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Studying Lipolysis in Adipocytes by Combining siRNA Knockdown and Adenovirus-Mediated Overexpression Approaches

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Abstract

3T3-L1 adipocytes are widely used as a model system for studying hormone-stimulated lipolysis. However, these cells were limited in their utility for gain- and loss-of-function studies due to the low efficiency of their transfection with plasmid DNA or small interfering RNA (siRNA) oligos. In this chapter, we provide a review of two methods established for manipulation of protein expression in differentiated mature adipocytes. The use of electroporation allows a high-efficiency delivery of siRNA oligos and subsequent knockdown of specific gene expression. A centrifugation-assisted infection with recombinant adenovirus, on the other hand, enables robust overexpression of ectopic proteins. Most importantly, by combining siRNA electroporation with adenovirus infection, simultaneous manipulation of levels of two different proteins can be achieved in differentiated adipocytes. Through subsequent analyses of lipase activity in cell extracts and fatty acid or glycerol release from living cells, mutual interdependence between the two proteins in the context of basal and hormone-stimulated adipocyte lipolysis can be evaluated.

INTRODUCTION AND RATIONALE

Adipose tissue or fat is loose connective tissue consisting mainly of adipocytes (Nakajima, Yamaguchi, Ozutsumi, & Aso, 1998; Sethi & Vidal-Puig, 2007). There are two types of adipose tissues: white adipose tissue (WAT) and brown adipose tissue (BAT) (Saely, Geiger, & Drexel, 2012). While BAT consumes energy (Cannon & Nedergaard, 2004; Frontini & Cinti, 2010), WAT is the main energy store in the body of mammals. Upon food intake, excess dietary fatty acids (FAs) are esterified to triacylglycerols (TGs) and stored in the lipid droplets of adipocytes (Oller do Nascimento, Ribeiro, & Oyama, 2009; Saely et al., 2012). In response to cold exposure, TG stores in brown adipocytes are mobilized via a hydrolytic process called lipolysis to supply FAs for β -oxidation and subsequent heat production (Cannon & Nedergaard, 2004; Frontini & Cinti, 2010). During chronic fasting or extended physical exercise, lipolysis in white adipocytes is stimulated to release free FAs (FFAs) into circulation for the purpose of providing energy to other peripheral tissues (Ahmadian, Wang, & Sul, 2010; Greenberg et al., 2011; Watt & Spriet, 2010). Although nonadipocyte cells are able to esterify FFAs into TGs and rehydrolyze the latter upon demand, they do not secrete FFAs but instead utilize them locally for ATP production and/or lipid synthesis. Thus, the provision of FFAs as systemic energy substrate is a unique

function of lipolysis in white adipocytes (Lass, Zimmermann, Oberer, & Zechner, 2011). In obesity, alterations in lipolysis contribute to elevated circulating FAs and the development of peripheral lipotoxicity and insulin resistance (Arner et al., 2008; Deng et al., 2012; Langin et al., 2005; Moro et al., 2009). Accordingly, the precise mechanisms for lipolytic regulation have attracted immense research interest over the years.

Lipolysis in adipocytes is mediated by a complex proteome comprised of specific acylglycerol lipases and their regulators (Kolditz & Langin, 2010). The adipose lipases include adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoacylglycerol lipase (Lass et al., 2011). ATGL favors TG substrates and catalyzes the rate-limiting first step of lipolysis. HSL, on the other hand, is believed to function as a diacylglycerol lipase *in vivo* (Haemmerle et al., 2002; Osuga et al., 2000; Schweiger et al., 2006; Zimmermann et al., 2004). Research conducted in the past decade has revealed that phosphorylation of lipid droplet coat protein perilipin 1 by cAMP-dependent protein kinase (PKA) plays a critical role in controlling the lipid droplet localization of HSL in adipocytes (Brasaemle, Levin, Adler-Wailes, & Londos, 2000; Egan et al., 1992; Sztalryd et al., 2003). Others and we have demonstrated that ATGL also undergoes perilipin 1-dependent lipid droplet translocation upon β -adrenergic stimulation (Bezaire et al., 2009; Wang et al., 2011; Yang et al., 2010). While HSL is mainly regulated by PKA phosphorylation (Brasaemle et al., 2000; Egan et al., 1992; Sztalryd et al., 2003; Wang et al., 2009), ATGL's activity is coactivated by the protein comparative gene identification-58 (CGI-58) and inhibited by the protein G0/G1 switch gene 2 (Lass et al., 2006; Yang et al., 2010). Recent reports have indicated a mechanism for ATGL activation in adipocytes that involves the dissociation of CGI-58 from phosphorylated perilipin 1 (Granneman et al., 2007; Granneman, Moore, Krishnamoorthy, & Rathod, 2009; Miyoshi et al., 2007; Subramanian et al., 2004; Yamaguchi, Omatsu, Matsushita, & Osumi, 2004). Experiments reconstituting interactions using ectopically expressed proteins have demonstrated that in unstimulated cells, CGI-58 is in complex with perilipin 1 at the surface of LDs and therefore is separated from ATGL. Upon stimulation, perilipin is phosphorylated on Ser492 or Ser517 by PKA, thereby releasing CGI-58 to act on ATGL (Granneman, Moore, Krishnamoorthy, & Rathod, 2009).

Although the basic mechanisms for lipolysis have been elucidated, the regulative signaling pathways are much more complicated. Lipolysis is now known to be regulated by various extracellular signals, namely adenosine, atrial natriuretic peptide, β -hydroxybutyrate, endothelin-1, insulin, glucocorticoids, growth hormone, lactate, leptin, melanocortins, neuropeptide Y, peptide YY, prostaglandin E2, thyroid-stimulating hormone, and tumor necrosis factor α (Carmen & Victor, 2006; Chaves, Frasson, & Kawashita, 2011; Duncan, Ahmadian, Jaworski, Sarkadi-Nagy, & Sul, 2007). When and how these signals exert their regulation remains largely unclear. Moreover, comparative mass spectrometry analysis has revealed proteins differentially associated with lipid droplets in adipocytes under basal and lipolytically stimulated conditions. For example, 17-hydroxysteroid dehydrogenase (type 7) was identified only in basal condition, whereas ACSL3/4 and two short-chain reductase/dehydrogenases were found on lipid droplets upon stimulation (Brasaemle, Dolios, Shapiro, & Wang, 2004). The function of these proteins in lipolysis is still unknown. Furthermore, it is likely that the development of new technologies such as mass spectrometry of higher

sensitivity and small interfering RNA (siRNA) library screening will lead to future discovery of more and more proteins involved in the lipolytic control in adipocytes. Consequently, a reliable system model for the functional characterization of proteins regulating lipolysis in mature adipocytes is highly desirable if not imperative.

During the past decade, loss- and gain-of-function studies using genetically engineered animals have greatly advanced our knowledge on lipolysis. Most notably, the lack of obese phenotypes in HSL-null mice prompted an intense search for a separate TG lipase (Haemmerle et al., 2002; Okazaki et al., 2002; Wang et al., 2001; Zimmermann et al., 2003) and led to the eventual discovery of ATGL (Jenkins et al., 2004; Villena, Roy, Sarkadi-Nagy, Kim, & Sul, 2004; Zimmermann et al., 2004). Knockout and transgenic mouse models of ATGL have provided valuable information that helps to establish ATGL as a key TG lipase in both adipose and nonadipose tissues (Ahmadian et al., 2009; Haemmerle et al., 2006, 2011; Ong, Mashek, Bu, Greenberg, & Mashek, 2011; Reid et al., 2008; Wu et al., 2011). Despite the obvious advantages, however, there are several limitations to the use of mouse models for studying adipose lipolysis. Firstly, it routinely takes 1–2 years to construct such an animal model, especially knockout mice. The process can be longer in the case of creation of an adipose tissue specific knockout mouse. Secondly, hormone-stimulated lipolysis only occurs in mature adipocytes, which originate from preadipocytes via adipogenic differentiation. It is possible that the effect of loss or gain of a particular gene of interest on lipolysis as revealed in a mouse model may be secondary to that on adipocyte differentiation other than lipolysis *per se*. Thirdly, the phenotypic changes observed in transgenic or knockout mice often reflect a chronic and systemic influence of a protein on lipolysis. Impact on lipolysis could be a result of complementation during the course of development and thus indirect. Lastly, lipolysis is mediated via a coordinated action of multiple enzymes and regulators. Given that they are usually separate models for one individual gene or protein, knockout and transgenic mice are not suitable for studies of interplay between two or more proteins involved in the lipolytic control. Though technically possible, it is costly and time-consuming to manipulate expression of more than one gene in mice. Accordingly, a new cell-based model is needed to provide a setting for studying the function of multiple proteins in adipocyte lipolysis.

HeLa cells loaded with exogenous sources of FAs (oleate or palmitate) can form lipid droplets mimicking those in adipocytes (Smirnova et al., 2006; Soni et al., 2009; Yang et al., 2010). Due to their readiness for transfection with exogenous DNA or siRNA, these cells have become a widely employed model for studying the roles of proteins involved in lipid droplet turnover. However, despite valuable information derived from these studies, the protein makeup of lipid droplets in HeLa cells does not allow hormone-responsive lipolysis like the one observed in adipocytes. For example, ATGL, HSL, perilipin, and other proteins essential for adipocyte lipolysis are expressed at low levels endogenously in HeLa cells (Miyoshi, Perfield, Obin, & Greenberg, 2008; Yang, Heckmann, Zhang, Smas, & Liu, 2013). In addition, in HeLa cells ATGL is constitutively located at the surface of lipid droplets (Smirnova et al., 2006; Soni et al., 2009), whereas in adipocytes it resides in cytosol in the basal state and translocates to lipid droplets in response to β -adrenergic stimulation (Bezaire et al., 2009; Wang et al., 2011; Yang et al., 2010).

3T3-L1 cell line, a highly adipogenic clone derived from Swiss mouse embryo tissue (Green & Meuth, 1974; Vogel & Pollack, 1973), is one of the most commonly used model for the studies of WAT. 3T3-L1 preadipocytes can differentiate into mature adipocytes when stimulated with an appropriate hormonal regimen (Madsen et al., 2003; Sadowski, Wheeler, & Young, 1992). During the process of adipocyte conversion, these preadipocytes lose their primitive mesenchymal character, accumulate TG-containing lipid droplets, and acquire adipocyte-like phenotypes (Ducharme & Bickel, 2008). In particular, differentiated 3T3-L1 cells are known to possess the complete LDs and protein machinery that support hormone-stimulated lipolysis (Liu, Zhou, Abumrad, & Su, 2010; Snyder, Esselstyn, Loughney, Wolda, & Florio, 2005; Yang et al., 2010). To make them a feasible model for functional studies on lipolytic regulators, however, it is essential to develop effective methods for manipulation of gene expression in these cells. In this regard, the well-recognized difficulty in introducing DNA or siRNA-targeting genes of interest into mature 3T3-L1 adipocytes has presented a major technical challenge (Ross, Song, Burney, Kasai, & Orlicky, 2003). For ectopic gene expression, some investigators used electroporation method to deliver plasmid DNA in differentiated cells (Jiang, Chawla, Bose, Way, & Czech, 2002; Martin, Lee, & McGraw, 2006; Okada, Mori, & Pessin, 2003; Watson et al., 2003). While it produces a sufficient number of positive cells for imaging analysis, the technique has limited usage due to the low transfection efficiency and thus is often unreliable for assays that are dependent on examining a majority of the cell population. Other investigators also attempted adenoviral gene transfer with varying degree of success. To enhance the infection efficiency, Ross et al. have generated stable clones of 3T3-L1 preadipocytes that exogenously express the coxsackie-adenovirus receptor (Ross et al., 2003). However, how efficiently cells from those stable clones can be differentiated into mature adipocytes with full lipolytic capacity remains unclear.

This chapter describes methods to effectively overexpress or knockdown specific gene of interest in differentiated 3T3-L1 adipocytes. Knockdown is accomplished by electroporation of cells in suspension with siRNA oligos. A centrifugation method is employed to enhance the overexpression mediated by recombinant adenovirus. Additionally, we describe the usage of these two methods in combination to achieve manipulation of two different proteins simultaneously for the purpose of studying the relevance of their interplay in the regulation of lipolysis.

6.1 MATERIALS AND REAGENTS

6.1.1 Equipment and materials

- Gene Pulser Xcell™ (Bio-Rad)
- Electroporation cuvette (4-mm gap, Molecular Bioproducts, #5540)
- Misonix sonicator (Qsonica, LLC, #XL-2000)
- Scintillation counter (Beckman, #LS6500)
- 15- and 50-mL conical tubes (BD Falcon, #352097 and #352098)

- Six-well and 15-cm tissue culture plate (vacuum gas plasma treated, BD Falcon, #3506 and #353025)
- 12 × 75 mm glass test tube (VWR, #47729-570)
- 5-mL polypropylene round-bottom tubes (BD Falcon, #352063)

6.1.2 Reagents

- Dulbecco's phosphate-buffered saline/modified (DPBS, –calcium–magnesium, Thermo Scientific, # SH30028)
- Dulbecco's modified Eagle's medium (DMEM with 4.5 g/L of glucose, Cellgro®, #10-013-v)
- DMEM without phenol red (4.5 g/L of glucose, Invitrogen, #31053-028)
- Fetal bovine serum (FBS, Invitrogen, #10437)
- Newborn calf serum (NCS, Invitrogen, #16010)
- Collagen type I (250 ×, 4.05 mg/mL, BD Biosciences, #354249)
- Trypsin–EDTA (0.05% and 0.25% solutions, Invitrogen, #25300 and #25200)
- Isobutylmethylxanthine (IBMX; Sigma I-7018)
- Dexamethasone (Sigma, D-4902)
- Insulin (bovine; Sigma, I-5500)
- Penicillin/streptomycin (100 × P/S, Thermo Scientific, # J120051)
- L-Glutamine (100 × G, Invitrogen, #25030)
- Polybrene (Sigma, #107689)
- Triolein (Sigma, #T7140)
- [³H]-triolein (PerkinElmer, #NET431001MC)
- Phosphatidylcholine (Sigma, #P3556)
- Phosphatidylinositol (Sigma, #P5954)
- Protein inhibitor mini tablet (Roche, # 11836170001)
- Sucrose (Fisher, #S5-500)
- DTT (Sigma, #43815)
- FFA detection kit (Zenbio, #LIP-2-NC-L1)
- Glycerol detection kit (Zenbio, #LIP-1-NC-L1)
- Bovine serum albumin, essentially FA free (Sigma, A6003)
- Custom and GC-matched control Stealth siRNA oligos (Invitrogen)
- Recombinant adenovirus (custom generated)

6.1.3 Media and stock solutions

- DMEM/10% NCS
 - 50 mL NCS
 - 5 mL 100× P/S
 - 5 mL 100× G
 - 440 mL DMEM
- DMEM/10% FBS
 - 50 mL FBS
 - 5 mL 100× P/S
 - 5 mL 100× G
 - 440 mL DMEM
- IBMX stock solution
 - 0.25 mM IBMX in 0.5 N KOH solution
 - Sterilize through a sterile 0.22-μm filter and store at –20°C
- Insulin stock solution
 - 167 μM (1 mg/mL) in 0.02 M HCl
 - Sterilize through a sterile 0.22-μm filter
 - Store at –20°C for long term, 4°C for 1 month.
- Dexamethasone stock solution
 - 10 mM in 100% ethanol (store at –20°C)
- Differentiation Media I
 - DMEM/10% FBS
 - 1:500 IBMX stock solution
 - 1:1000 insulin stock solution
 - 1:10,000 dexamethasone stock solution
- Differentiation Media II
 - 10% FBS/DMEM
 - 1:1000 insulin stock solution
- DMEM/2% BSA
 - 2% FA-free BSA in DMEM without phenol red
- DMEM/0.5% BSA
 - 0.5% FA-free BSA in DMEM without phenol red

- Polybrene stock solution
 - 10 mg/mL in water
 - Sterilize through a sterile 0.22- μ m filter
- Stealth siRNA solution
 - 100 μ M in sterile DEPC-treated water (aliquots stored at -20°C)

6.2 METHODS

6.2.1 Adipocyte culture and differentiation

3T3-L1 preadipocyte cells have a fibroblast-like morphology and differentiate into an adipocyte-like phenotype under appropriate conditions. Differentiated cells are sensitive to lipogenic and lipolytic hormones and drugs, including epinephrine, isoproterenol, and insulin. For culturing and differentiating 3T3-L1 preadipocytes, we have adapted a protocol originally developed by Rubin, Lai, and Rosen (1977).

1. Preadipocyte maintenance and passage

Mouse 3T3-L1 preadipocytes are maintained in DMEM/10% NCS in a humidified atmosphere containing 10% CO_2 at 37°C . They are generally split at a ratio from 1:5 to 1:10 depending on need, using 0.05% trypsin-EDTA. During passage preadipocytes should be plated evenly and maintained at low density (i.e., less than 70% confluence) at all times unless induction of differentiation is planned. Spontaneous and partial differentiation would be triggered in preadipocytes when high confluence is reached. If grown past 70% confluence and subsequently passaged, preadipocytes that have experienced partial differentiation will lose their ability to fully differentiate following hormonal stimulation. Moreover, most cell lines are maintained in 5% CO_2 atmosphere. However, 10% CO_2 appears to enhance the differentiation efficiency of preadipocytes in comparison to 5% CO_2 .

2. Induction of adipogenic differentiation

1. Prior to differentiation, 3T3-L1 preadipocytes are routinely maintained in 15-cm plates with 25–30 mL of DMEM/10% NCS.
2. Upon reaching confluence, cells are replaced in fresh DMEM/10% NCS and incubation is allowed to continue for 2 more days. This step further promotes preadipocyte proliferation so that high cell density is achieved to induce growth arrest and as a result improves the differentiation efficiency. At the end of day 2, preadipocytes should appear tightly packed with individual cells assuming narrow spindle-like morphology. Flat and spread out cell shape often predicts a low efficiency differentiation after hormonal induction.
3. To initiate differentiation, postconfluent preadipocytes are replaced in 25 mL of Differentiation Media I (Day 0) freshly made from stock solutions of different components. After thawing, insulin solution may be kept at 4°C for up to 1 month. IBMX solution should be stored at -20°C continually, which

can be thawed and frozen up to three times. Since dexamethasone is dissolved in ethanol, it will remain in liquid form even at -20°C and is stable up to 1 year.

4. Three days later (Day 3), the media become more viscous possibly due to increased accumulation of FFAs and chondroitin sulfate proteoglycan-I (versican) (Okada et al., 2008) secreted by the cells. The media is then carefully removed and changed to 25 mL of Differentiation Media II.
5. Two days later (Day 5), media is removed and cells are replaced in 25 mL of normal DMEM/10% FBS.
6. Two to three days later (Day 7–Day 8), cells should be fully differentiated and ready for subsequent functional experiments. For siRNA knockdown or adenoviral overexpression experiments, at least 90% of the cells present should be mature adipocytes with full and well-developed lipid droplets. When plating cells into six-well plates after knockdown or before virus infection, undifferentiated fibroblasts will quickly attach to the six-well plate and begin proliferating prior to the attachment of mature adipocytes, resulting in a large portion of the cells on the plates being fibroblasts rather than adipocytes. In addition, usage of adipocytes that have exceeded Day 8 of differentiation is not recommended, as they are overly mature, do not easily attach to the plates, and are prone to clumping.

6.2.2 Manipulation of gene expression

For knockdown of expression of particular genes, we routinely deliver specific siRNA oligos into adipocytes by using an electroporation method adapted from a plasmid transfection protocol (Okada et al., 2003). For ectopic gene expression, we have established an adenovirus-mediated method that is enhanced by polybrene and centrifugation.

1. siRNA knockdown via electroporation

1. DPBS and DMEM/10% FBS media are prewarmed to 37°C in water bath prior to conducting the experiment.
2. Differentiated adipocytes (Day 7 or 8) in 15-cm plates are washed twice with DPBS. The protocol below is optimized for one 15-cm plate of adipocytes. Corresponding volumes of reagents or materials need to be used for multiple plates of cells.
3. 5 mL of ice-cold 0.25% trypsin–EDTA solution is added followed by gentle swirling to ensure even coating of the cells. We recommend using a high concentration of trypsin (i.e., 0.25%) to detach adipocytes so that cell clumping is minimized and a complete dispersion can be achieved.
4. The trypsin–EDTA solution is removed by aspiration and the cells are placed in the culture incubator for 5–10 min. To gauge the degree of detachment, the cells are examined under a phase-contrast light microscope

every 2 min. Once detachment of the cells is complete, 10 mL of DMEM/10% FBS is added to wash off the cells.

Note: Tilt the plate and add the media to the top so that the cells flow to the bottom of the plate. Make sure to NOT pipet cells, as this is a potential avenue for cell damage.

5. Cells along with the media are transferred to a 50-mL conical tube and more DMEM/10% FBS is added to adjust the total volume to approximately 30 mL.
6. Cells are centrifuged for 5 min at 200×g at room temperature. To ensure the formation of a firm pellet, the deceleration speed of the centrifuge should be set to its minimum value (i.e., slowest speed).
7. The supernatant is carefully removed by aspiration without disturbing the cell pellet. After the addition of 30 mL of DPBS, the cell pellet is resuspended by gentle inversion of the tube for five to six times.
8. The tube is placed in a tube rack for 1–3 min at room temperature. The clumped cells would settle to the bottom of the tube, which then can be removed by gentle aspiration with a 5-mL pipette. Ensure to remove as many of the cell clumps as possible, as they would create a nonuniform mixture altering the dynamics of the cell suspension during electroporation.
9. The cell suspension is centrifuged for another 5 min at 200×g at room temperature.
10. Repeat steps (7)–(9).
11. The final cell pellet is resuspended in 500 µL of DPBS after supernatant is removed. Use a P-1000 pipettor to mix the cells as gently and thoroughly as possible. It is important to have a uniform suspension, ensuring even current distribution during electroporation.
12. In a separate tube, add 25 µL of 100 µM control or gene-specific siRNA solution to the bottom.

Note: Make sure to use RNase-free tubes to prevent possible degradation of siRNA. We typically use a 5-mL polypropylene round-bottom tube from BD Falcon (#352063).

13. Transfer the cell suspension from (11) into the tube containing siRNA and mix by gentle pipetting three times using the P-1000 pipettor. The final concentration of siRNA in the electroporation mixture is 5 µM. *Note:* siRNA concentration necessary for efficient knockdown is gene dependent. We suggest varying siRNA doses from 2 to 10 µM during the initial tries. Additionally, a better knockdown effect may be achieved by mixing siRNA oligos targeting different sequences in a gene of interest.

14. The mixture of cells/siRNA is transferred into an electroporation cuvette. Immediately place the cuvette into the Bio-Rad Gene Pulser electroporator and deliver a single pulse at 950 μ F and 160 V.
 15. After pulse delivery, 1 mL of DMEM/10% FBS is added to the cells in the cuvette. Immediate addition of the media is essential for readjusting the pH following current delivery and ensuring continued cell viability.
 16. Transfer the electroporated cell suspension to a 15-mL conical tube containing 11 mL of DMEM/10%, gently mix three times and incubate for 15 min at room temperature. This incubation period aids in both cell recovery and separation of cellular debris (i.e., dead cells) that will float to the surface of the suspension.
 17. Carefully remove the floating cell debris with a P-1000 pipettor.
 18. Split the cell suspension into four wells of a six-well plate by adding 3 mL/well. Distribute cells across the plate evenly by shaking gently and then place in a tissue culture incubator.
 19. The following day, carefully change the media and incubate for another 2 days. *Note:* If there appears to be a loss or lack of cell attachment, plates may be precoated with collagen solution to promote adherence as described in (2) of “Adenovirus-mediated overexpression assisted by centrifugation” in Section 2.2.
 20. Three days following electroporation, cells are ready for lipolysis assays. While 3 days is typically good for maximal knockdown, genes do vary slightly and it may be necessary to adjust this time frame according to the particular gene of interest.
2. Adenovirus-mediated overexpression assisted by centrifugation
 1. DPBS and DMEM/10% FBS are prewarmed to 37 °C in water bath before the experiment.
 2. Six-well plates are precoated with collagen solution. Make a 1:250 dilution of collagen type I solution in DPBS and add 2.5 mL/well diluted collagen solution to the plates. To allow establishment of a collagen base, plates are placed in a tissue culture incubator for 1 h and then washed with DPBS prior to the seeding of cells. It is an important step for the subsequent centrifugation and virus infection protocols, since the presence of collagen helps cells to remain adherent under stressful circumstances.
 3. Differentiated adipocytes (Day 7 or 8) in 15-cm plates are washed twice with DPBS.
Note: Again, differentiation efficiency needs to be at least 90% for adipocytes to be useful.
 4. 5 mL of 0.25% trypsin–EDTA solution is added to each plate followed by gentle swirling to evenly coat cells.

5. The trypsin–EDTA solution is removed by aspiration and the cells are placed in the tissue culture incubator for 3–5 min.
6. 10–15 mL of DMEM/10% FBS is added into each plate. Cells are dispersed by gentle pipetting and then transferred to a 50-mL conical tube. The volume is adjusted to ~40 mL. After further mixing by inversion, the cells are then split (3 mL/well) into the six-well plates precoated with collagen. Gentle shaking is applied to distribute the cells evenly.

Note: One 15-cm plate of adipocytes can be divided into 12 wells of 2 six-well plates.

7. After 2 days of standard incubation at 37 °C, cells are washed twice with room temperature DPBS.
8. 2.0 mL serum-free DMEM containing 5 µg/mL of polybrene and an appropriate amount of adenovirus (2×10^8 pfu) is added into each well.

Note: Please ensure that no serum is present since it may reduce the infection efficiency of the virus. Moreover, we recommend varying the titer of virus between 5×10^7 and 2×10^8 pfu/well during the initial tries, as this parameter can differ substantially between different genes.

9. The plates are sealed with parafilm, placed securely in microplate adaptors, and centrifuged for 1 h at $800 \times g$ at room temperature. *Note:* Make sure to tightly seal each plate with parafilm to prevent the entrance of bacteria and other possible pathogens as well as the overspill of virus. Be sure to set the deceleration speed of centrifuge to its minimum value. In order to ensure the same centrifugal force of each well in the plate, following 30 min of centrifugation the plate can be inverted for another 30 min of centrifugation.
10. After centrifugation, parafilm is removed and the plates are placed in the tissue culture incubator.
11. After 4 h, 2.0 mL of DMEM/10% FBS is added to each well in a step to aid cell recovery. *Note:* Do not remove the virus-containing media at this point.
12. After an overnight incubation, the virus-containing media is removed and replaced with fresh DMEM/10% FBS. This media change is essential to ensure the health of the cells as adenovirus and polybrene both have an overall negative impact on cell viability during/following long-term incubation.
13. After 2 more days of incubation, the cells are ready for functional analyses. While the given time of 3 days is typical for efficient expression of adenovirus-encoded proteins, anywhere in the range of 2–4 days following infection could be ideal depending on the particular gene of interest.

3. Combinational siRNA knockdown and adenoviral overexpression

1. Adipocytes are transfected with siRNA by electroporation and plated in six-well plates as described in “siRNA knockdown via electroporation” of Section 2.2.
2. After 1 day following siRNA transfection, cells are washed twice with DPBS and adenoviral infection is performed as detailed in “Adenovirus-mediated overexpression assisted by centrifugation” of Section 2.2.
3. After 1 day of adenovirus treatment, the media is replaced by DMEM/10% FBS.
4. After a total 3 days following siRNA transfection or 2 days following adenovirus infection, cells are ready for functional studies (Fig. 6.1).

6.2.3 Functional assays for lipolysis

To assay lipolysis, we typically measure both TG hydrolase activity in cell extracts and FA or glycerol release from living cells. For TG hydrolase activity assay, we have adapted an assay protocol published by Holm and Osterlund (Okada et al., 2003). Specifically, the TG hydrolase activity is measured in adipocyte extracts by using emulsified triolein as substrate. Free oleic acid released during the hydrolytic reaction is captured onto BSA, separated into the aqueous phase, and quantified as the readout for the enzyme activity. Since lipolysis in adipocytes produces both FFAs and glycerol, the lipolytic rates can be easily measured by determining the per hour release of FFA and glycerol from the cells into the culture media.

1. TG hydrolase activity assay

Substrate preparation

1. Prepare 10 mL of 20% FA-free BSA in 0.1 M potassium phosphate buffer (pH 7.0).

Note: The inclusion of BSA in the reaction mixtures serves the purpose of capturing free oleic acid released from triolein hydrolysis in the water phase.

2. Prepare a 0.1 M boric acid solution in potassium bicarbonate and adjust pH to 10.5.

Note: Final concentration of potassium bicarbonate should also be 0.1 M.

3. For 4 mL of substrate solution, add the following to a 12 × 75 mm glass test tube: 5.9 mg of unlabeled triolein, 5 × 10⁷ cpm of [³H]-triolein, and 30 μL of phospholipid solution (20 mg/mL phosphatidylcholine: phosphatidylinositol, 3:1). Mix thoroughly by pipetting.
4. Evaporate the solvents by placing the 10-mL glass test tube under a constant nitrogen (N₂) stream.
5. Following complete evaporation, transfer the test tube to a desiccator and dry under vacuum for an additional 15 min. It is imperative to completely remove residual of any organic solvents.

6. After complete drying, add 2 mL of 0.1 M potassium phosphate buffer (pH 7.0) and sonicate for two cycles using a Misonix sonicator at an output setting of 1–2. Each cycle should be 1 min in duration at room temperature with a 1-min recovery interval on ice between cycles.
7. Add an additional 1 mL of 0.1 M potassium phosphate buffer (pH 7.0) and continue to sonicate for four cycles of 30 s each at room temperature, with 30-s recovery intervals on ice between cycles. Output setting remains at 1–2.
8. Following sonication, add 1 mL of 20% FA-free BSA in 0.1 M potassium phosphate buffer (pH 7.0) and mix thoroughly by pipetting.

Adipocyte extraction

9. Prepare cell extraction buffer consisting of 0.25 M sucrose, 1 mM EDTA, 1 mM DTT, and protease inhibitors (1 mini tablet/10 mL) as desired in molecular biology grade water.
10. Add 0.5 mL/well of extracts buffer to adipocytes in six-well plates and scrape cells into a 1.5-mL microcentrifuge tube.
11. Sonicate the cells using the Misonix sonicator for three cycles of 10 s each at an output setting of 5, ensuring a recovery interval of at least 30 s between cycles.
Note: Keep extracts on ice during and after sonication to maintain protein integrity.
12. Following sonication, centrifuge the adipocyte extracts at $5000 \times g$ for 10 min at 4 °C. After collecting the supernatants, determine and normalize protein concentration among different samples.

Enzyme assay

1. Begin the assay by combining 100 μ L of adipocyte extract with 100 μ L of substrate solution in a 15-mL conical tube. Thoroughly mix by pipetting. Incubate the reaction mixture at 37 °C for 1 h.
Note: As with any enzyme reaction, timing of the substrate addition and length of the incubation are critical in producing reliable results. Be sure to accurately time the reactions.
2. As the reaction is proceeding, prepare the MCH/STOP buffer by thoroughly mixing 200 mL of methanol, 180 mL of chloroform, and 140 mL of heptane (10:9:7).
3. At the end of the reaction period, add 3.25 mL of MCH/STOP buffer and 1.0 mL of boric acid solution (described in Section 1.3) to each sample. Mix thoroughly by vortexing for 15 s. Again please ensure that timing is correct.
4. Spin all tubes at $800 \times g$ for 15 min at room temperature.

5. Transfer 1.0 mL of the clear supernatant to a scintillation vial containing 10 mL of scintillation (counting) cocktail. Be careful to avoid the lower phase.
 6. Determine radioactivity using a scintillation counter.
2. Lipolysis assay
1. Adipocytes in six-well plates are washed twice with DPBS and then incubated in 2 mL/well of serum-free DMEM/0.5% BSA.
 2. After 3 h of serum starvation, the media is replaced with 2 mL/well of serum-free, phenol red-free DMEM/2% BSA either containing or not containing 10 μ M of isoproterenol (ISO). Serum starvation ensures quenching of cAMP levels and 2% BSA in DMEM is important for capturing any FFAs released from the cells into the media.
 3. Without disturbing the cells, remove 100 μ L of the media at 0.5, 1, 2, or 3 h time points following ISO treatment. Aliquots of the media can be stored at -20°C if FFA or glycerol content is not measured immediately.
 4. At the conclusion of the media collection, wash the cells with ice-cold DPBS and proceed to lysis/extraction of proteins for immunoblotting analysis.
 5. Determine the glycerol or FFA content released into the media using commercially available reagent kits as described in Section 1.2.

6.2.4 Case study—evaluating the roles of perilipin 1 and CGI-58 in ATGL-mediated lipolysis

The goal of this study was to examine the respective impact of perilipin 1 and CGI-58 on ATGL action in differentiated 3T3-L1 adipocytes (Yang et al., 2013). To this end, we first subtracted endogenous perilipin 1 or CGI-58 through siRNA knockdown and then added ectopic ATGL using a recombinant adenovirus encoding mouse ATGL (Ad-ATGL). Compared with endogenous protein levels in cells receiving a GC-matched control siRNA, less than 15% of perilipin 1 and less than 10% of CGI-58 were present in cells electroporated with siRNA-targeting respective proteins (Figs. 6.2A and 6.3A). Infection with Ad-ATGL, on the other hand, led to a profound increase in the expression of ATGL when compared with the levels of endogenous protein in null virus (Ad-null)-infected cells (Figs. 6.2A and 6.3A). In the control knockdown cells, overexpression of ATGL increased the TAG hydrolase activity in the total cell extracts by ~80% (Fig. 6.2B). Knockdown of perilipin 1 further increased the activity derived from overexpressed ATGL (Fig. 6.2B), indicating an inhibitory effect of endogenous perilipin 1 on ATGL activity. Moreover, overexpression of ATGL was able to significantly increase both basal and isoproterenol-stimulated glycerol release in control knockdown cells (Fig. 6.2C). In cells with reduced expression of perilipin 1, overexpression of ATGL drastically enhanced basal glycerol release without significantly affecting the stimulated lipolysis (Fig. 6.2C). In contrast to perilipin 1 knockdown, depletion of CGI-58 resulted in a pronounced decrease in the basal glycerol release from cells infected with both Ad-null and Ad-ATGL (Fig. 6.3B). Interestingly, the impact of CGI-58 reduction was modest on stimulated lipolysis (Fig. 6.3B). Taken together, these data suggest that under basal conditions, perilipin 1 suppresses

and CGI-58 activates the enzyme action of ATGL in adipocytes. In response to β -adrenergic stimulation, the further activation of ATGL-mediated lipolysis is dependent on the presence of perilipin 1 but less on that of CGI-58.

CONCLUDING REMARKS

Here we describe some techniques that allow studying the function of proteins, individually or in combination, in the lipolytic pathways of mature adipocytes. For knockdown of specific gene expression, siRNA oligos are effectively delivered into differentiated 3T3-L1 adipocytes in suspension via an electroporation-based method. The method was originally developed by Pessin laboratory as an easy and fast way for transfection of adipocytes with plasmid DNA (Okada et al., 2003). However, a large amount of purified plasmid (i.e., up to 600 μ g per electro-poration) is usually required to achieve an expression efficiency that is greater than 50%. As a result, the cell survival rate is extremely low after plasmid electroporation and severe damage is often observed in adipocytes that remain viable. In comparison, siRNA electroporation is much less damaging to the cells due to the fact that the duplex oligos are usually only 20–25 base pairs in length and thus a considerably smaller amount of siRNA is needed. Over the years we have had great success in knocking down various genes in 3T3-L1 adipocytes using Stealth siRNA custom designed and generated by Invitrogen (Heckmann, Yang, Zhang, & Liu, 2013; Langlais et al., 2012; Yang et al., 2010; Yang, Zhang, Heckmann, Lu, & Liu, 2011). The testing results provided by Invitrogen indicate that the Stealth modification increases both the siRNA stability and their specificity for targets when compared to conditional, unmodified siRNA.

For adenovirus-mediated overexpression, a centrifugation step is employed to achieve high-efficiency infection of adherent adipocytes. The spin method has been used widely for promoting the infection of cells in suspension culture by recombinant adenovirus and lentivirus. For differentiated 3T3-L1 adipocytes adhered to the plate surface, we have found that the spin infection method works surprisingly well in enhancing the infection of adenovirus complexed with the positively charged polybrene molecules. It is possible that the centrifugation promotes the binding of the virus–polybrene complex to the adipocyte surface and thereby increases the uptake of the virus into the cells. We have also found that the adherent adipocytes are highly enduring of any potential stress caused by such centrifugation, as they stay healthy with intact lipid droplets and responsive to hormones such as insulin and isoproterenol after the procedure. Therefore, of all methods developed for ectopic gene expression in adipocytes, centrifugation-mediated enhancement of adenoviral infection demonstrates considerable promise because it permits a high-efficiency transduction in mature 3T3-L1 adipocytes known to express receptors for adenovirus at low levels.

The fact that cells can easily reattach to culture plates after electroporation has made it possible to conduct siRNA knockdown and adenoviral overexpression in a sequential manner. This is especially useful when the interplay between two different proteins needs to be interrogated. As described in the case study in Section 2.4, we were able to evaluate the impact of CGI-58 or perilipin 1 on ATGL action through overexpression of ATGL with or without simultaneous knockdown of endogenous CGI-58 or perilipin 1. Since the studies are

conducted in fully differentiated adipocytes, these methods enable the investigators to exclude any confounding effects of potential developmental or adipogenic defects caused by loss- or gain-of-function manipulations. Furthermore, though they were specifically developed for studying hormone-stimulated lipolysis, the techniques should be equally useful for elucidating protein functions related to many other aspects of adipocyte biology.

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FIGURE 6.1.

Summary of the experimental procedures for (A) knockdown, (B) overexpression, or (C) knockdown plus overexpression of genes of interest in fully differentiated 3T3-L1 adipocytes.

FIGURE 6.2.

Effects of overexpression adipose triglyceride lipase (ATGL) on TAG hydrolase activity and lipolysis in adipocytes with reduced perilipin 1. 3T3-L1 adipocytes were electroporated with either control or perilipin 1 specific small interfering RNA (siRNA) oligos. Twenty-four hours after electroporation, cells were infected with either a null adenovirus (Ad-null) or a virus-containing ATGL cDNA (Ad-ATGL) followed by incubation for another 48 h. (A) Cells were lysed and then protein expression was analyzed by immunoblotting with antibodies specific for perilipin 1, ATGL, and β -actin. (B) The TAG hydrolase activity in the cell extracts was measured using ^3H -labeled triolein as substrate. The activity was normalized with the total protein levels. (C) Cells were pretreated for 0.5 h in serum-free Dulbecco's modified Eagle's medium (DMEM)/0.5% BSA and then were switched to fresh medium without or with isoproterenol for another 1-h incubation. The glycerol concentrations in the medium were measured, and the data are presented as the means $\pm$ S.E. of three independent experiments. *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$. For details please refer to Yang et al. (2013).

FIGURE 6.3.

Effect of overexpression ATGL on lipolysis in the absence of comparative gene identification-58 (CGI-58). 3T3-L1 adipocytes were electroporated with either control or CGI-58 specific siRNA oligos. Twenty-four hours after electroporation, cells were infected with either a null adenovirus (Ad-null) or a virus-containing ATGL cDNA (Ad-ATGL) followed by incubation for another 48 h. (A) Cells were lysed and then protein expression was analyzed by immunoblotting with antibodies specific for CGI-58 and ATGL. (B) Cells were pretreated for 0.5 h in serum-free DMEM/0.5% BSA and then were switched to fresh medium without or with isoproterenol for another 1-h incubation. The glycerol concentrations in the medium were measured, and the data are presented as the means±S.E. of three independent experiments. *, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$. For details please refer to Yang et al. (2013).