Coordinated transcriptional control of adipocyte triglyceride lipase (Atgl) by transcription factors Sp1 and peroxisome proliferator–activated receptor γ (PPARγ) during adipocyte differentiation

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The breakdown of stored fat deposits into its components is a highly regulated process that maintains plasma levels of free fatty acids to supply energy to cells. Insulin-mediated transcription of Atgl, the enzyme that mediates the rate-limiting step in lipolysis, is a key point of this regulation. Under conditions such as obesity or insulin resistance, Atgl transcription is often mis-regulated, which can contribute to overall disease progression. The mechanisms by which Atgl is induced during adipogenesis are not fully understood. We utilized computational approaches to identify putative transcriptional regulatory elements in Atgl and then tested the effect of these elements and the transcription factors that bind to them in cultured preadipocytes and mature adipocytes. Here we report that Atgl is down-regulated by the basal transcription factor Sp1 in preadipocytes and that the magnitude of down-regulation depends on interactions between Sp1 and peroxisome proliferator–activated receptor γ (PPARγ). In mature adipocytes, when PPARγ is abundant, PPARγ abrogated transcriptional repression by Sp1 at the Atgl promoter and up-regulated Atgl mRNA expression. Targeting the PPARγ–Sp1 interaction could be a potential therapeutic strategy to restore insulin sensitivity by modulating Atgl levels in adipocytes.

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4 The abbreviations used are: TG, triglyceride; FA, fatty acid; NEFA, non-esterified fatty acid; Atgl, adipocyte triglyceride lipase; PPARγ, peroxisome proliferator–activated receptor γ; RXRα, retinoid X receptor α; MEF, mouse embryonic fibroblast; RIPA, radioimmune precipitation assay; RT-qPCR, quantitative RT-PCR.
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Figure 1. Atgl promoter-driven luciferase expression is inhibited by an Sp1-binding site. A, identification of an evolutionarily conserved putative Sp1-binding site in the Atgl promoter. All sites shown were identified by JASPAR as putative Sp1-binding sites (p < 0.05), with a higher score indicative of a stronger prediction of Sp1 binding. B, 3T3-L1 preadipocytes were co-transfected with luciferase reporter constructs of varying 5′-end deletions of the Atgl promoter and an Sp1 expression vector (pcDNA_Sp1). C, diagram of the putative Sp1-binding site at the Atgl minimal promoter (−50 to −36 bp) and the mutation (MUT) generated in the Atgl promoter constructs to disrupt this binding site. D, 3T3-L1 preadipocytes were co-transfected with luciferase reporter constructs of varying 5′-end deletions of the Atgl promoter bearing mutations in the predicted Sp1-binding site and an Sp1 expression vector. E, 3T3-L1 preadipocytes were co-transfected with variable-length Atgl promoter–luciferase reporter constructs and incubated with mithramycin A for 24 h. pgl2, empty vector luciferase control. not significant, p > 0.05; *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001.

Results

Sp1 binding to the Atgl promoter negatively regulates Atgl transcription

Analysis of approximately 3 kb of the DNA sequence immediately upstream of the Atgl transcription start site by Genomatix and TRANSFAC matrices revealed a putative Sp1-binding site at the minimal promoter (−50 to −36 bp). This site is conserved across numerous species, including mice and humans, suggesting an important biological function (Fig. 1A). Therefore, to test the involvement and mechanism of Sp1 in regulating the expression of Atgl, 3T3-L1 preadipocytes were co-transfected with luciferase reporter constructs of varying 5′ end deletions of the Atgl promoter and an Sp1 expression vector. Sp1 overexpression significantly decreased luciferase activity across all Atgl promoter constructs, indicating that Sp1 binding at the minimal promoter region (−192 to +21) can negatively affect Atgl expression (Fig. 1B). We examined the predicted Sp1-binding site by selectively mutating five conserved nucleotides (−46 5′-CCGCC-3′ −42), as shown in Fig. 1C. These mutations completely prevented the inhibitory effect of Sp1 on the minimal promoter of Atgl (Fig. 1D), confirming the involvement of this site in the transcriptional inhibition of Atgl. When 3T3-L1 preadipocytes were transfected with variable-length Atgl promoter–luciferase reporter constructs and incubated with mithramycin A, an Sp1 inhibitor, for 24 h, it also abrogated the inhibitory effect of endogenous Sp1 when transfected with the −192/+21 and −373/+21 constructs contain-
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The significance of Sp1 on endogenous Atgl gene expression was further investigated by Sp1 depletion and overexpression studies in 3T3-L1 cells. Knockdown of Sp1 in preadipocytes by a lentiviral vector producing shRNA targeting Sp1 increased the mRNA levels of Atgl relative to a control shRNA (Fig. 2, A and B). Conversely, overexpression of Sp1 in cultured adipocytes by electroporation (Fig. 2, C–F) resulted in lower expression levels of ATGL mRNA and protein (Fig. 2, E and F) with a concomitant reduction in basal glycerol release in the medium (Fig. 2, G). In addition, cultured preadipocytes treated with mithramycin A (10 μM) showed significant increase in ATGL levels both in the presence and absence of insulin (Fig. 2H). Also, in the presence of insulin, mithramycin A decreased Sp1 mRNA levels and increased Atgl mRNA levels (Fig. 2G). Collectively, this demonstrates that Sp1 mediates the negative regulation of Atgl in preadipocytes. The data further support that Sp1 reduces glycerol release in adipocytes via mechanisms that involve Atgl.

**Sp1-mediated negative regulation of Atgl is abrogated by PPARγ in adipogenesis**

Levels of PPARγ protein increase dramatically during adipogenesis, correlating with an increase in Atgl mRNA and protein levels (supplemental Fig. 1). Bioinformatic analyses identified putative PPARγ-binding sites in the Atgl promoter at −2424, −1674, and −1573 bp (14). In the absence of ChIP data in the literature (11–13), we speculated that the profound up-regulation of Atgl transcription is due to the binding of the PPARγ/ RXRα heterodimer (32–34) at the abovementioned sites.

Of note, PPARγ and Sp1 proteins both functionally and physically interact with each other (25). Thus, we hypothesized that PPARγ may abrogate the Sp1-mediated negative control of Atgl transcription. To test this, PPARγ and Sp1 overexpression

**Figure 2. Sp1 mediates the inhibition of Atgl expression.** A and B, RT-qPCR analysis of mRNA isolated from 3T3-L1 preadipocytes infected with viral particles expressing shRNA targeting Sp1 or a control shRNA. C–F, Western blot and RT-qPCR analyses of differentiated adipocytes transfected with Sp1 overexpression plasmids via electroporation. G, glycerol release in cultured adipocytes transfected with an Sp1 overexpression plasmid via electroporation. H, Western blot analysis of differentiated adipocytes treated with mithramycin A (10 μM) in the presence or absence of insulin. I, RT-qPCR analysis of differentiated adipocytes treated with mithramycin A (10 μM) in the presence of insulin. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
plasmids were co-transfected with wild-type or Sp1 site–mutated full-length Atgl promoter constructs in 3T3-L1 preadipocytes. PPARγ overexpression alone or in the presence of Sp1 increased the reporter activity compared with Sp1 overexpression alone in cells transfected with the wild-type full-length Atgl promoter luciferase construct (Fig. 3A). Upon mutation of the Sp1-binding site in the full-length Atgl promoter, PPARγ overexpression no longer induced Atgl expression (Fig. 3A). These data suggest that the Sp1 site (−50 bp to −36 bp) contributes to the regulation of Atgl by PPARγ in the context of the −2979 to +21 promoter construct. The Sp1-mediated transcriptional repression of Atgl in preadipocytes allows transcriptional activation by PPARγ during adipocyte differentiation. Furthermore, the presence of the Sp1 site has a positive effect on PPARγ-mediated transcriptional activation of the Atgl promoter. To further support this hypothesis, PPARγ overexpression alone or with Sp1 did not induce reporter activity of the shorter Atgl promoter fragment (−192/+21) in either the presence or absence of the Sp1 mutation (Fig. 3B). Taken together, we speculate that induction of PPARγ expression during adipogenesis reverses the negative control of Sp1 over Atgl transcription, presumably via direct interaction with Sp1.

The fact that PPARγ increases luciferase activity in the presence of Sp1 in the full-length promoter but not in the minimal promoter fragment shows that PPARγ stimulates Atgl transcription via binding sites upstream of −192 and by interacting directly with the Sp1-binding site at −50 bp to −36 bp. We speculate that the Sp1-mediated transcriptional down-regulation that is reversed by PPARγ is a stoichiometric transcriptional phenomenon in which there is a reversal of the role of the transcription complex, depending upon the available transcription factor levels. To further test this hypothesis, we knocked down PPARγ in preadipocytes via shRNA (Fig. 3C, left panel). PPARγ deficiency decreased Atgl mRNA levels (Fig. 3C, right panel), further supporting the conclusion that PPARγ is a positive transcriptional regulator of Atgl in preadipocytes via mechanisms that involve its interaction with Sp1 and inhibition of the Sp1-mediated negative regulation of Atgl transcription.

**PPARγ-mediated transactivation of Atgl is Sp1-dependent**

As the data suggested that Sp1 is a negative regulator of Atgl transcription in preadipocytes and that PPARγ overrides this process, we further investigated the importance of the Sp1-binding site on PPARγ-mediated transactivation of Atgl tran-
During adipogenesis, there is a decrease in the nuclear levels of Sp1 and an increase in PPARγ levels in 3T3-L1 cells (supplemental Fig. 1). We co-transfected 3T3-L1 preadipocytes with full-length Atgl luciferase reporter constructs containing mutations at both the Sp1 (−50 bp to −36 bp) and PPARγ-binding sites (−2428 bp to −2408 bp). We did not observe an increase in reporter activity by overexpression of PPARγ or PPARγ and Sp1 combined (Fig. 4A). PPARγ alone did not increase luciferase activity either in the presence or absence of the PPARγ agonist rosiglitazone. This indicates that the effect of the PPARγ-binding site is to positively drive transcription of Atgl.

In mature adipocytes, expression from the full-length Atgl promoter (−2979/+21) containing the PPARγ- and Sp1-binding sites was significantly greater than that from the minimal promoter (−192/+21) containing only the Sp1-binding site (Fig. 4B), suggesting a positive regulatory role of PPARγ. Interestingly, mutation of the Sp1-binding site in the full-length promoter that contains the PPARγ-binding site decreased Atgl reporter expression (Fig. 4B), suggesting a positive role of Sp1 in Atgl transcription in mature adipocytes, where PPARγ is abundant. This effect is lost in the minimal promoter lacking the PPARγ-binding site (Fig. 4B) and is in contrast to the data from preadipocytes, in which mutation of the Sp1 site in the full-length promoter increased expression of the Atgl reporter construct (Fig. 3A).

To examine the physical interaction between Sp1 and PPARγ proteins, co-immunoprecipitations were performed from 3T3-L1 protein lysates; they demonstrated that Sp1 interacts with PPARγ in this cell type (Fig. 4E). In addition, ChIP..
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Figure 5. Interaction between PPARγ and Sp1 leads to stage-specific increase in transcription of Atgl. We propose a model in which the regulation of Atgl transcription is dependent on the relative abundance of and interaction between the transcription factors Sp1 and PPARγ. Although Sp1 negatively regulates Atgl expression in preadipocytes, Sp1 coordinates with PPARγ in mature adipocytes to positively regulate Atgl transcription.

Discussion

It has been well-documented that active transcriptional complexes can adopt alternative functions when bound to additional co-activators or co-repressors (35–37). For example, Klf1 is a transcription factor that exhibits alternate functions in hematopoietic cells, depending on the stage of development. In erythroid cells, a primitive cell type, Klf1 functions as an activator of β-globin expression. However, in hematopoietic cells, a definitive cell type, Klf1 switches to function as a repressor of β-globin expression (38). As Sp1 is a prominent member of the KLF family of transcription factors, similar mechanisms could explain the transcriptional switch identified in this study.

Our results showed that the shortest fragment of the Atgl promoter (−192/+21) contains an evolutionarily conserved Sp1-binding site that is capable of inhibiting Atgl transcription when Sp1 is overexpressed exogenously. This negative impact of Sp1 on Atgl transcription is modulated by PPARγ. In addition, cell type–specific responses were observed. In MEFs, which produce negligible amounts of PPARγ, a positive regulation of the Atgl promoter was observed when Sp1 was expressed exogenously, further supporting the critical nature of the cellular transcription factor milieu in Atgl gene regulation. In adipocytes, we found that Sp1 allows PPARγ-mediated transactivation via a protein–protein interaction between Sp1 and PPARγ (Fig. 4E). PPARγ-mediated transactivation of the Atgl promoter decreased when the conserved Sp1 overlapping binding site at the proximal promoter was mutated (Fig. 4B). We propose that, although Sp1 maintains a steady promoter binding at the minimal promoter, as identified by ChIP analysis (Fig. 4F), and PPARγ and Sp1 directly interact (Fig. 4E), there is a complete reversal of the repressive transcription complex to an activating transcription complex during adipogenesis that governs the fate of Atgl transcription (Fig. 5).

PPARγ heterodimerizes with RXRα, binds to peroxisomal proliferator response elements, and exhibits ligand-induced transactivation of gene expression (39). Our results show transcriptional suppression of the Atgl promoter reporter by the Sp1-binding site at −192/+21 in the presence of exogenous PPARγ (Fig. 3A). As consistent binding of Sp1 at this smaller fragment is observed in both preadipocytes and mature adipocytes (Fig. 4E), we propose that physical interaction of Sp1–PPARγ contributes to Sp1-mediated repression and PPARγ-mediated transactivation of the Atgl gene in adipocytes. A similar mechanistic role of PPARγ–Sp1 interaction has been shown to regulate thromboxane gene expression (25). The increased abundance of PPARγ in mature adipocytes seems likely to explain this reversal of Sp1 function, warranting further investigation into potential Sp1-binding partners.

Sp1–nuclear hormone receptor interaction is found to enhance Sp1-induced gene transcription (40–42). This study demonstrates the possibility that other interacting partners of Sp1 play a role in mediating this process, in this case PPARγ. However, there are a number of other proteins that interact with Sp1, including Egr1. It has been determined that both Sp1 and Egr1 occupy overlapping binding sites and can compete with each other for DNA binding, with Egr1 displacing Sp1 when the local concentration is more than that of Sp1 (43). Therefore, additional cofactors, such as Egr1, may be involved in the repression of Sp1 target gene expression. Another potential interacting partner is C/ebp β, which, like PPARγ, increases during adipogenesis and has been shown to functionally interact with Sp1 (44, 45). In fact, the presence of additional transcription factors such as C/ebp β in mature adipocytes may contribute to the finding that, in preadipocytes, mutation of the Sp1-binding site in the Atgl promoter increased transcription, even in the presence of PPARγ (Fig. 3A), whereas mutation of this site in mature adipocytes led to an overall decrease in transcription (Fig. 4B).

In conclusion, Sp1 negatively regulates Atgl transcription in a PPARγ-dependent fashion. It is interesting to note how, being part of the same transcription complex, the increased abundance of PPARγ during adipogenesis leads to a reversal in the transcriptional action of Sp1, which acts as a repressor in prea-
dipocytes and as an activator in fully differentiated adipocytes (Fig. 5). Understanding the alternating functions of these transcription factors in regulating Atgl in adipocytes is relevant to the treatment of insulin resistance and type 2 diabetes. Obesity is a predominant risk factor for insulin resistance, and insulin action is highly impaired in adipocytes of individuals with metabolic syndrome (46–48). In these individuals, there is a reduction in hormone-induced lipolysis and an increase in the basal lipolysis of adipocytes (6, 49), most likely because of the changes in the levels of Atgl. Therefore, targeting the specific PPARγ–Sp1 interaction could possibly modulate Atgl levels in metabolic syndrome and restore whole-body insulin sensitivity.

**Experimental procedures**

**Cells and reagents**

HEK 293T, MEF (C57BL/6), C2C12, and 3T3-L1 cells were grown in DMEM with high glucose (4.5 mM) supplemented with 10% FBS (Gibco), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. 3T3-L1 preadipocytes were induced to differentiation 2 days after reaching 100% confluence via treatment with a differentiation mixture (5 μg/ml insulin (Sigma, 91077C), 1 μM dexamethasone (Sigma, D-2915), and 0.5 mM isobutylmethylxanthine (Sigma, 15879)) for 3 days. Cells were maintained in medium containing 5 μg/ml insulin until harvested 8 days after treatment with the differentiation mixture. Antibodies were used against the following proteins: ATGL (Cell Signaling Technology, 2138S), Sp1 (Santa Cruz Biotechnology, sc-59), and tubulin (Sigma, T9026). The following drugs were used: rosiglitazone (Sigma-Aldrich, R2408) and mithramycin A (Cayman Chemicals, 11434).

**RNA isolation and RT-qPCR**

Total RNA was isolated from 3T3-L1 cells using TRIzol reagent (Invitrogen). RNA was then reverse transcribed using SuperScript III First-Strand Synthesis SuperMix (Invitrogen), and levels of cDNA were quantified by RT-qPCR using Veri- SuperScript III First-Strand Synthesis SuperMix (Invitrogen), and levels of cDNA were quantified by RT-qPCR using Veri- Quest SYBR Green qPCR Master Mix (Affymetrix) with the StepOnePlus RealTime PCR System (Applied Biosystem). Primers used in the study are listed in supplemental Table S1. Relative mRNA levels were normalized to Gapdh using the ΔΔCt method.

**Immunoblotting and nuclear localization**

For total protein lysates, cells were washed with cold PBS twice and lysed with RIPA buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, and 0.1% SDS) supplemented with Complete EDTA-free protease inhibitor and PhosSTOP phosphatase inhibitor tablets (Roche, 1 tablet/10 ml of RIPA buffer). Lysates were sonicated and centrifuged at 13,000 rpm for 10 min at 4 °C. Supernatants were collected, and the amount of protein was quantified using the DC Protein Assay Kit (Bio-Rad).

For nuclear fractionation experiments, nuclei were isolated as described previously (50). Briefly, cells were pelleted, washed in PBS, and lysed with a mild detergent buffer (10 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, and 0.3% Nonidet P-40). Nuclei were collected by centrifugation, washed in this buffer without detergent, and resuspended in a high-salt buffer (20 mM HEPES, pH 7.9, 0.45 M NaCl, 1 mM EDTA, and 0.5 mM DTT). Nuclei were lysed via sonication, and protein was quantified as described above.

Equal quantities of protein were loaded and separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore) following standard procedures. Membranes were blocked with 5% nonfat milk in Tris-buffered saline with Tween 20 (TBST) for 1 h, rinsed in TBST, and incubated with a primary antibody overnight at 4 °C. Membranes were then probed for respective HRP-conjugated secondary antibody for 1 h at room temperature. A Western Lightning ECL substrate kit (PerkinElmer Life Sciences) was used for detection, and bands were quantified using densitometry by Adobe Photoshop CS 5.0.

**JASPAR analysis of Sp1-binding site**

The Sp1 consensus motif in the promoter region of Atgl in mice was aligned to other species using the LastZ track in the Ensembl database (51). Conserved sequences were identified in a number of species. Analysis of these sequences using the JASPAR database (52) identified putative Sp1 motifs in all species shown in Fig. 1A.

**Transient transfection, electroporation, and luciferase reporter assay**

3T3-L1 preadipocytes were transiently transfected with plasmids using X-tremeGENE HP transfection reagent (Roche) according to the instructions of the manufacturer. Briefly, cells were grown to 70–80% confluence and transfected with 500 ng of Atgl luciferase constructs (14), 500 ng of a plasmid encoding Sp1, 500 ng of a plasmid encoding PPARγ, and 50 ng of a plasmid encoding Renilla luciferase in a 6-well plate format. After 48 h of transfection, cells were lysed using passive lysis buffer (Promega). Luciferase activities were determined by Dual Glo Luciferase Reporter Assay Kit (Promega), with values expressed in relative light units and normalized to Renilla luciferase levels.

For electroporation, 3T3-L1 differentiated adipocytes were washed with calcium- and magnesium-free warm PBS and trypsinized. Post-trypsinization, cells were washed twice with PBS (containing Ca2+ and Mg2+) and centrifuged at 2000 rpm for 5 min. Cells were resuspended in 500 μl of PBS and electroporated with 50 μg DNA using the Gene Pulser Xcell electroporation system (Bio-Rad) with a pulse setting of 160 V and 950 microfarad. Following electroporation, cells were resuspended in growth medium in 6-well plates. 24 h after electroporation, cells were collected for the luciferase activity assay or immunoblot analysis.

**Site-directed mutagenesis**

The putative Sp1-binding site at the Atgl minimal promoter region was identified using TRANSFAC matrices. Five conserved residues in this putative Sp1-binding site were mutated to thymine residues using the following set of primers: forward, 5’-CGACC AGGCC ttttt CTCAC CCCGC ACTAA AACAC-3’; reverse, 5’-GAGG GGGCA GGAC TGG-3’. A putative PPARγ-binding site was also located at −2408 to −2428 bp upstream of the transcriptional start site, and five conserved
residues were mutated to thymine residues by using the following set of primers: forward, 5'-CTGAG TCTGA tttt GCCGG GTCTA CAAAT TGAGT TC-3'; reverse 5'-AATAC CACCT GCCTC TGC-3'. Mutagenesis was carried out using the Q5 site-directed mutagenesis kit (New England Biolabs) following the recommended protocol. Mutations were confirmed by Sanger sequencing.

**Lipolysis assay**

Differentiated 3T3-L1 adipocytes were incubated with phenol red–free DMEM with 2% fatty acid–free BSA for 6 h. Glyceral release was measured as a function of NADH consumption via absorbance at 340 nm using the Free Glycerol Kit (Megazyme).

**Co-immunoprecipitation**

3T3-L1 preadipocytes were transfected with human FLAG-Sp1 and human HA-PPARγ. Twenty-four hours post-transfection, cells were harvested in RIPA buffer (50 mm Tris-HCl (pH 8.0), 150 mm NaCl, 2 mm EDTA, 1% Nonidet P-40, and 1% Triton X-100) with protease inhibitor mixture (Roche). Lysates were incubated with anti-FLAG M2 affinity beads (Sigma, A2220) overnight at 4 °C on an orbital shaker. Beads were washed with RIPA buffer, and proteins were eluted with 30-min incubation in 1× glycine and analyzed by immunoblot.

**ChIP**

3T3-L1 preadipocytes and differentiated adipocytes were cross-linked by adding formaldehyde to the culture medium at a final concentration of 1% and incubated for 10 min at 37 °C in a CO₂ incubator. The medium was then aspirated and washed twice with cold PBS (pH 7.4) containing protease inhibitors (1 mm phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, and 1 µg/ml pepstatin A). Cells were collected, lysed with buffer containing 0.2% SDS, and then sonicated using a Bioruptor (Diagenode) with eight pulses of 15 s at high setting. Tubes were centrifuged for 10 min, and the supernatant was immunoprecipitated with 2 µg of anti-Sp1 antibody (Cell Signaling Technology) using Dynabeads protein A (Invitrogen). DNA was obtained by reverse cross-linking, purified, and analyzed via RT-qPCR with primers to detect the Sp1-binding site (forward, 5'-CGCGCG GAGGC GGAGA CGCT-3'; reverse, 5'-TCCCT GCTTG ATCCA GTTGG AT-3') using Premix TaqDNA Polymerase, Hot Start (catalog no. R028A, Takara) with 0.1% input.

**shRNA-mediated knockdown**

HEK-293T cells were grown to 70% confluence in 150-mm diameter dishes and then transfected with 5 µg of psPAX2 vector (Addgene), 5 µg of PMD2g vector (Addgene), 10 µg of shSp1 (Sigma, TRCN0000017603), 10 µg of shPPARγ (Sigma, TRCN000001660 (shPPARγ-1) and TRCN000001657 (shPPARγ-2)) using X-tremeGENE HP reagent (Roche) in serum-free medium. 8 h post-transfection, the medium was supplemented with fresh DMEM containing 10% FBS. 24 h post-transfection, medium containing a high titer of lentiviral particles was harvested and filtered with a 0.45-µm PVDF syringe filter. 3T3-L1 cells were infected with lentiviral particles expressing Sp1-specific shRNA for 48 h.

**Statistical analysis**

All data are presented as mean ± S.E. To evaluate statistical significance, two tailed Student’s t test or one-way analysis of variance (followed by post tests, Dunnett’s multiple comparison test and Welch correction test) were performed using the GraphPad Prism software package. p < 0.05 was considered to be statistically significant and is presented as follows: not significant, p > 0.05; *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001.

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