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TITLE:

Adipocyte cell culture model to manipulate proteins and micro-RNAs expression and study the impact on adipocyte function.

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KEYWORDS:

Adipocytes, cell culture, transfection, micro-RNA (miR), small interfering RNA (si-RNA).

SUMMARY:

Presented here is a protocol to deliver oligonucleotides such as small interfering RNA (si-RNA), micro-RNA mimic (miR), or anti-micro-RNA (anti-miR) into mature adipocytes to manipulate proteins and micro-RNAs expression.

ABSTRACT:

Alteration of adipocyte function plays a key role in the pathogenesis of metabolic diseases such as insulin resistance and Type 2 Diabetes. This highlights the need to better understand the molecular mechanism involved in adipocyte dysfunction in order to develop new therapies against obesity-related diseases. Manipulating the expression of proteins and micro-RNAs *in* adipocytes remains highly challenging. This manuscript describes a protocol to differentiate murine fibroblasts into mature adipocytes and, to manipulate the expression of proteins and micro-RNAs in mature adipocytes through reverse transfection using small interfering RNA and micro-RNA mimic oligonucleotides. Functional experiments such as insulin signaling, glucose uptake, lipogenesis, and lipolysis can be performed on the transfected 3T3-L1 mature adipocytes to study the impact of proteins or micro-RNAs 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 diseases¹. Current therapies have failed to stop the constant 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 in order to develop new therapies against obesity-related diseases. Many research studies have focused on understanding protein coding RNAs and their involvement in the regulation of adipocyte physiology and subsequent role in obesity. More recently the discovery of non-coding RNAs (ncRNAs), especially micro-RNAs (miRs) has forged novel concepts related to the mechanism of regulation of gene expression programs. Studies have shown that ncRNAs are important regulators of adipocyte function and their dysregulation is involved in metabolic diseases⁴. Thus, the manipulation of proteins and ncRNAs in adipocytes is crucial to decipher their roles in adipocytes function and their impact in 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.

The murine 3T3-L1 fibroblasts easily differentiate into mature, functional, and insulin-responsive adipocytes which are a well-characterized cell line model used to study adipocyte function such as insulin signaling, glucose uptake, lipolysis and adipokines secretion⁵⁻⁹. These properties make 3T3-L1 adipocytes an attractive model to manipulate protein-coding and ncRNAs 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 traditional commercially available reagents, the differentiated 3T3-L1 adipocytes are one of the most difficult cell lines to transfect. This is the reason why numerous studies manipulating 3T3-L1 gene expression were focused on adipocyte differentiation rather than adipocyte function.

For a long time, the only efficient technique to transfect adipocytes was electroporation⁵, which is tedious, expensive and can cause cell damage.

Here we report 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 transfection of si-RNA and other oligonucleotides such as micro-RNA mimic and anti-micro-RNA (antagomirs). 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 the mature adipocytes are seeded 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 to study the role of protein-coding and micro-RNAs in adipocyte function and their potential role in obesity-related diseases.

PROTOCOL:

General note: All steps of the protocol should be performed in a laminar flow cell culture hood using sterile technique.

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

- 1.1. Grow the 3T3-L1 fibroblasts in 100 mm dishes (see **table of materials**) in culture media DMEM, without pyruvate, 25 mM glucose, 10% newborn calf serum, and 1% penicillin and streptomycin (see **table of materials**), place the dishes in a tissue culture incubator (7% CO₂ and 37°C).
- 1.2. Two days after confluence (see **Note 1**), change the culture media for 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, 0.25 µM dexamethasone, 5 µg/ml insulin, and 10 µM rosiglitazone (see **table of materials**).
- 1.3. Two days after, replace the culture media for DMEM without pyruvate, 25 mM glucose, 10% FCS, and 1% penicillin and streptomycin supplemented with 5 µg/ml insulin and 10 µM rosiglitazone for two days.
- 1.4. Then, feed the cells every two days with DMEM without pyruvate, 25 mM glucose, 10% FCS, and 1% penicillin and streptomycin.
- 1.5. The 3T3-L1 adipocytes are transfected 7-8 days after the beginning of the differentiation protocol (see **Note 2**).

Note 1: it takes five days to reach confluency when the cells are seeded at 300,000 cells per 100 mm dishes.

Note 2: It is important to reach a high level of differentiation (>80%) before the transfection to avoid the proliferation of remaining fibroblasts after the transfection which would lead to a mixed population of cells and bias the results.

2. Preparation of the precoated plates

- 2.1. The day before or a few hours before the transfection, prepare a solution of collagen type I (see **table of materials**) at 100 µg/ml in 30% ethanol from a stock solution at 1mg/ml.
- 2.2. Add 250 µl of collagen per well of a 12-well plate, 125 µl per well of a 24-well plate and, 62.5 µl per well of a 48-well plate (see **table of materials**) and spread the solution over the surface of the well.
- 2.3. Leave the plate without the lid under the culture hood until the collagen dry.
- 2.4. Wash twice with PBS.

Note 3: Precoated plates are available for purchase.

3. Preparation of the transfection mix

Note 4: The final concentration of si-RNA (see **table of materials**) is between 1 and 100 nM (1 to 100 pmol of si-RNA per well of a 12-well plate). The final concentration of miR mimic (see **table of materials**) is 10 nM (10 pmol/well).

Note 5: The best concentration of each si-RNA, miR mimic or other oligonucleotides must be tested prior the experiment to avoid off-target effects.

Note 6: Performing transfection experiments in triplicate is recommended to allow statistical analysis of the results.

Note 7: To account for normal loss during pipetting, all reagents are prepared in excess.

- 3.1. Mix by pipetting (volume/volume) the si-RNA (or other oligonucleotides) with reduced serum media (see **table of materials** and **Table 1** for the reagent amount).
- 3.2. Incubate 5 minutes at room temperature.
- 3.3. Add the transfection reagent (see **table of materials**) and the reduced serum media to the si-RNA, and pipet to mix (see **Table 1** for the reagent amount).
- 3.4. Incubate 20 minutes at room temperature (during this time proceed with step 4).
- 3.5. Add the transfection mix to each well of the collagen-coated plate.

Table 1: Reagent amount for each plate settings.

	Per well (12-well plate)	Per well (24-well plate)
Oligonucleotides (si-RNA, miR...)	20 μ l	10 μ l
Reduced serum media	20 μ l	10 μ l
Transfection reagent	5.6 μ l	2.8 μ l
Reduced serum media	154.4 μ l	77.2 μ l
Total volume of transfection mix	200 μ l	100 μ l

4. Preparation of the 3T3-L1 adipocytes

- 4.1. Wash the cells in the 100 mm petri dish twice with D-PBS.
- 4.2. Add 5x trypsin (see **table of materials**) to the cells (1 ml per 100 mm dish), ensure to cover all the surface with the trypsin.

- 4.3. Wait 30 seconds and carefully remove the trypsin.
- 4.4. Incubate the petri dish for 5-10 min at 37°C in the incubator.
- 4.5. Tap the 100 mm dish to detach the cells.
- 4.6. Add 10 ml of DMEM without pyruvate, 25 mM glucose, 10% FCS, and 1% penicillin and streptomycin to neutralize the trypsin.
- 4.7. Pipet up and down the media to detach the cells and homogenize the cell suspension.
- 4.8. Count the cells using a Malassez counting chamber or an automated cell counter and adjust the concentration of the cells to 6.25×10^5 cells/ml of media.
- 4.9. Seed 800 μ l of the cell suspension/well of a 12-well plate (5×10^5 cells), or 400 μ l of the cell suspension/well of a 24-well plate (25×10^4 cells) containing the transfection mix (see **Note 8**).
- 4.10. Incubate the plates in a cell culture incubator (7% CO₂ and 37°C) and do not touch the cells for 24 hours.
- 4.11. The next day carefully replace the culture media for fresh DMEM without pyruvate, 25mM glucose, 10% FCS, and 1% penicillin and streptomycin (see **Note 9**).

Note 8: One 100 mm petri dish of adipocytes allows to prepare one 12-well plate or one 24-well plate. A 100 mm dish usually contains $6-7 \times 10^6$ adipocytes, which correspond to 5×10^5 adipocytes per well of a 12-wells plate.

Note 9: It is also possible to seed the cells into 48 and 96-well plates, but one should take more precautions when replacing the media to avoid the adipocytes to detach.

5. Functional experimentation on transfected 3T3-L1 adipocytes

- 5.1. Typical time points for detecting target knockdown after si-RNA or miR mimic delivery are 24-48 hours for mRNA and 48-96 hours for protein.
- 5.2. Functional experiments can be performed on transfected adipocytes including insulin signaling, glucose uptake, adipokine secretion, lipolysis and lipogenesis.

REPRESENTATIVE RESULTS:

Using the procedure of reverse transfection described here to manipulate the expression of proteins or micro-RNAs in 3T3-L1 adipocytes, we showed that the adipocytes preserve their morphology after the transfection (**Figures 1B-C**). Indeed, two days after the transfection, the adipocytes are well spread and attached to the plate, and present multilocular lipid droplets characteristic of mature 3T3-L1 adipocytes. We showed that the lipid content was not different between the transfected and non-transfected adipocytes (**Figures 1D-E**). Moreover,

the mRNA expression of differentiation markers such as PPAR γ (*Ppar γ 2*), adiponectin (*Adipoq*), GLUT4 (*Slc2a4*), IRS1 (*Irs1*), PLIN1 (*Plin1*) was unchanged in transfected compared to non-transfected adipocytes (**Figure 1F**). We showed that our protocol using reverse transfection is efficient since 70-80 % of the adipocytes were transfected (**Figures 1G-H**).

Perilipin 1 is an adipocyte-specific lipid-droplet associated protein that promotes lipid droplet formation and inhibits adipocyte lipolysis. Here 3T3-L1 adipocytes were transfected with scrambled si-RNA (si-SCR) or si-RNA against perilipin-1 (si-PLIN1). We showed that three days after the transfection of the si-RNA against perilipin-1 the mRNA level of *Plin1* was downregulated by 70% (**Figure 2A**) and the protein level by 63% (**Figures 2B-C**). We also analyzed the expression of PLIN1 using fluorescence microscopy four days after the transfection and, showed that PLIN1 expression was decreased by 92% compared to control adipocytes (**Figure 2D-F**) demonstrating the efficacy of both the transfection protocol and the the si-RNA against PLIN1.

We also used our protocol to reverse transfect adipocytes with mimic micro-RNA oligonucleotides to upregulate the expression of miR-34a (**Figure 3A**). We showed that the overexpression of miR-34a led to the downregulation of VAMP2 protein expression by 50% (**Figures 3B-C**), a confirmed target of miR-34a^{11,12}.

Finally, we showed that the reverse transfection of 3T3-L1 adipocytes preserves their function and responsiveness to insulin stimulation. Indeed, we showed that the knockdown of PLIN1 in 3T3-L1 adipocytes led to an increase of the basal lipolysis (**Figure 4A**). Moreover, the overexpression of miR-34a in 3T3-L1 adipocytes led to the inhibition of insulin-induced PKB phosphorylation (**Figures 4B-C**) and glucose uptake (**Figure 4D**).

FIGURE LEGENDS:

Figure 1. Differentiation of 3T3-L1 fibroblasts into mature adipocytes.

(A) 3T3-L1 fibroblasts were seeded at 3×10^5 cells per 100 mm dish. Representative 10x brightfield image of 3T3-L1 fibroblasts two days later. (B) Two days after confluency (day 0), the 3T3-L1 fibroblasts were differentiated into adipocytes using a differentiation cocktail mix for four 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 siRNA on day 7. Representative 10x brightfield image of transfected 3T3-L1 adipocytes (day 9). The morphology of the transfected adipocytes is comparable to non-transfected adipocytes, the transfection method is gentle and not toxic to the adipocytes. (D-E) The 3T3-L1 adipocytes were transfected or not with scrambled siRNA on day 7. Two days after, the cells were incubated with Oil Red O to stain lipids. (D) Representative pictures of the stained cells in the plate and representative 10x brightfield image are shown. (E) The Oil Red O incorporated into the cells was eluted with isopropanol and quantified using a spectrophotometer. Data are expressed in arbitrary units, with the non-transfected cells taken as 1. Results are expressed as the means \pm SEM of three independent experiments. Statistical analysis was performed by Student's *t* test. (F) The 3T3-L1 adipocytes were transfected or not with scrambled si-RNA (si-SCR). Three days after the transfection, the cells were harvested to isolate total RNA. The expression of makers of adipocyte differentiation was measure by RT-qPCR and normalized using 36B4 RNA levels. The graphs represent the mRNA expression in transfected cells relative to non-transfected cells (taken as 1) and, are expressed as the means \pm SEM of three independent experiments. Statistical analysis was performed by Student's *t* test. (G-H) The 3T3-L1 adipocytes were

transfected with scrambled siRNA or fluorescent dye (FAM) labeled si-RNA and plated on cover slips. 3T3-L1 adipocytes were analyzed 8 hours later by fluorescence microscopy. (G) Representative single plan image of transfected 3T3-L1 are shown. (H) Quantification of the FAM positive cells normalized to the total number of cells. Data are expressed as percentage of cells containing fluorescent si-RNA. Statistical analysis was performed by Mann-Whitney test, **** $p < 0.0001$.

Figure 2: Protein silencing in 3T3-L1 adipocytes.

3T3-L1 adipocytes were transfected with scrambled si-RNA (si-SCR) or si-RNA against perilipin-1 (si-PLIN1). (A) Three days after the transfection, mRNA expression of *Plin1* was measured by RT-qPCR. The mRNA expression was normalized using 36B4 RNA levels and expressed in arbitrary units, with the si-SCR-treated cells taken as 1. Results are expressed as the means \pm SEM of four independent experiments. Statistical analysis was performed by 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) PLIN1 amount was quantified by densitometry scanning analysis and normalized using HSP90 amount. Data are expressed in arbitrary units, with the si-SCR-treated cells taken as 1. Results are expressed as the means \pm SEM of four independent experiments. Statistical analysis was performed by Student's *t* test, * $p < 0.05$. (D-F) The 3T3-L1 adipocytes were transfected with scrambled siRNA or si-RNA against perilipin-1 (si-PLIN1) and plated on cover slips. The expression of PLIN1 was analyzed 96 hours later by fluorescence microscopy. (D) Representative single plan images of 3T3-L1 adipocytes stained with anti-perilipin antibody and an anti-rabbit-alexa647-conjugated antibody are shown. (E) The quantification of PLIN1 intensity signal was normalized to the total number of cells. Data are expressed in arbitrary units, with the si-SCR-treated cells taken as 100%. Statistical analysis was performed by Mann-Whitney test, **** $p < 0.0001$. (F) 3D volume rendering of 3T3-L1 adipocytes segmented in 3D by a commercial software.

Figure 3: micro-RNA overexpression in 3T3-L1 adipocytes.

3T3-L1 adipocytes were transfected with control mimic micro-RNA (miR-control) or mimic micro-RNA 34a (miR-34a). Three days after the transfection, the cells were harvested to prepare RNA (A) or protein lysates (B). (A) The expression of miR-34a was measured by RT-qPCR. The miR expression was normalized using U6 small RNA levels and expressed in arbitrary units, with the miR-control-treated cells taken as 1. Results are expressed as the means \pm SEM of three independent experiments. Statistical analysis was performed by 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) VAMP2 amount was quantified by densitometry scanning analysis and normalized using TUBULIN amount. Data are expressed in arbitrary units, with the miR-control cells taken as 1. Results are expressed as the means \pm SEM of three independent experiments. Statistical analysis was performed by Student's *t* test, * $p < 0.05$.

Figure 4: Effect of protein or micro-RNA modulation in 3T3-L1 adipocytes on adipocyte functions.

(A) 3T3-L1 adipocytes were transfected with scrambled si-RNA (si-SCR) or si-RNA against perilipin 1 (si-PLIN1). The media was changed 24 hours after the transfection, and then collected 48 hours later to measure basal lipolysis. Results are expressed as glycerol released

in the media ($\mu\text{g/ml}$), and as the mean \pm SEM of four independent experiments. Statistical analysis was performed by Student's *t* test, *** $p < 0.001$. (B) 3T3-L1 adipocytes were transfected with control mimic micro-RNA (miR-control) or mimic micro-RNA 34a (miR-34a). The media was changed 24 hours after the transfection, and then 48 hours later the media was changed to depletion media for 6 hours. Then the cells were treated or not 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) Phospho-PKB amount was quantified by densitometry scanning analysis and normalized using total PKB amount. Data are expressed in arbitrary units, with the miR-control cells treated with insulin taken as 1. Results are expressed as the means \pm SEM of three independent experiments. Statistical analysis was performed by Two-way ANOVA test, * $p < 0.05$ compared to miR-control cells treated with insulin. (D) 3T3-L1 adipocytes were transfected with control mimic micro-RNA (miR-control) or mimic micro-RNA 34a (miR-34a). The media was changed 24 hours after the transfection, and then 48 hours later the media was changed to depletion media for 6 hours. Then the cells were treated or not with 0.5 nM insulin for 20 min. Uptake of (^3H)deoxyglucose was measured during a 3 min period. Data are expressed in arbitrary units, with the basal glucose uptake in miR-control-treated cells taken as 1. Results are expressed as the mean \pm SEM of three independent experiments. Statistical analysis was performed by Two-way ANOVA test, * $p < 0.05$ compared to miR-control cells treated with insulin.

DISCUSSION:

Here we have presented 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, si-RNA, micro-RNA mimic, and anti-micro-RNA into 3T3-L1 adipocytes, 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 the transfection with plasmid DNA, which limit the utility of this technic for gain-of-function studies. Although, murine cell lines, such as the 3T3-L1 cell line, are the predominant *in vitro* models used to study adipocytes, it should be noted, that micro-RNA expression patterns and actions in murine tissue cannot always be extrapolated to humans, and differences in micro-RNA expression have been observed between murine and human, and between primary cells and cell lines. Moreover, the media 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, for example they present multilocular lipid droplets instead of an unilocular lipid droplet. This could explain some differences in genes expression and cellular responses between *in vitro* and *in vivo* studies.

One benefit of using the reverse transfection protocol compared to electroporation is that this method is cheaper. Indeed, the reagents are less expensive, the high efficiency of the reverse transfection decreases the quantity of oligonucleotides needed, and there is no need of expensive equipment such as an electroporator. Moreover, using a lower concentration of si-RNA is beneficial since it avoids off-target effects. Furthermore, the reverse transfection is an easy, fast, gentle and straightforward method of transfection that requires lower number of cells and will ensure an excellent cell viability and more robust data.

The procedure detailed above has been optimized for the differentiation and reverse transfection of mouse 3T3-L1 cells, however, human preadipocytes, can also be differentiated into adipocytes⁵ and easily subjected to the reverse transfection using this protocol.

Here we have demonstrated that adipocytes remain viable, healthy and responsive to insulin after the reverse transfection using our transfection reagent. The reverse transfection could also be performed using other popular transfection reagents. However, the amounts of oligonucleotides and transfection reagents would need to be optimized to ensure a good modulation of expression and no side effect on cell viability.

We demonstrated here that this protocol allows for the study of the role of proteins and micro-RNAs in adipocyte functions, but this protocol could be used to manipulate 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. Attention should be given to the differentiation of the 3T3-L1 fibroblasts into adipocytes. 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 correspond to 3-4 days after removing the differentiation cocktail, it will allow to reach a robust transfection efficacy and to favor a good reattachment of the adipocytes to the plate. Finally, one should also carefully monitor the treatment of adipocytes with trypsin in order to detach the adipocytes without damaging the cells.

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DISCLOSURES:

The authors have nothing to disclose.

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