RNAi-based gene silencing in primary mouse and human adipose tissues

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Abstract Cultured adipocyte cell lines are a model system widely used to study adipose function, but they exhibit significant physiological differences compared with primary cells from adipose tissue. Here we report short interfering RNA-based methodology to selectively attenuate gene expression in mouse and human primary adipose tissues as a means of rapidly validating findings made in cultured adipocyte cell lines. The method is exemplified by depletion of the PTEN phosphatase in white adipose tissue (WAT) from mouse and humans, which increases Akt phosphorylation as expected. This technology is also shown to silence genes in mouse brown adipose tissue. Previous work revealed upregulation of the mitochondrial protein UCP1 in adipose cells from mice lacking the gene for the transcriptional corepressor RIP140, whereas in cultured adipocytes, loss of RIP140 has a little effect on UCP1 expression.■ Application of our method to deplete RIP140 in primary mouse WAT elicited markedly increased oxygen consumption and expression of UCP1 that exactly mimics the phenotype observed in RIP140-null mice. This ex-vivo method of gene silencing should be useful in rapid validation studies as well as in addressing the depot- and species-specific functions of genes in adipose biology.—Puri, V., A. Chakladar, J. V. Virbasius, S. Konda, A. M. Powelka, M. Chouinard, G. N. Hagan, R. Perugini, and M. P. Czech. **RNAi-based gene silencing in primary mouse and human adipose tissues.** *J. Lipid Res.* 2007. 48: 465–471.

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Adipocytes are insulin-responsive energy-storing cells that also exhibit endocrine functions. They are recognized as central regulators of whole-body metabolism (1). Many long-established cell lines used to study adipocyte functions differ genetically and metabolically from both the original euploid cells and from early-established cell lines (2–4). Widely used 3T3-L1 and 3T3-442FA cells are good model systems for studying the differentiation process, yet there are several potential drawbacks to their use (1). One caveat is their different chromosomal number (aneuploid), and a second concern is that establishing these cells involves an alteration in cell permeability properties (5–7). Another significant limitation of cell culture models is their inability to mimic depot-specific differences in adipocyte behavior. Regional variations in metabolic functions of adipocytes from regional adipose depots are significant, and depot-related differences in protein expression have been identified (8). Hence, the sole use of cultured preadipocyte lines to generate adipocytes in culture may result in conclusions that do not apply to primary cells under physiological conditions.

One approach to confirm the physiological relevance of studies done with established cell lines is to generate transgenic and gene knock-out mice, but this is a relatively tedious process. Moreover, this genetic engineering approach cannot be extended to human adipose tissue. Another approach is to use isolated primary adipocytes, but primary adipocytes have been shown to lose their endocrine properties within hours of culturing. Several groups have reported downregulation of various genes within a few hours of isolating and culturing primary adipocytes (9, 10). A detailed study (9) revealed that the standard isolation of primary adipose cells from mouse epididymal fat pads induces inflammatory cytokines, leading to the downregulation of adipocyte genes. These changes in gene expression contribute to the development of insulin resistance in primary adipose cells in prolonged culture. Isolation of primary rat adipocytes results in a 20-fold decrease in GLUT4 glucose transporter expression and a 70-fold increase in GLUT1 mRNA levels within a 24 h time period (10).

We adopted an approach to keep adipocytes in their normal immediate physiological environment and then manipulate the expression of the gene of interest. In the present study, we have developed a technology for silencing genes in mouse white and brown adipose tissues, as

Abbreviations: BAT, brown adipose tissue; siRNA, short interfering RNA; WAT, white adipose tissue.
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well as in human visceral and subcutaneous adipose tissues, using short interfering RNA (siRNA). This method of gene silencing is useful to quickly study the physiological functions of various genes in different fat depots of both mouse and humans.

MATERIALS AND METHODS

Materials

C57 BL/6J (male, 10 weeks old) mice were obtained from Jackson Labs. Rabbit polyclonal antibodies against phospho Akt/PKB (ser 473) and Akt were from Cell Signaling Technology (Beverly, MA). Rabbit anti-PTEN antibody was from Upstate Cell Signaling Solutions (Lake Placid, NY). Monoclonal antibodies against Vti1a were from BD Transduction Laboratories (San Diego, CA). Goat anti-GLUT4 antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and rabbit anti-GLUT1 was a kind gift from Paul Pilch, Boston University, Boston, MA. Human insulin was obtained from Eli Lilly Co. Horseradish peroxidase-conjugated goat anti-rabbit was purchased from Santa Cruz Biotech.

siRNA

The siRNA species purchased from Dharmacon Research were designed to target the following cDNA sequences: scrambled, 5′-CAGTCGCGTTTGCGACTGG-3′; PTEN siRNA, 5′-GTATAGA-GCGTGCAGATAA-3′; Akt1 siRNA, 5′-AACCAGGACCACGAGA-AGCUG-3′; Akt2 siRNA, 5′-GAGAGGACCUUCCAUGUAG-3′; and RIP140 siRNA, 5′-GGAATGAGCTCGATTATAA-3′.

Tissue samples and culture

Epididymal fat pads and interscapular brown adipose tissues (BATs) were obtained from 10-week-old male C57 BL/6J mice. These were washed three times with sterile PBS and minced to a size of 1–2 mm under sterile conditions. The tissue explants were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 μg/ml streptomycin, and 50 U/ml penicillin (complete medium). Medium was changed after 2 h, then after 3 h, and thereafter every 24 h of culturing.

Human adipose tissue

Fresh human omental and subcutaneous tissues were procured according to procedures outlined by the University of Massachusetts Medical School Institutional Review Board, with the informed consent of patients undergoing gastric bypass surgery. Human tissues were processed for experiments using a method similar to that used for mouse adipose tissues.

Transfection of siRNAs in primary tissue

Freshly procured adipose tissue was washed with PBS under sterile conditions. For each transfection, 60–80 mg of tissue was minced to a size of approximately 1–2 mm. The tissue explants were resuspended in 200 μl PBS mixed with 16 nmol siRNA duplexes in an electroporation cuvette (0.4 cm) and subjected to sixteen shocks of 50 V and 950 μF with a time constant of 30 msec on a Bio-Rad Gene Pulser II system. Immediately after electroporation, DMEM supplemented with 10% fetal bovine serum, 50 μg/ml streptomycin, and 50 U/ml penicillin was added, and the explants were incubated at 37°C in 5% CO_2 for about 65 h. The medium was changed after 2 h, then after 5 h, and thereafter every 24 h. For Akt knock-down, siRNA for Akt1 and Akt2 were mixed in the ratio 2:3 and used for transfection.

Western blotting

Tissue explants were homogenized in 150 μl of lysis buffer (1% SDS in phosphate buffer saline containing protease inhibitors), frozen in methanol-dry ice, and thawed over ice. Samples were centrifuged at 13,000 rpm for 5 min. The top fat layer was discarded, and the middle clear layer of solublized proteins was collected. After protein estimation, Western blots were performed as described previously (11). Briefly, the tissue lysates were subjected to SDS-PAGE, followed by transference to nitrocellulose membranes. The membranes were incubated with antibodies specific for protein and were visualized with an enhanced chemiluminescence reagent (New England Nuclear; Boston, MA). Vti1a staining was done to confirm equal loading.

Glucose transport in primary adipocytes

Primary adipocytes were isolated from either fresh or cultured white adipose tissue (WAT) from epididymal fat pads of C57 BL/6J mice by collagenase treatment. Glucose uptake in primary mouse adipocytes was carried out as described elsewhere (12). Briefly, primary adipocytes were incubated at 37°C with constant shaking in Krebs-Ringer-Hepes (KRH) buffer (25 mM Hepes, 130 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, and 1.3 mM $MgSO_4$, pH 7.4) supplemented with 2.5% fatty acid-free BSA and 2 mM sodium pyruvate. Cells were then stimulated with 100 nM insulin for 30 min at 37°C. Glucose uptake was initiated by addition of U-[^{14}C]glucose to a final assay concentration of 0.01 μCi for 20 min at 37°C. The reaction was terminated by adding 0.05 mM cytochalsin B and separating cells from the medium by spinning the suspension through dinonyl phthalate oil (Eastman Kodak Co.).

Insulin stimulation in adipose tissue

Forty-eight hours after electroporation with siRNA, the tissue explants were serum-starved for 16 h in DMEM containing 0.5% BSA (starvation medium) before stimulation with 10 nM insulin for 30 min.

Quantitative RT-PCR

Total RNA was isolated from 3T3-L1 adipocytes or from WAT using TRIzol reagent (Invitrogen Corp.). cDNA was synthesized using oligo-dT primers and AMV reverse transcriptase (Roche Diagnostics Corp.) according to the manufacturer's instructions. Quantitative RT-PCR was performed in a LightCycler (Roche Diagonostics Corp.) using the SYBR Green 1 PCR kit (Roche Diagnostics Corp.) and specific primers to amplify the genes. Expression levels for all genes were correlated to that for the HPRT gene. Primer sequences may be obtained upon request.

Oxygen consumption assay

Oxygen consumption by tissues under various conditions was measured using the BD Oxygen Biosensor System (BD Biosciences, San Diego, CA). Equal weights of tissue samples were resuspended in KRH buffer in separate wells of the BD Oxygen Biosensor System plate. Oxygen consumption was measured in a fluorescence plate reader at 485 nm excitation and 630 nm emission. Fluorescence values for each well were normalized to their initial value and then to the values of no-tissue controls at each time point.

RESULTS AND DISCUSSION

The aims of present study were 2-fold: 1) to develop conditions for culturing isolated adipose tissue from mouse

and human sources that maintain functional integrity, and *2*) to develop methods for gene silencing in primary adipose tissue using RNA interference (RNAi). Therefore, we attempted to develop technology using adipose tissue explants for selective gene knock-down using RNAi. RNAi is now an established technology that is being widely used for gene silencing in various cell types (13), but has not yet been generally applied to primary adipose tissues. Initial studies in our lab (14, 15) and others (16) identified conditions for siRNA-based gene silencing in cultured adipocytes, but these and other methods are not effective in primary tissues. In the present study, we first optimized the culture conditions for adipose tissue to maintain near-normal physiological functions for at least 72 h as revealed from GLUT4 and GLUT1 expression, oxygen consumption, adiponectin secretion, and glucose uptake. This 72 h period is sufficient for silencing most genes using RNAi.

Conditions for culturing primary adipose tissue

Epididymal fat pads from C57 BL/6J mice were washed three times with sterile PBS and then minced to a size of approximately 1–2 mm and cultured in DMEM. Numerous changes of media under well-oxygenated conditions were necessary for best results. **Figure 1A** shows GLUT4 and GLUT1 protein expression in WAT cultured for 72 h. A moderate decrease in GLUT4 and a slight increase in GLUT1 expression levels were observed. The isolated adipocytes from cultured tissue showed the typical phenotype of primary adipocytes, with a large triglyceride globule, the nucleus bulging at the periphery, and a highly compressed cytosol. Viability of the isolated adipocytes was found to be ≥90%, based on trypan blue staining. To confirm that the tissue remained viable under the culture conditions used, we studied adiponectin secretion by the tissue explants at various time points. Figure 1B depicts adiponectin secretion normalized to the number of adipocytes in equally weighed tissue samples. Adiponectin secretion was maintained to a significant level after 72 h in culture. As shown in Fig. 1C, the tissue was consuming oxygen at a near-normal rate even after remaining in culture for 72 h. In fact, very little difference in oxygen consumption was observed when the tissue was switched to starvation media during the culture (data not shown). We next examined
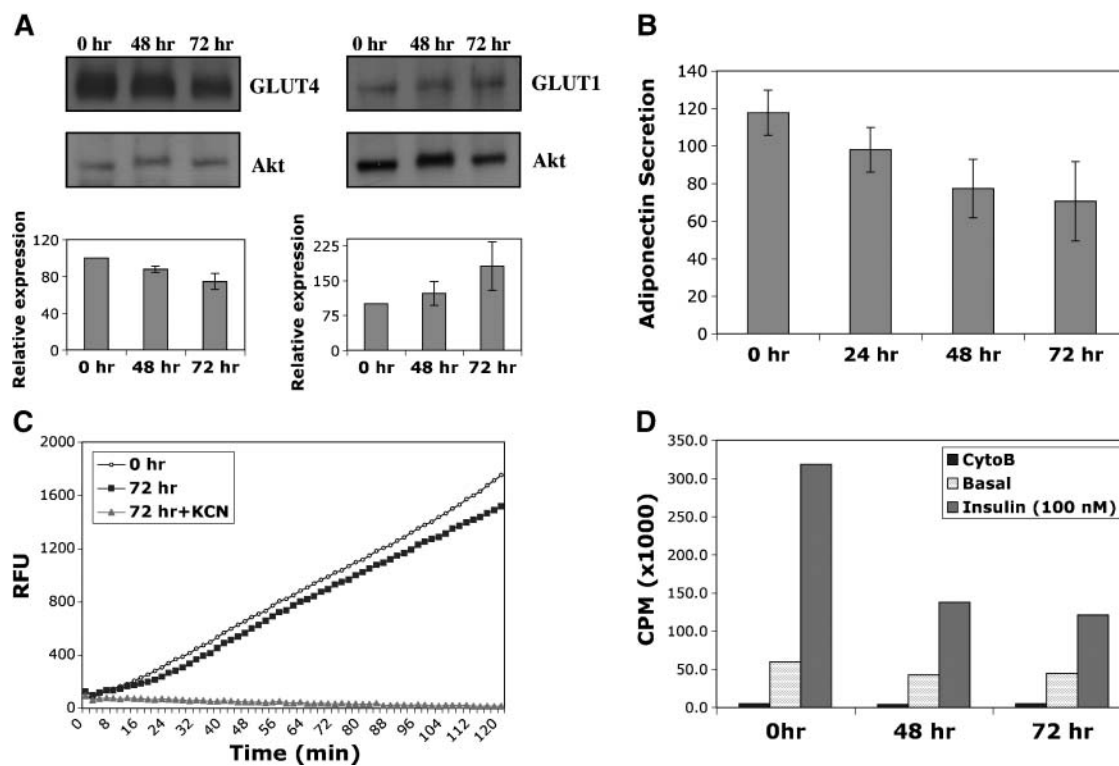

Fig. 1. Comparison of GLUT4 and GLUT1 expression, adiponectin secretion, oxygen consumption, and insulin-stimulated glucose uptake in freshly procured white adipose tissue (WAT) and WAT cultured for 72 h. A: Western blot analysis of GLUT4 and GLUT1 proteins in WAT explants cultured for 0 h (freshly procured), 42 h, and 72 h as described in Materials and Methods. Bar graphs represent the average of GLUT4 and GLUT1 expression from at least three independent experiments. B: Adiponectin secretion by cultured WAT explants. For each time point, media was collected 3 h after adding fresh media. Adiponectin secretion was then analyzed by Western blot analysis and normalized to the adipocyte cell number. Shown is the average quantification of adiponectin secretion in three independent experiments. Error bars indicate standard errors. C: Tissue was assayed for respiration using the oxygen biosensor fluorescence assay described in Materials and Methods. Open circle, 0 h (freshly procured tissue); closed square, tissue cultured for 72 h; closed triangle, tissue cultured for 72 h + KCN. The tissue was poisoned with KCN to confirm that it terminates the respiration and that the oxygen consumption that we were measuring was due to the respiring tissue. RFU, relative fluorescence units. D: Bar graph showing glucose uptake under basal and insulin-stimulated conditions in fresh and cultured WAT. The study was performed as described in Materials and Methods. Representative experiments of three or more were performed with similar results. CPM, counts per minute.

glucose uptake levels in adipocytes isolated from tissue explants that were cultured for a 48 h and 72 h time period. As shown in Fig. 1D, even after 72 h of culturing the adipose tissue, significant stimulation of glucose uptake with insulin was observed. GLUT4 protein levels were reduced by about 25% after 48 h or 72 h of culturing the adipose tissue, whereas insulin-stimulated glucose uptake was reduced by more than 50%. This indicates that other components of the insulin-signaling pathway or the membrane-trafficking pathway whereby GLUT4 is translocated to the plasma membrane may be compromised by the tissue culture conditions. Nonetheless, we still observed a 3-fold effect of insulin on deoxyglucose uptake under these conditions.

PTEN and Akt knock-down in WAT and its effect on Akt phosphorylation

PTEN and Akt were selected for siRNA-mediated depletion because of their well-established roles as negative and positive regulators of insulin-induced glucose uptake, respectively (14, 15). In this method, 60–80 mg of WAT was used for each transfection. WAT was minced to pieces approximately 2 mm in diameter, resuspended in 200 µl PBS, and electroporated with 16 nmol siRNA duplexes against PTEN or Akt using the settings detailed in the Materials and Methods section. Immediately after electroporation, complete medium was added and the tissue was incubated at 37°C at 5% CO_2. The medium was changed after 2 h and again at 5 h and thereafter every 24 h. **Figure 2A** shows the expression profile of PTEN and Akt proteins 65 h after transfection. There was more than 70% depletion of PTEN and Akt protein in the WAT (Fig. 2C), illustrating that this method of gene depletion is highly efficient.

Adipose tissue is composed of various cell types, including endothelia cells, macrophages, monocytes, and adipocytes. To confirm that gene silencing occurs in adipocytes comprizing the adipose tissue, we isolated primary adipocytes from WAT that had been cultured for 65 h after transfection with PTEN-directed siRNA. More than 70% depletion of PTEN protein was observed in isolated primary adipocytes derived from cultured adipose tissue (data not shown). It is likely that gene silencing also occurs in other cell types present in cultured adipose tissue.

PTEN and Akt are negative and positive regulators of insulin signaling in adipocytes, respectively. In cells stimulated with insulin or growth factors, Akt is phosphorylated and activated by phosphoinositide-dependent protein
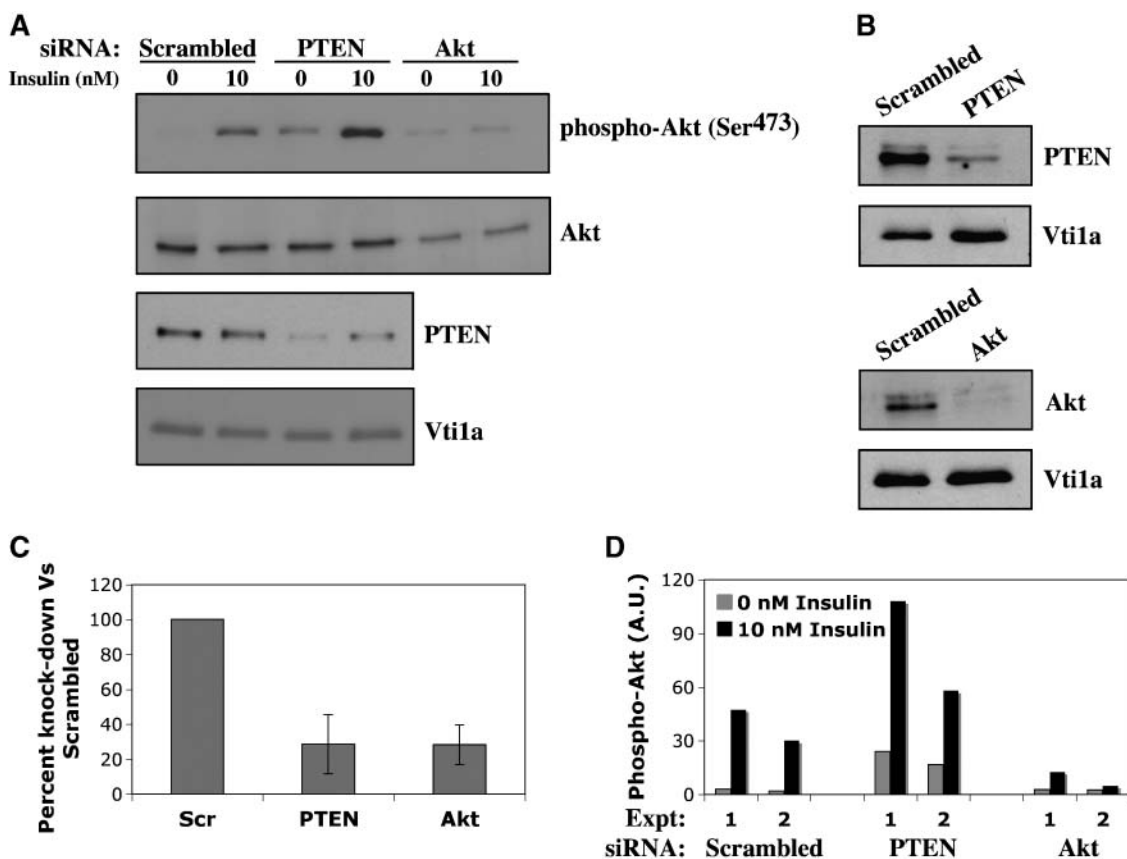

Fig. 2. Gene silencing in mouse WAT and brown adipose tissue (BAT). A: PTEN and Akt short interfering RNA (siRNA) was transfected in WAT as described in Materials and Methods. The Figure shows the Western blot analysis of PTEN and Akt knock-down, as well as the effect of PTEN and Akt depletion on Akt phosphorylation. B: Western blot analysis of PTEN and Akt knock-down in BAT. C: Percent of PTEN and Akt knock-down in WAT as compared with scrambled, where scrambled is taken as 100. Bar graphs are an average of at least three independent experiments. D: Relative quantification of Akt phosphorylation, in two independent experiments, in WAT transfected with scrambled, Akt, and PTEN siRNA. Error bars indicate standard errors.

kinase 1, downstream of PI3K (17). Activation of Akt has an important role in mediating the metabolic and mitogenic functions of insulin (17, 18). On the other hand, PTEN is a dual-function lipid and protein phosphatase that dephosphorylates $PI(3,4,5)P_3$, which leads to decreased levels of this phospholipid and simultaneous reduction of Akt activity (19, 20). Thus, PTEN suppresses insulin signaling through the PI3K/Akt pathway and acts as a negative regulator of insulin-induced metabolic actions (21). As shown in Fig. 2A, D, PTEN depletion in WAT results in the expected increase of Akt phosphorylation, whereas Akt depletion decreases the effect of insulin on Akt phosphorylation. Attenuation of Akt expression in WAT was also associated with a decrease in phosphorylation of the Akt substrate GSK3 (data not shown). To examine whether this technology of siRNA-based gene silencing is applicable to other depots of adipose tissue, we performed PTEN and Akt knock-down in BAT isolated from the interscapular region of mice. As shown in Fig. 2B, we observed an approximate 80% loss of PTEN protein and Akt protein in BAT following transfection of their respective siRNA species. These results demonstrate that the functional aspects of insulin signaling can be effectively studied in WAT and BAT by using the method of gene silencing described here.

PTEN knock-down in human omental adipose tissue leads to increased Akt phosphorylation

We next applied this gene-silencing method to human adipose tissue. Fresh human omental and subcutaneous adipose tissue was procured from patients undergoing gastric bypass surgery. Human adipose tissues were processed for siRNA-mediated protein depletion in the same manner as described for mouse WAT. As shown in **Fig. 3A, B**, approximately 70% depletion of PTEN protein was achieved in both human omental and subcutaneous adipose tissue explants. Similarly, using siRNA for another test protein, ILK, resulted in diminution of ILK protein levels by about 90% (data not shown). As expected, PTEN depletion in human omental tissue resulted in increased Akt phosphorylation (Fig. 3C, D), indicating that functional analysis of gene silencing can be achieved in human adipose tissues.

RIP140 knock-down upregulates UCP1 mRNA levels in WAT

RNAi-based gene silencing was then applied to address an unresolved question related to the function of the RIP140 protein in adipose cells. RIP140 is a transcriptional corepressor that binds to the ligand binding domain of nuclear receptors in the presence of agonists, and regulates fat accumulation in mice (22). RIP140 functions in isolated adipocytes and in intact mice as both a major suppressor of oxidative metabolism and as a regulator of glucose homeostasis due to its control of the expression of genes in these pathways (23, 24). Previous work using cultured 3T3-L1 adipocytes and adipose tissue from RIP140-null mice showed that RIP140 depletion increases expression of many proteins that have a role in glucose oxidation, mitochondrial biogenesis, and oxidative phosphorylation, resulting in an increase of mitochondrial oxygen consumption (23, 24). Remarkably, the BAT-specific uncoupling protein UCP1 is also expressed in the WAT of RIP140-null mice, potentially enhancing energy expenditure. However, RIP140 depletion in 3T3-L1 adipocytes fails
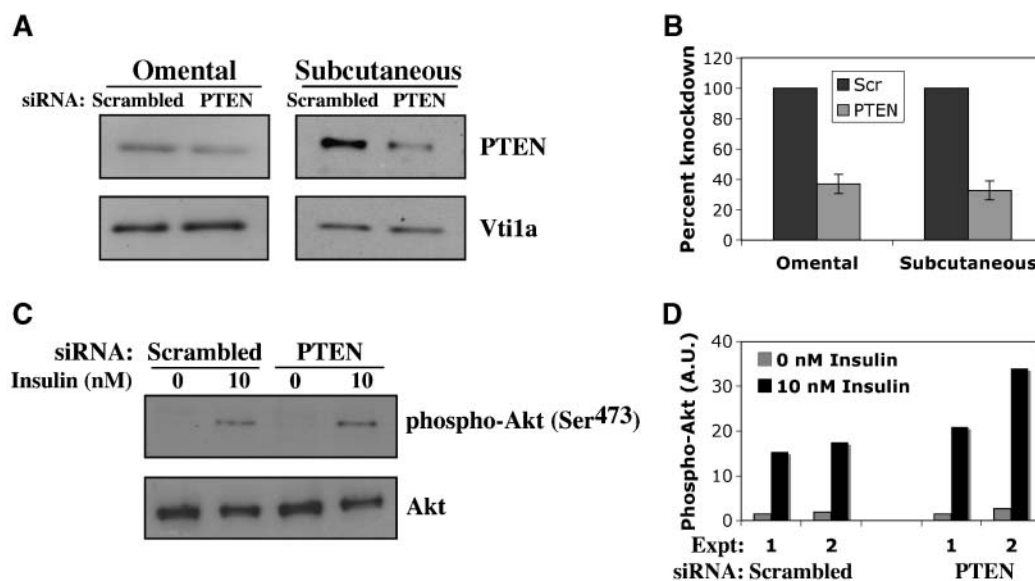

Fig. 3. Gene silencing in human omental and subcutaneous adipose tissues. A: Western blot analysis of PTEN knock-down in human omental and subcutaneous adipose tissues. B: Percent of PTEN knock-down as compared with scrambled siRNA, where scrambled siRNA is taken as 100. C: Top panel shows the insulin-stimulated Akt phosphorylation after PTEN depletion in human omental adipose tissue, and lower panel shows total Akt levels. Note the increase in insulin-stimulated Akt phosphorylation upon PTEN depletion. D: Relative quantification of Akt phosphorylation, in two independent experiments, in human omental adipose tissue transfected with scrambled siRNA and PTEN siRNA.

to upregulate UCP1 significantly. Taken together, these observations raise the question of whether UCP1 upregulation in vivo is a direct effect of the loss of RIP140 in adipose tissue or an indirect effect of RIP140 loss in other tissues.

To investigate this question, mouse WAT was transfected with RIP140 siRNA and the expression of UCP1 examined to evaluate how our ex vivo model correlates with the effects observed in RIP140 knock-out mice. We also measured the effect of RIP140 knock-down on mRNA levels of Cox8b, Cpt1, Pdk4, and Cidea in WAT. These genes previously have been shown to be targets for repression by RIP140 in 3T3-L1 adipocytes and in vivo (22–24), and are representative of dozens of nuclear genes encoding proteins in mitochondria that are regulated by
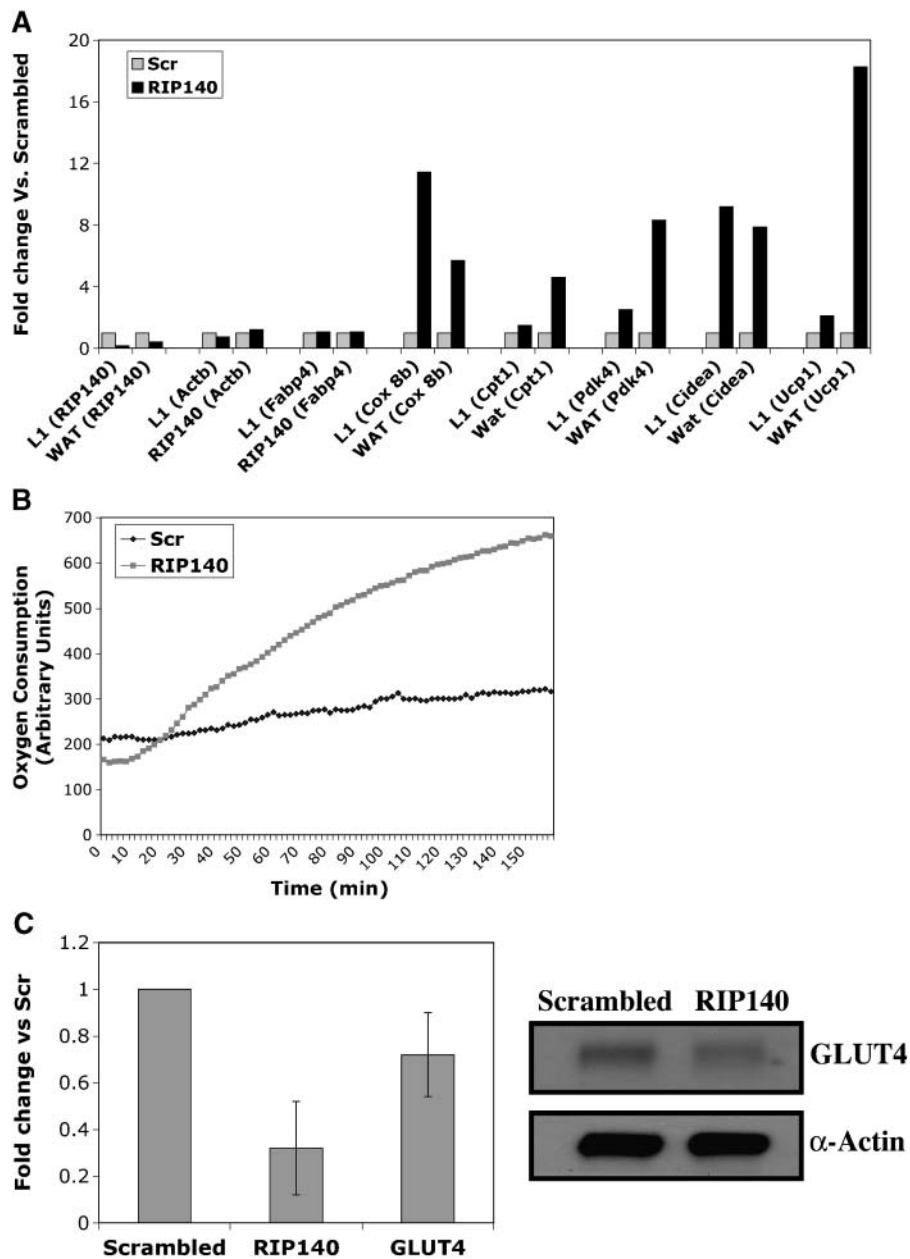
Fig. 4. Mitochondrial protein expression and oxygen consumption is enhanced in RIP140-depleted WAT. A: Bar graphs showing fold change in mRNA levels of genes in 3T3-L1 adipocytes and WAT transfected with siRNA for RIP140 or scrambled siRNA. 3T3-L1 adipocytes transfected and cultured as described previously (20). mRNA levels were determined by RT-PCR as described in Materials and Methods. Values are the averages of two or more independent experiments. B: Tissue was transfected with scrambled or RIP140 siRNA and assayed for oxygen consumption after 65 h using the oxygen biosensor fluorescence assay described in Materials and Methods. Equal weights of tissue were taken for both conditions. Results shown are representative of at least three experiments performed with similar results. C: Bar graph shows fold changes in RIP140 and GLUT4 mRNA levels in WAT transfected with siRNA for RIP140 or scrambled siRNA (average of three independent experiments). Right panel represents a Western blot showing a decrease in GLUT4 protein expression upon RIP140 depletion in mouse WAT. Error bars indicate standard errors.

RIP140. These include many classes of proteins, including cytochromes, transporters such as the fatty acid transporter Cpt1 (25, 26), and the uncoupling protein Ucp1. As shown in **Fig. 4A**, we observed that attenuating RIP140 expression in isolated WAT using RNAi results in an approximately 60% decrease in RIP140 mRNA levels. This led to significant increases in the mRNA levels of Cox8b, Cpt1, Pdk4, and Cidea, as well as Ucp1. This latter effect contrasts with the failure of RIP140 silencing to markedly upregulate UCP1 mRNA in 3T3-L1 adipocytes. Actb and Fabp4 were studied as negative controls and showed no change in expression upon RIP140 depletion. We confirmed by Western blot that several of the genes upregulated by RIP140 depletion showed increased expression at the protein level (data not shown). Because these proteins are involved in mitochondrial function, we also measured oxygen consumption in isolated WAT after RIP140 knock-down. As shown in Fig. 4B, RIP140 depletion also resulted in a marked enhancement of oxygen consumption in WAT, which is in agreement with the increased oxygen consumption found in vivo in RIP140-null mice and in cultured adipocytes (22–24). Taken together, these data indicate that the upregulation of UCP1 in RIP140-null mice is a direct consequence of RIP140 depletion in adipose tissue. Furthermore, these data confirm that the present system can be effectively used to assess the physiological function of adipose genes.

RIP140 knock-down in WAT decreases the mRNA and protein levels of the glucose transporter GLUT4

RIP140 depletion in 3T3-L1 adipocytes increases the expression of the GLUT4 protein (23), whereas GLUT4 expression is decreased in the WAT of RIP140-null mice (22). To elucidate this discrepancy, we examined the effect of RIP140 depletion on GLUT4 expression in isolated WAT by using our system. As shown in Fig. 4C, an RNAi-mediated decrease in RIP140 mRNA expression in mouse WAT resulted in a decrease of both mRNA and protein levels of GLUT4. Thus, as in the case of Ucp1, GLUT4 regulation is different in 3T3-L1 adipocytes compared with primary adipose tissue. These data further illustrate the utility of using primary adipose tissue for RNAi-mediated silencing in order to validate results obtained using the 3T3-L1 adipocyte system.▮▮

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