Lentiviral Short Hairpin Ribonucleic Acid-Mediated Knockdown of GLUT4 in 3T3-L1 Adipocytes

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Adipose tissue is an important insulin target organ, and 3T3-L1 cells are a model cell line for adipocytes. In this study, we have used lentivirus-mediated short hairpin RNA (shRNA) for functional gene knockdown in 3T3-L1 adipocytes to assess the molecular mechanisms of insulin signaling. We chose to target GLUT4 to validate this approach. We showed that lentiviruses efficiently delivered transgenes and small interfering RNA (siRNA) into fully differentiated 3T3-L1 adipocytes. We established a strategy for identifying efficient siRNA sequences for gene knockdown by transfecting 293 cells with the target gene fluorescent fusion protein plasmid along with a plasmid that expresses shRNA. Using these methods, we identified highly efficient siGLUT4 sequences. We demonstrated that lentivirus-mediated shRNA against GLUT4 reduced endogenous GLUT4 expression to almost undetectable levels in 3T3-L1 adipocytes. Interestingly, insulin-stimulated glucose uptake was only reduced by 50–60%, suggesting that another glucose transporter mediates part of this effect. When siGLUT1 was introduced into GLUT4-deficient adipocytes, insulin-stimulated glucose uptake was essentially abolished, indicating that both GLUT4 and GLUT1 contribute to insulin-stimulated glucose transport in 3T3-L1 adipocytes. We also found that GLUT4 knockdown led to impaired insulin-responsive aminopeptidase protein expression that was dependent on whether GLUT4 was knocked down in the differentiating or differentiated stage. We further found that GLUT4 expression was not required for adipogenic differentiation but was necessary for full lipogenic capacity of differentiated adipocytes. These studies indicate that lentiviral shRNA constructs provide an excellent approach to deliver functional siRNAs into 3T3-L1 adipocytes for studying insulin signaling and adipocyte biology. (Endocrinology 147: 2245–2252, 2006)

Effects in insulin signaling lead to insulin resistance, a common characteristic of type 2 diabetes, Syndrome X, as well as other human diseases (1–3). Adipose tissue is an important insulin target organ. 3T3-L1 adipocytes are a frequently used cell line for studies of insulin signaling and insulin resistance (4–6). These cells display the full repertoire of the insulin signaling cascade that leads to insulin-stimulated glucose transport. First, our studies demonstrated that lentivirus can efficiently deliver transgenes and siRNAs into fully differentiated 3T3-L1 adipocytes. Second, we established a strategy for identifying efficient siRNA sequences by transfecting 293 cells with the target gene fluorescent fusion protein plasmid along with a plasmid that expresses shRNA. Using these methods, we identified highly efficient siGLUT4 sequences. We demonstrated that lentivirus-mediated shRNA against GLUT4 reduced endogenous GLUT4 expression to almost undetectable levels in 3T3-L1 adipocytes. Interestingly, insulin-stimulated glucose uptake was only reduced by 50–60%, suggesting that another glucose transporter mediates part of this effect. When siGLUT1 was introduced into GLUT4-deficient adipocytes, insulin-stimulated glucose uptake was essentially abolished, indicating that both GLUT4 and GLUT1 contribute to insulin-stimulated glucose transport in 3T3-L1 adipocytes. We also found that GLUT4 knockdown led to impaired insulin-responsive aminopeptidase protein expression that was dependent on whether GLUT4 was knocked down in the differentiating or differentiated stage. We further found that GLUT4 expression was not required for adipogenic differentiation but was necessary for full lipogenic capacity of differentiated adipocytes. These studies indicate that lentiviral shRNA constructs provide an excellent approach to deliver functional siRNAs into 3T3-L1 adipocytes for studying insulin signaling and adipocyte biology. (Endocrinology 147: 2245–2252, 2006)
lated glucose uptake was essentially abolished, demonstrating that both GLUT4 and GLUT1 are important in transporting glucose in 3T3-L1 cells. These studies indicate that lentiviral shRNA constructs provide an excellent approach for delivering siRNA into 3T3-L1 adipocytes. Moreover, because the constructs are stably integrated into the host genome, they should be useful for a variety of studies of insulin signaling and adipocyte biology.

Materials and Methods

Materials

Mouse anti-phosphotyrosine (PY20) was from BD Biosciences (Lexington, KY). Rabbit anti-phospho-AKT (Ser473) antibody was from Cell Signaling Technology (Beverly, MA). Rabbit anti-GFP (green fluorescent protein) antibody, mouse anti-PPARα (peroxisome proliferator-activated receptor-γ) monoclonal antibody, rabbit anti-β2 (anti-IRS1, and anti-annexin II were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-RFP (red fluorescent protein) antibody was from BD Biosciences (Palo Alto, CA). Rabbit anti-IRS2 was from Upstate (Waltham, MA). Rabbit anti-phospho-AKT (Ser473) antibody was from Cell Signaling Technology (Beverly, MA). Rabbit anti-GLUT4 polyclonal antibody was from Sigma (St. Louis, MO). Mouse anti-GFP (green fluorescent protein) antibody, mouse anti-IRS2 was from Upstate (Waltham, MA). Rabbit antiactin was from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-IRAP (insulin-responsive aminopeptidase) antibody was from Dr. Steve Waters (Metabolix Inc., Hayward, CA). DMEM and fetal bovine serum were from Invitrogen (Carlsbad, CA). All reagents were purchased from Sigma (St. Louis, MO).

Cell culture

Culture and differentiation of 3T3-L1 cells were described previously (8, 9). In brief, the cells were grown and maintained in high-glucose DMEM containing 10% fetal bovine serum and 10% CO2 environment. The cells were allowed to grow for 2 d after confluence and then differentiated by the addition of isobutylmethylxanthine (50 μM), a stop signal for RNA polymerase III, and a 19-nucleotide antisense sequence, five thymidines (a derlined pBluescript SK plasmid as described (11). Each hairpin construct was stably integrated into the host genome with siGLUT1 (SmartPool; Dharmacon, Lafayette, CO; 2.5 nmol per 1 × 10^6 cells) as described previously (11) using two primers that contained XhoI site: forward primer (CGAGGTCAAGCG GTTCAAGATAAGGCACTGAGCGATGAGTCGTCTTAATTCTCGTTAAGAACTTGGAAA), no. 2 reverse (ACTGGTGCTCTTTGAAACAATTTCCGGTTCACACTGATCCGGG); no. 4 forward (GATCCCCCAATGTCTGGCGGGTGTTGCTCAAGAGAACACCGGCAAGAATCACTGTTTGGAAA), no. 4 reverse (AGCTTTTCAAAATTGATCAACGCAGGTTTACAGTGTAA). Oligo pairs for GFP shRNA were described previously (11).

To subclone the shRNA cassettes into the lentiviral vector, the hairpin along with the H1-RNA promoter was PCR-amplified as described previously (11) using two primers that contained XhoI site: forward primer (CGAGGTCAAGCG GTTCAAGAT GAGTCGTCTTAATTCTCGTTAAGAACTTGGAAA), no. 2 reverse (AAATTACCTCACTAAGGGG); the PCR products were then digested by XhoI and inserted into the XhoI site in the 3′-terminal repeat of the lentiviral expression plasmid that contains a cytomegalovirus promoter and GFP (11). Correct insertions of shRNA cassettes were confirmed by restriction mapping and DNA sequencing.

Lentivirus production and infection of 3T3-L1 adipocytes

Recombinant lentiviruses were produced by cotransfecting 293T cells with the lentiviruses expressing plasmid and packaging plasmids using the calcium phosphate method (12–14). Infectious lentiviruses were harvested at 48 and 72 h after transfection and filtered through 0.22-μm cell culture filters (0.22). Recombinant lentiviruses were concentrated by ultracentrifugation (2 h at 50,000 × g) and subsequently purified on a 20% sucrose cushion (2 h at 46,000 × g). The infectious titer was determined by FACS analysis of GFP positive in 293 cells.

The infection of 3T3-L1 adipocytes (8–10 d after differentiation) with siGLUT4 lentivirus was carried out by adding lentivirus into the cell culture at the MOI of approximately 100. The controls were infected with siLUC (siRNA against lucerase). Four hours after the incubation, the medium was changed.

Identification of efficient siRNA sequences

Optimal siRNA targets for GLUT4 knockdown were identified by cotransfecting 293T cells with GLUT4 reporter plasmid and siGLUT4 plasmid by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instruction. Three days after transfection, reporter gene expression was examined using fluorescent microscopy and anti-GFP or anti-RFP immunoblots. We also cotransfected 293 cells with the GLUT4-GFP reporter plasmid and PCR-derived siGLUT4 cassettes that contain the H1 promoter and shRNA templates (forward primer, AAATTACCTCACTAAGGGG; reverse primer, CGAGGTCAAGCG GTTCAAGATGAGTCGTCTTAATTCTCGTTAAGAACTTGGAAA). This facilitated the identification of optimal siRNA sequences as 293T cells are highly transfectable using routine lipid-based reagents.

Immunoblot and glucose uptake analysis

For immunoblot analysis, the cells were lysed in a buffer containing 25 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 25 mM NaCl, 1% Nonidet P-40, 1 mM Na2HPO4, 10 mM NaF, 0.2 mM leupeptin, 1 mM benzamidine, and 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride and rocked for 40 min at 4 °C. Insoluble material was removed by centrifugation at 12,000 × g for 10 min at 4 °C. Cell lysates were separated by SDS-PAGE for immunoblot analysis. The proteins were detected by enhanced chemiluminescence with horseradish peroxidase-labeled secondary antibodies.

The procedure for 2-[3H] deoxyglucose uptake was described previously (16). 3T3-L1 adipocytes were serum-starved for 3 h, and the cells were stimulated with insulin for 20 min at 37°C. Glucose uptake was determined after the addition of 2-[3H] deoxyglucose (0.1 μCi, final concentration 0.1 μM) in KRP-HEPES buffer [10 mM HEPES (pH 7.4), 131.2 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 2.5 mM CaCl2, 2.5 mM NaH2PO4] for 10 min at 37°C.

Electroporation of siRNA into 3T3-L1 adipocytes

Six days after differentiation, 3T3-L1 adipocytes were electroporated with siGLUT1 (SmartPool; Dharmacon, Lafayette, CO; 2.5 nmol per 1 × 10^6 cells) as described previously (11).
10^7 cells) or siLC oligonucleotides using the Gene Pulser Xcell (Bio-Rad, Hercules, CA; 250 V and 950 μF). Forty-eight hours after electroporation, glucose uptake assays were performed as described (16).

**Oil Red O staining of triglycerides**

3T3-L1 cells were fixed with 3.7% formaldehyde for 10 min and then stained with Oil Red O for 1 h followed by washing with 70% methanol and water (17). For fat quantitation, the plates were dried for 1 h at 37 °C, and Oil Red O was extracted with isopropanol and quantitated as described (18).

**Results**

**Lentivirus-mediated high-efficiency delivery of exogenous genes into 3T3-L1 adipocytes**

Because lipid-based transfection methods transduce genes into 3T3-L1 adipocytes poorly, we have adapted lentivirus-mediated expression of shRNA as an alternate approach to study insulin signaling in these cells. As shown in Fig. 1A, 3 d after transfection of GFP plasmids using Lipofectamine 2000, GFP was readily detectable in 3T3-L1 preadipocytes but barely detectable in 3T3-L1 adipocytes (Fig. 1A). In contrast, infection of cells with a lentivirus construct encoding GFP led to highly efficient expression in both cell types. Specifically, 3T3-L1 adipocytes were infected with lent GFP 9 d after differentiation. Two days later, GFP expression was readily detected, and 5 d later more than 95% of cells exhibited fluorescence (Fig. 1B). The transgene expression was quite stable because GFP was detected as late as 44 d after infection (Fig. 1B). An additional experiment was performed by infecting the cells on d 20 of adipogenic differentiation. As shown in Fig. 1C, 5 d after infection, most cells expressed GFP. Thus, lentiviruses provide an efficient system for delivering exogenous genes into fully differentiated 3T3-L1 adipocytes.

**Lentivirus-mediated gene expression persists through proliferation and differentiation**

We infected 3T3-L1 preadipocytes with lent GFP to determine whether transgene expression can be maintained throughout the proliferation and differentiation processes in 3T3-L1 cells. Cells were infected at approximately 75% confluence and as shown in Fig. 1D, GFP expression was detected before and after differentiation. As evaluated by Oil O Red staining of lipid droplets, the lentivirus-transduced preadipocytes differentiated normally into adipocytes in response to the differentiation mix.

**Lentiviral transduction does not interfere with insulin signaling in 3T3-L1 adipocytes**

To determine whether lentiviral infection interferes with insulin action, the adipocytes were treated with lent GFP, and insulin signaling was assessed 5 d later. As shown in Fig. 1E, protein levels of IRβ, IRS1, and AKT in the lentivirus-transduced cells were similar to those in the nontransduced control cells. The lentivirus-transduced cells also responded to insulin stimulation normally as assessed by insulin-stimulated phosphorylation of IRβ, IRS1, and AKT. In addition, the lentivirus did not alter basal or insulin-stimulated 2-[3H] deoxyglucose uptake (Fig. 1F).
Lentivirus-mediated siGFP efficiently blocks GFP expression

Fully differentiated 3T3-L1 adipocytes were infected with lentivirus, either alone or in combination with a lentivirus expressing siGFP (siRNA directed against GFP). Five, 10, and 20 d after infection, GFP was highly expressed in the lentivirus cells but was barely detectable in the cells coinfected with lentivirus (Fig. 2). These results show that lentiviruses provide efficient delivery of exogenous genes, as well as functional siRNAs into 3T3-L1 adipocytes.

Construction of GLUT4-GFP and GLUT4-RFP reporter plasmids for identification of siRNA targets

To assess the role of GLUT4 in insulin-stimulated glucose transport, we used the lentivirus method to delete GLUT4 from 3T3-L1 adipocytes. Because not all sequences in a given mRNA are equally sensitive to siRNA, we selected several potential target sequences along mouse GLUT4 mRNA. To identify the most efficient siRNA sequences, we constructed reporter plasmids expressing GLUT4-GFP and GLUT4-RFP fusion proteins. Transfection of 293 cells with the reporter plasmids led to high levels of GLUT4-GFP and GLUT4-RFP expression (Fig. 3, a-1 and b-1) as observed by fluorescence microscopy. In addition, immunoblot analysis with anti-GFP or anti-RFP antibodies showed bands corresponding to the expected size of the fusion proteins (Fig. 3, a-2 and b-2). These bands were also recognized by anti-Glut4 antibody (data not shown).

We then subcloned several siRNAs against GLUT4 into expression plasmids and transfected them into 293T cells along with the GLUT4-GFP reporter plasmid. Control cells were transfected with the reporter plasmid alone or with the reporter plasmid plus the siRNA expression plasmid. As shown in Fig. 3C, the siRNA expressing plasmid itself had no effect on GLUT4-GFP expression, whereas two siGLUT4 (i.e. 1 and 3) markedly silenced GLUT4-GFP expression, one had a moderate silencing effect (i.e. 4), and one had no effect (i.e. 2) as observed by fluorescence microscopy and immunoblot analysis. We also cotransfected 293T cells with the GLUT4-GFP reporter plasmid together with PCRP-directed siGLUT4 cassettes that contain H1 promoter and hairpin shRNA templates. A similar effect was observed (Fig. 3D): two siGLUT4 (i.e. 1 and 3) markedly silenced protein expression (fluorescence microscopy and immunoblot analysis), one had a moderate silencing effect (i.e. 4), and one had no effect (i.e. 2).

We further confirmed the silencing effect of siGLUT4 by cotransfecting 293T cells with the GLUT4-RFP reporter plasmid and siGLUT4 plasmids (data not shown). We then subcloned the most effective siGLUT4 siRNA together with the H1 promoter into a lentivirus expressing plasmid. Cotransfection of 293T cells with GLUT4-RFP and siGLUT4 plasmids showed that siGLUT4 markedly silenced GLUT4-RFP expression as observed by fluorescence microscopy and determined by immunoblot analysis (data not shown).

Silencing endogenous GLUT4 partially blocks insulin-stimulated glucose uptake

Fully differentiated 3T3-L1 adipocytes were infected with lenti siGLUT4 to assess the role of GLUT4 in glucose transport. As shown in Fig. 4A, lenti siGLUT4 markedly reduced endogenous GLUT4 expression (>90%) but did not affect other proteins such as GLUT1, IRβ, IRS1 and 2, PPARγ, annexin II, or actin. Insulin-stimulated 2-[3H] deoxyglucose uptake was reduced by approximately 55% in the lentivirus-infected adipocytes compared with cells infected with control lentivirus (Fig. 4B).

We also infected 3T3-L1 preadipocytes with lenti siGLUT4 and then differentiated them into adipocytes for glucose uptake analysis. As shown in Fig. 4C, lentivirus siGLUT4 reduced endogenous GLUT4 expression by more than 95% but did not affect GLUT1, IRβ, IRS1 and 2, PPARγ, or annexin II expression. When glucose transport was assessed, basal 2-[3H] deoxyglucose uptake was not affected, whereas insulin-stimulated uptake was decreased by approximately 60% in the lenti siGLUT4 adipocytes (Fig. 4D).

It should be noted that although GLUT4 protein was reduced to undetectable levels in the lenti siGLUT4 cells, a significant proportion of insulin-stimulated glucose uptake (~40%) remained. This suggests that another glucose transporter(s) plays an important role in insulin-stimulated glucose transport in 3T3-L1 adipocytes. Because it is well known that 3T3-L1 adipocytes also express GLUT1, we electroporated siGLUT1 into the GLUT4-deficient adipocytes to assess glucose uptake. Again, siGLUT4 lentivirus reduced GLUT4 to an undetectable level (Fig. 5A) and attenuated insulin-stimulated glucose uptake by 60% (Fig. 5B). Electroporation of siGLUT1 reduced GLUT1 protein expression by approximately 70% (Fig. 5A). As expected, this led to a marked decrease in basal glucose transport. We further found that GLUT1 depletion resulted in an approximately 68% reduction in insulin-stimulated glucose transport (Fig. 5B). When GLUT1 was knocked down in the GLUT4-deficient adipocytes, insulin-stimulated glucose transport was almost completely abolished (Fig. 5B). These data suggest that both GLUT4 and GLUT1 are of importance in the insulin-stimulated component of glucose uptake in 3T3-L1 adipocytes.
Effects of GLUT4 silencing on IRAP expression

It has previously been shown that IRAP expression is decreased in GLUT4 knockout animals (19–21). To assess this in vitro, we measured IRAP levels in the lenti siGLUT4 infected cells. When GLUT4 was knocked down after the 3T3-L1 adipocytes were fully differentiated, no effect on IRAP expression was noted (Fig. 6A). In contrast, IRAP expression in adipocytes was reduced by approximately 30% when lenti siGLUT4 was expressed before adipogenic differentiation (Fig. 6B).

Effect of GLUT4 silencing in 3T3-L1 differentiation

The differentiation of 3T3-L1 preadipocytes into adipocytes appeared not to be influenced by GLUT4 silencing.
because cells infected with control lentivirus vs. siGLUT4 lentivirus showed no appreciable difference for up to 9 d after differentiation as observed under the microscope (Fig. 7A). This is consistent with normal expression of PPARγ (an adipocyte differentiation marker) in GLUT4-deficient adipocytes (Fig. 4, A and C). Also, on d 9 after differentiation, there were no differences in the accumulation of intracellular lipid between the two groups (Fig. 7, A and B) as determined by quantitation of Oil Red O staining. However, more prolonged culture showed that lipid accumulation plateaued in the GLUT4-deficient adipocytes but continued to increase in control cells. Lipid content was reduced by 33% on d 15 and 45% on d 21 after differentiation compared with controls.

**Discussion**

The 3T3-L1 adipocyte has long been used as a model cell system for studies of insulin action (4–6). Once fully differentiated into adipocytes, these cells contain high levels of GLUT4, exhibit a robust degree of insulin responsiveness for stimulation of GLUT4 translocation and glucose transport, and exhibit all of the signaling molecules and intracellular machinery involved in insulin action. Dissection of the complex interconnections within the insulin signaling pathway has been a difficult and evolving field of study, as evidenced by the huge number of papers published over the past 20 yr using these cells for such studies.

Recently, RNA interference has emerged as a powerful tool to assess mechanisms of intracellular signaling. As widely reported, this approach allows one to knock down (or knock out) selected gene products in a highly specific fashion (22, 23). Although this method has been applied to 3T3-L1 cells, unfortunately, once adipogenic differentiation occurs, these cells become very refractory to standard methods of transfection and only negligible efficiencies are achieved. Some successful studies have been reported using single cell microinjection (24) or electroporation of siRNAs into 3T3-L1 adipocytes (25–27), but each of these techniques has certain drawbacks. For example, microinjection can only be performed in a limited number of cells (several hundreds) so that only single cell, visual assays, can be used, precluding biochemical assays of global cell populations. Whole culture electroporation has been used in several papers (25–27), but this approach can only be used in early stage (5–6 d after differentiation), the electroporation process itself may have
effects on cell viability or function, and the amount of siRNA needed is costly. A shortcoming common to both of these methods, and also with standard transfection, is that the target gene knockdown is transient over a period of 2–4 d. In this report, we have used a lentiviral vector to express specific shRNA in 3T3-L1 adipocytes. This method achieves near 100% efficiency and, importantly, because the lentivirus integrates into the cell genome, shRNA expression is permanent, leading to long-term knockdown of the targeted protein. As demonstrated in the current studies, this has important advantages when the experimental cell type manifests different stages (differentiation, cell cycle, apoptosis, etc.) with distinct cellular phenotypes. With the lenti-shRNA approach, targeted gene knockdown can be achieved at any phase of the cell cycle and maintained indefinitely.

In the current studies, we have used a lenti-shRNA vector targeted to GLUT4 to achieve a more than 90% knockdown of this protein and the effect appears permanent. Our results have led to several new insights into the role of GLUT4 in adipocyte biology and glucose transport. It is of interest that IRAP is colocalized to recycling vesicles that display high expression levels of GLUT4 and is considered a component of the GLUT4-containing vesicle. In previous studies, it has been shown that GLUT4 knockout mice demonstrate reduced levels of IRAP expression (19–21). In the current studies, we find that lentivirus-mediated introduction of GLUT4 shRNA, depending on the timing, affects IRAP expression differently. When introduced at the preadipocyte stage, GLUT4 shRNA leads to decreased IRAP expression after adipogenic differentiation. This is consistent with the KO mouse study (20, 21). However, in GLUT4 knockout mice, the IRAP expression in adipocytes was reported to be decreased in one study (21) but not in another study (19). By contrast, when introduced after the completion of adipogenesis, GLUT4 shRNA does not alter IRAP expression. This appears to dissociate the signals to impair IRAP expression, depending on the developmental stage of the cell. These results also illustrate the advantage of being able to time the deletion of a particular protein in understanding its full biological effects.

We also noted that lentivirus-mediated siGLUT4 expression in predifferentiated cells essentially abolished GLUT4 expression after differentiation in adipocytes but had no effect on the subsequent development of the fully differentiated adipocyte phenotype. However, once full differentiation occurred, GLUT4-deficient cells accumulated less lipid than the control cells, indicating some influence of GLUT4 activity on maintenance of the full lipogenic capacity of adipocytes, even though various other markers of adipocyte differentiation remained fully expressed.

Another important aspect of these studies was the relationship between GLUT4 content and overall insulin-stimulated glucose transport. We found that nearly complete deletion of GLUT4 had little effect on basal glucose transport. This was to be expected given the low GLUT4 concentration at the cell surface under basal conditions. Interestingly, however, despite near complete deletion of GLUT4 protein, insulin-stimulated glucose transport was only decreased by approximately 50%. This indicated that some other glucose transport species was largely responsible for basal glucose transport and for a large component of the insulin-stimulated glucose transport, which is consistent with a previous report (28). Our data strongly indicate GLUT1 as this alternate glucose transporter. Thus, we found no effect of GLUT1 knockdown on the expression of GLUT4 protein, but, nevertheless, GLUT1 contributed in an important way to both basal and insulin-stimulated glucose transport. The results showed that, after siRNA-mediated knockdown of GLUT1, approximately 70% of basal glucose transport was lost. More interestingly, knockdown of GLUT1 led to a marked impairment of overall insulin-stimulated glucose transport. Furthermore, when GLUT1 was knocked down in adipocytes infected with the lentiviral GLUT4 shRNA, essentially no residual insulin-stimulated glucose transport was observed. These results lead us to conclude that GLUT1 is the major glucose transporter in 3T3-L1 adipocytes responsible for basal glucose transport, whereas both GLUT4 and GLUT1 contribute in a relatively comparable way to the overall effect of insulin to stimulate glucose transport. These studies raise questions concerning previous interpretation of many studies examining insulin stimulated glucose transport in these cells. Clearly, in future studies, both GLUT1- and GLUT4-mediated events need to be considered in interpreting overall glucose transport in insulin stimulated 3T3-L1 adipocytes. It is quite possible that, even though insulin stimulated translocation of both GLUT1 and GLUT4 appear to be phosphatidylinositol 3-kinase dependent, there may be more subtle differences in insulin signaling cascades that recruit GLUT4 vs. GLUT1 to the cell surface. This remains a question for future study.

In summary, we have used a lentiviral vector to transduce specific shRNA sequences into 3T3-L1 adipocytes. This method is highly efficient leading to near complete depletion of the target protein GLUT4. The lentiviral infection can be timed to different stages of the cell life cycle and, taken together, this method should prove useful in further studies of insulin action and adipocyte biology. In the current studies, we found that GLUT4 knockdown can lead to impaired IRAP protein expression, but this effect is dependent on whether GLUT4 is knocked down in the differentiating or differentiated stage. We also find that GLUT4 is necessary for the full lipogenic capacity of differentiated adipocytes, and importantly the data show that a relatively large component of insulin stimulated glucose transport is mediated by GLUT1.

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