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Prolonged efficiency of siRNA-mediated gene silencing in primary cultures of human preadipocytes and adipocytes

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Abstract

Objective—Primary human preadipocytes and differentiated adipocytes in culture are valuable cell culture systems to study adipogenesis and adipose function in relation to human adipose biology. To use these systems for mechanistic studies, we studied siRNA-mediated knockdown of genes for its effectiveness.

Design and Methods—Methods were developed to effectively deliver siRNA to for gene silencing in primary preadipocytes isolated from human subcutaneous adipose tissue and newly-differentiated adipocytes. Expression level of genes and proteins was measured using quantitative RT-PCR and western blotting. Lipid droplet morphology was observed using microscopy and glycerol release was quantified as a measure of lipolysis.

Results—siRNA-mediated knockdown of genes in primary human preadipocytes resulted in prolonged silencing effects, suppressing genes throughout the process of their differentiation. In newly differentiated adipocytes, siRNA-mediated gene knockdown allowed proteins to stay depleted for at least 5 days. It was possible to re-express a protein after its siRNA-mediated depletion. Importantly, siRNA transfected human adipocytes remained metabolically active, responding to β-adrenergic stimulation to increase lipolysis.

Conclusions—Our study describes the methods of gene silencing in primary cultures of human preadipocytes and adipocytes and their prolonged effectiveness.

Keywords

siRNA; adipose; gene knockdown; adipogenesis; adipose tissue

Introduction

Obesity, the excess accumulation of fat mass, is increasing worldwide. Expansion of adipose tissue depends on both new adipocyte formation (recruitment of progenitors and

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Authorship

M.L. and V.P. designed, performed, and analyzed experiments. R.T.P. performed experiments. M.L. and V.P. wrote the manuscript; and all authors agreed on the final manuscript.

differentiation into adipocytes) and hypertrophy of existing adipocytes. In addition to their important metabolic function, adipocytes secrete a myriad of peptide hormones and cytokines that regulate systemic energy homeostasis and metabolism. Thus, mechanistic studies of how new fat cells are formed and their metabolic and endocrine functions are altered are central to understanding the etiology of obesity and its metabolic complications. Widely used mouse 3T3-L1 and 3T3-F442A cell lines provide invaluable model systems, yet there is a growing need for human preadipocytes and adipocytes that have clear translational relevance. While several human adipose cell lines are available, including SGBS and hMAD (1, 2), primary preadipocytes isolated from adipose tissues can be differentiated into adipocytes *in vitro* and are useful for studies of donor or depot dependent effects (3–12). In addition, newly-differentiated human adipocytes in culture respond to physiologically relevant concentrations of hormones, insulin and beta-adrenergic agonists (13–16). Thus, primary cultures of human preadipocytes and adipocytes are valuable tools for the studies of adipocyte development and function and their use is expected to increase.

Both loss and gain of function studies are utilized to test the roles of genes of interest. RNA interference (RNAi) is a robust gene silencing mechanism with which knockdown of genes can be easily achieved for functional studies. Overexpression of genes in human adipocytes is readily achieved through adenovirus mediated gene delivery (17). Although RNAi-mediated gene knockdown in primary human adipocytes and hMAD cells have been used in several studies (18–20), its efficacy in primary human preadipocytes or adipocytes have not been clearly described. Previously, we described an *ex-vivo* method to deliver siRNA into primary mouse and human adipose tissue explants (21). In the current study, we describe methods to effectively deliver siRNA to primary cultures of human preadipocytes and newly-differentiated adipocytes for gene silencing. We demonstrate that RNAi-mediated knockdown of genes can easily be used for the studies to test their roles in adipogenesis and metabolic functions in human preadipocytes and adipocytes, respectively. In addition, the expression of proteins can be repleted with lenti-virus delivery. The methods described are easily applicable to many genes, further increasing the use of primary cultures of human preadipocytes and adipocytes for mechanistic studies.

Materials and Methods

Materials

All chemicals were purchased from Sigma, except Rosiglitazone (Enzo) and recombinant human insulin (Lilly). Collagenase type I was purchased from Worthington Biochemical. Fetal bovine serum (FBS) and culture media were obtained from Life Technologies. siRNAs were purchased from Qiagen, Dhamarcon or Santa Cruz Biotechnologies. Transfection reagents were purchased from Qiagen (HiPerFect) and Life Technologies (Lipofectamine and PLUS reagents).

Human subjects

Adipose tissues were obtained from 5 subjects (mean age 48 ± 4.1 years and BMI 34 ± 3.9 kg/m^2) during panniculectomy in reduced obesity. All subjects provided informed consent.

The protocol was approved by Institutional Review Board of Boston University Medical Center.

Isolation of adipose stromal vascular cells

Stromal vascular cells were isolated with collagenase digestion (type 1, 1 mg/ml in HBSS) for 2 hours (22, 23). After digestion, the mixture was filtered with a 250 micron mesh and centrifuged at 500g for 10 minutes. Cell pellets were treated with erythrocyte lysis buffer (0.154 mM NH_4Cl, 10 mM K_2HPO_4 and 0.1 mM EDTA, pH 7.3) and repelleted with centrifugation. Cells were resuspended in growth media (α-MEM with 10% FBS) and then plated for culturing. Cells were subcultured up to 6 passages. Cells from individual subjects were used without pooling. Experiments were repeated in cells derived from at least 3 independent donors.

Differentiation of human preadipocytes into adipocytes

Preadipocytes plated in 12-well plates (5,000 to 15,000 cells/cm^2) were cultured and differentiated as previously described (23). Briefly, 2d-post confluent cells were induced to differentiate in the complete differentiation media (DMEM/F12 with 500 μM IBMX, 100 nM insulin, 100 nM dexamethasone, 2 nM T_3, 10 μg/ml transferrin, 1 μM Rosiglitazone, 33 μM biotin and 17 μM pantothenic acid) for 7 days followed by maintenance in DMEM/F12 supplemented with 33 μM biotin, 17 μM pantothenic acid, 10 nM insulin and 10 nM dexamethasone.

Gene silencing in preadipocytes

a. In the morning, preadipocytes were trypsinzed and seeded at 15,000 cells/cm^2 in 12-well cell culture plates.

b. At late afternoon, cells were transfected with siRNA using HiPerFect reagent (Qiagen). For one well of a 12 well plate, siRNA (5 to 40 nM final concentration) was mixed with 6 Hl HiPerFect reagent in total 200 μl serum free α-MEM (without antibiotics) and incubated for 15 min at room temperature.

c. During the incubation, cells were refed with 200 μl growth media (α-MEM with 10% FBS without antibiotics). To prevent cells from drying out, the plate was swirled intermittently during refeeding.

d. The pre-incubated siRNA-HiPerFect mix was added drop-wise to the wells, resulting in total 400 μl of α-MEM with 5% FBS. After gently rocking to mix, the plate was transferred to cell culture incubator.

e. After overnight transfection, cells were re-fed with regular growth media and allowed to proliferate until they were ready for differentiation. Cells generally reach post-confluent state between 4–5 days after transfection.

Gene silencing in differentiated adipocytes—Between day 9–14 of differentiation, adipocytes were transfected with siRNA using Lipofectamine and PLUS reagents (Life Technologies). Serum and antibiotics free DMEM/F12 is used for transfection.

a. For one well of a 12 well plate, siRNA (5 to 40 nM final concentration) was diluted in 50 Hl DMEM/F12 and mixed with 5 µl PLUS reagent. The mixture was incubated at room temperature for 15 min.

b. In a separate tube, 3 µl Lipofectamine reagent was diluted with 50 µl DMEM/F12.

c. Preincbuated siRNA-PLUS and diluted Lipofectamine were combined and incubated for 15 min at room temperature.

d. Meanwhile, cells were re-fed with 300 µl maintenance media. The siRNA-PLUS-Lipofectamine mixture was added drop-wise to the cells and plate was gently rocked to mix the solutions.

e. After overnight transfection, cells were refed with regulator maintenance media and maintained for additional 4 to 5 days with refeeding every 2 to 3 days.

Lentivirus production and transduction

293T cells were seeded in 10 cm plates. Recombinant lentiviruses were produced by a five-plasmid transfection procedure as described (24). The packaged recombinant lentiviruses were harvested from the supernatant of cell cultures 48 h after transfection and filtered through 0.45-µm filters. 500 Hl supernatant and 10 µg/ml Polybrene was added to each well of 12 well plate containing differentiated human adipocytes for overnight infection. Cells were refed with the maintenance medium on the following day and protein expression was measured with immunoblotting 4 days after transduction.

Lipolysis

Glycerol accumulation in culture media during the final day of culture was used as a measure of lipolysis. To provide a better assessment of changes in lipolytic rates, we also performed an 2h acute incubations in Krebs-Ringer bicarbonate buffer with 5 mM glucose and 4% bovine serum albumin (KRB+4% BSA) under basal and β-adrenergically-stimulated (Isoproternol, 1 µM) conditions as previously described (23). Glycerol concentration in the incubation media was measured fluormetrically (14).

Lipid droplet staining

Cells plated on glass cover slips were washed twice with PBS, fixed in 4% formaldehyde for 20 minutes and quenched with 0.1 M glycine. Cells were then incubated with 0.5 µg/ml of Nile Red for 30 minutes and washed with PBS. The cover slips were mounted on glass slides with VectaShield (Vector Labs, CA) mounting medium.

Microscopy

Microscopy was performed using a Zeiss LSM 710-Live Duo scan (Carl Zeiss, Oberkochen, Germany) with a 100X oil immersion objective. Images were processed using Metamorph imaging software, version 6.1 (Universal Imaging, Downingtown, PA).

RNA extraction and gene expression

Total RNA was extracted using Qiazol (Qiagen). After assessing quantity and quality with a Nano-Drop, 0.5~1 µg total RNA was reverse transcribed using Transcriptor First Strand

cDNA synthesis kit (Roche). qPCR was performed on Light Cycler 480 (Roche) using Taqman probes (Applied Biosystems). 18S rRNA was used as a reference gene.

Immunoblotting

Cells were washed with ice-cold PBS and scraped into cell lysis buffer (Cell Signaling) supplemented with 5% SDS and protease inhibitor cocktails (Pierce). Cell lysates were processed and 5~10 μg proteins was resolved in 10% or 15% Tris-HCl gels (Biorad) and transferred to PVDF membranes. After blocking in 5% milk, blots were probed for VDR (D-6, Santa Cruz), CIDEC (25) and loading controls (HSP90, α-tubulin, total AKT, Santa Cruz). Chemiluminescent images were captured using a Luminescent Image Analyzer (LAS4000, Fuji).

Statistics

Data were expressed as mean ± SE. Differences between groups were determined by analysis of variance (ANOVA) with repeated measures and Student t-test using GraphPad Prism.

Results

Efficient knockdown of genes in primary human preadipocytes

siRNA-mediated knockdown is a useful tool to study the role of various proteins in cells. We describe methodologies of depleting proteins effectively in primary cultures of human preadipocytes and adipocytes using commercially available transfection reagents. Initially, we silenced COUP-TFII (a known adipogenic inhibitor (26, 27)) in human subcutaneous preadipocytes and studied the effects of its knockdown on adipogenesis. As described in the methods section, preadipocytes were transfected with control (scrambled siRNA) or COUP-TFII-siRNA using HiperFect reagents. After overnight transfection, cells were grown for an additional 4–5 days till they were ready for differentiation. 60–90% knockdown of COUP-TFII was achieved in preadipocytes derived from 4 independent donors 4–5 days after transfection (Figure 1A) ($p=0.02$, $n=4$).

To test whether knockdown of COUP-TFII affected adipogenesis, preadipocytes were induced to differentiate following our published protocol (23) and expression markers of adipogenesis were measured during different period of adipogenesis. As expected, silencing of COUP-TFII enhanced the degree of differentiation as demonstrated by higher expression levels of adipocyte markers, PPARγ and FABP4 mRNA (Figure 1B). These data show that we can effectively silence genes of interest in primary human preadipocytes and study its down-stream effects, i.e. adipogenesis.

We performed siRNA-mediated depletion of additional 10 randomly picked genes in preadipocytes (5–40 nM final concentration of siRNA). 50–95% reduction in the mRNA and protein levels was generally achieved (data for all the genes is not shown). siRNA transfection caused about 65% and 75% reduction of VDR at mRNA (Figure 2A) and protein levels, respectively (Figure 2B). These data show that our methodology of siRNA-mediated gene knockdown is efficient and applicable to a wide variety of genes.

Intriguingly, we found a prolonged effect of siRNA-mediated knockdown in primary human preadipocytes. siRNA against CIDEC, a differentiation regulated lipid droplet associated protein which regulates lipid droplet morphology and lipolysis in adipocytes (28–30), was transfected in preadipocytes. As expected CIDEC levels increased during adipogenesis. Its expression however, remained at the reduced levels throughout differentiation process in CIDEC knockdown cells (Figure 2C). At even day 14 of differentiation, CIDEC protein levels were lower, about 20% of its level in control cells (Figure 2D). Similar data were observed for other genes we have tested (data not shown).

Knockdown of genes in newly-differentiated human adipocytes

We next developed a method to silence genes in differentiated adipocytes. On day 9 of differentiation, newly-differentiated human adipocytes were transfected with siRNA using Lipofectamine and PLUS reagents as described in the methods. About 85% reduction of VDR protein was observed after 5 days of transfection (Figure 3A). Knockdown of at least 10 other genes was also tested where 60–95% reduction in protein expression was achieved (data not shown). We also tested our method of gene silencing in fully differentiated adipocytes (day 14) and found that it was less efficient and 15–20% lower levels of knockdown was achieved compared to the transfection on day 9 of differentiation (data not shown).

We next tested the functional outcomes of gene knockdown in differentiated human adipocytes. Human adipocytes were transfected with control or CIDEC siRNA on day 9 of differentiation, and maintained for up to an additional 10 days. CIDEC protein was reduced by more than 90% after 5 days of transfection (Figure 3B) and remained about 80% depleted even after 10 days post siRNA transfection, showing that the gene silencing effects were maintained for at least 5 days. As expected, lipid droplets were smaller when CIDEC was silenced compared to the control cells (Figure 3C).

In order to study the metabolic consequences of CIDEC knockdown in human adipocytes, we measured lipolytic rates. Glycerol accumulation in culture media over 24 h period was increased by 3.2-fold in CIDEC silenced cells ($p<0.01$, $n=5$) (Figure 3D) compared to the control cells. To better assess the lipolytic capacities, cells were incubated in the KRB buffer with 4% albumin in both basal and beta-adrenergically-stimulated (Isoproterenol, 1 μM) conditions for 2 hours. Similar to the data in culture media, CIDEC knockdown increased basal lipolytic rates by 2.4-fold (Figure 3E). In addition, β-adrenergically-stimulated rates were also increased when CIDEC levels were reduced. These data show that CIDEC knockdown increases overall lipolytic rates without altering responsiveness to β-adrenergic stimuli.

CIDEC repletion in CIDEC-depleted human adipocytes

Since siRNA-mediated knockdown showed prolonged effectiveness, we wanted to investigate whether we could restore the expression of a protein after its depletion. CIDEC levels were reduced with RNAi in preadipocytes and cells were differentiated. On day 12 of differentiation, CIDEC lentivirus was transduced and cells were harvested on d17. As shown in Figure 4, CIDEC expression was restored with CIDEC lentivirus after its depletion. Our

CIDEC-siRNA targets the 3′-untranslated region (UTR) of the CIDEC mRNA and the lentivirus contains cDNA sequence which does not have the 3′-UTR. Therefore the lentivirus-mediated CIDEC expression vector was not targeted by the CIDEC siRNA.

Discussion

Primary preadipocytes isolated form human adipose tissue and the adipocytes differentiated from them are important tools to study adipose biology pertaining to human physiology. The main purpose of this study was to design and study the effectiveness of siRNA-mediated gene silencing in human primary preadipocytes and differentiated adipocytes. We first demonstrated that gene silencing can be effectively achieved in primary human preadipocytes and adipocytes, and then used it for testing its functional effects by silencing genes involved in adipogenesis and lipolysis. In addition, we present data showing repletion of a protein after silencing, thus demonstrating that expression levels of proteins can be easily modulated in human adipocytes.

Unexpectedly we found that the silencing effects in preadipocytes are maintained throughout the differentiation at least till day 14 of differentiation (more than 20 days after transfection). We were also able to achieve an efficient and prolonged knockdown of genes in mature adipocytes. Gene and protein knockdown were observed after about 5 days of transfecting siRNA in differentiated adipocytes and remained effective at least for another 5 days. We did not test the effect after 5 days because we believe that this much time is enough to perform most of the biochemical studies in these cells. Gene silencing via transient tranfection of siRNA is short lived in various cell types including 3T3-L1 (28, 31, 32). In fact the siRNA effect starts fading away after about 3 days or so, thus limiting the time to perform various functional studies. We believe the prolonged silencing effects is at least in part due to the fact that siRNA is not diluted through extensive cell divisions during the differentiation process since human primary preadipocytes do not undergo cell division to enter differentiation (33) and cell numbers remain same during differentiation (23). In addition, another possibility could be that siRNA is stable in primary preadipocytes and adipocytes. Further studies are needed to determine the reason of prolonged effect of siRNA in these cells.

Of note, we found subject dependent variations in the degree of knockdown with siRNA (data not shown). Although we do not completely understand the sources of these variations, we speculate the differences in extracelluar matrix composition may affect the transfection efficiency. Nonetheless, the effects of gene silencing were observed in all of the subjects we used.

We present data showing siRNA mediated gene silencing can be easily utilized for the functional studies, adipogenesis and lipolysis. COUP-TFII is an inhibitor of adipogenesis (26, 27). As expected, we found that siRNA-mediated knockdown of COUP-TFII had a significant effect on differentiation of human primary preadipocytes. CIDEC knockdown in 3T3-L1 adipocytes caused lipid droplet fragmentation and increased lipolysis (28–30). As anticipated, siRNA-mediated CIDEC depletion in mature human adipocytes showed fragmentation of lipid droplets and an increased rate of lipolysis. These data showed that

siRNA transfected cells are metabolically active and responding to the β-adrenergic stimulation to increases lipolysis.

Overall, in the current study, we describe methods to effectively silence genes of interest in primary preadipocytes and adipocytes. We believe these gene silencing methods can be readily used for the functions studies. Primary cultures of adipose derived preadipocytes and adipocytes differentiated from them provide an additional cell culture system and can be used to study the potential donor or depot specific cell autonomous effects in human preadipocytes and adipocytes (3, 5, 10–12). When combined with modifications in proliferation and differentiation protocols (23, 34), our gene silencing methods are expected to increase the use of the primary human adipose cells.

Acknowledgments

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Abbreviations

siRNA	short-interfering RNA
CIDEC	cell death-inducing DNA fragmentation factor, alpha subunit-like effector C
VDR	vitamin D receptor
COUP-TFII	chicken ovalbumin upstream transcription factor II
PPARγ	peroxisome proliferator activating receptor gamma
FABP4	fatty acid binding protein 4

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What is already known about this subject

1. RNAi-mediated knockdown is an efficient method of gene silencing for functional studies of genes of interest.
2. RNAi-mediated gene knockdown in primary human adipocytes and hMAD cells have been used in other studies but its effectiveness has not been demonstrated yet.

What this study adds

Our manuscript is novel and unique in showing

1. that siRNA transfection in human primary preadipocytes leads to prolonged gene silencing which stays effective even after their differentiation.
2. that siRNA-mediated gene silencing in mature human adipocytes stays effective up to 5 days after the depletion of genes, thus giving ample time to perform various functional studies.
3. that proteins could be repleted using lenti-virus after their siRNA-mediated depletion in human adipocytes

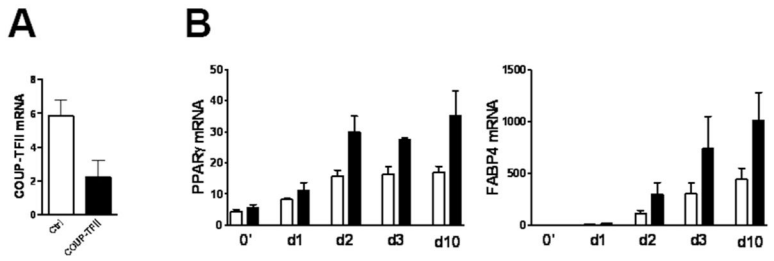

Figure 1. siRNA-mediated knockdown of COUP-TFII in human primary preadipocytes enhanced differentiation

(A) Transfection of siRNA against COUP-TFII in primary human preadipocytes resulted in 65% decrease in COUP-TFII mRNA expression levels ($p<0.5$, n=4). (B) Knockdown of COUP-TFII enhanced differentiation of human adipocytes. After confirming knockdown of COUP-TFII in preadipocytes, cells were induced to differentiate and harvested at indicated days in the figure. Expression levels of PPARγ and FABP4 were measured as indicators of differentiation degree (n=3 to 4).

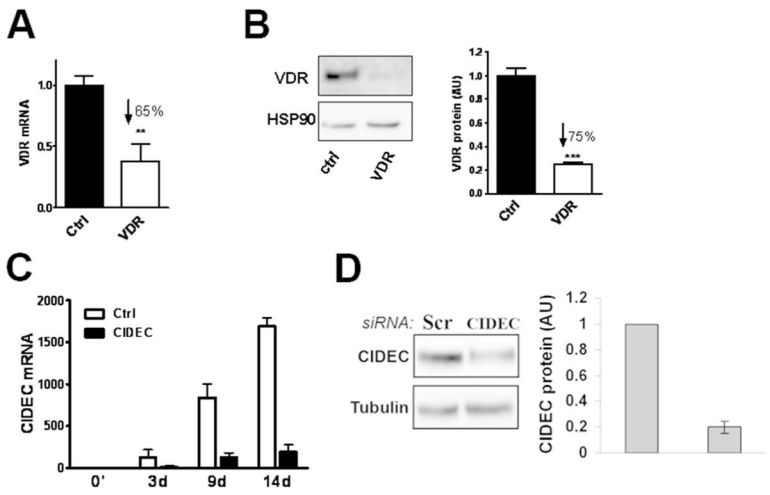

Figure 2. Prolonged effects of gene silencing in human preadipocytes

VDR siRNA transfection in primary human preadipocytes caused 65% decrease in VDR mRNA levels (A, $p<0.01$, n=5) and 75% decrease in VDR protein levels (B, $p<0.001$, n=5). CIDEC-siRNA transfection in primary preadipocytes maintained the mRNA levels depleted on day 3, 9 and 14 of differentiation (C) and about 80% decrease in CIDEC protein expression in mature adipocytes (D).

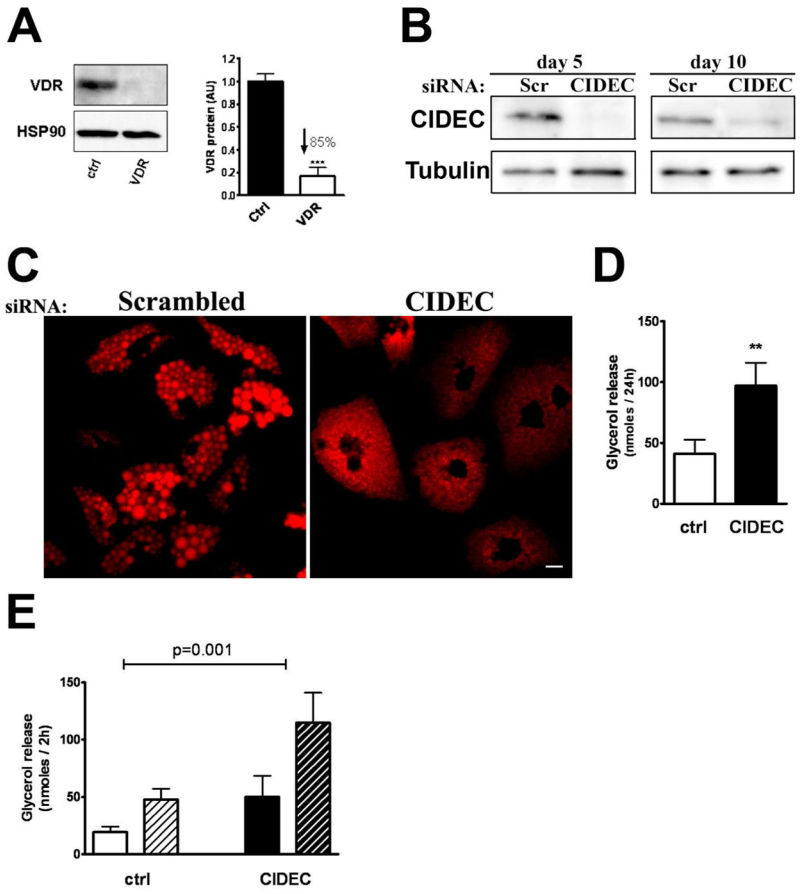

Figure 3. siRNA-mediated CIDEC depletion caused fragmentation of lipid droplets and increased lipolysis in cultured human adipocytes

(A) CIDEC siRNA was transfected day 9 of differentiation and CIDEC protein expression levels were analyzed after 5 days and 10 days with immunoblotting. (B) Lipid droplet fragmentation in CIDEC depleted human adipocytes. (C) CIDEC silencing increased glycerol accumulation during the final 24h period of culture ($p<0.01$, n=5). (D) CIDEC silencing increased both basal and beta-adrenergically stimulated lipolytic rates measured during an 2h incubation in KRB+4% BSA in human adipocytes ($p<0.001$ by two way ANOVA, n=4).

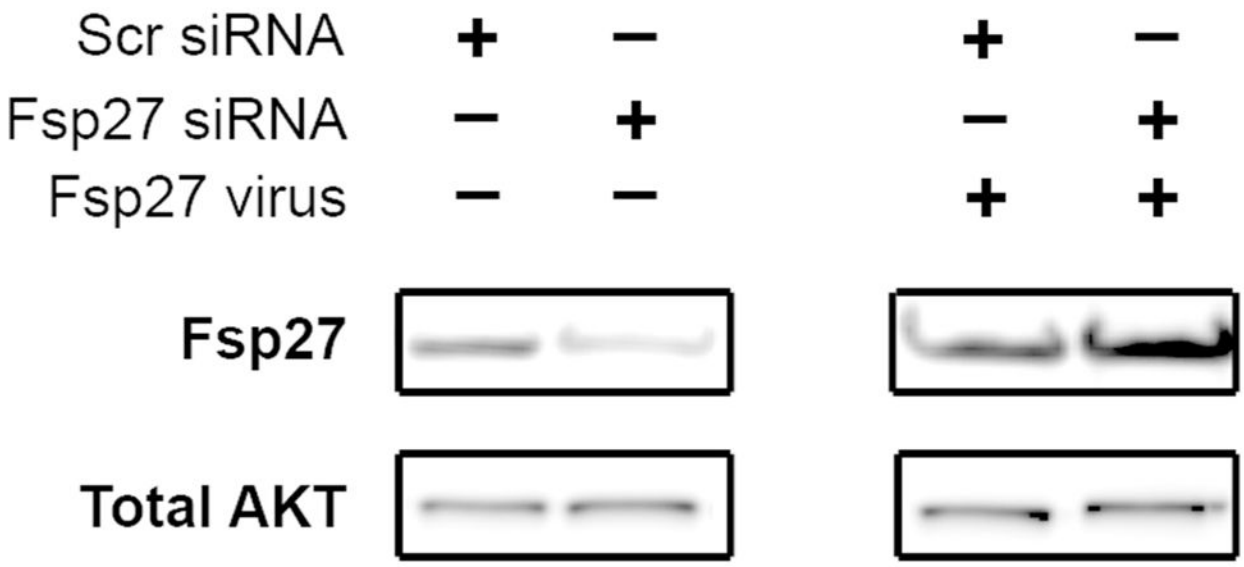

Figure 4. Re-expression of CIDEC in CIDEC-depleted human adipocytes

siRNA-mediated depletion of CIDEC in human preadipocytes resulted in about 80% decrease in protein levels on day 17 of adipocytes. CIDEC protein was repleted using lentivirus (empty-virus was used as a control). Total AKT was used as a loading control. The data is a representation of three independent experiments.