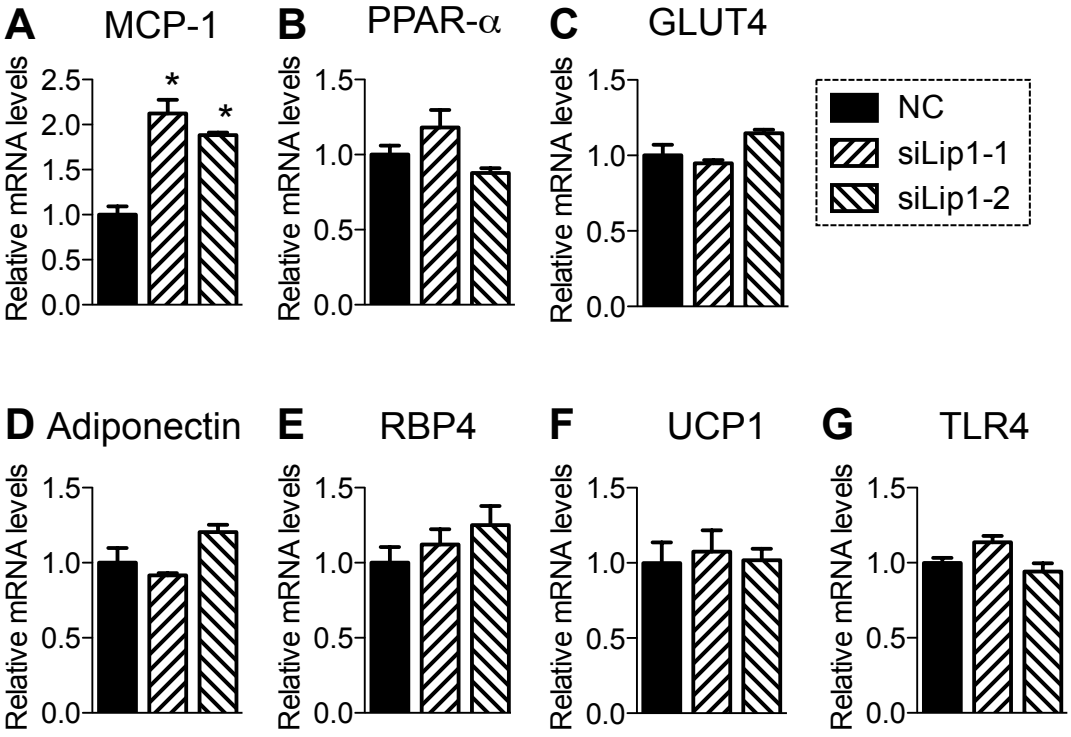
relative to the vehicle (NC). Data are expressed as mean ± S.E. (n = 4). *$P < 0.05$ vs. vehicle (NC).

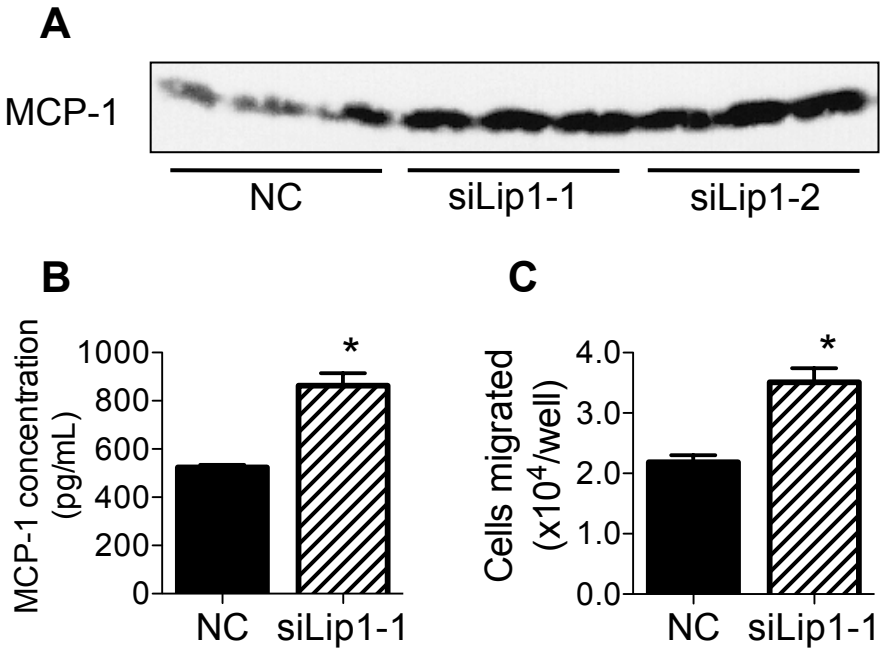
Supplementary Table 1 Analyzed genes and TaqMan assays

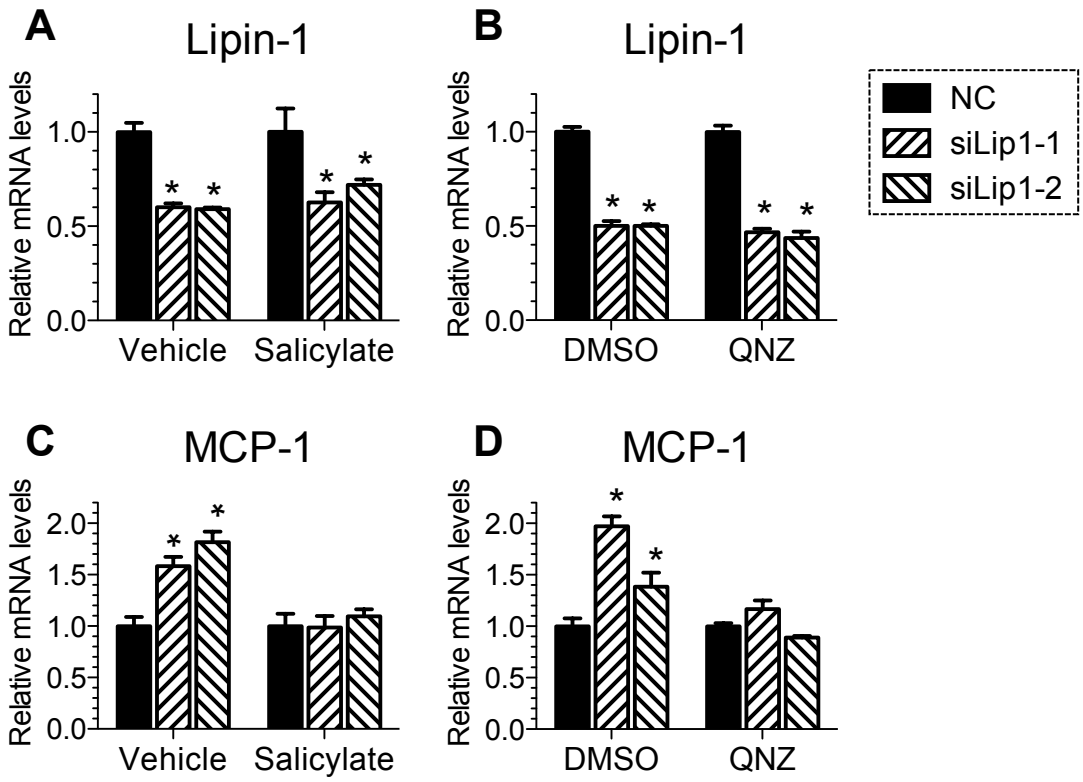
Gene (GenBank ID)	TaqMan gene expression assay
Lipin-1 (NM_172950+NM_015763)	Mm00550511_m1
Lipin-2 (NM_001164885)	Mm00522390_m1
Lipin-1α (NM_172950)	Mm00522205_m1
Lipin-1β (NM_015763)	Mm01276800_m1
MCP-1 (NM_011333)	Mm00441242_m1
PPAR-α (NM_001113418)	Mm00440939_m1
GLUT4 (NM_009204)	Mm00436615_m1
Adiponectin (NM_009605)	Mm00456425_m1
RBP4 (NM_011255)	Mm00803266_m1
UCP1 (NM_009463)	Mm01244861_m1
TLR4 (NM_021297)	Mm00445273_m1
18S rRNA (X03205)	Hs99999901_s1

MCP-1, monocyte chemoattractant protein-1; PPAR, peroxisome proliferator-activated receptor; GLUT, glucose transporter; RBP, retinol binding protein; UCP, uncoupling protein; TLR, toll-like receptor.


A
NC
siLip1-1
siLip1-2
Relative mRNA levels
1.0
0.5
0.0
*
*
Lipin-1
Lipin-2
B
Relative mRNA levels
1.0
0.5
0.0
*
*
*
*
Lipin-1α
Lipin-1β
C
NC
siLip1-1
siLip1-2
Lipin-1
β-actin


A
MCP-1
B
PPAR-α
C
GLUT4
Relative mRNA levels
2.5
2.0
1.5
1.0
0.5
0.0
*
*
NC
siLip1-1
siLip1-2
D
Adiponectin
E
RBP4
F
UCP1
G
TLR4


A
MCP-1
NC
siLip1-1
siLip1-2
B
MCP-1 concentration (pg/mL)
1000
800
600
400
200
0
*
NC
siLip1-1
C
Cells migrated (x10^4/well)
4.0
3.0
2.0
1.0
0.0
*
NC
siLip1-1


A
Lipin-1
Relative mRNA levels
1.0
0.5
0.0
Vehicle
Salicylate
B
Lipin-1
Relative mRNA levels
1.0
0.5
0.0
DMSO
QNZ
NC
siLip1-1
siLip1-2
C
MCP-1
Relative mRNA levels
2.0
1.5
1.0
0.5
0.0
Vehicle
Salicylate
D
MCP-1
Relative mRNA levels
2.0
1.5
1.0
0.5
0.0
DMSO
QNZ