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TITLE:

An Adipocyte Cell Culture Model to Study Impact of Protein and Micro-RNA Expression on Adipocyte Function

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KEYWORDS:

adipocytes, cell culture, transfection, micro-RNA (miR), small interfering RNA (siRNA).

SUMMARY:

Presented here is a protocol to deliver oligonucleotides such as small-interfering RNA (siRNA), micro-RNA mimics (miRs), or anti-micro-RNA (anti-miR) into mature adipocytes to modulate protein and micro-RNA expression.

ABSTRACT:

Alteration of adipocyte function contributes to the pathogenesis of metabolic diseases including Type 2 diabetes and insulin resistance. This highlights the need to better understand the molecular mechanism involved in adipocyte dysfunction to develop new therapies against obesity-related diseases. Modulating the expression of proteins and micro-RNAs *in* adipocytes remains highly challenging. This paper describes a protocol to differentiate murine fibroblasts into mature adipocytes and to modulate the expression of proteins and micro-RNAs in mature adipocytes through reverse-transfection using small-interfering RNA (siRNA) and micro-RNA mimicking (miR mimic) oligonucleotides. This reverse-transfection protocol involves the incubation of the transfection reagent and the oligonucleotides to form a complex in the cell culture plate to which the mature adipocytes are added. The adipocytes are then allowed to reattach to the adherent plate surface in the presence of the oligonucleotides/transfection reagent complex. Functional analyses such as the study of insulin signaling, glucose uptake, lipogenesis, and lipolysis can be performed on the transfected 3T3-L1 mature adipocytes to study the impact of protein or micro-RNA manipulation on adipocyte function.

INTRODUCTION:

Obesity is considered a major risk factor for numerous metabolic diseases, including insulin resistance (IR), Type 2 Diabetes (T2D), and cardiovascular diseases[1]. Current therapies have failed to stop the constantly rising prevalence of these diseases, and the management of the IR of obese and diabetic patients remains an important clinical issue. Adipose tissue plays a crucial role in the control of energy homeostasis, and its pathological expansion during obesity contributes to the development of IR and T2D[2,3]. This highlights the need to better understand the molecular mechanism involved in adipocyte dysfunction to develop new therapies against obesity-related diseases. Many research studies have investigated the role of protein-coding RNAs in adipocyte physiology and their association with obesity.

More recently, the discovery of non-coding RNAs (ncRNAs), especially micro-RNAs (miRs), has forged novel concepts related to the mechanism of the regulation of gene expression programs. Studies have shown that ncRNAs are important regulators of adipocyte function, and that their dysregulation plays an important role in metabolic diseases[4]. Thus, the manipulation of proteins and ncRNAs in adipocytes is crucial to decipher their roles in adipocyte function and their impact on pathologies such as T2D. However, manipulating the expression of proteins and ncRNAs in vivo as well as in primary adipocytes remains highly challenging, favoring the use of in vitro adipocyte models.

Murine 3T3-L1 fibroblasts easily differentiate into mature, functional, and insulin-responsive adipocytes, which are a well-characterized cell line used to study adipocyte function (e.g., insulin signaling, glucose uptake, lipolysis and adipokines secretion)[5–10]. These properties make 3T3-L1 adipocytes an attractive model to modulate the expression of protein-coding and nc-RNAs to decipher their role in adipocyte function and their potential role in obesity-related diseases. Unfortunately, whereas 3T3-L1 fibroblasts are easy to transfect using commercially available reagents, differentiated 3T3-L1 adipocytes are one of the most difficult cell lines to transfect. This is why numerous studies manipulating gene expression in 3T3-L1 cells have focused on adipocyte differentiation rather than on adipocyte function.

For a long time, the only efficient technique to transfect adipocytes was electroporation[5], which is tedious, expensive, and can cause cell damage. This paper reports a reverse-transfection technique using a common transfection reagent, which reduces hands-on time for transfection, has no effect on cell viability, and is much less expensive than electroporation. This protocol is perfectly suited for the transfection of siRNA and other oligonucleotides such as micro-RNA mimics (miR mimics) and anti-miRs. The principle of the reverse-transfection protocol is to incubate the transfection reagent and the oligonucleotides to form a complex in the cell culture plate and then seed the mature adipocytes into the wells. Then, the adipocytes reattach to the adherent plate surface in the presence of the oligonucleotides/transfection reagent complex. This simple, efficient, and inexpensive methodology permits the study of the role of protein-coding RNAs and miRs in adipocyte function and their potential role in obesity-related diseases.

PROTOCOL:

NOTE: Use sterile techniques to perform all the steps of the protocol in a laminar flow cell culture hood. See **Table of Materials** for details about all reagents and equipment.

1. Differentiation of murine 3T3-L1 fibroblasts into adipocytes

1.1. Grow the 3T3-L1 fibroblasts in 100 mm dishes in culture medium—DMEM without pyruvate, 25 mM glucose, 10% newborn calf serum, and 1% penicillin and streptomycin (**Figure 1A**). Place the dishes in a tissue culture incubator (7% CO_2 and 37 °C).

1.2. Two days after confluence, change the culture medium, replacing with DMEM without pyruvate, 25 mM glucose, 10% fetal calf serum (FCS), and 1% penicillin and streptomycin supplemented with 0.25 mM 3-Isobutyl-1-methylxanthine (IBMX), 0.25 µM dexamethasone, 5 µg/mL insulin, and 10 µM rosiglitazone.

NOTE: It takes 5 days to reach confluency when the cells are seeded at 300,000 cells per 100 mm dish.

1.3. Two days later, replace the culture medium with DMEM without pyruvate, 25 mM glucose, 10% FCS, and 1% penicillin and streptomycin supplemented with 5 µg/mL insulin and 10 µM rosiglitazone and incubate for 2 days. Then, feed the cells every 2 days with DMEM without pyruvate, 25 mM glucose, 10% FCS, and 1% penicillin and streptomycin (**Figure 1B**).

1.4. Transfect the 3T3-L1 adipocytes 7–8 days after the beginning of the differentiation protocol.

NOTE: It is important to reach a high level of differentiation (>80%) before the transfection to avoid the proliferation of the remaining fibroblasts after the transfection, which would lead to a mixed population of cells that might bias the results.

2. Preparation of precoated plates

2.1. On the day before or a few hours before the transfection, prepare a solution of collagen type I at 100 µg/mL in 30% ethanol from a stock solution at 1 mg/mL. Add 250 µL of collagen per well of a 12-well plate and 125 µL per well of a 24-well plate, and spread the solution over the surface of the well.

2.2. Leave the plate without the lid under the culture hood until the collagen dries. Wash twice with Dulbecco's phosphate-buffered saline (D-PBS).

NOTE: Precoated plates are available for purchase.

3. Preparation of the transfection mix

NOTE: The final concentration of siRNA is between 1 and 100 nM (1 to 100 pmol of siRNA per well of a 12-well plate). The final concentration of the miR mimic is 10 nM (10 pmol/well). Determine the best concentration of each siRNA, miR mimic, or other oligonucleotide prior to starting the experiment to avoid off-target effects. Perform transfection experiments in triplicate to facilitate statistical analysis of the results. Prepare all reagents in excess to account for normal loss during pipetting.

3.1. Mix by pipetting (volume/volume) the siRNA (or other oligonucleotides) with improved Minimal Essential Medium (**Table 1**). Incubate for 5 min at room temperature.

3.2. Add the transfection reagent and the improved Minimal Essential Medium to the siRNA, and pipet to mix (**Table 1**). Incubate for 20 min at room temperature (during this time, proceed to section 4). Add the transfection mix to each well of the collagen-coated plate.

4. Preparation of the 3T3-L1 adipocytes

4.1. Wash the cells in the 100 mm Petri dish twice with D-PBS. Add 5x trypsin to the cells (1 mL per 100 mm dish), making sure to cover all of the surface with the trypsin. Wait for 30 s and carefully remove the trypsin.

4.2. Incubate the Petri dish for 5–10 min at 37 °C in the incubator. Tap the 100 mm dish to detach the cells.

4.3. Add 10 ml of DMEM without pyruvate, 25 mM glucose, 10% FCS, and 1% penicillin and streptomycin to neutralize the trypsin. Carefully pipet the medium up and down to detach the cells and homogenize the cell suspension.

4.4. Count the cells using a Malassez counting chamber or an automated cell counter, and adjust the concentration of the cells to 6.25 x 10^5 cells/mL of medium. Seed 800 µL of the cell suspension/well of a 12-well plate (5 x 10^5 cells) or 400 µl of the cell suspension/well of a 24-well plate (2.5 x 10^5 cells) containing the transfection mix.

NOTE: One 100 mm Petri dish of adipocytes will allow the preparation of one 12-well plate or one 24-well plate. A 100 mm dish usually contains 6–7 x 10^6 adipocytes, which correspond to 5 x 10^5 adipocytes per well of a 12-well plate.

4.5. Incubate the plates in a cell culture incubator (7% CO_2 and 37 °C), and do not disturb the cells for 24 h. On the next day, carefully replace the supernatant with fresh DMEM without pyruvate, 25 mM glucose, 10% FCS, and 1% penicillin and streptomycin.

NOTE: It is also possible to seed the cells into collagen-precoated 48- and 96-well plates but take more precautions when replacing the media to avoid detachment of the adipocytes.

5. Functional analysis of transfected 3T3-L1 adipocytes

5.1. Study target knockdown 24–48 h and 48–96 h after siRNA or miR mimic delivery for mRNA and protein, respectively.

5.2. Perform functional analyses of transfected adipocytes to study insulin signaling, glucose uptake, adipokine secretion, lipolysis, and lipogenesis.

REPRESENTATIVE RESULTS:

Using the procedure of reverse-transfection described here to modulate the expression of proteins or micro-RNAs in 3T3-L1 adipocytes, the adipocytes have been shown to preserve

their morphology after the transfection (**Figure 1B,C**). Indeed, 2 days after the transfection, the adipocytes were well-spread and attached to the plate and presented multilocular lipid droplets that are a characteristic of mature 3T3-L1 adipocytes. The lipid content was not different between the transfected and non-transfected adipocytes (**Figure 1D,E**). Moreover, the mRNA expression of differentiation markers such as peroxisome proliferator-activated receptor gamma 2 (*Pparγ2*), adiponectin (*Adipoq*), glucose transporter 4 or solute carrier family 2 member 4 (*Slc2a4*), insulin receptor substrate 1 (*Irs1*), perilipin-1 (*Plin1*) was unchanged in transfected cells compared to that in non-transfected adipocytes (**Figure 1F**). Thus, this reverse-transfection protocol is efficient as >70% of the adipocytes were transfected (**Figure 1G,H**).

Perilipin-1 is an adipocyte-specific protein known to promote lipid droplet formation and inhibit lipolysis. Here, 3T3-L1 adipocytes were transfected with scrambled siRNA (si-SCR) or siRNA against *Plin1* (si-PLIN1). Three days after the transfection with si-PLIN1, the mRNA level of *Plin1* had decreased by 70% (**Figure 2A**) and the protein level by 63% (**Figure 2B,C**). PLIN1 expression was also analyzed by fluorescence microscopy 4 days after the transfection and was found to have decreased by 92% compared to its expression in control adipocytes (**Figure 2D–F**), thus demonstrating the efficacy of both the transfection protocol and the si-PLIN1.

This protocol was also used to perform reverse-transfection of adipocytes with micro-RNA mimicking (miR mimics) oligonucleotides to upregulate the expression of miR-34a (**Figure 3A**). The overexpression of miR-34a led to the decrease in VAMP2 protein expression by 50% (**Figure 3B,C**), a confirmed target of miR-34a[11,12]. Finally, this study shows that reverse-transfection of 3T3-L1 adipocytes preserves their function and responsiveness to insulin stimulation. Indeed, knockdown of *Plin1* in 3T3-L1 adipocytes led to an increase in basal lipolysis (**Figure 4A**). Moreover, the overexpression of miR-34a in 3T3-L1 adipocytes led to the inhibition of insulin-induced protein kinase B phosphorylation (**Figure 4B,C**) and glucose uptake (**Figure 4D**).

FIGURE AND TABLE LEGENDS:

Figure 1: Differentiation of 3T3-L1 fibroblasts into mature adipocytes. (**A**) 3T3-L1 fibroblasts were seeded at a density of 3 x 10^5 cells per 100 mm dish. Representative 10x brightfield image of 3T3-L1 fibroblasts 2 days later. (**B**) Two days after confluency (day 0), the 3T3-L1 fibroblasts were differentiated into adipocytes using a differentiation cocktail mix for 4 days (until day 4). Representative 10x brightfield image of 3T3-L1 fibroblasts differentiated into adipocytes (day 7). Adipocytes with multilocular lipid droplets are easily discernable. (**C**) The 3T3-L1 adipocytes were transfected with si-SCR on day 7. Representative 10x brightfield images of transfected 3T3-L1 adipocytes (day 9). The morphology of the transfected adipocytes is comparable to that of the non-transfected adipocytes, implying that the transfection method is gentle and not toxic to the adipocytes. Scale bars: 50 µm. (**D–E**) The 3T3-L1 adipocytes were transfected with si-SCR on day 7 (upper panels); non-transfected adipocytes (lower panels). Two days later, the cells were incubated with Oil Red O to stain lipids. (**D**) Representative images of the stained cells in the plate and representative 10x brightfield images are shown. Scale bars: 50 µm. (**E**) The Oil Red O incorporated into the cells was eluted with 2-propanol and quantified using a spectrophotometer. Data are expressed in arbitrary units, with the absorbance of the non-transfected cells normalized to 1. Results are

expressed as the mean ± SEM of three independent experiments. Statistical analysis was performed by Student's *t*-test. (**F**) The 3T3-L1 adipocytes were transfected with si-SCR. Three days after the transfection, the cells were harvested to isolate total RNA. The expression of adipocyte differentiation markers was measured by qRT-PCR and normalized using 36B4 RNA levels. The data represent the mRNA expression in transfected cells relative to that in non-transfected cells (normalized to 1, represented by the dotted line) and are expressed as the mean ± SEM of three independent experiments. Statistical analysis was performed by Student's *t*-test. (**G–H**) The 3T3-L1 adipocytes were transfected with si-SCR or fluorescent dye (FAM)-labeled si-RNA and plated on coverslips. 3T3-L1 adipocytes were analyzed 8 h later by fluorescence microscopy. (**G**) Representative single-plane image of transfected 3T3-L1 cells is shown. (**H**) Quantification of the FAM-positive cells relative to the total number of cells. Data is expressed as percentage of cells containing fluorescent si-RNA. Statistical analysis was performed using Mann-Whitney test, ****$p < 0.0001$. Abbreviations: si-SCR = scrambled siRNA; SEM = standard error of the mean; qRT-PCR = quantitative reverse-transcription polymerase chain reaction; FAM = fluorescein amidite; DAPI = 4',6-diamidino-2-phenylindole; si-FAM = FAM-labeled si-RNA.

Figure 2: Protein-silencing in 3T3-L1 adipocytes. 3T3-L1 adipocytes were transfected with scrambled si-RNA (si-SCR) or si-RNA against *Plin1* (si-PLIN1). (**A**) Three days after the transfection, mRNA expression of *Plin1* was measured by qRT-PCR. The mRNA expression was normalized using 36B4 RNA levels and expressed in arbitrary units, with the si-SCR-treated cells normalized to 1. Results are expressed as the mean ± SEM of four independent experiments. Statistical analysis was performed using Student's *t*-test, **$p < 0.01$. (**B–C**) Three days after the transfection, protein lysates were subjected to western blotting with antibodies directed against PLIN1 and HSP90 (loading control). Representative immunoblots are shown. (**C**) The amount of PLIN1 was quantified by densitometry scanning analysis and normalized using the amount of HSP90. Data are expressed in arbitrary units, with the si-SCR-treated cells normalized to 1. Results are expressed as the mean ± SEM of four independent experiments. Statistical analysis was performed using Student's *t*-test, *$p < 0.05$. (**D–F**) The 3T3-L1 adipocytes were transfected with si-SCR or si-PLIN1 and plated on coverslips. The expression of PLIN1 was analyzed 96 h later by fluorescence microscopy. (**D**) Representative single-plane images of 3T3-L1 adipocytes stained with anti-perilipin antibody and an anti-rabbit-Alexa647-conjugated antibody are shown. Scale bars = 10 µm. (**E**) Three-dimensional (3D) volume-rendering of 3T3-L1 adipocytes segmented in 3D using commercial software. Scale bars = 10 µm. (**F**) The quantification of PLIN1 signal intensity relative to the total number of cells. Data are expressed in arbitrary units, with the si-SCR-treated cells normalized to 100%. Statistical analysis was performed using Mann-Whitney test, ****$p < 0.0001$. Abbreviations: si-SCR = scrambled siRNA; si-PLIN1 = siRNA against *Plin1;* Plin1 = perilipin-1; HSP90 = heat shock protein 90; SEM = standard error of the mean; qRT-PCR = quantitative reverse-transcription polymerase chain reaction; FAM = fluorescein amidite; DAPI = 4',6-diamidino-2-phenylindole; IB = immunoblotting.

Figure 3: micro-RNA overexpression in 3T3-L1 adipocytes. 3T3-L1 adipocytes were transfected with control micro-RNA mimic (miR-control) or micro-RNA 34a mimic (miR-34a). Three days after the transfection, the cells were harvested for (**A**) RNA extraction or (**B**) preparation of protein lysates. (**A**) The expression of miR-34a was measured by qRT-PCR. The miR expression was normalized using U6 small RNA levels and expressed in arbitrary units,

with the miR-control-treated cells normalized to 1. Results are expressed as the mean ± SEM of three independent experiments. Statistical analysis was performed using Student's *t*-test, **p* < 0.05. (**B**) Protein lysates were subjected to western blotting with antibodies directed against VAMP2 and TUBULIN (loading control). Representative immunoblots of three independent experiments are shown. (**C**) The amount of VAMP2 was quantified by densitometry scanning analysis and normalized using the amount of TUBULIN. Data are expressed in arbitrary units, with the miR-control cells normalized to 1. Results are expressed as the mean ± SEM of three independent experiments. Statistical analysis was performed by Student's *t*-test, **p* < 0.05. Abbreviations: SEM = standard error of the mean; qRT-PCR = quantitative reverse-transcription polymerase chain reaction; VAMP2 = vesicle-associated membrane protein 2; IB = immunoblotting.

Figure 4: Effects of protein or micro-RNA modulation in 3T3-L1 adipocytes on adipocyte functions. (**A**) 3T3-L1 adipocytes were transfected with si-SCR or si-PLIN1. The medium was changed 24 h after the transfection and then collected 48 h later to measure basal lipolysis. Results are expressed as glycerol released in the media (µg/mL), and as the mean ± SEM of four independent experiments. Statistical analysis was performed using Student's *t*-test, ****p* < 0.001. (**B**) 3T3-L1 adipocytes were transfected with miR-control or miR-34a. The medium was changed 24 h after the transfection, and then, 48 h later, the medium was changed to the depletion medium (DMEM without pyruvate, 25 mM glucose, 1% penicillin and streptomycin, and 0.5% BSA) for 6 h. Then, the cells were treated with 0.5 nM insulin for 5 min. Cells were harvested to prepare protein lysates for western blotting with antibodies directed against phospho-PKB and PKB (loading control). Representative immunoblots of three independent experiments are shown. (**C**) The amount of phospho-PKB was quantified by densitometry scanning analysis and normalized using the total amount of PKB. Data are expressed in arbitrary units, with the miR-control cells treated with insulin normalized to 1. Results are expressed as the mean ± SEM of three independent experiments. Statistical analysis was performed using the two-way ANOVA test, **p* < 0.05 compared to miR-control cells treated with insulin. (**D**) 3T3-L1 adipocytes were transfected with miR-control or miR-34a. The media was changed 24 h after the transfection, and then, 48 h later, the medium was changed to the depletion medium for 6 h. Then, the cells were treated with 0.5 nM insulin for 20 min. Uptake of (2-^{3}H)deoxyglucose was measured over 3 min. Data are expressed in arbitrary units, with the basal glucose uptake in miR-control-treated cells normalized to 1. Results are expressed as the mean ± SEM of three independent experiments. Statistical analysis was performed using the two-way ANOVA test, **p* < 0.05 compared to miR-control cells treated with insulin. Abbreviations: si-SCR = scrambled siRNA; si-PLIN1 = siRNA against *Plin1;* SEM = standard error of the mean; PKB = protein kinase B; p-PKB = phosphor-PKB; miR-CTL = miR-control; DMEM = Dulbecco's modified Eagle's medium; BSA = bovine serum albumin; ANOVA = analysis of variance; IB = immunoblotting.

Table 1: Transfection reagents required for 12-well and 24-well plate formats.

DISCUSSION:

This paper presents a detailed protocol for the differentiation and transfection of mature adipocytes. This reverse-transfection method is a simple, economical, and highly efficient method to transfect oligonucleotides such as, but not limited to, siRNAs, micro-RNA mimics, and anti-micro-RNAs into 3T3-L1 adipocytes, which is one of the most difficult cell lines to

transfect. This method has some limitations that need to be considered. This protocol is not efficient for transfection with plasmid DNA, which limits the utility of this technique for gain-of-function studies. Although murine cell lines, including the 3T3-L1 cell line, have been typically used to study adipocyte function in vitro, micro-RNA expression patterns and activities in murine tissue are often different from those observed in humans; this is also the case with primary cells and cell lines. Moreover, the medium used for the differentiation of 3T3-L1 fibroblast into adipocytes requires an unphysiological hormone cocktail (insulin, dexamethasone, IBMX, and rosiglitazone), and the differentiated cells are morphologically distinct from in vivo mature adipocytes: they present multilocular lipid droplets instead of a unilocular lipid droplet. This could explain some differences in gene expression and cellular responses between in vitro and in vivo studies.

One benefit of using this reverse-transfection protocol compared to electroporation is that this method is cheaper. Indeed, the reagents are less expensive, and the high efficiency of the reverse transfection decreases the quantities of oligonucleotides needed, and there is no need of expensive equipment such as an electroporator. Moreover, using a lower concentration of siRNA is beneficial as it avoids off-target effects**.** Furthermore, this reverse-transfection is an easy, fast, gentle, and straightforward method of transfection that requires fewer cells and will ensure excellent cell viability and more robust data compared to electroporation. Although the procedure detailed above has been optimized for the differentiation and reverse-transfection of mouse 3T3-L1 cells, human preadipocytes can also be differentiated into adipocytes[5] and easily subjected to reverse-transfection using this protocol. This report shows that adipocytes remain viable, healthy, and responsive to insulin after the reverse-transfection using a new generation of a non-liposomal cationic amphiphile transfection reagent. The reverse-transfection could also be performed using other popular transfection reagents. However, the amounts of oligonucleotides and transfection reagent would need to be optimized to ensure good modulation of expression and no adverse effects on cell viability.

This protocol facilitates the study of the role of proteins and micro-RNAs in adipocyte function, but it could also be used to modulate other non-coding RNAs such as lnc-RNAs, Y-RNAs, or eRNAs. There are critical steps in the protocol that can impact the efficiency of the procedure. The differentiation of 3T3-L1 fibroblasts into adipocytes should be carefully monitored. It is important to reach a high level of differentiation to avoid the proliferation of remaining fibroblasts after the transfection, which would bias the results of the experiment. Another important point is to perform the transfection on newly differentiated mature adipocytes; the best timing is 7–8 days after the beginning of the differentiation protocol, which corresponds to 3–4 days after removing the differentiation cocktail. This will ensure robust transfection efficacy and favor good reattachment of the adipocytes to the plate. Finally, the treatment of adipocytes with trypsin should be monitored carefully to ensure detachment of the adipocytes without damaging the cells.

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DISCLOSURES:

The authors have nothing to disclose.

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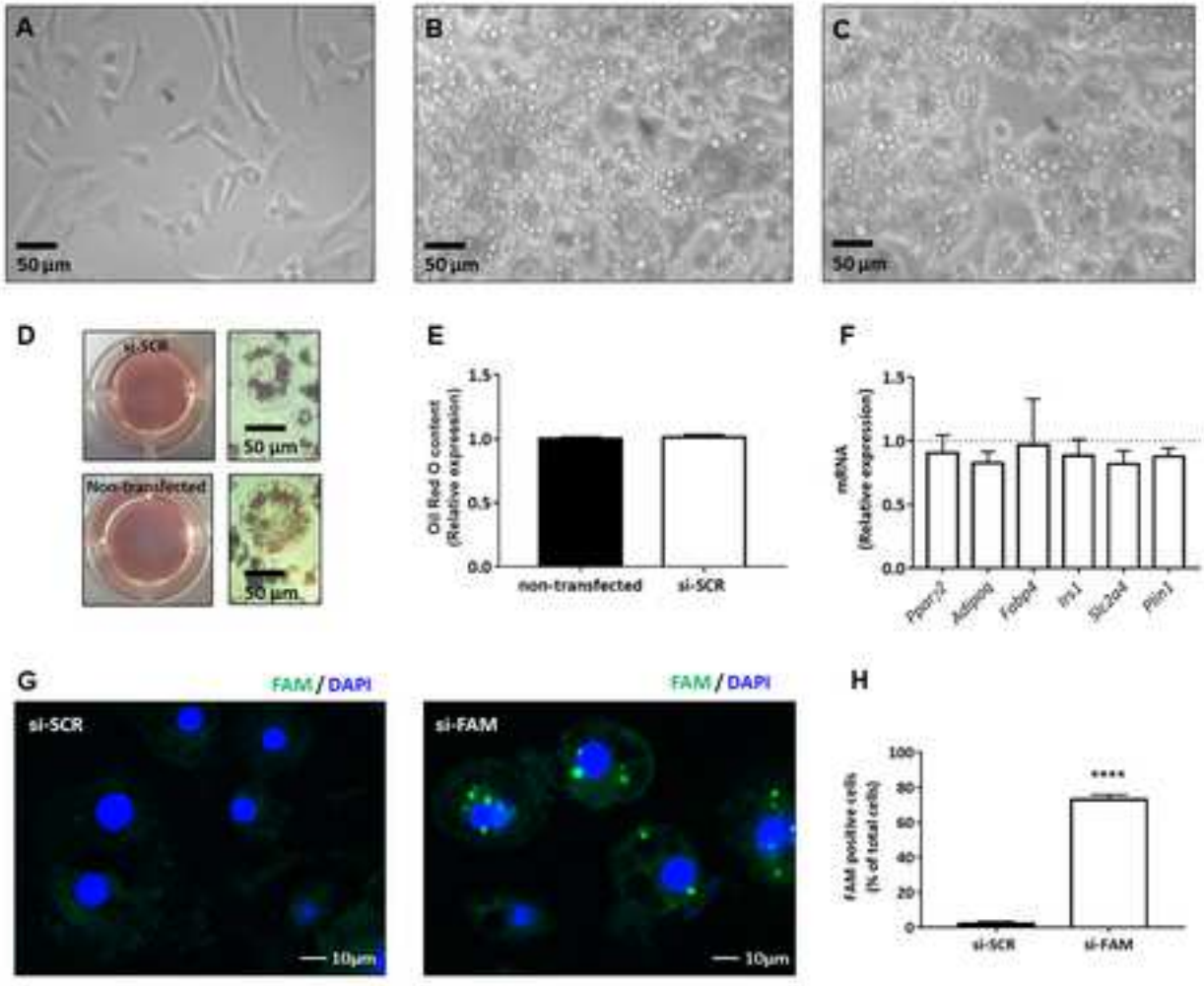
Figure 1



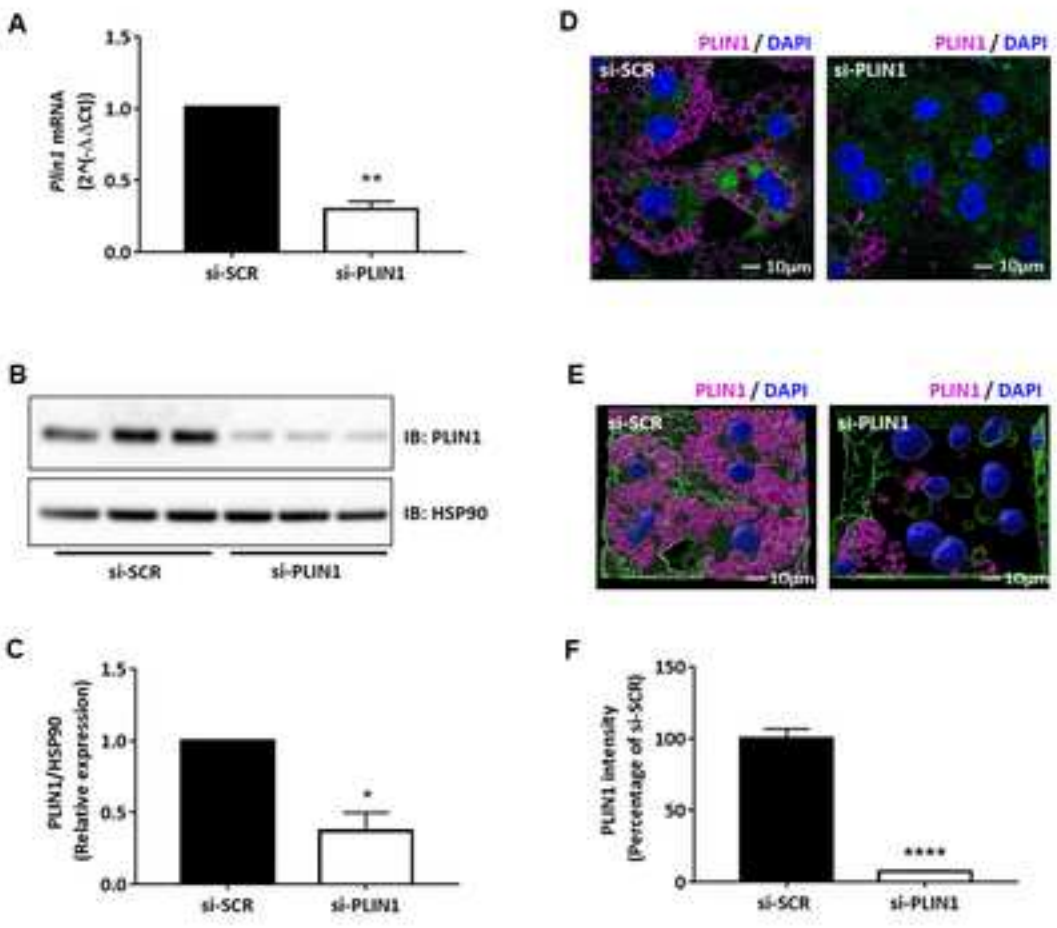


Figure 3

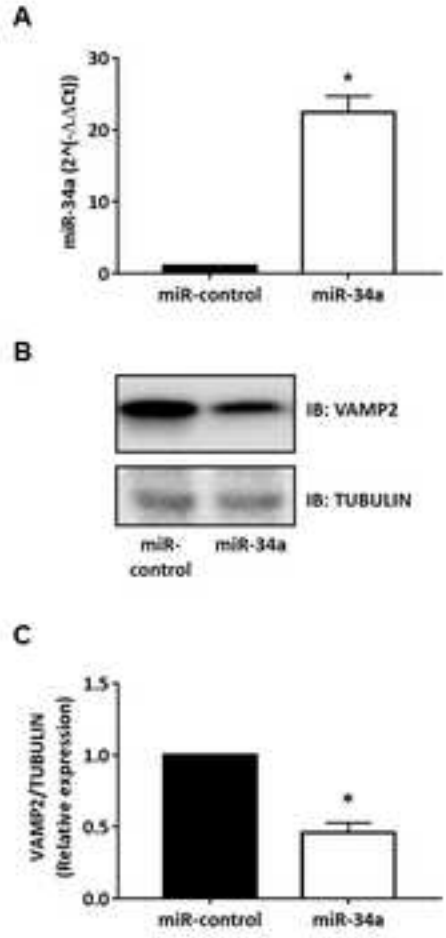
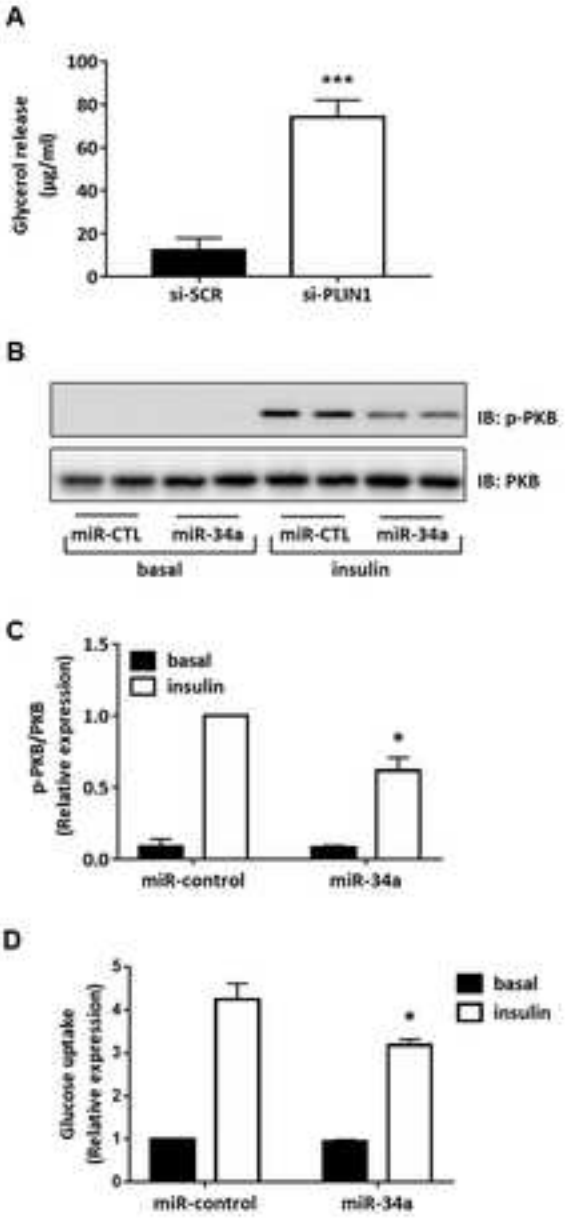


Figure 4



	Per well (12-well plate)	**Per well (24-well plate)**
Oligonucletides (si-RNA, miR…)	20 µL	10 µL
Improved Minimal Essential Medium	20 µL	10 µL
Transfection reagent	5.6 µL	2.8 µL
Improved Minimal Essential Medium	154.4 µL	77.2 µL
Total volume of transfection mix	200 µL	100 µL

Name of Material/Equipment	Company
12 well Tissue Culture Plate	Dutscher
2.5% Trypsin (10x)	Gibco
2-Propanol	Sigma
3-Isobutyl-1-methylxanthine	Sigma-Aldrich
Accell Non-targeting Pool	Horizon Discovery
Bovine Serum Albumin (BSA)	Sigma
Collagen type I from calf skin	Sigma-Aldrich
Dexamethasone	Sigma-Aldrich
D-PBS	Gibco
Dulbecco's Modified Eagles's Medium (DMEM)	Gibco
Ethanol	Sigma
FAM-labeled Negative Control si-RNA	Invitrogen
Fetal Bovine Serum	Gibco
Free Glycerol Reagent	Sigma-Aldrich
Glycerol Standard Solution	Sigma-Aldrich
HSP90 antibody	Santa Cruz
Improved Minimal Essential Medium (Opti-MEM)	Gibco
Insulin, Human Recombinant	Gibco
miRIDIAN micro-RNA mimics	Horizon Discovery
miRNeasy Mini Kit	Qiagen
miScript II RT Kit	Qiagen
miScript Primer Assays Hs_RNU6-2_11	Qiagen
miScript Primer Assays Mm_miR-34a_1	Qiagen
miScript SYBR Green PCR Kit	Qiagen
Newborn Calf Serum	Gibco
Oil Red O	Sigma
ON-TARGETplus Mouse Plin1 si-RNA SMARTpool	Horizon Discovery
Penicillin and Streptomycin	Gibco
Perilipin-1 antibody	Cell Signaling
Petri dish 100 mm x 20 mm	Dutscher
PKB antibody	Cell Signaling
PKB Phospho Thr308 antibody	Cell Signaling
Rosiglitazone	Sigma-Aldrich
Transfection reagent (INTERFERin)	Polyplus
α-tubulin antibody	Sigma aldrich
Vamp2 antibody	R&D Systems

Catalog Number	Comments/Description
353043	
15090-046	diluted to 5x with D-PBS
I9516	
D5879	
D-001910-10-05	
A7030	
C8919	
D1756	
14190144	
41965062	4.5 g/L D-Glucose; L-Glutamine; no Pyruvate
51976	
AM4620	
10270-106	
F6428	
G7793	
sc-131119	Dilution : 0.5 µg/mL
31985-047	
12585-014	
217004	
218161	
MS00033740	
MS00001428	
219073	
16010-159	
O0625	
L-056623-01-0005	
15140-122	
3470	Dilution : 1/1000
353003	
9272	Dilution : 1/1000
9275	Dilution : 1/1000
R2408	
409-10	
T6199	Dilution : 0.5 µg/mL
MAB5136	Dilution : 0.1 µg/mL

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Monday November the 30th 2020,

To the Editor,

We would like to thank you and the Reviewers for the valuable feedback. Our protocol manuscript has now been significantly strengthened by the thorough review and constructive suggestions we received. We have included new experiments and clarified the text as requested by the reviewers. You will find a point-by-point response to each reviewer's concerns below (in blue) and our changes to the text are marked with red font.

We hope that with all these changes, our manuscript will be now considered for publication in *JoVE.*

Please, do not hesitate to contact me if any further information is required.

Best regards,

Dr. Jennifer Jager

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Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
Answer: We went through the manuscript and did our best to check the spelling and the grammar.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points
Answer: We made these changes.

3. Please provide an email address for each author.
Answer: We added the email address for each author.

4. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Presented here is a protocol …"
Answer: We made these changes.

5. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.
Answer: We made these changes.

6. Please ensure the Introduction include all of the following with citation:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application
Answer: We made these changes.

7. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: INTERFERin, Opti-MEM, etc.
Answer: We have removed all the commercial language from the manuscript and added all the commercial products to the Table of Materials and Reagents.

8. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end we ask you to use a generic term for INTEFERin.
Answer: We have removed all the commercial language from the manuscript and replaced the generic term "INTERFERin" by "transfection reagent".

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9. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."
Answer: We made the changes when needed.

10. The Protocol should contain only action items that direct the reader to do something.
Answer: We made the changes when needed.

11. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.
Answer: We made the changes when needed.

12. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?
Answer: We made the changes when needed.

13. 1.1: How many cells are seeded.
Answer: We have added the number of cells seeded per cm^2.

14. 1.2: So the cells are not divided after 100% confluency?
Answer: It is right, at confluency the fibroblasts stop to proliferate (divide) and after two days of confluency the fibroblasts are ready to differentiate into adipocytes.

15. Only one note can follow one step. In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Other details can be moved to the discussion.
Answer: We made the changes when needed.

16. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Answer: We made the changes when needed. The Protocol's length is 3 pages.

17. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
Answer: There are no reuse of any figures.

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18. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

19. Figure: For all images with microscope please include a scale bar.
Answer: We made the changes when needed.

20. Please upload each Figure individually to your Editorial Manager account. Please combine all panels of one figure into a single image file.
Answer: We made the changes.

21. Please sort the materials table in alphabetical order.
Answer: We made the changes.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
Jager et al describe a method to reverse transfect mature 3T3-L1 with siRNAs and miRNA mimics, a protocol that is notably challenging. To overcome the challenges, they used a transfection reagent called INTERFERin and seeded differentiated 3T3-L1 onto collagen-coated plates containing the transfection complex. These settings appeared to successfully deliver small RNAs to the cells as evidenced by expression of target genes/proteins and functional assays. Although it is clear that the method works, I have some concerns that should be address.

Major Concerns:
1) Since fully differentiated adipocytes are expected to float, I am concerned that during the process of trypsin treatment and seeding, some of the adipocytes might be lost, thus creating a bias. Did the authors compare the number of cells before and after seeding? Did they notice cell loss? Did they notice any change in lipid accumulation in the cells after seeding?
Answer: It is true that mature adipocytes isolated from adipose tissue are floating cells due to high amount of lipid content. However, this is not the case for differentiated 3T3-L1 adipocytes that contains multilocular lipid droplets with lower amount of lipids compared to primary adipocytes. When we change the media 24 hours after the transfection, the cells are seeded and well attached. It can happen sometimes that the adipocytes accumulate at the center of the well and are not well attached to the plate, but this happens when the trypsin treatment is not well performed and, the cells are not well separated and homogenized before seeding.
We did compare the number of cells before and 24 hours after seeding, and did not notice any cell loss, most of the cells were still there and attached.

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We showed that the lipid content was not different between the transfected and non-transfected adipocytes (Figure 1D-E).

2) The authors should try to determine the proportion of cells that are infected with the small RNAs. Although there is a clear effect, that could be due to transfection of just a subpopulation of cells. One possibility to address this concern is by transfecting cells with a fluorescence-labelled siRNA and looking at the microscope for fluorescence distribution.
Answer: We thank the Reviewer for this comment and the suggestion. We have performed transfection with a fluorescent-labelled si-RNA (Figure 1G-H) and showed that 70-80% of the cells were transfected. We also performed experiments to knock-down perilipin-1 and analyzed the expression of perilipin using fluorescent microscopy. We showed 92% of knockdown and that most of the adipocytes were almost completely depleted of perilipin-1 and only a few cells were still expressing perilipin-1 (Figure 2D-F).

3) The conclusion regarding cell viability is based on cell function, however, cell viability may be significantly compromised despite the fact that some remaining cells continue to function. I would measure cell number and markers of cell viability.
Answer: Dying adipocytes detach and float. We did compare the number of cells before and 24 hours after seeding, and did not notice any cell loss, most of the cells were still there and attached. Moreover, we think that it is important to check adipocyte function such as lipolysis, glucose uptake, and insulin signaling because it reflects the health of the cells.

Minor Concerns:
1) Correct "adipocytes function" for "adipocyte function".
Answer: We thank the Reviewer for this comment. We have made the correction when needed.

2) In item 3.5, I assume they are referring to the collagen-coated plates. It seems obvious but I believe they should add it for the sake of accuracy.
Answer: We thank the Reviewer for this comment. We have made the correction.

3) One limitation of the assay which was pointed out by the authors is the necessity of cells reaching close to 100% differentiation prior to transfection. However, my experience working with 3T3-L1 and seeing many people work with these cells is that rarely one reaches almost 100% differentiation.
Answer: Indeed, in the discussion part of the manuscript we mentioned that one limitation of the assay is the necessity to reach a really high level of differentiation for 2 reasons:

- It is crucial to have a good differentiation into adipocyte to study adipocyte function
- If the differentiation is not good (less than 80%), after the trypsin treatment and the seeding of the cells, the remaining fibroblasts would proliferate and lead to a mixed population of cells which would have an impact on the experiments.

When using our protocol of differentiation, most of the fibroblast differentiate into adipocytes but we do use rosiglitazone though. I guess the confluency and the duration of confluency before inducing the differentiation are important points as well as the composition of the differentiation cocktail, especially the addition of rosiglitazone. We have changed the “almost 100% differentiation” by the “a high level of differentiation (>80%)”.

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Reviewer#2:

Manuscript Summary:

In this manuscript, Jennifer et al. detail a transfection protocol to manipulate the expression of proteins and non-coding RNAs in maturely differentiated adipocytes. They first use a standard procedure to differentiate 3T3-L1 fibroblasts into adipocytes and then perform a reverse transfection using INTERFERin to manipulate the levels of protein and non-coding genes. Growing evidence support the critical role of non-coding RNAs in adipose biology so this protocol covers an area of significant interest. However, there are a number of issues that need to be addressed before further consideration.

Major Concerns:

1. The authors need to confirm that their transfection protocol does not substantially alter the differentiation. They have made some attempts by examining the morphology of the adipocytes before and after transfection, but morphology is a very crude approach to evaluate cell's identity and integrity. Instead, they should use known protein, miRNA and lncRNA markers associated with adipogenesis to carefully ascertain the differentiation status after transfection.

Answer: We agree on the Reviewer's comment. We have performed additional analysis presented in Figure 1D, 1E and, 1F to confirm that our transfection protocol does not substantially alter the differentiation. Figure 1D and 1E showed that the transfection does not alter the lipid content of the adipocytes. Figure 1F showed that the mRNA expression of key proteins associated with adipogenesis and/or adipocyte function is not changed by the transfection.

2. The authors need to provide a detailed introduction of INTERFERin's chemical characteristics which is a useful information for user to understand if this reagent will interfere with their functional study after the transfection.

Answer: Since we had to remove all the commercial language from the manuscript, we have replaced "INTERFERin" by "transfection reagent". We have added some information on INTERFERin's chemical characteristics in the discussion section.

3. Reduced toxicity and enhanced efficacy are often the primary reasons for a lab to switch to a new transfection protocol. The authors need to make a comparison between INTERFERin and other popular transfection approaches such as Lipofectamine to confirm that their approach is superior in both or either category.

Answer: The main point of our assay is the reverse transfection not the transfection reagent. We have added some information on INTERFERin's chemical characteristics in the discussion section and discussed the possibility to use our protocol with other transfection reagents.

4. There are substantially more lncRNAs than miRNAs in mammalian genomes. If the authors want to claim that their approach is suitable for non-coding RNAs, they should test the knockdown efficacy of a lncRNA.

Answer: We do agree that we shouldn't claim that our approach is suitable for all non-coding RNAs since we have presented data only for micro-RNA. One should have also performed experiments with lncRNA, eRNA, Y-RNA… to make general conclusion about the efficacy of the protocol to manipulate non-coding RNA expression. We have not performed additional experiments by lack of tools and good non-coding RNA candidates (that we know and have previously manipulated) and their targets to validate their manipulation. In the revised manuscript we have replaced non-coding RNA by micro-

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RNA, and we have discussed the possibility to use this protocol for modulating other non-coding RNA such as lncRNA.

5. Most experiments were performed in duplicate which is not sufficient. The authors need to repeat their experiment in triplicate, perform statistical analyses between samples and add error bars to their figures.
Answer: We thank the Reviewer for this comment. In the revised manuscript all the presented data in the figures are the mean ± SEM of at least three independent experiments, and we have included the error bars and the statistical analysis.

Minor Concerns:
1. Is the DMEM in step 1.1 with or without pyruvate? Also, the DMEM w/o pyruvate is not in the table of materials.
Answer: We thank the Reviewer for pointing out this lack of information. The DMEM in step 1.1 is without pyruvate, we have now added this information in the text and in the table of materials.

2. The description from 3.1 to 3.5 is not clear. In section 3.3, the authors include the reagent amount used in both the 12 and 24-well plates. However, in section 3.5, the adding amount (200ul) seems to be applied only to the 12-well-plate. I recommend the authors draw a table to specify the reagent amount for 12/24-well-plate settings.
Answer: we thank the Reviewer for this comment. We have made changes to clarify the description from 3.1 to 3.5. and we have also included a table describing all the reagent amount for 12 and 24-well-plate settings.